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Pharmacological Characterization of Endothelin-stimulated Phosphoinositide Breakdown and Cytosolic Free Ca^{2+} Rise in Rat C_6 Glioma Cells

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Because increasing evidence indicates that glial cells are a target of endothelin, we have characterized endothelin-induced phosphoinositide (PI) turnover and Ca^{2+} homeostasis in C_6 glioma cells. Endothelin-1 (ET) increased formation of ^{3}H-inositol phosphate (IP) from PI and elicited an increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) in rat C_6 glioma. In the presence of Li^+, the increase in ^{3}H-inositol trisphosphate formation was rapid, reaching its peak at 5 min after stimulation. ET also elicited a rapid and sustained increase in $[Ca^{2+}]_i$ in a dose-dependent manner (1–100 nM). The rank orders of efficacy for ET-related peptides in increasing $[Ca^{2+}]_i$ were ET = ET-2 > sarafotoxin > ET-3. Both ET-mediated stimulation of IP formation and $[Ca^{2+}]_i$ increase were largely inhibited in the absence of external Ca^{2+} but unaffected by the depletion of external Na^+ and the presence of dihydropyridine derivatives or verapamil. Inorganic Ca^{2+} channel blockers Cd^{2+}, La^{3+}, and Mn^{2+} at 1 mM inhibited both responses induced by ET. Cross-desensitization and nonadditivity were observed for both events among ET-related peptides tested, but not between ET and ATP. Pretreatment of cells with pertussis toxin (PTX) attenuated the PI response to ET, but had no effect on ET-elicited $[Ca^{2+}]_i$ increase. ET-induced Ca^{2+} mobilization (measured in Ca^{2+}-free medium) was only transient and was inhibited by 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate. Moreover, the intracellular Ca^{2+} pools mobilized by ET and ATP appeared to overlap, as indicated by their partial heterologous desensitization. Pretreatment with phorbol dibutyrate (PDBu) for 10 min reduced the PI response and Ca^{2+} mobilization but potentiated the $[Ca^{2+}]_i$ increase induced by ET. In cells pretreated with PDBu for 24 hr, both responses to ET were attenuated. Our results suggest that, in C_6 glioma, ET induces PI hydrolysis and causes Ca^{2+} influx. The former response is mediated by a PTX-sensitive G-protein, leading to intracellular Ca^{2+} mobilization, while the latter response results in further increase in $[Ca^{2+}]_i$.

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The transmembrane biochemical signals of endothelin have been demonstrated in a variety of cell types. Endothelin-1 (ET) and its related peptides are capable of raising intracellular free calcium concentrations ($[Ca^{2+}]_i$) in several cell lines and tissues such as vascular smooth muscle cells (Kai et al., 1989; Marsden et al., 1989; Meyer-Lehnert et al., 1989; Simpson and Ashley, 1989), glial cells (Suppattapone et al., 1989; Marsault et al., 1990), endothelial cells (Emori et al., 1990; Vigne et al., 1990a), fibroblasts (Gardner et al., 1989; Takuwa et al., 1989; Ohnishi-Suzaki et al., 1990), ciliary muscle cells (Korbmacher et al., 1989), neuroblastoma NG 108-15 cells (Fu et al., 1989; Yue et al., 1990), and rat cardiocytes (Hirata et al., 1989; Vigne et al., 1990b).

In general, two events have been shown to occur during ET-induced increase of $[Ca^{2+}]_i$: an initial mobilization of Ca^{2+} induced by inositol trisphosphate (IP_3) and a sustained Ca^{2+} increase due to Ca^{2+} entry from the extracellular medium. The pharmacological characteristics of signal transduction mechanisms mediated by ET receptors have not been fully explored. The present study was undertaken to investigate mechanisms underlying ET-induced phosphoinositide (PI) hydrolysis and $[Ca^{2+}]_i$ increase in C_6 glioma cells, which have been used as a model to study cellular events after ET stimulation in neurally related cell types (Lin et al., 1990a,b; MacCumber et al., 1990; Zhang et al., 1991). Our results suggest that ET induces Ca^{2+} mobilization and Ca^{2+} influx by distinct mechanisms. The former involves a pertussis toxin (PTX)-sensitive GTP-binding protein that mediates ET-induced PI hydrolysis, while the latter may be due to opening of receptor-operated cation channels. Moreover, the influx of extracellular Ca^{2+} plays an essential role in maintaining the PI response to ET.

Materials and Methods

Cell culture. Rat C_6 glioma cells with an original passage number of 39 (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells with additional passages of 13–35 were subcultured into 35 mm dishes for PI turnover assay or grown on glass coverslips (9 × 35 mm^2) for intracellular Ca^{2+} measurement.

Intracellular Ca^{2+} measurement. Cells were grown on the coverslip to confluence (10^6 cells/slip) and then placed in physiological saline solution (PSS; 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 0.5 mM EDTA, 10 mM glucose, and 20 mM HEPES, pH 7.4) with 5 μM fura-2 acetoxymethylester (AM) and pluronic F-127 (0.25% v/v) at 37°C for 60 min. After the incubation time, cells were washed twice with PSS before fluorescence measurement. Cells on

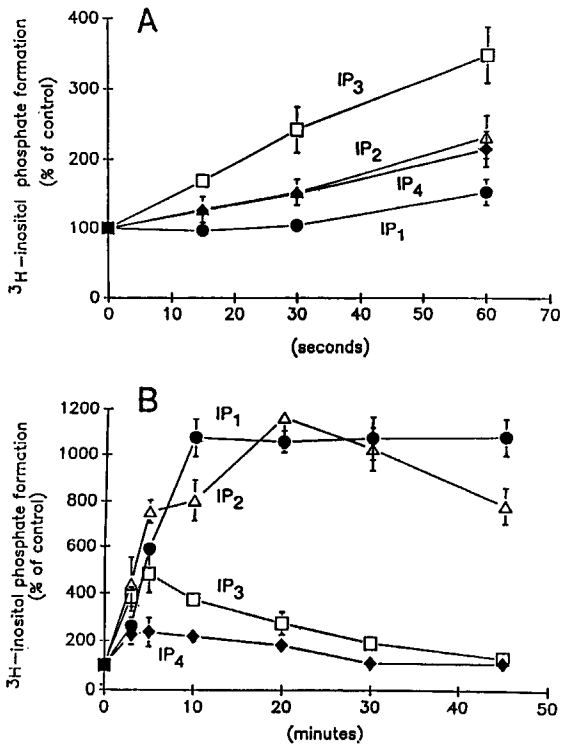

Figure 1. Time course of ET-induced PI hydrolysis in C_6 glioma cells. Cells prelabeled with ^{3}H-myo-inositol were stimulated with 30 nM ET for indicated periods of time in the presence of lithium. Various forms of ^{3}H-IPs were separated by anion-exchange chromatography as described in Materials and Methods. Data presented are means ± SEM of a representative experiment performed in triplicate. The 100% values were derived from samples incubated in parallel in the absence of any stimulant. These basal values did not significantly change within the incubation time of 45 min. The experiment was repeated three times with similar results.

the coverslip were placed in a continuously stirred thermostatic cuvette (37°C) containing 2 ml of PSS. The fluorescence was monitored on a PTI Delta Scan spectrofluorometer (Photon Technology International Inc., South Brunswick, NJ) with dual excitation wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm. Both excitation and emission spectra were recorded with 4 nm slit widths. Determinations of $[Ca^{2+}]_i$ were calculated from the fluorescence ratio (R) obtained according to the following formula (Grynkiewiez et al., 1985):

$$[Ca^{2+}]_i = \frac{Kd(R - R_{min})(Sf)}{(R_{max} - R)(Sb)},$$

where R_{min} is the ratio at zero calcium and R_{max} is the ratio at saturating calcium. K_d is the dissociation constant for fura-2 (224 nM) at 37°C. Sf and Sb are the fluorescence intensities of the dye measured at 380 nm in the absence of Ca^{2+} and with saturating Ca^{2+}, respectively. Agents were added to the cuvette in 5–20 μl aliquots.

Measurement of PI turnover. The procedure was essentially as described previously (Lin et al., 1989). Confluent cells on 35 mm Petri dishes (4×10^6 cells/dish) were labeled with ^{3}H-myo-inositol (2.5 μCi/ml) in the growth medium for 16–18 hr. Cells were then washed with PSS containing 20 mM LiCl (at this concentration, the greatest stimulation of ET-induced PI turnover was observed) and incubated at 37°C for 45 min. Following this preincubation, indicated drugs were added and incubated for another 45 min or as indicated. The reaction was stopped by an addition of ice-cold methanol, and the accumulation of ^{3}H-inositol phosphate (IP) was measured by AG1×8 resin (formate form, 100–200 mesh) according to the method of Berridge et al. (1982). In most experiments, ^{3}H-IPs containing predominantly inositol monophosphate (IP_1) were eluted with 0.2 *N* ammonium formate/0.1 *N* formic acid. When indicated, inositol bisphosphate (IP_2), inositol trisphosphate (IP_3), and inositol tetrakisphosphate (IP_4) were eluted with 0.4 *N*, 0.8 *N*, and 1.2 *N* ammonium formate (containing 0.1 *N* formic acid), respectively. The elutions of these inositol phosphates from columns were confirmed by using various forms of ^{3}H-inositol phosphates (New England Nuclear, Boston, MA) as standards. In our studies, the term "ET" refers to ET-1, unless otherwise stated.

Materials. ET-1, -2, and -3 and sarafotoxin S_{6b} (S_{6b}) were purchased from Peptide Institute, Inc. (Osaka, Japan). Fura-2 AM and BAPTA-AM were from Molecular Probes (Eugene, OR). ^{3}H-myo-inositol (16.5 Ci/mmol) was from New England Nuclear, and fetal calf serum was

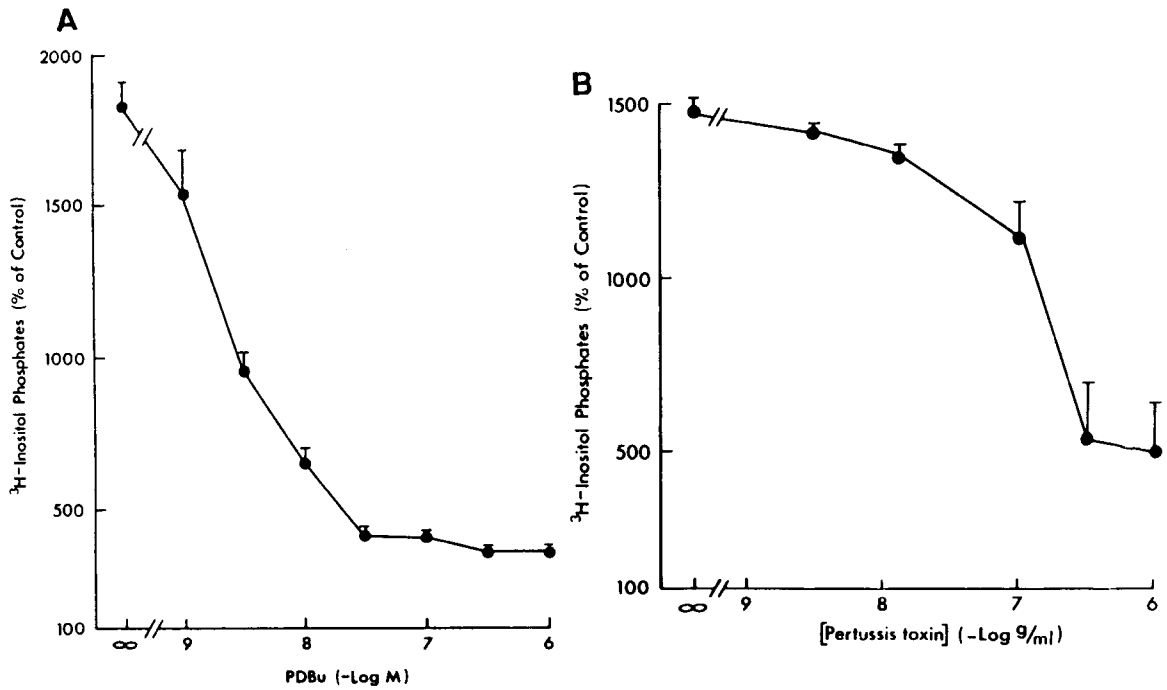

Figure 2. Concentration-dependent effects of PDBu and PTX on ET-induced PI turnover. C_6 glioma cells were pretreated with various concentrations of PDBu for 10 min (*A*) or PTX for 24 hr (*B*) before exposure to ET (30 nM) for 45 min. Data are expressed as mean ± SEM from three independent experiments, each performed in triplicate.

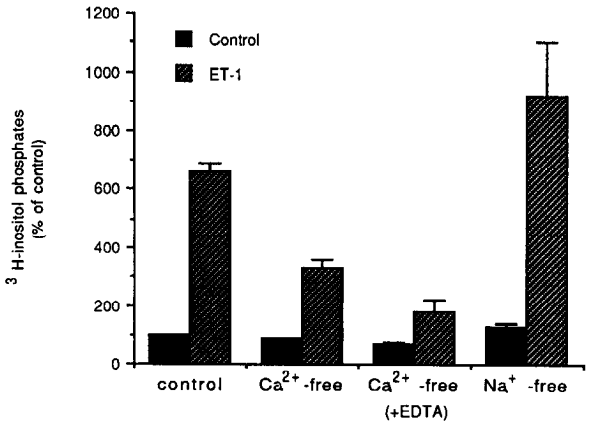

Figure 3. Effects of depletion of extracellular Ca^{2+} and Na^+ on ET-induced PI turnover. After labeling, cells were preincubated in Ca^{2+}-free medium (in the absence of $CaCl_2$ and EDTA), Ca^{2+}-free medium containing 0.5 mM EDTA, or Na^+-free medium (external NaCl was displaced with equal molarity of choline chloride) for 45 min before the stimulation of PI breakdown by ET (30 nM). Data presented are means ± SEM of three independent experiments, each performed in triplicate.

purchased from GIBCO (Grand Island, NY). Nisoldipine and nimodipine were generous gifts from Miles Pharmaceuticals Inc. (West Haven, CT). All other chemicals were products of Sigma Chemical Co. (St. Louis, MO).

Results

Characteristics of ET-induced PI turnover in C_6 glioma

In the presence of 20 mM LiCl, ET (30 nM) induced a rapid formation of ^{3}H-IP_3, ^{3}H-IP_4, ^{3}H-IP_2, and ^{3}H-IP_1 (Fig. 1*A*). These increases were detected at 15 sec after stimulation at 37°C, and the percentage of the increase for IP_3 was greater than that for other IPs within the first 60 sec measured. The amounts of IP_3 and IP_4 reached their peaks at 5 min; after this time their levels declined (Fig. 1*B*). The level of IP_2 peaked at 20 min, whereas IP_1 maintained at a plateau between 10 and 45 min.

The effects of short-term (10 min) exposure with phorbol dibutyrate (PDBu) and long-term (24 hr) treatment with PTX on ET-induced ^{3}H-IP accumulation within 45 min incubation are shown in Figure 2, *A* and *B*. PDBu attenuated the response to ET by about 80%, while PTX inhibited the effect of ET to about 30% of the control. The IC_{50} values of PDBu and PTX were 2.4 nM and 0.13 μg/ml, respectively.

The ET-induced PI breakdown was dependent on extracellular Ca^{2+}. As shown in Figure 3, removal of Ca^{2+} from the medium resulted in approximately 50% inhibition of the ET-induced IP accumulation, and inclusion of 0.5 mM EDTA in the Ca^{2+}-free medium produced a further reduction of the ET response. However, sodium depletion did not reduce ET-induced PI hydrolysis. The data in Table 1 show the effects of inorganic Ca^{2+} entry blockers on ET-induced IP formation. At 1 mM, Cd^{2+}, Mn^{2+}, and La^{3+} inhibited the ET-stimulated IP formation with a potency of $Cd^{2+} > Mn^{2+} > La^{3+}$, while Co^{2+} and Zn^{2+} had no effect. At a lower concentration of 0.3 mM, none of these cations produced a significant effect on the ET-induced response (data not shown). The ET-induced PI turnover was insensitive to 1 μM nimodipine and nisoldipine (L-type Ca^{2+} channel antagonist), 30 μM indomethacin (cyclooxygenase inhibitor), 30 μM nordihydroguaiaretic acid (lipoxygenase inhibitor), 10 μM ouabain (Na^+,K^+-ATPase inhibitor), and 1 mM 3,4-diaminopyridine (a nonselective K^+ channel blocker) (data not shown).

Table 1. Effects of inorganic Ca^{2+} entry blockers on ET-induced PI breakdown in C_6 glioma cells

Ca^{2+} entry blockers (1 mM)	^{3}H-IP formation (% of control)	
	Basal	ET (30 nM)
–	100	1978 ± 216
$CoCl_2$	94 ± 4	1821 ± 135
$MnCl_2$	92 ± 7	984 ± 108*
$LaCl_3$	98 ± 7	1325 ± 94*
$CdCl_2$	143 ± 19	722 ± 71*
$ZnCl_2$	167 ± 13	1617 ± 199

Cells prelabeled with ^{3}H-IP were incubated for 15 min with 1 mM Ca^{2+} channel blockers before ET was added. Results are means ± SEM from three independent experiments that were performed in triplicate. Mn^{2+}, La^{3+}, and Cd^{2+} at 0.3 mM did not change basal and ET-induced IP formation.

* PI turnover elicited by ET is significantly ($p < 0.05$, Student's t test) different from that in control cells.

The maximal PI response induced by ET was nonadditive to that elicited by S_{6b} but was additive to that produced by ATP, which was reported to activate PI turnover in C_6 glioma (Lin et al., 1990a) (Table 2). Pretreatment of cells with either ET or S_{6b} for 30 min resulted in cross-desensitization of the PI response to each peptide (Table 3), consistent with the notion that ET and S_{6b} act at the same population of ET receptors in neurons (Lin et al., 1989, 1991).

Increase of $[Ca^{2+}]_i$ elicited by ET and its homologs

Exposure of fura-2–preloaded C_6 cells to ET induced a rapid increase of $[Ca^{2+}]_i$ (Fig. 4*A*). The effect was concentration dependent, with a detectable increase at 1 nM ET, and the increment was approximately linear with up to 100 nM of this peptide (Fig. 4*B*). The onset of the increase of $[Ca^{2+}]_i$ was more rapid with an increasing concentration (Fig. 4*C*). The sustained $[Ca^{2+}]_i$ increase by 3 and 10 nM was maintained for at least 3 min, and the net increases of $[Ca^{2+}]_i$ after stimulation for 10 min were 170 ± 1 and 195 ± 33 nM (n = 4–6), respectively. In the presence of 100 nM ET, the net increase of $[Ca^{2+}]_i$ reached its maximum of 533 ± 71 nM (n = 14) within 30 sec and declined rapidly thereafter to about one-third of the maximal concentration at 3 min. The resting $[Ca^{2+}]_i$ level in C_6 cells was 151 ± 6 nM (n = 99).

Three ET-related peptides, ET-2, ET-3, and S_{6b} also induced an increase in $[Ca^{2+}]_i$ (Fig. 5). The rank orders of efficacy for these peptides in inducing $[Ca^{2+}]_i$ increase were ET = ET-2 > S_{6b} > ET-3. The net values of maximal $[Ca^{2+}]_i$ increase elicited

Table 2. Additivity of ET-, S_{6b}- and ATP-induced PI turnover in C_6 glioma cells

	^{3}H-IP formation (% of control)	
	Control	ET (30 nM)
None	100	1460 ± 99
S_{6b} (30 nM)	1849 ± 74	1614 ± 88 (3309)
ATP (100 μM)	1238 ± 121	2549 ± 35 (2698)

Data presented are means ± SEM of a typical experiment, which was performed in triplicate. Data in parentheses are theoretical additive values for agonist-induced PI responses. The experiment was repeated three times with similar results.

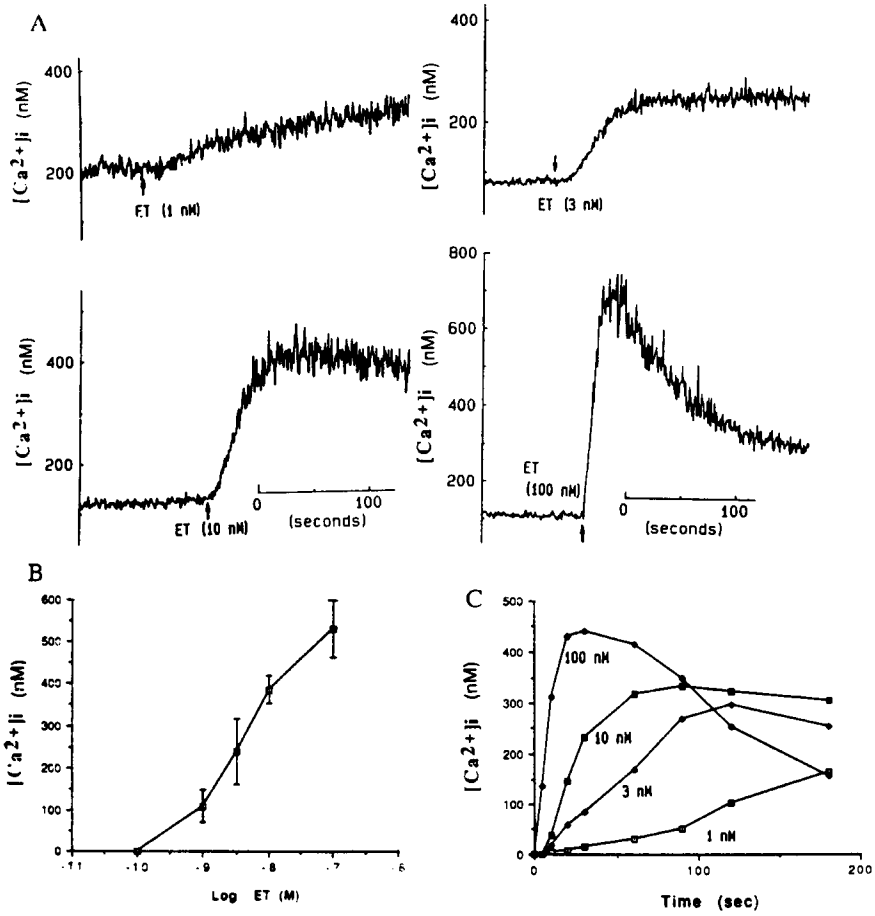

Figure 4. Effects of ET on $[Ca^{2+}]_i$ in C_6 glioma cells. *A*, Concentration–response relationship of ET-induced increase in $[Ca^{2+}]_i$. *B*, Maximal net $[Ca^{2+}]_i$ increase elicited by different concentrations of ET. *C*, Time course of net $[Ca^{2+}]_i$ increase induced by ET at 1, 3, 10, and 100 nM. The maximal responses of ET shown in *B* are the mean ± SEM of 5–18 independent experiments. Data shown in *C* are the mean values from 4–16 traces. Details of procedures are as described in Materials and Methods.

by 100 nM of these four peptides were 533 ± 71 nM (n = 14), 499 ± 91 nM (n = 3), 399 ± 75 nM (n = 5), and 140 ± 35 nM (n = 5), respectively. A rise of ET-3 concentration to 1 μM resulted in a net increase of $[Ca^{2+}]_i$ of 338 ± 120 nM (n = 5), but substantially declined with time after $[Ca^{2+}]_i$ reached its peak.

Homologous desensitization of ET- and S_{6b}- induced increase of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ increase in C_6 cells produced by 10 nM ET was desensitized to stimulation with 10 nM S_{6b} applied 4 min later, while the $[Ca^{2+}]_i$ response to subsequent application of 100 μM ATP remained intact (Fig. 6). In a similar experiment, pretreatment with S_{6b} induced a cross-desensitization of the $[Ca^{2+}]_i$ response to ET, but not that to ATP. Moreover, pretreatment of cells with ATP to elevate $[Ca^{2+}]_i$ did not abolish the subsequent increase of $[Ca^{2+}]_i$ induced by ET.

The homologous desensitization was further studied in cells pretreated with 10 nM ET for 24 hr. This long-term ET pretreatment abolished the $[Ca^{2+}]_i$ responses to 10 nM ET and 100 nM S_{6b} (Fig. 7). It also markedly attenuated the effects produced by 100 nM ET and 1 μM ET-2. However, the ATP-induced $[Ca^{2+}]_i$ increase remained unchanged.

Table 3. Cross-desensitization of ET- and S_{6b}-induced PI turnover in C_6 glioma cells

Agonist	^{3}H-IP formation (% of control)		
	Untreated	ET pretreated	S_{6b} pretreated
None	100	106 ± 8	130 ± 15
ET (30 nM)	1218 ± 146	112 ± 2	167 ± 17
S_{6b} (100 nM)	1047 ± 117	125 ± 15	171 ± 17

Cells were pretreated with ET (30 nM) or S_{6b} (100 nM) for 30 min before rechallenge with ET or S_{6b}. Data presented are means ± SEM of a typical experiment, which was performed in triplicate.

Characteristics of ET-induced $[Ca^{2+}]_i$ increase

ET-induced $[Ca^{2+}]_i$ increase was unaffected by the depletion of sodium ion (sodium chloride was replaced with choline chloride) (Fig. 8). The presence of BAPTA (an intracellular calcium chelator) lowered the resting state of $[Ca^{2+}]_i$ and markedly inhibited the ET (10 nM)-induced net $[Ca^{2+}]_i$ increase to about 100 nM (Fig. 8). In the absence of exogenous calcium and presence of 5 mM EGTA, the net $[Ca^{2+}]_i$ increase elicited by 10 nM ET was reduced by approximately 75% (Table 4). Nisoldipine (10 μM) added before (Table 4) or after (Fig. 8) ET application did not affect the steady state of $[Ca^{2+}]_i$ levels. In contrast, La^{3+} (1 mM) added 15 min before or after ET administration markedly inhibited the ET-induced $[Ca^{2+}]_i$ increase (Table 4, Fig. 8). Mn^{2+} at 1 mM inhibited the $[Ca^{2+}]_i$ response to ET by about 80% (Table 4); the IC_{50} of Mn^{2+} was approximately 95 μM (data not shown). Interestingly, this IC_{50} value is comparable with that for Mn^{2+} to inhibit K^+-induced Ca^{2+} influx in synaptosomes (Nachshen, 1984). Cd^{2+} (1 mM) also significantly inhibited the Ca^{2+} response, while Zn^{2+} (1 mM) and verapamil (10 μM) were

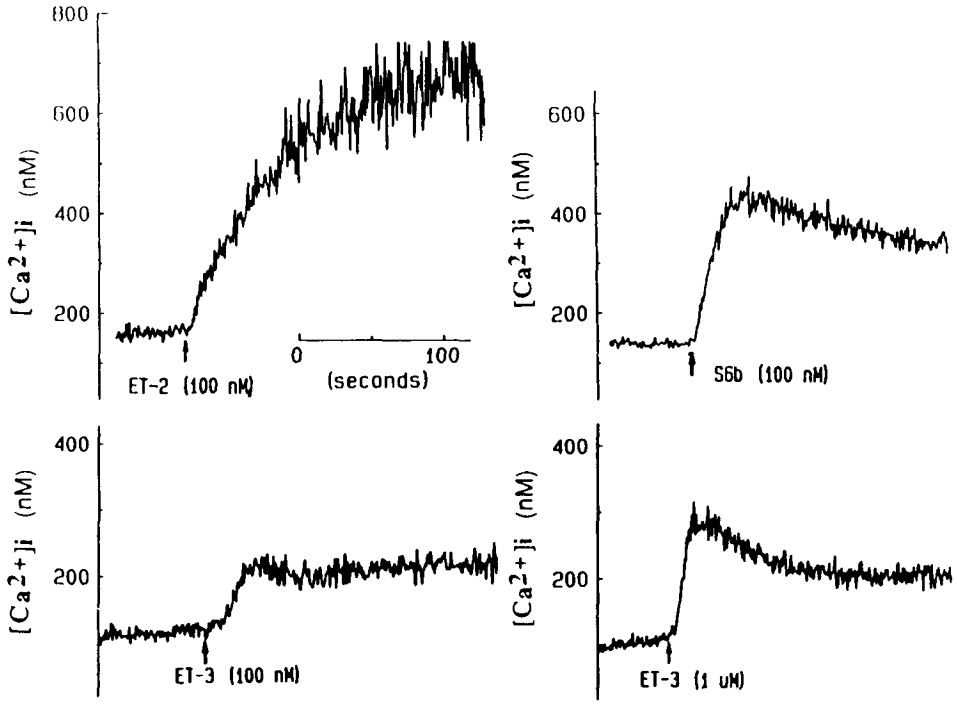

Figure 5. Fluorometer tracings of $[Ca^{2+}]_i$ in C_6 glioma cells in response to stimulation with ET homologs. Experimental details are as described in Materials and Methods. Tracings shown are from a typical experiment repeated three to six times with nearly identical results.

ineffective (Table 4). Short-term (10 min) pretreatment with PDBu facilitated, while long-term (24 hr) pretreatment attenuated, the effect of ET (Table 4, Fig. 8). Preexposure of cells to PTX for 3 hr had no apparent effect on ET-induced $[Ca^{2+}]_i$ increase (Table 4), as opposed to its inhibitory effect on ET-induced PI turnover (see Fig. 2). Prolongation of the PTX pretreatment time to 24 hr still failed to change ET-induced $[Ca^{2+}]_i$ increase.

Calcium mobilization induced by ET and ATP

To investigate the role of Ca^{2+} release from intracellular stores in the $[Ca^{2+}]_i$ increase, cells were exposed to a Ca^{2+}-free medium

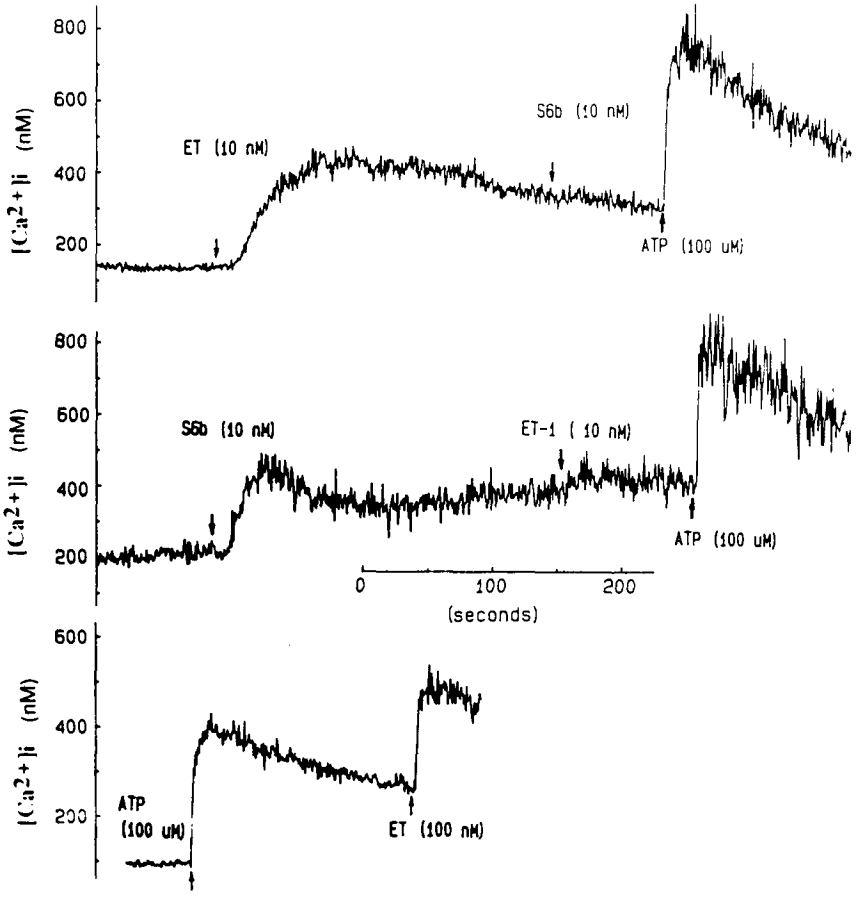

Figure 6. Fluorometer tracings of $[Ca^{2+}]_i$ in response to sequential addition of ET, S_{6b}, and ATP. Experimental details are as described in Materials and Methods and shown in the figure. Tracings shown are from a typical experiment repeated at least three times with similar results

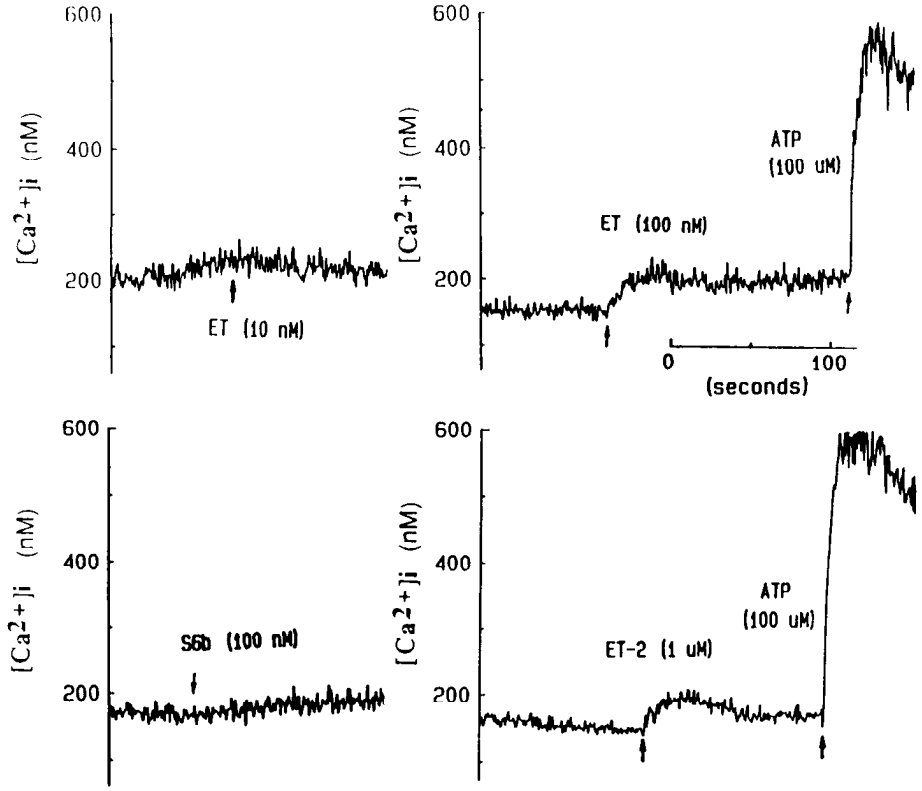

Figure 7. Fluorometer tracings of $[Ca^{2+}]_i$ increase induced by ET and its homologs in cells pretreated with ET for 24 hr. Cells pretreated with 10 nM ET for 24 hr were processed for the measurement of $[Ca^{2+}]_i$ in response to indicated concentrations of ET, S_{6b}, ET-2, or ATP. Details of procedures are as described in Materials and Methods. Results shown are from a representative experiment performed at least three times.

(containing 5 mM EGTA and 0.5 mM EDTA) for 5 min before stimulation with 100 nM ET or 100 μM ATP (Fig. 9). The prestimulating level of $[Ca^{2+}]_i$ was decreased from 151 ± 6 nM (n = 99) to 50 ± 5 nM (n = 21) due to Ca^{2+} depletion. Although desensitization of Ca^{2+} mobilization to ET or ATP was noted in the Ca^{2+}-free medium, the initial rise of Ca^{2+} induced by ET or ATP was only transient and its peak value decreased to 48 ± 7% (n = 9) or 43 ± 6% (n = 6) of the control obtained in the presence of Ca^{2+}, respectively. Moreover, ET pretreatment markedly attenuated Ca^{2+} mobilization induced by ATP [from 180 ± 26 (n = 6) to 83 ± 17 (n = 6) nM] (Fig. 9). Similarly, ATP pretreatment decreased Ca^{2+} mobilization induced by ET [from 256 ± 38 (n = 9) to 66 ± 2 (n = 3) nM]. In the Ca^{2+}-free medium, pretreatment of cells with 500 nM PDBu or 100 μM 8-(*N,N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8; a purported inhibitor of Ca^{2+} mobilization) reduced to less than 30% the $[Ca^{2+}]_i$ increase elicited by ET and ATP.

Table 4. Effects of pharmacological manipulations on ET-induced $[Ca^{2+}]_i$ increase in C_6 glioma cells

Pretreatment	Net ET (10 nM)-induced $[Ca^{2+}]_i$ increase (nM)
None	387 ± 34 (18)
Ca^{2+}-free (5 mM EGTA)	96 ± 7 (3)*
PDBu (500 nM, 10 min)	617 ± 70 (7)*
PDBu (500 nM, 24 hr)	200 ± 23 (4)*
PTX (500 ng/ml, 3 hr)	420 ± 81 (3)
Nisoldipine (10 μM, 15 min)	490 ± 76 (3)
Verapamil (10 μM, 15 min)	482 ± 35 (4)
$MnCl_2$ (1 mM, 15 min)	81 ± 13 (3)*
$LaCl_3$ (1 mM, 15 min)	164 ± 17 (4)*
$CdCl_2$ (1 mM, 15 min)	252 ± 52 (3)*
$ZnCl_2$ (1 mM, 15 min)	366 ± 41 (3)

Details of experimental conditions are as described in Materials and Methods and in the table. Data presented are means ± SEM of net $[Ca^{2+}]_i$ increase from the number of experiments shown in parentheses.

* $p < 0.05$ when compared with the control response.

Discussion

In C_6 glioma cells, ET caused a rapid and sustained $[Ca^{2+}]_i$ rise in a concentration-dependent manner. Similar to effects found in other cell lines and tissues, our results suggest that the $[Ca^{2+}]_i$ increase induced by ET is derived from two main sources; one is an influx of extracellular Ca^{2+}, and the other is the release of Ca^{2+} from intracellular storage sites. In the absence of extracellular Ca^{2+}, the initial $[Ca^{2+}]_i$ is diminished and the sustained phase is abolished, suggesting that the main source for the ET-induced sustained $[Ca^{2+}]_i$ response is the Ca^{2+} influx. In agreement with this notion, the intracellular Ca^{2+} chelator BAPTA, which is generated by hydrolysis of BAPTA-AM (cell-permeant form) within the cell by the action of esterases, failed to prevent completely the changes in $[Ca^{2+}]_i$ caused by ET.

To determine the underlying mechanism involved in the Ca^{2+} influx, we found that inorganic Ca^{2+} antagonists (Mn^{2+}, La^{3+}, Cd^{2+}) but not nisoldipine or verapamil, inhibited this ET-induced $[Ca^{2+}]_i$ increase, indicating that L-type Ca^{2+} channels are not activated. This observation is contradictory to reports using A7r5 cells (Van Renterghem et al., 1988), A_{10} cells (Simpson and Ashley, 1989; Xuan et al., 1989), primary cultured aortic smooth muscle cells (Kai et al., 1989), and cerebellar type I astrocytic glial cells (Suppattapone et al., 1989). However, L-type channel antagonist-resistant Ca^{2+} influx is also reported in cultured aortic smooth muscle cells (Wallnofer et al., 1989), glomerular mesangial cells (Simonson et al., 1989), and rat atrial

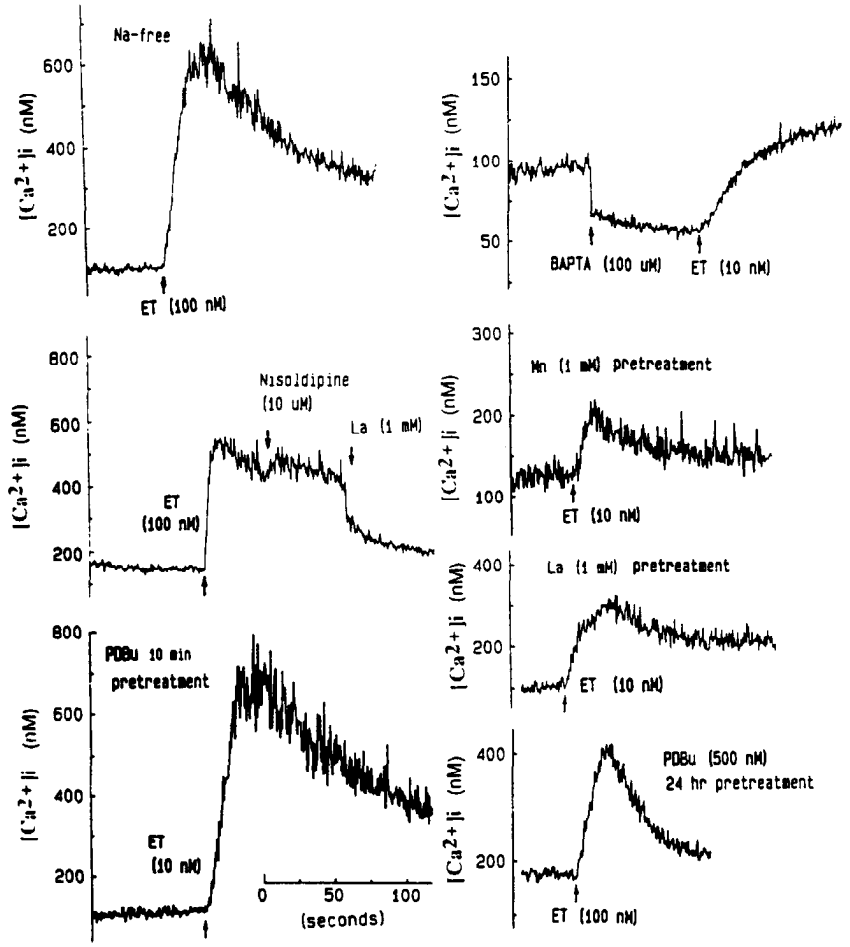

Figure 8. Fluorometer tracings of ET-induced $[Ca^{2+}]_i$ increase measured in Na^+-free buffer, in the presence of BAPTA-AM or nisoldipine, or after pretreatment with PDBu, Mn^{2+}, or La^{3+}. Experimental conditions are as described in Materials and Methods and as shown in the figure. In Na^+-free medium, equal molarity of choline chloride was used to replace NaCl. When pretreated, cells were preincubated with PDBu (500 nM) for 10 min or 24 hr or with inorganic cations (1 mM $MnCl_2$ or $LaCl_3$) for 15 min followed by addition of indicated concentrations of ET. When used, 100 μM BAPTA-AM was added 90 sec before ET application. Results shown are from a typical experiment of three to seven such experiments.

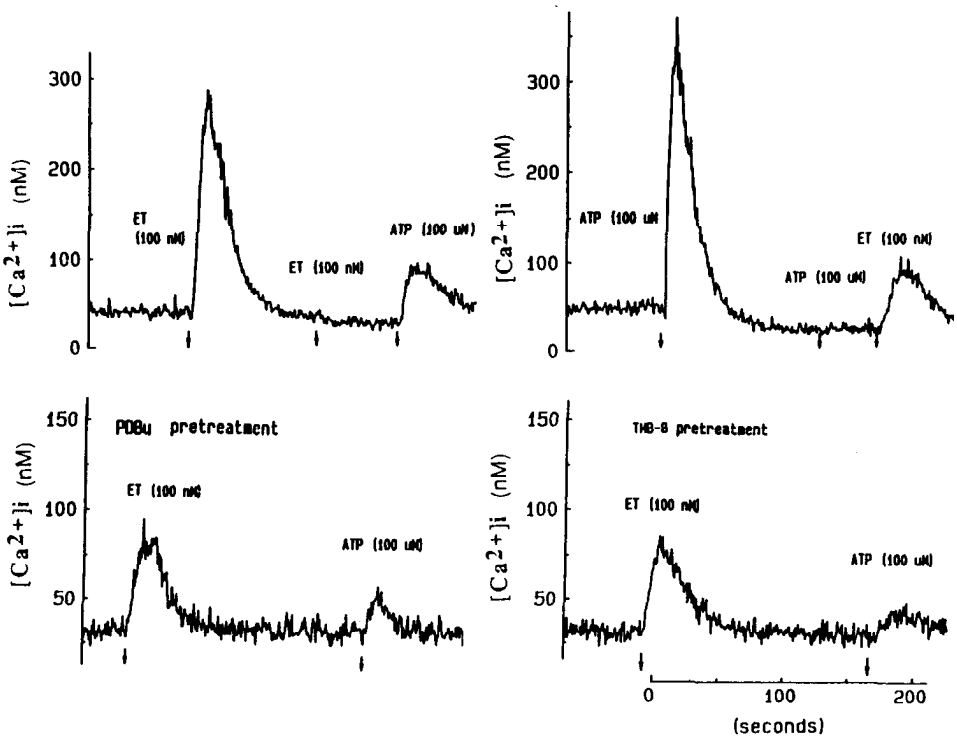

Figure 9. Fluorometer tracings of ET- or ATP-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium. $[Ca^{2+}]_i$ measurements were performed in cells preincubated for 5 min in Ca^{2+}-free PSS containing 5 mM EGTA before agonist stimulation. When indicated, cells were pretreated with PDBu (500 nM) or TMB-8 (100 μM) for 10 min before stimulation with ET (100 nM) or ATP (100 μM). Results shown are from a typical experiment of three such experiments.

cells (Vigne et al., 1990b). Conflicting results on the existence of L-type voltage-sensitive Ca^{2+} channels in cultured glial cells have been reported, possibly due to differences in the culturing conditions (Newman, 1985; Noronha-Blob et al., 1988; Barres et al., 1989; Glaum et al., 1990). However, using C_6 glioma, we found that high (55 mM) KCl did not elicit a significant change in $[Ca^{2+}]_i$ (data not shown), suggesting that L-type channel is not expressed in this cell line. A possible explanation for the $[Ca^{2+}]_i$ increase in C_6 cells is that ET induces an opening of nonselective cation channels that are permeable to Ca^{2+}, as reported in vascular smooth muscle cells (Van Renterghem et al., 1988; Wallnofer et al., 1989). An increase in $[Ca^{2+}]_i$ may result in opening of potassium channels, which was recently shown to occur in C_6 cells stimulated with endothelins (Suppattapone and Ashley, 1991).

ET-induced Ca^{2+} mobilization can also be demonstrated in C_6 glioma in Ca^{2+}-free medium, as expected from our results indicating that ET induced the rapid synthesis of IP_3. A pretreatment of cells with PDBu for 10 min attenuated ET-stimulated PI hydrolysis and Ca^{2+} mobilization to 25 ± 3% (n = 3) and 29 ± 8% (n = 3), respectively. Although PI turnover and Ca^{2+} mobilization were measured in the presence and absence of extracellular Ca^{2+}, respectively, these results are consistent with a major role of ET-induced IP_3 production in the mobilization of intracellular Ca^{2+}. However, a similar PDBu pretreatment enhanced the $[Ca^{2+}]_i$ increase. These results suggest that Ca^{2+} influx is positively regulated by protein kinase C but does not require IP_3-dependent Ca^{2+} mobilization. ET-induced Ca^{2+} entry that is independent of PI breakdown has also been suggested in studies using other cell types (Yue et al., 1990; Wilkes et al., 1991). The enhancement of $[Ca^{2+}]_i$ increase by short-term PDBu treatment is likely due to activation of protein kinase C, and this may explain our previous finding that ET-elicited release of ^{3}H-D-aspartate from cerebellar granule cells is stimulated by a similar pretreatment with the phorbol ester (Lin et al., 1990a). However, long-term (24 hr) treatment of C_6 glioma with PDBu markedly attenuated the effect of ET on PI turnover (Lin et al., 1990b), as well as $[Ca^{2+}]_i$ rise (Table 4). This effect may be due to depletion of protein kinase C and/or down-regulation of ET receptors known to occur after long-term phorbol ester treatment (Roubert et al., 1989; Resink et al., 1990). In this context, it is interesting to note that ET was shown to increase diacylglycerol formation in C_6 glioma due to activation of phospholipase C or D and to stimulate mitogenesis possibly through activation of protein kinase C by diacylglycerol (Zhang et al., 1991). The finding that PTX pretreatment markedly inhibited the PI response with no effect on $[Ca^{2+}]_i$ suggests that a PTX-sensitive G-protein is involved in ET receptor–mediated PI turnover, but not the Ca^{2+} influx. Thus, it may be hypothesized that these two ET-induced effector responses are mediated by distinct G-proteins.

Several observations support the view that Ca^{2+} influx plays an important role on the sustained PI response to ET in the C_6 cells. First, the depletion of extracellular Ca^{2+} resulted in reduction of ET-induced PI turnover and $[Ca^{2+}]_i$ rise. Second, inorganic cations, which potently inhibited the Ca^{2+} influx, attenuated the PI response. The discrepancies in the rank order potency of these inorganic cations for blocking the PI response ($Cd^{2+} > Mn^{2+} > La^{3+}$) and $[Ca^{2+}]_i$ increase ($Mn^{2+} > La^{3+} > Cd^{2+}$) might be related to the observation that these cations have additional sites of action such as inhibition of IP_3-stimulated Ca^{2+} release (Palade et al., 1989) or inhibition of ET binding to their receptors (Wada et al., 1991). Third, KCl at high concentrations (15–55 mM) attenuated the ET-induced Ca^{2+} influx and PI hydrolysis (data not shown). Fourth, there is a synergistic effect on PI turnover when C_6 cells are stimulated with ET in the presence of Ca^{2+} ionophores such as A23187 and ionomycin (Lin et al., 1990c). Our conclusion regarding the role of Ca^{2+} influx on the sustained formation of IP is consistent with results obtained using other cell types and tissues (Charest et al., 1985; Eberhard and Holz, 1988; Diamant and Atlas, 1989). Moreover, stimulation of PI breakdown by the general phospholipase C activator maitotoxin appears to be secondary to Ca^{2+} influx in many cell types including cerebellar granule cells (Gusovsky et al., 1990; Lin et al., 1990d).

The PI responses to ET-related peptides were nonadditive when applied simultaneously to the cells. Cross-desensitization of PI responses and Ca^{2+} signaling were noted among these related peptides, suggesting that they act on a common population of receptors. A similar conclusion was reached in studies using other cell types (Hirata et al., 1988; Lin et al., 1989; Suppattapone et al., 1989; Fabregat and Rozengurt, 1990). The intracellular Ca^{2+} pools mobilized by stimulation with ET and ATP appear to overlap, as indicated by their partial heterologous desensitization. Ca^{2+} mobilization induced by ET and ATP was attenuated by inhibiting PI hydrolysis with short-term PDBu treatment and by the presence of TMB-8, a purported intracellular Ca^{2+} antagonist (Malagodi and Chiou, 1974; Kojima et al., 1985) that was shown to inhibit IP_3-induced Ca^{2+} release in brain microsomes (Palade et al., 1989). C_6 glioma may provide a model system to elucidate molecular mechanisms involved in ET-induced Ca^{2+} mobilization and entry and the neurophysiological role of these events in cells of glial origin.

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