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BQ-123, CYCLO(-D-Trp-D-Asp-Pro-D-Val-Leu), IS A NON-COMPETITIVE ANTAGONIST OF THE ACTIONS OF ENDOTHELIN-1 IN SK-N-MC HUMAN NEUROBLASTOMA CELLS

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SK-N-MC cells, derived from a human neuroblastoma, respond to endothelin (ET) peptides with an increase in the free intracellular calcium concentration. The response is biphasic, with the secondary plateau phase being abolished or reduced by removal of extracellular Ca^{2+} or by the presence of 100nM nitrendipine. Restoration of Ca^{2+} to the bathing solution in cells stimulated by ET-1 in the absence of Ca^{2+} caused the plateau to reappear. The order of potency of ET family peptides was ET-2≥sarafotoxin S6b≥ET-1>>ET-3, suggesting that ET_A receptors mediate the response. Sarafotoxin S6c and the C-terminal hexapeptide endothelin (16-21) were inactive in these cells. [$Ala^{1,3,11,15}$]ET-1, a linear analogue of ET-1 which has been suggested to be a selective ET_B receptor agonist, was a weak competitive antagonist of the actions of ET-1 in these cells. However, BQ-123, recently introduced as a selective and competitive antagonist at ET_A receptors, was a potent non-competitive antagonist of ET-1 giving a 50% reduction in the maximum response at 6nM.

The endothelins (ETs) are a group of peptides characterized by containing 21 amino acids and by having two disulphide bridges between cysteine residues 1-15 and 3-11. In the human genome there is coding for 3 of these peptides which have been designated ET-1, ET-2 and ET-3 (1). Although ET-1 was first isolated from cultured vascular endothelial cells (2) there is evidence that the ETs might have physiological roles in many other tissues, including the central nervous system (CNS). There are binding sites for the ETs in the brain (3), mRNA for ET-1 is expressed in several brain regions (4, 5) and ET-like immunoreactivity for ET-1 and ET-3 has been found in the CNS (5, 6).

Activation of ET receptors in many tissues causes an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (7-9) and there is an increase in phosphatidylinositol turnover in various regions of the CNS (11). Thus it is likely that there might be an increase in $[Ca^{2+}]_i$ in nerve cells as a result of stimulation by the ETs although ET-1 has been reported to have no effect on $[Ca^{2+}]_i$ or $^{45}Ca^{2+}$ uptake in rat brain synaptosomes (9). However, $[Ca^{2+}]_i$ is increased by ET-1 and ET-3

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in glial cells such as cultured C6 glioma cells and cerebellar astrocytes (12) as well as in NG108-15 neuroblastoma x glioma cells (13).

Two types of receptor for the ETs have been cloned (14, 15). One, designated the ET_A receptor (16), is highly selective between endothelin isopeptides, with ET-1 being much more potent than ET-3 (14), whereas the second, ET_B, receptor, has equal affinity for ET-1 and ET-3 (15). On this basis, the receptors mediating the increase in phosphatidylinositol turnover and the binding sites in the CNS appear to be of the ET_B type (11, 17). The unambiguous identification of two receptor types has renewed interest in the production of selective ligands and it has been reported that a linear analogue of ET-1 containing no disulphide bridges, [$Ala^{1,3,11,15}$]ET-1 (17), is a selective ET_B receptor agonist (18). A selective ET_A antagonist, BE-18275B, has been isolated from the culture medium of *Streptomyces misakiensis* (19). As a result of structure activity studies based on BE-18275B, a more potent and selective ET_A receptor antagonist, BQ-123 (cyclo[-D-Trp-D-Asp-Pro-D-Val-Leu]), has been described (20).

With these compounds it should become easier to identify ET receptor types mediating the responses to the ETs. Thus, we have taken the opportunity to reinvestigate the types of ET receptor that occur in tissues of CNS origin, in particular of neural origin, and in this paper we report the use of ET isopeptides and of selective ligands to show that cultured SK-N-MC human neuroblastoma cells contain ET_A receptors which mediate increases in $[Ca^{2+}]_i$ but that BQ-123 is a non-competitive antagonist of ET-1 in these cells.

MATERIALS AND METHODS

Cell culture: SK-N-MC cells were obtained from the American Type Culture Collection and grown for 20-24h at a density of 60 000 cells/well on Lab-Tek Type 4808 8-well tissue culture/chamber slides using MEM medium containing fetal calf serum (10%), L-glutamine (1%), non-essential amino acids (1%) and penicillin/streptomycin (100 IU and 100 µg/ml respectively).
$[Ca^{2+}]_i$ measurements: Culture medium was removed from the cells by washing twice with Krebs solution of the following composition (mM): NaCl, 112; KCl, 4.9; $NaHCO_3$, 25; $CaCl_2$, 1.25; $MgSO_4$, 1.2; KH_2PO_4, 1.0; D-glucose, 11.5. The cells were then incubated for 30 min at 37°C with 2.5 µM fura-2 (Molecular Probes, OR, USA) in Krebs solution. The cells were then washed twice with Krebs solution and the second wash was left in contact with the cells for 20 min at 37°C. The cells were then kept at 30°C and changes in $[Ca^{2+}]_i$ were determined by placing the slides on a Nikon Diaphot inverted microscope for measurement of epifluorescence from 15-25 cells using an SLM-Aminco 8000C spectrofluorimeter (SLM Instruments Inc, Urbana, IL, USA). The cells were alternately stimulated at 300 Hz at 340 and 380 nM and emission was measured at 510nM. $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz *et al.* (21).

Peptide antagonists or nitrendipine were added to the wells 10 min before the addition of agonists. All drugs were added in either normal Krebs solution or Ca^{2+}-free Krebs solution, as appropriate. Ca^{2+}-free Krebs had the same composition as the normal Krebs except that the Ca^{2+} was omitted.
Data processing: Concentration/response data were fitted to a logistic equation, *i.e.* response = $R_{max}/\{1-(EC_{50}/\text{agonist concentration})^{n_H}\}$ where EC_{50} is the concentration giving 50% of the maximal response, R_{max} is the maximal response and n_H is a slope factor, using GraFit (Version 2.0; Erithacus Software, London) on a Compaq 286 personal computer. Values are given as means±SEM and values of n show either the number of wells examined at a particular dose of peptide or the range of the number of wells used at the various doses used to construct a concentration/response curve. Comparison between means was made by Student's t-test or analysis of variance as appropriate and $P < 0.05$ was taken as indicating statistical signifance.
Peptides and drugs: ET-1, ET-2, ET-3 and sarafotoxin S6b (STX) were obtained from The Peptide Institute, Osaka, Japan. [$Ala^{1,3,11,15}$]ET-1 was from Neosystem Laboratoire (Strasbourg,

France) and sarafotoxin S6c and endothelin (16-21) were from Peninsula Laboratories (Europe) Ltd (St Helens, Merseyside, England). BQ-123 was synthesized by standard solid phase synthesis techniques and cyclized with diphenylphosphoryl azide. All peptides were dissolved in Krebs solution, with or without Ca^{2+}, as required. Nitrendipine was from Bayer (Leverkusen, Germany) and was dissolved to give a stock solution of 1mM in ethanol; this solution was then diluted in Krebs solution. EGTA was from Sigma Chemical Co. (La Verpillière, France).

RESULTS

ET-1 caused biphasic changes in $[Ca^{2+}]_i$ which were characterized by a peak response, occurring within 10-20s after addition of the peptide, followed by a small, but sustained plateau (Fig. 1a). Replacement of Ca^{2+}-containing Krebs with the Ca^{2+}-free Krebs solution containing 2mM EGTA significantly reduced $[Ca^{2+}]_i$ from 72.3±4.6nM to 29.5±3.8nM ($n = 21$; $P < 0.001$) but the initial peak response was not dependent on the presence of extracellular Ca^{2+} since it occurred in Ca^{2+}-free Krebs solution containing 2mM EGTA (Fig. 1b) nor was it affected by the presence of 100nM nitrendipine in the bathing fluid (Fig. 1c). Fig. 2 shows that, although the peak responses to three concentrations of ET-1 were not affected by removal of external Ca^{2+} or the presence of nitrendipine, both the L-type Ca^{2+}-channel blocker and removal of external Ca^{2+} greatly reduced or abolished the plateau phase. In those cells stimulated with ET-1 in the absence of external Ca^{2+}, readdition of Ca^{2+} restored the plateau phase (Fig. 1c).

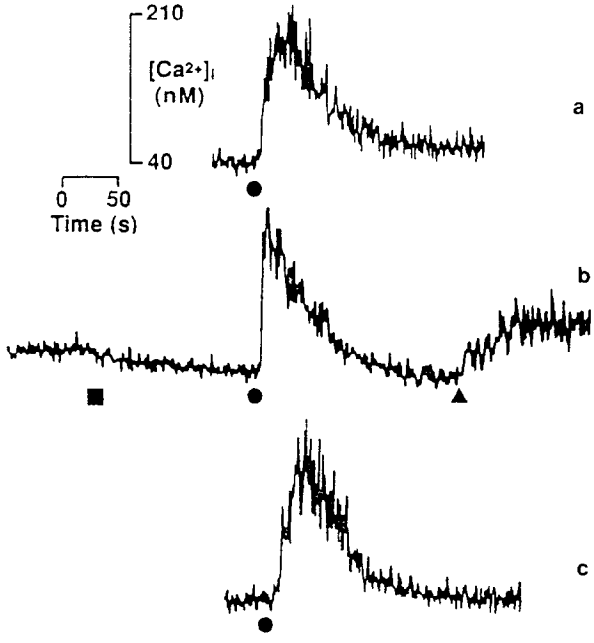

Figure 1. Traces showing the responses obtained to 300nM ET-1 in (a) normal Krebs solution, (b) in Ca^{2+}-free Krebs solution containing 2mM EGTA, and (c) in normal Krebs solution containing 100nM nitrendipine. (●) shows when the ET-1 was added; (■) shows when the Ca^{2+}-containing buffer was replaced with Ca^{2+}-free/EGTA buffer; and (▲) shows the point of readdition of Ca^{2+}-containing buffer.

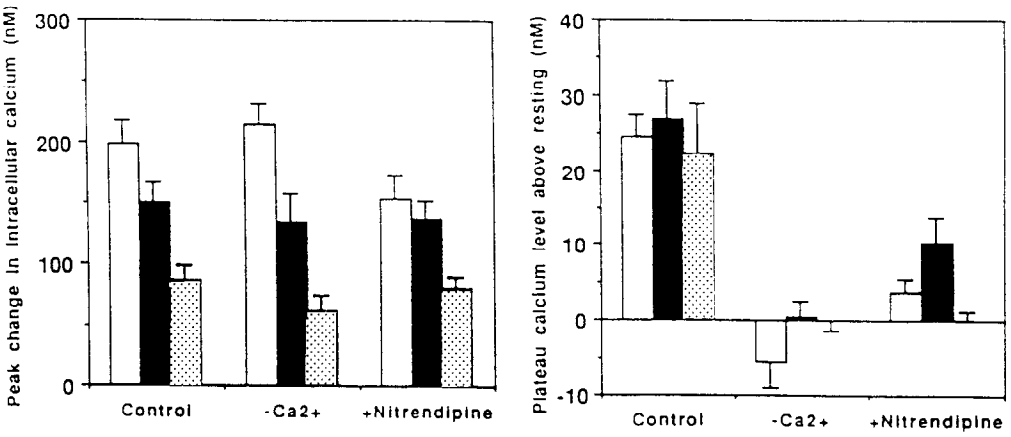

Figure 2. Effects of 100nM nitrendipine and removing extracellular Ca^{2+} with 2mM EGTA on the peak (left panel) and the plateau (right panel) responses to 3 concentrations of ET-1. Open, filled and stippled columns respectively show the responses to 300nM ET-1 (n values: 12, control; 4, nitrendipine; 6, without Ca^{2+}), 100nM ET-1 (n = 15; 7; 4) and 30nM ET-1 (n = 4; 4; 6).

The peak responses in $[Ca^{2+}]_i$ to ET-1 were concentration-related, as were those to ET-2, ET-3 and STX (Fig. 3) but the plateau responses showed no clear relationship to the concentration of agonist (e.g. see control responses in Fig. 2). Table 1 and Fig. 3 show that ET-1 and ET-2 gave approximately the same maximum responses but that ET-3 and STX were partial agonists in

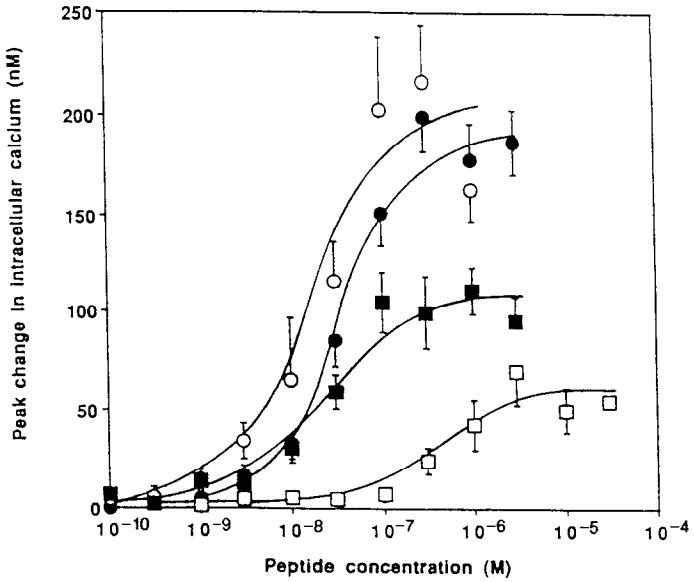

Figure 3. Log concentration/response curves for the increase in $[Ca^{2+}]_i$ caused by ET-1 (●; n = 4-15), ET-2 (○;n =3-4), ET-3 (□;n =4-6) and STX (■; n =4-6) in cultured SK-N-MC cells.

Table 1. Maximal peak changes in intracellular Ca^{2+} (R_{max}) and potencies (EC_{50}) of ETs and sarafotoin S6b in SK-N-MC cells

Peptide	EC_{50} (nM)	R_{max} (nM)	n_H
ET-1	33.5 ± 4.5	191 ± 6	1.3 ± 0.2
ET-2	17.6 ± 5.0	200 ± 16	1.2 ± 0.4
ET-3	376 ± 12**	57.9 ± 4.9**	1.5 ± 0.1
STX	20.0 ± 4.6	106 ± 6*	1.2 ± 0.3

Significant difference from the corresponding value for ET-1: $*P < 0.05$; $**P < 0.01$.

relation to the first two peptides. The order of potency of the peptides was ET-2≥STX≥ ET-1>>ET-3 (Table 1). Sarafotoxin S6c (n = 5) and ET (16-21) (n = 4) had no effect on $[Ca^{2+}]_i$ at concentrations of 10μM although at 3μM sarafotoxin S6c there was a non-significant increase in $[Ca^{2+}]_i$ of 4.8±3.7nM (n = 5).

Incubation of the SK-N-MC cells for 10 min with 30μM [$Ala^{1,3,11,15}$]ET-1 caused a parallel shift to the right of the ET-1 concentration/response curve (Fig. 4a). Fitting the data to the logistic equation showed that the maximum response in the presence of [$Ala^{1,3,11,15}$]ET-1 was a change in $[Ca^{2+}]_i$ of 191±6.4nM (n = 5-9) and the mid-point of the curve was at 211±60nM, giving a concentration ratio of 6.3. Using the Schild equation, this gives a K_d for [$Ala^{1,3,11,15}$]ET-1 of 5.6 μM. In none of the cells tested did [$Ala^{1,3,11,15}$]ET-1 increase $[Ca^{2+}]_i$. In contrast, Fig. 4b shows that incubation of the cells for 10min with 6 or 12nM BQ-123 caused no change in the mid-points of the ET-1 concentration/response curves (26.4±2.0nM and 38.6±8.9nM, respectively) but the maximal responses were significantly reduced to 123±4.4 and 111±7.2nM, respectively ($P < 0.05$) .

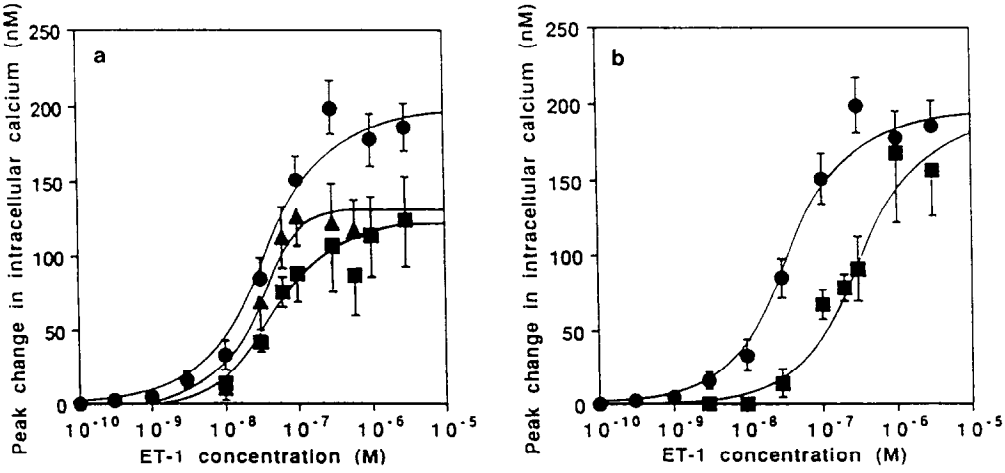

Figure 4. Effects on the peak changes in $[Ca^{2+}]_i$ in response to ET-1 in cultured SK-N-MC cells of (a) 6nM (▲; n = 4-8) or 12nM (■; n = 3-6) BQ-123 and (b) 30μM [$Ala^{1,3,11,15}$]ET-1 (■; n = 5-9). (●) represents the control responses to ET-1 (n = 5-15).

DISCUSSION

The results show that SK-N-MC cells respond to stimulation by members of the endothelin/sarafotoxin family of peptides with an increase in $[Ca^{2+}]_i$. Although the maximal responses obtained with ET-1 and ET-2 were only of the order of 200nM, this is much greater than the maximal response to neuropeptide Y (50nM) by the same cells (22). The responses to ETs were similar to those to neuropeptide Y, and those to ETs in many other cells (7-9), in that they were biphasic and the peak response was not dependent on the presence of extracellular Ca^{2+}. However, the plateau response was dependent on the presence of Ca^{2+} which, since it was also greatly reduced by the presence of nitrendipine, largely entered the cells through L-type voltage-sensitive Ca^{2+} channels. Readdition of Ca^{2+} to the bathing medium restored the plateau responses showing that the Ca^{2+} entry channels were open even in the absence of extracellular Ca^{2+}.

Comparison of the potencies of 4 members of the endothelin/sarafotoxin family of peptides shows that ET-3 was much less potent (10-20 fold) than ET-1, ET-2 and STX. This suggests that the receptors mediating the response in $[Ca^{2+}]_i$ to the ETs are of the ET_A type and this is confirmed by the weak potency of $[Ala^{1,3,11,15}]$ET-1 as an antagonist and by the absence of agonist activity in sarafotoxin S6c, which is only weakly active at contracting rat aorta and has also been proposed to be a selective ET_B agonist (23). Indeed, the estimated K_d for $[Ala^{1,3,11,15}]$ET-1 in this study represents an affinity for the ET_A receptors in SK-N-MC cells which is less than that (0.57μM) reported for inhibition of binding of $[^{125}I]$ET-1 to pig aortic smooth muscle membranes (18). It has recently been proposed that $[Ala^{1,3,11,15}]$ET-1 peptide is a selective agonist at ET_B receptors (18) and it is interesting to note that the present results show it apparently to be a competitve antagonist at ET_A receptors.

The absence of activity in ET (16-21) shows that SK-N-MC cells do not contain receptors similar to those through which this hexapeptide causes contraction of guinea-pig bronchus (24). These receptors were originally termed "ET_B" receptors but it seems likely that, in the bronchial tree, the hexapeptide does not interact with the same receptors as ET-1 since it does not inhibit $[^{125}I]$ET-1 binding (25).

The behaviour of BQ-123 was more complex. This cyclic pentapeptide has been developed as a selective and potent antagonist at ET_A receptors (20) and it causes parallel shifts to the right of the log concentration/response curve for ET-1 in pig isolated coronary arteries with a pA_2 of 7.4. It also inhibits the binding of $[^{125}I]$ET-1 to porcine aortic smooth muscle membranes with a K_i of 22nM whereas the K_i in cerebellar membranes, a source of ET_B receptors, is 23μM. However, in this study the peptide did not act as a competitive antagonist against ET-1 but as a non-competitive antagonist active in the low nanomolar range. The reason for this different behaviour is unclear since the results with $[Ala^{1,3,11,15}]$ET-1 show that parallel shifts in the log concentration/effect curve to ET-1 can be obtained. This suggests that BQ-123 might have an effect on the mobilization of $[Ca^{2+}]_i$ by interfering with the stages after interaction of ET-1 with its receptors, for example by interfering with G-protein coupling or by affecting the ability of the intracellular stores to release their Ca^{2+}.

Nevertheless, the data presented here show that use of selective ligands, as well as potency ratios for agonists, can now be used to identify receptors subtypes mediating the responses to

ETs. The results are of interest since they show that cells of CNS origin can contain receptors of the ET_A subtype since most of the previously reported responses to ETs in the CNS appeared to be mediated by ET_B receptors.

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