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DOES ENDOTHELIN MOBILIZE CALCIUM FROM INTRACELLULAR STORE SITES IN RAT AORTIC VASCULAR SMOOTH MUSCLE CELLS IN PRIMARY CULTURE ?

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SUMMARY: In the presence of endothelin, there was a rapid increase in the $^{45}Ca^{++}$ efflux from primary cultured rat vascular smooth muscle cells, both in physiological salt solution and in calcium free medium containing 2 mM EGTA . The $^{45}Ca^{++}$ influx was not affected. The endothelin-induced, transient increase in cytosolic calcium concentration is probably mainly due to release of calcium from the intracellular store in vascular smooth muscle cells.

Yanagisawa et al (1) isolated a potent vasoconstrictor peptide, endothelin, from the conditioned medium of cultured porcine aortic endothelial cells, determined its amino-acid sequence and molecularly cloned the peptide precursor (2). Since it is generally accepted that the contraction of vascular smooth muscle cells (VSMC) is initiated by mobilization of Ca^{++} into the cytosol (3), we considered that endothelin may mobilize Ca^{++} from the extracellular space and/or intracellular Ca^{++} stores to induce contraction of VSMC. To test this hypothesis we examined the effects of endothelin on $^{45}Ca^{++}$ fluxes in primary cultured rat aortic VSMC, a preparation which is suitable for investigating Ca^{++} homeostasis (4,5). We found that, even in the absence of extracellular Ca^{++}, endothelin induced a rapid increase in $^{45}Ca^{++}$ efflux from cultured VSMC in a concentration-dependent manner. Thus, endothelin probably mobilizes Ca^{++} from intracellular store sites.

ABBREVIATIONS : VSMC, vascular smooth muscle cells; PSS, physiological salt solution; EGTA, ethylene glycol-bis (β-aminoethylether) N,N,N',N'-tetraacetic acid; HEPES, N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid.

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MATERIALS AND METHODS

Primary cultures were obtained from the aortic media of male Wistar rats, as described(6). The cells were used at confluence after 10-11 days.

$^{45}Ca^{++}$ Efflux. After rinsing five times with PSS, the cultured VSMC were equilibrated with $^{45}Ca^{++}$ (15 μCi/ml) in 1 ml of PSS for 3 hours at 37°C. The cells were then incubated in 1.4 ml of PSS or Ca^{++}-free PSS containing 2 mM EGTA at 37°C, which was changed every minute for 25 min and trapped in a vial. Endothelin was added at 10 min of the efflux time. The amount of $^{45}Ca^{++}$ released by the cells in each time interval was measured by liquid scintillation counting with 10 ml of ACS II (Amersham Corporation, Arlington Heights, IL,USA). The radioactivity of $^{45}Ca^{++}$ remaining in the cells was also counted, using trypsin and collagenase solution. The number of cells per dish was counted in separate dishes from those cultured at the same time. Fractions lost or $^{45}Ca^{++}$ released per minute per 10^6 cells were calculated and plotted as a function of incubation time (7).

$^{45}Ca^{++}$ Influx. To measure the $^{45}Ca^{++}$ influx the cultured VSMC were rinsed five times with PSS and incubated for 15 min in PSS at 37°C. The cells were then preincubated with endothelin (10^{-7} M) for 0, 2, 5 and 10 min. After the preincubation, the cells were incubated in 1 ml of the solution containing endothelin and $^{45}Ca^{++}$ (1 μCi/ml) for 2 min. To terminate $^{45}Ca^{++}$ uptake and remove external $^{45}Ca^{++}$ the cells were rinsed with Ca^{++}-free PSS containing 2 mM EGTA at 4°C for 10 min (7).

Solutions. The millimolar composition of the normal PSS (pH 7.4 at 25°C) was: NaCl, 140; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; glucose, 10; HEPES, 10. The composition of Ca^{++}-free PSS was similar to normal PSS, except that it contained 2 mM EGTA instead of 1.5 mM $CaCl_2$. High K^+ solution was prepared by replacing NaCl with KCl isosmotically.

Chemicals. Collagenase (type I), elastase (type I) and trypsin (type III) were purchased from Sigma Chemical Co.,St. Louis, MO, USA, endothelin was purchased from Peptide Institute Inc., Osaka, Japan, Dulbecco's modified Eagle's medium was purchased from Gibco Laboratories, Grand Island, NY, USA, and $^{45}CaCl_2$ (32 mCi/mg) was purchased from NEN Research Products (Billerica, MA, USA). All other reagents were of the highest grade commercially available.

RESULTS AND DISCUSSION

The effect of endothelin on $^{45}Ca^{++}$ efflux from cultured VSMC in the presence of 1.5 mM extracellular Ca^{++} (PSS) is shown in Fig. 1. In the control cells, a nearly constant fraction of $^{45}Ca^{++}$ was released from the cells in each 1 min interval from 10-25 min. Addition of 10^{-7}M endothelin at 10 min markedly increased the $^{45}Ca^{++}$ efflux, which reached the maximum within a 1 min interval. Thereafter, the $^{45}Ca^{++}$ efflux rate gradually returned to control levels. This result suggests that endothelin induced the movement of $^{45}Ca^{++}$ in the cultured VSMC.

To determine whether the increase in $^{45}Ca^{++}$ efflux rate from VSMC by endothelin was due to either the release of intracellular Ca^{++} or

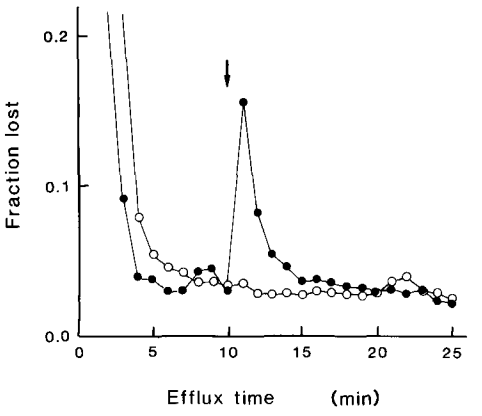
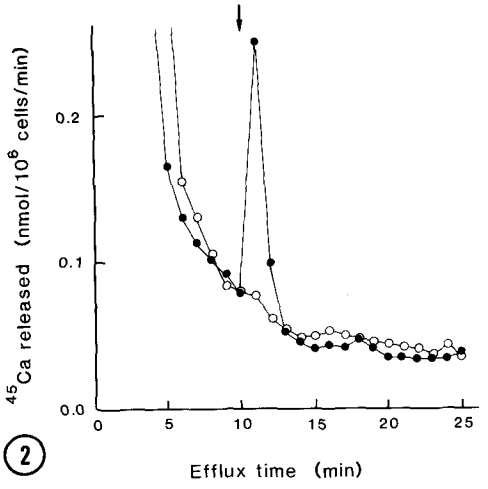


Figure 1. Effect of endothelin on $^{45}Ca^{++}$ efflux from primary cultured rat aortic VSMC in PSS containing 1.5 mM Ca^{++}. 10^{-7} M endothelin was added at 10 min as indicated by the arrow (closed circles). Open circles represent the control cells.

extracellular Ca^{++} influx or both , $^{45}Ca^{++}$ efflux was also determined in Ca^{++}-free medium in the presence of 2 mM EGTA, to chelate the external Ca^{++}. The high extracellular K^{+} (100 mM) under this condition had no effect on $^{45}Ca^{++}$ efflux (data not shown). On the other hand, endothelin (10^{-7}M) under this condition, did increase the $^{45}Ca^{++}$ efflux (Fig. 2). In the first 1 min interval after the addition of endothelin, the amount of $^{45}Ca^{++}$ appearing in the external medium increased three fold over the control and reached a peak. Thereafter, the $^{45}Ca^{++}$ efflux rapidly reverted to control levels. As shown in Fig. 3, this rapid increase in the $^{45}Ca^{++}$ efflux was dose-dependent.

The total amount of $^{45}Ca^{++}$ releasable by endothelin was calculated as a sum of the difference between $^{45}Ca^{++}$ released in the presence of endothelin and that in the control cells. 10^{-6} M endothelin released approximately 238 pmol/10^{6} cells or 79 µmol/liter cells when the volume of 10^{6} cells was assumed to be approximately 3 µl (4). Bond and co-workers reported that the increase in cytoplasmic Ca^{++} during maintained maximal contractions was 1.0 ± 0.2 mmol/kg dry cytoplasm or approximately 235 µmol/liter cell water(8). Thus, the above value estimated as the endothelin-releasable $^{45}Ca^{++}$ seems reasonable.



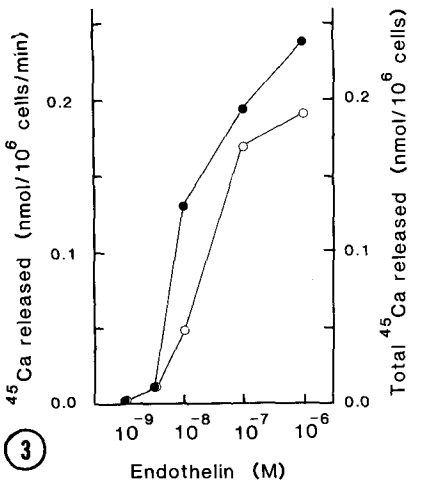


Figure 2. Effect of endothelin on $^{45}Ca^{++}$ efflux from primary cultured rat aortic VSMC in Ca^{++}-free PSS containing 2 mM EGTA. 10^{-7} M endothelin was added at 10 min as indicated by the arrow (closed circles). Open circles represent the control cells.

Figure 3. Dose-response curve for endothelin-stimulated $^{45}Ca^{++}$ efflux. The amount of $^{45}Ca^{++}$ released by endothelin within 1 min after addition was calculated as the difference between $^{45}Ca^{++}$ released into the Ca^{++}-free medium in the presence and absence (the control cells) of various concentrations of endothelin and plotted as a function of endothelin concentration (open circles). The total $^{45}Ca^{++}$ releasable by endothelin was also calculated as a sum of $^{45}Ca^{++}$ released by endothelin every minute (closed circles). The data are means of 2 experiments.

On the other hand, in the presence of 1.5 mM extracellular Ca^{++}, the $^{45}Ca^{++}$ influx of VSMC observed after 2 min of treatment with 10^{-7} M endothelin (including 0, 2, 5 and 10 min of pre-incubation) showed no increase, as compared to findings with the control cells (Fig. 4). The high K^{+} depolarization increased significantly ($p<0.005$) the $^{45}Ca^{++}$ influx (Fig. 4), a finding consistent with the presence of voltage-dependent Ca^{++}-channels in cultured rat aortic VSMC (5).

Yanagisawa et al (1) reported that the endothelin-induced contraction was marked and long lasting and was completely inhibited when it was given in Ca^{++}-free Krebs-Ringer solution containing 1 mM EGTA. The vasoconstriction was markedly attenuated with nicardipine, a Ca^{++}-channel blocker. These findings suggested to them that the influx of extracellular Ca^{++} was required for the action of endothelin. However, we found no detectable increase in Ca^{++} permeability of the plasma membrane, even with a long exposure to endothelin.

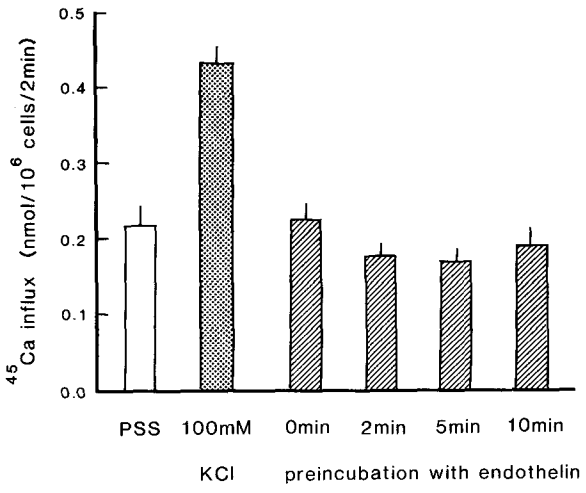


Figure 4. Effect of 10^{-7} M endothelin and 100 mM KCl on $^{45}Ca^{++}$ influx. The cell cultures were pre-incubated with endothelin for 0, 2, 5 and 10 min in normal PSS and then exposed to solution containing both 10^{-7} M endothelin and $^{45}Ca^{++}$ (1 μCi/ml) for 2 min. For 100 mM KCl, the cells were exposed to $^{45}Ca^{++}$ in 100 mM KCl-PSS for 2 min. 100 mM KCl significantly increased ^{45}Ca influx ($p<0.005$ by Student t-test). Each column represents mean ± SD of 6-7 experiments.

The present study shows that endothelin dose-dependently stimulates $^{45}Ca^{++}$ efflux in Ca^{++}-free medium containing 2 mM EGTA (Fig. 3). There are several possible interpretations for this increase in $^{45}Ca^{++}$ efflux by endothelin, including: 1) mobilization of Ca^{++} from intracellular store sites followed by extrusion, 2) primary activation of Ca^{++} extrusion or 3) release of extracellularly bound Ca^{++} not removed by EGTA containing solution. However, the primary activation of Ca^{++} extrusion by endothelin itself is not likely because we observed that the porcine coronary artery contracted and did not relax, in Ca^{++}-free medium containing 2 mM EGTA (unpublished observations). In addition, the endothelin-induced increase in $^{45}Ca^{++}$ efflux depended on temperature with a Q_{10} of 5 (data not shown), hence may be mediated by energy dependent cellular mechanisms. We tentatively conclude that endothelin elevates cytosolic Ca^{++} level mainly by releasing Ca^{++} from intracellular Ca^{++} stores, which in turn activates Ca^{++} extrusion mechanisms and contractile proteins.

The contraction induced by the release of Ca^{++} from the intracellular store, without Ca^{++} influx from extracellular spaces is usually not marked

and is transient (9), thereby differing from that reported in case of endothelin (1). To clarify mechanisms related to the characteristic contraction induced by endothelin, processes of contraction other than a Ca^{++}-mediated ones will have to be given attention.

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