Heterogeneity of ATP Receptors in Aortic Endothelial Cells

Involvement of P2y and P2u Receptors in Inositol Phosphate Response

S. Motte, S. Pirotton, and J.M. Boeynaems

Extracellular ATP plays an important role in the regulation of prostacyclin and nitric oxide release from vascular endothelial cells. These cellular responses to ATP are generally attributed to the stimulation of the P2y subtype of P2 purinergic receptors. However, it has recently been suggested that two types of ATP receptors might coexist on endothelial cells. To evaluate this hypothesis, we examined the effects of P2y2 receptor agonists 2-methylthioadenosine 5'-triphosphate (2MeSATP) and 2'- and 3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (BzATP) and of UTP on the accumulation of inositol phosphates in bovine aortic endothelial cells. BzATP, 2MeSATP, and UTP produced a smaller maximal effect than ATP. The effects of 2MeSATP and UTP were additive, whereas the effects of ATP and either UTP or 2MeSATP were not. Prior exposure to UTP reduced the subsequent response to UTP by 12% of the control response, whereas the response to 2MeSATP was decreased to 61%. Reciprocally, preincubation with 2MeSATP reduced the subsequent response to 2MeSATP to 23% of the control response, whereas the response to UTP was reduced to 73%. Pertussis toxin pretreatment decreased the response to both ATP and UTP (65% and 70% inhibition, respectively), whereas the response to 2MeSATP was not modified. Our data support the hypothesis that two classes of receptors recognizing ATP are expressed on bovine aortic endothelial cells. (Circulation Research 1993;72:504-510)

KEY WORDS: • ATP • receptors • endothelial cells • inositol phosphates

Extracellular ATP and ADP play an important role in the interaction between platelets (a rich source of adenine nucleotides) and the vessel wall, mainly through the stimulation of prostacyclin and nitric oxide release from vascular endothelial cells.1,2 The cellular responses to ATP are mediated by specific receptors, called P1, as opposed to P2 purinergic receptors, which interact preferentially with adenine nucleotides. Further subclassification of vascular P2 purinergic receptors into P2y and P2u has been proposed on the basis of the effects of several chemical analogues of ATP.3 P2y responses are characterized by the following order of potency: α,β-methylene ATP=β,γ-methylene ATP=2-methylthioadenosine 5'-triphosphate (2MeSATP). The P2y subtype displays a different rank order of potency: 2MeSATP>ATP>α,β-methylene ATP=β,γ-methylene ATP. The responses of vascular endothelial cells to adenine nucleotides are generally attributed to the stimulation of P2y receptors. This conclusion is supported by studies on the endothelium-dependent relaxation of the pig aorta,4 the prostacyclin production by pig aortic endothelial cells5 and human umbilical vein endothelial cells,6 inositol phosphate accumulation in bovine aortic endothelial cells (BAECs) of the AG476 cell line,7 and calcium mobilization in human endothelial cells.6 However, a careful analysis of the data suggests the possibility of a heterogeneous population of ATP-sensitive receptors on vascular endothelial cells. Indeed, in some cell preparations, 2MeSATP, a key agonist in the identification of P2y receptors, induced a smaller maximal effect than ATP, although it was more potent. Examples include the release of prostacyclin from pig aortic endothelial cells,5 the [Ca2+]i rise in human endothelial cells,6 and the endothelium-dependent relaxation of the rat aorta.8 Moreover, the exclusive role of P2y receptors in mediating endothelial responses to ATP is challenged by studies in which the pyrimidine nucleotide UTP appeared to be active and as potent as ATP. Examples include the release of prostacyclin from pig aortic endothelial cells5 or from bovine pulmonary artery endothelial cells9 and the endothelium-dependent relaxation of the rat aorta9 and the rat mesenteric arterial bed.10 Whether UTP and ATP are acting at the same site in the vessel wall is still controversial. Ralevic and Burnstock10 recently suggested that distinct purinergic and pyrimidinergic receptors are present in the rat mesenteric arterial bed. Their conclusion is in agreement with that of Seifert

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This work was made possible by a contract of the Ministère de la Politique Scientifique (Sciences de la Vie B 10/04) and was supported by a grant of the Fonds de la Recherche Scientifique Médicale. S.P. is an "Aspirant" of the Fonds National de la Recherche Scientifique.

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Received July 10, 1992; accepted November 11, 1992.
and Schultz,11 who reviewed data available from studies on the effects of ATP and UTP in other systems such as HL-60 cells, neutrophils, and macrophages. On the other hand, recent data obtained with isolated rat hepatocytes,12 NCB-20 cells,13 and a model of human airway epithelial cells14 suggest that ATP and UTP may activate a common receptor distinct from the classically defined P2\(_x\) and P2\(_\mathrm{Y}\) purinergic receptors. Taken together, these data led us to reconsider the hypothesis that endothelial responses to ATP are mediated exclusively by P2\(_x\) receptors. O’Connor et al14 proposed recently that two types of ATP receptors coexist on endothelial cells: P2\(_x\) receptors and receptors that recognize both ATP and UTP, termed P2\(_\mathrm{Y}\) receptors. The aim of the present study was to evaluate this hypothesis by examining the effects of P2\(_x\) receptor agonists 2MeSATP and 2\(-\) and 3\(-\)O-(4-benzoylbenzoyl)adenosine 5\(-\) -triphasphate (BzATP)15 and the effect of UTP on the primary biochemical event induced by ATP in endothelial cells, the accumulation of inositol phosphates.16,17

**Materials and Methods**

**Materials**

Collagenase type IA, BzATP, ATP, and UTP were from Sigma Chemical Co., St. Louis, Mo.; trypsin was from Flow Laboratories, Bioggio, Switzerland; and all other culture media and reagents were purchased from Gibco, Grand Island, N.Y. LiCl was from Merck, Darmstadt, FRG. 2MeSATP was from RBI, Natick, Mass. Pertussis toxin was from Janssen Chimica, Geel, Belgium. The radioactive product myo-D-[2\(^{3}\)H]inositol (10–20 Ci/mmol) was from Amersham, Ghent, Belgium. Dowex AG1X8 (formate form) was from Bio-Rad Laboratories, Richmond, Calif.

**Cell Culture**

BAECs were obtained by collagenase digestion of the aorta excised from a freshly slaughtered cow and cultured in a medium composed of minimum essential medium (MEM) D-valine (80% [vol/vol]), fetal calf serum (20% [vol/vol]), 2 mM glutamine, 100 units penicillin/ml, 100 \(\mu\)g streptomycin/ml, and 2.5 \(\mu\)g amphotericin B/ml. MEM D-valine was used to prevent survival of contaminating smooth muscle cells. The cells were incubated at 37°C in a humidified air-CO\(_2\) (19:1) incubator. When the primary culture formed a confluent monolayer, the cells were harvested with trypsin (0.1% [wt/vol]) in a Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks’ buffer and subcultured in 35-mm-diameter Petri dishes. For the second and the following passages, the medium was replaced by Dulbecco’s modified Eagle’s medium (DMEM, 60% [vol/vol]), Ham’s F-12 medium (20% [vol/vol]), fetal calf serum (20% [vol/vol]), antibiotics, amphotericin B, and glutamine at the same concentrations. The cells were used between the second and fourth passages.

**Incubation of BAECs**

Subconfluent cells (approximately \(8 \times 10^5\) cells per dish) were incubated for 24 hours in a medium containing inositol-free DMEM and Ham’s F-12, fetal calf serum (5% [vol/vol]), antibiotics, and amphotericin B as described above, supplemented with 10 \(\mu\)Ci D-[\(^{3}\)H]inositol (10–20 Ci/mmol)/ml. We have previously shown that ATP induces in BAECs a transient increase of inositol trisphosphate (InsP\(_3\)) that is maximal after 15 seconds; on the other hand, the time course of inositol monophosphate (InsP\(_1\)) in Li\(^+\)-treated cells showed that InsP\(_1\) was continuously accumulated over a 10-minute incubation.16,17 Therefore, when InsP\(_3\) accumulation was tested in the present study, cells were washed twice with DMEM and incubated in this medium for 30 minutes before the addition of the agonist; the incubation was stopped 15 seconds later. At that time, most of the InsP\(_3\) fraction represents inositol 1,4,5-trisphosphate.17 When the accumulation of InsP\(_3\) was tested, prelabeled cells were washed twice with DMEM and incubated for 40 minutes in DMEM with 10 mM LiCl; the agonist was added for the last 10 minutes. In these conditions, the InsP\(_1\) fraction is a mixture of inositol 1-monophosphate and inositol 4-monophosphate, the two end products of inositol 1,4,5-trisphosphate metabolism.17 Incubations with BzATP were performed in the dark. Boyer and Harden15 have shown that, in the absence of photolysis, BzATP interacts with P2\(_x\) receptors in a reversible way.

**Separation of Inositol Phosphates on Dowex Columns**

The inositol phosphates were extracted as described previously.16 Briefly, the medium was rapidly replaced by 1 ml HClO\(_4\) (3% [wt/vol]). The dishes were rinsed with 1 ml HClO\(_4\) (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) with increasing concentrations of ammonium formate: inositol was eluted...
with water (20 ml), glycerophosphoinositol, with 0.06 M ammonium formate/5 mM sodium tetraborate (8 ml); InsP₁, with 0.15 M ammonium formate/5 mM sodium tetraborate (20 ml); inositol bisphosphate, with 0.4 M ammonium formate/0.1 M formic acid (18 ml); and InsP₃, with 0.7 M ammonium formate/0.1 M formic acid (12 ml). The inositol phosphates were quantitated by liquid scintillation counting of a 4-ml portion of each fraction.

Statistical Analysis

Results are given as mean±SD. Statistical significance was evaluated by Student’s t test. A value of p<0.05 was taken as significant.

Results

Inositol Phosphate Accumulation in BAECs

Stimulated by BzATP, 2MeSATP, UTP, and ATP: Comparison of Concentration–Action Curves

2MeSATP was more potent than ATP at low concentrations, but it produced a smaller maximal response than ATP (Figure 1). The InsP₃ response to 10 μM 2MeSATP was 46% (mean of five experiments; range, 30–54%) of the response to 200 μM ATP. UTP was as potent as ATP, but it was slightly less effective than ATP (Figure 1). The amplitude of the maximal InsP₃ response to UTP (200 μM) was 75% (mean of six experiments; range, 61–87%) of the response to 200 μM ATP. Similar results were obtained in experiments in which InsP₁ was measured (Figure 2): the InsP₁ responses to 30 μM 2MeSATP represented 37% of the response to 200 μM ATP (mean of four experiments; range, 26–46%), and the response to UTP was 75% (mean of four experiments; range, 67–84%) of the response to ATP. Finally, BzATP was much less effective than ATP. Concentration–response curves showed that the maximal InsP₁ response was obtained with 100 μM BzATP (data not shown). This maximal response was only 13% of the ATP response (mean of three experiments; range, 11–16%) (Figure 2).

Additivity of the Responses to 2MeSATP and UTP

To investigate possible additivity, we added 2MeSATP or UTP simultaneously with ATP, and the responses were compared with those induced by each agonist alone. Figure 3 shows that the responses to 200 μM ATP were not significantly affected by the addition of either UTP (200 μM) or 2MeSATP (10 μM). On the contrary, the combination of 2MeSATP and UTP gave an additive response (p<0.05, Student’s t test).

To exclude the possibility that 2MeSATP is a partial agonist of the ATP receptor, we examined the ability of high concentrations of 2MeSATP to antagonize ATP. We added increasing concentrations of 2MeSATP (up to 200 μM) simultaneously with ATP (200 μM); as shown in Figure 4, the response to ATP was not inhibited by these high concentrations of 2MeSATP.

Cross Desensitization of the Responses to ATP, UTP, and 2MeSATP

It is well established that exposure of endothelial cells to ATP induces a refractoriness to a further stimulation...
that mostly results from a process of homologous desensitization.\textsuperscript{18,19} Therefore, we investigated the possible cross desensitization between ATP and its analogues. After an initial stimulation with either ATP, 2MeSATP, or UTP, the cells were again challenged by either 2MeSATP or UTP (Figure 5, left panel). Whereas pretreatment with bradykinin resulted in a minor reduction of the response to subsequent stimulation by either ATP, 2MeSATP, or UTP, pretreatment with 200 \( \mu \)M ATP reduced the subsequent response to both 2MeSATP and UTP (a 67\% and 74\% decrease in the response, respectively; mean of two experiments; Figure 5, right panel). Pretreatment with UTP reduced the subsequent response to UTP to 12\% of the control response (mean of three experiments; range, 10–14\%), whereas the response to 2MeSATP was reduced to 61\% of the control response (range, 50–71\%). Reciprocally, preincubation with 2MeSATP reduced the subsequent response to 2MeSATP to 23\% of the control response (mean of three experiments; range, 20–30\%), whereas the response to UTP was reduced to 73\% of the control response (range, 69–78\%). Similar results were obtained whether the kinase C inhibitor staurosporine was included in the medium or not (not shown).

**Effect of Pertussis Toxin Pretreatment**

We have previously reported the involvement of a pertussis toxin-sensitive G protein in ATP-stimulated inositol phosphate formation in endothelial cells.\textsuperscript{20} Therefore, we compared the effects of pertussis toxin on the responses to 2MeSATP and UTP (Figure 6).

**Figure 4.** Bar graph showing lack of competitive antagonism between high concentrations of 2-methylthioadenosine 5'-triphosphate (2MeSATP) and ATP. Bovine aortic endothelial cells, prelabelled with \([\text{H}]\text{inositol, were incubated for 40 minutes with 10 mM LiCl. 2MeSATP and ATP were added simultaneously for the last 10 minutes. The inositol monophosphate (InsP\(_1\)) fraction was isolated as described in "Materials and Methods." Data are from one experiment and are expressed as mean±SD of triplicate dishes. Bars indicate the following conditions: control (○), 200 \( \mu \)M ATP (●), 2MeSATP alone (■), and 2MeSATP+200 \( \mu \)M ATP (△).**

**Figure 5.** Left panel: Bar graph showing inositol trisphosphate (InsP\(_3\)) accumulation in bovine aortic endothelial cells: study of cross desensitization between 2-methylthioadenosine 5'-triphosphate (2MeSATP) and UTP. \([\text{H}]\text{inositol-labeled cells were preincubated with either 2MeSATP (10 \( \mu \)M), UTP (200 \( \mu \)M), or no agent for 2 minutes. The cells were then washed twice with Dulbecco's modified Eagle's medium and incubated in this medium for 2 minutes before the addition of 2MeSATP or UTP for 15 seconds. InsP\(_3\) was then extracted and isolated as described in "Materials and Methods." The results are expressed as mean±SD of triplicate determinations in one representative experiment of three. Bars indicate the following conditions at first challenge: control (○), 2MeSATP (●), and UTP (△). Right panel: Bar graph showing the effect of ATP pretreatment on 2MeSATP- and UTP-induced InsP\(_3\) accumulation. \([\text{H}]\text{inositol-labeled cells were preincubated with ATP (200 \( \mu \)M) or no agent for 2 minutes. The cells were then washed twice with Dulbecco's modified Eagle's medium and incubated in this medium for 2 minutes before the addition of 2MeSATP (10 \( \mu \)M) or UTP (200 \( \mu \)M) for 15 seconds. The results are expressed as mean±SD of triplicate determinations in one representative experiment of two. Bars indicate the following conditions at first challenge: control (○) and ATP (△).**
Pertussis toxin pretreatment (20 ng/ml for 16 hours) produced a comparable decrease of responsiveness to both ATP and UTP (65% and 70% of inhibition, respectively; mean of two experiments). Conversely, the response to 2MeSATP was not modified (93% of the control response; mean of two experiments; range, 85–100%).

This differential effect of pertussis toxin treatment was not related to the magnitude of the InsP₃ responses. As shown in Figure 7, no significant effect of pertussis toxin could be detected over the entire concentration-response curve of 2MeSATP, whereas it inhibited responses to UTP of comparable magnitude.

**Discussion**

Although the characterization of ATP receptors is handicapped by the lack of potent and selective antagonists, investigations of inositol phosphate accumulation, which is the earliest event that follows receptor occupation in endothelial cells, can provide information regarding the different classes of receptors that are involved in that response. The results reported here show that the Pₛ agonists 2MeSATP and BzATP produced a smaller maximal effect than ATP on inositol phosphate production. The relative inefficacy of 2MeSATP cannot be due to higher susceptibility to hydrolysis, because 2MeSATP appears to be hydrolyzed less well than ATP itself. Moreover, our experimental design regarding InsP₃ measurements was based on short incubation times so that hydrolysis of different agonists by ecto-ATPases should be minimal. Differences in the effects of 2MeSATP could arise because of differences in the efficiency of receptor coupling to phospholipase C, suggesting that 2MeSATP might act as a partial agonist. However, according to that hypothesis, a potent but partial agonist should act as a competitive antagonist of a full agonist acting at the same receptor. Our data show that 2MeSATP failed to act in this way: responses to ATP were not inhibited, even in the presence of high concentrations of 2MeSATP. This finding is in agreement with data of Needham et al. regarding prostacyclin production by pig aortic endothelial cells and those of O'Connor et al. regarding endothelium-dependent relaxation of rat aorta. A lower efficacy of 2MeSATP has been previously reported in other systems, including prostacyclin production in piglet aorta endothelial cells, elevation of free intracellular Ca²⁺ in rabbit aorta.
lial cells,\textsuperscript{6} and endothelium-dependent relaxation of rat aorta.\textsuperscript{8} Conversely, other authors reported that 2MeSATP was equipotent with ATP in other preparations of endothelium. Examples include the relaxation of piglet aorta\textsuperscript{4} and the inositol phosphate production in a monoclonal cell line derived from bovine aorta.\textsuperscript{7} These last findings suggest that in some preparations of endothelium ATP acts on a homogeneous population of receptors of the P\textsubscript{2Y} subtype, whereas in other types of endothelial cells the response to ATP is not mediated exclusively by P\textsubscript{2Y} receptors.

Our data also show that in endothelial cells BzATP was much less effective than ATP. On the contrary, when it was used in the dark, this photocactive compound behaved as a potent and full agonist of the P\textsubscript{2Y} receptors of turkey erythrocytes.\textsuperscript{15} After photolysis, it became covalently bound to the P\textsubscript{2Y} receptor.\textsuperscript{15} Interestingly, when Boyer et al\textsuperscript{21} used \textsuperscript{32}P]BzATP to label P\textsubscript{2Y} receptors, they detected only a faint radioactive band in bovine pulmonary arterial endothelial cells, in contrast to the intense signal obtained with turkey erythrocytes and rat astrocytes or hepatocytes.

Our experiments comparing the effects of ATP and UTP were an attempt to solve some unsettled questions regarding the expression on BAECs of distinct purinergic and pyrimidinergic receptors. Although UTP may be released from blood platelets (a rich source of uracid nucleotides) and may play an important physiological role in the local regulation of vascular tone, there are only few reports of its effects on vascular endothelial cells, and it does not emerge unambiguously from the literature whether UTP is acting at a same site as ATP. Seifert and Shultz\textsuperscript{21} have proposed in their review that the effects of ATP and UTP could be mediated through distinct purinergic and pyrimidinergic receptors. In accord with this hypothesis, Ralevic and Burnstock\textsuperscript{10} recently confirmed the presence of distinct purinergic and pyrimidinergic receptors in the rat mesenteric artery smooth muscle. Their results show that the vasoconstrictor response to ATP, which is mediated via P\textsubscript{2Y} receptors on smooth muscle cells, is abolished by prior desensitization with a P\textsubscript{2Y} purinergic receptor agonist, whereas the response to UTP was unaffected, suggesting the presence of distinct receptors.\textsuperscript{10} Results from a number of experiments reported herein are consistent with the hypothesis that 2MeSATP and UTP act through two distinct subtypes of receptors and that ATP acts on both of them. In particular, the maximal effect of UTP was additive to that of 2MeSATP but not to that of ATP. Similar results regarding the lack of additive between the effects of ATP and UTP have been reported in rat isolated hepatocytes\textsuperscript{12} and in human airway epithelial cells.\textsuperscript{14} Preincubation of cells with ATP resulted in a marked desensitization of the effects of subsequently added UTP and 2MeSATP, but we observed a differential desensitization after pretreatment with UTP and 2MeSATP. Prior exposure to either UTP or 2MeSATP resulted in a marked homologous desensitization, whereas only a minor cross desensitization was observed between both agonists. Minor cross desensitization was also observed after pretreatment with bradykinin and may not involve the receptor itself. Possible explanations include a decreased activity of phospholipase C or a depletion of a common pool of phosphatidylinositol bisphosphates.

Finally, pretreatment with pertussis toxin resulted in a parallel loss of responsiveness to ATP and UTP, without affecting the capacity of 2MeSATP to stimulate inositol phosphate formation. This last observation is consistent with the insensitivity to pertussis toxin of the P\textsubscript{2Y}-mediated stimulation of phospholipase C in turkey erythrocytes.\textsuperscript{22}

Taken together, our data support the hypothesis that two classes of receptors are expressed on BAECs: a classical P\textsubscript{2Y} receptor and a nucleotide receptor, which is recognized by both ATP and UTP and is termed P\textsubscript{2}u.\textsuperscript{1} In the future, this conclusion should be strengthened by additional experiments, including radioligand binding data.

Acknowledgments

We thank Mrs. N. Galand for her excellent technical assistance in performing the experiments and Mrs. E. Ledent and C. Piesen for typing the manuscript.

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Heterogeneity of ATP receptors in aortic endothelial cells. Involvement of P2y and P2u receptors in inositol phosphate response.
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Circ Res. 1993;72:504-510
doi: 10.1161/01.RES.72.3.504

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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