Evidence that a form of ATP uncomplexed with divalent cations is the ligand of P_{2y} and nucleotide/P_{2u} receptors on aortic endothelial cells

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1 The response of bovine aortic endothelial cells to adenosine 5'-triphosphate (ATP) is mediated by both P_{2y} and nucleotide/P_{2u} receptors. In order to determine which form of the nucleotide is the true ligand of these receptors, we have investigated the effects of divalent cations on ATP-, uridine 5'-triphosphate (UTP)- and 2-methylthioadenosine 5'-triphosphate (2MeSATP)-induced inositol phosphate accumulation in these cells.

2 Omission of Mg²⁺ from a calcium-free incubation buffer caused a shift to the left of the ATP concentration-action curve.

3 In the presence of EDTA (1 mM), the basal level of inositol trisphosphate (InsP₃) was markedly increased and the absolute maximal response to ATP was decreased; however, the response to low concentrations of ATP was enhanced.

4 When the results were plotted in terms of calculated ATP⁴⁻ concentrations, the concentration-response curves obtained in the presence of 1.25 mM Mg²⁺ lay closer to the respective curves obtained when Mg²⁺ was omitted from the medium or when Mg²⁺ was omitted and EDTA (1 mM) was added. The curves became almost superimposable when the baseline value was subtracted.

5 A similar shift to the left of the concentrations-action curves was also observed with both UTP and 2MeSATP.

6 Our data provide evidence that a form of ATP uncomplexed with divalent cation is the preferential agonist of both the nucleotide/P_{2u} and the P_{2y} receptors expressed on bovine aortic endothelial cells.

Keywords: ATP; UTP; 2-methylthioadenosine 5'-triphosphate (2MeSATP); magnesium; calcium; purinoceptors; inositol phosphates; endothelium

Introduction

Extracellular adenosine 5'-triphosphate (ATP) plays an important role in the regulation of many biological processes including the control of vascular tone and haemostasis. The cellular responses to ATP are mediated by specific receptors called P₂ as opposed to P₁ receptors which interact preferentially with adenosine. According to the rank order of potency of several ATP analogues, a subclassification of P₂ purinoceptors into 2 major classes (P_{2x}, P_{2y}), both present on blood vessels, has been proposed by Burnstock & Kennedy in 1985. P_{2x} responses are characterized by the following order of potency: α,β -methylene ATP (APCPP) = β,γ -methylene ATP (APPCP) > ATP = 2-methylthioadenosine 5'-triphosphate (2MeSATP). The P_{2y} subtype is more specific for 2MeSATP than for ATP and α,β -methylene ATP. Other P₂ purinoceptor subtypes have been described (Gordon, 1986). The P_{2i} type is present on platelets and megakaryocytes and responds only to ADP. The P_{2z} subclass is expressed on mast cells and macrophages, where it is coupled to histamine release and an increase in membrane permeability (Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988; Steinberg & Silverstein, 1987). In addition to these four subclasses of purinoceptors (P_{2x}, P_{2y}, P_{2i}, P_{2z}), the existence of another receptor which is called P_{2u} or nucleotide receptor has been recently proposed (Seifert & Schultz, 1989; O'Connor *et al.*, 1991). This receptor has a high affinity for both ATP and UTP and a low affinity for 2MeSATP. The existence of nucleotide/P_{2u} receptors is supported by studies on calcium mobilization in human poly-

morphonuclear neutrophils (Walker *et al.*, 1991), pituitary cells (Davidson *et al.*, 1990), human fibroblast (Fine *et al.*, 1989) and NCB-20 cells (Garritsen *et al.*, 1992), on prostacyclin production in bovine pulmonary artery endothelial cells (BPAE) (Lustig *et al.*, 1992), and on inositol phosphate accumulation in renal mesangial cells (Pfeilschifter, 1990), human epithelial cells (Brown *et al.*, 1992) and bovine aortic endothelial cells (BAEC) (Motte *et al.*, 1993).

ATP⁴⁻ forms complexes with the cations Mg²⁺, Ca²⁺, Na⁺, K⁺ and H⁺, the percentage distribution of these various forms is dependent upon the ionic composition of the solution, its pH, the temperature and the concentration of ATP (Bartfai, 1979). Using dissociation constants published in the literature (Fabiato, 1981), Fine *et al.* have calculated that in a physiological extracellular medium (140 mM Na⁺, 4 mM K⁺, 1 mM Ca²⁺, 1 mM Mg²⁺, pH 7.4) the distribution is as follows: MgATP²⁻ (71%), CaATP²⁻ (22%), NaATP³⁻ (3%), KATP³⁻ (0.8%), HATP³⁻ (1.2%) and ATP⁴⁻ (2.4%) (Fine *et al.*, 1989). Similar values have been reported by Lustig *et al.* (1992). Which of these forms constitutes the true or preferential agonist has not been determined for all classes of P₂ receptors. It is now well established that the P_{2z} receptors selectively recognize the tetrabasic form of ATP: these receptors have indeed been called 'ATP⁴⁻ receptors' (Cockcroft & Gomperts, 1980; Tatham *et al.*, 1988). ATP⁴⁻ also seems to be the best ligand for the nucleotide/P_{2u} receptors identified on human fibroblasts (Fine *et al.*, 1989), pituitary cells (Davidson *et al.*, 1990), neutrophils (Walker *et al.*, 1991) and BPAE (Lustig *et al.*, 1992), as well as for P_{2x} receptors on vas deferens smooth muscle (Fedan *et al.*, 1990). The primary biological event coupled to receptor activation induced by ATP in BAEC has been characterized in our

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laboratory as the hydrolysis of phosphatidylinositol bisphosphate by phospholipase C resulting in a rapid accumulation of inositol trisphosphate (InsP₃) (Pirrotton *et al.*, 1991). We have recently established that InsP₃ accumulation in BAEC in response to ATP involved both P_{2y} and nucleotide/P_{2u} receptors (Motte *et al.*, 1993).

In order to determine whether the P_{2y} receptor preferentially recognizes ATP⁴⁻ or a form of ATP complexed with monovalent cations, as does the nucleotide/P_{2u} receptor, we have evaluated the effects of changes in Mg²⁺ concentration and the effect of the Ca²⁺ and Mg²⁺ chelator ethylenediaminetetraacetic acid (EDTA) on the accumulation of InsP₃ induced by ATP, UTP and 2MeSATP in BAEC.

Methods

Cell incubation

BAEC were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow and cultured as previously described (Pirrotton *et al.*, 1991). Subconfluent cells were labelled for 24 h in inositol-free Dulbecco's Modified Eagle's Medium (80%, v/v), with Ham's F₁₂ (20%, v/v), foetal-calf serum (5%, v/v), penicillin 100 units ml⁻¹, streptomycin 100 µg ml⁻¹ and amphotericin B 2.5 µg ml⁻¹, supplemented with *myo*-D-[³H]-inositol 10 µCi ml⁻¹.

The cells were washed twice with buffer containing Mg²⁺ and Ca²⁺ (see Materials) and incubated in this medium for 30 min. Before addition of the agonist, cells were washed twice with Ca²⁺-free buffer containing various concentrations of Mg²⁺ or EDTA and then stimulated in the same buffer containing the agonist.

The inositol phosphates were extracted as described previously (Pirrotton *et al.*, 1991). Briefly, the medium was rapidly replaced by 1 ml of HClO₄ (3%, w/v). The dishes were rinsed with 1 ml of HClO₄ (1%) and the lysate was neutralized with KOH (0.765 M) and HEPES (0.375 M).

The inositol phosphates were separated on Dowex AG1X8 columns (0.8 ml wet bed volume) as previously described (Pirrotton *et al.*, 1991).

Calculation of ATP⁴⁻ concentration

We have assumed that the predominant forms of ATP under our experimental conditions were: MgATP²⁻, CaATP²⁻, NaATP³⁻, HATP³⁻, KATP³⁻ and ATP⁴⁻. Therefore, the relevant equation is as follows: ATP total = ATP⁴⁻ + MgATP²⁻ + CaATP²⁻ + NaATP³⁻ + HATP³⁻ + KATP³⁻. The equilibrium involved in the formation of complex of ATP and a cation (C) may be expressed as follows:

$$[C]_{\text{free}} + [ATP^{4-}]_{\text{free}} \rightleftharpoons [CATP]$$

$$K_C = \frac{[CATP]}{[C]_{\text{free}} \times [ATP^{4-}]_{\text{free}}}$$

Moreover, the ionic concentration may be described by: [C]_{total} = [C]_{free} + [CATP]. We have assumed that [Na]_{free} and [K]_{free} approximate [Na]_{total} and [K]_{total} respectively, as these cations were in large excess over ATP. When MgSO₄ and CaCl₂ were omitted from the bathing solution, the actual concentrations of Mg²⁺ and Ca²⁺ were measured by a colorimetric method using methylthymol blue and calmagite as indicators for Ca²⁺ and Mg²⁺ respectively (BioMérieux, Marcy-l'Etoile, France). Mg²⁺ concentrations were 45 µM ± 15 and 52 µM ± 35 (mean ± s.d.), and Ca²⁺ concentrations were 5 µM ± 2 and 9 µM ± 4, respectively before and after incubation with the cells. Finally, the following association constants were taken from the literature: K_{Mg}: 10^{4.28} M⁻¹, K_{Ca}: 10^{3.94} M⁻¹, K_H: 10^{6.95} M⁻¹, K_{Na}: 5.82 M⁻¹, K_K: 5.30 M⁻¹ (Taqui Khan & Martell, 1966; Fine *et al.*, 1989). As [ATP]_{total}, [Mg²⁺]_{total}, [Ca²⁺]_{total}, [Na⁺], [K⁺] and [H⁺] were known, we derived by

substitution the following third-degree polynomial which was used to determine ATP⁴⁻ concentration:

$$K_{Mg} \times K_{Ca} \times [ATP^{4-}]^3 \times (1 + K_H \times [H^+] + K_{Na} \times [Na^+] + K_K \times [K^+]) + [ATP^{4-}]^2 \times (K_{Mg} + K_{Ca} + K_{Mg} \times K_{Ca} \times [Mg^{2+}]_{\text{total}} + K_{Ca} \times K_{Mg} \times [Ca^{2+}]_{\text{total}} + K_H \times K_{Mg} \times [H^+] + K_H \times K_{Ca} \times [H^+] + K_{Na} \times K_{Mg} \times [Na^+] + K_{Na} \times K_{Ca} \times [Na^+] + K_K \times K_{Mg} \times [K^+] + K_K \times K_{Ca} \times [K^+]) - K_{Mg} \times K_{Ca} \times [ATP]_{\text{total}} + [ATP^{4-}] \times (1 + K_{Mg} \times [Mg^{2+}]_{\text{total}} + K_{Ca} \times [Ca^{2+}]_{\text{total}} + K_H \times [H^+] + K_{Na} \times [Na^+] + K_K \times [K^+] - K_{Mg} \times [ATP]_{\text{total}} - K_{Ca} \times [ATP]_{\text{total}}) - [ATP]_{\text{total}} = 0$$

ATP⁴⁻ concentrations were calculated, according to the polynomial, by successive iterations.

Materials

Collagenase type IA and ATP (disodium salt) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; trypsin was from Flow Laboratories, Bioggio, Switzerland; all the other culture media and reagents were purchased from GIBCO, Grand Island, NY, U.S.A. EDTA (disodium salt) was from Boehringer Mannheim, Germany. Dowex AG1X8 (formate form) was from Bio-Rad Laboratories, Richmond, CA, U.S.A. The radioactive product *myo*-D-[³H]-inositol (10–20 Ci mmol⁻¹) was from Amersham, Gent, Belgium. UTP (disodium salt) was from Sigma; 2 MeSATP was from RBI, Natick, MA, U.S.A. The basic composition of the buffer used for the incubation of the cells was (mM): NaCl 124, KCl 5, MgSO₄ 1.25, CaCl₂ 1.45, KH₂PO₄ 1.25, D-glucose 8 and Na-HEPES 25, pH 7.4. In most experiments, the cells were stimulated in a medium from which CaCl₂ was omitted, and which contained MgSO₄ (1.25 or 5 mM) or no MgSO₄, or no MgSO₄ plus EDTA (1 mM).

Results

The major form of ATP in a physiological medium is MgATP²⁻; therefore the most crucial experiment was to evaluate the effect of Mg²⁺ omission from the incubation buffer on the cell responsiveness to ATP. Since preliminary results indicated that the maximal response to ATP was maintained in a calcium-free buffer (data not shown), we chose like others (Steinberg & Silverstein, 1987) to perform these experiments in a calcium-free buffer. As shown in Figure 1a, omission of Mg²⁺ from the incubation buffer caused a shift to the left of the ATP concentration-action curve without altering the maximal response. The magnitude of the response to 200 µM ATP was unaffected (1.5 ± 0.5 versus 1.6 ± 0.3 c.p.m. × 10³/dish in Mg²⁺-free medium and in medium with 1.25 mM Mg²⁺ respectively; mean ± s.d. of 3 independent experiments). When the results were plotted in terms of calculated ATP⁴⁻ concentration, the concentration-response curve obtained in the presence of 1.25 mM Mg²⁺ lay closer to the one obtained when Mg²⁺ was omitted from the medium (Figure 1b). A similar shift of the concentration-response curve was observed with ATPγS, which is not hydrolyzed by aortic endothelial cells (Cusack *et al.*, 1983) (data not shown).

In order to prevent the complexing of ATP by Ca²⁺ or Mg²⁺ released from the endothelial cells, the chelator EDTA was used. Although EDTA itself had a significant effect on the basal level of InsP₃ and on the absolute maximal response, it appears clearly from Figure 2 that the endothelial cells remained responsive to ATP in the presence of EDTA. Furthermore, the response to a low concentration of ATP was enhanced by EDTA. Plotting the results against calculated ATP⁴⁻ rather than total ATP brought closer to each other the concentration-action curves obtained in the presence of 1.25 mM Mg²⁺ or in the absence of Mg²⁺ plus EDTA, respectively (Figure 2b). When the baseline value was subtracted, the two curves became superimposable (Figure 2c).

Figure 1 [^3H]-inositol-labelled BAEC were washed twice with 1 ml Ca^{2+} -free buffer with 1.25 mM Mg^{2+} ($\blacktriangledown$) or without Mg^{2+} ($\bullet$). Cells were then challenged for 15 s in the same medium, with various concentrations of ATP as indicated. Results represent the radioactivity in the inositol trisphosphate (InsP_3) fraction (mean $\pm$ s.d. of triplicate determinations in one representative experiment out of two). (a) Data plotted in terms of the total concentration of ATP; (b) data plotted in terms of calculated ATP^{4-} concentration as described in the Methods section.

Finally, omission of Mg^{2+} from the incubation fluid produced a similar leftward shift of the UTP (Figure 3) and 2 MeSATP (Figure 4) concentration-action curves.

Discussion

Omission of MgSO_4 and CaCl_2 from the incubation fluid enhanced the responsiveness of endothelial cells to low concentrations of ATP. However, such a medium still contains some Mg^{2+} ($\pm 50 \mu\text{M}$). Solving the equation derived in the methods section shows that the distribution of the predominant forms of total ATP ($50 \mu\text{M}$) is as follows: ATP^{4-} (34%), MgATP^{2-} (25%), NaATP^{3-} (25%) and HATP^{3-} (12%). The endothelial cells remained responsive to ATP in a

Figure 2 [^3H]-inositol-labelled cells were washed twice with 1 ml Ca^{2+} -free buffer containing 1.25 mM Mg^{2+} ($\bullet$) or 1 mM EDTA ($\blacktriangledown$). Cells were then challenged for 15 s in the same medium containing ATP. Data are expressed as means $\pm$ s.d. of triplicate determinations in one representative experiment out of three. (a) Data plotted in terms of the total ATP concentration; (b) data plotted in terms of the calculated ATP^{4-} concentration; (c) data from (b) expressed as the InsP_3 production above the basal level.

Ca^{2+} - and Mg^{2+} -free medium (no addition of CaCl_2 and MgSO_4 , 1 mM EDTA). In these conditions, ATP^{4-} represents 48% of total ATP ($50 \mu\text{M}$), NaATP^{3-} 34% and HATP^{3-} 17%. The interpretation of concentration-response curves in the presence of EDTA is somewhat complicated because the basal level of InsP_3 was notably increased and the absolute maximal response was reduced. This might explain why the curve replotted in terms of ATP^{4-} in the presence of Mg^{2+} is not superimposable on that obtained in the presence of EDTA (Figure 2b). When the data were expressed as the absolute InsP_3 production above the basal level, the curves were almost superimposable (Figure 2c). These effects on the basal level and on the absolute maximal response probably result from the fact that chelation of extracellular divalent cations may affect the intracellular levels of Mg^{2+} and Ca^{2+}

Figure 3 [³H]-inositol-labelled BAEC were washed twice with 1 ml Ca²⁺-free buffer with 5 mM Mg²⁺ (▼) or without Mg²⁺ (●). Cells were then challenged for 15 s in the same medium, with various concentrations of UTP as indicated. Results represent the radioactivity in the inositol trisphosphate (InsP₃) fraction (mean ± s.d. of triplicate determinations in one representative experiment out of three).

Figure 4 [³H]-inositol-labelled BAEC were washed twice with 1 ml Ca²⁺-free buffer with 5 mM Mg²⁺ (▼) or without Mg²⁺ (●). Cells were then challenged for 15 s in the same medium, with various concentrations of 2-methylthioadenosine-5'-triphosphate (2MeSATP) as indicated. Results represent the radioactivity in the inositol trisphosphate (InsP₃) fraction (mean ± s.d. of triplicate determinations in one representative experiment out of four).

which are both involved in several steps of phosphoinositides metabolism (Abdel-Latif, 1986). For example, Lambert *et al.* (1986) studying the bradykinin stimulation of inositol phosphate production in aortic endothelial cells have observed differences between basal and stimulated values in calcium-free versus calcium-supplemented buffer, although the degree of stimulation was not different. Nevertheless, our results indicate that the active agonist which induces phosphoinositide hydrolysis in BAEC is neither MgATP²⁻, the form present in highest concentration in physiological conditions, nor CaATP²⁻. The active species might thus be ATP⁴⁻ or a form of ATP complexed with monovalent cations. We have excluded the possibility that the shift of the concentration-response curve in the absence of Mg²⁺ is due to a reduced hydrolysis of ATP by Mg²⁺-dependent ecto-ATPase, since the same shift was observed with ATPγS, which is not a substrate of endothelial ecto-ATPase (Cusack *et al.*, 1983). Interestingly, a similar shift was observed with both UTP and 2 MeSATP; it appears therefore that both P_{2y} and nucleotide/P_{2u} receptors, recently shown to coexist on aortic endothelial cells (Motte *et al.*, 1993), recognize a form of ATP uncomplexed with bivalent cations. One implication of these studies is that the affinity of endothelial P₂-receptors for their true ligand is much larger than the value which can be derived from the concentration-action curve obtained in physiological medium. It has been initially assumed that the role of ATP forms uncomplexed with divalent cations as receptor agonists was restricted to the P_{2z} receptors expressed on mast cells and macrophages (Gordon, 1986). Recent data indicate that this situation is far more general. First, studies have shown that Mg²⁺ removal enhanced the ATP responses

mediated by the nucleotide/P_{2u} receptor. Examples include the ATP-induced InsP₃ production in pituitary cells (Davidson *et al.*, 1990), the effects of ATP and UTP on Ca²⁺ translocation in human fibroblasts (Fine *et al.*, 1989) and in neutrophils (Walker *et al.*, 1991), and the PGI₂ production in BPAE (Lustig *et al.*, 1992). Our data regarding UTP further extend this concept to InsP₃ production in BAEC. Second, Fedan *et al.* (1990) have shown that ATP⁴⁻ was the active agonist of the P_{2x} purinoceptors of the smooth muscle cells of the guinea-pig vas deferens. Finally, our data regarding 2MeSATP further extend these observations to the P_{2y} subtype purinoceptor and suggest that all the P₂ subtype ATP-receptors, including the nucleotide/P_{2u} receptor, are preferentially activated by ATP⁴⁻ or a form of ATP complexed with a monovalent cation rather than by MgATP²⁻ or CaATP²⁻. In that respect, the behaviour of ATP receptors is thus basically different from that of enzymes such as ATPases and kinases, for which the physiological substrate is MgATP²⁻.

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