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Characterization of endothelin receptors on a human neuroblastoma cell line: evidence for the ET_A subtype

Lesley C. Wilkes & Michael R. Boarder

Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN

1 Specific binding sites for synthetic endothelin (ET) isoforms were studied on intact cells of the SK-N-MC cell line, derived from a human neuroblastoma.

2 [^{125}I]-ET-1 (2.5×10^{-11} M) specifically bound to a single class of binding sites on these cells (Hill coefficient of 1.06 ± 0.04, $n = 3$) with an apparent K_d of $1.4 \pm 0.3 \times 10^{-9}$ M and a B_{max} of 3.1 ± 1.0 pmol mg^{-1} protein. [^{125}I]-ET-3 (2.5×10^{-11} M), did not specifically bind to SK-N-MC cells.

3 The binding of [^{125}I]-ET-1 was competitively inhibited by other ET isoforms, the order of potency being ET-1 > sarafotoxin S6b > ET-3.

4 Association of 1 nM [^{125}I]-ET-1 at 37°C reached apparent equilibrium at 60–80 min, with half-maximal binding being achieved at 12 min.

5 Dissociation was measured after both 10 min and 60 min of association with 64% and 30% respectively of specifically bound [^{125}I]-ET-1 dissociating. The actual amounts of [^{125}I]-ET-1 dissociated were similar in both cases.

6 Incubation of [^{125}I]-ET-3 with SK-N-MC cells at 37°C for 60 min did not result in significant degradation of this peptide. However, [^{125}I]-ET-1 was broken down by incubation with SK-N-MC cells, the pattern of degradation of dissociable [^{125}I]-ET-1 (and that found in the supernatant) being different from that of non-dissociable [^{125}I]-ET-1.

7 ET-1 concentration-dependently induced an increase in total inositol phosphate accumulation in sub-confluent (but not in confluent) cultures of SK-N-MC cells ($EC_{50} = 6.43 \pm 1.9 \times 10^{-10}$ M). ET-3 was without effect.

8 These results show that ET-1 specifically binds to SK-N-MC cells with the characteristics of an ET_A receptor. Our earlier finding that adrenal chromaffin cells express an ET_B receptor indicates the existence of multiple ET receptor types on neuronal cells.

Keywords: Endothelin; sarafotoxin; endothelin receptors; human neuroblastoma cell line; inositol phosphates

Introduction

Endothelin-1 (ET-1), a potent vasoconstrictor isolated from the culture medium of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988) is one of a family of structurally homologous peptides which includes ET-2, ET-3 and the sarafotoxins of the venom of the snake *Actractaspis engaddensis* (Yanagisawa & Masaki, 1989). The endothelins have distinct pharmacological activities including effects on vascular and non-vascular smooth muscle (Auguet *et al.*, 1988; Secrest & Cohen, 1989; Huang *et al.*, 1990), modulation of hormone secretion (Fukuda *et al.*, 1988; Takagi *et al.*, 1988; Morishita *et al.*, 1989) and may act as a growth factor (Brown & Littlewood, 1989). It has been found that endothelin is synthesized in tissues other than endothelial cells, including neural tissues such as the spinal cord (Shinmi *et al.*, 1989) and brain (Matsumoto *et al.*, 1989). We have previously characterized the endothelin binding site on bovine adrenomedullary chromaffin cells, a cell type widely used as a model for neuronal cells in culture (Wilkes & Boarder, 1991). This binding site was found to differ from that observed on A10 cells, a vascular smooth muscle cell line, leading to speculation on the existence of neuronal-type and non-neuronal-type endothelin receptors. Recent cloning of endothelin receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990) has defined two receptor types. The binding site on the A10 cell line has the characteristics of the ET_A receptor, which shows high selectivity for ET-1, while the ET_B receptor, which is non-selective for the ET isoforms, appears to be present on the chromaffin cell.

The present study was undertaken in order to characterize the endothelin binding site(s) on the SK-N-MC cell line, which was derived from a human neuroblastoma (Biedler *et al.*, 1973) and serves as a model for the peripheral nervous system, in order to investigate further the existence of a neuronal-type endothelin receptor. Earlier reports on the binding of endothelin *in vitro* (Hirata *et al.*, 1988) have shown that this binding is, at least in part, non-dissociable. Therefore reversibility of binding of endothelin to SK-N-MC cells, and the differential breakdown of ET-1 and ET-3, was investigated. In addition, a possible receptor-effector mechanism, that of intracellular inositol phosphate turnover, in response to ET-1 and ET-3, was investigated in the SK-N-MC cells.

Methods

Cell culture

SK-N-MC cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, streptomycin 100 μg ml^{-1}, penicillin 100 iu ml^{-1} and fungizone 2.5 μg ml^{-1} in a humidified atmosphere of 5% CO_2 at 37°C. Confluent flasks were passaged weekly. For experimental use cells were seeded into 24-well multiwell plates and grown to confluence. Experiments were performed in a balanced salts solution (BSS) consisting of (mM): NaCl 120, KCl 5.4, $NaHCO_3$ 16.2, $MgSO_4$ 0.8, NaH_2PO_4 1, $CaCl_2$ 1.8, D-glucose 5.5 and 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid 30; at pH 7.4.

Competition binding studies

Whole cell monolayer cultures were incubated (200 μl BSS + 0.1% bovine serum albumin (BSA)) in the presence of 2.5×10^{-11} M [^{125}I]-ET-1 or [^{125}I]-ET-3 and various concentrations (3×10^{-12} to 10^{-7} M) of unlabelled ET-1, ET-3 or

sarafotoxin S6b (STX S6b). The cell cultures were incubated at 37°C for 60 min. After aspiration of the incubation medium and extensive washing (4 × 1 ml of cold BSS) the cells were solubilized with 0.1 M NaOH and cell-bound radioactivity determined by gamma counting. Protein determination via the Lowry method (Lowry *et al.*, 1951) was performed on four wells of each plate.

Time course of association/dissociation

ET-1 was iodinated via the chloramine T method (Hunter & Greenwood, 1962) and purified on sephadex G10 columns to produce [^{125}I]-ET-1 with a specific activity of 400–500 Ci mmol^{-1}. Whole cell monolayer cultures were incubated (200 μl of BSS + 0.1% BSA) with 1 nM [^{125}I]-ET-1 (specific activity 400 Ci mmol^{-1}) for up to 90 min at 37°C. Non-specific binding was determined by the addition of 100 nM unlabelled ET-1. At the end of the incubation the cell monolayers were extensively washed (4 × 1 ml cold BSS) solubilized and counted. To determine the time course of dissociation whole cell monolayer cultures were incubated with 200 μl of 1 nM [^{125}I]-ET-1 for 10 or 60 min at 37°C. The labelled medium was then removed and replaced with 100 nM unlabelled ET-1. The cells were further incubated at 37°C, for up to 60 min, then washed, solubilized and counted.

Breakdown of endothelin

Whole cell monolayer cultures were incubated with 200 μl of 0.1 nM [^{125}I]-ET-1 or [^{125}I]-ET-3 (specific activity 2000 Ci mmol^{-1}) at 37°C for 60 min. The supernatant was removed and the cells incubated for a further 30 min with 200 μl of unlabelled ET (100 nM) to allow the [^{125}I]-ET to dissociate. Remaining cell-bound [^{125}I]-ET was extracted by the addition of 100 μl of 1% acetic acid followed by freezing/thawing. These three fractions (supernatant, dissociable and acid-extractable) were subjected to reverse-phase high-performance liquid chromatography (h.p.l.c.). The samples were injected onto a Vydac C_{18} column (25 × 4.6 mm) and eluted through with 0.1% trifluoroacetic acid (v/v in water) and acetonitrile, the concentration of acetonitrile initially being 10% v/v rising to 30% by 10 min and thereafter to 45% by 30 min. The flow rate was 1 ml min^{-1} and 1 min fractions were collected and subjected to gamma spectroscopy.

Total inositol phosphate production

Sub-confluent SK-N-MC cells were incubated with 1 μCi/well of *myo*-[^{3}H]-inositol in 0.5 ml of inositol-free Dulbecco's modified Eagle's medium supplemented with streptomycin 100 μg ml^{-1}, penicillin 100 i.u. ml^{-1}, fungizone 2.5 μg ml^{-1}, glutamine 27 mg 100 ml^{-1} and 1% non-essential amino acids for 48 h at 37°C. At the end of this loading period the monolayer cultures were washed twice and incubated in 0.5 ml of BSS + 10 mM lithium chloride for 10 min at 37°C. The test agents (ET-1 or ET-3 to a final concentration of 0.03–100 nM) were added and the cells incubated for a further 30 min. At the end of this period 0.5 ml of ice-cold methanol was added, followed by chloroform extraction and purification of total inositol phosphates on Dowex-1 (Cl^-) (Rooney & Nahorski, 1986).

Materials

ET-1, ET-3 and STX S6b were from Peptide Research Institute (Osaka, Japan). [^{125}I]-ET-1, [^{125}I]-ET-3 (specific activity of 2000 Ci mmol^{-1}) and Na^{125}I were from Amersham International (Buckinghamshire), *myo*-[2-^{3}H]-inositol was from New England Nuclear (Stevenage, Herts.). All tissue culture reagents were from Gibco (Paisley, Scotland); h.p.l.c. columns were from Technicol, Cheshire. Other chemicals were from Sigma (Poole, Dorset) or Fisons (Loughborough, Leicestershire).

Results

[^{125}I]-ET-1 specifically bound to SK-N-MC cells: non-specific binding (in the presence of 100 nM unlabelled ET-1) was typically less than 10% of total binding. The binding of [^{125}I]-ET-1 was competitively inhibited by unlabelled ET-1, ET-3 and STX S6b, the order of affinity was ET-1 > STX S6b > ET-3 (Figure 1a). The IC_{50} value for inhibition by ET-1 was 3.3 ± 0.9 × 10^{-9} M (mean ± s.e.mean, n = 3). Computer analysis of isotopic dilution binding data for ET-1 demonstrated an apparent K_d of 1.4 ± 0.3 × 10^{-9} M and an apparent B_{max} of 3.1 ± 1.0 pmol mg^{-1} protein. A typical Scatchard plot, with data consistent with a single affinity binding site, is shown in Figure 1b. This was confirmed by a Hill coefficient of 1.06 ± 0.04 (mean ± s.e.mean, n = 3). In contrast, ET-3 and STX S6b inhibited binding only weakly. In the presence of 100 nM unlabelled ET-3, 80% of specific binding of [^{125}I]-ET-1 to SK-N-MC cells remained and in the presence of 100 nM unlabelled STX S6b, 30% of specific binding was present (Figure 1a). Further experiments employing [^{125}I]-ET-3 demonstrated very little evidence of specific binding of 2.5 × 10^{-11} M [^{125}I]-ET-3 to SK-N-MC cells.

Specific binding of [^{125}I]-ET-1 to SK-N-MC cells reached apparent equilibrium at 60–80 min, with half-maximal binding being achieved at 12 min (Figure 2). The rate of dissociation of [^{125}I]-ET-1 from SK-N-MC cells on addition of 100 nM ET-1 is shown in Figure 2. After 10 min of association the addition of 100 nM unlabelled ET-1 resulted in a loss of 64% bound [^{125}I]-ET-1 after 20 min incubation. The pattern of dissociation after association for 60 min was similar, with 30% of specifically bound [^{125}I]-ET-1 dissociating over 20 min. Although the relative proportion of dissociable bound [^{125}I]-ET-1 differed between the two times of association, the actual

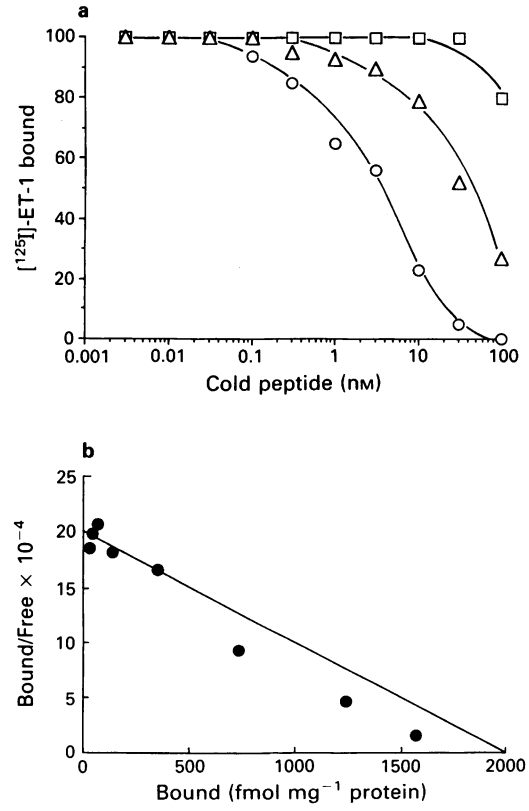

Figure 1 Competitive inhibition of binding of 2.5 × 10^{-11} M [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to SK-N-MC cell monolayer cultures. (a) Competitive inhibition by unlabelled ET-1 (○), ET-3 (□) or STX S6b (△). Each point represents the mean of three separate experiments each performed in duplicate. (b) Scatchard transformation of competitive inhibition of [^{125}I]-ET-1 binding by unlabelled ET-1. Data are from a typical experiment.

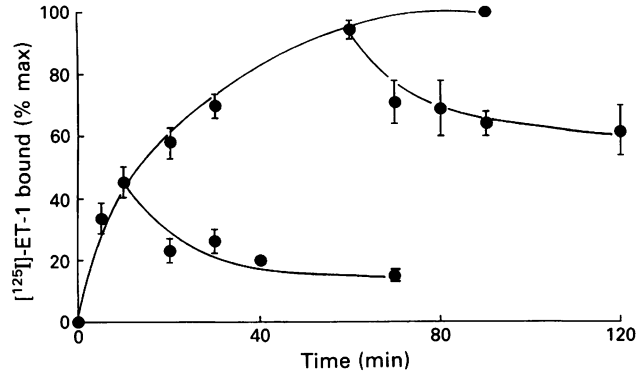

Figure 2 Time course of association/dissociation of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) to SK-N-MC cells. Association was observed by incubation of the cells with 1 nM [^{125}I]-ET-1 for up to 90 min. Dissociation was observed after association for 10 min and 60 min by the removal of radiolabel and the addition of 100 nM unlabelled ET, and incubation for a further 60 min. Data are mean of three experiments, each performed in duplicate; s.e.mean shown by vertical bars.

amount of radioactivity dissociating in each case was similar. This suggests that the binding of [^{125}I]-ET-1 to SK-N-MC cells consists of two components; an irreversibly bound component which increases with time of incubation, and a reversibly bound component which remains constant.

The breakdown of [^{125}I]-ET-1 and [^{125}I]-ET-3 by SK-N-MC cells was investigated by reverse-phase h.p.l.c. Recovery from the column both of original [^{125}I]-ET-1 and of the products of the incubation was typically around 50%. Significant breakdown of [^{125}I]-ET-1 occurred during incubation with SK-N-MC cells (Figure 3). In the supernatant 54% of radioactivity was associated with the native [^{125}I]-ET-1 peak (Figure 3b). There were three other main peaks: one consisting of 9% of total radioactivity eluting just behind the solvent front; one immediately prior to the native peptide peak (13% of total radioactivity); and one several fractions after the native peptide peak (10% of total radioactivity). The elution profile of the dissociable fraction was similar to that of the supernatant (Figure 3b,c). However, the elution profile of the cell-bound acid-extractable fraction (Figure 3d) exhibited only two peaks; the native [^{125}I]-ET-1 peak consisting of 50% of radioactivity, and a peak immediately prior to the native peptide peak, consisting of 38% of radioactivity. The results shown are a representative of three experiments exhibiting similar results. Overall, the percentages of radioactivity eluting with the native peptide were: supernatant, 57 ± 2%; dissociable, 40 ± 8%; acid-extractable, 58 ± 9% ($n = 3$ in each case). The elution profile of [^{125}I]-ET-3 was largely unchanged after incubation with SK-N-MC cells at 37°C for 60 min (Figure 4).

The effects of endothelins on total inositol phosphate accumulation by SK-N-MC cells were studied. ET-1 present during a 30 min incubation stimulated an increase in total labelled inositol phosphate content in sub-confluent SK-N-MC cells to 220% of control (in the absence of ET-1) with an EC_{50} value of $6.43 \pm 1.9 \times 10^{-10}$ M. However, on occasions when the SK-N-MC cells had attained confluence during the 48 h loading period, the total inositol phosphate response to ET-1 was very much attenuated (Figure 5a). ET-3, up to 100 nM, had no effect on total inositol phosphate production by either confluent or sub-confluent SK-N-MC cell cultures (Figure 5b).

Discussion

We have previously described the characteristics of the binding site for endothelin on chromaffin cells, a model for neuronal cell function (Wilkes & Boarder, 1991). Here we describe the characteristics of the endothelin binding site on another model of neuronal cell function, the SK-N-MC cell

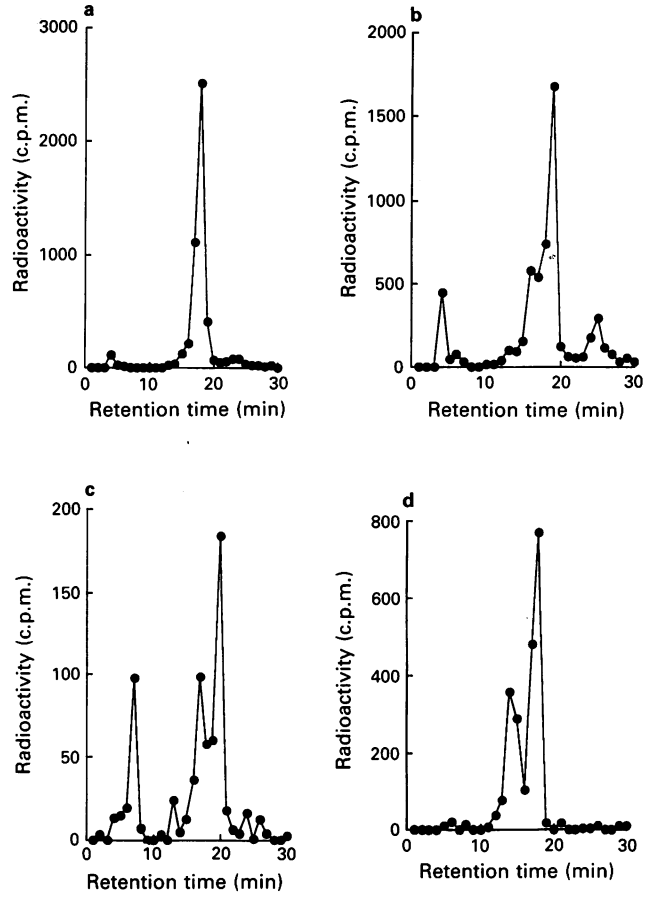

Figure 3 Analysis of [^{125}I]-endothelin-1 ([^{125}I]-ET-1) fractions by reverse phase h.p.l.c. (a) Native [^{125}I]-ET-1 before incubation with SK-N-MC cells. (b) Supernatant after incubation of 10^{-10} M [^{125}I]-ET-1 with SK-N-MC cells for 60 min. (c) Dissociable [^{125}I]-ET-1, after removal of supernatant and incubation with 100 nM unlabelled ET-1 for a further 30 min. (d) Acid-extractable [^{125}I]-ET-1, after extraction of the SK-N-MC cells with 1% acetic acid. Results are from a typical experiment.

line. The results presented demonstrate the existence of high-affinity binding sites for ET-1 on the SK-N-MC cell line. The observed order of affinity of the inhibiting ET isoforms of ET-1 > STX S6b > ET-3 has been described for other cell types (Galron *et al.*, 1989; Wilkes & Boarder, 1991). Recently the cloning of two separate endothelin receptors has been reported. The first, designated ET_A, shows high selectivity for ET-1, with the order of affinity of the ET isoforms being ET-1 > ET-2 > STX S6b > ET-3 (Arai *et al.*, 1990). The other endothelin receptor (ET_B) is non-selective for the ET isoforms (Sakurai *et al.*, 1990). The endothelin binding site on chromaffin cells exhibits equal affinity for the ET isoforms, suggesting that the ET_B receptor is expressed on these cells. However, it appears that the endothelin receptor expressed on SK-N-MC cells resembles the ET_A type. It is therefore evident that there exists heterogeneity of endothelin receptors on neuronal type cells in culture, even though the two cell types studied are both of peripheral neuronal origin. Other studies indicating types of endothelin receptors in brain preparations (Crawford *et al.*, 1990; Hiley *et al.*, 1990) are complicated by the likelihood that part of the response or binding may be due to non-neuronal elements, such as glial cells. Studies on cells in culture, such as those described here, avoid this complication.

The early reports of endothelin binding to whole cells indicated that binding may be complex in that only part of the binding was in equilibrium (Hirata *et al.*, 1988). To pursue these characteristics of binding we examined both association and dissociation in human neuroblastoma cells. The binding of [^{125}I]-ET-1 on incubation at 37°C reached apparent equilibrium at 60–80 min. Dissociation was observed after removal

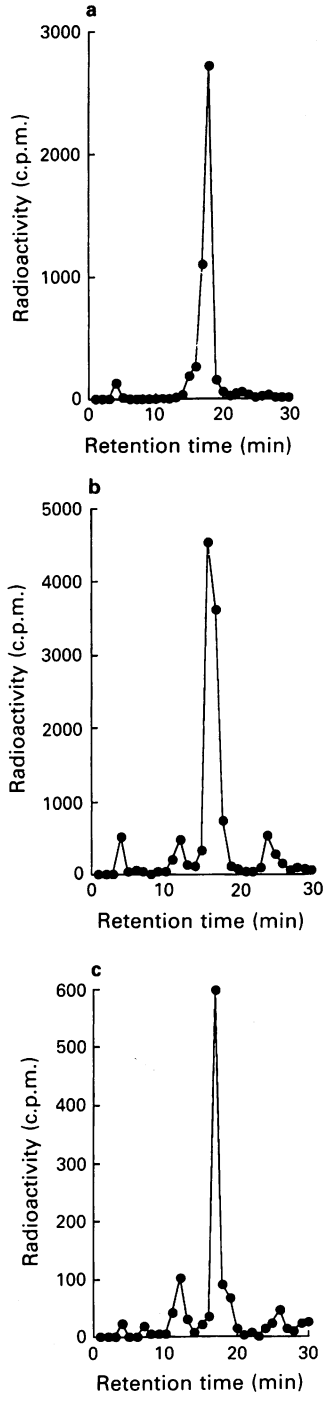

Figure 4 Analysis of [^{125}I]-endothelin-3 ([^{125}I]-ET-3) fractions by reverse phase h.p.l.c. (a) Native [^{125}I]-ET-3 before incubation with SK-N-MC cells. (b) Supernatant after incubation of 10^{-10} M [^{125}I]-ET-3 with SK-N-MC cells for 60 min. (c) Dissociable [^{125}I]-ET-3, after removal of supernatant and incubation with 100 nM unlabelled ET-3 for a further 30 min. The radioactivity in the acid-extractable fraction was too low to be analysed. Results are from a typical experiment.

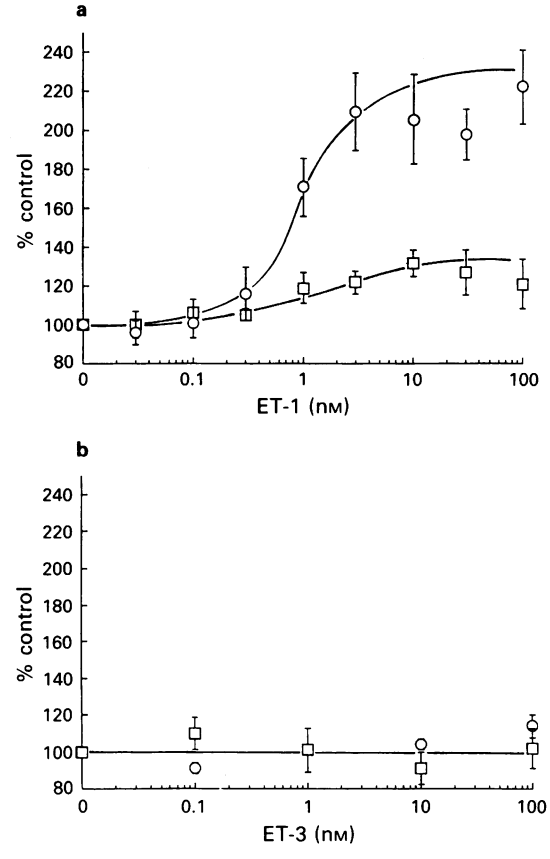

Figure 5 Dose-response curves for accumulation of labelled total inositol phosphates in response to a 30 min incubation with (a) endothelin-1 (ET-1) or (b) ET-3, by sub-confluent (○) or confluent (□) monolayer cultures of SK-N-MC cells. Each point represents the mean of three separate experiments, each performed in quadruplicate; s.e.mean shown by vertical bars.

of the label and addition of 100 nM unlabelled ET-1, over 60 min after incubation for both 10 min and 60 min. Only a proportion of the cell-associated radioactivity could be dissociated, with the absolute amount in equilibrium being constant after both 10 and 60 min incubation. However, the proportion of non-dissociable cell-associated radioactivity increased with time. This suggests that cell-associated [^{125}I]-ET-1 is compartmentalized, possibly with internalization of the receptor-agonist complex, and that the pool of dissociable cell-associated ET-1 is constant despite increasing amounts of bound ET-1. It is possible therefore, that the number of receptors on the cell surface remains constant despite a sustained loss due to internalization, implying a level of supply of receptors at the cell surface which is equal to the rate of agonist-induced loss. This model assumes that all the receptors on the cell surface bind ET-1 in a reversible manner in equilibrium with the peptide in solution.

Reverse-phase h.p.l.c. was employed to study metabolites of [^{125}I]-ET during incubation with SK-N-MC cells. The lack of high-affinity binding sites for ET-3 on these cells, and the observation that little degradation of [^{125}I]-ET-3 occurred after incubation at 37°C for 60 min, suggests that interaction of the peptide with an ET binding site may be required for degradation.

[^{125}I]-ET-1 was substantially broken down on incubation with SK-N-MC cells, with only 57% of radioactivity being associated with the native peptide in the incubation supernatant, and 34% in the dissociable fraction. The pattern of breakdown of [^{125}I]-ET-1 was similar between the supernatant and dissociable fractions, presumably reflecting the equilibrium that exists between them. The elution profile of the non-dissociable fraction was different from the supernatant and dissociable fractions, reflecting the non-equilibrium nature of this fraction. The breakdown product eluting in a peak of radioactivity just prior to the native peptide appears to be common to all three fractions. However, in the acid-extractable fraction this is apparently the only breakdown product, while in the dissociable and supernatant fractions further breakdown products are observed.

The reason for the disparity between the breakdown products of the dissociable and non-dissociable fractions is unclear. It is possible that the binding of ET-1 to the cell surface receptor is in simple equilibrium, and that breakdown

of the endothelin occurs on interaction of the peptide with the receptors by an ectoyrotease. This would be consistent with the breakdown of non-dissociable peptide being a consequence of internalization, since it would be by different proteolytic activity located within the cell.

SK-N-MC cells exhibit specific high-affinity binding sites for ET-1, with several pools of bound ET-1 existing within the cell membrane or cell itself. These binding sites appear to be coupled with inositol phosphate turnover in subconfluent SK-N-MC cells. However, when these cells attain confluence this response is greatly reduced. The reason for this is unclear. It is not because the confluent cell no longer expresses the endothelin receptor; the binding studies were performed on confluent cells which demonstrated a B_{max} of $3.1 \pm 1.0\,pmol\,mg^{-1}$ protein. It therefore appears that the receptor becomes uncoupled from turnover of inositol phosphates with increasing cell density, although the point at which this uncoupling occurs was not investigated in this study.

The data appear to indicate that occupation of only a small proportion of the receptor population appears to be necessary for stimulation of inositol phosphate turnover. The EC_{50} for this response was 6.43×10^{-10} M; at this concentration of ET-1 approximately 20% of the binding sites were occupied. However, this interpretation is complicated by the fact that the binding data were collated from studies on confluent cells, and data on total inositol phosphate turnover were from studies on sub-confluent cells.

These data demonstrate that SK-N-MC cells possess high-affinity binding sites for ET-1, and that the characteristics of these binding sites differ from those of another peripheral neuronal cell model (Wilkes & Boarder, 1991), suggesting that both ET_A and ET_B receptors may be present on peripheral neuronal cells. Further work is needed in order to assess the physiological significance of this observation.

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