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Comparison of P_2 Purinergic Receptors of Aortic Endothelial Cells with Those of Adrenal Medulla: Evidence for Heterogeneity of Receptor Subtype and of Inositol Phosphate Response

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SUMMARY

Vascular endothelial cells from different parts of the circulation are known to show different functional responses, presumably corresponding to physiological roles. Previous studies have shown that ATP acts on P_2 purinergic receptors of endothelial cells of major blood vessels, stimulating the formation of inositol phosphates. Here we have compared the action of ATP and congeners acting on endothelial cells of bovine thoracic aorta with cells derived from the microvasculature of bovine adrenal medulla. With measurement of total inositol phosphates, cells from the aorta showed a rank order of agonist potency of 2-methylthio-ATP > adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) > ADP > ATP > β,γ-imido-ATP > β,γ-methylene-ATP, consistent with action at receptors of the P_{2Y} subtype. However, with adrenal cells the rank order of potency was ATPγS > ATP > β,γ-imido-ATP > ADP > β,γ-methylene-ATP = 2-methylthio-ATP. This profile is not consistent with either P_{2x} or P_{2Y} receptors. When the nature of this inositol phosphate response was analyzed with anion exchange chromatography, it was found that the aortic cells showed an inositol trisphosphate stimulation that peaked within a few seconds and rapidly declined, whereas the response of the adrenal medulla cells continued to rise through 5 min. Analysis of isomers of inositol phosphates revealed a different pattern of metabolism between the two cell types, which may account for the different time course of response. With adrenal cells, ATP at low micromolar concentrations caused a dose-dependent increase in levels of cyclic AMP and had a greater than additive effect on cyclic AMP levels when combined with submaximal stimulation by prostaglandin E_2. These results suggest the presence of a P_{2Y} receptor on aortic endothelial cells, with an 'atypical' purinocepter, i.e., neither P_{2x} nor P_{2Y}, on adrenal cells. Furthermore, they show that activation of P_2 receptors on the two cell types has different functional consequences.

Over recent years it has become apparent that the vascular endothelium plays a complex physiological role involving, for example, interactions with the blood [e.g., by production of prostacyclin (1)] and regulation of the associated smooth muscle layers [e.g., by production of endothelium-derived relaxing factor and endothelin (2, and 3)]. The endothelium is itself subject to regulation by locally active substances; these include ATP (and ADP) derived principally from platelets and possibly from local elements of the nervous system. Following the proposals of Burnstock (4), purinergic receptors have been divided into P_1-purinoceptors (relatively sensitive to adenosine and AMP) and P_2-purinoceptors (for which ADP and ATP are more potent agonists). Subsequently, P_2 receptors have been divided into two subtypes (5). A rank order of potency of ATP analogues acting on vas deferens and urinary bladder of α,β-methylene-ATP = β,γ-methylene-ATP > ATP = 2MeSATP is characteristic of interaction with P_{2x} receptors. An order of potency for relaxation of guinea pig taenia coli of 2MeSATP > ATP > α,β-methylene-ATP = βγ-methylene-ATP is ascribed to interaction with P_{2Y} receptors. This working hypothesis for subdivision of P_2 receptors based upon relative agonist potency has received widespread acceptance, although some further subtypes of receptors have been proposed (6).

With respect to vascular endothelium, it was found that endothelium-dependent relaxation of preconstricted arterial vasculature showed an agonist potency profile of 2MeSATP > ATP > α,β-methylene-ATP (7, 8), consistent with a P_{2Y} receptor subtype. Other studies with major vessel endothelial cells have been broadly in agreement with this conclusion, including studies of purinoceptor-stimulated prostacyclin production (9,

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ABBREVIATIONS: 2MeSATP, 2-methylthioadenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $InsP_1$, inositol monophosphate of unspecified isomerism; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_4$, inositol tetrakisphosphate; Ins(1,3,4)P_3, D-*myo*-inositol-1,3,4-trisphosphate; PtdIns(4,5)P_2, D-phosphatidyl-*myo*-inositol-4,5-bisphosphate; PGE_2, prostaglandin E_2; HPLC, high performance liquid chromatography; G protein, GTP-binding protein; Ins(1,4,5)P_3, D-*myo*-inositol-1,4,5-trisphosphate; Ins(1,4)P_2, D-*myo*-inositol-1,4-bisphosphate; Ins(1,3,4,5)P_4, D-*myo*-inositol-1,3,4,5-tetrakisphosphate.

10), calcium mobilization (10), and phospholipid breakdown (11, 12).

Stimulation of bovine aortic endothelial cell P_{2Y} receptors has been shown to elevate phosphoinositide breakdown (12), with the generation of calcium-mobilizing inositol phosphates and protein kinase C-stimulating diacylglycerol. Breakdown of phosphatidylcholine by activation of endothelial P_{2Y} receptors has been described (13). It has been suggested that prostacyclin release is downstream of $InsP_3$ production and mobilization of calcium (13), and it has also been suggested that both stimulation of endothelial-derived relaxing factor and production of prostacyclin are linked at the level of protein kinase C activation (14). These proposals put phospholipid hydrolysis at center stage with respect to endothelial cell function.

The endothelial cells of the adrenal medulla are in an unusual environment with respect to ATP, due to the very large amounts of ATP that are released from adjacent chromaffin cells. It seems likely, therefore, that they may have an unusual response to ATP, when compared with endothelial cells derived from the major blood vessels. Here we have used a cell preparation from adrenal medulla that was characterized by others as endothelial (15). We show that, compared with bovine thoracic aortic endothelial cells, the medullary cells show a different rank order of agonist potency with respect to stimulation of production of total inositol phosphates and a different pattern of mobilization of individual inositol phosphates.

Materials and Methods

Cell culture. Bovine adrenal glands were retrogradely perfused with a solution of collagenase (1 mg/ml, Sigma type 1), protease (0.3 mg/ml, Sigma type IV), and DNase (25 μg/ml, Sigma type I) in 154 mM NaCl, 5 mM KCl, 5 mM glucose, 5 mM HEPES. Medullae were then removed, finely chopped, and stirred with enzyme mixture for two or three consecutive periods. Liberated cells were pooled and sequentially centrifuged at 200 × *g*, 160 × *g*, 120 × *g*, and 80 × *g*, after which they were finally suspended in medium and placed in 175-cm^2 Nunc tissue culture flasks at 10^6 cells/ml, 50 ml/flask, for 3–4 hr at 37°. Medium was Dulbecco's modified Eagle's medium with 10% fetal calf serum, 27 mg/100 ml glutamine, and 1 ml/100 ml 100× gentamycin, fungizone, penicillin, and streptomycin. After 3 hr the unattached chromaffin-rich cells were decanted off and the adherent cells were washed and then cultivated in medium until confluent. These apparently homogeneous cell cultures showed exactly the same morphological characteristics and most unusual growth patterns described in an earlier paper (15), in which they were identified as endothelial on the basis of Factor VIII immunoreactivity and ultrastructural criteria. They could be clearly distinguished from cultured vascular smooth muscle cells and fibroblasts. Cells were used, after one passage, in 6- or 24-well NUNC multiwell plates.

The cell line AG4762, a nonclonal line derived from bovine aorta endothelial cells, was obtained from the National Institute of Ageing (Bethesda, MD) and used at passages 17–19. Cells were cultivated under the same conditions as the adrenal cells. These cells exhibited the typical "cobblestone" morphology characteristic of bovine thoracic aorta endothelial cells in culture, which others (16) have identified ultrastructurally by the presence of Weibel-Palade bodies, pinocytotic vesicles, microfilaments, thrombosthenin-like contractile proteins, and Factor VIII antigen.

With each of these cultures, the coefficients of variation in cell protein content between wells in the same experiment were less than 5% (standard error as percentage of mean); for this reason protein determinations were not always undertaken, and results were expressed as response per well. However there was a larger variation in protein content between wells of different batches of cells, as for example, with the adrenal cell cultures with 24 well plates, from 27.03 ± 1.21 μg/well (n = 72) to, with a different batch of cells, 55.23 ± 1.25 μg/well (n = 72).

Incorporation of label into total inositol phosphates. Multiwell plates with confluent cells were incubated at 37° for 40–48 hr with 0.037 MBq of *myo*-[2-^{3}H]inositol in 0.5 ml of loading medium (medium M199, 27 mg/100 ml glutamine, and 1 ml/100 ml 100× fungizone, penicillin, and streptomycin). Following the loading period, cells were incubated in balanced salt solution (in mM: NaCl, 125; KCl, 5.4; $NaHCO_3$, 16.2; HEPES, 30; NaH_2PO_4, 1; $MgSO_4$, 0.8; $CaCl_2$, 1.8; and glucose, 5.5; buffered to pH 7.4 and gassed with 95% O_2/5% CO_2) in the presence of 10 mM lithium chloride for 30 min at 37°, in the presence of drugs as indicated. Subsequent extraction of inositol phosphates onto Dowex-1 (Cl^-) was as described in Ref. 17. Briefly, cells were scraped and extracted into methanol/H_2O, lipids were removed by chloroform extraction, and the aqueous phase was extracted batchwise onto Dowex-1, from which it was eluted with 1 mM HCl before scintillation counting. In some experiments the lipid phase in chloroform was dried and counted to provide an estimate of incorporation of label into total lipids. Where appropriate, values for EC_{50} were computed using an ALLFIT program.

Incorporation of label into individual inositol phosphates. Cells cultured in 35-mm wells were incubated with 0.22 MBq of [^{3}H]-inositol in 1.5 ml of loading medium for 40–48 hr at 37°. The cells were then washed and incubated with 1 ml, with or without drugs, of the balanced salt solution described above for the time indicated. The cells were extracted by scraping into 1 M trichloroacetic acid, the acid was removed by ether extraction, and the extract, adjusted to pH 7 with $NaHCO_3$, was applied to an anion exchange column. In some cases this was AG1 ×8 (200–400 mesh formate form) in 0.6 × 3 cm glass columns, prepared and eluted essentially as in Ref. 18. Elution was with the following protocol: 14 ml of H_2O eluted *myo*-inositol; 12 ml of 25 mM ammonium formate, glycerophosphoinositides; 14 ml of 200 mM ammonium formate, $InsP_1$; 14 ml of 500 mM ammonium formate/0.1 M formic acid, $InsP_2$; 14 ml of 750 mM ammonium formate/0.1 M formic acid, $InsP_3$; and 14 ml of 1 M ammonium formate/0.1 M formic acid, $InsP_4$. In other cases the column was a 25-cm Partisil 10 SAX column, which was used to separate isomers of inositol phosphate by adaptation of the method of Batty *et al.* (19). Elution was with the following gradient of $(NH_4)H_2PO_4$ (1.4 M, pH 3.7): 0 min, 0%; 40 min, 40%; 50 min, 40%; 60 min, 100%; 70 min, 100%; and 80 min, 0%. Nucleotides (AMP, GMP, ADP, ATP, and GTP) were used as internal standards mixed with each sample; the elution positions of inositol phosphates and their isomers were characterized by the elution pattern of a previously characterized extract of carbachol-stimulated brain slice prepared and kindly donated by Dr. I. Batty (Department of Biochemistry, Dundee University, Scotland) and were further confirmed by the elution of [^{3}H]Ins(1,4,5)P_3 purchased from New England Nuclear.

Measurement of cyclic AMP and protein. Cyclic AMP was measured using a protein-binding assay, as described elsewhere (20). Protein was estimated by the method of Lowry *et al.* (21).

Materials. Cell culture medium, additives, and plastics were from GIBCO (Paisley, Scotland). *myo*[2-^{3}H]Inositol (15 Ci/mmol) was from New England Nuclear (Stevenage, Herts, UK). 2MeSATP was from Research Biochemicals (Semat Ltd., St. Albans, UK). Other chemicals and drugs were from Sigma Chemical Co. (Poole, Dorset, UK) or Fisons plc (Loughborough, UK). Partisil 10 SAX columns were purchased from Techincol (Stockport, Cheshire, UK).

Results

Total inositol phosphate accumulation in adrenal-derived cells. Incubation of adrenal cells with [^{3}H]inositol as described above for different periods of time up to 72 hr, followed by counting of radioactivity in the lipid (chloroform) phase, showed that no additional counts accumulated in the

cell lipids after 48 hr, an indication that by this time equilibrium of labeling had been achieved (Fig. 1A).

In the presence of 10 mM lithium, incubation of adrenal cells with 30 μM ATP stimulated a linear accumulation of inositol phosphates over a period of 90 min (Fig. 1B). In the three such experiments undertaken, there was no significant depletion of label in the lipid phase of the cell extracts. The stimulation of inositol phosphate accumulation was concentration dependent (Fig. 2) and partially dependent on addition of calcium to the medium (data not shown). In the presence of added calcium, ATP was active at a concentration of 3 μM (142.1 ± 9.7% above basal). At 100 μM ATP there was a 272.4 ± 8.5% increase above

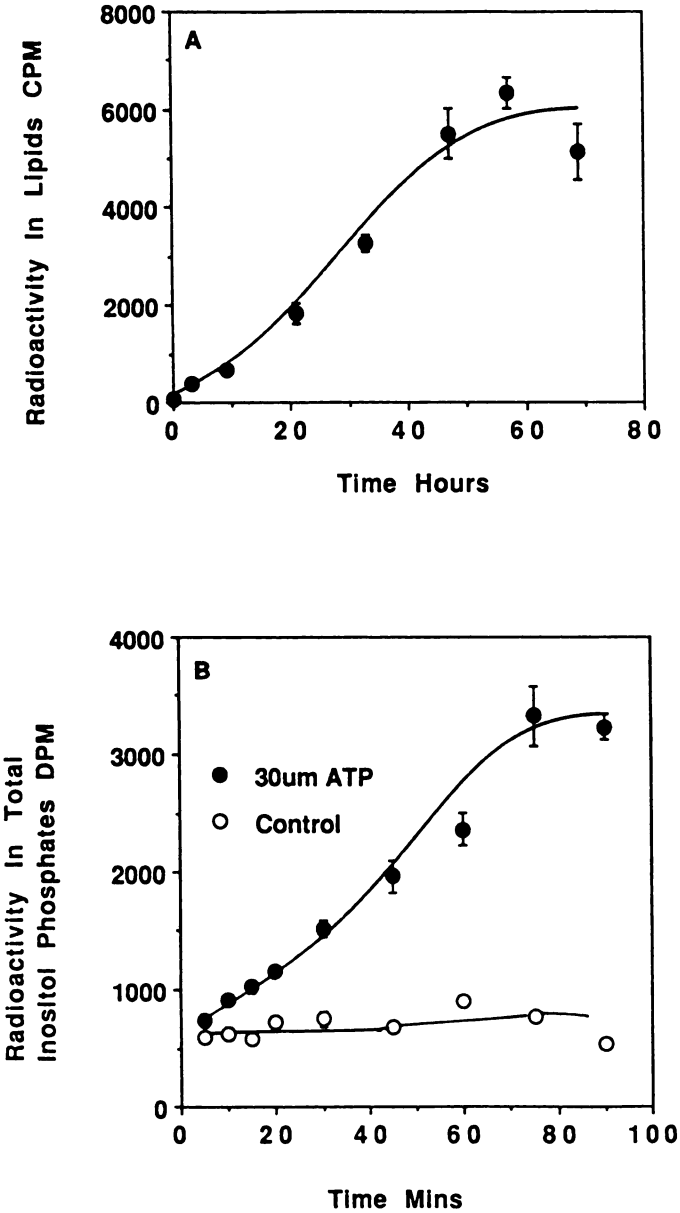


Fig. 1. A, Cells were incubated with 0.037 MBq/ml tritiated *myo*-inositol for the time indicated on the *abscissa*. Cells were then extracted and the radioactivity in the chloroform extracts was measured. Each point is the mean ± standard error of triplicate determinations in one representative experiment of three. B, Time course of accumulation of radioactivity in total inositol phosphates in adrenal-derived cells, in the presence (●) and absence (○) of 30 μM ATP. Each point is the mean ± standard error of quadruplicate determinations in one representative experiment of three.

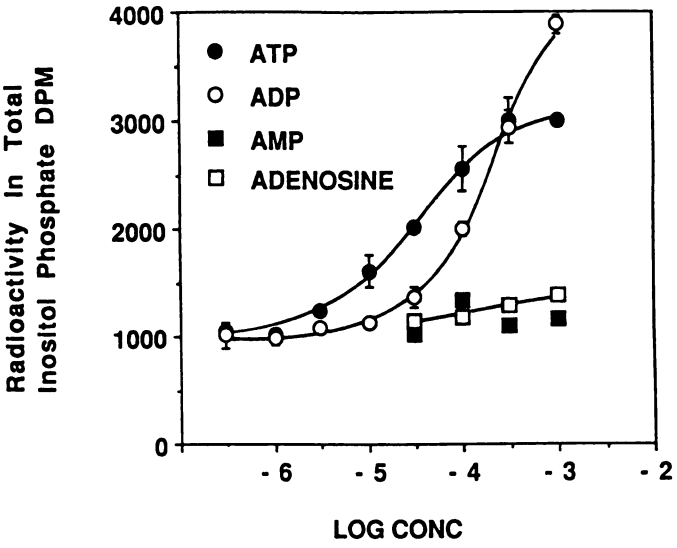


Fig. 2. Adrenal medulla cells dose-response curves for accumulation of radioactivity in total inositol phosphates in response to incubation with increasing concentrations of ATP (●), ADP (○), AMP (■), and adenosine (□) for 30 min. The basal value (no drug) was 1014 ± 57 dpm. Values are mean ± standard error of three determinations in one representative experiment of three.

basal. In the absence of added extracellular calcium there was overall a reduced response to ATP. Most of this reduced response was accounted for by a reduced basal level (54.5 ± 10.6% of that when calcium was added). Expressed as a percentage of basal, the effect of 100 μM ATP was similar in the presence or absence of added calcium (289.1 ± 11.1% and 272.4 ± 8.4%, respectively). The EC_{50} for ATP in the absence of added extracellular calcium was 68.5 μM (mean of two determinations). The EC_{50} for ATP in the presence of added calcium could not be reliably determined, because the response often continued to rise at the highest concentrations used (1 mM), failing to reach a plateau.

Further experiments with 1.8 mM calcium in the medium showed that ATP and ADP were both effective in stimulating inositol phosphate production, whereas AMP and adenosine were ineffective (Fig. 2). The dose-response curve for ADP was 1 order of magnitude to the right of the ATP curve and, like the ATP curve, it failed to form a plateau.

Fig. 3 shows the dose-response curves for three ATP congeners in adrenal medulla-derived cells. Both β,γ-methylene-ATP and 2MeSATP produced no response below 100 μM. ATPγS stimulated inositol phosphates at 1 μM; the dose-response curve produced a plateau and from three such experiments gave an EC_{50} of 2.81 ± 0.22 μM. Dose-response curves for β,γ-imido-ATP were also constructed in the same series of experiments. These showed a small response at 10 μM, which continued to rise to 1 mM, with no plateau.

Total inositol phosphate accumulation in AG4762 cells. Incubation of prelabeled AG4762 bovine aortic endothelial cells with 30 μM ATP in the presence of 10 mM lithium gave a linear increase in accumulation of inositol phosphate for 20–30 min, after which the rate of increase declined, to reach a plateau at 60 min (Fig. 4). Corresponding to this increasing label in inositol phosphates, we were also able to see in most experiments a fall in label in the lipid phase (data not shown). ATP and ADP both stimulated inositol phosphate accumulation dose dependently, with neither response plateauing at high

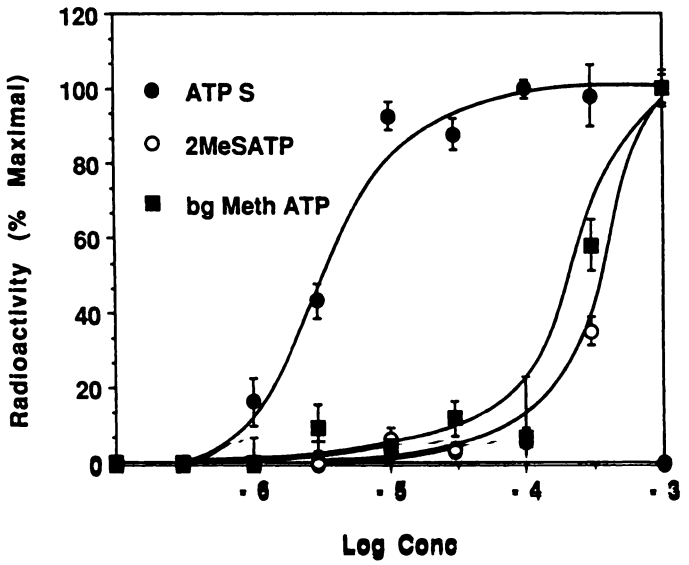


Fig. 3. Dose-response curves for accumulation of radioactivity in total inositol phosphates in cells from the adrenal medulla in response to incubation for 30 min with increasing concentrations of agonists. *ATPS*, ATPγS; *bg Meth ATP*, β,γ-methylene-ATP. Basal counts in total inositol phosphates were about 1000 dpm in each case. For the ATPγS curve the basal was 1177 ± 47 dpm, and the maximal response was 2803 ± 68 dpm. Each point is the mean ± standard error of triplicate determinations in one representative experiment of three.

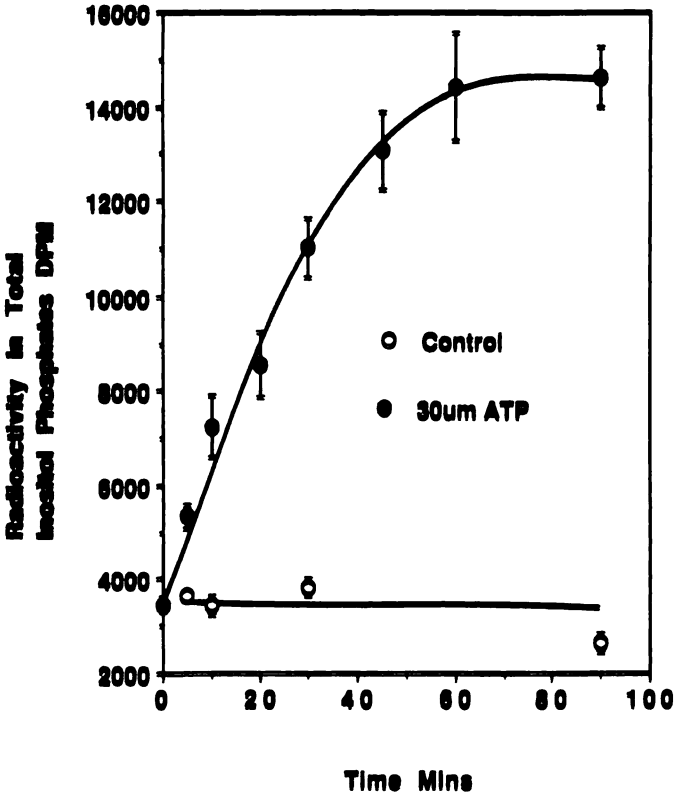
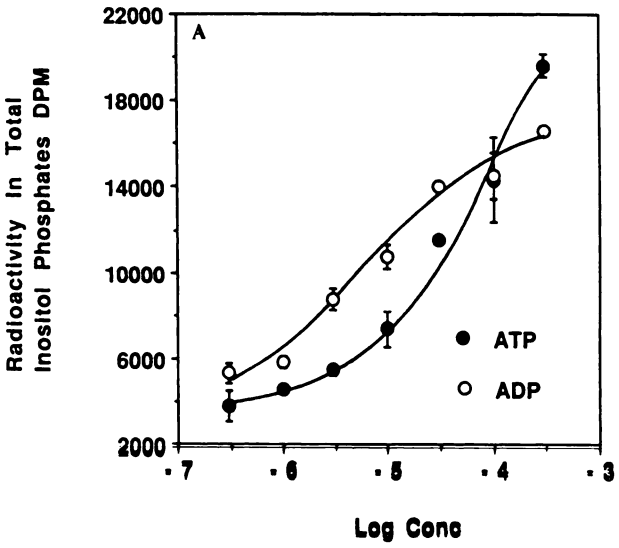


Fig. 4. Accumulation in aorta (AG4762) cells of radioactivity in total inositol phosphates in response to incubation, for the time shown, in the presence (●) or absence (○) of 30 μM ATP. Values are mean ± standard error of four determinations in one representative experiment of three.



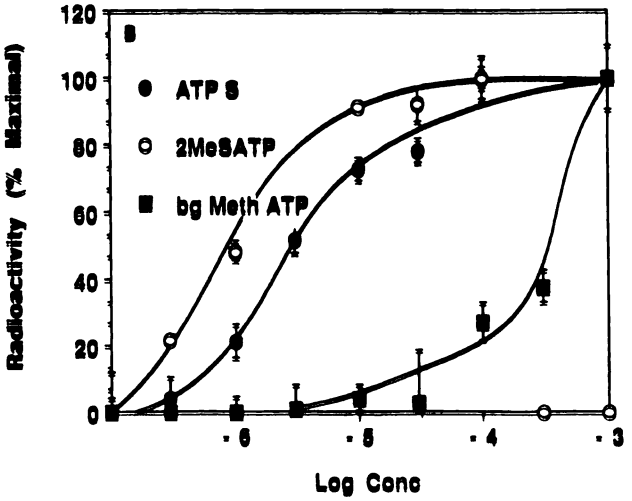


Fig. 5. A, Accumulation of radioactivity in aorta (AG4762) cells in response to increasing concentrations of ATP (●) and ADP (○). Basal accumulation was 3781 ± 391 dpm. Values are mean ± standard error of three determinations in one representative experiment of three. B, Dose-response curves for accumulation of radioactivity in total inositol phosphates in AG4762 cells in response to incubation for 30 min with increasing concentrations of agonists. The abbreviations are as for Fig. 3. Basal counts in each case were about 2000 dpm. For the 2MeSATP curve, the basal was 2054 ± 90 dpm and the maximal response was 11986 ± 781 dpm. Each point is the mean ± standard error of triplicate determinations in one representative experiment of three.

concentrations (Fig. 5A). ATP at 3 μM stimulated a 144.5 ± 14.0% increase above basal, whereas ADP at 0.3 μM produced a rise of 140.8 ± 19.4% above basal, reflecting an ADP curve 1 order of magnitude to the left of the ATP curve.

Fig. 5B shows the response of the AG4762 cells to increasing concentrations of three ATP congeners. β,γ-Methylene-ATP produced little response below 100 μM, whereas 2MeSATP and ATPγS both stimulated in a dose-dependent manner below 100 μM. Of these, only 2MeSATP formed a plateau at higher concentrations, which over three such experiments generated an EC_{50} of 0.9 ± 0.4 μM, with a maximum response at 10 μM. Again, β,γ-imido-ATP was also tested, giving a small response at 10 μM with no plateau.

The action of ATP on AG4762 cells consistently produced a larger response than on adrenal-derived cells, both in terms of absolute amounts of radioactivity accumulating and in terms of fold stimulation over basal.

Separation of individual inositol phosphates. A series of experiments were undertaken in which cells were stimulated with 30 μM ATP for increasing periods of time and the inositol phosphates in the resulting cell extracts were separated on small AG1 anion exchange columns. The results of one of three such experiments for adrenal cells are shown in Fig. 6. At 30 μM, ATP produced a sustained increase in $InsP_1$, $InsP_2$, $InsP_3$, and $InsP_4$, with no change in incorporation into glycerophosphoinositols and higher inositol phosphates (inositol pentakisphosphate plus inositol hexakisphosphate). At the shortest time measured, 5 sec, label in $InsP_3$ had risen to 207 ± 21% above basal; however, this continued to rise until it had reached 1174 ± 8% above basal after 5 min. In two experiments, label in $InsP_3$ continued to rise between 2 and 5 min, whereas in the third $InsP_3$ fell slightly after 2 min. The response in $InsP_2$ and $InsP_1$ lagged behind, in a manner consistent with the initial production of $InsP_3$ which is metabolized to other inositol phosphates. $InsP_4$ showed no clear sustained stimulation through these experiments.

The results of similar experiments with the AG4762 cells revealed a quite different pattern of inositol phosphate formation in response to 30 μM ATP (Fig. 7). Here the formation of label in $InsP_3$ had reached a maximum by 5 sec; by 60 sec the label in $InsP_3$ had fallen to a small fraction of this maximum but maintained a plateau above basal for the duration of the experiment. The $InsP_2$ and $InsP_1$ again showed stimulations that lagged behind, consistent with their sequential formation, but again the elevations reached maximum much more quickly in AG4762 cells than in adrenal cells. An increase in labeled $InsP_4$, which was sustained with no clear maximum, was seen in two of three experiments.

In both cell types, the time course of accumulation of counts into the total inositol phosphates was more sustained than that for accumulation into the total sum of separated inositol phosphates. This is presumably largely due to the presence of lithium in the former, but not the latter, experiments.

We subsequently proceeded to separate isomers of some inositol phosphates using HPLC with an anion exchange column. Typical results of several experiments are shown in Fig. 8. In each series the pattern of elution of labeled inositol phosphates from a carbachol-stimulated brain slice extract was established. This is well characterized and was used to indicate the elution position of various isomers of inositol phosphates. The elution of nucleotide internal standards was also monitored, both in this calibration run and with the samples. Fig. 8A shows the pattern when extracts of stimulated (15 sec of 30 μM ATPγS) and unstimulated adrenal cells were separated. Agonist-stimulated increases in label in $Ins(1,4)P_2$, $Ins(1,3,4)P_3$, and $Ins(1,4,5)P_3$ can be seen, whereas there was no increase in $InsP_1$. When the AG4762 cells were stimulated (15 sec of 30 μM 2MeSATP), the pattern was quite different (Fig. 8B). Agonist-dependent increases in label in $InsP_1$, $Ins(1,4)P_2$, and $Ins(1,4,5)P_3$ were large, compared with a barely detectable increase in label eluting with $Ins(1,3,4)P_3$. The most remarkable difference in response between the two cell types is the equal response of the two $InsP_3$ isomers in the adrenal cells, compared with the very unequal response in the aortic cells. In both cell types a very small increase in label eluting in the position of $InsP_4$ was seen (fractions 64 and 65).

ATP effects on cyclic AMP production in adrenal cells. Six experiments were performed in which adrenal medulla cells were exposed to increasing concentrations of ATP and cell extracts were subsequently assayed for cyclic AMP content. In each case, the level of cyclic AMP rose in a dose-dependent manner to about 3-fold over basal at about 100 μM ATP.

In order to investigate the effect of ATP on elevated as well as basal cyclic AMP levels, the cells were stimulated with a submaximal concentration of PGE_2 (1 μM), with and without

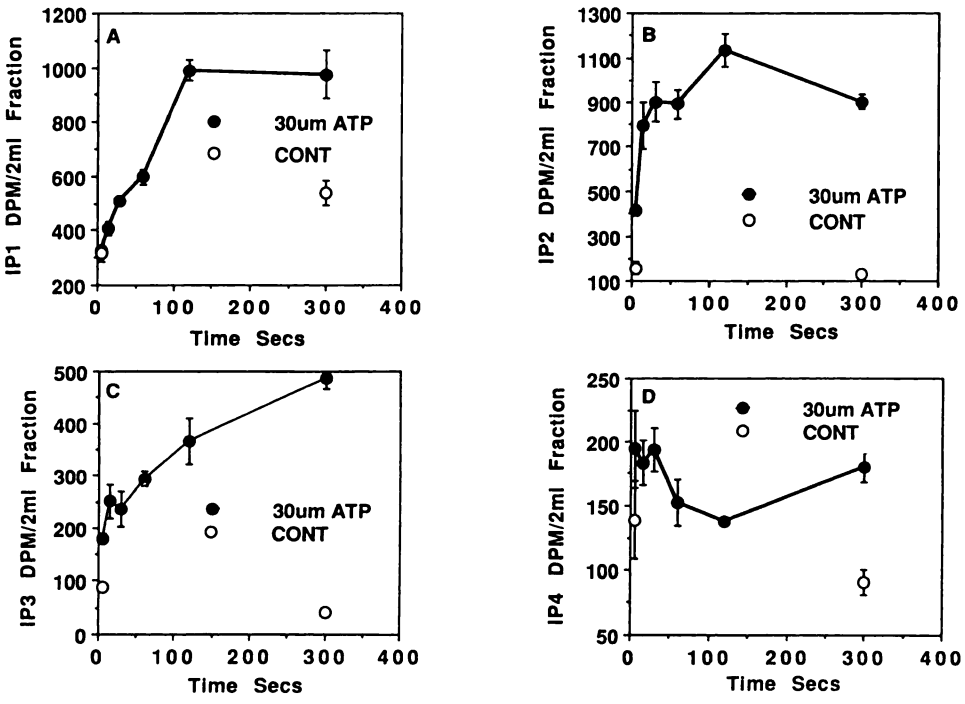


Fig. 6. Time course in adrenal medulla cells for the formation of radioactive inositol phosphates, separated on small anion exchange columns, in response to 30 μM ATP (●) or in unstimulated control (○). The initial data point in each case is for an incubation of 5 sec and the next point is 15 sec. A 2-ml aliquot of each fraction from the columns was counted and the results are expressed as dpm/2 ml. The panels correspond to elution of different inositol phosphates: A, $InsP_1$; B, $InsP_2$; C, $InsP_3$; D, $InsP_4$. Each determination was in triplicate, and this is one representative experiment of three.

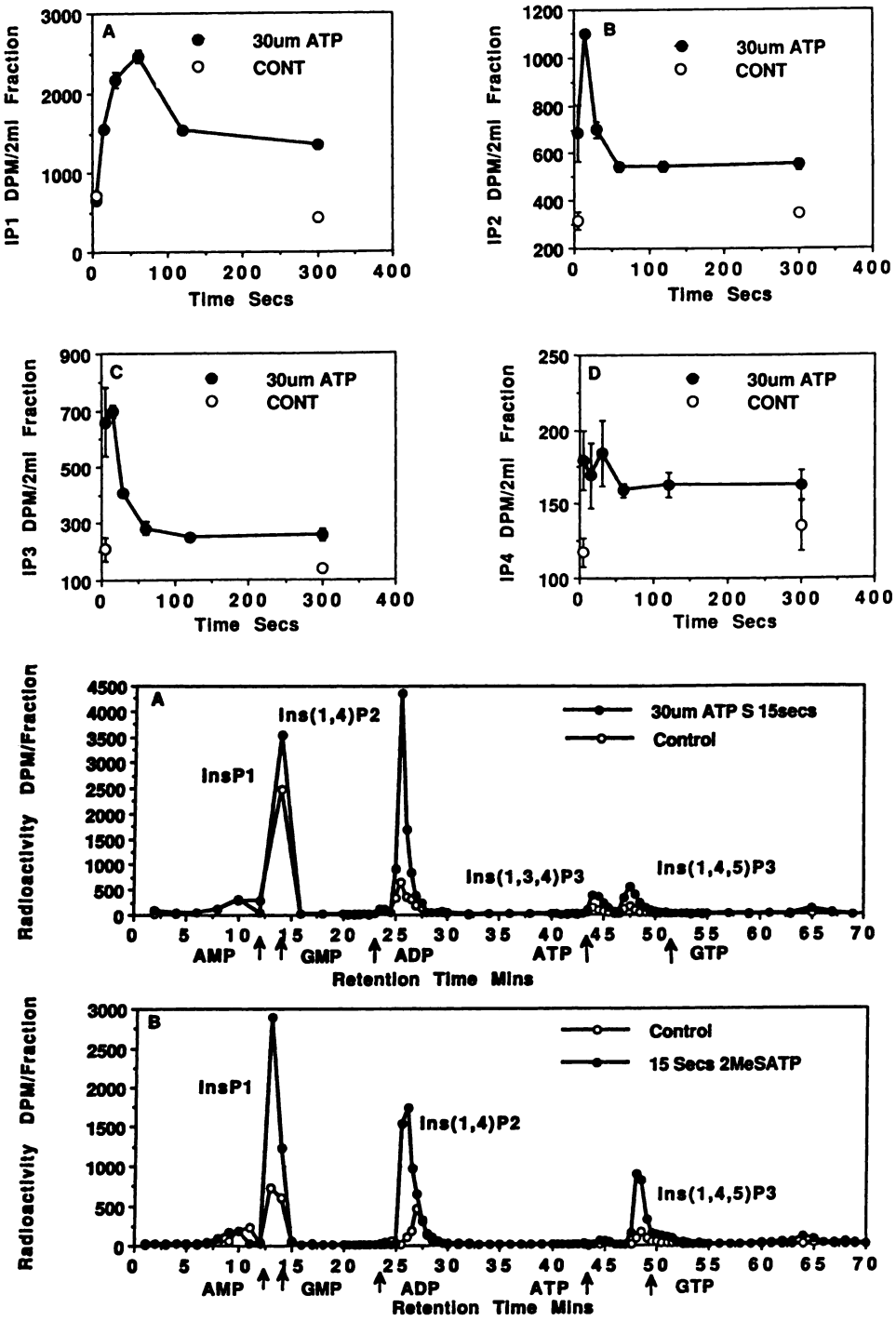


Fig. 7. Time course in aorta (AG4762) cells for the formation of radioactive inositol phosphates, separated on small anion exchange columns, in response to 30 μM ATP (●) or in unstimulated controls (○). Details are as in legend to Fig. 6. Each determination was in triplicate and this is one representative experiment of three.

Fig. 8. Separation of labeled inositol phosphates on anion exchange HPLC. Extracts of adrenal cells stimulated for 15 sec with 30 μM ATPγS (A) or aorta (AG4762) cells stimulated for 15 sec with 30 μM 2MeSATP (B) are shown. ●, Stimulated extracts; ○, unstimulated controls, in each case. This is one of four essentially similar experiments. The positions of elution of internal nucleotide standards are shown.

100 μM ATP. In each of two such experiments performed, ATP produced a greater than additive effect on cyclic AMP when combined with PGE_2, as for example (expressing the data as percentage of basal, mean ± SE, four experiments), 100 μM ATP, 385 ± 21; 1 μM PGE_2, 2362 ± 27; 1 μM PGE_2 plus 100 μM ATP, 3679 ± 23%.

Breakdown of ATP and congeners. We used anion exchange HPLC and UV absorption detection to monitor breakdown of ATP and related compounds by the two cell types. As expected β,γ-methylene-ATP and ATPγS were stable when incubated at 30 μM over 30 min. However, under these conditions both ATP and 2MeSATP were broken down to about 50% of their initial concentrations. The degree of breakdown did not vary between the two cell types (data not shown).

Permeabilization of cells. Because high concentrations of ATP^{4-} have been reported to compromise the integrity of cell membranes, we looked for evidence of permeabilization using incubation with ethidium bromide, which is excluded from cells with an intact plasmalemma but which will enter permeabilized cells and form a fluorescent complex with nucleic acids. We found, using this procedure, no evidence for permeabilization using ATP in the range of 100 μM to 3 mM (data not shown).

Discussion

The notion that endothelial cells from the adrenal medulla may have different P_2 receptors than those from major blood

vessels derives from the very different local concentrations of ATP with which the cells are in contact. This is a consequence of release of ATP from the chromaffin cells of the adrenal medulla at a molar concentration of 25% of the catecholamine concentration (22). One possible adaptation of adrenal cells would be a reduced potency of P_2 receptor agonists. This, however, was not seen in our experiments, but there was a difference in the size of response; the response of aortic cells to P_2 agonists was much larger (both in fold over basal and in absolute counts) than the response of adrenal cells.

Although differences in response between the two cell preparations used here may be ascribed to the difference in local ATP levels, i.e., specific to the adrenal medulla, it is also possible that the differences are due to a comparison between microvasculature and major blood vessels. The adrenal medulla is a highly vascularized tissue composed principally of adrenal chromaffin cells and elements of the microvasculature. The adrenal cells used in the current study originate from this microvasculature and are being compared with cells derived from the largest artery in the body. It seems likely that there may be major functional differences in the endothelium from these two sources.

During completion of this paper, another study was published that compares agonist-stimulated inositol phosphate accumulations in endothelial cells from bovine aorta and human umbilical vein (23). They found differential responses to bradykinin and thrombin, presumably the result of a different receptor population on the two cell types, giving rise to different physiological responses.

The results reported here are perhaps best explained by two differences between the cell types, firstly, a difference in receptor subtype (the results are consistent with the presence of P_{2Y} receptors on aorta cells and 'atypical' i.e., neither P_{2x} nor P_{2Y}, receptors on adrenal cells) and, secondly, a difference in the predominant route of $InsP_3$ metabolism.

The first quantitative difference in response revealed by the studies reported here was a difference in relative agonist potency between the two cell types, when the response being measured was the accumulation of total inositol phosphates in the presence of lithium. The aorta-derived AG4762 cells showed an overall rank order of agonist potency of 2MeSATP > ATPγS > ADP > ATP > β,γ-imido-ATP > β,γ-methylene-ATP. Combined with data showing insensitivity to adenosine and AMP, this profile is consistent with the presence of a P_{2Y}-purinoceptor, according to the criteria of Burnstock and Kennedy (5). However, with cells of adrenal origin the rank order of agonist potency was ATPγS > ATP > β,γ-imido-ATP > ADP > β,γ-methylene-ATP = 2MeSATP. Here the greater sensitivity to ATP than to ADP, combined with the essential lack of response to β,γ-methylene-ATP and 2MeSATP at concentrations at which ATP was effective, is inconsistent with the presence of either P_{2x} or P_{2Y} receptors, whereas the ineffectiveness of adenosine and AMP clearly suggests a P_2 receptor. One possible explanation, therefore, is that, whereas aorta endothelial cells possess a P_{2Y} receptor linked to phosphoinositide hydrolysis, in adrenal medulla the receptor is neither P_{2x} nor P_{2Y} but is of a novel subtype. A recent report on calcium mobilization and inositol phosphate production in human fibroblasts has also described an ATP-activated receptor that is not sensitive to 2MeSATP or β,γ-methylene-ATP (24).

In a previous report on P_2 receptors on adrenal medulla endothelial cells (25), it was concluded that the subtype for stimulation of formation of inositol phosphates was of the P_{2Y} subtype, on the basis of a smaller response to β,γ-methylene-ATP than to ATP. However, no experiments with the P_{2Y} agonist 2MeSATP were reported. The more extensive characterization carried out in the present report indicates clearly that this earlier classification of adrenal medulla receptors cannot be substantiated.

When we examined the nature of the response to P_2 agonists in the two cell types, the stimulation of label in the $InsP_3$ fraction preceded that in the $InsP_2$ and then the $InsP_1$ fractions, consistent with the notion that the lower phosphates were formed by phosphatase activity on the higher phosphates. However, in aorta cells the $InsP_3$ response reaches maximum within a few seconds, followed by a rapid fall, whereas in adrenal cells, although a substantial response can be seen within a few seconds, the response continues to rise and has not reached a maximum at the longest time studied (5 min).

It is possible that this difference in time course of response is a reflection of the different pattern of response revealed when individual isomers of some of the inositol phosphates are studied by HPLC. When $PtdIns(4,5)P_2$ is cleaved by the agonist-stimulated phospholipase C, it generates $Ins(1,4,5)P_3$, which can then be metabolized either to $Ins(1,4)P_2$ by the 5-phosphatase or to $Ins(1,3,4,5)P_4$ by the 3-kinase. This $InsP_4$ product may then be dephosphorylated to $Ins(1,3,4)P_3$. Both these isomers of $InsP_3$ will appear in the $InsP_3$ fraction in the above experiments using small anion exchange columns. However, studies on parotid glands (26, 27) have shown that $Ins(1,3,4)P_3$ has a half-life that is much longer (8.6 min) than that of $Ins(1,4,5)P_3$ (7.6 sec). It follows, therefore, that a cell that metabolizes $Ins(1,4,5)P_3$ via the 1,3,4-isomer will generate a more sustained increase in levels of $[^3H]InsP_3$ than a cell that directly dephosphorylates $Ins(1,4,5)P_3$ to $InsP_2$.

When the isomers were separated by HPLC in the present study, it was seen that stimulation of aorta cells for a few seconds resulted in production of $Ins(1,4,5)P_3$ and $Ins(1,4)P_2$, with only low levels of $Ins(1,3,4)P_3$ and $Ins(1,3,4,5)P_4$. This is consistent with the metabolism of $Ins(1,4,5)P_3$ principally by the 5-phosphatase pathway. However, in the adrenal cells label in both $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ was stimulated to a similar degree. This suggests that metabolism of $Ins(1,4,5)P_3$ is largely by phosphorylation to $Ins(1,3,4,5)P_4$, followed by phosphatase acting to generate $Ins(1,3,4)P_3$. Thus, $Ins(1,3,4)P_3$ is a dominant metabolite in the adrenal cells but not in the aorta cells; by virtue of its long half-life, this may lead to a long time course of accumulation of radioactivity in the $InsP_3$ fraction from the adrenal cells. Because $InsP_2$ and $InsP_1$ are generated by sequential loss of phosphates from $InsP_3$, it follows that radioactivity in these fractions also follows a very extended time course. In this way, the different routes of metabolism in the two cell types may account for the different time courses of accumulation following stimulation by agonist acting at P_2 receptors. This account is dependent upon the relative rates of breakdown of the two $InsP_3$ isomers in the endothelial cells being similar to those in the parotid gland. There are no studies to date that indicate whether this is so.

The P_2 receptor linkage to production of inositol phosphates has been best studied in one very different preparation, the turkey erythrocyte. Using erythrocytes prelabeled with *myo*-$[^3H]$inositol, these studies show that the inositol phosphates

that accumulate in response to stimulation by ATP acting at P_{2Y} receptors originate from inositol hydrolysis, by phospholipase C, of PtdIns(4,5)P_2 (28). The characteristics of this P_{2Y} stimulation of phospholipase C in turkey erythrocyte membranes show a dependency for GTP and analogues, with kinetics suggesting a similar role for a G protein as is apparent in adenylate cyclase-linked receptors (29). It is possible that the stimulation by ATP and analogues of the formation of inositol phosphates reported here in aortic and adrenal cells may be by a similar G protein-dependent linkage. Certainly the EC_{50} values for some of these analogues acting on intact erythrocytes (30) are similar to those reported here for bovine aortic and adrenal cells.

Complicating the characterization of P_2-linked second messenger systems, it has also been suggested that two types of P_2 receptors may be present on rat hepatocytes (31), one stimulating phospholipase C and the other inhibiting cyclic AMP with an inhibitory G protein (G_i). We have found no such P_2-mediated effect in adrenal medulla-derived cells. On the contrary, we found that ATP at 100 μM or below is able to stimulate small increases in cyclic AMP levels and acts synergistically with submaximal stimulation by PGE_2. Although the mechanism for this greater than additive effect of combining PGE_2 with ATP is not known, it is reminiscent of an earlier series of observations in which angiotensin II was seen to act in a synergistic manner with prostaglandin on cyclic AMP accumulation (32). The characteristics of this action of angiotensin II (which also activates phospholipase C) were consistent with a role for diacylglycerol-mediated stimulation of protein kinase C.

References

1. Moncada, S. Biological importance of prostacyclin. *Br. J. Pharmacol.* **76**:3–31 (1982).
2. Furchgott, R. F. Role of endothelium in response of vascular smooth muscle to drugs. *Annu. Rev. Pharmacol. Toxicol.* **24**:175–197 (1984).
3. Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Koboyashi, Y. Mitsui, Y. Yasaki, K. Goto, and T. Masaki. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond.)* **322**:411–415 (1988).
4. Burnstock, G. A basis for distinguishing two types of purinergic receptor, in *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach* (L. Bolis and R. W. Straub, eds.). Raven Press, New York, 107–118 (1978).
5. Burnstock, G., and C. Kennedy. Is there a basis for distinguishing two types of P_2 receptors. *Gen. Pharmacol.* **16**:433–440 (1985).
6. Gordon, J. L. Extracellular ATP: effects, sources and fate. *J. Biochem.* **233**:309–319 (1986).
7. Houston, D. A., G. Burnstock, and P. M. Vanhoutte. Different P_2 purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J. Pharmacol. Exp. Ther.* **241**:501–509 (1987).
8. Martin, W., N. J. Cusack, J. S. Carleton, and J. L. Gordon. Specificity of P_2 purinoceptor that mediates endothelium dependent relaxation of the pig aorta. *Eur. J. Pharmacol.* **108**:295–299 (1985).
9. Needham, L., N. J. Cusack, J. D. Pearson, and J. L. Gordon. Characteristics of the P_2 purinoceptor that mediates prostacyclin production by pigs aortic endothelial cells. *Eur. J. Pharmacol.* **134**:199–209 (1988).
10. Carter, T. D., T. J. Hallam, N. J. Cusack, and J. D. Pearson. Regulation of P_{2Y}-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.* **95**:1181–1190 (1988).
11. Boeynaems, J.-M., S. Pirotton, A. Von Coevorden, E. Raspe, D. Demolle, and C. Erneux. P_2-purinergic receptors in vascular endothelial cells: from concept to reality. *J. Recept. Res.* **8**:121–132 (1988).
12. Pirotton, S., E. Raspe, D. Demolle, C. Erneux, and J.-M. Boeynaems. Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J. Biol. Chem.* **262**:17461–17466 (1987).
13. Martin, T. W., and K. Michaelis. P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidyl choline in endothelial cells: evidence for activation of phospholipase D. *J. Biol. Chem.* **264**:8847–8856 (1989).
14. De Nucci, G., R. J. Gryglewski, T. D. Warner, and J. R. Vane. Receptor-mediated release of endothelium derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc. Natl. Acad. Sci. USA* **85**:2334–2338 (1988).
15. Banerjee, D. K., R. L. Ornberg, M. B. H. Youdim, E. Heldman, and H. B. Pollard. Endothelial cells from bovine adrenal medulla develop capillary like growth patterns in culture. *Proc. Natl. Acad. Sci. USA* **82**:4702–4706 (1985).
16. Booyse, F. M., B. J. Sedlak, and M. E. Rafelson. Culture of arterial endothelial cells: characterization and growth of bovine aortic cells. *Thromb. Diath. Haemorrh.* **34**:825–839 (1975).
17. Plevin, R., and M. R. Boarder. Stimulation of formation of inositol phosphates in primary cultures of bovine adrenal chromaffin cells by angiotensin II, histamine, bradykinin and carbachol. *J. Neurochem.* **51**:634–641 (1988).
18. Rooney, T. A., and S. R. Nahorski. Regional characterization of agonist and depolarization induced phosphoinositide hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.* **239**:873–879 (1987).
19. Batty, I. R., A. J. Letcher, and S. R. Nahorski. Accumulation of inositol phosphate isomers in agonist stimulated cerebral cortex slices. *J. Biochem.* **258**:23–32 (1989).
20. Brown, B. L., J. D. M. Albano, R. P. Ekins, A. M. Sgherzi, and W. Tampian. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121**:561–562 (1971).
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
22. White, T. D., J. E. Bourke, and B. E. Livett. Direct and continuous detection of ATP secretion from primary monolayer cultures of bovine adrenal chromaffin cells. *J. Neurochem.* **49**:1266–1273 (1987).
23. Bartha, K., R. Muller-Peddinhaus, and L. A. A. Van-Rooijen. Bradykinin and thrombin effects on polyphosphoinositide hydrolysis and prostacyclin production in endothelial cells. *Biochem. J.* **263**:149–155 (1989).
24. Fine, J., P. Cole, and J. S. Davidson. Extracellular nucleotides stimulate receptor-mediated calcium mobilization and inositol phosphate production in human fibroblasts. *Biochem. J.* **263**:371–376 (1989).
25. Forsberg, E. J., G. Feuerstein, E. Shomani, and H. B. Pollard. Adenosine trisphosphate stimualtes inositol phospholipid metabolism and prostacyclin formation in adrenal medullary endothelial cells. *Proc. Natl. Acad. Sci. USA* **84**:5630–5634 (1987).
26. Irvine, R. F., B. E. Anggard, A. J. Letcher, and C. P. Downes. Metabolism of inositol 1,4,5-trisphosphate and inositol 1,3,4-trisphosphate in rat parotid glands. *Biochem. J.* **229**:505–511 (1985).
27. Hughes, A. R., and J. W. Putney. Source of ^{3}H-labeled inositol bis- and monophosphates in agonist-activated rat parotid acinar cells. *J. Biol. Chem.*, **264**: 9400-9407 (1989).
28. Harden, T. K., P. T. Hawkins, L. Stevens, J. L. Boyer, and C. P. Downes. Phosphoinositide hydrolysis by guanosine 5′-[5-thio]trisphosphate-activated phospholipase C of turkey erythrocyte membrane. *Biochem. J.* **252**:583–593 (1988).
29. Boyer, J. L., C. P. Downes, and T. K. Harden. Kinetics of activation of phosphlipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.* **264**:884–890 (1989).
30. Berrie, C. P., P. T. Hawkins, L. R. Stephens, T. K. Harden, and C. P. Downes. Phosphatidylinositol 4,5-bisphosphate hydrolysis in turkey erythrocytes is regulated by P_{2Y} purinoceptors. *Mol. Pharmacol.* **35**:526–532 (1989).
31. Okajima, F., Y. Tokumitsu, Y. Kondo, and M. Ui. P_2-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocyes. *J. Biol. Chem.* **262**:13483–13490 (1987).
32. Boarder, M. R., R. Plevin, and D. B. Marriott. Angiotensin II potentiates prostaglandin stimulation of cyclic AMP levels in intact bovine adrenal medulla cells but not adenylate cyclase in permeabilized cells. *J. Biol. Chem.* **263**:15319–15324 (1988).

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