

Endothelin stimulates both cAMP formation and phosphatidylinositol hydrolysis in cultured embryonic bovine tracheal cells

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Embryonic bovine tracheal (EBTr) cells were found to possess receptors for endothelin (ET) of ET-1-selective (ET_A) subtype with a K_d for ET-1 of 114 pM and a B_{max} of 12.9 fmol/10⁶ cells. Stimulation of EBTr cells with 100 pM to 100 nM ET-1 increased the contents of both inositol phosphates and cAMP in a concentration-dependent manner, indicating that the receptors are coupled to both phosphatidylinositol hydrolysis and cAMP formation in EBTr cells.

Endothelin; Receptor; cAMP; Phosphatidylinositol turnover

1. INTRODUCTION

Endothelin, now known as ET-1, was first purified from the culture medium of porcine endothelial cells and is the most potent and long-acting vasoconstrictor [1]. Subsequently, ET-1 has been shown to be a member of a family consisting of three isopeptides (ET-1, ET-2 and ET-3) [2]. These ET isopeptides exhibit various pharmacological actions including bronchoconstriction and vasodilation [3], which are mediated by two receptor subtypes, i.e. ET-1- and ET-2-selective ET_A and non-isopeptide-selective ET_B [4–7].

Both ET-receptors have been shown to mediate the stimulation of phosphatidylinositol (PI) hydrolysis in many tissues and different cell types [3]. However, intracellular signal transduction involved in the ET receptors seems to be more complex, because ETs reportedly inhibit cAMP formation in several tissues and cells [8–10] and even stimulate the cAMP generation in a few systems such as cultured rat epididymal cells [11] and glomerular mesangial cells [12].

Abbreviations: EBTr cells, embryonic bovine tracheal cells; ET, endothelin; PI, phosphatidylinositol; IBMX, 3-isobutyl-1-methylxanthine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PG, prostaglandin; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HBSS, HEPES-buffered salt solution; [Ca²⁺]_i, intracellular Ca²⁺ concentration; IP, inositol phosphate; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate.

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In this paper, we show first that ET_A receptors are abundant in EBTr cells, a cell line derived from the embryonic bovine trachea. We also reveal that the ET_A receptor is coupled to both PI hydrolysis and cAMP formation in EBTr cells. The present study is the first to show that ET_A receptors are coupled to both PI hydrolysis and cAMP formation.

2. MATERIALS AND METHODS

2.1. Materials

ET-1 and ET-3 were obtained from Peptide Institute Inc. (Osaka, Japan). 3-Isobutyl-1-methylxanthine (IBMX) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma and fura-2-acetoxymethyl ester was from Dojin (Kumamoto, Japan). Prostaglandin (PG) D₂ and AH6809 were generous gifts from Ono Pharmaceuticals (Osaka, Japan) and Glaxo (Hertfordshire, UK), respectively. [¹²⁵I]ET-1 (74 TBq/mmol), [¹²⁵I]ET-3 (74 TBq/mmol) and [³H]inositol (699 GBq/mmol) were purchased from Amersham. All other chemicals were of reagent grade.

2.2. Cell culture

EBTr cells (27th passage) were obtained from the American Type Cell Culture Collection (ATCC CCL 44) and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a CO₂ incubator (5% CO₂/95% air) as previously reported [13]. EBTr cells of the 30th to 35th passage were used for experiments.

2.3. Binding assay

EBTr cells were cultured to confluence on 24-well plates (2×10⁵ cells/well). The cells were washed with 20 mM HEPES (pH 7.4), containing 140 mM NaCl, 4 mM KCl, 1 mM K₂HPO₄, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM D-glucose and 0.05% bovine serum albumin (HBSS) and incubated at 37°C for 1 h in HBSS with various concentrations of [¹²⁵I]ET-1 to obtain total binding. Nonspecific binding was determined in the presence of 100 nM unlabeled ET-1. Specific binding was defined as total binding minus nonspecific binding. The

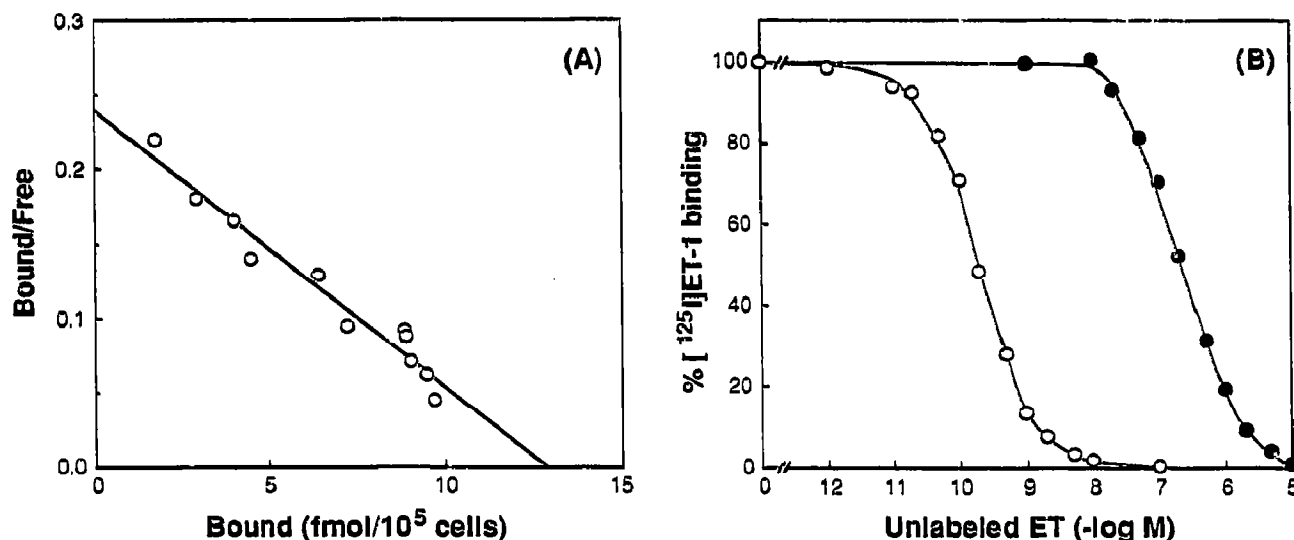


Fig. 1. Scatchard analysis of the specific binding of [¹²⁵I]ET-1 to EBTr cells (A). Competitive binding of 50 pM [¹²⁵I]ET-1 to EBTr cells by unlabeled ET-1 (○) and ET-3 (●) (B). Results are expressed as percentages of the specific binding. Values are the means of triplicate determinations.

cell-associated radioactivity was dissolved in 1 N NaOH and measured in a Wallac-1470 Wizard autogamma counter (Pharmacia).

2.4. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

EBTr cells cultured on quartz slide-glass were washed three times in a serum-free DMEM and incubated with 5 μ M fura-2-acetoxymethyl ester at 37°C for 1.5 h in a CO₂ incubator. After washing three times in HBSS, the fura-2-loaded EBTr cells were transferred to a chamber on the stage of a Mu-10 Ca^{2+} analyzer (Inter Dec, Osaka, Japan). The change of $[Ca^{2+}]_i$ was monitored fluorimetrically with the emission wavelength set at 510 nm and the excitation wavelengths at 340 and 380 nm. The $[Ca^{2+}]_i$ was estimated from the ratio of fluorescence at 340 nm to that at 380 nm by using a standard curve obtained with various concentrations of free Ca^{2+} .

2.5. Measurement of inositol phosphate (IP) formation

EBTr cells were cultured in 6-well plates at a density of 9×10^5 cells/well. The cells were labeled with [³H]inositol (74 kBq/well) for 1 day in inositol-free DMEM supplemented with 10% dialyzed fetal bovine serum. The formation of IP₁, IP₂ and IP₃ was determined as described previously [14].

2.6. Measurement of cAMP formation

The cAMP content of cells was determined in the presence of 0.5 mM IBMX, an inhibitor of phosphodiesterase, as reported previously [13]. EBTr cells grown to confluence in 24-well plates were washed twice with 1 ml of HBSS before experiments. After preincubation at 37°C for 10 min with 1 ml of HBSS containing 0.5 mM IBMX, the cells were incubated with various amounts of ET-1 or ET-3 at 37°C for 10 min except when otherwise indicated. The reaction was stopped by addition of 10% (w/v) trichloroacetic acid. The cAMP content was measured by radioimmunoassay with an [¹²⁵I]cAMP assay kit (Amersham).

3. RESULTS AND DISCUSSION

We detected the specific binding of [¹²⁵I]ET-1 to EBTr cells. The Scatchard analysis (Fig. 1A) revealed a single component of high affinity binding sites for ET-1 with a dissociation constant (K_d) of 114 pM and a maximum

binding capacity (B_{max}) of 12.9 fmol/10⁵ cells. In contrast, when [¹²⁵I]ET-3 was used as a ligand, no specific binding was detected on EBTr cells. In competitive binding assays (Fig. 1B), unlabeled ET-1 dose-dependently and completely inhibited the binding of [¹²⁵I]ET-1 to EBTr cells, showing a K_i of 140 pM. Although unlabeled ET-3 also competed with [¹²⁵I]ET-1 on the same binding sites, the K_i value of ET-3 (160 nM) was 1,100-fold higher than that of unlabeled ET-1, indicating that the ET-1 selective subtype of ET receptor (ET_A) is responsible for the binding of ET-1 to EBTr cells.

The K_d value of the ET_A receptor on EBTr cells is ~2-fold higher than that of the ET_A receptor (58 pM) on A-10 cells derived from rat aortic smooth muscle, which are used for a model of ET-induced vascular contraction, and ~7-fold that of endothelial ET_B receptor (17 pM), which is coupled to ET-induced vasodilation [15]. This may explain, in part, pharmacological observations that bronchoconstriction requires higher amounts of ETs [16] as compared with vasoconstriction or vasodilation [17]. On the other hand, the number of ET_A receptors of EBTr cells (78,000 sites/cell) is ~4-fold more than that of the ET_A receptor on A-10 cells (21,000 sites/cell) and ~16-fold that of the ET_B receptor on endothelial cells (>5,000 sites/cell).

ET-1 induced a rapid and transient increase in $[Ca^{2+}]_i$, accompanied with PI hydrolysis and cAMP formation in EBTr cells. Fig. 2A shows typical time courses of increases in those intracellular second messengers induced by 10 nM ET-1. After addition of 10 nM ET-1, $[Ca^{2+}]_i$ increased immediately from the basal level (120 nM) to a peak (450 nM) at about 20 s, decreased rapidly to a sustained level (200 nM) by 1 min, and maintained

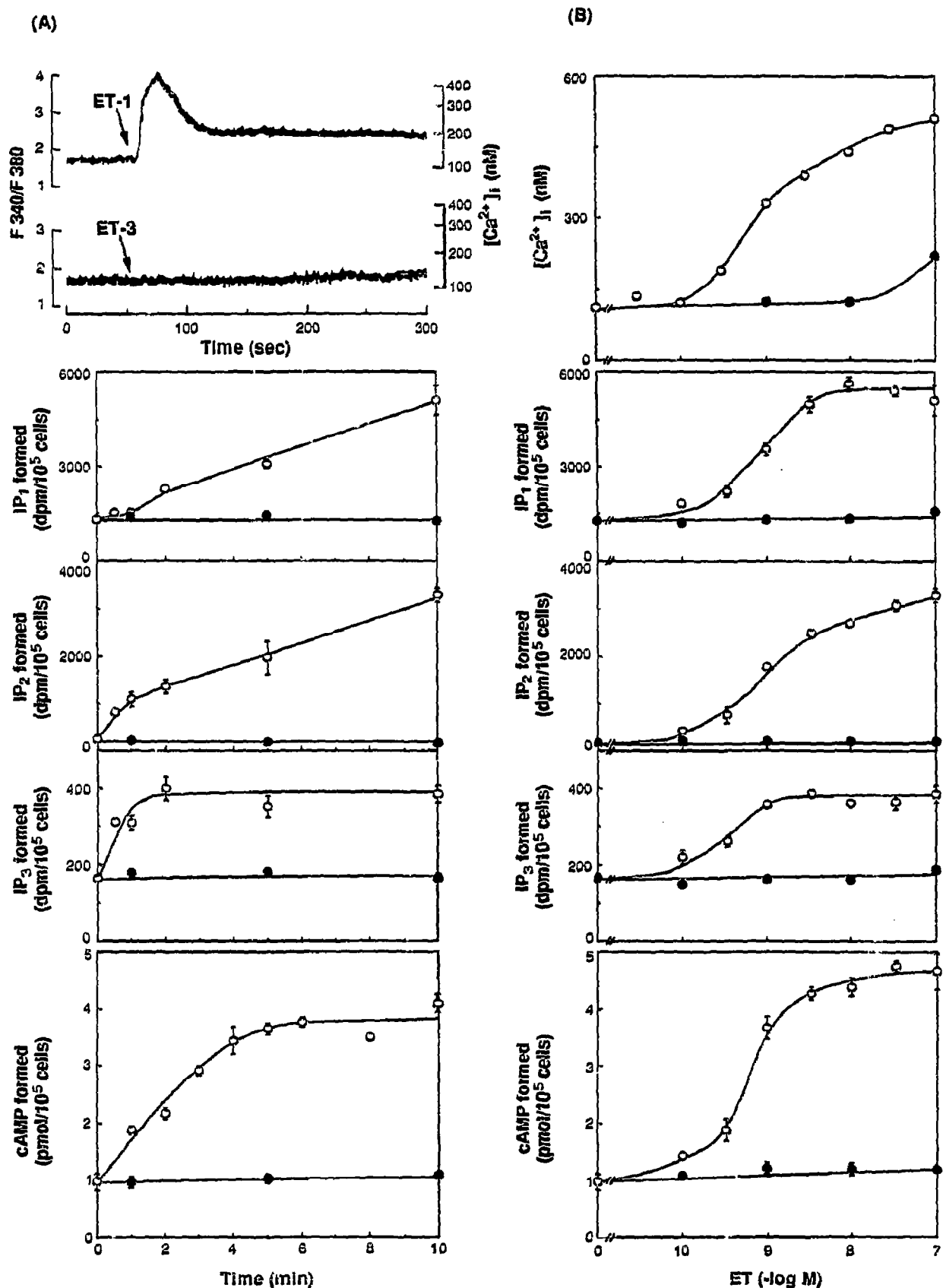


Fig. 2. (A) Time course of $[Ca^{2+}]_i$, PI hydrolysis, and cAMP content in EBTr cells after incubation with 10 nM ET-1 (O) and ET-3 (●). (B) Dose-response curves of ET-1 (O) and ET-3 (●) for increases in $[Ca^{2+}]_i$, PI hydrolysis and cAMP formation in EBTr cells. The $[Ca^{2+}]_i$ was estimated at the peak value after addition of ETs. The formation of IPs and cAMP was determined after incubation for 10 min. The values of IPs and cAMP are means $\pm$ S.D. of four determinations.

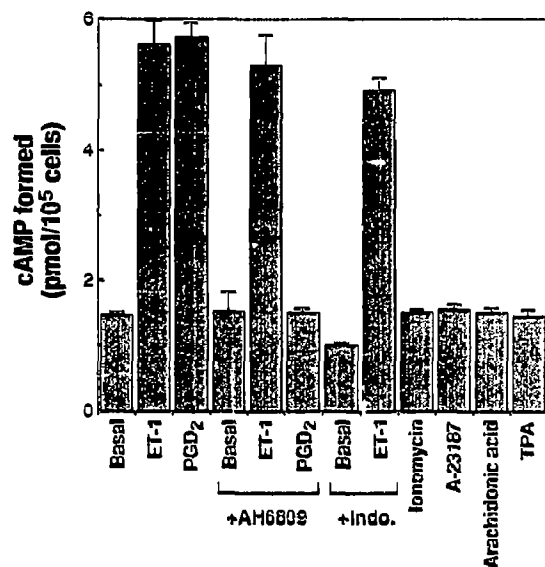


Fig. 3. Effects of various compounds on the increase in the cAMP content in EBTr cells. The cAMP content was determined after incubation for 10 min with 10 nM ET-1, 100 nM PGD₂, 1 μ M ionomycin, 1 μ M A-23187, 10 μ M arachidonic acid, or 100 nM TPA. AH6809 (100 μ M) or indomethacin (Indo.) (50 μ M) was added 10 min before various stimulation. The values are means $\pm$ S.D. of four determinations.

the sustained level for a further 10 min. A rapid rise in [³H]IP₃ formation was observed at 30 s, reaching a maximum level of 243% of the control at 2 min. [³H]IP₂ formation could be also detected at 30 s and increased continuously over a 10-min period. In contrast, [³H]IP₁ accumulation was detectable only after 2 min and then increased linearly. The cAMP content increased almost linearly after addition of ET-1 and reached to a plateau level of ~4-fold of the basal level by 5 min. As shown in Fig. 2B, the increases in [Ca²⁺]_i, IP₃ and cAMP were dose-dependent over the concentrations from 100 pM to 100 nM of ET-1. The half-maximal stimulation of IP₃ and cAMP contents occurred at ~500 pM, which is in the same order of the K_d value (114 pM) of ET_A receptors on EBTr cells. However, ET-3 did not affect [Ca²⁺]_i, PI hydrolysis, nor cAMP content in EBTr cells at concentrations up to 10 nM. At the maximum concentration (100 nM) of ET-3 used in this study, [Ca²⁺]_i and IP₃ increased only slightly while the cAMP content remained unchanged.

EBTr cells also have receptors for PGD₂ coupled to adenylate cyclase [13]. However, the ET-1-induced increase in the cAMP content was not at all affected by the presence of 50 μ M indomethacin, a cyclooxygenase inhibitor, nor of 100 μ M AH6809, an antagonist for the PGD₂-receptor (Fig. 3). The indomethacin-treatment inhibits the biosynthesis of PGs in various types of cells and AH6809 blocked completely the PGD₂-receptor-mediated increase in the cAMP content in EBTr cells [18]. Furthermore, the cAMP content in EBTr cells was

unchanged by incubation with Ca²⁺ ionophores (1 μ M ionomycin, 1 μ M A-23187), arachidonic acid (10 μ M) or phorbol ester (100 nM TPA) (Fig. 3). These results indicate that the cAMP formation in EBTr cells induced by ET-1 was not a secondary effect mediated by the increase in [Ca²⁺]_i and activation of protein kinase C resulting from PI hydrolysis.

The present study is the first to show that ET_A receptors are coupled to both PI hydrolysis and cAMP formation. In rat epididymal epithelial cells, ET-1 reportedly causes a rise in cAMP with no effect on [Ca²⁺]_i or IP contents [11]. In rat glomerular mesangial cells, ET-1 potentiates β -adrenergic-stimulated cAMP accumulation by a PGE₂-dependent mechanism, which is completely blocked by a 10-min preincubation with 10 μ M indomethacin [12]. Similar dual coupling to PI hydrolysis and cAMP formation has been demonstrated to be involved in the signal transduction of thyrotropin receptors [19], tachykinin receptors [20], and recombinant chimeric receptors of β -adrenergic and m1 muscarinic acetylcholine receptors [21]. On the other hand, secondary cAMP formation, which is mediated by PI hydrolysis, has been found in the case of m1 muscarinic acetylcholine receptors in human kidney cells and A9L cells [22,23].

It is likely that the ET_A receptor in EBTr cells is linked through two different GTP-binding proteins, G_p (Gq) and G_s, to two effector systems, phospholipase C and adenylate cyclase. Further evidence for the coupling of the ET_A receptor to GTP-binding proteins will be provided in studies with membranes of EBTr cells. Studies along these lines are now in progress.

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REFERENCES

- [1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) *Nature* 332, 411-415.
- [2] Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) *Proc. Natl. Acad. Sci. USA* 86, 2836-2867.
- [3] Rubanyi, G.M. and Botelho, L.H.P. (1991) *FASEB J.* 5, 2713-2720.
- [4] Sakurai, T., Yanagisawa, M., Takawa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) *Nature* 348, 732-735.
- [5] Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) *Nature* 348, 730-732.
- [6] Hosoda, K., Nakao, K., Arai, H., Suga, S., Ogawa, Y., Mukoyama, M., Shirakami, G., Saito, Y., Nakanishi, S. and Imura, H. (1991) *FEBS Lett.* 287, 23-26.
- [7] Ogawa, Y., Nakao, K., Arai, H., Nakagawa, O., Hosoda, K., Suga, S., Nakanishi, S. and Imura, H. (1991) *Biochem. Biophys. Res. Commun.* 178, 248-255.
- [8] Xuan, Y.-T., Watkins, W.D. and Whorton, A.R. (1991) *Am. J. Physiol.* 260, C492-C502.
- [9] Ladoux, A. and Frelin, C. (1991) *Biochem. Biophys. Res. Commun.* 180, 169-173.

- [10] Courand, P.-O., Durieu-Trautmann, O., Le-Nguyen, D., Martin, P., Glibert, F. and Strosberg, A.D. (1991) *Eur. J. Pharmacol.* 206, 191-198.
- [11] Wong, P.Y.D. and Huang, S.J. (1990) *Exp. Physiol.* 75, 321-337.
- [12] Simonson, M.S. and Dunn, M.J. (1990) *J. Clin. Invest.* 85, 790-797.
- [13] Sugama, K., Tanaka, T., Yokohama, H., Negishi, M., Hayashi, H., Ito, S. and Hayaishi, O. (1989) *Biochim. Biophys. Acta* 1011, 75-80.
- [14] Yokohama, H., Tanaka, T., Ito, S., Negishi, M., Hayashi, H. and Hayaishi, O. (1988) *J. Biol. Chem.* 263, 1119-1122.
- [15] Fujitani, Y., Oda, K., Takimoto, M., Inui, T., Okada, T. and Urade, Y. (1992) *FEBS Lett.* (in press).
- [16] Henry, P.J., Rigby, P.J., Self, G.J., Preuss, J.M., Goldie, R.G. (1990) *Br. J. Pharmacol.* 100, 786-792.
- [17] Sakata, K., Ozaki, H., Kwon, S.-C. and Karaki, H. (1989) *Br. J. Pharmacol.* 98, 483-492.
- [18] Ito, S., Okuda, E., Sugama, K., Negishi, M. and Hayaishi, O. (1990) *Br. J. Pharmacol.* 99, 13-14.
- [19] van Sande, J., Raspe, E., Perret, J., Lejeune, C., Maenhaut, C., Vassart, G. and Dumont, J.E. (1990) *Mol. Cell. Endocrinol.* 74, 6219-6224.
- [20] Nakajima, Y., Tsuchida, K., Negishi, M., Ito, S. and Nakanishi, S. (1992) *J. Biol. Chem.* (in press).
- [21] Wong, S. K-F., Parker, E.M. and Ross, E.M. (1990) *J. Biol. Chem.* 265, 6219-6224.
- [22] Peralta, E.G., Ashkenazi, A., Winslow, J.W., Ramachandran, J. and Capon, D.J. (1988) *Nature* 334, 434-437.
- [23] Felder, C.C., Kanterman, R.Y., Ma, A.L. and Axelrod, J. (1989) *J. Biol. Chem.* 264, 20356-20362.