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Effects of vasoactive intestinal contractor (VIC) and endothelin on intracellular calcium level in neuroblastoma NG108-15 cells

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Effects on $[Ca^{2+}]_i$ levels of endothelin-1 (ET) and vasoactive intestinal contractor peptide (VIC), which is a novel member of the endothelin family, were examined in fura 2-loaded neuroblastoma NG108-15 cells. VIC was found to be a very effective stimulus for intracellular Ca^{2+} mobilization and to be more potent than ET. Intracellular calcium response to sequential addition of two stimulants exhibited the homologous desensitization of either ET or VIC, but no heterologous desensitization between ET and VIC. This indicates evidence suggesting that these two peptides act through distinct receptors.

Vasoactive intestinal contractor; Ca^{2+} level, intracellular; Endothelin-1; Fura 2; Inositol 1,4,5-triphosphate; (Neuroblastoma NG108-15)

1. INTRODUCTION

Endothelin has been isolated from the vascular endothelium as a polypeptide consisting of 21 amino acid residues that exerts a potent vasoconstrictive action [1]. In addition to this characteristic function, the peptide displays several other biological effects [2–5]. Cloning studies of genomic DNA have shown three distinct members of the endothelin family [6,7]. A newly discovered peptide, VIC, differed from ET in 3 amino acid residues and had in vivo pressor activity similar to that of ET [8]. However, the findings that the VIC gene is expressed in intestine but not in other tissues or endothelial cells and that VIC induces strong contraction of mouse ileum, suggested that VIC might act as a gastrointestinal hormone [6,8]. Since gastrointestinal peptides such as VIP act as brain-gut peptides, effects of VIC on neuronal cells are worth studying.

In the present study, we examined the effects of VIC and ET on the $[Ca^{2+}]_i$ level and $IP_3(1,4,5)$ production in neuroblastoma NG108-15 cells.

2. MATERIALS AND METHODS

Fura 2, fura 2/AM were purchased from Dojindo Laboratory (Kumamoto, Japan). VIC and ET were made using a solid-phase peptide synthesizer as described [8]. All other chemicals were obtained from commercial sources and were of the highest quality available.

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Abbreviations: VIC, vasoactive intestinal contractor; ET, endothelin-1; VIP, vasoactive intestinal polypeptide; $IP_3(1,4,5)$, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; DMEM, Dulbecco's modified Eagle's medium

2.1. *Cell culture*

NG108-15 cells were kindly supplied by Dr H. Higashida (Neuroinformation Research Institute, Kanazawa University, Japan) and cultured in DMEM with 5% fetal calf serum as described [9].

2.2. *Measurement of $[Ca^{2+}]_i$ in single cells*

The cells were plated at a density of 2×10^4 cells/chamber on the glass coverslip which adhered to the smooth lower side of the Flexiperm-Disc (Heraeus Biotechnology) and cultured for 48 h. The collected cells were washed twice with serum-free DMEM and loaded with fluorescence indicator fura 2/AM (2 μM) for 40 min at 37°C in 0.3 ml of serum-free DMEM, then rinsed free of extracellular dye and incubated for 15 min to allow deesterification of the dye. Fluorescence was measured for single cells at 37°C using continuous alternating excitation (5 s/reading) from dual monochromators set at 340 and 360 nM with ARGUS-100/CA Software no. 1 (Hamamatsu Corp., Japan). Fluorescent emission greater than 500 nM was measured by photon counting. The linear 360 nM interpolation was used and the corrected fluorescent emission intensity ratio, using 340 and 360 nM excitation, was monitored continuously. Each trace represents measurements recorded from two separate experiments and is representative of at least 5 similar traces from a single field of about 20 cells.

2.3. *Determination of mass content of $IP_3(1,4,5)$*

$IP_3(1,4,5)$ was quantitatively measured in ET- and/or VIC-stimulated NG108-15 cells using the $IP_3(1,4,5)$ assay kit (Amersham) as described [10].

3. RESULTS AND DISCUSSION

3.1. *Increase of intracellular Ca^{2+} level by VIC and ET*

As shown in the representative tracings (fig.1A,B), exposure of fura 2-loaded NG108-15 cells to 1×10^{-8} M of VIC or ET induced a rapid rise in $[Ca^{2+}]_i$ level. The maximal response of $[Ca^{2+}]_i$ to VIC was

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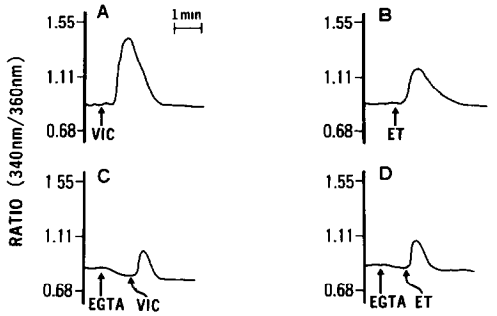

Fig.1. Effects of VIC and ET on $[Ca^{2+}]_i$ level in NG108-15 cells. $[Ca^{2+}]_i$ levels were measured in single cells as described in section 2. VIC (1×10^{-8} M) or ET (1×10^{-8} M) was added to fura 2-loaded NG108-15 cells in the presence of 1.8 mM Ca^{2+} (A,B) or in the presence of 1 mM EGTA (C,D).

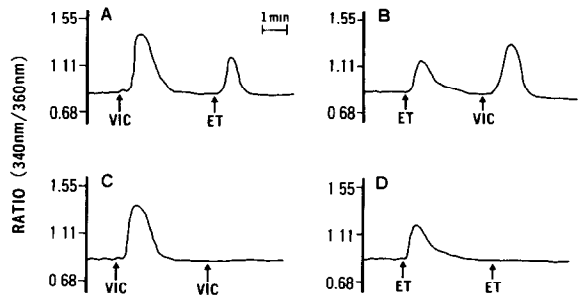

Fig.2. $[Ca^{2+}]_i$ response to sequential addition of VIC and ET. (A) 3-min stimulation with VIC (1×10^{-8} M) was followed by addition of ET (1×10^{-8} M) at the points indicated by the arrows. (B) 3-min stimulation with ET (1×10^{-8} M) was followed by addition of VIC (1×10^{-8} M). (C) VIC or (D) ET (final concentration 1×10^{-8} M) was added at the points indicated by arrows.

greater than that to ET. Minimum effective dose of VIC and ET were about 5×10^{-11} M and 2.5×10^{-10} M, respectively. The EC_{50} value of VIC (4.5×10^{-10} M) was also lower than that of ET (1×10^{-9} M), indicating that VIC is more potent in the ability to induce $[Ca^{2+}]_i$ increase.

ET has been shown to induce $[Ca^{2+}]_i$ transiently in several cell lines and tissues [5,11,12]. Two pathways have been considered to produce the increased $[Ca^{2+}]_i$ level; influx from outside the cell and intracellular mobilization by $IP_3(1,4,5)$ generated via PIP_2 hydrolysis. When porcine coronary strips were stimulated with ET, both pathways were involved in the increase of $[Ca^{2+}]_i$ [13]. Swiss 3T3 fibroblasts exposed to ET showed an initial $[Ca^{2+}]_i$ peak due to the intracellular mobilization and a subsequent substained increase by influx from extracellular medium [12].

In NG108-15 cells, the rise in $[Ca^{2+}]_i$ induced by either ET or VIC was suppressed in the presence of EGTA and the extent of inhibition was greater with VIC than with ET (fig.1C,D). This observation lead us to assume that the main source of increased $[Ca^{2+}]_i$ level was the uptake of extracellular Ca^{2+}. However, since it has been known that a bradykinin-induced $[Ca^{2+}]_i$ transient increase was largely dependent on intracellular mobilization by $IP_3(1,4,5)$ in NG108-15 cells [10], we measured the mass contents of $IP_3(1,4,5)$ in cells stimulated with VIC and ET (table 1). VIC produced much larger amounts of $IP_3(1,4,5)$ than did ET, indicating that VIC is a very potent stimulant for PIP_2 hydrolysis. In the presence of EGTA, $IP_3(1,4,5)$ generation by either VIC or ET was inhibited by 72% of 50%, respectively (data not shown). It should be noted that the degrees of inhibition in $IP_3(1,4,5)$ production were well compatible with those in the $[Ca^{2+}]_i$ rise in EGTA-treated cells. Thus, phosphoinositide breakdown by phospholipase C in this neuronal cell was dependent on extracellular Ca^{2+}, as reported in other cell systems [14–16]. From data of $IP_3(1,4,5)$ production and $[Ca^{2+}]_i$ rise, it was more likely that $[Ca^{2+}]_i$ increases caused by VIC and ET resulted largely from intracellular mobilization.

Table 1

$IP_3(1,4,5)$ generation in response to VIC and/or ET

	$IP_3(1,4,5)$ (pmol/10^6 cells)
Control	8.3 ± 1.0
ET 10 nM, 10 s	23.5 ± 4.3
VIC 10 nM, 10 s	73.8 ± 7.3
ET 10 nM, 3 min and VIC 10 nM, 10 s	68.8 ± 3.8
ET 10 nM, 3 min and ET 10 nM, 10 s	8.7 ± 1.4

The cell suspension (2×10^5 cells/assay) preincubated with 10 mM LiCl for 10 min was stimulated by adding each stimulant. The mass contents of $IP_3(1,4,5)$ were measured as described under section 2. Results represent the mean + SE of four experiments

3.2. *$[Ca^{2+}]_i$ increase and $IP_3(1,4,5)$ generation in response to sequential addition of VIC and ET*

Neither pretreatment with VIC nor ET at 10^{-8} M prevented the $[Ca^{2+}]_i$ response to subsequent addition of stimulant, which was different from that used for pretreatment (fig.2A,B). However, when the same stimulant as that used for pretreatment was employed for the second stimulation, the $[Ca^{2+}]_i$ increase was completely abolished (fig.2C,D). These findings indicate that a homologous but not heterologous desensitization of $[Ca^{2+}]_i$ response occurred between VIC and ET stimulations. Such desensitization profile was also observed for $IP_3(1,4,5)$ production (table 1), suggesting that VIC and ET act through the distinct receptors for the signal transduction.

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