Control of Intracellular Calcium Flux in Rat Submandibular Acinar Cells

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INTRODUCTION

The rat submandibular gland is an exocrine gland rich in mucous acinar cells. The major stimulus for mucin secretion in the gland is β-adrenergic and up to 70% of the total acinar cell mucin is released on exposure of submandibular cells to the β-adrenergic agonist, isoproterenol, (IPR) (2). A lesser, but significant secretory response of around 35% mucin can also be elicited by agonists of different classes which operate by increasing the concentration of cytosolic calcium to produce their physiological effects. Studies in this laboratory have shown that the Ca^{2+}-mobilizing secretagogues carbachol (muscarinic) substance P (SP, peptidergic) and methoxamine (α_1-adrenergic) release mucin from dispersed submandibular acinar cells (2).

Such Ca^{2+}-associated agents act via the phosphoinositide (PI) effect (see Fig. 1). Briefly, their receptors are coupled, through a GTP-binding, G regulatory protein (12,13), to the enzyme phospholipase C (PLC) which hydrolyzes the specific membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to release the products inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) (11,17). Both IP_3 and DG then act as second messengers in the cell. IP_3 has been shown to mobilize Ca^{2+} from cytosolic stores in a range of cell types, including pancreas, platelets and kidney cells (3-9). The released Ca^{2+} may then form a complex with calmodulin and instigate Ca^{2+}-dependent kinase activation and protein phosphorylation, leading to physiological responses such as secretion.

Diacylglycerol activates the enzyme protein kinase C (PKC) which stimulates phosphorylation of different proteins, again controlling the physiological responses of the cell. The DG activation of kinase C requires Ca^{2+} so there is an obvious interaction between the IP_3/DG limbs of the phosphoinositide signal transduction pathway.

Earlier studies in this laboratory established that rat submandibular acinar cells secreted mucin when both the IP_3- and DG- legs of the PI effect were activated (10). Subsequent work confirmed that calcium-elevating agonists (e.g. carbachol, substance P) increased IP_3 levels in the model (2), and that a G regulatory protein, which could be activated by GTP analogs, coupled receptors to lipase C (23).

The present project was undertaken to extend these observations to the next proposed stage of the coupling pathway by investigating whether IP_3, or any of the metabolites capable of generating endogenous IP_3, caused

the release of sequestered Ca^{2+} from cytosolic stores. The general approach was to load cells to equilibrium with radioactive $^{45}Ca^{2+}$ then to determine if the stored $^{45}Ca^{2+}$ was released by agonists, IP_3 or guanine nucleotides. Since many of these agents are highly polar and do not cross the plasma membrane of intact cells, it was necessary to use permeabilized cells in the study. A novel technique of electrical permeabilization by a high voltage capacitor discharge was used. In cells prepared by this technique the cytosol is continuous with the extracellular fluid so that changes in $^{45}Ca^{2+}$ content can be instantly monitored by scintillation counting of the culture medium.

A second aim was to investigate whether guanine nucleotides caused the release of stored $^{45}Ca^{2+}$ independent of their action in generating IP_3, as has been recently proposed in parotid gland and liver (14,15). In these studies, the antibiotic, neomycin, was used to inhibit the generation of endogenous IP_3.

The project has produced new information on control of Ca^{2+}-flux in submandibular acinar cells and has resulted in a publication and two abstracts (see Appendices).

MATERIALS AND METHODS

Materials

Purified collagenase was obtained from Cooper Biochemical, Mississauga, Ontario, Canada. Boehringer Mannheim, Dorval, Quebec, Canada supplied ATP, phosphocreatinine, creatinine kinase, guanosine-5'-trisphosphate (GTP), guanosine-5'-O-(3-thiotriphosphate) (GTPγS) and guanosine-5'-O-(2-thiodiphosphate) (GDPβS). Myo-[2-^{3}H] inositol and $^{45}Ca^{2+}$ were products of Amersham, Oakville, Ontario, Canada. Hank's Balanced Salt Solution (HBSS) was obtained from Gibco, Burlington, Ontario, Canada. Unlabelled inositol phosphates, carbachol, substance P, neurokinin B, neomycin sulphate B and all other reagents were from Sigma, St. Louis, MO, U.S.A..

Preparation of Cells

The submandibular glands of 2 or 3 rats weighing from 200-250 g were removed and chopped into small pieces. The fragments were dissociated at 37°C for 60 min by enzymatic dissociation with 2000 units of purified collagenase and 25 mg hyaluronidase in 50 ml modified Hank's Balanced Salt Solution, which was Ca^{2+} free but did not contain a chelating agent (2). The medium was supplemented with MEM amino acids, 0.2% bovine serum albumin, 5 mM beta-hydroxybutyrate, 5 mM inosine, 0.5 mM adenine and 4.8 mM $NaHCO_3$. Cell suspensions were buffered to pH 7.3 with 15 mM Hepes and maintained at 37°C in a gyrotary water bath shaker under atmospheric conditions. Mechanical shearing forces were applied to the tissue by repeated pipetting at 15 min intervals and the preparation was filtered through a 320 μm nylon mesh and washed twice and resuspended in an appropriate culture medium, depending on the experimental technique to be applied. Cell viability of over 90% was confirmed by trypan blue exclusion.

Calcium Studies

a). Cell permeabilization: Cells were washed in a cytosolic type culture medium with the following composition (mM): KCl, 100; NaCl, 20; $NaHCO_3$, 25; NaH_2PO_4, 0.96; $MgSO_4$, 5; ATP, 1.5; phosphocreatine, 1.5; creatine kinase, 5 u/ml; bovine serum albumin, 0.2%; soybean trypsin inhibitor, 0.01%. The pH was 7.2 (15 mM Hepes buffer) and the temperature 37°C. The cells were then resuspended in the same medium at a concentration of approximately 25 mg Lowry protein/ml, chilled on ice to 4°C, and placed

in a plastic cuvette fitted with electrodes, which was inserted into the Bio Rad Gene Pulser apparatus (Bio Rad Ltd., Mississauga, Ontario). Cells were permeabilized by being subjected to an intense electric field which was generated by a high voltage capacitor discharge across the electrodes as described by Knight and Baker (16). The experimental conditions, derived empirically, were two pulses of 0.5 sec at 2 KV/cm and 25 μF capacitance. For experimental treatment, the permeabilized cell preparation was diluted to 4 mg cell protein/ml in the same culture medium. More than 99% of cells were permeable to trypan blue after capacitor discharge, and remained so during the experimental period.

b). Calcium flux studies: Permeabilized acinar cells (4 mg protein/ml) were incubated in cytosolic culture medium with $^{45}Ca^{2+}$ (Amersham, Oakville, Ontario; 2.1 mCi/ml) at a final calcium concentration of approximately 2 μmol/litre (2 nmol/ml). Uptake experiments were carried out in which 300 μl samples of suspension were withdrawn at 2 min intervals. Cells and culture medium were separated by 0.45 μm Millipore filtration and $^{45}Ca^{2+}$ in both was estimated by scintillation counting. Measured counts were converted into molar concentrations of $^{45}Ca^{2+}$ by using data on the radioactive concentration, specific activity and decay curve of supplied isotope. Cells accumulated $^{45}Ca^{2+}$, reaching equilibrium in 20-25 min at a final medium concentration of about 0.2 μmol/1.

After equilibrium, the release of stored $^{45}Ca^{2+}$ in response to a range of doses of several test substances, was examined. These included IP_3, IP_2, IP, GTPγS, GDPβS, carbachol (a muscarinic agonist), isoproterenol (IPR, a β adrenergic agonist) and neurokinin B (a tachykinin/peptidergic agonist). In these experiments culture medium $^{45}Ca^{2+}$ levels were measured at 30 sec intervals after agonist treatment for periods of up to 10 minutes.

To try and differentiate between the endoplasmic reticulum (ER) and mitochondria as a potential source of released calcium, the following mitochondrial inhibitors were included in the culture medium during both the $^{45}Ca^{2+}$ uptake and release phases - antimycin (10^{-5} M), oligomycin (5×10^{-6} M) and azide (10^{-2} M).

In additional experiments, test substances were used after a 2 min incubation period with 20 mM neomycin sulphate, an antibiotic that inhibits IP_3 synthesis. The neomycin dose was determined in dose/inhibition experiments as described below.

Cell protein was estimated by the Lowry technique (24), with bovine serum albumin as standard, and calcium fluxes expressed as $^{45}Ca^{2+}$/mg cell protein.

Inositol Phosphate Studies

Dispersed intact, submandibular acinar cells were incubated for 60 min in culture medium containing 35 μCi myo-[2-^{3}H]inositol (12.3 Ci/mmol) per ml. The cells were then washed twice in culture medium containing 5 mM unlabelled inositol and once in the cytosolic-type culture medium described above. The cells were electropermeabilized as in the calcium studies, then transfered in 1 ml aliquots to 25 ml Erlenmyer flasks. The preparations were exposed to carbachol (10^{-5} M), GTPγS (10^{-7} - 10^{-4} M) or GDPβS (10^{-7} - 10^{-4} M) for 5 min, then precipitated with ice cold trichloroacetic acid (final concentration 6%). The precipitate was removed by centrifugation at 2000 g and the supernatant fluid was neutralized with 5 N NaOH. Water soluble, radiolabelled inositol phosphates were extracted by anion exchange chromatography as described by Berridge (1983) (17). Aliquots of 0.5 ml were applied to 0.7 x 4 cm columns of Bio Rad AG1 X8 resin, 100-200 mesh, formate form. Free inositol and glycerophosphoinositol were eluted with 15 ml of distilled water and 15 ml 5 mM disodium tetraborate/60 mM sodium formate respectively. Inositol 1-phosphate (IP), inositol 1,4-bisphosphate (IP_2) and inositol trisphosphate (IP_3) were then sequentially eluted with 15 ml of the respective buffers, 0.1 M formic acid/0.2 M ammonium formate; 0.1 M formic acid/0.4 M ammonium formate; 0.1 M formic acid/1.0 M ammonium formate. In this technique the IP_3 fraction is believed to contain I(1,3,4)P_3 and I(1,3,4,5)P_4 in addition to the immediate phospholipase C hydrolysis product of PIP_2, I(1,4,5)P_3 (18). However, since I(1,4,5)P_3 is thought to be the only precursor of I(1,3,4)P_3 and I(1,3,4,5)P_4 (19), the technique still reflects lipase C activity. For convenience, the fraction eluted with 0.1 M formic acid/1.0 M ammonium formate in this study is simply termed IP_3. The ^{3}H-labelled inositol phosphates present in the 15 ml eluates were quantitated by scintillation counting. In some of the inositol phosphate studies, cell preparations were pretreated for 2 min with a range of doses of neomycin to establish whether this putative inhibitor of PIP_2 hydrolysis actually blocked IP_3 formation in the submandibular experimental model.

RESULTS

Calcium Studies

Addition of permeabilized acinar cells to cytosolic medium containing $^{45}Ca^{2+}$ resulted in a decrease in labelled ion in the medium. Steady-state conditions were reached in 20-25 min with a reduction in $^{45}Ca^{2+}$ concentration from 2 µmol/l to 0.2 µmol/l (Fig. 2). The amount of $^{45}Ca^{2+}$ removed from the culture medium and sequestered within the cells was approximately 400 pmol/mg cell protein. Measurement of $^{45}Ca^{2+}$ in cells over the same time course confirmed the accumulation of ion as it was depleted from the culture medium (Fig. 2). After equilibration, an optimal dose of 10^{-6} IP_3 caused an immediate release of stored $^{45}Ca^{2+}$ that was measured as 26.0 ± 5.1 (mean ± SEM) pmol/mg protein within 30 sec (Fig. 3). Released calcium was quickly taken up again over the subsequent 2-3 min and the original equilibrium conditions were re-established. A second stimulation of the permeabilized cells with IP_3 produced a smaller release of $^{45}Ca^{2+}$ (17.2 ± 4.3 pmol/mg protein).

Carbachol, neomycin and GTPγS all elicited a $^{45}Ca^{2+}$ release of comparable order to that caused by IP_3 (Table 1). However, IP, IP_2 and GDPβS had no effect on the mobilization of cellular calcium. The responses caused by carbachol and GTPγS were significantly reduced in the presence of neomycin, an inhibitor of endogenous IP_3 synthesis (Table 1).

In some experiments the inhibitors of mitochondrial metabolism, antimycin, oligomycin and azide were included in acinar cell preparations during the $^{45}Ca^{2+}$ uptake and release phases. These agents did not inhibit the release of $^{45}Ca^{2+}$ in response to agents tested (Table 1).

Inositol Phosphate Studies

The guanine nucleotide GTPγS stimulated PIP_2 hydrolysis in permeabilized acinar cells to cause an increase in IP_3 levels (Fig. 4). The maximal response of 246 ± 17% (mean ± SEM) of control level was reached at a GTPγS concentration of 10^{-4} M. The muscarinic agonist carbachol (10^{-5} M) stimulated IP_3 concentrations to 283 ± 13.5% of control values over 5 min. The GDP analog, GDPβS, did not elevate cellular IP_3.

The effect of a range of doses of neomycin on IP_3 levels stimulated by test substances is shown in Fig. 5. Neomycin caused a concentration dependent reduction in IP_3 elevated by carbachol, GTPγS and GTP. IP_3 was reduced to control levels at 10-20 mM neomycin, confirming the inhibitory effect of this antibiotic on the PI effect.

DISCUSSION

The results obtained in this project confirm the validity of the electropermeabilized rat submandibular cell model for studies on the control of intracellular calcium ion flux. After the rigorous processes of enzymatic dissociation, high voltage treatment, centrifugation and extnesive handling, the model was still capable of mounting significant responses to several agents associated with IP_3 generation or calcium ion movement. The observation that IP_3 generated by carbachol treatment was reduced in the present study compared with carbachol-induced levels in previous work on intact cells (2) indicates that signal transduction mechanisms may be partially impaired after electroporation, but capable of qualitatively normal responses.

The findings on IP_3-induced $^{45}Ca^{2+}$ flux extend earlier work from this laboratory and now make it possible for the first time in a mucous gland model to detail the coupling sequence in the phosphoinositide signal transduction mechanism, i.e. receptor occupation -> G protein-GTP binding -> phospholipase C activation -> PIP_2 hydrolysis -> IP_3 generation -> release of sequestered Ca^{2+}. Our observations are consistent with results from work on other cell types which have shown the Ca^{2+}-mobilizing capacity of IP_3. These include the pancreas (3,4), kidney (9), liver (7) and platelets (6).

The specificity of the response was demonstrated in that additional inositol phosphates, IP and IP_2, were not capable of duplicating the IP_3 calcium-mobilizing action. Agents which are known to enhance endogenous IP_3, however, (carbachol, GTPγS, neurokinin B) did cause the elevation of cytosolic $^{45}Ca^{2+}$. There was a brief time lag of a few seconds for detection of released $^{45}Ca^{2+}$ with these agents compared with the almost instant IP_3-$^{45}Ca^{2+}$ response. This may reflect the period required for these stimulants to initiate coupling reactions and generate IP_3. The specificity of the control of coupling was again demonstrated by the failure of GDPβS, a GDP analog, to elicit a response, confirming GTP requirement.

It is generally accepted that the two major subcellular compartments associated with calcium concentration are the endoplasmic reticulum (ER) and the mitochondria. In an attempt to identify the source of sequestered Ca^{2+} liberated, cells were incubated with a battery of inhibitors known to block normal mitochondrial function (antimycin, oligomycin, azide). The presence of these inhibitors did not impair the agonist-generated

release of $^{45}Ca^{2+}$, indicating that the ER and not the mitochondria, was the source of mobilized ion. Again this is consistent with studies in other exocrine cell types such as pancreas (3). It was noted, however, that in the presence of inhibitors, cells required an extra 5-7 minutes to reach equilibrium. Streb and Schulz (25) suggested that initial Ca^{2+} uptake is mostly due to mitochondrial activity, while the equilibrium condition is controlled by a non-mitochondrial structure. Our finding that mitochondrial inhibitors increased the time required to reach equilibrium, but did not change the actual cytosolic calcium concentration at the steady state condition, suggests that this may also be the case in submandibular cells.

Our initial interpretation of GTPγS mobilization of calcium was that the nucleotide exerted its effects via generation of endogenous IP_3. However, recent studies by Henne and Soling (14) on guinea pig liver and parotid ER membranes, and by Chueh and Gill (15) on N1E-115 neuronal cells, indicated that guanine nucleotides may act directly on the ER to release Ca^{2+}, independent of their G protein/IP_3 generating action. We therefore used neomycin to block PIP_2 hydrolysis and examine guanine nucleotide effects in the absence of endogenous IP_3. Neomycin is a cationic aminoglycoside antibiotic which can bind to anionic polyphosphoinositides, especially PIP_2, thereby inhibiting PIP_2 hydrolysis and IP_3 generation (21). Preliminary experiments confirmed that neomycin completely blocked agonist-induced IP_3 formation to control levels at 10-20 mM concentration. However, GTP and GTPγS-induced $^{45}Ca^{2+}$ flux was not abolished by neomycin treatment, although their responses were significantly reduced (see Table 1). We therefore propose that the guanine nucleotide effect in mobilizing sequestered $^{45}Ca^{2+}$ in submandibular cells may be due partially to their stimulatory action on G proteins, leading to IP_3 synthesis, and partially to a direct action on ER membranes. Consistent with this idea was our observation that GTPγS and GTP were incapable of stimulating IP_3 as efficiently as was carbachol, yet they mobilized almost as much Ca as carbachol. This evidence supports the proposal that GTPγS and GTP may have a Ca^{2+} mobilizing potential directly from ER stores.

It has been suggested that in addition to the ability of neomycin to inhibit PIP_2 hydrolysis, this antibiotic can also bind to IP_3, thereby inhibiting IP_3-mediated Ca^{2+} release from permeabilized cells (22). The proposed mechanism for this inhibition is a chelation of anionic IP_3 to cationic neomycin. To test this theory the effect of IP_3 in the presence

of neomycin was investigated. IP_3 elicited a Ca^{2+} release which was approximately 70% of IP_3 mediated Ca^{2+} release without neomycin. Such results suggest that there may be some direct inhibitory effect on IP_3, although the major mechanism of neomycin inhibition is by way of PIP_2 hydrolysis.

Although a discrete IP_3 receptor on the ER which mediates Ca^{2+} efflux has been proposed, it is not yet known whether a specific guanine nucleotide receptor on ER membranes may exist to serve the same function, or if so whether it is completely independent of the IP_3 receptor. These preliminary studies provide some groundwork for future investigation on the guanine nucleotide control of cytosolic Ca^{2+} levels.

Accurate regulation of cell calcium controls many physiological processes in practically all cell types. It is increasingly recognized that at least the initial phase of this regulation is activated by the receptor-linked phosphoinositide effect. This study extends our knowledge of the coupling reaction sequence in the PI effect in mucus-secreting salivary glands and confirms the suitability of the submandibular model for extended investigation of stimulus-secretion coupling mechanisms.

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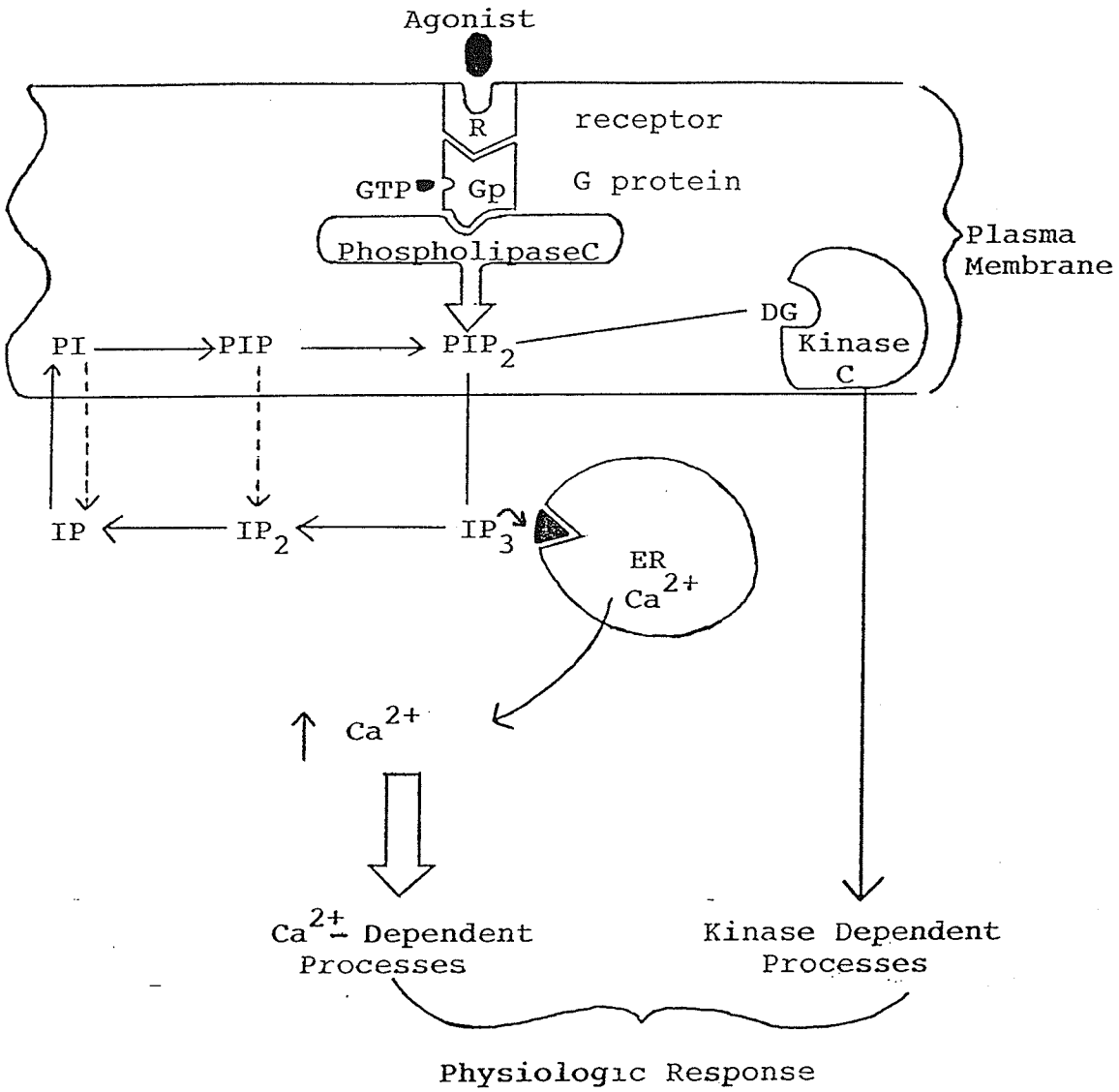

Fig 1 : The main features of the phosphoinositide signal transduction pathway leading to an elevation in cytosolic calcium levels. See Introduction for details.

PI - phosphatidylinositol ; PIP - phosphatidylinositol- 4-phosphate ; PIP_2 - phosphatidylinositol-4,5-bisphosphate.

IP - inositol phosphate ; IP2 - inositol-1,4-bisphosphate ; IP_3 - inositol-1,4,5-trisphosphate.

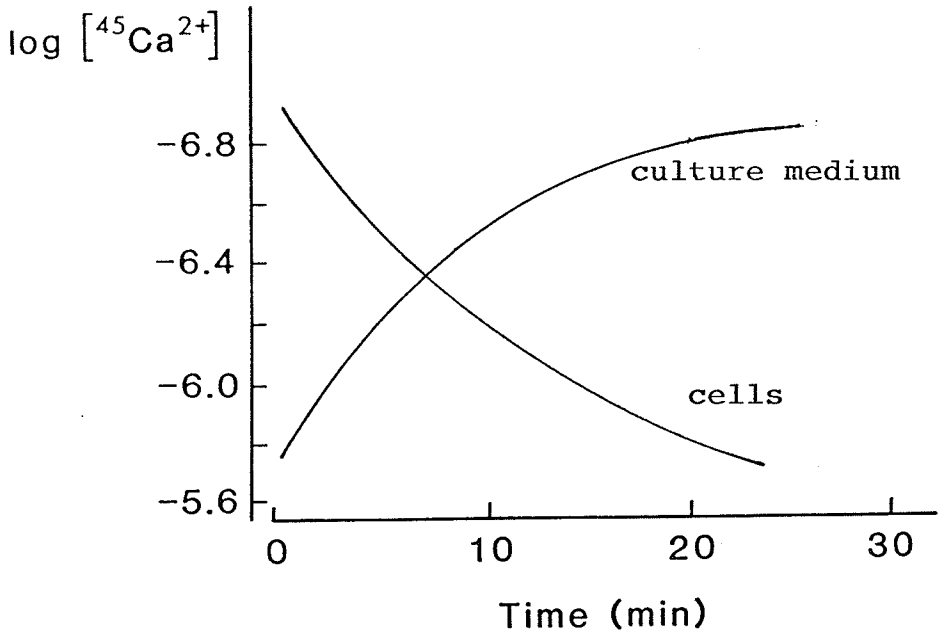

Fig 2 : Concentration of $^{45}Ca^{2+}$ in culture medium and permeabilized acinar cells over a time course. The system reached equilibrium in approx.25 min. Radioactive calcium taken up from the culture medium was stored in cell organelles.

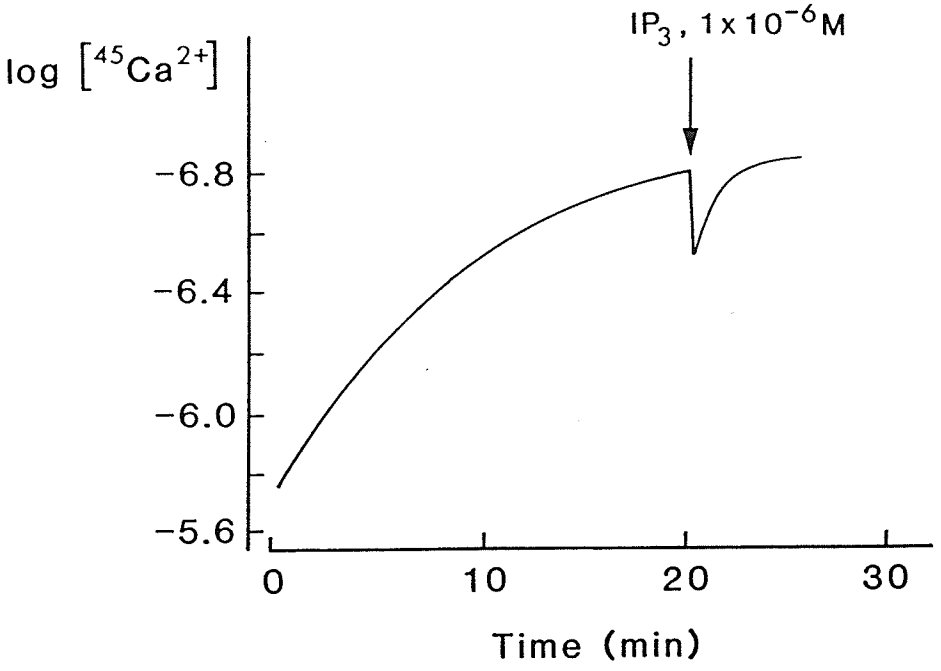

Fig 3 : The uptake of cytosolic $^{45}Ca^{45}$ into organelles of permeabilized submandibular acinar cells and its subsequent release in response to inositol 1,4,5-trisphosphate (IP_3).

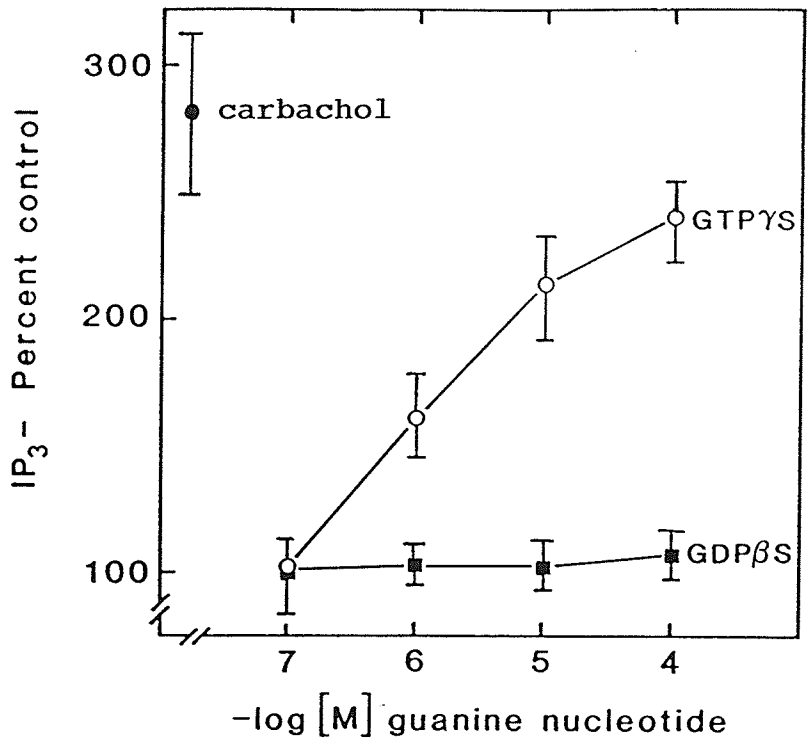

Fig 4 : Effect of a range of doses of GTPγS and GDPβS on IP_3 levels in permeabilized rat submandibular acinar cells after 5 min incubation. The response to 10^{-5}M carbachol is shown for comparison. Values are the means ± SEM of 4 or 5 experiments.

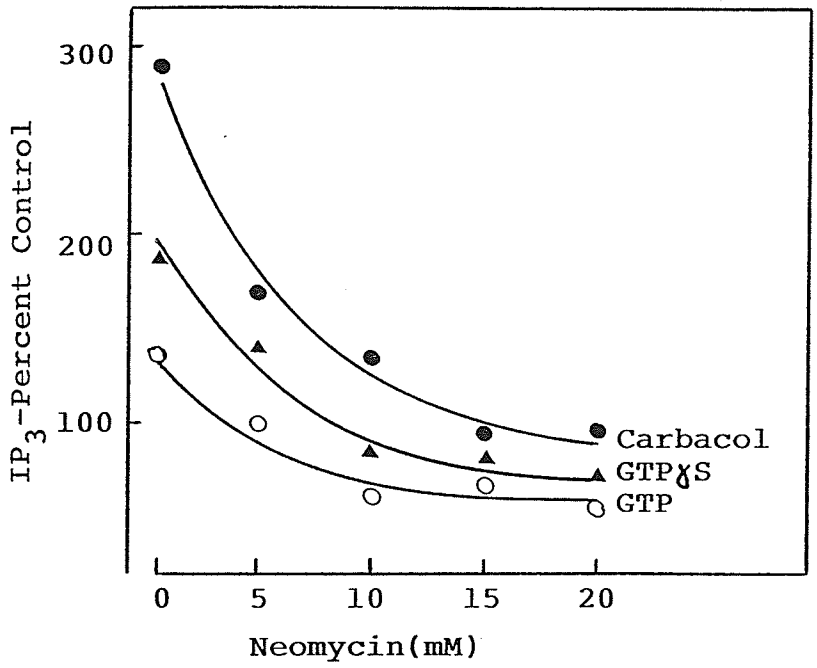

Fig 5 : Effect of a range of concentrations of neomycin in reducing IP_3 levels caused by carbachol (10^{-5}M) GTPγS (10^{-4}M) and GTP (10^{-4}M) in permeabilized rat submandibular cells.

TABLE 1

Release of $^{45}Ca^{2+}$ from permeabilized rat submandibular acinar cells in response to a range of test substances.

Test substance	Concentration	$^{45}Ca^{2+}$ release (pmol/mg cell protein)
IP_3	$10^{-6}M$	26.0 ± 5.1
IP_3 + Neomycin (20mM)	$10^{-6}M$	18.0 ± 3.2
IP_2	$10^{-6}M$	0
IP	$10^{-6}M$	0
Carbachol	$10^{-5}M$	29.3 ± 7.3
Substance P	$10^{-7}M$	30.5 ± 5.5
Neurokinin B	$10^{-7}M$	30.5 ± 3.0
GTP S	$10^{-4}M$	25.5 ± 3.0
GTP S + Neomycin (20mM)	$10^{-4}M$	14.7 ± 2.6
GDP S	$10^{-4}M$	0
GTP	$10^{-4}M$	24.8 ± 3.2
GTP + Neomycin (20mM)	$10^{-4}M$	16.5 + 3.6

Calcium release responses were not inhibited by mitochondrial inhibitors :- antimycin, $10^{-5}M$; oligomycin, $5 \times 10^{-6}M$; and azide, $10^{-2}M$. Values are means ± SEM. See text for details.

Appendix I
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G REGULATORY PROTEINS AND MUSCARINIC RECEPTOR SIGNAL TRANSDUCTION IN MUCOUS ACINI OF RAT SUBMANDIBULAR GLAND

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Summary

The involvement of G regulatory proteins in muscarinic receptor signal transduction was examined in electrically permeabilized rat submandibular acinar cells. The guanine nucleotide analog, GTPγS, caused the dose dependent hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate to release IP_3. This response was insensitive to pertussis toxin treatment and was duplicated by NaF but not by GDPβS. Enhanced IP_3 synthesis was observed with a combination of GTPγS and carbachol. Exogenous IP_3, as well as carbachol and GTPγS, provoked the release of sequestered $^{45}Ca^{2+}$ from non-mitochondrial stores. In intact cells, carbachol significantly reduced the level of cyclic AMP induced by the β-adrenergic agonist, isoproterenol, to 69% of its normal value. Pertussis toxin abolished this inhibitory action of carbachol on cyclic nucleotide levels. These results suggest that muscarinic receptors are coupled to two separate G regulatory proteins in submandibular mucous acini - the pertussis toxin - insensitive G_p of the phosphoinositide transduction pathway associated with elevated cytosolic calcium levels, and the pertussis toxin - sensitive G_i inhibitory protein of the adenylate cyclase complex.

It is now well established that Ca^{2+}-mobilizing agonists exert their physiological actions in many cell types via the phosphoinositide (PI) effect (1,2). Receptor-linked activation of phospholipase C leads to hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP_2), and the breakdown products, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG), act as separate second messengers in stimulus-response coupling pathways (1). Agonist-induced IP_3 generation has been demonstrated in exocrine cells of the pancreas (3) and parotid gland (4) and the capacity of IP_3 to mobilize Ca^{2+} from cytosolic stores has been confirmed in a range of cells (1). Recent studies in our laboratory indicated that both the DG (5)- and IP_3 (6)-associated pathways are involved in the control of mucin secretion in acinar cells of rat submandibular gland. We further found that muscarinic (carbachol) stimulation of submandibular cells not only elevated IP_3 levels and provoked mucin release, but also reduced the mucosecretory response caused by the β-adrenergic agonist isoproterenol (IPR) (6).

Evidence has accumulated that many signal transduction mechanisms are mediated by receptor-coupled, guanine nucleotide-binding, G regulatory proteins (7,8). The best defined are the G_s (stimulatory) and G_i (inhibitory) proteins which are activated respectively by β-adrenergic and muscarinic/α_2-adrenergic receptors to regulate adenylate cyclase/cAMP synthesis (9). G proteins also mediate signal transduction in brain cells (G_o) and in retinal rods and cones (G_t) (10). Studies with non-metabolizable GTP analogs in permeabilized cells of several organs, including parotid gland and pancreas, have indicated that G proteins (G_p) couple Ca^{2+}-mobilizing receptors to phospholipase C (11). In some cells (mast cells, neutrophils, mesangial cells), G_p is inhibited by pertussis toxin (11) suggesting that it is identical or closely related to the G_i protein of the adenylate cyclase system. In other cells (pancreas, liver, heart), however, G_p is pertussis toxin-insensitive (11). Heterogeneity in the structure of G_p is therefore indicated.

The present study was undertaken to investigate a potential coupling role for a G protein in the phosphoinositide effect in permeabilized rat submandibular acini; and to examine whether the previously observed muscarinic modulation of the β-adrenergic secretory response might operate at the G protein-adenylate cyclase level of signal transduction. Permeabilized mucous acinar cells also provided a suitable model for studies on the mobilization of stored Ca^{2+} by inositol phosphates and agonists.

Materials and Methods

Materials

Purified collagenase, CLSPA grade, was obtained from Worthington, Freehold, NJ, U.S.A.. Boehringer Mannheim, Dorval, Quebec, Canada, supplied ATP, phosphocreatine, creatine kinase, guanosine-5'-O-(3-thiotriphosphate) (GTPγS) and guanosine-5'-O-(2-thiodiphosphate) (GDPβS). Myo-[2-^{3}H]inositol and $^{45}Ca^{2+}$ were products of Amersham, Oakville, Ontario, Canada. Purified pertussis toxin was supplied by List Biological Laboratories, Campbell, CA, U.S.A.. New England Nuclear, Montreal, Quebec, Canada, provided ^{125}I-cAMP radioimmunoassay kits. Hank's Balanced Salt Solution was obtained from Gibco, Burlington, Ontario, Canada. Unlabelled inositol phosphates and all other reagents were from Sigma, St. Louis, MO, U.S.A..

Preparation and permeabilization of acinar cells

Mucous acini of rat submandibular glands were prepared by tissue dissociation with purified collagenase and maintained under normal amospheric conditions in supplemented Hank's Balanced Salt Solution (HBSS) at 37°C as described previously (6). This procedure produced a suspension in which more than 95% of the cells were assessed as viable by trypan blue exclusion.

Cells were concentrated to a density of approximately 25 mg protein/ml in a cytosolic-type culture medium with the following composition (mM): KCl, 100; NaCl, 20; $NaHCO_3$, 25; NaH_2PO_4, 0.96; $MgSO_4$, 5; ATP, 1.5; phosphocreatine 1.5; creatine kinase, 5 U/ml; bovine serum albumin, 0.2%; soybean trypsin inhibitor 0.01%. The preparation was buffered by 15 mM HEPES to pH 7.2 at 37°C. The cells were permeabilized by exposure to an intense electric field generated by high voltage capacitor discharge through the suspension as described by Knight and Baker (12). Each preparation was subjected to two pulses of 0.5 sec at 2 KV/cm and 25 μF capacitance, in the Bio-Rad Gene Pulser apparatus (Bio-Rad Canada Ltd., Mississauga, Ontario). For experimental treatment, the permeabilized cell suspension was diluted in the same culture medium to a concentration of 4 mg protein/ml. More than 99% of the cells remained permeable to trypan blue during all experiments.

IP_3 studies

Dispersed, intact cells were incubated for 1 hour in modified HBSS containing 35 μCi myo-[2-^{3}H]inositol per ml as described earlier (6). The cells were then transferred to cytosolic culture medium and permeabilized electrically. In some experiments, intact cells were treated with pertussis toxin (200 ng/ml, 1 h), which was also included in the cytosolic culture medium after electroporation. Permeabilized cells were exposed to a range of concentrations of GTPγS or GDPβS (10^{-7} - 10^{-4} M); to NaF (5-20 x 10^{-3} M) in the presence or absence of $AlCl_3$ (10^{-5} M); or to carbachol (10^{-5} M), for 5 min. Suspensions were then precipitated with 6% cold trichloroacetic acid and soluble inositol phosphates were extracted by anion exchange chromatography on columns of Bio-Rad AG 1 X 8 resin, formate form, as described previously (6). The ^{3}H-IP_3 fraction was quantitated by scintillation counting.

Cyclic AMP studies

The effect of carbachol on the β-adrenergic-induced elevation of cAMP was investigated in intact acinar cells. Suspensions were treated with carbachol (10^{-4} M), for 2 min, then isoproterenol (10^{-5} M) was added to the same medium and the cells incubated for a further 10 min. In additional experimental groups, cells were treated with carbachol alone (12 min), IPR alone (10 min) or remained untreated (controls). Samples of suspension were then precipitated in 6% cold TCA and centrifuged at 2500 g for 15 min. The supernatant fluid was extracted with water-saturated ether and assayed for cAMP by radioimmunoassay as described in an earlier study (13). In some experiments, cells were preincubated with pertussis toxin (200 ng/ml, 1 h) before agonist treatment.

Calcium studies

Permeabilized acinar cells were incubated with $^{45}Ca^{2+}$ (2.1 mCi/ml) at a concentration of approximately 2 μmol/l (2 nmol/ml). Samples of suspension were withdrawn at 2 min intervals, cells and culture medium were immediately separated by 0.45 μm Millipore filtration and the $^{45}Ca^{2+}$ content of cells and filtrate estimated by scintillation counting. Measured d.p.m. were converted to molar concentrations of calcium in calculations based on the specific activity, radioactive concentration and decay curve of $^{45}Ca^{2+}$. The system reached equilibrium in about 20 min at a culture medium $^{45}Ca^{2+}$ concentration of 0.2 μmol/l, reflecting uptake of the ion into intracellular stores.

The effects of a range of test substances, i.e. IP_3, IP_2, IP, GTPγS, GDPβS, carbachol and IPR on the release of stored $^{45}Ca^{2+}$ were monitored by measuring culture medium levels of labelled ion at 30 sec intervals. In some experiments, the mitochondrial inhibitors antimycin (10^{-5} M), oligomycin (5 x 10^{-6} M) and azide (10^{-2} M) were included in the culture medium during both the $^{45}Ca^{2+}$ uptake and release phases.

Results were examined statistically by two-way analysis of variance and Duncan's multiple range test. Values of $p < 0.05$ were considered significant.

Results

IP_3 studies

The effects of guanine nucleotides and NaF on IP_3 generation in permeabilized acinar cells are shown in Fig. 1. To normalize results, all observations are expressed as a percentage of untreated, control levels of ^{3}H-IP_3, which

varied from 1850-2400 d.p.m. per mg cell protein. GTPγS produced a concentration-dependent elevation of IP_3 to a maximum of 246 ± 17% (mean ± SEM) of control at 10^{-4} M. This increase was not affected by preincubation of cells with pertussis toxin. The GDP analog, GDPβS, had no effect on IP_3 concentration at any of the doses tested. NaF increased IP_3 to 282 ± 21% control level at an optimal concentration of 10 mM, where a plateau in the response was reached. Addition of 10^{-5} M $AlCl_3$ to NaF-treated preparation did not further increase the IP_3 concentration. The muscarinic agonist, carbachol (10^{-5} M) stimulated IP_3 to 187 ± 18% of control concentration ($p < 0.01$), a value significantly reduced from that of around 500% observed with the same concentration of agonist in an earlier study on non-permeabilized cells (6). This reduction in the carbachol response in permeabilized versus intact cells probably reflects an impairment in the coupling process caused by the electroporation procedure. The carbachol response was not inhibited by pertussis toxin. Treatment of permeabilized cells with a combination of carbachol (10^{-5} M) and GTPγS (10^{-5} M) produced an enhanced IP_3 response of 348 ± 32% which was again pertussis toxin-insensitive (result not shown).

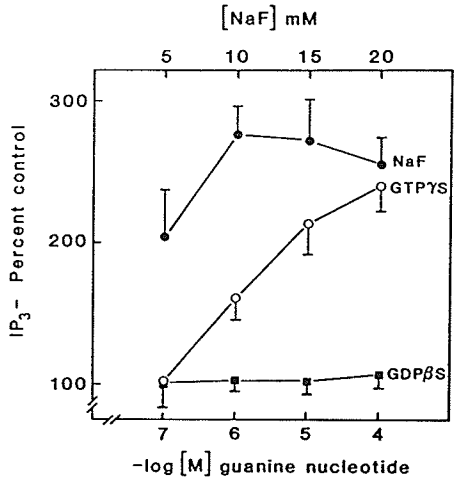

FIG. 1
Effect of a range of doses of GTPγS, GDPβS and NaF on IP_3 levels in electropermeabilized rat submandibular acinar cells after 5 min incubation. Values are means of 4 or 5 separate experiments and bars represent the S.E.M..

$^{45}Ca^{2+}$ studies

Addition of permeabilized acinar cells to cytosolic medium containing $^{45}Ca^{2+}$, resulted in a decrease in labelled ion in the medium. Steady-state conditions were reached in about 20 min with a typical reduction in $^{45}Ca^{2+}$ concentration from 2 μmol/l to 0.2 μmol/l (Fig. 2), representing an uptake of approximately 400 pmol/mg protein. Loss of labelled Ca^{2+} from the medium was matched by an appropriate increase in the $^{45}Ca^{2+}$ content of permeabilized cells, separated by Millipore filtration. After equilibrium of the system, an optimal concentration of 10^{-6} M IP_3 caused a rapid release of sequestered $^{45}Ca^{2+}$ that reached a maximum of 26.0 ± 5.1 pmol/mg cell protein within 30 sec (Fig. 2, Table 1). This release was equivalent to 15.4% of the sequestered $^{45}Ca^{2+}$. Released $^{45}Ca^{2+}$ was gradually taken up again over the subsequent 2-3 min to re-establish the original steady state (Fig. 2). Re-stimulation of the cells with IP_3 at this stage provoked a second but smaller release of 17.2 ± 4.3 pmol $^{45}Ca^{2+}$/mg protein. Similar release responses were elicited by GTPγS and carbachol, but not by IP, IP_2 or GDPβS or IPR (Table I). The

efflux of $^{45}Ca^{2+}$ from cytoplasmic stores was not affected by a combination of the mitochondrial inhibitors antimycin, oligomycin and azide, although cells incubated with these agents required an extra 5-7 min to reach equilibrium in the $^{45}Ca^{2+}$ uptake phase.

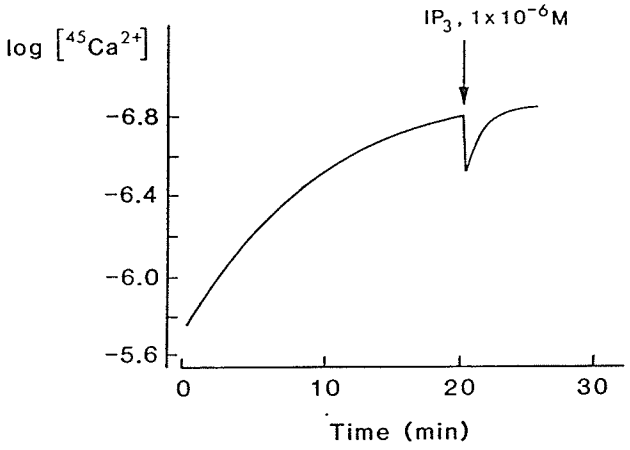

FIG. 2
Time course of the uptake of cytosolic $^{45}Ca^{2+}$ into organelles of electropermeabilized rat submandibular acinar cells and the subsequent release of stored $^{45}Ca^{2+}$ in response to IP_3.

TABLE I

Release of $^{45}Ca^{2+}$ from Electropermeabilized Rat Submandibular Acinar Cells in Response to a Range of Compounds

Test substance	Concentration	$^{45}Ca^{2+}$ released (pmol/mg cell protein)
IP_3	10^{-6} M	26.0±5.1
IP_3 + mitochondrial inhibitors*	10^{-6} M	34.2±7.6
IP_2	10^{-6} M	0
IP	10^{-6} M	0
Carbachol	10^{-4} M	29.3±7.3
Isoproterenol	10^{-4} M	0
GTPγS	10^{-4} M	25.5±3.0
GDPβS	10^{-4} M	0

* - antimycin, 10^{-5} M + oligomycin 5 x 10^{-6} M + azide 10^{-2} M, included in cell preparations during both the $^{45}Ca^{2+}$ uptake and release phases. Values are means ± S.E.M., n = 4. See text for abbreviations and incubation conditions.

Cyclic AMP studies

Isoproterenol (10^{-5} M, 10 min exposure) stimulated cAMP levels to 137 ± 22 pmol/mg protein in intact acini, compared with a value of 17.5 ± 2.9 in untreated control cells. Pretreatment of cells with carbachol for 2 min inhibited the IPR response to 69 ± 7% of its normal value ($p < 0.01$, Table II). Carbachol alone had no effect on cAMP levels in acinar cells. The carbachol-induced reduction of the β-adrenergic-cAMP response was abolished after preincubation of acini with pertussis toxin for 1 hour (Table II). Bacterial toxin, alone or in combination with carbachol, did not reduce control concentration of cAMP.

TABLE II

Effect of Pertussis Toxin on the Carbachol-Induced Inhibition of Elevated cAMP Levels Caused by β-Adrenergic Stimulation in Rat Submandibular Acinar Cells

Pretreatment	Control	Carbachol 10^{-4} M	Isoproterenol 10^{-5} M	Carbachol 10^{-4} M + isoproterenol 10^{-5} M
- Pertussis toxin	12.0±1.3	15.0±2.9	100	69.0±7.5*
+ Pertussis toxin	14.1±1.2	12.3±2.0	107±2.9	98.2±9.0

* $p < 0.01$ v. isoproterenol

Dispersed, intact cells were preincubated in the presence or absence of pertussis toxin (200 ng/ml; 1 hour) then exposed to carbachol (12 min); or isoproterenol (12 min); or carbachol (2 min) followed by isoproterenol (10 min). Cyclic AMP was measured by RIA as described in Materials and Methods. For normalization of the results of several experiments, values are expressed as percentages of the isoproterenol response. Means ± S.E.M., n = 6 or 7.

Discussion

The generation of IP_3 in permeabilized rat submandibular cells in response to GTPγS, in a concentration-dependent manner, indicates a role for a G regulatory protein in the activation of phospholipase C/PIP_2 hydrolysis in the model. The specificity of this response to the GTP analog was confirmed by the observation that GDPβS failed to elevate IP_3 levels in acinar cells. Enhanced IP_3 production on stimulation with a combination of GTPγS and carbachol suggests G protein mediation in the transduction of a muscarinic receptor signal to the lipase C effector. These findings are consistent with reports that G proteins couple Ca^{2+}-mobilizing agonist receptors to PLC activation in a range of cell types, including mast cells, platelets, liver and smooth muscle (11), as well as exocrine cells of the parotid gland (14) and pancreas (15). Moreover, Wang et al. (16, 17) have recently demonstrated that a G protein is physically and functionally associated with an inositol phospholipid-specific lipase C purified from calf thymocytes. In the present study, additional evidence for the involvement of a G protein in the transduction sequence is provided by the finding that NaF also stimulated the dose-dependent accumulation of IP_3. This effect of F^- was described previously in hepatocytes (18) and parotid gland (14). The probable mechanism of action of NaF first involves the formation of an AlF^-_4 complex, which then initiates the dissociation of the α from the βγ subunits of the G protein, permitting protein-effector coupling (19). Our observation that NaF elicited an optimal IP_3 response without the addition of Al^{3+} is compatible with that of Taylor et al. (14) on parotid cells. These authors proposed

that sufficient Al^{3+} is etched from laboratory glassware to produce the active AlF^-_4 species. F^- could enhance IP_3 levels, at least partially, by inhibiting the IP_3 5-phosphatase that dephosphorylates IP_3 to IP_2 (30). However, Taylor et al. (14) demonstrated in parotid cells that inhibition of IP_3 to IP_2 breakdown by 2,3 bisphosphoglyceric acid did not block the formation of IP_3 caused by F^-. It is now widely accepted that G proteins are a specific target of F^- in many cell types (2).

In earlier work, we found that carbachol not only enhanced IP_3 and stimulated mucin release in rat submandibular cells, but also partially inhibited the β-adrenergic (IPR) mucosecretory response (6). In the same study we also showed that adrenergic regulation of inositol phosphates was mediated by α_1 receptors but not β receptors. These findings are consistent with those of Doughney et al. on the same cell type (31) and with our present observation that isoproterenol did not cause $^{45}Ca^{2+}$ mobilization in the model. The β receptor is coupled to adenylate cyclase/cAMP generation via a G_s regulatory protein (7,8), and elevated nucleotide levels cause mucin secretion in the submandibular gland (26). Since muscarinic receptors are coupled to a second adenylate cyclase-associated G protein, G_i, that mediates inhibition of the enzyme (9), it is likely that carbachol-induced reduction of the β-adrenergic secretory response reflects reduced cAMP synthesis. We therefore investigated the possible role of the G_i protein in this coupling process by examining cAMP levels in cells after preincubation with pertussis toxin (PT), a bacterial toxin that inactivates G_i by causing ADP-ribosylation of its α subunit (7). While carbachol had no effect on cAMP levels in control cells, it did significantly reduce the IPR-cAMP response. This inhibition was abolished by pertussis toxin, indicating that the carbachol effect was mediated by the G_i protein. In some cell types, such as mast cells, neutrophils and lymphocytes (11), G_i also appears to be the regulatory protein that couples receptors to phospholipase C, whereas in astrocytoma cells, heart cells (20), pancreas (15) and liver (21), receptor activation of PIP_2 hydrolysis is pertussis toxin-insensitive. In the present study, the carbachol or GTPγS enhancement of IP_3 was not inhibited by pertussis toxin. We therefore propose that in rat submandibular cells, muscarinic receptors are coupled to two discrete G proteins, G_p (11) which mediates the phosphoinositide effect and G_i of the adenylate cyclase complex. A recent report by Lo and Hughes (22) suggests that this is the case in Flow 9000 cells derived from an embryonic pituitary tumor. We are currently investigating whether each G protein is selectively coupled to M_1 or M_2 muscarinic receptor subtypes.

There is strong evidence from studies on cells of several types that IP_3 stimulates cytosolic Ca^{2+} levels by mobilizing the ion from endoplasmic reticulum (ER) stores (1). Exogenous IP_3 has been shown to cause the release of Ca^{2+} in permeabilized cells of pancreas (23), kidney (24), liver (25), as well as in platelets (27). An IP_3 binding site has been demonstrated in rat liver microsomes (28) and it has been proposed that such a site is associated with an ER calcium channel (1). Consistent with these reports, we found that exogenous IP_3, but not IP_2 or IP, caused the rapid release of stored $^{45}Ca^{2+}$ from permeabilized rat submandibular cells. Using mitochondrial inhibitors in a study on permeabilized pancreatic acinar cells, Streb and Schulz (32) proposed that initial Ca^{2+} uptake is largely due to mitochondrial activity, whereas the steady state is determined by a non-mitochondrial structure. Our observation that mitochondrial inhibitors increased the time required to reach equilibrium, but did not alter the endpoint cytosolic $^{45}Ca^{2+}$ concentration, is compatible with this proposal. Moreover, the $^{45}Ca^{2+}$ release response was also unaffected by the inhibitors, indicating the ER as the probable source of the ion.
Similar levels of $^{45}Ca^{2+}$ release were elicited by carbachol and GTPγS. Since

these agents cause PIP_2 hydrolysis in the model, it is likely that their Ca^{2+} mobilizing effects are mediated by elevated endogenous IP_3. The possible direct affect of GTPγS on ER to mobilize Ca^{2+} cannot be ruled out at this stage. A guanine nucleotide Ca^{2+} regulatory mechanism that is independent of IP_3 has been demonstrated in permeabilized N1E-115 cells of neuroblastoma origin (29). While concentrations of agonists used to elicit the Ca^{2+} response or IP_3 generation in this study are above physiological levels, this is a common finding in in vitro work. The doses used here are comparable to those producing optimal responses in many other studies on exocrine cells (14,15,23,24,32).

These findings extend our earlier observations on the phosphoinositide effect in regulating mucin secretion in rat submandibular cells (6) by confirming the Ca^{2+}-mobilizing function of IP_3 in the model. In addition, the central role of separate G regulatory proteins in mediating muscarinic receptor signal transduction in both the PI and cAMP pathways has been established for the first time in this mucous cell type. Stimulation of α_1-adrenergic and peptidergic (substance P) receptors in the submandibular (6) and other exocrine glands (14, 15) also activates the PI effect and G proteins have been associated with such receptors in the parotid gland (14). It is not yet known whether a single G protein (G_p) couples all three receptor classes to phospholipase C activation, or whether discrete, receptor-specific G proteins are involved.

Acknowledgement

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Appendix II

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E.SLIWINSKI-LIS,D.BURKE and N. FLEMING*(Department of Oral Biology, University of Manitoba, Winnipeg,Canada): Role of a G Protein in Phosphoinositide-associated Signal Transduction in Submandibular Cells.

Previous work on rat submandibular cells has shown that the signal transduction mechanism of Ca^{2+}-mobilizing mucosecretagogues involves the phospholipase C-catalyzed hydrolysis of membrane polyphosphoinositides (PPI), leading to the generation of the second messenger inositol 1,4,5-trisphosphate (IP_3). The present study was undertaken to determine whether a GTP-binding, G regulatory protein may link receptor occupation to phospholipase C activation in the model. Collagenase-dispersed rat submandibular mucous acini were labelled with ^{3}-H-inositol (25 µCi/gland equivalent) for 1 h, transferred to cytosolic culture medium and electrically permeabilized by high voltage capacitor discharge (2 KV/cm;25 µF;x 2). Cells were exposed to GTPγS or other guanine nucleotides, or to the muscarinic agonist, carbachol for 5 min and inositol phosphates were quantitated. GTPγS stimulated IP_3 release in a dose dependent way to a maximum of 238+19% control value ($p<0.01$) at 10^{-4}M, but GDP and GTPβS had no effect. Carbachol (10^{-4}M) enhanced IP_3 to 181+18% control level ($p<0.01$). Carbachol plus GTPγS produced an additive response. In separate experiments, cells were labelled to equilibrium with ^{45}Ca then efflux of the ion was monitored in response to several test substances. IP_3 stimulated the release of 26.9 nmol ^{45}Ca/mg cell protein from cellular stores within 1 min. Similar responses were produced by carbachol and GTPγS and were not inhibited by mitochondrial poisons. These findings suggest the sequence:receptor occupation-G protein coupling-PIP_2 hydrolysis-IP_3-Ca^+ flux from ER. Supported by the Canadian Cystic Fibrosis Foundation.

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(q) ☐ Prosthodontics Research
(r) ☐ Pulp Biology
(s) ☒ Salivary Research
(t) ☐ Other (specify)

9. Is this research of interest to the general public and suitable for press coverage?
(1) ☐ yes (2) ☒ no
If yes, include (separately) a brief description in lay terms of the significance of this research.

10. Was any of this research supported by NIH? (If yes, print grant number as last line in abstract box.)
(1) ☐ yes (2) ☒ no
(NIH/NIDR employees should check *yes*.)

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12. PRESENTER/CO-AUTHOR INFORMATION: List Presenter on first line. Then list all co-authors.

(Last name/first name/middle initials)	IADR Member? (Y/N)	Institution
Presenter: Dr. N. Fleming	Y	U of Manitoba
Co-authors: E. Sliwinski-Lis	N	
D. Burke	N	

1989 IADR ABSTRACT FORM Type perfect original of abstract here: Appendix III

(FOR OFFICE USE)
R _____
P _____

Reviewer's Rating:
1 ☐ 4 ☐
2 ☐ 5 ☐
3 ☐

Disposition:
O ☐
P ☐
R ☐
W ☐

Average Score:

Guanine Nucleotides and Ca^{2+} Mobilization in Permeabilized Rat Submandibular Cells. N. FLEMING* and D. BURKE (Dept. of Oral Biology, University of Manitoba, Winnipeg, Canada):

Earlier work showed that GTPγS caused the release of sequestered $^{45}Ca^{2+}$ from non-mitochondrial stores in electrically permeabilized acinar cells of rat submandibular gland. In the present study we tested whether this effect was due to the generation of endogenous inositol 1,4,5-trisphosphate (IP_3), via a GTPγS-activated regulatory G_p protein, or to the direct action of nucleotide on cell organelles. Electrically permeabilized acinar cells were loaded to equilibrium with $^{45}Ca^{2+}$ (2 μmol/l) then exposed to guanine nucleotides in the absence or presence of neomycin (10 mM), an inhibitor of the phosphoinositide effect. GTP and GTPγS (10^{-5} M) stimulated the release of 24.8 ± 4.0 and 26.3 ± 4.9 pmol $^{45}Ca^{2+}$/mg protein respectively from cell organelles. These responses were reduced by neomycin to 16.5 ± 2.5 and 14.7 ± 2.6 ($p<0.05$). In studies on PIP_2 hydrolysis, however, it was demonstrated that neomycin completely inhibited the enhancement of IP_3 caused by GTP (160% control level) and GTPγS (198% control). These studies indicate that the guanine nucleotide effect in mobilizing sequestered Ca^{2+} may be due partially to G protein-associated IP_3 generation, and partially to the direct action of nucleotide on calcium-containing organelles.

Supported by the Canadian Cystic Fibrosis Foundation.

INFORMATION SECTION
(Type or print legibly in black or blue ink.)

mplete name and MAILING ADDRESS of the PRESENTER. Notification of acceptance will be mailed to this address. BE SURE IT IS CORRECT.

Name: Norman Fleming
Institution/ Company: University of Manitoba
Department: Oral Biology
Street Address: 780 Bannatyne Avenue
City: Winnipeg
State/Country: Manitoba, Canada
ZIP/Postal Code: R3E 0W2

2. Daytime Phone: (204)788-6794 (with area code)
(Students: give co-operating instructor's number)
Presenter's FAX #: 204-888-4113

3. Membership ID#: 0 0 1 0 7 9 9 7

4. IADR Member? (1) ☒ yes (2) ☐ no

5. Mode of Presentation Preferred (check only one):
(1) ☒ oral presentation
(2) ☐ poster presentation
(3) ☐ no preference

6. Give major source of funding for this research (check only one):
(1) ☐ school/university
(2) ☐ U.S. Government
☐ state/provincial government
(4) ☐ other national government
(5) ☐ industry
(6) ☒ foundation
(7) ☐ other

7. Are you applying for the Edward H. Hatton Awards Competition?
(1) ☐ yes (2) ☒ no

8. Is this research of interest to the general public and suitable for press coverage?
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If yes, include (separately) a brief description in lay terms of the significance of this research.

9. Was any of this research supported by NIH? (If yes, print grant number as last line in abstract box.)
(1) ☐ yes (2) ☒ no
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For American Division Only:

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Print grant number in abstract box.
Abstracts without grant numbers will not be considered. Grant must be in effect at time of meeting (July 1, 1989) to be considered.

12. Will you withdraw your paper if you do NOT receive a Bloc Travel Grant?
(1) ☐ yes (2) ☐ no

13. Area of Review (check only one):
(a) ☐ Behavioral Sciences/Health Services Research
(b) ☐ Cariology
(c) ☐ Craniofacial Biology
(d) ☐ Dental Materials: I—Polymers
(e) ☐ Dental Materials: II—Non-polymers
(f) ☐ Diagnostic Systems
(g) ☐ Experimental Pathology
(h) ☐ Geriatric Oral Research
(i) ☐ Implantology Research
(j) ☐ Microbiology/Immunology
(k) ☐ Mineralized Tissue
(l) ☐ Neuroscience/TMJ
(m) ☐ Oral & Maxillofacial Surgery
(n) ☐ Periodontal Research
(o) ☐ Pharmacology, Therapeutics & Toxicology
(p) ☐ Prosthodontics Research
(q) ☐ Pulp Biology
(r) ☒ Salivary Research

14. List five descriptors by number (see reverse) that apply to this research:
(1) 124 (4) 422
(2) 227 (5) 188
(3) 285

(For Office Use Only)
(1) ☐ yes (2) ☐ no

15. PRESENTER/CO-AUTHOR INFORMATION: List Presenter on first line. Then list all co-authors.

	Last name:	Full first name:	Middle Initial:
Presenter:	Fleming	Norman	
Co-authors:	Burke	Darryl	