British Journal of Pharmacology (1995) 115, 895–902
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A novel receptor for diadenosine polyphosphates coupled to calcium increase in rat midbrain synaptosomes

[1,*]Jesús Pintor & M. Teresa Miras-Portugal

Departmento de Bioquimica and *E.U. Optica, Fac. Veterinaria, Universidad Complutense 28040 Madrid, Spain

1 Diadenosine polyphosphates, Ap_4A and Ap_5A, as well as ATP, α,β-MeATP and ADP-β-S, were able to elicit variable intrasynaptosomal Ca^{2+} increases in rat midbrain synaptic terminals. The origin of the Ca^{2+} increment was the extrasynaptosomal space since the elimination of extracellular Ca^{2+} abolished the effect of all the agonists.

2 The P_2-purinoceptor antagonist, suramin, did not affect the Ca^{2+}-increase evoked by diadenosine polyphosphates but dramatically blocked the Ca^{2+} entry induced by ATP and its synthetic analogues.

3 The actions of Ap_5A and ATP on the intrasynaptosomal Ca^{2+} increase did not cross-desensitize.

4 Concentration-response studies for diadenosine polyphosphates showed pD_2 values of 54.5 ± 4.2 μM and 55.6 ± 3.8 μM for Ap_4A and Ap_5A, respectively.

5 The entry of calcium induced by diadenosine polyphosphates could be separated into two components. The first represented a selective voltage-independent Ca^{2+} entry; the second, a sustained phase which was voltage-dependent.

6 Studies on the voltage-dependent Ca^{2+}-channels involved in the effects of the diadenosine polyphosphates, demonstrated that ω-conotoxin G-VI-A inhibited the sustained Ca^{2+}-entry, suggesting the participation of an N-type Ca^{2+}-channel. This toxin was unable to abolish the initial cation entry induced by Ap_4A or Ap_5A. ω-Agatoxin IV-A, tetrodotoxin, or nifedipine did not inhibit the effects of the diadenosine polyphosphates.

7 The effect of ATP on Ca^{2+}-entry was abolished by nifedipine and ω-conotoxin G-VI-A, suggesting the participation of L- and N-type Ca^{2+}-channels in the response to ATP.

8 These data suggest that Ap_4A, Ap_5A and ATP activate the same intracellular Ca^{2+} signal through different receptors and different mechanisms. Ap_4A and Ap_5A induce a more selective Ca^{2+}-entry in a voltage-independent process. This is the first time that a selective action of diadenosine polyphosphate through receptors other than P_1 and P_2-purinoceptors has been described.

Keywords: Ap_4A; Ap_5A; calcium channels; channel toxins; diadenosine polyphosphates; P_2-purinoceptors; synaptic terminal

Introduction

Purinoceptors are responsible for the actions of adenosine as well as adenine nucleotides in many tissues. Receptors for adenosine 5′-triphosphate (ATP), termed P_2-purinoceptors, have been widely studied in neural and non-neural tissues (Burnstock, 1990; 1992). P_2-purinoceptors were initially divided into P_{2X} and P_{2Y} subtypes in accordance with the potency order of ATP and synthetic analogues and their differential sensitivity to tachyphylaxis (Burnstock & Kennedy, 1985). In platelets, as well as in macrophages, purinoceptors were termed P_{2T} and P_{2Z}, (Gordon, 1986) respectively, and both possess characteristic agonist and antagonist profiles (Cockcroft & Gomperts, 1979; Colman, 1990; Hoyle, 1992). Although ATP has been considered the main agonist, other nucleotides can exert actions via P_2-purinoceptors. UTP and α,ω-adenine dinucleotides are the most recent active nucleotidic substances with actions that have been demonstrated in epithelia and central nervous system (O'Connor *et al.*, 1991; Klishin *et al.*, 1994). Both UTP and diadenosine polyphosphates activate recently defined P_2-purinoceptors named P_{2U} and P_{2D}, respectively (O'Connor, 1992; Pintor *et al.*, 1993a). The broad group of P_2-purinoceptors can be classified into two families: ionotropic channels activated by ATP (Benham & Tsien, 1987), and metabotropic receptors which are coupled to G proteins (Harden *et al.*, 1990; Zimmermann, 1994).

Although it has been demonstrated that ATP is stored in synaptic vesicles and that it is exocytotically released, it is only recently that the role of ATP as a neurotransmitter in the central nervous system has been determined (White, 1977; Potter & White, 1980). Since then ATP has been shown to activate ionic currents in central neurones (Ueno *et al.*, 1992; Shen & North, 1993; Illes & Norenberg, 1993); only by means of the trypanocide, suramin, a P_2-purinoceptor antagonist (Hoyle, 1990), was it possible to demonstrate that central synapses use ATP as a fast excitatory neurotransmitter (Edwards *et al.*, 1992). Together with ATP, two diadenosine polyphosphates, Ap_4A and Ap_5A, have been identified in the synaptic terminals from rat brain. These dinucleotides, as well as ATP, are released, in a Ca^{2+}-dependent process, by the action of depolarizing agents (Pintor *et al.*, 1992a). Both diadenosine polyphosphates, Ap_4A and Ap_5A, exert an inhibitory effect on synaptic transmission in the hippocampus (Klishin *et al.*, 1994), this effect probably being mediated by a xanthine-sensitive purinoceptor through protein kinase C (PKC) activation (Pintor & Miras-Portugal, 1994).

In several tissues, it has been demonstrated that ATP and diadenosine polyphosphates can activate the same purinoceptor subtypes (MacKenzie *et al.*, 1988; Hoyle *et al.*, 1989; Pintor & Miras-Portugal, 1994; Ralevic *et al.*, 1994). In the vas deferens and urinary bladder their actions are through a P_{2X}-purinoceptor (MacKenzie *et al.*, 1988; Hoyle *et al.*, 1989; 1990); in bovine adrenal gland endothelial and chromaffin cells as well as in the rat mesenteric vascular bed the effects of Ap_4A and Ap_5A are mediated by P_{2U}, P_{2X} and P_{2Y}-purinoceptors, respectively (Pintor *et al.*, 1991; Castro *et al.*, 1994; Ralevic *et*

[1] Author for correspondence at present address: Dept. Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT.

al., 1994). The recent discovery of the P_{2D}-purinoceptor (Pintor *et al.*, 1993a), highly specific for diadenosine polyphosphates in synaptic terminals, reinforced the theory of the existence of different purinoceptors for nucleotides and dinucleotides (Stone & Perkins, 1981; Hoyle, 1990).

The aim of the present study was to determine whether or not the diadenosine polyphosphates, Ap_4A and Ap_5A, can act on rat isolated brain synaptic terminals, and if so through which receptor.

Methods

Synaptosomal preparation

Synaptosomes were prepared from middle brain of male Wistar rats that had been cervically dislocated and decapitated (Pintor *et al.*, 1992a). Synaptosomal pellets containing 1 mg of protein were resuspended in 1 ml incubation medium (composition mM: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5, $MgSO_4$ 1.2, glucose 10 and TES buffer 20, pH 7.4).

Calcium concentration measurements

The cytosolic free calcium concentration was determined with fura-2 as described by Grynkiewicz *et al.* (1985). Five minutes after resuspension $CaCl_2$ (1.33 mM) and fura-2-acetoxymethyl ester (5 μM) were added. Following incubation for 35 min the synaptosomes were pelleted (centrifuged at 800 r.p.m. for 1 min), washed twice and resuspended into fresh medium containing $CaCl_2$ (1.33 mM). Fluorescence was measured in a Perkin Elmer Spectrofluorimeter LS-50B, and monitored at 340 nm (excitation wavelength) and 510 nm (emission wavelength). Data were collected at 0.5 s intervals. Free calcium measurements were carried out with a mixture formed of 50 μM EGTA and 38 μM $CaCl_2$ as described by Verhage *et al.* (1989) and Carbone & Swandulla (1989).

Nucleotides and dinucleotides were tested at a final concentration of 100 μM. The adenosine receptor antagonist DPCPX, was used at a concentration of 250 nM. In the cross-desensitization studies the nucleotides (also at 100 μM) were preincubated for 3 min before the application of the corresponding agonist. Suramin was applied at a concentration of 100 μM, 1 min before application of the nucleotides.

Membrane potential measurements

Synaptosomal plasma membrane potential was monitored with a potential-sensitive cationic cyanide dye, 3,3-diethylthiadicarbocyanide iodine ($DiSC_2(5)$) as described by Simms *et al.* (1974). Synaptosomes were preincubated at 37°C for 1 h, then pelleted and resuspended (1 mg ml^{-1}) in incubation medium containing $DiSC_2(5)$ (5 μM) and $CaCl_2$ (1.33 mM). After 5 min equilibration, the nucleotide and dinucleotide compounds were added at a concentration of 100 μM. Fluorescence was determined at 643 and 680 nm. Data were collected at 2 s intervals.

Intrasynaptosomal sodium measurements

Synaptosomal sodium increments were performed with SBFI dye (Minta & Tsien, 1989) and following the same protocol as described for fura-2. Fluorescence was monitored at the same excitation and emission wavelength as for the fura assay (340 nm and 510 nm, respectively). Data were collected at 2 s intervals.

Toxins

Tetrodotoxin (TTX, 5 μM), ω-conotoxin G-VI-A (ω-CgTx, 5 μM), ω-agatoxin IV-A (ω-Aga, 5 μM) and nifedipine (200 nM) were incubated with synaptosomes 30 min before the application of the agonists.

Nucleotide stability

To assess the stability of nucleotides and dinucleotides, their purity was determined before the experimental studies were carried out, by using high performance liquid chromatography (h.p.l.c.). Chromatographic analysis was also performed at the end of the experiments to verify that they had not been degraded. The chromatographic system was equilibrated with the following mobile phase: 0.1 mM KH_2PO_4, 2 mM tetrabutyl ammonium and 15% acetonitrile pH 7.4, as described by Pintor *et al.* (1992b). Detection was monitored at 260 nm wavelength.

Ap_6A, diadenosine hexaphosphate, was not assayed because of its poor stability (Pintor *et al.*, 1992b).

Drugs used

ATP, synthetic analogues as well as diadenosine polyphosphates were from Sigma (St. Louis, MO, U.S.A.), except Ap_5A which was purchased from Boehringer (Mannheim, Germany). Suramin was kindly provided by Dr A. Ijzerman. Fura-2 as well as $DiSC_2(5)$ and SBFI were obtained from Molecular Probes (U.S.A.). Tetrodotoxin and nifedipine were purchased from Sigma (St. Louis, U.S.A.), ω-Conotoxin G-

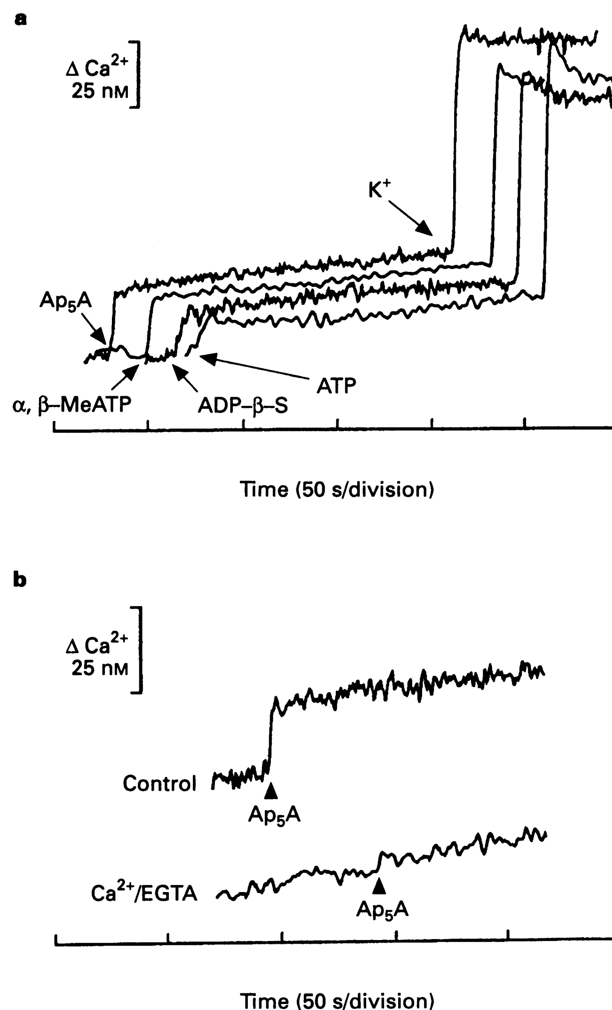

Figure 1 Effect of mono- and dinucleotides on rat brain intrasynaptosomal calcium levels: (a) different P_2-purinoceptor agonists induced an increase in intrasynaptosomal Ca^{2+} when applied at a final concentation of 100 μM. K^+ (30 nM) was used as a control of the synaptosomal functional response. (b) Response to diadenosine pentaphosphate (Ap_5A) in the presence of 1.33 mM extracellular Ca^{2+} and in the absence of extracellular Ca^{2+}. Traces are overlaid examples from different synaptosomal preparations.

VI-A and DPCPX were obtained from RBI (U.S.A.), ω-agatoxin IV-A from Alomone labs (Israel). Other reagents were analytical grade purchased from Merck (Darmstadt, Germany).

Statistical analysis

Data are presented as mean ± s.e.mean of at least four determinations in duplicate and in different synaptosomal preparations. Significant differences were determined by Student's two-tailed t test. When appropriate, single experimental traces are represented in the Figures; they are representative of at least four determinations in duplicate with equivalent results. Traces shown in figures are overlaid examples from different synaptosomal preparations.

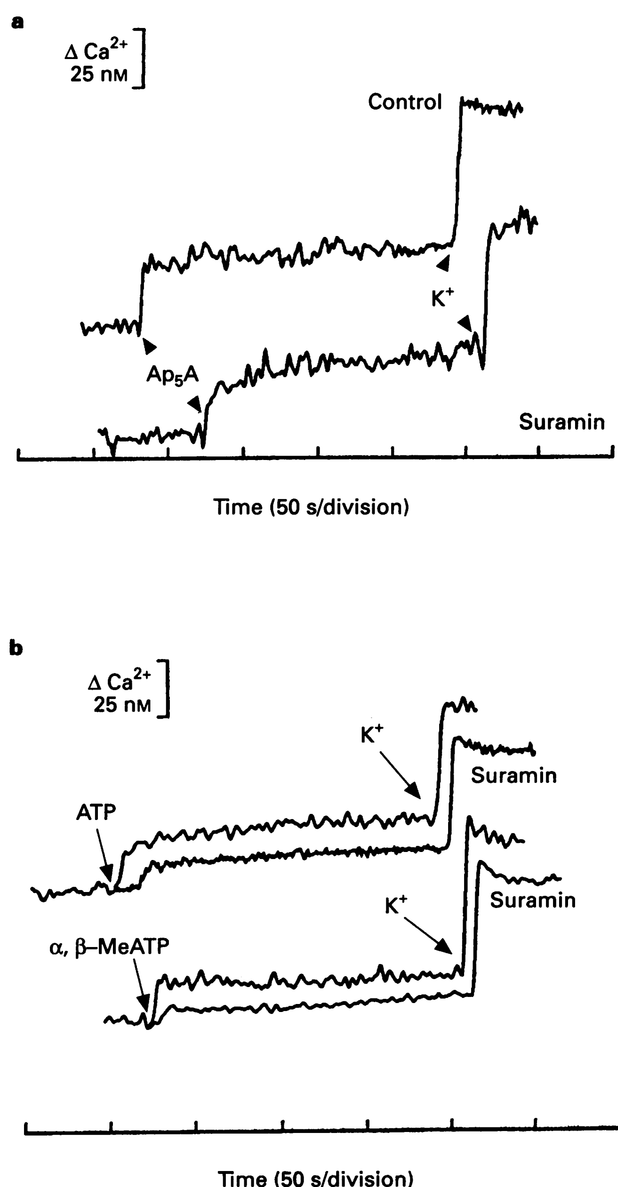

Figure 2 Effects of suramin on calcium entry into rat brain synaptosomes evoked by mono- and dinucleotides: (a) effect of diadenosine pentaphosphate (100 μM) on the intrasynaptomal calcium levels. The upper trace represents the effect in the absence of the P_2-purinoceptor antagonist, suramin. The lower record shows the effect in the presence of suramin (100 μM), under the conditions described in methods. (b) The effects of ATP (100 μM) and α,β-MeATP (100 μM), were blocked by preincubating synaptosomal preparations with suramin (100 μM). Traces are overlaid examples from different synaptosomal preparations.

Results

Effect of diadenosine polyphosphates and nucleotides on synaptosomal Ca^{2+} levels

The application of diadenosine polyphosphates, Ap_4A and Ap_5A, at final concentrations of 100 μM to synaptosomal preparations containing 1 mg protein ml^{-1}, induced an increase in cytosolic Ca^{2+} of 15 ± 1.4 nM and 26 ± 1.8 nM respectively.

ATP, as well as its synthetic analogues were able to induce the rise of intrasynaptosomal calcium levels. The amounts of calcium differed slightly among ATP and its analogues, the values being 18.1 ± 2.7 nM for ATP, 20.0 ± 2.4 nM for ADP-β-S and 23.7 ± 2.1 nM for α,β-MeATP ($P < 0.01$) (Figure 1a).

In the absence of extrasynaptosomal Ca^{2+}, Ap_5A was unable to induce any increase of the cytosolic calcium signal (Figure 1b). Ap_4A, as well as ATP and the synthetic analogues, behaved in a similar manner (data not shown). All the experiments were performed four times in duplicate.

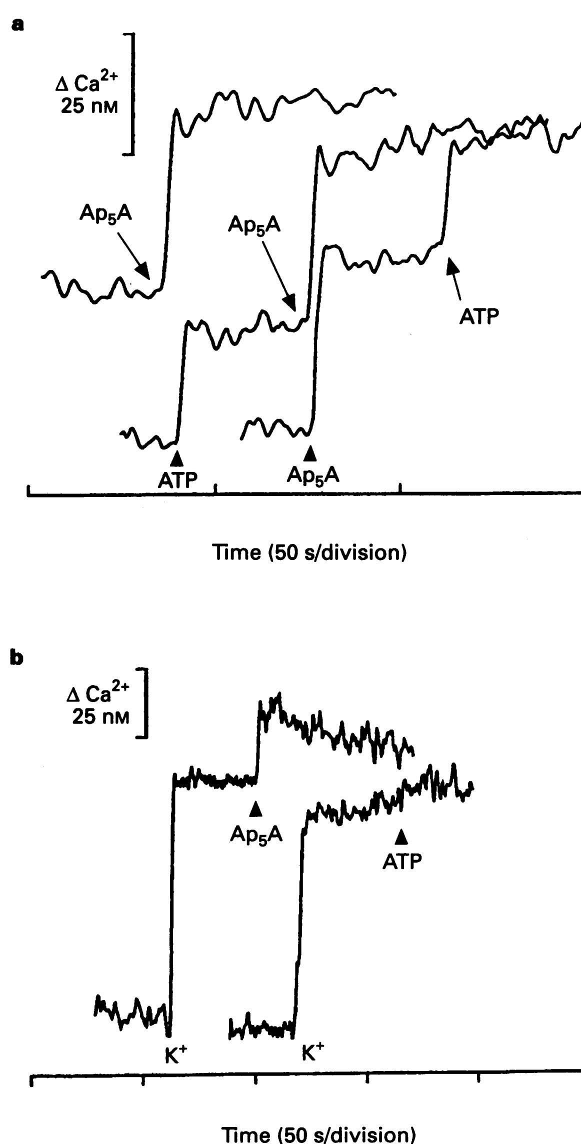

Figure 3 Cross-desensitization study between Ap_5A and ATP responses and effects of K^+ in rat brain synaptic terminals: (a) in the upper trace, Ap_5A (100 μM) was applied as control; application of ATP (100 μM) followed by Ap_5A (100 μM) (middle trace); application of Ap_5A (100 μM) and a further application of ATP (100 μM) (lower trace). (b) Effects of Ap_5A and ATP (both 100 μM) after application of K^+ (30 mM). Traces are overlaid examples from different synaptosomal preparations.

Pharmacology of the dinucleotide/nucleotide receptor action on Ca^{2+} influx

Preincubation of synaptosomal preparations with suramin (100 μM), a P_2-purinoceptor antagonist, did not affect the Ca^{2+}-transients evoked by Ap_5A (Figure 2a). In contrast, the Ca^{2+} transients elicited by ATP and its non-hydrolyzable analogues, were decreased to 9.5 ± 1.9 nM for ATP and 9.5 ± 1.7 nM for α,β-MeATP, when suramin (100 μM) was applied under the same conditions ($P < 0.01$). (Figure 2b).

Prior application of ATP, did not alter the Ca^{2+} increase induced by Ap_5A, nor was there any change in the Ca^{2+} signal evoked by ATP when synaptosomal samples were pretreated with Ap_5A (Figure 3a). These results could suggest that the receptors subserving the effects of ATP and Ap_5A are different. To develop this possibility further and to determine the role played by the membrane potential in signalling, both substances were assayed after applications of K^+. As shown in Figure 3b, ATP did not induce a further increase in intrasynaptosomal Ca^{2+} but Ap_5A did, although a decay in the Ca^{2+} signal induced by Ap_5A could be observed. These experiments also suggested the existence of two purinoceptors with differential selectivity for diadenosine polyphosphates and ATP.

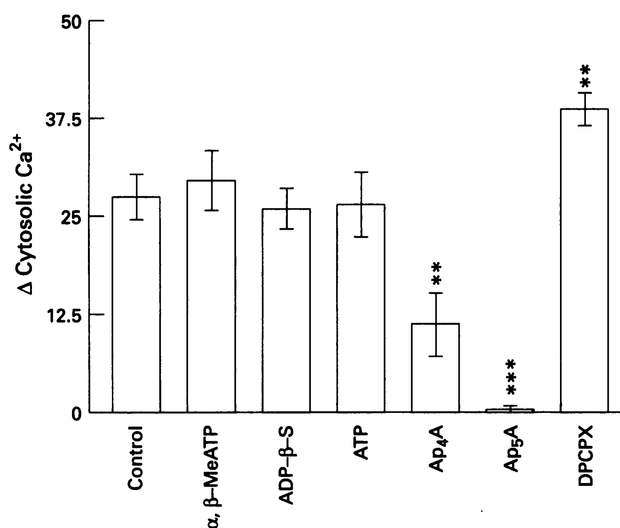

Figure 4 Desensitization studies with different mono- and dinucleotides in rat brain synaptic terminals: Effect of α,β-MeATP, ADP-β-S, ATP, Ap_4A and Ap_5A, preincubated in a final concentration of 100 μM, on the intrasynaptosomal Ca^{2+} increase induced by 100 μM Ap_5A. Data are means $\pm$ s.e.mean of four experiments in duplicate. $^{**}P < 0.01$, $^{***}P < 0.001$ vs control.

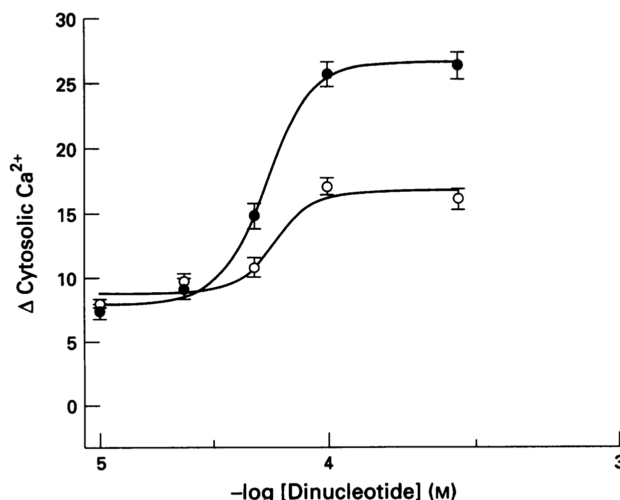

Figure 5 Concentration-response relationship for Ap_4A and Ap_5A on intracellular calcium increase in rat brain synaptosomes: concentration-dependence of Ap_4A (○) and Ap_5A (●) on the intrasynaptosomal Ca^{2+} levels. Data represent mean ($\pm$ s.e.mean) maximal increase in Ca^{2+} levels (four experiments in duplicate).

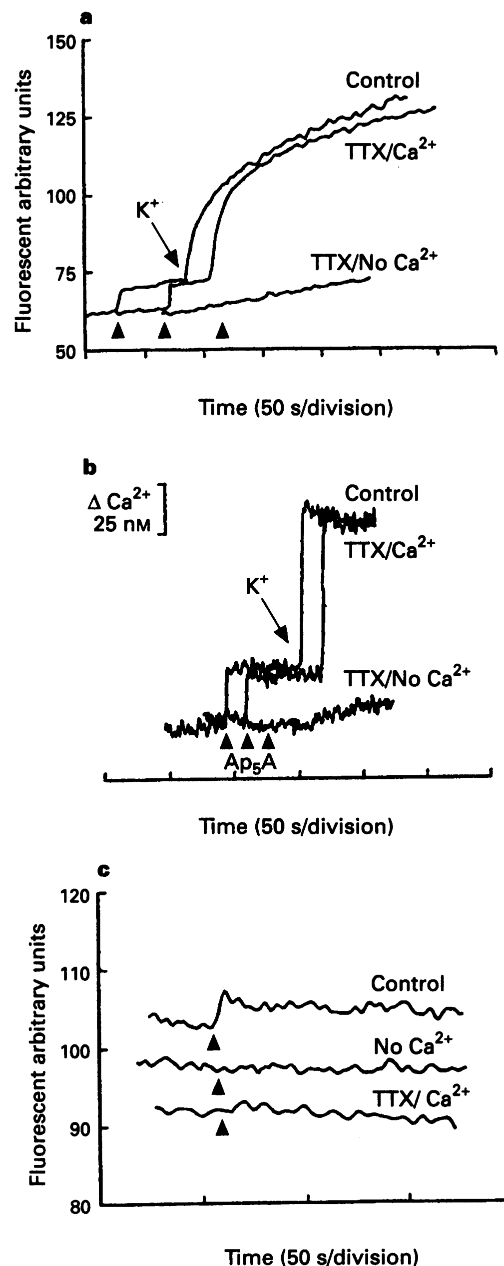

Figure 6 Effects of tetrodotoxin (TTX) on changes in membrane potential and intrasynaptosomal Ca^{2+} and Na^+ in rat brain synaptic terminals: (a) diadenosine pentaphosphate, 100 μM, induced depolarization in the presence and in the absence of 5 μM TTX (applications of the dinucleotide are indicated by the arrows). (b) TTX had no effect on the Ca^{2+}-signal evoked by Ap_5A. (c) Synaptosomal Na^+ increase induced by diadenosine pentaphosphate (indicated by the arrow). All the experiments are means of four experiments in duplicate. Traces are overlaid examples from different synaptosomal preparations.

Table 1 Effects of Ap_5A and ATP on intrasynaptosomal calcium signal, membrane potential, and sodium signals

	ΔCa^{2+} signal			$\Delta\Psi$			ΔNa^+ signal		
	Control	Ca^{2+} free	+ TTX	Control	$-Ca^{2+}$	+ TTX	Control	$-Ca^{2+}$	+ TTX
Ap_5A (100 μM)	26.0 ± 1.8	0	25.3 ± 2.2	7.5 ± 1.1	0	7.2 ± 1.4	4.6 ± 1.0	0	0
ATP (100 μM)	18.1 ± 2.7	0	10.8 ± 1.2	4.5 ± 0.8	0	3.7 ± 0.9	5.0 ± 1.2	3.6 ± 0.7	2.7 ± 0.5

ΔCa^{2+}, change in the intrasynaptosomal calcium signal expressed in nM; $\Delta\Psi$, change in the synaptosomal membrane potential expressed in arbitrary units; ΔNa^+, change in the intrasynaptosomal sodium signal expressed in arbitrary units. All values are mean ± s.e.mean of four experiments in duplicate.

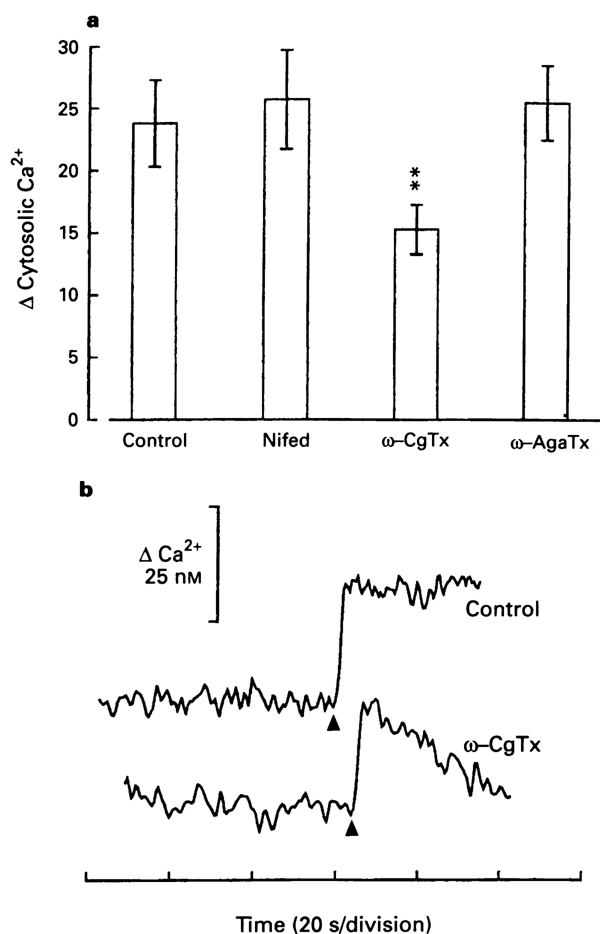

Figure 7 Effects of Ca^{2+}-channel antagonists on Ca^{2+}-signal evoked by Ap_5A in rat brain synaptic terminals: (a) effect of different calcium channel blockers on Ap_5A calcium signal. Nifedipine (Nifed), ω-conotoxin G-VI-A (ω-CgTx), ω-agatoxin IVA (ω-AgaTx) were preincubated for 30 min before the dinucleotide application. Results are means ± s.e.mean. All the experiments represent means of four different experiments in duplicate. $^{**}P<0.01$ vs control. (b) Differential kinetic response on Ca^{2+} sustained component to Ap_5A in the absence (control) and in the presence of ω-conotoxin G-VI-A. Arrows represent the application of the dinucleotide. These results are the means of four experiments in duplicate. Traces are overlaid examples from different synaptosomal preparations.

When synaptosomes were pre-incubated with ATP, ADP-β-S, α,β-MeATP, Ap_4A or Ap_5A, prior to application of Ap_5A, no inhibition of its effect was observed except in the cases of Ap_4A, which reduced the Ap_5A signal to 11.2 ± 4.0 nM and Ap_5A which reduced it to 1.2 ± 0.5 nM (Figure 4). The absence of cross-desensitization agrees with different purinoceptors for nucleotides and dinucleotides, their actions being independent and additive in the synaptosomal preparation.

To test whether diadenosine polyphosphates act through A_1-purinoceptors the synaptic terminals were pre-incubated with the A_1-purinoceptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 250 nM). In the presence of this compound Ap_5A produced a greater increase in the Ca^{2+}-signal than in its absence (Figure 4), excluding an action through A_1 receptors. All the experiments were performed four times in duplicate.

Concentration-response relationship for diadenosine polyphosphates

The concentration-dependency of the diadenosine polyphosphate purinoceptor was studied for both Ap_4A and Ap_5A. The pD_2 values were 54.5 ± 4.2 μM and 55.6 ± 3.8 μM respectively (Figure 5). It is noteworthy that although both dinucleotides have approximately the same pD_2, Ap_4A elicited a smaller maximum response than did Ap_5A. All the experiments were performed four times in duplicate.

Effects of TTX and Ca^{2+}-channel blockers

The diadenosine polyphosphates evoked depolarization of the membrane potential as shown in Figure 6a. This response was completely abolished in the absence of Ca^{2+} (Table 1, Figure 6a), but it was apparently unaffected by the presence of the voltage-dependent Na^+ blocker, tetrodotoxin, (TTX, 5 μM); nor did TTX modify the Ca^{2+}-signal (Figure 6b). The same experiments were performed by measuring the changes in the intrasynaptosomal Na^+-levels. Ap_5A in the presence of Ca^{2+} induced an increase in synaptosomal Na^+ which was abolished by either the absence of Ca^{2+} or the presence of TTX (Figure 6c). These results excluded the initial membrane depolarization via entry of Na^+ and Ca^{2+} through a non-specific receptor-operated cation channel, and agree with a more selective Ca^{2+} entry (Table 1).

To study whether voltage-dependent Ca^{2+} channels (VDCC) are involved in the action of diadenosine polyphosphate, different Ca^{2+} channel blockers were examined, as shown in Figure 7a. The channel blockers nifedipine (200 nM) and ω-agatoxin IVA (5 μM), specific for L- and P-types of Ca^{2+}-channels respectively (Catteral & Striessnig, 1992; Mintz *et al.*, 1992), did not modify the Ca^{2+} entry induced by diadenosine polyphosphates. ω-Conotoxin G-VI-A (5 μM), which blocks the N-type Ca^{2+}-channel (Miller & Fox, 1990; Tsien & Tsien, 1990), inhibited the Ca^{2+} influx evoked by Ap_4A and Ap_5A at 100 μM by 40% (Figure 7b). The results are summarized in Table 2.

The possible involvement of T-channels in the Ap_5A response was excluded since this type of channel is rapidly inactivated by a potential-dependent process (Carbone & Swandulla, 1989). After depolarization with K^+ there is still Ca^{2+} entry, excluding the participation of this channel (Figure 3b). In the presence of Ni^{2+} (40 μM) there was no change in the Ca^{2+} signal evoked by the diadenosine polyphosphates (Table 2). All the experiments were performed four times in duplicate.

ATP (100 μM) also modified the membrane potential but to a lesser extent than did Ap_5A at the same concentration (Figure 8a). The presence of TTX decreased both the membrane depolarization (Figure 8a) and the Ca^{2+} entry induced by ATP (Table 1, Figure 8b), but not the increase in intrasynaptosomal Na^+ (Figure 8c). These results suggest that ATP opened a non-specific cation channel. ATP calcium in-

Table 2 Effects of calcium antagonists on the intrasynaptosomal calcium levels in response to Ap_5A and ATP

	Control	*ω-Conotoxin G-VI-A*	*ω-Agatoxin IV-A*	*Nifedipine*	*Ni^{2+}*
Ap_5A (100 μM)	26.0 ± 1.8	15.1 ± 1.8	25.1 ± 3.0	25.4 ± 3.9	25.7 ± 2.8
ATP (100 μM)	18.1 ± 2.7	12.0 ± 3.6	20.4 ± 3.2	2.7 ± 1.3	19.4 ± 2.9

Intrasynaptosomal calcium levels are expressed in nM.
All values are mean ± s.e.mean of four experiments in duplicate.

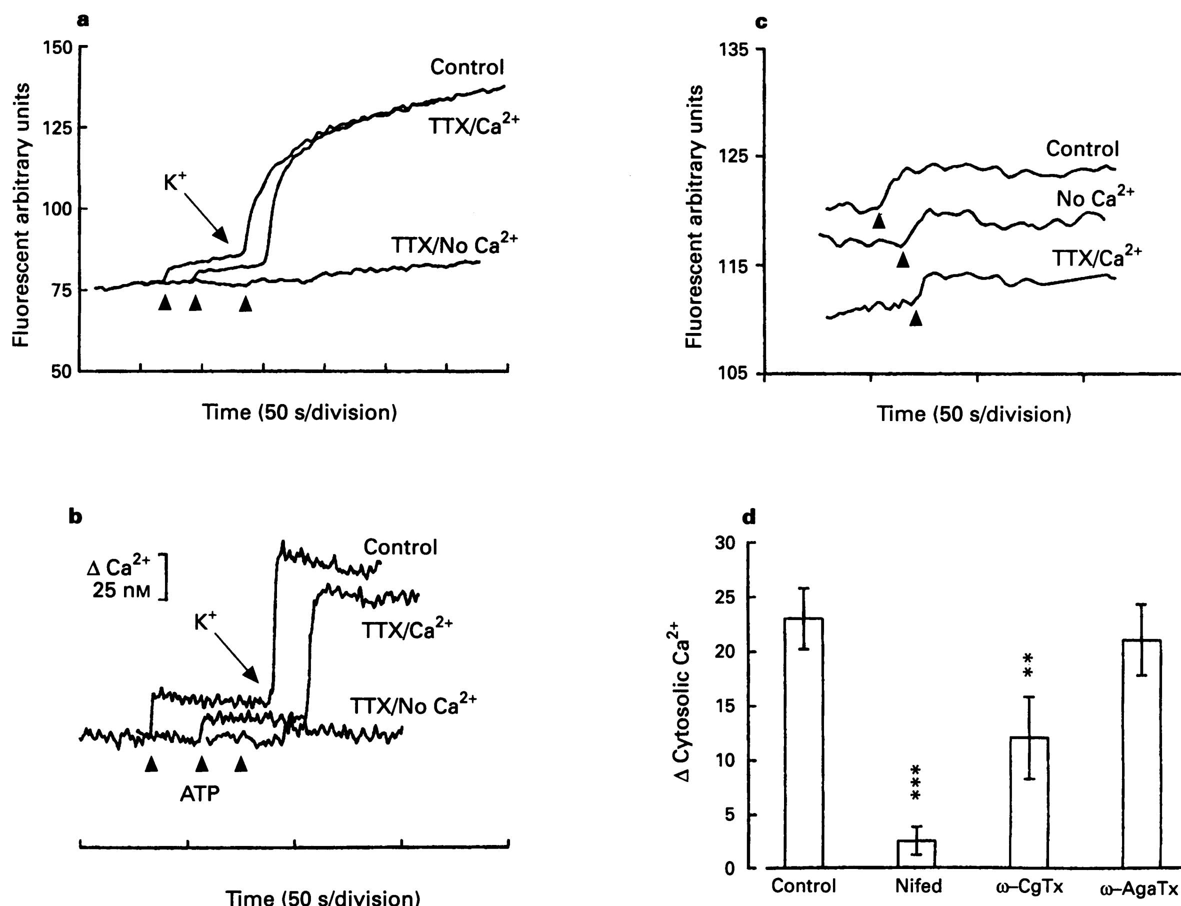

Figure 8 Effects of tetrodotoxin and Ca^{2+} antagonists on responses evoked by ATP in rat brain synaptic terminals: (a) ATP 100 μM induced depolarization which was partially sensitive to 5 μM TTX. (b) The Ca^{2+} influx induced by ATP was also partially inhibited by TTX. (c) Effect of 100 μM ATP on cytosolic Na^{+} levels. (d) Ca^{2+} channel blockers, nifedipine, ω-conotoxin G-VI-A and ω-agatoxin IVA, were assayed under the conditions described in Methods. All the experiments represent means of four different experiments in duplicate. $**P < 0.01$, $***P < 0.001$ vs control. Arrows indicate when ATP was applied. Traces are overlaid examples from different synaptosomal preparations.

crements were nevertheless abolished by nifedipine and by ω-conotoxin G-VI-A (Figure 8d). In conclusion, part of the ATP effects are probably mediated by L and N voltage-dependent calcium channels. The results are summarized in Table 2. All the experiments were performed four times in duplicate.

Discussion

The results described here, concerning the Ca^{2+} signalling, indicate that the diadenosine polyphosphates, Ap_4A and Ap_5A, act via different mechanisms and different receptors, from ATP in synaptosomal preparations of rat midbrain.

The first evidence suggesting the existence of an independent receptor for the dinucleotides from that of ATP is the lack of inhibition of responses to the dinucleotides by suramin. In contrast, the effects of ATP and its analogues were markedly inhibited by this compound. Although this is not a good antagonist for differentiating subtypes of P_2-purinoceptors, it is a useful tool for studying whether or not the effects of diadenosine polyphosphates are mediated by P_2-purinoceptors. Clearly suramin is useful in demonstrating distinct receptors for ATP and diadenosine polyphosphates.

The inability to cross-desensitize the diadenosine polyphosphate responses with ATP or its non-hydrolyzable analogues, together with their additive responses in the intrasynaptosomal Ca^{2+} increase is further evidence for separate receptor populations. The lack of cross-desensitization in rat midbrain synaptosomes, concerning Ca^{2+} signalling, deserves special comment, because the receptors so far described which mediate the actions of diadenosine polyphosphates have also been described as ATP receptors. The effects of Ap_nA compounds in the central nervous system have been described in cortical neurones (Stone & Perkins, 1981), in nodose ganglia neurones (Krishtal *et al.*, 1988) and in rat hippocampal neurones (Klishin *et al.*, 1994). In all these cases the actions described

were probably through P_2-purinoceptors which means they are activated not only by diadeosine polyphosphates but also by ATP. The actions of both compounds in the hippocampus appear to be mediated by PKC activation, implying a metabotropic receptor (Klishin *et al.*, 1994). In the peripheral nervous system, both P_{2X} and P_{2Y} subtypes are stimulated by adenine dinucleotides as well as the P_{2U} receptor present in blood vessel endothelial cells (MacKenzie *et al.*, 1988; Marchenko *et al.*, 1988; Hoyle, 1990; Castro *et al.*, 1994; Pintor & Miras-Portugal, 1994).

The Ca^{2+} signal induced by diadenosine polyphosphates mediated via the purinoceptor present in midbrain synaptic terminals is dependent on extracellular Ca^{2+} levels. Ca^{2+} entry can be split into two components. The first, is a rapid and voltage-independent process, which takes place by the direct action of the dinucleotide on the novel purinoceptor; the second component is sustained and voltage-sensitive. Two facts corroborate this. On the one hand, application of K^+ before an application of Ap_5A abolishes the sustained component but does not affect the initial transient indicating the loss of a voltage-sensitive component. On the other hand, treatment with ω-conotoxin G-VI-A, which blocks the N-type voltage-dependent Ca^{2+} channel (Miller & Fox, 1990; Tsien & Tsien, 1990), mimicks the effect produced by K^+, confirming the voltage-dependency of this second, sustained component.

The initial Ca^{2+} entry induces a depolarization inside the synaptic terminal which opens a voltage-dependent sodium channel as demonstrated by the experiments performed with the sodium fluorophore SBFI dye (Minta & Tsien, 1989). However, the blockade of this voltage-dependent Na^+ channel by tetrodotoxin did not affect either the initial or the sustained voltage-dependent Ca^{2+} entry, indicating the bypassing of this channel when TTX is present in the incubation medium. Thus, the initial Ca^{2+} entry induced by diadenosine polyphosphates is sufficient to activate the ω-CgTx-sensitive N-type Ca^{2+} channel although under normal conditions the voltage-dependent Na^+ channel is also activated. The N-type VDCC, appears to be playing a role in the sustained Ca^{2+} entry after diadenosine polyphosphate stimulation; however the L, P and T types were excluded because of the lack of effects of nifedipine, ω-agatoxin IV-A and Ni^{2+}, respectively (Carbone & Swandulla, 1989; Catteral & Striessnig, 1992; Mintz *et al.*, 1992). This is not the case for ATP, which induced Ca^{2+} entry that could be inhibited by ω-conotoxin G-VI-A and nifedipine. The differential action of calcium channel blockers in the ATP and diadenosine polyphosphate responses represent an additional support for the existence of different receptor populations mediating their responses.

Adenosine, a well known modulator of excitatory neurotransmission in the central nervous system (Barrie & Nicholls, 1993), was able, through A_1 receptors, to modulate the response to diadenosine polyphosphates. Moreover, the experiments carried out in the presence of DPCPX, a well known A_1 antagonist, showed a significant increase in the intrasynaptosomal Ca^{2+} signalling. These results exclude the action of the adenine dinucleotides through A_1 receptors. Although this is only a preliminary result, a careful study of the relationship between neurotransmission and neuromodulation by dinucleotides and nucleotides needs to be carried out.

The existence of a specific receptor for diadenosine polyphosphates in rat brain synaptic terminals supports the putative neurotransmitter role of these substances in the central nervous system. The possible physiological relevance of Ap_nA compounds is beginning to be elucidated both *in vitro* and *in vivo*. Experiments carried out *in vitro* with synaptosomes have demonstrated the presence and release of adenine dinucleotides after stimulation with depolarizing agents (Pintor *et al.*, 1992a). More recently, it has been possible to describe the release of diadenosine polyphosphates *in vivo* induced by amphetamine sulphate (Pintor *et al.*, 1993b). The levels found in the perfusates were 64.5 nM for Ap_4A and 57.5 nM for Ap_5A in the perfusion media. Due to the presence of ecto-enzymes degrading these compounds and their own polyanionic structure, the presence of concentrations several orders of magnitude higher can be assumed at the synaptic cleft (Pintor *et al.*, 1994). In a peripheral model such as chromaffin cells, an extracellular concentration for Ap_4A, Ap_5A and Ap_6A close to 30 μM, for each one, was estimated after stimulation (Pintor *et al.*, 1991; 1992b).

Diadenosine tetraphosphate shows a maximal Ca^{2+} increase, roughly half of that obtained with diadenosine pentaphosphate although in terms of dose-response, the pD_2 values were extremely close, perhaps acting as a partial agonist in this system. On the basis of the binding and displacement studies with labelled Ap_4A, the presence of high and medium affinity binding sites with K_d values in the nM and in the μM range have been described in several neural preparations (Pintor *et al.*, 1991; 1993a). These receptors were termed P_{2D} on the basis of non-agreement of the displacement order with that reported in the literature for the formerly characterized ATP receptors (Pintor *et al.*, 1993a). Nevertheless, on a functional basis, studying the Ca^{2+} response in the midbrain synaptic terminals there appears to be a clear difference between diadenosine polyphosphate- and ATP-mediated actions. The potential for purinergic transmission is in this way significantly increased.

In conclusion, the results reported here show that diadenosine polyphosphates induce Ca^{2+} entry in two ways: firstly, a sharp increase which is voltage-independent and secondly a sustained entry via N-type, voltage-dependent Ca^{2+} channels. The voltage-independent Ca^{2+} entry can be explained either by a receptor-operated calcium channel or through a G protein coupled to a Ca^{2+} channel. Due to the differences with respect to the action of ATP, the novel P-purinoceptor does not appear to be a member of the P_2-purinoceptor family. However, although we believe that the receptor for dinucleotides is different from that for ATP in this preparation, we do not have enough evidence to propose a new name. Ideally we must await the development of a specific or selective antagonist before a firm proposal can be made.

We would like to thank Dr Charles Hoyle for his helpful comments and kind criticism and Miss Jacqueline Rafia and Mr Erik Lundin for their help in the preparation of this manuscript. This work has been supported by a research grant of Dirección General de Investigación Cientifica y Tecnológica (DGICYT) number of PB 92 0230, the Areces Neuroscience programme, 1994 and INTAS no. 822 of the EU. J.P. is a Ramon Areces Foundation fellowship holder.

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(Received August 8, 1994
Received March 3, 1995
Accepted March 27, 1995)