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Ca^{2+}-stores mobilization by diadenosine tetraphosphate, Ap_4A, through a putative P_{2Y} purinoceptor in adrenal chromaffin cells

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1 Diadenosine tetraphosphate (Ap_4A) evoked a concentration-dependent increase in cytosolic [Ca^{2+}] in resting chromaffin cells. The EC_{50} value for this action was 28.2 ± 6.6 μM. This effect was also produced by diadenosine pentaphosphate (Ap_5A) with an EC_{50} of 50 ± 7 μM.

2 In contrast with this effect, pretreatment with Ap_4A or Ap_5A induced a 30% reduction in Ca^{2+} entry following 10 μM dimethylphenylpiperazinium.

3 The elevation in cytosolic [Ca^{2+}] induced by Ap_4A was persistent in $\approx$100 nM external [Ca^{2+}] and was sensitive to depletion of internal Ca^{2+} stores by a bradykinin prepulse or whole cell depletion in Ca^{2+}.

4 The effect of Ap_4A was mimicked and desensitized by the agonist adenosine 5'-O-(2-thiodiphosphate), and blocked by the P_{2Y}-receptor antagonist, cibachrome blue. The P_{2X}-receptor agonist α,β-methylene adenosine 5'-triphosphate was inactive both by itself or in combination with Ap_4A. This is compatible with a P_{2Y}-purinoceptor-mediated action.

Keywords: Cytosolic calcium; diadenosine polyphosphates; bradykinin; purinoceptors

Introduction

Diadenosine polyphosphates are a novel class of compounds with emerging extracellular actions being added to their known intracellular role. Diadenosine tetraphosphate (adenosine (5')tetraphospho (5') adenosine, Ap_4A), the major representative of this type of nucleotides, is found in eukaryotic cells at micromolar concentrations (Zamecnik, 1983; Coste *et al.*, 1987), fluctuating with the cellular growth rate (MacLennan & Prescott, 1984). The localization of Ap_4A is mainly nuclear (Weinmann-Dorsch & Grummt, 1986) inside the cells. Ap_4A has been implicated in the initiation of DNA replication (Weinmann-Dorsch *et al.*, 1984) through its binding to DNA polymerase α (Rapaport *et al.*, 1981) and in the processing of ADP-ribosylated histones (Surowy & Berger, 1983). So, this metabolite is now considered as a delayed intracellular messenger molecule involved in the regulation of the eukaryotic cell cycle (Baril *et al.*, 1983; Grummt, 1988) and in the response to cellular stress (Baker & Jacobson, 1986; Coste *et al.*, 1987).

Besides this role as a nuclear signal, diadenosine polyphosphates can also be found at high concentrations in the secretory granules of platelets (Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983) and adrenal chromaffin cells (Rodriguez del Castillo *et al.*, 1988), co-stored with ATP and 5-hydroxytryptamine or catecholamines respectively. These compounds are exocytotically released to the extracellular medium during platelet aggregation (Lüthje & Ogilvie, 1983) and chromaffin cells catecholamine secretion stimulation (Pintor *et al.*, 1991a). Acting from the extracellular side, diadenosine polyphosphates have been found to be active in the platelet aggregation process (Lüthje *et al.*, 1985) both inhibiting (Harrison *et al.*, 1975) and promoting it (Lüthje & Ogilvie, 1984); they are also known to have vasomotor properties in peripheral arteries (Busse *et al.*, 1988), and to modulate catecholamine release from chromaffin cells (Castro *et al.*, 1990).

Recently we have described high affinity binding sites for [^{3}H]-Ap_4A in chromaffin cells membranes (Pintor *et al.*, 1991b) showing a putative P_{2Y}-purinoceptor pharmacological profile according to the currently accepted classification of purinoceptors (Burnstock & Kennedy, 1985). Little is known about purinoceptors in chromaffin cells. In other tissues P_{2Y} receptors are linked to phospholipase C (Forsberg *et al.*, 1987; Boyer *et al.*, 1989). In chromaffin cells ATP is known to induce inositol phosphates mobilization and release of intracellular Ca^{2+} stores (Sasakawa *et al.*, 1989). Here we examine the action of Ap_4A on cytosolic Ap_4A in chromaffin cells to discover the mechanism of diadenosine polyposphate modulation of catecholamine release and to characterize further Ap_4A receptors in these cells.

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Methods

Preparation of chromaffin cells

The chromaffin cells were obained after digestion of adrenal glands with collagenase in retrograde perfusion as described by Miras-Portugal *et al.* (1985). Briefly, glands supplied by a local slaughterhouse were trimmed of fat, cannulated through the adrenal vein and washed with Ca^{2+}/Mg^{2+}-free saline containing (in mM) NaCl 154, KCl 5.6, $NaHCO_3$ 3.6, glucose 5.6 and HEPES 5.0, buffered at pH 7.4. Digestion was performed with a 0.2% collagenase plus 0.5% bovine serum albumin (BSA) solution in the above medium. After digestion glands were cut, soft medullae were removed, minced and dispersed cells filtered through a nylon mesh. Cells were purified by buoyant sedimentation in a Percoll gradient to greater than 95% chromaffin cells as assayed by neutral red staining. Purified cells were maintained in suspension cultures, dispersed in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum and standard antibiotics, with continuous stirring. Cells were used on 4th–6th culture day. Anchorage dependent cells do not grow, but gradually die, in these culture conditions.

Measurement of cytosolic [Ca^{2+}]

Cytosolic Ca^{2+} concentration was determined with the fluorescent indicator fura-2. The cells were dispersed in a Locke solution containing (in mM). NaCl 140, KCl 4.4, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 5.5 and HEPES 10, pH 7.4. Cells were loaded with 2.5 μM fura-2 AM for

45 min and washed by centrifugation. The recordings were performed in 1 ml sample containing 10^6 cells in thermostated and stirred cuvettes, in a Perkin-Elmer LS-50 fluorimeter. The probe was excited at 340 nm and fluorescence read at 510 nm. At the end of each experiment the cells were lysed in 0.4% Triton X-100 and the dye content calibrated from measurements at 2.5 mM Ca^{2+} and 7.5 mM EGTA/45 mM Tris ($[Ca^{2+}] < 0.2$ nM). The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was derived from fluorescence traces with the equation of Grynkiewicz *et al.* (1985). Additions to the cuvette were made with Hamilton syringes from at least 100 fold concentrated stock solutions, to avoid large volume variations.

Data expression and analysis

All data were interfaced to a computer to allow off-line analysis and record average. Data are presented as averaged traces of usually 3 records. Numerical values are presented as mean ± s.e.mean. Difference between averaged records were within noise range. Experiments with identical results were obtained in different batches of cells.

The EC_{50} ± s.e.mean estimates were derived from a logistic fit to the concentration-response curves. Six concentrations were used in the range from 1 to 300 μM, in triplicate. For 10 and 100 μM points, pooled data from nine different experiments were used. The fit was performed with a computer programme written by one of us (E.C.). This programme uses the Newton-Gauss-Mardquadt algorithm for least-squares fitting. Weighting proportional to experimental variance was introduced.

Materials

Inorganic salts were from Merk (F.R.G.). Ap_4A, Ap_5A (adenosine (5') pentaphospho (5') adenosine) and collagenase (EC 3.4.24.3) were from Boehringer (Mannheim, Germany). Adenosine 5'-O-(2-thiodiphosphate) (ADPβS), α,β-methylene adenosine-5'-triphosphate (α,β-MeATP) and dimethylphenylpiperazinium (DMPP) were from Sigma (Saint Louis, U.S.A.). Percoll is a registered mark of Pharmacia (Upsala, Sweden). Culture products and media were from Flow (Irvine, U.K.). Fura-2 was from Molecular Probes (Eugene, U.S.A.).

Results

Ap_4A and Ap_5A actions on resting and stimulated $[Ca^{2+}]_i$

The resting cytosolic $[Ca^{2+}]_i$ in chromaffin cells was found to be consistently low, usually in the 25–75 nM range (average 34 ± 5 nM). Challenging chromaffin cells with Ap_4A induced a rapid and transient rise in the cytosolic Ca^{2+} level, as shown in Figure 1a. The rise in $[Ca^{2+}]_i$ induced by Ap_4A was small, amounting to 80 ± 9 nM as a maximal effect. The action was dependent on the concentration of the agonist, with an EC_{50} of 28.2 ± 6.6 μM. Ap_5A was also active in increasing $[Ca^{2+}]_i$ levels, although somewhat less potent that Ap_4A, with an EC_{50} of 50 ± 7 μM.

In contrast, Ap_4A slightly reduced the Ca^{2+} peak evoked by the nicotinic agonist DMPP (Figure 2). The effect was small, a 30.5 ± 7.5% inhibition, but statistically significantly when tested in paired form within each experiment. A similar result was obtained with Ap_5A (not shown).

Source of Ca^{2+} during Ap_4A-induced $[Ca^{2+}]_i$ elevation

The $[Ca^{2+}]_i$ increasing effect of Ap_4A was not abolished by the absence of a Ca^{2+} gradient across the membrane. The response was present when extracellular $[Ca^{2+}]$ was buffered at ≈100 nM with a mixture of Ca/EGTA as employed in Figure 3. This fact suggests that $[Ca^{2+}]_i$ rise by Ap_4A was due

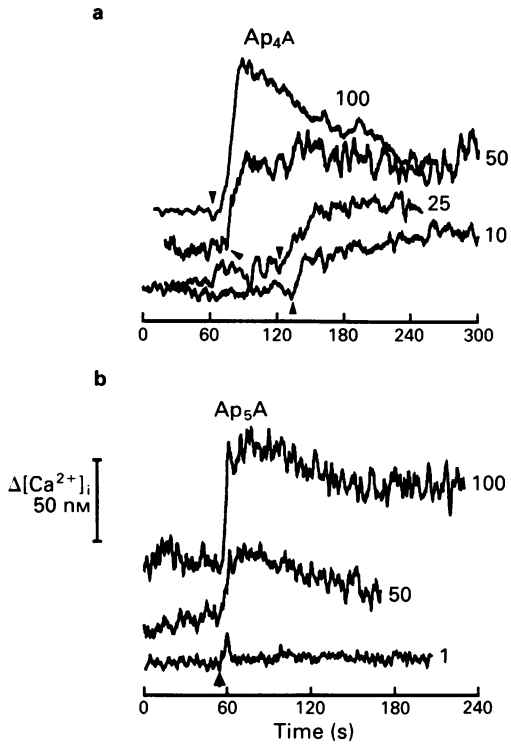

Figure 1 Elevation of cytosolic $[Ca^{2+}]$ by diadenosine tetraphosphate (Ap_4A) (a) and diadenosine pentaphosphate (Ap_5A) (b). At the time marked by the arrows Ap_4A or Ap_5A were added to the final concentration indicated (in μM).

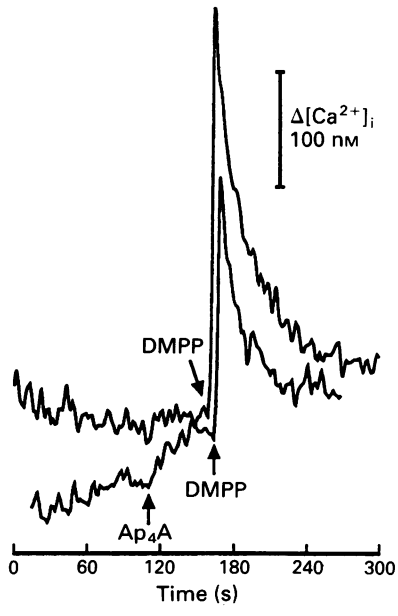

Figure 2 Reduction of 10 μM dimethylphenylpiperazinium (DMPP)-evoked Ca^{2+} entry by diadenosine tetraphosphate (Ap_4A) 100 μM. Peaks correspond to successive cuvettes.

to Ca^{2+} release from internal stores. To test this possibility further we tried to deplete these stores in two different ways. First, previous stimulation of the chromaffin cells with bradykinin severely compromises their ability to respond to Ap_4A (Figure 4). In contrast, Ap_4A stimulation had only a little effect on the response to bradykinin challenge. As a second strategy, the internal stores were emptied by incubating the cells for 45 min in a medium containing only ≈100 nM free $[Ca^{2+}]$ plus 10 mM caffeine to promote depletion by pump-

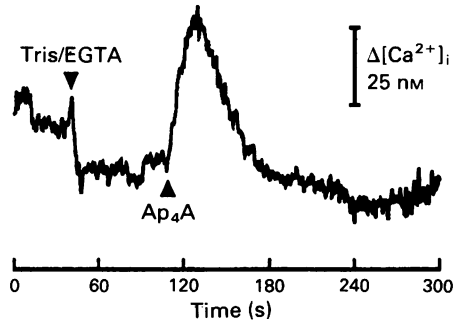

Figure 3 Response to diadenosine tetraphosphate (Ap_4A) in a nominal 100 nM $[Ca^{2+}]$ medium. $[Ca^{2+}]$ was buffered with a mixture of Ca/EGTA/Tris, $[EGTA]_t = 5$ mM. Ap_4A was 100 μM.

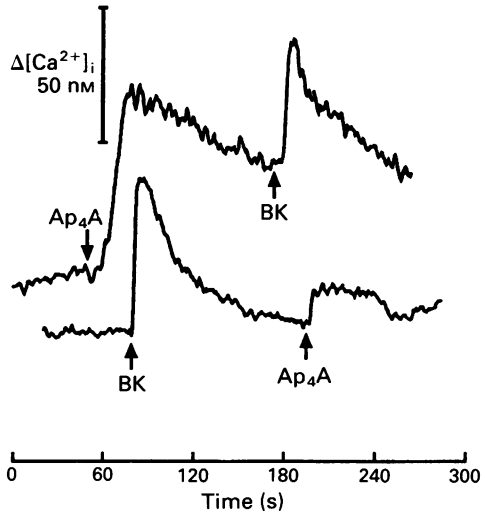

Figure 4 Response to diadenosine tetraphosphate (Ap_4A) after depletion of Ca^{2+}-stores. Inhibition of Ap_4A (100 μM) effect by a previous stimulation with 25 μM bradykinin (BK) and *vice versa*. Cells were incubated in normal medium including 2.5 mM Ca^{2+}.

ing out internal Ca^{2+} (Liu *et al.*, 1989). This treatment completely abolished the response to Ap_4A and profoundly diminished the effect of bradykinin, as shown in Figure 5. The reintroduction of Ca^{2+} to the medium restored the ability of the cells to respond to Ap_4A.

Interaction with P_2 purinoceptor drugs

The pharmacological identification of the Ap_4A receptor was assessed in the experiments summarized in Figure 6. Cibachrome Blue has been used as a selective P_{2Y}-receptor antagonist at moderate concentrations (Houston *et al.*, 1987). This agent completely and specifically blocked the Ca^{2+} rise induced by Ap_4A but not bradykinin. Furthermore, ADPβS is a potent P_{2Y}-receptor agonist which can induce receptor desensitization (Boyer *et al.*, 1989; Nanoff *et al.*, 1990). Figure 6 shows that ADPβS is a good agonist inducing $[Ca^{2+}]_i$ increase and that in the continuous presence of 100 μM ADPβS, the Ap_4A effect was lost, although a bradykinin-sensitive pool of Ca^{2+} was still present. In contrast, the selective P_{2X} agonist α,β-MeATP (Burnstock & Kennedy, 1985) produced only a minimal, possibly marginal, increase in $[Ca^{2+}]_i$, and failed to prevent the subsequent action of Ap_4A.

Discussion

In the present work we show how Ap_4A and Ap_5A stimulation of diadenosine polyphosphate receptors in chromaffin

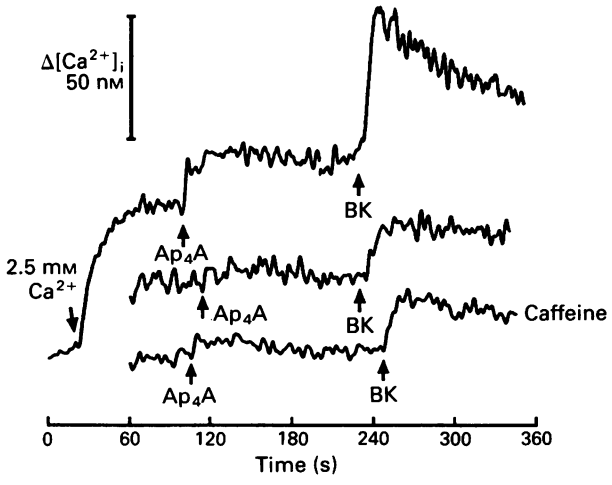

Figure 5 Response to diadenosine tetraphosphate (Ap_4A) after depletion of Ca^{2+}-stores. Depletion of Ca^{2+} from cells after incubation in 100 nM $[Ca^{2+}]_0$ plus 10 mM caffeine medium for 45 min. Lower trace, cells assayed with caffeine in the medium; middle trace, cells washed and resuspended for assay in the same medium without caffeine; upper trace, reintroduction of Ca^{2+} to a 2.5 mM final concentration. Ap_4A 100 μM, BK, bradykinin 25 μM.

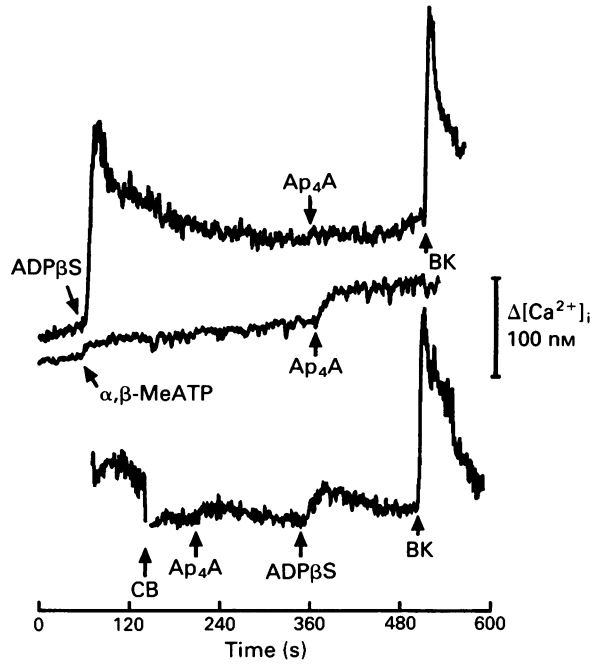

Figure 6 Pharmacology of diadenosine tetraphosphate (Ap_4A) effect. Upper trace, adenosine 5′-O-(2-thiodiphosphate) (ADPβS 100 μM) mimics and prevents the subsequent action of Ap_4A (100 μM) but not bradykinin (BK, 25 μM). Middle trace, α,β-methylene adenosine-5′-triphosphate (α,β-MeATP, 100 μM) shows a marginal action on $[Ca^{2+}]$ and does not prevent the Ap_4A (50 μM) effect. Lower trace, cibachrome blue (CB, 50 μM) effectively antagonizes the effects of Ap_4A (100 μM) and ADPβS (100 μM), without a major effect on the bradykinin (25 μM) peak. Drop produced by cibachrome blue is due to autoabsorption by the coloured dye, this effect was corrected in the calibration of $[Ca^{2+}]$.

cells is coupled to modulation of Ca^{2+}-signalling in these cells in a way parallel to diadenosine polyphosphate modulation of catecholamine release. The EC_{50} value for elevation of $[Ca^{2+}]_i$ is similar to that found for the action of diadenosine polyphosphates on catecholamine secretion and the potency order $Ap_4A > Ap_5A$ is the same (Castro *et al.*, 1990). This EC_{50} value is within an order of magnitude of the EC_{50} for

ATP stimulation of phosphoinositide turnover and Ca^{2+} mobilization (Sasakawa *et al.*, 1989). It is important to appreciate that the lifetime of nucleotides in the extracellular medium of the adrenal medulla may be very much shortened by the ectonucleotidase activity of chromaffin cells (Torres *et al.*, 1990). The ecto-phosphodiesterase (Ap_4Aase) activity present in the plasma membranes of these cells is three orders of magnitude lower than ecto-ATPase activity (Miras-Portugal *et al.*, 1990), so the lifetime of Ap_4A and Ap_5A will probably be much longer than that of ATP. This relative resistance of Ap_4A to ectonucleotidases present in chromaffin cells suggests that, after co-release with ATP (at approximately 1:10 dinucleotides:ATP ratio), Ap_4A-mediated effects become predominant with time and distance from release sites.

The source of Ca for the Ap_4A-induced increase in $[Ca^{2+}]_i$ seems to be the release of intracellularly stored Ca^{2+}, rather than Ca^{2+} entry through plasma membrane. This conclusion is supported by our findings that the Ca^{2+} signal remained in a low Ca^{2+} medium, when there is not a Ca^{2+} gradient across the membrane and so Ca^{2+} flux is negligible, and the sensitivity of this signal to store depletion. In addition to this, there is still a component of the response which is dependent on extracellular Ca^{2+}, as revealed by the smaller size and width of $[Ca^{2+}]_i$ peak of Figure 3 as compared with Figure 1 or 4. This component probably represents the replenishment of internal Ca^{2+} stores by extracellular Ca^{2+} entry. This same phenomenon has been observed in other tissues, although the precise pathway for this Ca^{2+} entry is not clear (Putney, 1987). However, this pathway is different from voltage-dependent Ca^{2+}-channels, since these are tightly coupled to secretion in chromaffin cells, while Ca^{2+} peaks induced by Ap_4A are not accompanied by corresponding secretory responses.

Diadenosine polyphosphates have only very small effects on basal catecholamine release (Castro *et al.*, 1990), which correlates perfectly with the size and origin of the Ap_4A-induced Ca^{2+} peak. A similar failure of intracellularly released Ca^{2+} to evoke catecholamine secretion has been described for bradykinin, angiotensin II, muscarinic agonists and nucleotides (O'Sullivan & Burgoyne, 1989; Cheek *et al.*, 1989b; Kim & Westhead, 1989). This fact is due to the spatial organization of the secretory machinery, which require $[Ca^{2+}]_i$ elevation just beneath the plasma membrane (i.e. Ca^{2+} flux through Ca^{2+} channels is fully active), while the release of intracellular Ca^{2+} takes place deep in the cytoplasm and becomes diluted (i.e. inactive) at secretory sites (Cheek *et al.*, 1989a,b). In contrast, Ap_4A slightly reduced the Ca^{2+} peak evoked by the nicotinic agonist DMPP, largely due to extracellular Ca^{2+} entry, which explains the small inhibitory action of diadenosine polyphosphates on nicotine-evoked catecholamine secretion (Castro *et al.*, 1990). The mechanism underlying this reduction, whether a direct modulation of Ca^{2+} channels, a modulation by cytosolic Ca^{2+}, or the activation of K^+ channels remains to be investigated.

The messenger linking receptor activation and Ca^{2+} release from intracellular stores is accepted as usually inositol (1,4,5)trisphosphate (IP_3) (Berridge, 1987). P_{2Y} receptors have been shown to be coupled to PLC/IP_3 pathway in vascular cells (Boyer *et al.*, 1989; Forsberg *et al.*, 1987). The [^{3}H]-Ap_4A binding site found previously in chromaffin cells showed a P_{2Y} displacing profile (Pintor *et al.*, 1990a). Therefore, our results are consistent with the view that Ap_4A exerts its effects through P_{2Y} purinoceptors, coupled to IP_3 formation, leading to release of intracellular Ca^{2+}, a mechanism which is common to other IP_3 generating agonists such as bradykinin (Challiss *et al.*, 1991). This functional assignment is reinforced by the pharmacological data. Although cibachrome blue is not an ideal antagonist, the reduction of Ap_4A and ADPβS responses selectively with respect to bradykinin, and the almost lack of Ca^{2+} response to α,β-Me-ATP, argue against a P_{2X}-purinoceptor as the site of action of Ap_4A.

However, the amplitude, kinetics, and sensitivity to acute or prolonged Ca^{2+}-depletion of Ap_4A and bradykinin were different. This probably reflects the complexity of the Ca^{2+} signalling pathway in chromaffin cells, which is more than a single messenger acting in a single target Ca^{2+} store. There are multiple intracellular Ca^{2+} pools in chromaffin cells, some sensitive to IP_3 and others responding to other (unknown) messengers. The non-IP_3-sensitive pools can be released experimentally by caffeine. These different Ca^{2+} pools are not completely separate, but are probably intercommunicated. We have had to include caffeine in order to abolish completely the response to Ap_4A, which probably means participation of different pools in the response to Ap_4A (and bradykinin). Since caffeine has P_1-purinoceptor antagonistic properties, an interfering effect could be suspected. However the result was identical whether caffeine was present during the assay or had been washed out beforehand, showing that its effect is not due to a competitive receptor blockade.

Altogether, these findings indicate that extracellular Ap_4A is a good ligand for a population of receptors present in chromaffin cell membranes, putatively identified as P_{2Y} type purinoceptors, and linked to the intracellular Ca^{2+} signalling pathway. To our knowledge this is the first time the mechanism of action of diadenosine polyphosphates has been directly addressed in neurosecretory cells.

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