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Endothelin-Stimulated Capacitative Calcium Entry in Enteric Glial Cells: Synergistic Effects of Protein Kinase C Activity and Nitric Oxide

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Abstract: Depletion of intracellular calcium stores by agonist stimulation is coupled to calcium influx across the plasma membrane, a process termed capacitative calcium entry. Capacitative calcium entry was examined in cultured guinea pig enteric glial cells exposed to endothelin 3. Endothelin 3 (10 n*M*) caused mobilization of intracellular calcium stores followed by influx of extracellular calcium. This capacitative calcium influx was inhibited by Ni^{2+} (89 ± 2%) and by La^{3+} (78 ± 2%) but was not affected by L-, N-, or P-type calcium channel blockers. Chelerythrine, a specific antagonist of protein kinase C, dose-dependently inhibited capacitative calcium entry. The nitric oxide synthase inhibitor N^G-nitro-L-arginine decreased calcium influx in a dose-dependent manner. The combination of chelerythrine and N^G-nitro-L-arginine produced synergistic inhibitory effects. Capacitative calcium entry occurs in enteric glial cells via lanthanum-inhibitable channels through a process regulated by protein kinase C and nitric oxide. **Key Words:** Glial cell—Calcium—Endothelin—Myenteric plexus.
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Initially identified as potent constrictors of vascular smooth muscle, endothelins (ETs) have been demonstrated to have a broader array of biological effects, ranging from smooth muscle contraction, to cell growth, to neuronal signal transduction (Stojilkovic and Catt, 1992; Battistini et al., 1993; Huggins et al., 1993; Stephenson et al., 1994). In the gastrointestinal system, ETs have been reported to influence acetylcholine-induced intestinal contractility, contraction of isolated smooth muscle cells (Yoshinaga et al., 1992), colonic secretion (Kiyohara et al., 1993), and development of the enteric nervous system (Baynash et al., 1994). Disruption of the endothelin B (ET-B) receptor gene in mice results in aganglionic megacolon resembling human Hirschsprung's disease (Puffenberger et al., 1994). These observations suggest potential roles for ETs in regulation of gastrointestinal function, although involved mechanisms remain undefined.

The enteric nervous system is composed of a mixture of cell types, including neurons, glial cells, and occasional fibroblasts. Glia outnumber neurons and are usually depicted as having supportive or nutritive roles for enteric neurons. Recent studies suggest that enteric glia may also function in information transfer. Enteric glia have cell-to-cell coupling and respond to a variety of neuroligands, including ETs, with calcium signaling (Kimball and Mulholland, 1996; Zhang et al., 1997). Enteric glia possess an ET-B receptor linked to phospholipase C.

In several cell types, the actions of ETs are associated with increases in intracellular calcium concentration ($[Ca^{2+}]_i$). Recent studies have demonstrated that ET-evoked increments in $[Ca^{2+}]_i$ are composed of an initial rapid, but transient, response caused by release of intracellular calcium stores and a subsequent prolonged elevation mediated via extracellular calcium entry (Supattapone et al., 1989; Highsmith et al., 1992; Stojilkovic and Catt, 1992). There is strong evidence that inositol trisphosphate-sensitive intracellular stores are responsible for the initial response (Highsmith et al., 1992; Stojilkovic et al., 1992; Zhang et al., 1997). The mechanisms controlling the prolonged elevation of $[Ca^{2+}]_i$ have not been determined. In nonexcitable cells, calcium released from intracellular stores induces calcium influx across the plasma membrane, a process

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Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; ET, endothelin; ET-B, endothelin B; ET_3, endothelin 3; fura-2/AM, fura-2 acetoxymethyl ester; Me-TC, *S*-methyl-L-thiocitrulline; D-NA, N^G-nitro-D-arginine; L-NA, N^G-nitro-L-arginine; L-NIO, L-N^5-(1-iminoethyl)ornithine; L-NMMA, N^G-monomethyl-L-arginine monoacetate; NO, nitric oxide; NOR3, (±)-(*E*)-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexeneamide; NOS, nitric oxide synthase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; SNAP, (±)-*S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

termed capacitative calcium entry (Putney, 1990; Putney and Bird, 1993; Berridge, 1995). Capacitative calcium entry can be observed in several cell types after experimental measures that deplete calcium from intracellular stores (Putney, 1991). In the present study, depletion of intracellular calcium stores by ET caused capacitative calcium entry in enteric glia. The current studies indicate that (1) ET evokes calcium influx in cultured enteric glia via activation of non-L-, non-N-, or non-P-type calcium channels; (2) capacitative calcium influx is regulated by protein kinase C (PKC) and nitric oxide (NO) synthase (NOS); and (3) PKC and NOS act synergistically to regulate calcium influx induced by ET.

MATERIALS AND METHODS

Materials

Endothelin 3 (ET_3) was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). N^G-Nitro-L-arginine (L-NA), N^G-nitro-D-arginine (D-NA), N^G-monomethyl-L-arginine monoacetate (L-NMMA), L-N^5-(1-iminoethyl)ornithine (L-NIO), *S*-methyl-L-thiocitrulline (Me-TC), (±)-(*E*)-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexeneamide (NOR3), (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP), and sodium nitroprusside (SNP) were from Calbiochem (San Diego, CA, U.S.A.). Fura-2 acetoxymethyl ester (fura-2/AM) and fura-2-free acid were from Molecular Probes (Eugene, OR, U.S.A.). Nickel chloride, lanthanum chloride, collagenase type V, trypsin-EDTA, trypsin inhibitor, penicillin–streptomycin solution, and HEPES were from Sigma Chemical (St. Louis, MO, U.S.A.). Phorbol 12-myristate 13-acetate (PMA), chelerythrine, conotoxin GIVA, agatoxin, and diltiazem were obtained from Research Biochemicals International (Natick, MA, U.S.A.). 4α-Phorbol 12-myristate 13-acetate (4α-PMA) was purchased from LC Laboratories (Woburn, MA, U.S.A.). Hanks' balanced salt solution, medium 199, and L-glutamic acid were from GibcoBRL (Grand Island, NY, U.S.A.). Neonatal male Duncan–Hartley guinea pigs were obtained from Simonsen Laboratories (Gilroy, CA, U.S.A.).

Solutions

All experiments were performed in standardized solutions except when noted. Standard control buffer was a modified Krebs–Ringer solution at pH 7.40, containing (in m*M*): NaCl 118, KCl 4.7, $CaCl_2$ 1.8, HEPES 10, $NaHCO_3$ 15, glucose 11, NaH_2PO_4 0.9, and $MgSO_4$ 0.8. For calcium-free control buffer, $CaCl_2$ was removed and 1 m*M* EGTA was added. In experiments where Ba^{2+} ions are used, HCO_3^-, $S_2O_4^{2-}$, and $H_2PO_4^-$ were removed from all solutions, as they form insoluble salts with Ba^{2+}.

Myenteric plexus isolation

Dispersed primary cultures of guinea pig myenteric plexus were prepared on collagen-coated coverslips and used for experiments within 5 days. Taeniae coli from 1-day-old male Duncan–Hartley guinea pigs were removed and placed in Hanks' balanced salt solution plus 0.1% collagenase for 16–20 h at 4°C. After a 35-min incubation at 37°C, the muscle layers of the taeniae coli were separated from the myenteric plexus by using a dissecting microscope. The plexus was trypsinized for 30 min at 37°C, using trypsin-EDTA solution, triturated with siliconized flamed Pasteur pipettes of decreasing tip diameter, and plated on collagen-coated coverslips. Cultures were exposed to complete medium 199 plus 5% NU serum and 0.001% trypsin inhibitor (type I-S from soybean). Penicillin–streptomycin solution was added for the first 48 h at a 2% concentration. Antimitotic agents were not added. Media were changed every other day. The cultures were incubated at 37°C with 5% CO_2.

Cell preparation for imaging

Cultured plexus was incubated at 37°C in fresh warmed media containing 2–3 μ*M* fura-2/AM for 45 min. Loaded coverslips were washed, resuspended in standard control buffer, and placed in a Lucite superfusion chamber. The superfusion rate of the control buffer and experimental solutions was 1 ml/min at 37°C.

Calcium measurements

A Zeiss Axiovert inverted microscope and Attofluor digital imaging system (Rockville, MD, U.S.A.) were used for single-cell $[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence intensities of fura-2 at 334- and 380-nm wavelengths with an emission wavelength of 500 nm. Calibration of the system was performed by using fura-2-free acid with the two-point standardization equation: $[Ca^{2+}]_i = K_D\{[R - R(\mathrm{Lo})]/[R(\mathrm{Hi}) - R]\}b$, where K_D = dissociation constant of the Ca^{2+}/fura-2 complex (225 n*M*), $R = F_{334}/F_{380}$, i.e., the fluorescence at 334-nm excitation divided by the fluorescence at 380-nm excitation, $R(\mathrm{Lo})$ = ratio at zero calcium (1 m*M* EGTA), $R(\mathrm{Hi})$ = ratio at high calcium (1 m*M* $CaCl_2$), and $b = F_{380}$ (zero Ca^{2+})/F_{380} (saturating Ca^{2+}). Frames were not averaged. A ratio pair was taken every second.

In some experiments, Ba^{2+} ions were substituted for Ca^{2+} ions to measure rates of ion entry into glia cells. Ba^{2+} ions cause fura-2 to produce fluorescence ratio changes in a fashion similar to Ca^{2+} ions. However, unlike Ca^{2+} ions, Ba^{2+} cannot be sequestered in internal stores and, therefore, provides an isolated measure of divalent cation entry in cells (Kwan and Putney, 1990). In the Ba^{2+} experiments, the imaging system was not calibrated, and data are expressed as the F_{334}/F_{380} ratio.

Data presentation

Capacitative calcium entry was experimentally defined as the rise in $[Ca^{2+}]_i$ that occurred after the return of calcium to the extracellular buffer after depletion of internal stores in calcium-free buffer. This was assessed by measuring the change in $[Ca^{2+}]_i$ before reintroduction of calcium relative to the peak value observed with the return of calcium. In inhibitory experiments, capacitative calcium entry was normalized to the control in which no inhibitor was added and was expressed as a percentage of control.

Results are presented as mean ± SEM values. Data were analyzed by using unpaired Student's *t* test. Significance was accepted as $p < 0.05$.

Dissection techniques, tissue preparation, media, and reagent vendors remained constant throughout the study. In this study, n = number of glial cells. At least three coverslips were used for each experimental condition. All experimental conditions were examined on glial cells derived from cell preparations performed on at least 2 different days.

Results have been calculated only from those responding glia having basal $[Ca^{2+}]_i$ levels of <150 n*M*, a criterion

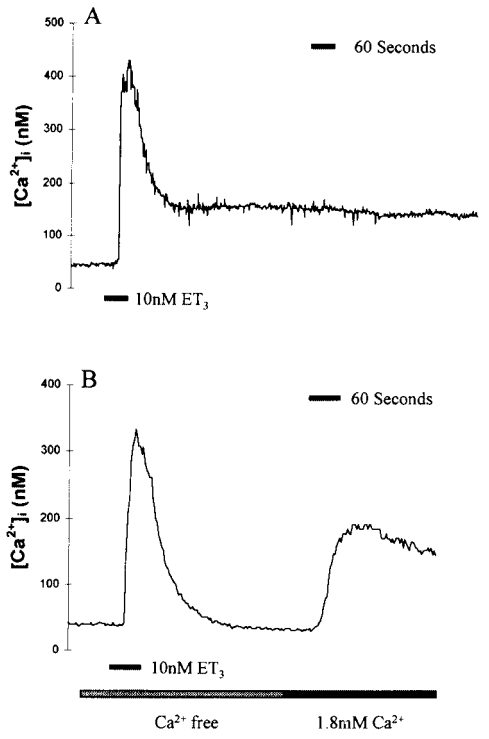

FIG. 1. ET caused capacitative calcium entry in enteric glia. **A:** In Ca^{2+}-containing buffer, ET_3 (10 n*M*) caused an initial $[Ca^{2+}]_i$ response followed by sustained elevation of $[Ca^{2+}]_i$. **B:** Application of ET_3 (10 n*M*) in Ca^{2+}-free buffer induced the release of calcium from intracellular stores and activation of calcium influx as indicated by the sustained increase of $[Ca^{2+}]_i$ after addition of 1.8 m*M* calcium to the superfusion medium (n = 325).

met by >95% of glial cells. Those glia with a high $[Ca^{2+}]_i$ before addition of agonist were considered damaged or leaky and were excluded from the study. There was only one microscope field examined per coverslip. Peak $[Ca^{2+}]_i$ was measured as the highest Ca^{2+} concentration achieved during agonist exposure.

At the time of the experiments, the following two criteria were used to determine whether cells of interest were glia as opposed to neurons: (1) morphology: myenteric neurons 2–7 days after plating are compact, phase bright, with few or no processes. Enteric glia have a larger, dense nucleus with wide surrounding cytoplasm; and (2) KCl depolarization: at the end of each experiment, the coverslip was superfused with 55 m*M* KCl. This exposure depolarizes neurons but not glia, increasing their $[Ca^{2+}]_i$.

RESULTS

ET-induced capacitative calcium entry in enteric glia

ET_3 (10 n*M*) caused a biphasic response in $[Ca^{2+}]_i$ that was composed of an initial rapid but transient increase and a subsequent prolonged elevation in $[Ca^{2+}]_i$ (Fig. 1A). To observe capacitative calcium entry in enteric glia, cells were exposed to 10 n*M* ET_3 in calcium-free buffer for 60 s, followed by calcium-free buffer for 200 s, and then were superfused with 1.8 m*M* calcium. Under these conditions, ET_3 caused a transient increase in $[Ca^{2+}]_i$ in calcium-free buffer; secondary increases in $[Ca^{2+}]_i$ were noted when 1.8 m*M* calcium was added to the perfusion medium (Fig. 1B). In 12 separate experiments (n = 325), the $[Ca^{2+}]_i$ increment noted on restitution of buffer Ca^{2+} was 176 ± 19 n*M*.

To determine if ET_3-induced capacitative calcium transients involve calcium entry from extracellular sources, barium was substituted for calcium in some experiments. As shown in Fig. 2, barium entered cells after depletion of calcium from the internal stores by ET_3, indicating that the source of calcium transients was via influx of extracellular calcium.

Calcium entry involves non-L-, non-N-, or non-P-type calcium channels

To characterize calcium channel types involved in ET_3-evoked capacitative calcium entry in enteric glia, a panel of calcium channel inhibitors was used. Ni^{2+} and La^{3+} are inorganic, nonspecific inhibitors of calcium channels and have been demonstrated to block capacitative calcium entry in other cell types (Berridge, 1995). The dihydropyridine compound diltiazem is an inhibitor of L-type voltage-gated Ca^{2+} channels; ω-conotoxin and agatoxin are specific inhibitors for N-type and P-type calcium channels, respectively (Tsien and Tsien, 1990; Olivera et al., 1994). In all the inhibition experiments, enteric glia were exposed to 10 n*M* ET_3 to mobilize internal calcium stores, followed by superfusion with calcium-free buffer plus inhibitor for 200 s, then buffer containing 1.8 m*M* Ca^{2+} with the same concentration of inhibitor.

As illustrated in Fig. 3, 1 m*M* Ni^{2+} inhibited ET_3-evoked capacitative calcium entry by 89 ± 2% (n = 68). The inhibitory effects of Ni^{2+} were reversible. In a similar manner, 1 m*M* La^{3+} produced 78 ± 2% (n = 57) inhibition of ET_3-stimulated capacitative calcium entry. In contrast, diltiazem, ω-conotoxin, and

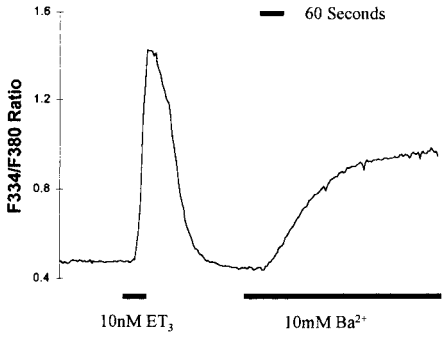

FIG. 2. Exposure of enteric glia to ET_3 (10 n*M*) in Ca^{2+}-free buffer caused a transient increase of fura-2 fluorescence ratio (F_{334}/F_{380} nm). When 10 m*M* Ba^{2+} was added to the Ca^{2+}-free buffer, the fluorescence ratio increased progressively, reflecting Ba^{2+} influx via the plasma membrane.

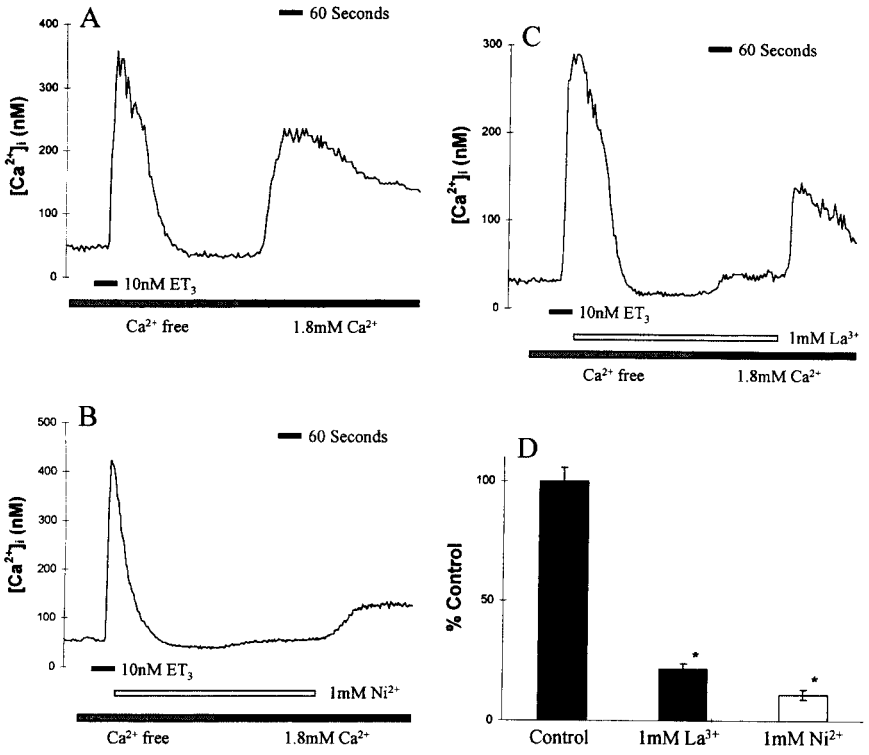

FIG. 3. Reversible inhibition by Ni^{2+} and La^{3+} of ET_3-stimulated capacitative calcium entry in enteric glia. **A:** Depletion of intracellular calcium stores caused a typical capacitative calcium entry on addition of Ca^{2+} to the perfusion buffer. **B:** Superfusion with 1 m*M* Ni^{2+} inhibited the increase of $[Ca^{2+}]_i$ when Ca^{2+} was added to the perfusion buffer. $[Ca^{2+}]_i$ increased when Ni^{2+} was removed. **C:** Similar to Ni^{2+}, La^{3+} reversibly inhibited capacitative calcium entry. **D:** Summary of inhibitory effect of Ni^{2+} and La^{3+}. n = 64, 68, and 57, for control, Ni^{2+}, and La^{3+}, respectively. $^*p < 0.05$, for Ni^{2+} or La^{3+} vs. control.

agatoxin had no effect on ET_3-induced capacitative calcium entry (Table 1).

Role of PKC

In some studies, PKC has been suggested to regulate capacitative calcium entry (Bode and Goke, 1994; Peterson and Berridge, 1994, 1995; Berridge, 1995; Xu and Ware, 1995; Ribeiro and Putney, 1996). To study the effect of PKC on ET_3-stimulated capacitative calcium entry in enteric glia, cells were pretreated with 100 n*M* PMA for 16 h to deplete PKC activity before being used for experiments. As shown in Fig. 4, pretreatment of cells with PMA produced a 67 ± 7% inhibition of ET_3-stimulated capacitative calcium entry (n = 38). In parallel studies, 100 n*M* 4α-PMA, an inactive analogue of PMA, demonstrated no effect (n = 59). In contrast, short-term pretreatment of glia with 100 n*M* PMA for 4 min to activate PKC activity produced a 6 ± 4% increase of ET_3-stimulated capacitative calcium entry ($p > 0.05$).

The effect of PKC on ET_3-stimulated capacitative calcium entry was further studied by using inhibitors of PKC. Staurosporine, a relatively nonspecific PKC antagonist, inhibited capacitative calcium entry by 55 ± 2% (n = 60) (Fig. 4). Chelerythrine is a newly described and more specific inhibitor of PKC (Herbert et al., 1990). Beginning 60 s before exposure to 10 n*M* ET_3, chelerythrine dose-dependently inhibited capacitative calcium entry. Chelerythrine at 1 and 5 μ*M* inhibited capacitative calcium entry by 32 ± 4% (n = 51) and 54 ± 2% (n = 58), respectively (Fig. 5A). As the effects of chelerythrine could be explained either by the inhibition of calcium influx from the extracellular sources or by effects on intracellular stores, barium was substituted for calcium in the superfusion solution. Figure 5B illustrates the effects of 1 μ*M* chelerythrine on barium entry into enteric glia after mobilization of internal calcium stores with 10 n*M* ET_3. Relative to controls, glia treated with chelerythrine demonstrated smaller increments in fura-2

TABLE 1. *Effect of calcium channel blockers on ET-stimulated capacitative calcium entry*

	Control	Diltiazem (5 μ*M*)	Diltiazem (50 μ*M*)	ω-Conotoxin (1 μ*M*)	Agatoxin (10 n*M*)
% of control	100 ± 6	102 ± 4	92 ± 4	108 ± 4	96 ± 5
n	57	89	59	86	44

In all inhibition experiments, a single dose of inhibitor was applied 200 s before 1.8 m*M* Ca^{2+}, and the same amount of inhibitor was added to the superfusion buffer. No significant difference ($p > 0.05$) in capacitative calcium entry was noted between the control and glia treated with diltiazem, conotoxin, or agatoxin.

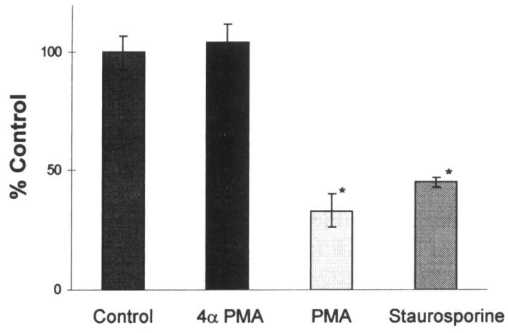

FIG. 4. Effect of PMA and staurosporine on capacitative calcium entry. Enteric glia were pretreated with 100 n*M* PMA for 16 h before exposure to ET_3 (n = 38). Pretreatment of glia with 100 n*M* staurosporine for 30 min attenuated capacitative calcium entry (n = 60). Pretreatment of glia with 100 n*M* 4α-PMA demonstrated no effect (n = 59). $^*p < 0.01$, vs. control.

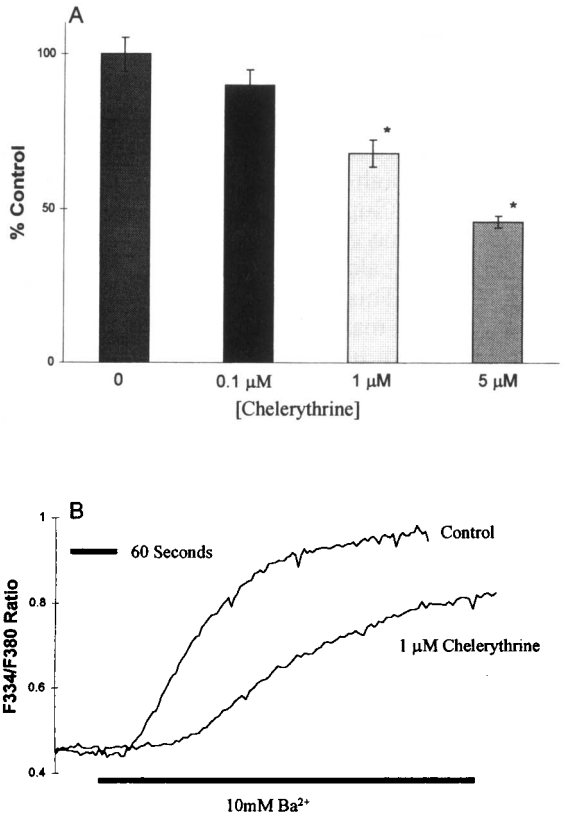

FIG. 5. A: Chelerythrine dose-dependently inhibited capacitative calcium entry induced by ET_3 (10 n*M*) in enteric glia. Beginning 60 s before exposure to 10 n*M* ET_3, a single dose of chelerythrine was used throughout each experiment. n = 43, 62, 51, and 58, for control, and 0.1, 1, and 5 μ*M* chelerythrine, respectively. $^*p < 0.01$, vs. control. **B:** The average of 20 tracings. Chelerythrine (1 μ*M*) inhibited the Ba^{2+} influx. Chelerythrine was applied to cells, beginning 60 s before exposure to 10 n*M* ET_3.

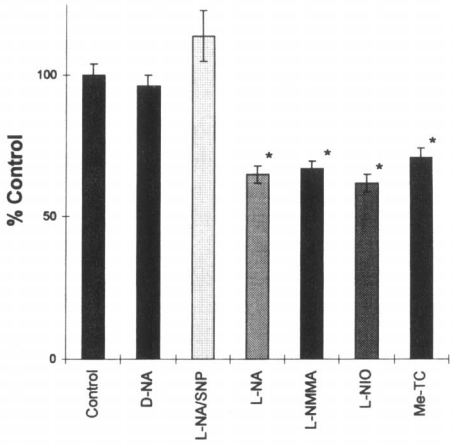

FIG. 6. Effect of NOS inhibitors on the capacitative calcium entry evoked by ET_3 in enteric glia. Cultured glia were pretreated with a single dose of NOS inhibitor for 5 min and then superfused with the inhibitor. All NOS inhibitors (1 m*M* L-NA, 100 μ*M* L-NMMA, 100 μ*M* L-NIO, and 100 μ*M* Me-TC) significantly attenuated capacitative calcium entry caused by ET_3. D-NA (1 m*M*), used as a negative control for L-NA, demonstrated no effect. In rescue experiments, 50 μ*M* SNP was superfused after enteric glia were exposed to 10 n*M* ET_3. The inhibition caused by 1 m*M* L-NA was reversed in the presence of 50 μ*M* SNP. $^*p < 0.01$.

fluorescent intensity, suggesting that chelerythrine acts to inhibit calcium entry via the plasma membrane.

Role of nitric oxide (NO)

Several studies have suggested that NO is involved in the regulation of capacitative calcium entry (Pandol and Schoeffield-Payne, 1990; Bischof et al., 1995). Enteric glia were pretreated with a series of inhibitors of NO synthase for 5 min and then superfused with the control buffer in the presence of inhibitor throughout the experiment. As illustrated in Fig. 6, pretreatment of glia with 1 m*M* L-NA attenuated capacitative calcium entry by 35 ± 3% (n = 59). Similar inhibition was found in experiments using L-NMMA (100 μ*M*, n = 89), L-NIO (100 μ*M*, n = 65), and Me-TC (100 μ*M*, n = 82). In parallel experiments, D-NA (1 m*M*), a negative control for L-NA, demonstrated no inhibitory effect (n = 54). In rescue experiments, simultaneous treatment of glia with 10 μ*M* SNP, an NO donor, restored L-NA-attenuated capacitative calcium entry (Fig. 6). The inhibitory effect of L-NA was dose dependent (Fig. 7A). None of the NO donors alone, i.e., SNP, NOR3, or SNAP, showed an effect on $[Ca^{2+}]_i$ in either resting or stimulated cells (data not shown).

When barium was substituted for calcium in the control experiments, rapid increases in intracellular fluorescent intensity was observed during capacitative calcium entry (Fig. 7B). In enteric glia pretreated with 1 m*M* L-NA, the rate of increment in intracellular fluorescent intensity was significantly attenuated, indicat-

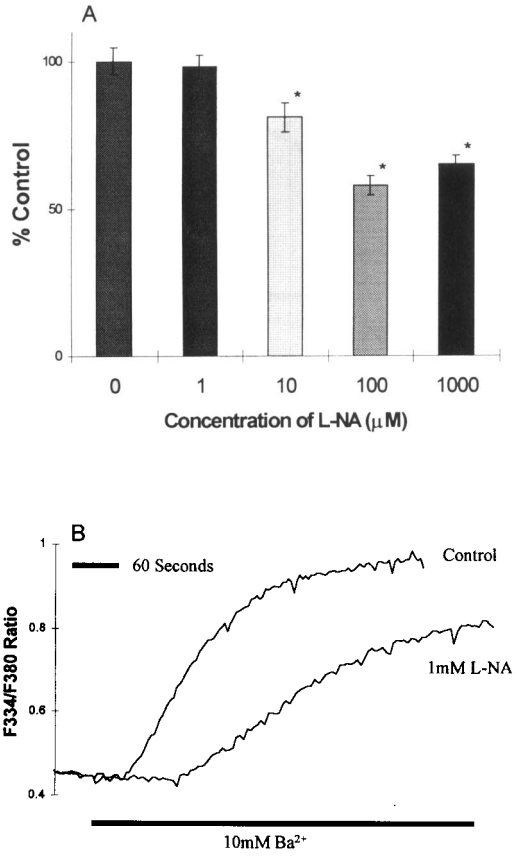

FIG. 7. A: The inhibition by L-NA of capacitative calcium entry elicited by ET_3 was dose dependent. *$p < 0.01$. **B:** Ba^{2+} influx was attenuated by the L-NA (1 m*M*), as indicated by the slower and smaller increments of fluorescence ratio. Shown is the average of 23 tracings.

ing an effect of L-NA on calcium entry across the plasma membrane.

Chelerythrine and L-NA act synergistically

As neither chelerythrine nor L-NA demonstrated complete inhibition of ET_3-stimulated capacitative calcium entry, experiments were performed to investigate the combined effects of these two inhibitors. Enteric glia were pretreated with 1 m*M* L-NA alone, 1 μM chelerythrine alone, or with 1 m*M* L-NA plus 1 μM chelerythrine before mobilization of internal calcium stores by 10 n*M* ET_3. As illustrated in Fig. 8, chelerythrine or L-NA alone inhibited ET-stimulated capacitative calcium entry by ~33%. The combination of chelerythrine and L-NA caused a synergistic 84 ± 1% inhibition in parallel experiments.

DISCUSSION

The present studies provide evidence for capacitative calcium entry in cultured enteric glial cells in which internal calcium stores are mobilized by ET_3. In these cells, capacitative calcium entry is mediated via non-L-, non-N-, or non-P-type calcium channels. ET_3-stimulated calcium entry was regulated by both PKC and NO. Inhibitory effects of PKC and NOS were synergistic, suggesting that PKC- and NO-dependent pathways are functionally separate.

Several studies have suggested a functional linkage between increases in $[Ca^{2+}]_i$ and the biological effects of peptides of the ET family (Supattapone et al., 1989; Stojilkovic and Catt, 1992; Battistini et al., 1993). Typically, ET activates a biphasic calcium signal, with an initial rapid, but transient, rise in $[Ca^{2+}]_i$ followed by a lower, sustained increment in $[Ca^{2+}]_i$. Previously, we have reported that ET_3-stimulated calcium signaling in enteric glial cells occurs via ET-B receptor activation of phospholipase C (Zhang et al., 1997). The initial rise in $[Ca^{2+}]_i$, originating from inositol trisphosphate-sensitive intracellular calcium stores, is followed by sustained elevation of $[Ca^{2+}]_i$ due to calcium entry across the plasma membrane. In these studies, elevation of enteric glial cell $[Ca^{2+}]_i$ induced by ET was reported to last for >10 min after agonist exposure. Prolonged elevation of $[Ca^{2+}]_i$ after exposure to ET has also been noted in other cell types and may have a key role in the regulation of DNA synthesis and cellular proliferation (Supattapone et al., 1989; Battistini et al., 1993). Prevention of calcium influx by calcium channel blockers inhibits the mitogenic effect of ET in smooth muscle cells and astrocytes (Nakaki et al., 1989; Supattapone et al., 1989).

The concept that calcium entry is regulated by the state of filling of calcium stores was first proposed by Putney (1990). This capacitative calcium entry has been observed in many cells and can be activated by several agonists or pharmacological agents that share the common property of releasing stored intracellular calcium. The current study is the first observation of

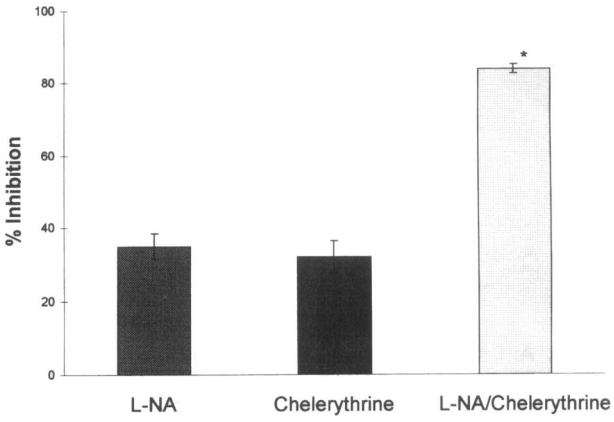

FIG. 8. Effects of 1 μM chelerythrine alone, 1 m*M* L-NA alone, or a combination of L-NA and chelerythrine on capacitative calcium entry stimulated by 10 n*M* ET_3 in enteric glia. *$p < 0.01$, vs. L-NA alone and chelerythrine alone.

capacitative calcium entry in enteric glia. In the current studies, depletion of intracellular calcium stores by ET activated calcium entry. Mobilization of intracellular calcium entry by ET in enteric glia caused barium influx, confirming that extracellular calcium influx occurring across the cell membrane was the source of the calcium transient. Increases in $[Ca^{2+}]_i$, which occurred after ET had been removed from the perfusion medium, demonstrated that the continuing presence of agonist is not required, typical of capacitative calcium entry in other cell types.

ET-induced capacitative calcium entry in enteric glia is mediated by Ni^{2+}- and La^{3+}-inhibitable calcium channels. The process is not sensitive to L-, N-, or P-type calcium channel blockers, as capacitative calcium entry evoked by ET_3 in enteric glia was not affected by diltiazem, ω-conotoxin, or agatoxin. Similar results have been reported for agonist-stimulated capacitative calcium entry in other nonexcitable cells (Peterson and Berridge, 1994; Berridge, 1995; Zhu et al., 1996). The existence of capacitative calcium entry in enteric glia with properties similar to those observed in other cell types suggests the presence of a calcium release-activated calcium channel. The calcium release-activated calcium channel has very low conductance and may be blocked by bivalent and trivalent cations such as Ni^{2+} and La^{3+} (Berridge, 1995). Recent studies imply that the transient receptor potential (*trp*) gene product might function as such a capacitative calcium entry channel (Friel, 1996; Zhu et al., 1996).

The role of PKC in regulation of capacitative calcium entry is uncertain. Recent studies using rat thyroid cells, human neutrophils, *Xenopus* oocytes, pancreatic cells, and RBL-2H3 cells have reported that PKC may either stimulate or inhibit capacitative calcium entry depending on cell type and experimental conditions (Montero et al., 1993; Tornquist, 1993; Bode and Goke, 1994; Peterson and Berridge, 1994, 1995; Parekh and Penner, 1995). The current studies indicate that, in enteric glia, PKC promotes capacitative calcium entry. Depletion of cellular PKC activity by PMA significantly inhibited calcium influx. In a similar manner, inhibition of PKC activity by chelerythrine suppressed calcium entry. These results suggest that PKC may be a factor in the regulation of ET-stimulated capacitative calcium entry in enteric glia. It is interesting that inhibition of PKC activity produced incomplete inhibition of calcium entry. Recent studies by Shibata et al. (1996) suggest that, in neutrophils, PKC may regulate the activity of calcium influx factor, proposed as a soluble mediator for capacitative calcium entry.

In enteric glia, capacitative calcium entry also appears to be regulated by NO. Similar to the effects of PKC inhibitors, inhibitory effects were observed in experiments using a series of specific NOS antagonists, L-NA, L-NMMA, L-NIO, and Me-TC. The effects of L-NA were reversed by SNP, a NO donor. L-NA was also able to inhibit the barium entry in enteric glia exposed to ET, indicating that the effects of NO are exerted at the level of plasma membrane calcium influx.

The role of NO in capacitative calcium entry is uncertain. Similar to our observations, inhibitory effects of L-NA on capacitative calcium entry have been reported in carbachol-treated pancreatic cells (Pandol and Schoeffield-Payne, 1990) and colonic epithelial cells (Bischof et al., 1995). Other authors have reported no effect of NO on capacitative calcium entry in rat pancreatic cells (Gilon et al., 1995) and in human platelets (Okamoto et al., 1995). It is noteworthy that although PKC or NOS inhibition alone caused only partial inhibition of ET-evoked calcium entry in enteric glia, the combination of inhibitors caused near-total suppression of calcium entry. The synergistic effect of PKC and NOS inhibitors suggests that PKC and NO may act on different pathways to regulate capacitative calcium entry.

In conclusion, the present studies demonstrate that depletion of internal calcium stores by ET induces capacitative calcium entry in enteric glia. Capacitative calcium entry is mediated by divalent or trivalent cation-sensitive calcium channels, which are not inhibited by L-, N-, or P-type calcium channel antagonists. PKC and NO act synergistically to regulate capacitative calcium entry stimulated by ET in these cells.

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