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P_2 Purinoceptor-Stimulated Conversion of Arginine to Citrulline in Bovine Endothelial Cells is Reduced by Inhibition of Protein Kinase C

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ABSTRACT. Bovine aortic endothelial cells contain two coexisting receptors for extracellular ATP, named the P_{2Y} and P_{2U} purinoceptors. Previous studies have shown that these receptors are linked to phospholipase C in a manner that is modulated in part by protein kinase C (PKC). In this study, we investigate the influence of PKC in the regulation of endothelial nitric oxide synthase (NOS) by these two purinoceptors. Activation of either P_{2Y} or P_{2U} purinoceptors by either 2-methylthio-ATP or UTP, respectively, stimulated the formation of [^{3}H]-citrulline in [^{3}H]-arginine-labelled cells in a concentration-dependent manner. This stimulation was sensitive to inhibition by N^G-nitro-L-arginine. Ten minutes of pretreatment with the PKC activator tetradecanoyl phorbol acetate (TPA) failed to affect NOS activity, either alone or when stimulated with 2-methylthio-ATP or UTP. However, under these conditions TPA caused almost complete translocation of PKC-α from the cytosol to the membrane. Ten minutes of pretreatment with the PKC inhibitor Ro 31-8220 significantly inhibited the agonist-induced stimulation of NOS. These results show that both P_{2Y} and P_{2U} purinoceptors stimulate endothelial NOS in a manner that is dependent on PKC activity. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;12:1849–1854, 1996.

KEY WORDS. P_2 purinoceptors; endothelial; nitric oxide synthase; protein kinase C; phorbol ester; N^G-nitro-L-arginine methyl ester

Genetic analysis has revealed the presence of three types NOS‡; these can be classified as the neuronal Ca^{2+}/calmodulin-dependent form (ncNOS or type I), the Ca^{2+}-independent inducible form (iNOS or type II) and the endothelial Ca^{2+}/calmodulin-dependent form (ecNOS or type III), as discussed recently by Nathan and Xie [1]. Endothelial Ca^{2+}-dependent NOS activity is present in both membrane-bound and cytosolic states in BAE cells but predominantly in particulate form [2]. Endothelial nitric oxide release is elevated in response to different agonists acting on G-protein-coupled receptors, including the P_2 purinoceptors, which respond to ATP and related agonists in a manner implicating a rise in intracellular Ca^{2+} [3–7]. We have recently shown that the P_2-purinergic response of bovine aortic endothelial cells is mediated by two coexisting receptors, P_{2Y} and P_{2U} purinoceptors, which are both differentially coupled to phospholipase C activation and vasorelaxation [8–10]. Of these two receptors, the response to stimulation of the P_{2Y} purinoceptor (but not the P_{2U} purinoceptor) is regulated by PKC activation and inhibition, such that activation of PKC inhibits agonist induced stimulation of $Ins(1,4,5)P_3$, and inhibition of PKC leads to an enhancement of agonist induced stimulation of $Ins(1,4,5)P_3$ in a manner consistent with the involvement of a short PKC-dependent inhibitory feedback mechanism [8, 9].

Besides its role in the regulation of induction of iNOS (e.g. [11]), PKC has been reported to modulate endothelial NOS activity. Some reports indicate that this is an inhibitory effect within the endothelial cell, such that both basal and agonist stimulated NOS activity are attenuated by mechanisms that include direct phosphorylation of the enzyme [12–14]. Other results with different cells show that PKC may exert a positive influence on agonist-stimulated NOS activity [15, 16]. In this report, we present evidence that conflicts with this earlier work, indicating that PKC activity is necessary for P_{2Y} and P_{2U} purinoceptor-stimulated endothelial NOS activity and that PKC activation does not exert an inhibitory influence on this activity.

MATERIALS AND METHODS

Materials

[^{3}H]-Arginine, secondary horseradish peroxidase-labelled antibodies and reagents for enhanced chemiluminescence

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‡ *Abbreviations:* NOS, nitric oxide synthase; PKC, protein kinase C; TPA tetradecanoyl phorbol acetate; L-NAME, N^G-nitro-L-arginine methyl ester; BSS, balanced salt solution; ECL, enhanced chemiluminescence; BAE cells, bovine aortic endothelial cells; ECL, enhanced chemiluminescence; 2MeSATP, 2-methylthio-ATP; SDS-PAGE, sodium dodecylsulphate polyacrilamide gel electrophoresis.

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detection were obtained from Amersham International (Buckinghamshire, UK). Rabbit polyclonal antibodies raised against PKC-ζ were a kind gift from Dr. P. J. Parker (ICRF, London UK). Mouse monoclonal antibody against PKC-α was purchased from UBI (TCS, Buckingham, UK). Ro 31-8220 was a kind gift from Dr. G. Lawton (Roche Products Ltd, Welwyn, Herts, UK). Cell culture supplies were obtained from GIBCO Life Technologies (Paisley, Scotland), 2MeSATP was obtained from Research Biochemicals (Semat, Herts, UK), and other biochemicals were obtained from Sigma (Poole, Dorset, UK) or Fisons (Loughborough, UK).

Cell Culture

BAE cells were prepared by the method of Booyse *et al.* [17] and, as described in Wilkinson *et al.* [9], were cultured in Minimum Essential Medium D-valine with 10% foetal calf serum, 25 IU · mL^{-1} penicillin, 25 μg · mL^{-1} streptomycin, 10 mg · mg^{-1} gentamycin, and 27 mg · mL^{-1} glutamine in 95:5% air:CO_2 at 37°C. The cells were essentially 100% positive for factor VIII:R immunofluorescence and were used in 24-well plates.

NOS assay

NOS activity was measured by a modification of the method of Bredt and Snyder [18]. Cells maintained at 37°C were washed twice with 1 mL of BSS [NaCl 125 mM, KCl 5.4 mM, $NaHCO_3$ 16.2 mM, N-2-hyroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 15 mM, NaH_2PO_4 1 mM, $MgSO_4$ 0.8 mM, $CaCl_2$ 1.8 mM, glucose 5.5 mM, pH 7.4] and gassed with 95% O_2, 5% CO_2. Cells were incubated with 1.5–3.0 μCi · mL^{-1} of [^{3}H]L-arginine (57 Ci $mmol^{-1}$) in 200 μL of BSS. This labelling period formed a preincubation of 10 min, followed by an incubation that was started by the addition of agonists at 10 times final concentration. When included, activators or inhibitors of PKC were present 10 min prior to and during the agonist stimulation period. Incubations were stopped by the aspiration of the incubate, washing with 3 × 1 mL of ice cold BSS without Ca^{2+} and addition of 0.5 mL of ice-cold 0.5 M trichloroacetic acid. After 2 hr on ice, the extract was washed 3 times with 2 mL of water saturated diethylether, and 0.25 mL was taken and buffered with 0.75 mL 20 mM HEPES (pH 6.0). The extract was then applied to a 1-mL column of Dowex 50 Na^+ form, citrulline was eluted with 4.0 mL of water and arginine was eluted with 4 mL of 0.1 M NaOH. Recovery of radioactivity through the columns was over 95%, and when [^{3}H]-arginine was applied directly to the columns over 95% was recovered in the NaOH wash.

PKC Western Blotting

Cultured endothelial cells were washed and maintained in culture for another 24-hr period in serum-free medium. Cells were then incubated with or without phorbol ester for the time periods indicated in the caption to Fig. 4. To obtain cytosolic or membrane fractions, the cells were washed with ice-cold PBS and scraped into ice-cold homogenisation buffer containing 25 mM Tris, 2.5 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 25 μg · ml^{-1} leupeptin, 1 mM phenylsulphonylfluoride, after which the cells were lysed in a teflon pestle tissue grinder and centrifuged for 10 min at 14,000*g*. The supernatants were used as a source for the cytosolic fraction of PKC. The pellets were sonicated in homogenisation buffer containing Triton X-100 and centrifuged at 14,000*g* for 10 min. The PKC fractions were subject to SDS-PAGE [19]. Separated proteins were then transferred to nitrocellulose paper by using a Bio Rad (Hercules, CA) transblot apparatus. After transfer, the nitrocellulose membranes were treated overnight with 10% (w/v) nonfat dried milk, which was used as a blocking agent. Membranes were then incubated for 4 hr at room temp with antibodies raised against the different PKC isoforms and incubated for another hour with donkey horseradish peroxidase-conjugated second antibodies. Immunoreactive proteins were detected using the ECL detection system (Amersham).

Data Analysis and Presentation

Data are expressed as mean ± SEM for the indicated number of experiments, with significance determined by Student's *t* test.

RESULTS

Preliminary experiments (data not shown) established that cells incubated in the presence of [^{3}H]-arginine rapidly accumulated radioactivity, reaching a maximum within 10 min; the accumulation of [^{3}H]-arginine was not affected by the presence of the PKC stimulator TPA. The formation of citrulline from arginine has been stoichiometrically linked to the synthesis of NO and therefore provides an assay for intracellular NOS activity. Figure 1 shows the accumulation of [^{3}H]-citrulline in these cells in response to 2MeSATP and UTP, selective agonists for P_{2Y} and P_{2U} purinoceptors, respectively, under these conditions [9]. Both agonists stimulated the accumulation of [^{3}H]-citrulline. For the first 5 min of stimulation, the response to maximally effective concentrations of the two agonists was the same (Fig. 1A), but by 10 min UTP reliably gave a larger response than did 2MeSATP. Figure 1B shows the concentration response relationship for responses to the two agonists with 5-min stimulations. The response to 2MeSATP was located to the left of the response to UTP (pA_{50} values were 5.96 ± 0.22 and 4.89 ± 0.22, respectively; mean ± SEM, n = 5), and the maximal response to UTP was 99.9 ± 5.0% of that seen with 2MeSATP. Figure 2 shows the effect of increasing L-NAME concentrations on the separate stimulation of P_{2Y} and P_{2U} purinoceptors by 2MeSATP and UTP, respectively, showing that the response to each receptor was inhibited in a similar manner

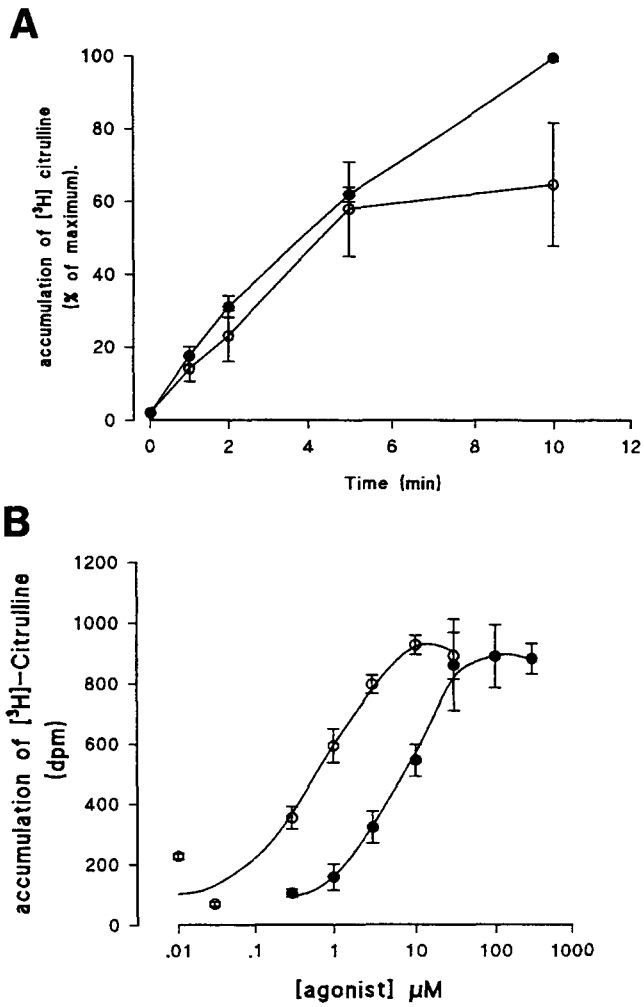

FIG. 1. (A) BAE cells preincubated with [^{3}H]-L-arginine (3 μCi/mL) for 10 min were stimulated with either 30 μM 2MeSATP (solid circle) or 300 μM UTP (open circle) for the times indicated. Data are mean ± SEM of three experiments performed in triplicate. (B) BAE cells preincubated with [^{3}H]-L-arginine as described in A were stimulated with either 2MeSATP (solid circle) or UTP (open circle) at the concentrations indicated for 5 min. Data are mean ± SEM of three experiments performed in triplicate.

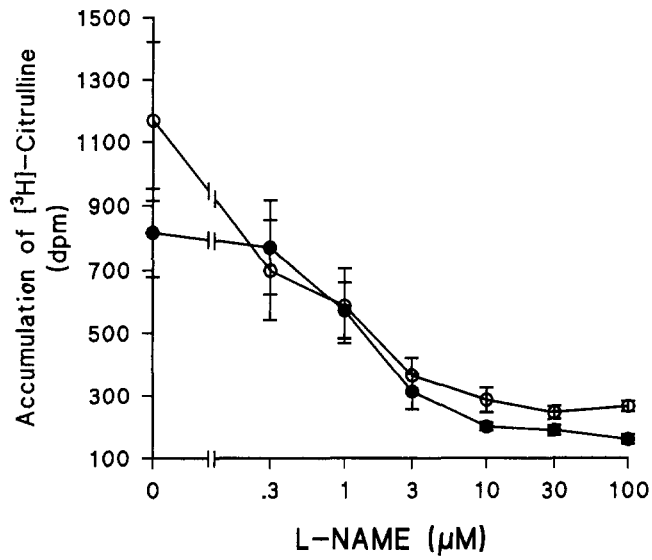

FIG. 2. Cells prelabelled with [^{3}H]-L-arginine were stimulated in the presence of either 30 μM 2MeSATP (solid circle) or 300 μM UTP (open circle) for 5 min in the presence of increasing concentrations of the NOS inhibitor L-NAME. Data are mean ± SEM of three experiments performed in triplicate.

and in a concentration range consistent with that reported by others for NOS inhibition.

To investigate the influence of PKC on these responses, we used PKC stimulation by TPA and the relatively selective PKC inhibitor Ro318220 [20]. In each experiment, there was a decrease in unstimulated NOS activity with 100 nM TPA to 47–84% of controls (68.5 ± 7.7%, mean ± SEM, n = 4), whereas Ro318220 had no affect on the unstimulated levels (83 ± 13%, mean ± SEM, n = 4). Figure 3A shows that TPA at 100 nM had no effect on the activity stimulated by maximally effective concentrations of 2MeSATP or UTP (n = 4). By contrast, inhibition of PKC with Ro318220 significantly attenuated agonist-stimulated NOS activity (*53.5 ± 4.7% and **50.8 ± 3.9% of the responses in the absence of Ro318220 for 2MeSATP and UTP, respectively; mean ± SEM, n = 4; *$p < 0.02$, **$p < 0.05$), with no effect on basal levels.

Figure 4 shows the effect of treatment with TPA for up to 10 min on the distribution of PKC-α and PKC-ζ immunoreactivity, as revealed by Western blots. A very substantial translocation of PKC-α occurs within 10 min of treatment with TPA, indicating that this isoform is activated under these conditions in these cells. However, there was no evidence of translocation of PKC-ζ in these experiments.

DISCUSSION

Endothelial synthesis and release of NO is one of several aspects of vascular endothelial cell function that are regulated by receptors for ATP and ADP (the P_2 purinoceptors), as summarised in a recent review [21]. In studies on bovine intercostal artery rings, we showed that endothelium-dependent vasorelaxation occurs when either P_{2Y} or P_{2U} purinoceptors were stimulated. The relaxation in response to P_{2Y}-purinoceptor stimulation was completely blocked by indomethacin, whereas the response to P_{2U}-purinoceptor activation was unaffected by this cyclooxygenase inhibitor [10]. This result suggests that the two receptors produce relaxation by release of different mediators and that the P_{2Y}-purinoceptor relaxation response was not predominantly due to release of NO. The data reported here, however, shows that both these receptors are linked to NOS stimulation in BAE cells in culture. The apparent discrepancy between this observation and the differential release of NO from intercostal endothelial cells *in situ* may be due to the different origin of the cells or to changes occurring in culture. However, aortic endothelial cells in

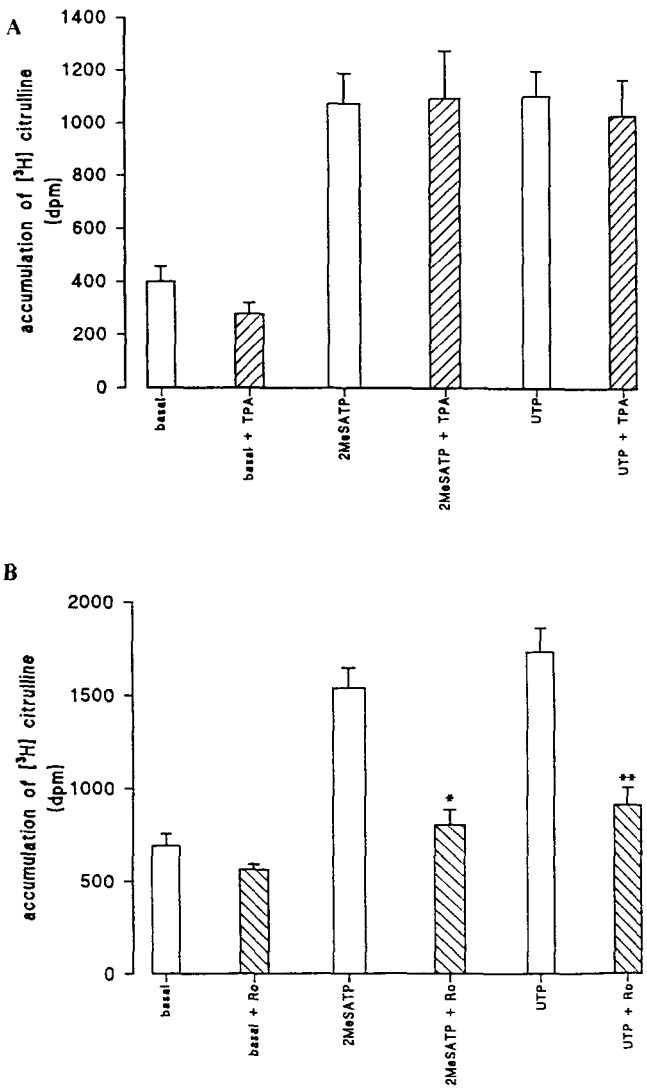

FIG. 3. (A) Cells prelabelled with [^{3}H]-L-arginine were stimulated with either 30 μM 2MeSATP or 300 μM UTP as indicated in the absence (open bar) or presence (hatched bar) of the PKC activator TPA (100 nM) for 5 min. Data are mean ± SEM of four experiments performed in triplicate. (B) BAE cells were stimulated as described in A except in the presence of the pKC inhibitor Ro318220 (10 μM). Data are mean ± SEM of four experiments performed in triplicate (*$p < 0.02$; **$p < 0.05$ significantly different from stimulation with agonist alone).

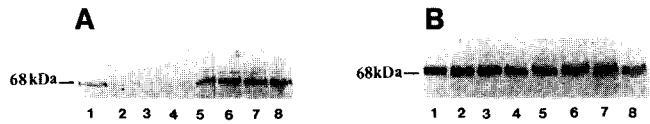

FIG. 4. Translocation of PKC isoforms α and ζ in response to TPA. Cells were exposed to 1 μM TPA for 0, 1, 5 or 10 min, and membrane and cytosolic fractions were separated and analysed by Western blot using antisera specific for PKC-α (A) or PKC-ζ (B). In A and B lanes 1–4 contain cytosolic PKC and lanes 5–8 contain membrane. Lanes 1 and 5 contain control without TPA treatment. The remaining lanes were exposed to TPA for 1 min (2 and 6), 5 min (3 and 7) and 10 min (4 and 8).

culture do maintain differential responses to the P_{2Y} and P_{2U} purinoceptors with respect to second messenger pathways [8].

2MeSATP stimulated NOS with a much higher potency than did UTP, which is similar to the stimulation of $Ins(1,4,5)P_3$ by these two agonists and is consistent with the notion that stimulation of NOS is downstream of stimulation of hydrolysis of inositol phospholipids by phospholipase C. Although a number of pathways may lead from phospholipid hydrolysis to NOS stimulation, initial considerations must concentrate on the elevation of intracellular Ca^{2+} and the stimulation of PKC. Endothelial cells contain both soluble and membrane-bound forms of Ca^{2+}/calmodulin-sensitive NOS [2], and evidence has been presented to suggest that an increase in Ca^{2+} downstream of phospholipase C stimulation provides the signal transduction to NOS [2, 5].

The present study used PKC activation and inhibition protocols, which have been well characterised in these cells, with an inhibitor that is both effective and relatively selective under the conditions used here [8, 19, 20, 22, 23]. We show that activation of PKC with TPA does not attenuate P_{2Y}- or P_{2U}-purinoceptor stimulation of BAE cells but that inhibition of PKC does remove most of this stimulated NOS. Taken together with our earlier work showing an inhibitory effect of activation of PKC on the P_{2Y}-mediated accumulation of $Ins(1,4,5)P_3$ [8], these results show that stimulation of NOS activity by 2MeSATP occurs under conditions where TPA profoundly reduces the accumulation of $Ins(1,4,5)P_3$. These results indicate an apparent dissociation between the accumulation of Ca^{2+} mobilising $Ins(1,4,5)P_3$ and stimulation of NOS. One possible explanation is the sensitization of NOS to Ca^{2+} mediated by PKC, which has previously been reported for NOS in cerebellar slices [15], leading to an increase in NOS activity despite the reduction in the Ca^{2+} response. However, this possibility is unlikely to be the case in this study because the effect of activation or inhibition of PKC is the same for both 2MeSATP and UTP, despite the differential effect of PKC on the $Ins(1,4,5)P_3$ response mediated by these receptors. These observations suggest that the P_2 purinoceptor control of NOS is not solely determined by the level of Ca^{2+} mobilising $Ins(1,4,5)P_3$, but that there is also a requirement for PKC in this response, although activation of PKC alone is not in itself sufficient to stimulate NOS activity. Another possibility that must be considered is that in the presence of Ro318220 there is altered metabolism or transport of citrulline or arginine, which may give rise to the observed effects of this compound. However, these effects of PKC are similar to those reported for P_2-purinoceptor-mediated stimulation of PGI_2 [23], where agonist-induced accumulation is enhanced by activation of PKC and abolished by inhibition of PKC. Understanding

these events and the apparent discrepancy between these results and others in the literature will require elucidation of the role of individual PKC isoforms.

In a separate study, we have undertaken a substantial investigation into PKC isoforms in these cells [23] in which we report that, of eight isoforms tested, only PKC-α, PKC-ε and PKC-ζ are present. Elsewhere it has been shown that in addition PKC-β is detectable in BAE cells [12]. Our observation that PKC-β is undetectable in our cells raises the possibility that different cell cultures of BAE cells contain different PKC isoforms. Our results with translocation of PKC-α confirm that this isoform is rapidly activated by the TPA treatment used elsewhere in this report, whereas parallel studies failed to show evidence of translocation of PKC-ε or of PKC-ζ. The report of rapid translocation of PKC-α is important in the present context because it provides a direct demonstration that PKC activation is occurring in those conditions in which TPA does not inhibit agonist-stimulated NOS.

The role of PKC in the control of agonist-stimulated NOS has been investigated in previous reports, with conflicting conclusions. Agonist activation of NOS through a PKC-dependent mechanism has been reported in cultures of rat striatal neurons [16], and activation of NOS by metabotropic glutamate receptors in rat brain cerebellar slices has also been reported to be PKC dependent [15]. However, a number of previous studies have reported that that PKC inhibits endothelium-dependent relaxation of arterial vascular rings [24–27]. Subsequently, Hecker *et al.* [12] showed that TPA attenuates and PKC inhibition potentiates bradykinin-stimulated NO release from cultured porcine and bovine endothelial cells. Davda *et al.* [13] presented evidence that both direct inhibition of endothelial NOS and receptor uncoupling were involved in the inhibition of the ATP-stimulated response in bovine pulmonary artery cells. This result was further clarified by Hirata *et al.* [14] who showed that phosphorylation of NOS by PKC occurs in a reconstituted cell-free system and in BAE cells stimulated with TPA and that in these intact cells this phosphorylation corresponds to a reduction in the NOS response to P_2-purinoceptor stimulation. None of these studies unequivocally demonstrated that agonist-stimulated endothelial NOS is inhibited by phosphorylation by PKC because each study with agonists involved intact cells in which receptor decoupling was likely to be taking place. Experiments with ionomycin show a partial inhibition of stimulated NOS activity on exposure of cells to TPA; however, this ionomycin stimulation of NOS is much larger than the agonist-stimulated NOS, even after partial loss following PKC activation [13].

It is difficult to explain the difference between our results and some of the earlier reports on NOS in endothelial cells. However, it is instructive to note that there is a similar degree of inconsistency in reports of TPA effects on agonist-stimulated phospholipase A_2, with both stimulation and inhibition being reported in endothelial cells [12, 28].

References

1. Nathan C and Xie Q-W, Nitric oxide synthases: Roles, tolls and controls. *Cell* **78:** 915–918, 1994.
2. Hecker M, Mulsch A, Bassenge E, Forstermann U and Busse R, Subcellular localisation and characterization of nitric oxide synthase(s) in endothelial cells: Physiological implications. *Biochem J* **299:** 247–252, 1994.
3. Martin W, Cusack NJ, Carleton JS and Gordon JL, Specificity of P_2-purinoceptor that mediates endothelium dependent relaxation of the pig aorta. *Eur J Pharmacol* **108:** 295–299, 1985.
4. Needham L, Cusack NJ, Pearson JD and Gordon JL, Characteristics of the P_2 purinoceptor that mediates prostacyclin production by pig aorta endothelial cells. *Eur J Pharmacol* **134:** 199–209, 1987.
5. Luckhoff A, Pohl U, Mulsch A and Busse R, Differential role of extra- and intra-cellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *Br J Pharmacol* **95:** 189–196, 1988.
6. Mitchell JA, Hecker M and Vane JR, The generation of L-arginine in endothelial cells is linked to the release of endothelium-derived relaxing factor. *Eur J Pharmacol* **176:** 253–254, 1990.
7. Mitchell JA, De Nucci G, Warner TD and Vane JR, Different patterns of release of endothelium-derived relaxing factor and prostacyclin. *Br J Pharmacol* **105:** 485–489, 1992.
8. Purkiss JR, Wilkinson GF and Boarder MR, Differential regulation of inositol 1,4,5-trisphosphate by co-existing P_{2Y}-purinoceptors and nucleotide receptors on bovine aortic endothelial cells. *br J Pharmacol* **111:** 723–278, 1994.
9. Wilkinson GF, Purkiss JR and Boarder MR, The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2Y}-purinoceptors and nucleotide receptors linked to phospholipase C. *Br J Pharmacol* **108:** 689–693, 1993.
10. Wilkinson GF, McKechnie K, Dainty IA and Boarder MR, P_{2Y}-purinoceptor and nucleotide receptor-induced relaxation of precontracted bovine aortic collateral artery rings: Differential sensitivity to suramin and indomethacin. *J Pharmacol Exp Ther* **268:** 881–887, 1994.
11. Fujihara M, Connolly N, Ito N and Suzuki T, Properties of protein kinase C isoforms (βII, ε, and ζ) in a macrophage cell line (J774) and their roles in LPS-induced nitric oxide production. *J Immunol* **152:** 1898–1906, 1994.
12. Hecker M, Luckhoff L and Busse R, Modulation of endothelial autacoid release by protein kinase C: Feedback inhibition or non-specific attenuation of receptor dependent cell activation? *J Cell Physiol* **156:** 571–578, 1993.
13. Davda RK, Chandler LJ and Guzman NJ, Protein kinase C modulates receptor-independent activation of endothelial nitric oxide synthase. *Eur J Pharmacol* **266:** 237–244, 1994.
14. Hirata KI, Kuroda R, Sakoda T, Katayama M, Inoue N, Suematsu M, Kawashima S and Yokoyama M, Inhibition of endothelial nitric oxide synthase activity by protein kinase c. *Hypertension* **25:** 180–185, 1995.
15. Okada D, Protein kinase C modulates calcium sensitivity of nitric oxide synthase in cerebellar slices. *J Neurochem* **64:** 1298–1304, 1995.
16. Marin P, Lafon-Cazal M and Bockaert J, A nitric oxide synthase activity selectively stimulated by NMDA receptors depends on protein kinase C activation in mouse striatal neurons. *Eur J Neurosci* **4:** 425–432, 1992.
17. Booyse FM, Sedlack BJ and Rafelson ME, Culture of arterial endothelial cells: Characterization and growth of bovine aortic cells. *Thromb Diath Haemorrh* **34:** 825–839, 1975.
18. Bredt DS and Snyder SH, Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. *Proc Natl Acad Sci USA* **86:** 9030–9033, 1989.

19. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
20. Davis PD, Hill CH, Keech E, Lawton G, Sedgwick AD, Wadsworth J, Westmacott D and Wilkinson SE, Potent selective inhibitors of protein kinase C. *FEBS Lett* **259:** 61–63, 1989.
21. Boarder MR, Weisman GA, Turner JT and Wilkinson GF, G-protein coupled P_2 purinoceptors: From molecular biology to functional responses. *Trends Pharmacol Sci* **16:** 133–139, 1995.
22. Purkiss JR and Boarder MR, Stimulation of phosphatidate synthesis in endothelial cells in response to P_2-receptor stimulation. *Biochem J* **287:** 31–36, 1992.
23. Patel V, Brown CA and Boarder MR, Protein kinase C isoforms in bovine aortic endothelial cells: Role in regulation of P_{2Y} and P_{2U} purinoceptor stimulated prostacyclin release. *Br J Pharmacol* **118:** 123–130, 1996.
24. Weinheimer G, Wagner B and Osswald H, Interference of phorbol esters with endothelium dependent vascular smooth muscle relaxation. *Eur J Pharmacol* **130:** 319–326, 1986.
25. Rubanyi GM, Desiderio D, Luisi A, Johns A and Sybertz EJ, Phorbol dibutyrate inhibits release and action of endothelium-derived relaxing factor(s) in canine blood vessels. *J Pharmacol Exp Ther* **249:** 858–863, 1989.
26. Morrison KJ and Pollock D, Impairment of relaxations to acetylcholine and nitric oxide by phorbol ester in rat isolated aorta. *Br J Pharmacol* **101:** 432–437, 1990.
27. Smith JA and Lang D, Release of endothelium-derived relaxing factor from pig aortic endothelial cells as assessed by changes in endothelial cell cycle cyclic GMP content is inhibited by phorbol ester. *Br J Pharmacol* **99:** 565–571, 1990.
28. Carter TD, Hallam TJ and Pearson JD, Protein kinase C activation alters the sensitivity of agonist stimulated endothelial cell prostacyclin production to intracellular Ca^{2+}. *Biochem J* **262:** 431–437, 1989.