BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF AIRWAY SMOOTH MUSCLE - MECHANISMS OF AIRWAY HYPERRESPONSIVENESS

HE JIANG

5272190

A Thesis Submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

ISBN 0-315-92211-7

Canada

Name _____

Dissertation Abstracts International is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

ENGLISH

SUBJECT TERM

0433 U·M·I

SUBJECT CODE

Subject Categories

THE HUMANITIES AND SOCIAL SCIENCES

COMMUNICATIONS AND THE ARTS

Architecture 0729
Art History 0377
Cinema 0900
Dance 0378
Fine Arts 0357
Information Science 0723
Journalism 0391
Library Science 0399
Mass Communications 0708
Music 0413
Speech Communication 0459
Theater 0465

EDUCATION

General 0515
Administration 0514
Adult and Continuing 0516
Agricultural 0517
Art 0273
Bilingual and Multicultural 0282
Business 0688
Community College 0275
Curriculum and Instruction 0727
Early Childhood 0518
Elementary 0524
Finance 0277
Guidance and Counseling 0519
Health 0680
Higher 0745
History of 0520
Home Economics 0278
Industrial 0521
Language and Literature 0279
Mathematics 0280
Music 0522
Philosophy of 0998
Physical 0523
Psychology 0525
Reading 0535
Religious 0527
Sciences 0714
Secondary 0533
Social Sciences 0534
Sociology of 0340
Special 0529
Teacher Training 0530
Technology 0710
Tests and Measurements 0288
Vocational 0747

LANGUAGE, LITERATURE AND LINGUISTICS

Language
- General 0679
- Ancient 0289
- Linguistics 0290
- Modern 0291

Literature
- General 0401
- Classical 0294
- Comparative 0295
- Medieval 0297
- Modern 0298
- African 0316
- American 0591
- Asian 0305
- Canadian (English) 0352
- Canadian (French) 0355
- English 0593
- Germanic 0311
- Latin American 0312
- Middle Eastern 0315
- Romance 0313
- Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY

Philosophy 0422
Religion
- General 0318
- Biblical Studies 0321
- Clergy 0319
- History of 0320
- Philosophy of 0322

Theology 0469

SOCIAL SCIENCES

American Studies 0323
Anthropology
- Archaeology 0324
- Cultural 0326
- Physical 0327

Business Administration
- General 0310
- Accounting 0272
- Banking 0770
- Management 0454
- Marketing 0338

Canadian Studies 0385
Economics
- General 0501
- Agricultural 0503
- Commerce-Business 0505
- Finance 0508
- History 0509
- Labor 0510
- Theory 0511

Folklore 0358
Geography 0366
Gerontology 0351
History
- General 0578
- Ancient 0579
- Medieval 0581
- Modern 0582
- Black 0328
- African 0331
- Asia, Australia and Oceania 0332
- Canadian 0334
- European 0335
- Latin American 0336
- Middle Eastern 0333
- United States 0337

History of Science 0585
Law 0398
Political Science
- General 0615
- International Law and Relations 0616
- Public Administration 0617

Recreation 0814
Social Work 0452
Sociology
- General 0626
- Criminology and Penology 0627
- Demography 0938
- Ethnic and Racial Studies 0631
- Individual and Family Studies 0628
- Industrial and Labor Relations 0629
- Public and Social Welfare 0630
- Social Structure and Development 0700
- Theory and Methods 0344

Transportation 0709
Urban and Regional Planning 0999
Women's Studies 0453

THE SCIENCES AND ENGINEERING

BIOLOGICAL SCIENCES

Agriculture
- General 0473
- Agronomy 0285
- Animal Culture and Nutrition 0475
- Animal Pathology 0476
- Food Science and Technology 0359
- Forestry and Wildlife 0478
- Plant Culture 0479
- Plant Pathology 0480
- Plant Physiology 0817
- Range Management 0777
- Wood Technology 0746

Biology
- General 0306
- Anatomy 0287
- Biostatistics 0308
- Botany 0309
- Cell 0379
- Ecology 0329
- Entomology 0353
- Genetics 0369
- Limnology 0793
- Microbiology 0410
- Molecular 0307
- Neuroscience 0317
- Oceanography 0416
- Physiology 0433
- Radiation 0821
- Veterinary Science 0778
- Zoology 0472

Biophysics
- General 0786
- Medical 0760

EARTH SCIENCES

Biogeochemistry 0425
Geochemistry 0996
Geodesy 0370
Geology 0372
Geophysics 0373
Hydrology 0388
Mineralogy 0411
Paleobotany 0345
Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
Physical Geography 0368
Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES

Environmental Sciences 0768
Health Sciences
- General 0566
- Audiology 0300
- Chemotherapy 0992
- Dentistry 0567
- Education 0350
- Hospital Management 0769
- Human Development 0758
- Immunology 0982
- Medicine and Surgery 0564
- Mental Health 0347
- Nursing 0569
- Nutrition 0570
- Obstetrics and Gynecology 0380
- Occupational Health and Therapy 0354
- Ophthalmology 0381
- Pathology 0571
- Pharmacology 0419
- Pharmacy 0572
- Physical Therapy 0382
- Public Health 0573
- Radiology 0574
- Recreation 0575
- Speech Pathology 0460
- Toxicology 0383

Home Economics 0386

PHYSICAL SCIENCES

Pure Sciences

Chemistry
- General 0485
- Agricultural 0749
- Analytical 0486
- Biochemistry 0487
- Inorganic 0488
- Nuclear 0738
- Organic 0490
- Pharmaceutical 0491
- Physical 0494
- Polymer 0495
- Radiation 0754

Mathematics 0405
Physics
- General 0605
- Acoustics 0986
- Astronomy and Astrophysics 0606
- Atmospheric Science 0608
- Atomic 0748
- Electronics and Electricity 0607
- Elementary Particles and High Energy 0798
- Fluid and Plasma 0759
- Molecular 0609
- Nuclear 0610
- Optics 0752
- Radiation 0756
- Solid State 0611

Statistics 0463

Applied Sciences

Applied Mechanics 0346
Computer Science 0984
Engineering
- General 0537
- Aerospace 0538
- Agricultural 0539
- Automotive 0540
- Biomedical 0541
- Chemical 0542
- Civil 0543
- Electronics and Electrical 0544
- Heat and Thermodynamics 0348
- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
- Nuclear 0552
- Packaging 0549
- Petroleum 0765
- Sanitary and Municipal 0554
- System Science 0790

Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994

PSYCHOLOGY

General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
Social 0451

Nom _____

Dissertation Abstracts International est organisé en catégories de sujets. Veuillez s.v.p. choisir le sujet qui décrit le mieux votre thèse et inscrivez le code numérique approprié dans l'espace réservé ci-dessous.

_____ ☐☐☐ U·M·I

SUJET — CODE DE SUJET

Catégories par sujets

HUMANITÉS ET SCIENCES SOCIALES

COMMUNICATIONS ET LES ARTS
Architecture 0729
Beaux-arts 0357
Bibliothéconomie 0399
Cinéma 0900
Communication verbale 0459
Communications 0708
Danse 0378
Histoire de l'art 0377
Journalisme 0391
Musique 0413
Sciences de l'information 0723
Théâtre 0465

ÉDUCATION
Généralités 515
Administration 0514
Art 0273
Collèges communautaires 0275
Commerce 0688
Économie domestique 0278
Éducation permanente 0516
Éducation préscolaire 0518
Éducation sanitaire 0680
Enseignement agricole 0517
Enseignement bilingue et multiculturel 0282
Enseignement industriel 0521
Enseignement primaire. 0524
Enseignement professionnel 0747
Enseignement religieux 0527
Enseignement secondaire 0533
Enseignement spécial 0529
Enseignement supérieur 0745
Évaluation 0288
Finances 0277
Formation des enseignants 0530
Histoire de l'éducation 0520
Langues et littérature 0279
Lecture 0535
Mathématiques 0280
Musique 0522
Orientation et consultation 0519
Philosophie de l'éducation 0998
Physique 0523
Programmes d'études et enseignement 0727
Psychologie 0525
Sciences 0714
Sciences sociales 0534
Sociologie de l'éducation 0340
Technologie 0710

LANGUE, LITTÉRATURE ET LINGUISTIQUE
Langues
- Généralités 0679
- Anciennes 0289
- Linguistique 0290
- Modernes 0291

Littérature
- Généralités 0401
- Anciennes 0294
- Comparée 0295
- Médiévale 0297
- Moderne 0298
- Africaine 0316
- Américaine 0591
- Anglaise 0593
- Asiatique 0305
- Canadienne (Anglaise) 0352
- Canadienne (Française) 0355
- Germanique 0311
- Latino-américaine 0312
- Moyen-orientale 0315
- Romane 0313
- Slave et est-européenne 0314

PHILOSOPHIE, RELIGION ET THÉOLOGIE
Philosophie 0422
Religion
- Généralités 0318
- Clergé 0319
- Études bibliques 0321
- Histoire des religions 0320
- Philosophie de la religion 0322

Théologie 0469

SCIENCES SOCIALES
Anthropologie
- Archéologie 0324
- Culturelle 0326
- Physique 0327

Droit 0398
Économie
- Généralités 0501
- Commerce-Affaires 0505
- Économie agricole 0503
- Économie du travail 0510
- Finances 0508
- Histoire 0509
- Théorie 0511

Études américaines 0323
Études canadiennes 0385
Études féministes 0453
Folklore 0358
Géographie 0366
Gérontologie 0351
Gestion des affaires
- Généralités 0310
- Administration 0454
- Banques 0770
- Comptabilité 0272
- Marketing 0338

Histoire
- Histoire générale 0578
- Ancienne 0579
- Médiévale 0581
- Moderne 0582
- Histoire des noirs 0328
- Africaine 0331
- Canadienne 0334
- États-Unis 0337
- Européenne 0335
- Moyen-orientale 0333
- Latino-américaine 0336
- Asie, Australie et Océanie 0332

Histoire des sciences 0585
Loisirs 0814
Planification urbaine et régionale 0999
Science politique
- Généralités 0615
- Administration publique 0617
- Droit et relations internationales 0616

Sociologie
- Généralités 0626
- Aide et bien-être social 0630
- Criminologie et établissements pénitentiaires 0627
- Démographie 0938
- Études de l' individu et de la famille 0628
- Études des relations interethniques et des relations raciales 0631
- Structure et développement social 0700
- Théorie et méthodes. 0344
- Travail et relations industrielles 0629

Transports 0709
Travail social 0452

SCIENCES ET INGÉNIERIE

SCIENCES BIOLOGIQUES
Agriculture
- Généralités 0473
- Agronomie. 0285
- Alimentation et technologie alimentaire 0359
- Culture 0479
- Élevage et alimentation 0475
- Exploitation des pâturages 0777
- Pathologie animale 0476
- Pathologie végétale 0480
- Physiologie végétale 0817
- Sylviculture et faune 0478
- Technologie du bois 0746

Biologie
- Généralités 0306
- Anatomie 0287
- Biologie (Statistiques) 0308
- Biologie moléculaire 0307
- Botanique 0309
- Cellule 0379
- Écologie 0329
- Entomologie 0353
- Génétique 0369
- Limnologie 0793
- Microbiologie 0410
- Neurologie 0317
- Océanographie 0416
- Physiologie 0433
- Radiation 0821
- Science vétérinaire 0778
- Zoologie 0472

Biophysique
- Généralités 0786
- Medicale 0760

SCIENCES DE LA TERRE
Biogéochimie 0425
Géochimie 0996
Géodésie 0370
Géographie physique 0368
Géologie 0372
Géophysique 0373
Hydrologie 0388
Minéralogie 0411
Océanographie physique 0415
Paléobotanique 0345
Paléoécologie 0426
Paléontologie 0418
Paléozoologie 0985
Palynologie 0427

SCIENCES DE LA SANTÉ ET DE L'ENVIRONNEMENT
Économie domestique 0386
Sciences de l'environnement 0768
Sciences de la santé
- Généralités 0566
- Administration des hôpitaux 0769
- Alimentation et nutrition 0570
- Audiologie 0300
- Chimiothérapie 0992
- Dentisterie 0567
- Développement humain 0758
- Enseignement 0350
- Immunologie 0982
- Loisirs 0575
- Médecine du travail et thérapie 0354
- Médecine et chirurgie 0564
- Obstétrique et gynécologie 0380
- Ophtalmologie 0381
- Orthophonie 0460
- Pathologie 0571
- Pharmacie 0572
- Pharmacologie 0419
- Physiothérapie 0382
- Radiologie 0574
- Santé mentale 0347
- Santé publique 0573
- Soins infirmiers 0569
- Toxicologie 0383

SCIENCES PHYSIQUES
Sciences Pures
Chimie
- Généralités 0485
- Biochimie 487
- Chimie agricole 0749
- Chimie analytique 0486
- Chimie minérale 0488
- Chimie nucléaire 0738
- Chimie organique 0490
- Chimie pharmaceutique 0491
- Physique 0494
- PolymÈres 0495
- Radiation 0754

Mathématiques 0405
Physique
- Généralités 0605
- Acoustique 0986
- Astronomie et astrophysique 0606
- Électronique et électricité 0607
- Fluides et plasma 0759
- Météorologie 0608
- Optique 0752
- Particules (Physique nucléaire) 0798
- Physique atomique 0748
- Physique de l'état solide 0611
- Physique moléculaire 0609
- Physique nucléaire 0610
- Radiation 0756

Statistiques 0463

Sciences Appliqués Et Technologie
Informatique 0984
Ingénierie
- Généralités 0537
- Agricole 0539
- Automobile 0540
- Biomédicale 0541
- Chaleur et thermodynamique 0348
- Conditionnement (Emballage) 0549
- Génie aérospatial 0538
- Génie chimique 0542
- Génie civil 0543
- Génie électronique et électrique 0544
- Génie industriel 0546
- Génie mécanique 0548
- Génie nucléaire 0552
- Ingénierie des systèmes 0790
- Mécanique navale 0547
- Métallurgie 0743
- Science des matériaux 0794
- Technique du pétrole 0765
- Technique minière 0551
- Techniques sanitaires et municipales 0554
- Technologie hydraulique 0545

Mécanique appliquée 0346
Géotechnologie 0428
Matières plastiques (Technologie) 0795
Recherche opérationnelle 0796
Textiles et tissus (Technologie) 0794

PSYCHOLOGIE
Généralités 0621
Personnalité 0625
Psychobiologie 0349
Psychologie clinique 0622
Psychologie du comportement 0384
Psychologie du développement 0620
Psychologie expérimentale 0623
Psychologie industrielle 0624
Psychologie physiologique 0989
Psychologie sociale 0451
Psychométrie 0632

BIOPHYSICAL AND BIOCHEMICAL PROPERTIES OF AIRWAY SMOOTH MUSCLE-MECHANISMS OF AIRWAY HYPERRESPONSIVENESS

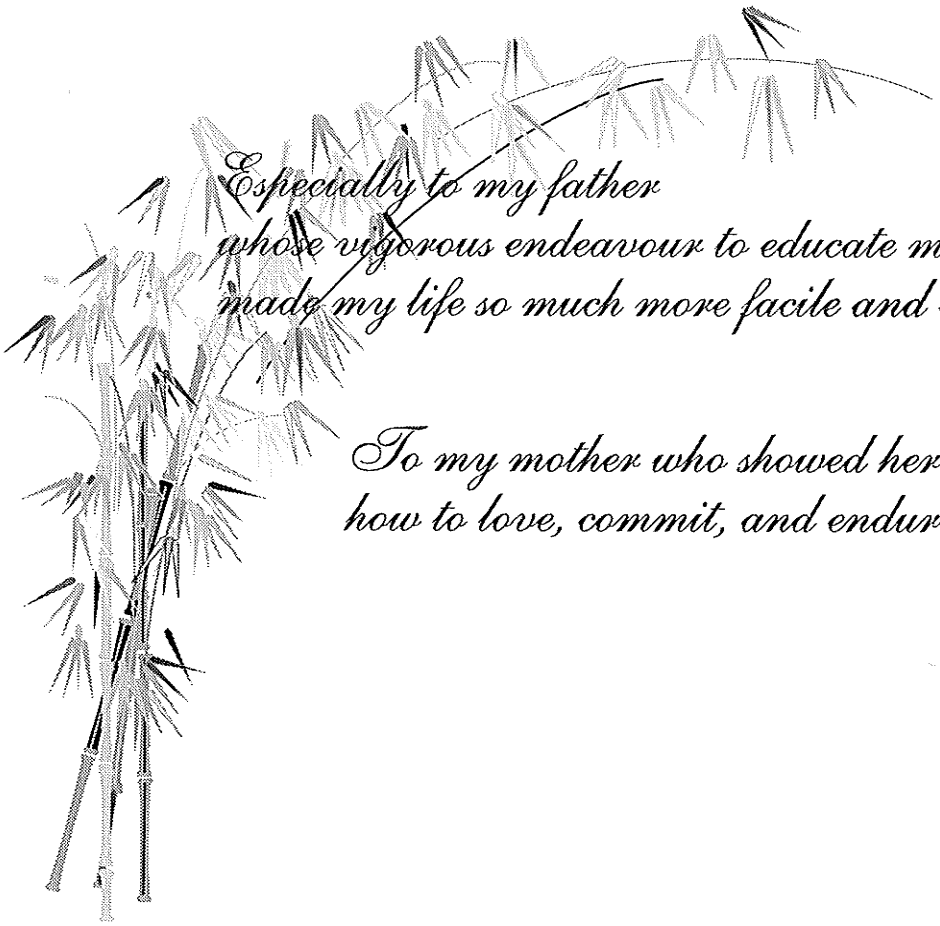
BY

HE JIANG

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Dedicated to my family
Who unconditionally supported
My pursuing for whatever I chose to do



Especially to my father
whose vigorous endeavour to educate me
made my life so much more facile and enjoyable

To my mother who showed her children
how to love, commit, and endure

何江

He Jiang

Spring, 1994

ACKNOWLEDGEMENTS

It is finally my turn, instead of his, to say something for a great advisor, **Dr. Newman L. Stephens**, who had so patiently encouraged and sharpened my intellect over the years, and edified me in almost everything, statistics, philosophy, downhill skiing, English, to list a few. Opportunities and freedom he provided had built up my energy and confidence. From him I have learnt the ways to canoe in *the Lake of the Woods* and to explore the secret of life. He had explicated to me an outstanding scientist and a wonderful friend, with whom I would never be hesitative to spend another five-year!

Likewise, my Ph.D. program would have just been a day-dream if it was not for the generosity of **Drs. Richard A. Murphy, Jaques Belik, William C. Cole, and Edwin A. Kroeger**, who responsibly guided me through discussing my research proposals and criticizing this manuscript (thanks for the good reference letters, too). Unquestionably I could not imagine where I might be wasting my ATP's if it was not for **Drs. Nicholas Anthonisen and Meir H. Kryger**, who recruited me into respiratory medicine and encouraged me for going into basic research. One man influenced my career the most was my mentor, **Prof. Bingyong Sun**, who had seriously, academically, and intellectually navigated my first five years of research pursuit in pulmonary and cardiovascular physiology and pathophysiology, where I began to perceive what the scientific research is about. Even though, I could have not finished this program without the benevolent financial support by the studentship and fellowships from **the Medical Research Council of Canada, the Manitoba Health Research Council, and the China State Educational Committee**.

Over the years my hard-working life as a student was made much easier by having people around who worked harder and smarter: **Andrew, Chun, Zhiping, Peter, Mark, Stefan, Pasquale, Qiying, and Weilong,** whose advice and help were best resembled as ion channels and pumps of the sarcolemma, which maintained my "muscle's" excitability and contractility! Listing the valuable help from **Judy, Maureen, and Gail** in the departmental office is impossible for me. Rarely, though, one could forget such things as disciplining him to the university regulations, typing up his manuscripts, or handling his pay cheques.

Venturing through the jungle of contractile protein molecules filled with the mist of ions, I was very fortunate to have old and new friends like: **Garry, Chao, Xia, Gang, Qiang, Heping, Joan, Lu, Weimin, Xiaoli, Yi, Weining, Xueliang, Ya, Jizhong, and Xiaoling,** who had so sincerely and honestly shared with me over the time their problems, frustrations, arguments, and disappointments, let alone their exhilaration, wisdom, trust, understanding, devotion, and encouragement! I was kept alert in such a stimulating environment all the time.

Especially at this moment, I tribute the honour that may accrue from the thesis to my mother and father. Notwithstanding my father could not criticize it any more, the invaluable and countless treasure from his scholarly education and fatherly love will be seen throughout this thesis and all my life, and my mother has always been tremendously patient and supportive throughout these years thinking that I am still 3 years old. Enormous support and motivation from my parents-in-law all along these years are also appreciated. Reward should also be given to my lovely sister, **Hong**, who shouldered my responsibilities to the family when I went to school.

Yearningly, I dedicate this thesis to my wife, **Kang**, who has been continuously strengthening me over these years believing that I could come home with the Nobel prize one day!? Grounding on her love and assistance, I was able to survive the endless nights and days of study. Vesting similar personalities as their parents, my daughter, **Kelen** and son, **Lan** had made the task of writing and defending the thesis more meaningful and challenging.

CONTENTS

LIST OF FIGURES

List of Tables

ABSTRACT

The mechanisms of airway smooth muscle (ASM) contraction from the biochemical and biophysical points of view have not been adequately described. The role of ASM in airway hyperresponsiveness such as asthma needs to be established as well. The objectives of this study were (1) to describe the biophysical changes of smooth muscle function in airway hyperresponsiveness; (2) to determine the cellular events underlying these changes to account for the hyperresponsiveness seen in such muscle. Length-tension and force-velocity relationships were thus studied in canine tracheal smooth muscle and bronchial smooth muscle (TSM; BSM); in the latter all cartilage plaques were carefully removed. The BSM from central airways was selected as it is these generations of airways that are active during the so called early asthmatic response. The latter being the site of asthmatic attack occurs. BSM without cartilage showed greater maximum shortening capacity (ΔL_{max}) and velocity (V_o) than, but similar maximum stress (P_o) to, that with cartilage, indicating that cartilage removal provides a valid mecahnical preparation. P_o normalized to the cross-sectional areas of strip, muscle tissue, and myosin content showed that normalization of force with respect to cross-section area of muscle tissue was adequate provided the myosin contents of the tissues compared were the same. Ragweed pollen sensitization resulted in an elevated ΔL_{max} and V_o at early contraction (2 sec) whereas P_o and V_o in late phase remained unchanged. Isotonic relaxation evaluated by half relaxation time, an index independent of load and contractile element length, was prolonged in SBSM. These results suggest that the abnormalities may be in early normally cycling crossbridges instead of latch bridges and justify the use of isotonic parameter over isometric. They also explain the excessive airway narrowing of asthma and maintained bronchoconstriction of asthma. The higher

myosin ATPase activity found in STSM and SBSM may result in higher V_o. Myosin light chain (MLC) phosphorylation level was also higher in STSM and SBSM, and this was due to primarily an increased amount of myosin light chain kinase (MLCK) rather than any changes in the intrinsic properties of MLCK. In conclusion, higher ΔL_{max} and V_o may play key roles in smooth muscle hyperresponsiveness. An increased quantity of MLCK in SASM, which results in increased MLC phosphorylation and myosin ATPase activity, may be the primary alteration in the development of airway hyperresponsiveness.

INTRODUCTION

Airway smooth muscle plays a central role in airway hyperresponsiveness of the type seen in the acute asthmatic attack[Hogg 1989], where increased smooth muscle contraction or failure of relaxation could account primarily for the excessive narrowing of airways and increase of resistance to ventilation[Stephens 1987]. Airway hyperresponsiveness is defined as the increased airway smooth muscle reactivity as well as sensitivity. Investigations of mechanisms underlying the pathogenesis of asthma or other airway hyperresponsive disease states have greatly increased probably because of the alarming observation that though heretofore the incidence of asthma was not high, it is now increasing[Sears 1986; Sly 1986; Barnes et al 1988]. The concomitant increase in mortality is also cause for concern. In addition, the role of airway smooth muscle in regulating ventilatory resistance remains unclear. The mechanisms underlying airway hyperresponsiveness are also poorly understood despite of the intensive research effort.

Our goals of research are: 1) to understand the physiology and biochemistry of airway smooth muscle; and 2) the role of smooth muscle in the development and maintenance of airway hyperresponsiveness such as asthma. It has been shown that ragweed pollen sensitized canine airway smooth muscle is a good model for studying airway hyperresponsiveness[Antonissen et al 1979; Becker et al 1989]. The objectives of present study were to 1) identify the mechanical abnormalities in airway hyperresponsiveness using biophysical approaches; 2) delineate the mechanisms underlying these changes; and 3) establish a working hypothesis of airway hyperresponsiveness for further investigation at cellular and molecular levels.

I. Bronchial Smooth Muscle Preparation

a. Development of canine model of allergic bronchoconstriction

Because human airway tissue is difficult to obtain, animal models have been employed consisting of airway smooth muscle; this is generally tracheal smooth muscle from small animals such as rats[Holme and Piechuta 1981], mice, guinea pigs[Dhillon and Rodger 1981], ferret[Coburn 1984], and rabbits[Moreno et al 1987]. With respect to studies of asthma, in most of these models the allergic state is IgG based and this does not match human allergy, which is IgE based. However, canine models developed by Kepron et al[1977] are eminently satisfactory. The airways of these animals are not defined to specific antigen challenge *in vivo*. *In vitro* antigen challenge demonstrated increased sensitivity and reactivity. The allergy is IgE based.

b. Development of bronchial smooth muscle preparation

Our objective has been to determine the primary cause of asthma and for this reason we have worked with tissues from animals that have only been antigen sensitized but never challenged. Neither smooth muscle hypertrophy and/or hyperplasia, nor inflammatory response such as infiltration with inflammatory cells was found in the airways suggesting that any changes detected in our model are unlikely to be influenced by these secondary disease changes. Thus the airway smooth muscle from the canine model may best serve the purpose of determining what is the primary cause in the pathogenesis of asthma. First of all, the mechanical properties of airway smooth muscle from such model should be examined to identify its functional abnormality of such muscle.

In this context, trachealis muscle has been used in mechanical and pharmacological studies purely because of the convenience[Aizawa et al 1988; Antonissen et al

1979; Mitchell and Stephens 1983; Stephens et al 1969). It was intended then to model the role of bronchial smooth muscle in the mechanisms underlying asthma. Unfortunately, such studies were not carried out directly on muscle from 3rd-5th order bronchi which are the chief site of airway resistance during normal ventilation(Pedley et al 1970), and of the bronchospasm of the acute asthmatic attack(Epstein et al 1948; Dulfano and Hewetson 1966). It has also been shown that striking bronchial smooth muscle hypertrophy occurs in bronchi from chronic asthmatics(Huber and Koessler 1922; Dunnill et al 1969), suggesting the involvement of bronchial smooth muscle in chronic disease. It is likely that early disease would stem from these cells also. As a matter of fact one of our hypothesis is that in early disease changes occur in mechanical function of the bronchial smooth muscle that precede any evidence of inflammation, hypertrophy or hyperplasia. The properties of bronchial smooth muscle and its role in the pathogenesis of airway hyperresponsiveness were not adequately investigated prior to the current study. In addition, there was no clear evidence to support the notion that tracheal smooth muscle would completely resemble its bronchial counterparts. In fact, heterogeneity exists with respect to pharmacological characteristics and neural control of smooth muscle along the tracheobronchial tree(Stephens and Hoppin 1986).

The difficulty in studying bronchial smooth muscle is that it contains considerable amount of connective tissues and the more troublesome cartilage plaques, which prevent us from describing the true mechanical properties of the smooth muscle itself. Initially the cartilage plaques were thought to be anchored to the smooth muscle directly, rendering the removal of the plaques impossible if the intactness of smooth muscle was of primary concern. However, on carefully checking the morphological arrangement of ASM vis-a-vis cartilage plaques, and referring to von Hayek(1960), we have found that the muscle bundles, instead of

fastening directly onto the cartilage plaques, connect with the plaques indirectly through loose connective tissues. Such an arrangement renders it possible to dissect away the cartilages without damaging the muscle.

II. Normalization of Smooth Muscle Tension

The importance of correctly normalizing contractile function in airway smooth muscle has come about because of the impact of asthma research, in which it has become necessary to compare contractile function of airway smooth muscle in different locations in the same respiratory tree, at the same location at different ages, and across animal species. Manifestations of asthma may vary with age as may the response to therapeutic measures. This has also led to an increase in research relating to the effect of maturation on airway smooth muscle[Russell 1978; Armour et al 1984; Shioya et al 1987; Mapp et al 1989; Murphy et al 1989; Panitch et al 1989]. However, considerable ambiguity in the use of normalizing parameters for isometric force is evident in the literature[Armour et al 1984; Shioya et al 1987; Mapp et al 1989], which could lead to erroneous conclusions. As the smooth muscle length-tension relationship shows, the force generated by smooth muscle behaves in the same way as skeletal muscle does, i.e. there is an optimal length, at which the muscle produces maximum force[Stephens et al 1969]. The accuracy of measurement of length and the determination of the optimal length is then crucial, which unfortunately, had been neglected in studies, where smooth muscle strips were simply stretched to certain lengths with selected loads. The maximum active isometric tension is a measure of muscle strength. It is also a variable predominantly used by investigators, chiefly because it is technically the easiest to measure. It is worthwhile to point out that the relevance of force to regulation of resistance of airway is moot, as its contribution is

relatively minor. Furthermore, Murphy's group has suggested that the late slow cycling or non-cycling crossbridges (latch bridges) may be responsible for force generation or maintenance in smooth muscle contraction[Dillon et al 1981]. Studies of maximum active isometric tension, therefore, only depict the properties of latch bridge, and the force is a poor index of activation in smooth muscle because of the very nonlinear dependence of force on crossbridge phosphorylation. So the claim that isometric studies can shed light on muscle shortening is not easy to defend. In any case, in understanding the subcellular process of smooth muscle contraction, the conversion of raw force developed to stress units is the first step. This is particularly important where contractile forces have to be compared. Comparing the force developed by a single crossbridge under physiological and pathophysiological conditions is what is really needed. This is not yet possible for smooth muscle. However, the mean content of myosin in the cross section of smooth muscle can serve as an indirect index of the numbers of crossbridges that may be present. The proportion of bronchial tissue cross sectional area occupied by muscle tissue could serve as a normalizing denominator only if the compared muscle tissues have the same concentration of contractile proteins. In order to evaluate force with above factors in mind, force generated by tracheal and bronchial smooth muscle strips is normalized as with respect to the cross-sectional areas of strip, muscle, and myosin heavy chain content.

III. Smooth Muscle Contraction

Although the pathophysiological changes leading to asthma are not yet well known, increased airway responsiveness to an important aspect of the disease[Cockcroft et al 1977]. Sensitized canine tracheal smooth muscle has been shown

to undergo nonspecific alterations in mechanical properties[Antonissen et al 1979; Antonissen et al 1980; Boushey et al 1980; Stephens et al 1988], which, along with the observation of Cockcroft et al[Cockcroft et al 1977], suggests that the essential defect in allergic bronchial hyperresponsiveness might be at muscle cell level. The role of airway smooth muscle as the effector in allergic bronchial hyperresponsiveness has been considerably investigated[Stephens et al 1985]. Studies in our laboratory have focused on the mechanical properties of tracheal smooth muscle, which we believe to be a mechanical model for airways down to those generations of bronchi where allergic bronchial hyperresponsiveness occurs. However, the tracheal smooth muscle is not a perfect model for bronchial smooth muscle, because we have shown that although the bronchial smooth muscle possesses the same qualitative mechanical properties as the trachealis, there are qualitative differences, as well as differences in pharmacology[Sigurdsson et al 1990]. Therefore it was necessary to employ the bronchial smooth muscle preparation[Jiang et al 1990] itself for mechanical study if elucidation of the pathogenesis of asthma was to be successful. This had not been attempted in the past because of the belief that attempts to remove cartilaginous plaques, a sine qua non if isotonic studies were to be undertaken, would seriously damage the attached smooth muscle. However, in an examination of histological slides of the bronchi, we noted that the muscle was not attached directly to cartilage at any point. Attachment was via connective tissue. We demonstrated that removal of the cartilage and provision of an intact muscle was feasible[Jiang et al 1990]. The present study was undertaken to determine whether the mechanical properties of bronchial smooth muscle from dogs are altered after ragweed pollen sensitization.

IV. Smooth Muscle Relaxation

Study of the process of smooth muscle relaxation has been largely neglected, which is surprising if one considers that impairment of relaxation may contribute significantly to the increase in resistance during bronchospasm or hypertension. Previous studies of relaxation related only to isometric relaxation[Johansson and Mellander 1975; Shibata and Cheng 1977; White et al 1988], which, by their nature, mostly provide information about the temporal diminution of stiffness of the airway wall. This may affect changes in resistance of airways by modifying airway reactance properties, but only to a very small extent. The major component of the resistance decrease during bronchodilation is controlled by isotonic or auxotonic relaxation of airway smooth muscle. Therefore, it is these parameters that must be studied *in vitro*. With one exception[Packer and Stephens 1985], neither in smooth muscle nor in skeletal has this been carried out *in vitro*. In striated muscle only the isometric phase of relaxation was studied, even though isotonic relaxation traces were published[Brutsaert et al 1978; Jewell and Wilkie 1960].

The hypothesis of a decreased ability of airway smooth muscle from asthmatics to relax has indirectly arisen from studies of isotonic relaxation in hypertensive vascular smooth muscle. Retarded isometric relaxation in the thoracic aorta of spontaneously hypertensive rats has been reported[Cohen and Berkowitz 1976]. Increased isometric and isotonic relaxation times have also been found in the caudal artery[Packer and Stephens 1985] and small mesenteric artery[Packer and Stephens 1987] of the spontaneously hypertensive rats. The failure of relaxation of vascular smooth muscle has thus been suggested to contribute to the initiation and maintenance of hypertension[Triner et al 1975; Shibata and Cheng 1977; Packer and Stephens 1985; Packer and Stephens 1987]. It is possible that this mechanism may be operative in the development and

maintenance of bronchoconstriction during an asthmatic attack in which the failure of smooth muscle relaxation could result in a sustained increase in airway resistance.

By shortening or elongating, smooth muscle in hollow organs such as blood vessels and airways regulates the calibre of the lumen and thus the resistance to flow through these vessels. Isometric force development, however, changes wall stiffness and contributes to the control of luminal resistance only minimally. It is significant that we have found that only the isotonic shortening capacity and maximum shortening velocity of ragweed pollen sensitized airway smooth muscle were increased, whereas isometric force remains unchanged[Stephens et al 1988; Jiang et al 1992a], making the delineation of isotonic shortening and relaxing properties of smooth muscle more relevant than that of the isometric in elucidation of the pathogenesis of diseases such as asthma or hypertension. A problem in the study of isotonic relaxation of smooth muscle is the lack of a valid index which should be independent of the load on the muscle and independent of the muscle's initial contractile element length because both the load on, and, the length of the muscle affect isotonic relaxation. The purposes of this project are to: 1) develop a reliable index for isotonic relaxation; 2) characterize the mechanical properties of airway smooth during muscle relaxation; 3) and to determine whether changes in properties of isotonic relaxation exist in ragweed sensitized bronchial smooth muscle as compared with control.

V. Activities of Myosin ATPase and Myosin Light Chain Kinase in Sensitized Airway Smooth Muscle

Functional studies on ragweed pollen sensitized dogs have revealed that

tracheal smooth muscle and bronchial smooth muscle possess increased maximum shortening capacity and elevated early maximum shortening velocity[Antonissen et al 1979; Jiang et al 1992a; Jiang and Stephens 1992;]. These changes in shortening variables may be responsible for the excessive airway narrowing observed in individuals displaying allergic airway hyperresponsiveness [Jiang et al 1992a]. Increase in shortening capacity of airway smooth muscle could directly account for abnormal bronchoconstriction. We have also shown that maximum shortening capacity can be influenced by early shortening velocity due to the fact that smooth muscle strips achieve to 70 to 80% of their maximum shortening capacity within the first 2 to 3 s[Jiang et al 1992a] of contraction, contraction time being 10 s. In fact, both the velocity of shortening and the extent of shortening during the early phase of contraction are significantly greater in sensitized airway smooth muscles than in control. The early shortening velocity is critical to maximum shortening as it provides a limitation to the latter. It has been suggested that maximum shortening velocity is an index of actomyosin ATPase activity in smooth muscle[Bárány 1967; Close 1972; Dillon et al 1981], which determines the rate of unloaded crossbridge cycling[Sobieszek and Bremel 1975; Bendall 1964]. Actomyosin ATPase activity is increased in smooth muscle from the pulmonary artery of sensitized dogs[Kong et al 1986]. It is important, therefore, to assess bronchial smooth muscle actomyosin ATPase, which governs the crossbridge cycling rate, maximal shortening velocity, and thus the extent of shortening, at precisely the site where acute asthmatic airway narrowing occurs[Epstein et al 1948; Dulfano and Hewetson 1966]. It is generally accepted that phosphorylation of a serine-19 on the regulatory myosin light chains (20,000 Da) by myosin light chain kinase is the major rate-limiting step for the activation of actomyosin ATPase[Kamm and Stull 1989]. Assaying the activities of actomyosin ATPase and myosin light chain kinase in sensitized airway smooth

muscles, therefore, becomes necessary if the cellular mechanisms of airway hyperresponsiveness are to be elucidated.

VI. Biochemical Mechanisms Underlying Smooth Muscle Isotonic Contraction and Relaxation

The regulatory mechanisms underlying smooth muscle contraction are not fully understood even though the current concept is that activation of smooth muscle actomyosin ATPase is mainly brought about by phosphorylation of the 20,000 dalton myosin light chain (MLC_{20}) by Ca^{2+} calmodulin dependent myosin light chain kinase[Marston 1982; Kamm and Stull 1989] and relaxation is the result of gradual MLC_{20} dephosphorylation by myosin phosphatase, which has recently been suggested to be regulated by protein kinase C[Somlyo et al 1989; Kitazawa et al 1991; Kitazawa et al 1992]. Previous studies have revealed a positive correlation between myosin light chain phosphorylation and tension development in skinned smooth muscle preparations[Barron et al 1979; Hoar et al 1979; Driska et al 1981]. Most of the data relating to phosphorylation of MLC_{20}, unfortunately, had been obtained during isometric tension development and relaxation[Butler et al 1983; Chatterjee and Murphy 1983; Dillon et al 1981; Gerthoffer 1987; Kamm and Stull 1985b; Merkel et al 1990; Moreland et al 1986; Silver and Stull 1982], which represent *in vivo* function of smooth muscle only partially because the regulatory function of smooth muscle is chiefly fulfilled by smooth muscle length change. Studies of smooth muscle intracellular Ca^{2+} are subject to the same criticism, viz, the data relate only to isometric contraction[Filo et al 1965; Morgan and Morgan 1982]. Furthermore, isometric studies cannot always provide insight into isotonic function as the two modes of contraction could be dissociated. For example we have reported that while the capacity for isotonic shortening is significantly increased in sensitized canine

airway smooth muscle, isometric force production may be completely normal[Jiang et al 1992a].

Shortening in skeletal[Taylor and Rudel 1970], cardiac[Jewell 1974], as well as smooth muscle[Stephens and Mitchell 1984] results in reduced activation of the muscle. The mechanisms underlying such desensitization were not entirely understood. In addition, a correlation between static muscle length and phosphorylation of the 20 kDa myosin light chain has been reported in arterial[Hai 1991] and tracheal[Jiang et al 1992b] smooth muscle. But it is not known how myosin light chain phosphorylation is influenced during smooth muscle shortening, in which the muscle length changes dynamically. The stoichiometry of mono-phosphorylation of the 20 kDa regulatory myosin light chain and the change in intracellular Ca^{2+} concentration, measured by Fura-2 fluorescence, were assessed throughout the course of isotonic and isometric contractions in order to shed light on the cellular mechanisms of smooth muscle regulation during actual length change.

LITERATURE REVIEW

Although interest in studying airway smooth muscle in order to elucidate the pathogenesis of asthma has resulted in progressive understanding of airway smooth muscle[Stein 1975; Nadel 1980; Hargreave 1980; Jenne and Murphy 1987; Kaliner and Barnes 1988; Barnes et al 1988; Coburn 1989, Agrawal and Townley 1990], the physiological role of airway smooth muscle remains unclear because most of the mechanics of ventilation can be accounted for on the premise that airways are passive pipes. Speculations about the role of airway smooth muscle are: 1) improvement of alveolar ventilation by narrowing airways i.e. by reducing anatomical dead space. This is useful only if ventilatory frequency does not increase too greatly as this would limit ventilation by increasing the airway resistance, 2) regulation of regional ventilation by shunting air from well-ventilated but poorly perfused areas to normal, 3) improvement of tracheobronchial clearance of mucus by rhythmically compressing mucous glands and augmenting flow of mucous on to the lining epithelium. Unfortunately, the focus has been overwhelmingly on bronchial hyperreactivity with emphasis on inflammatory changes in the mural tissues. Study of structural characteristics of airway has been neglected, apart from a few exceptions decades ago[von Hayek 1960; Krahl 1964; Weibel 1963; Weibel and Gomez 1962; Kirkpatrick 1975; Silva and Ross 1976]. Most recent reports of ultrastructure have dealt almost exclusively with the airway smooth muscle and its innervation.

Nevertheless, it is essential to understand the morphological characteristics of airway smooth muscle if one wishes to study airway smooth muscle at any other level.

I. Structure of Airway Smooth Muscle

I.1. Macroscopic Features

Tracheal muscle consists of a narrow thin sheet of smooth muscle running transversely in the dorsal (membranous) wall of the trachea. The muscle is arranged into bundles by thin septa of connective tissue carrying blood vessels. The bundles split and merge repeatedly; thus the muscle maintains its continuity along the length of the trachea. The bundles are compact and run approximately parallel to each other; however, at the ends of the muscle cells, they converge and insert into the ends of cartilage arches. Details of this insertion vary among species: in the rat the muscle is attached to the tips of the arches, whereas in the guinea pig the insertion occurs on the concave surface of the cartilages at certain distance from the tip. The canine trachealis inserts onto the external surface of the cartilage rings. In bronchi or bronchioli, the musculature is a network of bundles arranged as geodesic lines within the wall; their relationship with the cartilage was described some 30 years ago but neglected[von Hayek 1960]. The bronchial smooth muscle forms a layer surrounding the epithelium and attaches to the superjacent cartilage plaques via loose connective tissues, which appear to be rather compliant[Jiang and Stephens 1990] and would allow smooth muscle to shorten to a far larger extent[Stephens et al, 1991] (**Figure 1**) than if they were attached directly to cartilage. The magnitude of shortening is more than enough to shut the air passage totally. In the terminal bronchioli smooth muscle is seldom seen[von Hayek 1960].

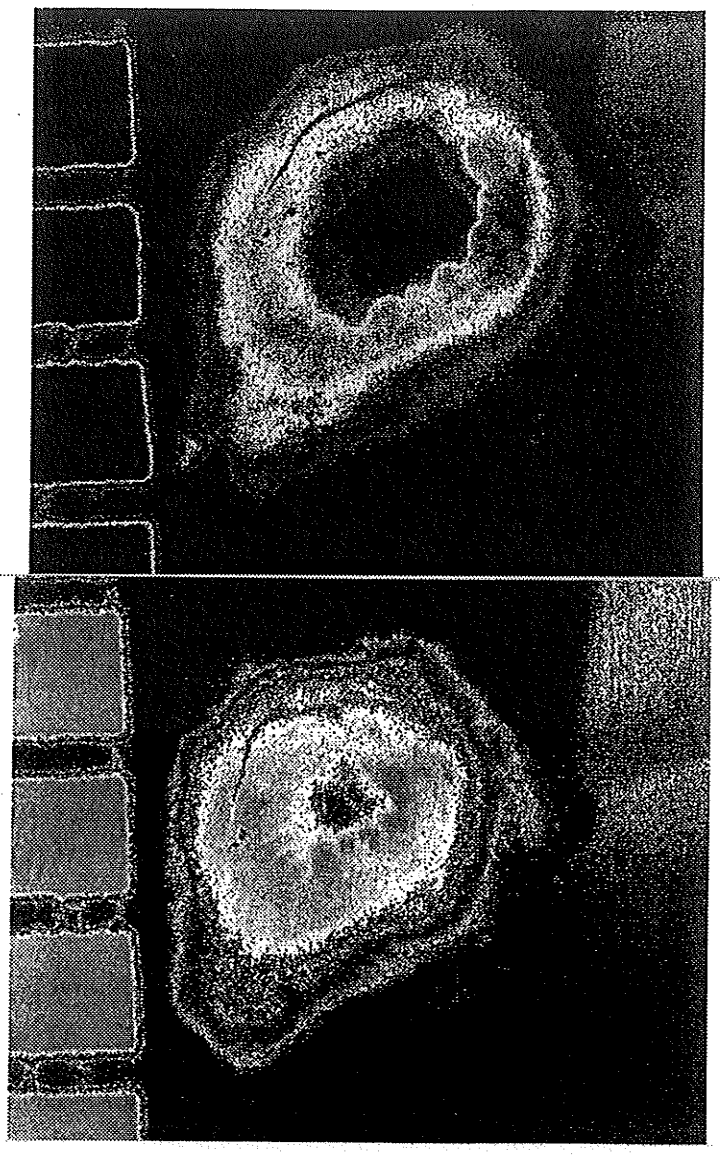


Figure 1. End-on view of 6th order bronchial segment at rest (A) and during full contraction with high concentration of KCl (B). The external perimeter of the bronchial ring, which contain cartilage plates, hardly moved when the lumen was totally closed, indicating that bronchial smooth muscle is able to shorten even with cartilage intact. This considerable degree of shortening of bronchial smooth muscle is provided for by the loose connective tissue attachment of the muscle to the cartilage.

I.2. General Microscopic Characteristics

Airway smooth muscle is so called because, like all other smooth muscle, it does not possess a regularly repeating sarcomere pattern of light and dark bands. In electron micrographs, while thin actin filaments are clearly seen filling the entire cytoplasm, the thick myosin filaments (the major components of the dark bands) are very seldom visualized. The canine tracheal smooth muscle cell is long and narrow, measuring about 750-1000 μm by 3-5 μm[Suzuki et al 1976; Stephens and Kroeger 1980]. Low power electromicrographs (**Figure 2**) reveal that the cells are arranged in bundles separated by fairly wide and variable interfascicular spaces. These spaces contain collagen, elastin (in both amorphous and fibrillar form), fibroblasts, neural axons, blood vessels, mast cells, macrophages, and lymphocytes. The muscle cells in canine trachealis represent 75-80% of the tissue's cross sectional area[Jiang et al 1991]. The cells maintain parallel orientation even at peak contraction. These features render it a suitable preparation for both biophysical and biochemical studies and could, with some reservations, serve as a model for bronchial smooth muscle[Jiang and Stephens 1990]. It must be pointed out that parallel alignment of the cells is not in itself, a sufficient condition for mechanical efficiency. The important factor is the orientation of the contractile filaments within the cell. In the toad stomach muscle these filaments are believed to traverse the cell obliquely[Bagby 1971] whereas in taenia coli they may run parallel to the longitudinal cellular axis[Gabella 1981]. From a functional point of view the maximum force and shortening developed by a muscle will be recorded when the measuring

device is orientated in the same axis as the force-generating filaments or as the muscle cells. This may be determined by cutting muscle strips from the tracheal muscular membrane at a variety of helical angles and noting in which strip maximum isometric force develops. In trachealis this is seen in the strip whose longitudinal axis coincides with the long axis of the cells, which proves that in this muscle the force generating cells may not obliquely oriented.

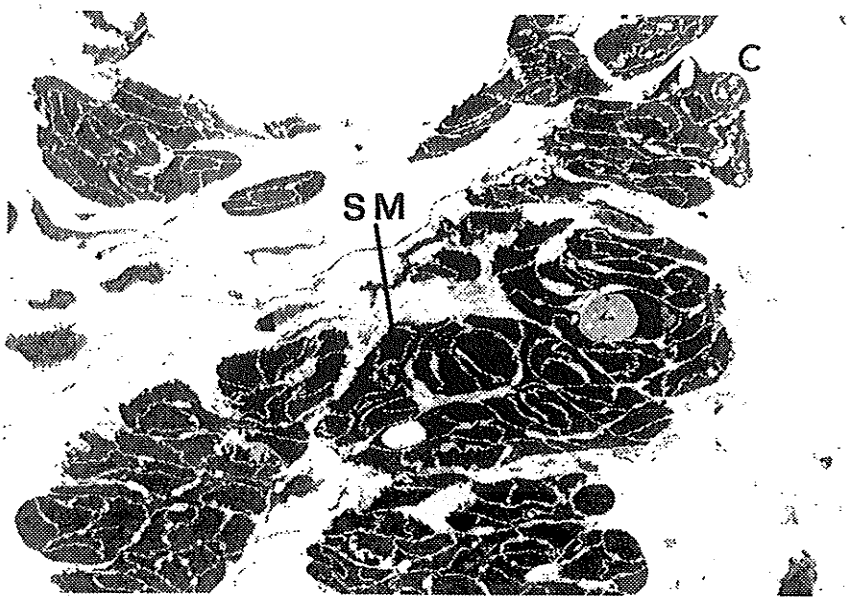


Figure 2 Cross-sectional view of tracheal smooth muscle under electromicroscope (× 2,000). The muscle cells are arranged in bundles and there are quite wide intercellular spaces.

I.3. Sarcolemma

The cell-membrane or sarcolemma has the classical "tram-line" lipid bilayer structure of any other cell membrane. Protein and glycoprotein molecules span this layer and act as ligand receptors, ion channels, and enzymes. Smooth muscle sarcolemma shows localized electron-dense areas in electron micrographs. These are called dense bands and may serve as attachment sites for actin filaments thus resembling the Z-discs of the striated muscle. To complete the analogy, proteins such as α-actinin and desmin which are usually found in the Z-line of skeletal muscle are also identified in these bands. Similar areas are found in the cytoplasm where they are called dense bodies. Actin filaments insert into these bodies, those on opposite sides of the bodies show polar reversal[(Somlyo et al 1984; Fay et al 1977)] of myosin binding sites. These bodies and the actin filaments, therefore, are considered as analogous to striated muscle sarcomeres. Arcuate cytoskeletal fibres (desmin) connect adjacent dense bodies and serve to support the arrangement of the filamentous network. While it is likely that these proteins are also present in airway smooth muscle, no specific studies have yet been conducted. Nevertheless, the evidence has suggested that in smooth muscle there are sarcomere-like structures, so-called mini-sarcomeres which serve as the morphological basis for contraction.

I.4. Pinocytotic Vesicles

Pinocytotic vesicles, also called caveolae, are flask-shaped small invaginations of the sarcolemma. Recent work suggests that the small membranous

structures are specially designed for conducting molecules and fluid into the cell and are possibly also centres for signal transduction[Travis 1993]. For example, the clathrin coated pits play an essential role in receptor-mediated endocytosis, a process in which the cell carries proteins and other large molecules into the cytoplasm. In longitudinal sections they seem to be separate from the sarcolemma, perhaps because of the plane of histological section. Actually they communicate with the extracellular space as evidenced by their filling with tracers such as colloidal lanthanum, ferritin, and peroxidase introduced into the extracellular space. Pinocytotic vesicles are lined with a continuation of the sarcolemma and the basement membrane is carried into the vesicular lumina. At the neck of the vesicles the basement shows electron dense particles. Gabella[1979] has shown that in the guinea pig taenia coli they are arranged in parallel rows in the longitudinal axis of the cell; they are interposed between dense bands. He also estimated that there are approximately 170,000 caveolae in each cell and they may serve to increase effective sarcolemmal area by as much as 70%. These caveolae may contain sarcolemmal Ca^{2+} pumps[Fujimoto 1993].

I.5. Sarcoplasmic Reticulum

The amount of sarcoplasmic reticulum present in smooth muscle makes up about 2-5% of the total cell volume. It has been suggested that the amount of sarcoplasmic reticulum present in smooth muscle is enough to provide Ca^{2+} for the needs of excitation-contraction coupling[Devine et al 1972]. In smooth muscle the amount of sarcoplasmic reticulum seems to depend on the extent to which the

muscle relies on intracellular calcium for contraction. Multiunit and intermediate types (trachealis) possess more reticulum than single-unit muscle (taenia coli). Sarcoplasmic reticulum is the major source and sink for intracellular calcium[Somlyo et al 1983; Bond et al 1984; Kowarski et al 1985]. In the lumen of the SR, low affinity and high capacity Ca^{2+} binding proteins (calsequestrin and calreticulin) which store large amounts of Ca^{2+} and help maintain a steep concentration gradient between the SR lumen and cytoplasm. The SR membranes contain a high affinity Ca^{2+}-ATPase which serves to maintain the Ca^{2+} gradient and initiate relaxation following an intracellular Ca^{2+} transient by pumping Ca^{2+} back in to the SR lumen. In smooth muscle the excitation-contraction coupling is not so well defined and structured as in striated muscle, and while considerable work has been carried out, it is still not known what the complete smooth-muscle pathway is, in structural terms. It has been suggested that connections of pinocytotic vesicles, sarcoplasmic reticulum, and mitochondria can often be found near the sarcolemma[Somlyo 1980]. Their proximity to each other suggests they could be acting as an excitation-contraction coupling apparatus.

I.6. Gap Junctions

Intercellular communications in smooth muscle may serve three functions: 1) as means of electrical communication between cells and facilitating propagation; 2) facilitating chemical communications between cells; 3) enabling transmission of mechanical force. Multiunit smooth muscle is, as stated before, generally not spontaneously contractile, does not fire action potentials and is devoid of a

myogenic reflex. Several investigators[Kroeger and Stephens 1975; Suzuki et al 1976] have shown that when it is treated with tetraethylammonium or 4-aminopyridine the muscle develops typical single unit characteristics: spontaneous rhythmic activity, action potentials, and a myogenic response. Daniel's group has shown that in such preparations a large number of gap junctions also develop[Daniel 1988]. Gap junctions are the most important structure for intercellular communication. These are areas of apposition of sarcolemmal membranes that are closer than those of the intermediate junctions. The gap is 2-3 nm in width and is composed of arrays of membrane proteins termed connexons, which provide intercellular channels that open and close. They are low in electrical resistance and capable of passing molecules up to 800-1200 daltons[Simpson et al 1977; Flagg-Newton and Loewenstein 1979; Flagg-Newton 1980]. The electrical conductance at the gap junction can be reduced by a decrease in pH. In humans[Richardson and Furguson 1980; Daniel et al 1980; 1986a], gap junctions are found in high density in trachea and the first and second order bronchi. However, when smaller (4th to 7th) bronchi are studied, typical gap junctions are absent[Daniel et al 1986a]. In dogs, the smooth muscle of both locations demonstrated gap junctions of average size down through the fourth order bronchi[Daniel 1988]. Therefore, canine airways appeared to be coupled throughout the bronchial tree studied. Bovine trachea resemble human and canine trachea in possessing numerous gap junctions[Cameron et al 1982]. Nevertheless, there is clearly a great variation in gap junction among species, and it seems that gap junctions are not more abundant when innervation is less dense[Gabella 1987].

I.7. Intermediate Junctions.

Another common and readily identified type of junction in smooth muscle is the intermediate junction, formed by two dense bands, from adjacent cells[(Henderson 1975)]. The morphological data suggest that intermediate junctions of this type provide a mechanical link between the cells concerned, and, being linked on both sides to myofilaments, they may provide a means of coupling mechanically adjacent muscle cells.

I.8. Mitochondria

The current view about the role of calcium regulation by mitochondria is not clear. They undoubtedly can take up calcium and in the process uncouple electron transport from oxidative phosphorylation, but this is only seen when Ca^{2+} levels are extremely high. Mitochondrial sequestration of calcium is therefore only seen under pathological conditions and plays a minor role under physiological conditions. The likelihood that the mitochondria are physiological sources of calcium is very small. It is well recognized, though, that the role of oxidative phosphorylation in oxidative ATP production is quantitatively less important than in striated muscle[(Stephens and Wrogemann 1970)]. Nevertheless, because oxidation yields almost 12 to 18 times as much ATP as that produced by glycolysis, its role in energy production is not negligible.

I.9. Innervation

Extensive work has been carried out on characterizing innervation of the human lung at the level of the light microscope, and the outline of the innervation

as derived from studies in the nineteenth and twentieth centuries was reviewed by Larsell and Dow[1933], and Gaylor[1934]. The general pattern consists of afferent and efferent pathways. The extensive afferent or sensory innervation originates in the epithelium of the airways, the interalveolar spaces, the muscle, and the submucosal layer[Nagaishi 1972]. The afferent fibres are myelinated and unmyelinated or nonmyelinated and terminate in the vagal nuclei. Axons occur with a frequency of 3-10 per muscle cell bundle, and are partially or completely surrounded by Schwann cell sheaths. The autonomic nerves (parasympathetic and sympathetic) controlling airway smooth muscle show a pattern consisting of sensory afferent nerves. The efferent parasympathetic fibres, which run to the smooth muscle and the glands, arise from ganglia. The latter are controlled preganglionically from the vagal nuclei and are part of the parasympathetic nervous system. The ganglia are situated close to the effector organ and lie external to the smooth muscle and the cartilage. The preganglionic fibres of the sympathetic nervous system leave the spinal cord and synapse with prevertebral ganglia. Postganglionic fibres emerge from the ganglia and are said to innervate the airway smooth muscle, glands, and pulmonary vasculature.

The accepted interpretation of this morphology is that the excitation of the glands and smooth muscle of the airways is under the control of the vagus nerve and is cholinergic. While the parasympathetic nerves are the major motor control system for airway smooth muscle, there is no certainty as to whether the sympathetics are responsible for relaxation. The inhibition of the glands and the

smooth muscle is presumed to be under control of the adrenergic system. This is somewhat controversial because though adrenoceptor certainly exist on the muscle cell membrane, their neural control has not been unequivocally demonstrated. In recent times nonadrenergic, noncholinergic inhibitory (putative transmitter is vasoactive intestinal polypeptide [VIP]) and excitatory nerves (putative transmitter is substance P) have been identified. In humans, perhaps the VIP system is the major relaxant system.

There is no evidence showing close contacts between nerve endings (varicosities) and muscle cells in the airway smooth muscles of all species investigated. It is somewhat difficult to define what constitutes a neuromuscular junction because of the variability of the width of the gap and the lack of prominent structural specializations. However, the local control of release of neurotransmitters from postsynaptic neurons innervating end organs can either result in augmentation or inhibition of the quanta of neurotransmitter released per action potential.

II. Neuropeptides, Mediators and Other Factors Regulating Airway Smooth Muscle in Physiological and Pathophysiological Situations

II.1. Neuropeptides

Recently a number of biologically active peptides have been identified, localized, and characterized by radioimmunoassay, immunofluorescence, and biochemical and bioassay techniques[Said 1982; 1984]. It is already clear that neuropeptides have an influence on airway smooth muscle function and that the

classic descriptions of autonomic and sensory innervation of the lung may have to be revised to include the "peptidergic". Some peptides, such as VIP, occur in sympathetic ganglia, as well as in preganglionic and postganglionic parasympathetic nerve fibres, and in intrapulmonary ganglia(Dey et al 1981; Lundberg et al 1978). There are identified neuropeptides (Said 1989) and some are listed below:

- *Bombesin, gastrin-releasing peptide*
- *cholecystokinin (CCK), gastrin, cerulein*
- *Neurokinins (tachykinins): neurokinin A, neurokinin B, substance P*
- *Neuropeptide Y (NPY), pancreatic polypeptide*
- *Opiod peptides: β-endorphin, enkephalins, dynorphins*
- *VIP, seretin, glucagon, peptide histidine isoleucin, helodermin, corticotropin- releasing factor, growth hormone-releasing factor, Sauvagine, urotensin, gastric inhibitory peptide.*

Peptides found in the parasympathetic nervous system include vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), and galanin, a 29-amino-acid peptide, whose name derives from glycine and alanine, amino acids present in galanin. VIP is a potent smooth muscle relaxant and its deficiency could contribute to the bronchospasm of asthma(Said 1991). At least one peptide, neuropeptide tyrosine (NPY), has been shown convincingly to be associated with the sympathetic innervation in many tissues including the respiratory tract(Lundberg et al 1983). NPY is particularly abundant around the vasculature of the respiratory

tract. It was, therefore, of interest to see a marked increase of NPY innervation in smaller blood vessels of children with pulmonary hypertension[Allen et al 1989]. Peptides like calcitonin gene-related peptide (CGRP), substance P, and some other neurokinins exist also in sensory nerves[Martling 1987; Nawa et al 1984]. CGRP and the neurokinins are not very abundant in the human respiratory tract in contrast to the abundance in animals. CGRP and substance P coexist within the same nerve fibre, and even within the same neurosecretory granules in the central and peripheral branches of sensory fibres, including those of the respiratory tract. CGRP is also present in a subclass of mucosal endocrine cells of the respiratory tract, in particular of animals. It has been suggested that the tracheal CGRP innervation arose principally in the jugular ganglion of the right vagus with that of the lung arising in the left vagus. In addition, CGRP-containing nerves arise from the ipsilateral dorsal root ganglia at spinal levels T1 to T6[Cadieux et al 1986; Springall et al 1987]. Other neuropeptides such as enkephalins which exert actions in airways have been shown by immunocytochemistry to be present in nerve fibres in guinea pig and rat lung[Shimosegawa et al 1989].

II.2. Epithelium-derived relaxing factor (EpDRF)

Removal of epithelium causes significant parallel leftward shifts in the concentration-response curves of airway smooth muscle to acetylcholine, histamine, and 5-hydroxytryptamine[Flavahan et al 1985]. These results were explained by the possible release from the epithelium of inhibitory substance(s) (the putative epithelium-derived relaxing factor - EpDRF) which may depress the tone of

bronchial smooth muscle. The identity of EpDRF remains controversial[Morrison and Vanhoutte 1991] even though it has been extensively studied. The mechanisms by which EpDRF regulate airway smooth muscle remain largely unresolved. It may modulate the neuronal innervation of the airways. For example, EpDRF in guinea pig trachea may inhibit the release of acetylcholine from intramural cholinergic nerve terminals[Murlas 1986], leading to a reduction in cholinergic tone. Neonatal treatment of guinea-pigs with capsaicin, which depletes sensory afferent fibres of tachykinins, decreases the augmentation of contractions to 5-HT in the absence of epithelium[Frossard and Muller 1986]. This suggests that EpDRF may interact with capsaicin-sensitive fibres. Furthermore, the hydrolysis of membrane phosphoinositides, and the subsequent generation of 1,2-diacyglycerol and inositol 1,4,5-triphophate induced by methacholine or histamine is enhanced by the removal of the epithelium[Hay et al 1988]. This indicates that EpDRF may depress smooth muscle contraction by inhibiting the rate of hydrolysis of phosphoinositides. Such effect may be mediated via inhibition of G proteins involved in the regulation of membrane phospholipase C activity. It is clear, nonetheless, that the epithelium has an important role to play in the modulation of airway smooth muscle tone. In asthma the epithelium may be damaged or dysfunctional and this balanced regulatory process is disrupted and bronchial hyperresponsiveness may develop.

II.3. Leukotrienes

The leukotrienes are a family of acid lipids formed mainly in leukocytes

from arachidonic acid that are implicated in the pathophysiology of inflammatory and immediate hypersensitivity reactions[Samuelsson 1983; Samuelsson et al 1987]. The first step in the synthesis of these compounds includes the oxygenation at site C-5 of arachidonic acid to yield 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE). This reaction, and the following transformation of 5-HPETE to the epoxide, 5,6-oxido-7,9,11,14-eicosatetraenoic acid (Leukotriene A_4, LTA_4) is catalyzed by 5-lipoxygenase[Rouzer et al 1986; Shimizu et al 1986; Ueda et al 1986], which is calcium and ATP-dependent[Rouzer and Samuelsson 1985] and recently has been shown that full activation of 5-lipoxygenase requires translocation of the enzyme from the cytosol to cellular membrane where it binds to an activating protein, 5-lipoxygenase activating protein (FLAP)[Miller et al 1990]. LTA_4 can be subsequently hydrolysed to LTB_4, a reaction catalyzed by LTA_4 hydrolase, or conjugated with glutathione to yield LTC_4. This compound and the metabolites LTD_4 and LTE_4 constitute the slow-reacting substance of anaphylaxis. There is now substantial evidence to suggest that LTs may indeed have a role in asthma and inflammatory lung diseases.

Leukotrienes are generated from human lung tissue *in vitro* by both immunological and nonimmunological stimuli[Conroy and Piper 1991] although the exact source of LTs in lung tissue remains unclear. The biological effects of LTs suggest that they may contribute to inflammatory changes in the lung, tissue damage and changes in airway calibre. When given by inhalation, leukotrienes C_4, D_4, and E_4 are potent bronchoconstrictors in normal man[Barnes et al 1984; Barnes and Piper

1986). A group of mild asthmatics were more than 4 times more sensitive to LTs than normals but 11 times more sensitive to histamine(Barnes et al 1985).

Leukotriene receptors have been extensively characterized(Crooke et al 1991). LTB_4 and LTD_4 receptors are clearly different, and employ signal transduction processes that are similar but differ in a number of important regards. The signal transduction process can be divided into immediate and late phases. Within seconds after LTD_4 interacts with its receptors, they couple to a number of signalling systems via at least two and probably more guanine nucleotide binding proteins. A rapid transient increase in intracellular calcium derived from both internal stores and extracellular calcium is induced by mechanisms that include activation of a receptor-operated calcium channel. Inositol phosphate metabolism is increased by activation of both a PIP_2-specific and a PIP_3-specific phospholipase C. Additionally, diacyglycerol is released. The second phase of the signalling process involves activation of topoisomerase 1 for induction of transcription of at least one gene for a protein that activates a PC-specific PLA_4, which has been called the phospholipase activating protein (PLAP). PLAP increases PLA_2 activity either through direct interactions with the enzyme, the substrate or an inhibitor of metabolized via the cyclooxygenase and lipoxygenase pathways in a variety of ways, depending on the phenotype of the cell and other factors. If the predominant metabolites are contractile, e.g., thromboxane A_2, the cells display increased contractile activity. If the predominant metabolites are relaxant, e.g., prostacyclin, the smooth muscle cells may relax.

II.4. Platelet activating factor

Platelet activating factor (PAF) is a highly potent lipid mediator which has been implicated in inflammation and allergy and particularly in allergic inflammation, including asthma[Braquet et al 1987; Barnes 1988]. It is released from sensitized guinea-pig lung after challenge with antigen[Fitzgerald et al 1986], and has been shown to be released from neutrophils, platelets, eosinophils, and macrophages. The receptors exist on the cell membrane[Dent et al 1989]. Binding appears to be specific and is inhibited by PAF antagonists. PAF is a potent bronchoconstrictor in guinea pig *in vivo*, yet has little effect on airway smooth muscle *in vitro*, suggesting that its effect is indirect[Vargaftig et al 1980]. Administration of high doses of PAF by intratracheal instillation in artificially ventilated cerebral-dead humans causes bronchoconstriction associated with hypoxaemia and a fall in cardiac output[Gateau et al 1984]. Nevertheless, the mechanisms by which PAF induces bronchoconstriction remain unclear.

II.5. Vasoactive intestinal polypeptide (VIP)

VIP has been shown to relax airway smooth muscle both *in vitro* and *in vivo*. It relaxes isolated tracheal or bronchial segments from guinea-pigs, rabbits, dogs, and humans and prevents or attenuates their contraction by a variety of constrictors, including histamine, $PGF_{2\alpha}$, kallikrein, leukotriene D4, neurokine A or B, and endothelin I-VII[Boomsma et al 1990; Cameron et al 1983; Hamasaki et al 1983; Ito and Takeda 1982; Piper et al 1970; Said et al 1974; Wasserman et al 1982] This action is long-lasting and is unaffected by blockade of adrenergic or cholinergic receptors or of cyclooxygenase activity[Altiere

and Diamond 1984; Said et al 1974). The principal localization of VIP-containing neurons in the tracheobronchial tree is in the smooth muscle layer (the surface of muscle bundles and also within the bundle), around submucosal mucous and serous glands, and in the walls of pulmonary and bronchial arteries(Dey et al 1981; Laitinen et al 1985; Uddman et al 1985; Uddman and Sundler 1979). Immunoreactive VIP is also present in neuronal cell bodies forming microganglia that provide a source of intrinsic innervation of pulmonary structures. In normal mammalian and human lungs and in human tumour cells, specific receptors for VIP have been identified. There is no direct evidence so far showing the existence of VIP receptor in airway smooth muscle. It is known, however, that VIP binding to receptors in all other cells and tissues(Amiranhoff and Rosselin 1982), its binding to airway sites is coupled to an adenylate cyclase. The resultant increase in cAMP levels is believed to mediate the airway relaxation and other biological effects of the peptide. At the same time, it is now known that the expression of VIP gene is stimulated by increased cAMP levels, meaning that VIP may be able to promote its own production(Gozes and Brenneman 1989).

VIP has been suggested to be the transmitter of the nonadrenergic, noncholinergic component of the autonomic nervous system in regulating airway smooth muscle function because of the following reasons: 1) the existence of rich innervation of VIP-containing nerve fibres and nerve terminals in the airway smooth muscle layer; 2) the potent relaxant activity of VIP on airway smooth muscle *in vitro* and *in vivo*; 3) the identification of its specific receptors.

III. Signal Transduction in Regulating Airway Smooth Muscle Contraction

Our knowledge about the intracellular signal transduction has developed into a very detailed picture of chemical reactions and a recent review article by Berridge[1993] has summarized in depth the mechanisms for inositol trisphosphate and calcium as secondary messengers. The majority of these reactions have been studied in systems other than airway smooth muscle, e.g. liver, neutrophils, blowfly salivary glands, secretory cells, platelets, iris and vascular smooth muscle. The early studies implicating agonist-stimulated inositol phospholipid turnover in pancreas and brain showed that [^{32}P]-P_i was incorporated into phosphatidylinositol (PI) to a very much greater extent than into other tissue phospholipids[Hokin and Hokin 1953; Hokin and Hokin 1955]. This was followed by similar studies in a variety of smooth muscles: rabbit iris[Abdel-Latiff, 1974], and guinea pig ileum[Jafferji and Michell, 1976]. In addition, [^{32}P]-P_i was found to be incorporated into the rabbit iris smooth muscle polyphosphoinositides, phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2), and phosphatidylinositol 4-phosphate (PI-4-P)[Abdel-Latiff et al 1977] and into toad stomach smooth muscle phosphatidic acid (PA)[Salmon and Honeyman 1979].

III.1 Receptors and G Protein Control of Second Messenger

Receptors linked to guanine nucleotide-binding proteins (G-proteins) have been studied in a variety of species and types of cells. There is a family of receptors, which are similar in having seven membrane-spanning domains connected by extracellular and intracellular loops (**Fig. 3**) and related by the fact that they are coupled to G-proteins. Among the receptors in this category are the α_1-, α_2-, β_1- and β_2-adrenergic receptors, muscarinic (M_1-M_5) receptors, serotonin

receptors, histamine, ATP, PAF, TXA_2, angiotensin II, substance P, bradykinin, vasopressin, neuropeptide Y, thrombin, endothelin, and the photon receptor rhodopsin[Gilman et al 1985; Raymond et al 1990; Berridge 1993]. Acetylcholine released from cholinergic nerves causes contraction of airway smooth muscle by activation of M_3 muscarinic receptors. The density of M receptors decreases as airways become smaller, so that terminal bronchioles are almost devoid of muscarinic receptors[Barnes et al 1983a]. Muscarinic receptors can be subclassified into several types according to their binding and function. It is important to note that, there is no M_1 receptor on the post-synaptic smooth muscle membrane. The smooth muscle muscarinic receptors have been designated as M_3 receptors, which have been identified in airway smooth muscle[Blaber et al 1985; Minette and Barnes 1988].

The G proteins couple agonist-activated receptors to intracellular effectors presumably by binding the intracellular domains of the receptor. The G proteins are heterotrimeric, consisting of α, β, and γ subunits[Birnbaumer et al 1987; Moss and Vaughan 1988; Weiss et al 1988]. The α subunits, which determine specificity for each type of G protein, range in size from 39-52 kDa. They bind and hydrolyse GTP and interact directly with both receptor and effector([Moss and Vaughan 1988; Weiss et al 1988]. The β subunits of 35-36 kDa are the products of at least two different highly homologous genes. Relatively little is known about the structure of the γ subunits (about 8 kDa), which are isolated in a tightly associated complex with the β subunits[Birnbaumer et al 1987]. The $\beta\gamma$ subunits are believed to participate in G-protein binding to the cell membrane, to promote interaction of the α subunit with receptor, and to modulate

the activity of the α subunit[Casey et al 1989].

The first step for G protein activation is the binding of agonist to its specific receptor. The agonist-receptor complex then forms a ternary complex with G protein. The process is favoured when G protein is in the αβγ form. G protein may be inactivated when it is the heterotrimeric αβγ species with GDP bound. Association of α(GDP)βγ with agonist-receptor promotes release of bound GDP and binding of GTP[Birnbaumer et al 1987; Moss and Vaughan 1988; Weiss et al 1988]. G protein with bound GTP dissociates from receptor, which then has a lower affinity for agonist, and α(GDP) dissociates from βγ. Both of these species participate in the subsequent steps of signal transduction (**Figure 3**). A G_{α}(GTP)has been implicated in the regulation of phospholipase activity[Birnbaumer et al 1987; Moss and Vaughan 1988; Weiss et al 1988]. The most important regulatory systems that are regulated by G-proteins are: the formation of cyclic GMP (for visual transduction)[Liebman 1986], the formation of cyclic adenosine monophosphate (cAMP)[Strulovici et al 1985; Gilman et al 1985], and the hydrolysis of PI-4,5-P_2[Cockcroft and Gomperts 1985].

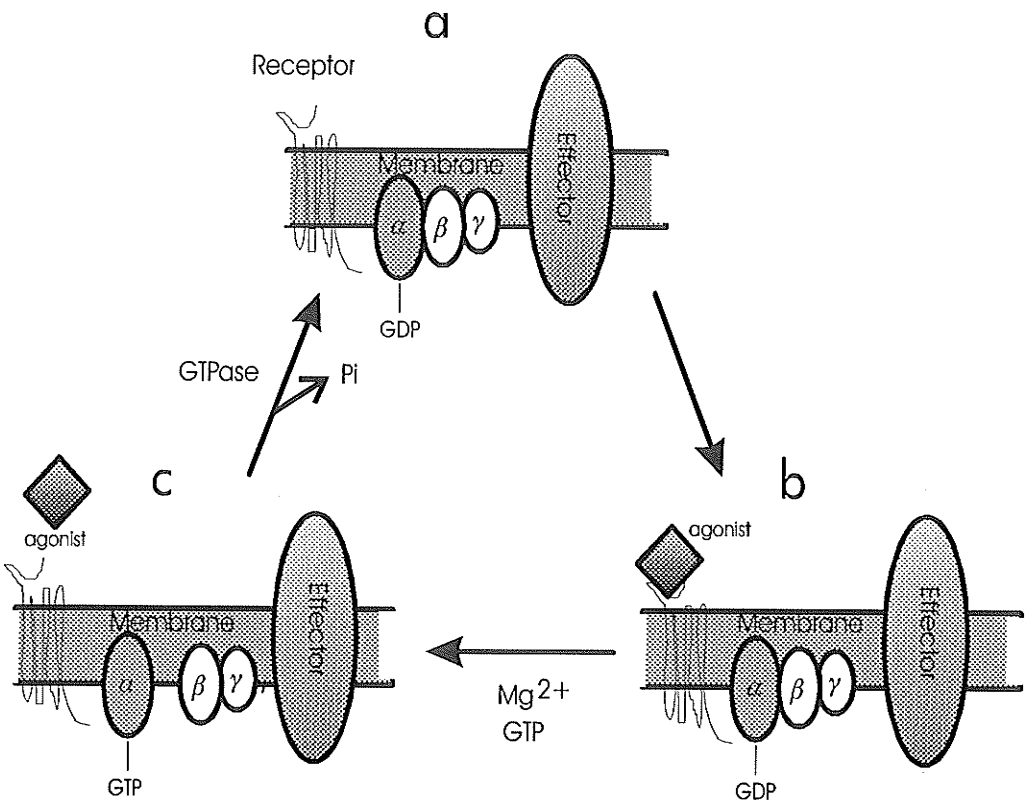


Figure 3. Scheme for G protein signal transduction. From the unliganded state (a), receptor binds agonist A (for example, acetylcholine, epinephrine) which produces a change (b) in receptor-G protein interaction, allowing GTP, in the presence of Mg^{2+}, to replace GDP on the α subunit. The activated α-GTP subunit and the βγ subunits dissociate and one or both interacts with effectors (for example, adenylyl cyclase, K^+ channel). Alternatively, free βγ may bind other α subunits. The intrinsic GTPase activity of the α subunit hydrolyses GTP to GDP, releasing inorganic phosphate (P_i), and α-GDP recombines with βγ (c), ending the activation cycle. Nonhydrolysable analogues of GTP such as Gpp(HH)p or GTP-γS produce persistent activation of α subunits and persistent dissociation of α from βγ because activation cannot be reversed by hydrolysis of these nucleotide analogues to GDP.

III.2. Metabolism of Inositol Phospholipid

The activation of receptors by agonist mobilizes the hydrolysis of the phospholipid PIP_2 into two moieties, water-soluble inositol 1,4,5-trisphosphate (IP_3) and lipid-soluble diacylglycerol (DAG), of which the fatty acid composition resembles that of the polyphosphoinositides[Cockcroft et al 1984]. The inositol phospholipid metabolism pathway is shown in **Figure 4**. Hydrolysis of PIP_2 has been found to occur in various smooth muscles including airway smooth muscle[Akhtar and Abdel-Latiff 1984; Baron and Coburn 1987], in which stimulation by agonist results in increases in the production of IP_3 and other inositol phosphates. The measurement of hydrolytic activity of inositol phospholipid-specific phospholipase C indicates that, when the intracellular calcium level is low (0.1 μM), PIP2 and PIP are preferred substrates[Litosch and Fain 1985; Lucas et al 1985; Melin et al 1986]. The simultaneous increases in IP_3 and cytosolic [Ca^{2+}] have been found in many systems[Streb et al 1983; Jean and Klee 1986; Portilla and Morrison 1986] including smooth muscle[Smith et al 1984; Nabika et al 1985]. Although there is no direct demonstration that generation of IP_3 precedes intracellular Ca^{2+} release, kinetic studies in vascular smooth muscle[Alexander et al 1985] showed that the rate of elevation of IP_3 after stimulation by agonist is fast enough to account for the cytosolic Ca^{2+} increase. Conclusive studies using permeabilized smooth muscle cells or tissues have shown that addition of IP_3 to such permeabilized cells increases fluorescence of the Ca^{2+}-sensitive dyes, quin-2, fura-2, as well as the efflux of $^{45}Ca^{2+}$[Suematsu et al 1984; Smith 1985; Somlyo et al 1985; Goldman et al 1986].

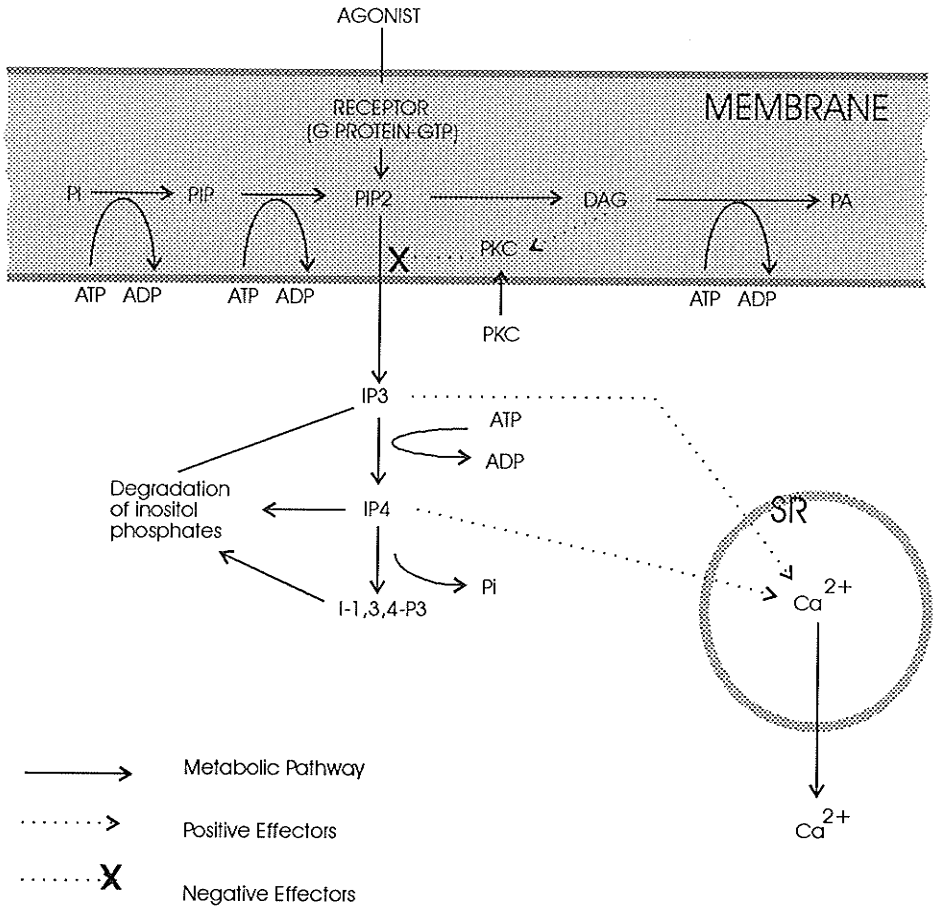


Figure 4. Pathway of inositol phospholipid metabolism. PI: phosphatidylinositol; PIP: phosphatidylinositol 4-phosphate; PIP_2: phosphatidylinositol 4,5-bisphosphate; IP_3: inositol 1,4,5-trisphosphate; IP_4: inositol 1,3,4,5-tetrakisphosphate.

Diacylglycerol (DAG), a lipid-soluble product of PIP_2 hydrolysis, has been found to increase rapidly and transiently, as well as sustainablly[Rebechhi et al 1983; Baron et al 1984; Berridge 1985; Sekar and Hokin 1986]. It has been demonstrated to stimulate the activity of the lipid-activated enzyme protein kinase C (PKC), which is involved in many cellular mechanisms including the tonic phase of smooth muscle contraction[Andrea and Walsh 1992].

III.3. β_2*-receptors and cAMP system*

β_2-adrenoceptors are present in the smooth muscle membrane of animal and human airways[Barnes et al 1982; Carstairs et al 1984; Carstairs et al 1985]. Functional studies have demonstrated that relaxation of both central and peripheral human airways is mediated solely via β_2-receptors[Zaagsma et al 1983; Goldie et al 1984]. β_2-agonists act as functional antagonists and inhibit or reverse the contractile response irrespective of the constricting stimulus. This is important in asthma, in which several spasmogens including leukotriene D_4, histamine, and acetylcholine are likely to be involved.

The intracellular mechanisms for mediating the relaxant effect of β_2-agonists in airway smooth muscle have recently been elucidated (**Figure 5**)[Barnes 1993]. β_2-Receptor stimulation increases intracellular cyclic adenosine monophosphate (cAMP) levels [Andersson et al 1978; Duncan et al 1980; Van den Brink 1972] which activate protein kinase A (PKA). PKA in turn phosphorylates serval proteins resulting in relaxation[Giembycz and Raeburn 1991] (phosphorylation of SR protein, etc.). In airway smooth muscle PKA may inhibit myosin light chain kinase activity[Gerthoffer

1986) and PI hydrolysis(Hall et al 1989; Jones et al 1987; McAtee and Dawson 1989), promote Na^+/Ca^{2+} exchange(Gunst and Stropp 1988), thereby lowering intracellular Ca^{2+}, and stimulate Na^+/K^+ ATPase activity. It has been shown that cholinergic agonists act to inhibit cAMP production(Hall and Chilvers 1989; Horwitz 1989). β_2-Agonists also open membrane K^+ channels. Charybdotoxin and iberiotoxin, which are selective blockers of large conductance Ca^{2+}-activated K^+ channels (maxi-K channels), inhibit the bronchodilator responses to β_2-agonists and other agents which elevate cAMP(Jones et al 1990; Jones and Chareste 1992). Patch clamp studies have confirmed that elevation of cAMP opens a maxi-K channel in airway smooth muscle(Kume et al 1989) probably via the α-subunit of G-protein (G_s)(Kume et al 1992). This suggests that relaxation of airway smooth muscle may occur independently of a rise in intracellular cAMP, and explains why there is a discrepancy between the low concentration of β-agonists needed to relax airway smooth muscle and the relatively high concentrations needed to elevate cAMP levels. In addition, when no contractile agonists are present, a rise in cAMP content acts to increase Ca^{2+} influx and increase the free intracellular Ca^{2+} concentration without causing an increase in muscle tone(Felbel 1988; Takuwa 1988). In sum, the relationship between intracellular cAMP and airway smooth muscle relaxation is still not completely clear.

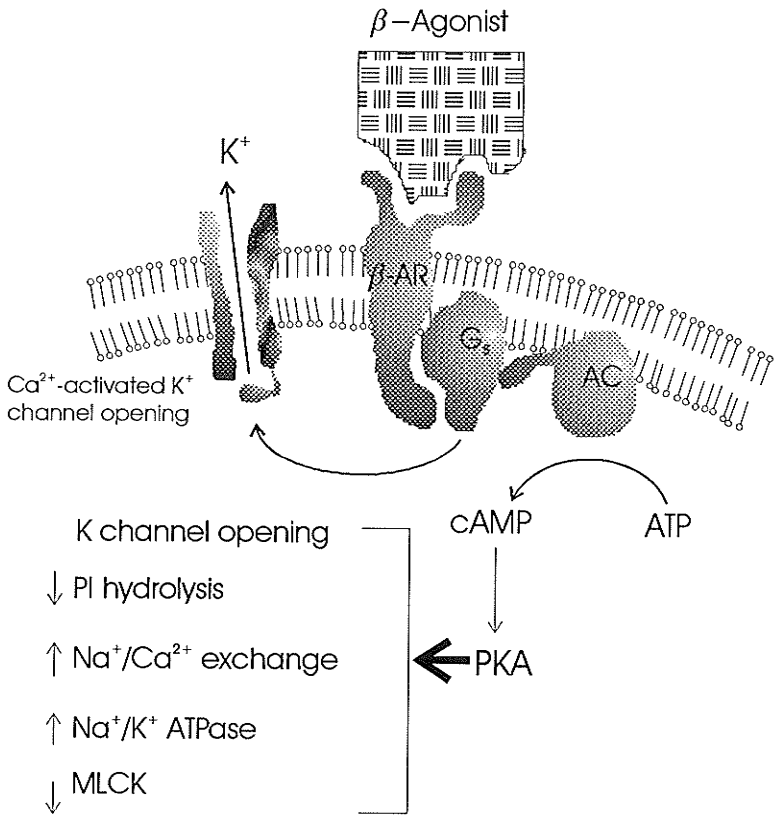


Figure 5. Molecular mechanisms involved in the bronchodilator response to β-agonists. β-adrenoceptor (β-AR) stimulation activates a stimulatory G-protein (G_s) which may couple directly to a large conductance Ca^{2+}-activated K^+ channel, or may activated adenylyl cyclase (AC), leading to an increase in cyclic adenosine monophosphate (AMP). Cyclic AMP activates protein kinase (MLCK) and enzymes involved in phospho-inositide (PI) hydrolysis. ATP = adenosine triphoshate; ATPase = adenosine triphosphatase.

III.4. Protein Kinase C

PKC is a widely distributed protein serine/threonine kinase with broad substrate specificity. It is actually a family of at least eight isoenzymes some of which are calcium dependent (α, β_I, β_{II}, and γ) and some calcium independent (δ, ε, ζ, and η). The smooth muscle PKC has a molecular weight of approximately 80 kDa and exists as a monomer under native conditions[De Vries et al 1989]. Chicken gizzard PKC activity is strongly dependent on Ca^{2+}, phospholipid, and DAG. Many studies have described the translocation of PKC from the cytosol to the plasma membrane on appropriate stimulation of a variety of cell types including smooth muscle[Haller et al 1990]. The typical slowly developing, sustained contraction of smooth muscle induced by phorbol ester has been reported. As alluded to earlier, the contractile response could result from direct or indirect effects of PKC at any one or more steps in the excitation-contraction coupling pathway, and evidence has accumulated indicative of several possible sites of action of PKC. Phorbol ester-induced contraction of vascular smooth muscle was accompanied by an increase in $[Ca^{2+}]_i$ with consequent phosphorylation of 20 kDa myosin light chain at serine 19[Rembold and Murphy 1988]. The potential sites of PKC phosphorylation would be the L-type voltage-dependent sarcolemmal Ca^{2+} channel, sarcolemmal receptor-operated Ca^{2+} channel, and the sarcolemmal and SR Ca^{2+} pumps and the sarcolemmal Na^+/Ca^{2+} exchanger. One could explain a phorbol ester-induced increase in $[Ca^{2+}]_i$ by PKC-catalyzed phosphorylation causing activation of one or more of the Ca^{2+} entry mechanisms or inactivation of one or more of the Ca^{2+}

removal mechanisms. No direct analysis of PKC-catalyzed phosphorylation of smooth muscle Ca^{2+} channels has been reported; however, the rabbit skeletal muscle dihydropyridine receptor was phosphorylated *in vitro* by PKC(Nastainczyk et al 1987). In other instances, phorbol ester-induced contractions of smooth muscle did not involve a change in cytosolic Ca^{2+} or in myosin light chain phosphorylation suggesting a site of action beyond Ca^{2+}(Jiang and Morgan 1987; Itoh and Lederis 1987). A great deal of effort has been devoted to identifying the substrate(s) of PKC that is/are involved in the contractile responses. The weight of evidence is against either myosin or MLCK being involved(Andrea and Walsh 1992). On the other hand, PKC-catalyzed phosphorylation of proteins responsible for the movement of Ca^{2+} across the sarcolemma or SR membrane, resulting in an increase in $[Ca^{2+}]_i$, is probably of importance, at least in specific smooth muscle cell types. In the phorbol ester-induced contractions that do not involve $[Ca^{2+}]_i$ and myosin light chain phosphorylation, it is necessary to invoke other PKC substrates. For example the intermediate filament proteins desmin and synemin are phosphorylated in bovine trachealis muscle treated with carbachol, but the functional significance of these phosphorylations is unknown, perhaps related to latch bridge formation. The thin filament-associated proteins caldesmon and calponin have both been implicated in the regulation of smooth muscle contraction, and both can be phosphorylated by PKC.

III.5. Pharmacomechanical and Electromechanical Coupling in Airway Smooth Muscle

Pharmacomechanical coupling mechanisms can be divided into two basic categories, one that contracts smooth muscle via IP_3 and one that relaxes smooth muscle via cAMP or cGMP systems. As discussed above, receptor-activated PIP_2 hydrolysis is mediated by G protein interaction with phospholipase C (PLC). There is direct evidence of GTPγS-activation of a membrane-bound PLC in an airway smooth muscle[Murray et al 1989]. IP_3 is an established second messenger that releases Ca^{2+} from nonmitochondrial intracellular organelles, most likely sarcoplasmic reticulum (SR), by binding to a Ca^{2+} channel protein[Ehrlich and Watras 1988], which increases the probability of the open state of this channel with resultant Ca^{2+} efflux into the cytoplasm. In smooth muscle, IP_3 has been shown to release Ca^{2+} from the SR[Somlyo et al 1985]. The superficial SR of smooth muscle may obtain its Ca^{2+} from extracellular space. It is not known if all of the calcium ions released by IP_3 originates in the SR, or if IP_3 can function in smooth muscle to mediate exchange of Ca^{2+} between different intracellular Ca^{2+} pools[Goldman et al 1989]. IP_3 activates surface membrane Ca^{2+} channels in mast cells[Penner et al 1988], and there may be a similar function in smooth muscle cells. In canine trachealis muscle, activation of inositol phospholipid metabolism occurs in two steps[Baron and Coburn 1987]. In the first step, which occurs during development of force and before onset of PI resynthesis, inositol phospholipid metabolism, with resultant production of second messengers, is driven by decreases in inositol phospholipid pool size. The second step, which occurs during force maintenance, is characterized by nearly constant total pool size of inositol phospholipids and a state where metabolic flux in inositol

phospholipids is nearly equal to the rate of PI resynthesis.

Electromechanical coupling includes two basic mechanisms: a) receptor-mediated events resulting in membrane depolarization and b) voltage-activated Ca^{2+} channels, which result in increase in free cytosolic [Ca^{2+}] because of the influx of Ca^{2+} across the plasma membrane via these channels. Agonist-induced membrane depolarization involves G-protein activation. It is also graded, does not evoke action potentials normally, and is associated with decreases in membrane resistance involving both inward and outward current. Ca^{2+}-activated, outwardly rectifying, voltage-dependent K^+ current have been described in canine bronchial smooth muscle[Sigurdsson et al 1992]. Possibilities to explain receptor-activated membrane depolarization are receptor activation of an inward current through a receptor-operated channel (ROC) or receptor-mediated inhibition of an outward K^+ current. Such activation seems likely to occur through G proteins. It is also possible because of the relatively slow onset of depolarization that activation of these putative channels involves cytoplasmic second messengers[Coburn and Baron 1990]. Although there are data that suggest, in vascular smooth muscle[Benham and Tsien 1987], the presence of non-voltage-operated Ca^{2+} channels that can provide inward current which may depolarize the surface membrane to threshold for activation of voltage-operated Ca^{2+} channels, little evidence has been found to prove the presence of ROCs in any airway smooth muscle. Inhibition of outward K^+ current induced by agonist has been postulated because of the observation that the M current, a late K^+ current, is inactivated in the presence of Ach[Cole and Sanders 1989,

Kotlikoff). So far, this current has not been identified in airway smooth muscle.

III.6. Intracellular Free Calcium Regulation

It is commonly accepted that contraction of smooth muscle, including airway smooth muscle, depends upon the level of calcium ions free within the cytoplasm of the cell ($[Ca^{2+}]_i$) (Filo et al 1965; Bolton 1979; van Breemen and Saida 1989). Two integrated membrane systems that play key roles in the regulation of smooth muscle $[Ca^{2+}]_i$ are: (a) the sarcolemma, which is under the control of the membrane potential and agonists, and (b) the sarcoplasmic reticulum (SR), controlled by second messengers. Both membranes establish a Ca^{2+} concentration gradient of about 10,000-fold. Studies using fluorescent Ca^{2+} indicators, such as quin-2, fura-2, indo-1, and fluo-3, have shown the sarcoplasmic free Ca^{2+} in the resting smooth muscle cell to fall within the range of 120-270 nM and in the activated smooth muscle to be about 500-700 nM(Williams and Fay 1986). The main sources of Ca^{2+} are now established as the extracellular space and the SR(Devine et al 1972), in each of which the free Ca^{2+} lies in the millimolar range. The cytosolic free Ca^{2+} is therefore controlled by the movement of Ca^{2+} across the sarcolemmal and SR membrane. The mechanisms for Ca^{2+} entry to the sarcoplasm from the extracellular milieu or the lumen of the SR, and Ca^{2+} extrusion from the sarcoplasm across the sarcolemma to the extracellular space or back into the SR are shown in **Figure 6**. Two major pathways exist at the level of sarcolemma for the entry of Ca^{2+} in response to appropriate stimuli: voltage-operated Ca^{2+} channels and receptor-operated Ca^{2+} channels(Hurwitz 1986). Membrane depolarization

and ligand binding of the receptor result in the opening of these channels, allowing Ca^{2+} to diffuse into the cell. The permeability of these channels may be modified in certain pathological situations such as in spontaneous hypertension rats, where the aortic smooth muscle Ca^{2+} uptake is increased[Yamada et al 1992]. At the level of the SR, the IP_3 receptor, also a Ca^{2+} release channel, mediates Ca^{2+} release into the sarcoplasm[Chadwick et al 1990]. As discussed earlier, IP_3 production results from the binding of ligand to receptors, which leads to the activation of polyphospholipase C by G-proteins. Plasmalemma bound Ca^{2+} has been suggested as an additional Ca^{2+} source[Janis et al 1987]. Hydrolysis of phosphatidylinositol phosphates during agonist stimulation can release Ca^{2+} from this source, functioning perhaps to trigger further SR Ca^{2+} release. This hypothesis, however, lacks direct evidence.

The SR membranes contain at least two distinct types of Ca^{2+} release channels: 1. Those that are sensitive to changes in the concentration of intracellular free Ca^{2+} [Ehrlich and Watras 1988], Mg^{2+}, and ATP on the *cis*-side, and are modulated by ryanodine (ryanodine receptor); 2. Those activated by IP_3[Ehrlich and Watras 1988].

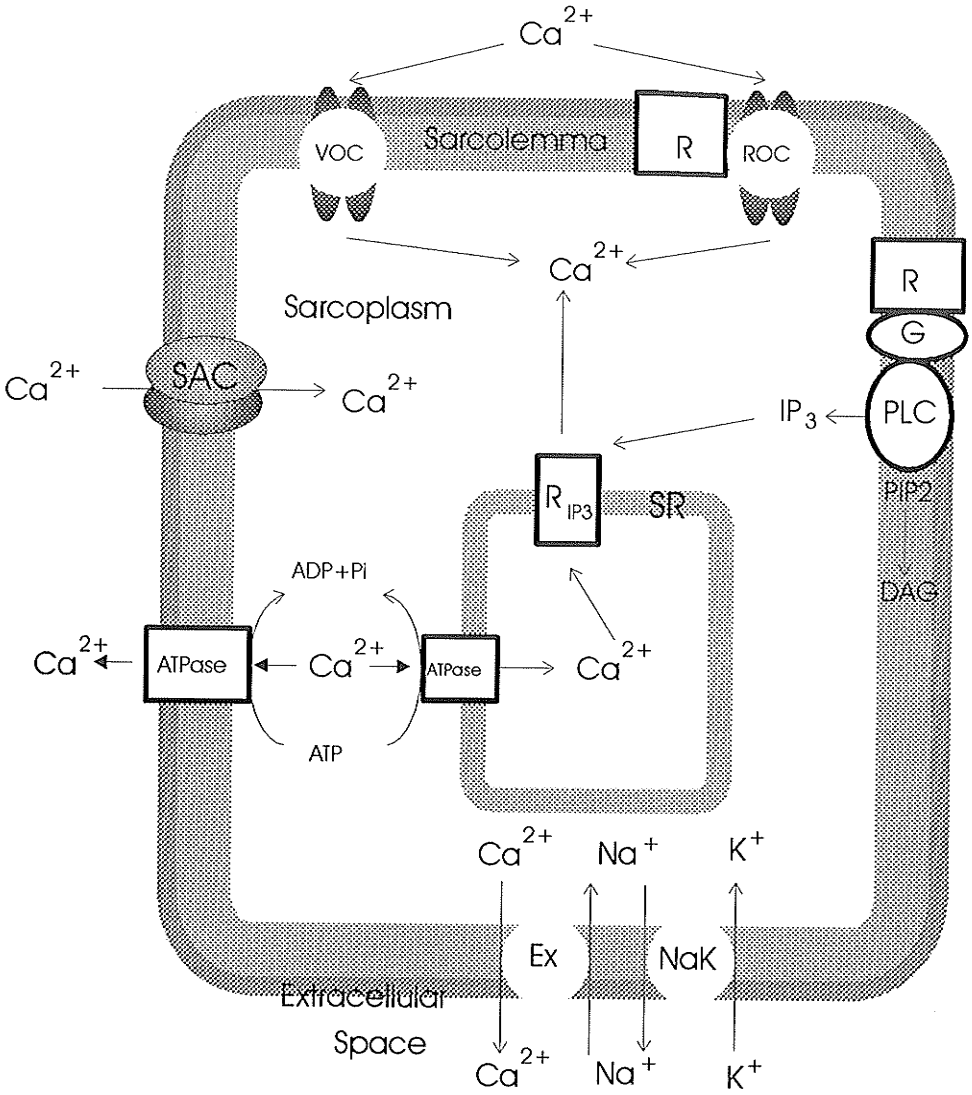


Figure 6. Mechanisms regulating intracellular [Ca^{2+}] via sarcolemal & sarcoplasmic reticulum membranes. VOC,voltage-operated channel; R, receptor; ROC, receptor-operated channel;G,guanine nucleotide binding protein; PLC, phospholipase C; PIP2, phosphatidylinositol 4-phosphate; DAG, diacylglycerol; RIP3, inositol 1,4,5-trisphosphate receptor, also a calcium channel; SR, sarcoplasmic reticulum; NaK, Na^+/K^+ transporting ATPase; Ex, Na^+/Ca^{2+} exchanger.

a. Ca^{2+} -Induced Ca^{2+} Release (CICR)

The influx of a small amount of Ca^{2+} through voltage-gated Ca^{2+}channels upon stimulation could release a much greater amount of Ca^{2+} from the intracellular store[van Breemen 1965]. Since insufficient Ca^{2+} for activation of contraction appeared to enter smooth muscle cells during action potentials, CICR was proposed in smooth muscle[Bolton 1979; Itoh et al 1981; Kuriyama et al 1982]. The use of saponin to permeabilize the plasmalemma while preserving SR function directly demonstrated CICR in smooth muscle[Saida 1981; Saida 1982]. The threshold for this process was estimated to be about 3 μM Ca^{2+}, which could be raised by high $[Mg^{2+}]_i$. Since ryanodine also releases Ca^{2+} in smooth muscle[Hwang and van Breemen 1987], it is conceivable that Ca^{2+} channels similar to the ones described for skeletal muscle and associated with the ryanodine-binding junctional foot proteins may also exist in smooth muscle.

There is evidence showing that the SR may, on the other hand, attenuate transsarcolemmal Ca^{2+} signalling. Portions of the SR were shown to make close contacts with specific areas of the surface membrane (gaps $<$ 50 nm) and were referred to as longitudinal or junctional SR. Such small gaps between the sarcolemma and the SR would predict that they could represent sites of restriction for the diffusion of ions, and more specifically Ca^{2+}. It has been postulated[van Breemen 1977; Casteels and Droogmans 1981] that a fraction of the Ca^{2+} that crosses the surface membrane of smooth muscle cells during depolarization is not directly available to bind to calmodulin and initiate contraction, but is instead captured by the

junctional SR to be preferentially released back toward the plasmalemmal membrane to be extruded via Na^+/Ca^{2+} exchange and the ATP-driven Ca^{2+}-ATPase[Chen and van Breemen 1993]. The SR could serve as a protective mechanism to limit the amount of Ca^{2+} available for contraction. Evidence in favour of the "superficial buffer barrier[Chen and van Breemen 1993; Sturek et al 1992; van Breemen 1977; van Breemen and Saida 1989] indicated temporal dissociation between Ca^{2+} entry into the cell and changes in $[Ca^{2+}]_i$ and/or tension, an effect which disappeared after perturbation of SR Ca^{2+} transport with caffeine or ryanodine (active SR Ca^{2+} release channels), and thapsigargin (specific inhibitor of SR Ca^{2+}-ATPase).

b. IP_3-Induced Ca^{2+} Release

The discovery of inositol 1,4,5-trisphosphate as the second messenger for Ca^{2+} release finally provides the links for excitation-function coupling[Berridge and Irvine 1984; Berridge 1993]. It was subsequently found that IP_3 activates Ca^{2+} release from smooth muscle SR[Suematsu et al 1984]. An IP_3 receptor from mammalian brain has been purified, cloned, and reconstituted in lipid vesicles[Supattapone et al 1988; Ferris et al 1989; Furuichi et al 1989; Mignery et al 1989]. It appears that the IP_3 binding site and the Ca^{2+} release channel reside in a single protein. More interestingly, increase in $[Ca^{2+}]_i$ could trigger IP_3-induced Ca^{2+} release from SR[Finch et al 1991] The IP_3 receptor contains typical membrane-spanning domains in the C-terminal region which anchor the protein in the membrane with four of the subunits combining to form the functional IP_3 sensitive calcium channel (**Fig. 6**). Upon binding IP_3, the receptor undergoes a large conformational change which is perhaps related to the coupling

process leading to channel opening[Mignery and Sudhof 1990]. The other major intracellular calcium channel, the ryanodine receptor (RYR) shows remarkable structural similarity to that of the IP_3 receptor. IP_3 or ryanodine-sensitive calcium stores coexist in smooth muscle and may interact with each other. The mean opening time for the IP_3 receptor was less than 10 ms and there was evidence of four conductance states. Meyer et al[1990] therefore proposed that IP_3 binds sequentially to the four putative binding sites of the tetrameric receptor (**Fig. 6**). Each binding step may cause a partial opening of the channel. However, it was also suggested that one molecule of IP_3 is sufficient to account for channel opening based on no evidence of co-operativity[Watras et al 1991; Finch et al 1991]. There are two main ways in which the sensitivity of IP_3-induced calcium release might vary. First, IP_3 sensitivity may change depending on the calcium content of the store. It seems that the sensitivity increases as the store charges up with calcium[Missiaen et al 1991; Tregear et al 1991]. The second possibility is that variations in sensitivity may depend upon receptor heterogeneity arising from the presence of different gene products, from alternative splicing or from post-translational modifications such as phosphorylation or autophosphorylation, since the IP_3 receptor can function as a protein kinase[Ferris et al 1992].

c. Stretch-activated Ca^{2+} channels

Another important Ca^{2+} source for smooth muscle activation is the so-called stretch activated channel (SAC), which was first identified in skeletal muscle cell[Guharay and Sachs 1984] and in almost all the tissues ever since studied

including smooth muscle[Kirber et al 1988; Davis et al 1992]. While the physiological significance of having such channels is not entirely clear, SAC current have been shown to be reversible, repeatable, blockable, incremental with applied stretch (suction), and specific for a single type of channel[Kirber et al 1988; Lansman et al 1987; Sachs 1989; Yang and Sachs 1989]. A proposed physiological role for SAC's in smooth muscle was given by Kirber et al[1988]. Under physiological conditions, inward current flowing through SAC's would depolarize the cell membrane. Unitary current through SAC's at the normal resting membrane potential is about 2-2.5 pA (pico ampere)[Davis et al 1992; Kirber 1988]. Because smooth muscle cells usually have a very high membrane impedance at rest ($>$1-2 GΩ), gating of only a small fraction of channels would significantly depolarize the membrane. This depolarization could in turn activate voltage gated calcium channels, which have also been identified in smooth muscle cells[Vivaudou et al 1988]. In addition, Ca^{2+} entering the cell directly through SAC could trigger release of Ca^{2+} from intracellular stores and/or directly activate the contractile machinery. Although the SAC's provide a good explanation for transduction of stretch into an ionic event that initiates myogenic contraction, they do not necessarily explain the maintenance of contraction following shortening, which would return SAC's to an unstretched state. Johnson[1980] proposed that a series arrangement between the contractile proteins and an excitable membrane could provide means for subjecting the membrane to sustained deformation. Thus a satisfactory model could include SAC's localized to discrete membrane regions in close proximity to the points of attachment between

contractile elements and the membrane. Pathophysiologically, myogenic response of airway smooth muscle, probably initiated by activation of SAC's, might be involved in the development of increased airway resistance in asthma[Martin and Woolcock 1989]. It is also worthwhile to point out that Ca^{2+}-activated K^+ channels[Kirber et al 1992] and L-type Ca^{2+} channels[Langton 1993] are known to be sensitive to deformation of the cell membrane in smooth muscle. The former would certainly depolarize the cell membrane and lead to Ca^{2+} influx.

d. Ca^{2+} extrusion mechanisms

Sarcoplasmic Ca^{2+} extrusion maintains and restores the low level of $[Ca^{2+}]_i$ in the resting muscle via the following two mechanisms: (a) Ca^{2+} pump and Na^+/Ca^{2+} exchanger in sarcolemma, (b) Ca^{2+} pump in the SR membrane[Casteels et al 1986]. The sarcolemmal Ca^{2+} pump, which is now believed to be localized in caveolae[Fujimoto 1993], is a Ca^{2+}-ATPase that transports Ca^{2+} out of the cell at the expense of ATP hydrolysis. The Na^+/Ca^{2+} exchanger is a sarcolemmal protein which, under physiological circumstances, allows three Na^+ ions to enter the cell in exchange for a Ca^{2+} ion and thus, plays a key role in regulating Ca^{2+} concentration[Reuter 1991]. The SR membrane of smooth muscle contains a Ca^{2+}-Mg^{2+}-ATPase (105 kDa) which, pumps Ca^{2+} from the cytosol into the SR at the expense of ATP. This enzyme differs from the sarcolemmal Ca^{2+} pump in structural and regulatory properties, e.g., only the SR Ca^{2+} pump undergoes Ca^{2+} dependent phosphorylation[Sumida et al 1984] and is regulated through cAMP-mediated phosphorylation of phospholamban[Reymaekers and Jones 1986]. It has recently been reported

that the smooth muscle Na^+/Ca^{2+} exchanger is largely co-distributed with the Na^+/K^+ pump on unique regions of the plasma membrane in register with, and close to, calsequestrin-sites where contractile filaments attach to the membrane[Moore et al 1993], suggesting a mechanism for the strong linkage seen in smooth muscle between Na^+/Ca^{2+} exchange and Ca^{2+} release from the SR. This molecular organization suggests that the plasma membrane is divided into at least two functional domains, and appear to provide a mechanism for the strong linkage seen in smooth muscle between Na^+/K^+ pumping and Na^+/Ca^{2+} exchange, and between Na^+/Ca^{2+} exchange and Ca^{2+} release form the SR[van Breemen 1977; Bird et al 1991; Moore et al 1993].

IV. Regulatory Mechanisms for Smooth Muscle Contraction

IV.1. Properties of Related Proteins

The organization of contractile filaments in smooth muscle is much less evident than in striated (skeletal and cardiac) muscles. There are three distinct types of filaments identified in various kinds of smooth muscle cell: **thin filaments** (6-8 nm in diameter) composed mainly of actin monomers, polymerized into a double-helical strand, and associated filamentous tropomyosin arranged along the length of the actin filament; **intermediate filaments** (10 nm in diameter) composed of desmin and vimentin; and **thick filaments** (12-18 nm in diameter) composed of aggregated myosin molecules[Somlyo 1980]. Intermediate filaments may be involved in formation of a cytoskeletal network in association with dense

bodies, distribution of tension throughout the cell, maintenance of the shape of the cell, and the structural integrity of the so-called mini-sarcomeres of smooth muscle[Somlyo 1980]. They may also contribute to the elastic recoil of the cell[Stephens et al 1988].

The contractile filaments may function in a similar way to that in striated muscle, i.e., production of contraction by the relative sliding of actin and myosin filaments. Actin filaments insert into the dense bands much as they do in Z-discs. Immunohistochemical studies with myosin heads have revealed "decoration" of actin exactly as seen in skeletal muscle. Reversal of polarity of the heads is seen at either side of the dense body[Somlyo 1980]. The similarity with striated muscle in thus quite striking. Electronmicrographs show myosin filaments interdigitating with the actins crossbridges are clearly seen extending from the thick filament to the thin. These structures, termed "mini-sarcomeres", as stated above, suggest that remarkable structural similarities exist between striated and smooth muscles.

The major proteins in smooth muscle that relate to contraction are contractile, regulatory, and cytoskeletal. The activities of most of them are, to some extent, regulated by the level of intracellular Ca^{2+}.

Contractile Protein

The thin, actin filaments constitute the bulk of the cytoplasm in electron micrographs. The orientation of these filaments has not been convincingly determined. Some report they may run obliquely across the cell[Bagby et al 1971], while others consider they run from one end of the cell to the other[Gabella, 1981]. The **actin**

molecule (Mr 42,000), isoforms: α (mainly in vascular smooth muscle), β (other smooth muscle and nonmuscle cells) and γ (enteric smooth muscle), are highly conserved in nature and actin from the muscle of a given species can interact with myosin from another [Tan et al 1992]. Actomyosin interactions are responsible, not only for shortening and force generation in muscle, but also for a variety of contractile events in non muscle cells. The biochemical properties of smooth muscle actin are quite similar to those of striated except that the ratio of thin-to thick filaments is 13-15 to 1 which differs markedly from that in striated muscle where it is 6 to 1[Stephens et al., 1992a]. Three properties of actin are particularly important for its involvement in contraction: first, the ability to form long filaments consisting of two strands of actin monomers wound around each other in a helical arrangement; second, the ability to bind to myosin and activate the Mg^{2+}-ATPase activity associated with myosin heads; third, the ability to bind regulatory proteins such as the troponin complex of striated muscles and tropomyosin. Actin serves to determine cell structure, cell motility, and force generation. The crystal structure of the actin monomer to atomic resolution has been solved[Kabsch et al 1990] and modelled into the actin filaments[Holmes et al 1990].

Thick filaments are not normally seen in light- and electron-micrographs of smooth muscle which is why the smooth muscle is so named. Smooth muscle **myosin** isoforms: MHC_1, MHC_2, and MHC_3 of 204, 200, and 196 kDa are a hexameric proteins each consisting of two heavy chains and two pair of light chains. The heavy chain forms an interesting structure whereby the carboxyl

terminal 130 KDa forms an elongated, dimeric, coiled-coil tail. The 90 KDa amino terminal of the heavy chain interacts with the light chain subunits to form an asymmetric, more globular "head" region which contains the ATP and actin binding sites. The amount of smooth muscle myosin is one-fifth of that in skeletal muscle which renders all the more remarkable fact that smooth muscle generates as much stress (force per unit cross-sectional area) as skeletal. Myosin filaments can be synthesized from smooth muscle myosin molecules. The length of the smooth muscle filaments (2 μ) is greater than that of the striated (1.6 μ) and the former does not possesses a central bare area. Recently it has become clear that the term "myosin" encompasses a large and diverse superfamily of actin based motor proteins. Nine classes of myosins have been described to date[Cheney et al 1993; Doberstein et al 1993; Bemeat and Mooseker 1993]. The myosin molecule has at least three functional domains. One of them is a motor domain that by sequence homology with characterized myosins should be capable of interacting with actin and ATP (subfragment I). Following the motor domain, there is a "neck" domain that is associated with low molecular weight light chain subunits or calmodulin (subfragment II). These two subfragments constitute heavy meromyosin (HMM). Following the neck there is a rod-like tail portion (light meromyosin-LMM) that anchors the myosin head to the thick filament. The head portion is subdivided, in the basis of protease activities, into 3 domains, a 25 kDa, a 50 kDa, and a 20 kDa. Recent work by Rayment's group[1993a; 1993b] has shed considerable light on the sequence structure and conformational shape of the myosin head (at 2.8 Å

resolution) by x-ray diffraction of the crystal obtained after treating the purified protein so as to convert lysines into methyllysines. These advances-the most important breakthroughs in the field in 1993-will help elucidate the ultimate mechanism of muscle contraction. Undoubtedly these mechanisms will apply to skeletal, cardiac, smooth muscles and nonmuscle contractile systems. Upon activation of the muscle, myosin undergoes cycling interactions with actin with relative sliding of the thick and thin filaments driven by energy derived from ATP hydrolysis.

Regulatory Proteins

In smooth muscle the major regulator of activation is the 20 kDa **myosin light chain** (MLC_{20}). A second light chain is one with molecular weight of 17 kDa (MLC_{17}), an alkali light chain whose function is not fully understood although there is evidence suggesting that the isoform of MLC_{17} influences the maximal shortening velocity of smooth muscle[Malmqvist and Arner 1991]. **Myosin light chain kinase** (MLCK) whose molecular weight is 105 kDa consists of four domains: calmodulin binding, pseudosubstrate, constitutive, and ATP binding. MLCK is considered as a rate-limiting enzyme in the activating cascade for smooth muscle contraction. **Calmodulin** (16,500 Da) has four so-called EF hand sequences, each of which binds one calcium ion. The combination of calcium to calmodulin during excitation leads to binding of this complex to MLCK with activation of the latter. The kinase phosphorylates MLC_{20}, which results in actin activation of myosin Mg^{2+}-ATPase[Sobieszek 1977; Kamm and Stull, 1989]. The unphosphorylated light chain acts as

a repressor of the actin-activated MgATPase activity[Sellers 1993]. Furthermore, phosphorylation of the light chains also has a significant effect on the stability of the myosin filaments[Trybus 1991].

Tropomyosin is present in smooth muscle, it is a dimer (Mr 66,000) of elongated, α-helical polypeptides coiled around each other and is located in the grooves between the two strands of the actin double helix. Smooth muscle tropomyosin exists in two isoforms, α and γ. Its function in smooth muscle, which lacks troponin, is not defined, though in vitro it seems to modify actomyosin ATPase activity[Miyata and Chacko 1986].

Caldesmon is a major smooth muscle protein that interacts with calmodulin in a Ca^{2+}-dependent manner and with actin, tropomyosin, and myosin in Ca^{2+}-independent manner[Sobue et al 1981; Walsh 1990]. Smooth muscle caldesmon (Mr 120,000 - 150,000) is a highly asymmetric monomer. It has been considered as an important thin-filament associated regulatory protein. The binding properties and effects of phosphorylation of caldesmon suggest that the protein is probably always bound to the thin filament but can undergo reversible interaction with myosin. Phosphorylation by calmodulin-dependent kinase II (CaM kinase II) has no significant effect on actin affinity[Ngai and Walsh 1987] and phosphorylation by PKC has only a modest effect on actin affinity[Tanaka et 1990]. Thus, rather than being involved in regulation of contraction, caldesmon may play a structural role in smooth muscle. Through cross-linking of actin and myosin, caldesmon may organize the contractile filaments into a three-dimensional network capable of

force development by crossbridges in response to appropriate stimuli[Kerrick et al 1989; Walsh 1991].

Calponin first described as a calmodulin- and F-actin-binding protein[Takahashi et al 1986] is another thin filament-associated, potential regulatory protein in smooth muscle. The protein also: binds F-actin and tropomyosin in a Ca^{2+}-independent manner and calmodulin in a Ca^{2+}-dependent manner[Takahashi et al 1986]; inhibits the actin-activated Mg^{2+}-ATPase activity of smooth muscle myosin; is phosphorylated by PKC and CaM kinase II; and loses the ability to inhibit the actomyosin ATPase upon phosphorylation. Therefore, it is postulated that calponin may play a role in regulating smooth muscle contraction[Winder et al 1991].

The specific protein **phosphatase** responsible for the rapid dephosphorylation of myosin light chain *in vivo* has not been clearly established. There are four general types of serine/threonine protein phosphatases referred to as types 1, 2A, 2B, and 2C[Cohen 1989]. Type 1 protein phosphatase binds to contractile proteins in striated muscle and dephosphorylates myosin light chain *in vitro*[Schlender et al 1989]. Smooth muscle contains multiple forms of protein phosphatases, which have broad overlapping substrate specificities. Bovine aortic smooth muscle myosin light chain phosphatases have been identified as soluble enzymes that have biochemical characteristics of type 2A phosphatase[Cohen 1989]. A multisubunit form of the isolated catalytic subunit of aortic phosphatase 2A dephosphorylates myosin and causes relaxation of skinned fibres from porcine carotid artery[Bialojan et al 1985] and uterine smooth muscle[Haeberle et al 1985]. Gizzard smooth

muscle contains four forms or protein phosphatases. In an attempt to identify the type of phosphatase in smooth muscle, phosphatase inhibitors have been applied. Calyculin A is more effective than okadaic acid in eliciting a contraction in smooth muscle fibres(Ishihara et al 1989). Calyculin A inhibits type 1 protein phosphatase more effectively than type 2, suggesting that type 1 may be the dominant protein phosphatase that dephosphorylates myosin in smooth muscle. Recent work by Cohen and Haystead have pretty well identified the smooth muscle myosin light chain phosphatase.

Cytoskeletal Proteins

The importance of cytoskeletal proteins in vascular smooth muscle was first recognized by Berber et al(1981). They found considerable increase in cytoskeletal filaments in vascular smooth muscle hypertrophy which preceded changes in thick and thin filaments. The major cytoskeletal proteins in smooth muscle are desmin, vimentin, α-actinin, vinculin, actin, gelsolin, talin, filamin, the 23 kDa protein, and nonerythrocytic spectrin. Their function is structural, although it has been suggested that cytoskeletal proteins may be responsible for force maintenance(Rasmussen et al 1987) during contraction.

As pointed out above, evidence from the laboratories of Fay(1984) and Somlyo(1984) suggests that in smooth muscle there are contractile units with a Z-I-A-I-Z element organization although they are not as well organized as in striated muscle.

IV.2. Thick-filament linked regulation: Ca^{2+}-Calmodulin-Myosin Light Chain

Kinase Regulatory Mechanism

Intracellular Ca^{2+} is a primary determinant for myosin light chain phosphorylation since the cell contents of calmodulin and myosin light chain kinase (MLCK) are approximately 40 and 4 μM, respectively, which are much greater than the affinity of Ca^{2+}/calmodulin for MLCK. Binding of four Ca^{2+} ions to one calmodulin molecule into a Ca^{2+}-calmodulin complex followed by binding of the complex to MLCK results in the activation of the latter, which, in turn, phosphorylates the regulatory light chain (20 kDa) on a serine 19 site leading to an increase in actin-activated myosin Mg^{2+}-ATPase activity and crossbridge cycling(Stull et al 1991b). **Figure 7** describes the regulatory mechanisms of smooth muscle contraction and relaxation. It has been proposed that the binding of Ca^{2+} to calmodulin produces conformational changes in calmodulin that expose hydrophobic regions on the surface of the molecule, and these regions may act as active sites for the interaction with target enzymes or calmodulin antagonists(Tanaka 1988). The binding of calmodulin to MLCK causes the removal of pseudosubstrate from the active site so that MLCK may phosphorylate the true substrate MLC_{20}.

The relaxation of smooth muscle is probably brought about by resequestration of calcium and dephosphorylation of the regulatory myosin light chain (20 kDa) by protein phosphatase. Whether or not the activity of protein phosphatase can be regulated remains controversial. Though originally postulated to be constitutively active at maximal rate, it is now believed regulation exists(Kitazawa et al 1991; 1992).

IV.3. Thin Filament Linked Regulatory Mechanism

Although most people agree that the Ca^{2+}-calmodulin-dependent phosphorylation of myosin is the key event in smooth muscle contraction, a considerable effort has been made to identify other regulatory mechanisms such as thin filament associated regulatory mechanism, that modulate the contractile state of the muscle(Kamm and Stull 1989) by altering interaction between actin and myosin. Intact and skinned fibres of smooth muscle can maintain their tension during prolonged stimulation even in the presence of dephosphorylation of myosin(Dillon et al 1981; Aksoy et al 1983; Chatterjee and Murphy 1983; Rembold and Murphy 1990). Such maintenance of force without myosin phosphorylation is felt to be subserved by the so-called latch bridges which are hypothesized to be noncycling or slowly cycling crossbridges. More recently, improvements in the methods of quantification of myosin phosphorylation levels in muscle fibres indicated that low levels of myosin phoshorylation (significantly above resting level) occur in the latch state(Hai and Murphy 1988). Thus, the latch bridges may be explained on the basis that dephosphorylation of an attached crossbridge gives rise to a long-lasting, noncycling latch bridge(Hai and Murphy 1988). However, Siegman et al(1989) showed a nonlinear relationship between myosin light chain phosphorylation and force production such that maximum force output occurred with 50% phosphorylation. Treatment with okadaic acid resulted in inhibition of MLC phosphatase but did not affect the force-MLC_{20} phosphorylation relationship. In skinned chicken gizzard fibre bundles, high levels of tension generated at low levels of MLC_{20} phosphorylation were associated with

high ATPase activities. This is also inconsistent with the latch bridge hypothesis that would require low ATPase activities at low MLC_{20} phosphorylation. Thus, it is possible that the latch bridge may be regulated by other Ca^{2+}-dependent system unrelated to MLCK-MLC phosphatase. There are a number of studies indicating regulatory mechanisms other than myosin phosphorylation, which control the contractile state of the muscle. Smooth muscle relaxation from carbachol[Tansey et al 1990] and K^+ induced[Ashizawa et al 1989] contraction was observed upon addition of okadaic acid accompanied by a marked decrease in $[Ca^{2+}]_i$ while MLC_{20} phosphorylation level remained high. Also, uterine smooth muscle contraction induced by oxytocin occurs in the Ca^{2+}-free medium without myosin light chain phosphorylation[Oishi et al 1991]. High levels of myosin light chain phosphorylation without any active tension development have been observed[Gerthoffer 1987]. Alteration in Ca^{2+} sensitivity of the contractile response might be able to explain the above data and this will be dealt with later.

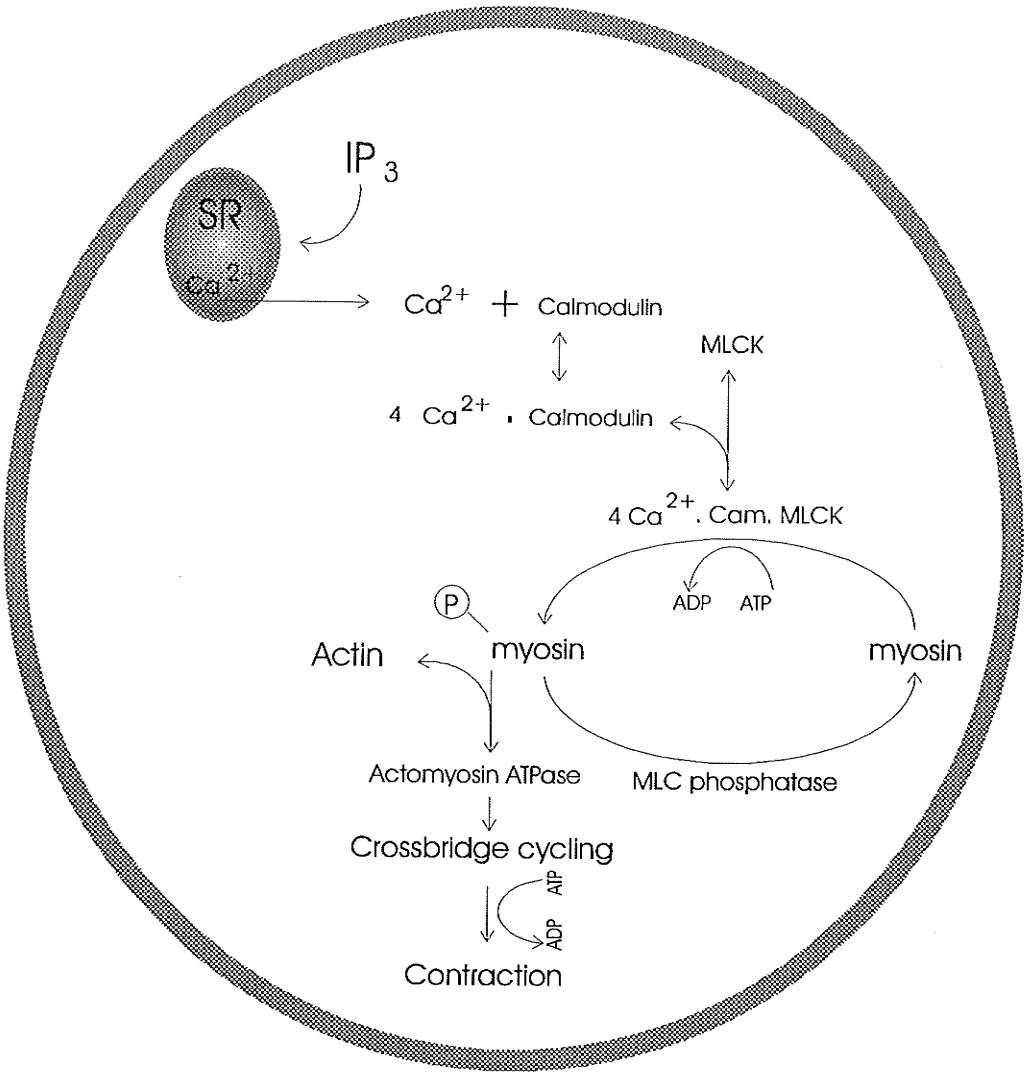


Figure 7. Schematic demonstration of the regulatory mechanisms underlie smooth muscle contraction and relaxation.

IV. 3.1 Role of Calponin

Early evidence suggested that thin filament-linked regulation of smooth muscle contraction also existed, but gave no clear indication of the regulatory molecules involved. In support of a possible role of regulatory role for calponin in smooth muscle, Winder and Walsh[1990] found that isolated calponin inhibited actin-activated myosin Mg^{2+}-ATPase in the *in vitro* reconstituted system composed of actin, myosin, tropomyosin, calmodulin, and MLCK without any effect on myosin phosphorylation. Calponin is a substrate *in vitro* of PKC and CaM kinase II. Phosphorylation by either kinase cancelled calponin-mediated inhibition of the myosin ATPase. Phosphorylated calponin retained the ability to bind to immobilized tropomyosin and calmodulin, but its affinity for F actin was dramatically reduced, suggesting that calponin inhibits the actin-activated myosin Mg^{2+} ATPase through its interaction with actin. A protein phosphatase that is capable of dephosphorylating calponin has also been identified[Winder et al 1991]. A model for thin filament-linked regulatory mechanism involving calponin has been described[Winder et al 1991], which states that at rest, calponin would be in the dephosphorylated state and therefore bound to the thin filament. An increase in free cytosolic [Ca^{2+}] would result in binding of Ca^{2+} to calmodulin and subsequent activation of CaM kinase II, possibly PKC, phosphorylation of calponin and its dissociation from the thin filament. Meanwhile, myosin light chain would be phosphorylated by MLCK and the number of attached cross-bridges and force development would be maximal. Since myosin and calponin are phosphorylated

by different Ca^{2+}-dependent kinases that have different Ca^{2+} sensitivities, myosin could be fully or partially phosphorylated at intermediate $[Ca^{2+}]_i$, while calponin was completely or predominantly dephosphorylated and therefore reassociated with the thin filament. Under these conditions, phosphorylated myosin heads would have limited access to actin if calponin functions by preventing the binding of myosin to actin, and therefore the number of attached crossbridges would be low. Alternatively, if calponin acts to inhibit a step in the ATPase catalytic cycle, the rate of crossbridge cycling and the velocity of shortening of the muscle would be reduced. A close correlation between the level of calponin phosphorylation and the shortening velocity of smooth muscle has also been found(Pohl et al 1991).

IV. 3.2 Role of Caldesmon

There is considerable evidence to show that caldesmon also regulates actin-myosin interactions in several types of *in vitro* actin-myosin preparations(Marston et al 1988). Three possible functions of caldesmon may need to be considered: (1) regulation of actin filament structure. Sobue et al(1985) suggested that unphysiologically high ratios of caldesmon to actin are required for bundling of actin filaments *in vitro*, therefore this may not account for inhibition of actomyosin ATPase activity by caldesmon; (2) formation of the "latch" state whereby force developed in a smooth muscle cell as a result of myosin phosphorylation is maintained while myosin is dephosphorylated. Latch state was postulated to explain the prolonged force maintenance, low energy expenditure, low level of myosin phosphorylation, and an intermediate $[Ca^{2+}]_i$ between resting and maximal

activation[Dillon et al 1981; Silver and Stull 1982; Chatterjee and Murphy 1983]. Caldesmon is an attractive candidate as a regulator of actin-myosin interaction and, the contractile state of the smooth muscle cells since caldesmon is located in the thin filament, it inhibits the actomyosin ATPase, and its effect is regulated by Ca^{2+}. It is suggested that caldesmon is always bound to the thin filament, but its interaction with myosin is regulated by phosphorylation[Walsh 1990]. If latch bridges are formed when caldesmon is dephosphorylated, they could be disrupted upon phosphorylation of caldesmon as the caldesmon-myosin interaction would be lost. The contractile state of the muscle would then be governed by the phosphorylation state of myosin: active force development would occur when myosin is phosphorylated, and relaxation when it was dephosphorylated; and (3) organization of actin and myosin filaments into a co-ordinated contractile network. The possibility that caldesmon plays a more structural role in smooth muscle, perhaps in the organization of actin and myosin filaments into a three-dimensional network is suggested by Hoar and Kerrick[Kerrick et al 1989]. Perhaps the protein-protein interactions serve to hold actin and myosin filaments in register in the muscle so that they have the proper orientation and spatial distribution for effective force development upon stimulation. Although there is evidence to suggest the role of caldesmon and calponin in modulating the myosin Mg^{2+}ATPase activity, the establishment of thin filament linked regulatory mechanism needs further investigation.

IV.4. Protein Kinase C and Smooth Muscle Contraction

As a result of agonists binding to a specific receptor, G proteins activate

phosphoinositide-specific phospholipase C, which catalyses the hydrolysis of membrane phosphatidylinositol 4,5-diphosphate (PIP_2), generating second messengers inositol triphosphate (IP_3) and 1,2-diacylglyerol (DAG)[Berridge 1984]. The lipid-soluble DAG remains within the sarcolemma where it activates PKC[Stabel and Parker 1991]. Phorbol ester, a PKC activator, can induce vascular smooth muscle contraction[Haller et al 1990], which is characterized by a slowly developing, sustained contraction. It has been noticed that PKC activation by phorbol ester results in elevation of $[Ca^{2+}]_i$[Rembold and Murphy 1988] probably because of the phosphorylation of L-type voltage-gated sarcolemmal Ca^{2+} channels, sarcolemmal receptor-operated Ca^{2+} channels, and the SR IP_3 receptor/Ca^{2+} release channels, and the Ca^{2+} extrusion pumps in sarcolemma and SR membrane. Furthermore, phorbol ester induced contraction did not involve a change in $[Ca^{2+}]_i$ or myosin light chain phosphorylation[Jiang and Morgan 1987; Itoh and Lederis 1987]. It has been suggested, for example, that PGF2α induced contraction is partly due to the activation of PKC and phosphatase inhibition[Katsuyama and Morgan 1993]. Nevertheless, the importance of PKC in mediating agonist-induced smooth muscle contraction remains debatable. Interestingly, an increase in smooth muscle MLC_{20} phosphorylation transiently induced by carbachol was accompanied by a slow and progressive increase in the extent of phosphorylation of desmin (α and β), synemin, caldesmon[Adams et al 1989], and four other small-molecular mass unidentified cytosolic proteins[Park and Rasmussen 1986; Takuwa et al 1988]. These proteins become phosphorylated later than does MLC_{20}, and their phosphorylation persists as long as agonist is present. Therefore, it is

postulated[Rasmussen et al 1987] that some of these late-phase phosphoproteins are the mediators of the late or sustained phase of contraction. The fact that intermediate and actin-binding proteins become phosphorylated during the sustained phase of the response raises the interesting possibility that a structural rearrangement of the components of the filamin-actin-desmin (F-A-D) domain, in distinction to the caldesmon-tropomyosin-actin-myosin domain, brings about the sustained change in smooth muscle tension as suggested from ultrastructural studies[Small et al 1986]. The phosphorylation of the late-phase proteins may lead to the stabilization of the F-A-D protein domain in a new configuration such that it is maintained in this configuration even when MLC_{20} phosphorylation ensues[Rasmussen et al 1990]. Nonetheless, the evidence in support of the PKC model is incomplete. The majority of the low-molecular-weight, late-phase phosphoproteins remain to be identified and functionally characterized. On the other hand, one of the identified phosphoproteins, caldesmon, may be of importance in modulating contraction as has been dealt with earlier.

IV.5. Ca^{2+} sensitivity of regulatory proteins

Studies in which force and $[Ca^{2+}]_i$ were simultaneously measured in intact smooth muscle tissue have suggested that the force/$[Ca^{2+}]_i$ ratio is greater in agonist-stimulated as compared to high K^+-stimulated contraction. The modulation of Ca^{2+} sensitivity has been identified not only in intact smooth muscle preparations[Morgan and Morgan 1982; Morgan and Morgan 1984] but also in permeabilized smooth muscle preparations[Kobayashi et al 1989]. Evidence shows that stimulation of receptors

significantly increases myofilament Ca^{2+}-sensitivity of smooth muscle[Nishimura et al 1991].

The inhibitory effect of GDPβS on agonist induced contractions and the preferential requirement of GTP for agonist-induced contraction of the α-toxin permeabilized tissue demonstrates that the enhanced Ca^{2+} sensitivity of contraction following agonist activation is mediated through G proteins[Kitazawa et al 1989; Nishimura et al 1990]. The fact that the α-toxin, permeabilized preparation can be activated by phorbol esters with a similar increase in myofilament Ca^{2+} sensitivity as that elicited by agonist stimulation indicates that PKC may be intimately involved in this process.

Another potential mechanism for changing the Ca^{2+} sensitivity of myosin light chain phosphorylation involves regulation of protein phosphatase activity. The activities of protein phosphatase types 1 and 2A are regulated in other tissue by inhibitory proteins and phosphorylation of inhibitory proteins and regulatory subunits[Cohen 1989]. A G protein may also be involved in regulation of protein phosphatase activity toward smooth muscle myosin. GTPγS increases the sensitivity of the contractile response to activation by Ca^{2+} in permeabilized smooth muscle[Fujiwara et al 1989; Kitazawa et al 1989; Nishimura et al 1988]. Recent experiments have shown that the increase in tension due to MLC_{20} phosphorylation appears to be mediated by inhibition of protein phosphatase[Kubota et al 1990].

Also, purified MLCK from smooth muscle is phosphorylated by cAMP-dependent protein kinase, PKC, and, the multifunctional Cam kinase II. Since

phosphorylation of a specific site (site A) by any one of the kinases desensitizes MLCK to activation by Ca^{2+}/calmodulin, phosphorylation of MLCK could play an important role in regulating smooth muscle contractility. Thus, study of these possibilities was carried out and revealed that phosphorylation of myosin light chain kinase at site A by Cam kinase II may play a role in reported desensitization of contractile elements in smooth muscle to activation by Ca^{2+}(Stull et al 1991). Furthermore, a protein of molecular weight in the range of 15-40 kDa distinct from calponin or caldesmon(Pritchard and Marston 1993), has been suggested to be the Ca^{2+}-sensitizing factor. Nevertheless, the functional role of changing the sensitivity of contractile response to Ca^{2+} remains unclear. It may be useful in obtaining a fine-tuned regulation of smooth muscle contraction.

It should be pointed out that through out this manuscript, the readers will see the concept of **normally cycling crossbridge and latch bridge** defined as the sequentially recruited mechinery. However, there is no evidence so far to support such idea. Therefore, we should deem latch and normally cycling crossbridges as co-existed crossbridges that predominate sequentially. There is also no theoretical justification for partitioning shortening attributable to phosphorylated crossbridges and force latchbridges.

METHODS AND MATERIALS

I. Biophysical Studies

Bronchial smooth muscle preparation

Lungs obtained from 10 mongrel dogs killed by intravenous injection of saturated KCl after pentobarbital anaesthesia were placed immediately in a beaker of ice-cold gassed (95% O_2-5% CO_2) Krebs-Henseleit solution composed (in mM) of 115 NaCl, 1.38 NaH_2PO_4, 25 $NaHCO_3$, 4.7 KCl, 2.46 $MgSO_47H_2O$, 1.91 $CaCl_2$, and 5.56 dextrose. The proximal bronchial airways were dissected from the lung and placed in cold, fresh Krebs-Henseleit solution. Since the muscle fiber orientation may be complex at a bifurcation[Krahl 1964; Macklin 1929], rings (0.7-1.5 mm in diameter) were cut from the non-branching segments of fifth generation bronchi. Bronchial generations were identified according to a method reported by Shioya et al[1987]. Each ring was cut to yield a rectangular strip that was then pinned to the bottom of a silicone tray filled with fresh Krebs-Henseleit solution. The cartilages and connective tissues exterior to smooth muscle in the bronchial wall were carefully removed under a binocular dissecting microscope **(Figure 8)**. The epithelial layer was removed because the removal of airway epithelium augments the responses of canine bronchial smooth muscle to contractile agonists[Aizawa et al 1988; Flavahan et al 1985]. Three muscle strips (tracheal and bronchial with and without cartilages) were obtained from each animal. A tracheal smooth muscle strip was dissected free of connective tissues and epithelium as previously described[Stephens et al 1969]. Each strip was mounted in a muscle bath (60 ml) that contained Krebs-Henseleit solution aerated with a 95% O_2-5% CO_2 mixture to maintain PO_2 of 600 Torr, PCO_2 of 40 Torr, and pH of 7.40 at 37 °C. The lower

end of the strip was anchored to a clamp at the bottom of the muscle bath, and the upper end was connected to an electromagnetic lever system[Brutsaert et al 1971; Mitchell and Stephens 1983] with a 6-mm length of braided 7-0 surgical thread. To obtain a steady level of maximal tetanic isometric contraction (P_o), the strip was stimulated at 6-min intervals by a 14-V 60-Hz current (pulse duration of 16 msec) applied to a pair of platinum wire electrodes positioned 2-mm from the muscle. In this system plate electrodes did not augment the contractile response.

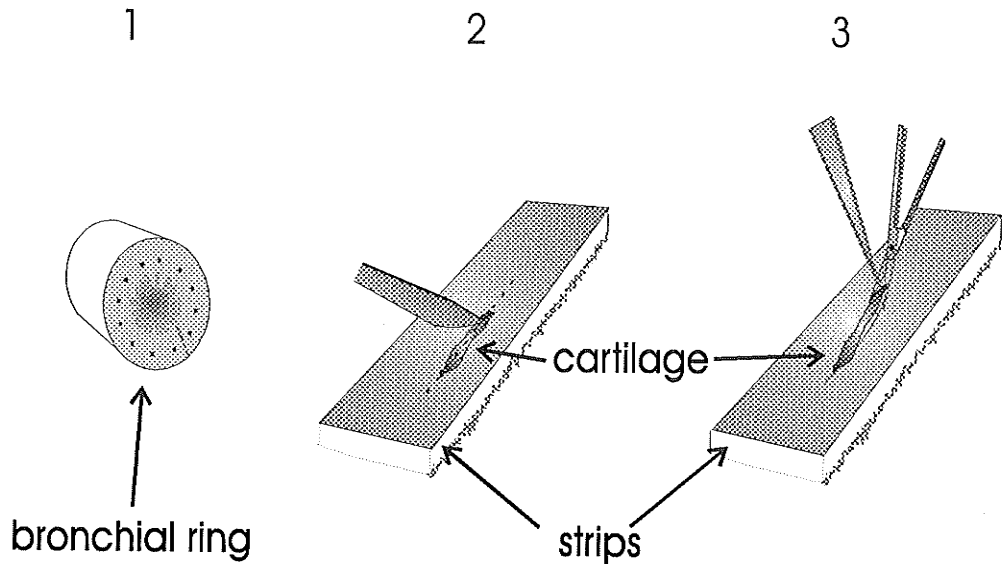


Figure 8. Schematic demonstration of cartilage removal from bronchial strip. Bronchial ring (1) from 5th generation of bronchial was cut open into a rectangular strip (2). Under dissecting microscope, cartilage was isolated from surrounding tissue and removed exteriorly from bronchial strip (3).

Determination of optimal length and maximum isometric force

Muscle strips were equilibrated in Krebs Henseleit solution at 37°C for 2 hrs which is found to be optimal. The strips were not stimulated for the first hour, since it made no significant difference to the ultimate force developed. At the end of equilibrium, force development was measured using a stimulation of voltage-regulated 60 Hz AC current at 5 min intervals until the maximum rate of tension development was steady. The voltage strength employed was 16 v, 10% higher than the voltage that actually elicited maximal muscle response. Conventional length-tension curves were determined as previously described[Stephens et al 1969; Stephens 1985; Jiang and Stephens 1990]. The length at which maximal isometric force (P_o) developed was identified as the optimal muscle length (l_o). A drop in P_o greater than 10% between the beginning and the end of the experiment led to the rejection of the individual data set.

The total compliance of the lever system was 0.2 μm/mN, and the total equivalent moving mass was 225 mg. Because the thread connecting the muscle strip to the lever possessed compliance, the latter was measured for correction of the L-T curve. Average compliance of the thread was 4.2 μm/mN. The time resolution of the lever system was 2 ms, and the sampling frequency used by the computer for recording the force and length signals was 200 Hz.

Determination of average cross-sectional area of muscle strip

This was required for the normalization of force developed by the muscle strip. Because of the small size and weight of the preparation, the conventional method of obtaining cross-sectional area, dividing the net wet weight of the muscle by its optimal length, may not be accurate and measurement errors could be prohibitive. Therefore, a Sony AVC-D5 black and white video camera was

used to capture images of muscle strips at l_o. A calibration grid with 100 μm resolution was placed in the bath beside the muscle strip. The glass wall of the bath incorporated two ports of non-distorting glass allowing the width and thickness of the muscle strip to be clearly visualized. The total cross-sectional area (CSA) of the strip was computed from the average of measurements of width and thickness made at the top, middle, and bottom levels of the muscle strip. In comparison, the CSAs of the same muscle strips were also calculated with the following equation:

$$\text{CSA } (mm^2) = \text{strip vol } (mm^3)/l_o \text{ (mm)}$$
$$= [\text{density } (mg/mm^3) \times \text{strip wet wt (mg)}]/l_o \text{ (mm)} \quad \text{(Eq. 1)}$$

which has been used in previous studies where the density of the smooth muscle strips was taken to be 1 (mg/mm^3)[Stephens 1984; Jiang and Stephens 1990].

The CSA obtained for strips is a composite of that of smooth muscle cells and nonmuscle components. To enable force normalization to smooth muscle CSA alone, the strips were fixed at l_o with formalin fixative for histological analysis. Longitudinal and transverse sections (5 μm in thickness) were made and treated with either haematoxylin-eosin or *Van Giesson* stain to discriminate between smooth muscle cells and other components. The proportion of smooth muscle CSA in the total cross section was determined by planimetry of multiple sections cut from the top, middle, and bottom of the strips. Sections were magnified and projected onto a sheet of paper via a projecting microscope and muscle tissue

outline traced. The tissue outline and the muscle cell component were subsequently cut out and weighed. The ratio of the latter to the former was used to estimate the proportion of muscle in the tissue's cross section. This proportion was applied to convert strip CSA to smooth muscle CSA. In addition, fractional water content of muscle strips was determined by comparing tissue dry wight with tissue wet weight.

Determination of optimal muscle fiber orientation for measurement of force

The maximum isometric tetanic tension (P_o) generated from a series of tracheal and bronchial strips cut at different helical angles, was measured and the transverse cut was found to yield the development of the maximum tension. Since the orientation of muscle fibres in smooth muscle strips is critical for the development of P_o[(Stephens et al 1984)], the arrangement of fibres in the transversely cut bronchial and tracheal strips used in mechanical studies was evaluated using electron microscopy.

Determination of isotonic variables

At 2 and 8 sec, isometrically contracted muscle strips were quickly released to a set of randomly chosen different loads, which were lighter than the maximal tension (P_o) that the muscle strips develop, so that the strips shortened to various extent. This resulted in a rapid transient due to shortening of the muscle's series elastic component (SEC) followed by artifactual oscillations and a slow transient. The maximum slope of the slow transient (the maximum slope in the period between 180-220 msec after quick release) was computed and identified as the maximum shortening velocity of contractile element (CE) for the given load at that time (**Figure 9**). The velocities and their respective loads

expressed as millinewtons per square millimetre of the cross-sectional area of each muscle were fitted with the Hill equation[Hill 1938], which states that $(P + a)(V + b) = (P_o + a)b$, where P is load, P_o is maximal isometric force developed by muscle, V is velocity, and a and b are constants with units of force and velocity, respectively. The maximum shortening velocities under zero load were obtained from the force-velocity curves. Also, the means of velocities and their respective loads, expressed as fractions of their P_o from each group were plotted.

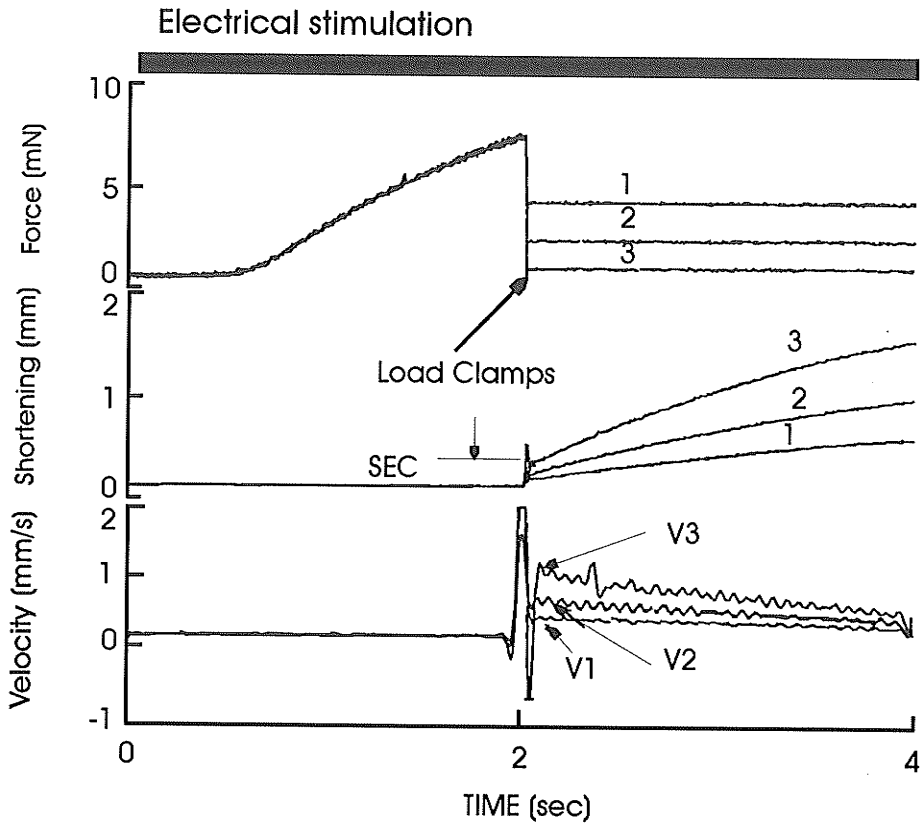


Figure 9. Original recordings of force, shortening, and derived velocity data obtained by quick release to various loads at 2s in a contraction whose contraction time is 10 s, Top: force traces, electrical stimulation, and load clamp. The muscle strip was quickly released to 3 different loads (1, 2, and 3). Middle: corresponding rapid length transient of series elastic component (SEC) recoil and slow transients of shortenings of contractile element (CE). With a customized computer program, maximum shortening velocities of CE were computer directly by obtaining average of 20 data points 180-220 ms after quick release when oscillation caused by quick release had ceased. Bottom: derived shortening velocity (V1, V2 and V3) as a function of time, where maximum shortening velocities of strip under different loads are pointed out by arrows. All the contractions were optimally damped.

The maximum shortening capacities (ΔL_{max}) of the muscles were directly measured from another set of isotonic shortenings under a load equal to the predetermined resting tension of that particular muscle. To determine the proportion of shortening contributed by early, normally cycling crossbridges and latch bridges, respectively, the percent shortenings developed within the first 2 sec, with respect to maximum shortening, were measured from isotonic contraction curves. The reason for studying the shortening development within the first 2 sec is that in the trachealis 75% of the total shortening (ΔL_{max}) is completed in this interval. This indicates that it may be chiefly the normally cycling crossbridges that subserve the shortening function.

The properties of the normal SEC that enable it to transmit force developed by the muscle's contractile element (force and shortening generator) to the outside environment, have been reported for canine tracheal smooth muscle[Stephens and Kromer 1971]. Because alterations in these properties could affect the maximum shortening velocity, it was necessary to examine them in control and sensitized muscles so as to obtain valid measurement of velocity of CE. The quick-release technique described above was used to measure the properties of the SEC (**Figure 9**). The active muscle was abruptly released at the selected time to a series of different loads. The rapid transient, as mentioned above, represented the elastic recoil of SEC. Because of the immediately artifactual oscillations following quick releases, the magnitude of the recoil could not be directly measured. However, the slow transient that followed and stemmed from contractile element shortening was graphically extrapolated backward to the point of intersection on the recoil trace. The magnitude of recoil so obtained was plotted against the isotonic load. The points for the data for the different loads delineated the length-tension curve for

the SEC.

To complete one experiment, a muscle strip would be stimulated approximately 30 times (at 5-6 min intervals). The stability of the muscle during the entire experiment was crucial in obtaining accurate and precise values. The P_o was assessed every 30 min to demonstrate the stability of the muscle. If P_o changed $>15\%$ between the beginning and the end of the experiment, the data were discarded.

Development of a relaxation index

At L_o, the muscle strip under a series of loads was supramaximally stimulated so as to elicit maximum isotonic shortening. The stimulus was turned off at peak shortening and the muscle strips were allowed to elongate. Relaxation depends on load, state of activation, and the length of the muscle's CE at the onset of relaxation. Thus to describe the mechanical characteristics of relaxation, a specific parameter (half relaxation time), which accounts for these variables, was developed. A similar analysis could also be carried out by using the appropriate rate constants for the different curves. The half-relaxation time represents the time needed for the muscle to re-elongate from peak shortening to half of its shortening length under the load (**Figure 10**) borne by the muscle. As this parameter was load dependent, it was plotted against load. A regression equation was fitted to the curve and from this the load-independent (zero load) half-relaxation time was computed.

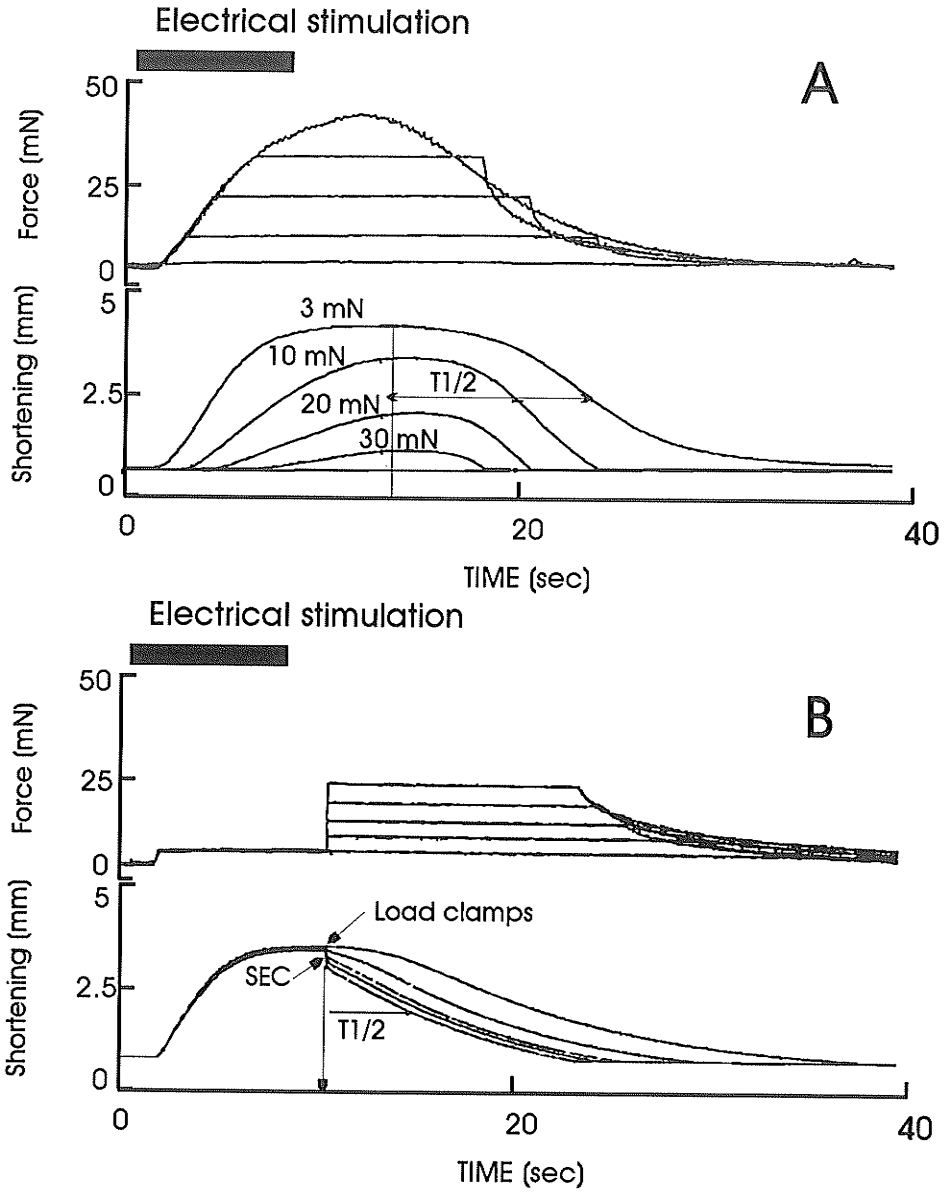


Figure 10. Isotonic relaxation of bronchial smooth muscle after 10 s of electrical stimulation. A: muscle strip was allowed to shorten and relax under different loads. Times needed for muscle strip to elongate to half of its shortening from l_o were designated as $T_{1/2,CE}$. B: muscle strip was allowed to shorten under same light preload (0.05 P_o) and at a specific point in time quickly clamped to different heavier loads. It can be seen that, muscle strips relax faster at heavier loads. Values of $T_{1/2,CE}$ for these relaxation curves were obtained and plotted against their respective loads.

Although the half-time of relaxation yielded a load-independent index, it must be pointed out that for each of the relaxation curves in **Fig. 10**, because of the different loads on the muscle, the initial lengths of the CE at the onset of relaxation were also different. This produced a second source of variability in the index of relaxation for which adjustment had to be made. To eliminate the effect of the varying initial CE lengths the muscle was allowed to shorten from L_o bearing the preload (0.05 P_o) required to set it at that length. At a desired time point a series of load clamps were applied (**Fig. 10**). The application of the load clamp at the same point in time for each contraction ensured that the muscle strip started to relax at the same CE length. The relaxation trajectories for the different load clamps are shown in the upper panel of **Figure 10**. The fast transients represent re-elongation of the series elastic component of the muscle, and the slow transients represent re-elongation of CE from the same initial length. The half-times for these slow transients under their respective loads were fitted with an exponential function (a coefficient of determination, R^2, of 0.9888 and $P < 0.01$ indicating a good fit) and the zero load half-time was extrapolated from the plot as shown in **Figure 10**. The coefficient of the equation gave the relaxation index, $T_{1/2,CE}$, which is load- and initial contractile element length-independent.

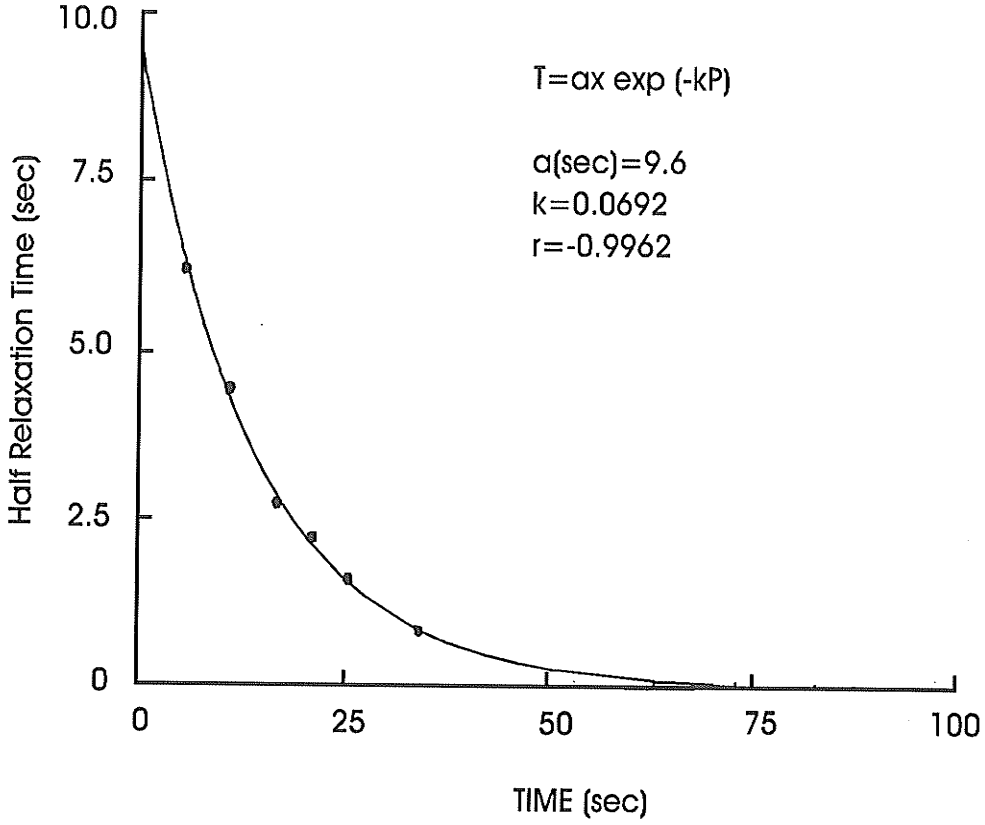


Figure 11. Typical $T_{1/2,CE}$ vs load curve fitted with exponential function. Coefficient of equation, (a), gives value of $T_{1/2,CE}$, which is a load- and initial contractile length-independent index of isotonic relaxation.

The muscle strips from sensitized and control bronchial smooth muscle were allowed to shorten isotonically under the preload, and then, during relaxation, quickly clamped to loads ranging from a preload (equal to resting tension at L_o) to P_o. To assess the relaxation properties of early normally cycling crossbridges and latchbridges separately, two load-clamping time points were chosen. First, the muscle strips were stimulated isotonically for only 1 sec and abruptly clamped to various loads at the 2-sec point to test the relaxation from early normally cycling crossbridges. Second, the muscle strips were stimulated for 10 sec and then released to different loads at 11 sec. This was intended to test the latchbridge relaxation as we have shown that the shortening velocities are quantitatively different at these two time points[Jiang and Stephens 1990]. Measurements were made from the relaxation curves with a computer program, and the half-times of relaxation under different loads were fitted into an exponential relationship (as shown in **Figure 11**) from which $T_{1/2,\,CE}$ values were obtained.

Muscle relaxation has been described as inactivation of the active state of the muscle[Brutsaert et al 1978]. This inactivation was estimated by quickly releasing the relaxing muscle strip to zero load at different points in relaxation period during isometric and isotonic contractions (**Fig. 12**). The zero load shortening velocity of the contractile element (the slow transient after quick releases in **Fig. 12**) is a good index of crossbridge cycling rate[Bárány 1967; Close 1972; Dillon et al 1981]. Therefore, shortening velocities after quick releases at different time points during relaxation were obtained when muscle strips were stimulated for 10 s for the estimation of the crossbridge cycling rate during isotonic relaxation.

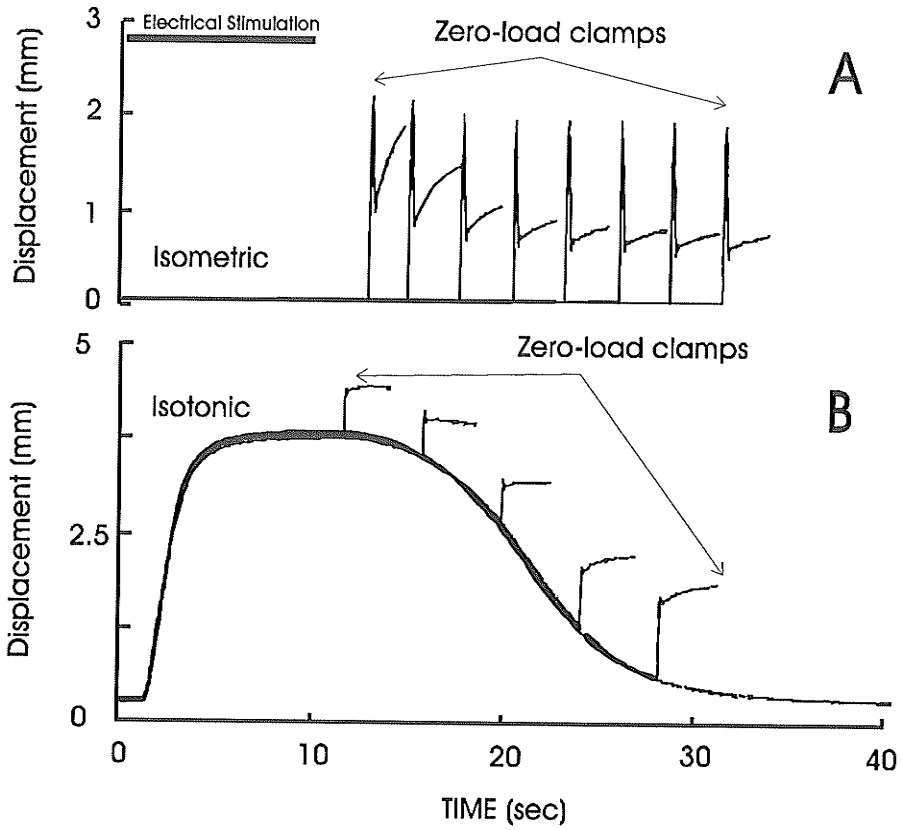


Figure 12. Zero-load velocities (V_o) during isometric and isotonic relaxation. A: isometrically contracted muscle strip was quickly released from afterload to zero load at different points in time during relaxation. V_o (maximum slope of slow transient after quick release) decreases with time after turning off the stimulator. B: zero load clamps were applied at various points after lightly loaded isotonic contraction. V_o decreased for the first 5s and then started to increase without any external stimulation, indicating a spontaneous reactivation of crossbridge cycling.

II. Biochemical Studies

Contractile protein quantification

Protein Extraction. Tracheal and fourth to sixth-order bronchial smooth muscle tissues stored frozen following dissection were used to extract myosin and actin. All the extractions were performed at 0-4 °C. Tissue was minced with scissors and homogenized in a Potter Elvehjem tissue grinder with 4 volumes of extraction buffer [40 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 20 μM leupeptin, 1 mM dithiothreitol, 250 μM phenylmethylsulfonyl fluoride, pH 8.8]. Crude extracts were centrifuged (15,000 g, 30 min), and the supernatant proteins were quantified[Bradford 1976] and then denatured with SDS-sample buffer[Laemmli 1970]. The pellets that remained after the initial tissue extraction and centrifugation were further extracted by resuspending them in SDS-sample buffer and heated at 95 °C for 15 min. Pellet extracts were then centrifuged (15,000 g, 10 min), and the supernatant was removed and filtered through 0.45 μm Nylon-66 membranes to remove unpelleted debris. No extractable myosin or actin remained in the pellets after this treatment.

Electrophoretic analysis/Western blotting. To quantify actin and myosin heavy chains, SDS-polyacrylamide electrophoresis (PAGE) was carried out according to the procedure of Laemmli[1970] using 4-20% linear polyacrylamide gradient separating gels with 3% stacking gels. Gels were stained with Coomassie blue R-250. Myosin heavy chain isoenzyme stoichiometry was assessed using 4% SDS-PAGE minigels (8 × 10 cm), which were stained using a commercial silver stain kit (Rapid-Ag-Stain, ICN Biomedicals, Cleveland, OH). Band identity of myosin and actin was confirmed by Western blotting[Towbin and Gordon 1984]. Blots were

incubated in PBS (1.3 mM NaH_2PO_4, 9 mM Na_2HPO_4, 500 mM NaCl, pH 7.6) containing either 5 μg/ml rabbit anti-chicken thrombocyte or fibroblast myosin IgG to confirm that the heavy chain bands were not non-smooth muscle in origin[Groschel-Stewart et al 1975; Groschel-Stewart et al 1985]. Blots were developed using goat anti-rabbit IgG conjugated with horseradish peroxidase, diluted 1:2000, as per manufacturers instructions (ICN ImmunoBiologicals, Cleveland, OH).

Laser densitometry. Myosin heavy chain and actin contents of supernatant and pellet extracts were determined by quantitative densitometry of the 4-20% SDS-PAGE gels using an LKB Ultroscan XL laser densitometer. With the use of this densitometer, entire widths of specific protein bands were scanned by consecutive parallel and adjacent passes, which covered 40 μm path width each sweep. Data for each scan was captured by using Gel Scan XL 2400 software. Absorbances within individual scanning profiles were collected, and customized software, **Scanplot**, was used to convert the raw data into X- and Y-plane positional data and Z-axis into absorbance data. After subtraction of background absorbance, integration of the three-dimensional raw data yielded a volume (absorbance units $\times$ mm^2) that corresponded to a specific amount of stained protein in a given band. Quantification was made possible by comparing densitometric profiles of myosin heavy chain and actin bands with those of known amounts of bovine serum albumin (BSA). The amount of myosin or actin in a given sample was therefore estimated by comparison with the band volumes of the BSA standard. BSA was run at several concentrations as an internal standard on each gel and subjected to an identical staining-destaining-storage regime as the tissue unknowns. The relative dye binding capacities of MHC, BSA, and actin were determined as described by Sutoh[1983]. For standards we used myosin and

actin purified from chicken gizzard[Walsh et al 1983; Ngai et al 1986]. The total amount of myosin heavy chain and actin in the supernatant and pellet extracts were summed and then normalized to the original wet weight of the tissue sample to obtain an indication of the content in either tracheal or bronchial tissues. In addition to the quantification of myosin heavy chain, the relative contents of the two isoforms of myosin heavy chain (MHC_1 and MHC_2) were determined by laser densitometry of sample lanes from the 4% SDA-PAGE mini-gels.

Determination of myofibrillar ATPase activity

Smooth muscle preparation. Dogs were sensitized to ragweed pollen according to methods described by Kepron and colleagues[1977]. Newborn littermate dogs were randomly divided into sensitized and control groups. The sensitized group received, intraperitoneally, ragweed pollen injection once a week until the day of experiment. The controls received adjuvant only. In previous studies[Kepron et al 1977; Becker et al 1989], sensitized dogs with passive cutaneous anaphylaxis titers > 1:64 developed marked increases in specific airway resistance upon bronchoprovocation with ragweed extract aerosol, as well as greater response to acetylcholine challenge. Eight sensitized and eight control dogs, 6 to 8 months of age, were killed with i.v. injection of saturated KCl after pentobarbital sodium anaesthesia. Smooth muscles from the third to sixth generation of bronchi and trachea were dissected out and stored at -70 °C for biochemical analysis.

ATPase assay. The activity of actomyosin ATPase was determined as described previously[Stephens et al 1991] in myofibrillar homogenates from sensitized and control TSM and BSM. Myofibrils were prepared according to the method of Sobieszek and Bremel[1975].

To initiate the reaction, Mg^{2+}-ATP (2 mM final concentration in 1 ml

assay medium) was applied to each assay tube containing homogenates. The reaction was stopped with the addition of trichloroacetic acid (TCA) at 10 sec to obtain maximum ATPase activity. The concentration of inorganic phosphorus (P_i) produced by myosin ATPase was obtained using the malachite green method of Lanzetta and associates[1979] with several modifications[Anner and Moosmayer 1975; Itaya and Ui 1966]. Absorbency was read at 620 nm with a PYE Unicam SP 1800 Ultraviolet Spectrophotometer. Aliquots of KH_2PO_4/10 % TCA were employed each time for preparation of a standard curve, which showed a linear increase in absorbency up to 18 nmol of KH_2PO_4.

The rates of ATP hydrolysis by actomyosin ATPase were standardized to myosin heavy chain (MHC) content in myofibrillar homogenates as described[Stephens et al 1991]. MHC content was obtained using a method described above[Jiang et al 1991]. Briefly, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separated MHC bands were scanned with a laser densitometer (Ultroscan XL, LKB) connected to a computer, which reconstructed the bands' images and quantified MHC contents by comparison with bovine serum albumin standard on the same gel. The activity of ATPase was then expressed as P_i liberated in nmol/μg MHC/min.

Determination of MLC phosphorylation

The 20 kDa regulatory myosin light chain (MLC_{20}) monophosphorylation was measured employing a method modified from those of Hathaway and Haeberle[1985] and Persechini et al[1986]. TSM strips were dissected free of connective tissues. In a 50 ml organ bath, the strips weighing 4 to 7 mg were allowed to equilibrate in Krebs Henseleit solution for 1 hr and then quickly frozen by immersion in a dry ice acetone slurry (-80 °C) containing TCA solution. The

two points in time at which strips were frozen were at rest and at 4 to 5 sec after onset of stimulation; this represents the time when electrical stimulation elicits maximal values of MLC phosphorylation[Kamm and Stull 1985]. The frozen strips were treated in a TCA/acetone mixture (5%, wt/vol, -80 °C) for 1 hr and in 10 mM 1,4 dithiothreitol/acetone (at room temperature) for another hour. The strips were frozen with liquid nitrogen and pulverized with a Crescent dental amalgam mixer for 30 sec and were then mixed in urea sample buffer containing 6.4 M urea, 17 mM Tris, 19.5 mM glycine, 10 mM 1,4 dithiothreitol, 0.04% bromophenol blue, 10 mM EGTA, 1 mM EDTA, 5 mM NaF, and 1 mM pheylmethylsulfonyl fluoride for 1.5 hr at room temperature. Sample tubes were centrifuged at 15,000 $\times$ g for 15 min.

The non-denaturing 10% polyacrylamide urea gel method, modified from those developed by Hathaway and Haeberle[1985] was employed to separate the monophosphorylated and unphosphorylated MLC_{20}, as the two possessed different electrical charges. The running tank buffer contained 20 mM Tris, 22 mM glycine, 1 mM sodium thioglycate, and 1 mM 1,4 dithiothreitol. All gels were subjected to pre-electrophoresis for 1 hr at 350 v. A crude extract of 50 μl sample was applied to each well. Electrophoresis was conducted at 350 v for 15 to 18 hrs at 15 °C and was stopped 1 hr after the bromophenol blue tracking dye exited the gel. The gels were then stained with a silver staining kit (ICN Biomedicals). The band identity was confirmed by Western blotting using anti-MLC_{20} monoclonal antibody (Sigma Immunochemicals).

Proteins were transferred to nitrocellulose sheets under buffer solution containing 25 mM Na_2HPO_4. The blotting was conducted at 27 v, 15 °C for 1 hr. Nitrocellulose sheets were shaken in blocking buffer of 10 mM Tris saline, 5%

bovine serum albumin, and 0.1% gelatin for at least 2 hrs and then were rinsed 3 times (3 min each) with Tris saline buffer + 0.1% Tween 20 (TBS-T) solution. The sheets were incubated in a monoclonal mouse IgM-anti-smooth muscle MLC_{20} antibody solution (1:500; Sigma Immunochemicals) for at least 1 hr before and then washed 3 times with TBS-T. Amersham blotting detection kit (RPN 22) for mouse antibodies (biotinylated, species-specific) was used for staining the blots. This method yields highly specific binding of MLC_{20} (**Figure 13**).

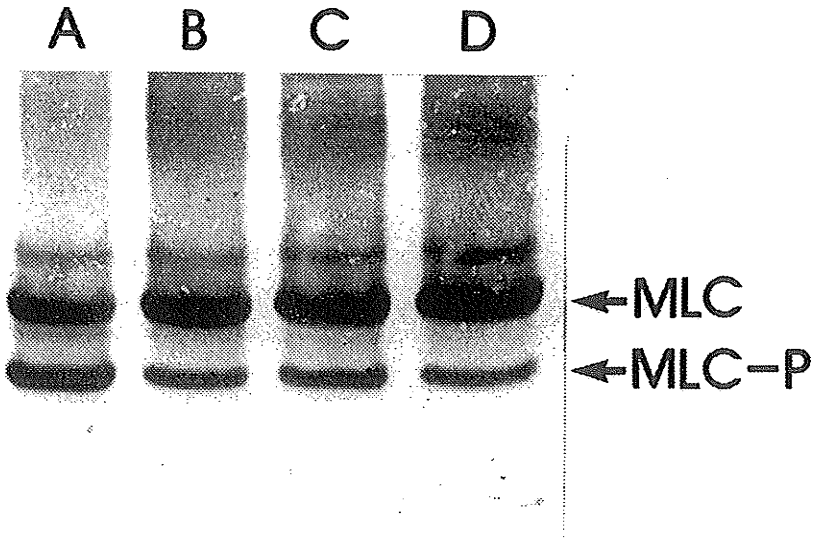


Figure 13. Western blot of phosphorylated and unphosphorylalated 20 kDa myosin light chains. Lane A: sensitized tracheal smooth muscle (TSM) at 4 s contraction; Lane B: sensitized TSM at rest; Lane C: control TSM at 4 s contraction; Lane D: control TSM at rest. MLC: unphosphorylated myosin light chain; MLC-P: monophosphorylated myosin light chain. The biphosphorylated band is only faintly seen in Lane A. The sensitized smooth muscle clearly demonstrates greater MLC_{20} phosphorylation both at rest and at 4 s after onset of electrical stimulation.

The developed nitrocellulose sheets were rendered transparent with a plastic embedding method described below (Pharmacia LKB Biotechnology). Polyvinyl chloride write-on films for the overhead transparency were used to sandwich the nitrocellulose sheets. Benzoin methyl ether (0.5 g) dissolved in 25 ml trimethylol propane trimethacrylate (2-ethyl-2-(hydroxymethyl)-1,3-propanediol trimethacylate) was applied to one half of the film. The nitrocellulose sheet and the other half of the film were then placed on it. The embedded sheet was pressed with a roller to squeeze out the air bubbles between the plastic films. The sandwich was then irradiated with long wave UV light for 3 min for each side to polymerize the sheet. The embedded transparent sheets containing band images were then scanned with an LKB laser densitometer and the images of MLC were analyzed using methods similar to those described before. The ratio of monophosphorylated to total MLC_{20} was obtained and expressed as the level of MLC phosphorylation.

MLCK quantification

MLCK content in tissue homogenates of sensitized and control TSM and BSM was obtained by quantitative 7.5% mini SDS-PAGE. The protein loads for both control and sensitized groups were the same (5, 10, and 15 μg in respective lanes). Bands of MLCK were identified and quantified by laser scanning and comparison with a smooth muscle MLCK standard obtained from chicken gizzard[(Walsh et al 1983)] on the same gel. As it was possible that bands identified as MLCK might consists of other proteins, Cleveland peptide mapping was performed in our preliminary experiment. The primary structure of MLCK bands was similar to that of MLCK standard, indicating that there was no significant contamination by other proteins in the MLCK band. The intensity of laser

scanning signal was linearly correlated with the loading of MLCK standards over range of 0 to 4 μg in our preliminary experiment. The identity of the bands was also confirmed by Western blotting employing a monoclonal anti-smooth muscle MLCK antibody (Sigma Immunochemicals). MLCK contents were determined by two-dimensional laser densitometry as described before. Digital data derived from the scanned bands' images were stored in a computer, and a custom derived programme enabled me to obtain the volumes of the bands. This technique is superior to one-dimensional scanning in that it analyzes the whole band instead of one peak, the latter having the potential of overestimation or underestimation of the band. Three-D scanning is now a standard procedure for us and has been proven reliable.

Determination of MLCK activity

TSM and BSM strips were homogenized in a buffer containing 20 mM imidazole, 60 mM KCl, 1 mM cysteine, 1 mM $MgCl_2$, 1 mM ouabain, 10 mM sodium azide, 0.001% leupeptin, 0.25 mM phenylmethylsulfonyl fluoride, 1 mM 1,4 dithiothreitol, and 2 mM $CaCl_2$. Myosin light chain phosphorylation is regulated by MLCK and myosin phosphatase, which can be experimentally inhibited by okadaic acid[Bialojan et al 1988]. In order to evaluate the roles of MLCK and myosin phosphatase separately, 5 μM okadaic acid (Molecular Probes) was added to each assay tube. Three samples from each group were also assayed without okadaic acid. Phosphorylation of MLC_{20} was initiated by adding Ca^{2+} ($CaCl_2$) and [γ-^{32}P]ATP (specific activity: 10 Ci/mmol; final concentration: 5 μM; NEN Research Products) into each tube and was stopped by ice-cold 10% TCA at 10 sec, by which time a time study had demonstrated the maximum level of MLC_{20} phosphorylation had been achieved. The samples were centrifuged at 13,000 $\times$

g for 30 min. The pellets were treated with acetone to eliminate TCA and to disrupt membranous structures, and pellets were solubilized in the same urea sample buffer as described before. The samples were centrifuged for 15 min after being fully mixed in the sample buffer. No traceable MLC_{20} was found in supernatant. The same non-denaturing urea gel as described above was employed to separate the monophosphorylated and unphosphorylated myosin light chains.

Monophosphorylated MLC_{20} bands containing radioactive phosphorus were excised after silver staining using a silver staining kit (ICN Biomedicals) and were digested in 0.5 to 1 ml NCS solubilizer (9:1, NCS:H_2O; Amersham) at 50 ^{0}C for 2 h. Acetic acid was added to neutralize NCS base. Scintillation solution (5 to 10 ml) was added to each counting vial and the activity was counted in an LKB Wallace 1216 Rackbeta Liquid Scintillation Counter. The activity and specific activity of MLCK were then expressed as the incorporation rate of ^{32}P into MLC/mg fresh tissue weight/min and the incorporation rate/μg MLCK/min, respectively.

MLCK peptide mapping

In order to compare the primary structure of MLCK from sensitized and control ASM, a peptide mapping method described by Cleveland and co-workers[Cleveland et al 1977] was employed. Homogenates from both groups were first electrophoresed in a 7.5% SDS-PAGE gel for 3 hr. MLCK bands, stained with Coomassie blue, cut out with a razor blade on a light box and then soaked for 30 min in 10 ml of an equilibration solution containing 0.125 M Tris/HCl, ph 6.8, 0.1% SDS, 10% glycerol, bromophenol blue, 0.03% β-mercaptoethanol, and 1 mM EDTA. The bands were then placed in the wells of a second 4-10% SDS gel (1.5 mm thickness) with a 3% stacking gel. Gel slices were further overlaid with

equilibration solution containing either 20 ng of Staphylococcus aureus V8 protease or 100 ng of chymotrypsin. The digestion of MLCK was enhanced in the stacking gel during the subsequent electrophoresis, turning the power off for 30 min after all samples had entered the gel. The gels were stained using a silver staining kit (ICN Biomedicals), and peptide maps were then scanned with an LKB laser densitometer and the profiles from both groups were compared.

MLC_{20} phosphorylation was also estimated during muscle isometric and isotonic contractions of the smooth muscle. Twenty to thirty muscle strips from each canine trachea were divided into isometric and isotonic groups and then equilibrated in Krebs-Henseleit solution for an hour before contractions were allowed. After determining l_o, we rapidly froze the strips at different points in time during isometric and isotonic contraction. The frozen tissues were then processed using the same method described above to obtain myosin light chain phosphorylation.

III. Measurement of Time Course of Intracellular [Ca^{2+}] during Isometric And Isotonic Contractions

Since the catalytic subunit of MLCK is activated by elevation of intracellular Ca^{2+}, and the binding of a $4Ca^{2+}$-calmodulin complex to MLCK, the intracellular Ca^{2+} concentration during both kinds of contractions was determined using a Muscle Research System (MRS, Scientific Instruments, Heidelburg, Germany, **Fig. 14**), which monitored the Ca^{2+} indicator fura-2 fluorescence signal, and isometric contraction or isotonic contraction signals simultaneously. The system is controlled by customized computer software. A muscle strip less than 1 mm in width and 4 mm in length was incubated in 250 μl Krebs Henseleit solution containing 8 μM of fura-2 AM (Mol Probes, Eugene, OR) for 2-3 hr at 30-37 °C before it was mounted into a quartz cuvette (3 ml) between the two tweezers connecting a force transducer at one end and a servo-motor at the other. Preheated, aerated Krebs Henseleit solution was constantly perfused through the cuvette. The muscle strip was stimulated every 6 min with an electrical stimulus (frequency=30 Hz; pulse width=3 ms; and 5 volts). Such parameters elicited submaximal muscle response (0.5 P_o) with a minimum generation of bubbles within the cuvette that could interfere with the emission light and fluorescence signals. The optimal muscle length (L_o) that generated the maximal response (P_o) was determined by changing the muscle length with the help of the servo-motor control. With the computer software, the MRS system allowed the load on the muscle to be changed at any desired time point within 5 ms. The servo-motor secured isotonic shortening under any pre-adjusted load.

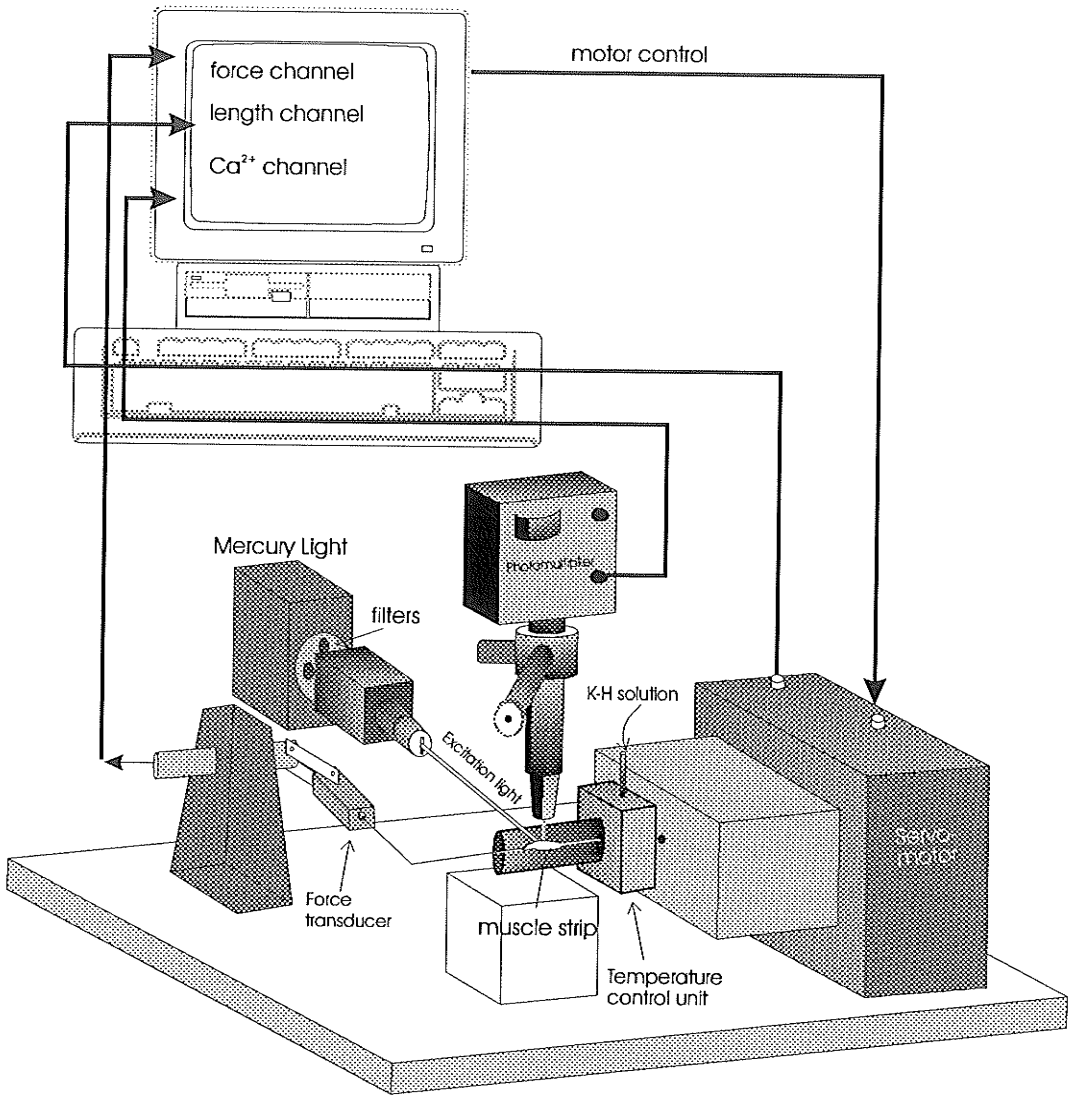


Figure 14. Schematic drawing of Muscle Research System (Scientific Instruments, Heidelburg, Germany). The system allows measurement of muscle force, length, and intracellular Ca^{2+} simultaneously. An IBM computer enables the data to be collected and stored. It also controls servomotor in the events of isotonic shortening.

The fluorescence signals excited at 340 and 380 nm by a mercury burner were received by a photometer and a monitor that were connected to signal amplifiers and sorters, which then sent out the ratio of the two wave lengths fluorescence to the data processing computer. The latter stored these data and allowed the analysis to be carried out later. The Ca^{2+} concentration was obtained by calibrating the changes in the ratio of 340 to 380 nm wave length against the Ca^{2+} standard using a calibration kit (Mol Probes, Eugene, OR). In order to delineate the mechanisms underlying smooth muscle activation, different kinds of stimulation including acetylcholine, KCl, histamine and electrical were employed under both isometric and isotonic conditions.

IV. Statistical Analysis

Group results were expressed as means $\pm$ SEs, which were compared using unpaired two-tailed Student's t test with P set at 0.05. A one way analysis of variance (fixed-constants model I) was performed on data obtained from the Hill equation. Individual means were compared using Duncan's new multiple range test, with P set at 0.05. The least significant difference method was used to compare values between sensitized and control as well as between 2 and 8 sec.

RESULTS

I. Bronchial Smooth Muscle Preparation

Morphological examination

As shown in **Figure 15A**, the smooth muscle cells lie subjacent to the epithelium. They are not attached to the overlying cartilage plates directly but by loose connective tissue. This anatomic arrangement facilitated removal of cartilage without muscle bundle damage. As shown in **Figure 15B**, the dense outlines (basal membranes of the outer layer of muscle cells were intact, illustrating that the muscle cells were not damaged. **Figure 16 (A and B)** shows cross sections of TSM and BSM. Analysis using planimetry demonstrated that smooth muscle tissue occupies 77, 30, and 12.9% of the total cross-sectional area of TSM, BSM, and BSM + C (cartilages), respectively. Upon removal of cartilage, the total cross-sectional area of bronchial strips was reduced by 57%. Morphometric study also indicates the parallel arrangement of smooth muscle fibres in tracheal and bronchial strips.

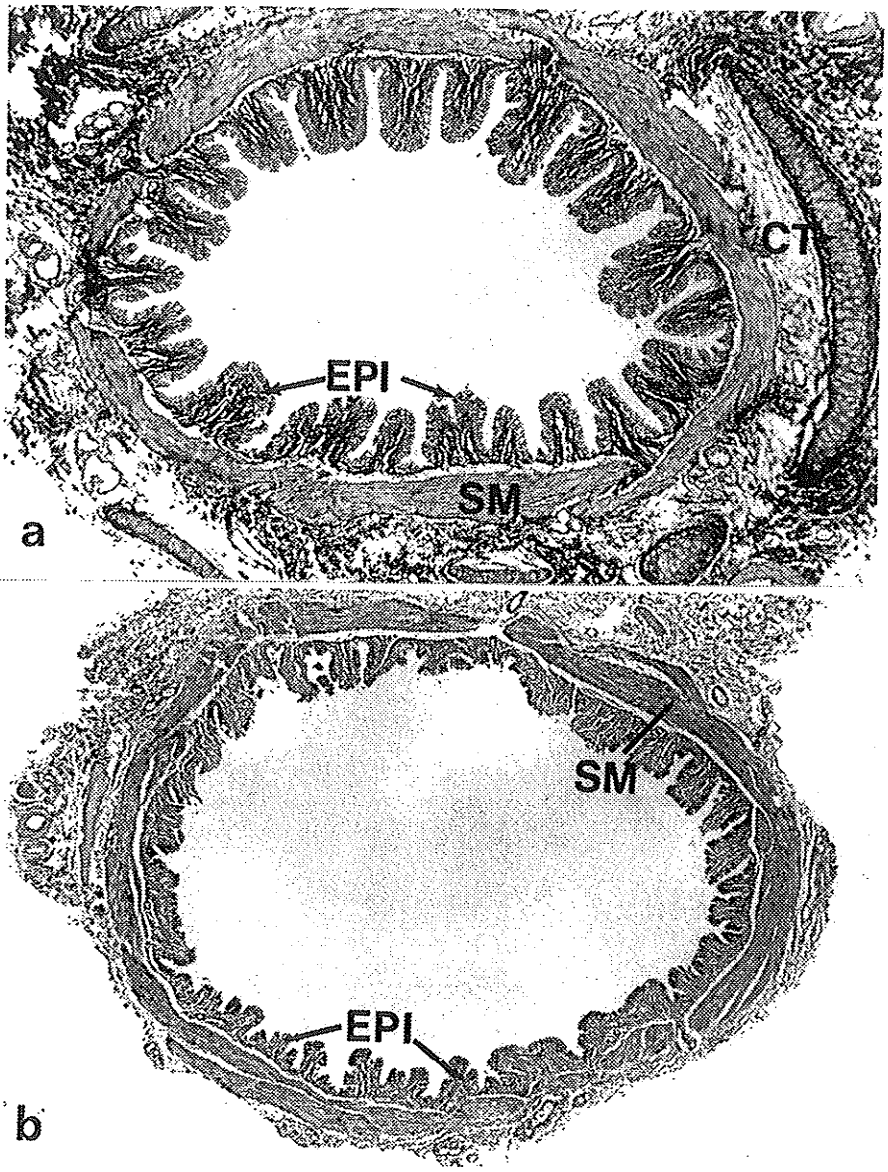


Figure 15. Histological sections of 5th order canine bronchi. A: before removal of cartilage, smooth muscle layer is located right beneath epithelium and does not directly attach to cartilage outside. B: after removal of cartilage, muscle layer is unimpaired. SM: bronchial smooth muscle; EPI: epithelium; CT: cartilage. Hematoxylin and eosin stain, ×200.

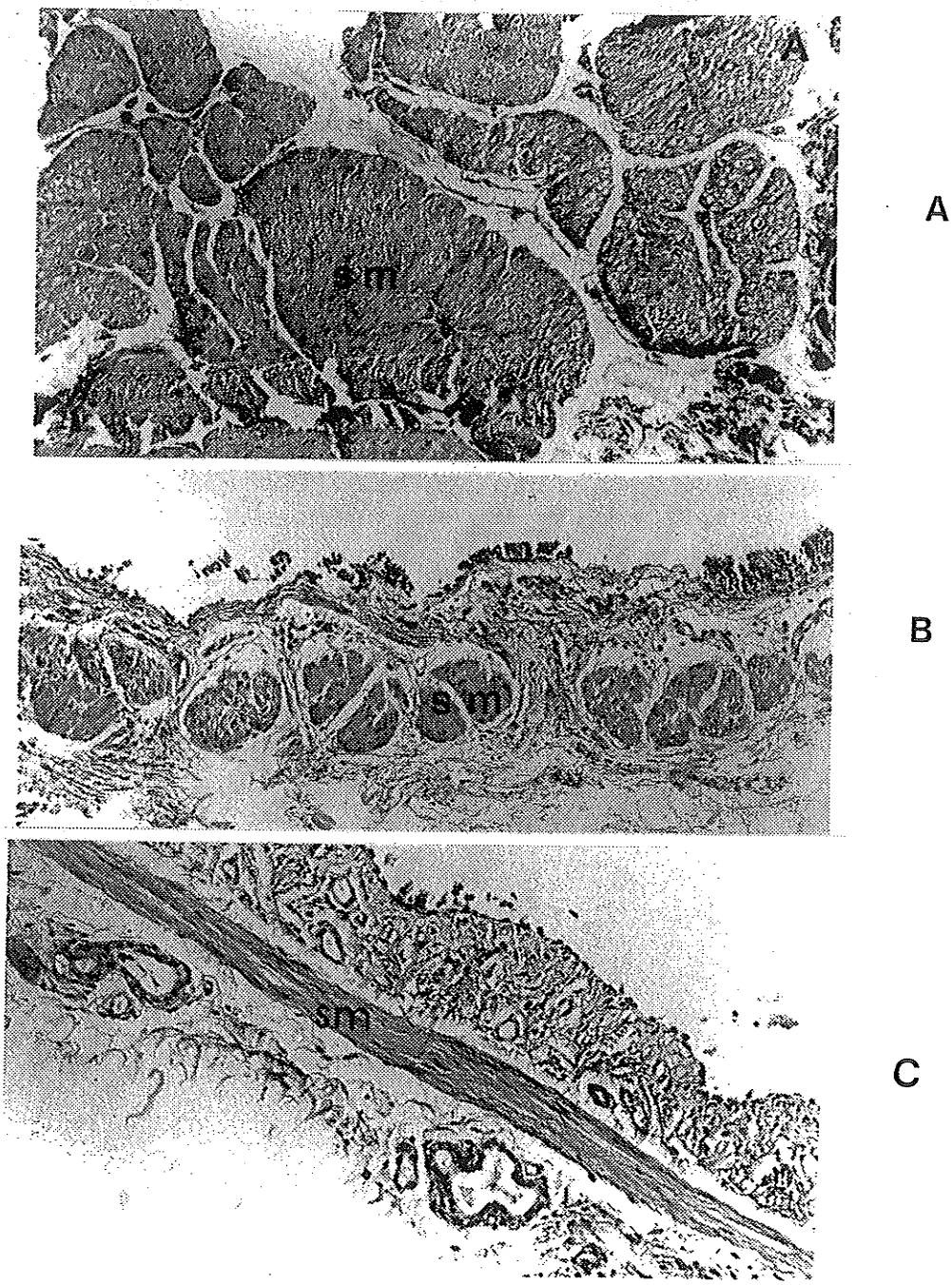


Figure 16. Cross sections of TSM (A, ×210) and BSM (B, ×200). Smooth muscle tissue occupied 77 and 30% of total cross-sectional area of strips for TSM and BSM, respectively. C: longitudinal sections of BSM (×200) showing parallel arrangement of smooth muscle fibers.

Length-tension relationship

Figure 17 shows the L-T relationships obtained from isometric contractions of cartilage-free TSM and BSM strips. The tensions developed by the tissue were normalized with respect to the area of microscopically identified muscle cells in the cross section. There was a considerable and significant difference between the two L-T curves. P_o generated by TSM was 18.2 $\pm$ 0.81 (SE) $\times$ 10^4 N/m^2 (n = 10), whereas that generated by BSM was 7.1 $\pm$ 0.19 $\times$ 10^4 N/m^2 (n = 10). As illustrated in **Figure 18**, the BSM and BSM + C showed no difference in force development. The active tension was 6.8 $\pm$ 0.21 $\times$ 10^4 N/m^2 for BSM + C. The resting tension, at a length of 1.1 L_o for BSM + C, was significantly higher than that of BSM (**Figure 18**). **Figure 19** shows that ΔL_{max}s were 0.76 $\pm$ 0.03, 0.41 $\pm$ 0.02, and 0.24 $\pm$ 0.02 L_o in TSM, BSM, and BSM + C, respectively. There are significant differences ($P < 0.05$) among the values of ΔL_{max} of the three groups as judged by **Duncan's new multiple-range test.**

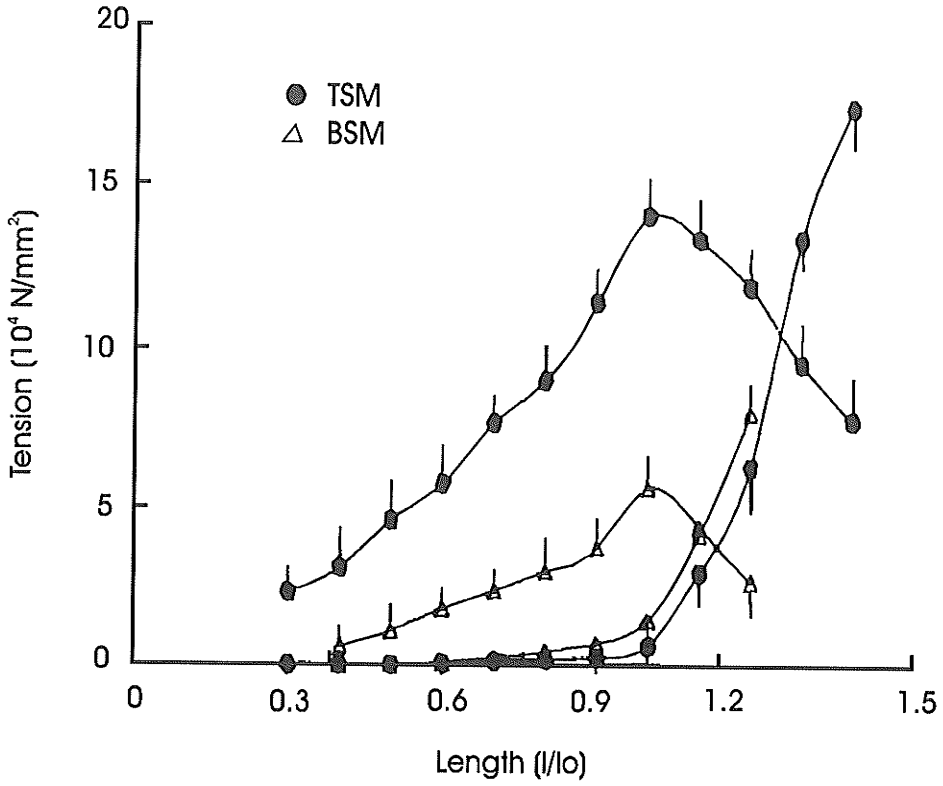


Figure 17. Superimposed length-tension curves of TSM and BSM. Tensions of muscle strips are expressed as $\times$ 10^4 N/m^2 cross-section of histologically identified muscle tissue. Lengths are normalized by dividing actual strip length by L_o. Active tensions of the 2 groups differed, whereas resting tensions were similar. a: active tension; r: resting tension.

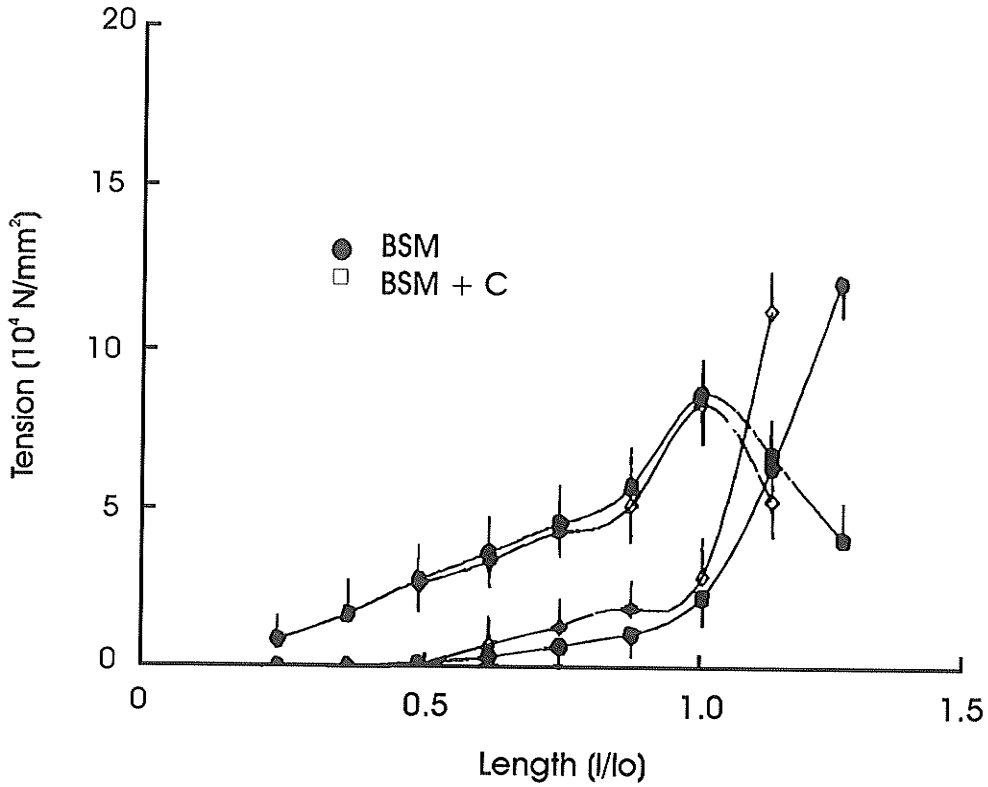


Figure 18. Length-tension curves of BSM without and BSM with cartilages (BSM+C). The two groups showed no difference between active and resting tensions.

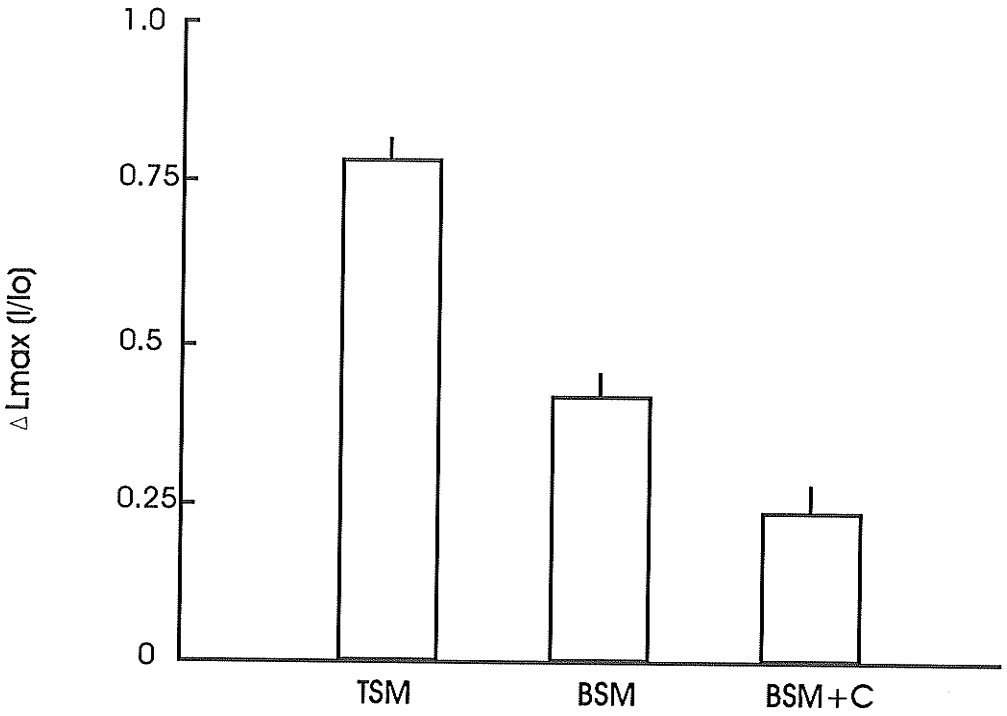


Figure 19. Maximum shortening capacities. Isotonic shortening (ΔL_{max}) of TSM was significantly greater than that of BSM. After removal of cartilage, BSM showed greater shortening than BSM+C.

. *Force-velocity properties*

Figure 20 displays the typical F-V curves of TSM and BSM at various time points. The linearized transform of the Hill equation was fitted to the data. Goodness of fit is shown by the r^2 values in **Figure 20**; they are significant ($P < 0.05$). The V_o's in the three groups at 2, 4, and 8 sec, obtained by the quick-release method, are shown in **Figure 21**. The quick releases were applied during the course of an isometric contraction to allow the muscle to shorten at various time points. All three groups demonstrated highest velocities at 2 sec, and all were slowest at 8 sec. Significant differences in V_o's were found among the three groups at all time points. In **Table 1**, muscle constants obtained from quick-release force-velocity experiments are shown. Constant *a* at 8 sec tended to be higher than at 2 sec, whereas *b* tended to decrease from 2 to 8 sec. The constant *a/b* increased significantly from 2 to 8 sec in all three groups. Constant *a* and *b* were asymptotes of X and Y axes with force and velocity units, respectively.

Table 1. Constants derived analysis of force-velocity data using the Hill equation

	TSM (n=8)		BSM (n=7)	
	2 sec	8 sec	2 sec	8 sec
a,mN/mm^2	11.06±1.51	12.20±1.96	12.88±1.56	14.46±1.65
b,L_o/s	0.052±0.008	0.040±0.009	0.064±0.007	0.032±0.006
a/P_o	0.197±0.003	0.195±0.004	0.206±0.004	0.201±0.003
a/b	246.9±37.97	385.1±71.38*	209.9±22.66	503.3±67.02*
$1/b$	24.44±5.083	38.89±10.31	16.99±1.733	31.17±5.351

Constants are means ± SE. TSM, tracheal smooth muscle; BSM, bronchial smooth muscle. * $P<0.05$, denotes significant differences compared with values at 2 seconds.

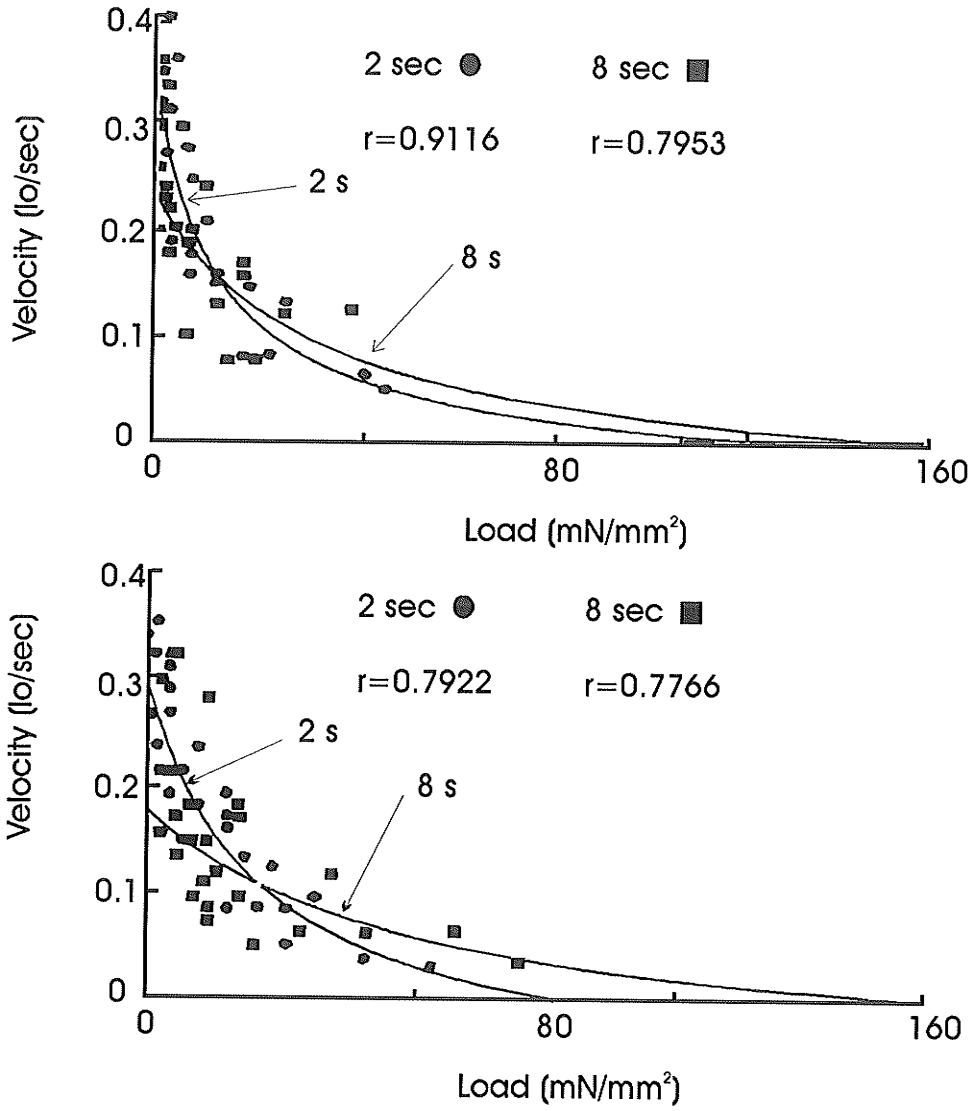


Figure 20. Force-velocity curves for TSM (A, n=6) and BSM (B, n=7) at 2 and 8 s of isotonic contraction. Analysis of variance indicated that r^2 values were high ($P < 0.05$)

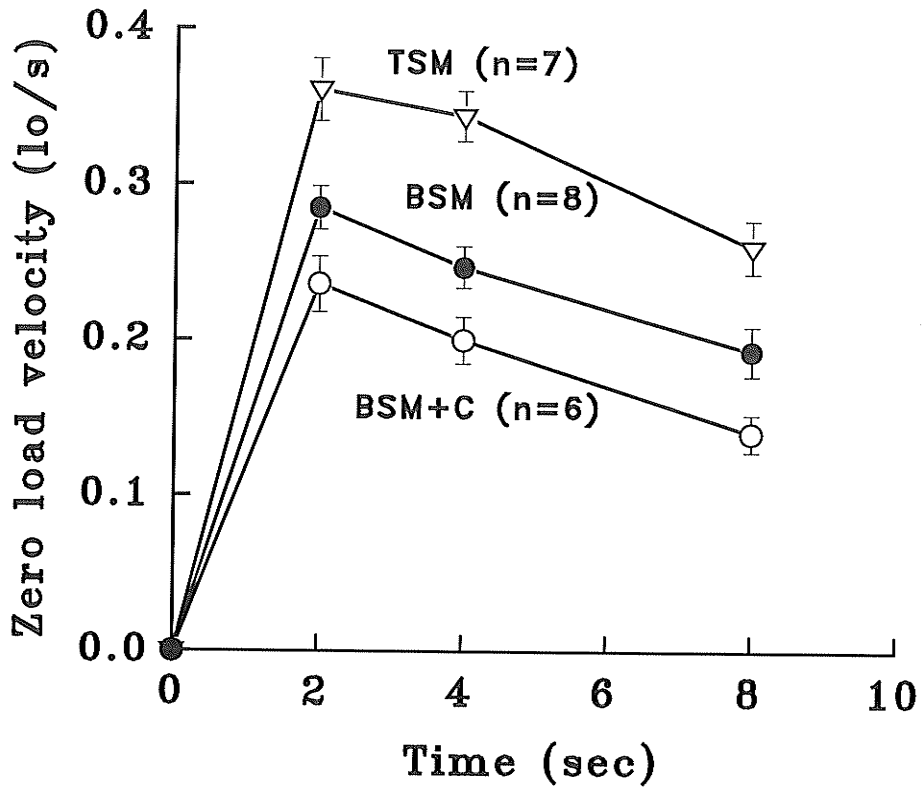


Figure 21. Changes of maximum shortening velocity as a function of time. All 3 groups showed a decline in V_o from 2 to 8 s. BSM demonstrated smaller values of V_o than TSM at 2, 4, and 8 s. BSM+C shortened more slowly than BSM at 2, 4, and 8 s.

II. Normalization of Force Generated by Airway Smooth Muscles

Morphological measurement

Photographs of cross sections of tracheal and bronchial strips obtained by light microscopy have already been shown in **Figure 16**. There was no statistical difference in the percent water content of tracheal and bronchial strips (**Table 2**). The mean total tissue cross-sectional areas, determined using the video camera system, for tracheal and bronchial strips were 0.299 ± 0.015 and 0.243 ± 0.019 mm^2, respectively, and were not statistically different (**Table 2**). In addition, when total tissue cross-sectional areas were calculated for the same strips using **Eq. 1**, the values obtained were not significantly different from those obtained via the video camera. On the contrary, the area identified

Table 2 Mechanical and morphometric data for TSM and BSM strips

	TSM	BSM	P
L_o, mm	7.16±0.468	7.50±0.54	0.28
P_o, mN	44.82±5.03	8.54±1.55	<0.01
CSA_T,mm^2	0.299±0.015	0.243±0.019	0.13
CSA_M,mm^2	0.23±0.012	0.082±0.006	<0.01
CSA_M/CSA_T	0.792±0.014	0.30±0.009	<0.01
Water content (% wet wt)	85.1±7.7	85.8±9.2	0.56

Values are means ± SE of 9 dogs. L_o, optimal length; P_o, maximal active force; CSA_T, total cross tissue cross-sectional area; CSA_M, muscle cross-sectional area.

as muscle in the total cross section of tracheal strips (0.23 $\pm$ 0.012 mm^2) was significantly greater ($P < 0.01$) than that in bronchial strips (0.082 $\pm$ 0.006 mm^2) (**Table. 2**). Hence the ratio of the area occupied by smooth muscle cells to the total cross-sectional area in tracheal tissue strips (0.79 $\pm$ 0.014) was significantly greater ($P < 0.01$) than in bronchial tissue strips (0.30 $\pm$ 0.009) (**Table 2**). Light and electron-microscopic analysis also showed that muscle fibres were oriented parallel to the longitudinal axis of the bronchial strips used in mechanical studies (**Figure 22**).

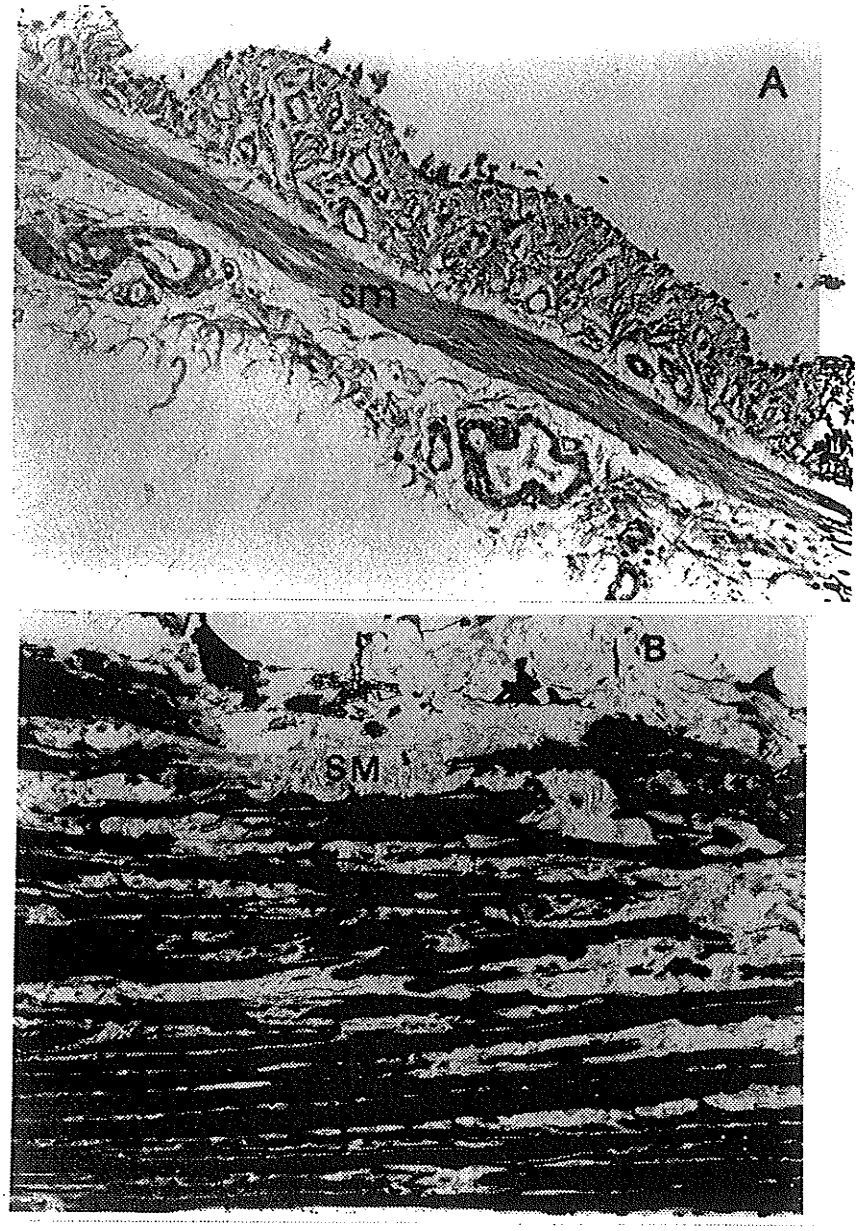


Figure 22. Longitudinal view of bronchial smooth muscle strip. A: light microscopy of hematoxylin-eosin-stained 5th generation bronchial strips (×198). B: an electronmicroscopic longitudinal section (×2,080) of bronchial smooth muscle fixed at optimal length. Parallel arrangement of bronchial smooth muscle cell fibers is confirmed.

Contractile protein quantification

The binding of Coomassie blue R-250 stain to BSA, myosin heavy chain, and actin standards in SDS-PAGE was linear for loads up to 10 μg of protein per lane. Linear regression analysis of dye binding (Sutoh 1983) yielded relative dye binding capacities of 0.88, 0.46, and 1 for BSA (r^2 = 0.9907), myosin heavy chain (r^2 = 0.9868), and actin (r^2 = 0.9959), respectively. MHC (200-204 kDa) and actin (43 kDa) bands were well resolved from other proteins using the 4-20% gradient gels employed (**Figure 23**).

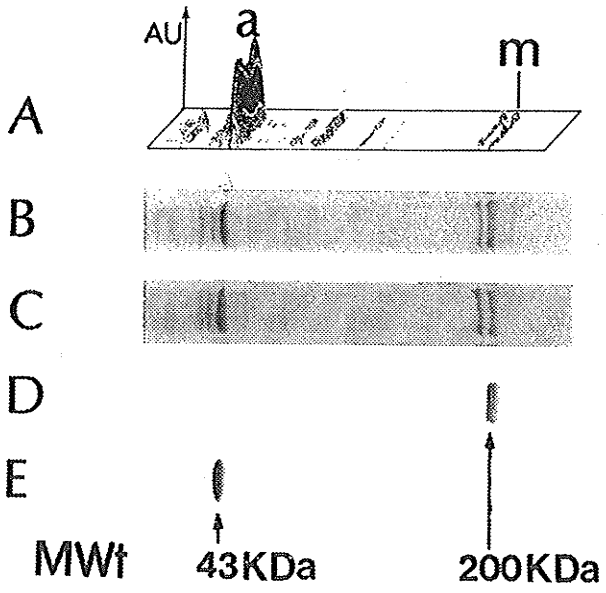


Figure 23. Typical 3-dimensional laser densitometric tracing of bands obtained by 4-7.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a tracheal sample lane (A) and actual Coomassie-stained gel patterns of tracheal (B) and bronchial (C) crude homogenates (sample loads = 10 μg). Purified chicken gizzard myosin heavy chain (D) and actin (E) are also illustrated (sample loads, 1.5 and 1.0 μg, respectively). AU, absorbance unit; a, actin; m, myosin heavy chain.

Table 3. Contractile protein values for tracheal and bronchial smooth muscle

	TSM	BSM	P
myosin, mg/g tissue	1.30±0.14	0.61±0.15	<0.05
actin, mg/g tissue	2.10±0.22	1.12±0.19	<0.05
actin:myosin, tissue	1.62±0.23	1.83±0.13	0.18
MHC_1:MHC_2, tissue	1.36±0.11	1.23±0.09	0.25
myosin, mg/g muscle	1.65±0.18	2.04±0.50	0.09
actin, mg/g muscle	2.68±0.27	3.72±0.62	0.08
actin:myosin, muscle	1.62±0.18	1.82±0.17	0.11

Values are means ± SE of 9 dogs. Actin:myosin ratios are by weight (mg/mg). MHC_1 and MHC_2, myosin heavy chain isoenzymes.

Positive immunodetection of MHC bands was displayed on Western blots using antibodies specific for smooth muscle MHC (not shown), however, no detection was shown using non-muscle (platelet and fibroblast) myosin antibodies. These results confirm that the MHC band was derived from the smooth muscle component of the tissues. The 4% SDS-PAGE minigels confirmed the existence of two MHC isoenzymes (MHC1 and MHC2) in both tracheal and bronchial tissues (**Table 3**). The bands corresponding to MHC were clearly distinguishable from other bands on both the 4% and 4-20% gradient gels, indicating that contaminating proteins[De Marzo et al 1987; Kawamoto and Adelstein 1987] had been separated. Tracheal strips possessed approximately 2.2 times as much myosin heavy chain as bronchial strips (**Table 3**).

The difference appears to be correlated with the difference in muscle cross section (CSAM)/tissue cross section (CSAT) (**Table 2**) between the tracheal and bronchial strips. This was indicated in that MHC content per unit muscle cell was not statistically different between tracheal and bronchial smooth muscle (**Table 3**). Quantitative measurement also revealed that the same situation exists for muscle cell actin content (**Table 3**). Data from densitometric analysis of the 4% SDS-PAGE minigels revealed that the relative ratio of MHC1:MHC2 in bronchial and tracheal tissue was not statistically different (**Table 3**).

Force generation and normalization

Fig. 24A shows stress-strain curves for tracheal and bronchial smooth muscle strips where force has been normalized with respect to total CSAT (tissue stress) determined with the video camera system. Tissue stress obtained from tracheal strips was 4.27 times that of bronchial strips (**Table 4**), and this difference was significant ($P < 0.01$). In **Figure 24B**, force has been normalized

to the proportion of smooth muscle in the total tissue cross-sectional area and represents muscle stress. Tracheal strips developed 1.60 times the muscle stress of bronchial strips, which represents a significant difference (**Table 4**). The stress-strain curves with force normalized to mean tissue MHC content, and myosin stress, are depicted in **Figure 24C**. Tracheal strip myosin stress was only 2.16 times that of the bronchial strips, a significant difference ($P < 0.05$) (**Table 4**). The 1.6-fold difference between tracheal and bronchial muscle stress was not statistically different from the 2.16-fold difference between tracheal and bronchial myosin stress ($P = 0.11$).

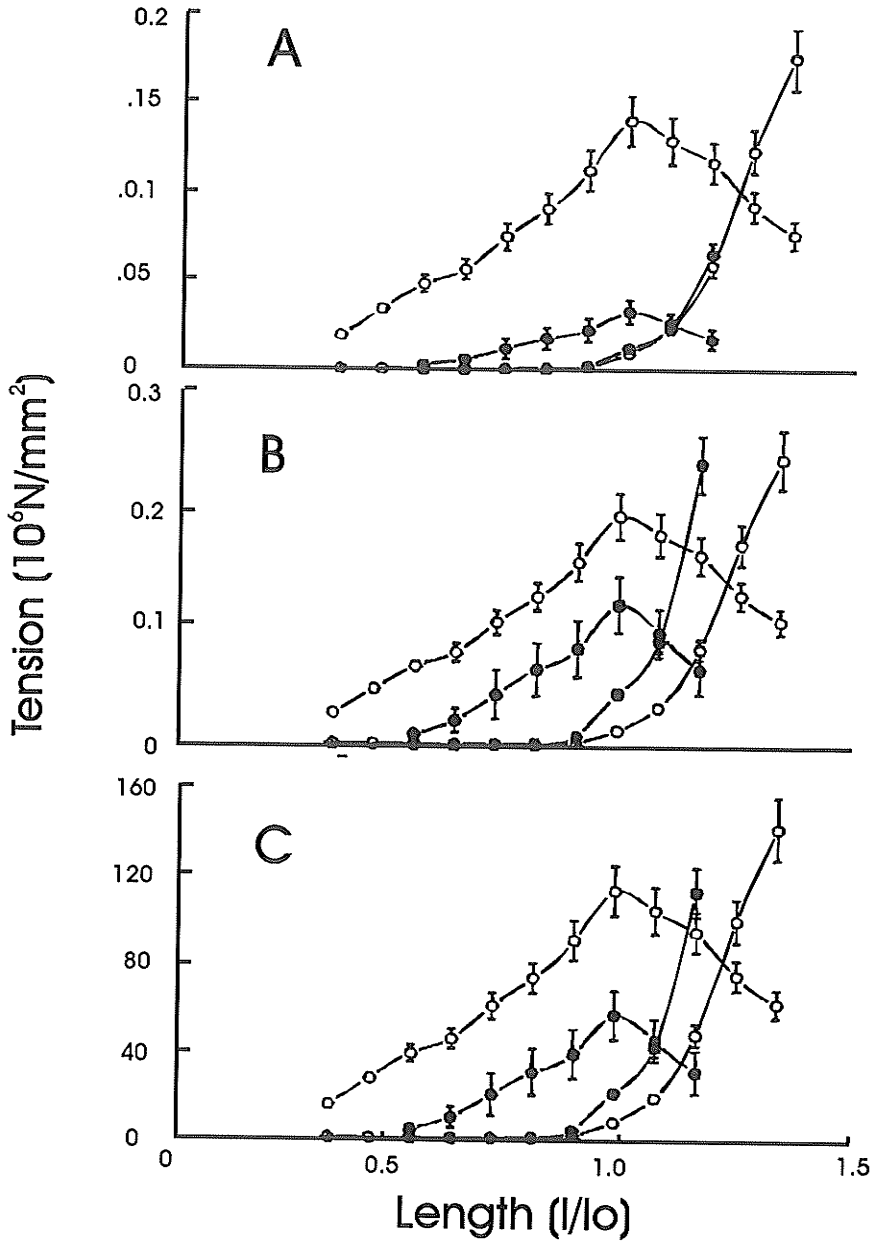


Figure 24. Length-tension relationships of tracheal (open circles) and bronchial (filled circles) smooth muscle computed employing different methods of normalization. A: active and resting tensions normalized with respect to total cross-sectional area of total strip were plotted against relative lengths of the tracheal (TSM) and bronchial (BSM) muscle strip (tissue stress). B: tensions were normalized with respect to actual muscle proportions in the muscle strips (muscle stress). C: tensions were normalized with respect to myosin heavy chain concentration of the tissue cross-section (myosin stress). Although the initial difference in unadjusted tension between TSM and BSM was 5.24-fold, after normalization with respect to tissue cross-sectional area (tissue stress) this difference was reduced to 1.60. Normalization with respect to myosin cross section as a fraction of total tissue cross section yielded a difference of 2.16-fold.

Table 4. Stress normalization using different parameters

	Tissue (CSA)	Muscle (CSA)	Myosin (CSA)
TSM	1.499 ± 0.1	1.893 ± 0.12	1153.08 ± 76.15
BSM	0.351 ± 0.05	1.171 ± 0.15	533.43 ± 75.99
P	< 0.01	< 0.05	< 0.05

Values are means $\pm$ SE; Stress is measured $\times 10^5$ N/m^2.

To demonstrate that tissue weight is not a suitable parameter for normalization of force (even at the expense of belabouring the obvious) as used by others[Shioya et al 1987; Mapp et al 1989], an experiment was conducted in which a single strip of canine tracheal smooth muscle was dissected out and cut into two smaller strips. The length of strip 1 (**Figure 25A**) was 8 mm and that of strip 2 was 4 mm. The figure shows that though their lengths were dissimilar, as were their weights (5.8 mg vs 2.8 mg), the maximum force was the same. The baselines in the figure were displaced with respect to each other simply to enable separation of the two records. In **Figure 25B** strip 3 was 2 mm wide while strip 4 was 1 mm wide; their length were the same. The records confirmed that force is a function of cross-sectional area rather than total weight of the muscle tested.

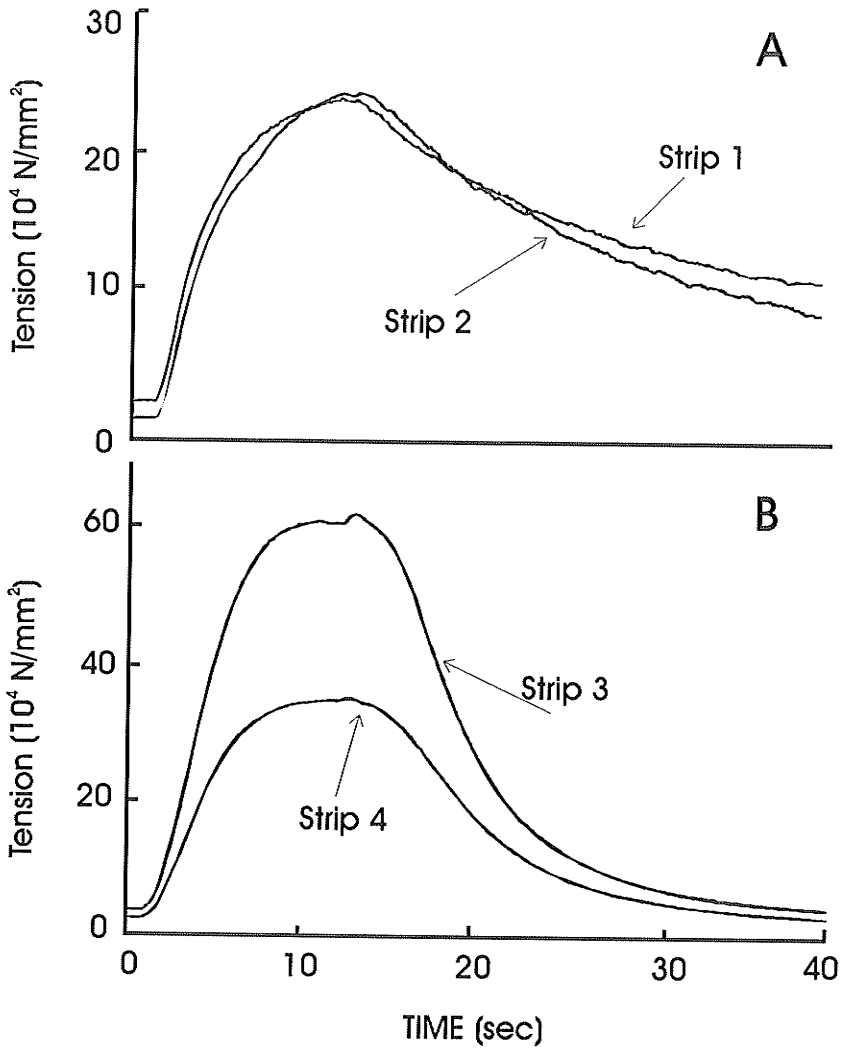


Figure 25. (A) Isometric myograms of strips 1 and 2 of canine tracheal smooth muscle. Strip 1 has a length of 10 mm and strip 2 only 5. The two showed similar force production ability; (B) Isometric myograms of strips 3 and 4. Strip 3 is twice the thickness of the strip 4. The former generated twice the force of the latter. This suggests that it is the cross-sectional area, rather than the length of the muscle that determines the force generation.

III. Mechanical Properties of Bronchial Smooth Muscle from Sensitized and Control dogs

Isometric studies

Length-tension relationships of the two groups are shown in **Figure 26**. No significant difference was found between sensitized and control when forces were normalized with respect to smooth muscle cross-sectional area in the total tissue cross section. The values of P_o for the two groups were not significantly different. The slopes of the two resting tension curves are also similar, indicating operatively similar static properties between sensitized and control bronchial smooth muscles at rest.

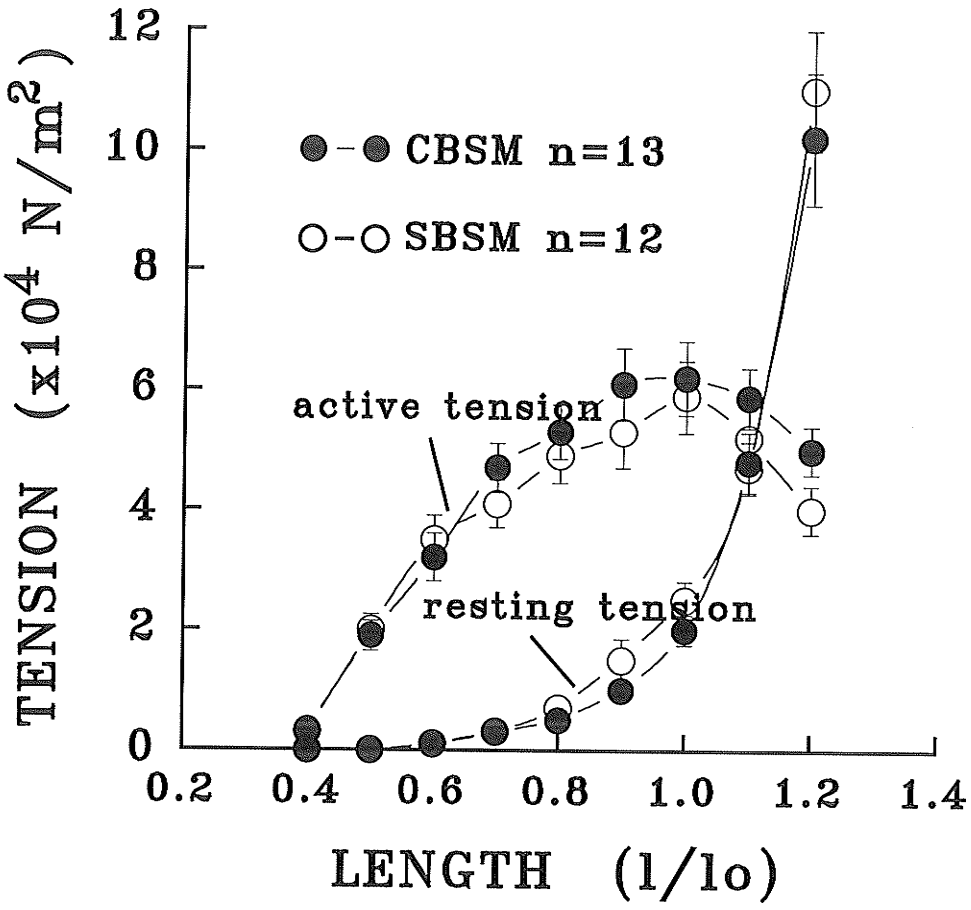


Figure 26. Length-tension relationships of sensitized (SBSM) and control bronchial smooth muscle (CBSM). Active and resting tensions are shown. Both groups demonstrated similar length-tension relationships. Maximum active tensions (P_o) are similar for both groups as well. Note that stresses were normalized to muscle fraction of total tissue cross-sectional area. L_o, optimal length.

Isotonic studies

Force-velocity relationships. **Figure 27** depicts the force-velocity curves for sensitized and control bronchial smooth muscle (SBSM and CBSM, respectively) at 2 and 8 sec. The force-velocity relationships were fitted with the **Hill equation** from which the muscle constants were obtained (**Table 5**). SBSM demonstrated greater maximum shortening velocity than the control at 2 sec ($P < 0.05$). Both groups showed higher shortening velocities 2 sec after the onset of stimulation than at 8 sec ($P < 0.05$). Also the value of *a/b*, reported to be an index of internal resistance to shortening[Stephens and Kromer 1971], was lower in SBSM than in CBSM.

The maximum elongations of SEC when the muscles developed their P_o's were 0.087 ± 0.004 and 0.087 ± 0.004 (SE) of L_o in CBSM and SBSM, respectively. No statistical difference was found between them. Furthermore these values are not statistically different ($P > 0.05$) from those we have previously reported for the trachealis[Stephens and Kromer 1971].

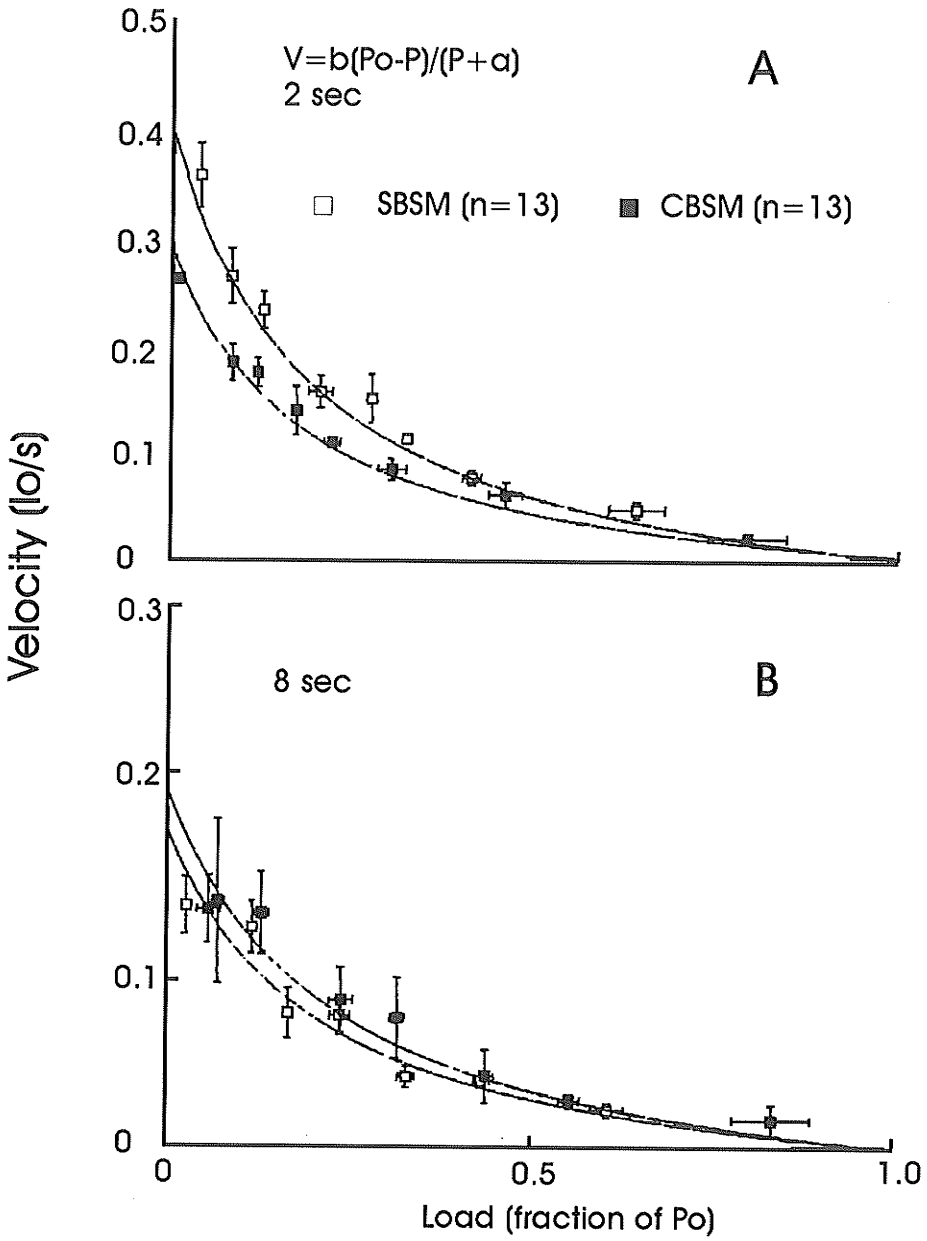


Figure 27. Mean force-velocity data elicited from SBSM and CBSM by quick-release load clamping technique. Mean shortening velocities are normalized with respect to optimal length per second; and their respective loads are expressed as fractions of P_o's, 2s (A) and 8s (B) after onset of stimulation. At 2s, sensitized groups showed greater maximum shortening velocity (V_o) than controls, whereas P_o's were the same.

Table 5. Bronchial smooth muscle constants obtained from Hill equation

	SBSM	(n=13)	CBSM	(n=11)
	2 sec	8 sec	2 sec	8 sec
V_o,L_o/s	0.35±0.02*§	0.19±0.02	0.27±0.02*	0.18±0.01
P_o,mN/mm^2	68.26±9.01	58.43±8.16	78.90±9.59*	68.24±8.29
a, mN/mm^2	13.25±1.81	10.03±1.4	16.96±1.98	13.84±1.83
b, L_o/s	0.062±0.007	0.036±0.007	0.060±0.005	0.039±0.005
a/P_o	0.193±0.007	0.173±0.007	0.219±0.009	0.201±0.007
a/b	209.6±30.85*§	291.7±39.01§	301.2±41.07*	374.4±43.62
1/b	15.90±0.83*	30.21±2.53	17.80±1.44*	30.13±4.53

Values are means ± SE; *a* and *b*, constants with units of force and velocity, respectively; * $P < 0.05$ compared with values at 8 sec; § $P < 0.05$ compared with control at the same time point.

The values of maximum shortening capacity (ΔL_{max}) under resting tension (0.05 P_o) are displayed in **Figure 28**. The ΔL_{max} for SBSM [0.67 $\pm$ 0.038 L_o, (SE)] was significantly greater than that for CBSM (0.51 $\pm$ 0.038, $P < 0.01$). A significant difference ($P < 0.01$) was also found in the proportion of isotonic shortening occurring within the first 2 sec between control (0.51 $\pm$ 0.049) and sensitized (0.81 $\pm$ 0.032) muscles. There is no difference between the two optimal lengths.

Morphological studies

Quantitative planimetric analysis showed no difference in the smooth muscle fraction in total tissue cross-sectional area of the strips. The proportions of muscle tissue in the total cross-sectional area were 0.301 $\pm$ 0.02 and 0.311 $\pm$ 0.03 for CBSM and SBSM, respectively. Hence, no correction for this factor was required in comparing tissue stresses developed by the CBSM and SBSM. Neither inflammation and cellular infiltration, nor smooth muscle hypertrophy or hyperplasia were found in the sections of bronchi from sensitized dogs.

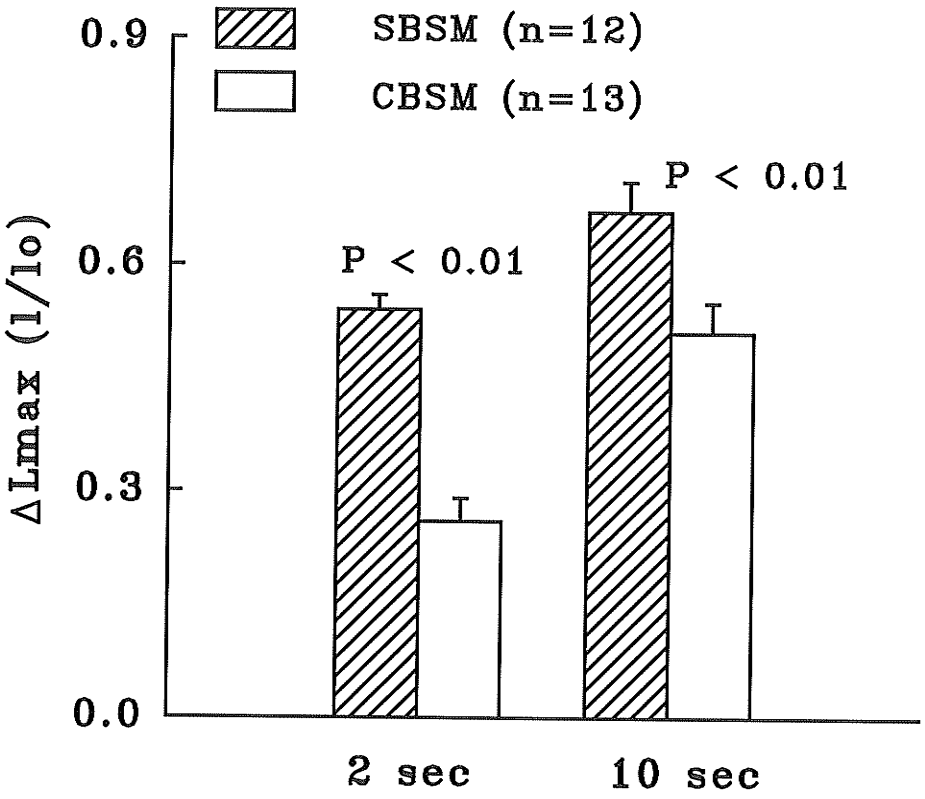


Figure 28. Maximum shortening capacities of SBSM and CBSM. SBSM showed a greater shortening capacity (ΔLmax) than CBSM. ΔLmax at 2s (L_{2sec}) is greater in SBSM than in CBSM. Values for SBSM are significantly higher ($P<0.01$) than for CBSM.

IV. Isotonic Relaxation of Bronchial Smooth Muscle

Half relaxation time

Figure 29 shows the values of $T_{1/2,CE}$ obtained from bronchial smooth muscle after 1 and 10 sec of stimulation. Sensitized bronchial smooth muscle was found to have a significantly increased $T_{1/2,CE}$ after 1 sec of stimulation when compared with that of the control. There is no difference between $T_{1/2,CE}$s in relaxations after 10 sec of stimulation of muscles from the two groups. Thus it is only the normally cycling crossbridges that show prolonged relaxation.

Mechanisms of relaxation: state of activation of crossbridge cycling

Figure 30A demonstrates that isotonic relaxation consists of an initial slow phase (phase i), the convexity of which is upward, followed by a linear phase (phase ii) and then a final slow phase (phase iii), the convexity of which is downward. Phase i represents the balance between the elongation effect of the load on the relaxing muscle and the retarding effect of residual activation. Phase ii represents the continued elongation, effected by the load on the muscle, the activation of which may have been almost absent. Whether passive recoil of the compressed internal resistor reported by us[Stephens et al 1988] also contributes to the relaxation is a possibility that needs future study. It would have contributed even more to relaxation in phase i but its effect would be obscured by the relatively greater activation of the muscle at that time.

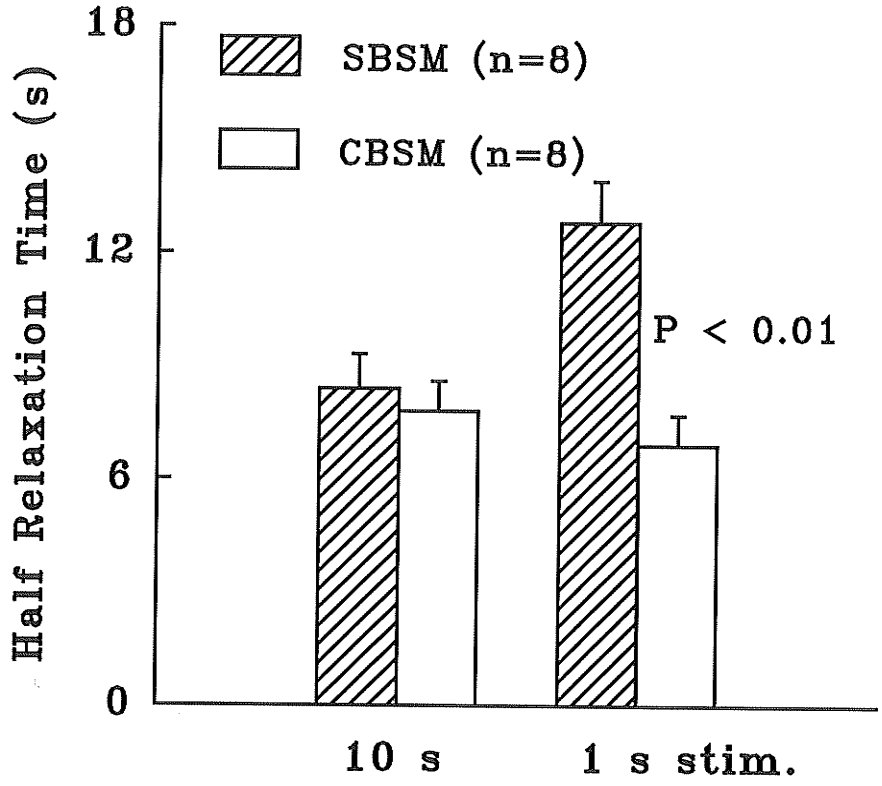


Figure 29. Mean half-relaxation time ($T_{1/2,CE}$) in sensitized and control bronchial smooth muscle. Value of $T_{1/2,CE}$ in SBSM was similar to that of CBSM after 10s of stimulation. However, after 1s of stimulation it was almost doubled in SBSM than in CBSM, indicating alterations in properties of early normally cycling crossbridges.

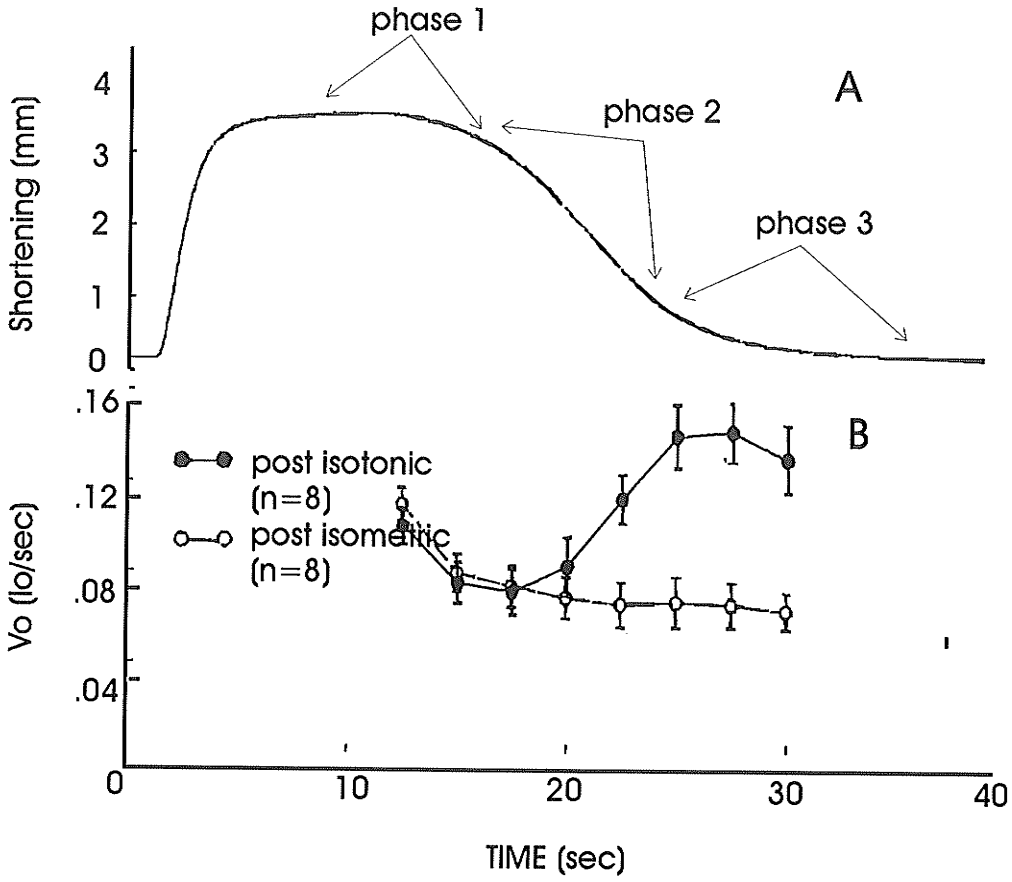


Figure 30. Inactivation of bronchial smooth muscle's active state during isotonic relaxation. A: isotonic shortening recording showing 3 phases during relaxation. B: zero load velocities (V_o) from quick release during isotonic relaxation (shown in **Figure 11B**). During phase 1, V_o decreased gradually as the relaxation became faster in A. In phase 2, V_o started spontaneously to increase and reached its peak in phase 3 accompanied by slowing down of relaxation.

The zero-load velocities (V_o) obtained by quick-release techniques during isometric and isotonic relaxation are shown in **Figure 30B**. The state of activation of the crossbridges in isometric relaxation, as judged by analysis of the temporal behaviour of V_o, decayed gradually after the withdrawal of stimulation. However, what was unexpected was that, during isotonic relaxation, activation decayed in the first 2-5 sec after turning off the stimulator and then started to increase again **(Figure 30B)**. This reactivation coincided with the final phase of relaxation (phase iii) in which the relaxing velocity of the muscle began to slow down. This spontaneous reactivation of the crossbridge cycling has been observed in tracheal smooth muscle also (unpublished observation). It has never been reported before in either striated or smooth muscle. No difference was found in the state of activation of crossbridges between sensitized and control bronchial smooth muscle.

V. Biochemical Properties of Sensitized and Control Airway Smooth Muscles

ATPase activity

As residual Ca^{2+} in homogenates could bind to calmodulin and activate MCLK and ATPase subsequently, EGTA was included in the assay buffer to eliminate it. There was no difference between ATPase activity with and without EGTA, indicating that there was no significant myosin ATPase activation before external trigger Ca^{2+} was applied. When maximally activated, actomyosin ATPase activity in sensitized ASMs was significantly higher (**Figure 31**) in both the tracheal and bronchial myofibrillar homogenates than in their control counterparts.

MLC phosphorylation

A Western blot of MLCs has already been shown in **Figure 13**. Ten percent polyacrylamide urea gel electrophoresis, as previously described by Hathaway and Haeberle[1985], was well suited to separation of the monophosphorylated and unphosphorylated MLC_{20} bands with high resolution. As shown in **Figure 13**, the unphosphorylated MLC_{20} bands are predominant whereas the biphosphorylated bands are hardly seen. The resting state phosphorylation of MLC_{20} in sensitized TSM was elevated to almost the same level as that of control at 4 sec. The maximum level with neurogenic stimulation (electrical field stimulation) in sensitized TSM was significantly increased when compared with that in control TSM (**Figure 32**). Also, the absolute increase in MLC_{20} phosphorylation after electrical stimulation in sensitized TSM is significantly higher (0.247 ± 0.03) when compared with that of the control (0.148 ± 0.02; $P < 0.05$).

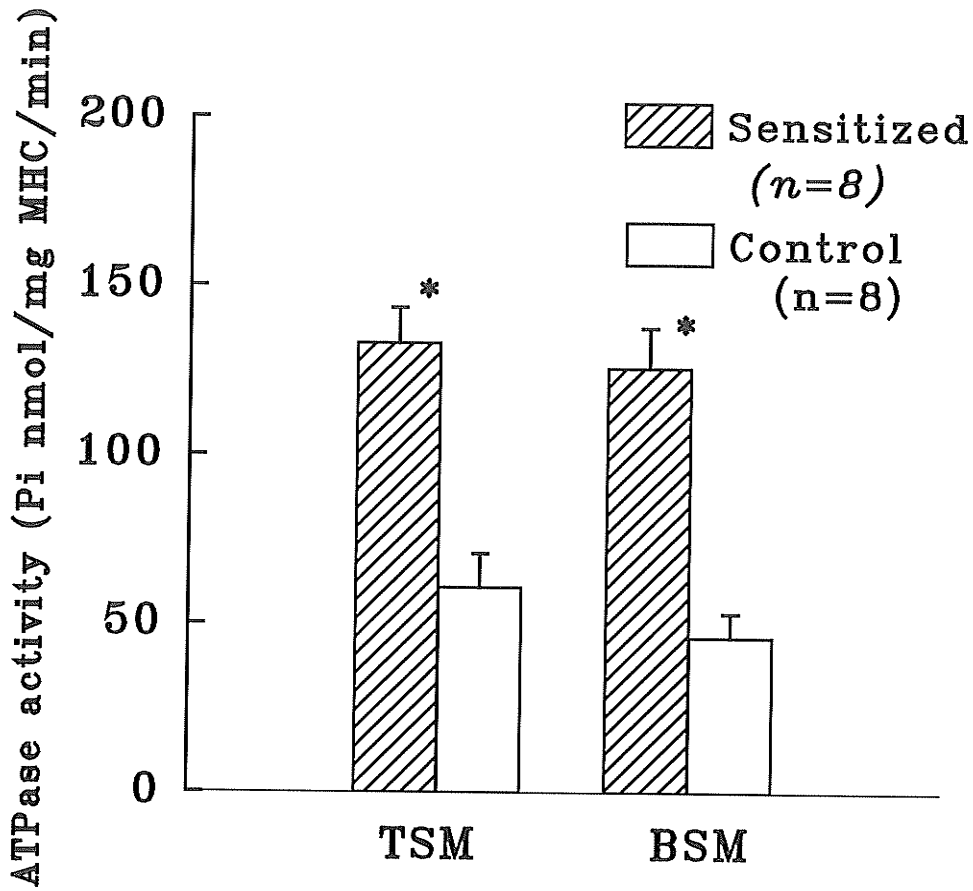


Figure 31. Actomyosin ATPase specific activity of airway smooth muscle in myofibrillar preparation. Both TSM and BSM from sensitized dogs show increased ATPase specific activity when normalized to myosin heavy chain (MHC) content. * $P < 0.01$.

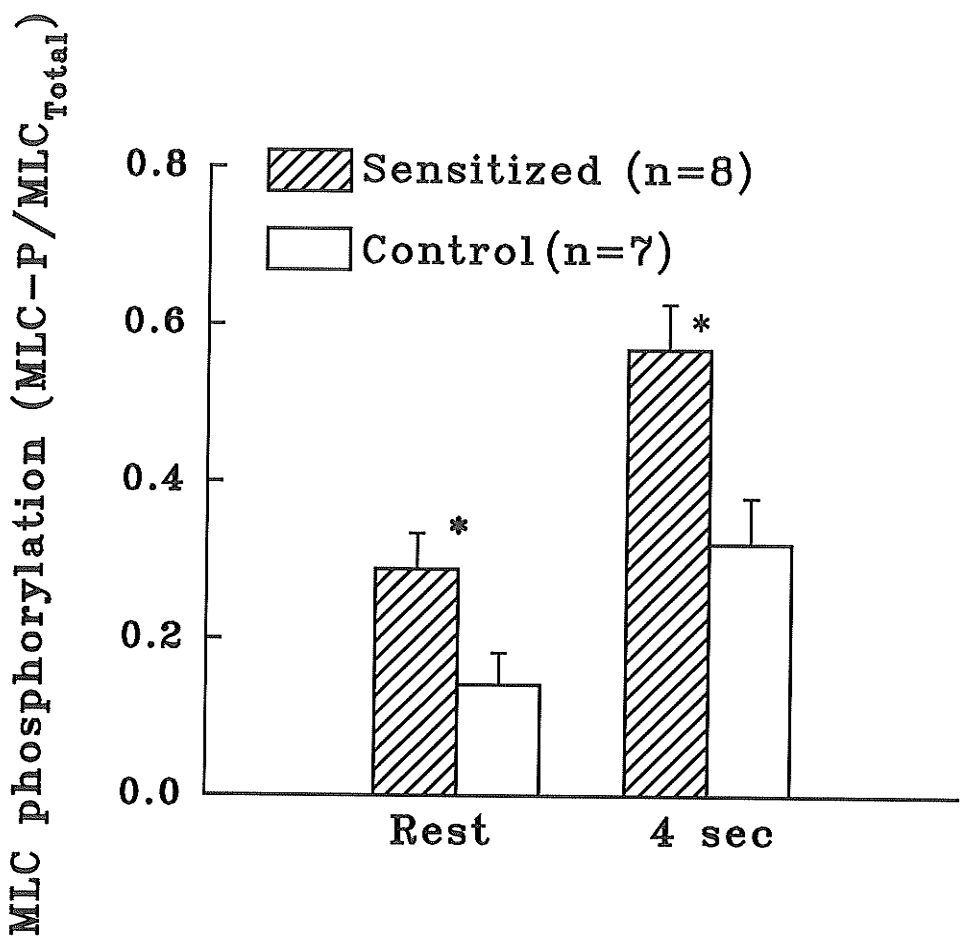


Figure 32. Myosin light chain (MLC_{20}) phosphorylation at rest and during electrical stimulation. The ratio of monophosphorylated MLC_{20} (MLC-P) to total MLC_{20} was found to be elevated at both time points. * $P<0.01$. The absolute increase in MLC_{20} phosphorylation after electrical stimulation in sensitized TSM is significantly higher (0.247 ± 0.03) when compared with that of the control (0.148 ± 0.02; $P<0.05$).

MLCK properties

Laser densitometric analysis of MLCK bands (**Figure 33**, upper panel) on 7.5% mini SDS-PAGE gel revealed a significant increase in its content in sensitized BSM and TSM (lower panel of **Figure 33**).

Okadaic acid inhibited phosphatase activity, thus significantly and considerably increasing the activity of MLCK in TSM from sensitized and control groups (**Table 6**). The activities of MLCK for sensitized and control TSM were intensified by similar amount after applying okadaic acid, indicating similar levels of myosin phosphatase inhibition for both groups. As the okadaic acid stock was dissolved in dimethyl sulfoxide (Me_2SO), the effect of Me_2SO on MLC_{20} phosphorylation was also tested. It was shown to have no effect. The incorporation rate of γ-^{32}P into MLC per minute was found significantly elevated in sensitized TSM and BSM in comparison with that in control (left panel in **Figure 34**). However, when the content of MLCK was taken into account, i.e., the γ-^{32}P incorporation rate was expressed as nmol $P_i/\mu g$ MLCK/min (specific activity), the difference between sensitized and control groups disappeared because of the increased amount of MLCK in sensitized groups (right panel of **Figure 34**).

Cleveland mapping disclosed no apparent change in the primary structures of MLCK in sensitized TSM and BSM when compared with those in control.

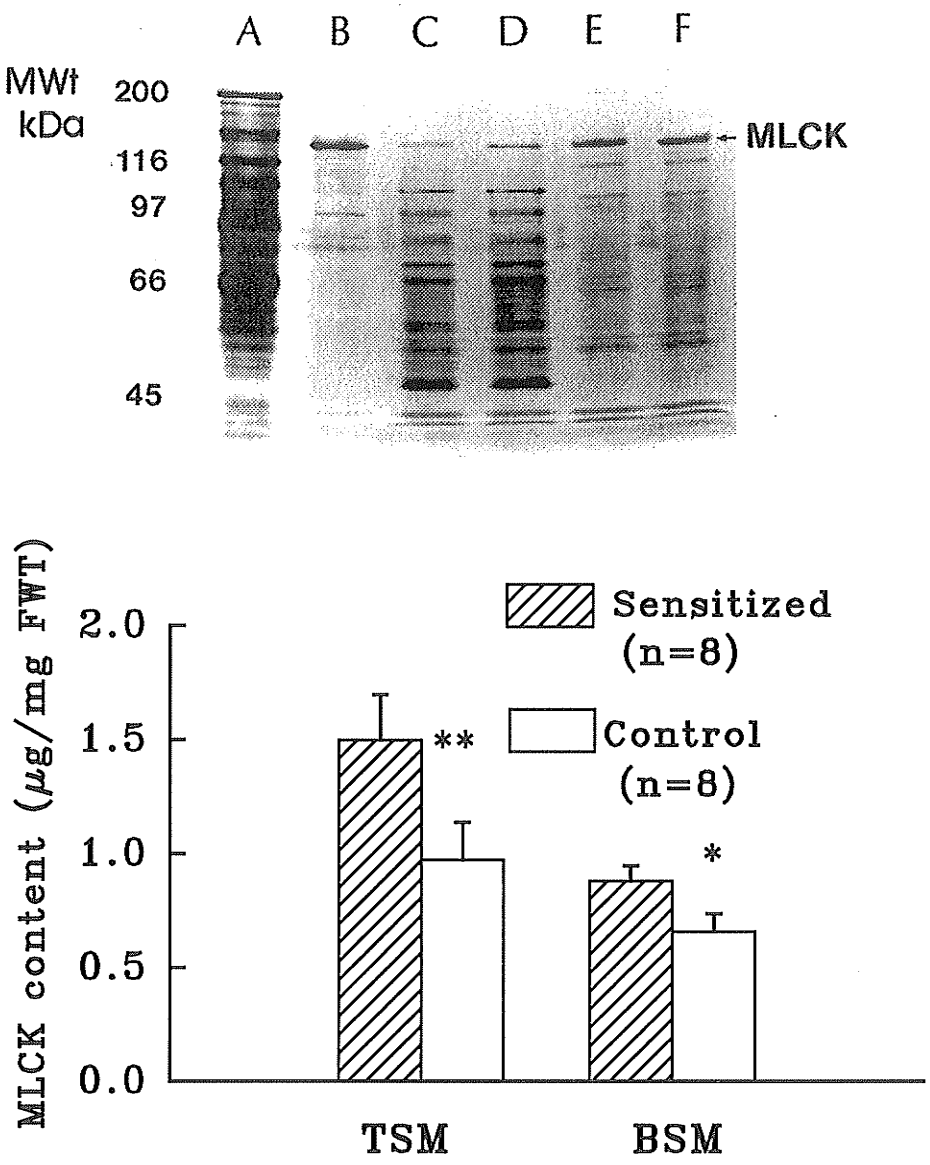


Figure 33. Myosin light chain kinase (MLCK) quantification. Upper panel: A typical 7.5 % mini SDS-PAGE gel showing MLCK bands. A = high molecular weight marker; B = smooth muscle MLCK standard; C,D = CTSM and CBSM (control TSM and BSM); E,F = STSM, SBSM (sensitized TSM and BSM). Lower panel: MLCK contents were found greater in sensitized airway smooth muscles than in controls. ** $P<0.05$; * $P<0.01$. FWT = fresh tissue weight.

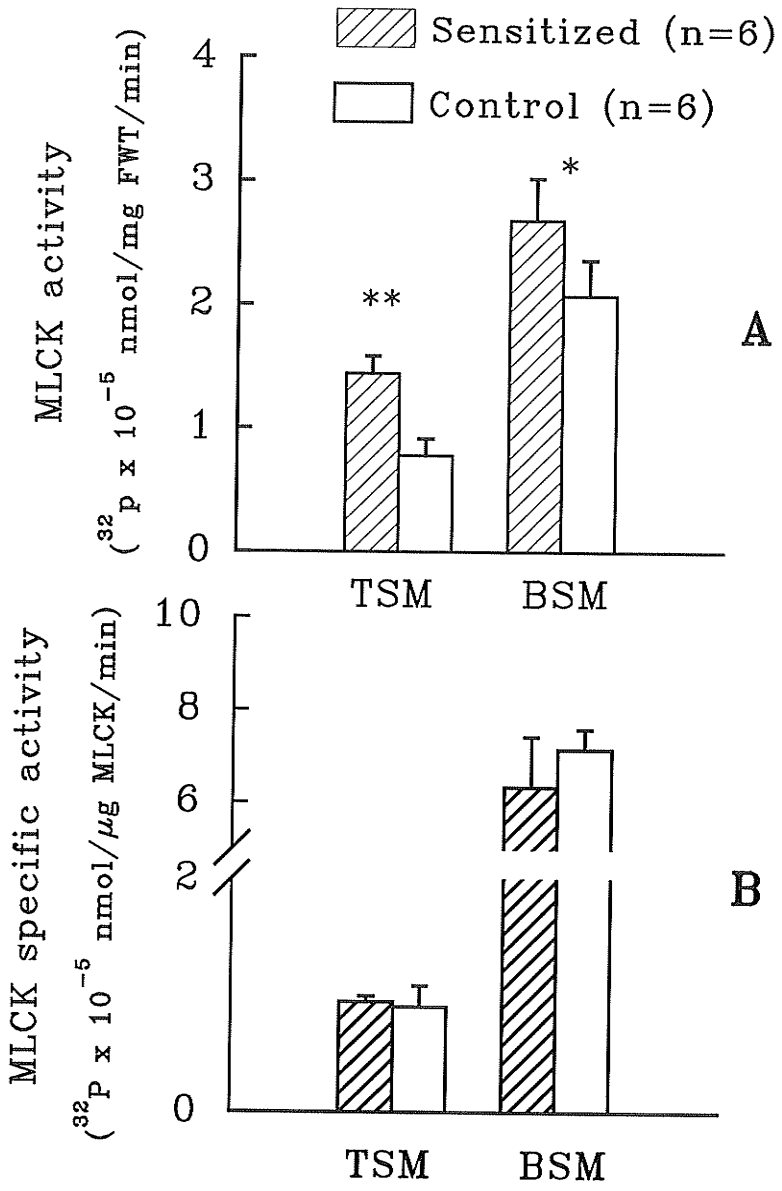


Figure 34. MLCK activity in sensitized and control TSM and BSM. Upper panel: the incorporation rate of ^{32}P into MLC (MLCK activity) in TSM and BSM was significantly higher than in control. Lower panel: when expressed as the incorporation rate of ^{32}P into MLC/μg MLCK/min, i.e., the MLCK specific activity, the difference between sensitized and control smooth muscles vanished. FWT = fresh tissue weight.

Table 6. Effect of okadaic acid on myosin light chain kinase activity*

	CTSM	STSM
OA-	28.5±5.7(n=3)	70.3±8.2(n=3)
OA+	46.99±8.33(n=8)	91.9±6.43(n=8)

Values are means ± SE; OA: okadaic acid; *: activity expressed as $^{32}P_i$ nmol/mg tissue/min; : $P < 0.01$ when compared with that of control.

VI. MLC_{20} phosphorylation and intracellular Ca^{2+} transient during isometric and isotonic contraction.

MLC_{20} phosphorylation

The resting value of MLC_{20} phosphorylation was the same for isotonic and isometric groups and was within the range of those reported by others(De Lanerolle and Paul 1991). The mean isometric MLC_{20} phosphorylation (**Figure 35**) reached its peak of 43% at 7 s after the onset of electrical stimulation. The level of MLC_{20} phosphorylation had already started to descend while stimulation of 10 s duration was still on and remained elevated above resting level for at least 200-250 s. Isotonic MLC_{20} phosphorylation, on the other hand, started at the same rate of MLC_{20} phosphorylation as that of the isometric, but did not reach to over 27% at 3 s under same electrical stimulation and fell back to almost resting level at about 6 s, while the muscle was still shortening with the maximum shortening being attained at about 12 s (**Figure 35**).

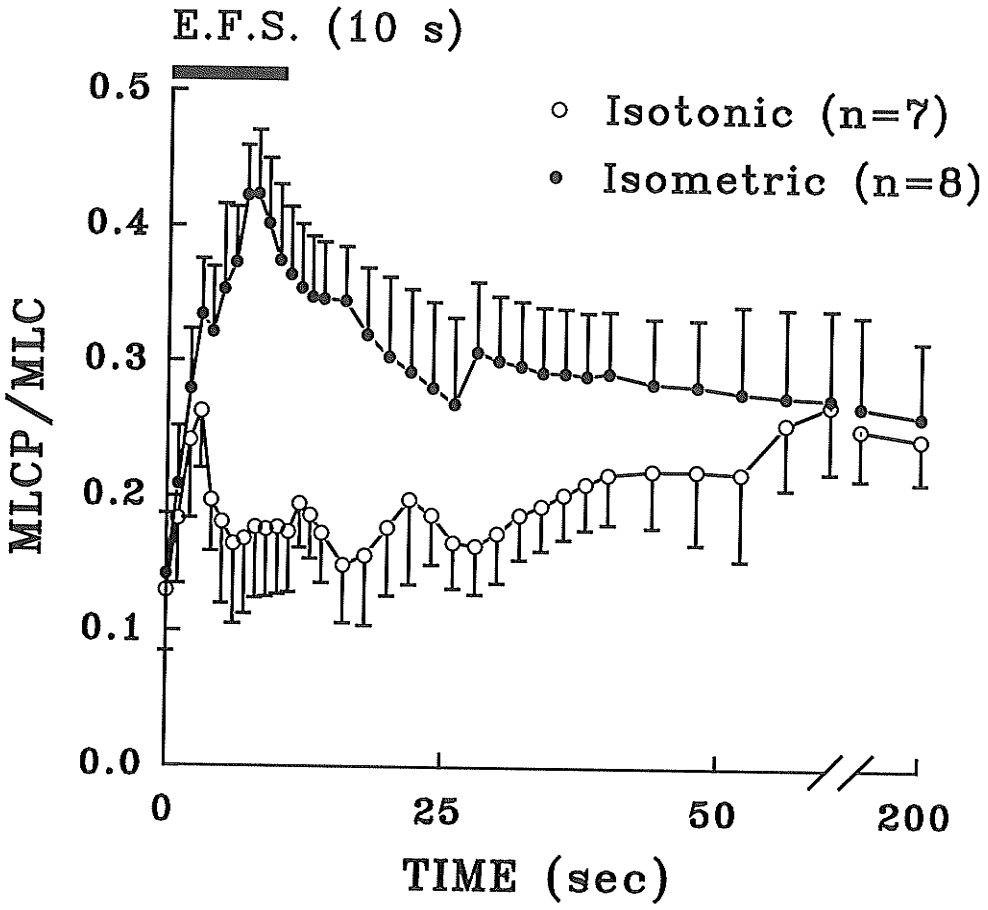


Figure 35. Myosin light chain phosphorylation during isometric (upper curve) and isotonic (lower curve) contraction. A significant difference was found between the levels of MLC_{20} phosphorylation under the same magnitude of electrical stimulation. Around 20 sec, isotonic MLC_{20} phosphorylation level started spontaneously to increase without stimulation.

MLC_{20} phosphorylation during isotonic relaxation showed spontaneous elevation for a relatively long period of time (**Figure 35**). Such spontaneous increase in MLC_{20} phosphorylation coincides with the spontaneous activation of crossbridge cycling assessed by maximum shortening velocities as mentioned before (**Figure 30**).

The relationship between muscle length and MLC_{20} phosphorylation was then investigated by measuring MLC_{20} phosphorylation of muscle strips stretched to different static lengths using the same techniques mentioned above. As shown in **Figure 36**, MLC_{20} phosphorylation correlated positively with the muscle lengths both at rest and during electrical stimulation. At 1.5 L_o, the resting value of MLC_{20} phosphorylation was even higher than the stimulated value at 0.5 L_o.

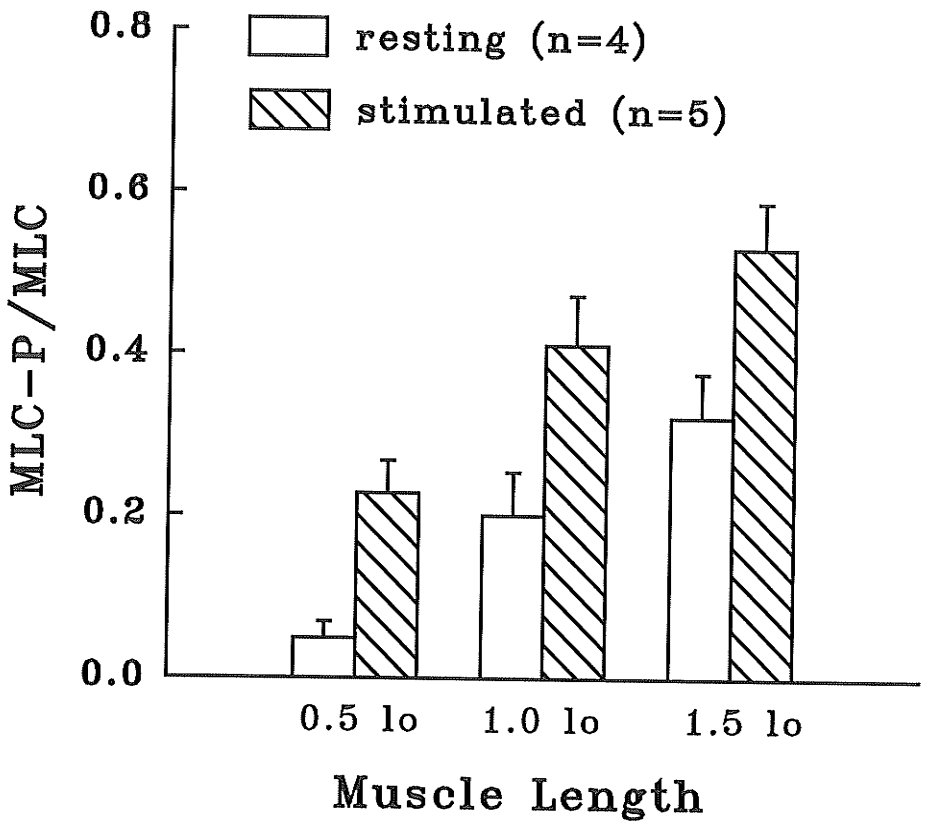


Figure 36. Myosin light chain phosphorylation levels at different lengths of muscle. MLC_{20} phosphorylation level correlates positively with the length of the muscle.

Intracellular Ca^{2+}

Upon stimulation, the fluorescence signals excited at 340 and 380 nm by a mercury lamp, increased initially and declined subsequently in a length-dependent manner during muscle shortening. Similar to the levels of MLC_{20} phosphorylation, $[Ca^{2+}]_i$ was less in isotonic shortening than in isometric contraction under the same magnitude of stimulation. The more the muscle shortened, the less was the intracellular Ca^{2+} concentration (**Fig 37**). Also, during isotonic relaxation, there was a slight spontaneous increase in $[Ca^{2+}]_i$, which corresponds with the spontaneous elevation of MLC_{20} phosphorylation during isotonic relaxation (**Figure 35**).

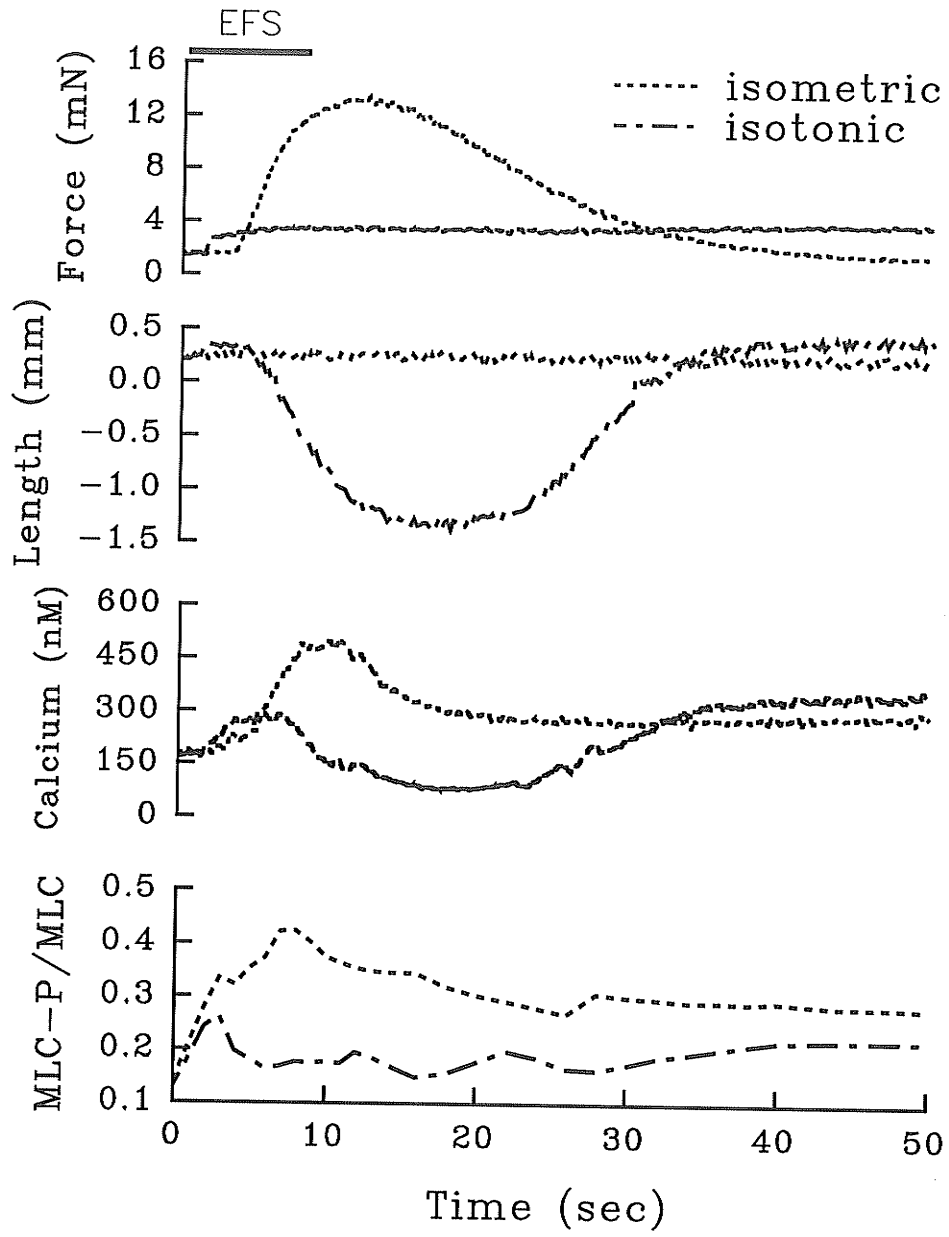


Figure 37. Typical Ca^{2+} transients during electrical stimulation (EFS) of sensitized and control tracheal smooth muscle. There was no difference found between sensitized and control. Isotonic shortening records in both sensitized and control groups showed lower Ca^{2+} transients than those of isometrics. Ca^{2+} concentration was normalized using standard Ca^{2+} solution from Molecular Probes.

DISCUSSION

I. Bronchial smooth muscle preparation

The majority of *in vitro* mechanical studies of airway smooth muscle has been carried out on trachea instead of bronchi[Aizawa et al 1988; Anthonissen et al 1979; Gunst and Mitzner 1981; Kroeger and Stephens 1971; Mitchel and Stephens 1983; Shioya et al 1987; Stephens et al 1969; Stephens 1973; Stephens 1985] because obtaining a strip of pure bronchial smooth muscle is technically difficult. Work in our laboratory has shown canine tracheal smooth muscle to be fairly suitable for mechanical studies[Kroeger and Stephens 1971; Mitchell and Stephens 1983; Seow and Stephens 1986; Seow and Stephens 1987; Stephens et al 1969] since it is easy to prepare, remains quite stable during the 3-hr experiment, possesses low resting tension at optimal length, and can be easily tetanized. However, the primary consideration in studying bronchial smooth muscle was that it was a direct approach to the study of allergic bronchoconstriction because the major locus of resistance to airflow is located in the third to seventh generations of the bronchial tree[Pedley et al 1970] and because it has been indicated that the early asthmatic response to antigen challenge is likely to involve constriction of the central airway[Epstein et al 1948; Dulfano and Hewetson 1966]. Furthermore, smooth muscle hypertrophy has been found in the segmental bronchi of asthmatics[Huber and Koessler 1922; Dunnill et al 1969]. These data suggest that it probably is the central airways that are constricted during the early asthmatic response even though the exact site of asthmatic attack is not established.

The main technical difficulty in studying bronchial smooth muscle was the presence of cartilaginous plates. Any study attempting to elucidate muscle contraction at crossbridge level should include delineation of force-velocity and instantaneous stiffness vs time curves. In presenting additional inertia and viscosity that would invalidate such studies, the plates were a major problem. On the other

hand if the behavior of intact airways were to be described, the removal of plates would be self-defeating. All the muscle strips for mechanical experimentation were also studied morphologically. The parallel arrangement of smooth muscle fibers in non-branching bronchial segments and the absence of any direct attachment of smooth muscle to cartilages rendered it possible to remove cartilage and connective tissues and to obtain an intact muscle strip preparation mechanically describable. Therefore the cartilages that were expected to exert inertial and viscous effects on the dynamic properties of the muscle were very carefully removed from the bronchial walls. Cartilage removal exerted no untoward damage on muscle contractility for the following reasons: 1) the normalized P'_os were similar in both bronchial smooth muscle strips with and without cartilage; 2) V_os of BSM were significantly greater than those of BSM+C; 3) no evidence of muscle damage was found in the histological study; and 4) the value of ΔL_{max} was greater in the BSM than in the intact groups (BSM+C).

The percentages of smooth muscle tissue in the cross-sectional area of tracheal and bronchial strips were obtained to normalize the length-tension and force-velocity relationships. This normalization allowed the effects of different quantities of muscle in TSM and BSM strips to be eliminated and comparison made between groups. However, the close to threefold difference between the active tensions of TSM and BSM cannot be totally accounted for by the difference in smooth muscle tissue content between these groups and this will be discussed later.

The isometric tension developed at the time when muscle was quickly released, $P_o(t)$, varied slightly from one contraction to another, mainly because of

the intrinsic instability of the muscle, which may be increased by application of load clamps (especially zero load clamp). The steady-state value of $P_o(t)$ is very important in fitting data to the hyperbolic Hill equation. Any significant decrease in $P_o(t)$ invalidates the experimental data because it may indicate progressive functional deterioration of the muscle preparation. Therefore only those results in which P_o varied $< 15\%$ between the beginning and the end of the experiment were accepted for analysis. From such carefully controlled experiments, the force-velocity relationship and maximum isotonic shortening capacity were computed.

The fact that ΔL_{max} was greater in BSM than in BSM+C proves that cartilaginous plates, though discontinuous and compliant, could have a significant role in preventing bronchial muscle from shortening maximally. Therefore, any study to elicit valid force-velocity relationship and maximum shortening capacity of BSM, where the data are to be analyzed in terms of crossbridges, should be conducted in tissue free of cartilage. Our preliminary experiment showed that the remaining connective tissue in the BSM strips, from which all cartilages had been removed, had no significant effect on the contractility of the muscle. No further effort was made to remove the remaining connective tissues because the possibility of muscle damage would increase.

Although the ΔL_{max} of the isolated strip of TSM was greater than that of the bronchus, it must be pointed out that the former muscle bridges between the two ends of the horseshoe-shaped cartilage, i.e., TSM extends for only about one-fifth of the tracheal circumference with the length of four-fifth of the that circumference unchangeable, whereas the BSM extends over the entire circumference of the bronchial wall. Therefore, one could expect that resistance to airflow in bronchi would be increased much more than that in the trachea for

equivalent degrees of muscle shortening. This is another important consideration in choosing bronchial smooth muscle.

Both bronchial and tracheal groups showed reduced maximum velocities at the 8 -s point of contraction. Our results agreed with that reported by Dillon et al[1981], who showed similar behavior in hog carotid arterial smooth muscle and suggested that the decreases are due to development of latch bridges. In fact, *a/b*, which was an index of an internal viscous resistance factor as reported by Seow and Stephens[1986], increased significantly from 2 to 8 sec in our study. The source of this internal resistance is not known but could be the so-called latch bridges. It has been suggested[Dillon et al 1981; Siegman et al 1976] that smooth muscle contraction is subserved by two types of crossbridges recruited sequentially. Early in contraction, normally cycling crossbridges are activated; later, slowly cycling ones become active. It is not clear whether they belong to the same population or two different ones. Slowly cycling crossbridges are believed to be responsible for the major portion of force development and force maintenance, whereas isotonic shortening may be effected mainly by normally cycling ones. Our results showed that the "latch bridges" are operative in BSM, just as they are in TSM. During the first 1-3 sec, normally cycling crossbridges were activated and produced more rapid isotonic contraction. When slowly cycling crossbridges were recruited later, the muscle strips presented decreased velocities.

It is likely that the slower velocity of BSM compared with TSM is due to the presence of connective tissue still remaining in the BSM after the cartilage has been removed. It is quite clear, however, that in removing the cartilage we have removed a considerable part (57% of cross-sectional area) of the extraneous load on the smooth muscle as estimated by planimetry. This extraneous load may have

been greater than estimated, in view of the fact that the cartilage is much denser than the connective tissues, which appear light and spongy. What is clear is that removal of the cartilage improves the mechanical performance of the BSM strip and allows better interpretation of the mechanical data in terms of crossbridges. Inasmuch as it presents a very loose structure, the aggregate amount of connective tissue is possibly very small and makes a very minor contribution to the visco-inertial properties of the strip. Confirmation of this will have to await the development of techniques that remove connective tissue totally without damaging the adjacent muscle.

The finding that maximum velocity of isotonic shortening at 2 sec in cartilage-free bronchial strips was greater than in those with cartilage intact suggested that bronchial cartilages prevented the muscle from shortening at maximal velocity. More important, it displayed that removal of cartilage did not impair contractile activity and that the preparation developed was a reliable one. Therefore, to better analyze the force-velocity characteristics and length-tension relationships of the BSM at the crossbridge level, the cartilages should be removed from the muscle. It must be pointed out that the use of bronchial strips is preferable to the use of bronchial ring preparations. When rings are not fully stretched, it is difficult to accurately measure muscle length and it is therefore not possible to accurately estimate cross-sectional area. This renders normalization of force almost impossible.

II. Force Normalization

Comparison of tension developed by smooth muscles from different loci or at different developmental stages mandates that techniques used yield valid

results and that reliable normalizations are employed. The former consideration entails the use of supramaximal stimulus, the ability of muscle to be easily tetanized, and the existence of parallel orientation of the contractile filaments to each other such that the cellular forces are exerted in the same axis[Jiang and Stephens 1990; Stephens et al 1984]. We have identified the optimal functional orientation of airway smooth muscle strips for force production by cutting them at a variety of angles and comparing the forces they develop. Transverse cuts of both tracheal and bronchial muscle tissues effected the greatest tissue stress; these values were, on average, 20-30% higher than those from strips cut at 45° from the transverse plane. In conjunction with mechanical data, histological data show that muscle cells are oriented parallel to each other and with the longitudinal axis of the transverse tracheal and bronchial strips. An electron-microscopy study of human airways also confirms that bundles of smooth muscles are arranged in a parallel fashion along a direction perpendicular to the longitudinal axis of subsegmental bronchi[Ebina et al 1990]. Based on these data, transversely cut strips were used in all experiments. These results also suggest that the contractile filaments are arranged parallel to the long axis of the cell and, not obliquely, at least in TSM and BSM.

The field of asthma research currently shows considerable activity in the contractile properties of airway smooth muscle, whereas, most investigations in the past have focused on evaluating force produced by whole tissues[Mapp et al 1989; Murphy et al 1989; Russell 1978; Shioya et al 1987]. This represents a switch in interest in the mechanics of the whole organ to one of analysis of cellular mechanisms of contraction. The measured force has been normalized to represent the properties of the muscle component of the tissue in a variety of ways[Armour et al 1984; Cohen and Murphy 1978; Jiang and Stephens 1990, Jiang et al 1991; Mapp et al 1989; Murphy et al 1989; Russell 1978; Shioya et al 1987],

rendering comparison of reported values difficult. Commonly used normalization is as grams force or tension developed per gram wet weight of tissue[Mapp et al 1989; Shioya et al 1987]. In addition, force has been related to the area of the longitudinal face of the muscle[Armour et al 1984]. Cohen and Murphy[1978] have employed stress and strain as normalizing parameters to analyze force development by smooth muscle. Their studies, however, dealt mainly with vascular rather than airway smooth muscle.

Theoretically, only the crossbridges in the half-sarcomeres of a given cross section of a skeletal muscle contribute to force generated by the tissue[Woledge et al 1985]. The rest of the crossbridges in the remainder of the muscle are in series and do not contribute to the force transduced externally. The force generated by adjacent crossbridges on both side of the Z-line neutralize each other as show in **Figure 38**. That is, of two muscle strips with equal weights, the one with the greater cross-sectional area (CSA) exerts more force if myosin filament lengths are equal. This theory is likely to be true for smooth muscle, in which mini-sarcomeres likely exist. The evidence in **Figure 25** supports these ideas and mandates the normalization of force as stress with respect to the cross-sectional area of smooth muscle tissue i.e. stress. Force normalization in terms of tissue weight or longitudinal side area may be based on the notion that all the crossbridges in a muscle strip contribute to the externally transduced force. However this assumption, (quite apart from being physiologically correct) as indicated above, renders such normalizations unsuitable for comparing tissues which are not of exactly the same dimension and origin. Given that cross-sectional area is an appropriate normalization parameter, when determining CSA using Eq. 1 one other desideratum to be satisfied is that tissue water contents should be the same, as tissue weight is used in the calculation. Our data confirm that there was

no difference in the water content of tracheal and bronchial strips.

Furthermore, normalization of force with respect to cross-section area has to consider the following issues. When force is normalized with respect to CSA_T (tissue stress) it is presumed that the entire CSA_T is made up of contractile units. Although this is almost true for striated muscle, it is certainly not so for smooth muscle[Cohen and Murphy 1978; Jiang and Stephens 1990]. In the current study I have assessed the fraction of the tissue cross section that is made up of muscle. The muscle fraction we report for canine trachealis (0.79) is very similar to that reported for pig trachealis (0.71;[Cohen and Murphy 1978]) where low-power electron microscopy was employed. On the other hand, muscle constituted a smaller fraction (0.3 ± 0.009) in bronchial tissue cross sections. In fact, when differences in cross-sectional muscle cell content were taken into account when normalizing the force (muscle stress) developed by tracheal and bronchial strips, greater than 62% of the difference in their respective tissue stresses was negated.

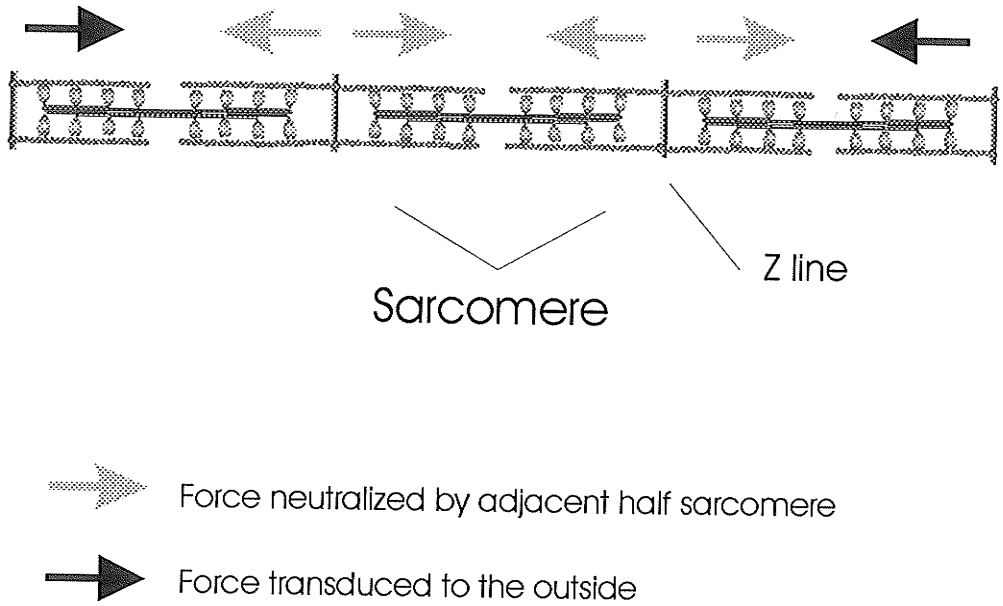


Figure 38. Schematic arrangement of crossbridges in sarcomeres. The vectors of force generated by the adjacent crossbridges at both side of Z-line are in opposite directions. Therefore, the force developed by these crossbridges are neutralized. Only the force generated by the last half sarcomere of each end of the muscle fibre is transduced to the outside world.

The correct normalization parameter is based critically on the mechanism by which isometric force is produced in the muscle. The common approach is to use that of skeletal muscle physiologists, which in turn is based on delineation of muscle structure. The unit of structure and function of skeletal muscle is the sarcomere, with the crossbridges being the force and shortening generators. The polarity of the crossbridges is such that the orientation of their power stroke is towards the centre of the sarcomere. Crossbridges in adjacent half-sarcomeres that exert force in opposite directions in the muscle can be thought of as being in series and therefore exert force on each other. It is only the crossbridges in the last half sarcomere that are unopposed and exert force on the outside world via tendons. These crossbridges transmit their force via the actin filament, and hence it is the force in the cross section to which these actins relate that is important. Normalization with respect to cross-sectional area yields, of course, stress.

Force is a function of the numbers and characteristics of the crossbridges operative in the muscle's cross section[Woledge et al 1985]. Normalization with respect to the crossbridges activated in the cross section is not yet possible for smooth muscle, however, as an immediate substitute we have employed the mean myosin content in the crossbridges present in a half sarcomere that operate in the muscles cross section. Considerable differences have been reported for smooth muscle tissue myosin and actin content from different tissues and species[Cohen and Murphy 1978; Murakami and Uchida 1985; Sobieszek and Bremel 1975; Tregear and Squire 1973]. Our estimates for MHC content in canine tracheal and bronchial tissue (**Table 3**) fall within range of other smooth muscle estimates, which have been as low as 0.01 mg/g wet wt in chicken gizzard[Murakami and Uchida 1985] and as high as 16.2 mg/g wet wt of pig oesophagus[Cohen and Murphy 1978]. A similar scenario is true for our actin measurements. The resolution

of MHC and actin bands by the gradient polyacrylamide gels showed and, in addition, Western blotting confirmed that the MHC band was not contaminated by non-smooth muscle myosin. In fact, a previous study[Cohen and Murphy 1978], which employed less-effective 5 mm-diameter tube gel electrophoretic methods, was able to achieve resolution high enough to show that actin and MHC estimates from various smooth muscles were not compromised by unknown proteins. Furthermore, the three-dimensional densitometric technique that we employ enabled us to ensure that band shapes and sizes did not introduce quantitative anomalies into our measurements. With the development of the *in vitro* motility assay[Sheetz et al 1986; Kron et al 1991; Umemoto and Sellers 1990] it may not be too long before we can measure the exact force generated by a single crossbridge of any muscle.

It has been suggested that higher actin-to-myosin ratios result in higher force generating ability[Cohen and Murphy 1978]. Smooth muscle actin contents have been reported ranging from 1.1 to 3.5 times those of MHC by weight[Cohen and Murphy 1978; Small and Sobieszek 1977; Sobieszek and Bremel 1975]. More specifically, Cohen and Murphy[1978] have shown a 1.8:1 (mg:mg) actin-to-MHC ratio for pig trachealis. This result is quite similar to those reported here for canine airway smooth muscle. From our measurements of actin and myosin content (**Table 3**) we estimate molar actin-to-MHC ratios (assuming the molecular weights of MHC and actin to be 200 and 45 kDa, respectively) in bronchial and tracheal smooth muscles to be about 8.2:1, which corresponds with previously accepted values[Small and Sobieszek 1977; Sobieszek and Bremel 1975]. Our data show that between canine tracheal and bronchial smooth muscle there are no differences in the relative amounts of actin and myosin; therefore, this factor does not account for the differences we have measured in force production between the two tissues.

Tissue MHC content for tracheal samples was 2.2-fold (**Table 3**) of that for bronchial samples. This difference was of similar magnitude to the difference found in histological studies where we estimated muscle content of tracheal strips to be 2.6-fold of that for bronchial strips (**Table 2**). Hence the content of myosin per unit of muscle cell was not significantly different in tracheal and bronchial muscles. In this situation, normalizing force as muscle stress is equivalent to using myosin stress, and the former would be sufficient to accurately compare force developed by airway smooth muscles from different loci and species. In fact, the 1.60-fold difference between tracheal and bronchial muscle stress was not significantly different from the 2.16-fold difference between tracheal and bronchial myosin stress. When comparing the mechanical properties of airway smooth muscle obtained from animals or humans at different levels of developmental or pathophysiological states (e.g., asthma), measurement of muscle cell contractile protein content is necessary to account for potential variations in muscle stress that may exist in these states.

After normalization with respect to cross-sectional smooth muscle content and contractile protein content, quantitative differences in force production by tracheal and bronchial strips exist, although the initial 4.3-fold difference between tracheal and bronchial tissue stress is considerably reduced to a 1.6-fold difference in their respective muscle stresses. The nature of this apparent difference in tracheal and bronchial smooth muscle properties is not obvious from our data. Subtle differences in fibre orientation could potentiate disparity seen for force development, such that normalization based on muscle content alone would be essentially incomplete. Our assessment of the muscle fibre orientation in the tissues used in this study was qualitative; that normalized tracheal smooth muscle

force is different from that for bronchial smooth muscle might warrant quantitative analysis of fibre orientation. Ultimately the nature of the crossbridges responsible for generating force in any smooth muscle will determine function, and it seems likely that properties of this type will need to be examined in some detail to fully understand differences between tissues.

III. Mechanical properties of sensitized and control airway smooth muscles

Post-mortem analysis from status asthmaticus patients have been reported to demonstrate bronchial smooth muscle hypertrophy[Dunnill et al 1969; Huber and Koessler 1922; Takizawa and Thurlbeck 1971]; only, one study[Sobonya 1990], however, reported that bronchial smooth muscle content was not significantly increased in asthmatic patients who had died from non-asthmatic causes. In the studies where smooth muscle hypertrophy was reported, the effective factor was likely the incidence of repeated asthmatic attacks which somehow resulted in the smooth muscle hypertrophy before death. Smooth muscle hypertrophy in either trachea or bronchi from our ragweed pollen sensitized dogs has never been indicated in morphometric study[Jiang et al 1992a] and in DNA, RNA quantification[Halayko et al 1993], probably because these animals have never been challenged. Inflammation and the concomitant cellular infiltration are not seen either. This suggests, in turn, that although inflammation may be responsible for the chronic asthmatic response, it likely plays almost no role in the acute response. Furthermore these young animals are likely to show only the early primary disease changes, which should facilitate the elucidation of primary causes of the disease.

The importance of investigating smooth muscle from bronchi has been dealt with in the **Introduction** section. It is obvious that the smooth muscle from

this segment be investigated. Therefore we developed a bronchial preparation from which the cartilage plates and connective tissue were removed while leaving smooth muscle structurally and functionally intact[Jiang and Stephens 1990]. From this preparation, the length-tension relationships of SBSM and CBSM were delineated. The P_o in sensitized dogs was unchanged, indicating that isometric parameters are insensitive indicators of disease.

Isometric properties of airway smooth muscle relate to the stiffness of the bronchial wall only, and their role in regulating resistance is minor. Inasmuch as the resistance to flow in a tube depends inversely on its diameter (a quartic function of its radius), flow regulation in airways or blood vessels, which are directly controlled by shortening or elongating rather than stiffening of smooth muscle, is best studied *in vitro* by evaluation of isotonic parameters such as the force-velocity relationship and ΔL_{max}. However, very few studies of these parameters[Stephens et al 1988] have been carried out.

An increase of 31.4% in maximum shortening capacity in SBSM has been found. This is significant with respect to allergic bronchoconstriction, because computations using Poiseuille's equation, which applies here because the flow in this part of the respiratory tree is laminar, indicate that this increase in shortening in the sensitized model (assuming that all other variables held constant) would translate into 386.1% increase in airflow resistance.

Because most of the shortening occurred within the first 2 sec, the early, normally cycling crossbridges are more important (than appears at first glance) to study as far as isotonic shortening is concerned. The increased shortening velocity and amount of shortening at 2 sec in SBSM suggest that early, normally cycling crossbridges may be responsible for the increased ΔL_{max}. That is, an increase in

the rate of normally cycling crossbridges would decrease the time that is needed for achievement of maximum isotonic shortening. These early crossbridges are activated by phosphorylation of myosin light chain[Chatterjee and Murphy 1983]. A concomitant increase in activity of myofibrillar adenosinetriphosphatase (ATPase) has been shown in the sensitized tracheal, as well as in bronchial and pulmonary arterial, smooth muscle[Kong et al 1986; Rao et al 1991; Jiang et al 1992c]. The level of myosin light chain (20 kDa) phosphorylation has been found to be increased in sensitized airway smooth muscle[Kong et al 1990; Jiang et al 1992c]. It is likely that the greater maximum shortening velocities found in SBSM are the result of increased myosin ATPase activation brought about by an elevated level of myosin light chain phosphorylation. We have also shown an increased cellular content of myosin light chain kinase in sensitized airway smooth muscle[Jiang et al 1992c].

Another factor that could affect the magnitude of shortening is the compliance of the so-called internal resistor. The evidence that it exists is straightforward. If a resting muscle, stretched to beyond L_o, is released, it returns to L_o. Furthermore, if stimulation is removed from a maximally shortened muscle, the muscle re-elongates to its original length. It is as if there were an elastic structure to resist shortening and stretching within the cell. In the shortening phase, this resistor is compressed and stores potential energy. When the stimulus is turned off, the resistor re-expands and restores the muscle to its original length. It has been reported that the internal resistance to shortening, inferred from the parameter a/b[Stephens et al 1987] or directly measured[Seow and Stephens 1987] of the sensitized tracheal smooth muscle is more compliant than that of the control[Stephens et al 1988]. This could partly account for the increased shortening in sensitized muscle. The mechanism for increased compliance of the internal resistor in sensitized muscle

is still unknown. Changes in properties of collagen, elastin, and other structures in the extracellular and extrafascicular spaces are strong contenders. However, the finding that the passive properties (resting tensions) from both groups were the same does not support the idea that changes in collagen and elastin are responsible for increased shortening. The cytoskeleton is thus another contender. It has been reported that low doses of ionizing radiation of skinned skeletal muscle cells induced changes in two cytoskeletal proteins, nebulin and titin, resulting in decreased passive and active tensions in response to calcium[Horowits et al 1986]. Although titin and nebulin have been found in smooth muscle, there is a large number of other smaller-molecular-weight cytoskeletal proteins present in smooth muscle[Rasmussen et al 1987] such as filamin, desmin, vimentin, vinculin, pectin, α-actinin, and synemin, all of which could contribute to the structure of the internal resistor. Additionally, Rasmussen et al[1987] have shown that these proteins are phosphorylated late in contraction. The purpose of this phosphorylation is not clear; however, it could alter the mechanical properties of the cytoskeletal network and, therefore, change the properties of the internal resistor. Presumably such properties could be quantitatively different in SASM to account for the increased shortening. Our recent study, intracellular free Ca^{2+} transient during isotonic shortening of airway smooth muscle does not reach the same level as that in isometric contraction[Jiang et al 1993]. This reduced Ca^{2+} elevation during isotonic shortening may result in a lower level of MLC_{20} phosphorylation[Jiang et al 1992b]. It is possible that there may be an intracellular system that senses the length change (shortening) and negatively feeds back to decrease the Ca^{2+} mobilization mechanisms, thus prevents excessive shortening, which is, in most cases, an undesirable situation that could lead to increase in airway or vascular resistance.

This last possibility might be another mechanism for the operation of the so-called internal resistance.

IV. Airway Smooth Muscle Relaxation

Antigen (ragweed pollen) sensitization of the newborn dogs has resulted in a series of mechanical alterations, such as increased maximum shortening velocity and capacity in early contraction in airway smooth muscle[Antonissen et al 1979; Jiang et al 1992a; Stephens and Kroeger 1980]. These changes in smooth muscle shortening occurred much earlier than any other mechanical changes that develop in airway hyperresponsiveness, for which the mechanisms are not understood. Neither changes in isometric force development nor evidence of inflammation, cellular infiltration, and muscle hypertrophy at this stage were observed[Jiang et al 1992a]. The elevated isotonic shortening velocity and capacity noticed in sensitized airway smooth muscle[Antonissen et al 1979; Jiang et al 1992a] may be responsible for the hyperresponsiveness reported in intact antigen sensitized dogs[Becker et al 1989; Kepron et al 1977].

All the studies of mechanical properties of airway smooth muscle in the past have focused on the interpretation of contractile response, whereas the properties of relaxation, the other equally important half of the whole contractile response, have not been systematically studied[White et al 1988], although the latter could play a key role in abnormality of airway hyperresponsiveness just as much as displayed by the shortening. The hypothesis that failure of airway smooth muscle relaxation could be partly responsible for allergic bronchospasm originated from the findings of a similar functional disorder in hypertensive vascular smooth muscle. The study of vascular smooth muscle from spontaneously hypertensive

rats (SHR) has suggested the arterial smooth muscle's failure to relax which may have contributed to the development of hypertension[Triner et al 1975]. Isometric relaxation of thoracic aorta from SHR has also been shown to be impaired[Cohen and Berkowitz 1976; Shibata and Cheng 1977], and caudal artery[Packer and Stephens 1985] and mesenteric artery[Packer and Stephens 1987] from SHR show prolonged isometric and isotonic relaxation, which may play roles in maintaining the hypertensive state[Packer and Stephens 1985; 1987].

It must be pointed out that any study intended to delineate the functional properties of smooth muscle has to look at the dynamic properties rather than static (isometric) ones. Smooth muscle in airways serves as an effector to regulate the calibre of the lumen and thus the resistance of airway to ventilation. Such regulation is subserved by smooth muscle shortening and elongation. Unfortunately the bulk of the research in the field has only described mechanical properties of smooth muscle in physiological and pharmacological studies, by measuring the isometric variable, an index that merely provides information about the stiffness of the vessel wall. It has been shown that isometric force development remained completely normal, whereas isotonic variables had undergone significant alterations[Antonissen et al 1979; Jiang et al 1992a] in sensitized canine airway smooth muscle. These have led us to conclude that isometric parameters are tardy indexes of disease, and further, they hardly relate to the parameter of relevance, viz, resistance. Therefore, it is only meaningful to study isotonic contraction and relaxation if one wants to depict the physiological and pathophysiological dynamic properties of smooth muscle of any hollow organ.

Goethals et al[1982] have shown that muscle relaxation is "load dependent". This referred only to the isometric phase of relaxation. Present study further

shows load-dependence of smooth muscle isotonic relaxation , which means the time course of relaxation is a function of the load that the muscle is bearing. The isometric phase of relaxation is, of course, of the "inactivation-dependent" type described by our previous study[Packer and Stephens 1985; 1987]. Another factor that has to be accounted for is the initial contractile element (CE) length from which the muscle starts to relax. The longer the CE length (less shortening), the shorter the relaxation time. Therefore, a load- and initial CE length-independent index of relaxation would ensure evaluation of the relaxation process without any influence from those factors.

In the present study, it was necessary to determine whether the relaxation process was also affected in sensitized airways smooth muscle given the fact that relaxation changes in vascular smooth muscle from spontaneously hypertensive rats (SHR) have been reported[Packer and Stephens 1985]. We have published evidence suggesting that early crossbridge cycling rate increases, while latch bridge cycling rate remains unchanged, which has provided the rationale for evaluation of the relaxation characteristics of early recruited, normally cycling crossbridges. In an attempt to functionally differentiate the two kinds of crossbridges, experiments were conducted in which muscles were stimulated for only 1 sec and 10 sec, respectively. It was anticipated, though the approach is simplistic, that with 1 sec stimulation we would be able to register the relaxation contributed mostly by early, normally cycling crossbridges, and with the 10 sec stimulation, that of predominantly latch bridges.

Under such an assumption the half-relaxation time ($T_{1/2,CE}$) from sensitized canine bronchial smooth muscle after 1 sec stimulation was found to be increased by 83% compared with control (**Fig 29**). The relaxation time of latch bridges after

10 sec stimulation showed no change. This again, supported the idea that it is the normally cycling crossbridge of airway smooth muscle that undergoes alterations with the development of sensitization[Jiang et al 1992a]. The increased myosin ATPase activity found in such preparations[Kong et al 1986; Rao et al 1991] may explain the increased maximum shortening velocity at 2 sec and the greater ΔL_{max}. It would therefore take more time for a sensitized muscle to elongate and relax than the control under the same load, as the former has shortened more. Another factor is the increased compliance of the muscle's internal resistance to shortening[Stephens et al 1988], which might also contribute to the increased ΔL_{max} of the sensitized muscle and prolonged relaxation time of the early crossbridge probably due to the reduced elastic recoiling force of the internal resistor. It is not clear at present what is the structural basis of such an internal resistor.

To obtain insight into the isotonic relaxation process, which could be arbitrarily divided into three phases as described in Results, the changes of V_o during the entire relaxation phase were delineated. This represented the temporal waning of the muscle's activation state. Relaxation-phase (phase i) was slow as the activation state was relatively high at this time. Phase ii was rapid because activation state was at minimal level and the load in the muscle was unopposed by muscle resistance. The unexpected finding was the redevelopment of partial activation in phase iii of relaxation. This occurred in a muscle essentially at rest, was therefore spontaneous, and suggests, the slowing in muscle elongation was due to reactivation of crossbridges opposing the effect of the load. The mechanisms of such spontaneous activation of airway smooth muscle are not understood at this time. Since the original work of Bülbring[1955], it has been known that stretch of smooth muscle can cause depolarization of the cell

membrane, increased frequency of action potentials, and subsequent contraction[Coburn 1987; Harder et al 1987; Johansson and Mellander 1975], i.e., the so-called myogenic response. Smooth muscle cells have been shown to contain a large number of stretch-activated, nonselective cationic channels which conduct Na^+, K^+, and Ca^{2+} upon mechanical stretching[Kirber et al 1988]. Coincidentally, stretching has been shown to induce maximal phosphorylation of the 20,000 Da myosin light chain[Bárány et al 1985, Jiang et al 1992b]. It is therefore postulated that during isotonic relaxation (but not during isometric relaxation) the stretch of airway smooth muscle strips produced by the preloads activated a myogenic response. It is obvious that the rate of elongation slows down at the period where the reactivation of crossbridges takes place as indicated by the two different slopes of phases ii and iii in **Fig. 30B**. The physiological significance of such reactivation is not clear but slowing produced by spontaneous reactivation of crossbridge cycling during relaxation would minimize the turbulence in flow that could result were the relaxation to have continued in the rapid linear fashion of the immediately preceding phase. The difficulties with these explanations are the following: 1) the stretch required to induce the biophysical and biochemical concomitant of the myogenic response is from 1.0 L_o to beyond; in our experiment the elongation was from 0.3 L_o (maximal shortening) to 1.0 L_o and thus not a stretch in strict sense; 2) the myogenic response is usually seen in single-unit smooth muscle[Kroeger and Stephens 1975] and not in multiunit, of which the tracheal and bronchial smooth muscles are examples. Perhaps the failure in the latter muscles is due to the fact that activation was judged by the development of active force (a relatively crude measure) rather than by measuring so called , zero load velocity, active state.

V. Activities of Myosin ATPase and Myosin Light Chain Kinase in Sensitized Airway Smooth Muscle

In vitro physiologic studies on airway smooth muscles from these ragweed pollen sensitized dogs have revealed increases in ΔL_{max} and V_o early in contraction[Antonissen et al 1979; Jiang et al 1992a]. The greater actomyosin ATPase activity of sensitized airway smooth muscle provides an explanation for the increase in V_o and the associated increase in ΔL_{max} seen in these animals.

The ATPase activities were obtained from myofibrillar homogenates, where the major regulatory and contractile proteins such as myosin heavy chain (MHC), light chain (MLC), actin, tropomyosin, calmodulin, caldesmon, and myosin light chain kinase (MLCK) are present in physiologic concentrations[Sobieszek and Bremel 1975]. Therefore, the possible explanations for the increased ATPase activity of airway smooth muscle from sensitized dogs could stem from any of the proteins mentioned above, although the protein involved in the rate-limiting step is most likely to be responsible. It is known that the globular subunit (S1) of MHC of skeletal and cardiac muscle carries the site for ATPase activity and exists in several isoforms. In cardiac muscle, for example, there are fast (V1), intermediate (V2), and slow forms (V3). Fast cardiac muscle (rat) contains a higher ratio of the V1 form and slow heart muscles, more V3[Hoh et al 1978; Hoh and Yeoh 1979; Schwartz et al 1981]. In smooth muscle, two isoforms of MHC have been identified as well[Rovner et al 1986]. However, previous investigation in this laboratory has failed to distinguish any modification of MHC isoforms in ragweed pollen-sensitized airway smooth muscle[Kong et al 1990] indicating that it is unlikely the shift of MHC isoforms that are responsible for increased ATPase activity.

It has been shown that both the ATPase activity and V_o are positively

correlated with the level of MLC_{20} phosphorylation[Sobieszek and Bremel 1975; Kong et al 1990], which is catalyzed by MLCK. It was necessary then to evaluate MLC_{20} phosphorylation and the properties of MLCK the enzyme responsible for activating actomyosin ATPase by phosphorylating the regulatory MLC_{20}; it was necessary also to study the properties of myosin light chain phosphatase, which is responsible for dephosphorylation of MLC_{20}[Kamm and Stull 1989]. Control MLC_{20} phosphorylation levels at basal (rest) and maximal activity (4 to 5 sec after onset of electrical stimulation) were of a similar order to that reported by others in bovine trachealis[Persechini et al 1986]. The levels of MLC_{20} phosphorylation in sensitized airway smooth muscle were significantly higher at both time points (0 and 4 sec) when compared with those of control, indicating either a higher MLCK activity or a lower phosphatase activity. As mentioned earlier, the level of MLC_{20} phosphorylation correlates positively with myosin ATPase activity and crossbridge cycling rate. The fact that the absolute increase in MLC_{20} phosphorylation after electrical stimulation is higher in sensitized TSM than in control suggests a greater magnitude of activation in sensitized muscle. There is no direct evidence to explain why the resting MLC_{20} phosphorylation level in sensitized TSM is almost the same as that of control at 4 sec while there was no mechanical activity (isometric) at 0 sec in sensitized TSM. However, this phenomenon indicates that the coupling mechanisms between MLC_{20} phosphorylation and smooth muscle contraction might also be altered, in addition to change in MLCK, by ragweed pollen sensitization.

The inhibition of myosin phosphatase by okadaic acid was significant in both control and sensitized TSM. The extent of inhibition was the same for both groups. Thus, it seems unlikely that phosphatase plays any role in the up-

regulation of MLC_{20} phosphorylation in sensitized ASM. A recent report supports such an idea[Liu et al 1993].

The most intriguing piece of data probably is that per minute incorporation rate of γ-^{32}P into the MLC_{20} per fresh tissue weight unit from the sensitized TSM and BSM was significantly higher than from their control counterparts. This higher MLCK activity could have resulted from (1) intrinsic structural modification of the enzyme that led to increased specific activity, (2) increased quantity of the enzyme, or (3) changes in other upstream regulatory mechanisms that regulate MLCK.

First, the greater activation of actomyosin ATPase could have resulted from altered intrinsic properties of MLCK. Therefore, Cleveland mapping of MLCKs was carried out. This did not reveal any notable change in the primary structure of sensitized MLCK, opposing the possibility of intrinsic modification of MLCK. Secondly, the specific activity of MLCK from sensitized TSM and BSM was the same as that from control. Neither the primary structure nor the specific activity of MLCK from sensitized airway smooth muscles evidenced any modification in intrinsic properties of MLCK. The second explanation for elevated MLCK activity in sensitized tissue was the increased content of the enzyme itself. Such an increase in airway smooth muscle MLCK content in an asthmatic animal model has never been reported before. The mechanism for the increase in MLCK from sensitized smooth muscle is not clear. The level of MLCK-mRNA has been shown unchanged[Halayko et al 1993] which suggests that translation and not transcription may be responsible. The increased MLCK concentration in airway smooth muscle cells from sensitized dogs seems to be sufficient to account for the elevated MLCK activity. We have recently reported that the shortening of normal TSM

inhibits MLC_{20} phosphorylation[Jiang et al 1992b], preventing smooth muscle from further shortening. Such a negative feedback mechanism, which may be one contender for the operation of the so-called internal resistor, could be inactivated by the increase in MLCK concentration in sensitized airway smooth muscles, thus resulting in more shortening. The last possibility is the influence from upstream regulatory mechanisms on MLCK activity. Such mechanisms, including increased Ca^{2+}, and calmodulin, or decreased caldesmon, calponin, protein kinase C, and cAMP-dependent kinase[Kamm and Stull 1989; Morgan et al 1991; Stocklet et al 1987; Winder and Walsh 1990] could be involved as well. Our recent study, however, does not provide supportive evidence that alteration in calmodulin content and activity is involved in the airway hyperresponsiveness of ragweed pollen sensitized dogs[Rao et al 1993; Jiang et al 1993b].

VI. Biochemical Mechanisms Underlying Smooth Muscle Isotonic Contraction and Relaxation

The resting value of MLC_{20} phosphorylation in control airway smooth muscle was the same for isotonic and isometric groups and was within the range of those reported by others[De Lanerolle and Paul 1991]. Maximum isotonic MLC_{20} phosphorylation was significantly lower than its isometric counterpart when maximally stimulated[Jiang et al 1992b; Hai 1991] indicating that shortening itself might trigger a negative feedback mechanism which off-sets excitation-contraction coupling. The relationship between resting muscle length and MLC_{20} phosphorylation was then investigated by measuring MLC_{20} phosphorylation of muscle strips stretched to different lengths and a positive correlation between the two was found. The resting value of MLC_{20} phosphorylation at the length of 1.5

L_o was even higher than the stimulated value at 0.5 L_o , suggesting that stretch is a strong activator of myosin light chain kinase or its upstream regulatory mechanisms. The elevated basal level of MLC_{20} phosphorylation in the absence of stimulation during late relaxation has not been reported before and its physiological role is not clear since there was no force production at this time (**Fig. 35A**). Such dissociation of force from low level of MLC_{20} phosphorylation indicates roles played by other regulatory factors, which might inhibit actomyosin ATPase activity. This is in contrast with the correlation of low level MLC_{20} phosphorylation with maintained tension in so-called latch bridge state as reported by others[Hai and Murphy 1988]. Physiologically, the importance of proportional relationship between muscle length and the state of activation might be that: 1) it serves as an internal resistor to prevent the muscle from extreme shortening; 2) it protects the muscle from being over-stretched by activating the contractile apparatus against stretching. Such speculations, of course, require further investigation.

The mechanism(s) behind the length regulation of MLC_{20} phosphorylation was/were not fully understood. The Ca^{2+} concentration of isotonically shortening muscle was much lower than that of the isometrically contracting one[Jiang et al 1993a], indicating that the shortening of the muscle may have uncoupled excitation-contraction coupling. It is then also possible that the Ca^{2+} release mechanism is inhibited or the sequestration of Ca^{2+} is enhanced. It is possible that the diminished intracellular Ca^{2+} concentration results in the subsequent lower level of MLC_{20} phosphorylation and the restriction of shortening. Spontaneous deactivation of smooth muscle at the lower levels of $[Ca^{2+}]_i$ and MLC_{20} may serve as a limitation to smooth muscle shortening. It may in fact be the internal resistor

described by Stephens et al[1988]. The mechanisms of deactivation are not fully understood. Isotonic shortening involves actual sliding movement of actin and myosin filaments whereas no, or very little, relative movement between the filaments occurs in isometric contraction. As muscle shortens, the arrangement of the proteins involved may undergo conformational changes. It is also possible that the ion channels on the membrane are modified by length change which may result in the modification of enzyme activities. Calcium-troponin C affinity in cardiac and skeletal muscles may be influenced by length change[Cheng et al 1992]. It is conceivable that similar alterations in the Ca^{2+}-calmodulin-MLCK mechanisms may occur when smooth muscle shortens. Mechanical stretching of the muscle result in changes in membrane properties such as the activation of a Ca^{2+} channel[Kirber et al 1988; Walsh and Singer 1990]. However, no study on MLC_{20} phosphorylation and intracellular Ca^{2+} during dynamic isotonic shortening and relaxation had been carried out. We postulate that an intracellular or membrane-related sensing system detects the direction and the extent of muscle length change and inactivates or activates Ca^{2+} regulatory mechanisms and subsequently the MLCK. Such a negative feedback mechanism may be physiologically significant since it prevents excessive smooth muscle shortening and keeps muscle from being over-stretched by sensing the length changes. In addition, it is logical for an isometrically contracting muscle to be activated maximally since it is, in effect, operating against a load that exceeds its maximum ability to develop force. In isotonic shortening, on the other hand, the muscle shortens under a load lighter than its maximum force producing ability, and therefore it would be more efficient for muscle not to be activated maximally.

CONCLUSIONS

1. This study showed that a novel, viable and quite stable BSM preparation can be obtained. It should be the preparation of choice in studying *in vitro* mechanical and pharmacological characteristics of BSM. The cartilage-free bronchial strip preparation affords the opportunity to pursue such isotonic experimentation.

2. Force normalized to the weight of tissue or the area of longitudinal face of the muscle strip as reported in the literature provided incorrect indexes of smooth muscle strength. Even tissue stress is not an adequate normalization when the muscle content of the two tissues being compared is not the same. Muscle cell stress is the most reliable index because the contractile protein content in the tissue cross section is the same in tissues being compared.

3. The maximum shortening capacity and early maximum shortening velocity of ragweed pollen-sensitized canine tracheal and bronchial smooth muscles are significantly greater than those of controls. Such increases may have resulted from the changes of properties of early, normally cycling crossbridges and could account for airway hyperresponsiveness seen in these animals. The maximum force generating ability is not changed by sensitization suggesting that it is an insensitive index and should not be used solely.

4. A new index of isotonic relaxation of smooth muscle that is independent of load and the initial length of muscle's contractile element has been developed; it validates comparison of relaxation processes in different

muscles. It has been shown that isotonic relaxation of early, normally cycling crossbridges of sensitized airway smooth muscle is prolonged when compared with that of muscle from littermate controls. The spontaneous development of reactivation of smooth muscle crossbridges during the late of relaxation phase has also been found and it may serve as a feedback regulatory mechanism to minimize mechanical disturbance during isotonic relaxation, as could occur during blood or air flow.

5. In sensitized airway smooth muscle, there is a higher MLCK activity due to the increased quantity of MLCK rather than the intrinsic properties of MLCK. This study supports the idea that the increased MLCK activity results in elevated actomyosin ATPase activity, which may be responsible for the increased maximum shortening velocity, and thus for the increased maximum shortening capacity seen in airway hyperresponsiveness.

6. Intracellular Ca^{2+} concentration and level of myosin light chain mono-phosphorylation, have been shown to correlate positively with smooth muscle length. During isotonic shortening, a postulated sensing system in the muscle cell may feed back the signal of length change and reduce the activation of muscle by lowering $[Ca^{2+}]_i$ concentration and thus the level of myosin light chain phosphorylation. Such a regulatory system prevents the smooth muscle cells from excessive shortening and serves to optimise the magnitude of constriction of airways or blood vessel. In addition, I have shown that late in isotonic relaxation, a spontaneous increase in intracellular Ca^{2+} and re-activation of myosin light chain phosphorylation and crossbridge cycling rate occurs simultaneously with a decrease in relaxation rate. This is a novel finding.

REFERENCES

Abdel-Latiff, A.A. 1974. Effects of neurotransmitters and other pharmacological agents on $^{32}P_i$ incorporation into phospholipids of the iris muscle of the rabbit. Life Sci 15:961-973.

Abdel-Latiff, A.A., R.A. Akhtar, and J.N. Hawthorne. 1977. Acetylcholine increases the breakdown of triphosphoinositide of rabbit iris muscle prelabelled with [^{32}P] phosphate. Biochem J 162:61-73.

Adams, L.P., J.R. Haeberle, and D.R. Hathaway. 1989. Phosphorylation of caldesmon in arterial smooth muscle. J Biol Chem 264:7698-7703.

Agrawal, D.K., and R.G. Townley. 1990. Airway Smooth Muscle. In: Modulation of Receptors and Response. Eds., CRC Press, Boston.

Aizawa, H., N. Miyazaki, N. Shigematsu, and M. Tomooka. 1988. A possible role of airway epithelium in modulating hyperresponsiveness. Br J Pharmacol 93:139-145.

Akhtar, R.A., and A.A. Abdel-Latiff. 1984. Carbachol causes rapid phosphodiesteratic cleavage of phosphatidylinositol 4,5-bisphosphate and accumulation of inositol phosphates in rabbit iris smooth muscle; prazosin inhibits noradrenaline- and inophore A23187-stimulated accumulation of inositol phosphates. Biochem J 224:291-300.

Aksoy, M.O., R.A. Murphy, and K.E. Kamm. 1982. Role of Ca^{2+} and myosin light chain phsophorylation in regulation of smooth muscle.

Am J Physiol 242:C109-C116.

Allen, K.M., J. Wharton, J.M. Polak, and S.G. Haworth. 1989. A study of nerves containing peptides in the pulmonary vasculature of healthy infants and children and of those with pulmonary hypertension. Br Heart J 62:353-360.

Alexander, R.W., T.A. Brock, M.A. Gimbrone, Jr., and S. Rittenhauser. 1985. Angiotensin increases inositol trisphosphate and calcium in vascular smooth muscle. Hypertension 7:447-451.

Altiere, R.J., and L. Diamond. 1984. Comparison of vasoactive intestinal peptide and isoproterenol relaxant effects in isolated cat airways. J Appl Physiol 56:986-???.

Amiranhoff, B., and G. Rosselin. 1982. VIP receptors and control of cyclic AMP production. In: Vasoactive Instestinal Pepetide. S.I. Said. ed., Raven Press, New York, pp307-322.

Andersson, R.G.G., G. Kovesi, and E. Ericsson. 1978. Beta adrenoceptor stimulation and cyclic AMP level in bovine tracheal muscle. Acta Pharmacol Toxicol 43:323-334.

Andrea, J.E., and M.P. Walsh. 1992. Protein kinase C of smooth muscle. Hypertension 20:585-595.

Anner, B., and M. Moosmayer. 1975. Rapid determination of inorganic phosphate in biological systems by a highly sensitive photometric method. Anal Biochem 65:305-309.

Antonissen, L.A., R.W. Mitchell, E.A. Kroeger, W. Kepron, K.S. Tse, and N.L.Stephens. 1979. Mechanical alterations of airway smooth muscle in a canine asthmatic model. J Appl Physiol 46:681-687.

Antonissen, L.A., R.W. Mitchell, E.A. Kroeger, W. Kepron, K.S. Tse, and N.L.Stephens, and J. Bergen. 1980. Histamine pharmacology in airway smooth muscle from a canine modle of asthma. J. Pharmacol. Exp. Ther. 213: 150-155.

Armour, C.L., J.L. Black., N. Berend, and A.J. Woolcock. 1984. The relationship between bronchial hyperresponsiveness to methacholine and airway smooth muscle structure and reactivity. Respir Physiol 58:223-233.

Ashizawa, N., F. Kobayashi, Y. Tanaka, and K. Nakayama. 1989. Relaxing action of okadaic acid, a black sponge toxin, on the arterial smooth muscle. Biochem Biophys Res Commun 162:971-976.

Bagby, R.M., A.M. Young, R.S. Dotson, A.B. Fisher, and K. McKinnon. 1971. Contraction of single smooth muscle cells from *bufo marinus* stomach. Nature 234:351-352.

Bárány, K., R.F. Ledvora, V. Mougios, amd M. Bárány. 1985. Stretch-induced myosin light chain phosphorylation and stretch-release-induced tension development in arterial smooth muscle. J Biol Chem 260:7126-7130.

Bárány, M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. J Gen Physiol 50(Suppl):197.

Barnes, N.C., P.J. Piper, and J.F. Costello. 1984. Comparative actions of inhaled leukotriene C_4, leukotriene D_4 and histamine in normal human subjects. Thorax 39:500-504.

Barnes, N.C., P.J. Piper, and J.F. Costello. 1985. Hyperresponsiveness

of asthmatics to inhaled leukotriene D_4. Am Rev Respir Dis 131(4):A24.

Barnes, N.C., and P.J. Piper. 1986. The actions of leukotrienes in human lung *in vitro* and *in vivo*. In: The Actions of Leukotrienes: Their Biological Significance. Raven Press, New York, pp199-212.

Barnes, P.J. 1993. β-adrenoceptors on smooth muscle, nerves and inflammatory cells. Life Sci 52:2101-2109.

Barnes, P.J., C.B. Basbaum, and J.A. Nadel. 1983. Autoradiographic localization of autonomic receoptors in airway smooth muscle: marked differences between large and small airways. Am Rev Respir Dis 127:758-762.

Barnes, P.J., C.B. Basbaum, J.A. Nadel, and J.M. Roberts. 1982. Localization of β-adrenoceptors in mammalian lung by light microscopic autoradiography. Nature 299:444-447.

Barnes, P.J., K.F. Chung, and C.P. Page. 1988. Platelet-activating factor as a mediator of allergic disease. J Allergy Clin Immunol 81:919-934.

Barnes, P.J., I.W. Rodger, and N.C Thomson. 1988. Future trends in asthma treatment. In Asthma: Basic Mechanisms and Clinical Management. Barnes P.J. I.W. Rodger, and N.C. Thomson, eds., Publ Acad Press, New York, pp751-769.

Baron, C.B., M. Cunningham, J.F.III. Strauss, R.F. Coburn. 1984. Pharmacomechanical coupling in smooth muscle may involve phosphatidylinositol metabolism. Proc Natl Acad Sci 81:6899-6903.

Baron, C.B., and R.F. Coburn. 1987. Inositol phospholipid turnover

during contraction of canine trachealis muscle. Ann NY Acad Sci 494:80-83.

Barron, J.T., M. Bárány, and K. Bárány. 1979. Phosphorylation of the 20.000-dalton light chain of myosin of intact arterial smooth muscle in rest and in contraction. J Biol Chem 254:4954-4956.

Becker, A.B., J Hershkovich, F.E.R. Simons, K.J. Simons, M.K. Liley, and W. Kepron. 1989. Development of chronic airway hyperresponsiveness in ragweed-sensitized dogs. J Appl Physiol 66:2691-2697.

Bement, W.M., and M.S. Mooseker. 1993. Keeping out the rain. Nature 365:785-786.

Bendall, J.R. 1964. The myofibrillar ATPase activity of various animals in relation to ionic strength and temperature. In: Biochemistry of Muscle Contraction. J. Gerogely, ed., Little Brown, Boston, pp448-452.

Benham, C.D., and R.W. Tsien. 1987. A novel receptor-operated Ca-permeable channel activated by ATP in smooth muscle. Nature 328:275-278.

Berner, P.F., A.V. Somlyo, and A.P. Somlyo. 1981. Hypertrophy-induced increase of intermediate filaments in vasuclar smooth muscle. J Cell Biol 88:96-101.

Berridge, M.J. 1984. Inositol trisphosphate and diacylglycerol as second messengers. Biochem J 220:345-360.

Berridge, M.J. 1985. Inositol trisphosphate and diacylglycerol as intracellular second messengers. In: Mechanisms of Receptor

Regulation, Poste, G., and S.T. Crooke, eds. Plenum, New York pp 111-130.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. Nature 361:315-325.

Berridge, M.J., and R.F. Irvine. 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312:315-321.

Bialojan, C., J.C. Rüegg, J. DiSalvo. 1985. Phosphatase-mediated modulation of actin-myosin interaction in bovine aortic actomyosin and skinned porcine carotid artery. Proc Soc Exp Biol Med 178:36-45.

Bialojan, C., J.C. Rüegg, and A. Takai. 1988. Effects of okadaic acid on isometric tension and myosin phosphorylation of chemically skinned guinea-pig taenia coli. J Physiol 398:81-95.

Bird, G. St.J., M.F. Rossier, A.R. Hughes, S.B. Shears, D.L. Armstrong, J.W.Jr. Putney. 1991. Activation of Ca2+ entry into acinar cells by a non-phosphorylatable inositol trisphosphate. Nature 352(6331): 162-165.

Birnbaumer, L., J. Codina, R. Mattera, A. Yatani, N. Scherer, M.J. Toro, and A.M. Brown. 1987. Signal transduction by G proteins. Kidney Int 32(Suppl 23):S14-S37.

Blaber, L.C., A.D. Fryer, and J. Maclagan. 1985. Neuronal muscarinic receptors attenuate vagally-induced contraction of feline bronchial smooth muscle. Br J Pharmacol 86:723-728.

Bolton, T.B. 1979. Mechanisms of action of transmitters and other

substances on smooth muscle. Physiol Rev 59:606-718.

Bond, M., T. Kitazawa, H. Shuman, A.P. Somlyo, and A.V. Somlyo. 1984. Calcium release from and recycling by the sarcoplasmic reticulum in guinea pig smooth muscle. J Physiol 355:677-694.

Boomsma, J.D., H.D. Foda, and S.I. Said. 1990. Vasoactive intestinal peptide (VIP) reverses endothelin-induced contractions of guinea pig trachea and pulmonary artery. Am Rev Respir Dis 141:A485.

Boushey, H. A. 1980. Acquired hyperreactivity. In: Airway Reactivity Hargreave, F. E. Mississauga, Ontario, Canada: Astra. p.190-197.

Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of dye binding. Anal Biochem 72:248-254.

Braquet, P., T.. Shen, L. Touqui, and B.B. Vargaftig. 1987. Perspectives in platelet-activating factor research. Pharmacol Rev 39:97-145.

Brutsaert, D.L., V.A. Claes, and M.A. Goethals. 1971. Velocity of shortening of unloaded heart muscle and the length-tension relation. Circ Res 29:63-75.

Brutsaert, D.L., V.A. Claes. and Goethals, M.A. 1978. Velocity of shortening of unloaded heart muscle and the length-tension relation. Cir. Res. 29(1):63-75.

Brutsaert, D.L., N.M. De Clerck, M.A. Goethals, and P.R. Housmans. 1978. Relaxation of ventricular cardiac muscle. J Physiol 283:469-480.

Butler, T.M., M.J. Siegman, and S.U. Mooers. 1983. Chemical energy usage during shortening and work production in mammalian

smooth muscle. Am J Physiol 244 (Cell Physiol 13): C234-C242.

Cadieux, A., D.R. Springall, P.K. Mulderry, J. Rodrigo, M.A. Ghatei, G. Terenghi, S.R. Bloom, and J.M. Polak. 1986. Occurrence, distribution and ontogeny of CGRP immunoreactivity in the rat lower respiratory tract: effect of capsaicin treatment and surgical denervations. Neurosci 19:605-627.

Cameron, A.C., C.G. Bullock, and C.T. Kirkpatrick. 1982. The ultrastructure of bovine tracheal muscle. J Ultrastruct Res 81:290-305.

Cameron, A.C., C.F. Johnson, C.T. Kirkpatrick, and M.C.A. Kirkpatrick. 1983. The quest for the inhibitory neurotransmitter in bovine tracheal smooth muscle. Quart J Exp Physiol 68:413-426.

Carstairs, J.R., A.J. Nimmo, and P.J. Barnes. 1984. Autoradiographic localization of beta-adrenoceptors in human lung. Eur J Pharmacol 103:189-190.

Carstairs, J.R., A.J. Nimmo, P.J. Barnes. 1985. Autoradiographic visualization of beta-adrenoceptor subtype in human lung. Am Rev Respir Dis 1332:541-547.

Casey, P.J., M.P. Graziano, and A.G. Gilman. 1989. G protein $\beta\gamma$ subunits from bovine brain and retina: equivalent catalytic support of ADP-ribosylation of α subunit by pertussis toxin but differential interactions with $G_{s\alpha}$. Biochemistry 28:611-616.

Casteels, R., and G. Droogmans. 1981. Exchange characteristics of the noradrenaline-sensitive calcium store in vascular smooth muscle cells of rabbit ear artery. J Physiol (London) 317:263-279.

Casteels, R., F. Wuytack, B. Himpens, and L. Raeymaekers. 1986. Regulatory systems for the cytoplasmic calcium concentration in smooth muscle. Biomed Biochim Acta 45:S147-S152.

Chadwick, C.C., A. Saito, and S. Fleischer. 1990. Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. Proc Natl Acad Sci 87:2132-2136.

Chatterjee, M., and R.A. Murphy. 1983. Calcium dependent stress maintenance without myosin phosphorylation in skinned smooth muscle. Science 221:464-466.

Chen, Q., and C. van Breemen. 1993. The superficial buffer barrier in venous smooth muscle: sarcoplasmic reticulum refilling and unloading. Br J Pharmacol 109:336-343.

Cheney, R.E., M.A. Riley, and M.S. Mooseker. 1993. Phylogenetic analysis of the myosin superfamily. Cell Motil Cytoskel 24:215-223.

Cheng, R., A. Babu, F. Fein, E. Sonnenblick, H. Su, and J. Gulati. 1992. Evidence for symmetric and asymmetric extractions using biotinylated TnC and rhodamine avidin: Polarity of the thin filament as the mechanism of length transduction in the modulation of Ca^{2+}-sensitivity. FASEB J 6(1):A294.

Cleveland, D.W., S.G. Fisher, M.W. Kirshner, and U.K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem 252:1102-1106.

Close, R.I. 1972. Dynamic properties of mammalian skeletal muscles.

Physiol Rev 52:129-197.

Coburn, R.F. 1984. Neural coordination of excitation of ferret trachealis muscle. Am J Physiol 246:C459-C466.

Coburn, R.F. 1987. Stretch-induced membrane depolarization in ferret trachealis smooth muscle cells. J Appl Physiol 62:2320-2325.

Coburn, R.F., ed., 1989. Airway Smooth Muscle in Health and Disease, Plenum Press, New York.

Coburn, R.F., and C.B. Baron. 1990, Coupling mechanisms in airway smooth muscle. Am J Physiol 258 (Lung Cell Mol Physiol 2):L119-L133.

Cockcroft, D. W., D. N. Kilian, J. J.A. Mellon, and F. E. Hargreave. 1977. Bronchial reactivity to inhaled histamine: a method and clinical survey. Clin. Allergy 7: 235-243.

Cockcroft, S., J.M. Baldwin, and D. Allan. 1984. The Ca^{2+}-activated polyphosphoinositide phosphodiesterase of human and rabbit neutrophil embranes. Biochem J 221:477-482.

Cockcroft, S., and B.D. Gomperts. 1985. Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. Nature 314:534-536.

Cohen, D.M., and R.A. Murphy. 1978. Differences in cellular contractile protein contents among porcine smooth muscles. J Gen Physiol 72:369-380.

Cohen, M.L., and B.A. Berkowitz. 1976. Decreased vascular relaxation in hypertension. J Pharmacol Exp Ther 196(2):396-406.

Cohen, P. 1989. The structure and regulation of protein phosphatases.

Annu Rev Biochem 58:453-508.

Cole, W.C., and K.M. Sanders. 1989. G-proteins mediate suppression of Ca-activated K current by acetylcholine in smooth muscle cells. Am J Physiol 257 (Cell Physiol 26):C596-600.

Conroy, D.M. and P.J. Piper. 1991. Metabolism and generation of systeinyl-containing leukotrienes by human airway prepartions. In: Advances in the Understanding and Treatment of Asthma, vol 629, P.J. Piper, and R.D. Drell, eds., N.Y. Acad Sci Publ, pp455-457.

Crooke, S.T., B.P. Monia, M.Y. Chiang, and C.F. Bennett. 1991. Leukotriene receptors and mechanisms of signal transduction. In: Advances in the Understanding and Treatment of Asthma vol 629, P.J. Piper and R.D. Krell, eds., Annl N.Y. Acad Sci, New York, pp120-124.

Daniel, E.E., C. Davis, W.R. Jones, and M.S. Kannan. 1980. Control of airway smooth muscle. In: Airway Reactivity, F.R. Hargreave, ed., Mississauga, Ontario, Astra Pharmaceuticals pp 80-109.

Daniel, E.E., M.S. Kannan, C. Davis, and V. Posey-Daniel. 1986. Ultrastructural studies on the neuromuscular control of human tracheal and bronchial muscle. Respir Physiol 63:109-128.

Daniel, E.E. 1988. Control of airway smooth muscle. In: The Airways, Neural Control in Health and Disease, M.A. Kaliner and P.J. Barnes eds., Vol 33 Lung Biology in Health and Disease, C. Lenfant, ed. pp485-521.

Davis, M.J., J.A. Donovitz, J.D. Hood. 1992. Stretch-activated single channel and whole cell currents in vascular smooth muscle cells.

Am J Physiol 262(4 pt 1)): C1083-C1088.

De Lanerolle, and R.J. Paul. 1991. Myosin phosphorylation/dephosphorylation and regulation of airway smooth muscle contractility. Am J Physiol 261(Lung Cell Mol Biol):L1-L14.

De Marzo, N., S. Sartore, L. Saggia, L.M. Fabbri, and S. Schiaffino. 1987. Myosin heavy chain isoforms in human smooth muscle. In: Regulation and Contraction of Smooth Muscle. M.J. Siegman, A.P. Somlyo, and N.L. Stephens, eds., Liss, New York p451.

Dent, G., D. Ukena, and P.J. Barnes. 1989. PAF receptors. In: PAF and Human Disease. P.J. Barnes, C.P. Page, and P.M. Henson, eds. Blackwell, Oxford, pp58-81.

Devine, C.E., A.V. Somlyo, and A.P. Somlyo. 1972. Sarcoplasmic reticulum and excitation-contractoin coupling in mammalian smooth muscle. J Cell Biol 52:690-718.

DeVries, G., E.D. Fraser, and M.P. Walsh. 1989. Protein kinase C from chicken gizzard: characterization and detection of an inhibitor and endogenous substrates. Biochem Cell Biol 67:260-270.

Dey, R.D., W.A. Shannon, Jr., and S.I. Said. 1981. Localization of VIP-immunocreative nerves in airways and pulmonary vessels of dogs, cats, and human subjects. Cell Tissue Res 220: 231-238.

Dhillon, D.S., and I.W. Rodger. 1981. Hyperreactivity of guinea-pig isolated airway smooth muscle. Br J Pharmacol 74:180p.

Dillon, P.F., M.L. Aksoy, S.P. Driska, and R.A. Murphy. 1981. Myosin phosphorylation and the crossbridge cycle in arterial smooth

muscle. Science 211:495-497.

Doberstein, S.K., I.C. Baines, G. Wiegand, E.D. Korn, and T.D. Pollard. 1993. Inhibition of contractile vacuole function *in vivo* by antibodies against myosin-I. Nature 365:841-844.

Driska, S.P., M.O. Aksoy, and R.A. Murphy. 1981. Myosin light chain phosphorylation associated with contraction in arterial smooth muscle. Am J Physiol 240:C222-C233.

Dulfano, M.J., and J. Hewetson. 1966. Radiologic contributions to the nosology of obstructive lung disease entities. Dis Chest 50:270-280.

Duncan, P.G., C. Brink, R.L. Adolphson, and J.S. Douglas. 1980. Cyclic nucleotides and contration/relaxation in airway muscle. H_1 and H_2 agonists and antagonist. J Pharmacol Exp Ther 215:434-442.

Dunnill, M.S., G.R. Massarella, and J.A. Anderson. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis and in emphysema. Thorax 24:176-179.

Ebashi, S. 1974. Regulatory mechanism of muscle contraction with special reference to the Ca-troponin-tropomyosin system. Essays Biochem 10:1-36.

Ebina, M., H. Yaegashi, T. Takahashi, M. Motomiya, and M Tanemura. 1990. Distribution of smooth muscles along the bronchial tree: A morphometric study of ordinary autopsy lungs. Am Rev Respir Dis 141:1322-1326.

Ehrlich, B.E., and J. Watras. 1988. Inositol 1,4,5-trisphosphate activates

a channel from smooth muscle sarcoplasmic reticulum. Nature 336:583-586.

Epstein, B.S., J. Sherman, and E.E. Walzer. 1948. Bronchography in asthmatic patients, with the aid of adrenalin. Radiology 50:96-97.

Fay F.S. 1977. Mechanics of single isolated smooth muscle cells. In: Muscle, Casteels. R., ed. Amsterdam, Elsevier/North Holland, pp433-439.

Fay, F.S., K. Fogarty, and K. Fugiwara. 1984. The organization of the contractile apparatus in single isolated smooth muscle cells. In: Smooth Muscle Contraction, N.L. Stephens ed., Marcel Dekker, New York, p75.

Felbel, J., B. Trodsur, T. Edser, W. Landgraf, and F. Hoffmann. 1988. Regulation of cyosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. J Biol Chem 263:16764-16771.

Ferris, C.D., R.L. Huganir, S. Supattapone, S.H. Snyder. 1989. Purified inositol 1,4,5-trisphosphate receptor mediates calcium flux in reconstituted lipid vesicles. Nature 342(6245): 87-89.

Ferris, C.D., A.M. Cameron, D.S. Bredt, R.L. Huganir, and S.H. Snyder. 1992. Autophosphorylation of inositol 1,4,5-trisphosphate receptors. J Biol Chem 267:7036-7041.

Filo, R.S., D.F. Bohr, and J.C. Rüegg. 1965. Glycerinated skeletal and smooth muscle: calcium and magnesium dependence. Science 147:1581-1583.

Finch, E.A., T.J. Turner, and S.M. Goldin. 1991. Calcium as a coagonist

of inositol 1,4,5-trisphosphate-induced calcium release. Science 252:443-446.

Fitzgerald, M.F., S. Moncada, and L. Parente. 1986. The anaphylactic release of platelet-activating factor from perfused guinea pig lungs. Br J Pharmacol 88:149-153.

Flagg-Newton, J. 1980, The permeability of the cell-to-cell membrane channel and its regulation in mammalian cell junction. In Vitro 16:1043-1048.

Flagg-Newton, J. and W.R. Loewenstein. 1979. Experimental depression of junctional permeability in mammalian cell culture. A study with tracer molecules in the 300 to 800 dalton range. J Membr Biol 5):65-100.

Flavahan, N.A., L.L. Aarhus, T.J. Rimele, and P.M. Vanhoutte. 1985. Respiratory epithelium inhibits bronchial smooth muscle tone. J Appl Physiol 58:834-838.

Frossard, N., and F. Muller. 1986. Epithelial modulation of tracheal smooth muscle responses to antigenic stimulation. J Appl Physiol 61:1449-1456.

Fujimoto, T. 1993. Calcium pump of the plasma membrane is localized in caveolae. J Cell Biol 120:1147-1157.

Fujiwara, T., T. Itoh, Y. Kubota, and H. Kuriyama. 1989. Effects of guanosine nucleotides on skinned smooth muscle tissue of the rabbit mesenteric artery. J Physiol (Lond) 408:535-547.

Furuichi, T., T. Furuichi, S. Yoshikawa, A. Miyawaki, K. Wada, N. Maeda, and K. Mikoshiba. 1989. Primary structure and functional

expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature. 342(6245): 32-38.

Gabella, G. 1979. Smooth muscle cell junctions and structural aspects of contraction. Br Med Bull 35(3):213-218.

Gabella, G. 1981. Structure of smooth muscle, In: Smooth Muscle: An Assessment of Current Knowledge, E. Bulbring, A.F. Brading, A.W. Jones, T. Tomita, eds., Edward Arnold, London, pp1-46.

Gabella, G. 1989. Structure of airway smooth muscle and its innervation. In: Airway Smooth Muscle in Health and Disease. R.F. Coburn, ed. Plenum, New York, pp1-16.

Gateau, O., B. Arnoux, H. Deriaz, P. Viars, and J. Benveniste. 1984. Acute effects of intratracheal administration of paf-acether (platelet-activating factor) in humans. Am Rev Respir Dis 129:A129.

Gaylor, J.B. 1934. The intrinsic nervous mechanim of the human lung, Brain 57:143-160.

Gerthoffer, W.T. 1986. Calcium dependence of myosin phosphorylation and airway smooth muscle contraction and relaxation. Am J Physiol 250:C597-C604.

Gerthoffer, W.T. 1987. Dissociation of myosin phosphorylation and active tension during muscarinic stimulation of tracheal smooth muscle. J Pharmacol Exp Ther 240:8-15.

Giembycz, M.A., and D. Raeburn. 1991. Putative substrates for cyclic nucleotide-dependent protein kinases and the control of airway smooth muscle tone. J Auton Pharmacol 166:365-398.

Gilman, A.G., M.D. Smigel, C.M. Bokoch, and J.D. Robishaw. 1985.

Guanine-nucleotide-binding regulatory proteins: membrane-bound information transducers. In: Mechanisms of Receptor Regulation, G. Poste and S.T. Crooke, eds., Plenum Press, New York, pp149-158.

Goethals, M.A., P.R. Housmans, and D.L. Brutsaert. 1982. Loading determintants of relaxation in cat papillary muscle. Am J Physiol 242 (Heart Circ Physiol 11):H303-H309.

Goldie, R.G., J.W. Paterson, D. Spina, and J.L. Wale. 1984. Classification of β-adrenoceptors in human isolated bronchus. Br J Pharmacol 81:611-615.

Goldman, W.F., W.G. Wier, and M.P. Blaustein. 1989. Effects of activation on distribution of Ca in single arterial smooth muscle cells. Cir Res 64:1019-1029.

Goldman, Y.E., G.P. Reid, A.P. Somlyo, A.V. Somlyo, D.R. Trentham, and J.W. Water. 1986. Activation of skinned vascular smooth muscle by photolysis of "caged inositol trisphosphate" to inositol 1,4,5-trisphosphate ($Insp_3$). J Physiol (Lond.) 377:100P.

Gozes, I., and D.E. Brenneman. 1989. VIP: molecular biology and neurobiological funciton. Mol Neurobiol 3:201-236.

Gröschel-Stewart, U., J.H. Chamley, J.D. McConnel, and G. Burnstock. 1975. Comparison of the reaction of cultured smooth muscle and cardiac muscle cells and fibroblasts to specific antibodies to myosin. Histochemistry 43:215-224.

Gröschel-Stewart, U., L. Rokousky, R. Franke, I. Peleg, I. Kahance, A. Eldor, and M. Muhlrad. 1985. Immunohistochemical studies with

antibodies to myosins from the cyoplasm and membrane fraction of human blood platelets. Cell Tissue Res 241:399-404.

Guharay, F., and F. Sachs. 1984. Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. J Physiol (London 352:685-701.

Gunst, S.T., and W. Mitzner. 1981. Mechanical properties of contracted canine bronchial segments in vitro. J Appl Physiol 50:1236-1247.

Gunst, S.T., and J.Q. Stropp. 1988. Effect of Na-K adenosine triphosphatase activity on relaxation of canine tracheal smooth muscle. J Appl Physiol 64:635-641.

Haeberle, J.R., D.R. Hathaway, and A.A. DePaoli-Roach. 1985. Dephosphorylation of myosin by the catalytic subunit of a type-2 phosphatase produced relaxation of chemically skinned uterine smooth muscle. J Biol Chem 260:9965-9968.

Hai, C.-M. 1991. Length-dependent myosin phosphorylation and contraction of arterial smooth muscle. Pflugers Arch 418:564-571.

Hai, C.-M., and R.A. Murphy. 1988. Cross-bridge phosphorylation and regulation of latch state in smooth muscle. Am J Physiol 254:C99-C106.

Hall, I.P., and E.R. Chilvers. 1989. Inositol phosphates and airway smooth muscle. Pulmonary Pharmacol 2:113-120.

Hall, I.P., J. Donaldson, and S.J. Hill. 1989. Inhibition of histamine-stimulated inositol phospholipid hydrolysis by agents which increase cyclic AMP levels in bovine tracheal smooth muscle. Br J Pharmacol 97:603-613.

Haller, H., J.I. Smallwood, H. Rasmussen. 1990, Portein kinase C translocation in intact vascular smooth muscle strips. Biochem J 270:375-381.

Hamasaki, Y., T. Saga, M. Mojarad, and S.I. Said. 1983. VIP counteracts leukotriene D_4-induced contractions of guinea pig trachea, lung, and pulmonary artery. Trans Asso Am Physicians 96:406-411.

Harder, D.R., R. Gilbert, and J.H. Lombard. 1987. Vascular muscle cell depolarization and activation in renal arteries on elevation of transmural pressure. Am J Physiol 253(Renal Fluid Electrolyte Physiol 22):F778-F781.

Hargreave, F.E., ed., 1980, Airway Reactivity, Mississauga, Ontario, Astra Pharmaceuticals.

Hathaway, D.R., and J.R. Haeberle. 1985. A radioimmunoblotting method for measuring myosin light chain phosphorylation levels in smooth muscle. Am J Physiol 249(Cell Physiol 18):C345-C351.

Hay, D.W.P., R.M. Muccitelli, and D. Raeburn. 1988. Does the epithelium-derived inhibitory factor (EpDIF) act via inhibition of phosphatidylinositol (PI) turnover in guinea-pig trachea (GPT)? FASEB J 2:A1057.

Henderson, R.M. 1975. Cell-to-cell contacts. In: Medthods in Pharmacology 3, Smooth Muscle, E.E. Daniel, and D.M. Paton. eds. Plenum, New York, pp47-77.

Hill, A.V. 1938. The heat of shortening and the dynamic constants of muscle. Proc R Soc Lond Ser B 126:136-195.

Hoar, P.E., E.G.L. Kerick, and P.S. Cassidy. 1979. Chicken gizzard: relation between calcium-activated phosphorylation and contraction. Science 204:503-506.

Hogg, J.C. Airway Hyperreactivity, Relationship to Disease States, In: Airway Smooth Muscle in Health and Disease, R.F. Coburn, Plenum, New York, pp267-276.

Hoh, J.F.Y., P.A. Mcgrath, and P.T. Hale. 1978. Electrophoretic analysis of multiple forms of rat cardiac myosin: effect of hypophysectomy and thyroxine replacement. J Mol Cell Cardiol 10:1053-1076.

Hoh, J.F.Y., and G.P.S. Yeoh. 1979. Rabbit skeletal myosin isoenzymes from fetal, fast-twitch and slow-twitch muscles. Nature 280:321-323.

Hokin, L.E., and M.R. Hokin. 1955. Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices, Biochim Biophys Acta 18:102-110.

Hokin, M.R., and L.E. Hokin. 1953. Enzyme secretion and the incorporation of ^{32}P into phospholipids of pancreatic slices. J Biol Chem 203:967-977.

Holme, G., and H. Piechuta. 1981. The derivation fo an inbred line of rats which develop asthma-like symptoms following challenge with aerosolized antigen. Immunology 42:19-24.

Holmes, K.C., D. Popp, W. Gebhard, W.A. Kabsch. 1990. Atomic model of the actin filament. Nature 347(6288): 44-49.

Horowits, R., E.S. Kempner, M.E. Bishor, and R.J. Podolsky. 1986. A physiological role for titin and nebulin in skeletal muscle. Nature 323:160-195.

Horwitz, J. 1989. Muscarinic receptor stimulation increases inositol-phospholipid metabolism and inhibits cyclic AMP accumulation in PC12 cells. J Neurochem 53:197-203.

Huber, H.L., and K.K. Koessler. 1922. The pathology of bronchial asthma. Arch Intern Med 30:689-760.

Hurwitz, L. 1986. Pharmacology of calcium channels and smooth muscle. Ann Rev Pharmacol Toxicol 26:225-258.

Hwang, K., and van Breemen. 1987. Ryanodine modulation of ^{45}Ca efflux and tension in rabbit aortic smooth muscle. Pflüger Arch 408:343-350.

Ishihara, H., H. Ozaki, K. Sato, M. Hori, H. Karaki, S. Watabe, Y. Kato, N. Tusetani, K. Hashimoto, D. Uemura, D.J. Hartshorne. 1989. Calcium-independent activation of contractile apparatus in smooth muscle by calyculin-A. J Pharmacol Exp Ther 250:388-396.

Itaya, K., and M. Ui. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. Clin Chim Acta 14:361-366.

Ito, Y., and K. Takeda. 1982. Non-adrenergic inhibitory nerves and putative transmitters in the smooth muscle of cat trachea. J Physiol 330:497-511.

Itoh, H., and K. Lederis. 1987. Contraction of rat thoracic aorta strips induced by phorbol 12-myristate 13-acetate. Am J Physiol

252:C244-C247.

Itoh, T., H. Kuriyama, and H. Suzuki. 1981. Excitation-contraction coupling in smooth muscle cells of the guinea-pig mesenteric artery. J Physiol 321:513-535.

Jafferji, S.S., and R.H. Michell. 1976. Muscarinic cholinergic stimulation of phosphatidylinoditol turnover in the longitudinal smooth muscle of guinea-pig ileum. Biochem J 154:653-657.

Janis, R.A., P.J. Silver, and D.J. Triggle. 1987. Drug action and cellular calcium regulation. Adv Drug Res 16:309-591.

Jean, T., and C.B. Klee. 1986. Calcium modulation of inositol 1,4,5-trisphosphate-induced calcium release from neuroblastoma X glioma hybrid (NG108-15) microsomes. J Biol Chem 261:16414-16420.

Jenne, J.W. and S. Murphy., eds., 1987. Drug Therapy for Asthma - Research and Clinicl Practice, Marcel Dekker, New York.

Jewell, B.R. and D.R. Wilkie. 1960. The mechanical properties of relaxing muscle. J Physiol 152:30-47.

Jewell, B.R. 1974. Decreased activation at short lenghths. In: The Physiological Basis of Starling's Law of the Heart, Ciba Symposium:24, Elsevier, Amsterdam, pp109-111.

Jiang, H., K. Rao, A.J. Halayko, W. Kepron, and N.L. Stephens. 1992a. Bronchial smooth muscle mechanics of canine model of allergic airway hyperresponsiveness. J Appl Physiol 72(1):39-45.

Jiang, H., K. Rao, A.J. Halayko, and N.L. Stephens. 1992b. Length-dependency of canine tracheal smooth muscle (TSM) myosin light

chain (MLC) phosphorylation. Am Rev Respir Dis 145(4):A389.

Jiang, H., K. Rao, A.J. Halayko, X. Liu, and N.L. Stephens. 1992c. Ragweed sensitization-induced increase of myosin light chain kinase content in canine airway smooth muscle. Am J Respir Cell Mol Biol 7:567-573.

Jiang, H., K. Rao, and N.L. Stephens. 1993a. Different levels of intracellular calcium ($[Ca^{2+}]_i$) between smooth muscle isometric and isotonic contractions. Biophy J 64(2 pt2):A260.

Jiang, H., K. Rao, G. Liu, and N.L. Stephens. 1993b. Intracellular Ca^{2+}, calmodulin activity, and myosin light chain phosphorylation in sensitized airway smooth muscle. Submitted to Am J Physiol (Lung Cell Mol Physiol).

Jiang, H., and N.L. Stephens. 1990. Contractile properties of bronchial smooth muscle with and without cartilage. J Appl Physiol 69(1):120-126.

Jiang, H., and N.L. Stephens. 1992. Isotonic relaxation of sensitized bronchial smooth muscle. Am J Physiol 262(Lung Cell Mol Physiol 6):L344-L350.

Jiang, H., and N.L. Stephens. 1993. Calcium and smooth muscle contraction (Invitied Review). Mol Cell Biochem, in press.

Jiang, M.J., K.G. Morgan. 1987. Intracellular calcium levels in phorbol ester-induced contractions of vascular smooth muscle. Am J Physiol 253:H1365-H1371.

Johnson, P.C. 1980. The myogenic response. In: Handbook of Physiology. The Cardiovascular System. Vascular Smooth Muscle.

Am Physiol Soc Bethesda, M.D. sect 2, vol II, chapt 15, pp409-442.

Johansson, B., and S. Mellander. 1975. Static and dynamic components in the vascular myogenic response to passive changes in length as revealed by electrical and mechanical recordings from the rat portal vein. Cir Res 36:76-83.

Jones, C.A., J.M. Madison, M. Tom-May, and J.K. Brown. 1987. Muscarinic cholinergic inhibition of adenylate cyclase in airway smooth muscle. Am J Physiol 253(Cell Physiol 22):C97-C104.

Jones, T.R., L. Charette, M.L. Garcia, and G.J. Kaczorowski. 1990. Selective inhibition of relaxation guinea-pig trachea by charybodotoxin, a potent Ca^{2+}-activated K^+ channel inhibitor. J Pharmacol Exp Ther 225:697-706.

Jones, T.R., L. Chareste. 1992. Interaction of the Ca^{2+}X-activated K^+ channel inhibitor iberiotoxin with beta adrenoceptor agonists on isolated guinea pig trachea. Am Rev Respir Dis 142:A203.

Kabsch, W., H.G. Mannherz, D. Suck, E.F. Pai, K.C. Holmes. 1990. Atomic structure of the actin:DNase I complex. Nature 347:37-44.

Kaliner, M.J. and P.J. Barnes, eds., 1988. The Airway Neural Control in Health and Disease, Marcel Dekker, New York.

Kamm, K.E., and J.T. Stull. 1985a, Myosin phosphorylation, force, and maximal shortening velocity in neurally stimulated tracheal smooth muscle. Am J Physiol 249 (Cell Physiol 18): C238-C247.

Kamm, K.E., and J.T. Stull. 1985b, The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Ann Rev

Pharmacol Toxicol 25:593-620.

Kamm, K.E., and J.T. Stull. 1989. Regulation of smooth muscle contractile elements by second messengers. Ann Rev Physiol 51:299-313.

Katsuyama, H., and K.G. Morgan. 1993. Mechanisms of Ca^{2+}-independent contraction in single permeabilized ferret aorta cells. Circ Res 72:651-657.

Kawamoto, S., and R.S. Adelstein. 1987. Characterization of myosin heavy chains in cultured aorta smooth muscle cells. J Biol Chem 262:7282-7288.

Kepron, W., J.M. James, B. Kirk, A.H. Sehon, and K.S. Tse. 1977. A canine model for reaginic hypersensitivity and allergic bronchoconstriction. J Allergy Clin Immunol 59:64-69.

Kerrick, W.G.L., M.P. Walsh, C. Sutherland, and P.E. Hoar. 1989. Caldesmon is required for the activation of smooth muscle contraction in skinned gizzard cells. Biophys J 55:70a.

Kirber, M.T., J.V. Walsh Jr., and J.J. Singer. 1988. Stretch-activated ion channels in smooth muscle: a mechanism for the initiation of stretch-induced contraction. Pflügers Arch 412:339-345.

Kirber, M.T. et al. 1992. FEBS Lett 297:24-28.

Kirkpatrick, C.T. 1975. Excitation and contraction in bovine tracheal smooth muscle. J Physiol (London) 244:263-281.

Kitazawa, T., S. Kobayashi, K. Horiuchi, A.V. Somlyo, and A.P. Somlyo. 1989. Receptor coupled, permeabilized smooth muscle: Role of the phosphatidylinositol cascade, G-proteins and modulation

of the contractile response to Ca^{2+}. J Biol Chem 264:5339-5342.

Kitazawa, T., M. Masuo, and A.P. Somlyo. 1991. G protein-mediated inhibition of myosin light chain phosphatase in vascular smooth muscle. Proc Natl Acad Sci (USA) 88(20):9307-9310.

Kitazawa, T., M. Masuo, and A.P. Somlyo. 1992. G protein-activation inhibits smooth muscle MLC phosphatase to increase the contractile sensitivity to Ca^{2+}. Jpn J Pharmacol 58: Suppl 2:348p.

Kobayashi, S., T. Kitazawa, A.V. Somlyo, and A.P. Somlyo. 1989. Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. Physiological role of inositol 1,4,5-trisphosphate in pharmacolmechanical coupling. J Biol Chem 264:17997-18004.

Kong, S.K., A.J. Halayko, and N.L. Stephens. 1990. Increased myosin phosphorylation in sensitized canine tracheal smooth muscle. Am J Physiol 259(Lung Cell Mol Physiol 3):L53-L56.

Kong, S.K., R.P.C. Shiu, and N.L. Stephens. 1986. Studies of myofibrillar ATPase in ragweed sensitized canine pulmonary smooth muscle. J Appl Physiol 60:92-96.

Kowarski, D., H. Shuman, A.P. Somlyo, A.V. Somlyo. 1985. Calcium release by noradrenaline from central sarcoplasmic reticulum in rabbit main pulmonary artery smooth muscle. J Physiol 366:153-175.

Krahl, V.E. 1964. Anatomy of the mammalian lung. In: Handbook of Physiology, Respiration, Washington, DC: Am Physiol Soc, section 3, Vol I, pp 213-284.

Kroeger, E.A., and N.L. Stephens. 1971. Effect of hypoxia on energy and

calcium metabolism in airway smooth muscle. Am J Physiol 220:1199-1204.

Kroeger, E.A., and N.L. Stephens. 1975. Effect of tetraethylammonium ion on airway smooth muscle: initiation of phasic electrical activity. Am J Physiol 228:633-636.

Kron, S.J., Y.Y. Toyoshima, T.Q.P. Uyeda, and J.A. Spudich. 1991. Assays for actin sliding movement over myosin-coated surfaces. Methods Enzymol 196:399-416.

Kubota, Y., K.E. Kamm, and J.T. Stull. 1990. Mechanism of GTP-gamma-S-dependent regulation of smooth muscle contraction. Biophys J 57:152a.

Kume, H., M.P. Graziano, and M.I. Kotlikoff. 1992. Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide binding proteins. Proc Natl Acad Sci (USA) 89:11051-11055.

Kume, H., A. Takai, H. Tokuno, and T. Tomita. Regulation of Ca^{2+}-dependent K^{+}-channel activity in tracheal myocytes by phosphorylation. Nature 341:152-154.

Kuriyama, H., Y. Ito, H. Suzuki, K. Kitamura, and T. Itoh. 1982. Factors modifying contractio-relaxation cycle in vascular smooth muscles. Am J Physiol 243:H641-H662.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 27:680-685.

Laitinen, A., M. Partanen, A. Hervonen, M. Pelto-Huikko, and L.A. Laitenen. 1985. VIP-like immunoreactive nerves in human

respiratory tract. Histochemistry 82:313-319.

Langton. 1993. J Physiol (Lond) 471:1-11.

Lansman, J.B., T.J. Hallam, and T.J. Rink. 1987. Single, stretch-activated ion channels in vascular endothelial cells as mechanotrasducers? Nature 325:811-813.

Lanzetta, P.A., L.J. Alvarez, P.S. Reinach, and O.A. Candia. 1979. An improved assay for nanomole amounts of inorganic phosphate. Anal Biochem 100:95-97.

Larsell, G., and R.S. Dow. 1933. The innervation of the human lung. Am J Anat 52(1):125-146.

Liebman, P.A. 1986. The role of cGMP control in visual receptor transduction. Neurosci Res 4(Suppl):S35-S43.

Litosch, I., and J.N. Fain. 1985. 5-Methyltryptamine stimulates phospholipase C-mediated breakdown of exogenous phosphoinositides by blowfly salivary gland membranes. J Biol Chem 260:16052-16055.

Liu, X., K. Rao, G. Liu, A.J. Halayko, and N.L. Stephens. 1993. Phosphatase activity of sensitized airway smooth muscle. In press: Am J Physiol (Lung Cell Mol Physiol).

Lucas, D.O., S.M. Bajjalieh, J.A. Kowalchyk, T.F.J. Martin. 1985. Direct stimulation of thyrotropin-releasing hormone (TRH) of polyphosphoinositide hydrolysis in GH_3 cell membranes by a guanine nucleotide-modulated mechanism. Biochem Biophys Res Commun 132:721-728.

Lundberg, J.M., T. Hökfelt, G. Nilsson, L. Terenius, J. Rehfeld, R.

Elde, and S.I. Said. 1978. Peptide neurons in the vagus, splanchnic, and sciatic nerves. Acta Physiol Scand 104:499-501.

Lundberg, J.M., L. Terenius, T. Hökfelt, and M. Goldstein. 1983. High levels of neuropeptide Y in peripheral noradrenergic neurons in various mammals including man. Neurosci Lett 42:167-172.

Macklin, C.C. 1929. The musculature of the bronchi and lungs. Physiol Rev 9:1-60.

Malmqvist, U., and A. Arner. 1991. Correlation between isoform composition of the 17 kDa myosin light chain and maximal shortening velocity in smooth muscle. Eur J Physiol 418:523-530.

Mapp, C.E., P. Chitano, N. De Marzo, P. Di Blasi, M. Saetta, A. Di Stefano, V.M. Bosco, L. Allegra, and L.M. Fabbri. 1989. Response to acetylcholine and myosin content of isolated canine airways. J Appl Physiol. 67(4):1331-1335.

Marston, S.B. 1982. The regulation of smooth muscle contractile proteins. Prog Biophys Mol Biol 41:1-41.

Marston, S.B., K. Pritchard, C. Rediwood, and M. Taggart. 1988. Ca^{2+} regulation of thin flaments: biochemical mechanisms and physiological role. Biochem Soc Trans 16:494-497.

Martin, R., and A.J. Woolcock. 1989. Is a myogenic response involved in deep inspiration-induced bronchoconstriction in asthmatics? Am Rev Respir Dis 140:1354-1358.

Martling, C.R. 1987. Sensory nerves containing tachykinins and CGRP in the lower airways. Functional implications for bronchoconstriction, vasodilation and protein extravasation. Acta Physiol Scand Suppl

563:1-57.

McAtee, P., and G. Dawson. 1989. Rapid dephosphorylation of protein kinase C substrates by protein kinase A activation results from inhibition of diacylglycerol release. J Biol Chem 264:11193-11199.

Melin, P-M., R. Sundler, and B. Jergil. 1986. Phospholipase C in rat liver plasma membranes. FEBS Lett 198:85-88.

Merkel, L., W.T. Gerthoffer, and T.J. Torphy. 1990. Dissociation between myosin phosphorylation and shortening velocity in canine trachea. Am J Physiol 259(Cell Physiol 27):C524-C532.

Meyer, T., T. Wensel, and L. Stryer. 1990. A site-directed mutagenesis study on Escherichia coli inorganic pyrophosphatase. Glutamic acid-98 and lysine-104 are important for structural integrity, whereas aspartic acids-97 and -102 are essntial for catalytic. Biochemistry 29:32-37.

Mignery, G.A., ad T.C. Sunhof. 1990. The ligand binding site and transduction mechanism in the inositol-1,4,5-triphosphate receptor. EMBO J 9:3893-3898.

Mignery, G.A., T.C. Sudhof, K. Takei, P. De-Camilli. 1989. Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature 342(6246): 192-195.

Miller, D.K., J.W. Gillard, P.J. Vickers, S. Sadowski, C. Leveille, J.R. Mancini, P. Charleson, R.A.F. Dixon, A.W. Ford-Hutchinson, R. Fortin, J-Y. Gauthier, R.C. Rosen, C. Rouzer, I.S. Sigal, C.D. Strader, and J.F. Evans. 1990. Identification and isolation of a membrane protein necessary for leukotriene production. Nature

343:278-281.

Minette, P., and P.J. Barnes. 1988. Prejunctional muscarinic receptors on cholinergic nerves to human and guinea pig airways. J Appl Physiol 64:2532-2537.

Missiaen, L., C.W. Taylor, and M.J. Berridge. 1991. Spontaneous calcium release from inositol trisphosphate-sensitive calcium store. Nature 352:241-244.

Mitchell, R.W., and N.L. Stephens. 1983. Maximum shortening velocity of smooth muscle: zero load-clamp vs. afterloaded method. J Appl Physiol 55:1630-1633.

Moore, E.D.W., E.F. Etter, K.D. Philipson, W.A. Carrington, K.E. Fogarty, L.M. Lifshitz, and F.S. Fay. 1993. Coupling of the Na^+/Ca^{2+} exchanger, Na^+/K^+ pump and sarcoplasmic reticulum in smooth muscle. Nature 365:657-660.

Moreland, S., R.S. Moreland, and H.A. Singer. 1986. Apparent dissociation between myosin light chain phosphorylation and maximal velocity of shortening in KCl depolarized swine carotid artery: effect of temperature and KCl concentration. Pflügers Arch 408:139-145.

Moreno, R.H., J.C. Hogg, T.M. MaLean, and P.D. Paré. 1987. Isovolumetric and isobaric rabbit tracheal contraction *in vitro*. J Appl Physiol 62:82-90.

Morgan, J.P., and K.G. Morgan. 1982. Vascular smooth muscle: the first recorded Ca^{2+} transients. Pflugers Arch. 395:75-77.

Morgan, J.P., C.L. Perreault, and K.G. Morgan. 1991. The cellular basis

of contraction and relaxation in cardiac and vascular smooth muscle. Am Heart J 121:961-968.

Morrison, K.J., and P.M. Vanhoutte. 1991. Airway epithelial cells in the pathophysiology of asthma. In: Advances in the Understanding and Treatment of Asthma. Ann New York Acad Sci Vol 629, P.J. Piper and R.D. Krell, eds. New York Acad Sic, New York. pp82-88.

Moss, J., M. Vaughan. 1988. ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. Adv Enzymol 61:303-379.

Murakami, U., and K. Uchida. 1985. Contents of myofibrillar proteins in cardiac, skeletal and smooth muscles. J Biochem 98:187-197.

Murlas, C. 1986. Effects of mucosal removal on guinea-pig airway smooth muscle responsiveness. Clin Sci 70:571-575.

Murray, R.K., C.S. Bennett, S.J. Fluharty, and M. Kotlikoff. 1989. Mechanisms of phorbol ester inhibition of histamine-induced IP_3 formation. Am J Physiol 257(Lung Cell Mol Physiol 1):L209-L216.

Murphy, T.M., R.W. Mitchell, J.S. Blake, M.M. Mack, E.A. Kelly, N.M. Munoz, and A.R. Leff. 1989. Expression of airway contractile properties and acetycholinesterase activity in swine. J Appl Physiol. 67(1):174-180.

Nabika, T., P.A. Velletri, W. Lovenberg, and M.A. Beavens. 1985. Increase in cytosolic calcium and phosphoinositide metabolism induced by angiotensin II and [Arg] vasopressin in vascular smooth

muscle cells. J Biol Chem 260:4661-44670.

Nadel, J.A., ed., 1980. Physiology and Pharmacology of the Airway, Marcel Dekker, New York.

Nagaishi, C., ed., 1972. Functional Anatomy and Histology of the Lung, Unversity Park Press, Baltimore.

Nastainczyk, W., A. Röhrkasten, M. Sieber, C. Rudolph, C. Schachtele, D. Marmé, and F. Hofmann. 1987. Phosphorylation of the purified receptor for calcium channel blockers by cAMP kinase and protein kinase C. Eur J Biochem 169:137-142.

Nava, H., H. Kotani, and S. Nakanishi. 1984. Tissue specific generation of two prepotachykinin mRNAs from one gene by alternative RNA splicing. Nature 312-729.

Ngai, P.K., U. Groschel-Stewart, and M.P. Walsh. 1986. Comparison of the effects of smooth and skeletal muscle actins on smooth muscle actomyosin Mg^{2+}-ATPase. Biochem Int 12:89-96.

Ngai, P.K., and M.P. Walsh. 1987. The effects of phosphorylation of smooth muscle caldesmon. Biochem J 244:417-425.

Nishimura, J., M. Kolber, C. van Breemen. 1988. Norepinephrine and GTP-gamma-S increase myofilaments Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. Biochem Biophys Res Commun 157:677-683.

Nishimura, J., R.A. Khalil, J.P. Drenth, and C. van Breemen. 1990. Evidence for increased myofilament Ca^{2+} sensitivity in norepinephrine-activated vascular smooth muscle. Am J Physiol 259 (Heart Circ Physiol 28):H2-H8.

Nishimura, J., S. Moreland, R.S. Moreland, and C. van Breemen. 1991. Regulation of the Ca^{2+}-force relationship in permeabilized arterial smooth muscle. In: Regulation of Smooth Muscle Contraction, R.S. Moreland, ed. Plenum Press, New York, pp 111-127.

Oishi, K., H. Takano-Ohmuro, N. Minakawa-Matsuo, O. Suga, H. Karibe, K Kohama, and M.K. Uchida. 1991. Oxytocin contracts rat uterine smooth muscle in Ca^{2+}-free medium without any phosphorylation of myosin light chain. Biochem Biophys Res Commun 176:122-128.

Packer, C.S. and N.L. Stephens. 1985. Mechanics of caudal artery relaxation in control and hypertensive rats. Can J Physiol Pharmacol 63:209-213.

Packer, C.S. and N.L. Stephens. 1987. Prolonged isobaric relaxation time in small mesenteric arteries of the spontaneously hypertensive rat. Can J Physiol Pharmacol 65:230-235.

Panitch, H.B., J.L. Allen, J.P. Ryan, M.R. Wolfson, and T.H. Shaffer. 1989. A comparison of preterm and adult airway smooth muscle mechanics. J Appl Physiol 66(4):1760-1765.

Park, S., and H. Rasmussen. 1986. Carbachol-induced protein phosphorylation changes in bovine tracheal muscle. J Biol Chem 261:15734-15739.

Pedley, T.J., R.C. Schroter, and M.F. Sudlow. 1970. The prediction of pressure drop and variation of resistance within the human bronchial airways. Respir Physiol 9:387-405.

Penner, R., G. Matthews, and E. Neher. Regulation of calcium influx by

second messengers in rat mast cells. Nature 334:499-504.

Persechini, A., K.E. Kamm, and J.T. Stull. 1986. Different phosphorylated forms of myosin in contracting tracheal smooth muscle. J Biol Chem 261:6293-6299.

Piper, P.J., S.I. Said, and J.R. Vane. 1970. Effects on smooth muscle preparations of unidentified vasoactive peptides from the intestine and lung. Nature 225:1144-1146.

Pohl, J., M.P. Walsh, and W.T. Gerthoffer. 1991. Calponin and caldesmon phosphorylation in canine tracheal smooth muscle. Biophys J 59:58a.

Pritchard, K., and S.B. Marston. 1993. The Ca^{2+}-sensitizing component of smooth muscle thin filaments properties of regulatory factors that interact with caldesmon. Biochem Biophys Res Commun 190:668-673.

Pritchard, K., and C.J. Moody. 1986. Caldesmon: a calmodulin-binding actin-regulatory protein. Cell Calcium 7:309-328.

Raeymaekers, L., and L.R. Jones. 1986. Evidence for the presence of phosphlamban in the endoplasmic reticulum of smooth muscle. Biochim Biophys Acta 882:258-265.

Rao, K., H. Jiang, A.J. Halayko, N. Pan, W. Kepron, and N.L. Stephens. 1991. Increased ATPase activity and myosin light chain kinase (MLCK) content in airway smooth muscle from sensitized dogs. In: Regulation of Smooth Muscle Contraction, R.S. Moreland, ed., Plenum Press, New York, pp 369-376.

Rao, K., A.J. Halayko, H. Jiang, and N.L. Stephens. 1993. Unaltered

calmodulin activity in ragweed pollen sensitized canine airway smooth muscle. Am Rev Respir Dis 147(Supl. Pt 2):A53.

Rasmussen, H., Y. Takuwa, and S. Park. 1987. Protein kinase C in the regulation of smooth muscle contraction. FASEB J 1:177-185.

Rayment, I., W.R. Rypniewski, K. Schmidt-Base, R. Smith, D.R. Tomchick, M.M. Benning, D.A. Winkelmann, G. Wesenberg, and H.M. Holden. 1993a. Three-dimensional structure of myosin subfragment-1: a molecular motor. Science 261(5117):50-58.

Rayment, I. H.M. Holden, M. Whittaker, C.B. Yohn, M. Lorenz, K.C. Holmes, and R.A. Milligan. 1993b. Structure of the actin-myosin complex and its implications for muscle contraction. Science 261:58-65.

Raymond, J.R., M. Hnatowich, M.G. Caron, R.J. Lefkowitz. 1990. Structure-function relationships of G-protein-coupled receptors. In: ADP-ribosylating toxins and G proteins: insights into signal transduction. Moss, J., and M. Vaughan. eds. Washington, D.C. Am Soc for Microbiol 163-188.

Rebecchi, M.J., R.N. Kolesnick, and M.C. Gershengorn. 1983. Thyrotropin-releasing hormone stimulates rapid loss of phosphatidylinositol and its conversion to 1,2-diacylglycerol and phosphatidic acid in rat mammotropic pituitary cells: Association with calcium mobilization and prolactin secretion. J Biol Chem 258:227-234.

Rembold, C.M., and R.A. Murphy. 1988. [Ca^{2+}]-dependent myosin phosphorylation in phorbol diester stimulated smooth muscle

contraction. Am J Physiol 255:C719-C723.

Rembold, C.M., and R.A. Murphy. 1990. Latch-bridge model in smooth muscle: $[Ca2+]_i$ can quantitatively predict stress. Am J Physiol 259:C251-C257.

Reuter, H. 1991. Sodium-calcium exchange. Ins and outs of Ca2+ transport. Nature 349:567-568.

Richardson, J.B., and C.C. Furguson. 1980. Neuromuscular structure and function in the airways. Fed Proc 38:202-208.

Rodger, I.W. 1985. Excitation-contraction coupling and uncoupling in airway smooth muscle. Br J Clin Pharmacol 20:255S.

Rouzer, C.A., T. Matsumoto, and B. Samuelsson. 1986. Single protein from human leukocytes possesses 5-lipoxygenase and leukotriene A_4 synthase activities. Proc Natl Acad Sci USA 83:857-861.

Rouzer, C.A. and B. Samuelsson. 1985. On the nature of the 5-lipoxygenase reaction in hyman leukocytes: enzyme purification and requirement for multiple stimulatory factors. Proc Natl Acad Sci USA 82:6040-6044.

Rovner, A.S., M.M. Thompson, and R.A. Murphy. 1986. Two different heavy chains are found in smooth muscle myosin. Am J Physiol 250(Cell Physiol 19):C861-C870.

Russell, J.A. 1978. Responses of isolated canine airways to electric stimulation and acetylcholine. J Appl Physiol: Respirat Environ Exercise Physiol 45(5):690-698.

Sachs, F. 1989. Ion channels as mechanical transducers. In: Cell Shape. Academic, New York, chapt. 3, pp63-92.

Said, S.I., S. Kitmatura, T. Yoshida, J. Preskitt, and L.D. Holden. 1974. Humoral control of airways. Ann N.Y. Acad Sci 221:103-114.

Said, S.I. 1982. Vasoactive substances in the lung, with special reference to vasoactive intestinal peptide. Exp Lung Res 3:343-348.

Said, S.I. 1984. Peptide hormones and neurotransmitters of the lung. In: The Endocrine Lung in Health and Disease, Vol 15. K.L. Becker and A. Gazdar, eds. W.B. Saunders, Philadelphia, pp267-276.

Said, S.I. 1989. Polypeptide-containing neurons and their function in airway smooth muscle. In: Airway Smooth Muscle in Health and Disease. R.F. Coburn, ed., Plenum, New York. pp55-76.

Said, S.I. 1991. Vasoactive intestinal polypeptide (VIP) in asthma. In: Advances in the Understanding and Treatment of Asthma. P.J. Piper, and R.D. Krell. eds. Annl N.Y. Acad Sci Vol 629, pp305-318.

Saida, K. 1981. Ca^{2+}- and depolarization-induced Ca^{2+} release in skinned smooth muscle fibers. Biomed Res 2:453-55.

Saida, K. 1982. Intracellular Ca release in skinned smooth muscle. J Gen Physiol 80:191-202.

Salmon, D.M., and T.W. Honeyman. 1979. Increased phosphatidate accumulation during single blowfly salivary glands exposed to 5-hydroxytryptamine. Biochem J 222:327-334.

Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitity reactions and inflammation. Science 220:568-575.

Samuelsson, B., S.-E. Dahlen, J.A. Lindgren, C.A. Rouzer, and C.N. Serhan. 1987. Leukotrienes and lipoxins: structures, biosynthesis,

and biological effects. Science 237:1171-1176.

Schlender, K.K., W. Wang, and S.E. Wilson. 1989. Evidence for a latent of protein phosphatase 1 associated with cardiac myofibrils. Biochem Biophys Res Commun 159:72-78.

Schwartz, K., Y. Lecarpentier, J.L. Martin, A.M. Lompre, J.J. Mercadier, and B. Swynghedauw. 1981. Myosin isoenzymic distribution correlated with speed of myocardial contraction. J Mol Cell Cardiol 13:1071-1075.

Sears, M.R. 1986. Why are deaths from Asthma increasing? Eur J Resir Dis 69 (Suppl 147):175-181.

Sekar, M.C., and L.E. Hokin. 1986. The role of phosphoinositides in signal transduction. J Membr Biol 89:193-210.

Sellers, J.R. 1993. Structure and function of sarcomeric and nonsarcomeric contractile proteins. Personal communication.

Seow, C.Y., and N.L. Stephens. 1986. Force-velocity curves for smooth muscle: analysis of internal factors reducing velocity. Am J Physiol 251(Cell Physiol 20):C362-C368.

Seow, C.Y., and N.L. Stephens. 1987. Time dependence of series elasticity in tracheal smooth muscle. J Appl Physiol 62:1556-1561.

Sheetz, M.P., S.M. Block, and J.A. Spudich. 1986. Myosin movement in vitro: A quantitative assay using oriented actin cables from Nitella. Methods Enzymol 134:531-544.

Shibata, S., and J.R. Cheng. 1977. Relaxation of vascular smooth muscle in spontaneously hypertensive rats. Blood Vessels 14:247-248.

Shimizu, T., T. Izumi, Y. Seyama, Y. Tadokoro, O. Radmark, and B.

Samuelsson. 1986. Characterization of leukotrine A_4 synthase from murine mast cells: evidence for its identity to arachidonate 5-lipoxygenase. Proc Natl Acad Sci USA 83:4175-4179.

Shimosegawa, T., H.D. Foda, and S.I. Sain. 1989. Immunohistochemical demonstration of enkephalin containing nerve fibres in guinea pig and rat lungs. Am Rev Respir Dis 140:441-448.

Shioya, T., E.R. Pollack, N.M. Munoz, and A.R. Leff. 1987. Distribution of airway contractile responses in major resistance airways of the dog. Am J Pathol 129(1):102-117.

Siegman, M.J., T.M. Butler, S.U. Mooer, and R.E. Davies. 1976. Crossbridge: attachment, resistance to stretch and viscoelasticity in resting mammalian mooth muscle. Science 191:383-385.

Siegman, M.J., T.M. Butler, S.U. Mooers. 1989. Phosphatase inhibition with okadaic acid does not alter the relationship between force and myosin light chain phosphorylation in permeabilized smooth muscle. Biochem Biophys Res Commun 161:838-842.

Sigurdsson, S., R., H. Jiang, W. Kepron, and N. L. Stephens. 1990. Sensitized canine bronchial smooth muscle as a model for allergic brinchospasm. FASEB J. 4: A616.

Sigurdsson, S., R. Jabr, A Miller, N.L. Stephens, and W.C. Cole. 1992. Whole-cell and single channel K^+ currents in isolated canine bronchial smooth muscle cells. Biophy J 61(2 pt2):A1446.

Silva, D.G., and G. Ross. 1974. Ultrastructural and fluorescence histochemical studies on the innervation of the tracheo-bronchial muscle of normal cells and cats treated with 6-hydroxydopamine.

J Ultrastuct Res 47:310-328.

Silver, P., and J.T. Stull. 1982. Regulation of myosin light chain and phosphorylase phosphorylation in tracheal smooth muscle. J Biol Chem 257:6145-6150.

Simpson, J., R. Rose, and W.R. Loewenstein. 1977. Size limit of molecules permeating the junctional membrane channels. Science 195:294-296.

Sly, R.M. 1986. Effects of treatment on mortality from asthma. Ann Allergy 56:207-212.

Small, J.V., D.O. Furst, and J. DeMeys. 1986. Localization of filamin in smooth muscle. J Cell Biol 102:210-220.

Small, J.V., and A. Sobieszek. 1977. Studies on the functiion and composition of the 10-nm (100 A) filaments of vertebrate smooth muscle. J Cell Sci 23:243-268.

Smith, J.B., L.S. Smith, E.R. Brown, D. Barnes, M.A. Sabir, J.S. Davis, and R.V. Farese. 1984. Angiotensin II rapidly increases phosphatidate-phosphoinoditide synthesis and phosphoinositide hydrolysis and mobilizes intracellular calcium in cultured vascular smooth muscle cells. Proc Natl Acad Sci 81:7812-7816.

Smith, J.B., L.S. Smith, and B.L. Higgins. 1985. Temperature and nucleotide dependence of calcium release by myo-inositol 1,4,5-trisphosphate in cultured vascular smooth muscle cells. J Biol Chem 260:14413-14416.

Sobieszek, A., and R.D. Bremel. 1975. Preparation and properties of vertebrate smooth muscle myofibrils and actomyosin. Eur J

Biochem 55:49-60.

Sobonya, R.E. 1990. Quantitative structural alterations in long-standing allergic asthma. Am Rev Respir Dis 130:289-292.

Sobue, K., Y. Muramoto, M. Fujita, and S. Kakiuchi. 1981. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. Proc Natl Acad Sci USA 78:5652-5655.

Sobue, K., K. Takahashi, T. Tanaka, K. Kanda, N. Ashino, S. Kakiuchi, and K. Maruyama. 1985. Crosslinking of actin filaments is caused by caldesmon aggregates, but not by its dimers. FEBS Lett 182:201-204.

Somlyo, A.P. 1980. Ultrastructure of vascular smooth muscle. In: The Handbook of Physiology, The Cardiovascular System. Vol 2, Vascular Smooth Muscle. D.F. Bohr, A.P. Somlyo, and H.V. Sparks, eds., Am Physiol Soc, Washington, pp33-67.

Somlyo, A.P., A.V. Somlyo, T. Kitazawa, M. Bond, H. Shuman, D. Kowarski. 1983. Ultrastructure, function and composition of smooth muscle. Ann Biomed Eng 11:579-588.

Somlyo, A.V., M. Bond, A.P. Somlyo, and A. Scarpa. 1985. Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. Proc Natl Acad Sci USA 82:5231-5235.

Somlyo, A.V., M. Bond, T.M. Butler, P.F. Berner, F.T. Ashton, H. Holtzer, and A.P. Somlyo. 1984. The contractile apparatus of smooth muscle: an update. In: Smooth Muscle Contraction, N.L. Stephens, ed., Marcel Dekker, New York, pp1-20.

Somlyo, A.P., T. Kitazawa, B. Himpens, G. Matthijs, K. Horiuti, S.

Kobayashi, Y.E. Goldman, and A.V. Somlyo. 1989. Modulation of Ca^{2+}-sensitivity and of the time course of contraction in smooth muscle: A major role of protein phosphatase? In: Advances in Protein Phosphatases, W. Merlevede and J. Di Salvo, eds., Leuven Univ Press, Vol 5:181-195.

Springall, D.R., A. Cadieux, H. Oliveira, H. Su, D. Royston, and J.M. Polak. 1987. Retrograde tracing shows that CGRP-immunoreactive nerves of rat trachea and lung originate from vagal and dorsal root ganglia. J Auton Nerv Syst 20:155-166.

Stabel, S., and P.J. Parker. 1991. Protein kinase C. Pharmacol Ther 51:71-95.

Stein, M. 1975; New Directions in Asthma, American College of Chest Physicians, Park Ridge, IL.

Stephens, N.L. 1973. Effect of hypoxia on contractile and series elastic components of smooth muscle. Am J Physiol 224:318-321.

Stephens, N.L. 1985. Force-velocity constants in smooth muscle: afterloaded isotonic and quick-release methods. Can J Physiol Pharmacol 63:48-51.

Stephens, N.L., and F.G. Hoppin, Jr. 1986. Mechanical properties of airway smooth muscle. In: Handbook of Physiol., The Respir System Vol III. Mechanics of Breathing, Pt 1, P.T. Macklem and J Mead, eds., Am Physiol Soc Bethesda.

Stephens, N.L. 1987. Airway smooth muscle. Am Rev Respir Dis 135:960-975.

Stephens, N. L.,S. K. Kong, and C. Y. Seow. 1988. Mechanisms of

increased shortening of sensitized airway smooth muscle. In: Mechanisms of Asthma: Pharmacology, Pysiology, and Management. C. L. Armour and J. L. black. eds, New York: Liss. p.231-251.

Stephens, N.L. 1990. Structure of airway smooth muscle. In: Airway Smooth Muscle: Modulation of Receptors and Response. Agrawal, D.K., and R.G. Townley, CRC Press, Boston. pp1-37.

Stephens, N.L., A.J. Halayko, and B. Swynghedauw. 1991. Myosin heavy chain isoform distribution in normal and hypertrophied rat aortic smooth muscle. Can J Physiol Pharmacol 69:8-14.

Stephens, N.L., and H. Jiang. 1993. Relaxation of smooth muscle. Can J Physiol Pharmacol, in press.

Stephens, N.L., H. Jiang, J. Xu, and W. Kepron. 1991. Airway smooth muscle mechanics and biochemistry in experimental asthma. Am Rev Respir Dis 143:1182-1188.

Stephens, N.L., S.K. Kong, C.Y. Seow. 1988. Mechanisms of increased shortening of sensitized airway smooth muscle. In: Mechanisms in Asthma. eds.: C.L. Armour, and J.L. Black, Alan Liss, New York, pp231-254.

Stephens, N.L., and E.A. Kroeger. 1980. Ultrastructure, biophysics, and biochemistry of airway smooth muscle. In: Physiology and Pharmacology of the Airways, Ed. Nadel, J.A., Lung Biology in Health and Disease, Marcel Dekker, New York, pp31-121.

Stephens, N.L., E.A. Kroeger, and S.K. Kong. 1984. Techniques for the study of airway smooth muscle. In: Techniques in the Life

Sciences, Vol P4/11, Techniques in Respiratory Physiology, Pt II, A.N. Otis, ed., County Clare, reland: Elsevier pp418-1-418-27.

Stephens, N.L., E.A. Kroeger, and J.P. Mehta. 1969. Force-velocity characteristics of respiratory airway smooth muscle. J Appl Physiol 26:685-692.

Stephens, N.L., and U. Kromer. 1971. Series elastic component of tracheal smooth muscle. Am J Physiol 220:1890-1895.

Stephens, N.L. and R.W. Mitchell. 1984. Shortening inactivation, maximum force protential, relaxation, contractility. In: Smooth Muscle Contraction, N.L. Stephens, ed. Marcel Dekker, New York, pp91-112.

Stephens, N.L., V. Nathaniel, W. Kepron, R.W. Mitchell, C.Y. Seow, and S.K. Kong. 1987. Anatomy and physiology of respiratory smooth muscle tone. In: Drug Therapy for Asthma: Research and Clinical Practice, J.W. Jenne and S. Murphy, eds., New York, Dekker, Vol 31 pp1-66 (Lung Biol Health Dis Ser).

Stephens, N.L., C.Y. Seow, A.J. Halayko, H. Jiang. 1992a, The biophysics and biochemistry of smooth muscle contraction. Can J Physiol Pharmacol 70:515-531.

Stephens, N.L., K. Wrogemann. 1970. Oxidative phosphorylation in smooth muscle. Am J Physiol 219(6):1796-1801.

Stocklet, J.-C., D. Gérard, M.-C. Kelhoffer, C. Lugnier, R. Miller, and P. Schaeffer. 1987. Calmodulin and its role in intracellular calcium regulation. Prog Neurobiol 29:321-364.

Streb, H., R.F. Irvine, M.J. Berridge, and I Schulz. 1983. Release of

Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. Nature 306:67-69.

Strulovici, B., J.M. Stadel, and R.J. Lefkowitz. 1985. Adenylate-cyclase-coupled beta-adrenergic receptors: Biochemical mechanisms of desensitization. In: Mechanisms of Receptor Regulation. Poste, G. and S.T. Crooke, eds., Plenum Press, New York. pp279-294.

Stull, J.T., P.J. Gallagher, B.P. Herring, and K.E. Kamm. 1991a. Vascular smooth muscle contractile elements, cellular regulation. Hypertension 17:723-732.

Stull, J.T., M.G. Tansey, A. Word, Y. Kubota, and K.E. Kamm. 1991b. Myosin light chain kinase phosphorylation: regulation of the Ca^{2+} sensitivity of contractile elements. In: Regulation of Smooth Muscle Contraction, R.S. Moreland, ed., Plenum Press, New York, pp 129-138.

Sturek, M., K. Kunda, and Q. Hu. 1992. Sarcoplasmic reticulum buffering of myoplasmic calcium in bovine coronary artery smooth muscle. J Physiol (London) 451:25-48.

Suematsu, E., M. Hirata, T. Hashimoto, and H. Kuriyama. 1984. Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of procine coronary artery. Biochem Biophys Res Commun 120:481-485.

Sumida, M., H. Okuda, and M. Hamada. 1984. Ca^{2+}, Mg^{2+}-ATPase of microsomal membranes from bovine aortic smooth muscle. Identification and characterization of an acid-stable phosphorylated intermediate of the Ca^{2+}, Mg^{2+}-ATPase. J Biochem 96:1365-1364.

Supattapone, S., P.F. Worley, J.M. Baraban, S.H. Snyder. 1988. Solubilization, purification, and characterization of an inositol trisphosphate receptor. J Biol Chem 263(3): 1530-1534.

Sutoh, K. 1983. Mapping of actin-binding sites on the heavy chain of myosin subfragment. Biochemistry 22:1579-1585.

Suzuki, H., K. Morita, and H. Kuriyama. 1976. Innervation and properties of the smooth muscle of the dog trachea. Jpn J Physiol 26:303-320.

Takahashi, K., K. Hiwada, and T. Kokubu. 1986. Isolation and characterization of a 34,000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle. Biochem Biophys Res Commun 141:20-26.

Takizawa, T., and W.M. Thurlbeck. 1971. Muscle and mucous gland size in the major bronchi of patients with chronic bronchitis, asthma, and asthmatic bronchitis. Am Rev Respir Dis 104:331-336.

Takuwa, Y., G. Kelley, N. Takuwa, and H. Rasmussen. 1988. Protein phosphorylation changes in bovine carotid artery smooth muscle during contraction and relaxation. Mol Cell Endocrinol 60:71-86.

Takuwa, Y., N. Takuwa, and H. Rasmussen. 1988. The effect of isoproterenol on intracellular calcium concentration. J Biol Chem 263:762-768.

Tan, J.L. S. Ravid, and J.A. Spudich. 1992. Control of nonmuscle myosins by phosphorylation. Annu Rev Biochem 61:721-759.

Tanaka, T. 1988. Calmodulin-dependent calcium signal tranduction. Jpn J Pharmacol 46:101-107.

Tanaka, T., H. Ohta, K. Kanda, T. Tanaka, H. Hidaka, K. Sobue. 1990. Phosphorylation of high-Mr caldesmon by protein kinase C modulates the regulatory function of this protein on the interaction between actin and myosin. Eur J Biochem 188:495-500.

Tansey, M.G., M. Hori, H. Karaki, K.E. Kamm, and J.T. Stull. 1990. Okadaic acid uncouples myosin light chain phosphorylation and tension in smooth muscle. FEBS Lett 270:219-221.

Taylor, S.R. and R. Rudel. 1970. Inactivation of contraction induced by shortening. Science 167:882-884.

Torphy, T.J. and W.T. Gerthoffer. 1986. Biochemical mechanisms of smooth muscle contraction and relaxation, In: Current Topics in Pulmonary Pharmacology and Toxicology, Vol 1, Hollinger, M.A., ed., Elsevier Science Publishing, New York, pp23-?.

Torphy, T.J. 1987. Biochemical regulation of airway smooth muscle tone: current knowledge and therapeutic implications. Rev Clin Basic Pharmacol 6:61-103.

Towbin, H., and J. Gordon. 1984. Immunoblotting and dot immunoblotting-current status and outlood. J Immunol Methods 72:313-340.

Travis, J. 1993. Cell biologists explore 'tiny caves'. Research News. Science 262:1208-1209.

Tregear, R.T., and J.M. Squire. 1973. Myosin content and filament structure in smooth muscle striated muscle. J Mol Biol 77:279-290.

Tregear, R.T., A.P. Dawson, and R.F. Irvine. 1991. Quantal release of Ca^{2+} from intracellular stores by $InsP_3$, tests of the concept of

control of Ca^{2+} release by intraluminal Ca^{2+}. Proc Roy Soc Lond B243:263-268.

Triner, L., Y. Vulliemoz, M. Verosky and W.M. Manger. 1975, Cyclic adenosine monophosphate and vascular reactivity in spontaneously hypertensive rats. Biochem Pharmacol 24:743-745.

Trybus,K.M. 1991. Assembly of cytoplasmic and smooth muscle myosins. Curr Opin Cell Biol 3:105-111.

Uddman, R., and F. Sundler. 1979. VIP nerves in human upper respiratory tract. Otorhinoloaryngology 41:221-226.

Uddman, R., A. Luts, and F. Sundler. 1985. Nerve fibres containing peptide histidin isoleucine (PIH) in the respiratory tract. Arch Otohinoloaryngol 242:189-193.

Ueda, N., S. Kaneko, T. Yoshimoto, and S. Yamamoto. 1986. Purification of arachidonate 5-lipoxygenase from porcine leukocytes and its reactivity with hydroperoxyeicosatetraenoic acids. J Biol Chem 261:7982-7988.

Umemoto, S., and J.R. Sellers. 1990. Characterization of in vitro motility assays using smooth muscle and cytoplasmic myosins. J Biol Chem 265:14864-14869.

van Breemen, C. 1965, Calcium distribution and exchange in rat uterus. PhD thesis, University of Alberta. 110pp.

van Breemen, C. 1977. Calcium requirement for activation of intact aortic smooth muscle. J Physiol (London) 272:317-329.

van Breemen, C., and K. Saida. 1989. Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. Annu Rev Physiol 51:315-329.

Van den Brink, F.G. 1972. The pharmacology of bronchiolar muscle relaxation. Bull Physiol-Pathol Respir 8:475-486.

Vargaftig, B.B., J. Lefort, M. Chignard, and J. Benveniste. 1980. Platele-activating factor induces a platelet-depedent bronchoconstriction unrelated to the formation of prostaglandin derivatives. Eur J Pharmacol 65:185-192.

Vivaudoui, M.B., L.H. Clapp, J.V. Walsh, Jr., and J.J. Singer. 1988. Regulation of one type of Ca^{2+} current in smooth muscle cells by diacylglycerol and acetylcholine. FASEB J 2:2497-2504.

von Hayek, H. (Translated by V.E. Krahl), 1960. The Human Lung, Hafner Publisher, New York.

Walsh, M.P. 1990. Smooth muscle caldesmon. In: Progress in Clinical and Biological Research. Vol 327, Frontiers in smooth muscle research. N. Sperelakis and J.D. Wood. eds., Wiley-Liss, New York. pp 127-140.

Walsh, M.P. 1991. Calcium-dependent mechanisms of regulation of smooth muscle contraction. Biochem Cell Biol 69:771-800.

Walsh, M.P., S. Hinkins, R. Salrowska, and D.J. Hartshorne. 1983. Smooth muscle myosin light chain kinase. Methods Enzymol 99:279-288.

Wasserman, M.A., R.L. Griffin, and P.E. Malo. 1982. Comparative *in vitro* tracheal-relaxant effects of porcine and hen VIP. In: Vasoactive Intestinal Peptide. S.I. Said, ed., Raven Press, New York, pp177-184.

Watras, J., I. Bezprozvanny, and B.E. Enrlich. 1991. Inositol 1,4,5-

trisphosphate-gated channels in cerebellum: presence of multiple conductance states. J Neurosci 11:3239-3245.

Weibel, E.R. and D.M. Gomez. 1962. A principle for counting tissue structures on random sections. J Appl Physiol 17:343-348.

Weibel, E.R. 1963. Morphometry of the Human Lung, Springer-Verlag, Berlin.

Weiss, E.R., D.J. Kelleher, C.W. Woon, S. Soparkar, S. Osawa, L.E. Heasley, and G.L. Johnson. 1988. Receptor activation of G proteins. FASEB J 2:2841-2848.

White, S.R., K.J. Popovich, R.W. Mitchell, S.M. Koenig, M.M. Mack, N.M. Munoz, and A.R. Leff. 1988. Antagonism of relaxation to isoproterenol caused by agonist interactions. J Appl Physiol 64(6):2501-2507.

Williams, D.A., and F.S. Fay. 1986. Calcium transients and resting levels in isolated smooth muscle cells as monitored with quin-2. Am J Physiol 250:C779-C791.

Winder, S.J., C. Sutherland, and M.P. Walsh. 1991. Biochemical and functional characterization of smooth muscle calponin. In: Regulation of smooth muscle: Progress in Solving the Puzzle. R.S. Moreland, ed., Plenum Press, New York, pp37-52.

Winder, S.J., and M.P. Walsh. 1990. Smooth muscle calponin, inhibition of actomyosin MgATPase and regulation by phosphorylation. J Biol Chem 265:10148-10155.

Woledge, R.C., N.A. Curtin, and E. Homsher. 1985, Initial facts and ideas. In: Energetic Aspects of Muscle Contraction. R.C.

Woledge, N.A. Curten, and E. Hamster, eds., Academic Press, New York, pp1-26.

Yamad, K., A. Goto, H. Matsuoka, and T. Sugimoto. 1992. Alteration of calcium channels in vascular smooth muscle cells from spontaneous hypertensive rats. Jpn Heart J 33(5):727-734.

Yang, X.-C., and F. Sachs. 1989. Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. Science 243:1068-1071.

Zaagsma, J., P.J.C.M. Van der Heijden, M.W.C. Van der Schaar, C.M.C. Bank. 1983. Comparison of functional β-adrenoceptor heterogeneity in central and peripheral airway smooth muscle of guinea pig and man. J Recept Res 3:89-106.

(Total Number of References: 413.)