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# Endothelin-1[1–31], acting as an ETA-receptor selective agonist, stimulates proliferation of cultured rat zona glomerulosa cells

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**Abstract Endothelin-1 (ET-1)[1–31] is a novel hypertensive peptide that mimics many of the vascular effects of the classic 21 amino acid peptide ET-1[1–21]. However, at variance with ET-1[1–21] that enhances aldosterone secretion from cultured rat zona glomerulosa (ZG) cells by acting via ETB receptors, ET-1[1–31] did not elicit such effect. Both ET-1[1–21] and ET-1-[1–31] raised the proliferation rate of cultured ZG cells, the maximal effective concentration being $10^{-8}$ M. This effect was blocked by the ETA-receptor antagonist BQ-123 and unaffected by the ETB-receptor antagonist BQ-788. Quantitative autoradiography showed that ET-1[1–21] displaced both [$^{125}$I]PD-151242 binding to ETA receptors and [$^{125}$I]BQ-3020 binding to ETB receptors in both rat ZG and adrenal medulla, while ET-1[1–31] displaced only [$^{125}$I]BQ-3020 binding. The tyrosine kinase (TK) inhibitor tyrphostin-23 and the p42/p44 mitogen-activated protein kinase (MAPK) inhibitor PD-98059 abolished the proliferogenic effect of ET-1[1–31], while the protein kinase-C (PKC) inhibitor calphostin-C significantly reduced it. ET-1[1–31] ($10^{-8}$ M) stimulated TK and MAPK activity of dispersed ZG cells, an effect that was blocked by BQ-123. The stimulatory action of ET-1[1–31] on TK activity was annulled by tyrphostin-23, while that on MAPK activity was reduced by calphostin-C and abolished by either tyrphostin-23 and PD-98059. These data suggest that ET-1[1–31] is a selective agonist of the ETA-receptor subtype, and enhances proliferation of cultured rat ZG cells through the PKC- and TK-dependent activation of p42/p44 MAPK cascade.** 

*Key words:* Endothelin-1[1–31]; Zona glomerulosa; Aldosterone; Cell proliferation; Protein kinase-C; Tyrosine kinase; p42/p44 mitogen-activated protein kinase

## 1. Introduction

Endothelin-1 (ET-1) is the prototype of a family of 21 amino acid peptides acting through two main receptor subtypes, called ETA and ETB. It is generated from big-ET-1 by cleavage at the Trp$^{21}$-Val/Ile$^{22}$ bond by the endothelin-converting enzyme (ECE)-1 [1]. However, evidence has been recently provided that big-ET-1 may be also selectively cleaved at the Tyr$^{31}$-Gly$^{32}$ bond by a chymase to produce a novel peptide, named ET-1[1–31] [2].

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 [3], raising of intracellular $Ca^{2+}$ concentration in cultured human vascular smooth-muscle cells (VSMC) [4–6], and stimulation of VSMC proliferation [7]. ETA receptors mediate the vasoconstrictor and VSMC proliferogenic effect of ET-1[1–31] [8]. However, it was contended that they are involved also in ET-1[1–31] effects [3,4,6].

We have demonstrated that ET-1[1–21] in addition to enhancing aldosterone secretion in the rat via the ETB receptor [9], exerts a marked proliferogenic action on the zona glomerulosa (ZG) of in situ perfused rat adrenal gland. This effect occurs via ETA receptors coupled with protein kinase (PK) C- and tyrosine kinase (TK)-dependent signaling pathways [10]. However, the effect of ET-1[1–31] on rat ZG has not been studied. Thus, the present study was undertaken to investigate whether ET-1[1–31] affects secretion and proliferation of rat ZG cells cultured in vitro.

## 2. Materials and methods

### 2.1. Cell cultures

Adult male Sprague–Dawley rats (260 ± 30 g body weight) were purchased from Charles-River (Como, Italy), and the protocol of the experiment was approved by the local Ethical Committee for Animal Studies. Rats were decapitated, and their adrenal glands were promptly removed and cleaned from adherent adipose tissue.

Adrenals were gently decapsulated to separate ZG from the inner zones, and dispersed ZG cells were obtained by sequential collagenase digestion and mechanical disaggregation [9]. Dispersed cells were suspended in Eagle's minimum essential medium (Paisley, UK), supplemented with 2% fetal bovine serum (Sigma, St. Louis, MO, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, and plated in 35-mm tissue culture dishes at a density of $5\times10^4$ cells/dish. They were cultured at 37°C in a humidified atmosphere of 95% air–5% $CO_2$, and employed after 24 h of culture.

### 2.2. Cell secretion

Culture medium was replaced with fresh medium, which was then collected after 120 min to evaluate basal cell secretion. New medium was then added which contained the following compounds: (i) ET-1[1–21] or ET-1[1–31] (Peptide Institute, Osaka, Japan) (from $10^{-11}$ to $10^{-7}$ M); and (ii) ET-1[1–21] or ET-1[1–31] ($10^{-8}$ M) alone or with $10^{-7}$ M BQ-123 and BQ-788 (Neosystem, Strasbourg, France), which are selective antagonists of the ETA and ETB receptors, respectively [2]. A further 120-min incubation was then performed (stimulated secretion). Some samples were incubated with $10^{-9}$ M ACTH.

Aldosterone was extracted from the incubation media and purified by HPLC [11]. Its concentrations were measured by specific RIA, as

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previously detailed [9]. Values were expressed as percent change from basal secretion, and six culture dishes for each experimental point were employed.

*2.3. Cell proliferation*

Cultures were incubated for 24 h as follows: (i) ET-1[1–21] or ET-1[1–31] (from $10^{-11}$ to $10^{-7}$ M); (ii) ET-1[1–31] ($10^{-8}$ M) alone or in the presence of $10^{-7}$ M BQ-123, $10^{-7}$ M BQ-788, $10^{-5}$/$10^{-4}$ M phosphoramidon (Sigma), $10^{-5}$ M H-89, $10^{-5}$ M calphostin-C, $10^{-5}$ M tyrphostin-23 and $10^{-4}$ M PD-98059 (Biomol, Milan, Italy). During the last 12 h of incubation, 5-bromo-2′-deoxyuridine (BrdU) (Sigma) was added to the culture medium to a final concentration of 20 mg/ml.

Cultures were fixed in 4% paraformaldehyde for 30 min, and BrdU-positive (S-phase) cells were detected by immunocytochemistry, using the Cell Proliferation kit™ of the Amersham Pharmacia (Aylesbury, UK). The percentage of positive cells was evaluated by counting 5000 cells per dish, and five dishes for each experimental point were employed.

*2.4. TK and mitogen-activated protein kinase (MAPK) activity*

Dispersed ZG cells were put in Medium 199 (Difco, Detroit, MI, USA) and Krebs–Ringer bicarbonate buffer with 0.2% glucose, containing 5 mg/ml of bovine serum albumin (BSA) (Sigma). They were incubated with ET-1[1–31] ($10^{-8}$ M) alone or in the presence of $10^{-7}$ M BQ-123 and BQ-788. Other ZG cell preparations were preincubated for 30 min with $10^{-5}$ M H-89, $10^{-5}$ M calphostin-C, $10^{-5}$ M tyrphostin-23 or $10^{-4}$ M PD-98059, and then exposed to ET-1[1–31] ($10^{-8}$ M). The incubation was carried out in a shaking bath at 37°C for 15 min, and was stopped by two quick washes with ice-cold PBS.

Dispersed cells were lysed as previously described [12], and then were Dounce homogenized (20 strokes; Kontes, Vineland, NJ, USA) on ice for 1 min. Homogenates were centrifuged at 4°C at $800\times g$ for 10 min, and then at $12\,000\times g$ for 15 min. Supernatants were removed, the protein concentration was determined by the Lowry method using BSA as a standard, and then stored at −80°C. TK activity was assayed, using poly ($Glu^4$,$Tyr^1$) (Sigma) as substrate [13]. MAPK activity was measured by immune complex kinase assay [14], using anti-MAPK p42/p44 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and myelinic basic protein (Sigma) as substrate. The procedures have been described previously in detail [12]. Each experimental point was the mean of four separate experiments.

*2.5. Quantitative autoradiography*

Adrenal glands of three rats 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 for autoradiography as previously detailed [9]. They 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 (Amersham), which are selective ligands of the ETA and ETB receptors, respectively [2]. The selectivity of binding was checked by addition of $10^{-7}$ M BQ-123 or BQ-788. The ability of ET-1[1–21] and ET-1[1–31] to displace binding was measured by adding each of them to a concentration ranging from $10^{-11}$–$10^{-7}$ M. 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 2 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.

For each adrenal gland, three unstained autoradiograms 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). In each autoradiogram, 10 areas of the ZG (about 36 000 pixels) were analyzed. The density value of the capsule was taken as the background value.

*2.6. Statistics*

Data were expressed as means ± S.E.M., and their statistical comparison was done by ANOVA, followed by Duncan's multiple range test.

## 3. Results and discussion

Under basal conditions ZG cell cultures produced sizeable amounts of aldosterone (20–25 pmol/well); they responded to $10^{-9}$ M ACTH stimulation (approximately 5-fold rise) (data not shown), thereby indicating that ZG cells maintain their native phenotype after the 24-h in vitro culture conditions of this study. ET-1[1–21] concentration-dependently increased aldosterone secretion, maximal effective concentration being $10^{-8}$ M (2-fold rise), while ET-1[1–31] was ineffective (Fig. 1). The stimulatory effect of $10^{-8}$ M ET-1[1–21] was abolished by the ETB-receptor antagonist BQ-788 ($10^{-7}$ M) and unaffected by the ETA-receptor antagonist BQ-123 (Fig. 1), thus confirming that the aldosterone secretagogue action of ET-1[1–21] is exclusively mediated by the ETB-receptor subtype [2].

Both ET-1[1–21] and ET-1 [1–31] concentration-dependently increased the percentage of BrdU-positive cells in ZG cell cultures; the maximal effective concentration eliciting an about 4.5-fold rise was $10^{-8}$ M (Fig. 2). The ECE-1 inhibitor phosphoramidon [15] did not blunt the proliferogenic action of ET-1[1–31] (Fig. 2), thereby ruling out the possibility that the effect of this peptide is due to its cleavage to ET-1[1–21], as reported to occur in cultured bronchial smooth muscle cells [16]. The proliferogenic effect of $10^{-8}$ M ET-1[1–31] was blocked by BQ-123 ($10^{-7}$ M), and unaffected by BQ-788 (Fig. 2). This finding indicates that the ZG proliferogenic

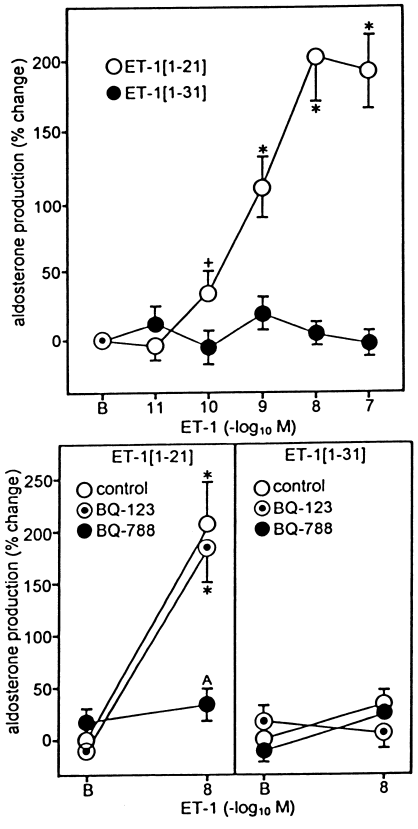


Fig. 1. Effects of ET-1[1–21] and ET-1[1–31] on aldosterone secretion from cultured rat ZG cells (upper panel), and of the ET-receptor antagonists ($10^{-7}$ M) on aldosterone response to $10^{-8}$ M ET-1[1–21] or ET-1[1–31] (lower panel). Data, expressed as percent change from baseline, are means ± S.E.M. ($n=6$). *$P<0.01$ versus the baseline value (B); $^{A}P<0.01$ versus the respective control value.

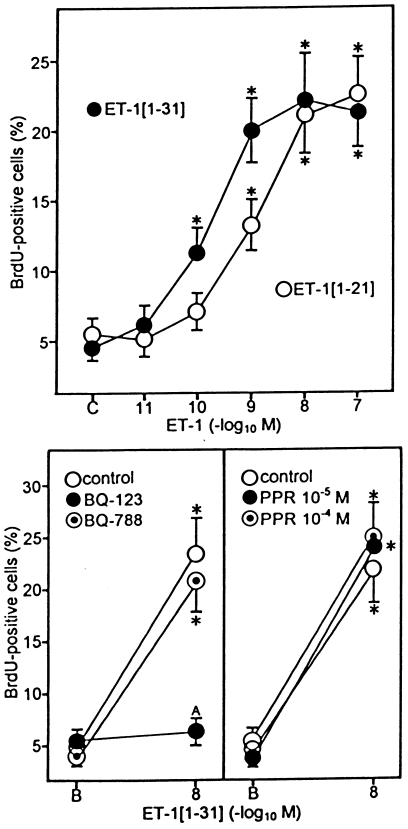


Fig. 2. Effects of ET-1[1–21] and ET-1[1–31] on the proliferation rate of cultured rat ZG cells (upper panel), and of the ET-receptor antagonists ($10^{-7}$ M) and phosphoramidon (PPR) ($10^{-5}$ or $10^{-4}$ M) on the proliferogenic response to $10^{-8}$ M ET-1[1–31] (lower panel). Data are means ± S.E.M. ($n=5$). $^{+}P<0.05$ and $^{*}P<0.01$ versus the respective baseline value (B). $^{A}P<0.01$ versus the respective control value.

effect of ET-1[1–31], like that of ET-1[1–21] [10], is mediated by the ETA-receptor subtype. Although the ZG proliferogenic actions of the two ET-1 peptides display similar efficacy, ET-1[1–31] was significantly more potent than ET-1[1–21] ($EC_{50}$: $1.1\pm0.3\times10^{-10}$ M versus $8.2\pm1.6\times10^{-10}$ M; $P<0.05$, $n=5$), an observation consistent with the possibility that ET-1[1–31], at variance with ET-1[1–21], exclusively binds the ETA-receptor subtype.

This last contention is supported by autoradiographic studies. As expected [9], ETA receptors ([$^{125}$I]PD-151242 BQ-123-displaceable binding sites) and ETB receptors ([$^{125}$I]BQ-3020 BQ-788-displaceable binding sites) were mainly present in both ZG and adrenal medulla (Fig. 3). The quantitative densitometric analysis of autoradiograms showed that ET-1[1–21] concentration-dependently displaced either [$^{125}$I]PD-151242 or [$^{125}$I]BQ-3020 binding, while ET-1[1–31] displaced only [$^{125}$I]PD-151242 binding in both ZG and adrenal medulla (Fig. 4). Again, ET-1[1–31] was more potent than ET-1-[1–21] in displacing ETA-receptor binding ($EC_{50}$: ZG, $0.6\pm0.3\times10^{-10}$ M versus $1.1\pm0.5\times10^{-9}$ M; adrenal medulla, $0.5\times10^{-10}$ M versus $0.7\times10^{-9}$ M; $P<0.05$, $n=3$).

The $10^{-8}$ M ET-1[1–31]-induced rise in the number of BrdU-positive cells was significantly reduced by calphostin-C ($10^{-5}$ M) and abolished by either tyrphostin-23 ($10^{-5}$ M) or PD-98059 ($10^{-4}$ M). In contrast, H-89 was ineffective (Fig. 5). ET-1[1–31] ($10^{-8}$ M) enhanced TK and MAPK activities (about 2-fold) in dispersed ZG cells, and the effect was annulled by $10^{-7}$ M BQ-123, but not BQ-788 (Fig. 6). Preincubation with $10^{-5}$ M tyrphostin-23, but not with H-89, calphostin-C or PD-98059, blocked the ET-1[1–31]-induced stimulation of TK activity (Fig. 6). The ET-1[1–31]-evoked rise in MAPK activity was annulled by preincubation with tyrphostin-23 ($10^{-5}$ M) or PD-98059 ($10^{-4}$ M), significantly blunted by calphostin-C ($10^{-5}$ M), and unaffected by H-89 (Fig. 6).

The TK receptor is involved in the activation of p42/p44 MAPKs, ubiquitous members of a family of serine/threonine kinases, that play a crucial role in cell proliferation [17,18]. Our results strongly suggest that ET-1[1–31] exerts its growth-promoting action by activating the TK–MAPK signaling

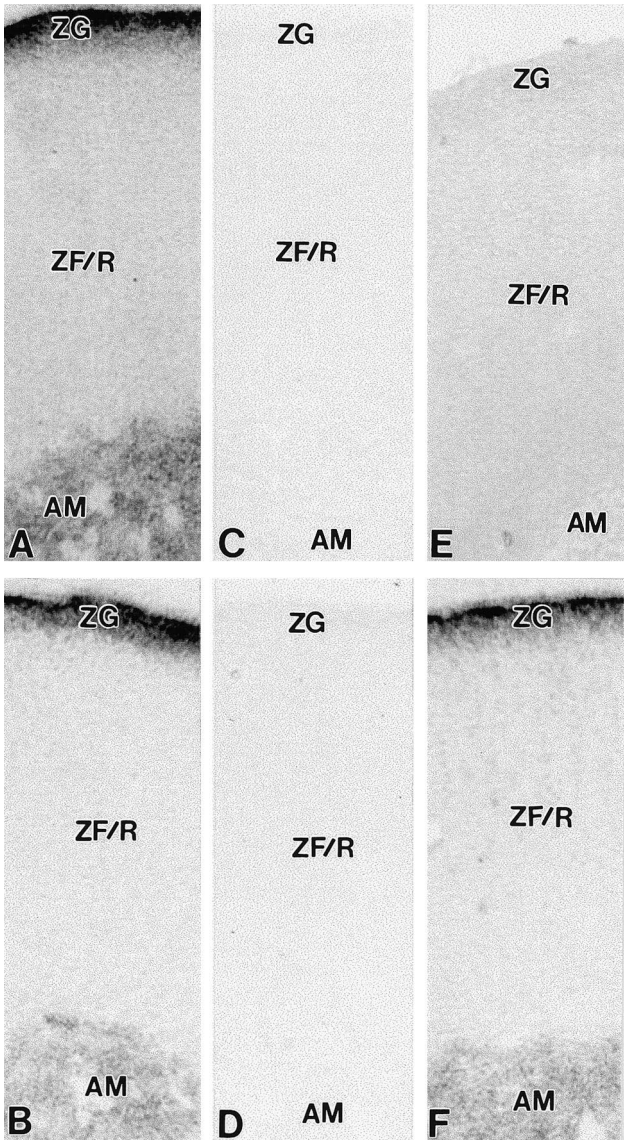


Fig. 3. Autoradiograms of unstained frozen sections of rat adrenal gland incubated with $10^{-9}$ M [$^{125}$I]PD-151242 (A) or $10^{-9}$ M [$^{125}$I]BQ-3020 (B). Binding of [$^{125}$I]PD-121542 to ETA receptors was eliminated by $10^{-7}$ M BQ-123 (C) and binding of [$^{125}$I]BQ-3020 to ETB receptors was completely displaced by $10^{-7}$ M BQ-788 (D). ET-1[1–31] eliminated binding to ETA receptors (E), without affecting binding to ETB receptors (F). c, Gland capsule; AM, adrenal medulla. 67×.

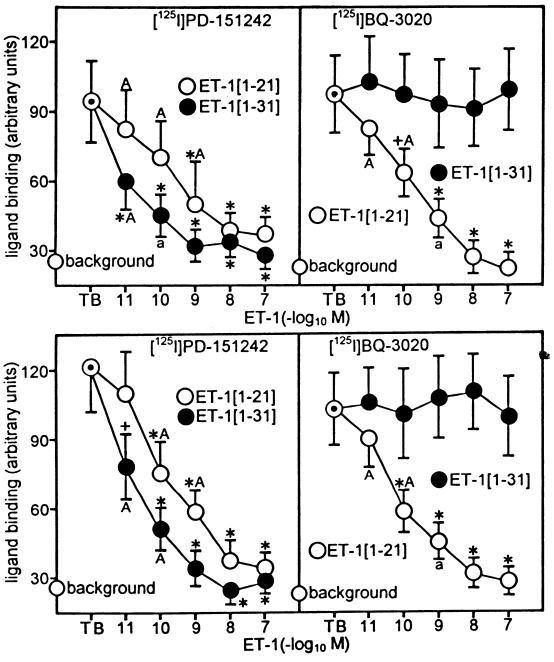


Fig. 4. Evaluation by quantitative densitometry of the displacement of [$^{125}$I]PD-151242 and [$^{125}$I]BQ-3020 (total binding, TB) by ET-1-[1–21] and ET-1[1–31] in the ZG (upper panels) and adrenal medulla (lower panels). Data are means ± S.E.M. ($n = 3$). $^{+}P < 0.05$ and $^{*}P < 0.01$ versus the respective TB value; $^{a}P < 0.05$ and $^{A}P < 0.01$ versus the respective background value.

pathway. This contention is based on the following pieces of evidence: (i) the TK inhibitor tyrphostin-23 [19] and the MEK1 inhibitor PD-98059 [20] block the ET-1[1–31]-induced rise in the number of BrdU-positive ZG cells; (ii) these inhibitors per se do not evoke any apparent effect on the basal rate of DNA synthesis, thus excluding the possibility that these results were due to a non-specific toxic effect on cultured ZG cells; (iii) ET-1[1–31] stimulates both TK and MAPK activity in dispersed rat ZG cells and the effect is blocked by BQ-123; and (iv) like PD-98059, tyrphostin-23 blocks the ET-1[1–31]-induced stimulation of MAPK activity.

Evidence indicates that, in addition to TK, G protein-coupled receptors can activate the MAPK cascade [21],

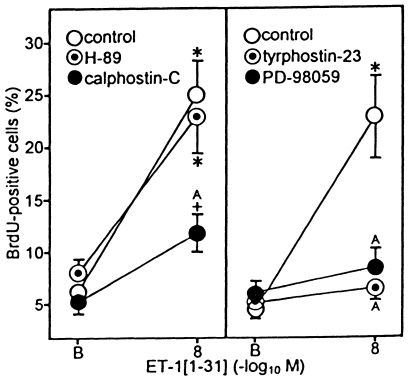


Fig. 5. Effects of H-89 ($10^{-5}$ M), calphostin-C ($10^{-5}$ M), tyrphostin-23 ($10^{-5}$ M) and PD-98059 ($10^{-4}$ M) on $10^{-8}$ M ET-1[1–31]-induced stimulation of the proliferation rate of cultured rat ZG cells. Data are means ± S.E.M. ($n = 5$). $^{+}P < 0.05$ and $^{*}P < 0.01$ versus the respective baseline value (B); $^{A}P < 0.01$ versus the respective control value.

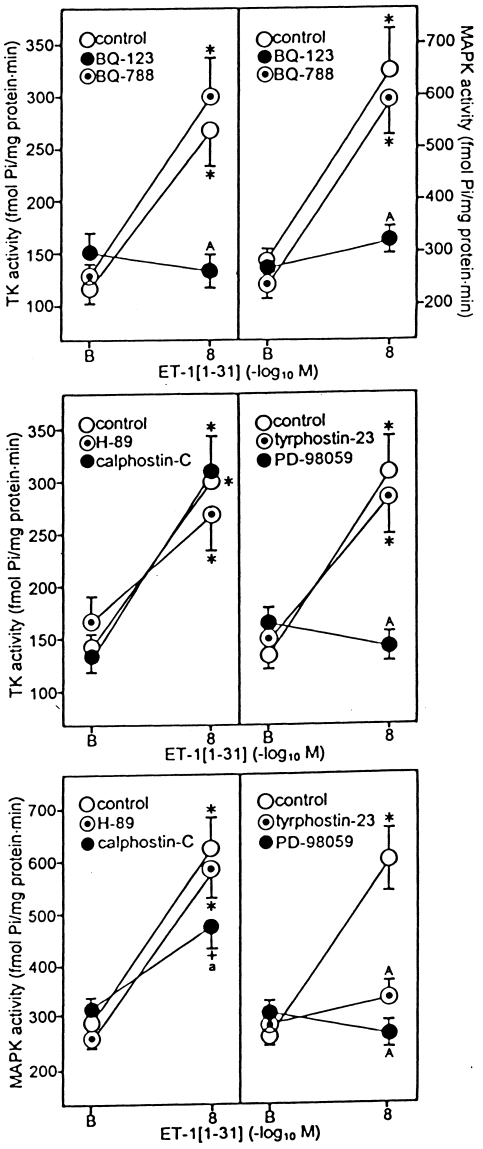


Fig. 6. Effects of the ET-receptor antagonists ($10^{-7}$ M) (upper panels), and of the preincubation with H-89 ($10^{-5}$ M), calphostin-C ($10^{-5}$ M), tyrphostin-23 ($10^{-5}$ M) or PD-98059 ($10^{-4}$ M) (middle and lower panels) on $10^{-8}$ M ET-1[1–31]-induced stimulation of TK and MAPK activity of dispersed rat ZG cells. Data are means ± S.E.M. ($n = 4$). $^{+}P < 0.05$ and $^{*}P < 0.01$ versus the respective baseline value (B); $^{a}P < 0.05$ and $^{A}P < 0.01$ versus the respective control value.

through both protein kinase-C (PKC)- [22–24] and PKA-dependent pathways [24,25]. Accordingly, angiotensin-II was found to stimulate mitogenesis and MAPK activity in bovine ZG cells via a PKC-dependent and a PKC-independent pathway [26], and cyclic-AMP was shown to activate MAPK and to enhance proliferation of ZG cells [27]. Our data demonstrate the involvement of the PKC pathway in the MAPK-mediated ZG proliferogenic action of ET-1[1–31]. In fact, the PKC inhibitor calphostin-C [28] partially reverses ZG cell proliferation and MAPK activation induced by ET-1[1–31]. PKC-dependent and TK-dependent MAPK activation in rat ZG cells are conceivably two independent pathways, because calphostin-C does not affect ET-1[1–31]-induced stimulation of TK activity. In contrast, adenylate cyclase/PKA pathway

does not appear to play a relevant role, inasmuch as the PKA inhibitor H-89 [29] affects neither ZG cell proliferogenic nor the TK- and MAPK-activating action of ET-1[1–31]. This finding agrees with the recent report of the inability of ACTH to stimulate MAPK in rat ZG cells [30].

In summary, the present results collectively indicate that ET-1[1–31] (i) is a selective agonist of the ETA receptor, and (ii) enhances proliferation of cultured rat ZG cells through both a primary TK-dependent and a secondary PKC-dependent activation of the p42/p44 MAPK cascade.

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