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ENDOTHELIN STIMULATES A SUSTAINED 1,2-DIACYLGLYCEROL INCREASE AND PROTEIN KINASE C ACTIVATION IN BOVINE AORTIC SMOOTH MUSCLE CELLS

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Endothelin is a long-lasting potent vasoconstrictor peptide. We report here that in bovine aortic smooth muscle cells, endothelin biphasically increased total cellular diacylglycerol (DAG) content. When cellular DAG was labeled with [^{14}C] glycerol for 48h, endothelin stimulated [^{14}C]DAG formation in a biphasic pattern. Only one prolonged phase of DAG accumulation was observed when cells were labeled with [^{3}H]glycerol for 2 h. Endothelin induced an increase in the membranous protein kinase C (PKC) activities, which lasted for more than 20 min. These data suggest that (i) endothelin stimulates a sustained generation of DAG, (ii) this accumulation of DAG results in a sustained translocation of cytosolic PKC activities to the membrane.

Endothelin is a novel potent vasoconstrictor peptide of vascular endothelial origin. Both the endothelin-induced contraction of arterial strips and the *in vivo* pressor effect last for more the 30 min (1). In renal glomerular mesangial cell, endothelin induces a transient increase in introcellular calcium and inositol 1,4,5-triphosphate levels probably derived from phospholipase C-mediated phosphoinositides breakdown (2). However, in cultured vascular smooth muscle cells, Hirata et al. failed to observe phosphoinositides turnover in response to endothelin (3).

Cellular diacylglycerol (DAG) has multiple metabolic roles. DAG can activate protein kinase C (PKC) (4) and can induce influx of extracellular calcium (5). Activation of PKC by phorbol esters induces contractions of different arterial smooth muscles from different species. These contractions were characteristically long-lasting and dependent on the extracellular calcium (6,7) as in endothelin-induced contractions. Furthermore, the presence of Ca^{2+} channel blockers attenuates both endothelin- and phorbol ester-induced contractions by about 60% (1,7), while the presence of α-adrenergic blockers does not block the contractile effect of either of them (1,6). In view of these similarities, it is interesting to investigate whether

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endothelin will stimulate DAG formation and PKC activities in vascular smooth muscle cells.

MATERIAL AND METHODS

Human endothelin was obtained from Peninsula Laboratories, Belmont, CA, and dissolved in 0.1% acetic acid at the concentration of 0.1 mM as stock solution.

Cell Culture - Bovine aortic smooth muscle cells were obtained by methods previously described (8) and were maintained in DME medium supplemented with 10% fetal bovine serum.

Extraction and Analysis of Lipids - Following preincubations with the appropriate isotopes for 2 or 48 h, cells were treated with designated concentrations of endothelin for indicated intervals. Reactions were terminated by aspirating the media and adding 1 ml of methanol and cellular lipids were extracted as described by Bligh (9). DAG was resolved on silica gel G t.l.c. plates developed together with standards in hexane:diethyl ether:acetic acid (60:40:1; v/v/v) (R_f sn-1,2 diacylglycerol, 0.27) (10). Spots of lipids on t.l.c. plates were visualized by either autoradiography or charring with H_2SO_4 and individual spots, identified by the standards, were scraped into vials and radioactivity counted by liquid scintillation spectrometry.

Analysis of Total Mass of DAG - Total cellular DAG levels were measured by the DAG kinase assay as described by Priess et al. (11) with DAG kinase purchased from Lipidex, Westfield, NJ. The standard curve showed a linear slope from 50 to 500 pmole DAG.

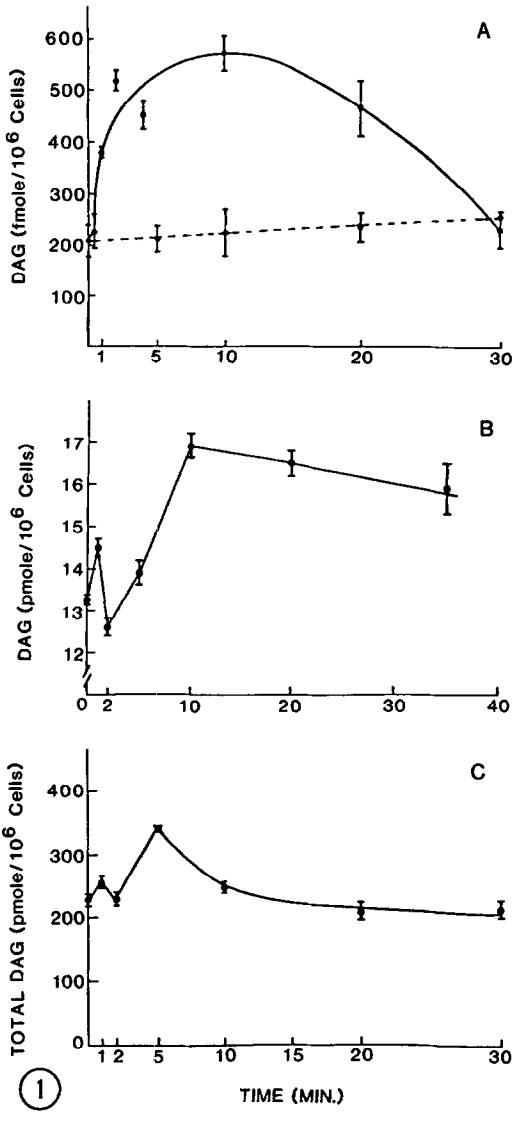
Subcellular Fractionation and Partial Purification of Protein Kinase C - Cells were fractionated and PKC partially purified by DE-52 chromatography as previously described (12) with the following modification: A 10-min centrifugation at 2500 g was performed after breaking the cells by homogenization with a Dounce homogenizer. Only the supernatant after this centrifugation was applied to the subsequent ultracentrifugation.

Protein Kinase Activity Assay - The activity of protein kinase C was measured by its ability to transfer ^{32}P from [γ-^{32}P]ATP into histone HI in the presence or absence of 0.5 mM Ca^{2+}, 6.4 µg/ml 1,2-dioleoylglycerol and 96 µg/ml phosphatidylserine as previously described (12).

RESULTS

[^{3}H] glycerol Labeling of DAG - Addition of 10^{-7} M endothelin to aortic smooth muscle cells prelabeled 2 h with [^{3}H] glycerol resulted in a rapid formation of diacyl [^{3}H] glycerol (Fig. 1A). The stimulation of DAG formation starts within 1 min of exposure, reaches a maximum at 10 min with a 160 ± 15% ($P < 0.01$) increase. The increase gradually declines to 100 ± 23% at 20 min and returns to basal level at 30 min. The dose dependence of endothelin action on diacyl [^{3}H] glycerol formation is shown in Fig. 2A. The plateau is approached at an endothelin concentration of 10^{-8} M and the half-maximal stimulation is reached by 10^{-9} M.

[^{14}C] Glycerol Labeling of DAG - DAG labeling with [^{14}C] glycerol reaches a steady state after 48 h (data not shown here). In smooth muscle cells prelabeled 48 h with [^{14}C] glycerol, endothelin causes a rapid 10 ± 3% ($P < 0.02$) increase of [^{14}C]DAG at 1 min, declining to slightly below basal level by 2 min and increasing maximally to 28 ± 5% ($P < 0.001$) at 10 min and thereafter remaining at least 20% elevated for more than 35 min (Fig. 1B).



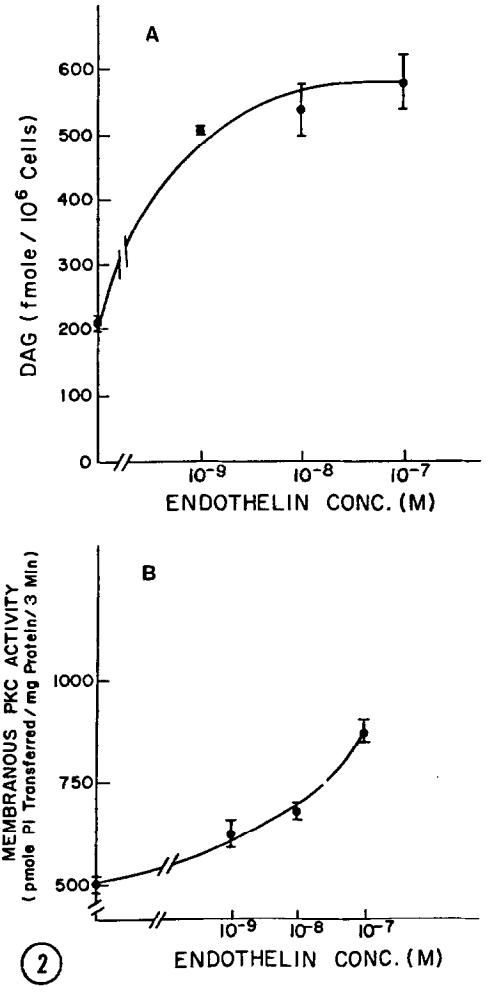


Figure 1: Time course of endothelin on DAG labeling from [^{3}H] glycerol (A), [^{14}C] glycerol (B), and DAG mass (C) in smooth muscle cells. Bovine aortic smooth muscle cells were cultured on 6-well plates. (A), After 2-h labeling with [^{3}H] glycerol (2 μCi/ml), cells were incubated for various time with 10^{-7} M endothelin (solid line) or no addition (dotted line), and the radioactivity in DAG was determined. Using the specific activity of [^{3}H] glycerol, results were expressed as fmole of [^{3}H]DAG per 10^6 cells. (B), After 48-h labeling with [^{14}C] glycerol, cells were incubated with 10^{-7} M endothelin and the radioactivity in DAG was determined and calculated into pmole [^{14}C]DAG per 10^6 cells. (C), Cells were incubated with endothelin for various interval of time and total cellular DAG was measured. Values are the mean ± S.E. of three experiments.

Figure 2: Dose response curve of endothelin effect on [^{3}H] DAG formation (A), and membranous protein kinase C activity in smooth muscle cells (B). (A), Cells were grown on 6-well plates and [^{3}H] DAG formation was measured after 10 min incubation with various concentrations of endothelin in the media at 37°C. (B), Confluent cells in P-150 dishes were incubated for 5 min at 37°C in the presence of various concentrations of endothelin. Protein kinase C activities of the cell pellets (centrifuged at 100,000 x g) were assayed after DE-52 chromatrography. Protein kinase C activities were calculated and corrected for protein content. Values were the mean ± S.D. of three experiments.

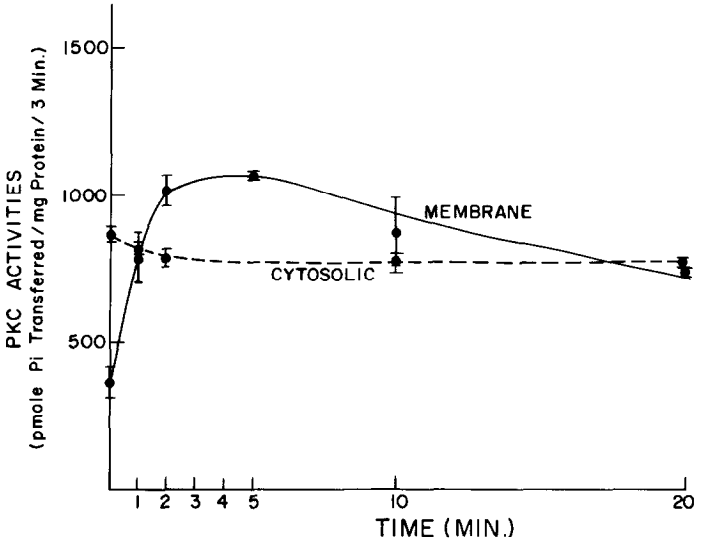


Figure 3: Time course of endothelin on membranous and cytosolic protein kinase C activities in smooth muscle cells. Cells were incubated in the presence of 10^{-7} M endothelin for various intervals of time. PKC activities were assayed as in Figure 2B in both the membranous (solid line) and cytosolic (dotted line) extracts from treated and control cells. Values are expressed as mean ± S.D. of three experiments.

Endothelin's Effect on Total DAG Mass - The time course of alteration in DAG mass during stimulation with 100 nM endothelin is shown in Fig. 1C. The total mass of DAG, increased 10 ± 7% ($P < 0.2$) at 1 min, returned to basal level by 2 min, increased again at 5 min by 50 ± 1% ($P < 0.01$), and returned and remained at basal level by 10 min.

Activation of Protein Kinase C - PKC activity in the particulate (membrane) fraction increased 110 ± 21% ($P < 0.02$) within 1 min after endothelin exposure and then continued to increased to 170% at 2 min (Fig. 3). There was a sustained 170 ± 14% ($P < 0.01$) increase of membranous PKC activity from 2 to 5 min. The increased membranous PKC activity gradually declined from 5 to 20 min, yet it was still 100 ± 4% ($P < 0.01$) above the basal level at 20 min. PKC activity in cytosol concomitantly diminished over the same period and remained decreased at 20 min. The increase in total PKC activity in the membranous fraction can be mostly explained by a shift of the enzyme from cytosol to membrane. However, this is not easily reflected in Fig. 3, because the enzyme activity unit utilized in Fig. 3 is specific activity, pmole Pi transferred per mg protein in 3 min, and the cytosol has 8-10 fold more total protein than the membranous fraction. Fig. 2B shows endothelin induces an increase of membranous PKC activity in a dose-dependent manner. This increase is clearly detected at 10^{-9} M endothelin and appears linear from 10^{-9} to 10^{-7} M.

DISCUSSION

The evidence presented here indicates that endothelin stimulates cellular DAG accumulation and membranous PKC activity in bovine aortic smooth muscle cells.

DAG can be derived from phosphoinositides hydrolysis together with the formation of inositol phosphates (4, 13). Alternatively, DAG formation could also result form an increase of *de novo* synthesis from phosphatidic acid (14-16), hydrolysis of glycolipid (17), hydrolysis of phosphatidylcholine (18). It is therefore possible that some hormones or agonists induce an increase of cellular DAG content without an increase in inositol phosphates levels. Thus, the observation by Hirata et al. (3) that endothelin does not induce changes in inositol phosphates levels in vascular smooth muscle cells do not contradict our findings that endothelin can increase DAG levels. These results suggest that the endothelin-induced DAG accumulation may result from pools other than phosphoinositides hydrolysis. It is possible that multiple pools of DAG exist in different subcellular sites, and endothelin can affect these various pools of DAG in different manners. Evidence for the existence of different DAG pools resides in the observations that endothelin alters DAG levels in different patterns when the labeling time varies. This can also explain the failure to observe an increase of total DAG mass between 10 to 30 min after exposure to endothelin because the metabolism of DAG in some subcellular pool(s) may be enhanced by endothelin whereas decreased in others.

The sustained stimulation of membranous PKC activity by endothelin can be attributed to a translocation of the enzyme from cytosol to membrane. The time course of this sustained stimulation of membranous PKC activity appears to correlate with [^{3}H]DAG formation, suggesting that the the pool(s) of DAG measured by [^{3}H]DAG formation may be related to PKC activation. However, the dose response curve of endothelin on increasing membranous PKC activity is linear from 10^{-9} to 10^{-7} M range while the dose response curve of endothelin on [^{3}H]DAG formation reaches a plateau at 10^{-8} M (Fig. 2). One possible explanation for this discrepancy is that the pools measured by [^{3}H]DAG formation are not stimulated by high concentration of endothelin.

Several hormones and agonists such as angiotensin II and phenylephrine induce a sustained contractile response in vascular smooth muscle with only a transient increase in intracellular calcium (19, 20). Rasmussen et al. proposed that the activation of PKC is involved in the mechanism regulating the sustained phase of contraction while intracellular free calcium regulates the early phase (6). Evidence for the involvement of PKC in regulating smooth muscle contraction is that direct activation of PKC by phorbol esters causes sustained contractions in vascular smooth muscles from different species (6,7). After exposure to endothelin a transient increase of intracellular free calcium was observed in vascular smooth muscle cells (3), while the pressor effect lasts for more than 30 min in both isolated arterial strips or anesthetized animals. The endothelin-induced PKC stimulation reported here, however, lasts for more than 20 min in smooth muscle cells. Given these findings, we propose that an increased intracellular free calcium probably together with the activation of PKC, regulates the early phase of contraction caused by endothelin and that the activation of PKC regulates the sustained phase of contraction.

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