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Desensitization of ET_A endothelin receptor-mediated negative chronotropic response in right atria – species difference and intracellular mechanisms

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1 Desensitization of ET_A endothelin receptor (ET_AR) was compared between the rat and guinea-pig with regard to negative chronotropic response (NC) in the right atria (RA).

2 ET-1 (100 nM) produced distinct NC in the presence of BQ788 (300 nM), and positive chronotropic response (PC) in the presence of BQ123 (1 μM) in both species, showing that ET_AR and ET_B endothelin receptor (ET_BR) mediate NC and PC, respectively.

3 Repetitive applications of ET-1 (50 nM) desensitized PC, and the second application only induced a strong NC in both species. Later applications of ET-1 produced virtually no response in the rat RA, whereas they produced BQ123-sensitive NCs repetitively in guinea-pig RA, exhibiting marked species difference in desensitization of ET_AR-mediated NC.

4 Pretreatment with staurosporine (100 nM) prevented desensitization of ET_AR in the rat RA altogether. However, phorbol 12-myristate 13-acetate (PMA, 300 nM) failed to induce, but rather hampered, desensitization of ET_AR.

5 Partial amino acid sequencing of ET_ARs, spanning from the 2nd through the 4th intracellular loops, revealed that all the potential Ser/Thr phosphorylation sites, including a protein kinase C (PKC) site, are conserved among guinea-pigs, rats, rabbits, bovines and humans.

6 In guinea pig RA, pretreatment with okadaic acid (1 $\mu g\,ml^{-1}$) and PMA did not facilitate desensitization of ET_AR whereas these agents successfully desensitized ET_AR during combined stimulation of β-adrenoceptor and ET_AR by isoproterenol (300 nM) and ET-1 (100 nM).

7 These results suggest that species differences in desensitization of ET_AR are not caused by differences in the site(s) of, but caused by differences in the environment for phosphorylation of the receptor. Desensitization of ET_AR appears to require phosphorylation of the receptor by PKC as well as a kinase stimulated by β-adrenoceptor activation.

Keywords: Desensitization; ET_A endothelin receptor; heart rate; negative chronotropic effect; amino acid sequence; phosphorylation; protein kinase C

Introduction

The endothelins (ETs) are a family of potent vasoactive peptides termed endothelin-1, -2 and -3 (ET-1, -2 and -3), all of which consist of 21 amino acid residues (Inoue *et al*., 1989). The first member of the family, ET-1, was initially described as a potent vasoconstrictor produced by vascular endothelial cells (Yanagisawa *et al*., 1988). ETs exert a wide variety of biological actions, mediated by specific cell surface receptors that belong to the superfamily of heptahelical G-protein coupled receptors (GPCRs; Masaki *et al*., 1992; Morello & Bouvier, 1996). To date, two subtypes of endothelin receptor, named ET_A and ET_B endothelin receptors (ET_AR and ET_BR), have been identified (Arai *et al*., 1990; Hosoda *et al*., 1991; Lin *et al*., 1991; Sakurai *et al*., 1990; Sakamoto *et al*., 1991). The ET_AR has an affinity rank order of ET-1 $\geqslant$ ET-2 $\gg$ ET-3, whereas the ET_BR exhibits similar affinities to all the three isopeptides. ET_AR and ET_BR have distinct cell-type/tissue distributions and thus mediate different physiological actions of ETs (Masaki *et al*., 1992). After full characterization of the ET_AR and ET_BR, a number of selective ligands have been developed. These include BQ123 and BQ788 which are, respectively, ET_AR- and ET_BR-selective antagonists (Ihara *et al*., 1992; Ishikawa *et al*., 1994), and BQ3020, an ET_BR-selective agonist (Kobayashi *et al*., 1993).

GPCRs play a key role in transmitting and regulating neuroendocrine stimuli through their three major functions: (1) ligand binding; (2) effector coupling and (3) desensitization. Recent recombinant DNA techniques have facilitated investigations into the structure-function relationships of ET receptors with particular regard to the selectivities of ligand binding and coupling to subclasses of G-proteins (Sakamoto *et al*., 1993; Krystek *et al*., 1994; Takagi *et al*., 1995; Koshimizu *et al*., 1995). For example, the 3rd intracellular loop of human ET_AR was found be important for the interaction with Gs protein when expressed in CHO cells (Takagi *et al*., 1995). Palmitoylation of one of the cysteine residues in the C-terminal tail of human ET_AR, which constructs the 4th intracellular loop in the receptor, was found to be necessary for activation of G_q protein (Horstmeyer *et al*., 1996). The C-terminal tail of human ET_AR was shown not to be responsible for its uncoupling from G_q protein (Cyr *et al*., 1993). Here one has to bear an important fact in mind, that all the three

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characteristics of GPCRs' functions are strongly influenced not only by their intrinsic structures and their ligands but also by the intracellular environment to which the receptors are exposed. For example, bovine and human ET_ARs couple with Gs protein when expressed in CHO cells (Aramori & Nakanishi 1992; Takagi *et al*., 1995), while ET_AR couples with G_i protein in hearts of guinea-pig (Ono *et al*., 1994; 1995a; James *et al*., 1994) and humans (Vogelsang *et al*., 1994).

It is well known that desensitization of ET receptors develops very rapidly and lasts much longer than in other GPCRs such as β-adrenergic and neurokinin A receptors (Cyr *et al*., 1993). However, the mechanisms underlying desensitization of ET receptors are only poorly understood. Following our previous discovery that ET_AR mediates NC in RA (Ono *et al*., 1994; 1995a, b), we encountered the surprising phenomenon that although NC in RA mediated by ET_AR desensitizes in the rat, it does not desensitize in the guinea-pig. This striking finding is reminiscent of the previous belief that ET receptors do not display homologous desensitization as evidenced by the uniquely long-lasting vasoconstricting activity of ETs. By making use of the guinea-pig RA as a model system that spontaneously lacks desensitization of ET_AR, we set out to investigate the molecular mechanisms for desensitization of ET_AR in terms of negative chronotropic response. The importance of phosphorylation of GPCRs for their desensitization has been elucidated recently (Sibley *et al*., 1987; Dohlman *et al*., 1991; Premont *et al*., 1995). ET_ARs of bovines, rats and humans carry in common a single potential phosphorylation site for PKC (XRXX**S**XRX) in their 3rd intracellular loops (Kemp & Pearson, 1990; Lin *et al*., 1991) whereas none of these carries Ser/Thr residue in its 1st intracellular loop (Arai *et al*., 1990; Hosoda *et al*., 1991; Lin *et al*., 1991), indicating structural importance of the region spanning from the 2nd through the 4th intracellular loops of ET_AR for its effector coupling and desensitization. We isolated and sequenced the corresponding region of cDNAs for guinea-pig ET_AR, as well as those for rabbit ET_AR for the reference, in order to search for a possible difference in amino acid sequence involved in phosphorylation, and investigated the role of PKC in desensitization of NC mediated by ET_AR. Preliminary results have been reported in abstract form (Ono *et al*., 1996).

Methods

Measurement of RA rate

Male Hartley guinea-pigs (350–450 g body weight) and Wistar rats (300–350 g body weight) were used. Under anaesthesia with pentobarbitone (50 mg kg^{-1}, i.p.), hearts were quickly removed and RA dissected out in modified Krebs'-Ringer solution of the following composition (mM); NaCl 113, KCl 4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and D-Glucose 5.5. The Krebs'-Ringer solution was maintained at 37°C and bubbled with 95% O_2–5% CO_2. RAs were suspended in organ baths containing 20 ml of the modified Krebs'-Ringer solution continuously bubbled with 95% O_2–5% CO_2, under the initial basic tensions of 0.5 g. After an equilibration period of at least 30 min, application of ET-1 was started. Spontaneous contractions of RA were detected with a force-displacement transducer (Nihon Kohden TB-611T, Tokyo, Japan) connected to an amplifier (Nihon Kohden AP-621G, Tokyo, Japan) and recorded on an Apple Macintosh computer using the MacLab® recording system (AD Instruments, Castle Hill, NSW, Australia). RA rate was counted 'on-line' using the software 'Chart'.

PMA, staurosporine and okadaic acid were dissolved in DMSO, and BQ788 in methanol, at concentrations 1000 fold greater than final desired concentrations in the organ bath. BQ123 was once dissolved in DMSO at a concentration of 0.5 mg/100 μl, and further diluted with distilled water to make a concentration 200 fold greater than the desired final concentration. ET-1, isoproterenol(ISO)·HCl and acetylcholine(ACh)·HCl were dissolved in distilled water, and BQ3020 once dissolved in methanol and diluted by 0.05% bovine serum albumin, at concentrations 200 fold greater than the desired final concentrations. Aliquots of these stock solutions were applied into the organ bath.

To record repeat-dependent desensitization of ET_AR, ET-1 was applied to the organ bath for 5 min, unless otherwise indicated, and the maximum magnitude of NC or PC attained during this period was measured over the basal RA rate. When ET-1 was applied in the presence of ISO, decline in RA rate from the rate in the presence of ISO was measured as the NC. After washing ET-1 throughly with Krebs'-Ringer solution, a 30 min period for recovery was given before the next application of ET-1 was started. Time for the pretreatments with BQ788, BQ123 or okadaic acid were 20 min, and that with PMA was 10 min. Staurosporine was added to the organ bath 20 min before the 1st application of ET-1 and was present throughout the experiment.

Determination of partial amino acid sequence of ET_A endothelin receptor

Ten μg of total RNA prepared from whole hearts of guinea-pigs or rabbits was reverse transcribed by Super Script II (GIBCO BRL, Gaithersburg, MD, U.S.A.) and subjected to RT-PCR. Guinea-pig or rabbit ET_AR cDNAs encompassing from the 2nd through the 4th intracellular loops were amplified by Thermal Cycler 480 (Perkin Elmer, Norwalk, CT, U.S.A.) with a primer set: 5′-TCTGCGCGCTAAGTGTTGACAGGT-3′ (upper primer) and 5′-TCATCAGGCTTTAGGACTGGTAAC-3′ (lower primer). The reactions were carried out under the following condition: amplified with 30 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 1 min, and extended at 72°C for 10 min. Based on the reading frame of cDNAs for ET_ARs of humans (Hosoda *et al*., 1991), rats (Lin *et al*., 1991) and bovines (Arai *et al*., 1990), the amino acid sequences for guinea-pig and rabbit ET_ARs were deduced from their cDNA sequences, which were directly determined on both strands by 373A DNA autosequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.). Multiple-alignment of the amino acid sequences for ET_ARs was performed by GeneWorks software (IntelliGenetics, Mountain View, CA, U.S.A.).

Materials

PMA and staurosporine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and okadaic acid from Wako Pure Chemical Industries Ltd. (Osaka, Japan). ET-1 was from Peptide Institute Co. (Ibaraki, Japan). BQ123 and BQ788 were from American Peptide Co. (Sunnyvale, CA, U.S.A.). BQ3020 was kindly provided by Drs M. Ihara and M. Yano of Banyu Pharmaceutical Co. (Tsukuba, Japan). All other chemicals were of analytical grade.

Statistical analyses

All values are expressed as means ± s.e.m.. Statistical analyses were performed by using the StatView software (Abacus Concepts, Inc. CA, U.S.A.). Repeat-dependency of the effect

of ET-1 was statistically approved by the repeated measure ANOVA. Statistical difference between two groups was tested by ANOVA and Student's *t*-test. A value of $P<0.05$ was considered to be statistically significant.

Results

Distinct roles of ET_AR and ET_BR in chronotropic responses induced by ET-1

ET-1 (100 nM) induced both NC and PC in spontaneously beating RA of the rat and guinea-pig (Figure 1a and b: left panels), producing biphasic and triphasic chronotropic profiles in the rat and guinea-pig, respectively. In the rat, ET-1 usually induced an initial rapid and profound decline of RA rate, followed by a slow and steady increase (Figure 1a and b: top left panels). In guinea-pig RA, on the other hand, ET-1 caused an initial decline in RA rate, which was relatively small in size, a following increase in RA rate which was relatively fast and a slow declining phase once the peak response was reached (Figure 1a and b: bottom left panels). BQ788 (300 nM), an ET_BR-selective antagonist (Ishikawa *et al*., 1994), eliminated the positive chronotropic component such that ET-1 produced a profound NC both in the rat and guinea-pig RAs in the presence of BQ788 (Figure 1a, b (centre panels) and c). On the other hand, BQ123 (1 μM), an ET_AR-selective antagonist (Ihara *et al*., 1992), almost completely blocked the initial negative chronotropic component in RAs from both the rat and guinea-pig (Figure 1a, b (right panels) and c; Ono *et al*., 1995a) and significantly enhanced the magnitude of PC (Figure 1c).

Repetitive applications of ET-1 in guinea-pig and rat RAs

Desensitizations of both NC and PC, which were found to be mediated by ET_AR and ET_BR respectively, were examined by repetitive applications of ET-1 (50 nM) following extensive washout and a 30 min recovery period. ET-1 did not elicit PC at the second application any more, either in the rat or guinea-pig RA, showing that ET_BR-mediated PC desensitized easily (Figures 2 and 3). Desensitization of the ET-1 response did not affect the strong positive chronotropic effect of ISO, indicating that the peptide did not heterologously desensitize β-adrenoceptor-mediated PC (Figures 2 and 3).

The second application of ET-1 caused a profound NC in both species. This NC, which we previously found to be mediated by ET_AR (Ono *et al*., 1994; 1995a,b), exhibited marked species difference in its repeat-dependency. In the rat RA, NC induced by ET-1 desensitized very easily (Figures 2 and 3a). In clear contrast, repetitive applications of ET-1 to guinea-pig RA invariably produced a marked decline in RA rate, which even tended to be augmented initially with the repetitive applications (Figures 2 and 3a). The magnitude of

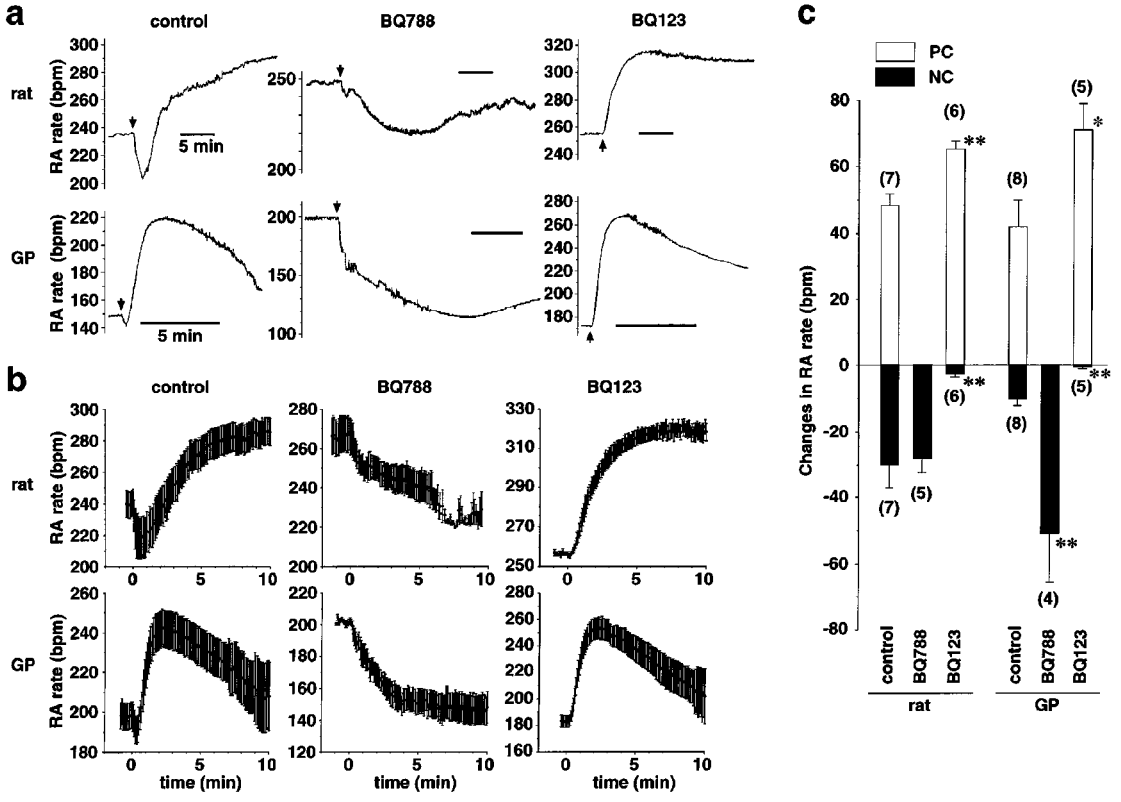

Figure 1 Distinct roles of ET_AR and ET_BR in the regulation of RA rate in rats and guinea-pigs. (a, b) Typical recordings (a) and mean time courses (b) of RA rate responses (bpm; beats per min) induced by ET-1 (100 nM) in the rat (upper panels) and guinea-pig (GP, lower panels) RAs. ET-1 was applied at points indicated by arrows in (a) or at time = 0 in (b), in the absence of antagonists (left), in the presence of BQ788 (ET_BR-selective antagonist, 300 nM, centre) and in the presence of BQ123 (ET_AR-selective antagonist, 1 μM, right). Horizontal bars in (a) indicate 5 min. In (b) data points were obtained every 5 s from five to eight independent experiments for each panel. Vertical bars denote s.e.m. (c) Mean of maximum changes in RA rate induced by ET-1 (100 nM) attained in 10 min. Vertical bars denote s.e.m.. In parentheses are the numbers of experiments. $*P<0.05$, $**P<0.01$; significantly different from the control values in each species.

NC in response to the 3rd application of ET-1 was almost comparable to that induced by ET-1 in the presence of BQ788 as measured at 5 min after the application of the peptide (Figures 1a, b and 3a). This desensitization-resistant NC in the guinea-pig RA was almost completely blocked by BQ123 (1 μM, Figures 2 and 3b). The repeated measure ANOVA indicated that the magnitude of NC induced by ET-1 was significantly repeat-dependent both in the rat ($P<0.01$) and guinea-pig ($P<0.001$) RAs (Figure 3a). However, the time-course of the decline in NC in guinea-pig RAs, along with the repeated applications of ET-1, was significantly slower than that in the rat RAs ($P<0.05$). In fact, NCs in response to the 3rd through the 8th applications of ET-1 were significantly larger in the guinea-pig RA than in the rat RA (Figure 3a).

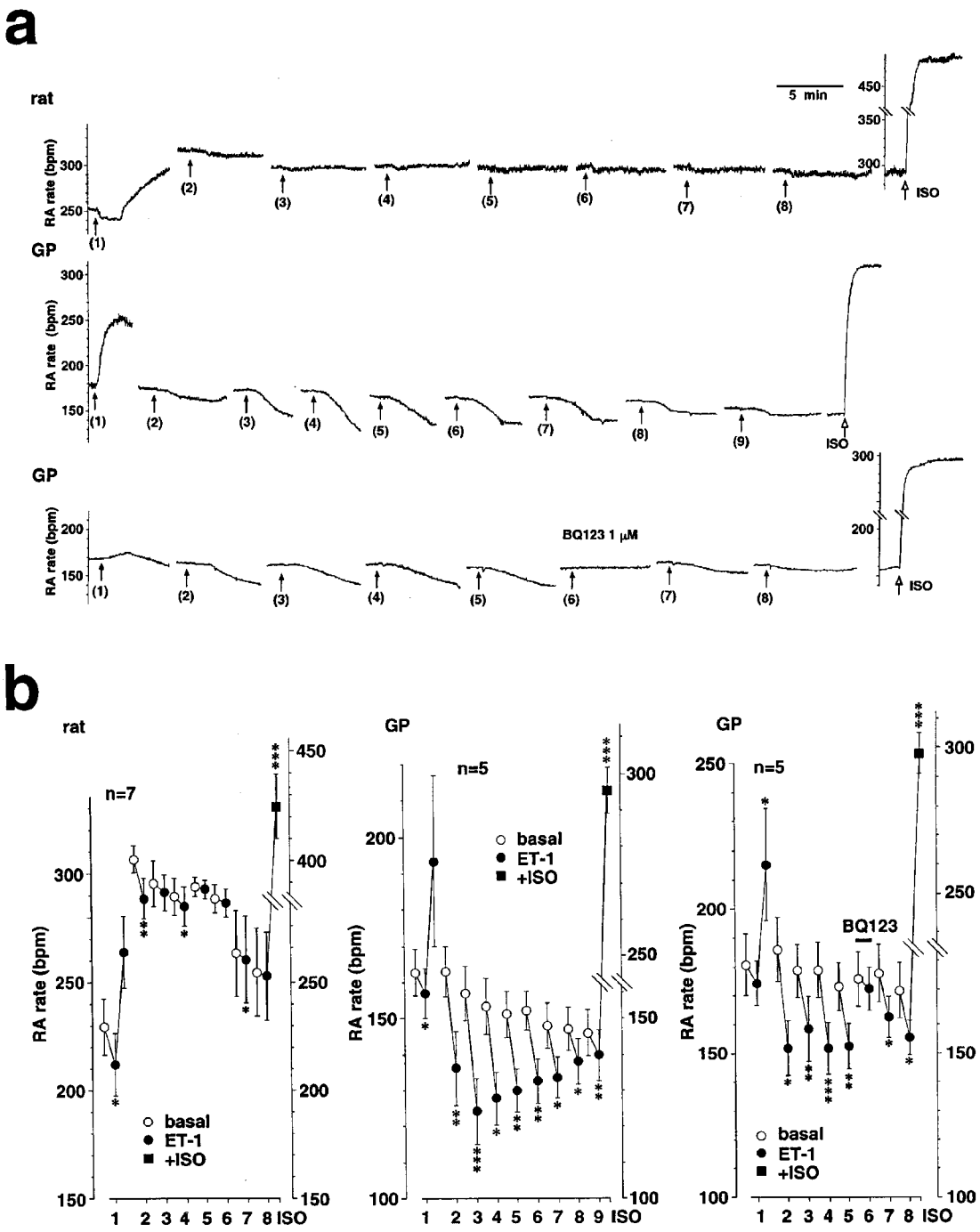

Figure 2 Species difference in the desensitization of chronotropic responses induced by repetitive applications of ET-1 (50 nM). (a) Typical recordings of RA rate in the rat (top panel) and guinea-pig (GP, middle and bottom panels), representative of five to seven independent experiments. In the bottom panel, BQ123 (1 μM) was present during the 6th application of ET-1. The numbers in parentheses denote application orders of ET-1. (b) Mean RA rate before (basal) and after the application of ET-1 and ET-1 plus ISO. Vertical bars denote s.e.m.. Numbers along the abscissas indicate application orders of ET-1. $^*P<0.05$, $^{**}P<0.01$ (paired t-test); significantly different from each basal value.

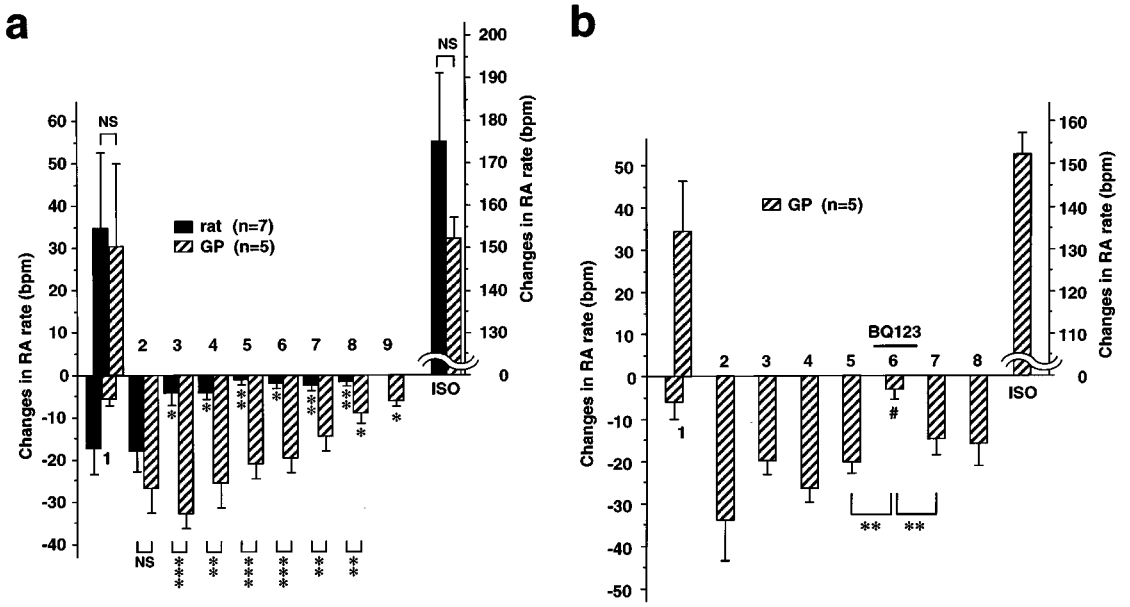

Figure 3 Repeat-dependency of chronotropic effects of ET-1 (50 nM) in the rat and guinea-pig RAs. Means ± s.e.m. of maximum changes in RA rate attained in 5 min after each application of ET-1 (50 nM), obtained from five to seven independent experiments as illustrated in Figure 2, are shown. (a) Species differences in the development of desensitization in ET_AR-mediated NC. $*P<0.05$, $**P<0.01$, and $***P<0.001$. Symbols below the bars denote statistical significance against the second response. Symbols on the bottom indicate significant difference between the species. NS; not significantly different. (b) Block by BQ123 (1 μM) of the desensitization-resistant NC in guinea-pig RAs. BQ123 was pretreated only for the 6th application of ET-1. $**P<0.01$. $\#P<0.01$, compared with the 6th response in guinea-pig RA shown in (a).

Long-term treatment with ET-1

Treatment of guinea pig RA with ET-1 for 30 min still failed to cause desensitization of the ET_AR-mediated NC (Figure 4). The initial application of ET-1 (50 nM) induced a typical chronotropic response consisting of two and three phases in the rat and guinea-pig RAs, respectively (Figure 4a, b, Ono *et al*., 1995a); initial rapid decline, following fast increase and then, in guinea-pig, a gradual decline in RA rate. During the continuous presence of the peptide for 30 min, RA rate was maintained almost constant in the rat RA, whereas it declined progressively in the guinea-pig RA, reaching almost the basal value recorded before the application of ET-1. After washing out the peptide, a minimum recovery period of 30 min failed to restore RA rate to the basal value in the rat RA but it restored the RA rate back to the basal level in the guinea-pig RA. Although the second application of ET-1 (50 nM) caused a detectable and significant NC in RAs of both species (Figure 4a and b), the magnitude of NC in the guinea-pig RA, which was almost comparable to that after the pretreatment with ET-1 for 5 min (Figure 3a), was significantly larger than that in the rat RA (Figure 4a and c).

Effect of BQ3020 treatment on ET-1 response

We further tested whether stimulation of ET_BR desensitizes ET_AR heterologously. BQ3020 (30 nM), an ET_BR-selective agonist, induced a slight but sustained increase in RA rate in the rat RA. Eight minutes after application of BQ3020, a higher concentration of BQ3020 (300 nM) did not cause any further response (data not shown), whereas addition of ET-1 (300 nM) in the continuous presence of BQ3020 caused a striking NC without accompanying a positive chronotropic component (Figure 5), showing that stimulation of ET_BR does not heterologously desensitize the ET_AR-mediated NC.

Treatment of the rat RA with staurosporine or PMA

A possibility was tested that phosphorylation of ET_AR by PKC might be involved in desensitization of ET_AR in the rat RA. Pretreatment with staurosporine at a PKC-selective concentration (100 nM; Tamaoki *et al*., 1986; Freedman *et al*., 1997) was found to effectively prevent the development of desensitization of ET_AR in the rat RA (Figure 6a and b); in the continuous presence of staurosporine, repeated applications of ET-1 (50 nM) successfully produced sustained NCs in the rat RA (Figure 6a and b), which was in contrast to the results obtained in the absence of staurosporine (Figures 2 and 3). Repeated measure ANOVA indicated that the magnitude of NC did not vary significantly with time from the 2nd to the 6th applications of ET-1 ($P \geqslant 0.05$). Thus, PKC was found to be necessary for desensitization of ET_AR.

Next, we examined a possibility that stimulation of PKC could desensitize ET_AR in the rat RA. In this particular experiment, BQ788 (300 nM) was present throughout the study to block ET_BR, for the purpose of testing (1) whether NC has already desensitized before the first application of ET-1 and (2) whether selective stimulation of ET_AR desensitizes the following NCs to ET-1. Pretreatment of the rat RA with PMA (300 nM), a selective activator of PKC (Manger *et al*., 1986; Kikkawa *et al*., 1989), however, did not cause desensitization of ET_AR (Figure 6c and d); single pretreatment with PMA not only failed to desensitize NC in response to the first application of ET-1, but also prevented desensitization of the following ET_AR-mediated NCs, as evidenced by repeatable NCs in response to ET-1 (Figure 6c and d). Repeated measure ANOVA indicated there is no significant time-dependent decay

in the magnitude of NC from the 1st through the 6th applications of ET-1 ($P \geqslant = 0.05$).

Partial amino acid sequence of guinea-pig ET_AR

Figure 7 illustrates the multiple alignment of amino acid sequences of ET_ARs from humans, bovines, rats, guinea-pigs (DDBJ accession No. D83953) and rabbits (DDBJ accession No. D83954). It was found that all the Ser/Thr residues, which are potential sites for phosphorylation, are conserved among these species (Figure 7). A single amino acid substitution, Arginine[306] in guinea-pig versus Lysine[306] in all the other species, was found near the PKC phosphorylation site, located at the 3rd intracellular loop. A NPXX(X)Y motif in the 7th transmembrane domain, a potential sequence involved in internalization of heptahelical GPCRs (Barak *et al*., 1994), was also completely conserved in all the species (Figure 7).

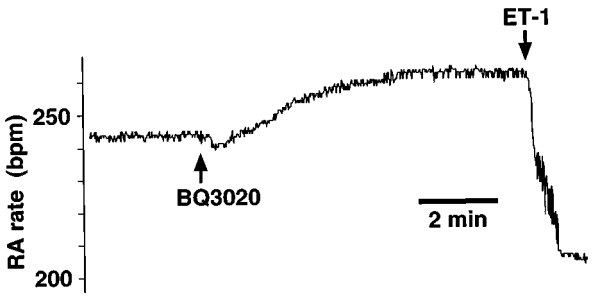

Figure 5 Effect of selective stimulation of ET_BR on the ET-1-induced chronotropic response in the rat RA. A representative trace of RA rate response, obtained from four independent experiments, is illustrated. After application of BQ3020 (30 nM) for 8 min, ET-1 (300 nM) was added to the bath, which caused sudden decline in RA rate by 35.8 ± 10.3 b.p.m. ($n = 4$) during the first 5 min.

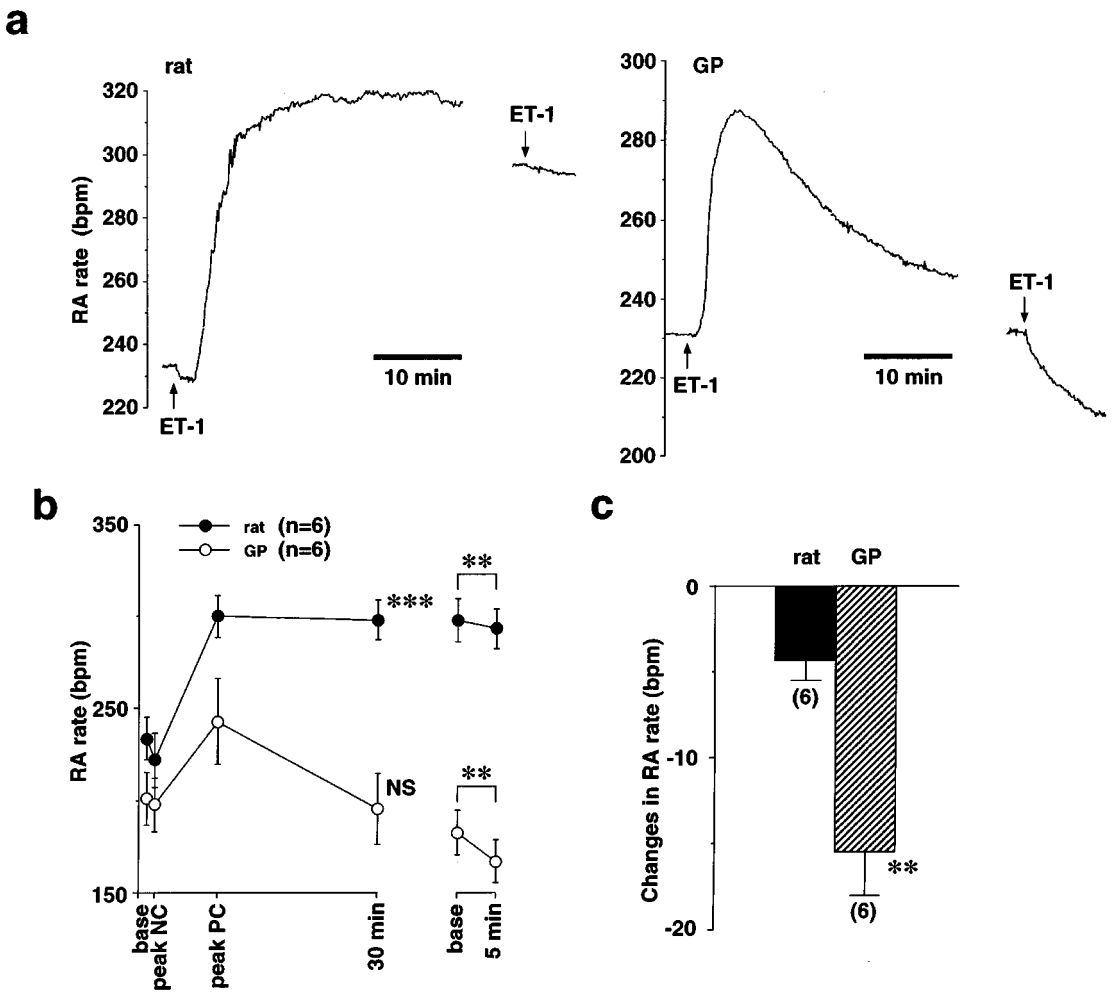

Figure 4 Species difference in the development of desensitization of ET_AR after a long-term treatment with ET-1. (a) Typical recordings of RA rate in the rat (left) and the guinea-pig (right) RAs. Arrows indicate application of ET-1 (50 nM). The duration of the treatment with ET-1 was 30 min for the first and 5 min for the second applications, respectively. (b) Summaries of the time courses of RA rate in the rat and guinea-pig RAs. Mean RA rates and s.e.m., obtained from six independent experiments in each species, are illustrated. base; basal RA rate before the application of ET-1. peak NC, peak PC; maximum NC and maximum PC attained in 30 min after the application of ET-1. $***P < 0.001$, compared with the basal RA rate (paired t-test). NS; not significantly different. $**P < 0.01$, by paired t-test. (c) Mean changes in RA rate induced by the second application of ET-1, obtained from six independent experiments. Vertical bars denote s.e.m.. $**P < 0.01$, compared with rat.

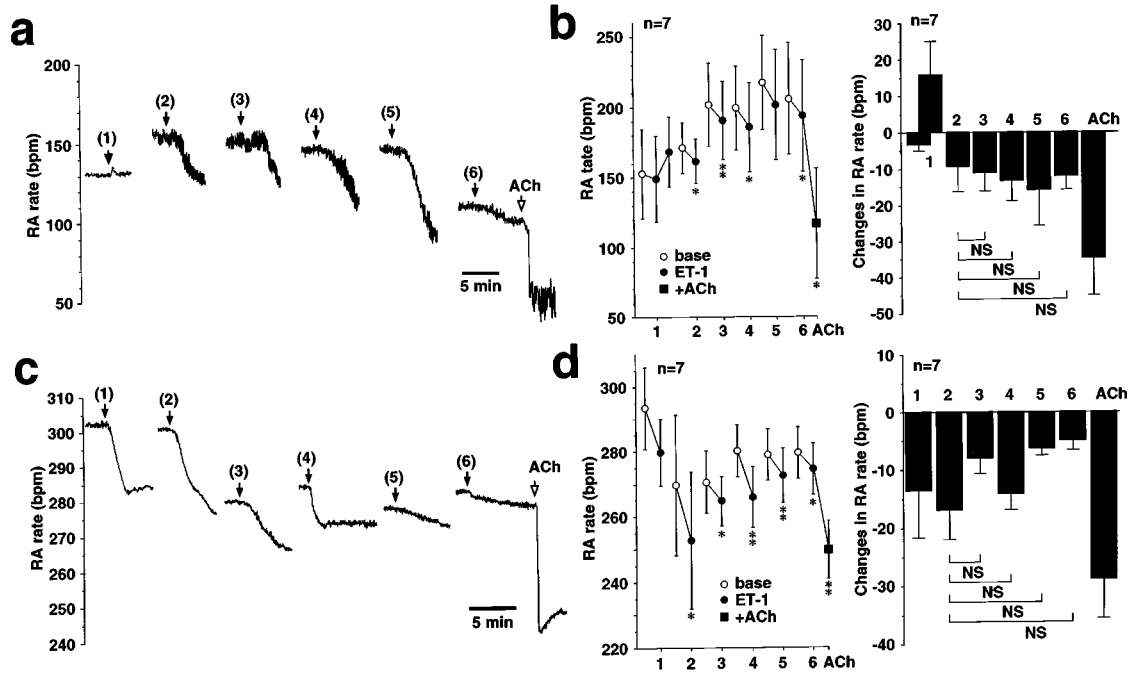

Figure 6 Involvement of PKC in the desensitization of ET_AR in the rat RA. (a, b) Prevention by staurosporine (100 nM) of the development of desensitization of ET_AR-mediated NC. (c, d) Effect of pretreatment with PMA (300 nM) on the desensitization of ET_AR-mediated NC. PMA was pretreated only before and during the 1st application of ET-1. (a, c) Typical RA rate responses to the repeated applications of ET-1 (50 nM), for which numbers in parentheses indicate application orders. Concentration of ACh was 3 μM. (b, d) Summaries of the RA rate responses to ET-1, obtained from seven independent experiments, as illustrated in (a) and (c) respectively. Left panels; means ± s.e.m. of RA rate before (base) and after the application of ET-1 and ET-1 plus ACh. $*P<0.05$, $**P<0.01$, compared with each basal value. Right panels; means ± s.e.m. of the maximum changes in RA rate induced by ET-1 or ACh in 5 min. Numbers along the abscissas indicate the application orders of ET-1. NS, not significantly different.

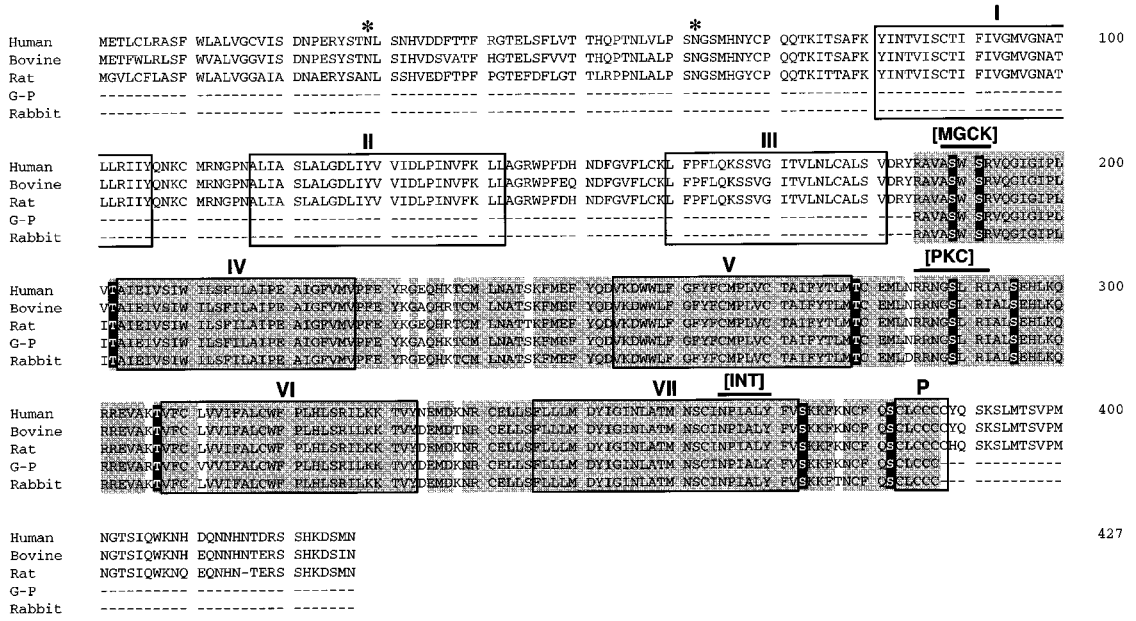

Figure 7 Multiple alignment of deduced amino acid sequences for ET_ARs of various species. Partial amino acid sequences of guinea-pig and rabbit ET_ARs, spanning from the 2nd through the 4th intracellular loops, were determined (see Methods) and compared with those of human, bovine and rat ET_ARs. Boxes numbered as I–VII indicate putative transmembrane domains. Shaded letters denote amino acid residues conserved among all the species. Note that all the Ser/Thr residues, indicated by reversed letters, are conserved among all the species. P; putative palmitoylation site(s) (Horstmeyer *et al.*, 1996). *Putative *N*-glycosylation sites. [MGCK], [PKC]; consensus sequences for phosphorylation by mammary gland casein kinase and PKC, respectively (Kemp & Pearson, 1990). [INT]; a putative motif for internalization of heptahelical GPCRs (Barak *et al.*, 1994). The nucleotide sequences of cDNAs for guinea-pig and rabbit ET_ARs were deposited in DDBJ under accession numbers D83953 and D83954, respectively.

Treatment with okadaic acid and phorbol ester in guinea-pig RA

We further tested whether alteration in phosphorylation state of ET_AR could facilitate desensitization of the receptor in guinea-pig RA. Pretreatment of guinea-pig RA with okadaic acid (1 μg ml^{-1}), an inhibitor of protein phosphatases (Cohen *et al*., 1989), and PMA (300 nM), a selective activator of PKC, did not affect the non-desensitizing property of ET_AR-mediated NC in guinea-pig RA (Figure 8a and b). Even after pretreatment with these two agents, ET-1 (50 nM) repeatedly induced NCs without developing desensitization. Repeated measure ANOVA indicated that the magnitude of NC did not change significantly with the repeated applications of ET-1 (Figure 8b, $P \geqslant 0.05$). NC induced by ACh was also not affected by pretreatment with PMA (Figure 8a and b).

We have recently reported that ET-1 exerts NC through stimulation of ET_AR in the presence of ISO (Ono *et al*., 1994; 1995a,b) which is known to activate cyclic adenosine 3′, 5′-monophosphate-dependent protein kinase (PKA) but not PKC (Stiles *et al*., 1984; Bylund *et al*., 1994). This NC induced by ET-1 during stimulation of β-adrenoceptors with ISO (300 nM) was also reproducible without the development of desensitization in the guinea-pig RA (Figure 8c and d). In this setting, however, pretreatment with okadaic acid (1 μg ml^{-1}) and PMA (300 nM) caused desensitization of ET_AR-mediated NC (Figure 8c and d). The NC induced by ET-1 was lost along with repetitive applications of ISO plus ET-1 whereas NC in response to ACh was not affected, which indicated establishment of desensitization of ET_AR-mediated NC in the guinea-pig RA. Repeated measure ANOVA indicated that the magnitude of NC significantly decayed with the repeated applications of ET-1 (Figure 8d, $P<0.05$).

Discussion

We have previously found that ET_AR mediates NC through a pertussis toxin-sensitive G_i protein (Ono *et al*., 1994; 1995a,b). In the present study, we have found that ET-1 induced NC in the presence of BQ788, whereas it produced PC in the presence of BQ123 in both rat and guinea-pig RAs. These results clearly indicated that ET_AR and ET_BR distinctly mediate negative and positive chronotropic effects of ET-1, respectively, in both species. While the ET_BR-mediated PC rapidly desensitized in both species, desensitization of NC mediated by ET_AR exhibited a marked species difference; ET_AR homologously desensitized in the rat RA, whereas it was found to be resistant to homologous desensitization in guinea-pig RA. Although we have not determined the subtype of G-protein responsible for the ET_BR-mediated PC in the present study, it is obvious that stimulation of ET_BR does not desensitize ET_AR heterologously. This is the first report to demonstrate an ET_AR-mediated response which lacks homologous desensitization.

The ET receptors, ET_AR and ET_BR, belong to a superfamily of GPCRs (Masaki *et al*., 1992; Morello & Bouvier, 1996), desensitization of which involves uncoupling from

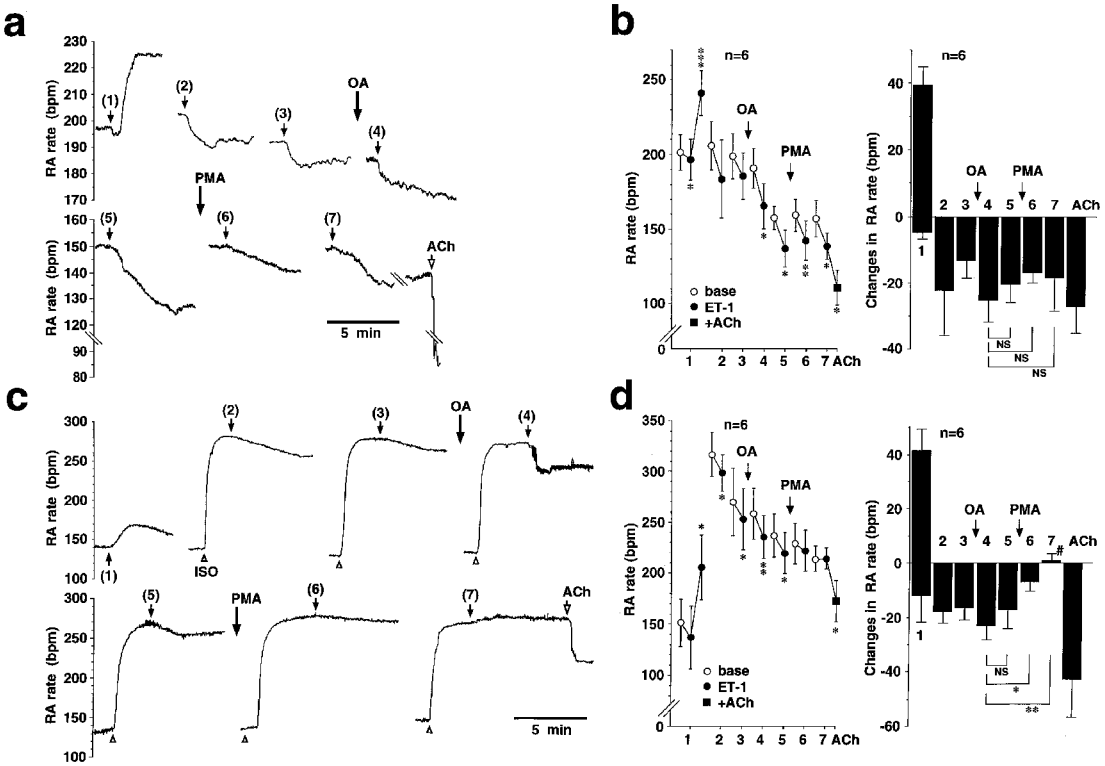

Figure 8 Attempts to desensitize ET_AR-mediated NC in guinea-pig RA by okadaic acid (OA, 1 μg ml^{-1}) and PMA (300 nM). Single application of ET-1 (50 nM, a and b) or combined applications of ISO (300 nM) plus ET-1 (50 nM, c and d) were repeated. (a, c) Typical recordings of RA rate responses. The numbers in parentheses indicate application orders of ET-1. ACh (3 μM) was added after the 7th application of ET-1. In (c), open triangles denote application of ISO. (b, d) Summaries of NCs induced by repeated applications of ET-1, obtained from six independent experiments, as illustrated in (a) and (c), respectively. Numbers along the abscissas indicate application orders of ET-1. Left panels; means ± s.e.m. of RA rate before (base) and after the application of ET-1 and ET-1 plus ACh. *$P<0.05$ and **$P<0.01$ (paired t-test), compared with each basal value. Right panels; means ± s.e.m. of the changes in RA rate induced by ET-1 or ACh in 5 min. *$P<0.05$, **$P<0.01$. #$P<0.05$, compared with the value at the corresponding time in (b). NS; not significantly different.

G-proteins followed by sequestration from plasma membrane and/or down-regulation (including degradation; Premont *et al*., 1995; Morello & Bouvier, 1996). Recent studies have revealed the importance of phosphorylation of GPCRs for the uncoupling and sequestration of GPCRs. Two major categories of enzymes are known to be involved in these processes: (1) the second messenger-regulated protein kinases such as PKC and PKA, which phosphorylate GPCRs in an agonist-independent manner and therefore lead to heterologous, as well as homologous, desensitization, and (2) G-protein coupled receptor kinases (GRKs) which phosphorylate only agonist-occupied GPCRs, causing homologous or receptor-specific desensitization (Sibley *et al*., 1987; Dohlman *et al*., 1991; Lefkowitz, 1993; Premont *et al*., 1995). The species differences in the desensitization of ET_AR found in the present study provided useful native organ systems in which to examine intracellular mechanisms involved in the phosphorylation of ET_AR which is both necessary for, and sufficient to cause desensitization of ET_AR. The rat RA showed rapid desensitization of ET_AR, providing a method in which to search for pathways necessary for desensitization of the receptor. The guinea pig RA did not display desensitization of ET_AR, providing us a reconstitution system in which to develop desensitization of ET_AR.

First, we examined the intracellular pathways leading to desensitization of ET_AR, by using the rat RA as a model of normal ET_AR desensitization. Phosphorylation of GPCRs by protein kinases such as PKC or GRK is known to play a crucial role in receptor desensitization (Sibley *et al*., 1987; Dohlman *et al*., 1991; Lefkowitz, 1993; Premont *et al*., 1995). Based on the fact that ET_ARs from bovine, human and rat origin have a potential phosphorylation site for PKC in the third intracellular loop (Arai *et al*., 1990; Hosoda *et al*., 1991; Lin *et al*., 1991), we tested the contribution of PKC to desensitization of ET_AR. Pretreatment of the rat RA with staurosporine (100 nM), a selective PKC inhibitor (Tamaoki *et al*., 1986; Freedman *et al*., 1997), prevented development of desensitization of ET_AR during repetitive applications of ET-1. Stimulation of PKC by PMA failed, however, to desensitize ET_AR in the rat RA; PMA rather prevented desensitization of the receptor, probably as a consequence of auto-phosphorylation and down-regulation of PKC (Darbon *et al*., 1987; Kikkawa *et al*., 1989). Thus, staurosporine most likely exerted its action through selective inhibition of PKC, but not through a non-selective action on other protein kinases such as GRKs (Lefkowitz, 1993; Premont *et al*., 1995; Freedman *et al*., 1997).

Activation of ET_AR produces NC by coupling with G_i proteins (Ono *et al*., 1994; 1995a,b; Vogelsang *et al*., 1994), which is also phosphorylated and inactivated by PKC (Katada *et al*., 1985). We assume, however, that the target for phosphorylation by PKC is not G_i protein but ET_AR, since PMA did not abolish ACh-induced NC, as discussed below. These results indicate that phosphorylation of ET_AR by PKC is necessary for desensitization of ET_AR-mediated NC. It is likely that activation of PKC through G_q protein/phospholipase C (PLC) intra-signaling cascade, which is known to be stimulated by ET_AR itself in the heart (Hilal-Dandan *et al*., 1992; Vogelsang *et al*., 1994), is responsible for homologous desensitization of ET_AR-mediated G_i protein pathway. Similar PKC-dependency of GPCR desensitization has been reported for α_1- (Leeb-Lundberg *et al*., 1985) and β_2- (Pitcher *et al*., 1992; Yuan *et al*., 1994) adrenoceptors, AT_{1B} (Tang *et al*., 1995), 5-HT_{1A} (Raymond 1991), 5-HT_2 (Weng *et al*., 1994), LTD_4 (Vegesna *et al*., 1988) and V_1 vasopressin receptors (Gallo Payet *et al*., 1991).

A requirement for phosphorylation of ET_AR by PKC in the genesis of desensitization prompted us to search for possible amino acid substitutions in guinea-pig ET_AR which did not desensitize, within or surrounding the potential recognition sequence for PKC. This region is conserved among ET_ARs from bovine, human and the rat origin (Arai *et al*., 1990; Hosoda *et al*., 1991; Lin *et al*., 1991). Sequencing of cDNA for guinea-pig and rabbit ET_ARs revealed, however, that not only the potential PKC sequence but also all the other Ser/Thr residues are totally conserved widely among all the species tested including the guinea-pig, implying the physiological relevance of this sequence. Furthermore, a NPXX(X)Y motif in the seventh transmembrane domain of ET_AR, a potential sequence for agonist-induced sequestration of GPCRs (Barak *et al*., 1994), was also well conserved in ET_ARs of all the species. In the present study measuring RA rate, treatment with okadaic acid, a potent phosphatase inhibitor (Cohen *et al*., 1989), did not promote desensitization of ET_AR in guinea-pig RA. These results suggest that phosphorylation of ET_AR at the PKC site is inefficient or inadequate in guinea-pig RA to induce desensitization, probably due to a difference in its intracellular environment for phosphorylation of the receptor at the PKC recognition site. Here, the possibility of augmented dephosphorylation of ET_AR in the guinea-pig RA was unlikely to be the cause of such difference. A defect in sequestration of the receptor was also unlikely to be the cause of the lack of desensitization of ET_AR in guinea-pig RA. Therefore, the lack of desensitization of ET_AR-mediated NC in guinea-pig RA is most probably due to impaired phosphorylation of ET_AR by PKC and/or other protein kinases such as GRKs. The amino acid substitution from $Lysine^{306}$ to $Arginine^{306}$ near the PKC site in the 3rd intracellular loop of guinea-pig ET_AR might cause a structural hindrance for the access of PKC to the potential phosphorylation site of guinea-pig ET_AR.

A similar PKC-dependency of ET_AR desensitization has recently been reported in *Xenopus* oocytes co-expressing human neurokinin A (NKA) receptors and human ET_AR (Cyr *et al*., 1996). By examining chloride current stimulated by intracellular calcium, the release of which is coupled to G_q/PLC cascade, they found that stimulation of the NKA receptors desensitized ET_AR through activation of PLC. Together with these findings, our present results indicate that phosphorylation of ET_AR by PKC is essential for desensitization of the receptor in terms of both G_i- and G_q-pathways, both of which are coupled to ET_AR in cardiac myocytes (Hilal-Dandan *et al*., 1992; Ono *et al*., 1994; 1995a; James *et al*., 1994; Vogelsang *et al*., 1994).

Next, we investigated the signaling pathway(s), activation of which is (are) sufficient to induce desensitization of ET_AR, by using guinea-pig RA which can be regarded as a naturally occurring reconstituting system for desensitizing the receptor. PMA alone failed to facilitate desensitization of ET_AR-mediated NC in the guinea-pig RA. ET-1-induced NCs did not desensitize in the presence of ISO. In contrast, ET-1 desensitized the ET_AR-mediated NC in the combined stimulation of PKC and β-adrenoceptors by PMA and ISO, which indicated that stimulation of PKC and β-adrenoceptors was sufficient to facilitate desensitization of ET_AR. Stimulation of PKC is known to phosphorylate and consequently inactivate G_i protein (Katada *et al*., 1985). However, this is unlikely to be involved in the facilitation of the desensitization of ET_AR by PMA, since PMA by itself failed to attenuate NCs induced by both ET-1 and ACh, which are mediated by G_i protein (Endoh *et al*., 1985; Boyer *et al*., 1986; Brown, 1990; Ono *et al*., 1994; 1995a). Therefore, the major target of

phosphorylation by PKC was suggested not to be G_i protein but to be ET_AR itself.

On the other hand, the β-adrenoceptor is known to activate PKA, as well as GRKs which phosphorylate and desensitize agonist-bound GPCRs (Benovic *et al*., 1986; Lefkowitz, 1993; Premont *et al*., 1995). However, PKA phosphorylation site does not exist in either ET_AR (present observation) or G_i protein (Katada *et al*., 1985). Moreover, ISO did not attenuate ACh-induced NC which is mediated by G_i (Endoh *et al*., 1985; Boyer *et al*., 1986; Brown, 1990). Therefore, phosphorylation of ET_AR or G_i protein by PKA is also unlikely to be the mechanism underlying the facilitation of the desensitization of ET_AR by β-adrenoceptor stimulation in guinea-pig RA, which raises the possibility that GRK is involved in the facilitation of desensitization of ET_AR by ISO. In our observation, pretreatment with ISO plus PMA did not attenuate ET_AR-mediated NC in response to the first application of ET-1 (data not shown), probably because GRK can desensitize ET_AR only when it is occupied by its agonist ET-1 (Lefkowitz, 1993; Premont *et al*., 1995). Upon these considerations, the facilitation by β-adrenoceptor stimulation of the desensitization of ET_AR seems to be most likely mediated by recruitment of GRKs. We assume that the combination of PKC and GRK are necessary for and sufficient to the development of ET_AR desensitization, in terms of NC.

The effect of ET-1 on guinea-pig RA was initially described as PC which exhibited extensive tachyphylaxis (Ishikawa *et al*., 1988); the chronotropic response obviously altered from PC into NC following repetitive applications of the peptide. In so far as PC and NC to ET-1 are mediated, respectively, by ET_BR and ET_AR, and since ET_BR does not desensitize ET_AR heterologously as shown in the present study, repeat-dependent changes in the relative contributions to the regulation of RA rate of the two receptor pathways, desensitization-sensitive ET_BR and desensitization-resistant ET_AR, would quite reasonably explain this repeat-dependent 'reversal' of the chronotropic effect of ET-1. ET_AR has been reported to be coupled with the PLC/PKC pathway through a PTX-insensitive G_q protein in cardiac myocytes (Hilal-Dandan *et al*., 1992; Vogelsang *et al*., 1994). Functional coupling of ET_AR with G_q/PLC has been reported to be regulated by palmitoylation of the receptor at its carboxy terminal tail; loss of palmitoylation hampers activation of PLC/PKC pathway *via* ET_AR (Horstmeyer *et al*., 1996). Weak or impaired coupling of ET_AR with G_q/PLC pathway might cause insufficient stimulation of PKC, rendering ET_AR resistant to desensitization, as observed in guinea-pig RA in the present study. Alteration in PKC activity during postnatal development of animals (Puceat *et al*., 1994; Clerk *et al*., 1995) might also dramatically affect the susceptibility of ET_AR to desensitization. Dynamic changes in such intracellular environments for ET_AR may affect desensitization of the receptors, which in turn could modulate not only quantitative but also qualitative regulations of cardiac functions by ETs.

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