Vol. 164, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1989 Pages 474-479

ENDOTHELIN-1 INCREASES INTRACELLULAR CALCIUM MOBILIZATION BUT NOT CALCIUM UPTAKE IN RABBIT VASCULAR SMOOTH MUSCLE CELLS

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Received August 10, 1989

SUMMARY: Conflicting evidence has been reported regarding the role of endothelin-1, a potent vasoconstrictor peptide, in stimulating extracellular calcium influx in rabbit vascular smooth muscle. The objective of this study was to elucidate the effects of endothelin-1 on transmembrane $^{45}Ca^{2+}$ influx and intracellular calcium mobilization in cultured rabbit aortic smooth muscle cells. In calcium containing buffer, endothelin-1 induced a concentration-dependent $^{45}Ca^{2+}$ efflux response over the range of 10 pM to 100 nM with an EC_{50} of approximately 60 pM. Maximum endothelin-stimulated $^{45}Ca^{2+}$ efflux was not affected by the absence of extracellular calcium or the presence of 1 μM verapamil. Endothelin-1 did not induce transplasmalemmal $^{45}Ca^{2+}$ uptake at times up to 30 min. These findings suggest that an alteration in intracellular calcium handling, rather than extracellular calcium influx, is responsible for the endothelin-stimulated increase in intracellular calcium concentration in rabbit aortic smooth muscle cells.

Endothelin-1, a 21 amino acid peptide isolated from the culture supernatant of porcine aortic endothelial cells, is a potent vasoconstrictor of a variety of blood vessels. Initial molecular characterization of ET by Yanagisawa et al. (1) demonstrated that this peptide has remarkable sequence homology with conotoxins and alpha-scorpion toxins which bind to tetrodotoxin-sensitive sodium channels. This information, combined with evidence indicating that ET-stimulated contraction of porcine aorta is dependent on extracellular calcium and inhibited by nifedipine, led the investigators to propose that ET is an endogenous agonist of voltage-dependent calcium channels. In rabbit aortic smooth muscle, the effect of ET on the influx of extracellular calcium is not clear. Although Marsden et al. (2) demonstrated that ET-stimulated contractions of rabbit aortic rings are inhibited by nifedipine, Hay et al.(3) found the response of similar ring preparations to be insensitive to inhibition by this calcium channel antagonist. Only one laboratory has described the direct measurement of $^{45}Ca^{2+}$ influx (2) in cultured rabbit vascular smooth muscle cells. However,

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The abbreviations used are: ET, endothelin-1; PSS, physiologic salt solution; IP_3, inositol trisphosphate; DAG, diacylglycerol.

0006-291X/89 $1.50

interpretation of their results is difficult due to the limited data presented. Thus, the principal objective of this study was to clarify the effects of ET on transplasmalemmal $^{45}Ca^{2+}$ influx and intracellular calcium mobilization in cultured vascular smooth muscle cells from the rabbit aorta.

MATERIALS and METHODS

Cell Culture: Rabbit aortic vascular smooth muscle cells were cultured from aortic medial tissue by an enzymatic dissociation method as previously described (4). Confluent cell monolayers employed in these experiments were from subculture 5 through 30 and were used for experiments 8 to 14 days after the time of passage.

$^{45}Ca^{2+}$ Efflux: Measurement of $^{45}Ca^{2+}$ efflux was performed as previously reported (5). Briefly, cell monolayers plated on 35 mm dishes were equilibrated with $^{45}Ca^{2+}$ for 18-24 hr in serum-free minimal essential medium containing 2 μCi/ml of $^{45}CaCl_2$. Following equilibration, the cells were washed rapidly 3 times with PSS at 37 °C. The millimolar composition of normal PSS was: NaCl 130; KCl 5; $CaCl_2$ 1.5; $MgCl_2$ 1; glucose 5; HEPES 20. Zero calcium (Ca^{2+}-free) PSS contained 2 mM EGTA instead of $CaCl_2$. Efflux of $^{45}Ca^{2+}$ was initiated by the addition of 1 ml PSS containing ET or vehicle and other agents under study. The assay was terminated after 6 min by washing the cells 5 times with ice-cold PSS containing 10 mM $LaCl_3$ and no $CaCl_2$. The cells were then dissolved with 1 ml of .1 N HNO_3 and cell associated $^{45}Ca^{2+}$ determined by counting the radioactivity within the acid digest using standard scintillation techniques. Data were calculated as described in the figure legends and the Student t-test was used for statistical comparison of the results. Differences were considered significant when $P<.05$.

$^{45}Ca^{2+}$ Uptake: Cell $^{45}Ca^{2+}$ uptake was also determined as previously described with minor modifications (6). The assay was initiated by adding 1 ml of PSS containing 2 μCi/ml of $^{45}Ca^{2+}$ with or without ET and the other agents under study. High K^+ (55 mM) solution was prepared by replacing NaCl with isosmotic KCl. Uptake was terminated at various time intervals using the same procedures described for the efflux assay.

Materials: Endothelin was purchased from Cambridge Research Biochemicals, Inc., Valley Stream, NY. Lyophilized ET was resuspended in .1 g/dl BSA to obtain a stock solution of 10 mM. $^{45}CaCl_2$ (13.4 mCi/mg) was obtained from ICN Biomedicals, Inc., Irvine, CA. Verapamil was purchased from Sigma Chemicals,Inc., St. Louis, MO. All other reagents were of the highest quality commercially available.

RESULTS

Synthetic ET induced a concentration dependent $^{45}Ca^{2+}$ efflux response from cultured rabbit vascular smooth muscle cells (Fig. 1) with an EC_{50} of 57 $\pm$ 5 pM. The threshold concentration for ET-stimulated $^{45}Ca^{2+}$ efflux was approximately 1 pM whereas maximum efflux occurred using 10 nM of the peptide. Maximum concentrations of ET (.1 μM) elicited the efflux of 36 $\pm$ 3% of total cell calcium. In comparison, 10 μM norepinephrine and .1 μM bradykinin stimulated the efflux of 20 $\pm$ 4% and 30 $\pm$ 6% of total cell calcium, respectively. These results demonstrate that ET is an effective activator of cell calcium efflux.

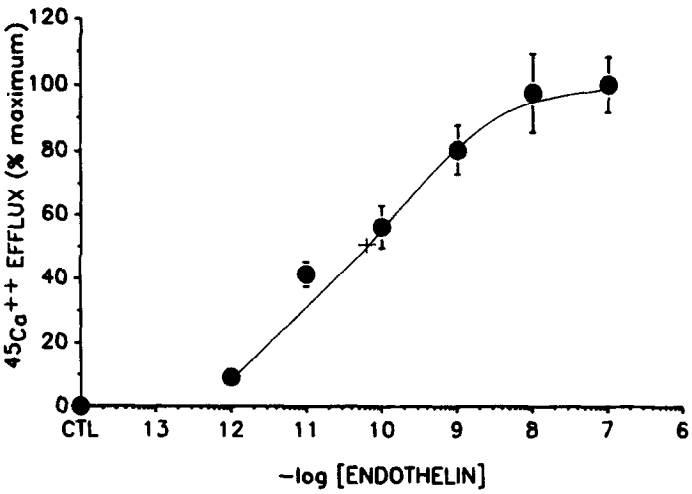

Fig. 1. Concentration-dependence of ET-stimulated $^{45}Ca^{2+}$ efflux from cultured rabbit aortic vascular smooth muscle cells in PSS containing 1.5 mM Ca2+. The amount of $^{45}Ca^{2+}$ released by ET within 6 min after addition was calculated as the percentage difference between $^{45}Ca^{2+}$ remaining in ET-stimulated cells and non-stimulated control cells. Points are expressed as the mean ± SEM of 3 experiments each performed in triplicate.

Several studies were undertaken to determine whether the ET-stimulated increase in $^{45}Ca^{2+}$ efflux was due to the mobilization of stores of intracellular calcium, to influx of extracellular calcium, or both. Maximum ET-stimulated calcium efflux was assessed after a 30 min preincubation with either 1 μM verapamil or Ca^{2+}-free PSS containing 2 mM EGTA. Neither verapamil nor Ca^{2+}-free buffer significantly reduced the ET-stimulated $^{45}Ca^{2+}$ efflux response (Table I). This data demonstrates that ET-induced $^{45}Ca^{2+}$ efflux is not dependent on the influx of extracellular calcium, but rather is due to mobilization of intracellular calcium stores.

The effect of ET on transplasmalemmal calcium influx was characterized using $^{45}Ca^{2+}$ uptake procedures. Fig. 2 compares the magnitude of $^{45}Ca^{2+}$ uptake at 4 min in response to .1 μM ET and 55 mM KCl. The uptake of $^{45}Ca^{2+}$ stimulated by ET (-1 ± 7%) was not significantly altered compared to non-stimulated control cells. In contrast, KCl stimulated $^{45}Ca^{2+}$ uptake by 38 ± 4% ($P<.05$) compared to similar

Table I.

Effect of verapamil (VP) and Ca^{2+}-free PSS on maximum ET-stimulated $^{45}Ca^{2+}$ efflux

	ET Control	ET +1 μM VP	ET + Ca^{2+}-free PSS
% Efflux	36 ± 2	31 ± 4	41 ± 3
n =	14	4	3

Cell monolayers were preincubated for 30 min in the presence of VP or Ca^{2+}-free PSS prior to initiating and throughout the efflux period.

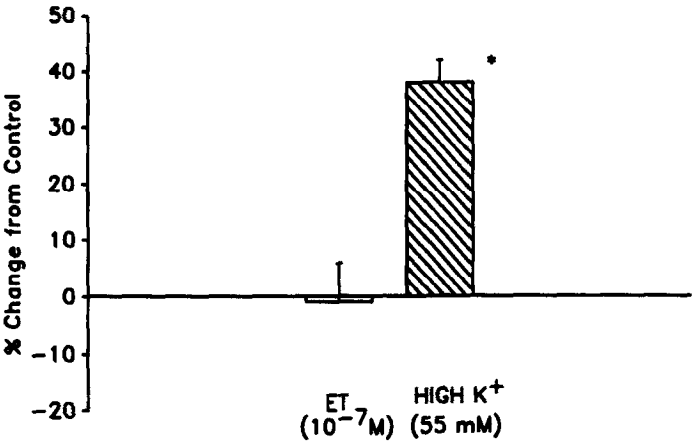

Fig. 2. Effect of .1 μM ET and 55 mM KCl on $^{45}Ca^{2+}$ uptake. Cell monolayers were incubated with 2 μCi/ml $^{45}Ca^{2+}$ for 4 min in the absence (control, CTL) or presence of agonist. Each column represents the mean ± SEM of at least 4 experiments each performed in triplicate. *P<.05 compared to non-stimulated control cells.

control cells. Fig. 3 shows the ET-stimulated $^{45}Ca^{2+}$ uptake response over 30 min as the percent change from control in rabbit smooth muscle cells. ET-stimulated responses were not different from controls at any time point from 30 sec to 30 min. These data suggest that ET does not induce measureable $^{45}Ca^{2+}$ uptake in subcultured rabbit vascular smooth muscle cells.

DISCUSSION

This study demonstrates that ET stimulates a concentration-dependent increase in cellular calcium efflux from cultured rabbit vascular smooth muscle

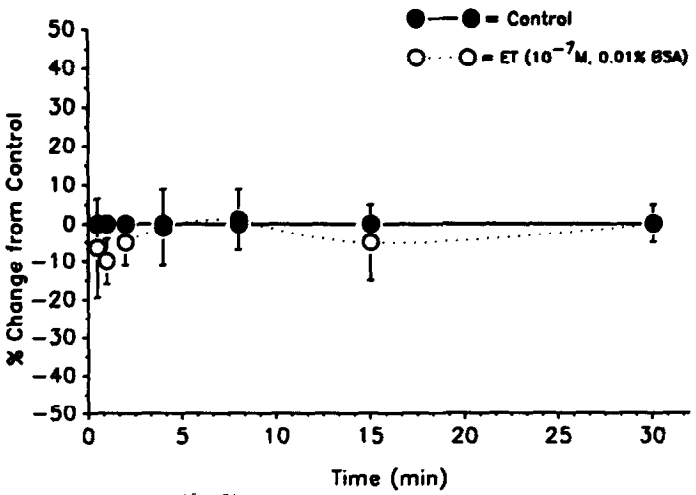

Fig. 3. Time course of $^{45}Ca^{2+}$ uptake in response to .1 μM ET expressed as the percentage change from non-stimulated control cells. Cell monolayers were exposed to 2 μCi/ml $^{45}Ca^{2+}$ in normal PSS with or without ET for 0, .5, 1, 2, 4, 8, 15, and 30 min. Points are expressed as the mean ± SEM and represent data from 4-7 experiments each performed in triplicate.

cells. The ET-stimulated increase in $^{45}Ca^{2+}$ efflux which we observed was not attenuated by the blockade of voltage dependent calcium channels or the absence of extracellular calcium. These data corroborate the findings of a previous report in which ET-stimulated $^{45}Ca^{2+}$ efflux from cultured rabbit vascular smooth muscle was not inhibited by exposure to Ca^{2+}-free buffer (2). Taken together, these results strongly suggest that in rabbit vascular smooth muscle ET affects the mobilization of intracellular calcium stores.

The increase in ET-stimulated $^{45}Ca^{2+}$ efflux was not accompanied by a demonstrable $^{45}Ca^{2+}$ uptake response over a wide range of times. This observation is consistent with the preliminary findings of Hay, et al. (3), who have shown that ET elicits a concentration-dependent vasoconstriction of rabbit aortic rings which is insensitive to inhibition by nifedipine, nicardipine, or nominally Ca^{2+}-free buffer. As noted above, the inability to demonstrate a significant $^{45}Ca^{2+}$ influx response in our studies is not in agreement with the results of Marsden, et al. (2). However, it is difficult to interpret the results of the latter study due to the limited data available. In contrast to our findings in rabbit vascular smooth muscle cells, it has been shown that in rat vascular smooth muscle cells ET increases transplasmalemmal $^{45}Ca^{2+}$ influx and that an increase in $[Ca^{2+}]_i$ is partially dependent on the presence of extracellular calcium (2, 7-12). However, at least one preliminary report (13) has found that nicardipine has no affect on the ET-stimulated rise in $[Ca^{2+}]_i$ of cultured vascular smooth muscle. We speculate that the lack of a significant ET-stimulated $^{45}Ca^{2+}$ uptake response in cultured rabbit cells may represent a fundamental species difference in the calcium response to ET. Alternatively, we can not exclude the possibility that the species differences in $^{45}Ca^{2+}$ influx are a consequence of variations in cell culture techniques.

The mechanism of action of ET is not fully understood. Recent reports indicate that in rabbit vascular smooth muscle ^{125}I-ET binds to specific cell surface receptors distinct from the voltage-dependent calcium channel (2). Endothelin binding initiates the phospholipase C-mediated hydrolysis of phosphatidyl inositol-bis-phosphate to IP_3 and DAG (2, 14, 15). Sunako, et al. (15) demonstrated that ET induced biphasic formation of IP_3 and DAG in rabbit vascular smooth muscle with an early transient peak at 30 sec and a late sustained phase at 5 min. Other investigators have used similar cells to show early transient and late sustained elevations of $[Ca^{2+}]_i$ which correlate with the formation of IP_3 and DAG (2, 16). In addition to the hydrolysis of inositol phosphates, Takayasu, et al. (16) have demonstrated that inhibitors of prostaglandin and thromboxane A_2 synthesis prevented the ET-stimulated increase in $[Ca^{2+}]_i$, suggesting that arachidonic acid metabolites may be involved in the

ET-induced mobilization of intracellular calcium stores. If production of inositol phosphate and arachidonic acid metabolites contribute to the elevation of $[Ca^{2+}]_i$ levels in rabbit vascular cells, it is possible that sustained release of intracellular calcium stores may explain the sustained ET-stimulated vasoconstriction of rabbit aorta observed in the absence of extracellular calcium.

ACKNOWLEDGMENTS

The authors would like to thank Ms. Shauna MacVeigh for expert technical assistance. This work was suported by N.I.H. Grant R01-HL-42539. R.A.B. and N.J.I. are supported in part by fellowships from the Massachusetts Heart Association. W.S.C. is an AHA-Sandoz Established Investigator of the American Heart Association.

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