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Diadenosine polyphosphates as antagonists of the endogenous $P2Y_1$ receptor in rat brain capillary endothelial cells of the B7 and B10 clones

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1 Diadenosine polyphosphates (Ap_nAs, $n=2-7$) are considered as stress mediators in the cardiovascular system. They act both *via* identified P2 purinoceptors and *via* yet to be characterized receptors. This study analyses the actions of Ap_nAs in clones of rat brain capillary endothelial cells that express $P2Y_1$ receptors (B10 cells) or both $P2Y_1$ and $P2Y_2$ receptors (B7 cells).

2 B10 cells responded to Ap_3A with rises in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). This response was prevented by adenosine-3′-phosphate-5′-phosphate, an antagonist of $P2Y_1$ receptors. It was largely suppressed by a treatment with apyrase VII or with creatine phosphokinase/creatine phosphate to degrade contaminating ADP.

3 Ap_nAs inhibited ADP induced increases in $[Ca^{2+}]_i$ mediated by $P2Y_1$ receptors by shifting ADP concentration-response curves to larger concentrations. Apparent K_i values were estimated to be 6 μM for Ap_4A, 10 μM for Ap_5A and 47 μM for Ap_6A. Ap_2A and Ap_3A were much less active.

4 Ap_nAs were neither agonists nor antagonists of the endogenous $P2Y_2$ receptor in B7 cells.

5 Ap_nAs are neither agonists nor antagonists of the G_i-coupled, ADP receptor in B10 cells.

6 The results suggest that most actions of Ap_nAs in B7 and B10 cells can be accounted for by endogenous $P2Y_1$ receptors. Ap_4A, Ap_5A and Ap_6A are specific antagonists of endogenous Ca^{2+}-coupled $P2Y_1$ receptors.

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Abbreviations: A2P5P, adenosine-2′-phosphate-5′-phosphate; A3P5P, adenosine-3′-phosphate-5′-phosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PPADS, pyridoxal phosphate-6-azophenyl-2′,4′-sulphonic acid

Introduction

Diadenosine polyphosphates (Ap_nAs, $n=2-7$) form a group of compounds that consist of two adenosine molecules bridged by 2–7 phosphate groups. They are stored in platelet dense granules, in chromaffin granules of the adrenal medulla, in *Torpedo* cholinergic vesicles and in rat brain synaptic terminals. Possible roles as neurotransmitters and stress signals have been proposed (Hoyle, 1990; Hoyle *et al*., 1996; Pintor *et al*., 1997). Actions of Ap_nAs on the cardiovascular system have recently been reviewed (Flores *et al*., 1999). Some of the compounds promote platelet aggregation, while others are inhibitory. Compounds with four or more phosphates are usually vasoconstrictors, while those with three or less phosphates are vasodilators (Ralevic *et al*., 1995). Ap_nAs also produce marked cardiac electrophysiological effects (Flores *et al*., 1999).

Ap_nAs are thought to act *via* known metabotropic and ionotropic purinoceptors of the P2 type. Ap_3A was reported to be an agonist of the chick $P2Y_1$ receptor (Pintor *et al*., 1996) and of the human $P2Y_2$ receptor (Lazarowski *et al*., 1995). Ap_4A was reported to be an agonist of the chick, turkey and human $P2Y_1$ receptors (Pintor *et al*., 1996; Schachter *et al*., 1996), of the human $P2Y_2$ receptor (Lazarowski *et al*., 1995) and of rat $P2X_2$ receptors (Pintor *et al*., 1996). Results obtained with endogenous receptors are less clear. Ap_3A and Ap_4A have been reported to be either active or inactive at the $P2Y_1$ receptor depending on the cell line used (Chen *et al*., 1996; Conant *et al*., 1998; Mateo *et al*., 1996; Stachon *et al*., 1998). Ap_4A was reported to be inactive at endogenous $P2Y_2$ receptors (Chen *et al*., 1996; Conant *et al*., 1998; Mateo *et al*., 1996). Different hypotheses may account for these conflicting results. (i) Contamination of commercially available Ap_nAs by mononucleotides may lead to spurious responses (Conant *et al*., 1998). (ii) P2 receptors may not have been correctly identified in cell lines and tissues. (iii) Properties of the P2 receptors in recombinant expression systems may be different from those in tissues (Hechler *et al*., 1998b; Palmer *et al*., 1998). (iv) Ap_nAs may act *via* receptors that are yet to be identified (Miras-Portugal *et al*., 1996; 1998).

This study analyses the actions of Ap_nAs in two clones of rat brain capillary endothelial cells: B7 and B10 cells. Previous results from this laboratory have shown that while B7 cells express mRNAs that code for $P2Y_1$ and $P2Y_2$ receptors, B10 cells only express $P2Y_1$ receptor transcripts (Feolde *et al*., 1995; Webb *et al*., 1996). Nucleotides have two major actions in B7 and B10 cells. They induce a mobilization of intracellular Ca^{2+} stores and inhibit cholera toxin stimulated adenylyl cyclase *via* a pertussis toxin-sensitive, G_i-mediated, mechanism (Frelin *et al*., 1993; Webb *et al*., 1996). Detailed pharmacological analyses using additivity experiments, desensitization experiments and specific inhibitors of $P2Y_1$ receptors allowed us to attribute these responses to three types of receptors. Intracellular Ca^{2+} rises ($[Ca^{2+}]_i$) in B10 cells have been attributed to the $P2Y_1$ receptor. ADP and 2-methyl-thio-ADP are agonists of this receptor. ATP and its derivatives, pyridoxal phosphate-

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6-azophenyl-2′,4′-sulphonic acid (PPADS), adenosine-2′-phosphate-5′-phosphate (A2P5P) and adenosine-3′-phosphate-5′-phosphate (A3P5P) and their sulphate derivatives are antagonists (Vigne *et al*., 1998b; Hechler *et al*., 1998a). $[Ca^{2+}]_i$ responses in B7 cells have been attributed to the $P2Y_1$ receptor and to an additional receptor that is specific for ATP and UTP on the basis of additivity and desensitization experiments (Frelin *et al*., 1993; Vigne *et al*., 1994). This pharmacological profile and the observation that B7 cells express $P2Y_2$ receptor mRNAs suggest the presence of functional $P2Y_2$ receptors. The G_i-coupled receptor in B10 cells was initially thought to be the $P2Y_1$ receptor (Webb *et al*., 1996) but this conclusion was challenged by more recent experiments using selective antagonists of the $P2Y_1$ receptors (Hechler *et al*., 1998b). Taken together these results have suggested that these clones of endothelial cells express three forms of purinoceptors: (i) a Ca^{2+}-coupled $P2Y_1$ receptor, (ii) a Ca^{2+}-coupled $P2Y_2$ receptor and (iii) a G_i-coupled ADP receptor. It should be noted that the properties of B7 and B10 cells may not be representative of those of native brain capillary endothelial cells which do not express $P2Y_1$ receptors and express an additional $P2Y_4$ receptor (Anwar *et al*., 1999).

Methods

Cell culture

Rat brain capillary endothelial cells of the B7 and B10 clones were cultured as described previously (Vigne *et al*., 1998a). The culture medium was Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Intracellular Ca^{2+} measurements

For intracellular Ca^{2+} measurements, suspended cells were incubated for 30 min in the presence of 5 μM indo-1/AM, centrifuged at $1000 \times g$ and resuspended into an Earle's salt solution (composition in mM): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 5, HEPES 25, pH 7.4) at a density of 10^6 cells ml^{-1}. Nucleotides (10 μl) were added to test tubes and the reaction was initiated by adding 90 μl of the cell suspension. These conditions ensured that cells were exposed at the same time to agonists and antagonists. After mild vortexing, tubes were inserted into a FacStar Plus cytometer (Becton Dickinson) (Vigne *et al*., 1994). Mean fluorescence ratios were determined for 1000 cells sampled between 8 and 10 s after the addition of nucleotides. This time corresponded to the peak of the $[Ca^{2+}]_i$ transient (Vigne *et al*., 1994). Fluorescence ratios were calculated in arbitrary units set to a value of 100 for unstimulated cells. This value takes into account the autofluorescence and the basal indo-1 fluorescence. Using these settings, ADP (1 μM) or UTP (0.1 mM, in B7 cells only) increased the indo-1 fluorescence ratio to 200–250 arbitrary units. Responses were calculated as the difference between the fluorescence units observed in stimulated cells and those observed in control unstimulated cells. Means of triplicate measurements were used. Controls were included in each experimental protocol. All responses were expressed as a percentage of the response to 1 μM ADP, typically 100–150 arbitrary units. Experiments were repeated at least three times using different batches of cells.

Adenylyl cyclase assays

B10 cells grown in 6-well tissue culture clusters were labelled with 1 μCi ml^{-1} of [^{3}H]-Adenine (Amersham, 26 Ci $mmol^{-1}$) overnight in complete culture medium. The culture medium was then removed, cells were rinsed with an Earle's salt solution and further incubated into an Earle's salt solution supplemented with 0.1 mM isobutylmethylxanthine. After 10 min at 37°C, cells were stimulated with 1 μM forskolin in the absence or the presence of diadenosine polyphosphates (0.1 mM) and or of ADP (5 μM). After 5 min, the incubation solution was aspirated off and the reaction was stopped by addition of 500 μl of ice cold 5% trichloracetic acid supplemented with 2 mM ATP and 2 mM cyclic AMP (used as carriers). After 15 min of extraction at 4°C, cell extracts were harvested and the tritium label associated with cyclic AMP was determined using sequential chromatographies on Dowex and alumina columns (Bozou *et al*., 1986). In some experiments a protein-binding assay (Immunotech, Marseille, France) was used.

Materials

Ap_nAs, adenosine-3′-phosphate-5′-phosphate, ADP, UTP, creatine, creatine phosphate, creatine phosphokinase (Type III from bovine heart), indo-1/AM, forskolin, isobutylmethylxanthine and apyrase VII were purchased from the Sigma Chemical Co. Reagents were dissolved into an Earle's salt solution. In some experiments it was necessary to purify solutions of Ap_nAs from contaminating ADP (see Results). Solutions of Ap_3A and Ap_6A (1 mM in Earle's salt solution) were treated for 20 min with 1 unit ml^{-1} apyrase VII or with a mixture of 20 units ml^{-1} creatine phosphokinase and 10 mM creatine phosphate. The mixture was then added to the cells without inactivation of the enzymes. We checked that these treatments abolished the Ca^{2+} mobilizing action of a 10 μM solution of ADP. To remove trace contaminants of ATP, solutions of Ap_nAs were treated for 30 min with a mixture of 20 units ml^{-1} creatine phosphokinase and 10 mM creatine.

Curve fitting and statistical analysis

Curves were fitted with a logistic equation using the SigmaPlot software (Jandel Scientifics). Mean ± s.e.mean from *n* indepen-

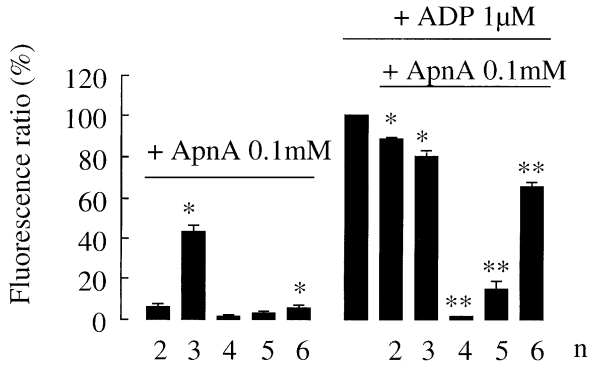

Figure 1 Actions of Ap_nAs on $[Ca^{2+}]_i$ in B10 cells. Indo-1 loaded cells were incubated in the presence of 0.1 mM Ap_nA or of 1 μM ADP and 0.1 mM Ap_nAs and mean indo-1 fluorescence ratio were recorded at 10 s. Means ± s.e.mean ($n = 3$) are shown. Increases in indo-1 fluorescence ratio were expressed relative to that produced by 1 μM ADP. $^*P < 0.05$ and $^{**}P < 0.01$ as compared to controls. *n* on the abscissa means the number of phosphate groups in the Ap_nAs tested.

dent experiments are indicated. Statistical analysis was performed by use of Student's *t*-test.

Results

Actions of Ap_nAs on $[Ca^{2+}]_i$ in B10 cells

Actions of Ap_nAs on intracellular Ca^{2+} were analysed using indo-1 loaded B10 cells. Figure 1 shows that only Ap_3A and Ap_6A increased $[Ca^{2+}]_i$ in B10 cells. Ca^{2+} transients induced by Ap_3A had the same shape and time course as ADP-induced Ca^{2+} transients and were largely independent of the presence of extracellular Ca^{2+} (data not shown). The efficacy of 0.1 mM Ap_3A was only 44% that of 1 μM ADP, the natural agonist of $P2Y_1$ receptors (Figure 1). Actions of Ap_3A were prevented by 0.1 mM A3P5P, an antagonist of $P2Y_1$ receptors. They were also highly variable from one batch of compound to the other. These suggested that the $[Ca^{2+}]_i$ responses to Ap_3A could be due to contaminating ADP. Considering that a concentration of 0.7 μM ADP induced half maximum activation of $P2Y_1$ receptors in B10 cells (see below), it can be calculated that the $[Ca^{2+}]_i$ rise induced by 0.1 mM Ap_3A could be accounted for by only a 0.2% contamination of Ap_3A by ADP. To check the hypothesis that actions of Ap_3A were due to contaminant ADP, solutions of Ap_3A were treated with ADP degrading enzymes. Creatine phosphokinase (in the presence of creatine phosphate) decreased the efficacy of 0.1 mM Ap_3A from 44 to 10% that of 0.1 mM ADP. Conversely apyrase decreased the efficacy of 0.1 mM Ap_3A from 44 to 7% that of 1 μM ADP. These results suggested that low amounts of ADP contaminated Ap_3A solutions and were probably responsible for most of the observed rises in $[Ca^{2+}]_i$. The action of 0.1 mM Ap_6A which was at the limit of detectability was not investigated.

We next looked for possible antagonistic actions of Ap_nAs on $P2Y_1$ responses using ADP, the natural agonist of $P2Y_1$ receptors. The EC_{50} value for the action of ADP was 0.7 ± 0.1 μM ($n=9$) in this series of experiments. Cells were stimulated with a submaximally effective concentration of ADP (1 μM) in the presence of 0.1 mM and Ap_nAs. These experiments do not require the use of enzymatically purified Ap_nAs for 1 μM ADP was already present in the assay medium to stimulate receptors. Figure 1 shows that all Ap_nAs inhibited the action of ADP. Inhibition was almost complete with Ap_4A and Ap_5A. Actions of Ap_2A and Ap_3A were not analysed.

Figure 2A shows concentration-response curves for the action of ADP in the absence or the presence of Ap_4A. Ap_4A (0.1 mM) shifted the ADP concentration-response curve to larger concentrations without modifying the maximum efficacy of ADP. This suggested an apparent competitive mechanism. The 13 fold shift led us to calculate a pA_2 value for Ap_4A of 5.9 corresponding to an apparent K_i value of 8 μM. Figure 2B shows the concentration-response curve for the inhibition by Ap_4A of ADP (1 μM) responses. In three independent experiments such as those presented in Figure 2B, the concentration of Ap_4A that produced half-maximum inhibition of the action of ADP (IC_{50}) was 11.4 ± 1.5 μM. An apparent K_i value for Ap_4A was deduced from the Cheng-Prusoff relationship:

$$IC_{50} = Ki * \left(1 + \frac{[ADP]}{K_{(ADP)}}\right)$$

Considering that $K_{(ADP)}$ was 0.7 μM, [ADP] was 1 μM and IC_{50} was 11.4 μM, a K_i value of 5 μM was calculated. It was close to the value found in the preceding experiment.

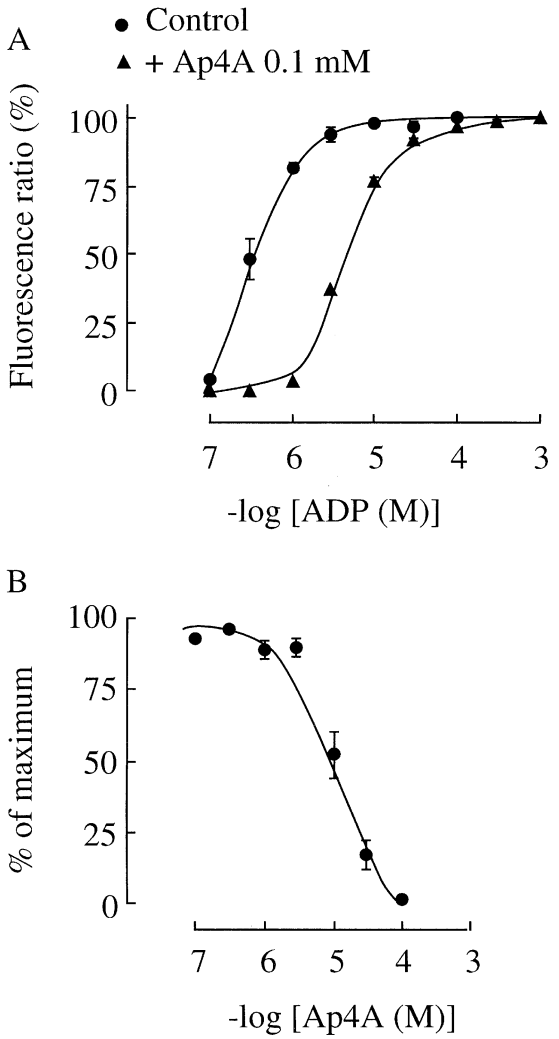

Figure 2 Antagonistic actions of Ap_4A in B10 cells. (A) Concentration-response curves for the action of ADP in the absence or the presence of 0.1 mM Ap_4A. EC_{50} values for the action of ADP were 0.40 and 5.2 μM in the absence and the presence of Ap_4A respectively. Data were normalized to the effect of 1 mM ADP in the absence of Ap_4A. (B) Concentration-response curve for the inhibition by Ap_4A of ADP (1 μM) responses. Means $\pm$ s.e.mean ($n=3$) are shown.

Figure 3A shows that Ap_5A (0.2 mM) shifted the ADP concentration-response curve to larger concentrations without modifying the maximum efficacy of ADP. The 16 fold increase in EC_{50} value indicated a pA_2 value for Ap_5A of 4.9 corresponding to an apparent K_i value of 13 μM. Figure 3B shows the concentration-response curve for the inhibition by Ap_5A of ADP (1 μM) responses. Half-maximum inhibition was observed at 20.0 ± 1.6 μM Ap_5A ($n=3$). Application of the Cheng-Prusoff relationship led to an apparent K_i value of 8 μM, close to the value found in the preceding experiment.

Figure 4A shows that Ap_6A (0.2 mM) shifted the ADP concentration-response curve to larger concentrations without modifying the maximum efficacy of ADP. The 9 fold increase in EC_{50} value indicated a pA_2 value for Ap_6A of 4.6 corresponding to an apparent K_i value of 25 μM. Figure 4B shows the concentration-response curve for the inhibition by Ap_6A of ADP responses. Half-maximum inhibition was observed at 168 ± 14 μM Ap_6A ($n=3$). Application of the Cheng-Prusoff relationship led to an apparent K_i value of 69 μM.

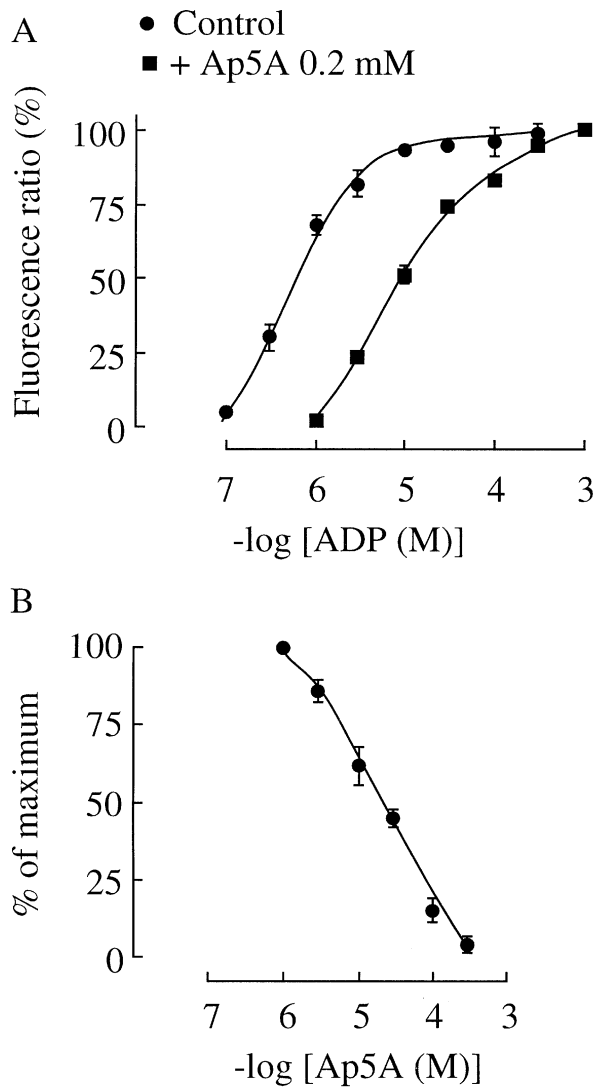

Figure 3 Antagonist actions of Ap_5A in B10 cells. (A) Concentration-response curves for the action of ADP in the absence or the presence of 0.2 mM Ap_5A. EC_{50} values for the action of ADP were 0.6 and 9.7 μM in the absence and the presence of Ap_5A respectively. Data were normalized to the effect of 1 mM ADP in the absence of Ap_5A. (B) Concentration-response curve for the inhibition by Ap_5A of ADP (1 μM) responses. Means$\pm$s.e.mean ($n=3$) are shown.

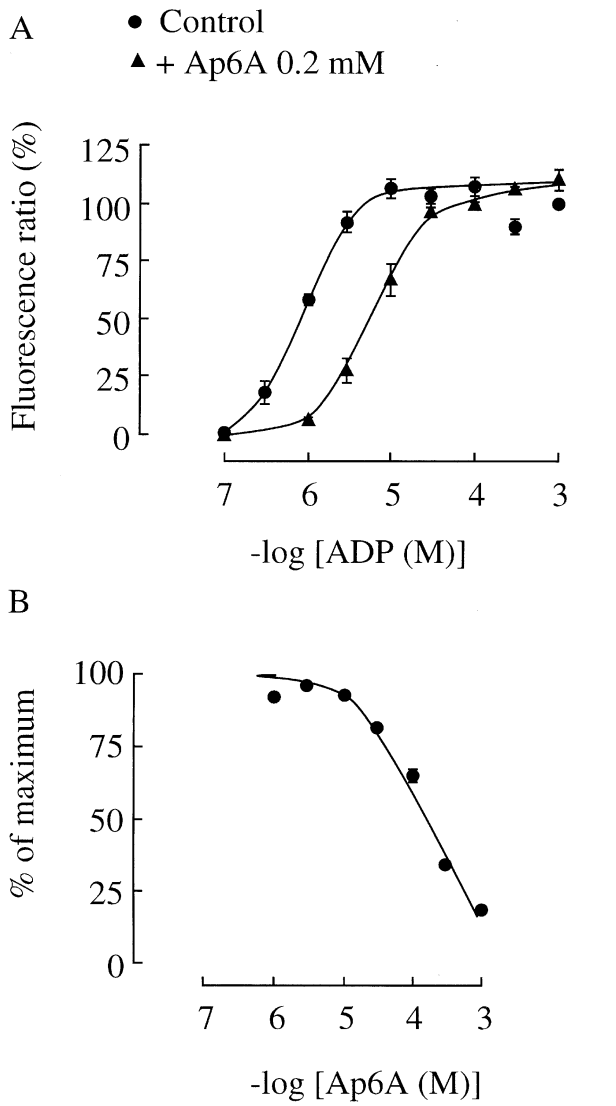

Figure 4 Antagonist actions of Ap_6A in B10 cells. (A) Concentration-response curves for the action of ADP in the absence or the presence of 0.2 mM Ap_6A. EC_{50} values for the action of ADP were 0.8 and 7.5 μM in the absence and the presence of Ap_6A respectively. Data were normalized to the effect of 1 mM ADP in the absence of Ap_6A. (B) Concentration response curve for the inhibition by Ap_6A of ADP (1 μM) responses. Means$\pm$s.e.mean ($n=3$) are shown.

A major difficulty encountered with purinergic ligands is that actions measured could be due to other nucleotides present in commercial powders or produced for instance by cellular ecto-enzymes. We checked that AMP and adenosine (up to 1 mM) did not inhibit ADP responses in endothelial cells and were thus unlikely to mediate inhibitory actions of Ap_4A, Ap_5A and Ap_6A. We also checked that purification of Ap_nA solutions from ATP using creatine phosphokinase and creatine did not modify the properties of antagonistic Ap_nAs. Taken together these indicated that inhibitory actions of Ap_nAs in endothelial cells were unlikely to be mediated by adenosine, AMP or ATP.

Actions of Ap_nAs in B7 cells

Identical experiments were performed using cells of the B7 clone. These cells were of interest because they express functional $P2Y_2$ receptors in addition to $P2Y_1$ receptors. Figure 5A shows that Ap_3A and Ap_6A induced increases in $[Ca^{2+}]_i$ that were of smaller amplitude than those induced by ADP. Other Ap_nAs were inactive. These actions were decreased to less than 10% of the amplitude of the ADP (1 μM) signal after enzymatic degradation of contaminating ADP using creatine phosphokinase or apyrase. Figure 5A further shows that Ap_nAs did not antagonize UTP responses. UTP and some Ap_nAs (Ap_3A, Ap_5A and Ap_6A) had additive actions on $[Ca^{2+}]_i$. The reasons for these differences was not investigated. It could simply be due to the fact that UTP and a contaminant present in Ap_nA solutions (presumably ADP) activated two independent receptors. Finally, Figure 5B shows that Ap_4A blocked ADP-responses mediated by $P2Y_1$ receptors with properties similar to those observed in B10 cells. UTP responses were insensitive to Ap_4A. These results indicate that Ap_nAs had neither agonistic nor antagonistic actions at the endogenous $P2Y_2$ receptor in B7 cells.

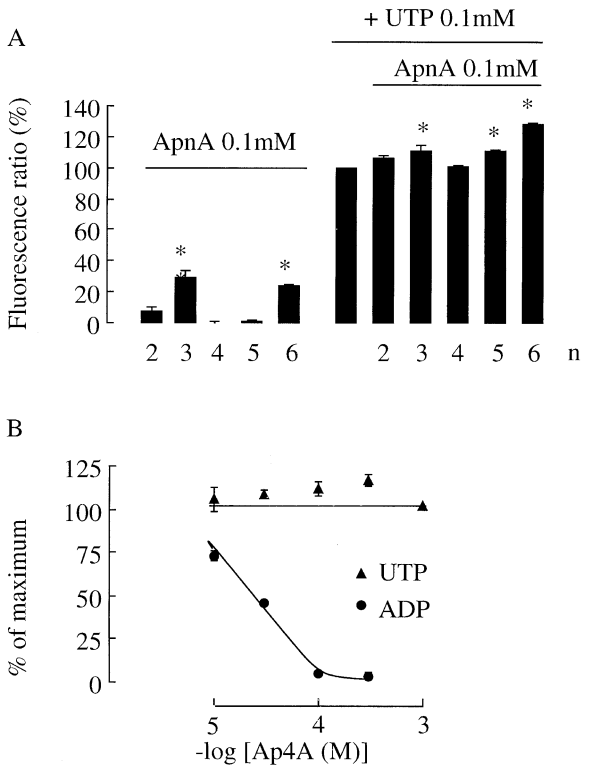

Figure 5 Actions of Ap_nAs in B7 cells in B10 cells. (A) Indo-1 loaded cells were incubated in the presence of 0.1 mM Ap_nAs or of 1 μM UTP and 0.1 mM Ap_nAs and mean indo-1 fluorescence ratio were recorded at 10 s. Increases in indo-1 fluorescence ratio were expressed relative to that produced by 0.1 mM UTP. (B) Concentration-response curves for the actions of Ap_4A on ADP (1 μM) and UTP (0.1 mM) responses. $^*P<0.05$ and $^{**}P<0.01$ as compared to controls. *n* on the abscissa means the number of phosphate groups in the Ap_nAs tested. Means$\pm$s.e.mean ($n=3$) are shown.

Actions of Ap_nAs on adenylyl cyclase in B10 cells

Formation of cyclic AMP can be assessed either by protein binding assays or by following the conversion of [^{3}H]-ATP into [^{3}H]-cyclic AMP. The first technique led us to the conclusion that Ap_4A, Ap_5A and Ap_6A (at concentrations >10 μM) stimulated adenylyl cyclase. Further studies showed however that this conclusion was erroneous and due to the fact that Ap_nAs recognized the antibody directed against cyclic AMP and interfered with the assay. When adenylyl cyclase activity was measured from the conversion of [^{3}H]-ATP into [^{3}H]-cyclic AMP, no stimulatory activity of Ap_nAs could be detected. We also observed that in contrast to ADP, Ap_nAs (0.1 mM) did not inhibit forskolin stimulated adenylyl cyclase activity. Finally, we checked that Ap_nAs (0.1 mM) did not inhibit ADP-induced inhibition of forskolin-stimulated adenylyl cyclase (data not shown). These results indicated that Ap_nAs are neither agonists nor antagonists of the G_i-coupled ADP receptor.

Discussion

This paper first shows that Ap_nAs are not agonists of endogenous $P2Y_1$ and $P2Y_2$ receptors in endothelial cell clones. Weak actions of Ap_3A and Ap_6A were observed but they were likely due to low amounts of contaminating ADP. The lack of clear agonist action of Ap_nAs was surprising. Ap_3A and Ap_4A have been reported to stimulate recombinant $P2Y_1$ and $P2Y_2$ receptors as well as the endogenous $P2Y_1$ receptors in ECV305 endothelial cells (Lazarowski *et al*., 1995; Pintor *et al*., 1996; Schachter *et al*., 1996; Mateo *et al*., 1996; Stachon *et al*., 1998; Conant *et al*., 1998). Our results are however in agreement with previous reports indicating no action of Ap_4A on other endogenous $P2Y_2$ receptors (Chen *et al*., 1996; Conant *et al*., 1998; Mateo *et al*., 1996). Reasons for these differences are discussed below.

A second observation of this paper is that Ap_4A, Ap_5A and Ap_6A inhibit ADP responses in B10 cells. Knowing that ADP responses in these cells are sensitive to PPADS (Vigne *et al*., 1998a,b), A2P5P and A3P5P (Hechler *et al*., 1998a) and that these cells only express mRNA species that code for $P2Y_1$ receptors (Webb *et al*., 1996), an obvious conclusion is that Ap_4A, Ap_5A and Ap_6A inhibit $P2Y_1$ receptors. This conclusion is consistent with the results of experiments using platelets. Ap_4A, Ap_5A and Ap_6A have been reported to prevent platelet aggregation to ADP (Harrison *et al*., 1975). Ap_5A was further described as a competitive antagonist of ADP-induced rises in $[Ca^{2+}]_i$ in human platelets (Hall & Hourani, 1993). This action was initially attributed to the platelet P_{2T} receptor. It is now known to be mediated by $P2Y_1$ receptors (Daniel *et al*.,1998; Fagura *et al*., 1998; Hechler *et al*., 1998a; Jin *et al*., 1998).

A recent paper (King *et al*., 1999) established that diinosine polyphosphates but not Ap_nAs were antagonists at $P2X_1$ and $P2X_3$ receptors. It would be of interest to test the possibility that Ap_nAs are deaminated by endothelial cells and that actions of Ap_nAs are in fact mediated by the corresponding diinosine polyphosphates.

We have previously described that ATP is a natural antagonist of the endogenous $P2Y_1$ receptor in B10 cells. The K_i value for ATP is 23 μM (Hechler *et al*., 1998b). Ap_4A and Ap_5A are slightly more potent than ATP with apparent K_i values of 6–10 μM. These values clearly rule out the possibility that the observed antagonistic actions were mediated by contaminating ATP. Ap_6A is less potent than ATP. Synthetic antagonists of the endogenous $P2Y_1$ receptor in B10 cells have previously been described. These are 2-chloro-ATP ($K_i=27$ μM), 2-methylthio-ATP ($K_i=36$ μM), benzoylATP ($K_i=5$ μM), A2P5P ($K_i=5$ μM) and A3P5P ($K_i=3$ μM) (Hechler *et al*., 1998a,b; Vigne *et al*., 1999). Ap_4A is thus as potent as adenosine biphosphates.

These results indicate that Ap_nAs ($n=4-6$) are antagonists of the endogenous $P2Y_1$ receptor in B7 in B10 cells and in platelets. They are agonists of $P2Y_1$ receptors expressed in recombinant expression systems (see Introduction). Similar discrepancies have already been noticed with ATP (Hechler *et al*., 1998b; Palmer *et al*., 1998), benzoyl ATP (Boyer *et al*., 1996; Vigne *et al*., 1999), A2P5P, A3P5P and their sulphate derivatives (Boyer *et al*., 1996; Hechler *et al*., 1998a; Jin *et al*., 1998). One possibility could be that many adenine nucleotides (except ADP and 2-methylthio-ADP) are in fact partial agonists of $P2Y_1$ receptors and that receptor densities determine whether nucleotides have agonist or antagonist properties (Palmer *et al*., 1998). An agonist activity may prevail in the presence of a large receptor reserve. An antagonist activity may prevail at low receptor densities. This hypothesis cannot be tested in the absence of suitable radioligands to titrate receptors.

Another proposed action of adenine nucleotides in B10 cells is to inhibit adenylyl cyclase (Webb *et al*., 1996). This paper shows that Ap_nAs have no action (either stimulatory or inhibitory) on adenylyl cyclase in B10 cells. This suggested that Ap_nAs do not recognise the G_i-coupled, ADP receptor in B10 cells. Thus it may be concluded that ADP recognises two types of receptors in B10 cells (i) a $[Ca^{2+}]_i$ coupled receptor which is

sensitive to Ap_nAs and (ii) a Gi-coupled receptor which is insensitive to Ap_nAs. A similar conclusion was drawn previously from studies using PPADS, A2P5P and A3P5P. These compounds inhibit ADP-induced intracellular Ca^{2+} mobilization in B10 cells. They do not inhibit ADP-induced inhibition of adenylyl cyclase (Webb *et al*., 1996; Hechler *et al*., 1998a).

In conclusion, this study indicates that all actions of Ap_nAs in B7 and B10 cells can be accounted for by the endogenous $P2Y_1$ receptors. Ap_4A, Ap_5A and Ap_6A are antagonists of this receptor.

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References

ANWAR, Z., ALBERT, J., GUBBY, S.E., BOYLE, J.P., ROBERTS, J.A., WEBB, T.E. & BOARDER, M.R. (1999). Regulation of cyclic AMP by extracellular ATP in cultured brain capillary endothelial cells. *Br. J. Pharmacol.,* **128,** 465–471.

BOYER, J.L., ROMERO-AVILA, T., SCHACHTER, J.B. & HARDEN, T.K. (1996). Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol. Pharmacol.,* **50,** 1323–1329.

BOZOU, J.C., AMAR, S., VINCENT, J.P. & KITABGI, P. (1986). Neurotensin mediated inhibition of cyclic AMP formation in neuroblastoma N1E115 cells: Involvement of the inhibitory GTP-binding component of adenylate cyclase. *Mol. Pharmacol.,* **29,** 489–496.

CHEN, B.C., LEE, C.M., LEE, Y.T. & LIN, W.W. (1996). Characterization of signaling pathways of P2Y and P2U purinoceptors in bovine pulmonary artery endothelial cells. *J. Cardiovasc. Pharmacol.,* **28,** 192–199.

CONANT, A.R., FISCHER, M.J., MCLENNAN, A.G. & SIMPSON, A.W.M. (1998). Characterization of the P2 receptors on the human umbilical vein endothelial cell line ECV304. *Br. J. Pharmacol.,* **125,** 357–364.

DANIEL, J.L., DANGELMAIER, C., JIN, J., ASHBY, B., SMITH, J.B. & KUNAPULI, S.P. (1998). Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. *J. Biol. Chem.,* **273,** 2024–2029.

FAGURA, M.S., DAINTY, I.A., MCKAY, G.D., KIRK, I.P., HUMPHRIES, R.G., ROBERTSON, M.J., DOUGALL, I.G. & LEFF, P. (1998). $P2Y_1$-receptors in human platelets which are pharmacologically distinct from P2YADP-receptors. *Br. J. Pharmacol.,* **124,** 157–164.

FEOLDE, E., VIGNE, P., BREITTMAYER, J.P. & FRELIN, C. (1995). ATP, a partial agonist of atypical P2Y purinoceptors in rat brain capillary endothelial cells. *Br. J. Pharmacol.,* **115,** 1199–1203.

FLORES, N.A, STAVROU, B.M. & SHERIDAN, D.J. (1999). The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc. Res.,* **42,** 15–26.

FRELIN, C., BREITTMAYER, J.P. & VIGNE, P. (1993). ADP induces inositol phosphate independent intracellular Ca^{2+} mobilization in brain capillary endothelial cells. *J. Biol. Chem.,* **268,** 8787–8792.

HALL, D.A. & HOURANI, S.M.O. (1993). Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P2T-purinoceptors on human platelets. *Br. J. Pharmacol.,* **108,** 728–733.

HARRISON, M.J., BROSSMER, R. & GOODY, R.S. (1975). Inhibition of platelet aggregation and the platelet release reaction by diadenosine polyphosphates. *FEBS Lett.,* **54,** 57–60.

HECHLER, B., LÉON, C., VIAL, C., VIGNE, P., FRELIN, C., CAZENAVE, J.P. & GACHET, C. (1998a). The $P2Y_1$ receptor is necessary for ADP induced platelet aggregation. *Blood,* **92,** 152–159.

HECHLER, B., VIGNE, P., LÉON, C., BREITTMAYER, J.P., GACHET, C. & FRELIN, C. (1998b). ATP derivatives are antagonists of the $P2Y_1$ receptor; similarities to the platelet ADP receptor. *Mol. Pharmacol.,* **53,** 727–733.

HOYLE, C.H.V. (1990). Pharmacological activity of adenosine dinucleotides in the periphery: possible receptor classes and transmitter function? *Gen. Pharmacol.,* **21,** 827–831.

HOYLE, C.H.V., ZIGANSHIN, A.U., PINTOR, J. & BURNSTOCK, G. (1996). The activation of P1- and P2-purinceptors in the guinea pig left atrium by diadenosine polyphosphates. *Br. J. Pharmacol.,* **118,** 1294–1300.

JIN, J., DANIEL, J.L. & KUNAPULI, S.P. (1998). Molecular basis for ADP-induced platelet activation. II The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J. Biol. Chem.,* **273,** 2030–2034.

KING, B.F., LIU, M., PINTOR, J., GUALIX, J., MIRAS-PORTUGAL, M.T. & BURNSTOCK, G. (1999). Diinosine pentaphosphate (IP_5I) is a potent antagonist at recombinant rat $P2X_1$ receptors. *Br. J. Pharmacol.,* **128,** 981–988.

LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the clones human P2U-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.,* **116,** 1619–1627.

MATEO, J., MIRAS-PORTUGAL, M.T. & CASTRO, E. (1996). Coexistence of P2Y- and PPADS-insensitive P2U-purinoceptors in endothelial cells from adrenal medulla. *Br. J. Pharmacol.,* **119,** 1223–1232.

MIRAS-PORTUGAL, M.T., CASTRO, E., MATEO, J. & PINTOR, J. (1996). The diadenosine polyphosphate receptors; P2D purinoceptors. *Ciba Found Symp.,* **198,** 35–47.

MIRAS-PORTUGAL, M.T., GUALIX, J. & PINTOR, J. (1998). The neurotransmitter role of diadenosine polyphosphates. *FEBS Lett.,* **430,** 78–82.

PALMER, R.K., BOYER, J.L., SCHACHTER, J.B., NICHOLAS, R.A. & HARDEN, T.K. (1998). Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol. Pharmacol.,* **54,** 1118–1123.

PINTOR, J., HOYLE, C.H.V., GUALIX, J. & MIRAS-PORTUGAL, M.T. (1997). Diadenosine polyphosphates in the central nervous system. *Neurosci. Res. Commun.,* **20,** 69–78.

PINTOR, J., KING, B.F., MIRAS-PORTUGAL, M.T. & BURNSTOCK, G. (1996). Selectivity and activity of adenine dinucleotides at recombinant $P2X_2$ and $P2Y_1$ purinoceptors. *Br. J. Pharmacol.,* **119,** 1006–1012.

RALEVIC, V., HOYLE, C.H. & BURNSTOCK, G. (1995). Pivotal role of phosphate chain length in vasoconstrictor versus vasodilator actions of adenine dinucleotides in rat mesenteric arteries. *J. Physiol.,* **483,** 703–713.

SCHACHTER, J.B., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human $P2Y_1$-purinoceptor. *Br. J. Pharmacol.,* **118,** 167–173.

STACHON, A., STEGEMANN, H., HOHAGE, H., RAHN, K.H. & SCHLATTER, E. (1998). Effects of diadenosine polyphosphates on the intracellular Ca^{2+} concentration in endothelial cells. *Cell. Physiol. Biochem.,* **8,** 175–184.

VIGNE, P., BREITTMAYER, J.P. & FRELIN, C. (1998a). Analysis of the influence of nucleotidases on the apparent activity of exogenous ATP and ADP at P2Y1 receptors. *Br. J. Pharmacol.,* **125,** 675–680.

VIGNE, P., FEOLDE, E., BREITTMAYER, J.P. & FRELIN, C. (1994). Characterization of the effects of 2-methylthio-ATP and 2-chloro-ATP on brain capillary endothelial cells. Similarities to ADP and differences from ATP. *Br. J. Pharmacol.,* **112,** 775–780.

VIGNE, P., HECHLER, B., GACHET, C., BREITTMAYER, J.P. & FRELIN, C. (1999). Benzoyl ATP is an antagonist of rat and human $P2Y_1$ receptors and of platelet aggregation. *Biochem. Biophys. Res. Commun.,* **256,** 94–97.

VIGNE, P., PACAUD, P., LOIRAND, G., BREITTMAYER, J.P. & FRELIN, C. (1998b). PPADS inhibits $P2Y_1$ purinoceptors in rat brain capillary endothelial cells and in rat ileal myocytes by an indirect mechanism. *Biochem. Biophys. Res. Commun.*, **244,** 332–335.

WEBB, T.E., FEOLDE, E., VIGNE, P., NEARY, J.T., RUNBERG, A., FRELIN, C. & BARNARD, E. (1996). The P2Y purinoceptor in rat brain microvascular endothelial cells couples to inhibition of adenylate cyclase. *Br. J. Pharmacol.*, **119,** 1385–1392.

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