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A nucleotide receptor in vascular endothelial cells is specifically activated by the fully ionized forms of ATP and UTP

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Extracellular ATP causes an increase in the concentration of cytoplasmic free calcium ($[Ca^{2+}]_i$) in bovine pulmonary-artery endothelial (BPAE) cells that results in the synthesis and release of prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet aggregation. We show here that PGI_2 release in BPAE cells correlates with the concentration of the fully ionized form of extracellular ATP (ATP^{4-}) and not with the concentration of other ionic forms of ATP. Concentrations as low as 10 nM-ATP^{4-} elicited an increase in PGI_2 release [EC_{50} (concn. giving half-maximal stimulation) 3 μM] in BPAE cells incubated in an iso-osmotic medium, pH 7.4, lacking Ca^{2+} and Mg^{2+}. When the pH or the Mg^{2+} concentration of the medium was varied so as to maintain a constant level of ATP^{4-}, while varying the concentration of proton-ATP ($HATP^{3-}$) or $MgATP^{2-}$ respectively, PGI_2 release remained constant. An inhibitory effect of extracellular Mg^{2+} on PGI_2 release could be attributed solely to a decrease in the concentration of ATP^{4-}. In contrast with Mg^{2+}, extracellular Ca^{2+} stimulated PGI_2 release induced by ATP. Several results suggest that extracellular Ca^{2+} modulates PGI_2 release by increasing Ca^{2+} uptake through an ATP^{4-}-activated plasma-membrane channel. In BPAE cells incubated in Ca^{2+}-free medium, ATP elicited a transient increase in $[Ca^{2+}]_i$ that declined to the basal level within 60 s. In cells incubated in Ca^{2+}-containing medium, ATP caused an increase in $[Ca^{2+}]_i$ that had two components: a transient peak in $[Ca^{2+}]_i$ (0–60 s) and a sustained increase in $[Ca^{2+}]_i$ that was maintained for several minutes after ATP addition. Increasing the concentration of extracellular calcium from 0.25 mM to 10 mM had no effect on the transient rise in $[Ca^{2+}]_i$ induced by ATP, but significantly enhanced the magnitude of the sustained increase in $[Ca^{2+}]_i$. Alterations in the magnitude of the sustained increase in $[Ca^{2+}]_i$ would likely modulate PGI_2 release, which was not complete until 2 min after ATP addition. Extracellular Ca^{2+} also stimulated PGI_2 release induced by bradykinin. Bradykinin caused a sustained increase in $[Ca^{2+}]_i$ in BPAE cells in the presence of extracellular Ca^{2+}. Finally, the magnitude of PGI_2 release induced by UTP, a more potent agonist than ATP, correlated with the concentration of extracellular fully ionized UTP (UTP^{4-}). These findings support the hypothesis that nucleotide receptors in BPAE cells recognize the fully ionized form of ATP and UTP and are coupled to signal-transduction pathways involving the mobilization of intracellular Ca^{2+}, the influx of extracellular Ca^{2+} and the subsequent release of PGI_2.

INTRODUCTION

There is considerable pharmacological and biochemical evidence supporting the hypothesis that the hormone-like effects of extracellular nucleotides in the cardiovascular system are mediated by plasma-membrane receptors (Gordon, 1986; Cooper *et al.*, 1989; Boyer *et al.*, 1990; Erb *et al.*, 1990). Nucleotide receptors in mammalian tissues are coupled to a variety of signal-transduction pathways involving the phospholipase C-dependent mobilization of cytoplasmic free Ca^{2+} (Charest *et al.*, 1985; Dubyak & De Young, 1985; Fine *et al.*, 1989; Gonzalez *et al.*, 1989), the activation of plasma-membrane channels for Ca^{2+} (Benham & Tsien, 1987; Greenberg *et al.*, 1988), K^+ (Gordon & Martin, 1983) or normally impermeant molecules (Rozengurt & Heppel, 1975; Weisman *et al.*, 1984; Gomperts, 1985; Heppel *et al.*, 1985; Steinberg *et al.*, 1987), the stimulation of a phospholipase A_2 (Boeynaems & Pearson, 1990) or the phospholipase D-dependent formation of phosphatidic acid (Martin & Michaelis, 1989; Pirotton *et al.*, 1990).

One of the more intensively studied nucleotide receptors, located in vascular endothelial cells, regulates the formation of prostacyclin (PGI_2), a vasodilator and inhibitor of platelet aggregation (Moncada, 1982). It has been postulated that the extracellular ATP-mediated release of PGI_2 from the endothelium prevents the excessive accumulation of platelets at a site of vascular injury, thereby preventing thrombosis, and that, through its vasodilatory actions, PGI_2 also helps alleviate the adverse effects of ischaemia and circulatory shock (Boeynaems & Pearson, 1990). The activation of a nucleotide receptor in endothelial cells leads to the phospholipase C-dependent mobilization of intracellular Ca^{2+}, the influx of extracellular Ca^{2+} and the subsequent activation of enzymes that degrade phospholipids to generate arachidonic acid (Boeynaems & Galand, 1983; Pearson *et al.*, 1983; Hallam & Pearson, 1986; Luckoff & Busse, 1986; Forsberg *et al.*, 1987; Pirotton *et al.*, 1987), the rate-limiting substrate for PGI_2 synthesis (Moncada & Vane, 1979). The ATP-mediated increase in the concentration of cytoplasmic free calcium ($[Ca^{2+}]_i$) is necessary and sufficient to induce the synthesis and release of PGI_2 (Carter *et al.*, 1988; Hallam *et al.*, 1988; Martin & Michaelis, 1990; Lustig *et al.*, 1992). In bovine pulmonary-artery endothelial (BPAE) cells, an increase in $[Ca^{2+}]_i$ elicited by ATP or UTP, a pyrimidine nucleotide, activates a calmodulin-dependent, phosphatidylcholine-specific phospholipase A_2 (Lustig *et al.*, 1992).

There are several possible mechanisms by which ATP and other nucleotides can be released into the blood and thereby

Abbreviations used: BPAE cells, bovine pulmonary-artery endothelial cells; PGI_2, prostacyclin; $[Ca^{2+}]_i$, the concentration of cytoplasmic free calcium; EC_{50}, concentration giving half-maximal stimulation; fura-2-AM, fura-2 penta-acetoxymethyl ester; 6-oxo-$PGF_{1\alpha}$, 6-oxo-prostaglandin $F_{1\alpha}$; HBS, Hepes-buffered saline solution; $HATP^{3-}$, proton-ATP; ATP^{4-}, the fully ionized form of ATP; UTP^{4-}, the fully ionized form of UTP.

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stimulate PGI_2 release. Two primary sources of extracellular nucleotides are platelets (Meyers *et al.*, 1982) and adrenal medullary cells (Rojas *et al.*, 1987), which store high millimolar levels of adenine nucleotides in cytoplasmic granules and release these stores into the bloodstream upon chemical stimulation. Other sources of blood ATP include dead and injured cells, myocardial cells (Forrester & Williams, 1977), and endothelial and smooth-muscle cells (Pearson & Gordon, 1979). Blood levels of ATP sufficient to cause PGI_2 release (20 μM) have been detected 3–5 min after a small incision in human skin (Born & Kratzer, 1984), and the ATP level immediately after injury may be considerably higher, since nucleotidases present in the serum (Ishii & Green, 1973; Weisman *et al.*, 1988) and on the surface of endothelial cells (Gordon *et al.*, 1986) serve to degrade extracellular ATP.

The predominant form of ATP in the blood is the magnesium–ATP complex ($MgATP^{2-}$), the substrate for many intracellular ATP-requiring enzymes. Although most studies to date have not determined whether nucleotide receptors are activated by a specific ionic form of extracellular ATP, it has been suggested that the fully ionized form of ATP (ATP^{4-}), and not $MgATP^{2-}$, is the receptor ligand that mediates the release of histamine from rat mast cells (Dahlquist & Diamant, 1974; Cockcroft & Gomperts, 1979) and the activation of plasma-membrane channels to normally impermeant metabolites in rat mast cells (Gomperts, 1985), mouse fibroblasts (Heppel *et al.*, 1985) and mouse macrophages (Steinberg *et al.*, 1987). In addition, it has recently been suggested that ATP^{4-}, and not $MgATP^{2-}$, induces an increase in $[Ca^{2+}]_i$ in mouse macrophages (Greenberg *et al.*, 1988), human skin fibroblasts (Fine *et al.*, 1989) and sheep pituitary cells (Davidson *et al.*, 1990). Finally, Pearson & Cusack (1985) have suggested, based on indirect evidence, that a nucleotide receptor in endothelial cells recognizes ATP uncomplexed to a metal cation.

The objective of the present study was to determine whether the receptor that mediates the nucleotide-induced release of PGI_2 in BPAE cells is activated by a specific ionic form of nucleotide. We demonstrate that nucleotide receptors in BPAE cells are activated by ATP^{4-} or the fully ionized form of UTP (UTP^{4-}) and that neither $MgATP^{2-}$, proton-ATP ($HATP^{3-}$), $NaATP^{3-}$, $KATP^{3-}$ nor $CaATP^{2-}$ acts as receptor agonist or antagonist. Our results also suggest that the stimulation of PGI_2 release by ATP is a consequence of both the ATP-mediated release of Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+} through an ATP^{4-}-activated plasma-membrane channel.

MATERIALS AND METHODS

Materials

BPAE cells (CCL 209, passage 14) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and used for experiments between passages 19 and 26. Fetal-bovine serum, Eagle's (1959) minimum essential medium, penicillin and streptomycin were obtained from GIBCO (Grand Island, NY, U.S.A.). The radioimmunoassay kit for 6-oxoprostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$) was obtained from New England Nuclear (Boston, MA, U.S.A.). Sterile disposable pipettes and polystyrene tissue-culture flasks and dishes were manufactured by Corning (New York, NY, U.S.A.) and obtained from Fisher Scientific (St. Louis, MO, U.S.A.). Ecolume scintillation cocktail was obtained from ICN (Irvine, CA, U.S.A.). Fura-2 pentaacetoxymethyl ester (fura-2-AM), nucleotides, nucleosides and other chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). All compounds were solubilized in deionized water purified by using a Milli-Q water-treatment system (Millipore Co., Bedford, MA, U.S.A.). Concentrations of nucleotide stock solutions were verified spectrophotometrically.

Methods

Cell culture. BPAE cells were grown on 75 cm^2 polystyrene flasks in 20 ml of growth medium, pH 7.4, consisting of Eagle's minimum essential medium, 15% (v/v) fetal-bovine serum, 10 mM-Hepes, 26 mM-$NaHCO_3$, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell cultures were maintained at 37 °C in a humidified (90%) atmosphere of air/CO_2 (19:1). The cells were removed from the flask at confluence by a 5 min incubation with 5 ml of phosphate-buffered saline [PBS; 8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4 (pH 7.4)/137 mM-NaCl/4 mM-KCl], containing 0.05% (w/v) trypsin and 0.3 mM-EDTA, and then subcultured at a density of 1×10^4 cells/cm^2 on 10 cm^2 polystyrene dishes in 2 ml of growth medium. Cell number was determined using a Coulter Model ZBI cell counter (Hialeah, FL, U.S.A.) or a haemocytometer. The growth medium was replaced 2 or 3 days after subculturing cells on dishes or flasks.

Measurement of prostacyclin release. At 6–10 days after subculturing BPAE cells on 10 cm^2 dishes, confluent monolayers were washed twice and incubated for 5 min at 37 °C in 1 ml of HBS (10 mM-Hepes (pH 7.4)/137 mM-NaCl/5 mM-KCl/5 mM-glucose), unless otherwise indicated. Nucleotides and other compounds were added to the medium as specified in the Figure and Table legends. After the indicated time period at 37 °C, the medium was removed and stored at −20 °C until assayed for PGI_2. The amount of PGI_2 in each sample was quantified by using a radioimmunoassay for 6-oxo-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2 (Johnson *et al.*, 1976), according to instructions in the radioimmunoassay kit (New England Nuclear).

Ca^{2+} measurements. Confluent cultures of BPAE cells on 75 cm^2 flasks were removed by trypsin treatment as described above. Dissociated cells were sedimented at 200 ***g*** for 5 min at 25 °C and washed once with 10 ml of HBS, pH 7.4. The washed cells were suspended in HBS, pH 7.4, supplemented with 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$ at 5×10^5 cells/ml, and stored (for up to 4 h) at room temperature. A 2 ml aliquot of the cell suspension was incubated with 2 μM-fura-2-AM for 20 min at 37 °C. The hydrolysis of the membrane-permeant fura-2-AM by non-specific cytoplasmic esterases traps fura-2, a fluorescent Ca^{2+}-binding probe, inside cells (Grynkiewicz *et al.*, 1985). Fura-2-loaded cells were sedimented, washed twice and suspended in 2 ml of HBS, pH 7.4, supplemented with 1 mM-$MgCl_2$ and the indicated concentration of $CaCl_2$. The cell suspension then was transferred to a quartz cuvette and placed in the temperature-regulated (37 °C) sample chamber of a dual-excitation beam spectrofluorimeter (SPEX, Edison, NJ, U.S.A.) and $[Ca^{2+}]_i$ was measured according to a method developed by Grynkiewicz *et al.* (1985) with slight modifications (Lustig *et al.*, 1992). During measurements of $[Ca^{2+}]_i$ the cell suspension was stirred continuously with a Teflon bar driven by a magnetic-stirring motor positioned beneath the sample chamber. The magnitude of the maximal increases in $[Ca^{2+}]_i$ and PGI_2 release elicited by ATP and UTP varied somewhat between different BPAE cell preparations, but was consistent within a single preparation (K. D. Lustig & G. A. Weisman, unpublished work).

Calculation of ionic forms of ATP and UTP at different nucleotide and cation concentrations. The concentrations of the different ionized forms of extracellular ATP and UTP were calculated by using a BASIC program (kindly supplied by Dr. Tom Berkelman, University of California, Davis CA, U.S.A.) that was based upon a FORTRAN program written by Perrin & Sayce (1967). In the present study it has been assumed that the predominant forms of extracellular ATP are $MgATP^{2-}$, ATP^{4-}, $CaATP^{2-}$, $HATP^{3-}$, $NaATP^{3-}$ and $KATP^{3-}$. The concentrations

Table 1. PGI_2 release correlates with the concentration of ATP^{4-} and not $MgATP^{2-}$

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with the indicated concentration of $MgCl_2$. After 5 min, ATP was added to the indicated concentrations of ATP^{4-} and $MgATP^{2-}$, calculated as described in the Materials and methods section. The medium was sampled 5 min later and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Results are means ± S.D. of triplicate determinations and are representative of results from five experiments.

$[MgATP^{2-}]$ (μM)	$[ATP^{4-}]$ (μM)	$[MgCl_2]$ (mM)	PGI_2 release (pmol/10^6 cells)
15.8	0.9	1.0	23.8±2.4
24.5	0.9	1.5	31.9±3.6
32.9	0.9	2.0	33.6±5.7
49.8	0.9	3.0	28.9±1.5
84.0	0.9	5.0	25.1±1.9
128.4	0.9	7.5	26.3±10.5
167.4	0.9	10.0	30.0±5.9
0	0	10.0	1.0±0.1
0	0.9	0	29.6±4.2
0	15.0	0	51.2±9.6

of other forms of ATP (e.g., H_2ATP^{2-}), which are presumably present at very low concentrations, have not been considered. The equilibria and conservation equations needed to calculate the concentration of ATP^{4-}, $MgATP^{2-}$ and $HATP^{3-}$ are described by Steinberg & Silverstein (1987). The concentrations of $CaATP^{2-}$, $NaATP^{3-}$, $KATP^{3-}$ or the different ionic forms of UTP were calculated by a similar approach. The following association constants were used for all calculations: Mg^{2+} for ATP^{4-}, $10^{4.28}$ M^{-1}; and for UTP^{4-}, $10^{4.35}$ M^{-1}; Ca^{2+} for ATP^{4-}, $10^{3.94}$ M^{-1}; H^+ for ATP^{4-}, $10^{6.95}$ M^{-1}; K^+ for ATP^{4-}, 5.30 M^{-1}; Na^+ for ATP^{4-}, 5.82 M^{-1} (Cockcroft & Gomperts, 1979; Sigel, 1987; Fine *et al.*, 1989).

RESULTS AND DISCUSSION

The nucleotide receptor that mediates PGI_2 release from BPAE cells recognizes ATP^{4-} but not $MgATP^{2-}$

Extracellular ATP can bind a variety of cations found in the blood, including Mg^{2+}, Ca^{2+}, Na^+, K^+ and H^+. In an iso-osmotic medium, pH 7.4, containing 100 μM-ATP, 137 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$, the predominant ionic forms of ATP, calculated as described in the Materials and methods section, are distributed as follows: $MgATP^{2-}$, 62.7%; ATP^{4-}, 3.5%; $HATP^{3-}$, 1.3%; $NaATP^{3-}$, 2.8%; $KATP^{3-}$, 0.1%; and $CaATP^{2-}$, 29.7%. Other ionic forms of ATP are found only at very low levels (< 0.01%) and have not been considered in the present study. Since the level of each ionic form of ATP is dependent upon the affinity of each cation for ATP, the concentration of each cation and the total ATP concentration, it is possible to manipulate experimental conditions so that the concentration of one ionic form of ATP is maintained at a fixed level while the concentration of another ionic form is varied. For example, by varying the Mg^{2+} concentration of Ca^{2+}-free medium in direct proportion to the total concentration of ATP, it is possible to vary the concentration of $MgATP^{2-}$ while maintaining a constant level of ATP^{4-}.

When the concentrations of Mg^{2+} and ATP in the medium were varied so as to maintain a fixed concentration of ATP^{4-} (0.9 μM) while varying the concentration of $MgATP^{2-}$, the magnitude of PGI_2 release remained constant, albeit submaximal (Table 1). The amount of PGI_2 release induced by 0.9 μM-ATP^{4-} was the same in the presence or absence of $MgATP^{2-}$. In the absence of ATP, Mg^{2+} had little effect on PGI_2 release at concentrations as high as 10 mM (Table 1).

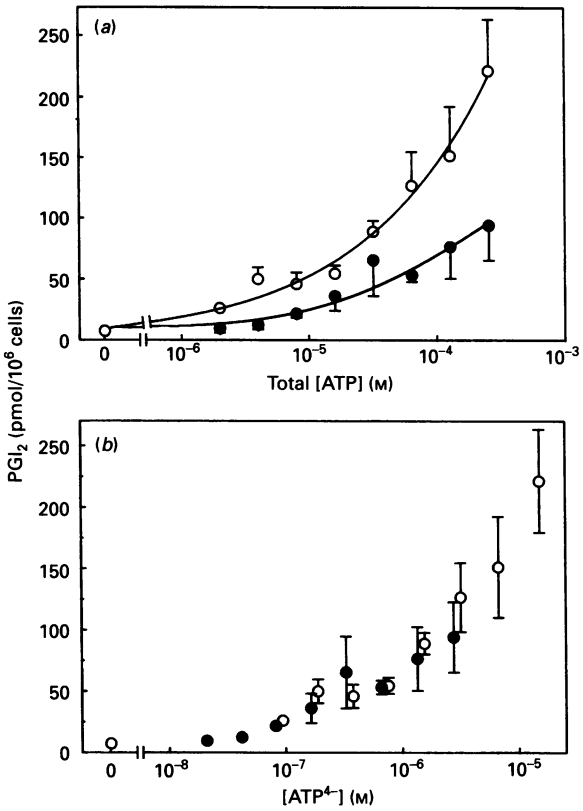

Fig. 1. Effect of extracellular Mg^{2+} on ATP-induced PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with either 1 mM-(○) or 5 mM-(●) $MgCl_2$. After 5 min, ATP was added at the indicated concentration, and 5 min later the medium was sampled and assayed for 6-oxo-$PGF_{1\alpha}$, the stable hydrolysis product of PGI_2 (see the Materials and methods section). (*a*) Blot of PGI_2 release as a function of the total concentration of ATP. (*b*) A replot of PGI_2 release as a function of the ATP^{4-} concentration, calculated as described in the Materials and methods section. Data are means ± S.D. of triplicate determinations and are representative of results from four experiments.

PGI_2 release was also measured over a wide range of ATP concentrations in BPAE cells incubated in iso-osmotic Ca^{2+}-free medium, pH 7.4, containing either 1 mM- or 5 mM-$MgCl_2$. The amount of PGI_2 release was significantly lower in BPAE cells incubated in medium containing 5 mM-$MgCl_2$ than in cells incubated with 1 mM-$MgCl_2$ (Fig. 1*a*). If the concentration of ATP^{4-} was calculated for each data point and then plotted against the amount of PGI_2 released, the results indicated that the extent of PGI_2 release varied directly with the concentration of ATP^{4-} in the medium (Fig. 1*b*). Thus the inhibitory effect of extracellular Mg^{2+} on ATP-induced PGI_2 release could be attributed solely to a reduction in the concentration of ATP^{4-} (Table 1; Fig. 1). These results suggest that $MgATP^{2-}$ is not an agonist or antagonist of the nucleotide receptor in BPAE cells.

The nucleotide receptor that mediates PGI_2 release from BPAE cells recognizes ATP^{4-}, but not $HATP^{3-}$, $NaATP^{3-}$ or $KATP^{3-}$

The results described above do not rule out the possibility that other ionic forms of ATP, including $HATP^{3-}$, $NaATP^{3-}$ and

Table 2. PGI_2 release correlates with the concentration of ATP^{4-} and not $HATP^{3-}$

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS supplemented with 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$ at the indicated pH. After 5 min, ATP was added to the indicated concentrations of ATP^{4-} and $HATP^{3-}$, calculated as described in the Materials and methods section. The medium was sampled 5 min later and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Results are means ± S.D. of triplicate determinations and are representative of results from four experiments.

$[HATP^{3-}]$ (μM)	$[ATP^{4-}]$ (μM)	pH	PGI_2 release (pmol/dish)
0.1	3.7	8.6	23.5±7.4
0.2	3.7	8.2	28.0±7.2
0.5	3.7	7.4	26.8±9.3
1.3	3.6	7.0	30.3±6.4
7.6	3.4	6.6	30.4±0.8
17.0	3.0	6.2	31.6±9.3
0	0	6.2	0.9±0.3
0	0	7.4	1.0±1.0
0	0	8.6	1.2±0.9

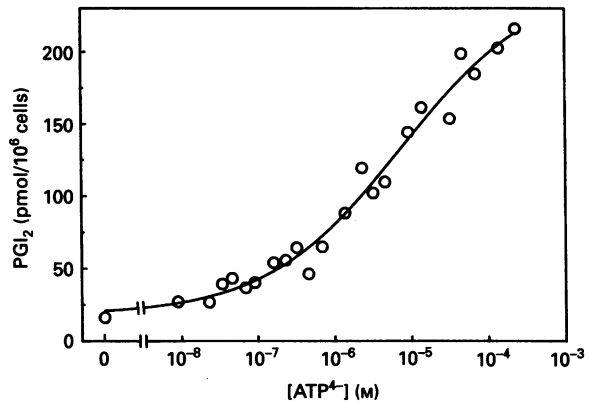

Fig. 2. Dose-dependence of ATP^{4-}-induced PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4. After 5 min, ATP^{4-} was added at the indicated concentration, and 5 min later the medium was sampled and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). The concentration of ATP^{4-} was calculated as described in the Materials and methods section. Data are means of triplicate determinations and are representative of results from three experiments.

$KATP^{3-}$, contribute to PGI_2 release in Ca^{2+}-free medium. Accordingly, it was determined whether varying the pH of the iso-osmotic medium altered PGI_2 release in BPAE cells treated with a constant concentration of ATP^{4-}. When the pH of the medium was varied so as to maintain the concentration of ATP^{4-} between 3 to 4 μM while varying the concentration of $HATP^{3-}$, the extent of PGI_2 release did not correlate with the concentration of $HATP^{3-}$ (Table 2). The pH of the medium had little effect on PGI_2 release in the absence of ATP (Table 2). ATP-induced PGI_2 release was not altered when BPAE cells were incubated in a Na^+-free medium containing choline or in a medium lacking K^+ (K. D. Lustig & G. A. Weisman, unpublished work), suggesting that neither $NaATP^{3-}$ nor $KATP^{3-}$ is the ionic form of ATP that induces PGI_2 release. Finally, ATP^{4-} induced a dose-dependent increase in PGI_2 release (EC_{50} 3 μM) from BPAE cells incubated in an iso-osmotic medium (pH 7.4) lacking Ca^{2+} and Mg^{2+} (Fig. 2). An increase in PGI_2 release was observed at ATP^{4-} concentrations as low as 10 nM (Fig. 2). These results indicate that

Table 3. Extracellular Ca^{2+} stimulates PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with the indicated concentration of $CaCl_2$. After 5 min, ATP was added to the indicated concentrations of ATP^{4-} and $CaATP^{2-}$, calculated as described in the Materials and methods section. The medium was sampled 5 min later and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Results are means ± S.D. of triplicate determinations and are representative of results from four experiments.

$[CaATP^{2-}]$ (μM)	$[ATP^{4-}]$ (μM)	$[CaCl_2]$ (mM)	PGI_2 release (pmol/10^6 cells)
6.0	1.4	0.5	72.8±5.0
12.0	1.4	1.0	77.5±14.0
36.0	1.4	3.0	89.1±10.7
60.0	1.4	5.0	93.9±4.1
119.0	1.4	10.0	108.7±12.9
0	0	10.0	7.6±1.6
0	1.4	0	33.8±2.0

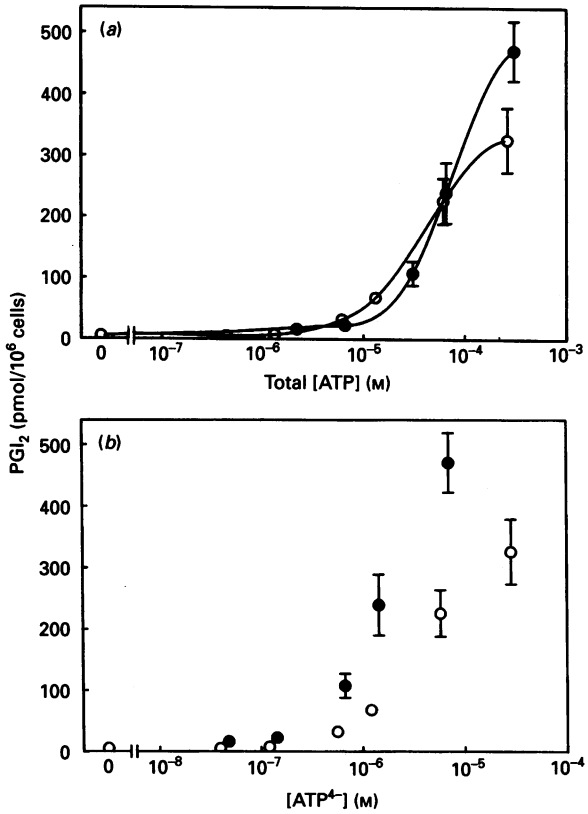

Fig. 3. Effect of extracellular Ca^{2+} on ATP-induced PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with either 1 mM-(○) or 5 mM-(●) $CaCl_2$. After 5 min, ATP was added at the indicated concentration, and 5 min later the medium was sampled and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). (*a*) Plot of PGI_2 release as a function of the total concentration of ATP. (*b*) Replot of PGI_2 release as a function of the ATP^{4-} concentration, calculated as described in the Materials and methods section. Data are means ± S.D. of triplicate determinations and are representative of results from four experiments.

the nucleotide receptor that mediates PGI_2 release in BPAE cells is activated by ATP^{4-} but not by $MgATP^{2-}$, $HATP^{3-}$, $NaATP^{3-}$ or $KATP^{3-}$.

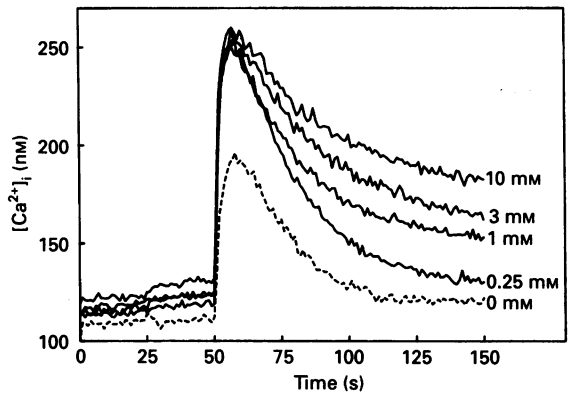

Fig. 4. Effect of extracellular calcium on the increase in $[Ca^{2+}]_i$ elicited by ATP

BPAE-cell suspensions were loaded with fura-2 (see the Materials and methods section), washed and incubated at 37 °C in 2 ml of HBS, pH 7.4, supplemented with 1 mM-$MgCl_2$ and the indicated concentration of $CaCl_2$ (broken line, Ca^{2+}-free medium). Then $[Ca^{2+}]_i$ was measured as described in the Materials and methods section, and 100 μM-ATP was added 50 s after measurements began. Each curve is a trace from the same experiment with a single cell preparation and the data are representative of results from three experiments.

Stimulation of nucleotide-induced PGI_2 release by extracellular Ca^{2+}

The Ca^{2+} and ATP concentrations in an iso-osmotic Mg^{2+}-free medium, pH 7.4, were varied so as to maintain a constant concentration of ATP^{4-} while varying the concentration of $CaATP^{2-}$. PGI_2 release increased as the concentration of $CaATP^{2-}$ in the medium was increased (Table 3). In the absence of ATP, Ca^{2+} had little effect on PGI_2 release at concentrations as high as 10 mM.

PGI_2 release was measured over a wide range of ATP^{4-} concentrations in BPAE cells incubated in Mg^{2+}-free medium containing 1 mM- or 5 mM-$CaCl_2$. The extent of PGI_2 release was the same in BPAE cells incubated in medium supplemented with 5 mM-$CaCl_2$ or 1 mM-$CaCl_2$ (Fig. 3*a*). If the concentration of ATP^{4-} was calculated for each data point and then plotted against the amount of PGI_2 release, the results indicate that the extent of PGI_2 release did not correlate with the concentration of ATP^{4-} (Fig. 3*b*).

One explanation for these results is that both ATP^{4-} and $CaATP^{2-}$ are agonists of the nucleotide receptor that mediates PGI_2 release in BPAE cells. Alternatively, since the increase in PGI_2 release induced by ATP in BPAE cells was dependent upon an increase in $[Ca^{2+}]_i$ that is partially mediated by an influx of extracellular Ca^{2+} (Lustig *et al.*, 1992), another plausible explanation is that the stimulation of PGI_2 release by extracellular Ca^{2+} is due to an alteration in the rate of Ca^{2+} uptake through an ATP^{4-}-activated plasma-membrane channel. Several different approaches were used to distinguish between these two possibilities. To determine whether alterations in the extracellular Ca^{2+} concentration affected the rate of Ca^{2+} uptake, the effect of ATP on $[Ca^{2+}]_i$ was measured in BPAE cells incubated with different concentrations of extracellular Ca^{2+}. In Ca^{2+}-depleted medium, ATP caused an increase in $[Ca^{2+}]_i$ that was maximal (2–3 times the basal level) within 5 s of ATP addition, but then declined within 60 s to the basal level (Fig. 4). The addition of ATP to BPAE cells incubated in media containing $\geqslant$ 0.25 mM-Ca^{2+}, however, caused a rapid increase in $[Ca^{2+}]_i$ that was greater and more sustained than in cells incubated in the absence of extracellular Ca^{2+} (Fig. 4). Moreover, the magnitude of the sustained increase in $[Ca^{2+}]_i$ was dependent upon the concentration of extracellular Ca^{2+} (Fig. 4). An alteration in the rate that $[Ca^{2+}]_i$ declines to the basal level after ATP addition would likely affect the extent of PGI_2 release, since the release of PGI_2 did not begin until 20–30 s after the addition of ATP^{4-} to BPAE cells and was not complete until 2 min after ATP^{4-} addition (Fig. 5). The Ca^{2+} concentration (< 0.25 mM) that modulated the transient increase in $[Ca^{2+}]_i$ induced by ATP was significantly different from the Ca^{2+} concentration ($\geqslant 0.25$ mM) that modulated the sustained increase in $[Ca^{2+}]_i$ (Fig. 4). These findings may indicate that ATP activates two different subclasses of nucleotide receptor in BPAE cells: one coupled to a phospholipase C and intracellular Ca^{2+} mobilization and the other coupled to plasma-membrane Ca^{2+} channels, as has been observed in mouse macrophages (Greenberg *et al.*, 1988).

Bradykinin, a peptide hormone, also induced Ca^{2+} uptake and

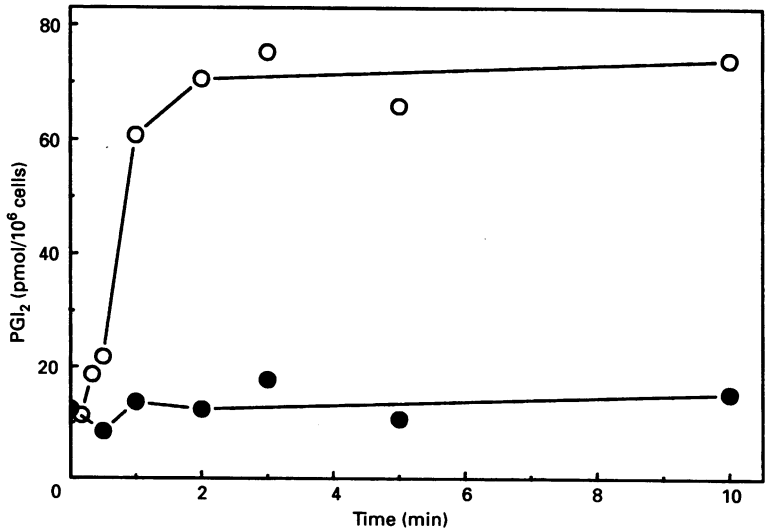

Fig. 5. Time course of ATP-induced PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4. After 5 min, 100 μM-ATP (○) was added and at the indicated time, the medium was sampled and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Cells incubated in the absence of ATP served as controls (●). Data are means of triplicate determinations and are representative of results from three experiments.

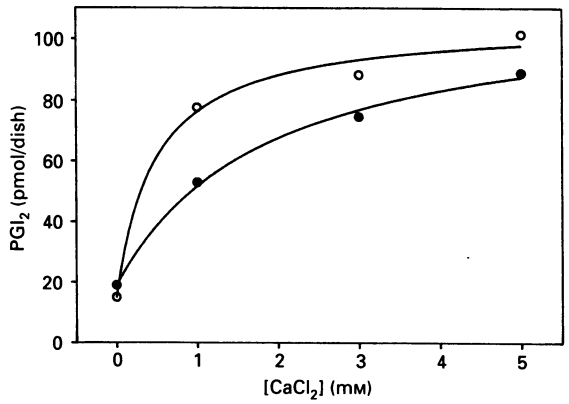

Fig. 6. Effect of extracellular Ca^{2+} on bradykinin-induced PGI_2 release

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with the indicated concentration of $CaCl_2$. After 5 min, 50 nM-bradykinin (●) or 500 nM-bradykinin (○) was added, and 5 min later the medium was sampled and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Data are means of triplicate determinations and are representative of results from two experiments. In the absence of Ca^{2+} and bradykinin, the level of PGI_2 release was 15.5 pmol/dish.

Table 4. PGI_2 release correlates with the concentration of UTP^{4-} and not $MgUTP^{2-}$

BPAE cells on 10 cm^2 dishes were washed and incubated at 37 °C in 1 ml of HBS, pH 7.4, supplemented with 1 mM-$CaCl_2$ and the indicated concentration of $MgCl_2$. After 5 min, UTP was added to the indicated concentrations of UTP^{4-} and $MgUTP^{2-}$, calculated as described in the Materials and methods section. The medium was sampled 5 min later and assayed for 6-oxo-$PGF_{1\alpha}$ (see the Materials and methods section). Results are means ± S.D. of triplicate determinations and are representative of results from three experiments.

[$MgUTP^{2-}$] (μM)	[UTP^{4-}] (μM)	[$MgCl_2$] (mM)	PGI_2 release (pmol/10^6 cells)
10.8	0.6	0.75	45.0±9.0
17.6	0.6	1.25	37.7±4.0
20.9	0.6	1.5	34.7±4.7
27.8	0.6	2.0	45.1±3.4
41.1	0.6	3.0	50.4±9.7
67.8	0.6	5.0	54.9±5.2
134.4	0.6	10.0	46.4±4.3
0	0	10.0	1.8±0.4
0	0.6	0	47.8±4.6

the release of PGI_2 in cultured porcine aortic endothelial cells (Whorton *et al.*, 1984). Similar to ATP, bradykinin induced a sustained increase in $[Ca^{2+}]_i$ in BPAE cells that was dependent upon the concentration of extracellular Ca^{2+} (K. D. Lustig & G. A. Weisman, unpublished work). Extracellular Ca^{2+} also modulated PGI_2 release induced by bradykinin in BPAE cells (Fig. 6). These results strongly suggest that extracellular Ca^{2+} modulates PGI_2 release in BPAE cells by altering the magnitude of the sustained increase in $[Ca^{2+}]_i$. Taken together, these findings suggest that the increase in PGI_2 release induced by extracellular Ca^{2+} is due to the stimulation of Ca^{2+} uptake through an ATP^{4-}-activated plasma-membrane channel rather than an increase in the concentration of $CaATP^{2-}$.

Stimulation of PGI_2 release in BPAE cells by UTP^{4-}

The pyrimidine nucleotide UTP elicits the inositol trisphosphate-dependent mobilization of intracellular Ca^{2+}, the influx of extracellular Ca^{2+} and the release of PGI_2 in BPAE cells (Lustig *et al.*, 1992). When the concentration of $MgUTP^{2-}$ in the medium was varied while maintaining a constant concentration of UTP^{4-} (0.6 μM), the extent of PGI_2 release remained constant (Table 4). The amount of PGI_2 release induced by 0.6 μM-UTP^{4-} was the same in the presence or absence of $MgUTP^{2-}$, suggesting that $MgUTP^{2-}$ is not an agonist or antagonist of the nucleotide receptor. As with ATP^{4-}, an increase in the extracellular Ca^{2+} concentration caused an increase in the amount of PGI_2 release induced by UTP^{4-} (K. D. Lustig & G. A. Weisman, unpublished work), presumably due to enhanced uptake of Ca^{2+} through a UTP^{4-} activated plasma-membrane channel. Although these results do not indicate whether ATP and UTP interact with the same nucleotide receptor in BPAE cells, they do suggest that the nucleotide receptor(s) that mediates PGI_2 release recognizes the fully ionized form of nucleotide.

Biological significance of ATP^{4-} and UTP^{4-}

The fully ionized forms of ATP and UTP may play a physiological role in mediating the release of PGI_2 from the endothelium. In addition to ATP, storage granules in adrenal-medullary cells (Goetz *et al.*, 1971; Van Dyke *et al.*, 1977) and platelets (Goetz *et al.*, 1971) also contain significant amounts of UTP (up to 8% of granule nucleotide content). A nucleotide translocator transports ATP^{4-} and UTP^{4-} into adrenal-medullary storage granules (Weber & Winkler, 1981), and both nucleotides are presumably released upon degranulation. The specific release of ATP^{4-} and UTP^{4-} into the bloodstream presumably would localize the effects of the nucleotides, since bivalent cations and other ions in the blood would rapidly reduce the concentration of the active, fully ionized, forms of both nucleotides. The binding of bivalent cations to ATP^{4-} and UTP^{4-} also would facilitate the degradation of both nucleotides, since ectonucleotidases on the surface of endothelial cells are likely to hydrolyse bivalent-cation–nucleotide complexes (Pearson & Gordon, 1985).

Steinberg *et al.* (1987) have postulated that bivalent cations inactivate ATP^{4-} after release from P_2 purinergic nerves in the peripheral and central nervous systems, where ATP has been postulated to act as a neurotransmitter (Stone, 1981; Gordon, 1986). Consistent with the hypothesis that P_2 purinergic receptors recognize ATP^{4-}, the increase in $[Ca^{2+}]_i$ induced by ATP in NG108-15 neuroblastoma × glioma cells correlated with the concentration of ATP^{4-} and not $MgATP^{2-}$ (T.-A. Lin, K. D. Lustig & G. A. Weisman, unpublished work). Thus nucleotide receptors, in general, may be activated by the fully ionized form of nucleotide. An intriguing recent study indicated that both ATP and UTP, in combination with amiloride, can correct a defect in chloride transport in cultured human-airway epithelia from cystic-fibrosis patients (Knowles *et al.*, 1991). It seems plausible that these therapeutic effects also may be mediated by ATP^{4-} and UTP^{4-}.

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