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Endothelin-1[1-31] acts as a selective ETA-receptor agonist in the rat adrenal cortex

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Summary. Endothelin-1 (ET-1) is a 21-amino acid residue (ET-1[1-21]) hypertensive peptide, which together with its receptor subtypes A and B (ETA and ETB) is expressed in the rat adrenal cortex, where it stimulates steroid-hormone (aldosterone and corticosterone) secretion through the ETB receptor and the growth (proliferative activity) of the zona glomerulosa (ZG) through the ETA receptor. ET-1[1-21] is generated from bigET-1 by the endothelin-converting enzyme (ECE-1). However, recent evidence indicates the existence of an alternative chymase-mediated biosynthetic pathway leading to the production of an ET-1[1-31] peptide, which was found to reproduce the ETA receptor-mediated vascular effects of ET-1[1-21]. We found that ET-1[1-21], but not ET-1[1-31], concentration-dependently raised steroid secretion from dispersed rat adrenocortical cells, its effect being blocked by the ETB-receptor selective antagonist BQ-788. Both ET-1s concentration-dependently increased the number of "S-phase" cells (as detected by the 5-bromo-2'-deoxyuridine immunocytochemical method) in capsule-ZG strips within a 240 min incubation. The ZG proliferogenic action of both ET-1s was blocked by the ETA-receptor antagonist BQ-123, and ET-1[1-31] was found to be significantly more potent than ET-1[1-21]. Autoradiography showed that in the rat adrenal ET-1[1-21] displaced the binding of selective ligands to both ETA ([^{125}I]PD-151242) and ETB receptors ([^{125}I]BQ-3020), while ET-1[1-31] eliminates only the binding to ETA receptors. Collectively, our findings provide strong evidence that ET-1[1-31] acts in the rat adrenal glands as a selective ETA-receptor agonist, mainly involved in the stimulation of ZG proliferative activity.

Key words: Endothelin-1, Endothelin-1[1-31], Endothelin receptors, Adrenal cortex, Steroid-hormone secretion, Cell proliferation, Rat

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Introduction

Endothelin-1 (ET-1) is the prototype of a family of 21-amino acid residue peptides, which act via two main receptor subtypes, named ETA and ETB. The 21-amino acid ET-1 (ET-1[1-21]) is generated from big-ET-1 through cleavage at the Trp^{21}-Val/Ile^{22} bond by a specific endothelin-converting enzyme (ECE)-1 (for review, see Rubanyi and Polokoff, 1994). More recently, evidence has been provided that big-ET-1 may also be selectively cleaved at the Tyr^{31}-Gly^{32} bond by a chymase to produce ET-1[1-31] (Nakano et al., 1997).

ET-1[1-31] has been found to reproduce many of the vascular effects of ET-1[1-21], including contraction of porcine coronary artery and rat aorta (Kishi et al., 1998), and a rise in intracellular Ca^{2+} concentration (Yoshizumi et al., 1998a, 1999; Inui et al., 1999) and proliferation rate of cultured human coronary smooth-muscle cells (Yoshizumi et al., 1998b). While the ET-1[1-21] vasoconstrictor and proliferogenic effects are mainly ETA receptor-mediated (for review, see Miyauchi and Masaki, 1999), it was assumed that those of ET-1[1-31] involve both ET-receptor subtypes, based on studies with the ETA-receptor antagonist BQ-485 and the ETB-receptor antagonist BQ-788 (Kishi et al., 1998).

ET-1[1-21], which is locally produced in the adrenal glands, is deemed to act as an autocrine-paracrine regulator of adrenocortical-cell function (for review, see Nussdorfer et al., 1999). In the rat, ET-1[1-21] was found to stimulate the secretion and proliferation of adrenocortical cells, acting through ETB and ETA receptors, respectively (Belloni et al., 1996; Mazzocchi et al., 1997). No investigations are at present available on the effect of ET-1[1-31] on the rat adrenal cortex. It, therefore, seemed worthwhile to compare the *in vitro* effects of ET-1[1-21] and ET-1[1-31] on rat adrenocortical-cell function, and to study by autoradiography to which subtype of receptors ET-1 binds in the rat adrenal gland.

Materials and methods

Reagents and animals

ET-1[1-21], the selective ETA-receptor antagonist

BQ-123 and the ETB-receptor antagonist BQ-788 (Ohlstein et al., 1996) were purchased from Neosystem Laboratories (Strasbourg, France), and ET-1[1-31] from Peptide Institute (Osaka, Japan). Medium 199 was obtained from Difco (Detroit, MI, USA), and the ECE-1 inhibitor phosphoramidone (Loffler, 2000), 5-bromo-2'-deoxyuridine (BrdU), human serum albumin (HSA), and all other laboratory reagents were provided by Sigma Chemical Company (St. Louis, MO, USA). [^{125}I]PD-151242 and [^{125}I]BQ-3020, which are selective ligands of ETA and ETB receptors (Ohlstein et al., 1996), were obtained from Amersham Laboratories (Aylesbury, UK). Adult male Sprague-Dawley rats (260±30 g in body weight) were purchased from Charles-River (Como, Italy). The experimental protocol was approved by the local Ethical Committee for Animal Studies.

Steroid-hormone secretion

Rat adrenals were gently decapsulated to separate zona glomerulosa (ZG), and then halved and enucleated to eliminate medullary chromaffin cells. Dispersed ZG and zona fasciculata-reticularis (ZF/R) cells were obtained by collagenase digestion and mechanical disaggregation (Belloni et al., 1996). Medullary chromaffin-cell contamination of adrenocortical-cell preparations, as evaluated by phase microscopy, was virtually absent, and the viability of dispersed cells, as checked by the trypan blue-exclusion test, was higher than 92%. Dispersed cells were put in Medium 199 and Krebs-Ringer bicarbonate buffer with 0.2% glucose, containing 5 mg/mL HSA. They were incubated (10^5 cells/mL) with increasing concentrations of ET-1[1-21] or ET-1[1-31] (from 10^{-11} to 10^{-7} M). Some samples were also incubated with ET-1[1-21] (10^{-8} M) alone and in the presence of 10^{-7} M BQ-123 or BQ-788. The incubation was carried out at 37 °C for 90 min in an atmosphere of 95% air-5% CO_2. At the end of the experiments, the incubation tubes were centrifuged and supernatants were stored at -80 °C.

Aldosterone and corticosterone were extracted from the incubation media and purified by HPLC (Mazzocchi et al., 1998), and their concentrations were measured by RIA, using the following commercial kits: ALDO-CTK2 (IRE-Sorin, Vercelli, Italy; sensitivity, 5 pg/mL) and CRTX-RIA (Eurogenetix, Milan, Italy; sensitivity, 25 pg/mL). Intra- and interassay variation coefficients were: aldosterone, 7.5% and 8.6%; and corticosterone, 6.1% and 7.8%, respectively.

ZG cell DNA synthesis

Capsule-ZG strips were obtained as described earlier (Bernet et al., 1994) and incubated with ET-1[1-21] or ET-1[1-31] (from 10^{-11} to 10^{-7} M); and ET-1[1-21] or ET-1[1-31] (10^{-11} M) plus or without 10^{-7} M BQ-123 , 10^{-7} M BQ-788 or 10^{-4} M phosphoramidone, in the presence of BrdU (20 mg/mL). The incubation was carried out at 37 °C for 240 min in an atmosphere of 95% air-5% CO_2. According to Mazzocchi et al. (1997), the duration of the incubation being sufficient for ET-1[1-21] to exert its proliferogenic effect on the ZG cells of *in situ* perfused rat adrenals. At the end of the experiment, the strips were fixed in 4% paraformaldehyde and embedded in paraffin. BrdU-positive cells were detected by immunocytochemistry (Cell Proliferation Kit™;Amersham), and their number evaluated by counting 2,000 parenchymal cells in each sample.

Autoradiography

Adrenal glands were immediately frozen at -30 °C by immersion in isopentane, and stored at -80 °C. Frozen sections (10-15 μm thick) were cut in a cryostat (Leitz 1720 Digital) at -20 °C, and processed as previously detailed (Belloni et al., 1996). ETA and ETB receptors were labeled *in vitro* by incubation for 120 min at 37°C with 10^{-9} M [^{125}I]PD-151242 or [^{125}I]BQ-3020, respectively. The following cold molecules were added to the incubation mixture at a concentration of 10^{-7} M: ET-1[1-21], ET-1[1-31], BQ-123 and BQ-788. The reaction was stopped by washing the samples three times in 50 mM Tris/HCl buffer. After rinsing, the sections were rapidly dried, fixed in paraformaldehyde vapors at 80 °C for 120 min, and coated with NTB2 nuclear emulsion (Eastman Kodak, Rochester, NJ, USA). Autoradiographs were exposed for two weeks at 4 °C, and then developed with undiluted Kodak D19 developer. They were stained with hematoxylin-eosin, and observed and photographed with a Leitz Laborlux microscope.

Three unstained autoradiograms obtained from three adrenals were analyzed by computer-assisted densitometry with a camera-connected microscope and an IBM-compatible computer equipped with a software specifically written for this purpose (Studio Casti Imaging, Venice, Italy). For each autoradiogram, 10 areas of ZG (about 36,000 pixels) were analyzed. The density value of the capsule was taken as the background value.

Statistics

Values were expressed as means ± SEM of six (steroid secretion and DNA synthesis) or three (autoradiography) separate experiments. The statistical comparison of the results was done by ANOVA, followed by the Multiple Range Test of Duncan.

Results

Steroid-hormone secretion

ET-1[1-21] concentration-dependently increased the production of both aldosterone and corticosterone from ZG and ZF/R cells, respectively. Minimal and maximal effective concentrations were: aldosterone, 10^{-10} M and

$10^{-8}/10^{-7}$ M (1.4-fold and 2.1-fold rises); and corticosterone, 10^{-9} M and $10^{-8}/10^{-7}$ M (2.1-fold and 2.8-fold rises) (Fig. 1). The secretagogue effect of 10^{-8} M ET-1[1-21] was annulled by 10^{-7} M BQ-788, and unaffected by 10^{-7} M BQ-123 (Fig. 2). ET-1[1-31] did not evoke any secretory response of dispersed rat adrenocortical cells (Fig. 1).

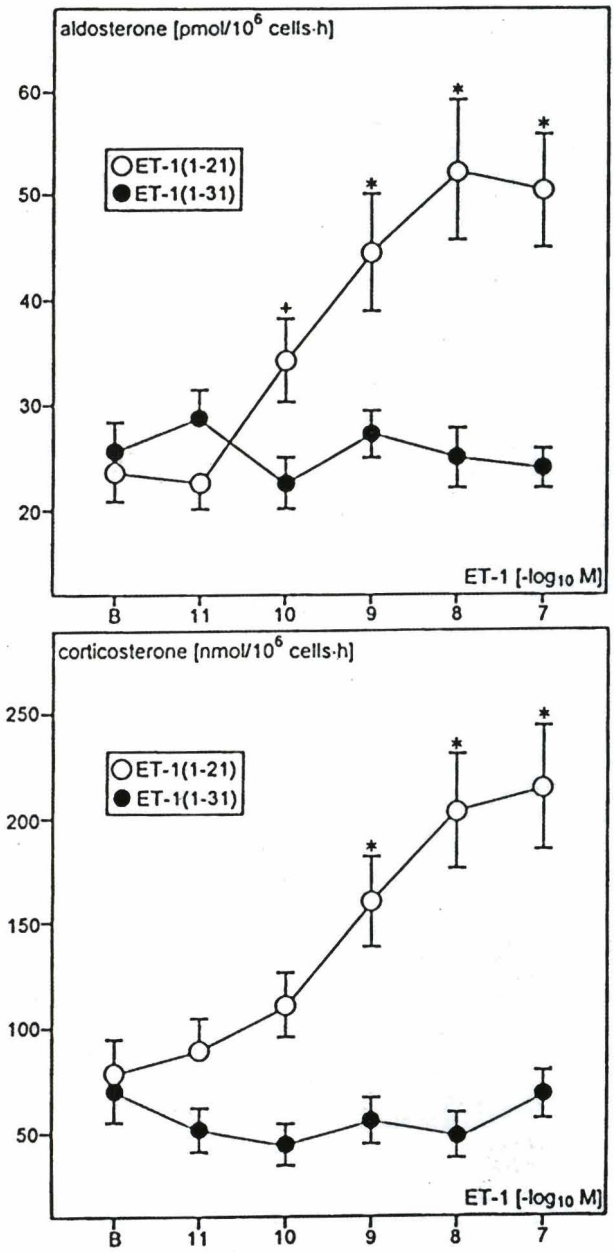

Fig. 1. Effects of ET-1[1-21] and ET-1[1-31] on aldosterone and corticosterone secretion from dispersed rat ZG and ZF/R cells, respectively. Data are means ± SEM of six independent experiments. +: p<0.05, and *: p<0.01 from the respective baseline (B) value.

ZG cell DNA synthesis

Both ET-1[1-21] and ET-1[1-31] concentration-dependently raised the number of BrdU-positive cells in ZG strips, minimal and maximal effective concentrations being 10^{-10} and $10^{-8}/10^{-7}$ M, respectively. Despite the fact that the efficacy was the same (the maximal effective concentration of both ET-1s elicited an about 5-fold increase), the potency of ET-1[1-31] was higher than that of ET-1[1-21] (EC50: $0.8\pm0.1\times10^{-10}$ M *versus* $7.2\pm1.0\times10^{-10}$ M; $p<0.05$) (Fig. 3). The effect of 10^{-8} M ET-1[1-21] and ET-1[1-31] was blocked by 10^{-7} M BQ-123, and unaffected by either 10^{-7} M BQ-788 or 10^{-4} M phosphoramidone (Fig. 4).

Autoradiography

As expected (Belloni et al., 1996, 1997), ETA receptors ([^{125}I]PD-151242 BQ-123- displaceable binding sites) were present in both ZG and adrenal medulla (Fig. 5 A,C), and ETB receptors ([^{125}I]BQ-3020 BQ-788-displaceable binding sites) in ZG, ZF/R and to a lesser extent in adrenal medulla (Fig. 5B,D). ET-1[1-21]

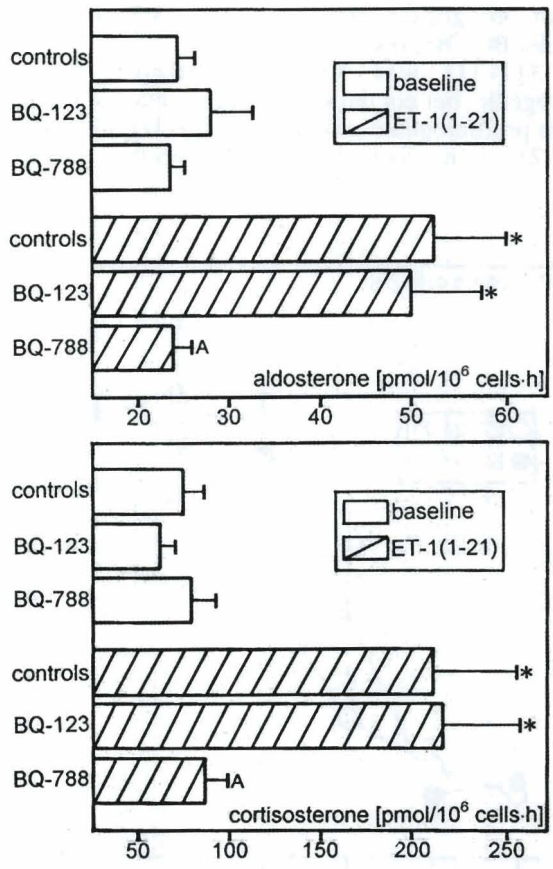

Fig. 2. Effects of BQ-123 and BQ-788 (10^{-7} M) on aldosterone and corticosterone response of dispersed rat ZG and ZF/R cells to 10^{-8} M ET-1[1-21]. Bars are means ± SEM of six independent experiments. *: p<0.01 from the respective baseline value; A: p<0.01 from the respective control (ET-1) value.

eliminated either [^{125}I]PD-151242 or [^{125}I]BQ-3020 binding (data not shown), while ET-1[1-31] abolished [^{125}I]PD-151242 binding, leaving unaffected [^{125}I]BQ-3020 binding (Fig. 5 E, F). Quantitative densitometry confirmed these qualitative descriptions, as far as ZG was concerned (Fig. 6).

Discussion

It is well demonstrated that ET-1[1-21], at variance with other peptides which stimulate adrenal steroid secretion and ZG proliferogenic activity only at high concentrations (Nussdorfer, 1996), exerts such effect at concentrations that are consistent with its possible physiological role (Nussdorfer et al., 1999). The present findings agree with this contention, because minimal and maximal effective concentrations are in the range of those that endogenous locally-produced ET-1[1-21] can conceivably reach in the adrenal gland (Nussdorfer et al., 1999). They also confirm that in the rat adrenals, the ZG proliferogenic and secretagogue effect of ET-1[1-21] are mediated by the ETA- and ETB-receptor subtypes, respectively (Belloni et al., 1996; Mazzocchi et al., 1997). In fact, they are selectively blocked by the ETA-receptor antagonist BQ-123 and the ETB-receptor antagonist BQ-788, respectively.

ET-1[1-31] was found to mimic the ZG proliferogenic, but not the secretagogue effect of ET-1[1-21]. The proliferogenic action of ET-1[1-31], like that of ET-1[1-21], is mediated by the ETA receptor subtype, inasmuch as it is blocked by BQ-123 and unaffected by BQ-788. The ECE-1 inhibitor phosphoramidone does not blunt the ZG proliferogenic action of ET-1[1-31], which indicates that this effect is not due to the cleavage of this peptide to ET-1[1-21], as reported to occur in cultured bronchial smooth muscle cells (Hayasaki-Kajiwara et al., 1999). This possibility is also made unlikely by the fact that ET-1[1-31] does not mimic the adrenocortical secretagogue effect of ET-1[1-21]. Taken together, these findings would suggest that ET-1[1-31] is a selective agonist of ETA receptors, a possibility in keeping with the observation that although the ZG proliferogenic actions of the two ET-1s display similar efficacy, ET-1[1-31] is significantly more potent that ET-1[1-21].

Our autoradiographic data provide strong support for this contention. In fact, besides confirming the currently accepted distribution of ETA and ETB receptors in rat adrenal gland (Belloni et al., 1996, 1997), they show that, at variance with ET-1[1-21], ET-1[1-31] displaces the binding to ETA receptors of the ligand [^{125}I]PD-151242, but not that to ETB receptors of the ligand [^{125}I]BQ-3020.

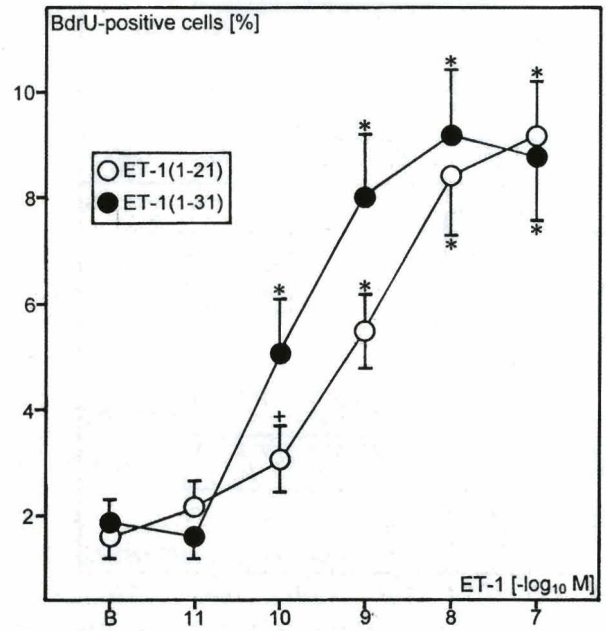

Fig. 3. Effects of ET-1[1-21] and ET-1[1-31] on the number of BrdU-positive cells in the ZG of rat adrenal glands. Data are means ± SEM of six independent experiments. +: $p<0.05$, and *: $p<0.01$ from the respective baseline (B) value.

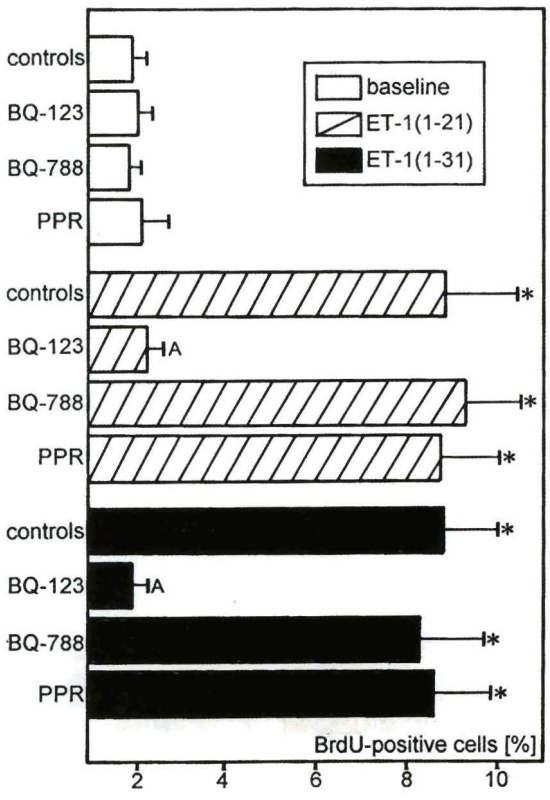

Fig. 4. Effects of BQ-123 (10^{-7} M), BQ-788 (10^{-7} M) and phosphoramidone (PPR) (10^{-4} M) on the 10^{-8} M ET-1[1-21] or ET-1[1-31]-induced increase in the number of BrdU-positive cells in the ZG of rat adrenal glands. Bars are means ± SEM of six independent experiments. *: $p<0.01$ from the respective baseline value; A: $p<0.01$ from the respective control (ET-1) value.

The chymase splitting big-ET-1 to ET-1[1-31] has been detected in humans, but not yet in the rat tissues (Sanker et al., 1997). Hence, the endogenous production of ET-1[1-31] in the rat remains to be demonstrated. However, our results raise the appealing possibility that in the adrenal gland the post-translational alternative cleavage of big-ET-1 by ECE-1 or chymase may lead, depending upon the local needs, to the production of either ET-1[1-21] exerting both secretagogue and growth effects via the ETA and ETB receptors, or ET-1[1-31] exclusively exerting a growth promoting effect via the ETA receptor. Be that as it may, our findings have a notable pharmacological relevance, because they seem to provide the first demonstration of the existence of a

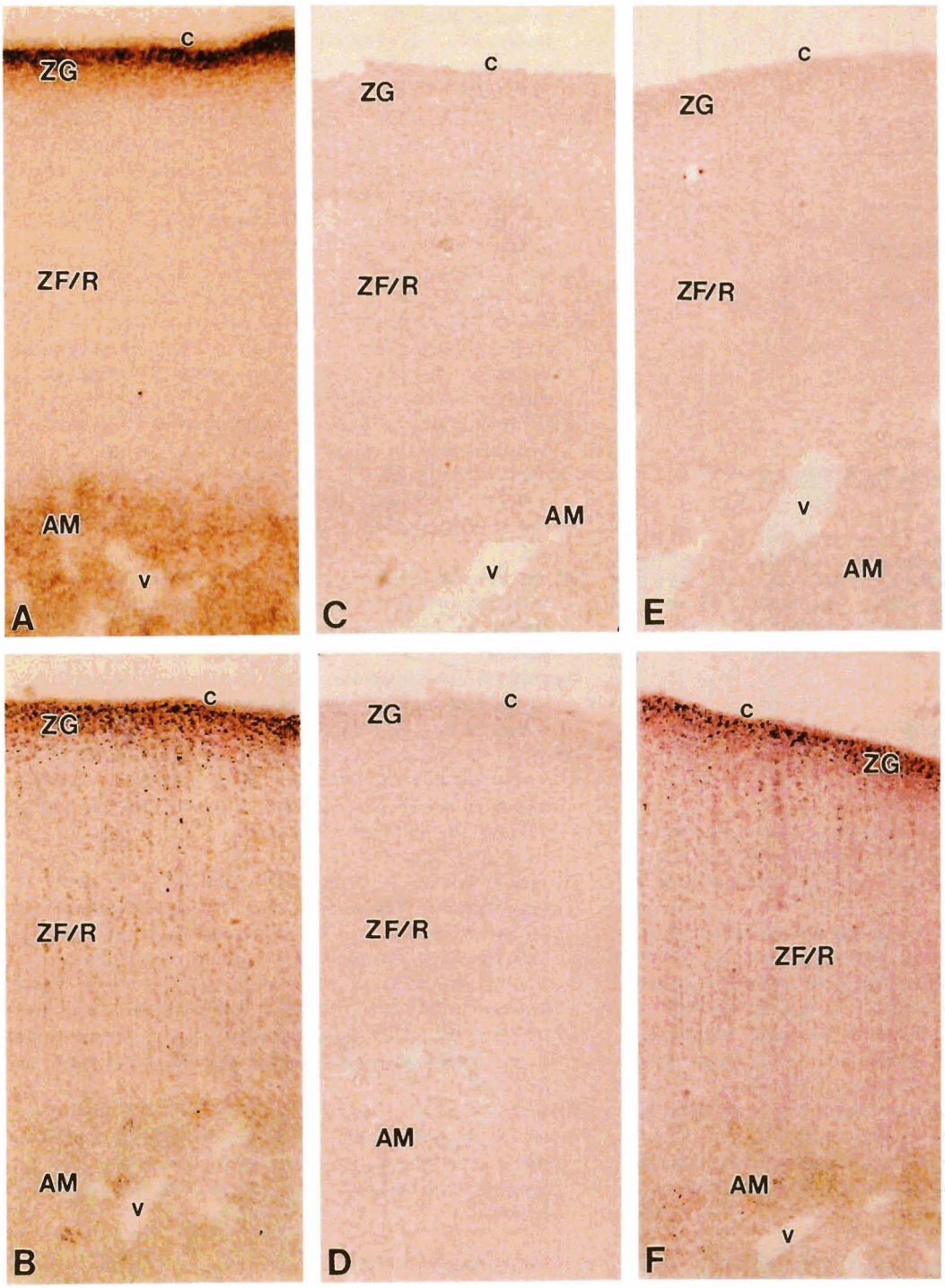

Fig. 5. Autoradiograms of hematoxylin-eosin-stained frozen sections of rat adrenal gland incubated with 10^{-9} M [^{125}I]PD-151242 **(A)** or 10^{-9} M BQ-3020 **(B)**. Binding of [^{125}I]PD-151242 to ETA receptors is completely displaced by 10^{-7} M BQ-123 **(C)** and binding of [^{125}I]BQ-3020 is eliminated by 10^{-7} M BQ-788 **(D)**. ET-1[1-31] (10^{-7} M) eliminated binding to ETA receptors **(E)**, without altering binding to ETB receptors **(F)**. c: gland capsule; AM: adrenal medulla; v: medullary blood vessels. x 80

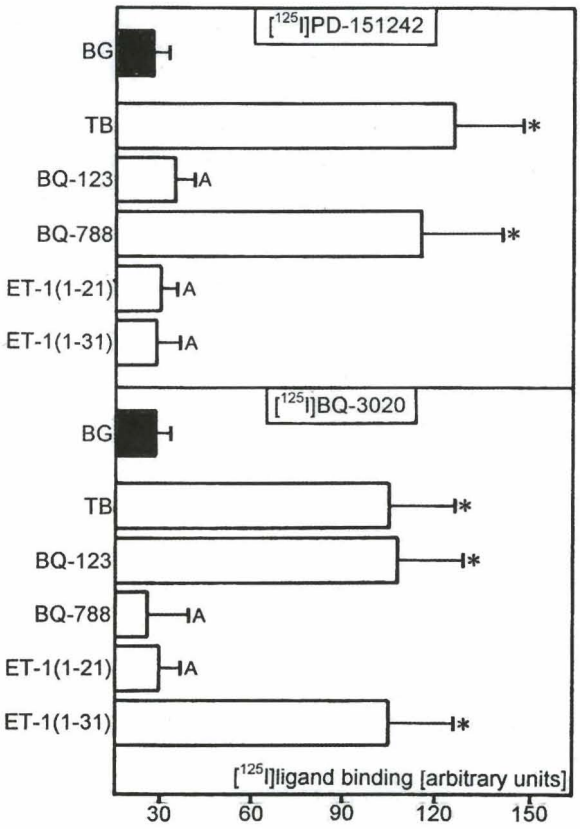

Fig. 6. Evaluation by quantitative densitometry of 10^{-9} M [^{125}I]PD-151242 or 10^{-9} M [^{125}I]BQ-3020 binding in the rat ZG (total binding, TB), and of its displacement by 10^{-7} M BQ-123, BQ-788, ET-1[1-21] and ET-1[1-31]. Bars are means ± SEM of three independent experiments. *: $p<0.01$ from the respective background (BG) value; ^{A}: $p<0.01$ from the respective TB value.

selective ETA receptor agonist.

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