Diadenosine Pentaphosphate Activates Purinoceptors and Releases Intracellular Calcium from Caffeine/Ryanodine-Sensitive Stores in Cultured Human Fetal Astrocytes

By

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A Thesis

Submitted to the Faculty of Graduate Studdies

in Partial Fulfillment of the Requirements

for the degree of

MASTER OF SCIENCE

Department of Pharmacology and Therapeutics

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Winnipeg, Manitoba, R3E OW3

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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DIADENOSINE PENTAPHOSPHATE ACTIVATES PURINOCEPTORS AND RELEASES INTRACELLULAR CALCIUM FROM CAFFEINE/ RYANODINE-SENSITIVE STORES IN CULTURED HUMAN FETAL ASTROCYTES

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

MASTER OF SCIENCE

I dedicate this thesis to my Mother and Father for their

eternal support and encouragement.

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ABBREVIATIONS

diadenosine polyphosphates (Ap_nAs, n= 2 to 6 phosphate groups)

diadenosine tetraphoshate (Ap_4A)

adenosine triphosphate (ATP)

phospholipase C (PLC)

cyclic AMP (cAMP)

phospholipase A (PLA)

inositol 1,4,5-trisphosphate (IP_3)

diacylglycerol (DAG)

free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

endoplasmic/sarcoplasmic reticulum (ER/SR)

inositol 1,4,5-trisphosphate (IP_3)

ryanodine receptors (RyRs)

cyclic ADP-ribose (cADPR)

ADP-ribose (ADPR)

β-nicotinamide - adenine dinucleotide (βNAD)

Ca^{2+} -induced Ca^{2+} release (CICR)

central nervous system (CNS)

periodate oxidized P^1, P^5-di(adenosine-5′) pentaphosphate (Ap_5A)

glial fibrillary acidic protein (GFAP)

bovine serum albumin (BSA)

pyridoxal phosphate 6-azophenyl 2', 4'-disulphonic acid (PPADS)

phenylmethylsulfonyl fluoride (PMSF)

ABSTRACT

A group of adenosine-based compounds termed diadenosine polyphosphates (Ap_nAs, n= 2 to 6 phosphate groups) act as agonists on P_2-type adenine nucleotide purinoceptors, increase levels of intracellular calcium ($[Ca^{2+}]_i$), and act as modulators of brain and muscle ryanodine receptor intracellular calcium release channel complexes (RyRs). In this study, using single cell fluorescence techniques and cultured human fetal astrocytes loaded with the calcium sensitive dye fura-2/AM to measure free $[Ca^{2+}]_i$, we determined the extent to which and the mechanisms by which P^1, P^5-di(adenosine-5') pentaphosphate (Ap_5A) increased $[Ca^{2+}]_i$. Ap_5A, pressure ejected from micropipettes filled with 100 μM Ap_5A, produced statistically significant increases in $[Ca^{2+}]_i$ from basal levels of 141 ± 12 nM to 980 ± 150 nM in 55 out of 95 cultured human fetal astrocytes tested. These actions of Ap_5A were mediated, at least in part, through activation of purinoceptors because the nonselective P_{2X}/P_{2Y} purinoceptor antagonist pyridoxal-phosphate-6-azophenel-2',4'-disuphonic acid tetrasodium (PPADS), at a final concentration of 300 μM, blocked, by 52%, Ap_5A-induced increases in $[Ca^{2+}]_i$. Initial studies showed that chelation of extracellular Ca^{2+} with 2 mM EGTA prevented Ap_5A-induced increases in $[Ca^{2+}]_i$. Subsequently, thapsigargin (5 μM), a Ca^{2+}- ATPase inhibitor that depletes intracellular stores of $[Ca^{2+}]_i$, was found to block, by 58%, Ap_5A-induced increases in $[Ca^{2+}]_i$. Thus, Ap_5A - induced increases in $[Ca^{2+}]_i$ originated extracellularly and intracellularly. The calcium released from intracellular stores was from RyR- and not inositol 1,4,5-trisphosphate (IP_3) - regulated stores because caffeine and ryanodine blocked by

approximately 66%, but bradykinin did not block Ap_5A-induced increases in $[Ca^{2+}]_i$. The presence of RyR - regulated stores of calcium was confirmed by results that pressure-applied ryanodine (100 nM) increased $[Ca^{2+}]_i$ by 723 ± 298 nM. Bath-applied ryanodine at concentrations of 1, 10, and 100 μM induced significant increases in baseline $[Ca^{2+}]_i$ from 150 ± 17 nM to 192 ± 18 nM ($p<0.05$), 354 ± 37 nM ($p<0.001$) and 389 ± 53 nM ($p<0.001$), respectively. The molecular presence of RyR-regulated intracelluar Ca^{2+} stores was confirmed through dot blot technique using a mouse monoclonal anti-RyR antibody. In cultured human fetal astrocytes, Ap_5A activates P_{2X} and possibly P_{2D} purinoceptors and significantly increases levels of Ca^{2+} by increasing influx of Ca^{2+} which stimulates release of $[Ca^{2+}]_i$ from ryanodine/caffeine sensitive intracellular stores. We are the first group to demonstrate the existence of RyR-regulated intracellular Ca^{2+} stores in human astrocytes.

INTRODUCTION

Diadenosine polyphosphates (Ap_nAs) consist of two adenosine molecules bridged by one to six phosphate groups and are present in both eukaryotic and prokaryotic cells. Ap_nAs are believed to be present in virtually all mammalian tissue and have been shown to be concentrated within thrombocytes (Ogilvie, 1992), chromaffin cells (Hoyle, 1990) and cholinergic neurons (Pintor et al., 1995). Diadenosine polyphosphates were first shown to be biologically active in 1978 when it was shown that diadenosine tetraphoshate (Ap_4A) induced DNA replication (Grummt et al., 1978a; 1978b). In the past several years, researchers have become very interested in the potential therapeutic significance of Ap_nAs in physiological processes such as vasotone regulation, hemostasis, neuromodulation, and neurotransmission.

The concentrations of Ap_nAs increase in response to cell activation, cellular stress, and during periods of cell proliferation and DNA replication/repair (Grummt et al., 1979; Baril et al., 1983). Intracellularly, the levels of Ap_nAs may increase over 100-fold to approximately 100 μM and within storage granules the levels have been estimated to be in the mM range (McLennan, 1992).

Although formed intracellularly, Ap_nAs have been shown to be released from thrombocytes (Pinto et al., 1991), adrenal chromaffin cells (Castillo et al., 1992), as well as brain cells where the release was shown to be calcium-dependent (Pintor and Miras-Portugal, 1995a). Enzymes metabolizing Ap_nAs, namely ectohydrolases, degrade extracellular Ap_nAs into ATP, ADP, or AMP, and AMP can be further degraded to adenosine by 5'-nucleotidase (Guranowski

et al., 1994). To varying degrees, Ap_nAs have been shown to interact with cell surface P_2 purinoceptors and ectohydrolases almost certainly function to control the levels of Ap_nAs available to act on these sites.

Considerable evidence supports the notion that adenosine triphosphate (ATP), an adenine nucleotide, functions as a transmitter-like compound in central and peripheral nervous system tissues, and as an important regulator of physiological functions controlled by the autonomic nervous system (Burnstock, 1990). The actions of nucleotides appear to be mediated through at least two families of receptors, each of which has multiple members - ligand-gated ion channel P_{2X} receptors and G-protein-coupled P_{2Y} receptors (Harden et al., 1995). In addition, nucleotide receptors termed P_{2Z}, P_{2T}, P_{2U} and P_{2D} have been identified (Harden et al., 1995; Castro et al., 1992).

P_2 Purinoceptors have been classified on the basis of rank order of agonist potencies (Table 1) and modern cloning technologies. The rank order of potency of adenine nucleotides for the P_{2X} purinoceptor is α,β-methylene ATP $\geq$ β,γ-methylene ATP $>$ ATP $=$ ADP $>$ 2-methylthio ATP. For P_{2Y} purinoceptors the rank order of potency is 2-methylthio ATP $\geq$ α,β-MeADP $\geq$ ATP $>>$ α,β-methylene ATP. In platelets, ADP is the most potent agonist while ATP acts as an antagonist. For P_{2Z} purinoceptors, ATP^{4-} is a highly selective agonist, and for P_{2U} purinoceptors the rank order of potency is UTP $\geq$ ATP. Recently, a putative P_2 purinoceptor named P_{2D} that preferentially binds Ap_4A has been added to the family of P_2 receptors (Castro et al., 1992).

Table 1. P_2 purinoceptors; agonist profile and distribution.

P_2 receptor	P_{2X}	P_{2Z}	P_{2Y}	P_{2U}	P_{2T}	P_{2D}
agonist profile	α,β-methylene ATP ≥ β,γ-methylene ATP > ATP = ADP > 2-methylthio ATP	ATP^{4-}	2-methylthio ATP ≥ α,β-MeADP ≥ ATP >> α,β-methylene ATP	UTP ≥ ATP	ADP	Ap_4A
distribution	neuron, muscle	immune cells	wide	wide	platelets, osteoblasts	wide

Following recent advances in molecular biology P_2 purinoceptors have subsequently been classified into two categories: P_{2X} purinoceptor ATP-gated channels (Suprenant et al., 1995) and P_{2Y} G-protein coupled purinoceptors (Barnard et al., 1994; Boarder et al., 1995). At present seven different P_{2X} and seven different P_{2Y} purinoceptor genes have been cloned. My research on P_2 purinoceptors does not encompass their genetic sequencing, therefore I have chosen to refer to the different P_2 purinoceptor subtypes according to nucleotide agonist profiles.

The most prominent signal transduction pathways mediated by the activation of P_2 receptors are summarized in Figure 1.

P_{2X} purinoceptors

The activation of the P_{2X} -gated ion channels allows for the non-selective influx of K^+, Na^+, and Ca^{2+} ions. Nucleotide stimulation of P_{2X} purinoceptors activates ion-gated channels in central neurons (Ueno et al., 1992), peripheral neurons (Bean, 1990; Bean et al., 1990; Fieber and Adams, 1991), cardiac muscle (Danziger et al, 1988; Scamps and Vassort, 1990; Christie et al., 1992), and smooth muscle (Benham et al., 1987; Benham and Tsien, 1987). Different cell types have very similar, but not identical ligand selectivity, which supports the finding that several P_{2X} purinoceptor subtypes exist..

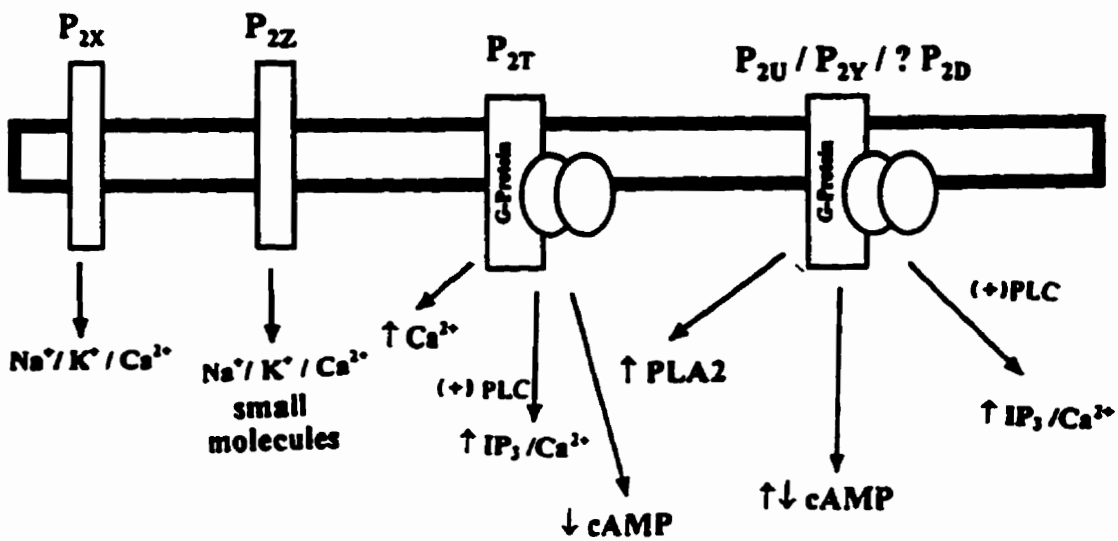

Fig. 1. Signal transduction pathways utilized by P_2 purinoceptors.

Abbreviations: PLC (phospholipase C), cAMP (cyclic AMP), PLA (phospholipase A), IP_3 (inositol 1,4,5-trisphosphate)

P_{2Z} purinoceptors

The P_{2Z} purinoceptor mechanism of action is still a mystery. It has been suggested that nucleotide activation of P_{2Z} purinoceptors leads to the formation of large membrane pores which allow the non-selective influx of K^+, Na^+, and Ca^{2+} ions as well as some low molecular weight molecules (Chen et al., 1994). P_{2Z} purinoceptors are present mainly in immune cells.

P_{2Y}, P_{2U}, P_{2T}, and P_{2D} purinoceptors

At least four purinoceptor subtypes, P_{2Y}, P_{2U}, P_{2T} and P_{2D} , are coupled to G proteins and are linked to the activation of phospholipase C (PLC) and the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Berridge, 1993). The accumulation of intracellular IP_3 can then cause a variety of secondary events including release of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores (see Fig. 1). In addition to its effects on IP_3, P_{2T} purinoceptors inhibit adenylate cyclase activity and decrease the production of cAMP. P_{2D} , and P_{2U} purinoceptors are distributed in a very wide range of tissues; P_{2T} purinoceptors are believed to be confined to platelets (Gordon, 1986).

CALCIUM

Calcium is utilized by cells as a second messenger to control many cellular processes including muscle contraction, secretion of neurotransmitters, metabolism of neurotransmitters, neuronal excitability, and cell proliferation. Excess Ca^{2+} can lead to cell death. The free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in resting cells ranges from 10 - 150 nM while the extracellular concentration of Ca^{2+} is usually around a few mM; thus a large concentration gradient across the plasma membrane exists which is maintained by Ca^{2+} pumps and Na^+ / Ca^{2+} exchangers. The cell has access to two potential sources of Ca^{2+}, influx from the extracellular space and release from internal stores.

Located in the plasma membrane are a variety of channels, described below, which allow the entry of extracellular Ca^{2+} into the cell.

Voltage - operated Ca^{2+} channels: This group of Ca^{2+} channels opens in response to membrane depolarization. Four types of voltage - operated Ca^{2+} channels have been classified on the basis of their electrophysiological and pharmacological characteristics:

- L-type - long lasting current, activated by high voltages and sensitive to 1,4-dihydropyridines
- N-type - neuronal, transient channel current activated by high voltage, sensitive to ω-conotoxin
- T-type - transient current, low-voltage activated

- P-type - long-lasting current, low-voltage activated, sensitive to ω-agatoxin

Receptor - operated channels: The binding of an agonist to its inotropic receptor will cause conformational changes in the receptor and as a result ions cross the membrane down their electrochemical gradient. Ca^{2+} channels can be opened by the binding of specific agonists usually neurotransmitters.

Ca^{2+} can also be released from intracellular Ca^{2+} stores. The endoplasmic/sarcoplasmic reticulum (ER/SR) has two families of intracellular channels, each responsible for releasing Ca^{2+} from separate and distinct pools of Ca^{2+} (Berridge, 1993): IP_3- and RyR/caffeine-sensitive pools.

Inositol 1,4,5-trisphosphate (IP_3) receptor. IP_3 released into the cytoplasm acts to liberate Ca^{2+} from pools of Ca^{2+} located on the ER/SR (Berridge, 1993). IP_3 is the only known physiological activator of the IP_3 receptor. Subsequent to IP_3 being metabolized or removed from the IP_3 receptor the channel closes and the receptor is now ready to be activated again (Ehrlich et al., 1994). Ca^{2+} and ATP can increase the sensitivity of the IP_3 receptor, but are unable to activate the channel in the absence of IP_3 (Bezprozvanny et al., 1991; Bezprozvanny and Ehrlich, 1993).

Ryanodine Receptors (RyRs): Ryanodine, a plant alkaloid, binds to so called ryanodine receptors (RyRs) that regulate Ca^{2+} release (Meissner, 1986). These receptors are widely distributed in skeletal, cardiac, and smooth muscle as well as brain (Sorrentino and Volpe, 1993). Three different molecular forms of mamalian RyRs are encoded by three different genes; skeletal muscle

(type 1 receptor), cardiac muscle (type 2 receptor), and brain (type 3 receptor) (Otsu et al., 1990; Sorrentino et al., 1993). The three RyR isoforms contain mutual regulatory sites which exist within the RyR -channel complex (Fig. 2). Ryanodine and caffeine stimulate Ca^{2+} release from types 1 and 2 RyRs. Type 3 RyRs are responsive to ryanodine, but not to caffeine (Giannini et al., 1992). The presence of intracellular and extracellular Ca^{2+} is required for the following compounds to function as regulators of RyR Ca^{2+} release channel activity.

Ryanodine

Ryanodine has been found to either stimulate or inhibit the activity of RyRs. Low micromolar concentrations have been shown to lock the channel in an open, reduced - conductance configuration, and millimolar concentrations irreversibly lock the channel in a closed state (Lai et al., 1989).

Adenine Nucleotides

Various adenine nucleotides including ATP, adenosine, cyclic ADP-ribose, ADP-ribose and β-nicotinamide-adenine dinucleotide have been shown to potentiate RyR-regulated Ca^{2+} release (Sitsapesan et al., 1995). AMP-PCP, ADP, AMP and adenine have also been proposed to bind to the adenine nucleotide binding site and potentiate release of RyR-regulated intracellular Ca^{2+} stores (Meissner, 1984; Morii and Tonomura, 1983; McGarry and Williams, 1994). The

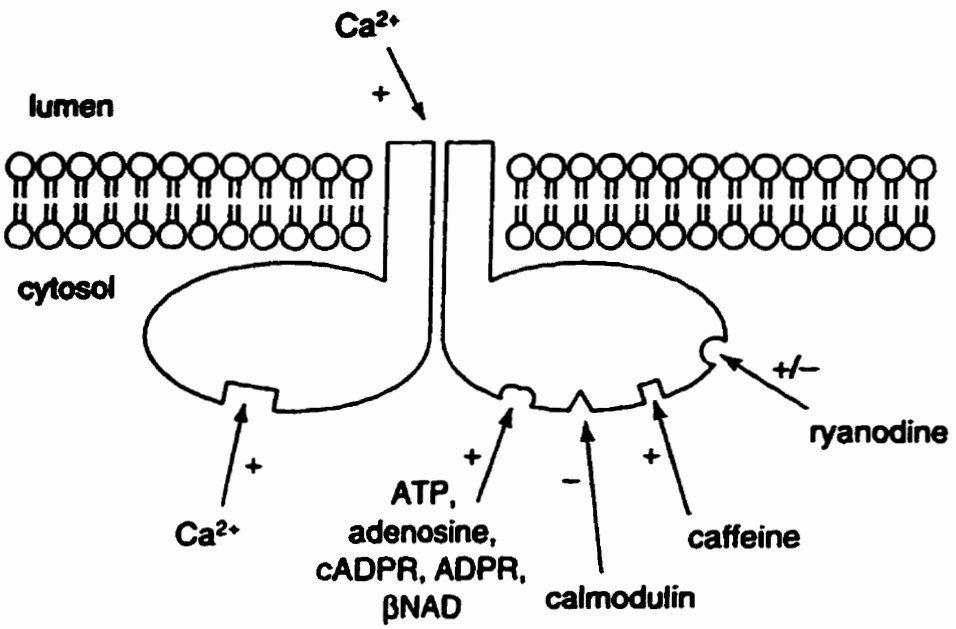

Fig. 2 Modulators of ryanodine receptor - channel complexes. Cytosolic Ca^{2+} is the primary activating ligand, being effective in the absence of all other ligands. Activaton of the channel by adenine nucleotides and caffeine is regulated by both cytosolic and luminal Ca^{2+}. Ligands proposed to act at the adenine nucleotide site include ATP, adenosine , cyclic ADP-ribose (cADPR), ADP-ribose (ADPR) and β-nicotinamide - adenine dinucleotide (βNAD). Depending upon the concentration applied, ryanodine will either increase or decrease open probability. Taken from Sitsapesan et al., 1995.

potentiation of channel activation by nucleotides can be inhibited by Mg^{2+} ions.

Caffeine

In the presence of cytosolic Ca^{2+} , millimolar concentrations of caffeine sensitize the channel to Ca^{2+} (Sitsapesan and Williams, 1994). The modulator sites activated by caffeine are distinct from those activated by adenine nucleotides (McGarry and Williams, 1994).

Calmodulin

Calmodulin has been shown to inhibit or potentiate RyRs. Calmodulin directly inhibits type 1 and type 2 RyRs. Type 2 RyRs phosphorylated by calmodulin - dependent protein kinases exhibit increased probabilities of channel opening.

Calcium

All the ligands mentioned above either will not activate RyR channels in the absence of Ca^{2+}, or require Ca^{2+} for maximum effect. Mg^{2+} is believed to compete for Ca^{2+} binding sites. Hence, when excess free Mg^{2+} is present RyR channel activity is inhibited (Kirino et al., 1983; Meissner, 1984; Nagasaki and Kasai, 1993).

IP_3 Rs and RyRs are sensitive to Ca^{2+} through positive feedback mechanisms. A positive feedback process involving Ca^{2+} -induced Ca^{2+} release (CICR) is very important in increasing $[Ca^{2+}]_i$ and is thought to be the primary activator of RyRs in many cell types. Once channels have been activated and Ca^{2+} has been released RyRs change conformation and the channels close.

Cyclic ADP ribose has been proposed to be a physiological agonist at the adenine nucleotide regulatory site on the RyR and hence in the presence of Ca^{2+} can activate RyR-regulated intracellular Ca^{2+} stores (Galione et al., 1991). Caffeine has also been shown to induce Ca^{2+} release by activiating RyRs (Endo M, 1985; Ehrlich and Watras, 1988).

An increase in $[Ca^{2+}]_i$ in healthy cells is counterbalanced by extrusion of intracellular Ca^{2+} via a variety of mechanisms including Na^+ / Ca^{2+} exchangers and plasmalemmal Ca^{2+} pumps.

DIADENOSINE POLYPHOSPHATES AND CALCIUM

One prominent cellular response to Ap_nAs is increased levels of the signal transducer, intracellular calcium. Ap_nA-induced increases in Ca^{2+} influx have been shown following P_2 receptor activation in rat brain synaptic terminals (Pintor and Miras-Portugal, 1995). P_{2X} receptor activation in guinea-pig urinary bladder (Usune et al., 1996) revealed a specific interaction with N-type calcium channels in rat CNS neurons (Panchenko et al., 1996). Nucleotides have been shown to increase $[Ca^{2+}]_i$ following mobilization from intracellular stores in a variety of cells (Walz et al., 1994; Pacaud and Loirand, 1995; Salter and Hicks, 1994; 1996; Palmer et al., 1994; Squires et al., 1994). Our hypothesis that Ap_nAs may be releasing Ca^{2+} from RyR-regulated pools of intracellular Ca^{2+} is supported by findings that ATP and other adenosine-based compounds have been found to increase ryanodine binding (McPherson et al., 1991; Padua et al., 1994; Sitsapesan et al., 1996), that Ap_5A and Ap_6A were both potent activators of RyR-regulated calcium-induced calcium release from skeletal muscle sarcoplasmic reticulum (Morrii

and Makinose, 1992), and that Ap_nAs significantly enhanced binding of [^{3}H]ryanodine to RyRs of rat brain, skeletal muscle and cardiac muscle (Holden et al., 1996).

ASTROCYTES

Glial cells are the most numerous cell type in the central nervous system (CNS) and are thought to play an important role in guiding brain development and in supporting adult brain function (Cserr and Bundgaard, 1986). Macroglia are classified into two groups within the CNS, astrocytes and oligodendrocytes. Processes from oligodendrocytes wrap many times around axons of neurons in the central nervous system to provide the myelin sheath. This sheath insulates axons from one another and inhibits current flow between axons. Schwann cells provide the same protection to axons in peripheral nerves.

Astrocyte cytoplasm contains glial filaments which provide mechanical support for CNS components and hypertrophy following a CNS insult. Astrocytes can limit the diffusion of substances and can actively take up K^+ ions and neurotransmitters (Berne and Levy, 1993). Thus, astrocytes by providing support, nutrients, and trophic factors, regulate the microenvironment of neurons in the central nervous system (Lindsay et.al., 1982; Varon and Somjen, 1979). Astrocytes do not form synapses with neurons, but they have do release neuroactive substances in the vicinity of neurons (Shain et.al., 1986; Shain et.al., 1989).

A wide range of receptors for neurotransmitters including P_2 purinergic receptors have been found on astrocytes (Neary et al., 1988). Nucleotide-activated P_2 purinoceptors mediate a wide range of biological responses including activation of RyR-regulated intracellular Ca^{2+} stores (Langley and Pearce, 1994), activation of IP_3-sensitive intracellular Ca^{2+} stores (Lin and Chung, 1994; Kastritsis et al., 1992; Salter and Hicks, 1994; Lin and Chuang, 1993), increased prostaglandin synthesis (Gebicke-Haerter et al., 1988), thromboxane release (Bruner and Murphy, 1993), inhibition of agonist - stimulated cAMP accumulation (Debernardi et al., 1993; Boyer et al., 1993), growth changes (Neary and Norenberg, 1992), stimulation of ion channels (Norenberg et al., 1994; Langosch et al., 1994; Walz et al., 1994; Walz et al., 1993), and increased glial fibrillary acidic protein and DNA synthesis (Neary et al., 1994a; Neary et al., 1994b; Abbracchio et al., 1994).

Here, using cultured human fetal astrocytes, for which little information is available on pools of intracellular calcium, we show that activation of IP_3- regulated pools of $Ca^{2+}{}_i$ by bradykinin receptor stimulation led to large increases in $[Ca^{2+}]_i$, but that Ap_nA-induced increases in $[Ca^{2+}]_i$ were not affected by bradykinin receptor agoinist. Caffeine and ryanodine increased $[Ca^{2+}]_i$ and significantly decreased Ap_nA-induced increases in $[Ca^{2+}]_i$. Therefore, we hypothesize the existence of RyR-regulated intracellular Ca^{2+} stores in human fetal astrocytes. Furthermore, we hypothesize, activation of P_2 purinergic membrane receptors on human fetal astrocytes by diadenosine pentaphosphate (Ap_5A) in the presence of Ca^{2+} stimulates release of RyR-regulated intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Cultured human fetal astrocytes: All procedures related to the acquisition and use of human fetal tissue were approved by the Human Ethics Committees of the Health Sciences Centre Hospital and the University of Manitoba Faculty of Medicine. Fetal tissue was obtained and astrocytes were cultured as previously described (Furer et al., 1993; Gu et al., 1996). Briefly, brain tissue obtained from 15 to 17 week old fetus' was washed in DMEM culture media containing 10% fetal calf serum (FCS), 1% L-glutamine and 0.2% antibiotic solution consisting of 1000 units/ml of penicillin G, 10 mg/ml streptomycin and 25 μg/ml amphotericin B in 0.9 % NaCl. Following trituration through a 20 gauge needle, cells were centrifuged at 270 Xg for 10 min, suspended in culture medium, plated in 75 cm^2 flasks, and incubated in 5% CO_2 at 37°C. Seven day old cultures were placed on a rotatory shaker for 2 hours at 330 rpm at room temperature, the supernatant was discarded, and the remaining cells, mainly astrocytes, were exposed for 10 min to 0.05% trypsin plus 0.53 mM EDTA. Cells were collected, centrifuged as above, resuspended in DMEM media containing 10% FCS, and placed into 75 cm^2 culture flasks. After 30 min, decanted cells, which were >98% astrocytes, were plated onto poly D-lysine coated 25 mm diameter cover slips at a density of 1000 cells/ml. Cover slips were placed in sterile 6 well containers (NUNC) and were incubated in 5% CO_2 at 37°C for no longer than 7 days. Fresh media was added at 3 to 4 days.

Immunohistochemical staining for GFAP: To test for purity, cultures of astrocytes were stained for glial fibrillary acidic protein (GFAP) as described previously [Bignami et al., 1972]. Cells plated on glass cover slips were fixed in acetone/methanol (1:1) for 30 min, washed three times with phosphate-buffered saline (PBS), and incubated with PBS plus 10% horse serum for 1 hr. Rabbit anti-GFAP (Boehringer Mannheim) was added at a dilution of 1:100 and cells were incubated for 90 min at 25°C. Goat-anti-rabbit IgG conjugated with rhodamine red (Boehringer Mannheim) was then added at a dilution of 1:50 for 90 min at 25°C followed by three washes with PBS.

Intracellular calcium ($[Ca^{2+}]_i$) measurements: Levels of $[Ca^{2+}]_i$ were determined in human fetal astrocytes loaded with the calcium sensitive fluorescent probe fura-2-acetoxymethyl ester (Fura-2/AM) (Molecular Probes). Cells were incubated for 1 h at 25°C with 2 μM Fura-2/AM in Krebs buffer consisting of (in mM) 111.0 NaCl, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, 4.7 KCl, 1.2 $MgCl_2$, 15 HEPES, 1.8 $CaCl_2$, 5 glucose, and 1.5 μM bovine serum albumin (BSA), pH 7.4. Cells were washed three times with Krebs buffer (no BSA) and incubated at 37°C for 5 min. Fura-2 loaded cells were transfered to an open perfusion microincubator Model PDMI-2 (Medical Systems Corp.) kept at 37°C on the stage of a Nikon Diaphot microscope equipped with a Nikon Fluor 40x objective. Ratiometric epifluorescence images, at excitation wavelengths 340 and 380 nm, and an emission wavelenth of 510 nm were obtained using a Hamamatsu CCD camera and the analog image data were digitized and stored using Image2/FL hardware and software (Universal Imaging Co.). Fluorescence values were corrected for background and $[Ca^{2+}]_i$ were calculated using the equation $[Ca^{2+}]_i = Kd\ ((R - R_{min})/(R_{max} - R_{min}))(F_{min} / F_{max})$ where R_{min} =

0.347, R_{max} = 8.29, F_{min} = 45.48, F_{max} = 7.75, and Kd = 224 nM. R is the measure of actual fluorescences measured at 340 nm and 380 nm, R_{max} is the value of R at saturating Ca^{2+}, R_{min} is the value of R at zero Ca^{2+}. F_{min} / F_{max} is the ratio of fluorescences measured at zero Ca^{2+} and saturating Ca^{2+} for 380-nm excitation (Grynkeiwicz et al., 1985).

Periodate oxidized P^1, P^5-di(adenosine-5') pentaphosphate (Ap_5A), was dissolved in Krebs buffer to a final concentration of 100 μM, and loaded into glass micropipettes pulled using a Flaming Brown Micropipette puller Model P-87 (Sutter Instrument Co.). The tip of the micropipette was placed using a Nikon Narishige MO-388 micromanipulator 30 - 50 μm away from the astrocyte(s) to be imaged, and Ap_5A was administered by pressure ejection using a Picospritzer IID (General Valve Co.) set at 3 x 100 ms at 10 psi. The targeted astrocyte was monitored for changes in $[Ca^{2+}]_i$ in the presence of Ca^{2+}-free buffer containing 2 mM EGTA, Ca^{2+} buffer, or Ca^{2+} buffer containing thapsigargin (5 μM), bradykinin (500 nM), caffeine (20 mM), ryanodine (10 μM) or the P_{2X}/P_{2Y} purinoceptor antagonist pyridoxal phosphate 6-azophenyl 2', 4'-disulphonic acid (PPADS, 300 μM). With the exception of Ap_5A, all drugs were bath applied.

For the final set of experiments (Table 2.), α,β-MeATP (α,β-methelene ATP), α,β-MeADP (α,β-methelene ADP), Ap_5A (diadenosine pentaphosphate), and UTP (uridine 5'-triphosphate) dissolved in Krebs buffer at a final concentration of 100 μM, were bath applied to cultured human fetal astrocytes. . The astrocytes within the field of view were monitored for changes in $[Ca^{2+}]_i$.

Data were reported as mean ± S.E.M values. Statistical analyses were performed using either Student's t-test or a one way analysis of variance (ANOVA) with a Student-Newman-Keuls multiple comparisons test (INSTAT 2, Biosoft). Statistical significance was considered at the $p < 0.05$ level.

Tissue preparations for dot blots:

Astrocyte preparations: Human fetal astrocytes were cultured in 75 cm^2 flasks as described above. Culture media was removed and cells were washed 3 times with PBS. Lysing buffer (20 mM Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol and 0.75% NP_{40} detergent) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) was placed into the flask and cells were mechanically scraped and placed on ice for 30 minutes. Lysate was centrifuged at 1100 Xg for 10 minutes, and supernatants were collected and centrifuged at 100,000 Xg for 60 minutes. Pellets were washed three times with ice cold PBS containing 1 mM PMSF. Pellets were resuspended in PBS containing 1 mM PMSF at a concentration of approximately 1 mg/ml.

Rat heart preparations: Sprague-Dawley rats were killed by decapitation. Hearts were removed, washed with PBS and homogenized in PBS containing 1 mM PMSF using a Polytron Tissuemizer and then placed on ice for 30 minutes. The sample was centrifuged at 12,000 Xg for 10 minutes, and supernatants were collected and centrifuged at 100,000 Xg for 60 minutes. Pellets were

washed three times with ice cold PBS containing 1 mM PMSF. Pellets were resuspended in PBS containing 1 mM PMSF at a concentration of approximately 1 mg/ml.

Dot blot analysis: Dot blot analysis was performed to verify, molecularly, the presence of RyR-regulated intracellular calcium stores in human fetal astrocytes. Binding of the monoclonal mouse anti - RyR antibody (Affinity Bioreagents, MA3916) to human fetal astrocytes and rat heart was performed as follows: Protein was transferred to a nylon membrane via a BioRad dot blot analysis apparatus. Transfer membranes were blocked with 5% nonfat dried milk in PBS for 90 minutes. Transfer membranes were then washed 3 x 15 minutes with 0.1% Tween 20 in PBS and incubated with monoclonal mouse anti - ryanodine receptor antibody (1 μg/ml), 1% nonfat dried milk and 0.1% Tween 20 in PBS overnight at 21°C. The blot was then developed with horse radish peroxidase - conjugated secondary antibody (1:1000) using 3,3' -diaminobenzidine tetrahydrochloride (0.05%) and H_2O_2 (0.2%).

RESULTS

Greater than 98 % of the cultured cells tested stained positively for the astrocyte marker protein, GFAP (Fig. 3). Of 95 astrocytes tested, which were identified morphologically by their shape and large nucleus containing one or more nucleoli, 55 responded to 100 μM Ap_5A with peak increases in $[Ca^{2+}]_i$ of 980 ± 150 nM; responses ranged from about 500 to 2000 nM. The remaining 40 astrocytes tested did not show any significant increase in $[Ca^{2+}]_i$. Ap_5A, when applied repetitively at intervals of 8 to 10 min, induced increases in $[Ca^{2+}]_i$ of 950 ± 130 nM (n=7); no statistically significant differences were detected between responses to the repeated applications of Ap_5A (Fig. 4a). However, when a second ejection of Ap_5A was applied immediately following the return of $[Ca^{2+}]_i$ to baseline, statistically significant ($p<0.05$) attenuations of Ap_5A-induced increases in $[Ca^{2+}]_i$ were observed; responses were 11 ± 3 % (n=7) of initial increases in $[Ca^{2+}]_i$ (Fig. 4B). When injections were spaced at ≥ 5 min intervals, no statistically different responses to Ap_5A were observed ($[Ca^{2+}]_i$ increases were 990 ± 160 nM, n=7).

To determine the extent to which the increases in $[Ca^{2+}]_i$ were due to Ap_5A interactions with purinoceptors, Ap_5A was applied through micropipettes to astrocytes in the absence or presence of bath applied pyridoxal-phosphate-6-azophenyl -2',4'- disulphonic acid tetrasodium salt (PPADS), a P_{2X}/P_{2Y} purinergic receptor antagonist. PPADS at a concentration of 300 μM significantly ($p<0.005$) reduced to 48.2 ± 2.2 % (n=5) of control responses Ap_5A-induced increases of $[Ca^{2+}]_i$ (Fig.5, Table 2). The effects of PPADS were completely reversible in that

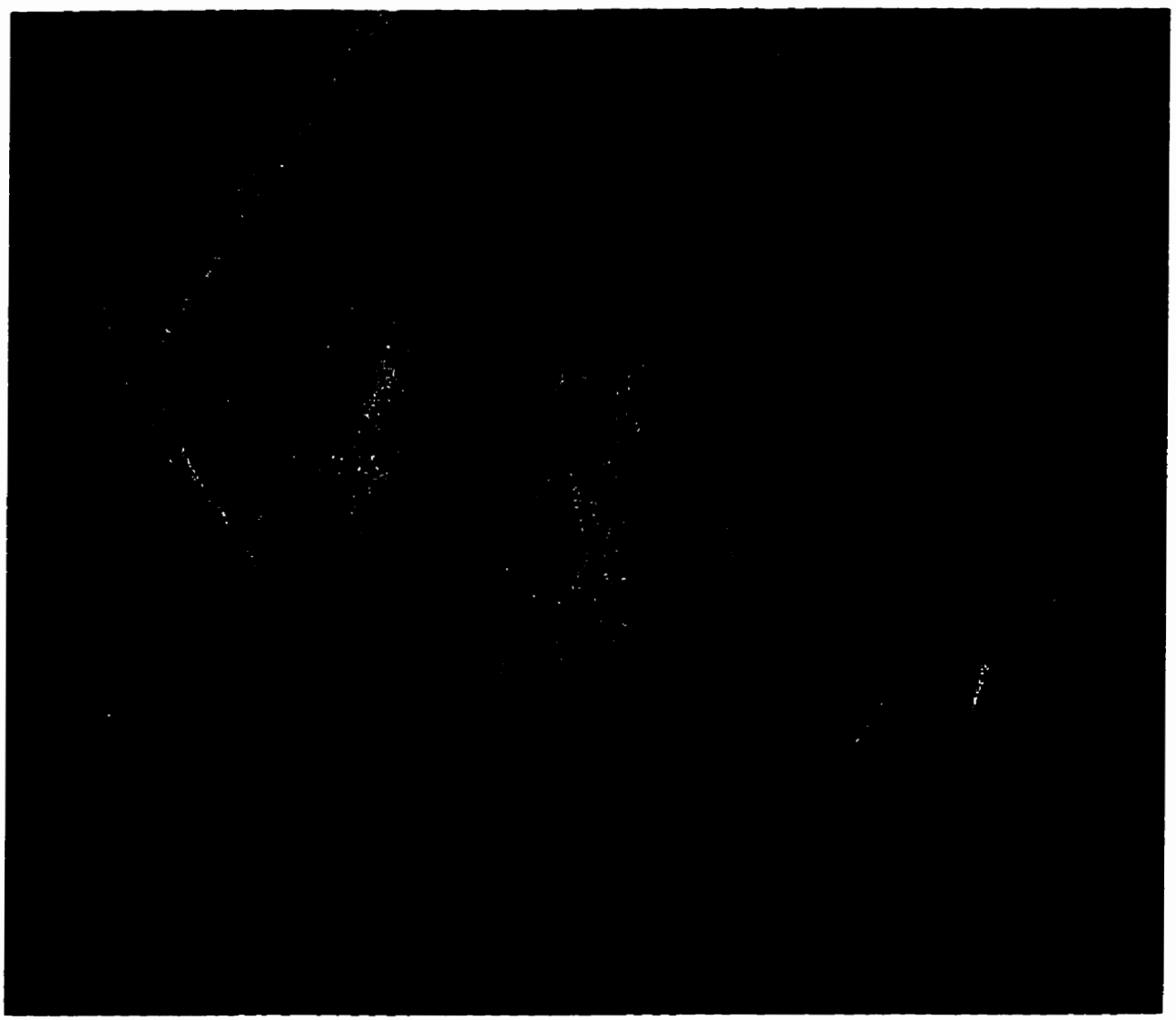

Fig. 3 Immunostaining of human fetal astrocytes with anti-glial fibrillary acidic protein antibody.

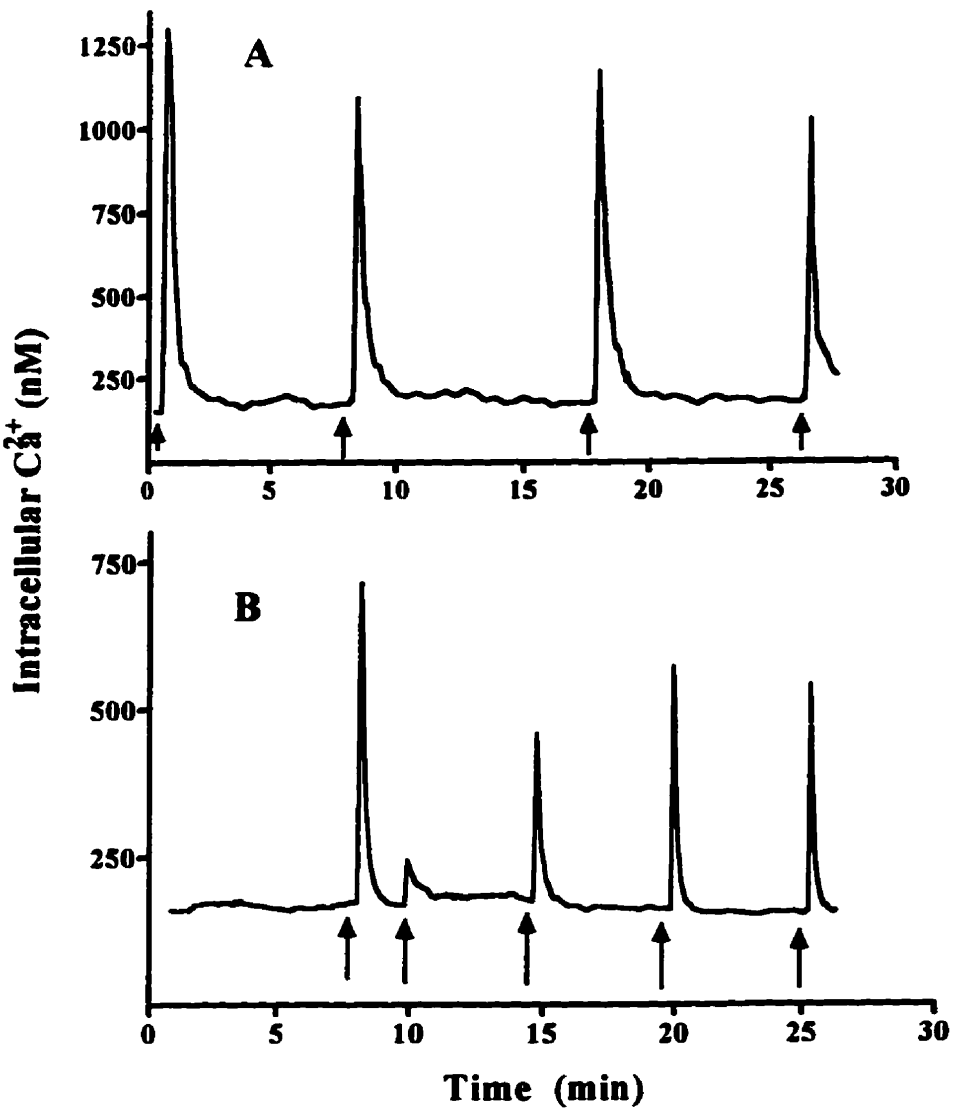

Fig. 4. Representative traces of Ap_5A - induced increases of $[Ca^{2+}]_i$. Ap_5A (100 μM in the micropipette) was pressure ejected (3 x 100 ms, 10 psi) onto isolated human fetal astrocytes as indicate by arrows. **A.** $[Ca^{2+}]_i$ responses to repeated applications of Ap_5A separated by 8-10 min recovery periods. No statistically significant differences in $[Ca^{2+}]_i$ responses were observed between responses. **B.** $[Ca^{2+}]_i$ responses to repeated applications of Ap_5A were significantly ($p<0.001$) decreased when Ap_5A was applied immediately after the initial $[Ca^{2+}]_i$ response returned to baseline.

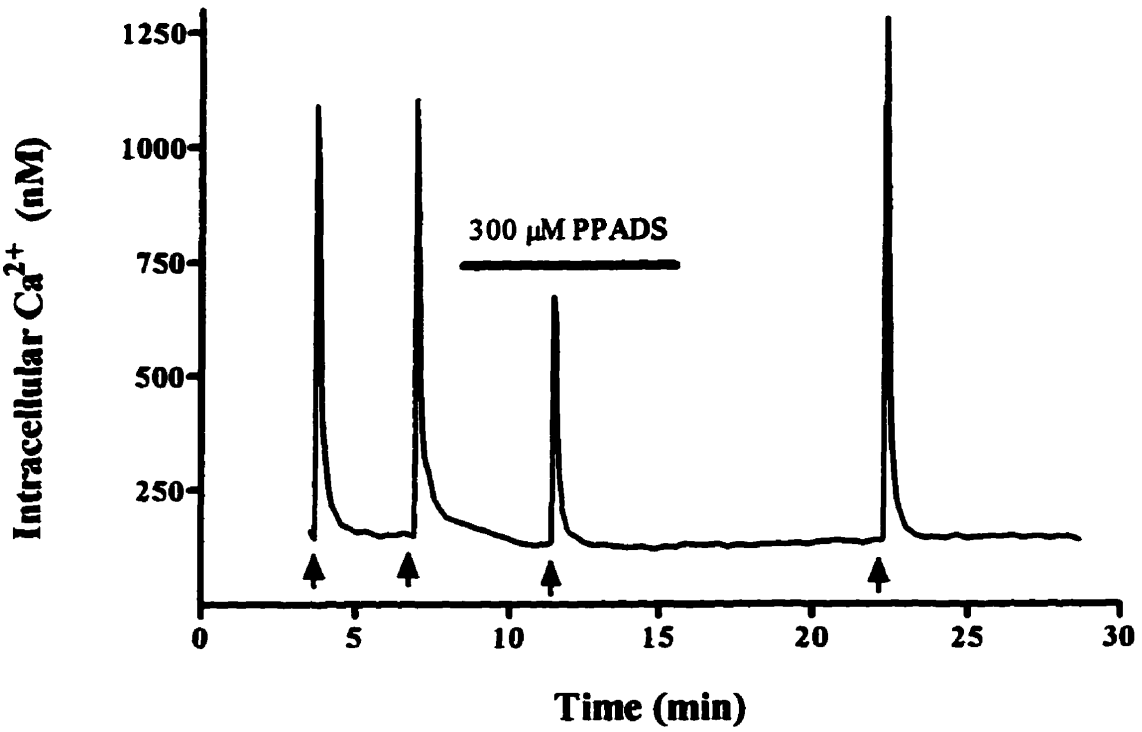

Fig. 5. Effects of the nonselective P_{2X}/P_{2Y} antagonist pyridoxal - phosphate - 6- azophenyl -2',4'-disulphonic acid tetrasodium salt (PPADS) on Ap_5A-induced increases in $[Ca^{2+}]_i$. Bath applied PPADS at 300 μM significantly ($p<0.005$) inhibited Ap_5A-induced increases in $[Ca^{2+}]_i$. Arrows represent pressure application (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

Table 2.

Summary of the effects of Ca^{2+}-free conditions, PPADS, thapsigargin, bradykinin, caffeine and ryanodine on Ap_5A-induced increases in $[Ca^{2+}]_i$.

Agent	Concentration	Response (% of control)	Time to Peak (min)	Recovery (min)
Control	N/A	N/A	0.11 ± 0.01	0.30 ± 0.02
Ca^{2+}-free	N/A	2.1 ± 0.4[a] (7)	N/A	N/A
PPADS	300 μM	48.2 ± 2.2[b] (5)	0.13 ± 0.05	0.31 ± 0.07
Thapsigargin	5 μM	41.9 ± 8.5[b] (7)	0.14 ± 0.02	0.43 ± 0.06
Bradykinin	500 nM	112 ± 10 (5)	0.14 ± 0.03	0.32 ± 0.02
Caffeine	20 mM	21.6 ± 26[b] (5)	0.15 ± 0.03	0.38 ± 0.04
Ryanodine	10 μM	34.1 ± 7.5[a] (8)	0.16 ± 0.03	0.24 ± 0.03

Ap_5A (100 μM in the micropipette) was pressure ejected (3 x 100 ms, 10 psi) onto isolated human fetal astrocytes. Increases in $[Ca^{2+}]_i$ by Ap_5A were 980 ± 150 nM and data summarized above were expressed as a percentage ± S.E.M. of these values. Numbers in parentheses indicate the number of astrocytes tested from different fetus'. Time to peak indicates the interval between ejection and peak increases in $[Ca^{2+}]_i$, and recovery indicates time between peak response and return to baseline levels of $[Ca^{2+}]_i$. N/A, not applicable. Significance was determined by student's t-test.

[a] $p<0.001$ compared with control Ap_5A response.

[b] $p<0.005$ compared with control Ap_5A response.

following washout of PPADS, Ap_5A - induced increases in $[Ca^{2+}]_i$ were not significantly different from control responses (Fig. 5).

The next series of experiments was performed to determine the extent to which Ap_5A - induced increases of $[Ca^{2+}]_i$ in human fetal astrocytes were due to $[Ca^{2+}]_i$ influx into cells or release from intracellular stores. In the absence of extracellular Ca^{2+}, repeated applications of Ap_5A failed to increase $[Ca^{2+}]_i$ levels above baseline levels of 141 ● 12 nM (Fig. 6). Eight to ten minutes after calcium containing buffer was re-introduced, $[Ca^{2+}]_i$ responses to Ap_5A were not significantly different from controls (Fig. 6). These data suggest an involvement of extracellular calcium in Ap_5A - induced increases of $[Ca^{2+}]_i$, but do not discount contributions from intracellular stores.

Thapsigargin (5 μM), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase that depletes intracellular stores of Ca^{2+}, increased $[Ca^{2+}]_i$ by 897 ± 301 nM (in 7 out of 7 cells), and in the continued presence of thapsigargin Ap_5A-induced increases of $[Ca^{2+}]_i$ were significantly ($p<0.005$) reduced to 41.9 ± 8.5 % of control values (Fig. 7, Table 2). To determine the extent to which the thapsigargin effect was due to depletion of ryanodine and/or IP_3-sensitive $[Ca^{2+}]_i$ pools, we examined responses to bradykinin, ryanodine, and caffeine. Bradykinin, a releaser of IP_3-sensitive Ca^{2+} stores, at 500 nM induced increases in $[Ca^{2+}]_i$ to 1090 ● 246 nM (in 5 out of 5 cells) and in its presence, responses to Ap_5A were not significantly affected; $[Ca^{2+}]_i$ levels were 112 ± 10 % ($n=5$) of control responses (Fig. 8 , Table 2). Ryanodine, a releaser of ryanodine - sensitive Ca^{2+} stores, at concentrations of 1, 10, and 100 μM, induced significant increases in baseline $[Ca^{2+}]_i$; 192 ± 18 nM ($p<0.05$), 354 ± 37 nM ($p<0.001$) and 389 ± 53 nM ($p<0.001$), respectively (Fig. 9).

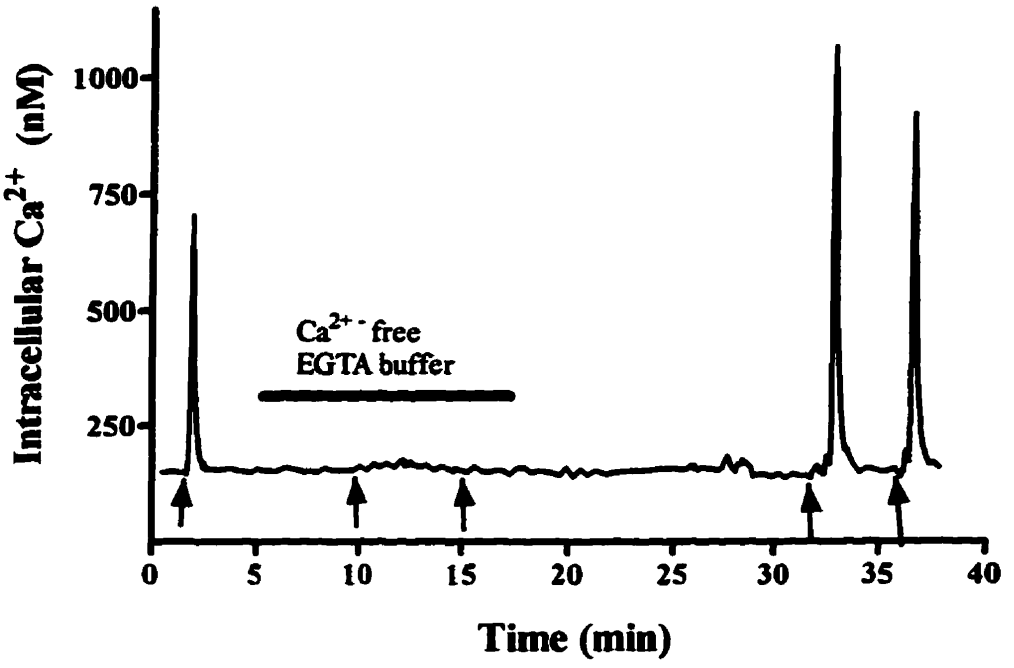

Fig. 6. Representative trace of Ap_5A-induced increases of $[Ca^{2+}]_i$ in the absence and presence of extracellular Ca^{2+}. Following initial Ap_5A-induced increases in $[Ca^{2+}]_i$, human fetal astrocytes were bathed in Ca^{2+}-free Krebs buffer containing 2 mM EGTA and responses to Ap_5A were 2.1 ± 0.4 % of control levels. Arrows represent pressure applications (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

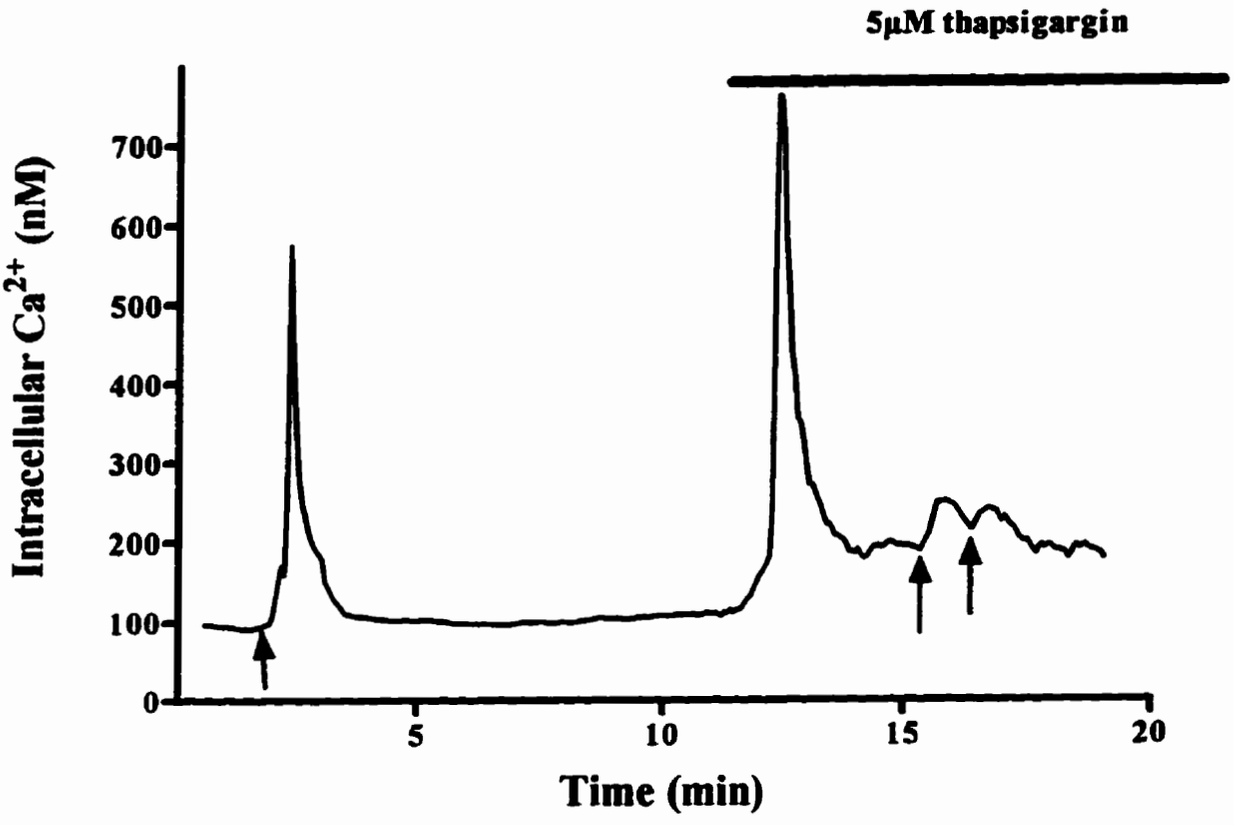

Fig. 7. Representative trace of effects of thapsigargin on $[Ca^{2+}]_i$ and on Ap_5A-induced increases in $[Ca^{2+}]_i$. Bath applied thapsigargin at 5 μM significantly ($p<0.005$) increased levels of $[Ca^{2+}]_i$ by 897 ± 301 nM, and significantly ($p<0.005$) inhibited Ap_5A-induced increases in $[Ca^{2+}]_i$. Arrows represent pressure application (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

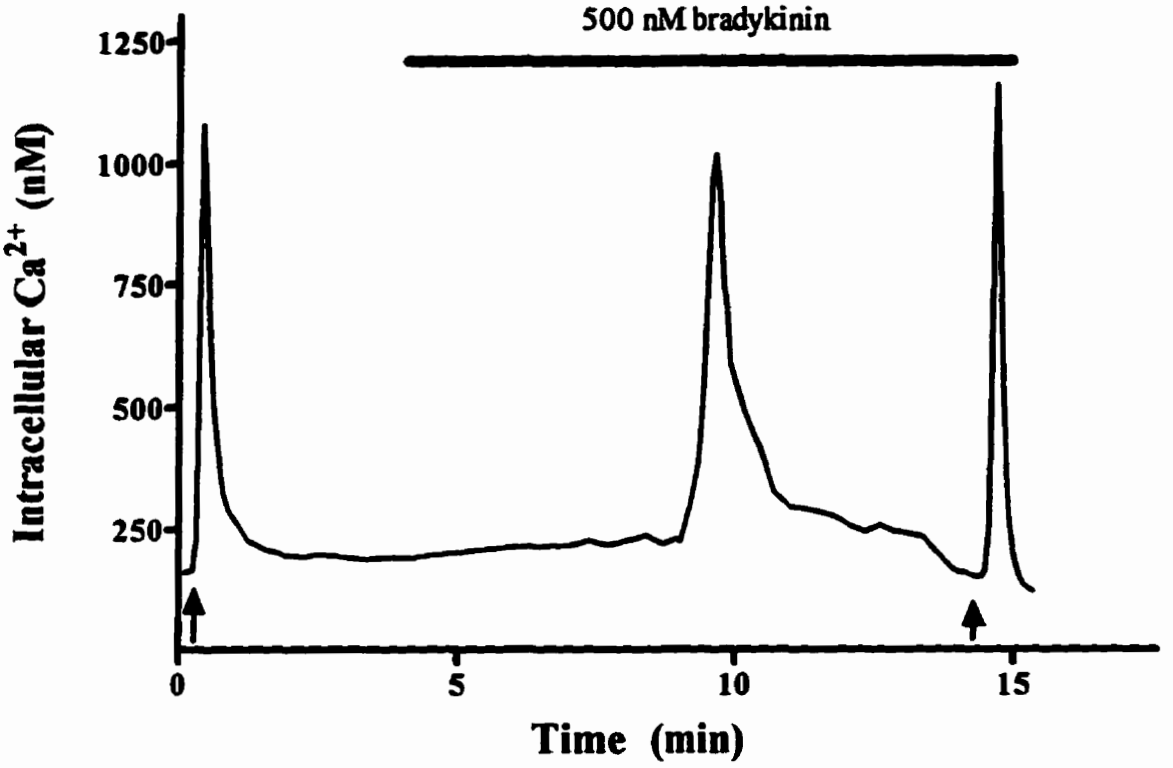

Fig. 8. Representative trace of effects of the IP_3-receptor agonist bradykinin on $[Ca^{2+}]_i$ and on Ap_5A-induced increases in $[Ca^{2+}]_i$. Bath applied bradykinin at 500 nM significantly ($p<0.005$) increased levels of $[Ca^{2+}]_i$, but did not significantly inhibit Ap_5A-induced increases in $[Ca^{2+}]_i$. Arrows represent pressure application (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

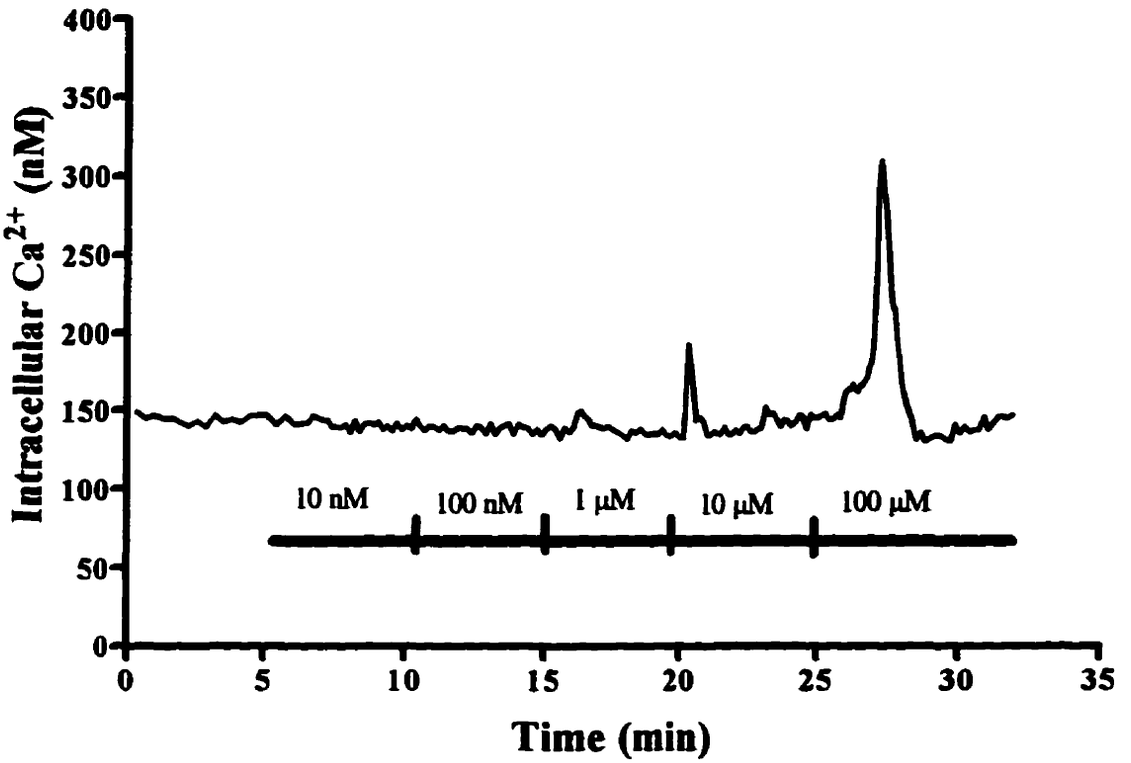

Fig. 9.. Representative traces of ryanodine, a releaser of ryanodine - sensitive Ca^{2+} stores, at concentrations indicated on the solid bar located below the trace. Ryanodine at 1, 10, and 100 μM significantly increased $[Ca^{2+}]_i$ from 150 ± 17 nM to 192 ± 18 nM ($p<0.05$), 354 ± 37 nM ($p<0.001$) and 389 ± 53 nM ($p<0.001$), respectively.

1 μM ryanodine induced an increase in $[Ca^{2+}]_i$ in 16 of 39 cells, 10 μM ryanodine induced an increase in $[Ca^{2+}]_i$ in 25 of 39 cells, and 100 μM ryanodine induced an increase in $[Ca^{2+}]_i$ in 36 of 39 cells.

Pretreatment of astrocytes with 10 μM ryanodine significantly inhibited Ap_5A - induced $[Ca^{2+}]_i$ by approximately 66% (Fig. 10, Table 2) (in 8 out of 8 cells). Similar to ryanodine, 20 mM caffeine, a releaser of ryanodine - sensitive Ca^{2+} stores, significantly increased $[Ca^{2+}]_i$ by 307 ± 172 nM ($p<0.05$) (in 5 out of 5 cells). Pretreatment of astrocytes with 20 mM caffeine significantly inhibited Ap_5A - induced increases in $[Ca^{2+}]_i$ by approximately 84% (Fig. 11, Table 2).

Three different subtypes of the mammalian RyR exist (RyR-1, RyR-2 and RyR-3), each originating from its own gene. The mouse monoclonal anti - RyR antibody (IgG_1) (MA3-916, Affinity Bioreagents) has been shown to react with RyR-1 and RyR-2 subtyes in a broad range of species and tissues (McPherson and Campbell, 1993) and was used to determine the presence of RyRs in cultured human fetal astrocytes. This dot blot consists of three different protein samples (Fig. 12). The negative control is demonstrated by the first arrow, this illustrates that no visible amount of RyR antibody is bound to bovine serum albumin. The second arrow indicating a positive control, showed RyR antibody bound to a light membrane fraction isolated from rat heart. The third arrow indicates RyR antibody bound to a microsomal preparation isolated from human fetal astrocytes. Visual examination of the results indicates that the amount of RyR antibody that bound to human fetal astrocyte microsomes was less than that for rat heart microsomes. The concentrations of the protein samples were 1 μg/μl.

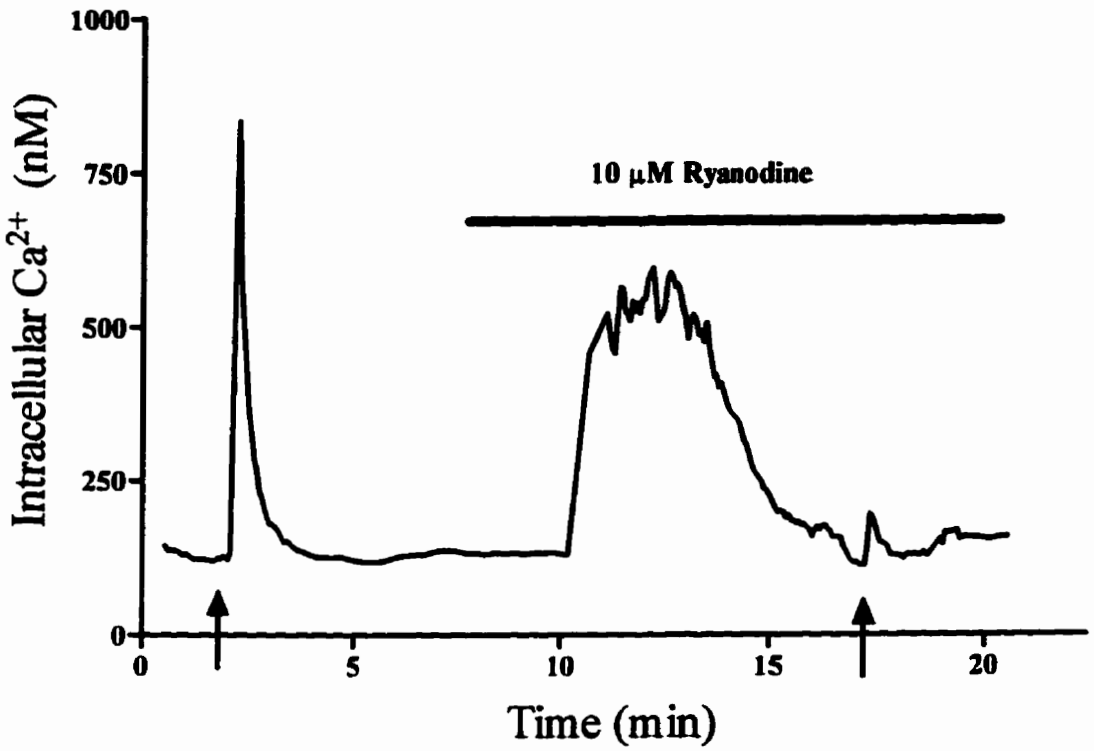

Fig. 10. Representative trace of the effects of ryanodine, a releaser of ryanodine - sensitive Ca^{2+} stores, on Ap_5A-induced increases in $[Ca^{2+}]_i$. Ryanodine (10 μM) significantly increased $[Ca^{2+}]_i$ and inhibited Ap_5A-induced increases in $[Ca^{2+}]_i$ ($p<0.001$). Arrows represent pressure application (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

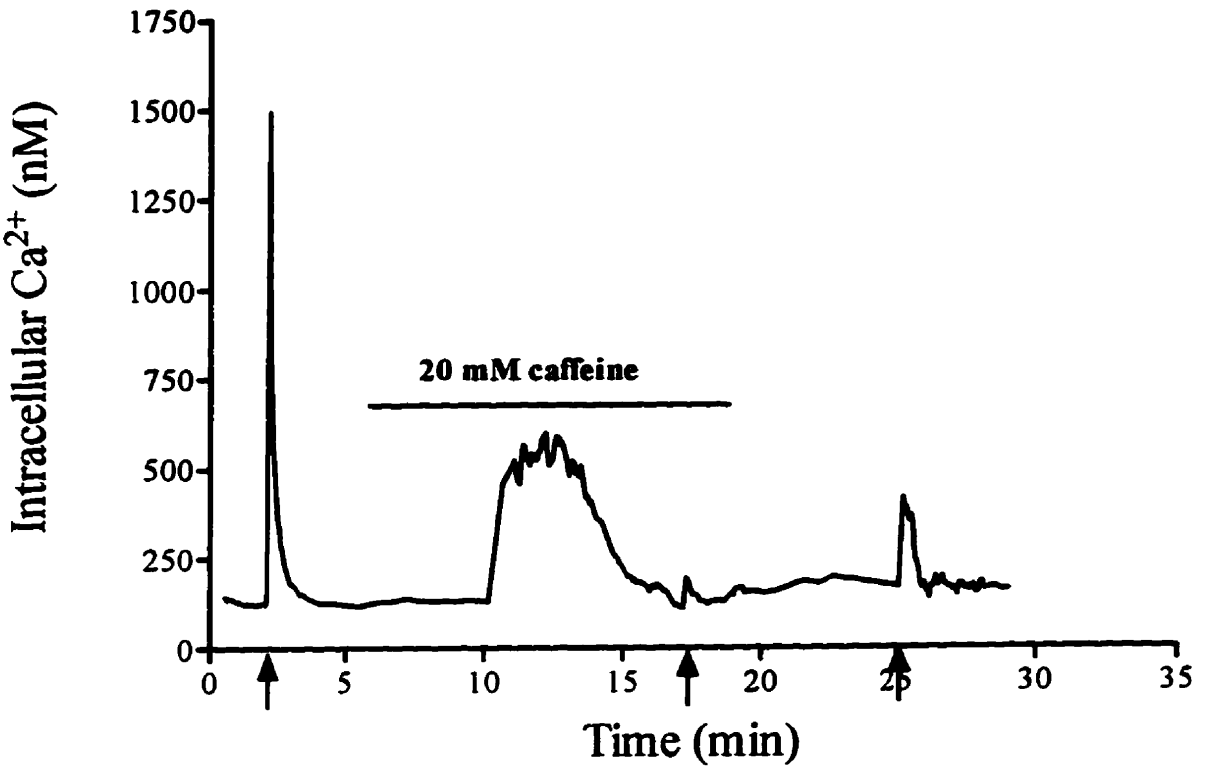

Fig. 11. Representative trace of the effects of caffeine, a releaser of ryanodine - sensitive Ca^{2+} stores, on Ap_5A-induced increases in $[Ca^{2+}]_i$. Caffeine (20 mM) significantly increased $[Ca^{2+}]_i$ by 307 ± 172 nM and inhibited Ap_5A-induced increases in $[Ca^{2+}]_i$ by approximately 84 % ($p<0.05$). Arrows represent pressure application (3 x 100 ms at 10 psi) of 100 μM Ap_5A.

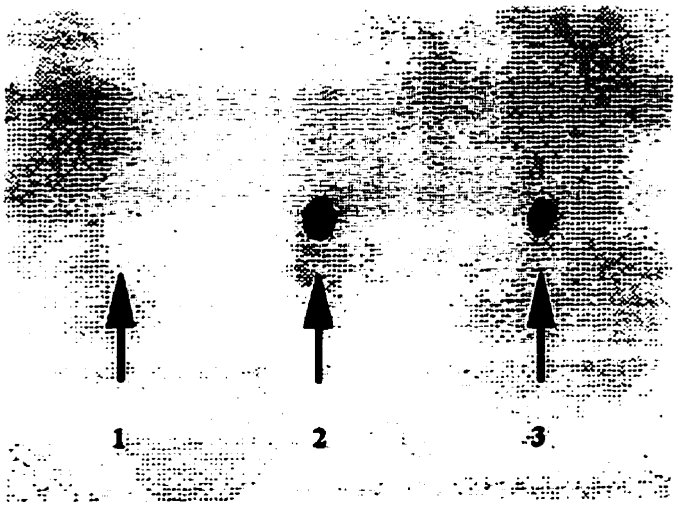

Fig. 12 Dot blot analysis using mouse monoclonal anti - RyR antibody (IgG_1) (MA3-916, Affinity Bioreagents). The first arrow (1) represents the relative amount of antibody bound to the negative control, bovine serum albumin. The second (2) arrow indicates the positive control where the RyR-antibody is bound to a light membrane fraction isolated from rat heart. The third arrow (3) indicates binding of RyR-antibody to microsomes isolated from human fetal astrocytes. Horse raddish peroxidase - conjugated secondary antibody was developed using 3,3' - diaminobenzidine tetrahydrochloride and H_2O_2 Protein sample concentrations were 1 μg/μl.

The final set of experiments was performed to determine if multiple P_2 purinoceptor subtypes exist on human fetal astrocytes. We selected our most selective nucleotide agonist for the P_{2X} purinoceptor (α,β-MeATP), P_{2Y} purinoceptor (α,β-MeADP) and P_{2U} purinoceptor (UTP). The nucleotides were bath applied at 100 μM and levels of Ca^{2+}_i were recorded (Table 2). All of the selective nucleotides and Ap_5A significantly increased $[Ca^{2+}]_i$ above resting levels. UTP induced the largest increase in $[Ca^{2+}]_i$, 393 ± 63 nM. α,β-MeADP increased $[Ca^{2+}]_i$ by 337 ± 77 nM. α,β-MeATP increased $[Ca^{2+}]_i$ by 132 ± 55 nM. Ap_5A increased $[Ca^{2+}]_i$ by 76 ± 10 nM.

Table 2. Effects of bath - applied nucleotides on $[Ca^{2+}]_i$.

	UTP	Ap_5A	α,β-MeATP	α,β-MeADP
P_2 receptor most selective for agonist	P_{2U}	?	P_{2X}	P_{2Y}
increase in $[Ca^{2+}]_i$ (nM) (mean ± SEM)	393 ± 63	76 ± 10	132 ± 55	337 ± 77
n	9	9	9	9

Increase in $[Ca^{2+}]_i$ following bath application of specified agonist at a final concentration of 100 μM.

Abbreviations: α,β-MeATP (α,β-methelene ATP), α,β-MeADP (α,β-methelene ADP), Ap_5A (diadenosine pentaphosphate), UTP (uridine 5'-triphosphate)

DISCUSSION

In cultured human fetal astrocytes, Ap_5A appears to activate P_{2X} and possibly P_{2D} purinoceptors. Purinoceptor activation opens plasma membrane Ca^{2+} channels increasing $[Ca^{2+}]_i$ which in turn stimulates the release of $Ca^{2+}{}_i$ from intracellular stores that are sensitive to both ryanodine and caffeine.

In the presence of extracellular Ca^{2+}, Ap_5A significantly increased $[Ca^{2+}]_i$ in 55 of 95 human fetal astrocytes tested. However, when astrocytes were repeatedly exposed to Ap_5A immediately following the return of $[Ca^{2+}]_i$ to baseline levels, the responses to Ap_5A were almost completely abolished. The significant attenuation in signal may have been due to purinergic receptor desensitization, depletion of the RyR-regulated intracellular Ca^{2+} stores, or a combination of both factors.

In previous experiments with astrocytes, Langely and Pearce (1994) found that consecutive ATP applications three minutes apart reduced responses that were almost identical to those observed in the present study. Salter and Hicks (1994) showed that three minute recovery periods between applications of ATP to astrocytes were required in order for maximal increases in $[Ca^{2+}]_i$ to be seen. Similarly, we found that 5 minute recovery periods were necessary following the initial applications of Ap_5A in order to achieve a second Ap_5A-induced maximal response. Therefore, a recovery period following the application of adenine nucleotides to astrocytes may

be required for purinergic receptors to re-sensitize and/or the intracellular Ca^{2+} pools to be refilled.

In the absence of extracellular Ca^{2+}, Ap_5A did not significantly increase $[Ca^{2+}]_i$ in any of the human fetal astrocytes tested. However, these findings alone were not sufficient for us to conclude that Ap_5A - induced increases in intracellular Ca^{2+} were solely due to an influx of extracellular Ca^{2+} across the plasma membrane. Therefore, in our next set of experiments we eliminated the possibility that Ca^{2+} was released from intracellular stores by incubating the astrocytes with 5 μM thapsigargin, a Ca^{2+} ATPase inhibitor, in order to deplete all intracellular stores of Ca^{2+}. By doing so, we found that Ap_5A - induced increases in $[Ca^{2+}]_i$ were reduced by about 62%. Because a significant Ap_5A - induced increase in $[Ca^{2+}]_i$ was present despite depletion of intracellular Ca^{2+} stores, we suggest that Ap_5A may be increasing $[Ca^{2+}]_i$ by two mechanisms; influx of extracellular Ca^{2+} across the plasma membrane and the release of Ca^{2+} from intracellular stores. In support of this hypothesis, Langely and Pearce (1994) demonstrated that P_2 purinoceptor stimulation in astrocytes induced both Ca^{2+} release from internal stores and Ca^{2+} influx from the extracellular space.

To determine the intracellular store from which Ap_5A stimulated the release of Ca^{2+}, we tested the involvement of IP_3- and RyR-regulated stores of $Ca^{2+}{}_i$. We examined the involvement of IP_3-sensitive intracellular Ca^{2+} stores by adding 500 nM bradykinin, a compound that following activation of its receptors induces the release of IP_3 -regulated stores of intracellular Ca^{2+}. With the application of bradykinin, significant increases in $[Ca^{2+}]_i$ were

observed. However, bradykinin did not significantly affect Ap_5A - induced increases in $[Ca^{2+}]_i$. These findings led us to conclude that Ap_5A was not inducing the release of Ca^{2+} from IP_3 stores.

To demonstrate the presence of RyR-regulated intracellular Ca^{2+} stores in human fetal astrocytes, we bath-applied a wide range of concentrations of ryanodine to astrocytes (Fig. 7). Previous work showed that concentrations of ryanodine less than 10 μM activated while concentrations greater than 10 μM inhibited the release of Ca^{2+} from RyR-regulated intracellular Ca^{2+} stores (Ehrlich et al., 1994). We found that ryanodine bath applied at concentrations ranging from 1 - 100 μM transiently increased levels of $[Ca^{2+}]_i$ in human fetal astrocytes. Initially, therefore, our results appear to be inconsistent with the literature. However, unlike studies that used ER and /or SR preparations, we applied ryanodine extracellularly to whole cells and were unable to measure the intracellular concentration. The intracellular concentration may have been less than the extracellular concentration, which could account for why we observed a release, as opposed to the previously described inhibition, of intracellular Ca^{2+} following the application of 100 μM ryanodine.

We next examined the possibility that Ap_5A was releasing Ca^{2+} from RyR-regulated intracellular Ca^{2+} stores. Both 20 mM caffeine and 10 μM ryanodine significantly increased $[Ca^{2+}]_i$ and reduced Ap_5A - induced increases in $[Ca^{2+}]_i$. We further confirmed the molecular presence of RyRs in human fetal astrocytes via dot blot analysis using a mouse monoclonal anti-RyR antibody specific for type 1 and type 2 RyRs (Affinity Bioreagents, MA3916). Thus, type 1

and/or type 2 RyRs appear to be present and to regulate intracellular Ca^{2+} stores in human fetal astrocytes. Although the presence of type 3 RyRs was not able to be determined by the RyR antibody used, it is unlikely that a major component of Ap_5A-induced Ca^{2+}_i release is activated by type 3 RyRs because type 3 RyRs are characteristically insensitive to caffeine. Therefore, type 1 and/or type 2 RyRs appear to be involved in Ap_5A-induced increases in $[Ca^{2+}]_i$.

The purinergic P_2 antagonist PPADS significantly inhibited Ap_5A - induced $[Ca^{2+}]_i$ in all astrocytes tested. PPADS has been shown to be an effective P_{2X} antagonist (Lambrech et al., 1992; Zinghanshin et al., 1994) however, it has also been found to block, at least in some tissues, P_{2Y} and P_{2U} purinoceptors (Charlton et al., 1996). Since no prior work has been done to characterize the effectiveness of PPADS on specific P_2 purinoceptors in human fetal astrocytes, and because PPADS inhibition was never greater than 60%, we hypothesize that Ap_5A may not only have actions on purinergic P_{2X} purinoceptors, but may also activate other subtype(s) of purinergic receptors that are insensitive to inhibition by PPADS. Nevertheless, by showing that Ap_5A does not induce the release of IP_3 Ca^{2+} stores, we ruled out the possibility that Ap_5A acted through P_{2Y} or P_{2U} purinoceptors. Furthermore, since P_{2Z} purinoceptors are located primarily on immune cells (Chen et al., 1994) and P_{2T} purinoceptors have been shown to be confined to platelets (Gordon, 1986), it is unlikely that Ap_5A had an effect at either of these purinoceptor subtypes in human fetal astrocytes. By the process of elimination, we favor the possibility that Ap_5A acted through P_{2D} and P_{2X} purinoceptors.

The P_{2X} purinergic receptor subtype has been shown to be coupled to activation of cell membrane Ca^{2+} channels (Bean 1992; Burnstock, 1990), while P_{2D} receptor activation has been shown to produce an influx of extracellular Ca^{2+} (Harden et al., 1995). Two other P_{2X} receptor antagonists, suramin and methylene blue, were tested but both caused autofluorescence when applied to astrocyte cultures and thus, the measurement of $[Ca^{2+}]_i$ following their application was not possible. Nevertheless, on the basis of the results obtained to date, we propose that Ap_5A stimulates at least P_{2X} and possibly P_{2D} purinoceptor subtypes. Since no selective antagonists are available for the purinergic receptor subtypes P_{2D}, P_{2U}, P_{2T}, P_{2Y}, and P_{2Z}, the interactions between Ap_5A and other receptor subtypes which may lead to Ap_5A-induced release of $[Ca^{2+}]_i$ remain to be determined.

In summary, Ap_5A activates P_{2X} and possibly P_{2D} purinoceptors in cultured human fetal astrocytes. The activated intrinsic cation channel then allows a relatively small amount of Ca^{2+} to enter the cell from the extracellular space, increasing the level of free cytosolic Ca^{2+}. The increase in $[Ca^{2+}]_i$ permits Ca^{2+} binding to Ca^{2+} modulatory sites on the RyR, activating the RyR-channel complex and inducing the release Ca^{2+}. In the absence of extracellular Ca^{2+}, the initial influx of Ca^{2+} from the extracellular space is lost, preventing Ca^{2+} binding to Ca^{2+} modulatory sites on the RyR, and thus no increase in $[Ca^{2+}]_i$ is observed. Therefore, the increase in $[Ca^{2+}]_i$ observed following the application of Ap_5A appears to be a combination of Ca^{2+} entry across the plasma membrane and Ca^{2+} release from RyR-regulated intracellular stores.

Tissue injury is known to cause release of ATP and other adenine nucleotides (El-Moatassim 1992; Gordon, 1986). Nucleotides have also been shown to be released from many types of healthy cells and have been suggested to be involved in intercellular signaling (Phillis and Wu, 1981; Stone, 1981; Burnstock, 1990). Ectonucleotidases that rapidly metabolize nucleotides and dinucleotides are present on the surface of most target cells (Slakey et al., 1990; Dubyak and El-Moatassim, 1993). Extracellular adenine nucleotide interactions with P_2 purinoceptors have been shown to be involved in several physiological processes (Table 4). Due to their longer half-life in comparison to mononucleotides such as ATP or ADP (Baxi and Vishwanatha, 1995), endogenous Ap_nAs may also activate P_2 purinoceptors and play an important role in these physiological processes,

Ultimately, the physiological changes induced by Ap_nAs must originate at the cellular level. Ap_nA-induced increases in $[Ca^{2+}]_i$ observed following purinoceptor activation may alter several physiological properties of the cell (Kostyuk and Verkhratsky, 1994). Alterations in $[Ca^{2+}]_i$ can modify membrane excitability by changing the activity of voltage- and ligand-gated plasmalemmal channels. Changes in $[Ca^{2+}]_i$ can alter voltage-gated calcium channels by binding to the Ca^{2+} channel or by dephosphorylation via Ca^{2+} -dependent enzymes (Armstrong et al., 1991; Chad and Eckert, 1986). Similarly, Ca^{2+} -dependent K^+ and Cl^- currents can also be directly controlled by $[Ca^{2+}]_i$ (Kostyuk and Verkhratsky, 1994; Currie and Scott, 1992; Ivanenko et al., 1993).

Table 4. Physiological effects of extracellular adenine nucleotides (taken from Harden et al., 1995).

Cell Type	Effect
vascular smooth muscle	contraction and relaxation
endothelial cells	release of vasorelaxing substances
urinary bladder smooth muscle	contraction
smooth muscle	contraction
cardiac muscle	inotropic and chronotropic effects
CNS and sensory neurons	rapid depolarization
peripheral and CNS neurons	modulation of neurotransmitter release
glial cells	regulation of second-messenger production
epithelial cells in airway, gastrointestinal, and other tissues	regulation of Cl^- secretion
human spermatozoa	stimulation of acrosome reaction in
hepatocytes	stimulation of glycogenolysis
airway cells	stimulation of mucin secretion and ciliary beat frequency
neutrophils, monocytes, and macrophages	stimulation of inflammatory responses
lymphocytes	modulation of immune responses of
endocrine and neuroendocrine tissues (pancreas,thyroid, parotid, and adrenal glands)	promotion of secretion
mast cells and platelets	promotion of secretions

Recent advances in Ca^{2+} research have revealed a connection between the filling state of Ca^{2+} stores and plasmalemmal Ca^{2+} channels referred to as Ca^{2+} release-activated Ca^{2+} channels (CRAC) (Hoth and Penner, 1992; Penner et al., 1993). Ap_nA-induced release of IP_3-R and/or RyR-regulated intracellular Ca^{2+} stores may potentially be one of several endogenous modulators of CRAC channels.

An increase in $[Ca^{2+}]_i$ can trigger the fusion of synaptic vesicles to the presynaptic membrane and induce neurotransmitter release (Llinas et al., 1992; Smith and Augustine, 1988; Zucker, 1993). Ap_nAs are present in cholinergic neurons and are released in conjunction with acetylcholine (Pintor et al., 1995). In this case, Ap_nAs may be stimulating pre-synaptic P_2 purinoceptors and function to enhance synaptic transmisison.

Synaptic plasticity can also be altered as a result of changes in $[Ca^{2+}]_i$. Such modification can be the result of a change in the postsynaptic membrane and/or a change in the quanta of presynaptic neurotransmitter released (Kostyuk and Verkratsky, 1994). Thus, Ap_nAs may be involved in the modulation of both long-term potentiation and long-term depression (Kasai, 1993; Linden and Connor, 1993; Klishin et al., 1994).

Ca^{2+} ions have also been shown to activate gene transcription and are crucial in the development and growth of cells. The expression of individual genes can be triggered by Ca^{2+} influx from the extracellular space or from the release of Ca^{2+} from intracellular stores (Spitzer, 1994; Spitzer et al., 1994). In fact, when Ap_nAs were initially discovered, they were shown to induce DNA replication, and thus may possibly be linked to gene transcription.

Ca^{2+} waves can be transmitted from glial cells to neurons through structures similar to gap junctions (Nedergaard, 1994). Changes in calcium homeostasis have been linked to neuronal aging (Kirischuk et al., 1992; Michaelis et al., 1984). In aged CNS neurons, the caffeine/ryanodine-sensitive intracellular stores contain a higher amount of calcium relative to young CNS neurons (Verhratsky et al., 1994). These overloaded calcium stores were not as efficient in the removal of cytoplasmic calcium after neuronal activity was stimulated (Vitorica and Satrustegui, 1986). Ap_nAs, as we have shown, play an important role in regulating the release of Ca^{2+} from caffeine-/ryanodine-sensitive stores and therefore, may also be involved in the aging process.

Glial cells can interact with neurons by direct contact through gap junction-like structures (Nedergaard, 1994), by altering neurotransmitter concentrations, or by altering the ionic composition of the interstitial environment (Kostyuk and Verkhratsky, 1994). Adenine nucleotides have been shown to be released from nerve terminals in peripheral and central neurons during tissue injury and nerve stimulation (Maire et al., 1982). Thus, the release of diadenosine polyphosphates from brain cells (Pintor and Miras-Portugal, 1995a) or from cholinergic neurons (Pintor et al., 1995) could be stimulating astrocytic P_2 purinoceptors, altering cellular $[Ca^{2+}]_i$ and modifying some of the physiological effects mentioned in Table 4.

In summary, Ap_5A activates P_{2X} and possibly P_{2D} purinoceptors in cultured human fetal astrocytes, induces Ca^{2+} to enter cells from the extracellular space, and increases the levels of free

cytosolic Ca^{2+}. The increase in $[Ca^{2+}]_i$ permits Ca^{2+} binding to Ca^{2+} modulatory sites on the RyR, activating the RyR-channel complex and induces the release Ca^{2+}. Therefore, the increase in $[Ca^{2+}]_i$ observed following the application of Ap_5A appears to be a combination of Ca^{2+} entry across the plasma membrane and Ca^{2+} release from RyR-regulated intracellular stores.

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