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ENDOTHELIN ACTION: INHIBITION BY A PROTEIN KINASE C INHIBITOR AND INVOLVEMENT OF PHOSPHOINOSITOLS

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Endothelin tightly bound to rabbit aortic strips and caused a prolonged vasoconstriction both in the presence and absence of extracellular Ca^{2+}, although only partial constriction (20-30%) developed in the latter case, indicating that its action may not be limited to the opening of a calcium channel. The endothelin-induced constriction was reversed by the protein kinase C inhibitor, 1-(5-isoquinolynylsulfonyl)-2-methylpiperazine (H-7). In contrast to the observation of Hirata et al (1), endothelin caused a robust phosphatidylinositol breakdown producing inositol mono-, bis-and trisphosphates in cultured rat vascular smooth muscle cells. It showed no effect on cyclic nucleotide levels in the same cultured cells. These results indicate that phosphatidylinositol turnover and protein kinase C activation are involved in endothelin-induced vasoconstriction.

Endothelin is a novel vasoconstrictor peptide, consisting of 21 amino acids, initially purified from the culture medium of porcine aortic endothelial cells(2). Specific high affinity binding sites for endothelin have been identified in cultured rat smooth muscle cells (1). Upon binding, endothelin increases intracellular calcium, resulting in a powerful and sustained vasoconstriction (1,3).

Full vasoconstriction by endothelin appears to depend on extracellular Ca^{2+}. However, the role of intracellular Ca^{2+} stores in the endothelin action has not been elucidated. Indeed, Hirata et al were unable to observe any increase in phosphatidylinositol (PI) turnover after endothelin stimulation (1). This is unusual since endothelin caused a transient increase in intracellular Ca^{2+} levels in the absence of extracellular Ca^{2+} (1,3). Such an increase is usually associated with inositol trisphosphate

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(IP_3) stimulated release of intracellular Ca^{2+} stores. Thus it will be intriguing to clarify the mechanism of endothelin-induced induced vasoconstriction in relation to intracellular and extracellular Ca^{2+} requirements. In the present study, we investigated the potential importance of the well-known phosphoinositol-protein kinase-C mechanism in mediating the action of endothelin.

We report here the effect of 1-(5-isoquinolynylsulfonyl)-2-methylpiperazine (H-7), a potent protein kinase C inhibitor, on endothelin-induced contraction of rabbit aortic strip and the effect of endothelin on PI response as well as cyclic nucleotide levels in cultured rat vascular smooth muscle cells.

Materials and Methods

Chemical: Synthetic endothelin was purchased from the Peptide International Inc. (Louisville, KY). Angiotensin II (ANG II) and atria natriuretic factor (ANF_{99-126}) were purchased from Peninsula Laboratory Inc. (Belmont, CA). 1-(5-Isoquinolynylsulfonyl)-2-methylpiperazine (H-7) was obtained from Sigma (St. Louis, MO) and myo-[2-^{3}H]inositol (20 Ci/mmol) from Amersham Corp. (Arlington Heights, IL). All other reagents were of analytical grade.

Strip Assay: The contractile activity of endothelin and the effect of H-7 were measured using rabbit aortic strips as described previously (4).

Culture of Vascular Smooth Muscle Cells (VSMC): Male, 6-8 week old Wistar-Kyoto rats were decapitated and the thoractic aorta removed immediately. VSMC were isolated according to the procedure of Chamley et al (5). VSMC (3rd passage, 2 x 10^5 cells/well) were seeded in microplate wells (24 wells, 1.6 cm diameter). The cells were allowed to grow for 4 - 5 days in 5% CO_2, 95% air at 37° C. The culture medium used was DMEM with 10% fetal calf serum (FCS).

The Measurement of Cyclic Nucleotide Levels: After the culture medium was removed, cells were washed once with serum free DMEM, then DMEM (0.5 ml) containing 0.1% bovine serum albumin and 1mM isobutylmethylxanthine (medium A) was added. After 10 minutes preincubation medium A was removed and medium A (0.5 ml) with or without a substance whose effect was to be tested was added. The reaction was continued for a period specified for each experiment. The treatment was terminated by the addition of trichloroacetic acid (TCA) to 5%. Diethylether extraction was repeated four times to remove TCA, then the aqueous layer lyophilized. The lyophilized material was dissolved in distilled water. After centrifugation at 2,700 rpm for 10 minutes, the supernatent was separated, acetylated then used for the measurement of cyclic nucleotide contents by radioimmunoassay.

Phosphatidylinositol (PI) Response: Culture medium of VSMC was changed to serum-free DMEM. After 16 - 24 hours, the medium was replaced with DMEM containing myo-[^{3}H]inositol (8-10μCi/ml, 0.5 ml/well). Cells were labelled for 20-24 hours. After treatment with a vasoactive substance, inositolphosphates were separated and counted according to Nabika et al (6).

Results

Endothelin caused a strong and sustained contraction of rabbit aortic strips (Fig. 1A). Such contraction was dose-dependent and persisted for at least 2 hours. Washing the endothelin-contracted strip with Ca^{2+} free buffer, containing 1mM EGTA, resulted in a profound vasorelaxation (Fig 1C). Subsequent replenishment of extracellular Ca^{2+} (2.5 mM) re-established the vasoconstriction. Endothelin also caused vasoconstriction in the absence of extracellular Ca^{2+} (Fig. 1B). Such constriction developed slowly, reaching a maximum tension about 20-30% to that observed in the presence of extracellular Ca^{2+} (Fig 1B). Addition of Ca^{2+} to 2.5 mM again restored the contraction (Fig. 1D).

The protein kinase C inhibitor H-7 (7) caused relaxation of endothelin-contracted strips in the presence or absence of extracellular Ca^{2+} as shown in Fig. 2 top and bottom panels, respectively.

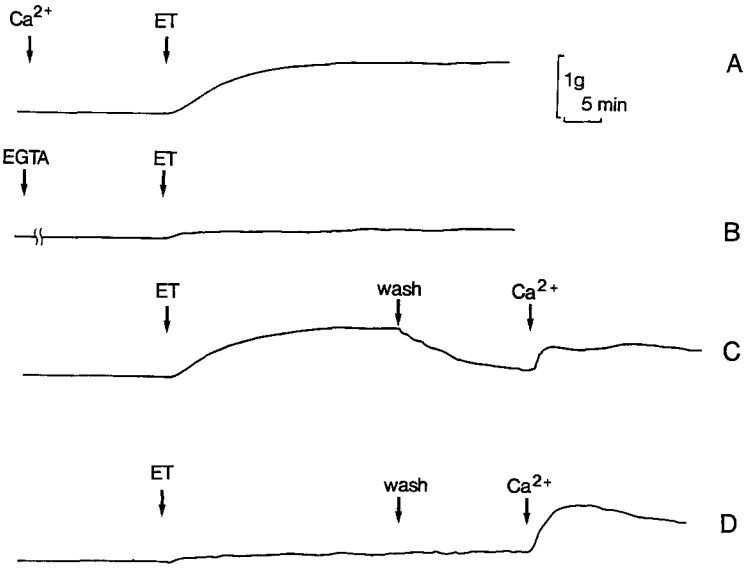

Fig. 1. A: Contraction of rabbit aortic strip by endothelin (ET)(10^{-8}M) in the presence of 2.5 mM Ca^{2+} in the bathing medium. B: Effect of endothelin(10^{-8}M) on rabbit aortic strips in the absence of Ca^{2+} with 1mM EGTA. Endothelin was added to the reaction chamber 20-60 min after the medium was changed to Ca^{2+}-free solution containing 1mM EGTA. C: Effect of Ca^{2+} (2.5 mM) on aortic strip after extensive washing of the strip with Ca^{2+}-free media containing 1mM EGTA. Endothelin had been added initially in the presence of Ca^{2+} as a positive control. D: Effect of Ca^{2+} (2.5 mM) after endothelin addition in the absence of Ca^{2+} then washing with Ca^{2+}-free buffer containing 1mM EGTA.

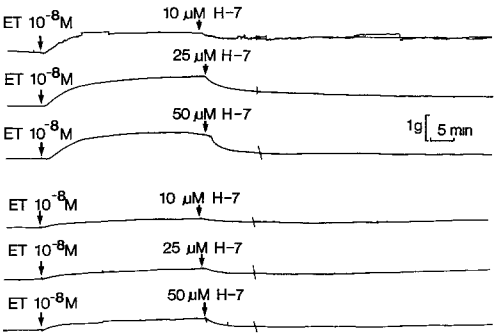

Fig. 2. Effect of H-7 on endothelin-induced contraction in the presence (top panel) and absence (bottom panel) of Ca^{2+} in the bathing medium. H-7 was added to medium after endothelin-induced contraction reached the maximum.

Endothelin (10^{-7}M) did not cause measurable changes either in the intracellualr cAMP (open circles, Fig. 3, left panel) or cGMP level (open circles, Fig. 3, right panel) of cultured smooth muscle cells. ANF significantly increased the intracellular cGMP (open triangles, Fig. 3, right panel) but had no effect on cAMP levels (open triangles, Fig. 3, left panel).

Both endothelin and ANG II stimulated robust increases in inositol phosphates in cultured smooth muscle cells (Fig. 4). ANG II elicited a more

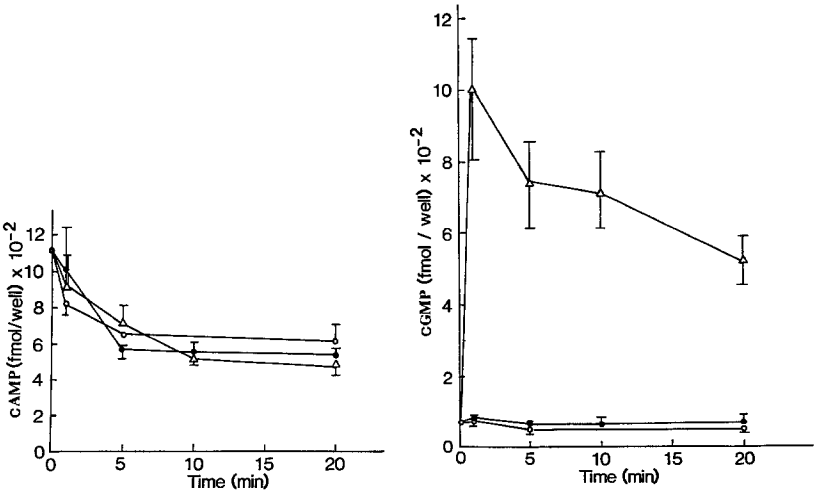

Fig. 3. Left Panel: Effect of endothelin on cAMP production in VSMC. Each point represents the mean value ± S. D. of 3 experiments. Right Panel: Effect of ET on cGMP formation in VSMC. Each point indicates the mean value ± S. D. of 3 experiments. o——o:ET(10^{-7}M); Δ——Δ:ANF(10^{-7}M); ●——● :control.

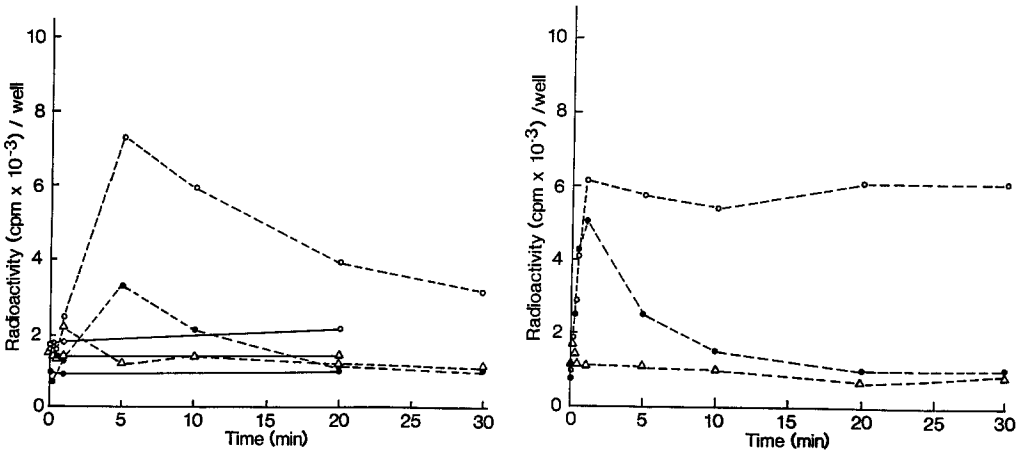

Fig. 4. Left Panel: Effect of ET(endothelin, 10^{-7}M) on IP, IP_2 and IP_3 formation. Each point is the mean value of 3 experiments. Right Panel: Effect of ANG II (10^{-7}M) on IP, IP_2 and IP_3 formation. Each point is the mean value of 3 experiments.
o:IP (inositol monophosphate); •:IP_2(inositol bisphosphate); Δ:IP_3 (inositol trisphosphate); solid line:control; broken line: endothelin or ANG II

rapid PI response (Fig. 4, right panel) than that observed after endothelin stimulation (Fig. 4, left panel). There was no PI response in the absence of endothelin or ANG II.

Discussion

Endothelin is the most potent vasoconstrictor yet discovered (2). The unusually prolonged vasoconstriction induced by endothelin in the presence or absence of extracellular Ca^{2+} (Fig. 1 A & B), suggests that the action of this peptide may profoundly influence blood pressure regulation under normal and pathophysiological conditions. Thus, elucidation of the mechanism by which endothelin induces vasoconstriction will be intriguing and worthwhile.

We observed a powerful and sustained contraction of rabbit aortic strips by endothelin lasting for more than 2 hours (3). Similar contraction was also observed in thoracic aortic strips from spontaneously hypertensive and Wistar-Kyoto rats (data not shown).

Development of full vasoconstriction depended upon the presence of extracellular Ca^{2+} (Fig. 1), as illustrated by the profound relaxation of endothelin-constricted vessels after washing with Ca^{2+} free buffer (1mM

EGTA). Replenishment of extracellular Ca^{2+} to the washed vessel restored vasoconstrictions suggesting that endothelin remained tightly bound to its receptor, allowing continual entry of external Ca^{2+} into the cells. This was confirmed by direct measurement of intracellular calcium using the Ca^{2+} sensitive dye fura-2 in cultured vascular smooth muscle cells (3).

Despite this dependence on extracellular Ca^{2+}, endothelin causes a slowly developing partial constriction of aortic strips in the absence of extracellular Ca^{2+} (Fig. 1 B & D). The same phenomena are observed by Auguet et al (8), using isolated rat aorta. This effect was probably not due to gradual entry of small amounts of external Ca^{2+} sequestered on the surface of vessels because it was observed even after pretreatment in Ca^{2+} free buffer (1mM EGTA) for 60 or 120 minutes. Furthermore, when direct intracellular Ca^{2+} measurements were performed in Ca^{2+} free buffer, endothelin caused a transient increase in intracellular Ca^{2+}, which returned to a stable baseline after 1-2 minutes (3). Although we do not yet know the mechanism by which endothelin causes such vasoconstriction, an increase in the sensitivity of the contractile machinery may be involved.

The protein kinase C inhibitor H-7 caused relaxation of endothelin contracted vessels, both in the presence and absence of extracellular Ca^{2+}, suggesting that protein kinase C may play an important role in mediating the observed vasoconstriction.

The effect of protein kinase C inhibition on vessels which had contracted in the absence of extracellular Ca^{2+} also suggests that protein kinase C may increase the sensitivity of the contractile machinery to Ca^{2+}.

Endothelin did not alter cyclic nucleotide levels suggesting that either cAMP or cGMP are not essential for vasoconstriction induced by endothelin. In fact, 8-Br-cGMP caused marked relaxation of endothelin-constricted vessels (data not shown) suggesting that cGMP stimulation by other hormones may regulate such vasoconstriction. These observations eliminate the possibility that H-7 may have exerted its relaxant effect through nonspecific inhibition of the cAMP or cGMP systems (7).

In contrast to the observations of Hirata et al (1), endothelin caused a robust PI response, comparable to that induced by ANG II. The time required to elicit the maximal response was slower than that by ANG II, a property in concordance with slower vasoconstriction by endothelin. This finding is also consistent with the transient increase in intracellular Ca^{2+} observed in vascular smooth muscle stimulated by endothelin in the absence of extracellular Ca^{2+} (3). Such a response is usually attributed to IP_3-induced release of intracellular Ca^{2+} stores into the cytoplasm may trigger the slow, protein-kinase C-dependent contraction observed in the absence of extracellular Ca^{2+}. Thus, the mechanism of endothelin-induced vasoconstriction may not be solely dependent on extracellular calcium as initially proposed (2). A PI response was also observed in cultured rat mesangial cells (9).

Taken together these observations indicate that the inositol phosphates formation and protein kinase C activation play an important role for endothelin-induced vasoconstriction.

Acknowledgements

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