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Pharmacological profile of the ATP-mediated increase in L-type calcium current amplitude and activation of a non-specific cationic current in rat ventricular cells

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1 The pharmacological profile of the ATP-induced increase in I_{Ca} amplitude and of ATP activation of a non-specific cationic current, I_{ATP}, was investigated in rat ventricular cells.

2 The EC_{50} values for I_{Ca} increase and I_{ATP} activation were 0.36 μM and 0.76 μM respectively. Suramin (10 μM) and cibacron blue (1 μM) competitively antagonized both effects of ATP.

3 The rank order of efficacy and potency of ATP analogues in increasing I_{Ca} amplitude was 2-methylthio-ATP ≈ATP≈ATPγS. The derivatives α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-imido-ATP up to 500 μM had no significant effects.

4 The rank order of efficacy of ATP analogues in activating a non-specific cationic current, I_{ATP}, was 2-methylthio-ATP >ATP >>ATPγS. The rank order of potency was 2-methylthio-ATP≈ATP. The EC_{50} of ATPγS could not be determined owing to its very low efficacy.

5 The ATP analogues α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-imido-ATP at 500 μM did not activate I_{ATP} but acted as antagonists of activation of I_{ATP} by ATP.

6 The results suggest that the increase in I_{Ca} amplitude induced by external ATP is due to activation of P_{2Y}-purinoceptors.

7 The mechanism of I_{ATP} activation remains to be determined before the receptor subtype involved can be deduced.

Keywords: Adenosine 5'-triphosphate; P_{2Y}-purinoceptor; suramin; cibacron blue 3GA; calcium current; non-specific cationic current; rat ventricular myocyte

Introduction

It is now well established that extracellular adenosine 5'-triphosphate (ATP) exerts physiological effects in many different tissues through P_2-purinoceptors, and at least five subtypes of P_2-purinoceptors are proposed: P_{2T}, P_{2X}, P_{2Y} and P_{2U} (Dubyak & El-Moatassim, 1993). In rat cardiac preparations, ATP is reported to have positive inotropic effects and to increase the Ca^{2+} transients of electrically stimulated cells (Danziger *et al.*, 1988; Legssyer *et al.*, 1988). It has also been reported that ATP transiently increases the intracellular Ca^{2+} concentration, Ca_i, of quiescent cells (De Young & Scarpa, 1987; Danziger *et al.*, 1988; Björnsson *et al.*, 1989; Pucéat *et al.*, 1991a; Hirano *et al.*, 1991). In addition, electrophysiological studies have shown that ATP activates a non-specific cationic current in frog, rat and guinea pig cardiac cells (Friel & Bean, 1988; Scamps & Vassort, 1990; Matsuura & Ehara, 1992), an inwardly rectifying K^+ channel in frog (Friel & Bean, 1990) and a chloride current in guinea pig (Matsuura & Ehara, 1992), increases the L-type calcium current amplitude in rat (Scamps *et al.*, 1990; 1992; Zheng *et al.*, 1993) and increases both the L- and T-type in frog cells (Alvarez & Vassort, 1992). However, in ferret ventricular myocytes, ATP decreases both Ca_i and I_{Ca} amplitude and does not induce the non-specific cationic current (Qu *et al.*, 1993a). This decrease in I_{Ca} has been attributed to occupancy of P_{2Y}-purinoceptors, with 2-methylthio-ATP being more potent than α,β-methylene-ATP (Qu *et al.*, 1993b). The transient increase in Ca_i (in rat) and the positive inotropism (in rat and guinea pig) induced by ATP have also been attributed to P_{2Y} purinoceptors (Björnsson *et al.*, 1989; Mantelli *et al.*, 1993; Wang *et al.*, 1993). In the present study we have investigated the pharmacological profile of ATP-activated non-specific cationic current and the increase in I_{Ca} in rat ventricular myocytes, which are supposed to be largely responsible for the changes in Ca_i and the positive inotropism.

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Methods

Cell isolation

Adult male Wistar rats (200–300 g) were anaesthetized with ethylcarbamate prior to excision of the hearts. The procedure for dissociating ventricular myocytes has been previously described (Scamps *et al.*, 1990).

Electrophysiological study

Membrane currents were recorded with the whole-cell patch-clamp (Hamill *et al.*, 1981). For routine monitoring of L-type Ca current, I_{Ca}, and of non-specific cationic current induced by external ATP, I_{ATP}, a ventricular cell was depolarized from −70 mV holding potential to 0 mV for 200 ms every 4 s. The cells were superfused with solution containing (mM) CsCl 20, NaCl 117, $CaCl_2$ 1.8, $MgCl_2$ 1.7, glucose 10 and HEPES 10; pH was adjusted to 7.4 with NaOH and 50 μM tetrodotoxin was added to block the sodium current. No T-type Ca current was observed in the rat ventricular cell. The internal solution in the patch electrode contained (mM) CsCl 120, $MgCl_2$ 4, Na_2ATP 5, Na_2-creatine phosphate 5, Na_2GTP 0.4, Cs_2EGTA 5, $CaCl_2$ 0.062 (intracellular free $Ca^{2+} = 2 \times 10^{-9}$ M) and HEPES 20; pH was adjusted to 7.2 with CsOH. Experiments were conducted at room temperature (20 ± 1°C).

Drugs

ATP and cibacron blue 3GA (cibacron blue) were from Sigma. Adenosine 5'-*O*-3-thiotriphosphate (ATPγS), 2-methylthio-ATP, α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-amido-ATP were from Boehringer Mannheim (France). Suramin was a gift from Dr Kazda, Bayer Leverkusen (Germany).

Statistical analysis

Data are given as means ± s.e.mean. Statistical analyses were based on a paired *t*-test or on a Student's *t*-test. The differences were significant when $P<0.05$. To determine the concentration–response curves, no more than two concentrations of ATP, one low and one high, were used for each cell.

Results

Effects of inhibitors

Figure 1 shows typical effects of external application of ATP on I_{Ca} amplitude and on holding current for a holding potential of −70 mV in rat ventricular cells. The first effect of external application of 1 μM ATP was to induce a transitory inward current at −70 mV which peaked more or less rapidly depending on the ATP concentration and then decreased (upper part of Figure 1 for current traces; lower part of Figure 1 and Figure 6 for kinetics of activation). This change in holding current has been attributed to the activation of a non-specific cationic current, I_{ATP} (Scamps & Vassort, 1990). In addition, application of 1 μM ATP induced a progressive increase of I_{Ca} amplitude up to a steady-state value reached after 2–3 min of ATP application (upper part of Figure 1 for current traces; lower part shows the kinetics of effects). The increase in I_{Ca} amplitude under ATP application has also been characterized (Scamps *et al.*, 1990; 1992). In 80% of cells ATP induced both effects, while in 20% ATP induced only the non-specific cationic current. The 20% of cells have not been analysed.

In the presence of 10 μM suramin, a non-specific blocker of P_2-purinoceptors, 3 μM ATP had no effects on both currents (Figure 2a). Increasing the ATP concentration to 100 μM could overcome the blocking effect of suramin (not shown). In the presence of 1 μM cibacron blue (reactive blue 2), a reported selective inhibitor of P_{2Y}-purinoceptors, ATP at 1 μM hardly increased I_{Ca} and activated a small but significant I_{ATP}, as shown in Figure 2b. A full response developed when 20 μM ATP was applied.

Figure 3 shows the concentration–response curves of the ATP-induced I_{Ca} increase in control cells and in the presence of the inhibitors. Under control conditions, the concentration of ATP giving half-maximal effect, EC_{50}, was 0.36 μM. This value was shifted to 2.2 μM and 12.4 μM in the presence of 1 μM cibacron blue or 10 μM suramin respectively. The Hill factor, n_H, was 1.9, 1.8 and 2.2, respectively, in control and cibacron blue- and suramin-treated cells. The concentration–response curves of ATP activation of a non-specific cationic current, I_{ATP}, are illustrated in Figure 4. Under control conditions, the EC_{50} was 0.76 μM. This value was shifted to 4.1 μM and 16.5 μM in the presence of 1 μM cibacron blue and 10 μM suramin respectively. n_H was 1.9, 2.6 and 3.1, respectively, in control and cibacron blue- and suramin-treated cells. At 10 μM, cibacron blue completely inhibited the effects of 10 μM ATP on I_{Ca} and I_{ATP} ($n=3$, not shown) and thus appears to be a more potent inhibitor of ATP effects than suramin.

Agonist efficacy and potency

To determine whether the I_{Ca} increase and I_{ATP} activation are due to occupation of the same subtype of purinergic receptor,

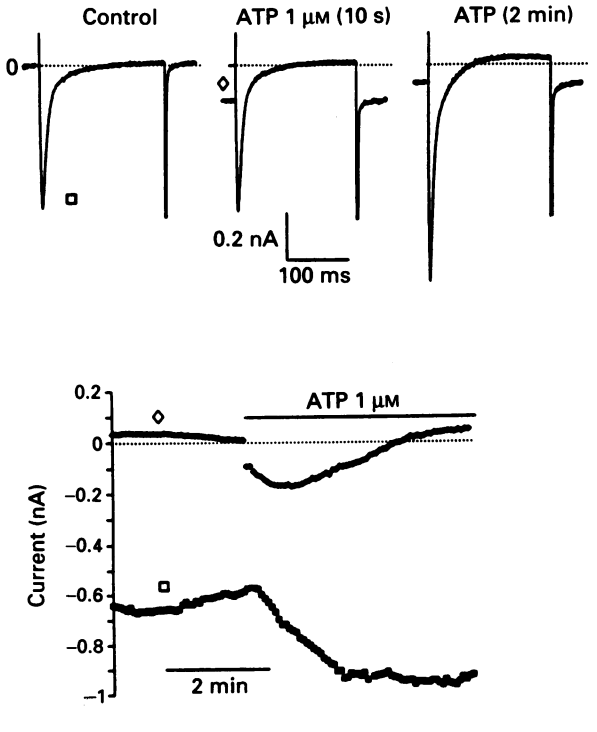

Figure 1 Effects of ATP on calcium current amplitude measured at 0 mV test pulse and on holding current flowing at −70 mV, every 4 s. In a control cell, 1 μM ATP increased I_{Ca} amplitude (□, measured as the difference between peak inward current and the current at the end of the 200 ms pulse) and the current measured at −70 mV became transiently more inward (◇, measured relative to the zero current). The upper part of figure shows current traces and the lower part of the kinetics of ATP effects.

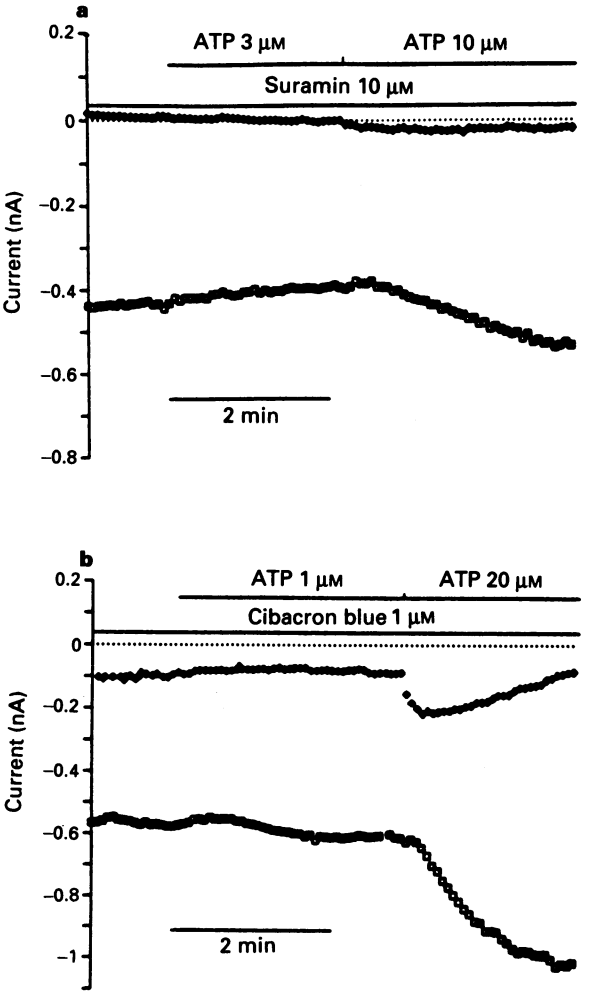

Figure 2 Effects of ATP on I_{Ca} amplitude and on holding current on a cell superfused with (a) 10 μM suramin and (b) 1 μM cibacron blue (same protocol as in Figure 1).

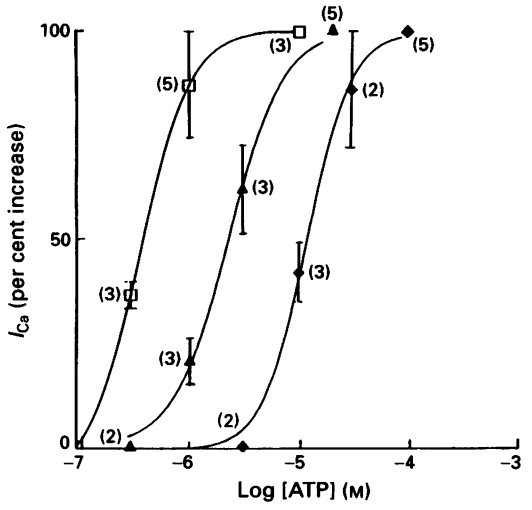

Figure 3 Concentration–response curves of ATP-induced I_{Ca} increase in control (□) cells and in the presence of 1 μM cibacron blue (▲) 10 μM suramin (◆). Curves were fitted according to a sigmoid shape: $y = EC_{max} \times [ATP]^{nH}/([ATP] + EC_{50}^{nH})$. The curves were normalized to EC_{max} (concentration giving maximal effect). Number of cells in brackets (as for the other figures).

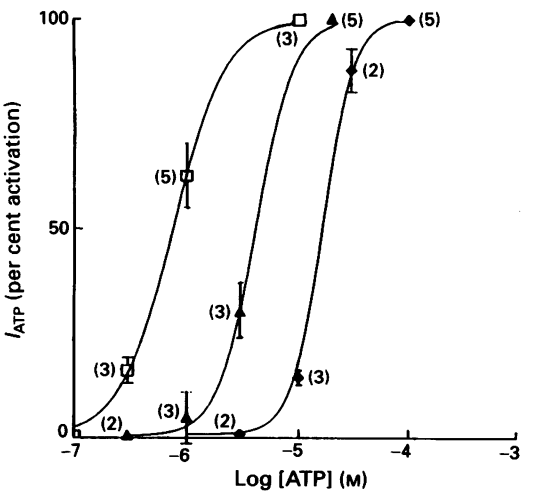

Figure 4 Concentration–response curves of ATP-activated non-specific cationic current in control (□), in the presence of 1 μM cibacron blue (▲) or 10 μM suramin (◆). Curves were fitted according to $y = EC_{max} \times [ATP]^{nH}/([ATP] + EC_{50}^{nH})$ and normalized to EC_{max}.

the rank orders of ATP analogues efficacy and potency were evaluated. Figure 5 gives the maximal percentage of I_{Ca} increase and the maximal amplitude of I_{ATP} elicited by applying various ATP analogues. For I_{Ca} increase, the rank order of efficacy was 2-methylthio-ATP≈ATP≈ATPγS. The ATP derivatives of α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-imido-ATP were without significant effects (Figure 5a). The EC_{50} observed was 0.23 μM and 0.41 μM for 2-methylthio-ATP and ATPγS respectively. Thus the rank order of potency for I_{Ca} increase was 2-methylthio-ATP≈ATP≈ATPγS. For I_{ATP} activation the rank order of efficacy was 2-methylthio-ATP > ATP >> ATPγS (Figure 5b). Similarly to the case of the I_{Ca} increase, α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-imido-ATP at 500 μM did not activate I_{ATP}. The EC_{50} for 2-methylthio-ATP was 0.71 μM. The EC_{50} for ATPγS could not be determined because of the small amplitude of the maximal effect. Thus the rank order of potency for activation of I_{ATP} was 2-methylthio-ATP≈ATP.

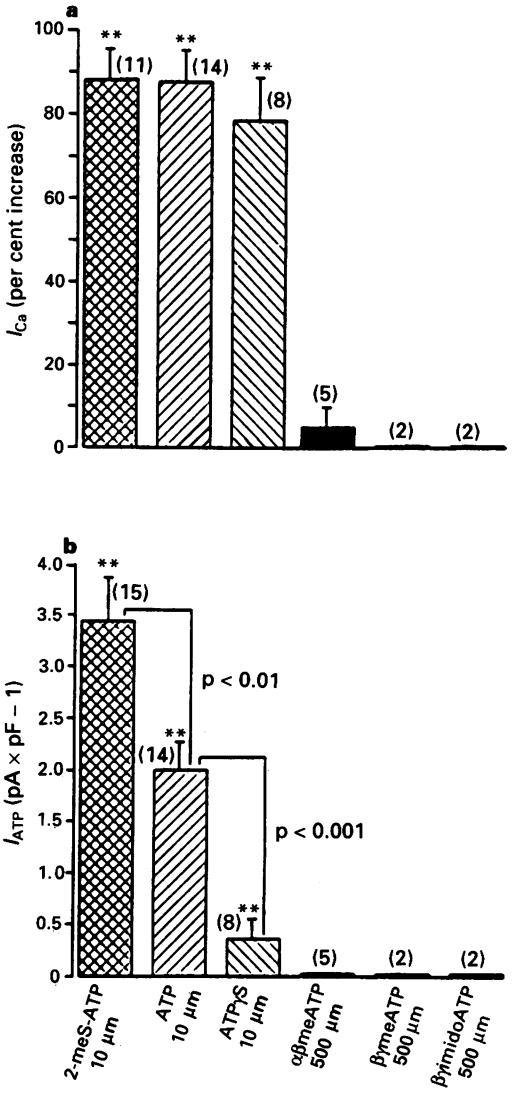

Figure 5 Rank order of agonist efficacy on the percentage of I_{Ca} increase (a) and the density of I_{ATP} (b). The values of I_{ATP} are normalized to the cell capacitance and thus given in density. $^{**}P < 0.01$ (paired test). In (b) a Student's *t*-test was used to compare 2-methylthio-ATP and ATPγS with ATP; *P*-values are given in the figure.

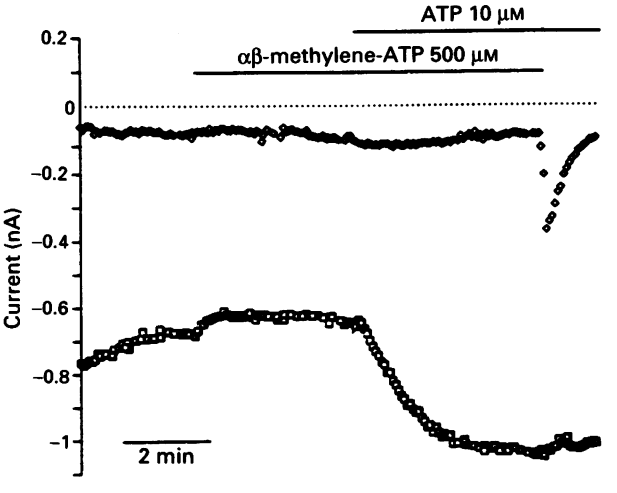

Figure 6 Antagonism of α,β-methylene-ATP on ATP activation of a non-specific cationic current. α,β-Methylene-ATP by itself did not activate I_{ATP} nor did it increase I_{Ca} amplitude (same protocol as in Figure 1).

As stated above, the analogues α,β-methylene-ATP, β,γ-methylene ATP and β,γ-imido-ATP did not increase I_{Ca} amplitude, even at 500 μM, and did not prevent the increase in I_{Ca} induced by adding 10 μM ATP in their presence (Figure 6). Furthermore, these analogues, which also did not activate I_{ATP}, prevented the activation of I_{ATP} with the further addition of 10 μM ATP. Note that on washout of these analogues, I_{ATP} instantaneously appeared. Figure 6 illustrates such an effect for α,β-methylene-ATP. Similar results were obtained with the three analogues mentioned above ($n = 6$, for two experiments in each condition). α,β-methylene-ATP, used at 100 μM, did not prevent the activation of I_{ATP} (not shown), which suggested a competitive antagonism. Superfusion of the cell for a longer period of time (10 min) with 500 μM α,β-methylene-ATP did not desensitize the ATP effects (two cells).

Discussion

In the present study, it is shown that the external application of ATP triggers an increase in I_{Ca} amplitude and activates a non-specific cationic current, I_{ATP}, with similar EC_{50}. Both currents are inhibited by the P_2-purinoceptor antagonists suramin and cibacron blue. However, the subtypes of purinoceptors involved should be different, as suggested by the variations in rank order of efficacy of ATP analogues and the observation that some of these analogues exert an antagonistic effect only on the ATP-activated non-specific cationic current.

Based on the rank order of efficacy and potency of ATP analogues, 2-methylthio≈ATP≈ATPγS>>α,β-methylene-ATP, the increase in I_{Ca} amplitude indicates activation of a P_{2Y}-purinoceptor. In support of this proposal, α,β-methylene-ATP (a potent P_{2X}-purinoceptor agonist) not only had no effect by itself, but it also did not induce desensitization of the receptor. The increase in I_{Ca} amplitude is probably responsible for part of the sustained positive inotropism reported for purinergic compounds in rat papillary muscle (Legssyer *et al.*, 1988; Scamps *et al.*, 1990). In agreement with this suggestion, a P_{2Y}-purinoceptor was recently proposed to mediate the positive inotropism induced by ATP in rat and guinea pig hearts (Wang *et al.*, 1993; Mantelli *et al.*, 1993). A discrepancy remains concerning the effect of α,β-methylene-ATP, which on the one hand did not significantly increase I_{Ca} amplitude (this study) and on the other hand had clear positive inotropic effects (Legssyer *et al.*, 1988; Mantelli *et al.*, 1993). A possible alternative is that α,β-methylene-ATP acts through another type of receptor, the P_{2X}-purinoceptor being a good candidate. Occupation of this receptor subtype could involve different intracellular events, such as an increase in phosphoinositide turnover, as reported by Legssyer *et al.* (1988), and expected to lead to a positive inotropism. Consequently, the positive inotropism induced by purinergic compounds should be related to occupation of different purinoceptors subtypes. One of them is linked to the increase in I_{Ca} amplitude and identified in the present study as a P_{2Y}-purinoceptor with an EC_{50} of around 1 μM, in the range of values usually reported for the effects of ATP. The best concentration–response curve fittings were obtained by assuming two binding sites. The same Hill coefficient was obtained for the inhibition of I_{Ca} of chromaffin cells by ATP (Gandia *et al.*, 1993), while for I_{Ca} inhibition in ferret ventricular cells a 1:1 binding of ligand to receptor was suggested (Qu *et al.*, 1993b). Suramin and cibacron blue were competitive antagonists, which suggests that they act at the ATP binding site. It should be mentioned that the concentration of antagonists required in rat ventricular cells (1 μM for cibacron blue and 10 μM suramin) used in this study was much lower than those reported to achieve inhibition in other cell types (usually in the 100–500 μM range).

In the present study, the EC_{50} values obtained for I_{Ca} increase and I_{ATP} activation during ATP application were not significantly different under control conditions or in the presence of suramin or cibacron blue, which rather suggests a common receptor for both effects. As for the I_{Ca} increase, two binding sites gave the best concentration–response curves fitting for I_{ATP} activation. Two binding sites were also reported for I_{ATP} in bullfrog atrial cells (Friel & Bean, 1988). However, the rank order of efficacy of the agonists is not identical for the I_{Ca} increase and I_{ATP} activation. While 2-methylthio-ATP, ATP and ATPγS are roughly equipotent in increasing I_{Ca}, there is a clear sequence of agonist efficacy in activating I_{ATP}, with 2-methylthio-ATP being the most efficient and ATPγS the least. Importantly, while high concentrations of the non-hydrolysable analogues prevented I_{ATP}, they did not prevent the increase in I_{Ca} amplitude and thus are not antagonists of this response. Such an antagonistic effect of α,β-methylene-ATP on I_{ATP} activation has been previously reported by Friel & Bean (1988) in bullfrog atrial cells. In addition, we show that the lack of ATP effect in the presence of these analogues was not due to a slow desensitization of the receptor since a full effect of ATP was observed on washout of these analogues. Consequently, α,β-, β,γ-methylene-ATP and β,γ-imido-ATP act as rather specific antagonists of I_{ATP}. We should point out that the activation of I_{ATP} that we report in this study and which was determined to be a non-specific cationic current as previously described, and not a chloride current, seems inconsistent with the hypothesis we initially proposed (Scamps & Vassort, 1990), i.e. I_{ATP} is the consequence of an internal acidosis, with α,β-methylene-ATP, β,γ-methylene-ATP and β,γ-imido-ATP being less potent but as efficient as ATP in inducing acidosis (Pucéat *et al.*, 1991b). However, it should be noted that in parallel experiments the cells did not show clear acidosis on applying ATP. Thus these results do not rule out the possibility that acidosis can activate an I_{ATP}, the nature of which (non-specific cationic current or chloride current) should be checked by applying the ATP analogues.

In view of the present results, two theories can be proposed concerning the type of receptors involved. First, the receptors involved in the I_{Ca} increase and in I_{ATP} activation are of two different subtypes of P_2-purinoceptors, which, however, share a close pharmacological profile. The presence of two types of P_2-purinoceptors on the same preparation has been reported previously based on photoaffinity labelling (Giannattasio *et al.*, 1992). Second, both types of effects are mediated by a common P_{2Y}-purinoceptor, but the effector involved in I_{ATP} is blocked by the non-hydrolysable analogues. A strong argument against the latter hypothesis of a common receptor is that the increase in I_{Ca} amplitude is mediated by a G-protein, while the activation of I_{ATP} is not (Scamps *et al.*, 1992; Zheng *et al.*, 1993). Indeed, it has been shown that internal perfusion of the cell with non-hydrolysable analogues of GTP modifies the ATP-induced I_{Ca} increase in a way that would be expected from involvement of a G-protein while having no effect on the I_{ATP} response. It has been proposed that P_{2Y}-purinoceptors can be subdivided in two subtypes (Illes & Nörenberg, 1993). The subtype $P_{2Y\alpha}$ (also called P_{2X}, see Edwards & Gibb, 1993) would be the ligand-activated channel while the $P_{2Y\beta}$ would be G-protein linked. Until now, cloning of G-protein-coupled receptors has demonstrated three types of P_{2Y}-purinoceptors, classified as P_{2Y1}, P_{2Y2} and P_{2Y3}, with P_{2Y1} having the closest pharmacological profile to the P_2-purinoceptor that induces I_{Ca} increase (Barnard *et al.*, 1994). However, P_{2X}-purinoceptor has been solubilized and found to have a pharmacology rather different from I_{ATP} (Bo *et al.*, 1992). A complementary hypothesis initially suggested by Björnsson *et al.* (1989) and developed by Christie *et al.* (1992) would be that I_{ATP} is the result of an external phosphorylation. According to this scheme, a kinase would be close to the P_{2Y}-purinoceptor and would utilize the appropriate substrate. This hypothesis would both meet the pharmacological profile and explain the competitive antagonism of the poorly hydrolysable analogues through a mass action law on the kinase reaction. However,

in that case ATPγS would be as efficient as ATP. Obviously, further experiments are required to understand the nature of I_{ATP} activation.

In conclusion, the increase in I_{Ca} amplitude caused by ATP application relates to occupancy of a P_{2Y}-purinoceptor. Despite a close pharmacological profile and antagonistic effects of suramin and cibacron blue, the activation of I_{ATP} cannot be attributed to simple P_{2Y}-purinoceptor occupancy. A better understanding of the mechanism of activation of I_{ATP} should help to clarify the receptor subtype involved.

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