Diadenosine polyphosphates evoke Ca^{2+} transients in guinea-pig brain via receptors distinct from those for ATP

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1. The ability of diadenosine polyphosphates, namely P^1,P^2-di(adenosine) pyrophosphate (Ap2A), P^1,P^3-di(adenosine) triphosphate (Ap3A), P^1,P^4-di(adenosine) tetraphosphate (Ap4A), P^1,P^5-di(adenosine) pentaphosphate (Ap5A) and P^1,P^6-di(adenosine) hexaphosphate (Ap6A) to evoke Ca^{2+} signals in synaptosomes prepared from three different regions of the guinea-pig brain was examined.

2. In synaptosomal preparations from the paleocortex (cortex), diencephalon/brainstem (midbrain) and cerebellum all the dinucleotides evoked Ca^{2+} signals that were concentration dependent over the range 1–300 μM. ATP and its synthetic analogues, α,β-methylene ATP, 2-methylthio ATP and adenosine 5′-*O*-(2-thio)diphosphate (all 100 μM) also evoked Ca^{2+} signals in these preparations.

3. In the midbrain and cerebellum preparations, responses to ATP and its analogues were attenuated or abolished by the P2 receptor antagonist suramin (100 μM) but responses to the dinucleotides were not. Also, desensitization by a dinucleotide blocked responses to dinucleotides but not mononucleotides, and desensitization by a mononucleotide blocked responses to mononucleotides but not dinucleotides.

4. In cortical preparations, suramin (100 μM) blocked responses to both classes of nucleotides. Furthermore, there was mutual cross-desensitization between the mono- and dinucleotides.

5. The adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine, did not affect responses evoked by the dinucleotides, nor did the pyrimidine UTP.

6. It is concluded that there are specific dinucleotide receptors, activated by diadenosine polyphosphates, but not ATP or UTP, on synaptic terminals in guinea-pig diencephalon/brainstem and cerebellum. These receptors bear a similarity to the dinucleotide receptor (P_4 receptor) in rat brain. In guinea-pig cerebral cortex synaptosomes, diadenosine polyphosphates appear to act via the same receptor as ATP.

There is increasing interest in the activity of diadenosine polyphosphate compounds acting as intracellular and extracellular signal molecules. They are involved in cell proliferation and cellular responses to stress, they have diverse pharmacological activity in the central nervous system and in the periphery, and they may even be neurotransmitter substances (for reviews see Hoyle, 1990; McLennan, 1992; Baxi & Vishwanatha, 1995; Chen, Levy & Lightman, 1995; Pintor & Miras Portugal, 1995*a*).

Diadenosine tetraphosphate (Ap4A), diadenosine pentaphosphate (Ap5A) and diadenosine hexaphosphate (Ap6A) have all been identified as being present in significant quantity in synaptosomes prepared from the rat brain (Pintor, Diaz-Rey & Miras Portugal, 1992; Pintor & Miras Portugal, 1995*a*). Their release from synaptosomes can be evoked by depolarizing stimuli in a Ca^{2+}-dependent manner (Pintor *et al.* 1992; Pintor & Miras Portugal, 1995*a*). Using a push–pull cannula technique, it has been shown in the rat brain, *in vivo*, that amphetamine-induced dopamine release stimulates the release of diadenosine polyphosphates from striatal nerve terminals (Pintor, Porras & Miras Portugal, 1995*c*).

It is not entirely clear whether or not the diadenosine polyphosphates act on specific dinucleotide receptors or on

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adenosine or P2 receptors. For example, in rat cortical slices Ap4A and Ap5A inhibit action potential discharge via a methylxanthine-sensitive P1-purinoceptor (Stone & Perkins, 1981). Similarly, in rat hippocampal slices Ap4A and Ap5A inhibit excitatory synaptic transmission via A_1 receptors (Klishin, Lozovaya, Pintor, Miras Portugal & Krishtal, 1994), while in cortical slices from rabbit brain Ap4A inhibits release of [^{3}H]noradrenaline via either A_1 or P2 receptors (von Kügelgen, Spath & Starke, 1994). However, in rat and deermouse brain, binding sites have been identified that are highly selective, or even specific, for adenine dinucleotides as opposed to mononucleotides (Hilderman, Martin, Zimmerman & Pivorun, 1991; Pintor, Diaz-Rey & Miras Portugal, 1993), and this possibly represents a P_{2D} receptor (Pintor *et al.* 1993) or $P2Y_{ApnA}$ receptor (see Pintor, Hoyle, Gualix & Miras Portugal, 1997). In the current provisional nomenclature from the Subcommittee of the International Union of Pharmacology and Clinical Pharmacology Committee on Drug Classification and Receptor Nomenclature (NC-IUPHAR) on Adenosine and P2 receptors, the $P2Y_{ApnA}$ receptor has been renamed $P2Y_{Ap4A}$ (Alexander & Peters, 1997). Furthermore, in a functional study, in synaptosomes prepared from subcortical regions of the rat brain, there is evidence that dinucleotides act through receptors (P_4 receptors) which are distinct from those activated by ATP, in order to induce an elevation in intrasynaptosomal Ca^{2+} levels (Pintor & Miras Portugal, 1995*b*; Pivorun & Nordone, 1996). The term 'P_4 receptor' does not fit in with the NC-IUPHAR system of naming P2 receptors. However, it is not the same as the $P2Y_{Ap4A}$ receptor because it appears to be a ligand-gated ion channel, and therefore of the P2X rather than the P2Y family. Until this receptor is defined at a molecular level it may be better to use the term 'dinucleotide receptor' instead (Pintor *et al.* 1997).

The aim of the present study was to examine the pharmacological activity of diadenosine polyphosphates in synaptosomes prepared from three different regions of the guinea-pig brain in order to determine whether or not they evoke calcium signals via dinucleotide receptors (similar to $P2Y_{Ap4A}$ or P_4 receptors) or via P2 receptors. Preliminary observations have been reported previously (Pintor, Hoyle, Abal, Gualix, Puche & Miras Portugal, 1995*a*; Pintor, Hoyle, Puche, Abal, Gualix & Miras Portugal, 1995*b*).

METHODS

Preparation of synaptosomes

Male guinea-pigs (approximately 250 g) were killed by cervical dislocation and exsanguination (in accordance with the guidelines of the International Council for Laboratory Science (ICLAS)). The brain was removed and placed in ice-cold medium. Synaptosomes were prepared from three different areas, namely: paleocortex (cortex), cerebellum and diencephalon/brainstem (midbrain), as described previously (Pintor *et al.* 1992). Synaptosomal pellets containing 1 mg of protein were suspended in 1 ml of incubation medium (composition (mM): NaCl, 122; KCl, 3·1; KH_2PO_4, 0·4; $NaHCO_3$, 5·0; $MgSO_4$, 1·2; glucose, 10; and TES buffer, 20; pH 7·4).

Measurement of calcium concentration

The synaptosomal free calcium concentration was determined using fura-2 as described by Grynkiewicz, Poenie & Tsien (1985). Five minutes after resuspension of synaptosomes, $CaCl_2$ (1·33 mM) and fura-2 acetoxymethyl ester (5 μM) were added. Following incubation for 35 min at 37 °C the synaptosomes were pelleted (centrifuged at 800 r.p.m. for 1 min), washed twice and resuspended into fresh medium containing 1·33 mM $CaCl_2$. Fluorescence was measured in a Perkin Elmer Spectrofluorimeter LS-50, and monitored at 340 and 510 nm. Data were collected at 0·5 s intervals.

Experimental procedures

Concentration–response relationships were established for the different adenine dinucleotides, by incubating 1 mg of synaptosomes with graded concentrations of ApnA compounds ranging from 1 to 300 μM. After an observed response, the integrity of the synaptosomal preparation was checked by applying KCl (60 mM). ATP and its synthetic analogues were tested at a final concentration of 100 μM. Suramin, a non-selective P2 receptor antagonist (Dunn & Blakeley, 1988; Hoyle, Knight & Burnstock, 1990) was applied at a concentration of 100 μM, 2 min before application of the agonists. In the cross-desensitization studies the first nucleotide (100 μM) was pre-incubated for 3 min before the application of the second nucleotide.

Nucleotide stability

The purity of the mononucleotides and dinucleotides was determined before the experimental studies were carried out, using high performance liquid chromatography. Chromatographic analysis was also performed at the end of the experiments to ensure that the compounds had not been degraded during the course of the experiment. 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.* (1992). Detection was monitored at a wavelength of 260 nm.

Drugs used

Diadenosine pyrophosphate (sodium salt; Ap2A), diadenosine triphosphate (ammonium salt; Ap3A), diadenosine tetraphosphate (ammonium salt; Ap4A), diadenosine pentaphosphate (sodium salt; Ap5A), diadenosine hexaphosphate (ammonium salt; Ap6A), ATP (sodium salt), α,β-methylene ATP (lithium salt; α,β-meATP) and UTP (sodium salt) were all obtained from Sigma. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-methylthio ATP (sodium salt; 2-MeSATP) and suramin were purchased from Research Biochemicals Incorporated. Fura-2 was obtained from Molecular Probes. Adenosine 5′-*O*-(2-thio)diphosphate (sodium salt; ADP-β-S) was obtained from Boehringer Mannheim. Other analytical grade reagents were purchased from Merck.

Data analysis

Data for calcium signals are presented as means ± S.E.M of at least four determinations in duplicate and in different synaptosomal preparations. Significant differences were determined at the level of $P \leqslant 0{\cdot}05$, by analysis of variance and Student's t test. For statistical analysis of EC_{50} values, these were converted to log(EC_{50}) before applying ANOVA and Student's *post hoc* t tests. Mean log(EC_{50}) and 95% confidence limits were antilogged for presentation in the text. When appropriate, single experiment traces are represented in the figures. They are representative of at least four determinations in duplicate with equivalent results. Graphs were drawn using the computer program FigP v. 2.7 (Biosoft, Cambridge, UK), which also fitted the curves to a sigmoidal regression using a subroutine called PFit.

Table 1. EC_{50} values and Hill coefficients (n_H) for diadenosine polyphosphates in synaptosomal preparations of guinea-pig cerebral cortex, cerebellum and midbrain

	Cortex		Cerebellum		Midbrain	
	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H	EC_{50} (μM)	n_H
Ap2A	14·9 (9·1, 24·5)	1·1 ± 0·2	1·0 (0·5, 1·7)	1·2 ± 0·1	16·7 (6·9, 40·6)	1·2 ± 0·5
Ap3A	8·8 (5·2, 14·9)	1·0 ± 0·2	7·0 (4·1, 12·0)	1·1 ± 0·2	6·1 (2·5, 14·7)	0·9 ± 0·3
Ap4A	8·7 (6·0, 12·5)	0·9 ± 0·1	18·0 (14·9, 21·6)	0·8 ± 0·4	7·1 (2·0, 26·0)	0·9 ± 0·4
Ap5A	8·7 (5·9, 12·8)	1·2 ± 0·2	11·2 (4·7, 12·5)	0·9 ± 0·3	8·8 (3·9, 19·8)	1·0 ± 0·3
Ap6A	11·8 (6·5, 21·2)	1·5 ± 0·4	6·1 (2·8, 13·3)	1·0 ± 0·3	6·1 (2·8, 13·3)	1·1 ± 0·2

Values are the means with lower and upper 95% confidences limits given within parentheses, from four experiments performed in duplicate. Significant differences are discussed in the text.

RESULTS

Midbrain synaptosomes: effects of adenine dinucleotides and ATP derivatives

Application of each of the diadenosine polyphosphates (100 μM) to midbrain synaptosomes elicited an increase in intrasynaptosomal Ca^{2+} (Fig. 1*A*). Because the adenine dinucleotides clearly evoked measurable Ca^{2+} transients, concentration–response relationships were constructed from 1 to 300 μM (Fig. 2). There were no significant differences between the EC_{50} values using one-way ANOVA (Table 1), and the Hill coefficients for each diadenosine polyphosphate did not significantly differ from 1 (Table 1). At the highest concentration tested (300 μM), the rank order for evoking the Ca^{2+} signal (nM) was: Ap2A (30·2 ± 1·1) = Ap4A (30·0 ± 0·9) = Ap6A (26·9 ± 1·9) > Ap3A (21·8 ± 1·0) > Ap5A (19·1 ± 1·3). Ap2A, Ap4A and Ap6A all evoked higher signals than Ap3A ($P < 0·05$), and Ap5A evoked a weaker response than any other dinucleotide ($P < 0·001$).

Three synthetic analogues of ATP, 2-MeSATP, ADP-β-S and α,β-meATP, together with ATP itself were also tested. ATP and these analogues also induced a Ca^{2+} increase when applied at a concentration of 100 μM (Fig. 1*B*). ATP and α,β-meATP were the most potent, evoking Ca^{2+} signals of 24·5 ± 0·2 and 23·0 ± 0·6 nM, respectively, followed by ADP-β-S and 2-MeSATP, which evoked signals of 19·0 ± 1·7 and 13·1 ± 0·8 nM, respectively. ATP evoked a significantly

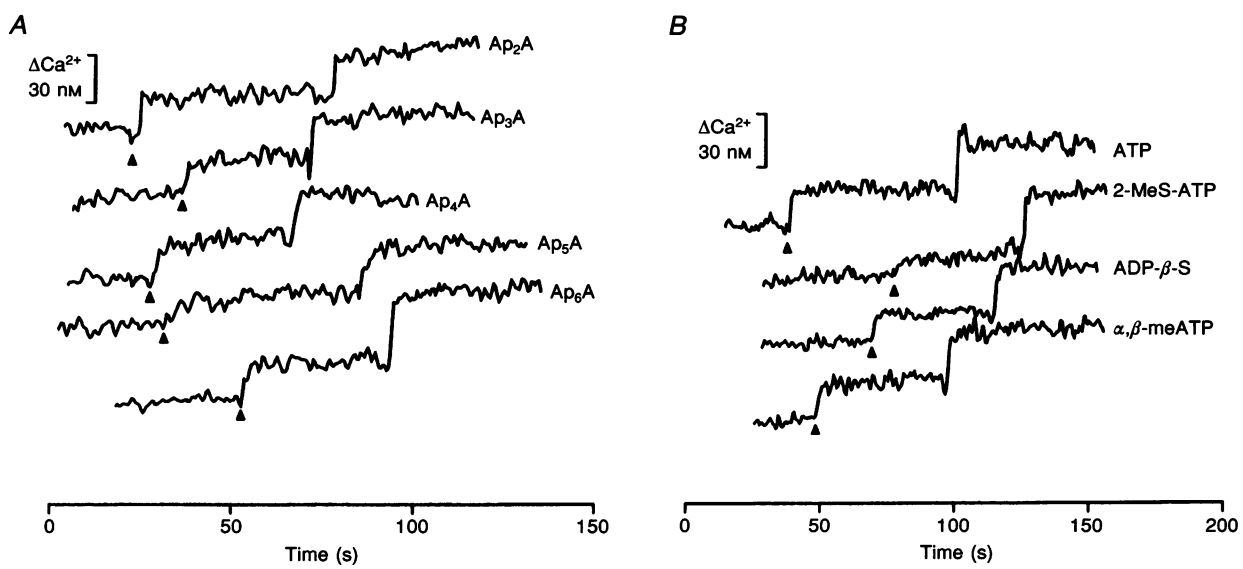

Figure 1. Effects of mono- and dinucleotides on guinea-pig midbrain synaptosomal Ca^{2+} levels

A, intrasynaptosomal Ca^{2+} responses elicited by diadenosine polyphosphates (Ap2A–Ap6A) at a concentration of 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal). *B*, synaptosomal Ca^{2+} increase elicited by ATP and its synthetic analogues, 2-methylthioATP (2-MeS-ATP), adenosine 5′-*O*-(2-thio)diphosphate (ADP-β-S) and α,β-methylene ATP (α,β-meATP), all at 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal).

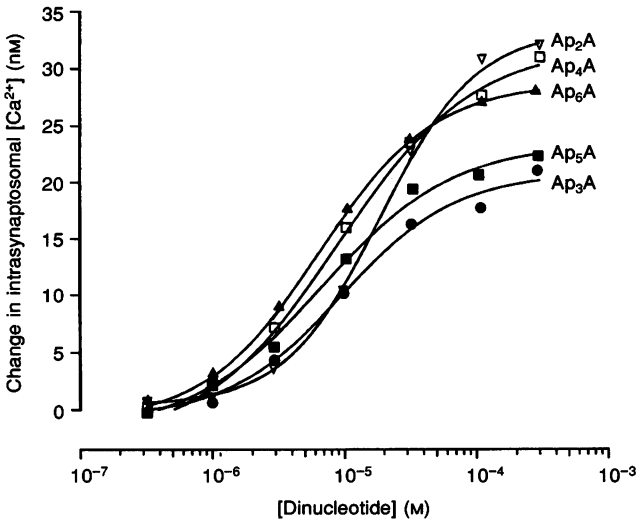

Figure 2. Concentration–response curves for diadenosine polyphosphates in guinea-pig midbrain synaptosomes

Ap2A (▽), Ap3A (●), Ap4A (□), Ap5A (■) and Ap6A (▲). Points show means of at least four experiments performed in duplicate.

greater signal than ADP or 2-MeSATP ($P < 0{\cdot}002$), and 2-MeSATP evoked a weaker signal than any other mononucleotide ($P < 0{\cdot}02$).

Midbrain synaptosomes: cross-desensitization studies and antagonism by suramin

In order to determine whether or not adenine dinucleotides were evoking the Ca^{2+} transients by acting on the same receptor as ATP, or by an independent receptor, antagonism by suramin and cross-desensitization studies were performed. Suramin, pre-incubated for 2 min before the application of 100 μM Ap4A, did not modify the Ca^{2+} transient elicited by this dinucleotide, but markedly diminished the response induced by 100 μM ATP (Fig. 3).

Pre-incubation for 2 min with the non-hydrolysable analogue of ATP, α,β-meATP, did not significantly modify the response to a subsequent application of 100 μM Ap4A. In the converse experiment, pre-incubation of the synaptosomes with Ap4A (100 μM) did not significantly modify the response to the subsequent application of ATP (100 μM) (Fig. 3). Similar results were obtained with all the diadenosine polyphosphates (data not shown). In contrast, pre-incubation with Ap4A almost abolished the response to Ap4A, and pre-incubation with α,β-meATP almost abolished the response to ATP (Fig. 3). Neither the adenosine receptor antagonist DPCPX nor the P_{2U} receptor agonist UTP (250 nM and 100 μM, respectively) evoked a calcium signal, nor did they affect the activity of any of the diadenosine polyphosphates (results not shown).

Cerebellar synaptosomes: effects of adenine dinucleotides and ATP derivatives

Application of each of the diadenosine polyphosphates (100 μM) to cerebellar synaptosomes elicited a Ca^{2+} signal (Fig. 4*A*). Concentration–response relationships were determined for all the diadenosine polyphosphates (Fig. 5). The potency order deduced from the EC_{50} values was Ap2A > Ap6A = Ap3A ⩾ Ap5A = Ap4A (Table 1). Ap2A was significantly more potent than any other dinucleotide ($P < 0{\cdot}001$), and Ap4A was significantly less potent ($P < 0{\cdot}01$) than any other dinucleotide except Ap5A. The Hill coefficient for each of these compounds did not differ

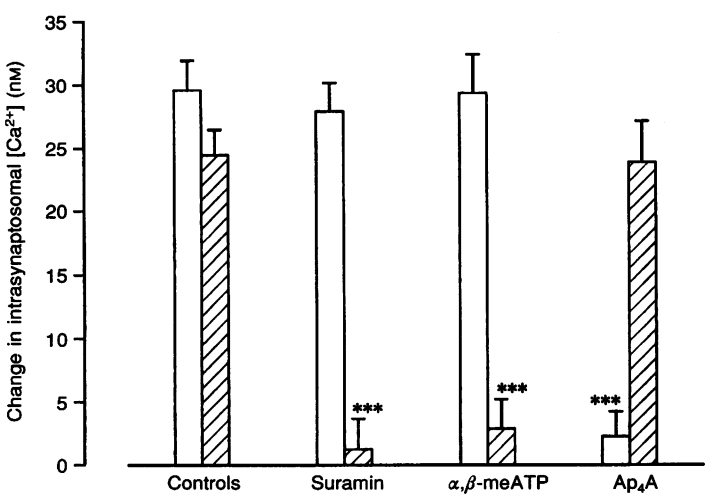

Figure 3. Antagonism and cross-desensitization studies on guinea-pig midbrain synaptic terminals

The Ca^{2+} signals elicited by diadenosine tetraphosphate (Ap4A, 100 μM; □) and ATP (100 μM; ▨) were assayed in the presence of the P2 receptor antagonist suramin (100 μM). Cross-desensitization studies were carried out by pre-incubating synaptic terminals with α,β-meATP (100 μM) and Ap4A (100 μM) as described in Methods. *** $P < 0{\cdot}001$ *vs.* control.

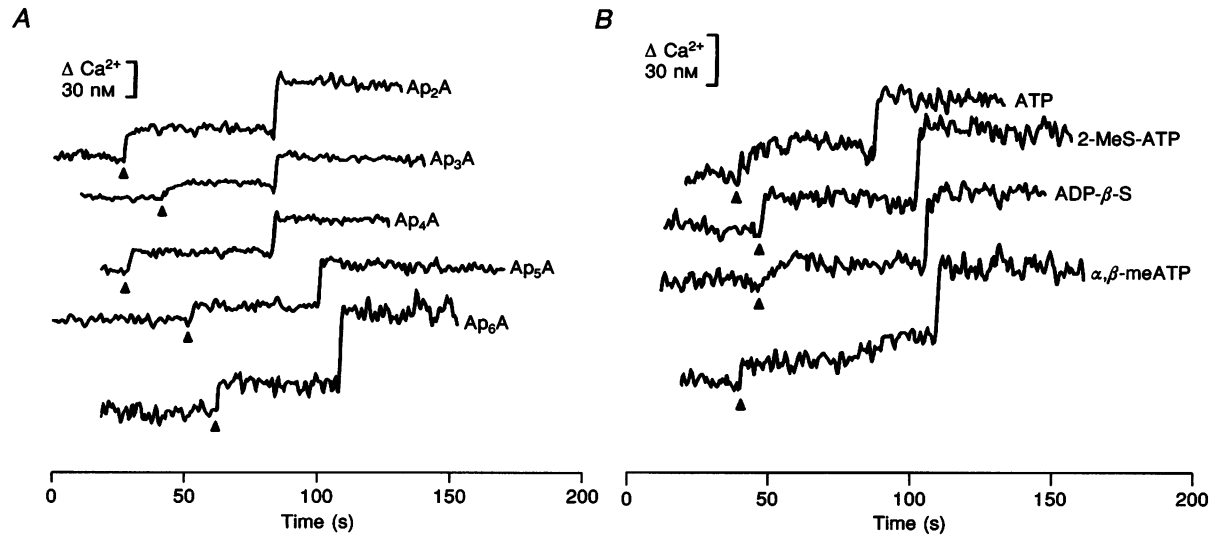

Figure 4. Effects of mono- and dinucleotides on guinea-pig cerebellar synaptosomal Ca^{2+} levels
A, Ca^{2+} responses elicited by diadenosine polyphosphates (Ap2A–Ap6A) at a concentration of 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal). *B*, synaptosomal Ca^{2+} increases elicited by ATP and its synthetic analogues, 2-MeS-ATP, ADP-β-S and α,β-meATP, all at 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal).

significantly from unity (Table 1). At the highest concentration tested (300 μM), the rank order for evoking the Ca^{2+} signal (nM) was: Ap2A (34·2 ± 1·2) > Ap3A (27·4 ± 0·8) = Ap6A (26·9 ± 1·1) = Ap4A (24·3 ± 1·2) > Ap5A (19·2 ± 1·0). Ap2A gave larger responses than any other dinucleotide ($P < 0·001$), and Ap5A gave the smallest responses ($P < 0·02$). There were no significant differences between Ap3A, Ap4A and Ap6A.

When ATP and its analogues were tested in the cerebellar synaptosomes, they also induced Ca^{2+} transients (Fig. 4*B*). At the single concentration of 100 μM, ATP and 2-MeSATP evoked the greatest Ca^{2+} signals of 29·9 ± 2·4 and 27·9 ± 0·2 nM, respectively, followed by ADP-β-S and α,β-meATP, which evoked signals of 23·0 ± 1·0 and 20·4 ± 0·6 nM, respectively. The values for ATP and 2-MeSATP were both significantly greater than either ADP-β-S or α,β-meATP ($P < 0·05$).

Cerebellar synaptosomes: cross-desensitization studies and antagonism by suramin

Suramin applied at a concentration of 100 μM, 2 min before the addition of Ap4A or ATP (both 100 μM), antagonized the responses to ATP but had no significant effect against Ap4A (Fig. 6). Cross-desensitization studies revealed that

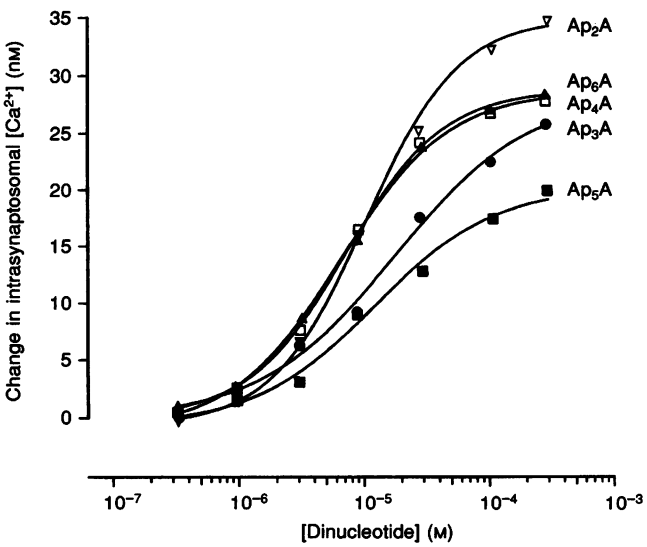

Figure 5. Concentration–response curves for diadenosine polyphosphates in guinea-pig cerebellar synaptic terminals
Ap2A (▽), Ap3A (●), Ap4A (□), Ap5A (■) and Ap6A (▲). Points show means of at least four experiments carried out in duplicate.

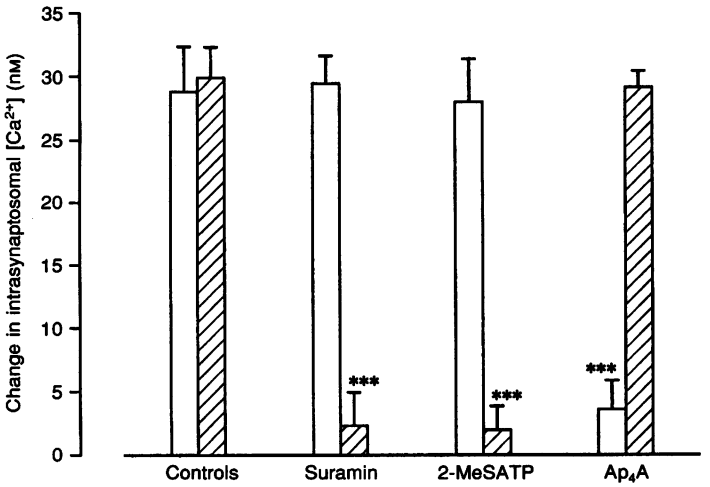

Figure 6. Antagonism and cross-desensitization studies on guinea-pig cerebellar synaptosomes
The Ca^{2+} signals elicited by Ap4A (100 μM; □) and ATP (100 μM; ▨) were assayed in the presence of the P2 receptor antagonist suramin (100 μM). Cross-desensitization studies were performed by pre-incubating synaptic terminals with 2-MeS-ATP (100 μM) and Ap4A (100 μM) as described in Methods. *** $P < 0.001$ *vs.* control.

pre-incubation with 2-MeSATP (100 μM) did not affect the response elicited by Ap4A (100 μM), but almost abolished the response to ATP. In converse experiments, an initial application of Ap4A (100 μM) did not significantly affect the response to a subsequent application of ATP (100 μM), while it severely attenuated the response to Ap4A (Fig. 6). Similar results were obtained with all the adenine dinucleotides (data not shown). Neither DPCPX (250 nM) nor UTP (100 μM) affected the activity of any of the diadenosine polyphosphates (results not shown).

Cortical synaptosomes: effects of diadenosine polyphosphates and ATP derivatives

Application of each of the diadenosine polyphosphates (100 μM) to cortical synaptosomes elicited a Ca^{2+} transient (Fig. 7*A*). Concentration–response relationships were determined for the diadenosine polyphosphates (Fig. 8). There were no significant differences between the EC_{50} values (Table 1). The Hill coefficient for each of these compounds did not differ significantly from 1 (Table 1). At the highest concentration tested (300 μM), the rank order for evoking the Ca^{2+} signal (nM) was: Ap4A (29.0 ± 1.0) = Ap3A (26.9 ± 0.9) $\geqslant$ Ap2A (25.3 ± 1.1) $\geqslant$ Ap6A (21.3 ± 1.3) = Ap5A (20.6 ± 1.0). Ap4A gave significantly greater responses than any other dinucleotide ($P < 0.05$) except Ap3A, and Ap5A gave significantly smaller responses than all others ($P < 0.02$) except Ap6A.

ATP and its synthetic analogues also increased Ca^{2+} levels when applied at a concentration of 100 μM (Fig. 7*B*). Among the mononucleotides, α,β-meATP evoked the greatest response (34.5 ± 2.9 nM, $P < 0.01$ against any

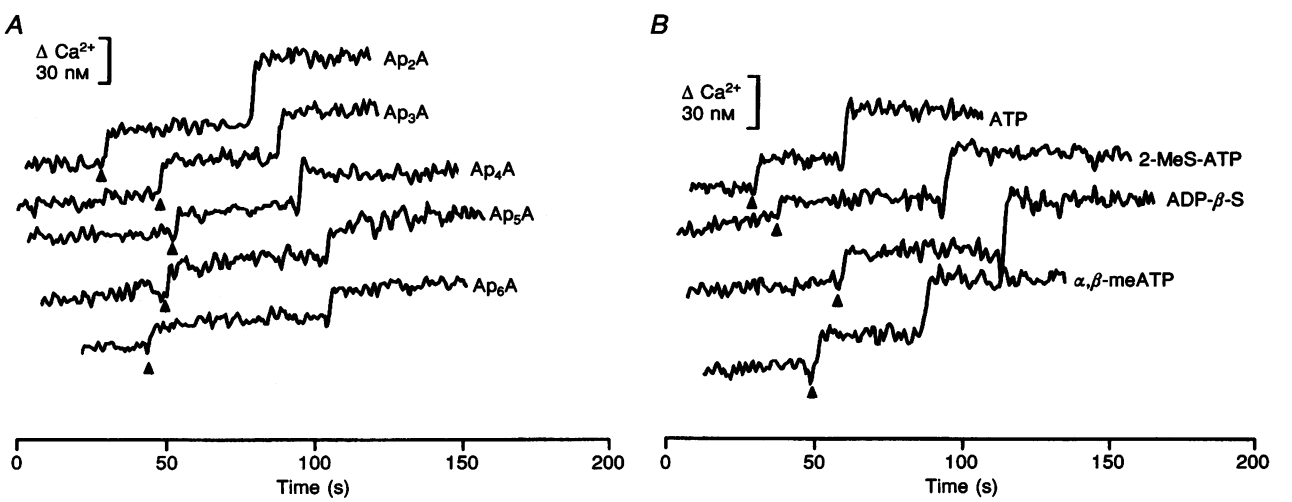

Figure 7. Effects of mono- and dinucleotides on guinea-pig cortical synaptosomal Ca^{2+} levels
A, Ca^{2+} signals elicited by diadenosine polyphosphates (Ap2A–Ap6A) at a concentration of 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal). *B*, synaptosomal Ca^{2+} increases elicited by ATP and its synthetic analogues, 2-MeS-ATP, ADP-β-S and α,β-meATP, all at 100 μM (arrowheads). Synaptosome integrity was tested by applying 60 mM KCl (second increment in Ca^{2+} signal).

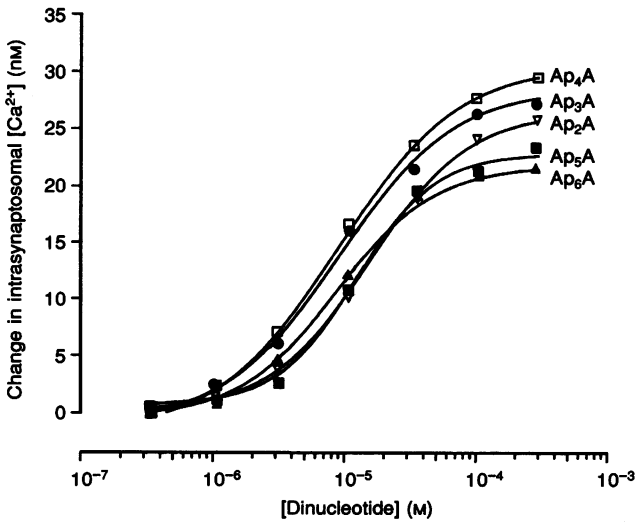

Figure 8. Concentration–response curves for diadenosine polyphosphates in guinea-pig cortical synaptosomes
Ap2A (▽), Ap3A (●), Ap4A (□), Ap5A (■) and Ap6A (▲). Points show means of at least four experiments carried out in duplicate.

other nucleotide), followed by ADP-β-S (23·1 ± 0·8 nM), which was equi-effective with ATP (21·6 ± 1·7 nM), while 2-MeSATP evoked the smallest response (15·7 ± 0·8 nM, $P < 0·02$ against any other nucleotide) in this preparation.

Cortical synaptosomes: cross-desensitization studies and antagonism by suramin

Pre-incubation with the P2 receptor antagonist suramin (100 μM) abolished the Ca^{2+} transients elicited by both Ap4A and ATP (100 μM) (Fig. 9). In cross-desensitization studies, an initial application to cortical synaptosomes of α,β-meATP (100 μM) markedly attenuated the response elicited by Ap4A (100 μM). Similarly, pre-incubation with Ap4A markedly attenuated responses evoked by ATP (Fig. 9).

DISCUSSION

The results show that diadenosine polyphosphates (Ap2A–Ap6A), ATP, α,β-meATP, 2-MeSATP and ADP-β-S can evoke calcium signals in synaptosomes prepared from guinea-pig midbrain, cortex and cerebellum. The pharmacological profile of the activity of the dinucleotides and mononucleotides suggests that these regions of the brain contain different populations of receptors.

In both the midbrain and cerebellar synaptosomal preparations the dinucleotides and mononucleotides appear to be acting via separate receptors. Suramin antagonized responses to ATP but not to Ap4A, and responses to Ap4A were not desensitized by mononucleotides, nor vice versa. In contrast, in the cortical preparations suramin blocked responses to dinucleotides and mononucleotides alike, and responses to these groups of compounds mutually cross-desensitized.

Although the cerebellar and midbrain synaptosomes appeared to contain dinucleotide receptors that were not activated by ATP the pharmacological profile of the dinucleotides was not the same in these two regions. Ap2A was the most potent dinucleotide in the cerebellum but was not in the midbrain; however, it evoked responses of the greatest maximal amplitude in both these regions. This

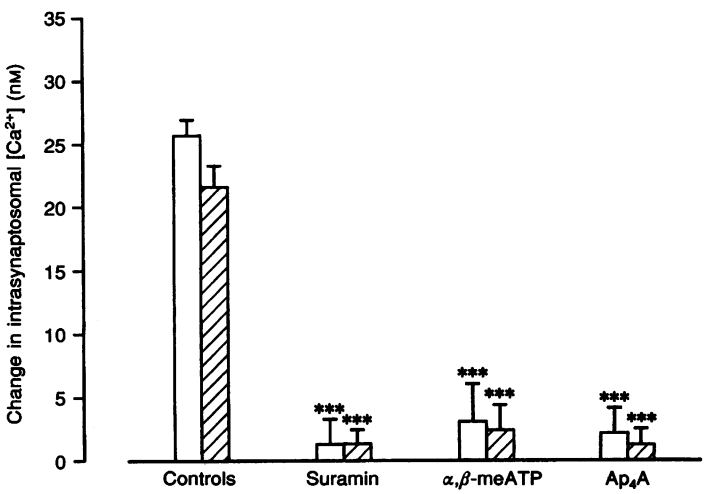

Figure 9. Antagonism and cross-desensitization studies on guinea-pig cortical synaptosomes
The Ca^{2+} signals elicited by Ap4A (100 μM; □) and ATP (100μM; ▨) were assayed in the presence of the P2 receptor antagonist suramin (100 μM). Cross-desensitization studies were performed by pre-incubating synaptic terminals with α,β-meATP (100 μM) and Ap4A (100 μM) as described in Methods. ***$P < 0·001$ *vs.* control.

difference in potency in these two regions, based on EC_{50} values, was approximately 18-fold. For the remaining dinucleotides (Ap3A–Ap6A) there were no significant differences between their respective potencies in these two regions of the brain. In all the synaptosomal preparations the Hill coefficients for each of the dinucleotides did not differ significantly from unity, implying that there is a straightforward 1 : 1 interaction between the agonist molecule and the receptor. However, it must be borne in mind that the Hill coefficient can only be interpreted as indicating the stoichiometry of agonist–receptor binding when the effects of potentially confounding factors are known, such as the receptor transduction mechanism or level of desensitization.

High affinity dinucleotide-specific binding sites have previously been identified in deermouse and rat brain. In deermouse brain membrane preparations the profile for dinucleotide binding is Ap4A > Ap6A = Ap5A > Ap3A ≫ Ap2A (Hilderman *et al.* 1991), while in rat brain subcortical synaptosomes the order is Ap4A > Ap5A ≫ Ap6A (Pintor *et al.* 1993). In the same rat brain preparation Ap4A and Ap5A are equipotent when eliciting Ca^{2+} transients, and they do so via a receptor distinct from that activated by ATP, being resistant to suramin and to desensitization by mononucleotides, and tentatively named 'P_4' (Pintor & Miras-Portugal, 1995*a*, *b*). None of these profiles bears a striking resemblance to any of those obtained in the present functional study, and although, based on EC_{50} values, Ap4A and Ap5A were equipotent in the midbrain and cortical preparations, Ap5A always produced the smallest maximum response.

The dinucleotide-specific receptors in the guinea-pig midbrain and cerebellum appear to be different from those described in other neural tissues. In rat cerebral cortex, hippocampus, cardiac vagal afferents, rabbit cortex and sympathetic nerve terminals in the rat and guinea-pig vas deferens, diadenosine polyphosphates act on an adenosine (or methylxanthine-sensitive) receptor (Stone, 1981; Stone & Perkins, 1981; Klishin *et al.* 1994; von Kügelgen *et al.* 1994; Hoyle, Posterino & Burnstock, 1995; Rubino & Burnstock, 1996). In all these neural systems the dinucleotides inhibit synaptic transmission via a prejunctional receptor. Adrenal medullary chromaffin cells are neural analogues, and these cells possess two dinucleotide binding sites. At the lower affinity site, the order of affinity is: Ap5A > Ap4A > Ap3A > Ap6A, and possibly represents an adenosine receptor since adenosine analogues can displace the dinucleotide binding. At the very high affinity binding site, Ap4A, Ap5A and Ap6A are equipotent, and have an affinity approximately two orders of magnitude greater than that of Ap3A (Pintor, Torres, Castro & Miras Portugal, 1991). Functionally the dinucleotides stimulate basal secretion of catecholamines from adrenal medullary chromaffin cells but inhibit cholinergically stimulated catecholamine release (Castro, Torres, Miras Portugal & Gonzalez, 1990).

In the guinea-pig midbrain and cerebellar synaptosomes the receptor at which the dinucleotides act cannot be interpreted as an adenosine receptor, because the responses to Ap4A were unaffected by the A_1 receptor antagonist DPCPX. Furthermore, UTP did not affect the responses elicited by Ap4A in cerebellar and midbrain synaptosomes. This indicates that the receptor in the present study is not like the Ap4A-sensitive P_{2U} receptor, renamed $P2Y_2$ (see Alexander & Peters, 1997), cloned from the human epithelial CF/T43 cell line (Lazarowski, Watt, Stutts, Boucher & Harden, 1995).

In summary, diadenosine polyphosphates activate a dinucleotide receptor in guinea-pig cerebellar and midbrain synaptic terminals. This receptor is insensitive to ATP, which acts via its own P2 receptor, which is insensitive to diadenosine polyphosphates. In contrast, in guinea-pig cerebral cortical synaptic terminals, diadenosine polyphosphates and ATP appear to act via the same type of P2 receptor.

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