Protein kinase C activation alters the sensitivity of agonist-stimulated endothelial-cell prostacyclin production to intracellular Ca^{2+}

Thomas D. CARTER,* Trevor J. HALLAM† and Jeremy D. PEARSON*‡

* Section of Vascular Biology, MRC Clinical Research Centre, Harrow, Middx. HA1 3UJ, U.K., and † Department of Cellular Pharmacology, Smith, Kline & French Research Ltd., Welwyn, Herts. AL6 9AR, U.K.

Agonist-stimulated release of prostacyclin (PGI_2) from endothelial cells requires elevation of the concentration of intracellular ionized calcium ($[\text{Ca}^{2+}]_i$) above a threshold value, and raised $[\text{Ca}^{2+}]_i$ provides a sufficient transduction signal to account for the extent of PGI_2 production. However, chronic activation of protein kinase C has been reported separately to potentiate PGI_2 release, but to depress agonist-induced elevations of $[\text{Ca}^{2+}]_i$. We show here that pretreatment with phorbol 12-myristate 13-acetate (PMA) dose-dependently induces PGI_2 release over many minutes after a significant lag period without any change in $[\text{Ca}^{2+}]_i$. In addition, PMA potentiates the transient release of PGI_2 in response to agonists in a complex manner depending on the time of pre-incubation and the concentrations of both PMA and agonist. Concomitant measurement of $[\text{Ca}^{2+}]_i$ and PGI_2 release demonstrates that PMA pretreatment dose-dependently inhibits both the peak $[\text{Ca}^{2+}]_i$ transient and the subsequent steady-state elevation of $[\text{Ca}^{2+}]_i$ in response to agonists. Determination of the quantitative $[\text{Ca}^{2+}]_i/\text{PGI}_2$ dose/response relationship, when PGI_2 release is driven purely by elevating $[\text{Ca}^{2+}]_i$ with ionomycin, demonstrates that PMA also enhances the Ca^{2+} -sensitivity of PGI_2 release. The observed effects of PMA on PGI_2 release can be explained quantitatively by its abilities to lower the threshold $[\text{Ca}^{2+}]_i$ required for PGI_2 synthesis and to depress the peak $[\text{Ca}^{2+}]_i$ evoked by agonist. We propose that these effects are due respectively to actions of PMA on phospholipase A_2 and on a G-protein (G_p) that couples activated receptors to phospholipase C.

INTRODUCTION

Activation of endothelial cells by a variety of stimuli, including adenine nucleotides, thrombin, histamine or bradykinin, leads to the transient synthesis and release of prostacyclin (PGI_2), a potent vasodilator and inhibitor of platelet function. There is considerable indirect evidence of a role for elevated intracellular calcium ion concentrations ($[\text{Ca}^{2+}]_i$) in the transduction of this event, since studies using endothelial cells loaded with Ca^{2+} -sensitive fluorescent dyes have shown that each of these agonists stimulates increases in $[\text{Ca}^{2+}]_i$ [1–5]. By concomitant measurements of PGI_2 release and $[\text{Ca}^{2+}]_i$ in adherent monolayers of human endothelial cells we have demonstrated that: (i) an elevation of $[\text{Ca}^{2+}]_i$, derived mainly from release of Ca^{2+} from internal stores, is necessary for PGI_2 release in response to ATP or thrombin; (ii) under these conditions $[\text{Ca}^{2+}]_i > 0.8\text{--}1\text{ }\mu\text{M}$ is required for significant stimulation of PGI_2 release; and (iii) the dose–response relationship between $[\text{Ca}^{2+}]_i$ and PGI_2 release is identical when thrombin, ATP or its more potent analogue 2-chloro-ATP, or a calcium ionophore is used to stimulate PGI_2 production [6,7].

These observations imply that elevation of $[\text{Ca}^{2+}]_i$ is a necessary and sufficient transduction signal. Because each of the agonists noted above, but not calcium ionophore, activates phospholipase C in endothelium,

activation of protein kinase C by diacylglycerol apparently does not modulate PGI_2 release under these conditions [3,6,8–13]. This idea is supported by the observation that, after removal of extracellular Ca^{2+} and depletion of intracellular stores, thrombin induces endothelial phosphoinositide turnover, but not PGI_2 synthesis [6]. Nonetheless, activation of protein kinase C by phorbol esters or synthetic diacylglycerols can induce PGI_2 release and can potentiate both receptor-mediated and ionophore-induced PGI_2 synthesis [9,14,15]. In addition, the same agents can attenuate phosphoinositide turnover and elevations in $[\text{Ca}^{2+}]_i$ induced in endothelial cells by thrombin or histamine [9,13].

Hence the aim of the present study was to understand how the apparently disparate effects of protein kinase C activation upon $[\text{Ca}^{2+}]_i$ and PGI_2 release can be reconciled, by investigating the time-, concentration- and dose-dependent effects of phorbol 12-myristate 13-acetate (PMA) on agonist-stimulated elevations of $[\text{Ca}^{2+}]_i$ and PGI_2 , measured concomitantly in monolayers of human endothelial cells.

METHODS

Cell culture

Endothelial cells were isolated and cultured from segments of human umbilical vein as previously described

Abbreviations used: PGI_2 , prostacyclin; $[\text{Ca}^{2+}]_i$, cytoplasmic ionized calcium concentration; PMA, phorbol 12-myristate 13-acetate; PDD, 4 α -phorbol didecanoate; PG, prostaglandin.

‡ To whom correspondence and reprint requests should be addressed.

[6]. For experiments, cells from confluent primary cultures were detached by brief trypsin treatment, resuspended in growth medium and seeded at near-confluent density in 16 mm-diameter plastic wells of 24-well trays (Nunc) or on glass coverslips (22 mm × 11 mm). Then 1–2 days later, when confluent, cells were used for determination of PGI₂ release (on coverslips or in wells) or for measurement of [Ca²⁺]_i (on coverslips).

Determination of PGI₂ release

Confluent monolayers of cells on 16 mm-diameter wells ($\approx 10^5$ cells/well) were used for the replicate determination of PGI₂ release. Basal release, or agonist-stimulated release over a 5 min period, was determined as previously described [7]. ATP, sodium arachidonate, bradykinin, histamine and human α -thrombin were obtained from Sigma. Ionomycin (Calbiochem) was diluted in buffer from a stock solution (1 mM) in dimethyl sulphoxide. Cells were pretreated with PMA or 4 α -phorbol didecanoate (PDD; Sigma) for specified times before addition of other agents. Phorbol esters were diluted from stock solutions (1 mg/ml) in dimethyl sulphoxide. In some experiments PGI₂ release was determined concomitantly with [Ca²⁺]_i by sub-sampling the medium above coverslips of fura-2-loaded cells (see below).

Determination of [Ca²⁺]_i

Confluent monolayers of cells on coverslips were loaded with fura-2 by incubating the cells with 1 μ M-fura-2 acetoxymethyl ester (Calbiochem), and [Ca²⁺]_i was determined by recording fluorescence changes as previously described [7]. All results are expressed as means $\pm$ S.E.M. of individual replicate observations, and in each Figure and Table results from a single representative experiment are given.

RESULTS

Effects of phorbol esters on basal PGI₂ release

PMA stimulated PGI₂ release in a time- and dose-dependent manner (Fig. 1). No significant release was induced by 0.1 nM-PMA when incubated with endothelial cells for up to 180 min; 1 nM-PMA increased PGI₂ release by less than 2-fold after > 60 min incubation, whereas 10 nM- or 100 nM-PMA substantially increased PGI₂ production, after a lag period of 5–10 min, for up to 180 min. PDD (100 nM) had no effect on PGI₂ release when cells were incubated for up to 180 min (results not shown).

Potentiation of agonist-stimulated PGI₂ release by PMA

The time- and dose-dependence of the effects of PMA were investigated in detail by using ATP as the agonist, which stimulates PGI₂ release in non-treated cells with a threshold active concentration of $\approx 1 \mu$ M [7]. Fig. 2 shows that incubation with 10 nM-PMA enhanced the response to a sub-maximal dose of ATP (10 μ M), the maximum effect being reached after 5–10 min pretreatment, whereas 100 nM-PDD was inactive. In similar experiments pretreatment with PMA (1–100 nM) for 5 min potentiated PGI₂ release in response to 10 μ M-ATP in a dose-dependent manner, with EC₅₀ (concn. giving 50% of maximum potentiation) ≈ 5 nM. Stimulated

Fig. 1. Time- and dose-dependent stimulation of PGI₂ release from human umbilical-vein endothelial cells by PMA

Points are means of three observations; bars represent S.E.M. where larger than the point size. This experiment was repeated three times with equivalent results.

Fig. 2. Potentiation of ATP-stimulated PGI₂ release by PMA

PGI₂ release in response to 10 μ M-ATP was measured after preincubation with 10 nM-PMA (●) or 100 nM-PDD (○) for various times. Points are means of 10–12 observations. Bars represent S.E.M.

release was measured over 5 min: PMA alone, as expected, did not induce PGI₂ release; moreover, the transience (< 5 min) of the release induced by ATP was unaltered (results not shown).

Table 1. Effects of phorbol esters on basal and agonist-stimulated PGI₂ release

Values are means $\pm$ S.E.M. (no. of observations). Stimulated PGI₂ release was measured 5 min after agonist addition, after a 10 min preincubation with phorbol ester. Basal release was measured by sub-sampling before and after the 10 min preincubation period.

Treatment	Agonist...	PGI ₂ release (ng/10 ⁵ cells)				
		None	ATP (10 μ M)	Thrombin (0.02 unit/ml)	Bradykinin (10 nM)	Histamine (0.5 μ M)
None		101 $\pm$ 24 (24)	615 $\pm$ 60 (6)	790 $\pm$ 190 (6)	1940 $\pm$ 240 (6)	1680 $\pm$ 180 (5)
PMA (10 nM)		81 $\pm$ 13 (30)	1360 $\pm$ 175 (7)	1760 $\pm$ 230 (8)	3650 $\pm$ 300 (8)	3410 $\pm$ 540 (6)
PDD (100 nM)		70 $\pm$ 30 (9)	595 $\pm$ 84 (3)	740 $\pm$ 140 (3)	1720 $\pm$ 90 (3)	1420 $\pm$ 280 (3)

The results from the experiments summarized in Table 1 demonstrate that PMA pretreatment similarly potentiated (by about 2-fold) the response to each of four agonists, tested at sub-maximal doses.

Effects of phorbol esters on basal [Ca²⁺]_i

[Ca²⁺]_i in non-stimulated cells was identical in the presence of 1 mM extracellular Ca²⁺ (103 $\pm$ 6 nM; mean $\pm$ S.E.M., 38 observations from four different experiments) or in the absence of added extracellular Ca²⁺ and in the presence of 1 mM-EGTA [106 $\pm$ 6 (20) nM]. Preincubation with up to 100 nM-PMA or -PDD for up to 10 min had no effect on resting [Ca²⁺]_i in either case, e.g. after 5 min pretreatment with 10 nM-PMA [Ca²⁺]_i was 99 $\pm$ 5 (34) and 109 $\pm$ 5 (16) nM in the presence or absence of extracellular Ca²⁺ respectively.

Effects of PMA on agonist-stimulated elevations of [Ca²⁺]_i

The effects of PMA were again studied in most detail with ATP as the agonist. Fig. 3(a) illustrates the time course of elevations in [Ca²⁺]_i in response to ATP in untreated cells in the presence of 1 mM extracellular Ca²⁺. The response consisted of a rapid, transient, peak elevation followed by a sustained steady-state elevation, the extent of both being dependent on the concentration

of added ATP. We have previously shown that the initial peak is mainly due to release of Ca²⁺ from internal stores, whereas the steady-state elevation requires influx of extracellular Ca²⁺ [7]. Fig. 3(b) gives examples of the effect of 5 min treatment with 10 nM-PMA before addition of ATP. Both the peak [Ca²⁺]_i and the subsequent steady-state [Ca²⁺]_i were decreased by comparison with non-treated cells. PDD (100 nM) had no effect (result not shown).

The results from experiments in which PGI₂ release and [Ca²⁺]_i were measured concomitantly in cells preincubated for 5 min with 10 nM-PMA are shown in Fig. 4. Peak [Ca²⁺]_i was significantly decreased by PMA for all tested concentrations of ATP; PGI₂ release was markedly potentiated with 10 μ M- or 100 μ M-ATP, but not significantly altered by a near-threshold concentration (1 μ M) of ATP.

The dose-dependence of the effect of PMA (1–100 nM; 5 min pretreatment) on peak [Ca²⁺]_i and PGI₂ release in response to a single dose of ATP (10 μ M) is shown in Fig. 5. Although [Ca²⁺]_i declined with increasing concentrations of PMA, PGI₂ release was more greatly potentiated in the presence of 10 nM- than 100 nM-PMA. The depression in ATP-stimulated peak [Ca²⁺]_i by PMA was not affected by carrying out experiments in the absence of extracellular Ca²⁺ and in the presence of 1 mM-EGTA, e.g. under these conditions peak [Ca²⁺]_i in response to

Fig. 3. Changes in [Ca²⁺]_i in response to ATP

(a) Untreated endothelial cells; (b) cells preincubated for 5 min with 10 nM-PMA. Arrows show time at which ATP was added.

Fig. 4. Concomitant measurement of PGI₂ release (○) and peak [Ca²⁺]_i (●) in response to ATP in untreated cells or cells preincubated for 5 min with 10 nM-PMA.

Points are means of 3–9 observations. Bars represent S.E.M.

10 μM-ATP was 1010 ± 60 (4) nM in control cells and 420 ± 90 (4) nM after 5 min preincubation with 10 nM-PMA.

As illustrated in Fig. 3(b), PMA also decreased the steady-state elevation of [Ca²⁺]_i after agonist stimulation. Fig. 6 demonstrates the effectiveness of preincubation with PMA (10 nM; 5 min) in inhibiting the steady-state elevation of [Ca²⁺]_i, measured 2–3 min after addition of ATP. The effect was also dependent on the dose of PMA: the changes in steady-state [Ca²⁺]_i in relation to basal values, measured 2–3 min after stimulation with 10 μM-ATP were +35 ± 7 (9), +13 ± 11 (5), +9 ± 6 (7) and -4 ± 4 (5) nM after preincubation with 0, 1 nM-, 10 nM- or 100 nM-PMA respectively. PMA was also able to decrease steady-state [Ca²⁺]_i rapidly when added 2–3 min after ATP (results not shown).

Table 2 indicates that analogous enhancement of PGI₂ production, with concomitant decreases in peak [Ca²⁺]_i, occurred when using sub-maximal concentrations of other agonists. In contrast, as shown in Table 3, PGI₂ release in response to exogenous arachidonate (4–16 μM) for up to 30 min was not affected by preincubation with 10 nM-PMA.

Effects of phorbol esters on ionomycin-stimulated elevations of [Ca²⁺]_i and PGI₂ release

To determine the effects of phorbol esters on Ca²⁺-

Fig. 5. Effect of PMA on peak [Ca²⁺]_i (●) and PGI₂ release (○) in response to 10 μM-ATP.

Cells were preincubated for 5 min with PMA. Points are means of 5–9 observations. Bars represent S.E.M.

driven PGI₂ release in the absence of receptor-mediated events, cells in medium with no added Ca²⁺ and in the presence of 1 mM-EGTA were exposed to graded concentrations of ionomycin (1 nM–1 μM) after preincubation for 5 min with vehicle control, 10 nM-PMA or 100 nM-PDD. Peak [Ca²⁺]_i and PGI₂ release (measured over 5 min) were determined concomitantly, and the dose-response relationships obtained are shown in Fig. 7. In untreated cells, or cells preincubated with PDD, ionomycin induced graded elevations in peak [Ca²⁺]_i to concentrations approaching the maximum quantifiable by using fura-2, but no significant PGI₂ release was observed until [Ca²⁺]_i > ≈ 800 nM was achieved. In contrast, in cells preincubated with PMA, the threshold for ionomycin-stimulated PGI₂ release was decreased to 250–300 nM.

DISCUSSION

Activation of protein kinase C enhances agonist-induced PGI₂ release while inhibiting peak [Ca²⁺]_i

Our results demonstrate that pretreatment of endothelial cells with PMA, a specific irreversible activator of protein kinase C [16], can significantly modulate PGI₂ synthesis. After a concentration-dependent lag period, PMA induced sustained PGI₂ release. In addition, under conditions where no effect was observed on basal PGI₂

Fig. 6. Effect of PMA on steady-state $[Ca^{2+}]_i$ in response to stimulation with ATP

Cells were untreated or preincubated with 10 nM-PMA for 5 min. $[Ca^{2+}]_i$ was determined 2–3 min after addition of ATP, when steady-state elevations were achieved. Points are means of 5–9 observations. Bars represent S.E.M.

release, PMA potentiated transient, agonist-induced, PGI_2 production to an extent that depended in a complex manner on the time of preincubation and the concentrations of both PMA and agonist.

It has been reported that protein kinase C activation in endothelial cells decreases agonist-stimulated elevations in $[Ca^{2+}]_i$ [13]. We have shown here that PMA potentiates

Table 2. Effects of PMA on agonist-stimulated peak $[Ca^{2+}]_i$ and PGI_2 release

Cells were either untreated or preincubated for 5 min with 10 nM-PMA before addition of agonist. Values are means $\pm$ S.E.M. (no. of observations).

Treatment	$[Ca^{2+}]_i$ (nM)	6-Oxo- $PGF_{1\alpha}$ (ng/ 10^5 cells)
Thrombin (0.05 unit/ml)	895 ± 50 (3)	431 ± 46 (3)
+ PMA	293 ± 49 (4)	523 ± 53 (4)
Bradykinin (10 nM)	793 ± 105 (5)	188 ± 19 (5)
+ PMA	356 ± 45 (6)	462 ± 65 (5)
Histamine (0.5 μ M)	801 ± 104 (4)	238 ± 18 (5)
+ PMA	378 ± 135 (3)	340 ± 36 (3)

agonist-induced PGI_2 release while suppressing $[Ca^{2+}]_i$ transients, although elevation of $[Ca^{2+}]_i$ is the only transduction pathway normally required to account for agonist-induced PGI_2 release, with a threshold $[Ca^{2+}]_i$ of $\approx 0.8 \mu$ M [6,7]. Recent studies indicated that PMA potentiates ionophore-induced PGI_2 release [9,14], and from those data it can be deduced that there was a decrease in the threshold dose of ionophore required, consistent with the hypothesis that PMA treatment enhances the sensitivity of PGI_2 release to $[Ca^{2+}]_i$. The results in Fig. 7 demonstrate directly that the $[Ca^{2+}]_i/PGI_2$ activation curve was shifted to the left by pretreatment for 5 min with 10 nM-PMA, thus producing a lower threshold ($[Ca^{2+}]_i = 0.2$ – 0.3μ M) for PGI_2 release.

Since ionophore- and agonist-induced PGI_2 synthesis exhibit the same dependence on $[Ca^{2+}]_i$ [6,7], we deduce that the ability of PMA to enhance agonist-stimulated release can be quantitatively explained by the balance of its two opposing actions; to lower peak $[Ca^{2+}]_i$, and to increase the Ca^{2+} -sensitivity for PGI_2 production. Thus 5 min preincubation with 10 nM-PMA does not enhance PGI_2 release in response to a minimally active agonist concentration (e.g. 1 μ M-ATP), because the lower peak $[Ca^{2+}]_i$ achieved in PMA-treated cells ($\approx 0.2 \mu$ M; Fig. 4) is similar to the lower threshold required, whereas with higher concentrations of agonist (e.g. 10–100 μ M-ATP)

Table 3. PMA does not alter PGI_2 release in response to added arachidonate

Values are means $\pm$ S.E.M. (no. of observations). PGI_2 release was measured, with or without 10 min preincubation with 10 nM-PMA, for the times specified.

Treatment	Time after addition of arachidonate...	PGI_2 release (ng/ 10^5 cells)		
		5 min	15 min	30 min
None		0.10 ± 0.02 (3)	0.13 ± 0.02 (3)	0.16 ± 0.03 (3)
+ PMA		0.08 ± 0.02 (4)	0.15 ± 0.05 (4)	0.67 ± 0.04 (4)
Arachidonate (4 μ M)		2.76 ± 0.74 (6)	6.54 ± 1.72 (6)	11.12 ± 1.20 (6)
+ PMA		2.31 ± 0.46 (6)	5.96 ± 0.70 (5)	11.36 ± 0.72 (6)
Arachidonate (8 μ M)		2.30 ± 0.74 (6)	9.00 ± 0.98 (6)	16.68 ± 1.58 (6)
+ PMA		2.08 ± 0.74 (6)	9.60 ± 1.84 (5)	15.28 ± 0.76 (6)
Arachidonate (16 μ M)		1.90 ± 0.16 (6)	12.32 ± 0.84 (5)	19.60 ± 1.28 (5)
+ PMA		2.20 ± 0.42 (9)	12.12 ± 1.14 (9)	19.72 ± 1.24 (9)

Fig. 7. Dose-response relationship between $[Ca^{2+}]_i$ and PGI_2 release in response to ionomycin, in untreated cells ($\circ$), cells preincubated for 5 min with 100 nM-PDD ($\blacktriangle$), or cells preincubated for 5 min with 10 nM-PMA ($\bullet$).

the decrease in peak $[Ca^{2+}]_i$ is more than compensated for by the leftward shift in the Ca^{2+} -activation curve.

Since PMA alone has no effect on basal $[Ca^{2+}]_i$, we conclude that its ability to induce PGI_2 release depends on a time- and dose-dependent increase in the Ca^{2+} -sensitivity of PGI_2 synthesis until it is within the basal $[Ca^{2+}]_i$ range.

Possible mechanisms by which activation of protein kinase C decreases peak $[Ca^{2+}]_i$

The rapid transient increase of $[Ca^{2+}]_i$ in response to agonists in endothelial cells is due predominantly to release of Ca^{2+} from intracellular stores, and is believed to be caused by the formation and action of inositol trisphosphate [17].

In several cell types, activation of protein kinase C accelerates the extrusion of Ca^{2+} from the cytoplasm by pumps stimulated by elevated $[Ca^{2+}]_i$ [18–21]. Although this may contribute to the more rapid fall in $[Ca^{2+}]_i$ after the initial peak (Fig. 3), PMA treatment is known to inhibit endothelial phosphoinositide turnover in response to agonists [9,13], and this may account for its ability to suppress peak $[Ca^{2+}]_i$. There is no evidence in endothelium for direct effects on receptor affinity or number after PMA treatment, which, although reported for certain receptors in some cell types (e.g. the α_1 receptor of vascular smooth-muscle cells [22]), do not generally take place (see [23] and refs. therein). This also seems unlikely to explain our results, because of the similar action of PMA on responses to a variety of agonists, which implies modulation of post-receptor events.

A likely regulatory site is a guanine-nucleotide-binding protein (G_p) that modulates the coupling between receptors and phospholipase C [24]. G-proteins have been

shown to be substrates for protein kinase C, and this phosphorylation can lead to uncoupling of the receptor-effector system [25,26]. Furthermore, PMA inhibits guanine-nucleotide-stimulated phosphoinositide turnover in several other cell types by an action on G_p [27–29], and the ability of guanine nucleotides to stimulate phosphoinositide turnover in permeabilized human endothelial cells implies the presence of G_p in this cell type [30].

Possible mechanisms by which activation of protein kinase C enhances Ca^{2+} -sensitivity of prostaglandin synthesis

We have previously argued that liberation of arachidonate from phospholipids by the action of Ca^{2+} -activated phospholipase A_2 is the Ca^{2+} -sensitive step in the pathway of PGI_2 production [6]. In agreement with this, our results (Table 3) show that PMA does not affect PGI_2 release in response to exogenous arachidonate, and indicate that protein kinase C activation increases agonist- or ionophore-induced PGI_2 synthesis by enhancing the sensitivity of phospholipase A_2 to Ca^{2+} . Whether this effect in endothelial cells is due to direct phosphorylation of phospholipase A_2 , or to indirect effects via phosphorylation of regulatory proteins such as lipocortin or a G-protein, as described in other cell types [31,32], remains to be determined.

Protein kinase C activation inhibits agonist-induced steady-state elevations in $[Ca^{2+}]_i$

The steady-state elevations observed in endothelial cells after agonist-induced peak $[Ca^{2+}]_i$ rises can be sustained for many minutes, and are dependent on influx of extracellular Ca^{2+} through non-voltage-gated cation channels [1,4–7,12,33,34]. As shown in Fig. 6, PMA inhibited this elevated $[Ca^{2+}]_i$, which is presumably maintained by a balance between Ca^{2+} influx and sequestration or extrusion of Ca^{2+} by pumps. In several other cell types protein kinase C activation enhances Ca^{2+} efflux [18–21]. In endothelium, it is not known whether PMA acts in this way and/or by inhibition of Ca^{2+} influx. Whichever mechanism is responsible, it is likely that steady-state $[Ca^{2+}]_i$ elevation is of functional importance, since agonist-induced release of endothelium-derived relaxing factor can be maintained for many minutes in the presence of an agonist, is dependent on extracellular Ca^{2+} , and is blocked by PMA [35–38].

Conclusions

Chronic activation of protein kinase C in human endothelial cells in response to PMA has opposing actions on agonist-stimulated transient PGI_2 synthesis, which is driven by elevating $[Ca^{2+}]_i$: it independently decreases peak $[Ca^{2+}]_i$, and enhances the Ca^{2+} -sensitivity of PGI_2 production. The latter effect is predominant for most supra-threshold concentrations of agonists, leading to potentiation of PGI_2 release, and also accounts for the ability of PMA alone to increase chronic PGI_2 release. PMA has a further effect on Ca^{2+} homeostasis in endothelium, resulting in significant depression of agonist-stimulated steady-state $[Ca^{2+}]_i$ elevations.

Our previous results [6,7] imply that elevation of $[Ca^{2+}]_i$, but not of diacylglycerol, regulates PGI_2 release in response to a single exposure to agonist. Although diacylglycerol is produced and is present at elevated levels for several minutes after addition of an agonist

[13,39], protein kinase C activation may be insufficient or too slow to alter the initial $[Ca^{2+}]_i$ transient. The results presented here demonstrate that prior activation of protein kinase C enhances release of PGI_2 in response to agonists. It remains unclear, however, what may be the physiological role of diacylglycerol, since rapid sequential challenge with an agonist leads not to potentiation of PGI_2 release but to homologous tachyphylaxis [40]. Our current experiments are therefore directed towards measuring how previous exposure to an agonist affects the subsequent ability of the same agonist to change cellular Ca^{2+} homeostasis, and to relate this to the effects that we have observed with PMA pretreatment.

T. D. C. holds an M.R.C./Smith, Kline & French Partnership research student award. We thank Dr. Lindsey Needham and Mrs. Val Toothill for stimulating our interest in this work. We are grateful for the continued expert help of the Delivery Suite, Northwick Park Hospital, in the collection of umbilical cords, and to Elsie Prestige for word processing.

REFERENCES

- Hallam, T. J. & Pearson, J. D. (1986) *FEBS Lett.* **207**, 95–99
- Luckhoff, A. & Busse, R. (1986) *J. Cell. Physiol.* **126**, 414–420
- Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) *J. Biol. Chem.* **262**, 8557–8565
- Rotrosen, G. & Gallin, J. I. (1986) *J. Cell Biol.* **103**, 2379–2380
- Morgan-Boyd, R., Stewart, J. M., Vavrek, R. J. & Hassid, A. (1987) *Am. J. Physiol.* **253**, C588–C598
- Hallam, T. J., Pearson, J. D. & Needham, L. A. (1988) *Biochem. J.* **251**, 243–249
- Carter, T. J., Hallam, T. J., Cusack, N. J. & Pearson, J. D. (1988) *Br. J. Pharmacol.* **95**, 1181–1190
- Lambert, T. L., Kent, R. S. & Whorton, A. R. (1986) *J. Biol. Chem.* **261**, 15288–15293
- Halldorsson, H., Kjeld, M. & Thorgeirsson, G. (1988) *Arteriosclerosis* **8**, 147–154
- Hong, S. L. & Deykin, D. (1982) *J. Biol. Chem.* **257**, 7151–7154
- Derian, C. K. & Moskowitz, M. A. (1986) *J. Biol. Chem.* **261**, 3831–3837
- Pirotton, S., Raspe, E., Demolle, D., Erneux, C. & Boeynaems, J.-M. (1987) *J. Biol. Chem.* **262**, 17461–17466
- Brock, T. A. & Capasso, E. A. (1988) *J. Cell. Physiol.* **136**, 54–62
- Demolle, D. & Boeynaems, J. M. (1988) *Prostaglandins* **35**, 243–257
- Pearson, J. D. & Carter, T. D. (1989) in *Vascular Endothelium: Receptors and Transduction Mechanisms* (Carravaso, J., Ryan, U. S. & Gillis, C. N., eds.), pp. 189–196, Plenum Press, New York
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *Nature (London)* **295**, 68–71
- Lagast, H., Pozzan, T., Waldvogel, F. A. & Lew, P. D. (1984) *J. Clin. Invest.* **73**, 878–883
- Drummond, A. H. (1985) *Nature (London)* **315**, 752–755
- Rink, T. J. & Sage, S. O. (1987) *J. Physiol. (London)* **393**, 513–524
- Cotecchia, S., Leeb-Lundberg, L. M. F., Hagen, P. O., Lefkowitz, R. J. & Caron, M. G. (1985) *Life Sci.* **37**, 2389–2398
- Brown, K. D., Blakeley, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) *Biochem. J.* **245**, 631–639
- Cockcroft, S. (1987) *Trends Biochem. Sci.* **12**, 75–78
- Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S. & Jakobs, K. H. (1985) *Eur. J. Biochem.* **151**, 431–437
- Zick, Y., Sagi-Eisenberg, R., Piner, M., Gierschik, P. & Spiegel, A. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 9294–9297
- Orellana, S., Solski, P. A. & Brown, J. M. (1987) *J. Biol. Chem.* **262**, 1638–1643
- Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) *J. Biol. Chem.* **262**, 6121–6127
- Llano, I. & Marty, A. (1987) *J. Physiol. (London)* **394**, 239–248
- Brock, T. A., Dennis, P. A., Griendling, K. A., Diehl, T. S. & Davies, P. F. (1988) *Am. J. Physiol.* **255**, C667–C673
- Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. L., Ramachandran, K. L. & Pepinsky, R. B. (1986) *Nature (London)* **320**, 77–81
- Axelrod, J., Burch, R. M. & Jelsema, C. L. (1988) *Trends Neurosci.* **11**, 117–123
- Hallam, T. J., Jacob, R. & Merritt, J. E. (1988) *Biochem. J.* **255**, 179–184
- Schilling, W. P., Ritchie, A. K., Navarro, L. T. & Eskin, S. G. (1988) *Am. J. Physiol.* **255**, H219–H227
- Singer, H. A. & Peach, M. J. (1984) *Hypertension* **4** (Suppl. 2), 19–25
- Weinheimer, G., Wagner, B. & Osswald, H. (1986) *Eur. J. Pharmacol.* **130**, 319–322
- Lewis, M. J. & Henderson, A. H. (1987) *Eur. J. Pharmacol.* **137**, 167–171
- De Nucci, G., Gryglewski, R. J., Warner, T. D. & Vane, J. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2334–2338
- Hong, S. L., McLaughlin, N. J., Tzeng, C.-Y. & Patton, G. (1985) *Thromb. Res.* **38**, 1–10
- Toothill, V. J., Needham, L., Gordon, J. L. & Pearson, J. D. (1988) *Eur. J. Pharmacol.* **157**, 189–196

Received 24 February 1989/28 April 1989; accepted 9 May 1989