Endothelium-derived relaxing factor inhibits the endothelin-1-induced increase in protein kinase C activity in rat aorta

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1 Particulate and cytosolic protein kinase C (PKC) activity was measured in rat aortae with and without endothelium, following exposure to endothelin-1 (10^{-8} M) for various time intervals.

2 Endothelin-1 induced two peaks of particulate PKC activity, occurring at 30 s and 10 min exposure times in both endothelium-intact and endothelium-denuded preparations. Cytosolic PKC activity fell below baseline at all incubation times studied.

3 In endothelium-denuded preparations, elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels with sodium nitroprusside (10^{-6} M) or atrial natriuretic peptide (10^{-6} M) and, in endothelium-intact preparations with the calcium ionophore A23187 (10^{-6} M), inhibited the activation of particulate PKC activity seen after incubation with endothelin-1 for 30 s. The inhibitory effect of A23187 was prevented by prior incubation of the endothelium-intact vessels with the nitric oxide synthetase inhibitor, L-N^G-nitro arginine (5×10^{-5} M).

4 These results indicate that EDRF acting via cyclic GMP can inhibit the activation of PKC induced by endothelin-1 in rat aorta.

Keywords: Rat aorta; EDRF; protein kinase C; endothelin; L-N^G-nitro arginine

Introduction

In vascular smooth muscle, protein kinase C (PKC) is present in relatively high concentrations (Nishizuka, 1984) and is presumed to play an important role in the regulation of tone, particularly during the tonic phase of contraction (Rasmussen *et al.*, 1987; Haller *et al.*, 1990). Several constrictor agonists including endothelin have now been shown to cause activation and translocation of PKC from the cytosolic to the particulate fraction of vascular smooth muscle cells (Haller *et al.*, 1990) and it has been shown that these events are responsible for the maintenance of tone during the tonic phase of contraction.

Nitric oxide is now known to be the active principle of endothelium-derived relaxing factor (EDRF; Palmer *et al.*, 1987). It induces vascular smooth muscle relaxation by the activation of soluble guanylate cyclase and elevation of intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (for review see Griffith *et al.*, 1988). However, the precise mechanism whereby cyclic GMP induces smooth muscle relaxation is not fully understood. It is known that cyclic GMP inhibits phosphatidylinositol (PI) hydrolysis in vascular smooth muscle (Rapoport, 1986) and platelets (Takai *et al.*, 1981). More recently it has been shown that in both vascular smooth muscle and endothelium this effect of cyclic GMP leads to inhibition of agonist-induced inositol 1,4,5-trisphosphate (IP₃) production (Lang & Lewis, 1989; 1991). However, this effect of cyclic GMP on IP₃ production does not adequately explain the mechanism of inhibition of vascular smooth muscle tone during the tonic phase of contraction, which involves PKC activation.

In the present study therefore we have investigated the effects of EDRF and other cyclic GMP elevating agents, on endothelin-1-induced activation of PKC in rat aorta.

Methods

Tissue preparation

Male Wistar rats (250–300 g) were killed by cervical dislocation and their thoracic aortae removed and placed into

Krebs-Ringer bicarbonate (KRB) solution of the following composition (mM): NaCl 95.5, KCl 4.8, MgSO₄ 1.2, CaCl₂ 2.5, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11 and indomethacin 0.001. The vessels were cleaned of all fat and connective tissue and each divided into 4 equal lengths. Paired lengths were used in each experiment. The endothelium was removed from some of the vessels by gentle rubbing of the intimal surface with a wooden stick. The sections were placed in KRB gassed with 95% O₂:5% CO₂ and left to equilibrate for at least 60 min at 37°C.

Protein kinase C assay

The PKC activity was measured with a commercially available kit (Amersham International, U.K.). The assay is dependent on PKC catalysing the transfer of a radiolabelled [³²P]-phosphate of ATP to the threonine group of a substrate peptide which is specific for PKC. The assay is a modification of a previously described technique (Hannun *et al.*, 1985).

At the end of each experiment each vessel was homogenized in an ice-cold hypotonic lysis buffer and the cytosolic and particulate PKC fractions separated by ultracentrifugation. These fractions were stored on ice and their PKC activity measured within 4 h of the initial homogenization. The protein content of each sample was measured immediately following the assay of PKC activity, as described previously (Collins *et al.*, 1988). The cytosolic fraction was also assayed for its cyclic GMP content with a commercially available radioimmunoassay kit (New England Nuclear Research Products, Germany), after being stored at –20°C for up to 1 month. PKC activity is expressed as fmol phosphate transferred min⁻¹ μg⁻¹ protein and the cyclic GMP content as fmol μg⁻¹ protein.

Experimental protocol

Endothelin-1 was chosen as the constrictor agonist and all experiments were carried out in KRB gassed with 95% O₂:5% CO₂, pH 7.4 at 37°C. Vessels with and without endothelium were exposed to endothelin-1 (10^{-8} M) for 30 s, 1, 2, 5, 10, 20 and 30 min. The time showing peak PKC activity was chosen for all subsequent experiments. In all experiments, untreated vessels were included for measurement of basal PKC activity.

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For investigation of the effects of cyclic GMP on PKC activity, two series of experiments were performed. In the first series, endothelium-denuded vessels were exposed for 30 s, 1, 2, 5 and 10 min to sodium nitroprusside (10^{-5} M) or atrial natriuretic peptide (ANP, 10^{-6} M) alone. In the second series vessels denuded of endothelium were incubated with either agent for the stated times followed by a 30 s exposure to endothelin-1 (10^{-8} M), the time at which PKC activity is maximally induced by endothelin-1.

To study the effect of EDRF on endothelin-1-induced PKC activity, endothelium-intact preparations were exposed to the calcium ionophore A23187 (10^{-6} M) alone for 30 s, 1, 2, 5 and 10 min. In other experiments endothelium-intact vessels were incubated with A23187 for the stated times, followed by a 30 s exposure to endothelin-1 (10^{-8} M). This second series of experiments were repeated to include a 30 min pre-incubation with the nitric oxide synthetase inhibitor L- N^G -nitro arginine (L-NNA, 5×10^{-5} M; Moore *et al.*, 1990), before each addition of A23187. Also, endothelium-intact vessels were exposed to endothelin-1 (10^{-8} M) alone for 30 s, 1, 2, 5 and 10 min following a 30 min pre-incubation with L-NNA (5×10^{-5} M).

Calculation and statistics

PKC activity and cyclic GMP levels were determined as described in the respective kits.

Each data point represents the mean $\pm$ standard error of the mean (s.e.mean) of at least 4 separate experiments. Control measurements were made repeatedly throughout the course of the study.

For analysis of within-group data a one-way analysis of variance was used followed by Dunnett's multiple range test to identify significant differences where $P < 0.05$. For between-group data, a one-way analysis of variance was followed by Tukey's multiple range test to identify significant differences where $P < 0.05$.

Drugs

All drugs were purchased from Sigma Chemical Co., Dorset, England. Endothelin-1 and human sequence atrial natriuretic peptide were used. All other chemicals used were analytical reagent standard.

Results

Endothelin-1

Figure 1a and 1b show the changes in cytosolic and particulate PKC activity respectively following incubation of endothelium-intact and -denuded vessels with endothelin-1 for the times indicated. As can be seen, in the intact preparations cytosolic PKC activity was significantly different from baseline levels only after 20 and 30 min exposure to endothelin-1. The particulate enzyme showed two distinct peaks of activity which were significantly different from basal. These occurred after 30 s and 10 min of agonist exposure.

In the denuded preparations cytosolic activity was significantly reduced below baseline values after 5 and 10 min exposure to endothelin-1, returning to baseline levels thereafter. Like the endothelium-intact preparations, the particulate activity in the denuded preparations showed significant increases above basal at 30 s and 10 min.

Figure 1c shows that endothelin-1 caused no significant changes in intracellular cyclic GMP levels at any of the times studied. The data do show, however, that basal levels of cyclic GMP in endothelium-intact preparations (Figure 1c) are approximately 9 times greater than in endothelium-denuded preparations.

For all subsequent experiments a 30 s exposure to endothelin-1 was chosen to investigate the effects of the cyclic GMP-elevating interventions on PKC activity.

Figure 1 Changes in cytosolic (a) and particulate (b) protein kinase C (PKC) activity in endothelium-intact (closed columns) and -denuded (open columns) rat aortae following incubation with endothelin-1 for 0.5, 1, 2, 5, 10, 20 and 30 min. Changes in cyclic GMP levels in the same experiment are also shown (c). Values are the mean of 4–6 determinations from 8 separate experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ compared with basal values (B).

Sodium nitroprusside

Endothelium-denuded preparations only were used for these studies. As shown in Figures 2a and 2b, neither cytosolic nor particulate PKC activities were altered from basal values following exposure of the tissues to sodium nitroprusside alone.

The changes in cytosolic and particulate PKC activities induced by endothelin-1 in the presence of sodium nitroprusside are also shown in Figures 2a and 2b. As can be seen, cytosolic activity was also unchanged by endothelin-1 in the presence of sodium nitroprusside at any of the incubation times studied. Likewise, the increase in particulate PKC activity following exposure to endothelin-1 was inhibited by prior incubation with sodium nitroprusside for 30 s, 1, 2, 5 and 10 min, though at 10 min values were significantly greater than basal.

Pre-incubation of the vessels with sodium nitroprusside alone for 30 s and also when followed by endothelin-1, resulted in an approximate 180 fold increase in the levels of cyclic GMP above basal values (Figure 2c); exposure to sodium nitroprusside for longer periods also significantly increased cyclic GMP levels but the extent of the increase was less than that seen at 30 s, being about 25 times the basal values at 10 min in both experiments.

Figure 2 Changes in cytosolic (a) and particulate (b) protein kinase C (PKC) activity in endothelium-denuded rat aortae following incubation with sodium nitroprusside alone for 0.5, 1, 2, 5 and 10 min (closed columns) and following similar incubations with sodium nitroprusside before exposure to endothelin-1 for 30 s (open columns). Changes in cyclic GMP levels in the same experiments are also shown (c). Values are mean of 4–6 determinations from 5 separate experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ compared with basal values (B); † $P < 0.05$ compared to exposure to endothelin-1 (ET-1) alone for 30 s in endothelium-denuded preparations (stippled column).

Atrial natriuretic peptide

In the presence of ANP alone neither the cytosolic nor particulate PKC activities were altered significantly at any of the incubation periods studied (Figure 3a and 3b). The particulate activity in the presence of ANP alone was approximately double the values found in the presence of endothelin-1, though none of the points reached was significantly different. Pre-incubation with ANP for up to 10 min significantly inhibited the changes in particulate PKC activity induced by a 30 s exposure to endothelin-1, levels remaining unchanged from basal (Figure 3b).

A significant increase in cyclic GMP levels above basal values was observed following incubation with ANP alone or in the presence of endothelin-1 (Figure 3c).

Endothelium-derived relaxing factor

Calcium ionophore-induced EDRF release in endothelium-intact preparations resulted in a significant reduction in basal cytosolic PKC activity following the 5 and 10 min incubation periods (Figure 4a). A significant reduction in basal particu-

Figure 3 Changes in cytosolic (a) and particulate (b) protein kinase C (PKC) activity in endothelium-denuded rat aortae following incubation with atrial natriuretic peptide (ANP) alone for 0.5, 1, 2, 5 and 10 min (closed columns) and following similar incubations with ANP before exposure to endothelin-1 for 30 s (open columns). Changes in cyclic GMP levels in the same experiments are also shown (c). Values are mean of 4–6 determinations from 5 separate experiments; vertical bars show s.e.mean. ** $P < 0.01$ compared with basal values (B); † $P < 0.05$ compared to exposure to endothelin-1 (ET-1) alone for 30 s in endothelium-denuded preparations (stippled column).

late activity was also observed following 1 min incubation with A23187 (Figure 4b).

Changes in cytosolic and particulate PKC activity in endothelium-intact vessels pre-incubated for various times with A23187 followed by endothelin-1 exposure, are also shown in Figures 4a and 4b respectively. Cytosolic activity fell gradually throughout the whole time course of the experiment, reaching significance compared with basal activity at the 10 min incubation period. Particulate activity also tended to fall compared with basal values though significance was not achieved at any incubation time studied.

When compared to the data obtained in the presence of endothelin-1 alone (Figure 1a), cytosolic activity in the presence of both A23187 and endothelin-1 was significantly reduced at all A23187 incubation times studied. A similar significant reduction was observed in particulate activity when compared to that seen with endothelin-1 alone (Figure 1b).

A23187, alone and in the presence of endothelin-1, significantly increased cyclic GMP levels above basal values, at each incubation time studied (Figure 4c). Peak cyclic GMP levels were achieved after 5 min incubation with A23187 both in the absence and presence of endothelin-1.

The effect of L-NNA on the changes induced by A23187 in endothelium-intact vessels in the presence of endothelin-1 is

Figure 4 Changes in cytosolic (a) and particulate (b) protein kinase C (PKC) activity in endothelium-intact rat aortae following incubation with A23187 alone for 0.5, 1, 2, 5 and 10 min (closed columns) and following similar incubations with A23187 before exposure to endothelin-1 for 30 s (open columns). Changes following preincubation with L-N^G-nitro arginine before A23187 and endothelin-1 are also included (hatched columns). Changes in cyclic GMP levels in the same experiments are also shown. Values are mean of 4–6 determinations from 8 separate experiments; vertical bars show s.e.mean. * $P < 0.05$; ** $P < 0.01$ compared to basal values (B); † $P < 0.05$ compared to exposure to endothelin-1 (ET-1) alone for 30 s in endothelium-intact preparations (stippled column).

again shown in Figure 4. Cytosolic PKC activity, compared to basal values, was unaffected by the presence of this nitric oxide synthetase inhibitor. Particulate activity however was significantly enhanced at all time points when compared to basal values following incubation with A23187 and endothelin-1 in the presence of L-NNA.

Compared to the data obtained in the absence of L-NNA, cytosolic PKC activity was not significantly altered by L-NNA. Particulate activity was however significantly different from that obtained in the absence of L-NNA at the 30 s and 2 min incubation periods with A23187.

Figure 4c shows that in the presence of L-NNA, A23187 did not increase cyclic GMP levels above basal values at all incubation times studied.

To test the effect of L-NNA itself on endothelin-1-induced changes in PKC activity, endothelium-intact preparations were incubated for 30 min prior to exposure to endothelin-1 for various times. The PKC response to endothelin-1 was similar to that observed in the absence of L-NNA (Figure 1a and 1b) but with no significant increase in particulate PKC occurring after 10 min exposure to endothelin-1 (data not shown).

Discussion

Endothelin-1 is a potent vasoconstrictor peptide producing a contractile response in vascular smooth muscle which is slow in onset and unusually long-lasting (Yanagisawa *et al.*, 1988). It has been suggested that PKC plays an important part in mediating this contractile response (Danthuluri & Brock, 1990). The data presented here show that exposure of rat vascular smooth muscle to endothelin-1 results in the production of two temporally separated, transient increases in particulate, or membrane bound, PKC activity. The first and largest, increase occurs after only 30 s exposure to endothelin-1 with the second increase developing later at 10 min. This transient, biphasic response occurs both in the presence and absence of endothelium.

It is interesting to note that endothelin-1 did not significantly increase cyclic GMP levels in the aortic preparations. A recent report by Topouzis and colleagues (1991) showed a small, transient, early increase (1 min) in cyclic GMP in rat aortic rings in response to endothelin-1 (10^{-8} M) which was dependent on the presence of endothelium. The reasons for the discrepancy between their data and those of the present study are difficult to explain. One possibility is the different experimental conditions used in the two studies.

It has been suggested (Nishizuka, 1984) that under basal conditions PKC is found located mainly in the cytosol (cytosolic fraction). Then, after cell activation, it is rapidly translocated to the membrane (particulate fraction), where it becomes associated with membrane phospholipids. If this is the case then an increase in particulate PKC activity should be associated with a corresponding decrease in cytosolic PKC activity. However, to date, opinion on this matter is conflicting. For example, prolactin stimulation of rat aortic smooth muscle has been shown to lead to simultaneous increases in both particulate and cytosolic PKC activity (Sauro *et al.*, 1989). In contrast, agonist-induced decreases in cytosolic PKC activity with corresponding increases in particulate PKC activity have been shown by a number of workers (Wheeler-Jones *et al.*, 1989; Chambers *et al.*, 1990; Haller *et al.*, 1990). An unusual situation occurs in platelets in that either dual activation of both cytosolic and particulate enzymes can occur or, alternatively, the cytosolic activity decreases while the particulate activity increases depending on which aggregating agent is used (Salari *et al.*, 1990).

The biphasic activation of particulate PKC shown here is not accompanied by a simultaneous decrease in cytosolic PKC activity. In the presence of endothelin-1 and in response to endothelin-1, cytosolic activity is decreased significantly only after 20 and 30 min of agonist exposure. In the absence of endothelium however cytosolic activity is decreased after 5 and 10 min of endothelin-1 exposure, thereafter returning to levels comparable with basal activity. It appears therefore, that in the absence of endothelium this return to basal levels may well represent a re-translocation of PKC from the membrane to the cytosol. Why this difference between endothelium-intact and denuded preparations should occur is not at all evident. It is possible that endothelial cells release a substance, perhaps EDRF, that prevents the retranslocation of the PKC. Further work is needed to explain this phenomenon.

The lack of any real association between increases in particulate PKC activity and decreases in cytosolic PKC activity in the presence of endothelin-1 leads us to suggest that in this model, the two pools of the PKC isoenzymes may be acting independently of one another. There is no reason to believe that the response to other agonists would be similar however. Other workers have examined the effects of endothelin-1 exposure on PKC activity in vascular smooth muscle. One such study (Haller *et al.*, 1990) in intact strips of bovine carotid artery showed endothelin-1 to cause a sustained elevation in particulate PKC activity over 50 min. This was accompanied by a corresponding decrease in cytosolic PKC activity. These results would therefore seem to disagree with

those of the present study. Why this discrepancy should exist is not clear. One explanation is that a species difference may exist. A possible difference between aortic and carotid artery tissue could also be responsible.

If PKC is responsible for the sustained phase of agonist-induced vascular smooth muscle contraction, changes in the intracellular levels of diacylglycerol (DAG), the specific activator of PKC (Nishizuka, 1984) are also of importance. The DAG response of vascular smooth muscle to endothelin-1 has been studied by a number of groups. For example, cultured cells from bovine and rat aortae were shown to develop maximum levels of DAG at 1–2 min after exposure, decaying to basal levels thereafter (Resink *et al.*, 1988). In contrast, in cultured aortic smooth muscle cells of rat (Griendling *et al.*, 1989) and rabbit (Sunako *et al.*, 1989; 1990) endothelin-1 has been shown to produce a biphasic increase in DAG levels with an early transient increase being followed by a more sustained increase. This biphasic production would correlate well with the biphasic activation of particulate PKC shown in the present study.

Another vasoconstrictor, angiotensin II, also produces a biphasic DAG response in vascular smooth muscle cells (Griendling *et al.*, 1986; Sunako *et al.*, 1989; 1990). As inositol 1,4,5-trisphosphate (IP_3) is produced from phosphatidylinositol 4,5 bisphosphate (PIP_2) at the same time as DAG (Berridge & Irvine, 1984) some of these workers (Griendling *et al.*, 1986; Sunako *et al.*, 1989) also measured the IP_3 response of these cells to angiotensin II. Both groups found the IP_3 response to be rapid and transient compared to the biphasic DAG response. This suggests that the early transient DAG phase is derived from PIP_2 hydrolysis while a large part of the late sustained DAG phase could come from other major membrane-associated phospholipids such as PI (Griendling *et al.*, 1986) or phosphatidylcholine (PC) (Griendling *et al.*, 1989).

The present study also clearly shows that an elevation of

intracellular cyclic GMP levels by either EDRF, sodium nitroprusside or ANP causes an inhibition of the particulate PKC response to endothelin-1. No discernible changes in cytosolic PKC activity were noted under the same conditions. We have previously shown that cyclic GMP causes inhibition of IP_3 production in both vascular smooth muscle (Lang & Lewis, 1989) and cultured endothelial cells of the pig (Lang & Lewis, 1991). An inhibition of DAG production from PIP_2 could account for the effect of cyclic GMP on the early rise in endothelin-1-induced particulate PKC, since cyclic GMP is thought to exert its effect at the site of the G protein coupling receptor to phospholipase C (Hirata *et al.*, 1990).

Our observations on the effects of cyclic GMP on PKC activity confirm those of other groups using bovine vascular smooth muscle (Ahlner *et al.*, 1988), and rat aortic smooth muscle (Sauro & Fitzpatrick, 1990a,b).

Although inhibition of PIP_2 hydrolysis by cyclic GMP can account for inhibition of the early activation of PKC by endothelin-1, cyclic GMP elevating agents have been shown to cause a reversal of the sustained or tonic phase of endothelin-1-induced contraction in vascular smooth muscle (Yanagisawa *et al.*, 1988; Bonhomme *et al.*, 1989; Miller *et al.*, 1989). This suggests that cyclic GMP may also inhibit the sustained production of DAG and the late phase of PKC activation.

In conclusion, endothelin-1 causes a biphasic activation of particulate PKC activity in rat aortic smooth muscle. The study also shows that EDRF, acting via cyclic GMP, inhibits the first phase of particulate PKC activation, thus providing further evidence for the mechanism of EDRF-induced relaxation in vascular smooth muscle.

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