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Localization of endothelin ET_B receptors on the myenteric plexus of guinea-pig ileum and the receptor-mediated release of acetylcholine

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1 The type of endothelin (ET) receptor located on the myenteric neurones of guinea-pig ileum was determined by receptor autoradiography and function of the receptor was examined by release experiments of acetylcholine (ACh) from the longitudinal muscle myenteric plexus (LM-MP) preparations.

2 Specific [^{125}I]-ET-1 binding sites were distributed in muscle layers, myenteric and submucous plexuses, and mucosa layers. High-grain densities were detected in both myenteric and submucous plexuses.

3 Binding in the myenteric plexus was abolished by incubation with either IRL 1620 (endothelin ET_B receptor agonist) or BQ 788 (endothelin ET_B receptor antagonist), but not with BQ 123 (endothelin ET_A receptor antagonist). The [^{125}I]-IRL 1620 binding sites were evident in the myenteric plexus. Thus, the endothelin receptor located on the myenteric neurones is of the ET_B type.

4 ET-1 ($10^{-10}-3\times10^{-8}$ M) and ET-3 ($10^{-10}-3\times10^{-8}$ M) evoked ^{3}H outflow from LM-MP preparations of ileum preloaded with [^{3}H]-choline, in a concentration-dependent manner. There was no significant difference between maximum amounts of ET-1-evoked and ET-3-evoked ^{3}H outflow.

5 ET-1 and ET-3 evoked outflow of ^{3}H was BQ 788-sensitive, but BQ 123-insensitive. Both evoked outflows of ^{3}H were Ca^{2+}-dependent and tetrodotoxin-sensitive.

6 These results indicate that the endothelin ET_B receptor is located on the enteric cholinergic neurones and that stimulation evokes the release of ACh.

Keywords: Endothelin receptor; guinea-pig ileum; myenteric plexus; acetylcholine release; receptor autoradiography

Introduction

Endothelin (ET) was detected in the rat gastrointestinal tract by radioimmunoassay (Takahashi *et al.*, 1990), and an immunohistochemical study demonstrated the presence of ET in the enteric neurones of the human gut (Inagaki *et al.*, 1991; Escrig *et al.*, 1992). ET binding sites have been identified in the intestine by receptor binding assay, in membrane preparations (Takahashi *et al.*, 1990) and by receptor autoradiography (Inagaki *et al.*, 1991). Functional studies revealed biphasic responses of the intestine to endothelins: a transient relaxation followed by a contraction of isolated ileum from the guinea-pig (Lin & Lee, 1990; Wollberg *et al.*, 1991; Guimaraes & Rae, 1992). The relaxant response is due to activation of Ca^{2+}-activated K^+ channels (Lin & Lee, 1992) and to inhibition of cholinergic neuronal activity (Wiklund *et al.*, 1989). The contractile responses to ETs are thought to be due to a direct action on smooth muscle cells (Lin & Lee, 1990; Yoshinaga *et al.*, 1992; Hori *et al.*, 1994; Okabe *et al.*, 1995; Smith *et al.*, 1995).

Receptors for ETs are classified into two types, ET_A (selective for ET-1 and ET-2) and ET_B (nonselective) receptors (Masaki *et al.*, 1994). In the intestine, endothelin ET_A and ET_B receptors are located on smooth muscle cells (Yoshinaga *et al.*, 1992; Hori *et al.*, 1994; Okabe *et al.*, 1995; Smith *et al.*, 1995). The ET binding sites are present in the myenteric ganglia (Inagaki *et al.*, 1991), and ET-1, ET-2 and ET-3 evoke the release of acetylcholine (ACh) from guinea-pig ileal preparations (Kan *et al.*, 1994). However, we found no documentation on the type of ET receptors located on the myenteric neurones. In the present work, we attempted to elucidate the type of ET receptors located on the myenteric neurones of guinea-pig ileum, by use of receptor autoradiographic and release experiments.

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Methods

Adult guinea-pigs of either sex, weighing between 300 and 350 g, were killed by cervical dislocation. Strips of ileum were excised 10 cm proximal to the ileocaecal sphincter.

Receptor autoradiography

Six animals were used for receptor autoradiographic experiments. The ileal tissues were immediately immersed in isopentane at −30°C. Frozen tissues were cut into 20 μm-thick round sections on a cryostat, thaw-mounted onto gelatin-coated glass slides and stored overnight under vacuum at 4°C. Related tissue sections were incubated *in vitro* with 1 nM [^{125}I]-endothelin-1 (ET-1) in the incubation buffer at 4°C for 48 h, as described (Niwa *et al.*, 1992). In brief, after preincubation in incubation buffer (50 mM Tris-HCl buffer (pH 7.4) containing: NaCl 100 mM, EDTA-2Na 10 mM, phosphoramidon 10 μM, bacitracin 1 mg ml^{-1}, leupeptin 4 μg ml^{-1}, chymostatin 2 μg ml^{-1} and 0.3% bovine serum albumin (protease-free)) at 23°C for 10 min, tissue sections were incubated in 2 ml of incubation buffer containing [^{125}I]-ET-1 at 4°C for 48 h. Consecutive tissue sections were labelled to characterize [^{125}I]-ET-1 binding in the presence of 1 μM unlabelled ET-1 (nonspecific binding), 10 μM BQ 123, a highly selective antagonist for the endothelin ET_A receptor (Ihara *et al.*, 1991), 10 μM BQ 788, a highly selective antagonist for the endothelin ET_B receptor (Ishikawa *et al.*, 1994) or 10 μM IRL 1620, a highly selective agonist for the endothelin ET_B receptor (Takai *et al.*, 1992).

Then, the labelled sections were washed three times (for 1 min each) at 4°C in 50 mM Tris-HCl buffer (pH 7.2), tapped in ice-cold distilled water and dried under a stream of cold air. To obtain autoradiograms of a higher resolution, the dry-labelled sections were apposed against Hyperfilm-^{3}H (Amersham, U.K.) and the films were developed with a D19 developer (Eastman Kodak, USA) for 7 min at 4°C (Niwa *et al.*, 1992).

To determine whether the endothelin ET_B receptors are present in the myenteric plexus, the tissue sections were incubated with 1 nM [^{125}I]-IRL 1620, under the same conditions as described for [^{125}I]-ET-1 binding. After the film image had been obtained, the labelled sections were coated with NTB-3 liquid autoradiographic emulsion (Eastman Kodak, USA) at 42°C. Slides were exposed at 23°C in a desiccated dark box. After exposure, the slides were developed with a D19 developer (4°C, for 5 min), rinsed briefly in distilled water, and fixed. After washing in distilled water for 1 h, slides were counter-stained with hematoxylin.

Cholinesterase in related ileal tissue sections was stained according to Karnovsky's method (Karnovsky & Roots, 1964) for anatomical orientation.

Measurement of acetylcholine release

Seven animals were used for release experiments. The longitudinal muscle-myenteric plexus (LM-MP) preparations were dissected from strips of ileum. The methods of incubation and superfusion were as described by Nakamoto *et al.* (1987). The LM-MP preparations were incubated at 35°C for 60 min with [^{3}H]-choline at a final concentration of 200 nM in Krebs solution of the following composition (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and glucose 11. After being washed in fresh Krebs solution for 30 min, the preparations were mounted in the superfusion apparatus and superfused at 1.2 ml min^{-1} with Krebs solution gassed with 95% O_2–5% CO_2, maintained at 35–37°C. Krebs solution containing 10 μM hemicholinium-3 to prevent the uptake of choline formed from ACh was the superfusion medium used. The superfusate was collected every 1 min. Radioactivity of the superfusates and of the tissue dissolved in Soluene at the end of the release experiment was counted in a liquid scintillation spectrometer (Packard Instrument Co., IL, USA). Experiments were begun 60 min after the spontaneous ^{3}H outflow had approached a plateau.

The proportion of unchanged [^{3}H]-ACh to total ^{3}H in the superfusates was estimated, as described previously (Potter & Murphy, 1967). The superfusates were collected in 1 ml of 3-heptanone-tetraphenylboron (10 mg ml^{-1}) on ice. [^{3}H]-choline and [^{3}H]-ACh were extracted with 1 N HCl, dried and dissolved in 1 N formic acid:acetone 15:85 (v/v). The samples were then subjected to electrophoresis to separate ACh and choline. The spots were identified with iodine and the radioactivity was determined in a liquid scintillation spectrometer. When the recovery of added [^{3}H]-ACh was measured after electrophoresis, at least 96% of applied [^{3}H]-ACh was present in the spot. Over 72% of the total radioactivity released from ET-1-or ET-3-stimulated preparations proved to be [^{3}H]-ACh, the value being similar to that of electrical stimulation-evoked release from isolated ileal longitudinal muscle strips (Vizi *et al.*, 1984). The total radioactivity in the superfusates from the stimulated preparation was considered to approximate the amount of [^{3}H]-ACh, and therefore was denoted as [^{3}H]-ACh release.

The outflow of ^{3}H was represented as the fractional rate obtained by dividing the amount of ^{3}H in the superfusate by the respective amount of ^{3}H in the tissue. The ^{3}H content of the tissue at each period was calculated by adding cumulatively the amount of each fractional ^{3}H outflow, to the ^{3}H content of the tissue at the end of the experiment. From each of the outflow curves obtained by plotting the fractional outflow of ^{3}H against time, the peak outflow of ^{3}H evoked by stimulation in each condition was calculated as the percentage increase over the basal outflow. When ETs were applied successively four times to the preparation at 30 min intervals, there were no significant differences between the outflow of ^{3}H evoked by the second to the fourth applications. Therefore, the outflow of ^{3}H evoked by the first application of ETs was disregarded and the outflow evoked by the second application of ETs was used as control. Data were analyzed by Dunnett's t test and a P value of 0.05 or less was considered statistically significant.

Chemicals

Substances used were as follows: [^{125}I]-endothelin-1 (81.4 TBq mmol^{-1}), [^{125}I]-IRL 1620 (81.4 TBq mmol^{-1}) and [^{3}H]-choline (3.33 TBq mmol^{-1}) (New England Nuclear, Boston, Mass, U.S.A.), hemicholinium-3 and EGTA (Sigma, St. Louis, MO, U.S.A.), Soluene (Packard, Downers Groves, IL, U.S.A.), endothelin-1 (ET-1) and endothelin-3 (ET-3) (Peptide Institute, Osaka, Japan), tetrodotoxin (Wako, Osaka, Japan). Other chemicals used were of reagent grade. BQ 123 and BQ 788 were generously provided by Banyu Pharmaceutical, Tsukuba, Japan and IRL 1620 was generously provided by Ciba-Geigy, Takarazuka, Japan.

Results

In vitro *receptor autoradiography*

Figure 1 and Figure 2(a–c) show typical receptor autoradiograms of [^{125}I]-ET-1 binding sites of guinea-pig ileal round sections. The [^{125}I]-ET-1 binding sites were distributed in the muscle layers, myenteric and submucous plexuses, and mucosa layers (Figures 1a and 2a), and the densities of all layers were abolished by addition of unlabelled 1 μM ET-1 (Figure 1d). Specific [^{125}I]-ET-1 binding sites in the presence of

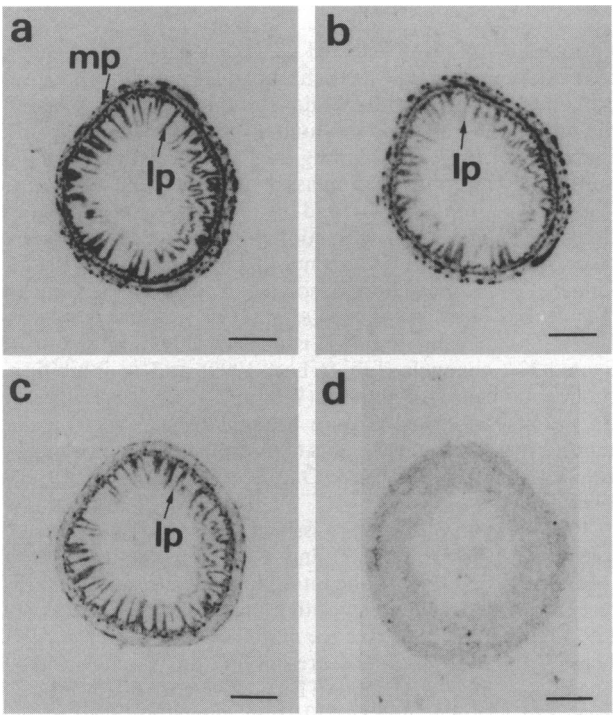

Figure 1 Typical receptor autoradiographic localization of [^{125}I]-endothelin-1 binding (a–d) sites in guinea-pig ileal round sections. Consecutive, 20 μm-thick sections were labelled with 1 nM [^{125}I]-endothelin-1 (ET-1) in the absence (total binding) (a) and presence of 10 μM BQ 123 (b), 10 μM IRL 1620 (c) or 1 μM unlabelled ET-1 (non-specific binding) (d), *in vitro*. mp: myenteric plexus, smp: submucous plexus, lm: longitudinal muscle layer, cm: circular muscle layer, lp: lamina propria. Scale bar: 1 mm.

10 μM ET-1 (Figure 1d). Specific [^{125}I]-ET-1 binding sites in the presence of 10 μM BQ 123, an endothelin ET_A receptor antagonist, were seen in the longitudinal muscle layer (lm), myenteric plexus (mp), submucous plexus (smp) and lamina propria (lp) (Figures 1b and 2b). IRL 1620 at 10 μM, an endothelin ET_B receptor agonist, decreased the densities of specific [^{125}I]-ET-1 binding sites dramatically in the myenteric plexus (mp) and moderately in the submucous plexus (smp) (Figures 1c and 2c). Some of the densities in longitudinal muscle layers (lm) remained in the presence of IRL 1620 (Figures 1c and 2c). Similar findings were seen in the case of BQ 788, an endothelin ET_B receptor antagonist (data not shown). Specific [^{125}I]-ET-1 binding sites in the lamina propria (lp) were noted in the presence of either BQ 123 or IRL 1620 (Figure 2b and c), but were abolished by both BQ 123 and IRL 1620 (data not shown). The following experiments focussed on [^{125}I]-ET-1 binding to the myenteric plexus. Cholinesterase was stained to visualize the myenteric plexus, submucous plexus

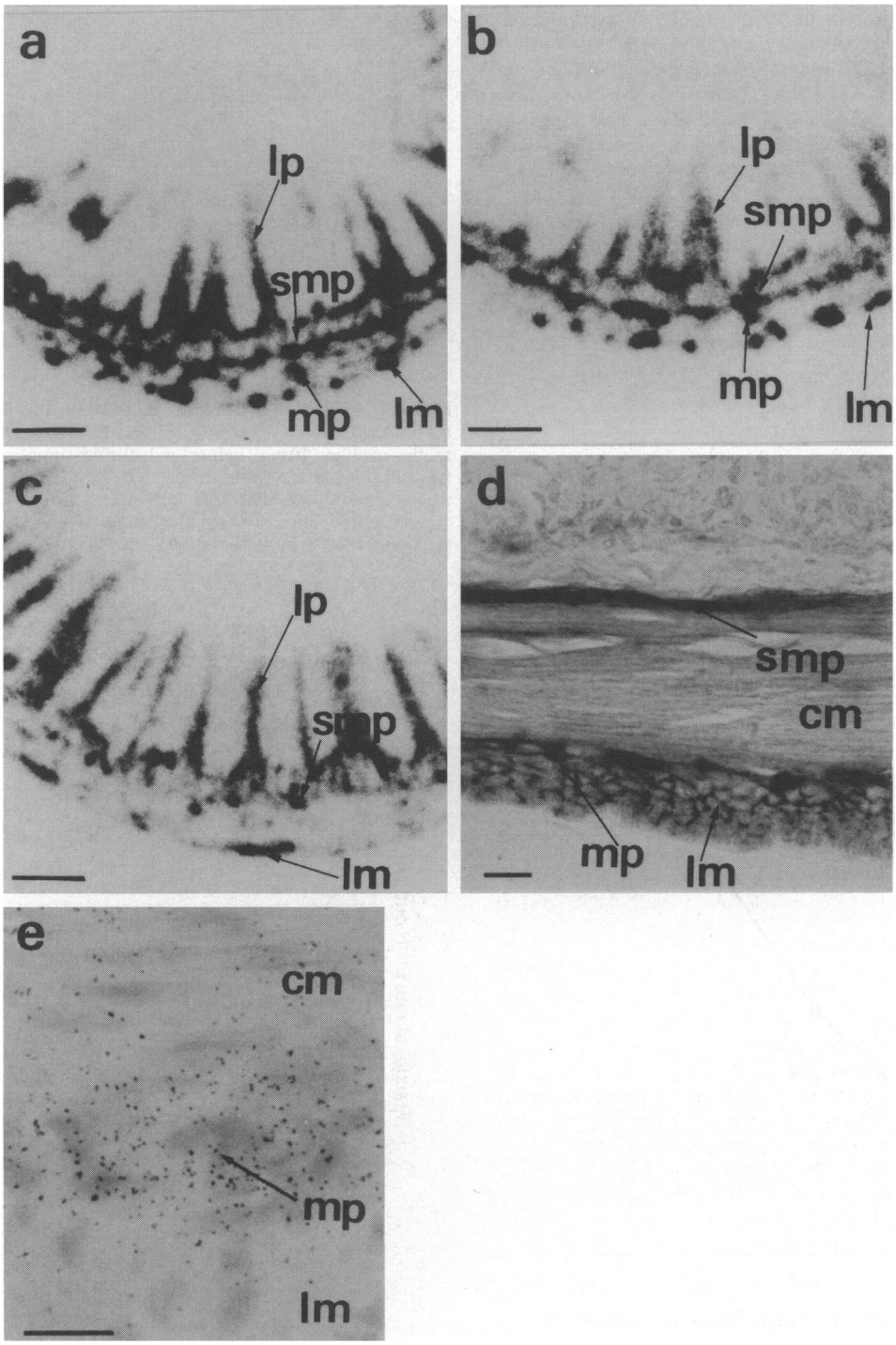

Figure 2 Typical high-magnification of receptor autoradiograms of [^{125}I]-endothelin-1 binding (a–c) sites in guinea-pig ileal round sections, and cholinesterase staining (d) and [^{125}I]-IRL 1620 binding (e) in the guinea-pig ileal myenteric plexus. Consecutive, 20 μm-thick sections were labelled with 1 nM [^{125}I]-endothelin-1 (ET-1) in the absence (total binding) (a) and presence of 10 μM BQ 123 (b), 10 μM IRL 1620 (c), *in vitro*. (d) Histochemical staining of cholinesterase by Karnovsky's staining method. (e) Emulsion autoradiograms of 1 nM [^{125}I]-IRL 1620 binding to myenteric neurones. mp: myenteric plexus, smp: submucous plexus, lm: longitudinal muscle layer, cm: circular muscle layer, lp: lamina propria. Scale bar in (a), (b) and (c): 0.5 mm, and in (d) and (e): 20 μm.

and muscle layers (Figure 2d). The distribution of cholinesterase staining in the myenteric plexus was similar to that of the [^{125}I]-ET-1 binding site in the presence of BQ 123. [^{125}I]-IRL 1620 binding grains were clearly visible in the myenteric plexus, by use of an emulsion autoradiographic method (Figure 2e).

Endothelin-evoked outflow of ^{3}H from longitudinal muscle with myenteric plexus preparations

The spontaneous ^{3}H outflow from the LM-MP preparations preloaded with [^{3}H]-choline reached a fairly constant level after 60 min of superfusion with Krebs medium at 1.2 ml min^{-1}, the fractional rate being 0.342±0.019% min^{-1} (mean±s.e.mean from 30 preparations). Repeated application of 10^{-8} M ET-1 or ET-3 at 30 min intervals to the same preparation produced a reliable response. ET-1 and ET-3 at 10^{-10} M to 3×10^{-8} M evoked ^{3}H outflow, in a concentration-dependent manner (Figure 3). The maximum amount of ET-3-evoked ^{3}H outflow was not significantly different from that of ET-1-evoked ^{3}H outflow. Pretreatment with 10^{-7} M BQ 788, an endothelin ET_B receptor antagonist for 10 min, but not 10^{-7} M BQ 123, an endothelin ET_A receptor antagonist inhibited the ^{3}H outflow evoked by ET-1 (10^{-8} M) and ET-3 (10^{-8} M) (Figure 4a and b). Either removal of external Ca^{2+} or pretreatment with 3×10^{-7} M tetrodotoxin inhibited the ^{3}H outflow evoked by ET-1 and ET-3 (Figure 5a and b).

Discussion

Receptor autoradiograms demonstrated that [^{125}I]-ET-1 binding sites were abundant in the myenteric and submucous plexuses, as noted in the human colon (Inagaki *et al.*, 1991).

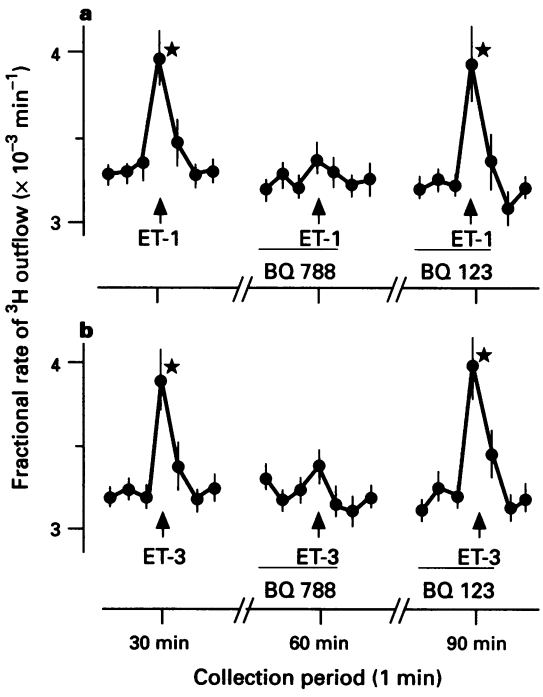

Figure 4 Effects of BQ 123 and BQ 788 on the ^{3}H outflow evoked by ET-1 (a) and ET-3 (b). BQ 123 and BQ 788 at 10^{-7} M were added to the medium 10 min before and during stimulation by ET-1 and ET-3 at 10^{-8} M for 1 min, respectively. Each column represents the mean from 7 animals with s.e.mean shown by vertical lines. *Significantly different from the value of basal outflow calculated by Dunnett's t test, at the <0.05 level of probability.

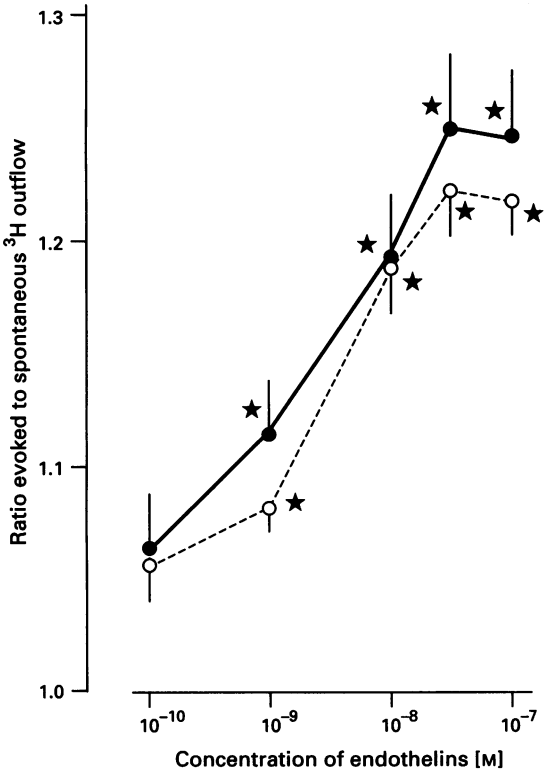

Figure 3 Concentration-response curves of ET-1-and ET-3-evoked ^{3}H outflow from longitudinal muscle with myenteric plexus preparations preloaded with [^{3}H]-choline. The ^{3}H outflow evoked by ET-1 (○ - - - - ○) and ET-3 (●———●) was represented as a ratio of evoked release to spontaneous release. Each point represents the mean from 7 animals with s.e.mean shown by vertical lines. *Significantly different from the value of basal outflow calculated by Dunnett's t test, at the <0.05 level of probability.

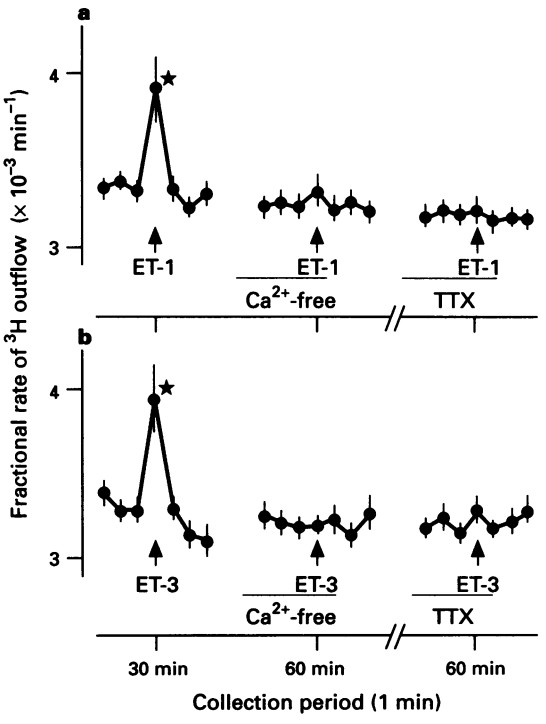

Figure 5 Ca^{2+}-dependent (a) and tetrodotoxin-sensitive (b) ^{3}H outflow evoked by ET-1 and ET-3. The normal medium was changed to the EGTA (1 mM)-containing Ca^{2+}-free medium or tetrodotoxin (300 nM)-containing medium 15 min before and during stimulation by 10^{-8} M ET-1 or 10^{-8} M ET-3 for 1 min. Each point represents the mean from 6 animals with s.e.mean shown by vertical lines. *Significantly different from the value of basal outflow calculated by Dunnett's t test, at the <0.05 level of probability.

Incubation with either unlabelled ET-1 or a combination of endothelin ET_A receptor antagonist and endothelin ET_B receptor agonist or antagonist abolished the [^{125}I]-ET-1 binding, thereby indicating that the binding sites are specific ET receptors and the type of receptor involved is either ET_A or ET_B. The present study focussed on the ET receptor located on the myenteric plexus. As the specific [^{125}I]-ET-1 binding in the myenteric plexus was abolished by either the endothelin ET_B receptor agonist (IRL 1620) (Takai *et al.*, 1992) or antagonist (BQ 788) (Ishikawa *et al.*, 1994), but not by an endothelin ET_A receptor antagonist (BQ 123) (Ihara *et al.*, 1991), the receptor is the ET_B type. The concept that the ET_B type of endothelin receptor is located on myenteric neurones was confirmed by the radioligand binding experiments; the endothelin ET_B receptor agonist, [^{125}I]-IRL 1620 bound to the myenteric plexus. Immunohistochemical studies have shown that ET-1-containing neurones are present in the intestine and that nerve terminals innervate myenteric neurones (Inagaki *et al.*, 1991; Escrig *et al.*, 1992). Thus, the endothelin ET_B receptor on the myenteric neurones may be the target of ET-containing nerve fibres. Since the distribution of cholinesterase staining in the myenteric plexus was similar to that for the endothelin ET_B receptor, the endothelin ET_B receptor may be located on the cholinergic neurones.

The function of the endothelin ET_B receptor was examined by experiments involving the release of ACh. ET-1 and ET-3 evoked the release of ACh from LM-MP preparations of guinea-pig ileum in a concentration-dependent manner, and these evoked releases were Ca^{2+}-dependent, thereby indicating that the released ACh may originate from enteric cholinergic nerve terminals. There was no significant difference between maximum amounts of ET-1-evoked and ET-3-evoked ^{3}H outflow. Since the endothelin ET_B receptor, but not the endothelin ET_A receptor, shows equal affinity for ET-1 and ET-3 (Masaki *et al.*, 1994), the endothelin receptors located on the cholinergic neurones may be the ET_B type. This hypothesis was confirmed by the finding that both ET-1- and ET-3-evoked release of ACh were inhibited by an endothelin ET_B antagonist, BQ 788 (Ishikawa *et al.*, 1994), but not by an endothelin ET_A receptor antagonist, BQ 123 (Ihara *et al.*, 1991). In cultured cholinergic neurones co-existing with smooth muscle cells from the guinea-pig trachea, the endothelin ET_B receptor is predominant and mediates an increase in intracellular Ca^{2+} concentration in neurones (Takimoto *et al.*, 1993).

The ET-1 and ET-3 evoked release of ACh were both tetrodotoxin-sensitive. As the tetrodotoxin-sensitive release of neurotransmitter is considered to be induced by the stimulation of somato-dendritic regions of the neurones (Gonella *et al.*, 1980), both ET-1 and ET-3 may stimulate the endothelin ET_B receptor localized on the somato-dendritic regions of cholinergic neurones. The functional endothelin ET_B receptor may correspond to the ET binding sites in the myenteric plexus demonstrated in the receptor autoradiographic study.

Both endothelin ET_A and ET_B receptors are present in intestinal smooth muscle cells and both may mediate contractions (Yoshinaga *et al.*, 1992; Hori *et al.*, 1994; Okabe *et al.*, 1995; Smith *et al.*, 1995). The endothelin ET_B receptor has been postulated to participate partially in ET-induced relaxation of guinea-pig ileum (Hori *et al.*, 1994), to be responsible for ion transport in rabbit colonic mucosa (Smith *et al.*, 1995), and to play an essential role in the normal development of enteric ganglion neurones, based on the finding that a null mutation induced by targeted disruption of the mouse endothelin ET_B receptor gene produced an aganglionic megacolon (Hosoda *et al.*, 1994). The present study showed that endothelin receptors are localized on myenteric cholinergic neurones and are of the ET_B type. These receptors could modulate intestinal motility through cholinergic neuronal activity, being different from the receptors located on the smooth muscle cells.

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