Receptor-linked early events induced by vasoactive intestinal contractor (VIC) on neuroblastoma and vascular smooth-muscle cells

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Vasoactive intestinal contractor (VIC) caused a series of biochemical events, including the temporal biphasic accumulation of 1,2-diacylglycerol (DAG), transient formation of $\text{Ins}(1,4,5)\text{P}_3$, and increase in intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) in neuroblastoma NG108-15 cells. In these cellular responses, VIC was found to be much more potent in NG108-15 cells than in cultured rat vascular smooth-muscle cells. The single cell $[\text{Ca}^{2+}]_i$ assay revealed that in the presence of nifedipine (1 μM) or EGTA (1 mM), the peak $[\text{Ca}^{2+}]_i$ declined more rapidly to the resting level in VIC-stimulated NG108-15 cells, indicating that the receptor-mediated intracellular Ca^{2+} mobilization is followed by Ca^{2+} influx through the nifedipine-sensitive Ca^{2+} channel. Pretreatment with pertussis toxin only partially decreased $\text{Ins}(1,4,5)\text{P}_3$ generation as well as the $[\text{Ca}^{2+}]_i$ transient induced by VIC, whereas these events induced by endothelin-1 were not affected by the toxin, suggesting involvement of distinct GTP-binding proteins. The VIC-induced transient $\text{Ins}(1,4,5)\text{P}_3$ formation coincident with the first early peak of DAG formation suggested that $\text{PtdIns}(4,5)\text{P}_2$ is a principal source of the first DAG increase. Labelling studies with $[^3\text{H}]\text{myristate}$, $[^{14}\text{C}]\text{palmitate}$ and $[^3\text{H}]\text{choline}$ indicated that in neuroblastoma cells phosphatidylcholine (PtdCho) was hydrolysed by a phospholipase C to cause the second sustained DAG increase. Down-regulation of protein kinase C (PKC) by prolonged pretreatment with phorbol ester markedly prevented the VIC-induced delayed DAG accumulation. Furthermore, chelation of intracellular Ca^{2+} completely abolished the second sustained phase of DAG production. These findings suggest that PtdCho hydrolysis is responsible for the sustained production of DAG and is dependent on both Ca^{2+} and PKC.

INTRODUCTION

A potent mammalian vasoconstrictive peptide, endothelin-1 (ET-1), was first isolated from the conditioned culture medium of pig aortic endothelial cells [1], and genetic analyses of ET-1 revealed that there is a group of structurally related peptides, named the ET family [2]. A wide spectrum of biological effects of ET-1 has been reported in various cell types, such as vascular smooth-muscle cells (VSMCs), fibroblasts, glomerular mesangial cells, cardiomyocytes and nervous tissues [3–7]. A vasoactive intestinal contractor (VIC), which differs from ET-1 in three amino acid residues and is expressed in intestinal cells, but not in endothelial cells, has differential contractile activities on mouse ileum and pig coronary artery: VIC has a stronger effect on intestinal contraction, but a weaker effect on vasoconstriction, than ET-1 [8,9]. Several investigations have provided evidence that ET-1 causes the receptor-mediated activation of phosphoinositide-specific phospholipase C (PLC) to produce two second messengers, diacylglycerol (DAG) and $\text{Ins}(1,4,5)\text{P}_3$ [10–12]. DAG serves as an endogenous activator of protein kinase C (PKC) [13], and $\text{Ins}(1,4,5)\text{P}_3$ induces Ca^{2+} mobilization from intracellular stores [14]. Also, influx of extracellular Ca^{2+} through a plasma-membrane channel may contribute to the increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [1,15]. Despite these abundant observations, the precise mechanism of signal transduction by ET-1 remains to be explored.

On the other hand, as for VIC, much less information is available about the signal pathway. We have recently demonstrated that both VIC and ET-1 triggered a $[\text{Ca}^{2+}]_i$ transient in neuroblastoma NG108-15 cells, with VIC being more potent than ET-1 [16]. In the present study, in order to gain more insight into the signalling mechanism of VIC, we have examined phosphoinositide turnover by measuring the mass contents of $\text{Ins}(1,4,5)\text{P}_3$ and DAG, and also measured the $[\text{Ca}^{2+}]_i$ level in NG108-15 cells and VSMCs stimulated with VIC. Furthermore, since a GTP-binding protein (G-protein) has been suggested to be involved in the receptor-mediated signal-transduction system including $\text{PtdIns}(4,5)\text{P}_2$ breakdown by phosphoinositide-specific PLC [17], the effects of pertussis toxin (PT) on the biochemical events were examined in VIC-stimulated NG108-15 cells and VSMCs.

The temporal biphasic accumulation of DAG was reported in several cell types, including ET-1-stimulated VSMCs [12,18–20]. The first transient phase is considered to be derived from phosphoinositide turnover, but the source(s) of the second sustained phase is still unclear. Several studies suggested that phosphatidylcholine (PtdCho) is a likely precursor source for DAG accumulation of the second phase [21–24]. In order to determine the source(s) of the second large DAG accumulation, we investigated VIC-induced changes in radioactivity distribution in NG108-15 cells labelled with $[^3\text{H}]\text{myristic acid}$, $[^{14}\text{C}]\text{palmitic acid}$ and $[^3\text{H}]\text{choline}$.

Abbreviations used: VSMCs, vascular smooth-muscle cells; ET, endothelin; VIC, vasoactive intestinal contractor; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hepes-buffered salt solution; PT, pertussis toxin; PtdOH , phosphatidic acid; PtdEtOH , phosphatidylethanol; PtdCho , phosphatidylcholine; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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EXPERIMENTAL

Materials

VIC and ET-1 were synthesized by using a solid-phase peptide synthesizer as described in ref. [9]. Fura-2, fura-2 acetoxymethyl ester (fura-2/AM) and quin 2 acetoxymethyl ester (quin 2/AM) were purchased from Dojindo Laboratories (Kumamoto, Japan), phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and PT was from Funakoshi Corp. (Tokyo, Japan); [$1\text{-}^{14}\text{C}$]palmitic acid (59 mCi/mmol) was from Amersham, [$9,10\text{-}^3\text{H}$]myristic acid (39.3 Ci/mmol) from Du Pont-New England Nuclear and [$\text{methyl-}^3\text{H}$]choline chloride (85 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.); silica gel 60 plates were from Merck (Darmstadt, Germany), and silica gel LK60 was from Whatman Chemical Separation Inc. (Clifton, NJ, U.S.A.). All other chemicals were obtained from commercial sources and were of the highest quality available.

Methods

Cell cultures. NG108-15 cells were kindly supplied by Dr. H. Higashida (Neuroinformation Research Institute, Kanazawa University, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY, U.S.A.) with 100 μM -hypoxanthine, 1 μM -aminopterin, 16 μM -thymidine (all from Sigma) and 5% (v/v) fetal-calf serum as described by Higashida *et al.* [25]. Routinely, the media were changed on day 3 after plating and every other day thereafter. For experiments, NG108-15 cells were seeded in 24-well culture trays (Coaster) precoated with polyornithine, at a density of 2.5×10^4 cells/well, and grown to near confluency and then assayed for $\text{Ins}(1,4,5)\text{P}_3$. For DAG assay, cells were washed twice with Hepes-buffered salt solution (HBSS) (148 mM-NaCl/10 mM-Hepes/5.8 mM-KCl/2 mM- CaCl_2 /1 mM- MgCl_2 , pH 7.35) and detached by gentle pipetting in HBSS to prepare the cell suspension (10^6 cells/assay), which gave essentially the same results as those obtained in adhered cells.

VSMCs were obtained from Dr. H. Arita (Shionogi Research Laboratories, Osaka, Japan) and cultured in DMEM plus 10% fetal-calf serum under 10% CO_2 in air at 37 °C. The cells were passaged weekly, and cells after 9–12 passages were used for the present study. VSMCs were seeded in 24-well culture trays at a density of 10^5 cells/well for $\text{Ins}(1,4,5)\text{P}_3$ assay and seeded in 35 mm-diam. Corning plastic dishes at a density of 2×10^5 cells/dish for DAG assay. These cells were grown in the serum-containing growth medium for 4 days to confluency, and were then serum-starved for 2 days before stimulation with VIC or ET-1. It has been suggested that endothelin has an effect on proliferation of VSMCs in culture [3,15]. Our observation that VSMCs grown in the presence of serum failed to cause endothelin-induced phosphoinositide turnover (results not shown) was similar to those of Araki *et al.* [10] and Badr *et al.* [6]. On the other hand, in NG108-15 cells, phosphoinositide turnover occurred in either the absence or the presence of serum, but cells tended to detach from culture trays on serum deprivation.

Determination of mass contents of DAG. NG108-15 cells and VSMCs were washed twice with HBSS and stimulated with 0.6 ml of VIC or ET-1 at 37 °C for the indicated times. The reaction was terminated by adding 0.6 ml of cold methanol. The cells were transferred to silicone-treated glass tubes containing 1.8 ml of chloroform/methanol (4:5, v/v). Lipids were then extracted essentially as described by Bligh & Dyer [26], except that 0.2 M-KCl/5 mM-EDTA was used instead of 2 M-KCl. The mass content of DAG in the cellular extracts was measured by using DAG kinase (Amersham) by the procedure of Preiss *et al.*

[27]. Briefly, the DAG in lipid extracts prepared from cells was quantitatively converted into [^{32}P]phosphatidic acid (PtdOH) by DAG kinase in the presence of [^{32}P]ATP. After extraction steps to remove [^{32}P]ATP that has not reacted, [^{32}P]PtdOH was separated by t.l.c. (silica gel 60) with the solvent system chloroform/methanol/acetic acid (12:3:1, by vol.). The area corresponding to [^{32}P]PtdOH was located by autoradiography and then scraped off plates, and the radioactivities were determined in a liquid-scintillation counter (Beckman LS7500).

Determination of mass contents of $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,4,5)\text{P}_3$ was quantitatively measured in ET-1- or VIC-stimulated NG108-15 cells and VSMCs by using the $\text{Ins}(1,4,5)\text{P}_3$ assay system (Amersham) as described previously [28]. Briefly, before stimulation with ET-1 or VIC, both NG108-15 cells and VSMCs were preincubated with DMEM containing 10 mM-LiCl for 5 min at 37 °C. It was reported that Li^+ prolonged and/or enhanced not only agonist-induced accumulation of inositol monophosphate, but also that of inositol bisphosphates and inositol trisphosphates in a neuroblastoma cell line or primary cultures of cerebella granule cells [29,30]. The reaction was terminated at designated times by adding 10% (w/v) HClO_4 , and the mixture was kept on ice for 20 min, then neutralized with ice-cold 1.53 M-KOH/75 mM-Hepes for 20 min. The samples were centrifuged at 2000 g for 10 min to remove the KClO_4 precipitate. The supernatant samples (100 μl each) were assayed for $\text{Ins}(1,4,5)\text{P}_3$ with the InsP_3 assay kit. The standard curve was linear from 0.19 to 25 pmol of $\text{Ins}(1,4,5)\text{P}_3$.

Extraction and analysis of lipids from NG108-15 cells prelabelled with [^{14}C]palmitic acid or [^3H]myristic acid. After preincubation with [^{14}C]palmitic acid (0.15 $\mu\text{Ci/ml}$) for 48 h at 37 °C, NG108-15 cells (2×10^6 cells/assay) were treated with 0.6 ml of the designated concentrations of VIC in HBSS for the indicated times. Reactions were terminated by adding 0.6 ml of ice-cold methanol. After lipid extraction by the method of Bligh & Dyer [26], phospholipids were separated by two-dimensional t.l.c. The mobile phase for the first dimension was chloroform/methanol/28% (v/v) NH_3 (65:35:6, by vol.) and for the second dimension was chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.). Authentic standards were added to the extracted lipids to enhance their detection by I_2 vapour. After labelling with [^3H]myristic acid (0.5 $\mu\text{Ci/ml}$) for 24 h at 37 °C, NG108-15 cells (10^6 cells/assay) were incubated with designated concentrations of VIC for the indicated times. Reactions were terminated by adding ice-cold methanol. The lipid extraction and analysis were same as above. For phosphatidylethanol (PtdEtOH)-formation experiments, NG108-15 cells labelled with [^3H]myristic acid were preincubated with 0.5% ethanol in HBSS for 5 min before stimulation with VIC.

Extraction and analysis of choline metabolites from NG108-15 cells prelabelled with [^3H]choline. NG108-15 cells were exposed to 0.5 μCi of [^3H]choline/ml for 48 h. The prelabelled cells were then rinsed twice with DMEM and incubated in the medium for 1 h before incubation with VIC in HBSS. The incubation was arrested with chloroform/methanol (1:2, v/v) and the aqueous and lipid phases were separated as described by Bligh & Dyer [26]. The aqueous phase was dried by centrifuging under vacuum and resuspended in 50 μl of 50% (v/v) ethanol, spotted on to silica gel LK60 and run in chloroform/0.5% NaCl/28% NH_3 (50:50:1, by vol.) to separate choline metabolites in accordance with the method of Yavin [31]. Standards of choline, phosphocholine and glycerophosphocholine were also run and identified with I_2 vapour. Phospholipids were separated and analysed as described above.

Measurement of $[Ca^{2+}]_i$ in single cells. For this, both types of cells were plated at a density of 2×10^4 cells/chamber on a glass coverslip which adhered to the smooth lower side of a Flexiperm-Disc (Heraeus Biotechnology, Hanau, Germany). After culture for 48 h at 37 °C, VSMCs were serum-deprived as described above, whereas NG108-15 cells were not, because the serum deprivation did not affect the $[Ca^{2+}]_i$ responses induced by these agonists in NG108-15 cells. Before stimulation with VIC or ET-1, these cells were washed twice and loaded with the fluorescence indicator fura 2/AM ($2 \mu M$) for 40 min at 37 °C in 0.3 ml of serum-free DMEM, then rinsed free of the extracellular dye and incubated for 15 min in HBSS to allow de-esterification of the dye. Fluorescence images were obtained at alternating excitation wavelengths of 340 and 360 nm through a SIT vidicon camera, and processed by an ARGUS-100 image analyser (Hamamatsu Photonics Corp., Hamamatsu, Japan). The linear 360 nm interpolation was used and the corrected fluorescence-emission-intensity ratio, by using 340 and 360 nm excitation with background subtraction, was monitored continuously in single cells. Conversion from the ratio into the absolute value of $[Ca^{2+}]_i$ was done by a micro-droplet assay as described by Kudo & Ogura [32]. In the range 50–850 nm $[Ca^{2+}]_i$, the ratio was logarithmically proportional to $[Ca^{2+}]_i$.

Data analysis. Statistical differences between control and test values were analysed by Student's *t* test.

RESULTS

Effects of VIC on $[Ca^{2+}]_i$

The mean basal $[Ca^{2+}]_i$ in NG108-15 cells and VSMCs, measured by the single-cell $[Ca^{2+}]_i$ assay, was 147 ± 14 nm (mean $\pm$ S.E.M., $n = 21$) and 62 ± 5 nm ($n = 21$) respectively. VIC caused transient increases in $[Ca^{2+}]_i$. The maximal response of $[Ca^{2+}]_i$ was observed at 10 nm-VIC in NG108-15 cells, but little rise in $[Ca^{2+}]_i$ occurred at the same concentration in VSMCs. At this concentration, more than 90 % of NG108-15 cells showed the rapid $[Ca^{2+}]_i$ rises. Compared with the Ca^{2+} spike response in NG108-15 cells, VIC (100 nm) elicited a weak and less transient response in VSMCs; the declining phase after the peak was much slower than in NG108-15 cells (Fig. 1).

Nifedipine ($1 \mu M$) had no effects on the basal level of $[Ca^{2+}]_i$, the lag time of response to VIC (10 nm), and the number of cells responding. The peak $[Ca^{2+}]_i$ (468 ± 42 nm, $n = 21$) was attained at about 9 s after stimulation in nifedipine-treated cells (Fig. 2b). At the same time point in VIC-stimulated cells without nifedipine

Fig. 1. Effects of VIC on $[Ca^{2+}]_i$ in fura-2-loaded NG108-15 cells and VSMCs

$[Ca^{2+}]_i$ was measured as described in the Experimental section: 10 nm-VIC was added to NG108-15 cells and 100 nm-VIC was added to VSMCs. Traces shown are representative examples of typical responses of single cells from four similar separate experiments.

Fig. 2. Effects of EGTA and nifedipine on VIC-induced $[Ca^{2+}]_i$ increase in fura-2-loaded NG108-15 cells

(a) VIC (10 nm) was added in the incubation buffer containing 1 mM- Ca^{2+} . (b) Nifedipine ($1 \mu M$) was added 5 min before addition of VIC (10 nm). (c) EGTA (1 mM) was added in Ca^{2+} -free condition 2 min before addition of VIC (10 nm). $[Ca^{2+}]_i$ was measured as described in the Experimental section. Traces shown are representative examples of typical $[Ca^{2+}]_i$ responses of individual cells from at least three similar experiments.

(Fig. 2a), the $[Ca^{2+}]_i$ rose to an equivalent level (471 ± 46 nm, $n = 21$), but it increased to a peak (641 ± 59 nm, $n = 21$) thereafter. Thus the agent affected the peak height and the sustained phase of $[Ca^{2+}]_i$. The declining phase after the peak was more rapid in nifedipine-treated cells (50 ± 3 s, $n = 21$) than in control cells (128 ± 11 s, $n = 21$). In the presence of EGTA, the response of $[Ca^{2+}]_i$ to 10 nm-VIC was greatly decreased (314 ± 52 nm, $n = 21$) and the increased level rapidly returned to the basal value (32 ± 3 s, $n = 21$) (Fig. 2c), although the lag time as well as the number of cells responding were unaffected. These data indicated that VIC induced the $[Ca^{2+}]_i$ increase by dual mechanisms: the initial $[Ca^{2+}]_i$ peak is caused by intracellular Ca^{2+} mobilization, and the subsequent sustained increase is due to Ca^{2+} influx through a nifedipine-sensitive Ca^{2+} channel.

Furthermore, it was found that the pretreatment with PT, which inhibits GTP-binding proteins (G_i , G_o) by ADP-ribosylation, decreased the VIC-induced $[Ca^{2+}]_i$ rise in NG108-15 cells and VSMCs (Fig. 3). However, little or no inhibitory effects were observed in both cells stimulated with ET-1.

Ins(1,4,5) P_3 production induced by VIC

Incubation of NG108-15 cells with VIC resulted in the rapid accumulation of Ins(1,4,5) P_3 in a concentration-dependent manner (Fig. 4). The minimum effective doses of VIC and ET-1 were about 50 pM and 100 pM respectively. The 50 % effective doses were 0.52 nM for VIC and 1.25 nM for ET-1. The maximal responses were observed at a dose of 10 nM for both VIC and ET-1 stimulation. Combined application of 10 nm-ET-1 and 10 nm-VIC produced an additive Ins(1,4,5) P_3 response (results not shown). The time course showed that the cellular Ins(1,4,5) P_3

Fig. 3. Differential effects of PT on $[Ca^{2+}]_i$ in VIC- or ET-1-stimulated NG108-15 cells and VSMCs

NG108-15 cells and VSMCs were incubated in the absence ($\square$) or presence ($\square$) of PT (100 ng/ml) for 3 h, and were then stimulated with the designated concentration of VIC or ET-1 (C, control). $[Ca^{2+}]_i$ was measured in single cells as described in the Experimental section. Values of peak $[Ca^{2+}]_i$ are cumulative of three separate experiments and are presented as means $\pm$ S.E.M. ($n = 21$; * $P < 0.05$; ** $P < 0.01$).

Fig. 4. Dose-dependence of $Ins(1,4,5)P_3$ formation in VIC- or ET-1-stimulated NG108-15 cells

NG108-15 cells (10^5 /assay) were preincubated with medium containing 10 mM-LiCl for 5 min at 37 °C and were then stimulated with various concentrations of VIC ($\bullet$) for 10 s or of ET-1 ($\blacktriangle$) for 30 s. $Ins(1,4,5)P_3$ content was determined as described in the Experimental section. Values are the means of duplicate determinations from two separate experiments.

Fig. 5. Time courses of VIC or ET-1-induced $Ins(1,4,5)P_3$ formation in NG108-15 cells and VSMCs

(a) NG108-15 cells (10^5 cells/assay) were stimulated with 10 nM-VIC ($\bullet$) or 10 nM-ET-1 ($\blacktriangle$) for the indicated times after pretreatment with 10 mM-LiCl for 5 min at 37 °C. (b) VSMCs (2×10^5 cells/assay) were pretreated with 10 mM-LiCl for 5 min at 37 °C, and were then stimulated with 100 nM-VIC ($\bullet$) or 100 nM-ET-1 ($\blacktriangle$) for the indicated times. $Ins(1,4,5)P_3$ content was determined as described in the Experimental section. Values are the means of duplicate determinations from three separate experiments.

content increased about 9-fold above the control (from a resting value of 8.3 ± 1.1 pmol/ 10^6 cells to 73.8 ± 7.3 pmol/ 10^6 cells) within 10 s after stimulation with 10 nM-VIC, whereas a smaller peak (30.8 ± 4.2 pmol/ 10^6 cells) was observed 30 s after stimulation with 10 nM-ET-1 (Fig. 5a). In contrast, VSMCs showed a much lower level of $Ins(1,4,5)P_3$ after VIC stimulation (Fig. 5b). As depicted in Fig. 6, pretreatment with PT (100 ng/ml) for 3 h suppressed VIC-mediated generation of $Ins(1,4,5)P_3$, but not $Ins(1,4,5)P_3$ production induced by ET-1. Furthermore, we confirmed that after pretreatment with PT, $Ins(1,4,5)P_3$ formation induced by 0.3 nM-VIC, which elicited a similar production of $Ins(1,4,5)P_3$ to that induced by 10 nM-ET-1, was decreased to $63.4 \pm 8.4\%$ ($n = 4$) in NG108-15 cells. These findings indicate the selective effect of PT on VIC responses.

Biphasic DAG production induced by VIC

As shown in Fig. 7, the resting levels of cellular DAG in NG108-15 cells and VSMCs were about 200 and 50 pmol/ 10^6 cells respectively. Upon stimulation with VIC (10 nM), NG108-15 cells showed the biphasic accumulation of DAG: a first transient phase and a second sustained phase. At the same concentration of VIC, there was no increase in DAG content in VSMCs. However, at a higher concentration (100 nM), a small but distinct biphasic DAG generation was observed. The time courses of the first peaks of DAG production were consistent with those of $Ins(1,4,5)P_3$ formation, indicating that the second sustained phase of agonist-induced DAG accumulation originates from phospholipid(s) other than PtdIns. Cells were incubated with 40 μ M quin 2/AM for 10 min to chelate intracellular Ca^{2+} , and then stimulated with VIC. The initial transient of DAG accumulation was partially decreased, whereas the second phase of DAG production was completely abolished (Fig. 7). Thus the Ca^{2+} -dependency of the second DAG accumulation also supports the possibility of another source than PtdIns.

Next, to determine the source of the second sustained accumulation of DAG, we examined changes in the radioactivity distribution of VIC-stimulated NG108-15 cells prelabelled with [^{14}C]palmitic acid (Table 1). About 56 % of the total radioactivity

Fig. 6. Differential effects of PT on Ins(1,4,5) P_3 formation in VIC- or ET-1-stimulated NG108-15 cells and VSMCs

NG108-15 cells (10^5 /assay) were incubated in the absence ($\square$) or presence ($\boxplus$) of PT (100 ng/ml) for 3 h and treated with 10 mM-LiCl for 5 min, and were then stimulated with 10 nM-VIC for 10 s or 10 nM-ET-1 for 30 s. VSMCs (2×10^5 cells/assay) were incubated in the absence or presence of PT (100 ng/ml) for 3 h, then stimulated with 100 nM-VIC for 10 s or 100 nM-ET-1 for 30 s. Ins(1,4,5) P_3 content was measured as described in the Experimental section. Each point represents the mean $\pm$ S.E.M. from four separate experiments (* $P < 0.01$; ** $P < 0.05$). C, control.

was incorporated into PtdCho. Exposure of labelled cells to VIC (10 nM) for 5 min, but not for a short time (10 s), resulted in a significant increase in both DAG and PtdOH, which was concurrent with the loss of ^{14}C radioactivity from PtdCho. In contrast, no discernible changes were observed in other phospholipid fractions. Similar findings of an increase in DAG accompanied by a decrease in PtdCho were obtained in [^3H]myristic acid-labelled NG108-15 cells (Table 2). These results strongly suggest that PtdCho may serve as a source for the second phase of DAG production.

Table 1. VIC-induced changes in radioactivity distribution in lipids from NG108-15 cells labelled with [^{14}C]palmitic acid

NG108-15 cells were labelled to equilibrium with 0.15 μCi of [^{14}C]palmitic acid/ml for 48 h. The cell suspension (2×10^6 cells/assay) was incubated with 10 nM-VIC for the indicated times. After extraction of cellular lipids, neutral lipids were separated on t.l.c., and DAG containing ^{14}C radioactivity was determined as described under 'Methods'; PtdOH, PtdCho, phosphatidylethanolamine (PtdEtn) and PtdIns were separated by two-dimensional t.l.c. and analysed as described under 'Methods'. Results are means ($\pm$ S.E.M.) of d.p.m./assay and percentage of total radioactivity in each cellular phospholipid ($n = 4$): * $P < 0.05$; $\dagger P < 0.01$.

	DAG	PtdOH	PtdCho	PtdEtn	PtdIns
Control					
d.p.m./assay	1458 $\pm$ 55	554 $\pm$ 10	48218 $\pm$ 529	10338 $\pm$ 389	4440 $\pm$ 75
(%)	1.71	0.65	56.72	12.10	5.23
10 s					
d.p.m./assay	1465 $\pm$ 32	656 $\pm$ 52	47869 $\pm$ 672	10427 $\pm$ 121	4227 $\pm$ 227
(%)	1.72	0.77	56.33	12.32	5.01
5 min					
d.p.m./assay	2131 $\pm$ 79 $\dagger$	783 $\pm$ 32 $\dagger$	44655 $\pm$ 829*	10451 $\pm$ 131	4335 $\pm$ 84
(%)	2.51	0.87	52.51	12.26	5.06

Fig. 7. VIC-mediated biphasic DAG generation in NG108-15 cells and VSMCs

NG108-15 cells (10^5 /assay) or VSMCs (4×10^5 cells/assay) were preincubated with Ca^{2+} -free HBSS ($\bullet$) or with the buffer containing 40 μM quin 2/AM ($\circ$) for 10 min, and were then stimulated with 10 nM-VIC or 100 nM-VIC respectively. The mass of DAG was measured as described in the Experimental section. Each point represents the means from at least four separate experiments.

Table 2. Effects of prolonged PMA treatment on VIC-induced PtdCho loss and DAG accumulation in NG108-15 cells labelled with [^3H]myristic acid

NG108-15 cells were seeded on a 60 mm-diam. dish at a density of 10^5 cells/dish. After culture for 48 h, the cells were labelled with [^3H]myristic acid (0.5 μCi /dish) for 12 h and then treated with or without PMA (100 ng/ml) in the medium for 12 h. After harvest, the cell suspension was incubated with or without 10 nM-VIC in HBSS for 5 min. After extraction of cellular lipids, lipids were separated on t.l.c. and analysed as described under 'Methods'. Results (d.p.m./assay) represent means $\pm$ S.E.M. from four separate experiments: * $P < 0.05$; $\dagger P < 0.01$.

	No PMA		+PMA	
	PtdCho	DAG	PtdCho	DAG
Control	108994 $\pm$ 1644	2933 $\pm$ 197	107301 $\pm$ 2702	2570 $\pm$ 483
VIC (10 nM)	100308 $\pm$ 2544*	4725 $\pm$ 286 $\dagger$	105346 $\pm$ 1963	3128 $\pm$ 393

Fig. 8. VIC-mediated production of choline metabolites in $[^3\text{H}]$ choline-labelled NG108-15 cells

Cells were labelled with $[^3\text{H}]$ choline ($0.5 \mu\text{Ci}/\text{ml}$) for 48 h and then treated with 10 nM-VIC in HBSS for the indicated times. Choline metabolites were extracted and analysed for $[^3\text{H}]$ choline and $[^3\text{H}]$ phosphocholine as described in the Experimental section. Results are means $\pm$ S.E.M. ($n = 6$).

In order to know whether PtdCho-specific PLC or/and PtdCho-specific phospholipase D (PLD) activation is responsible for PtdCho breakdown, we examined metabolites of PtdCho in NG108-15 cells. Cells were labelled to equilibrium with $[^3\text{H}]$ choline ($0.5 \mu\text{Ci}/\text{ml}$) for 48 h. The relative distribution of label among the metabolites of the water-soluble fraction in unstimulated cells was phosphocholine 78.0%, choline 1.5%, glycerophosphocholine 14.5%, others 6%. As shown in Fig. 8, the metabolite produced by VIC stimulation was entirely $[^3\text{H}]$ phosphocholine, and the increase in $[^3\text{H}]$ phosphocholine preceded that in $[^3\text{H}]$ choline, suggesting that PtdCho-specific PLC plays an important role in VIC-induced PtdCho hydrolysis.

Furthermore, we have examined PLD activation by VIC in NG108-15 cells labelled with $[^3\text{H}]$ myristic acid. The addition of 10 nM-VIC to the cells (for 5 min) provoked a significant elevation of $[^3\text{H}]$ PtdOH (results not shown). In the presence of ethanol, it induced a 1.8-fold increase (from 470 to 850 d.p.m./assay) in $[^3\text{H}]$ PtdEtOH, and this increase was accounted for by a decrease in $[^3\text{H}]$ PtdOH. $[^3\text{H}]$ PtdEtOH is formed from $[^3\text{H}]$ PtdCho by a transphosphatidyl transfer reaction unique to PLD in the cells [33].

As shown in our previous report [28], the long-term (12 h) pretreatment of NG108-15 cells with PMA (100 ng/ml) resulted in down-regulation of PKC. In the PKC-depleted cells, VIC-induced PtdCho breakdown and DAG accumulation did not occur (Table 2), which implies that activation of PKC is required for the sustained DAG formation owing to PtdCho hydrolysis by a PLC.

DISCUSSION

The results obtained here demonstrated that VIC induced much greater responses in $\text{Ins}(1,4,5)\text{P}_3$ formation, $[\text{Ca}^{2+}]_i$ transient and DAG accumulation in NG108-15 cells compared with those in VSMCs, suggesting that VIC, a member of the ET family, may act not only as a vasoconstrictor and gastrointestinal hormone but also as a neuromodulator.

Our previous experiments with NG108-15 cells showed that the sequential addition of the same stimulant, either VIC or ET-1, failed to cause the second response [16]. This homologous desensitization may be due to receptor down-regulation, as observed in VSMCs which had been sequentially challenged

with ET-1 [34]. But ET-1 followed by VIC, or vice versa, did not cause attenuation of Ca^{2+} as well as of the $\text{Ins}(1,4,5)\text{P}_3$ response induced by the second agonist [16]. As demonstrated in the present study, similar phenomena were also observed with VSMCs. In addition, combined application of maximal doses of VIC (10 nM) and ET-1 (10 nM) to NG108-15 cells produced the additive response of $\text{Ins}(1,4,5)\text{P}_3$. Thus these two structurally related peptides, VIC and ET-1, are considered to act through distinct receptors. Recently it was reported that different profiles were observed in specific binding affinity for various ETs and a group of structurally related peptide toxins, sarafotoxins S6, in several tissues, including aorta and brain [35].

It was found in the present study that the responses of $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ to VIC and ET-1 were different in sensitivity to PT treatment; PT had no significant effects on the ET-1-induced responses, but it suppressed the VIC-induced ones with either a maximal or a sub-maximal dose of VIC. We and others have previously obtained evidence which indicates that PT-sensitive and -insensitive GTP-binding proteins would be present in NG108-15 cells [24,36,37]. Thus one would expect that distinct GTP-binding proteins may be involved in VIC- and ET-1-mediated phosphoinositide turnover.

Apart from transient $[\text{Ca}^{2+}]_i$ increase and $\text{Ins}(1,4,5)\text{P}_3$ formation, DAG accumulation showed a typical biphasic pattern in NG108-15 cells and VSMCs: the first transient phase was followed by the second sustained and large phase. The first initial peak of DAG accumulation coincided with that of $\text{Ins}(1,4,5)\text{P}_3$ formation, indicating that DAG was derived from $\text{PtdIns}(4,5)\text{P}_2$. When cells were pretreated with quin 2/AM to chelate intracellular Ca^{2+} , the initial DAG phase was partially decreased, whereas the second phase was completely abolished. Differential Ca^{2+} -dependency of the two DAG phases suggested that phospholipid(s) other than PtdIns contribute to the second phase. Recently, the biphasic accumulation of DAG upon stimulation has been shown in various cell systems [38], and several lines of evidence showed that the agonist-induced hydrolysis of PtdCho is responsible for the second DAG accumulation. In the present study, we labelled NG108-15 cells with $[^{14}\text{C}]$ palmitate or $[^3\text{H}]$ myristate to determine the source(s) of the second DAG phase. An appreciable decrease in PtdCho and significant increases in DAG and PtdOH were observed in VIC-stimulated cells labelled with radioactive fatty acids, indicating that PtdCho is a likely candidate for the source of the second DAG phase. However, loss of radioactivity from PtdCho was much greater than the increment of DAG and PtdOH. Although we are unable to offer a clear explanation for the discrepancy, it is possible that a phospholipase A_2 hydrolysing PtdCho may be activated in these cells, as reported in ET-1-stimulated VSMCs [39,40].

Two metabolic pathways for DAG formation from PtdCho have been proposed: PtdCho-specific PLC and PtdCho-specific PLD/PtdOH phosphohydrolase [23,24,41–45]. In the current experiment, when $[^3\text{H}]$ choline-labelled NG108-15 cells were stimulated with VIC, the metabolite produced was almost entirely $[^3\text{H}]$ phosphocholine. Only a small amount of $[^3\text{H}]$ choline was produced. This finding, taken together with the time course for the two types of metabolites (Fig. 8), led us to assume that PtdCho breakdown upon VIC stimulation occurred mainly by the action of PtdCho-specific PLC. When the cells were stimulated with VIC in the presence of ethanol, a small but significant amount of PtdEtOH was produced, and the comparable amount of PtdOH was decreased. The results indicate enhancement of PLD phosphatidyltransferase activity when ethanol acts as a phosphatidyl-group acceptor.

Furthermore, we have found that down-regulation of PKC by long-term exposure to PMA prevented the VIC-induced delayed

DAG accumulation in NG108-15 cells. Although PLC-catalysed hydrolysis of PtdCho is known to be activated by PKC in various types of cells [21,23], a recent investigation has shown that PMA also caused the activation of PtdCho-specific PLD in NG108-15 cells [33]. As demonstrated in the present study, the VIC-induced second large accumulation of DAG was completely abolished in the Ca^{2+} -depleted NG108-15 cells. We have previously shown that the Ca^{2+} -depleted cells failed to cause translocation of PKC in neuroblastoma NCB-20 cells [19]. These findings indicate the requirement for PKC in the activation of PtdCho breakdown catalysed by either PLC or PLD.

This study was supported in part by a research grant from the Ministry of Education, Science and Culture of Japan.

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Received 22 March 1990/28 June 1990; accepted 16 July 1990