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Studies on endothelin receptors in the zonae fasciculata/reticularis of the rat adrenal cortex: contrast with the zona glomerulosa

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Abstract This study investigated the ET_A and ET_B receptor subtypes in rat adrenal cortex. The ET_A antagonist, BQ-123, inhibited the zona glomerulosa (zg), but not the inner zone (iz) response to ET-1. RES-701-1, the ET_B antagonist, abolished the iz response to ET-1, but had less effect on the zg. [^{125}I]ET-1 binding studies revealed two receptor subtypes in both zones, with ET_A predominating in the zg, and ET_B in the iz. These data suggest that the ET_A subtype is functionally more important in the zg while the ET_B receptor is the major subtype in the inner zones.

Key words: Aldosterone; Corticosterone; Endothelin receptor; Zona glomerulosa; Adrenal

1. Introduction

The potent vasoactive peptide endothelin-1 has been shown to stimulate adrenal steroidogenesis in several different mammalian species, including rat, cow and human [1–6]. The effects of endothelin have been demonstrated both in vivo and in vitro. Furthermore, it has been reported that this peptide is synthesised within the adrenal cortex [7], and is released following stimulation with corticotrophin (ACTH), the major regulator of glucocorticoid secretion [2,8].

Endothelin acts through two distinct receptor subtypes, designated ET_A and ET_B. The ET_A receptor is selective for endothelin-1 and is specifically antagonised by BQ-123, while the ET_B receptor binds both endothelin-1 and the related peptide, endothelin-3, and is specifically antagonised by RES-701-1. Autoradiographic studies have demonstrated high levels of [^{125}I]ET-1 binding in the adrenal glands from several different species, with the highest levels of binding in the zona glomerulosa [9–11]. Studies of endothelin receptors in the adrenal gland have therefore focussed on this region of the gland. The presence of both subtypes has been described in the rat adrenal gland [12] and in normal and adenomatous human zona glomerulosa tissue but it is not clear which is involved in mediating the response to stimulation [7,11]. To date the nature of the ET receptors in the inner adrenocortical zones has not been established. The present study was designed to investigate the distribution of the ET_A and ET_B receptor subtypes in the different zones of the rat adrenal cortex, and to determine their functional significance in mediating the adrenocortical response to endothelin stimulation.

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2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma Chemical Co, Poole, Dorset, UK, with the following exceptions: ACTH(1–24) (Synacthen) and angiotensin II amide (Hypertensin) were supplied by Ciba-Geigy, Horsham, Sussex, UK; collagenase (Worthington type I) was purchased from Lorne Diagnostics, Reading, Berks, UK; endothelin and BQ-123 were purchased from Peninsula Laboratories Europe Ltd, St. Helens, Merseyside, UK; RES-701-1 was supplied by Kyowa Hakko Kogyo Co, Ltd, Tokyo, Japan. All radiolabels were purchased from Amersham International plc, Amersham, Bucks, UK. Male and female Wistar rats, body weight 350–450 g, were obtained from A. Tuck and Sons, Battlesbridge, Essex, UK and maintained at Queen Mary and Westfield College for a minimum of 5 days prior to use. They were fed standard rat chow and water was available ad libitum.

2.2. Effects of endothelin receptor antagonists on steroidogenesis

Zona glomerulosa and zonae fasciculata/reticularis cells were prepared by collagenase digestion, as described previously [2]. Zona glomerulosa cells contained less than 10% contamination with inner zone cells, while zonae fasciculata/reticularis cells were contaminated with less than 5% of zona glomerulosa cells. Cells (5×10^5 cells/tube) were incubated for 60 min at 37°C with 10^{-7} mol/l ET-1 in the absence or presence of endothelin antagonists RES-701-1 or BQ-123 (10^{-8} mol/l). After incubation the samples were centrifuged at 10000 rpm to pellet the cells, and stored at −20°C. Unextracted aliquots were assayed for aldosterone (zona glomerulosa cells) and corticosterone (inner zone cells) by direct radioimmunoassay as previously described [2].

2.3. [^{125}I]ET-1 binding assay

The binding assay was a modified form of previously described methods [13,14]. Briefly, aliquots of cells (10^6 cells/tube) were incubated (in KRBGA with 20 mM HEPES, 150 mM NaCl and 1 μg/ml each of aprotinin and soybean trypsin inhibitor) with 3-([^{125}I]iodotyrosyl^{10})ET-1 (2000 Ci/mmol; final concentration 0.1 nmol/l) for 60 min at 37°C. Non-specific binding was determined by incubating labelled cells with 100-fold excess of unlabelled peptide. Incubations were terminated by the addition of 800 μl of ice-cold buffer and the tubes were centrifuged at 10000 rpm for 5 min at 4°C. Supernatants were discarded and the pellets washed twice. After washing, radioactivity bound to the cells was estimated using a 1272 Clinigamma counter (LKB Wallac, St Albans, Herts, UK). Displacement studies were carried out using ET-1, BQ-123, RES-701-1, ACTH or angiotensin II.

2.4. Statistical analysis

Arithmetic means and standard deviations were calculated. One-way analysis of variance was used to test whether peptides had a significant effect on basal (control) levels of steroids. Student's *t*-tests were used to test whether the above-mentioned responses were affected by the presence of antagonists. Saturation data were analysed by Hill and Scatchard analysis using the computer programme LIGAND.

3. Results

3.1. Effect of endothelins on steroid secretion

Endothelin-1 caused a significant increase in aldosterone secretion by collagenase dispersed zona glomerulosa cells

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(Fig. 1A). This effect was slightly attenuated by the ET_B receptor subtype antagonist, RES-701-1 ($p < 0.05$) and very significantly attenuated in the presence of the ET_A receptor antagonist, BQ-123 ($p < 0.001$). In the presence of both receptor antagonists, ET-1-stimulated aldosterone secretion was not significantly different from basal levels (Fig. 1A). Neither of these antagonists had any effect on basal or ACTH-stimulated aldosterone production (data not shown).

Fig. 1B illustrates the effects of ET-1 on corticosterone production by collagenase-dispersed zonae fasciculata/reticularis cells in the presence or absence of either BQ-123 or RES-701-1. In the absence of the antagonists, ET-1 (10^{-8} mol/l) caused significant stimulation of corticosterone secretion from zonae fasciculata/reticularis cells. The ET_A antagonist, BQ-123 (10^{-8} mol/l) had no effect on the corticosterone response to ET-1. However, 10^{-8} mol/l RES-701-1 significantly inhibited the corticosteroid response to ET-1 ($p < 0.001$). The response to the combination of these receptor antagonists on endothelin-stimulated corticosterone secretion resulted in a further reduction of the ET-1 response. Neither antagonist had any effect on basal or ACTH-stimulated corticosterone secretion (data not shown).

3.2. Binding studies

The binding kinetics of [^{125}I]ET-1 are shown in Fig. 2. Binding of ET-1 to the zona glomerulosa was saturable at 75–120 pmol and 175–200 pmol [^{125}I]ET-1 and non-specific binding was less than 10% of bound radioactivity (Fig. 2A). Binding data were analysed by the curve-fitting programme LIGAND and Hill analysis predicted the probability of a 2 binding site model versus a single population ($p < 0.05$) of endothelin receptors in the rat adrenal zona glomerulosa. Subsequent Scatchard analysis revealed the presence of 2 populations of binding sites (Fig. 2B). One receptor population of high-affinity binding sites had an apparent dissociation constant (K_d) of 1.87 nmol/l with a concentration of binding sites (B_{max}) of 535 fmol/10^6 zona glomerulosa cells (Hill coefficient of 0.593). The second population of receptors were low-affinity binding sites with an apparent K_d of 10.25 nmol/l and a B_{max} of 1047 fmol/10^6 zona glomerulosa cells (Hill coefficient of 0.774). Displacement of [^{125}I]ET-1 binding by unlabelled peptides showed that ET-1 displaced the tracer in a concentration-dependent manner with an IC50 of 5×10^{-9} mol/l (Fig. 2C). BQ-123, an ET_A receptor antagonist, displaced [^{125}I]ET-1 in a dose-dependent manner, similar to that of unlabelled ET-1 (Fig. 2C). RES-701-1 caused some displacement of the labelled ligand, although to a lesser extent than BQ-123 (Fig. 2C). Neither angiotensin II nor ACTH displaced [^{125}I]ET-1 binding in zona glomerulosa cells.

The binding kinetics of [^{125}I]ET-1 in inner zone cells are illustrated in Fig. 3. ET-1 binding was saturable at 175–200 pmol [^{125}I]ET-1 (Fig. 3A) and Hill analysis predicted there was a single population of endothelin receptors (Hill coefficient of 0.943; $p < 0.05$). The Scatchard plot (Fig. 3B) revealed a single class of low-affinity binding sites with an apparent K_d of 4.95 nM and B_{max} of 521 fmol/10^6 zonae fasciculata/reticularis cells.

Displacement studies with ET-1, RES-701-1, BQ-123, ACTH or angiotensin II revealed a difference in the receptor subtype present in this tissue compared to the zona glomerulosa. In these cells RES-701-1 had a potency comparable to ET-1, whereas BQ-123 was not very effective at displacing

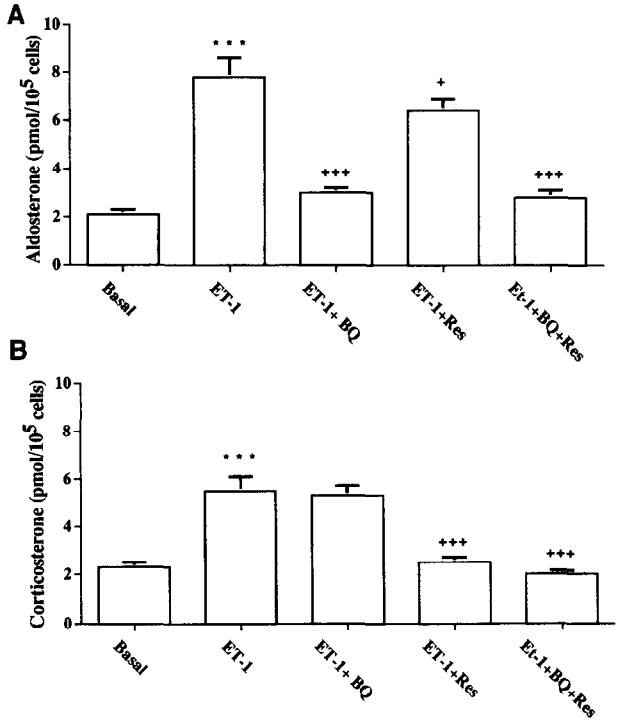

Fig. 1. Effect of endothelin receptor antagonists on endothelin-stimulated (10^{-8} mol/l) steroid secretion. (A) Effects on aldosterone secretion by zona glomerulosa cells and (B) effects on corticosterone secretion by inner zone cells. Effect of 10^{-8} mol/l BQ-123, 10^{-8}mol/l RES-701-1 or both BQ-123 and-RES-701-1 together. Values are means ± S.E.M., $n = 4$. $^{***}p < 0.001$ compared with control levels of aldosterone (analysis of variance). $^{+}p < 0.05$, $^{+++}p < 0.001$ compared with ET-1 alone (Student's t-test).

labelled ET-1 from the receptor. Neither ACTH nor angiotensin II had any effect on [^{125}I]ET-1 binding to zonae fasciculata/reticularis cells.

4. Discussion

The data presented here strongly suggest that different subtypes of the endothelin receptor are expressed in the zona glomerulosa and the zonae fasciculata/reticularis of the rat adrenal gland. Furthermore, these receptor subtypes appear to be functionally important in mediating the responses of the different zones to stimulation by endothelin-1.

The finding that the aldosterone response to endothelin-1 stimulation was significantly inhibited by both BQ-123 and RES-701-1 strongly suggests that both ET receptor subtypes have a role in mediating the zona glomerulosa response to endothelin stimulation. As it was shown that BQ-123 had a much greater inhibitory effect, it appears likely that the ET_A receptor plays the major role. This conclusion is supported by the receptor binding studies which suggest that of the two forms of the endothelin receptor in the zona glomerulosa, the most abundant is the ET_A subtype.

As much of the research on the adrenal actions of endothelin have focused on the effects of this peptide on aldosterone secretion, so previous studies have concentrated almost exclusively on the endothelin receptors in the zona glomerulosa. The results of the present investigation confirm the findings of other studies which have described both ET_A and ET_B receptor subtypes in both the human and bovine adrenal cor-

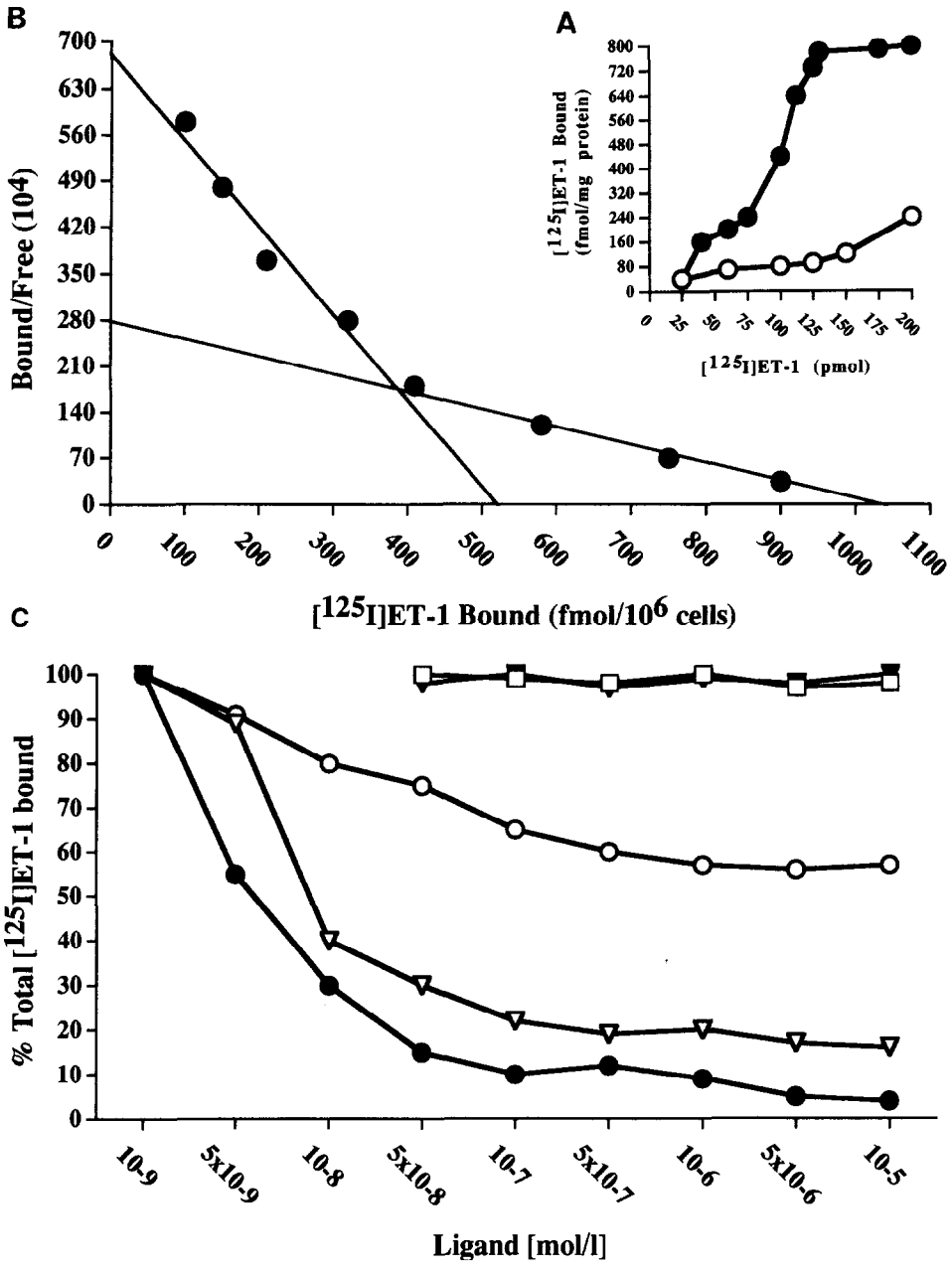

Fig. 2. Scatchard analysis, saturation curves and inhibition of [^{125}I]ET-1 binding to zona glomerulosa cells. (A) Concentration dependence of [^{125}I]ET-1 binding to membrane preparations from adrenal zona glomerulosa cells showing specific (●) and non-specific (○) binding. (B) Scatchard analysis of specific [^{125}I]ET-1 binding. (C) Inhibition of total [^{125}I]ET-1 binding by increasing concentrations of ET-1 (●), BQ-123 (▽), RES-701-1 (○) ACTH (▼) or angiotensin II (□). Each point represents the means from two experiments conducted in triplicate.

tex. Rossi and co-workers [11] characterised endothelin receptors in the human adrenal zona glomerulosa. The receptor K_ds they described are within the same range as those described in the present study, although others, notably Vesci et al [15], described a receptor with a higher affinity in the rat adrenal.

The functional significance of the different receptor types in the adrenal gland has been unclear. Gomez-Sanchez and coworkers [16] reported that the ET_A receptor was mainly responsible for mediating the aldosterone response in the calf, while the same group later found that the ET_A antagonist BQ123 had no effect on the aldosterone response to endothelin-1 stimulation in the rat, suggesting that endothelin was acting through the ET_B receptor in this species [6]. In this study, however, Pecci and co-workers removed the adrenals from rats treated in vivo, and measured the zona glomerulosa content of aldosterone, rather than either the circulating concentration or the subsequent in vitro response. As the rat adrenal gland does not store significant amounts of aldosterone the validity of this measurement is questionable. It is not clear why the rat adrenal zona glomerulosa would require two receptor subtypes to mediate the same response. However, the results of the present study strongly suggest that of the two endothelin receptor subtypes the ET_A receptor mediates the steroidogenic effects of endothelin-1 in the zona glomerulosa.

While the presence of endothelin receptors in the inner zones of the adrenal cortex has been clearly demonstrated,

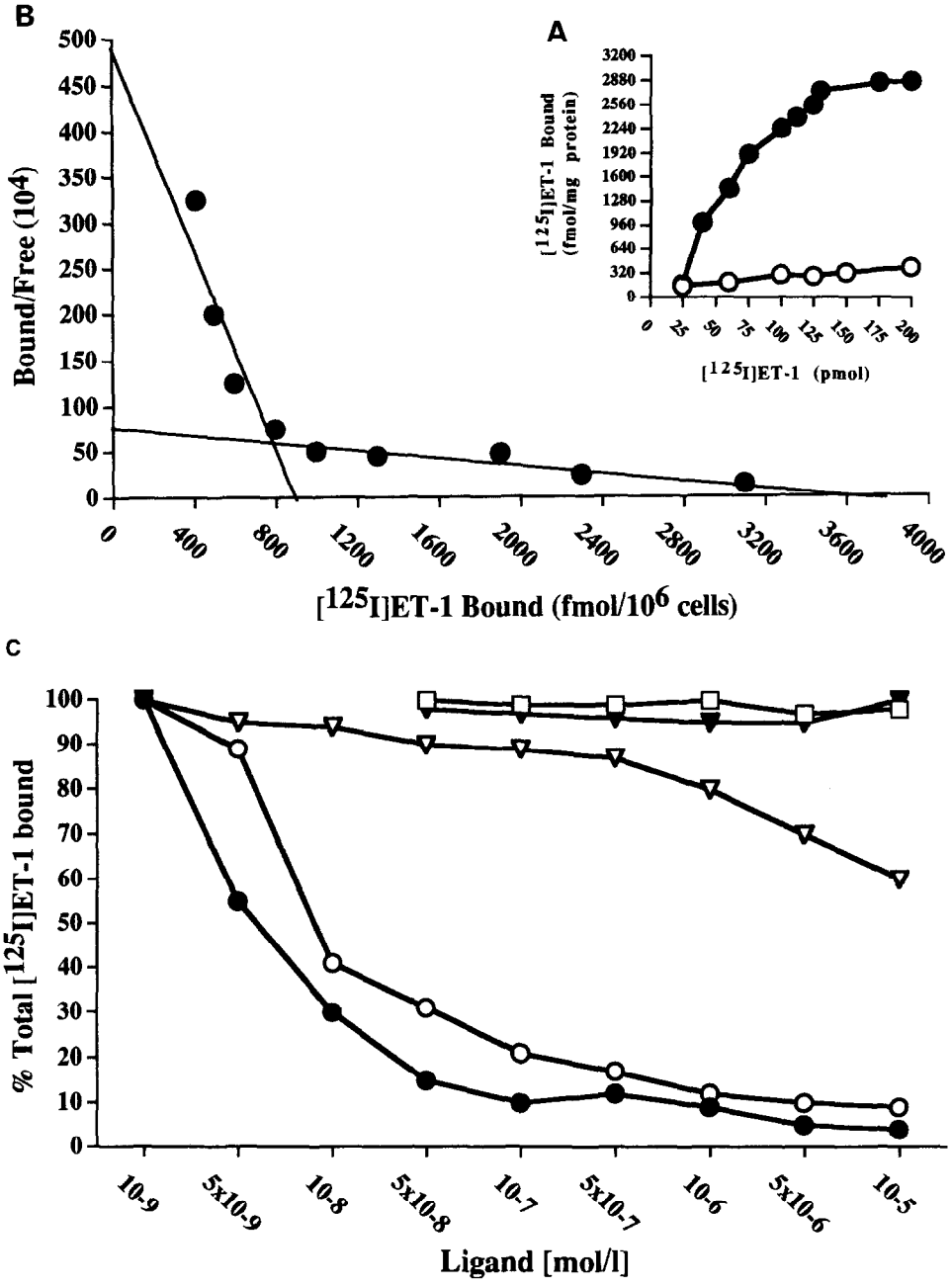

Fig. 3. Scatchard analysis, saturation curves and inhibition of [^{125}I]ET-1 binding to inner zone cells. (A) Concentration dependence of [^{125}I]ET-1 binding to membrane preparations from adrenal zonae fasciculata/reticularis cells showing specific (●) and non-specific (○) binding. (B) Scatchard analysis of specific [^{125}I]ET-1 binding. (c) Inhibition of total [^{125}I]ET-1 binding by increasing concentrations of ET-1 (●), BQ-123 (▽)), RES-701-1 (○), ACTH (▼) or angiotensin II (□). Each point represents the means from two experiments conducted in triplicate.

no attempt has been made to characterise these receptors. Immunohistochemical studies have described the ET_B receptor around the blood vessels of the bovine adrenal cortex, but not associated with the steroidogenic cells of the zona glomerulosa [17]. Using autoradiography, Rossi and co-workers demonstrated binding of labelled endothelin to specific receptors throughout the human adrenal cortex [11]. They found that BQ123 reduced binding in the zona glomerulosa but did not significantly attenuate binding in the zona fasciculata. This would suggest the presence of significant ET_A binding in the zona glomerulosa and ET_B binding in the inner zones, in good agreement with the present study. In previous studies we demonstrated a potent effect of endothelin-1 on corticosterone secretion by dispersed rat adrenal zona fasciculata/reticularis cells [2]. Thus, there is a clear functional significance to the presence of endothelin receptors in the inner zones of the rat adrenal cortex.

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References

[1] Cozza, E.N., Gomez-Sanchez, C.E., Foecking, M.F. and Chiou, S. (1989) J. Clin. Invest. 84, 1032–1035.

[2] Hinson, J.P., Vinson, G.P., Kapas, S. and Teja, R. (1991) J. Endocrinol. 128, 275–280.
[3] Mazzochi, G., Malendowicz, L. and Nussdorfer, G.G. (1990) Peptides 11, 763–765.
[4] Mazzochi, G., Rebuffat, P., Meneghelli, V., Malendowicz, L.K., Kasprzak, A. and Nussdorfer, G.G. (1990) Peptides 11, 767–772.
[5] Morishita, R., Higaki, J. and Ogihara, T. (1989) Biochem. Biophys. Res. Commun. 160, 628–632.
[6] Pecci, A., Gomez-Sanchez, C.E., De Bedners, M.E.O., Lantros, C.P. and Cozza, E.N. (1993) J. Steroid Biochem. Mol. Biol. 45, 555–561.
[7] Imai, T., Hirata, Y., Eguchi, S., Kanno, K., Ohta, K., Emori, T., Sakamoto, A., Yanagisawa, M., Masaki, T. and Marumo, F. (1992) Biochem. Biophys. Res. Commun. 182, 1115–1121.
[8] Cameron, L.A., Kapas, S. and Hinson, J.P. (1994) Biochem. Biophys. Res. Commun. 202, 873–879.
[9] Koseki, C., Imai, M., Hirata, Y., Yanagisawa, M. and Masaki, T. (1989) Am. J. Physiol. 256, R858–R866.
[10] Kohzuki, M., Johnston, C.I., Chai, S.Y., Casley, D.J., Rogerson, F. and Mendelsohn, F.A. (1989) Clin. Exp. Pharmacol. Physiol. 16, 239–242.
[11] Rossi, G., Albertin, G., Belloni, A., Zanin, L., Biasolo, M.A., Prayer-Galetti, T., Baderm, M., Nussdorfer, G.G., Palu, G. and Pessina, A.C. (1994) J. Clin. Invest. 94, 1226–1234.
[12] Hori, S., Komatsu, Y., Shigemoto, R., Mizuno, N. and Nakanishi, S. (1992) Endocrinology 130, 1885–1895.
[13] Barker, S., Marchant, W., Clark, A.J.L., Jimenez, E., Marsigliante, S., Montiel, M. and Vinson, G.P (1993) Biochem. Biophys. Res. Commun. 192, 392–398.
[14] Yoshida, A., Nishikawa, T., Tamura, Y. and Yoshida, S. (1991) J. Biol. Chem. 266, 4288–4294.
[15] Vesci, L., Mattera, G.G., Botarelli, M., Tobia, P., Corsico, N. and Martelli, E.A. (1994) Life Sci. 55, PL421–424.
[16] Gomez-Sanchez, C.E., Cozza, E.N., Foecking, M.F., Chiou, S. and Ferris, M.W. (1990) Hypertension 15, 744–747.
[17] Hagiwara, H., Nagasawa, T., Yamamoto, T., Lodhi, K.M., Ito, T., Takemura, N. and Hirose, S. (1993) Am. J. Physiol. 264, R777–R783.