Vol. 167, No. 1, 1990
February 28, 1990

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 161-167

VASOACTIVE INTESTINAL CONTRACTOR, A NOVEL PEPTIDE, SHARES A COMMON RECEPTOR WITH ENDOTHELIN-1 AND STIMULATES Ca^{2+} MOBILIZATION AND DNA SYNTHESIS IN SWISS 3T3 CELLS

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Received December 27, 1989

SUMMARY: Vasoactive intestinal contractor peptide (VIC), a novel member of the endothelin family, stimulated a rapid increase in the intracellular Ca^{2+} concentration in fura-2-loaded Swiss 3T3 cells. Sequential addition of VIC and endothelin-1 (ET_1) demonstrated the induction of both homologous and heterologous desensitization. VIC was as potent as ET_1 in displacing the binding of ^{125}I-ET_1 and in stimulating mitogenesis in Swiss 3T3 cells. These findings suggest that VIC and ET_1 share a common receptor in Swiss 3T3 cells.

Vasoactive intestinal contractor (VIC) has recently been identified as a new member of the peptides of the endothelin family (1,2). VIC differs from porcine or human endothelin (ET_1), a 21-residue vasoconstrictor peptide (3), in three amino acid residues (nos 4, 6, 7). The peptide is expressed in the intestine but not in other tissues or endothelial cells (1) and is more effective than ET_1 in inducing contraction of mouse ileum, suggesting that VIC may function as a gastrointestinal hormone (2). Binding competition measurements and chemical cross-linking studies have identified at least three distinct endothelin receptor subtypes (4-8). It is not known whether the biological effects of VIC are mediated by any of these receptors or by a distinct receptor subtype.

Abbreviations: ET_1, porcine or human endothelin; VIC, vasoactive intestinal contractor; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; EGF, epidermal growth factor; PBt_2, phorbol 12,13-dibutyrate; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin.

0006-291X/90 $1.50

A recent report showed that VIC, like ET_1, caused a rapid increase in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in neuroblastoma NG-108-15 cells (9). Sequential addition of these peptides induced homologous but not heterologous desensitization, suggesting that VIC and ET_1 act via distinct receptors, but more direct evidence (e.g. binding competition) was not presented.

Quiescent cultures of Swiss 3T3 cells have provided a useful model system to elucidate the signal transduction pathways in the action of growth factors and mitogenic peptides (10-12). ET_1 stimulates phosphoinositide breakdown, Ca^{2+} mobilization and DNA synthesis in these cells (4,13,14). Here we report that the novel peptide VIC can also act as a mitogen for Swiss 3T3 cells. VIC shares a common receptor with ET_1 and induces a rapid increase in $[Ca^{2+}]_i$ and subsequently, stimulates DNA synthesis in the presence of either epidermal growth factor (EGF) or insulin.

MATERIALS AND METHODS

Swiss 3T3 cells were maintained in culture and assays of DNA synthesis were performed by [^{3}H]-thymidine incorporation as described (15).

Determination of $[Ca^{2+}]_i$: Confluent and quiescent Swiss 3T3 cells grown in 90 mm dishes were washed twice with Dulbecco's modified Eagle's medium (DMEM) and incubated for 10 min in 5 ml DMEM containing 1 µM fura-2 tetraacetoxymethyl ester. After this incubation the cells were washed three times with electrolyte solution which contained 120 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM Hepes, 6 mM Tris and a mixture of amino acids at the same concentrations as those in DMEM (pH 7.2). The cells were suspended in electrolyte solution (2 ml) by gently scraping and transferred to a 1 cm^2 quartz cuvette. The cell suspension was stirred continuously at 37°C. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrophotometer with an excitation wavelenght of 336 nm and emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated as described (16).

Measurement of ^{125}I-ET_1 binding: Swiss 3T3 cells were incubated at 4°C with 0.5 nM ^{125}I-ET_1 in phosphate buffered saline containing 25 mM N-2-Hydroxyethylpiperazine-N'2-ethanesulfonic acid, 0.1% fatty acid free bovine serum albumin (BSA) (pH 7.0). After 3 hours, unbound radioactivity was removed by washing the cells three times with cold (4°C) phosphate buffered saline (pH 7.4) containing 0.1% BSA. The cells were extracted (20 min at 37°C) with 0.5 ml of 2% Na_2CO_3, 1% sodium dodecyl sulfate, 0.1N NaOH and cell associated radioactivity was determined in a LKB γ-counter. Under these conditions, non-specific binding, determined as cell-associated radioactivity in the presence of 1 µM unlabelled ET_1, was about 5% of the total.

Materials: ET_1 and sarafotoxin S6b were purchased from Peninsula Laboratories, Inc (U.K.). VIC was obtained from American Peptide Co.

(U.S.A.). Bovine insulin, EGF, bombesin, vasopressin, phorbol 12,13-dibutyrate (PBt_2), BSA were from Sigma Chemical Co., St. Louis, Mo. ^{125}I-ET_1 was purchased from Amersham International, Amersham, U.K.

RESULTS AND DISCUSSION

Addition of 10 nM VIC to quiescent Swiss 3T3 cells loaded with the fluorescent Ca^{2+}-indicator fura-2 increased $[Ca^{2+}]_i$ without any measurable delay (Fig. 1). $[Ca^{2+}]_i$ reached peak values at 15 s and subsequently declined towards the basal level. Subsequent addition of

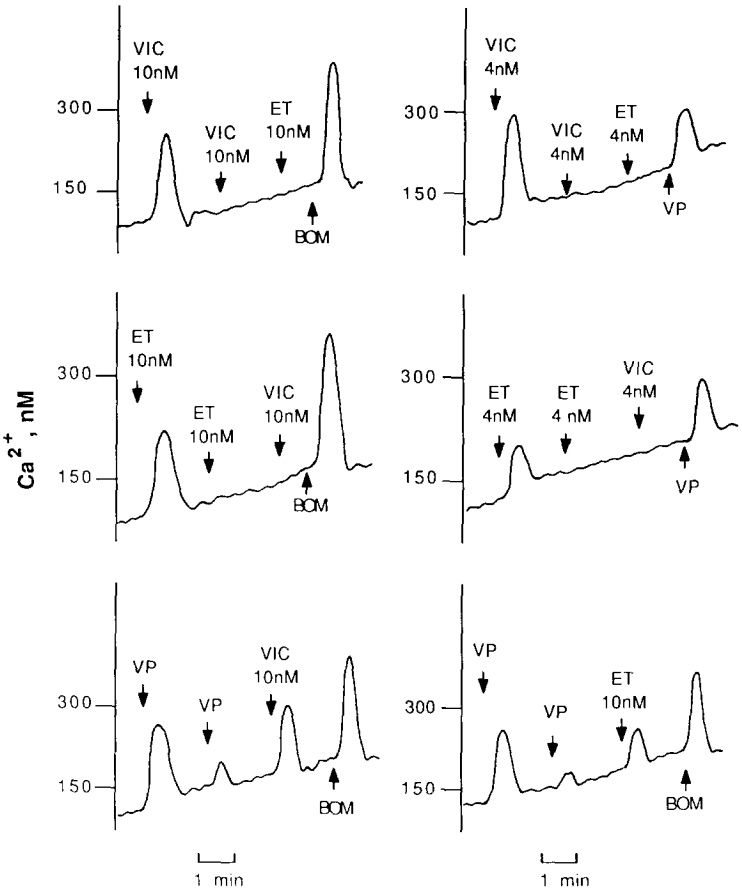

Figure 1. Effect of repeated additions of VIC and ET_1 on $[Ca^{2+}]_i$. Quiescent Swiss 3T3 cells in 90 mm dishes were loaded with fura-2 as described in Materials and Methods. Fluorescence was recorded continuously while additions were made at the points and concentrations indicated by the arrows: VIC, vasoactive intestinal contractor peptide; ET_1, endothelin-1; VP, 10 nM vasopressin; BOM, 6 nM bombesin. After a suitable control period, the peptides were added and fluorescence followed until it returned to near base line levels. After this, addition of the same or a different peptide was performed. Measurements of $[Ca^{2+}]_i$ were performed after sequential addition of Triton X-100 and ethylene glycol-bis(β-aminoethyl ether) as previously described (16).

either 10 nM VIC or 10 nM ET_1 did not elicit any further rise in $[Ca^{2+}]_i$. Bombesin, which acts through a distinct receptor (17,18 and below) caused a marked and rapid increase in $[Ca^{2+}]_i$ in cells previously exposed to both VIC and ET_1 (Fig. 1, upper). Similar sequential responses were elicited when the concentration of VIC was 4 nM instead of 10 nM and when the cells were finally challenged with vasopressin instead of bombesin. Thus, VIC not only caused homologous desensitization but also blocked the subsequent response to ET_1. Identical changes in cellular responsiveness could be induced when the order of peptide addition was reversed i.e. ET_1 either at 10 or 4 nM completely blocked the subsequent responses to VIC but did not prevent the increase in $[Ca^{2+}]_i$ caused by either bombesin or vasopressin (Fig. 1, middle).

A possible interpretation of these results is that the ET_1 and VIC responses can be blocked by any agonist that causes Ca^{2+} mobilization and activation of protein kinase C. The experiment shown in Fig. 1 (lower) using vasopressin (19,20) was designed to test this possibility. Treatment of Swiss 3T3 cells loaded with fura-2 with 10 nM vasopressin promoted a transient increase in $[Ca^{2+}]_i$ equivalent to that induced by either VIC or ET_1 and markedly attenuated the Ca^{2+} response to a subsequent addition of vasopressin. Crucially, exposure to vasopressin did not abolish the increase in $[Ca^{2+}]_i$ caused by either VIC or ET_1 (Fig. 1, lower). The results shown in Fig. 1 are different from those recently reported with neuroblastoma NG108-15 cells (9) and raised the possibility that ET_1 and VIC bind to a common receptor in Swiss 3T3 cells.

The preceding results prompted a study of the binding of $^{125}I\text{-}ET_1$ to cultures of Swiss 3T3 cells. When cultures of these cells were incubated with $^{125}I\text{-}ET_1$ at 4°C, cell-associated radioactivity reached a maximum after 3 h (results not shown). $^{125}I\text{-}ET_1$ binding to 3T3 cells was saturable (Fig. 2) and Scatchard analysis indicated the presence of a single class of high-affinity sites (K_d = 0.96 ± 0.23

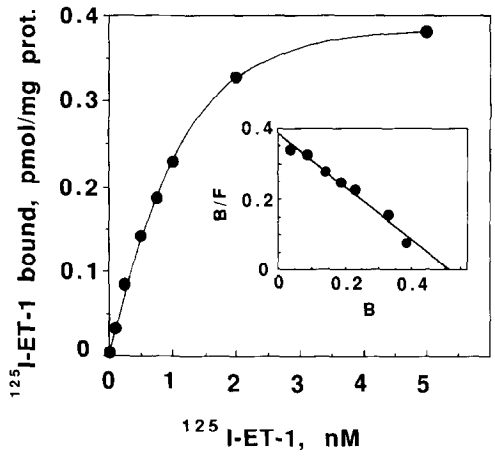

Figure 2. Concentration dependence of ^{125}I-ET$_1$ binding to intact quiescent Swiss 3T3 cells. Specific cell-associated ^{125}I-ET$_1$ binding was measured in quiescent cells incubated for 3 hours at 4°C in binding medium containing various concentrations of ^{125}I-ET$_1$. Each point represents the mean of two determinations. Specific binding is expressed as pmol/mg of protein. Inset: Scatchard plot of the same data. Bound (B) radiolabelled ligand is expressed in pmol/mg of protein; the concentration of ^{125}I-ET$_1$ in the medium (F) is in nM. All other experimental details were as described in Materials and Methods.

nM; n=3) and a maximum binding capacity of 0.5 pmol/mg of protein (Fig. 2, inset). Binding of ^{125}I-ET$_1$ to Swiss 3T3 cells was competed by unlabelled ET$_1$ and by the cardiotoxic peptide sarafotoxin S6b in a dose-dependent manner (Fig. 3). In contrast, neither bombesin nor vasopressin (up to 10 μM) reduced the specific binding of ^{125}I-ET$_1$. The salient feature shown in Fig. 3 is that VIC displaced ^{125}I-ET$_1$ binding as potently as unlabelled ET$_1$ (IC_{50} = 2 nM). These findings demonstrate for the first time that VIC and ET$_1$ share a common receptor that recognises both peptides with virtually identical apparent affinities.

Recent reports have shown that ET$_1$ can stimulate DNA synthesis in Swiss 3T3 cells (4,13) and other cell types (21,22) acting synergistically with other growth promoting factors. In view of the results depicted in Figs. 1 and 3, we tested whether VIC can stimulate DNA synthesis in quiescent cultures of Swiss 3T3 cells incubated in the absence or presence of EGF. Fig. 4 shows that VIC acted synergistically with EGF in eliciting DNA synthesis in Swiss 3T3

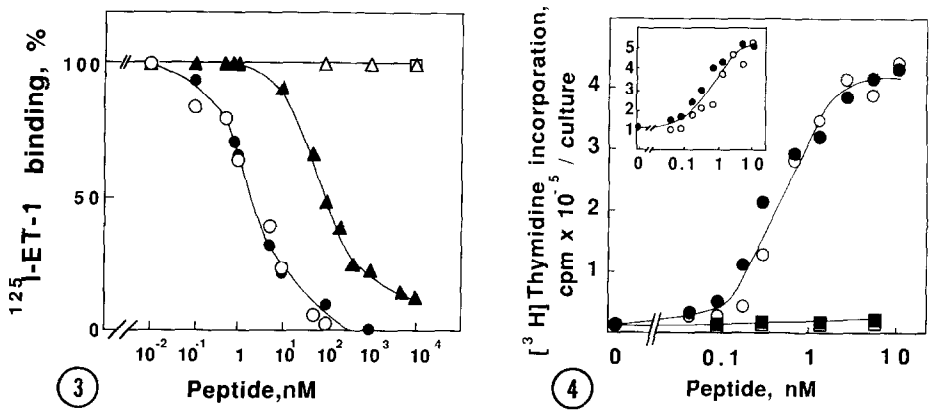

Figure 3. Inhibition of specific ^{125}I-ET$_1$ binding in intact, quiescent Swiss 3T3 cells by ET$_1$ (●), VIC (o), sarafotoxin S6b (▲), bombesin (△) and vasopressin (□). Confluent and quiescent cultures of 3T3 cells were incubated at 4°C for 3 hours with 0.5 nM ^{125}I-ET$_1$ either in the presence or in the absence of the related or unrelated peptides at the concentrations indicated. Each point represents the mean of at least two determinations. Binding of ^{125}I-ET$_1$ is expressed as a percentage of the specific binding in the absence of any unlabelled peptide (100% = 0.147 ± 0.014 pmol/mg of protein).

Figure 4. Stimulation of DNA synthesis in intact quiescent Swiss 3T3 cells by VIC and ET$_1$. The assay for DNA synthesis was performed in the absence (□ , VIC; ■ , ET$_1$) or in the presence of 5 ng/ml EGF or 1 µg/ml insulin (inset) (o, VIC; ●, ET$_1$). Confluent and quiescent cultures of Swiss 3T3 cells were washed and incubated in 2 ml DMEM/Waymouth's medium containing [^{3}H]-thymidine, with or without insulin or EGF and various concentrations of VIC or ET$_1$. The incorporation of radioactivity into acid-precipitable material was measured after 40 h. Each point represents the mean of 2 determinations. In this experiment, values obtained in the presence of either 10% fetal bovine serum or 5 ng/ml EGF plus 1 µg/ml insulin were 8.65 x 10^{-5} and 7.77 x 10^{-5} cpm culture, respectively.

cells. Maximum and half-maximal effects were achieved at 2.5 and 0.4 nM, respectively. An identical dose-response relationship was obtained when ET$_1$ was added instead of VIC. In other experiments, we observed that VIC, like ET$_1$, stimulated DNA synthesis in synergistic combination with insulin (Fig. 4, inset) or agents that either activate protein kinase C (e.g. 10-100 ng/ml PBt$_2$) or increase the cellular level of cAMP (e.g. forskolin and IBMX).

VIC is a novel peptide of the endothelin family which is expressed in the intestine but not in endothelial cells (1,2). Although recent studies concerning contractile responses (2) and Ca^{2+} mobilization in neuroblastoma cells (9) suggested the existence of separate receptors for VIC and ET$_1$, this question remained to be elucidated. Here we

show, for the first time, that VIC and ET_1 interact with a common receptor in Swiss 3T3 cells, as judged by ^{125}I-ET_1 binding competition and cross-desensitization of Ca^{2+} signals. Our results demonstrate that VIC, like ET_1, is a potent mitogen for 3T3 cells acting synergistically with other growth promoting factors. Since VIC is preferentially expressed in the intestine (1), these findings raise the possibility that this novel peptide may act as a growth factor for target cells in the gastrointestinal tract.

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