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ATP, a partial agonist of atypical P_{2Y} purinoceptors in rat brain microvascular endothelial cells

Erick Feolde, Paul Vigne, *Jean Philippe Breittmayer & [1]Christian Frelin

Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, Université de Nice-Sophia Antipolis, 660 Route des Lucioles, 06560 Valbonne, France and *Inserm U343, Hopital de l'Archet, BP 79,06202 Nice Cedex 3, France

1 Purinoceptor responses were analyzed in B10 cells, a clonal population of rat brain capillary endothelial cells.

2 B10 cells lack P_{2U} receptors as evidenced by the lack of UTP responses and the failure to amplify P_{2U}-related sequences by polymerase chain reaction.

3 B10 cells responded to adenine nucleotides by large increases in $[Ca^{2+}]_i$. Half maximum effective concentrations were 2-methylthio-ATP: 180 nM > 2-chloro-ATP: 310 nM = ADP: 330 nM > adenosine 5'-O-(3-thiotrisphosphate): 2.3 μM = ATP: 2.7 μM. The maximum response to ATP was only 55% of that to ADP while that to ATP derivatives was 75%.

4 The actions of adenine nucleotides were not associated with a measurable activation of phospholipase C.

5 Cross desensitizations of the actions of ADP and ATP were observed.

6 In additivity experiments, ADP superposed its action on top of that of ATP and ATP partially inhibited the action of ADP.

7 It is concluded that ATP acts as a partial agonist of the P_{2Y}-like receptor of brain capillary endothelial cells.

Keywords: Purinoceptor; ADP; 2-methylthio-ATP; 2-chloro-ATP; endothelial cells

Introduction

Purinoceptors of the P_2 type constitute a new class of receptors that recognize adenine nucleotides and UTP and that mediate the actions of nucleotides in many cell types. Their structures and the intracellular signalling pathway they control are only partially known. Five subtypes of P_2 purinoceptors are now recognized (Dubyak & El-Moatassim, 1993; Dalziel & Westfall, 1994; Fredholm *et al.*, 1994). P_{2X} receptors are receptor-operated channels that are selectively activated by α,β methylene ATP (Brake *et al.*, 1994; Valera *et al.*, 1994). P_{2Y} receptors are seven transmembrane domain receptors coupled to phospholipase C (Webb *et al.*, 1993; Filtz *et al.*, 1994) that recognize 2-methylthio-ATP (2MeSATP), ADP and ATP and that probably comprise several members. P_{2U} receptors are seven transmembrane domain receptors coupled to phospholipase C and that mainly recognize ATP and UTP (Lustig *et al.*, 1993; Parr *et al.*, 1994). The nature of P_{2T} receptors that specifically recognize ADP is still unclear (Hourani & Hall, 1994). Finally, P_{2Z} receptors are receptor-operated channels.

In previous papers, we showed that adenine nucleotides and UTP increase $[Ca^{2+}]_i$ in rat brain capillary endothelial cells (BCEC) and that these responses could be accounted for by the presence of two distinct receptors: a P_{2U} purinoceptor and a P_{2Y} like receptor (Frelin *et al.*, 1993; Vigne *et al.*, 1994). In subsequent experiments we analyzed the purinoceptor responses of different clones of rat BCEC in order to find cells that lack P_{2U} receptors and that only express the P_{2Y} like receptor. This paper describes the properties of such a cell clone and defines the properties of interaction of adenine nucleotides with the atypical P_{2Y} receptor. The results show that while ADP is a full agonist at this receptor, ATP and its derivatives are partial agonists.

[1] Author for correspondence.

Methods

Clonal populations of BCEC were prepared as previously described (Vigne *et al.*, 1989) and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% foetal bovine serum (Dutscher, Strasbourg, France), 100 units ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin.

Total RNA was isolated as described by Chomczynski & Sacchi (1987). cDNA was synthesized from 1 μg of total RNA using the Moloney Murine Leukaemia Virus reverse transcriptase (GIBCO BRL, 200 units/assay) in the presence of 1 μg oligo dT, dNTPs (0.4 mM each), and 40 units RNAse inhibitor (RNasin, Promega). After a 5 min denaturation at 85°C, dNTPs and the enzyme were added, the reaction was allowed to proceed for 1 h at 37°C and stopped by heating to 95°C for 5 min. Amplification was performed using 1/10 volume of the reverse transcription reaction mixture, 100 ng of each primer, 400 μM of each dNTPs and 1 unit of Goldstar DNA polymerase (Eurogentec) in a total volume of 20 μl and using a thermo cycler (Biometra) programmed with the following conditions: 3 min denaturation at 98°C followed by 35 cycles of 30 s at 94°C, 90 s at 62°C, 90 s at 72°C. The sense primer (primer No. 1) was 5'-CACSTGCATMAGCGTGCA-3'. Antisense primers were primer No. 2: 5'-ACGTGGAARG-GSAGRWAG-3' or primer No. 3: 5'-CTCTACTTCCTGG-CMGGG-3'. Products from the polymerase chain reaction were resolved on an agarose gel by electrophoresis, transferred to Nytran nylon membrane (Schleicher & Schnell) and hybridized with a P_{2U} receptor probe kindly provided by Dr D. Julius. Washes were performed at 42°C, for 30 min with different concentrations of SSPE solution (180 mM NaCl, 10 mM NaH_2PO_4. H_2O, 0.025 mM EDTA) buffered at pH 7.5 with NaOH and containing 0.1% sodium dodecyl sulphate: 2X SSPE, 1X SSPE, 0.5X SSPE and 0.2X SSPE, one time each. Amplification products, purified by electrophoresis on low melting agarose gels (NuSieve GTG agarose, FMC Bioproducts), were also subcloned into the SmaI site of bluescript SK(−) plasmid (Stratagene). Insert positive plasmids were sequenced as double strand using T7 sequencing DNA kits (Pharmacia).

To measure the production of total inositol phosphates, confluent monolayers of cells in 6 well Falcon plates were labelled to equilibrium with 2 μCi ml^{-1} *myo*-[2-^{3}H]-inositol in complete culture medium. After washing with an Earle's salt solution (composition mM: NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgSO_4$ 0.8, glucose 5, buffered at pH 7.4 with HEPES-NaOH 25), cells were incubated for 10 min at 37°C in a 100 mM NaCl, 40 mM LiCl, modified Earle's salt solution and then exposed for 5 min to agonists. Reaction was stopped by aspiration of the incubation solution and addition of 750 μl ice cold 10 mM formic acid (pH 3.0). Plates were then kept at 4°C for 30 min. The radioactivity incorporated into total inositol phosphates was determined as follows. The cell extract was neutralized with 3 ml of 5 mM NH_3 and loaded onto an AG 1X8 anion exchange column in the formate form (Biorad, 200-400 mesh). Inositol, glycerophosphoinositol and inositol phosphates were sequentially eluted with 5 ml water, 4 ml 40 mM ammonium formate/formic acid (pH 5.0) and 900 mM ammonium formate/formic acid (pH 5.0). The radioactivity incorporated into the last fraction was then determined by liquid scintillation.

Intracellular Ca^{2+} was measured by flow cytometry as previously described (Frelin *et al.*, 1993; Vigne *et al.*, 1994). The technique measures indo-1 fluorescence ratio in suspended cells at a rate of 1000 cells s^{-1}. Mean indo-1 fluorescence ratio from 1000 to 2000 cells were collected at regular time intervals following the addition of agonists to the cells. To define dose-response curves, cells were exposed to agonists and the mean indo-1 fluorescence ratio from 1000 cells was measured 15 s later. Acquisition time was <2 s. Each experiment was repeated at least 3 times.

Nucleotides, indo-1/AM and endothelin-1 (Et-1) were from the Sigma Chemical Co. 2-Methylthio-ATP (2MeSATP) and 2-chloro-ATP (2ClATP) were purchased from Research Biochemicals Inc. All enzymes were from Promega. *myo*-[2-^{3}H]-inositol (19 Ci mmol^{-1}) was from Amersham.

Data are represented as mean ± s.e. A logistic curve fit programme (Sigma Plot, Jandel Scientific, U.S.A.) was used to define EC_{50} values.

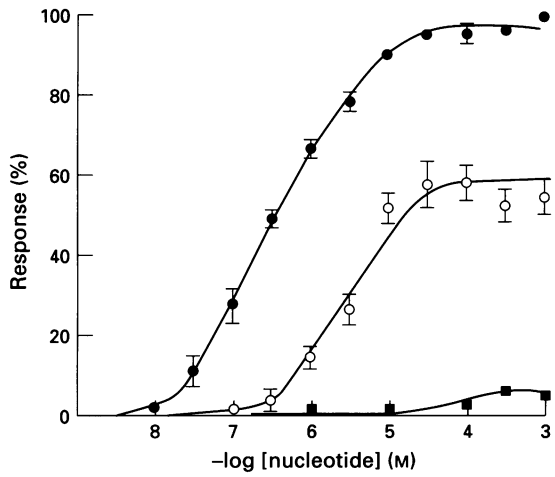

Figure 1 Dose-response curves for the actions of ATP, ADP and UTP on $[Ca^{2+}]_i$ in B10 cells. The cells were exposed to the indicated concentrations of ADP (●), ATP (○) and UTP (■) and the mean indo-1 fluorescence ratio was measured 15 s later. Means ± s.e. ($n=6$) (ATP and ADP) or 3 (UTP)) are shown. All data are expressed relative to the maximum effect of ADP.

Results

Seventeen clonal populations of rat BCEC were prepared and analyzed. In all cases except one (the B10 clone), responses were identical to those previously described for the B7 clone (Frelin *et al.*, 1993; Vigne *et al.*, 1994). Figure 1 shows that in B10 cells, ADP, ATP but not UTP increased $[Ca^{2+}]_i$. Dose-response curves for ATP and ADP were monophasic. EC_{50} values were 0.33 ± 0.4 μM and 2.72 ± 0.36 μM for ADP and ATP respectively. The maximum response to ATP was only 55% that to ADP. Figure 2 further shows that 2MeSATP, 2ClATP and adenosine 5'-O-(3-thiotrisphosphate) (ATPγS) also increased $[Ca^{2+}]_i$ in B10 cells. Their maximum response was however less than that of ADP. EC_{50} values were 0.18 ± 0.03 μM, 0.31 ± 0.10 μM and 2.3 ± 0.4 μM for 2MeSATP, 2ClATP and ATPγS respectively. All nucleotides tested induced intracellular Ca^{2+} transients that were partially de-

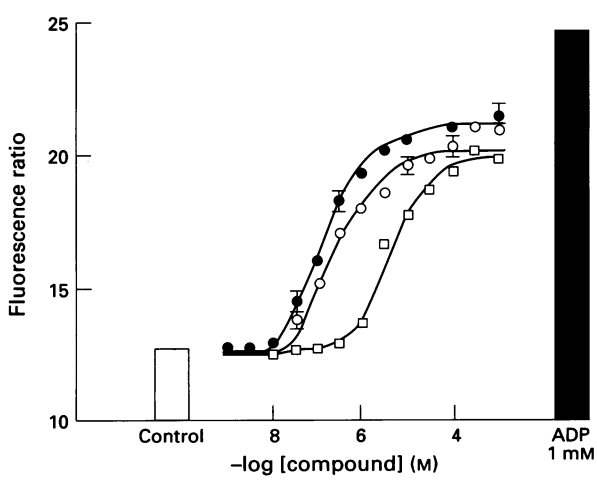

Figure 2 Dose-response curves for the actions of 2MeSATP, 2ClATP and ATPγS on $[Ca^{2+}]_i$ in B10 cells. The cells were exposed to the indicated concentrations of 2MeSATP (●), 2ClATP (○) and ATPγS (□) and the mean indo-1 fluorescence ratio was measured 15 s later. Means ± s.e. ($n=3$) are shown. The columns indicate indo-1 fluorescence ratio (arbitrary values) observed in control (open column) and ADP (1 mM)-treated cells (solid column). For abbreviations, see text.

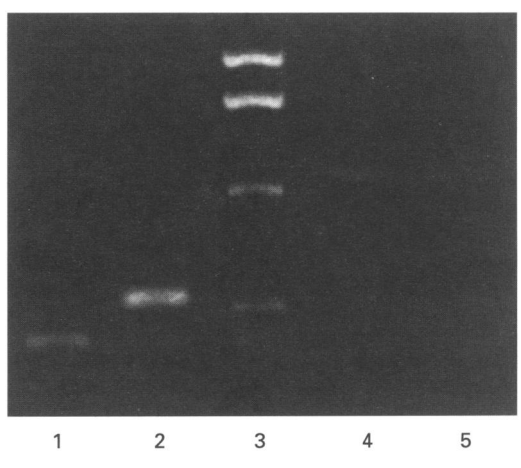

Figure 3 Agarose gel electrophoresis of PCR amplification products. Lanes: 1 and 2, amplification products produced using primers No. 1 and No. 2 (lane 1) or No. 1 and No. 3 (lane 2) and RNA isolated from B7 cells that express functional P_{2U} receptors. Amplification products of 420 and 555 bp were detected after staining with ethydium bromide. Lane 3: markers. Lanes: 4 and 5, amplification products produced using primers No. 1 and No. 3 (lane 2) or No. 1 and No. 3 (lane 5) and RNA isolated from B10 cells.

pendent on the presence of extracellular Ca^{2+} and that were similar to those previously described for the B7 clone (Frelin *et al.*, 1993; Vigne *et al.*, 1994). AMP and α,β methylene ATP, a selective agonist of P_{2X} receptors, were ineffective.

The lack of UTP response suggested that P_{2U} receptors were not expressed in B10 cells. To confirm this conclusion, polymerase chain reaction (PCR) experiments were performed on reverse transcribed mRNAs using primers selected to amplify both P_{2U} and P_{2Y} purinoceptor sequences. Figure 3 shows that only one product could be amplified using the two combinations of primers in the B7 cells that expressed functional P_{2U} receptors. Their size (420 bp and 555 bp respectively) was expected from the mouse P_{2U} receptor sequence. The two amplification products had a single restriction site for Bgl-1 as expected from the mouse P_{2U} receptor sequence and strongly hybridize with a mouse P_{2U} receptor probe (data not shown). The 555 bp product was subcloned and partial sequences were obtained from 4 independent clones. Three other partial sequences were obtained by direct sequencing of the PCR amplification product. In all cases, sequences were highly homologous to the mouse P_{2U} receptor sequence. Figure 3 further shows that when the same experiments were repeated with mRNA isolated from B10 cells, no amplification product could be detected. This confirmed the previous experiments showing the lack of functional responses to UTP (Figure 1).

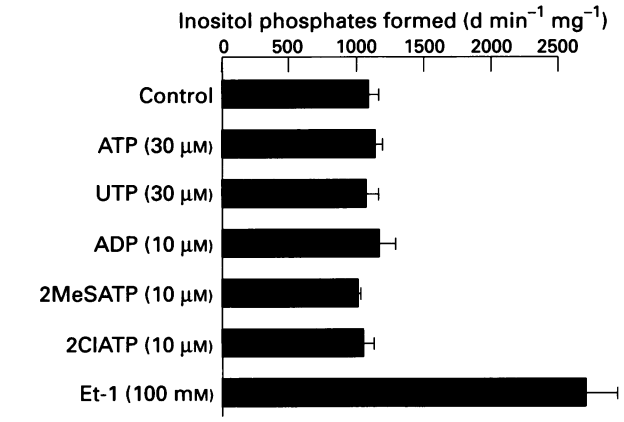

Figure 4 Nucleotides did not activate phospholipase C in B10 cells. Cells that had been labelled to equilibrium with [^{3}H]-inositol were exposed to nucleotides or to endothelin-1 (Et-1) and the production of inositol phosphates was measured. Means ± s.e.mean ($n=4$) are indicated. This experiment is representative of 3 independent experiments.

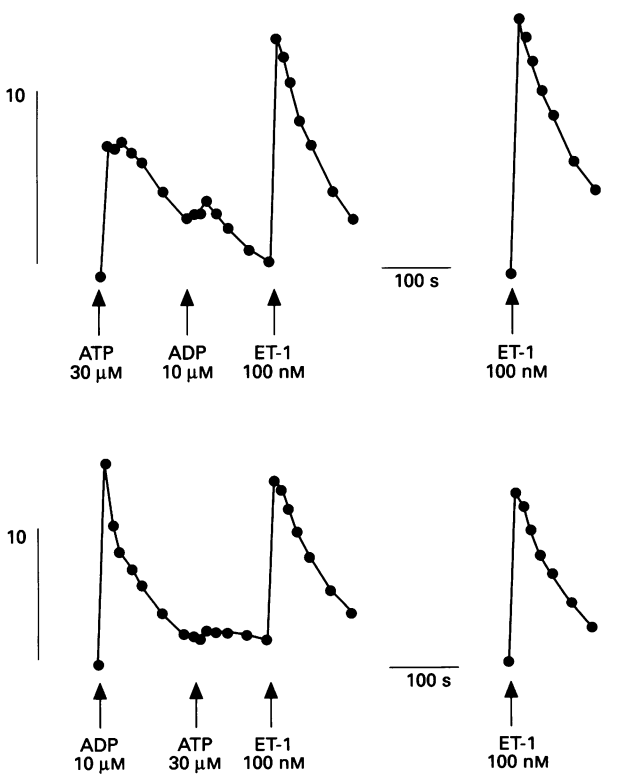

Figure 5 Cross-desensitization of ATP and ADP actions: typical tracings showing the changes in indo-1 fluorescence ratio observed after the sequential addition of ATP, ADP and endothelin-1 (Et-1) as indicated. The two experiments shown were performed on the same batch of cells and are directly comparable. The right panels show control Et-1 (100 nM) traces. Identical results were obtained in 2 other independent experiments.

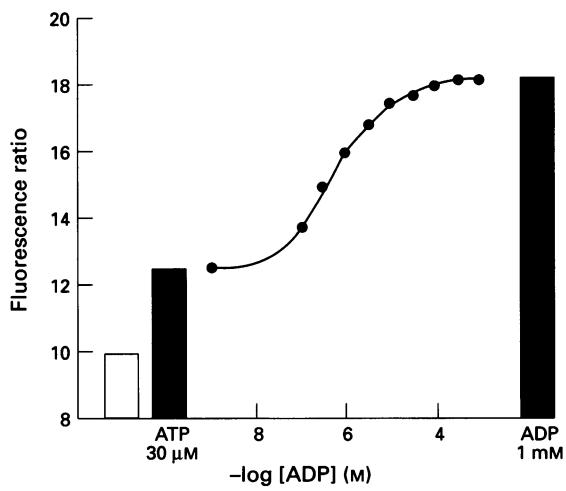

Figure 6 Additivity experiments: dose-response curve for the action of ADP on $[Ca^{2+}]_i$ measured in the presence of 30 μM ATP. Cells were exposed at the same time to ATP and ADP. Also shown for comparison are indo-1 fluorescence ratio in control (open column), ATP (30 μM) and ADP (1 μM)-treated cells. Standard errors ($n=3$) were smaller than the size of the points.

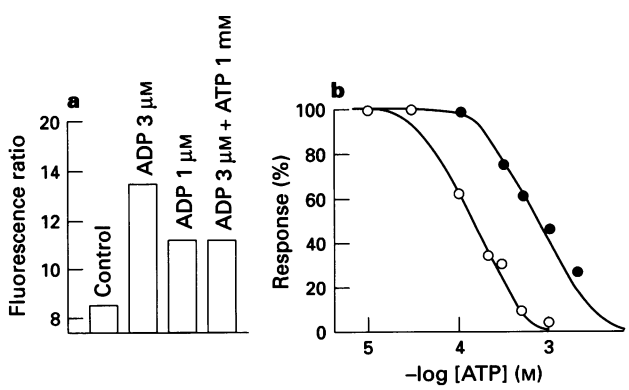

Figure 7 Partial inhibition by ATP of ADP responses. (a) Cells were treated with the indicated nucleotides and the mean indo-1 fluorescence ratio determined 15 s later; s.e. was <2% and was not represented. (b) Dose-response curve for the inhibition by ATP of the responses to 3 μM ADP (○) and 30 μM ADP (●). The zero point on the y axis represents the effect of 1 mM ATP alone. The 100% point represents the effects of 3 μM or 30 μM ADP alone. Standard errors ($n=3$) were smaller than the size of the points.

A characteristic feature of the action of nucleotides in B7 cells is their ability to mobilize intracellular Ca^{2+} stores in the absence of measurable activation of phospholipase C (Frelin *et al.*, 1993; Vigne *et al.*, 1994). Figure 4 shows that in B10 cells, nucleotides, at concentrations that increased $[Ca^{2+}]_i$ to a large extent, did not activate phospholipase C as measured from the production of inositol phosphates. Under the same conditions, Et-1 (100 nM), a potent agonist of phospholipase C in rat BCEC (Vigne *et al.*, 1990) increased the production of inositol phosphates 2.5 fold (Figure 4). Identical results were obtained with suspended cells. The Ca^{2+} mobilizing action of 10 μM ADP being $75 \pm 10\%$ ($n=3$) that of 100 nM Et-1, an obvious conclusion is that intracellular Ca^{2+} mobilization did not correlate with phospholipase C activation. Intracellular Ca^{2+} mobilization in response to adenine nucleotides may be mediated by minute, undetectable amounts of inositol (1,4,5)trisphosphate or by a yet unidentified second messenger.

A useful way to assess receptor heterogeneity is to perform desensitization experiments. Figure 5 shows a cross desensitization of ATP and ADP actions. Cells that had been exposed to ADP did not respond any more to ATP and conversely, cells that had been exposed to ATP did not respond to ADP. Desensitized cells still responded to Et-1, indicating that the lack of response was not due to a depletion of intracellular Ca^{2+} stores. These suggested that ATP and ADP probably shared a common receptor site.

Another useful way to assess receptor heterogeneity is to look for additive responses. Additive responses of maximally effective concentrations of agonists are expected if they bind to different receptor sites. Non additive actions are expected if they bind to the same receptor. They are also expected if they bind to different sites but maximally increase $[Ca^{2+}]_i$ by depleting intracellular Ca^{2+} stores. The latter situation was unlikely in B10 cells for the Ca^{2+} mobilizing action of ADP was less than that of Et-1, a powerful agonist of phospholipase C and for the actions of ADP and Et-1 were partially additive (data not shown). Figure 6 shows that when 30 μM ATP and various concentrations of ADP were added simultaneously to the cells, it was possible to see an effect of ADP on the top of 30 μM. The EC_{50} value for ADP action was 0.53 ± 0.09 μM. Yet the combined action of 30 μM ATP and 1 mM ADP was not greater than that of 1 mM ADP. This result was consistent with the previous conclusion that ATP and ADP probably shared the same receptor site. Figure 7 further shows that when cells were exposed at the same time to a maximally effective concentration of ADP (3 μM) and various concentrations of ATP, ATP inhibited ADP responses with an IC_{50} value of 0.15 ± 0.05 mM (Figure 7). An identical result was obtained in the presence of 30 μM ADP but the dose-response curve for ATP action was shifted by 10 fold to larger concentrations ($IC_{50} = 1.0 \pm 0.2$ mM). This observation suggested that ATP and ADP share the same receptor site.

Discussion

The identification of a BCEC clone that did not express functional P_{2U} receptors allowed us to characterize P_{2Y}-like responses in these cells. All responses to adenine nucleotides could be accounted for by the presence of a single receptor that controls the intracellular Ca^{2+} concentration. Its pharmacological profile can be defined as 2MeSATP (180 nM) > 2-ClATP (310 nM) = ADP (330 nM) > ATPγS (2.3 μM) = ATP (2.7 μM). It suggests the presence of a P_{2Y} receptor. The properties of this receptor are however distinct from those of the cloned meleagrid P_{2Y} receptor (Filtz *et al.*, 1994) in three respects. (i) The P_{2Y} receptor of B10 cells is 10 times more sensitive to 2ClATP than the meleagrid receptor, (ii) it does not couple to phospholipase C (Figure 4) and (iii) maximum responses of adenine nucleotides are different. Whether these differences reflect species differences or the existence of multiple subtypes of P_{2Y} receptors cannot be ascertained at the present time.

Irrespective of the type of receptor involved, the results presented in this paper suggest that while ADP is a full agonist of P_{2Y} like responses in B10 cells, ATP and its derivatives behave as partial agonists. First, the Ca^{2+} mobilizing action of maximally effective concentrations of ATP, 2MeSATP, 2ClATP and ATPγS was less than that of ADP (Figures 1 and 2). Second, high concentrations of ADP increased intracellular Ca^{2+} transients on the top of a maximally effective concentration of ATP (Figure 6). Third, high concentrations of ATP partially inhibit intracellular Ca^{2+} transients induced by maximally effective concentrations of ADP (Figure 7). Partial agonism is a property of many ligands of seven transmembrane domain receptors but it has not yet been described for P_{2Y} receptors. Undoubtedly partial agonism can be a major difficulty when assessing the specificity of purinoceptors from additivity experiments. For instance additive actions shown in Figure 6 could be interpreted as evidence for the presence of an ADP-specific receptor distinct from the ATP receptor. This type of result led us to conclude previously that brain capillary endothelial cells of the B7 clone expressed an ADP specific receptor that did not recognize ATP (Frelin *et al.*, 1993; Vigne *et al.*, 1994). The results obtained in this study suggest that cells of the B10 and B7 clones probably express the same receptor but that its interaction with ATP could not be defined in B7 cells due to the partial agonistic action of ATP and the presence of P_{2U} receptors.

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