British Journal of Pharmacology (1996) 117, 1572-1578

Evidence for a discrete UTP receptor in cardiac endothelial cells

[1]Shumei Yang, Iain L.O. Buxton, Charles B. Probert, Jeffery N. Talbot & [2]Michael E. Bradley

Department of Pharmacology, University of Nevada, Reno, Reno, NV 89557, U.S.A.

1 We have examined the effects of various purine and pyrimidine nucleotides upon cells cultured from guinea-pig cardiac endothelium (CEC), and find the P_{2Y}-agonist 2-methylthioadenosine triphosphate (2MeSATP) to be a potent ($EC_{50} = 85 \pm 10.2$ nM) stimulator of increases in intracellular calcium concentrations, while uridine 5'-triphosphate (UTP) and adenosine 5'-triphosphate (ATP) are less potent but equipotent with one another (EC_{50}s $= 2.1 \pm 0.3$ and 1.8 ± 0.2 μM, respectively).

2 While the P_{2Y} receptor exhibited rapid homologous desensitization, this had no effect upon subsequent responsiveness of CEC to either ATP or UTP. Effects of maximal concentrations of ATP and UTP were not only additive, but did not cross-desensitize. Responses to UTP (but not to ATP or 2MeSATP) were blocked by treatment with pertussis toxin (PTX); all three nucleotides appeared to liberate calcium from an intracellular pool.

3 Suramin (30 μM) significantly ($P < 0.05$) increased the EC_{50} for ATP-dependent increases in intracellular calcium (5.3 ± 2.2 μM *vs.* 2.0 ± 0.9 μM in the absence of suramin), while it completely blocked the response to 2MeSATP. Suramin had no effect upon responses to UTP at concentrations of 100 μM.

4 We conclude that in addition to the P_{2Y} and P_{2U} subtypes of the ATP receptor, an additional receptor responsive to UTP but exhibiting no affinity for purine nucleotides is present in CEC; this 'pyrimidine receptor' liberates intracellular calcium *via* a G-protein, and may partly mediate the contractile response to UTP in the coronary vasculature.

Keywords: Pyrimidine receptor; UTP; ATP; cardiac endothelial cells; intracellular calcium

Introduction

Extracellular purines such as adenosine and adenine nucleotides are known to be capable of eliciting a variety of responses from cells making up almost all tissues (Gordon, 1986). Based primarily upon rank order of potency, purine receptors responding to adenosine 5'-triphosphate (ATP) and adenosine 5'-diphosphate (ADP), P_2 receptors, have been classified into an ever-growing number of subtypes (Burnstock & Kennedy, 1985). There is some current debate regarding classifications of P_2 receptors (Boarder *et al.*, 1994; Barnard *et al.*, 1994); in general, receptors with high affinity for the adenine nucleotide analogue, 2-methylthioadenosine 5'-triphosphate (2MeSATP), are classified as P_{2Y} receptors, while those responding best to $\alpha\beta$-methyleneadenosine 5'-triphosphate (α,βMeATP) have traditionally been termed P_{2X}, although recent evidence suggests that in the absence of extracellular purine nucleotide metabolism, ATP and 2MeSATP may in fact be more potent agonists at the P_{2X} receptor than α,βMeATP (Kennedy & Leff, 1995). Activation of the P_{2Y} receptor, which has been found (among other places) in liver (Häussinger *et al.*, 1987), mesenteric artery (Ralevic & Burnstock, 1991), and aortic endothelial cells (Motte *et al.*, 1993) is usually associated with an increase in phospholipase activity *via* its coupling by G-protein (Wilkinson *et al.*, 1994); in the case of endothelial cells, this activation has been shown to result in the release of vasodilator compounds such as nitric oxide (Vials & Burnstock, 1993) and adenyl purines (Yang *et al.*, 1994).

Uridine nucleotides can also function as extracellular signals. Effects of uridine 5'-triphosphate (UTP) have been demonstrated in vascular tissues (Urquilla, 1978), vas deferens (Urquilla *et al.*, 1978; von Kügelgen *et al.*, 1990), airway epithelial cells (Brown *et al.*, 1991), and endothelia from a variety of sources, including aorta (Motte *et al.*, 1993), brain microvasculature (Purkiss *et al.*, 1994), and cardiac blood vessels (Vials & Burnstock, 1993; Mannix *et al.*, 1993). As is the case with studies of purine nucleotide action, the virtual absence of specific receptor antagonists has severely hampered characterizations of the receptor(s) mediating the effects of pyrimidine treatment of tissues and cells; as a result, rank order of potency profiles have played a key role in elucidating differences in responses to various agonists. In several cell types, UTP has now been shown to be equipotent with ATP; the rank order of potency for nucleotides interacting with these cells usually resembles UTP $\geqslant$ ATP > 2MeSATP. Until very recently it has been difficult to determine whether the activities of ATP and UTP were mediated by the same or different receptors in the target tissues. With the cloning and expression of the P_{2U} subtype (previously 'nucleotide') receptor, however, the evidence for a single membrane protein with the ability to bind both purine and pyrimidine nucleotides is at last convincing (Lustig *et al.*, 1993). There remains, however, evidence to suggest that UTP is capable of interacting with receptors in responsive cells which do not fit into the classification schemes of either P_{2U} or P_{2Y} receptors (O'Connor *et al.*, 1991).

The purpose of the studies reported here was to examine the effects of UTP upon intracellular calcium concentrations ($[Ca^{2+}]_i$) in cultured cardiac endothelial cells (CEC), and to characterize the receptor(s) mediating any responses obtained. We found that in addition to ATP responses believed to be mediated by P_{2Y} and P_{2U} receptors, UTP was capable of eliciting changes in $[Ca^{2+}]_i$ in CEC by means of a receptor with no significant affinity for purine nucleotide; this 'pyrimidine receptor' may not fit the classification of the P_2 class of ATP receptors.

Methods

Cardiac endothelial cell isolation and culture

Endothelial cells from the cardiac vasculature (CEC) were isolated essentially as described by Nees *et al.* (1981). Briefly, hearts were removed from 250 g female guinea-pigs (Si-

[1]Present address: Division of Biomedical Science, University of California, Riverside, CA 92521, U.S.A.
[2]Author for correspondence.

monsen, Gilroy, CA) which had been killed by CO_2 asphyxiation in keeping with protocols approved by the University of Nevada's Animal Care and Use Committee. Retrograde perfusion of the aorta was then accomplished by cannulation; the perfusate which was forced as a result through the cardiac vasculature consisted of a modified Dulbecco's Modified Eagle Medium (DMEM) lacking Ca^{2+} and supplemented with penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}), collagenase (CLSII, 1.5 mg ml^{-1}), and trypsin (1:250, 1 mg ml^{-1}). The heart perfusion solution was filtered through a 0.7 μm filter before use. Cells liberated into the perfusate were harvested by differential sedimentation (500 $g^{\bullet}$min) and loaded onto pre-formed 50% Percoll gradients and separated by centrifugation at 30,000 $g^{\bullet}$min. Endothelial cells were removed from the layer equilibrating at a density of 1.033 g ml^{-1}, residual Percoll was removed by dilution and centrifugation, and cells were plated onto specially-modified 35 mm culture dishes which had the bottoms replaced with glass cover slips. Cells were allowed to grow to confluency in a growth medium of DMEM supplemented with penicillin: streptomycin and 10% (v/v) foetal bovine serum in a water-saturated atmosphere of 95% O_2:5% CO_2; confluency was usually achieved within 1 week. Only CEC in primary culture were used for these experiments.

The endothelial origin of cultured cells was routinely confirmed by positive staining in immunofluorescent assays using FITC-labelled antibodies directed against clotting Factor VIII (Fry *et al.*, 1984). Cultured uterine smooth muscle cells and lung fibroblasts served as negative controls.

Intracellular Ca^{2+} detection

Confluent primary cultures of CEC in glass-modified culture dishes were rinsed of growth medium with a MOPS-buffered 'Solution A' consisting of (in mM): NaCl 120, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 6, Na-MOPS 10 and $NaHCO_3$ 5. CEC were then incubated in a loading solution consisting of Solution A into which 0.1 mg ml^{-1} bovine serum albumin, 0.02% cremophor EL, 10 nM neostigmine bromide, and 2 μM fura-2/AM were dissolved by sonication; the incubation was performed for 60 min at 37°C in room atmosphere. Cells were rinsed twice with warm Solution A and allowed to incubate in the latter for an additional 15 min to permit complete hydrolysis of any intact ester linkages on intracellular fura-2.

Modified culture dishes containing CEC were mounted in a specially-modified, thermostatically-controlled chamber affixed to the stage of a Nikon Diphot inverted phase-contrast microscope. Excitation light was provided by a DeltaScan Model 1 (PTI, Inc., Princeton, New Jersey, U.S.A.) scanning fluorometer *via* bifurcating fibre optical cables connected to the microscope. Samples were alternatively illuminated by light with wavelengths of 340_{nm} and 380_{nm} by means of a mirrored chopper wheel. Emitted light was filtered first by means of a high-pass filter, followed by a restriction filter selective for 510_{nm}. Both incident and emitted light were passed through a 40× objective equipped with quartz optics. Emissions were quantified by means of a photomultiplier tube connected to a computer running acquisition software supplied by PTI, and were collected as both the 340_{nm} and 380_{nm} signals for post-hoc ratioing operations. The sampling rate was 10 Hz.

During the course of experiments CEC were incubated at 35°–36°C in a salt solution of the following composition (in mM): NaCl 120, KCl 5, KH_2PO_4 0.587, Na_2HPO_4 0.598, $MgCl_2$ 2.5, glucose 20, $CaCl_2$ 2.5 and HEPES 10. Addition of drugs to culture dishes was accomplished by replacement of the bathing media *via* modified Pasteur pipettes clamped to the microscope stage; in this way agents were always present at the correct final concentrations.

Data analysis

The amount of light emitted at 510_{nm} when cells were excited at 340_{nm} was quantified by photomultiplication and normalized to the signal obtained following excitation at 380_{nm}. While fluorescent maxima and minima were obtained during each experiment (by means of sequential addition of 5 μM ionomycin and 2 mM Mn^{2+} addition), no attempt was made to calibrate fluorescent signals to $[Ca^{2+}]_i$ due to the variability and inaccuracy inherent in such manipulations; all results are therefore presented in simple ratiometric units, or as a percentage of the maximum response elicited by ionomycin.

Except for those associated with pertussis toxin (PTX) effects, data presented are tracings representative of those obtained from cells in n culture dishes; each dish, in turn, was obtained from a different cell isolation to which CEC from 4 different animals contributed. Statistical evaluations given in the text were performed by either a two-tailed Student's t test (unpaired) or (for PTX experimental results) one-way analysis of variance followed by Student's t test. Analysis of suramin effects upon the ATP concentration-response relationship was performed by use of a commercially-available programme (Graphpad Prism, Graphpad Software, San Diego, CA, U.S.A.). Standard error of the mean is represented as s.e.mean.

Drugs

Fura-2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.), Na-MOPS and ionomycin were from Calbiochem (Eugene, OR, U.S.A.), collagenase was CLSII from Worthington (Freehold, NJ, U.S.A.), foetal bovine serum was from Gibco (Santa Clara, CA, U.S.A.), and anti-Factor VIII antibodies were from Vector Labs (Burlingame, CA, U.S.A.). Percoll was obtained from Pharmacia (Alameda, CA, U.S.A.). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Concentration-response relationships

Increases in $[Ca^{2+}]_i$ in fura-loaded CEC incubated with increasing, noncumulative concentrations of 2MeSATP, ATP, UTP, or the P_{2X}-selective agonist α,βMeATP, revealed a rank order of potency of 2MeSATP > UTP = ATP (EC_{50}s in nM ± s.e.mean of 85 ± 10.2, 2100 ± 273, and 1800 ± 194, respectively), with α,βMeATP exhibiting no significant effect upon $[Ca^{2+}]_i$ in these cells (Figure 1). Maximal responses to 2MeSATP, ATP, and UTP were (on average) the same, suggesting that all three agonists act with equal efficacy with respect to the liberation of $[Ca^{2+}]_i$ in CEC. At a concentration of 1 mM, responses to both ATP and UTP were somewhat reduced, suggesting either rapid desensitization, extracellular metabolism, or that both occur when nucleotides are present at this high concentration.

Desensitization and additivity

Both purine and pyrimidine nucleotide agonists exhibited a high degree of homologous desensitization in CEC. Incubation with maximal concentrations (10 μM) of ATP, for example, resulted in greatly attenuated response to subsequent addition of this agent after 2 min (Figure 2); similarly rapid desensitizations to UTP were also demonstrated. Importantly, there was little evidence of heterologous desensitization between the purine and pyrimidine agonists in our experiments; as seen in Figure 2, for example, desensitization of the UTP response did not obliterate the response to subsequent addition of ATP. The converse experiment was also performed with similar results - desensitization of the ATP response had no apparent effect upon the ability of cells to respond to UTP (Figure 2b). Statistical analysis of the relative magnitudes of the response to ATP before and after treatment with UTP revealed no significant change in peak height; similarly, no statistically significant alteration in the peak response to UTP was seen following ATP treatment ($n = 6$ for both types of experiments).

The response to ATP in CEC can be dissociated into two distinct components. Addition of 2MeSATP to CEC produced a response which rapidly (<60 s) desensitized (Figure 3a). Addition of ATP to 2MeSATP-desensitized cells resulted in a fluorescent response which was unaffected by the prior desensitization to 2MeSATP (Figure 3a); the average peak response to ATP following 2MeSATP treatment was 70% ± 32% of the original ATP response ($n = 5$). The failure to find a statistically significant reduction in the ATP response indicates a lack of heterologous desensitization of the ATP response by the P_{2Y}-selective 2MeSATP. However, the converse was not the case: desensitization of the ATP response was shown also to abolish the response to 2MeSATP (Figure 3b); the mean peak response to 2MeSATP following ATP treatment was 12% ± 11% of the original response, a statistically significant ($P < 0.05$) reduction. Desensitization of the P_{2Y} receptor with 2MeSATP had no apparent effect upon responsiveness of CEC to UTP treatment, nor was UTP desensitization able to affect subsequent responsiveness to 2MeSATP (data not shown). Also illustrated in Figure 3 is our occasional finding of oscillatory changes in $[Ca^{2+}]_i$ in the continued presence of agonist (Figure 3a), a characteristic which we have not observed in CEC examined while in a subconfluent state (M.E. Bradley, unpublished observations).

Separate receptor systems for UTP and ATP were also suggested by results demonstrating additivity of the effects of these two agonists. Addition of a saturating concentration of UTP to CEC produced a maximal response which could not be enhanced upon further addition of UTP (control experiments not shown), but to which a subsequent ATP response added (Figure 4a); the converse was also true, whereby responses to UTP were seen in the presence of a demonstrably maximum response to ATP (Figure 4b). The sum of the responses to ATP and UTP when added sequentially averaged 110% ± 19% of the sum of the responses when taken separately. These values (110% *vs.* 100%) were not statistically different from one another, and the order of treatments (*i.e.* as presented in Figure 4a *vs.* those presented in Figure 4b) had no apparent effect on

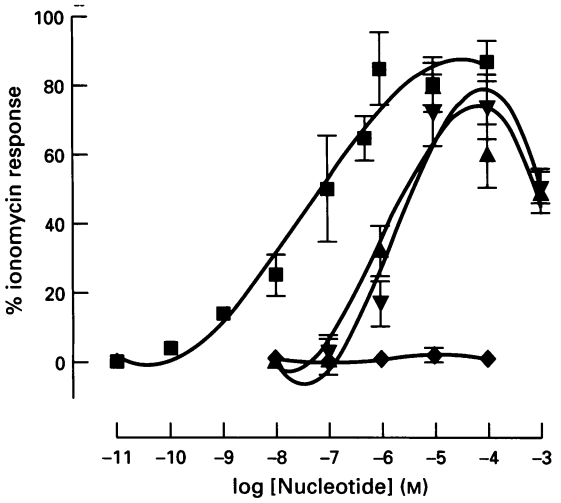

Figure 1 Concentration-response relationships for purine (2MeSATP, ATP, α,βMeATP) and pyrimidine (UTP) nucleotides in cultured cardiac endothelial cells. Cells in primary culture were loaded with fura-2 and challenged with increasing (noncumulative) concentrations of a single nucleotide for approx. 2 min at 37°C; responses were quantified by peak height, and are presented as a percentage of the maximum response elicited at the conclusion of the experiment by the addition of 30 μM ionomycin. Each point represents an n of at least 4 (see Methods); s.e.mean are represented by error bars: (■) 2MeSATP; (▲) ATP; (▼) UTP; (◆) α,β-MeATP.

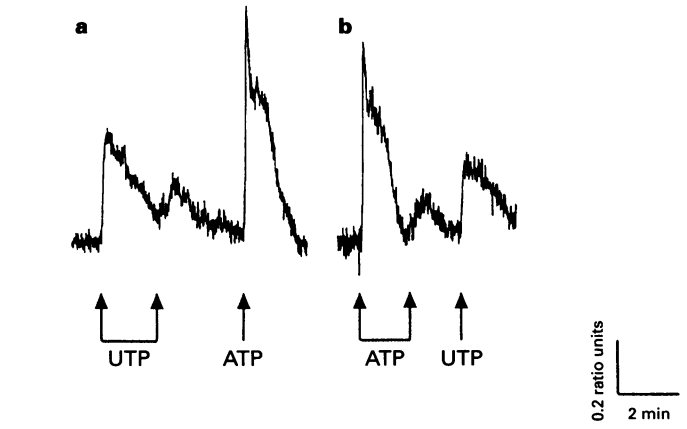

Figure 2 ATP and UTP do not cross-desensitize. Desensitization of the UTP response by repeated treatments with maximal (10 μM) concentrations did not abolish the response to subsequent addition of ATP (a); conversely, ATP desensitization had no effect upon UTP responses (b). Tracings are representative of those obtained from culture dishes obtained from 6 different preparations ($n = 6$) in which similar results were obtained.

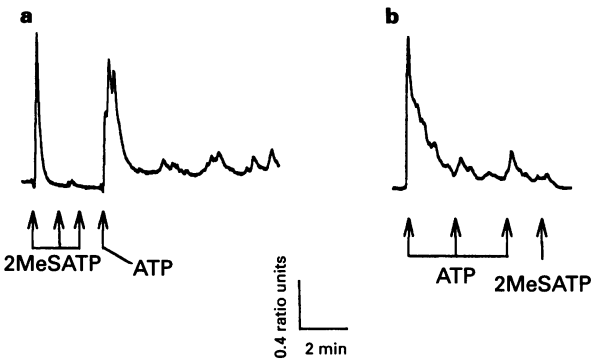

Figure 3 Evidence for multiple ATP receptors in CEC. Fluorescence microscopy of fields of 1-6 cells revealed a rapid desensitization to maximal (noncumulative) concentrations of 2MeSATP (10 μM) which had no effect upon subsequent responsiveness to 10 μM ATP (a). Addition of maximal concentrations of ATP (10 μM) also resulted in desensitization (b), but in this case the response to subsequent addition of 2MeSATP was obliterated. Results are representative recordings from $n = 5$ preparations.

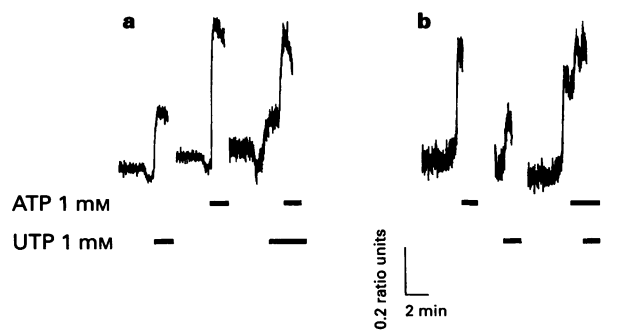

Figure 4 ATP and UTP responses are additive. Addition of maximal concentrations of either UTP or ATP resulted in the first two responses in (a), respectively. Addition of ATP to cells which had already been allowed to respond to UTP Resulted in the additional response seen in the third tracing in (a). (b) Represents the converse experiment, in which UTP responses were allowed to add to ATP responses. Tracings are representative of those obtained in 4 other experiments ($n = 4$).

the magnitude of the responses, suggesting that while the effects of ATP and UTP are clearly additive in these cells, the mechanisms responsible for mediating the responses to ATP and UTP do not exhibit any significant synergy.

Given the complexity of CEC responses to adenine nucleotides, experiments were performed in which responses mediated by receptors with affinity for ATP were eliminated; in these experiments the P_{2Y} receptor was desensitized by repeated application of 2MeSATP, and any remaining responses to ATP were then desensitized immediately following the 2MeSATP treatments. As can be seen in Figure 5, addition of UTP immediately following the desensitization of both the 2MeSATP and ATP responses in CEC resulted in a maximal fluorescent response which appeared to have been unaffected by the pretreatments with adenine nucleotides. In $n=8$ experiments of this sort, the average response to ATP following 2MeSATP desensitization was $60\% \pm 10\%$ of the ATP response prior to 2MeSATP treatment; this significant reduction ($P<0.05$) was probably due to the loss of the contribution to the total ATP response of the (now desensitized) P_{2Y} receptors in these cells, and the remaining ATP response is therefore probably due to the response mediated by the P_{2U} receptor. The response to UTP following desensitization of both the ATP and 2MeSATP responses was on average 51% ($\pm 13\%$) of its initial response; once again, this statistically-significant ($P<0.05$) reduction in UTP responsiveness is consistent with the loss of part of the receptor population mediating the response to UTP (due to desensitization of the P_{2U} receptors by ATP treatment).

Effects of suramin

Responses to UTP and ATP differed with respect to sensitivity to the non-specific P_2 antagonist, suramin. Responses to ATP at maximal concentrations were significantly reduced ($P<0.05$) when the agonist was added to cells pretreated with 30 μM suramin; the response to ATP was completely blocked by suramin at 100 μM, yet this response was readily recovered following a 5 min washout of the suramin (Figure 6b). Concentration-response curves describing the effect of ATP upon $[Ca^{2+}]_i$ in the presence and absence of 30 μM suramin were also performed (not shown); these studies revealed an EC_{50} in the presence of suramin of 5.2 ± 2.2 μM *vs.* an EC_{50} of 2.0 ± 0.9 μM in its absence. Statistical analysis of these curves revealed a significant ($P<0.05$) difference in EC_{50} values due to the presence of the suramin. Suramin was even more potent in inhibiting the response to 2MeSATP in these cells (Figure 6c). However, UTP responses were unaffected by suramin pretreatment of CEC, even at concentrations as high as 100 μM (Figure 6a). The effect of suramin on the ATP and 2MeSATP responses was not due to its apparent ability to chelate ATP (Yang *et al.*, 1994), as suramin was used only as a pretreatment agent and was removed from the bathing medium immediately (~3 s) prior to agonist additions; preliminary experiments in which suramin was present during the addition of UTP produced results identical to those obtained in its absence.

Pertussis toxin sensitivities

The involvement of guanine nucleotide-binding proteins of the G_i subclass in the transduction of pyrimidine and purine nucleotide effects in CEC was investigated by incubating cells with PTX for 12 h before treatment with maximal concentrations of UTP, ATP, or 2MeSATP. PTX treatment reduced the UTP response of CEC to a value 17% of that seen in appro-

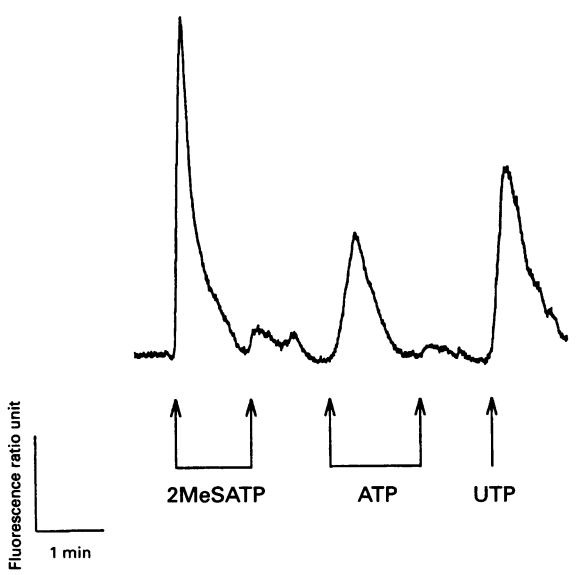

Figure 5 Evidence for a distinct pyrimidine receptor. Desensitization of all ATP responses in CEC was accomplished by sequential treatment of cells with maximal concentrations (10 μM for each) of 2MeSATP and ATP; addition of 10 μM UTP immediately following this treatment resulted in a pronounced response amounting to approximately 50% of the UTP response prior to purine treatment. Tracing is representative of those obtained from $n=8$ preparations.

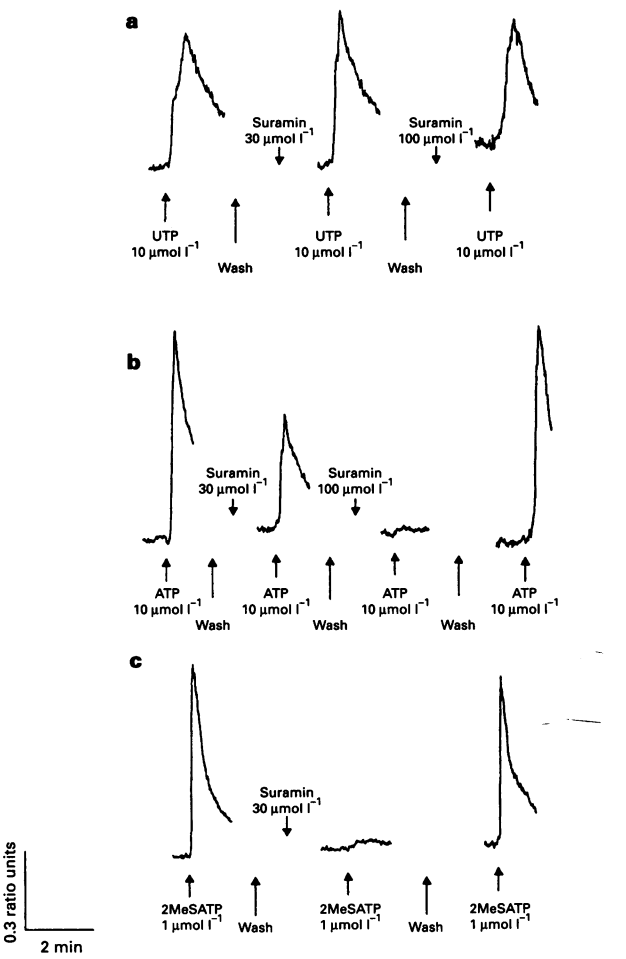

Figure 6 Suramin inhibition of ATP and 2MeSATP but not UTP responses. Incubation of CEC with either 30 μM or 100 μM suramin for 30 min followed by treatment with either ATP (b) or 2MeSATP (c) resulted in a concentration-dependent decrease in responsiveness to purine nucleotides; responses were immediately restored, however, following washout of suramin. Suramin had no effect upon UTP responses even when used at 100 μM for 30 min (a). Tracings in each panel are continuous recordings from the same cells, and are representative of findings obtained in 4 other experiments ($n=5$).

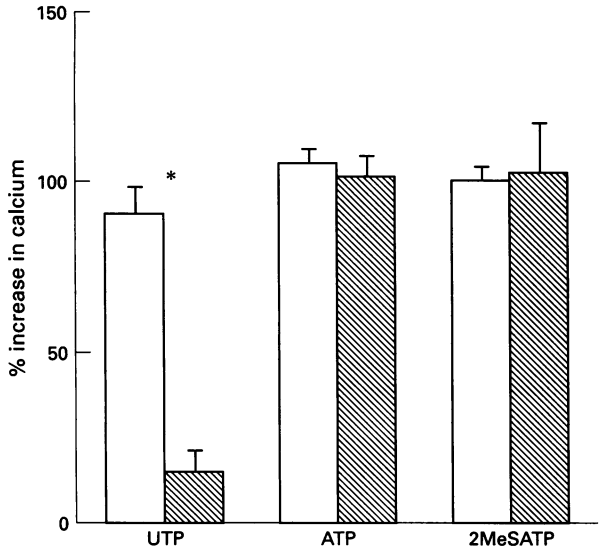

Figure 7 Effects of PTX on nucleotide responses. Cells were incubated with PTX (100 ng ml^{-1}) for 12 h prior to challenge with maximal concentrations of agonists (each at 10 μM); data are presented as means ± s.e.mean. *$P<0.05$, $n=4$ for each condition. Control (open) columns represent cells maintained under identical conditions but for the absence of PTX.

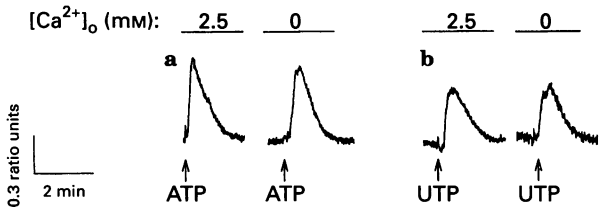

Figure 8 Role of $[Ca^{2+}]_i$ in ATP and UTP responses. Cells were treated with 10 μM of either ATP (a) or UTP (b) in normal (2.5 μM) Ca^{2+}, and then incubated in the absence of extracellular Ca^{2+} (no added Ca^{2+}, 50 μM EGTA) for 5 min before a second addition of nucleotide at 10 μM. Results represent those obtained in 6 other experiments ($n=7$).

priate control cells; no effect of PTX treatment was seen upon fluorescent responses to either ATP or 2MeSATP (Figure 7).

Role of $[Ca^{2+}]_i$

The possibility that pyrimidine and purine nucleotides effect increases in $[Ca^{2+}]_i$ by accessing different sources of Ca^{2+} was evaluated by comparing responses to ATP and UTP in the presence and absence of extracellular Ca^{2+}. Responses to both nucleotides following chelation of extracellular Ca^{2+} were virtually identical to those seen immediately prior to Ca^{2+} removal (Figure 8), suggesting that Ca^{2+} release in response to ATP and UTP is from intracellular stores.

Discussion

Evidence has been accumulating for a number of years for the existence of a plasma membrane-associated receptor capable of responding to extracellular pyrimidine nucleotides in a number of tissue types (Burnstock, 1993). With the cloning and expression (Lustig *et al.*, 1993) of the P_{2U} receptor subtype, the evidence that pyrimidines such as UTP are capable of interacting with specific, high-affinity receptor proteins located within the plasma membranes of a variety of cell types would now appear to be incontrovertible. This 'nucleotide receptor' appears to exhibit almost identical affinity for ATP and UTP, couples to its effects by means of at least one G-protein, and is sensitive to cross-desensitization by ATP and UTP (Brown *et al.*, 1991).

Many tissues have now been found to possess a P_{2U} receptor; some of these appear to contain P_{2U} as the sole purine nucleotide receptor (*e.g.* liver, Keppens, 1993), while most express more than one type of receptor for ATP. However, the existence of a specific receptor mediating responses to pyrimidine nucleotides but exhibiting no affinity for purines has occasionally been suggested (Brown *et al.*, 1991) but not, until quite recently (Lazarowski & Harden, 1994) demonstrated, and there has yet to be presented any convincing evidence for the existence of such a receptor in an intact tissue. The evidence for a 'UTP receptor' has been inconsistent and (at times) confusing. For example, as described in the review by O'Connor *et al.* (1991), the presence of both P_{2Y} and P_{2U} receptors has been established in a number of tissue types, and there have been findings in certain tissues of rank order of potencies (2MeSATP > UTP = ATP) which are suggestive of a 'mixed' or heterogeneous receptor population to which these two receptor types (P_{2Y} and P_{2U}) contribute. The conclusion reached by O'Connor *et al.* (1991) regarding the mixed receptor population was not, however, presented as evidence for a separate and distinct 'pyrimidinergic receptor', as has been suggested elsewhere (*e.g.* Keppens, 1993).

Evidence (if indirect) for a pyrimidine receptor does, however, exist, and this work has been summarized in the reviews by Seifert & Schultz (1989) and O'Connor *et al.* (1991). Most notably, Lazarowski & Harden (1994) recently determined that cells in a transformed rat glial cell culture model responded well to treatment with pyrimidine nucleotides, but were insensitive to incubation with either purine or purine nucleotides. This is the first clear description of a receptor capable of binding to UTP but not to ATP, and to these findings we now add those contained in the present paper. We find that in endothelial cells from the cardiac vasculature grown in primary culture, ATP and UTP effects appear to be mediated by separate and distinct receptors. This conclusion is based upon the absence of significant cross-desensitization between the responses to ATP and UTP, upon the additivity of effects seen when maximal concentrations of ATP and UTP are present simultaneously, and upon differential sensitivities of the responses to these two nucleotides to suramin and PTX treatment. It therefore appears that, in addition to the P_{2Y} and P_{2U} receptors already characterized in endothelial cells (Vials & Burnstock, 1993; Mannix *et al.*, 1993), a distinct receptor for UTP which is incapable of responding to purine nucleotides is present and coupled to Ca^{2+} release from intracellular stores.

The findings upon which we have based our conclusions differ from those of Motte *et al.* (1993). Although their studies (performed in subcultured endothelial cells derived from bovine aorta) detected the presence of P_{2Y} and P_{2U} receptors, they failed to detect a response to pyrimidine which could not be explained by the presence of the P_{2U} receptor. We find in CEC that ATP and UTP effects are additive, do not cross-desensitize, and are unaffected by prior desensitization with the P_{2Y}-specific agonist, 2MeSATP. In contrast, in cultured aortic endothelial cells, ATP and UTP are not additive, their effects cross-desensitize, and the response to UTP is partially blocked by pretreatment with 2MeSATP (Motte *et al.*, 1993). There was, therefore, no evidence obtained in the studies by Motte *et al.* (1993) for the existence of a purine nucleotide-insensitive response to UTP. It is possible that the differences between the findings of Motte *et al.* (1993) and our own can be explained on the basis of differences in origin of the endothelial cell cultures (cardiac *vs.* aortic), or by the fact that the aortic cells used by Motte *et al.* (1993) were repeatedly subcultured before use, resulting in a change in receptor phenotype; indeed, the rapid loss of G-protein-coupled receptors in endothelial cells following a single passage has been reported previously (Tracey & Peach, 1992) and has been observed in this laboratory

(M.E. Bradley, unpublished data). In principle, however, our results would appear to extend rather than contradict the findings of Motte *et al.* (1993) in that we propose not only the existence of P_{2Y} and P_{2U}, but an additional pyrimidine nucleotide receptor not seen in the aortic cell cultures.

Given the high potency of 2MeSATP in our CEC and the previously-acquired evidence for its existence, there is little doubt that CEC express the P_{2Y} receptor. Cross-desensitization did not occur with respect to UTP effects upon 2MeSATP; we can therefore conclude that UTP interacts with CEC by a receptor distinct from the P_{2Y} receptor. We find, however, a pronounced heterologous desensitization of ATP upon the 2MeSATP receptor, a finding consistent with the intermediate affinity of the latter receptor for ATP. Furthermore, desensitization of all responsiveness to adenine nucleotides by sequential addition of 2MeSATP and ATP had no effect upon subsequent CEC responses to UTP addition (Figure 5), perhaps the most convincing evidence in this paper for a distinct UTP receptor. Clearly, were UTP binding to the same receptor(s) as ATP in these cells, the response to UTP should have been significantly reduced if not abolished, and it was neither.

Our observation of differential sensitivities of nucleotide responses to PTX is consistent with the work of others which demonstrates that both the P_{2U} (Brown *et al.*, 1991; Sipma *et al.*, 1994) and the pyrimidine receptor of C6-2B cells (Lazarowski & Harden, 1994) exhibit sensitivity to pertussis toxin. We find no significant effect of PTX treatment upon either ATP or 2MeSATP responses in CEC, a finding which suggests that, if ATP- or 2MeSATP-activated receptors exist in these cells, they do not (in contrast to ATP receptor transduction in other cell types, *e.g.* Boyer *et al.*, 1989) couple to a PTX-sensitive G-protein. Finally, our finding of a significant effect of suramin treatment upon the $[Ca^{2+}]_i$ response to ATP and 2MeSATP but not UTP would appear to be consistent with the notion that suramin is interacting primarily with the P_{2Y} receptor present in our cultures, but does not significantly interact with either the P_{2U} or the separate pyrimidine receptor which also binds to UTP; that suramin may not be capable of interacting with the P_{2U} receptor has been suggested by Wilkinson *et al.* (1993, 1994).

The potential would appear to be great for ultimately elucidating a physiological role for pyrimidines in the regulation of cardiac vascular tone. Indeed, several important criteria for elucidating such a role appear to have been met by UTP: (1) UTP can affect vascular tone in general, both in an endothelium-dependent and independent manner (Vials & Burnstock, 1993), and there are therefore physiologically-relevant consequences to the presence of extracellular UTP in vascular smooth muscle; (2) UTP-stimulated release of vasodilator compounds such as NO from the endothelium of intact cardiac blood vessels has been demonstrated (Vials & Burnstock, 1993), suggesting that UTP effects upon vascular smooth muscle may be both direct and indirect, and (3) a source for UTP at physiological concentrations has been found in platelets, which can release UTP in concentrations equal to those of purine nucleotide during the course of degranulation reactions (Goetz *et al.*, 1971). Along these lines, we have observed in preliminary experiments performed upon intact rings (+ endothelium) of coronary artery (M.E. Bradley, unpublished data) that UTP addition elicits a strong contraction, while ATP addition results in a potent relaxation of the vascular smooth muscle. These findings may provide further support for a role for UTP in the regulation of coronary blood flow *in situ*.

This work was funded by an American Heart Association (Nevada Affiliate) Grant-in-Aid of research (to M.E.B.) and fellowship (to S.Y.), and by NIH HD26227 (to I.L.O.B.). We thank Dr L. Zhang for critically reviewing this manuscript.

References

BARNARD, E.A., BURNSTOCK, G. & WEBB, T.E. (1994). Barnard, Burnstock and Webb reply. The cloning of novel P_2 purinoceptor subtypes requires the re-classification of P_{2Y} receptors. *Trends, Pharmacol. Sci.*, **15,** 280–281.

BOARDER, M.R., TURNER, J.T., ERB, L. & WEISMAN, G.A. (1994). Classification of P_2 purinoceptors. Not all G protein-coupled P_2 purinoceptors can be classed as P_{2Y}. *Trends Pharmacol. Sci.*, **15,** 280–281.

BOYER, J.L., DOWNES, C.P. & HARDEN, T.K. (1989). Kinetics of activation of phospholipase C by P_{2Y} purinergic receptor agonists and guanine nucleotides. *J. Biol. Chem.*, **264,** 884–890.

BROWN, H.A., LAZAROWSKI, E.R., BOUCHER, R.C. & HARDEN, T.K. (1991). Evidence that UTP and ATP regulate phospholipase C through a common extracellular 5′-nucleotide receptor in human airway epithelial cells. *Mol. Pharmacol.*, **40,** 648–655.

BURNSTOCK, G. (1993). Physiological and pathological roles of purines: An update. *Drug Dev. Res.*, **28,** 195–206.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16,** 433–440.

FRY, G., PARSONS, T., HOAK, J., SAGE, H., GINGRICH, R.D., ERCOLANI, L., NGHIEM, D. & CZERVIONKE, R. (1984). Properties of cultured endothelium from adult human vessels. *Arteriosclerosis*, **4,** 4–13.

GOETZ, U., DA PRADA, M. & PLETSCHER, A. (1971). Adenine-, guanine- and uridine-5′-phosphonucleotides in blood platelets and storage organelles of various species. *J. Pharmacol. Exp. Ther.*, **178,** 210–215.

GORDON, J.L. (1986). Extracellular ATP: effects, sources, and fate. *Biochem. J.*, **233,** 309–319.

HÄUSSINGER, D., STEHLE, T. & GEROK, W. (1987). Actions of extracellular UTP and ATP in perfused rat liver. A comparative study. *Eur. J. Biochem.*, **167,** 65–71.

KENNEDY, C. & LEFF, P. (1995). How should P_{2X} purinoceptors be classified pharmacologically? *Trends Pharmacol. Sci.*, **16,** 168–174.

KEPPENS, S. (1993). The complex interaction of ATP and UTP with isolated hepatocytes. How many receptors? *Gen. Pharmacol.*, **24,** 283–289.

LAZAROWSKI, E.R. & HARDEN, T.K. (1994). Identification of a uridine nucleotide-selective G-protein-linked receptor that activates phospholipase C. *J. Biol. Chem.*, **269,** 11830–11836.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90,** 5113–5117.

MANNIX, R.J., MOATTER, T., KELLEY, K.A. & GERRITSEN, M.E. (1993). Cellular signaling responses mediated by a novel nucleotide receptor in rabbit microvessel endothelium. *Am. J. Physiol.*, **265,** H675–H680.

MOTTE, S., PIROTTON, S. & BOEYNAEMS, J.M. (1993). Heterogeneity of ATP receptors in aortic endothelial cells. Involvement of P_{2Y} and P_{2U} receptors in inositol phosphate response. *Circ. Res.*, **72,** 504–510.

NEES, S., GERBES, A.L. & GERLACH, E. (1981). Isolation, identification, and continuous culture of coronary endothelial cells from guinea pig heart. *Eur. J. Cell. Biol.* **24,** 289–297.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12,** 137–141.

PURKISS, J.R., WEST, D., WILKES, L.C., SCOTT, C., YARROW, P., WILKINSON, G.F. & BOARDER, M.R. (1994). Stimulation of phospholipase C in cultured microvascular endothelial cells from human frontal lobe by histimine, endothelin and purinoceptor agonists. *Br. J. Pharmacol.*, **111,** 1041–1046.

RALEVIC, V. & BURNSTOCK, G. (1991). Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Circ. Res.*, **69,** 1583–1590.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. *Trends Pharmacol. Sci.*, **10,** 365–369.

SIPMA, H., DEN HERTOG, A. & NELEMANS, A. (1994). The phospholipase C activating P2U purinoceptor also inhibits cyclic AMP formation in DDT1 MF-2 smooth muscle cells. *Eur. J. Pharmacol. Mol. Pharmacol.*, **268,** 431–437.

TRACEY, W.R. & PEACH, M.J. (1992). Differential muscarinic receptor mRNA expression by freshly isolated and cultured bovine aortic endothelial cells. *Circ. Res.*, **70,** 234–240.

URQUILLA, P.R. (1978). Prolonged contraction of isolated human and canine cerebral arteries induced by uridine 5'-triphosphate. *Stroke*, **9,** 133–136.

URQUILLA, P.R., WESTFALL, D.P., GOTO, K. & FLEMING, W.W. (1978). The effects of ouabain and alterations in potassium concentration on the sensitivity to drugs and the membrane potential of the smooth muscle of the guinea-pig and rat vas deferens. *J. Pharmacol. Exp. Ther.*, **207,** 347–355.

VIALS, A.J. & BURNSTOCK, G. (1993). Effects of pyrimidines on the guinea-pig coronary vasculature. *Br. J. Pharmacol.*, **110,** 1091–1097.

VON KÜGELGEN, I., BÜLTMANN, R. & STARKE, K. (1990). Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. *Naunyn-Schmied Arch. Pharmacol.*, **342,** 198–205.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1993). The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P2y-purinoceptors and nucleotide receptors linked to phospholipase C. *Br. J. Pharmacol.*, **108,** 689–693.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1994). Differential heterologous and homologous desensitization of two receptors for ATP (P_{2y} purinoceptors and nucleotide receptors) coexisting on endothelial cells. *Mol. Pharmacol.*, **45,** 731–736.

YANG, S., CHEEK, D.J., WESTFALL, D.P. & BUXTON, I.L.O. (1994). Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ. Res.*, **74,** 401–407.

(Received July 24, 1995
Revised November 20, 1995
Accepted December 8, 1995)