Differential regulation of inositol 1,4,5-trisphosphate by co-existing P_{2Y} -purinoceptors and nucleotide receptors on bovine aortic endothelial cells

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- 1 We have examined the inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) responses in bovine aortic endothelial (BAE) cells to purines (ATP, ADP and analogues) and the pyrimidine, uridine triphosphate (UTP).
- 2 Exchange of medium on BAE cells in the absence of agonist was found to be a stimulus for $\text{Ins}(1,4,5)\text{P}_3$ generation. BAE cells stimulated with $100\text{ }\mu\text{M}$ ATP, $30\text{ }\mu\text{M}$ 2MeSATP (an agonist at P_{2Y} -purinoceptors but not nucleotide receptors) or $100\text{ }\mu\text{M}$ UTP (an agonist at nucleotide receptors but not P_{2Y} -purinoceptors) gave $\text{Ins}(1,4,5)\text{P}_3$ responses above that caused by exchange of medium. The time course was rapid, with peak response within the first 5 s and levels returning close to basal after 30 s of stimulation.
- 3 Significant differences in $\text{Ins}(1,4,5)\text{P}_3$ responses to $100\text{ }\mu\text{M}$ UTP and $30\text{ }\mu\text{M}$ 2MeSATP stimulation were observed. The response to UTP was reproducibly more sustained than that to 2MeSATP.
- 4 Stimulation of BAE cells with $100\text{ }\mu\text{M}$ UTP plus $30\text{ }\mu\text{M}$ 2MeSATP produced a response statistically indistinguishable from that predicted by addition of the responses to the two agonists in isolation.
- 5 The $\text{Ins}(1,4,5)\text{P}_3$ response to UTP was attenuated to 25% of control by pretreatment of BAE cells with pertussis toxin. Responses to 2MeSATP and ADP were essentially unaffected. ATP stimulation was reduced to 65% of control.
- 6 Activation of protein kinase C with tetradecanoyl phorbol acetate (TPA) profoundly inhibited $\text{Ins}(1,4,5)\text{P}_3$ responses to 2MeSATP and ADP but had no effect on UTP stimulation. The protein kinase C inhibitor, Ro 31-8220, enhanced responses to 2MeSATP, ADP and ATP but no effect was observed on UTP stimulation.
- 7 These observations show that nucleotide and P_{2Y} -receptors mobilise the second messenger $\text{Ins}(1,4,5)\text{P}_3$ by separate routes resulting in different patterns of generation and suggest that while ATP activates both receptors, ADP principally influences these cells by interacting with the P_{2Y} -purinoceptors.

Keywords: Purinoceptors; endothelial cells; nucleotide receptors; phospholipase C; inositol phosphates; UTP receptors

Introduction

Regulation of free intracellular Ca^{2+} in vascular endothelial cells contributes to the control of several aspects of local vascular function, such as vascular permeability, smooth muscle contraction and platelet aggregation. One of the mechanisms controlling Ca^{2+} is the generation of inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) by receptors which are linked to hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC). $\text{Ins}(1,4,5)\text{P}_3$, the primary hydrophilic product of this activity, mobilizes intracellular Ca^{2+} stores (see Berridge, 1993, for review); in addition it is the precursor to $\text{Ins}(1,3,4,5)\text{P}_4$, which in endothelial cells is reported to play a role in entry of extracellular Ca^{2+} (Luckhoff & Clapham, 1992). It is well established that ATP and ADP act on cell surface receptors to regulate vascular endothelial cells by this PLC-linked mechanism (Boynaems & Pearson, 1990). Following the proposals of Burnstock & Kennedy (1985) it is also widely accepted that this is by action at P_{2Y} -purinoceptors. The principal evidence for this is the potency of the P_{2Y} -selective agonist 2-methylthioadenosine 5'-triphosphate (2MeSATP) and the lack of effect of β,γ -methylene ATP (a P_{2X} -purinoceptor agonist) on aortic endothelial cells (Martin *et al.*, 1985; Houston *et al.*, 1987; Piroton *et al.*, 1987; Allsup & Boarder, 1990).

Recently it has become apparent that the situation is more complex, with the recognition that the pyrimidine nucleotide, UTP can also elicit a PLC response from these endothelial cells. Work of ours and others has established that there are

two populations of co-existing receptors for ATP on these cells, the P_{2Y} -purinoceptors and nucleotide receptors. The P_{2Y} -purinoceptors respond to ATP and 2MeSATP but not to UTP, while the nucleotide receptors respond to both ATP and UTP but not to 2MeSATP (Wilkinson & Boarder, 1992; Wilkinson *et al.*, 1993; Motte *et al.*, 1993). ADP β S has been shown to be an agonist at P_{2Y} -purinoceptors but not nucleotide receptors and β,γ -methylene ATP acts at neither (Wilkinson *et al.*, 1993).

To pursue the significance of regulation of responses to ATP and ADP by two receptor populations, we have investigated the accumulation of $\text{Ins}(1,4,5)\text{P}_3$. Here we describe for the first time the mass measurement of $\text{Ins}(1,4,5)\text{P}_3$ in endothelial cells in response to purinoceptor activation and provide evidence that this primary second messenger is differentially regulated by P_{2Y} -purinoceptors and nucleotide receptors co-existing on bovine cultured aortic endothelial cells.

Methods

Cell culture

BAE cells were prepared from fresh aortae by digestion with 2 to 5 mg ml^{-1} collagenase (Booyse *et al.*, 1975) and cultured in Minimum Essential Medium D-valine with 10% foetal calf serum, 10% new-born calf serum, 25 iu ml^{-1} penicillin, 25 $\mu\text{g ml}^{-1}$ streptomycin, 10 mg ml^{-1} gentamycin, 250 $\mu\text{g ml}^{-1}$ fungizone and 27 mg ml^{-1} glutamine in 95:5% air:CO₂ at 37°C. Cells formed a confluent monolayer of homogeneous cells

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positive for Von Willebrand factor immunofluorescence and were used up to passage nine in 24 well plates.

Measurement of $\text{Ins}(1,4,5)\text{P}_3$

Cells cultured in 24 well multiwells were gently washed twice with balanced salt solution (BSS) with the following composition (mM): NaCl 125, KCl 5.4, NaHCO_3 16.2, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid 15, NaH_2PO_4 1, MgSO_4 0.8, CaCl_2 1.8, glucose 5.5 and gassed with 95% O_2 , 5% CO_2 . Agonists and drugs were all made up in this solution. Preincubations when included were for 5 min (at 37°C) with tetradecanoyl phorbol acetate (TPA), Ro 31 8220 or control balanced salt solution. The preincubation was in place of the second wash. When present during the preincubation, TPA and Ro 31 8220 were included in the 0.2 ml incubation, which was for 5 s (except where indicated) in the presence of the appropriate purine or pyrimidine. Where there was no preincubation the final wash was left for 5 min before removal and addition of incubate. The incubation was stopped by addition of 0.1 ml of cold (4°C) trichloroacetic acid (1.5 M). The acid extract was washed with 3×2 ml of H_2O saturated diethyl ether, buffered to pH 7 with NaHCO_3 and assayed for mass of $\text{Ins}(1,4,5)\text{P}_3$ by the procedure of Challiss *et al.* (1991). Results are given as mean $\pm$ s.e.mean.

Materials

$[\text{H}^3]\text{-Ins}(1,4,5)\text{P}_3$ for the mass assay was purchased from NEN Du Pont (UK) Ltd., Stevenage, Herts. 2MeSATP was from Research Biochemicals, Semat, St. Albans, Herts. Other chemicals were from Sigma, Poole, Dorset or Fisons, Loughborough, Leics. Ro 31 8220 (a bis-indolymaleimide derivative, compound 3 in Davis *et al.*, 1989) was a kind gift from Dr G. Lawton, Roche Products Ltd., Welwyn Garden City, Herts.

Results

Figure 1 shows results of experiments in which the concentration-effect relationship for various nucleotides were determined, stimulating for 5 s and measuring accumulation of $\text{Ins}(1,4,5)\text{P}_3$. Data were pooled from three separate experiments, resulting in computer generated (Graph-Pad) EC_{50} values (in μM) as follows, in order of decreasing potency: (2MeSATP (1.11 ± 0.54); ADP βS (2.07 ± 0.56); ADP (3.17 ± 0.88); ATP γS (31.0 ± 12.3); ATP (36.3 ± 12.9); UTP (64.2 ± 6.84)).

Experiments were set up to examine the time course of the $\text{Ins}(1,4,5)\text{P}_3$ response to some of these nucleotide agonists. Preliminary experiments indicated that there was a rapid fluctuation in levels in control wells to which no agonist was added (Figure 2). In these experiments the culture medium was removed and the cells gently washed twice. After 5 min the final wash was removed and BSS with no agonist added for the times shown, followed by addition of TCA. The zero time point was determined by removal of wash and direct addition of 0.3 ml of 0.5 M TCA. Without drugs there was a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ levels at 5 and 10 s, followed by a gradual decline. This pattern was followed when 100 μM ATP was present but was enhanced approximately 2 fold (Figure 2a). In all the data reported in this paper a control (no agonist added) value was established in each experiment for each condition of stimulation and has been deducted from the level obtained on stimulation with agonist. Subtraction of the basal from the ATP stimulation illustrated in Figure 2a is shown in Figure 2b.

The time course of response to 30 μM 2MeSATP and 100 μM UTP is shown in Figure 3. We characteristically obtained a fast response to 2MeSATP, peaking within 5 s followed by a rapid decline to control levels by 20–30 s. Stimulation with UTP gave a reproducibly more sustained

response. In some experiments this was a slower response (maximal at 15 s), in others (Figure 3) it was rapid (maximal at 5 s) and in all cases sustained compared with the response to 2MeSATP. The statistically significant differences were not due to differential breakdown: both these agonists were susceptible to ectonucleotidase attack, but this was not significant within the time course of these experiments (determined by high performance liquid chromatography – data not shown).

Figure 4 shows that the response to a mixture of 2MeSATP (30 μM) and UTP (100 μM) was rapid and followed by a decline. This response to a maximally effective concentra-

Figure 1 Concentration-effect curves for inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) responses in bovine aortic endothelial (BAE) cells stimulated for 5 s with increasing concentrations of: (a) ATP ($\blacktriangle$) and ADP ($\triangle$), (b) 2MeSATP ($\bullet$) and ADP βS ($\circ$) and (c) ATP γS ($\blacksquare$) and UTP ($\square$). Stimulations of $\text{Ins}(1,4,5)\text{P}_3$ are expressed as fold increases over basal $\pm$ s.e.mean. Data are pooled from 3 separate experiments, each carried out in triplicate. Typical values were basal, 11.06 ± 0.49 ; 300 μM ATP, 32.13 ± 0.30 ; 100 μM ADP, 29.11 ± 0.81 ; 30 μM 2MeSATP, 30.74 ± 1.00 ; 30 μM ADP βS , 29.28 ; 300 μM ATP γS , 40.84 ± 1.85 and 1 mM UTP, 38.41 ± 0.84 pmol $\text{Ins}(1,4,5)\text{P}_3$ per well.

tion of 2MeSATP and submaximal UTP was additive and was statistically indistinguishable from that predicted by addition of the two individual responses (Figure 3).

Experiments were undertaken to determine whether the

Figure 2 Time courses of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) generation in bovine aortic endothelial (BAE) cells. (a) Responses to BSS alone (○) and 100 μM ATP (●). (b) Response to 100 μM ATP with BSS response (basal) subtracted. Data expressed as pmol Ins(1,4,5)P₃ per well ± s.e.mean, *n* = 6 pooled from two separate experiments.

Figure 3 Time courses of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) generation in bovine aortic endothelial (BAE) cells in response to 100 μM UTP (○) and 30 μM 2MeSATP (●) with basal response subtracted. Data expressed as pmol Ins(1,4,5)P₃ per well ± s.e.mean, *n* = 6 pooled from two separate experiments. Significant differences in the response to the two agonists indicated by ***P < 0.001 or *P < 0.01.

responses to the agonists differed in the involvement of a pertussis toxin sensitive G-protein. The data in Table 1 show that the responses to 2MeSATP were unaffected by pertussis toxin at 5, 15 and 30 s of stimulation. The response to UTP was significantly reduced by toxin pretreatment at each of three times. The inhibition of the ATP response by pertussis toxin varied somewhat between experiments, but assumed in general, an intermediate position between UTP and 2MeSATP (e.g. Table 1). Over three separate experiments (each in triplicate) the responses to 5 s stimulation after pertussis toxin treatment as a percentage of control stimulations were: ATP (64.5 ± 5.0%); 2MeSATP (90.2 ± 9.4%); UTP (19.5 ± 2.1%). Further experiments with ADP at 5 s stimulation showed no effect of pertussis toxin (102 ± 18.7%) (*n* = 4). After pertussis toxin the basal (no agonist) response fell to 48.6 ± 9.7%.

It is a common observation that activation of protein kinase C with TPA can attenuate agonist-stimulated PLC responses. In some cases this has been related to a short feedback loop: agonist stimulation of PLC leads to protein kinase C activation which attenuates the PLC response. In this case downregulation or inhibition of protein kinase C augments the response to the agonist. The extent to which this occurs is very variable (see for example Helper *et al.*, 1988; Pfeilschifter *et al.*, 1989; Boarder & Challiss, 1992; Challiss *et al.*, 1993). Here we looked at the effect of protein

Figure 4 Time course of inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) generation in bovine aortic endothelial (BAE) cells in responses to 100 μM UTP plus 30 μM 2MeSATP (○, solid line) and the response predicted for the two agonists acting additively (●, dotted line). The predicted response is the addition of response from paired wells into which either 30 μM 2MeSATP or 100 μM UTP were added. Basal response (no agonist) was subtracted in each case. No significant difference was seen between observed and predicted values. Data expressed as pmol Ins(1,4,5)P₃ per well ± s.e.mean, *n* = 6 pooled from two separate experiments.

Table 1 The effect of pertussis toxin treatment on the inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) responses

Time	Treatment	ATP	2MeSATP	UTP
5 s	Control	15.36 ± 4.26	10.18 ± 0.70	9.73 ± 0.10
	PTX	8.37 ± 1.50*	9.34 ± 0.84	2.28 ± 1.34*
15 s	Control	6.84 ± 3.07	4.99 ± 1.55	8.7 ± 1.10
	PTX	3.56 ± 1.89	5.87 ± 1.01	4.15 ± 1.35*
30 s	Control	3.97 ± 1.72	4.74 ± 1.88	6.03 ± 2.26
	PTX	1.35 ± 0.09*	5.16 ± 0.78	0.08 ± 0.07*

Stimulation with ATP (300 µM), 2MeSATP (30 µM) and UTP (300 µM) were for the times indicated. Basal values were determined for each condition and have been deducted. Data are pmol per well of Ins(1,4,5)P₃, mean ± s.e.mean, *n* = 3, from a single experiment in each case (see text for data pooled across experiments). PTX = pertussis toxin treated (100 ng ml⁻¹ for 16 h). The asterisks denote difference from control (no PTX) values was significant at the *P* < 0.05 level or greater (Student's *t* test).

Table 2 The effect of tetradecanoyl phorbol acetate (TPA) on the inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) responses

Agonist	Control stimulation (pmol/well)	Stimulation + TPA (pmol/well)	<i>P</i> value
ATP (300 µM)	20.20 ± 1.62	12.47 ± 1.8	< 0.05
UTP (300 µM)	9.69 ± 2.37	14.87 ± 1.46	NS
2MeSATP (30 µM)	13.79 ± 1.68	4.86 ± 0.55	< 0.01
ADP (100 µM)	9.09 ± 0.34	- 0.66 ± 1.01	0.001

Stimulation was for 5 s. Unstimulated values (control = 6.29 ± 1.61, TPA = 3.36 ± 1.39) have been deducted. TPA was at 100 nM. Data (pmol per well of Ins(1,4,5)P) are mean ± s.e.mean (*n* = 3) from a single representative experiment (see text for data pooled across experiments). The *P* values are by Student's *t* test, NS = non significant.

kinase C stimulation with TPA, and inhibition with Ro 31 8220 on the stimulation of Ins(1,4,5)P₃ levels by purinoceptor agonists and UTP. There was a significant attenuation of response to the purinoceptor agonists by TPA: the response to ADP and to 2MeSATP was substantially attenuated while the response to ATP was down to 61% of control stimulation (Table 2). By contrast the UTP response was not inhibited by TPA. Over three such experiments (each in triplicate) the percentage of original response left by TPA was: ADP (12 ± 4.1%); 2MeSATP (41 ± 15%); ATP (68 ± 4%); UTP (123 ± 27%) (*n* = 3). TPA treatment reduced the control (no agonist) response to 51.4 ± 10.4% (*n* = 4).

The effect of the protein kinase C inhibitor Ro 31 8220 on Ins(1,4,5)P₃ responses was largely the inverse of the TPA effect: responses to 2MeSATP, ADP and ATP were increased by the protein kinase C inhibitor. For example, the response to ATP was increased by 10 µM Ro 31 8220 from 24.1 ± 4.4 to 48.6 ± 4.0; to 2MeSATP from 21.9 ± 3.8 to 46.7 ± 6.3 and to ADP from 22.4 ± 3.0 to 35.9 ± 2.7 (data are pmol/well, *n* = 3). However, there was no effect on the UTP response (112 ± 15% of response with no Ro 31 8220, *n* = 5 separate experiments). Ro 31 8220 raised the control (no agonist) response to 132 ± 12% (*n* = 6 separate experiments).

Discussion

In the absence of selective antagonists or cloning studies, the P₂-purinoceptors are classified on the basis of rank order of agonist potency. While this can yield only working hypotheses for classification, the significance of this approach can be seen in the value which has been attached to the subdivision into P_{2X} and P_{2Y} subtypes, based principally on the relative potencies of 2MeSATP and β,γ-methylene ATP (Burnstock & Kennedy, 1985). A number of receptors responding to ATP and ADP have been described which do not fall into this classification: recently interest has focussed on nucleotide receptors, which respond to both the purine ATP (or its stable analogue ATPγS) and the pyrimidine UTP, but do not respond to either 2MeSATP or β,γ-methylene ATP.

This pattern has been described in a variety of cell types (e.g. Demolle *et al.*, 1988; Fine *et al.*, 1989; Pfeilschifter,

1990; Davidson *et al.*, 1990; Brown *et al.*, 1991; Murrin & Boarder, 1992). In vascular endothelial cells, nucleotide receptors are known to be present on endothelial cells from aorta (Wilkinson & Boarder, 1992; Wilkinson *et al.*, 1993; Motte *et al.*, 1993) and from microvasculature of the adrenal medulla (Purkiss *et al.*, 1993). While in adrenal medulla cells nucleotide receptors are the sole PLC linked receptors for ATP (Allsup & Boarder, 1990; Purkiss *et al.*, 1993), in BAE cells we have shown that ATP regulates vascular function by interacting with two co-existing receptor populations, P_{2Y}-purinoceptors and nucleotide receptors, linked to both PLC (Wilkinson *et al.*, 1993) and phospholipase D (Purkiss & Boarder, 1992). Using precontracted rings of bovine aortic collateral artery (vessels supplying the intercostal muscles), we have shown that both receptors mediate relaxant responses to ATP, but with differential sensitivity to indomethacin: the response to P_{2Y} stimulation (2MeSATP) was indomethacin-sensitive while the response to nucleotide stimulation (UTP) was not (Wilkinson *et al.*, 1994). This shows that the two receptors generate differential release of relaxant factors. Since they co-exist on the same cells and both are PLC-linked, this suggests that the pattern of second messenger generation must differ according to which receptor population is activated. Here we have initiated the study of this difference by investigating the generation of Ins(1,4,5)P₃, the primary product of phosphoinositide(4,5)bisphosphate hydrolysis by PLC.

The principal observations to emerge from this study are: (i) there was differential sensitivity to pertussis toxin depending on whether P_{2Y} or nucleotide receptors were stimulated; (ii) the pattern of Ins(1,4,5)P₃ accumulation was dependent on the type of receptor stimulated; (iii) there was differential regulation of this stimulation by protein kinase C.

The observations on pertussis toxin sensitivity unequivocally show that the P_{2Y} response is not mediated by pertussis toxin-sensitive G-proteins. We can therefore exclude a role for the G_i family of G-proteins, which includes G_i. It seems likely that a member of the G_q family is involved: G_{q/11} has been shown to stimulate phosphoinositide directed PLC activity (Smrcka *et al.*, 1991). The observations for stimulation of nucleotide receptors by UTP shows pertussis toxin-sensitivity, from which we can conclude that a member of the

G_i family is involved in linking the receptor to PLC. The two receptor populations acting on the same cell therefore differ in the G-proteins used to elicit the PLC response. With respect to the two principal endogenous agonists of these receptors (ATP and ADP), the data show an intermediate sensitivity of the ATP response to pertussis toxin, consistent with this being a natural agonist for both receptors. However, ADP was unaffected by pertussis toxin, suggesting that the influence of ADP was principally via the P_{2Y}-purinoceptors.

With respect to the different time course of Ins(1,4,5)P₃ accumulation, it is of importance to consider how this comes about if we are to understand how activation of the two PLC linked receptor populations can effect different physiological responses. Three possibilities will be introduced: (1) Activation of P_{2Y}-purinoceptors may result in more rapid desensitization than activation of nucleotide receptors. Rapid desensitization (i.e. within a few seconds) has been described for PLC linked receptors (Tobin *et al.*, 1993) and so may be involved.

(2) The rate at which the G-protein activation of PLC is terminated by GTPase activity may vary. When an effector such as PLC is activated by a G-protein linked receptor, the activation is terminated by the intrinsic GTPase activity of the α -subunit converting PLC* $\cdot\alpha_{GTP}$ to PLC and α_{GDP} , where PLC* is active and PLC is inactive. The hydrolysis rate for GTP bound to purified α -subunits is slow, e.g. 50 s for G_{q/11} (Berstein *et al.*, 1992a) and may therefore play a role in the events shown in Figure 3. This rate varies according to the specific G-proteins involved, but it is also regulated by the GTPase activating characteristics of PLC (Berstein *et al.*, 1992b). The involvement of G-proteins with different intrinsic GTPase activity, or of PLC species with differing GTPase activating characteristics, could therefore generate within the same cells the differential kinetics seen in Figure 3.

(3) Activation of P_{2Y}-purinoceptors may generate conditions in which breakdown of Ins(1,4,5)P₃ is faster than that which occurs following nucleotide receptor activation. For example it has been shown that elevation of Ca²⁺ may cause enhanced 3-kinase activity, leading to increased metabolism of Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄ (Shears, 1991). Both the 3-kinase and 5-phosphatase pathways for Ins(1,4,5)P₃ metabolism are subject to regulation by protein kinase C (King & Rittenhouse, 1989; Sim *et al.*, 1990). Therefore if there is differential control of Ca²⁺ or protein kinase C by P_{2Y}-purinoceptors or nucleotide receptors it may result in differential rates of Ins(1,4,5)P₃ metabolism.

The final observations from this paper which require discussion are those involving protein kinase C. It is apparent that the nucleotide response is unusual amongst receptor stimulation of G-protein-regulated PLC in that TPA does

not attenuate the agonist-stimulated PLC activity. The P_{2Y}-purinoceptor stimulation is much more typical in that activation of protein kinase C by TPA severely attenuated the response. The consequences of protein kinase C inhibition on agonist-stimulated PLC described in the literature show considerable variation, with instances of major enhancement (e.g. Challiss *et al.*, 1993) indicating an active short inhibitory feedback loop, alongside reports in which only a small effect is seen (e.g. Boarder & Challiss, 1992). The outcome of the present paper is that the P_{2Y}-purinoceptor agonists activate this short protein kinase C feedback loop while the nucleotide agonist, UTP, does not. The observation that TPA does not attenuate the UTP response suggests that this is not because UTP fails to activate protein kinase C. Possibly the reason for the differential sensitivity to protein kinase C activation lies in the involvement of different G-proteins.

Of the principal endogenous agonists, the responses to ATP and ADP were both decreased by TPA and enhanced by Ro 31 8220. The effect of TPA on ADP was more profound than the effect on ATP. These results are consistent with ATP acting at both receptors (as suggested by our earlier work), but also with ADP acting principally at the P_{2Y}-purinoceptor and not at the nucleotide receptor. The notion that ADP, unlike ATP, is not an effective agonist at nucleotide receptors is supported by other evidence, including the pertussis toxin data discussed above, our work on relaxant responses of aortic collateral artery rings (Wilkinson *et al.*, 1994) and a recent publication describing a cloned receptor with the characterization of a nucleotide receptor (Lustig *et al.*, 1993).

In conclusion, the data presented here show that there are two separate routes for activation of PLC by co-existing nucleotide receptors and P_{2Y}-purinoceptors on BAE cells, leading to a different pattern of second messenger generation. This may involve modulation of separate populations or subtypes of PLC, but the interpretation discussed here suggests that the differential responses could arise from different receptors acting through different G-proteins on a common pool of PLC activity. The different pattern of second messenger generation which we have described here may provide an explanation for the differential physiological responses elicited by two receptors for ATP co-existing on the same cells. They may also contribute to understanding how ATP and ADP may differentially regulate vascular endothelial cells.

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