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DEPOLARIZATION POTENTIATES ENDOTHELIN-INDUCED EFFECTS ON CYTOSOLIC CALCIUM IN BOVINE ADRENAL CHROMAFFIN CELLS

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SUMMARY:The effects of endothelin on intracellular calcium concentrations ($[Ca_i^{2+}]$) in primary cultures of bovine adrenal chromaffin cells (BAM) were measured using Fura 2. Endothelin had minimal effects on $[Ca_i^{2+}]$ over a broad dose range (1 nM to 1 μM). However, in conjunction with K^+ depolarization there was a synergistic increase in $[Ca_i^{2+}]$. This effect was dependent on extracellular calcium as was the response to KCl alone. A partial synergistic effect was evident with endothelin and nicotinic stimulation. The effects of endothelin and angiotensin II on $[Ca_i^{2+}]$ are only additive. Blockade of voltage sensitive calcium channels failed to alter the synergistic effects. Our results indicate that endothelin influences BAM calcium mobilization through sites regulated by membrane depolarization but differing from traditional voltage sensitive calcium channels.

Endothelin, a 21-residue peptide isolated from endothelial cells (1), exhibits significant structural homology to peptide neurotoxins (α-scorpion toxins) that modulate tetrodotoxin and voltage sensitive sodium channels (2) and has been proposed to act directly on voltage sensitive (i.e., dihydropyridine sensitive) calcium channels (1). It has been shown that endothelin and sarafotoxin-b bind with high affinity to the same population of sites in the rat atrium and brain, and that both stimulate phosphoinositide hydrolysis that is, in part, calcium-independent (3). Controversy exists as to the second messengers involved in mediating endothelin's mechanism of action on vascular smooth muscle. While there is agreement that endothelin increases cytosolic calcium $[Ca_i^{2+}]$ in smooth muscle and other tissues (4-8), some have argued that the source of the calcium is extracellular (1,7) while others propose that it originates from an intracellular store through inositol phosphate sensitive sites (4,5,8). Even less is known about the effects of endothelin on neurons despite its presence in the central nervous system (9). Since bovine adrenal chromaffin cells (BAM) are peripheral postganglionic sympathetic neurons and considered cellular models for neurosecretion, we investigated the effects of endothelin on $[Ca_i^{2+}]$,

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catecholamine secretion, and phosphoinositide turnover, alone and in combination with known secretagogues, in order to elucidate the mechanism of action of endothelin in these cells.

MATERIALS AND METHODS

Chemicals: Endothelin was graciously provided by Dr. T. Watanabe of the Peptide Institute (Osaka, Japan), [^{3}H]-norepinephrine (13 Ci/mmol) and [^{3}H]-myoinositol (14.3 Ci/mmol) from New England Nuclear (Boston, MA), Dowex AG1-X8 from BioRad (Richmond, CA) and Fura 2-AM from Molecular Probes (Eugene, OR).

Cell Culture and Calcium Measurement: Chromaffin cells were isolated from bovine adrenal glands obtained locally using modifications of the method of Greenberg and Zinder (10) using Percoll centrifugation to obtain a chromaffin cell enriched preparation. Differential plating did not alter secretion, calcium mobilization or angiotensin receptor binding results indicating minimal contamination by non-chromaffin cells, including endothelium. Cells were maintained in suspension cultures at 37°, 5% CO_2 overnight in MEM media containing 10% fetal calf serum, 1% l-glutamine and 1% non-essential amino acids. For [Ca_i^{2+}] measurements, cells were resuspended at $4x10^6$ viable cells/ml in Locke's buffer with 2.2 mM $CaCl_2$ and 5 mg/ml BSA, loaded for 20-30 minutes with 1 μM Fura 2-AM at room temperature, washed and resuspended in Locke's with 2.2 mM $CaCl_2$. Fluorescence measurements of suspended cells used continuous stirring to prevent bleaching of the Fura 2-AM in a SPEX fluorimeter (excitation at 340 and 380 nm, emission at 505 nm) at room temperature and cation calculation was performed according to the method by Grynkiewicz, et. al.(11).

Catecholamine Secretion and Phosphoinositide Turnover: Chromaffin cells were isolated as above but plated in 12 well plates at a density of 4 x 10^5 cells/well and cultured for 5-7 days. To measure catecholamine secretion, cells were prelabelled with [^{3}H]-norepinephrine (1 μCi/ml for 1-3 hours) and secretion measured by the method of Kenigsberg and Trifaro, 1980 (12). Data are expressed as percent catecholamine secreted (into the media) compared to that retained in the cells. Turnover of inositol phosphates was determined after prelabelling the cells with [^{3}H]-myoinositol (10 μCi/ml) for 72 hours, the reaction terminated and inositol phosphates separated on Dowex AG1-X8 (100-200 mesh columns (13,14).

RESULTS

Intracellular Calcium Response: The effects of endothelin in doses of 1 nM to 1 μM on [Ca_i^{2+}] are evident in Figure 1. The kinetics reflect a slow accumulation of [Ca_i^{2+}] but a sustained response. No effect of endothelin is observed at 1 nM while the maximal increase in [Ca_i^{2+}] is only 42 $\pm$ 2.1 nM (n=17) at the highest doses of endothelin, 100 nM or 1 μM. This contrasts with most other peptides (e.g., angiotensin, bradykinin), secretagogues (histamine) or depolarizing agents, which exhibit a rapid accumulation (spike) to much higher levels of [Ca_i^{2+}] (increases of 400-600 nM [Ca_i^{2+}]) and a more rapid return to baseline.

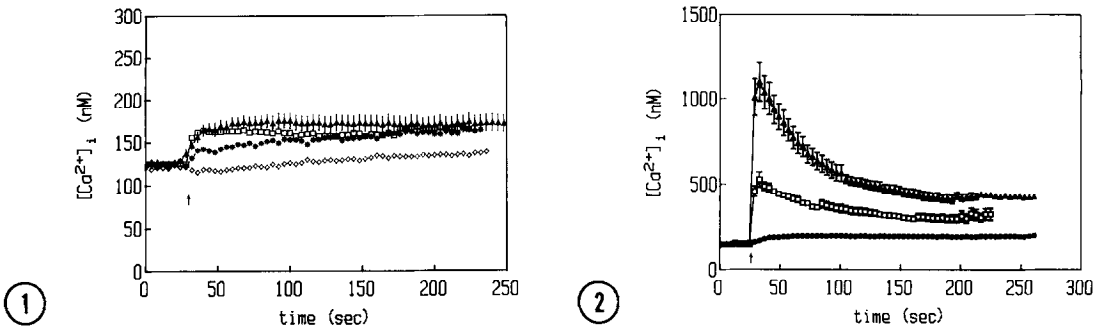

Figure 1. Kinetics of $[Ca_i^{2+}]$ in response to varying concentrations of endothelin. Arrow denotes time of addition of peptide. Symbols are as follows: (◇) 1 nM; (✱) 10 nM; (▲) 100 nM; ([]) 1 μM. Data points are averages from multiple experiments: mean $\pm$ standard deviation, n = 15.

Figure 2. Kinetics of $[Ca_i^{2+}]$ in response to 55 mM KCl ([]), 100 nM endothelin (●) and the simultaneous addition of both agents (▲). Arrow denotes time of addition. Results are expressed as mean $\pm$ standard deviation, n = 12.

A depolarizing dose of KCl (55 mM) produces a strong transient of $[Ca_i^{2+}]$ as shown in Figure 2. A marked synergism is evident when 100 nM endothelin is added with 55 mM KCl. The synergism is absolutely dependent on extracellular calcium (data

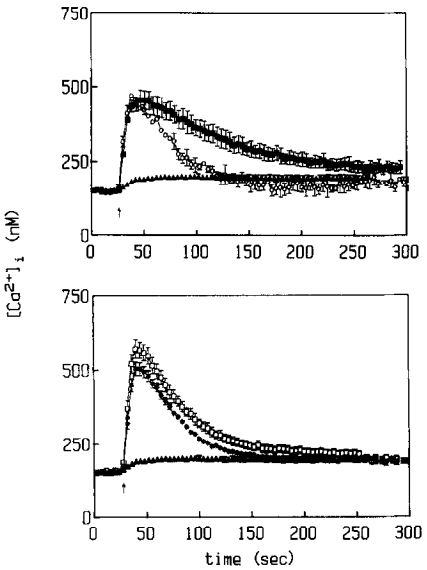

Figure 3. Kinetics of $[Ca_i^{2+}]$ in response to combinations of endothelin and either angiotensin II or nicotine. Arrow denotes time of addition. Top panel: Response to 5 μM nicotine (o) alone, 100 nM endothelin (▲) alone and the combination (■). Endothelin potentiates the duration of the nicotine $[Ca_i^{2+}]$ response. Bottom panel: Simple additivity of the combination of angiotensin II and endothelin on $[Ca_i^{2+}]$; 100 nM angiotensin II (✱) alone, 100 nM endothelin (▲) alone and the combination when added simultaneously ([]). Results are shown as mean $\pm$ standard deviation for each panel, n = 12.

not shown) as is the response to KCl alone. The addition of endothelin and nicotine (Figure 3 top panel) results in a potentiation of the plateau phase without changing the magnitude of the spike. In contrast, the combination of endothelin and angiotensin II results merely in simple additivity (Figure 3 bottom panel).

In order to identify the calcium channels involved in the response, nifedipine, nitrendipine and verapamil were added to block voltage-dependent channels. While we observed some attenuation of the KCl-mediated calcium response, there was no inhibition of the synergistic interaction between endothelin and KCl.

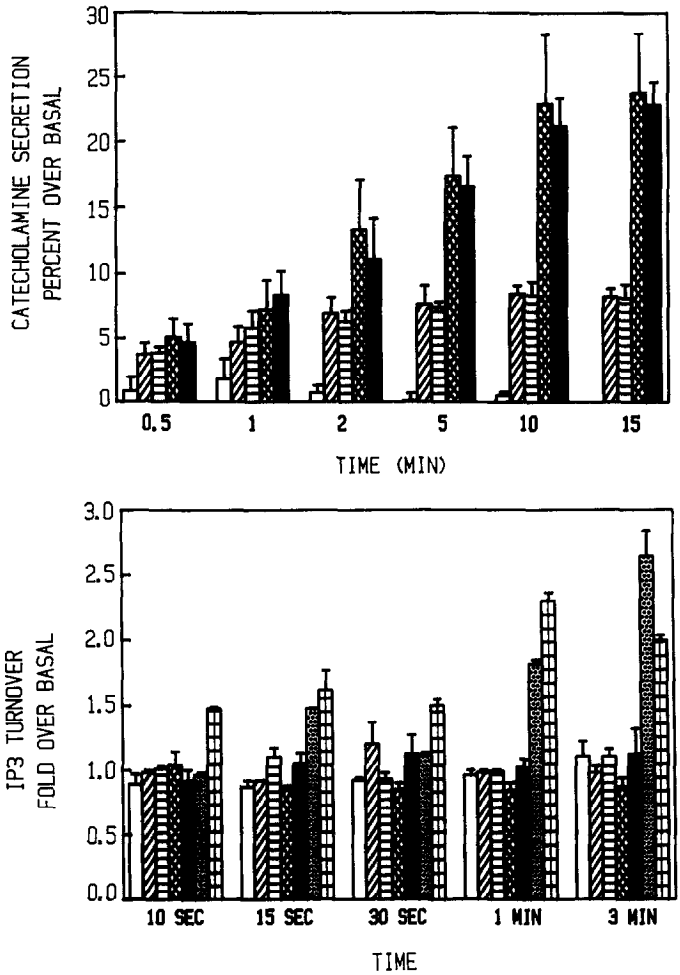

Figure 4. Effects of endothelin and other secretagogues, alone and in combination, on kinetics of norepinephrine secretion and inositol triphosphate turnover from labelled chromaffin cells. Top panel: catecholamine secretion ([^{3}H]-norepinephrine) expressed as percent over basal. Bottom panel: [^{3}H]-Inositol trisphosphate turnover expressed as fold increase over basal. Bar patterns are: 100 nM endothelin ([]); 55 mM KCl (//); 55 mM KCl plus endothelin (≡); 10 μM nicotine (X); 10 μM nicotine plus 100 nM endothelin (■); 1 μM angiotensin II alone (▒); 100 nM bradykinin alone (+).

Catecholamine Secretion and Inositol Phosphate Production: Endothelin (100 nM) is a very weak secretagogue by itself (1-2% over basal) and does not alter secretion induced by KCl or nicotine (Figure 4 top panel). There was no significant production of IP_3 induced by endothelin alone or in combination with either KCl or nicotine (Figure 4 bottom panel).

DISCUSSION

The adrenal medulla is highly vascularized with blood perfusing from the cortex through the medulla and into the venous circulation. The content of endothelium in the medulla is therefore very high and potential release of endothelin, a product of endothelial cells and the most potent endogenous vasoconstrictor isolated (1), could potentially influence chromaffin cell function. However, the dose-response curve for endothelin indicates that it has minimal effects, by itself, on either $[Ca_i^{2+}]$ or catecholamine secretion at doses much greater than physiological. This could reflect few receptors on the chromaffin cell. However, when endothelin is added in conjunction with a depolarizing agent (55 mM KCl or nicotine) synergism or potentiation of enhanced $[Ca_i^{2+}]$ is evident. Despite the synergistic increase in $[Ca_i^{2+}]$, there does not appear to be a functional correlation to the potentiation of the intracellular calcium signal in BAM cells. For example, there is no increase in catecholamine secretion associated with endothelin alone nor potentiation of secretion in response to KCl or nicotine. Thus, $[Ca_i^{2+}]$ levels and secretory activity are uncoupled.

It has been proposed that endothelin exerts its action on vascular smooth muscle through a binding site on the dihydropyridine (voltage-sensitive) calcium channel (1,15,16). For example, Goto, et. al. (15), showed that in a high K^+ depolarizing solution endothelin augments a calcium-induced contraction in porcine coronary artery smooth muscle. They proposed that endothelin acts by activating voltage-dependent calcium channels through an increase in the number or availability of channels. However, we were unable to inhibit the synergism between endothelin and KCl with voltage-dependent, calcium channel blockers.

Our results suggest that endothelin binds to a site other than a voltage-dependent calcium channel. Since there was synergism between endothelin and KCl, this may indicate that membrane depolarization exposes additional endothelin binding sites. Alternatively, there may be positive cooperative interactions between voltage-dependent calcium channels and endothelin-gated calcium channels. In this regard, Fenwick et. al. (17) found a that a facilitation of Ca^{2+}-channel currents occurs in BAM cells

with a prior depolarization pulse. Further, Hoshi et. al. (18) showed that the facilitation of Ca^{2+}-channel current is dependent on a change in voltage and not on Ca^{2+} entry.

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