Dual effects of endothelins on the muscarinic K⁺ current in guinea pig atrial cells

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Yamaguchi, Hiroshi, Naoya Sakamoto, Yasuhide Watanabe, Toshihiro Saito, Yoshiaki Masuda, and Haruaki Nakaya. Dual effects of endothelins on the muscarinic K⁺ current in guinea pig atrial cells. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1745–H1753, 1997.—Effects of endothelins (ETs) on the acetylcholine receptor-operated K⁺ current (IKACh) were examined in isolated guinea pig atrial cells using patch-clamp techniques. ET-1 or ET-3 produced a transient activation of IKACh in atrial cells held at −40 mV. When IKACh was preactivated by 1 µM carbachol, however, both ETs produced a transient potentiation followed by a sustained inhibition of the current. When IKACh was maximally activated by 10 µM carbachol or 100 µM adenosine, these ETs produced only a sustained inhibition of the IKACh. Their inhibitory effects on the preactivated IKACh were concentration dependent, and the half-maximal effective concentrations were 314 pM for ET-1 and 1.13 nM for ET-3. The inhibitory effect of ET-1 was antagonized by BQ-485, a specific ETA receptor antagonist, but not by BQ-788, a specific ETB receptor antagonist, indicating that the ET-1 effect is mediated by ETA receptors. On the other hand, the inhibitory effect of ET-3 was antagonized by BQ-788 and more effectively by BQ-485, suggesting the involvement of “atypical” ET receptors. Both ETs partly reversed the carbachol-induced shortening of the action potential recorded in the current-clamp mode. Inhibitory effects of ET-1 and ET-3 on the preactivated IKACh may contribute to the positive inotropic and chronotropic effects of ETs in atrial tissues.

inorganic 1,4,5-trisphosphate; protein kinase C; action potential; patch clamp

ENDOTHELIN (ET), a potent vasoconstrictor polypeptide consisting of 21 amino acids, was originally isolated from the culture medium of porcine aortic endothelial cells (36). Three distinct ET-related genes were cloned by genomic DNA analysis, and encoded peptides were named ET-1, ET-2, and ET-3 (8). ET-1 and ET-2 are similar in terms of their biologic activities, whereas ET-3 has quite different effects (8, 11, 22). Cloning of cDNA encoding the ET receptor showed at least two ET receptor subtypes, ETA and ETB receptors (1, 28). It has been reported that ET-1 and ET-2 activate these receptor subtypes nonselectively, whereas ET-3 is relatively ETA receptor specific (27, 28).

Among the diverse effects of ET in various organs and tissues, it should be emphasized that ET is one of the most potent positive inotropic endogenous substances in mammalian heart (6, 12, 15, 18, 23). In addition, ETs induce a positive chronotropic response. In rat atrial preparations, ET-1 and ET-3 induced positive inotropic and chronotropic responses (11). The ET-1-induced positive inotropic response was accompanied by a prolongation of action potential duration (APD) in guinea pig left atria (6, 12). On the other hand, Kim (16) reported that all three isoforms of ETs induced a negative chronotropic response in isolated rat atrial cells. More recently, Ono and colleagues showed that ET-1 shortened the APD in isolated atrial cells (25) and transiently decreased the beating rate of isolated right atria of guinea pigs (24). In these studies the negative chronotropic effects of ETs have been attributed to the activation of muscarinic acetylcholine (ACH) receptor-operated K⁺ current (IKACh) through the pertussis toxin (PTX)-sensitive GTP-binding protein. To gain greater insight into the cause of the apparent discrepancy, this study was designed to examine the effects of ETs on IKACh in the absence and presence of muscarinic stimulation, because IKACh is an important determinant of APD in atrial cells and there may be a tonic stimulation of muscarinic receptors in situ heart.

MATERIALS AND METHODS

Cell Isolation

Single atrial cells of the guinea pig heart were isolated by an enzymatic dissociation method, as previously described (31). Briefly, female guinea pigs, weighing 250–350 g, were anesthetized with an intraperitoneal injection of pentobarbital sodium. Their hearts were removed, immediately mounted on a Langendorff apparatus, and retrogradely perfused with 1) normal N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-Tyrode solution for 10 min, 2) nominally Ca²⁺-free Tyrode solution for 10 min, and then 3) Ca²⁺-free Tyrode solution containing 0.2 mg/ml collagenase (Wako, Osaka, Japan) for 20–30 min. After digestion, the hearts were perfused with a high-K⁺-low-Cl⁻ solution [modified Kraftbrühe (KB) solution] (9, 31). Atrial tissue was cut into small pieces in the modified KB solution, and the pieces were gently agitated to isolate cells. The cell suspension was stored in a refrigerator (4°C) and used on the same day.

Solutions

The composition of the normal HEPES-Tyrode solution was (in mM) 143 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.33 NaH₂PO₄, 5.5 glucose, and 5 HEPES-NaOH buffer (pH 7.4). The composition of the modified KB solution was (in mM) 70 KOH, 50 L-glutamic acid, 40 KCl, 20 taurine, 20 KH₂PO₄, 3 MgCl₂, 10 glucose, 1 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), and 10 HEPES-KOH buffer (pH 7.4). For unitary current recordings in cell-attached mode, a depolarizing bath solution containing (in mM) 140 KCl, 1.8 MgCl₂, 1 EGTA, 5.5 glucose, and 5 HEPES-KOH buffer (pH 7.4) was used. The composition of the pipette solution for whole cell clamp was (in mM) 110 potassium aspartate, 20 KCl, 1 MgCl₂, 5 potassium ATP, 5 potassium phosphocreatine, 10 EGTA, and 5 HEPES-KOH.

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buffer (pH 7.4). GTP (sodium salt, 100 µM) or guanosine 5’-O-(thiotriphosphosphate) (GTPγS; lithium salt, 100 µM) was also added to the pipette solution. The free Ca\(^{2+}\) concentration in the pipette solution was adjusted to pCa 8.0 according to the calculation by Fabiato and Fabiato (3) with the correction of Tsien and Rink (33). For unitary current recordings, a pipette solution containing (in mM) 140 KCl, 1.8 CaCl\(_2\), and 5 HEPES-KOH buffer (pH 7.4) was used. Carbachol (1 µM) was added to the solution. In some of the experiments, 30 nM ET-1 or 30 nM ET-3 was added to the solution instead of carbachol. In these experiments, 10 µM atropine and 100 µM theophylline were also included in the pipette solution to block muscarinic and adenosine receptors.

**Data Acquisition and Analysis**

Whole cell voltage clamp. Whole cell membrane currents were recorded by the patch-clamp method (5). Single atrial cells were placed in a recording chamber (0.4 ml volume) attached to an inverted microscope (model IMT-2, Olympus, Tokyo, J apan) and superfused with the HEPES-Tyrode solution at a rate of 2 ml/min. The temperature of the bath solution was kept constant at 36 ± 1.0°C. Patch pipettes were made from borosilicate glass capillaries (1.5 mm OD) using a vertical microelectrode puller (model PB-7, Narishige, Tokyo, Japan). The tip resistance was 3–5 MΩ when filled with the solution described above. The electrode was connected to a patch-clamp amplifier (model CEZ-2300, Nihon Koden, Tokyo, J apan). Command pulse signals were generated by a 12-bit digital-to-analog converter controlled by pCLAMP software (Axon Instruments, Foster City, CA). Current signals were filtered at 1 kHz, digitized, and stored on the hard disk of an IBM-compatible computer with a 200-Mbyte hard disk (Compaq Prolinea 4/50, Houston, TX). A liquid junction potential between the pipette solution and the bath solution of −8 mV was corrected. After a gigahm seal between the tip of the electrode and the cell membrane was established, the cell membrane was ruptured by negative pressure to make the whole cell configuration. The outward K\(^+\) current was activated by the extracellular application of 1–10 µM carbachol or 100 µM adrenaline to the GTP-loaded cells or by the intracellular loading of 100 µM GTPγS, a nonhydrolyzable analog of GTP, in atrial cells held at −40 mV. Effects of various concentrations of ETs on the preactivated I\(_{\text{K,ET}}\) were examined. To calculate percent inhibition of I\(_{\text{K,ET}}\), the difference between the steady-state current in the solution containing 10 µM carbachol, which maximally activated I\(_{\text{K,ET}}\) in atrial cells held at −40 mV, and the current level in the absence of any agonists was taken as 100%. In addition, to clarify the receptor subtype(s) involved in the effects of ETs, influences of selective ET\(_A\) and ET\(_B\) receptor antagonists BQ-485 (perhydrolazepin-1-yl-L-leucyl-d-tryptophanyl-d-tryptophan) (13) and BQ-788 [N-cis-2,6-dimethylpiperidinocarboxyl-L-γ-MeLeu2-d-Trp(CooMe)-d-Nle-ONa] (10) were also studied. In some experiments, BQ-123 [cyclo-(d-Trp-d-Asp-L-Pro-d-Val-L-Leu)]

**Drugs**

ET-1 and ET-3 were obtained from the Peptide Institute (Osaka, J apan), BQ-485 from Banyu Pharmaceutical (Ibaraki, J apan), BQ-123 from Funakoshi (Tokyo, J apan), BQ-788 and sarafotoxin S6c from NovoBiochem (Laufelfingen, Switzerland), PTX from Kaken Pharmaceutical (Tokyo, J apan), carbachol chloride, adenosine, glibenclamide, staurosporine, phorbol 12-myristate 13-acetate (PMA), and d-myo-inositol 1,4,5-trisphosphate (IP\(_3\)) from Sigma Chemical (St. Louis, MO), atropine sulfate monohydrate and theophylline from Wako, and 1-[N,O-bis(1,5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) from Seikagaku (Tokyo, J apan). ET-1, ET-3, BQ-485, BQ-123, BQ-788, and sarafotoxin S6c were dissolved in sodium phosphate buffer as a stock solution. Glibenclamide, staurosporine, PMA, and KN-62 were dissolved in dimethyl sulfoxide. PTX was dissolved in the modified KB solution with albumin (1 mg/ml). All other compounds were dissolved in distilled water.

**Statistics**

Values are means ± SE. Analysis by Student’s t-test was performed for paired and unpaired observations. P < 0.05 was taken as significant. Half-maximal effective concentration (EC\(_{50}\)) was obtained using a Macintosh computer (Apple Computer, Cupertino, CA) and Kaleida Graph program (Synergy Software, Reading, PA).

**RESULTS**

Effects of ETs on the Whole Cell Outward Current

ET-1 (30 nM) induced a transient outward current in atrial cells held at −40 mV (Fig. 1A). A similar increase in the outward current was observed in eight cells...
exposed to 30 nM ET-1. However, ET-1 produced a transient potentiation followed by a sustained inhibition of the outward current when \( I_{K_{ACh}} \) was preactivated by an application of carbachol (1 \( \mu \)M) to the bath (Fig. 1B). ET-1 (30 nM) transiently increased the carbachol (1 \( \mu \)M)-induced \( I_{K_{ACh}} \) by 57.7 ± 3.8% of the preactivated level and then decreased it by 64.7 ± 5.8% of the preactivated level in three cells. Effects of ET-3 on \( I_{K_{ACh}} \) were qualitatively similar. ET-3 also induced a transient outward current in six cells (Fig. 1C) and produced a biphasic effect on the preactivated \( I_{K_{ACh}} \) (Fig. 1D). ET-3 (30 nM) transiently increased the carbachol (1 \( \mu \)M)-induced \( I_{K_{ACh}} \) by 12.3 ± 4.7% and decreased it by 55.0 ± 3.7% in five cells. However, the potentiating effect of ET-3 on \( I_{K_{ACh}} \) was weaker than that of ET-1 when effects of the same concentration (30 nM) of ETs were compared (Fig. 1, B and D). The inhibitory effects of ET-1 and ET-3 on the preactivated \( I_{K_{ACh}} \) were sustained, and the outward current recovered only slightly on washout of ETs. Even with the addition of ET antagonists such as BQ-485 and BQ-788, the inhibition of \( I_{K_{ACh}} \) could not be reversed promptly.

In atrial cells preincubated with PTX (5 \( \mu \)g/ml, 36°C, 120 min), neither 30 nM ET-1 (\( n = 4 \)) nor 30 nM ET-3 (\( n = 4 \)) induced the transient outward current. Recent reports have indicated that ACh can activate the ATP-sensitive \( K^+ \) current in atrial cells (34) and ET-1 can inhibit the current in ventricular cells (17). Therefore, we conducted similar experiments in the presence of the ATP-sensitive \( K^+ \) channel blocker glibenclamide. ET-1 (30 nM, \( n = 2 \)) and ET-3 (30 nM, \( n = 2 \)) still induced a transient outward current in the presence of 10 \( \mu \)M glibenclamide. In addition, the ETs produced a transient potentiation followed by a sustained inhibition of the outward current induced by 1 \( \mu \)M carbachol in glibenclamide-treated atrial cells (\( n = 2 \) each). In some experiments, ramp pulse protocol (repolarization or hyperpolarization pulse from +100 to −100 mV with a slope of −1.2 mV/ms) was used to record a quasi-steady-state current. In carbachol (1 \( \mu \)M)-treated cells the reversal potential of the outward current (around −80 mV) was not influenced by the addition of ET-1 or ET-3. Taken together, the current activated and inhibited by ET-1 or ET-3 would be \( I_{K_{ACh}} \).

When \( I_{K_{ACh}} \) was maximally activated by a higher concentration (10 \( \mu \)M) of carbachol, only inhibitory effects of ETs on the outward current were observed (Fig. 2, A and B). ET-1 (30 nM) and ET-3 (30 nM) inhibited the \( I_{K_{ACh}} \) by 76.7 ± 10.3% (\( n = 6 \)) and 73.7 ± 6.5% (\( n = 7 \)), respectively. Although carbachol and adenosine act on different membrane receptors, i.e., \( M_2 \)-muscarinic ACh receptors and A1-adenosine receptors, adenosine can also induce \( I_{K_{ACh}} \) through the activation of PTX-sensitive GTP-binding protein in atrial cells (20). ET-1 (30 nM) elicited an almost identical inhibitory effect on the adenosine (100 \( \mu \)M)-induced \( I_{K_{ACh}} \), which was also persisted after the washout (Fig. 2C). ET-3 also inhibited the adenosine-induced outward current. ET-1 (30 nM) and ET-3 (30 nM) inhibited the adenosine-induced \( I_{K_{ACh}} \) by 69.4 ± 6.7% (\( n = 8 \)) and 62.0 ± 2.8% (\( n = 4 \)), respectively. Intracellular loading of GTP\( \gamma \)S (100 \( \mu \)M), a nonhydrolyzable GTP analog, gradually activated a persistent outward current, even in the absence of any agonists. Addition of 30 nM ET-1 inhibited the GTP\( \gamma \)S-induced outward current by 87.5 and 90.0% without transient activation in two cells. ET-3 (30 nM) also inhibited the GTP\( \gamma \)S-induced current by 88.9 and 76.9% in two cells without activation of the outward current. Because ETs commonly inhibited carbachol-, adenosine-, and GTP\( \gamma \)S-induced \( I_{K_{ACh}} \), it is likely that ETs inhibit \( I_{K_{ACh}} \) by mechanism(s) other than blocking the muscarinic receptors. Although ET-3 inhibited the preactivated \( I_{K_{ACh}} \), sarafotoxin S6c (30
nM), a specific ET<sub>B</sub> receptor agonist (27), failed to inhibit the carbachol (10 µM)-induced <i>I<sub>Kach</sub></i> (Fig. 2D). Similar results were obtained in four other cells.

Figure 3 summarizes the concentration-response relationships of the inhibitory effects of ETs on the carbachol (10 µM)-induced <i>I<sub>Kach</sub></i> in the absence and presence of ET receptor antagonists in the atrial cells held at −40 mV. This high concentration of carbachol was used to minimize the potentiating effects of ETs and quantify only their inhibitory effects on <i>I<sub>Kach</sub></i>. ET-1 and ET-3 inhibited <i>I<sub>Kach</sub></i> in a concentration-dependent fashion, and the maximal inhibition of <i>I<sub>Kach</sub></i> (~75%) was achieved with 30 nM ET-1 and 30 nM ET-3. The EC<sub>50</sub> values of ET-1 and ET-3 for inhibiting the carbachol-induced <i>I<sub>Kach</sub></i> were 314 pM and 1.13 nM, respectively.

Pretreatment with 30 nM BQ-485, a selective ET<sub>A</sub> antagonist (13), shifted the concentration-response curve for the inhibitory effect of ET-1 to the right ~30-fold. In contrast, 30 nM BQ-788, a selective ET<sub>B</sub> antagonist (10), slightly shifted the curve to the right, but this effect was not statistically significant. The EC<sub>50</sub> values of ET-1 were 10.1 nM in the presence of 30 nM BQ-485 and 830 pM in the presence of 30 nM BQ-788 (Fig. 3A). The inhibitory effect of ET-3 was more sensitive to BQ-485. The concentration-response curve for the inhibitory effect of ET-3 on <i>I<sub>Kach</sub></i> was shifted by 30 nM BQ-485 to the right ~900-fold. Moreover, the curve was shifted by 3 nM BQ-485 by 60-fold. In addition, 30 nM BQ-788 also shifted the concentration-response curve for the ET-3 effect to the right ~30-fold. The EC<sub>50</sub> values of ET-3 in the presence of 3 nM BQ-485, 30 nM BQ-485, and 30 nM BQ-788 were 72.7 nM, 1.04 µM, and 28.7 nM, respectively (Fig. 3B). BQ-123 (30 nM), another selective ET<sub>A</sub> antagonist (7), also blocked the inhibitory effect of 30 nM ET-3 (n = 3, data not shown). These results suggest that ET-1 inhibits <i>I<sub>Kach</sub></i> through the activation of ET<sub>A</sub> receptors, whereas ET-3 inhibits <i>I<sub>Kach</sub></i> through the activation of BQ-485- and BQ-788-sensitive atypical ET receptors.

To elucidate the subcellular mechanism(s) by which ETs inhibit <i>I<sub>Kach</sub></i>, we evaluated influences of various compounds modulating the signal transduction system on ET-induced <i>I<sub>Kach</sub></i> inhibition. Because ETs are known to facilitate phosphatidylinositol hydrolysis (6, 15), we examined whether activation of protein kinase C (PKC) or production of IP<sub>3</sub> was involved in the inhibitory effects of ETs on <i>I<sub>Kach</sub></i>. ET-1 (3 nM) and ET-3 (3 nM) inhibited the carbachol (10 µM)-induced <i>I<sub>Kach</sub></i> by 62.0 ± 8.0% (n = 9) and 48.7 ± 7.2% (n = 10), respectively. Pretreatment with 30 nM staurosporine, a PKC inhibitor, failed to affect the inhibition of <i>I<sub>Kach</sub></i> by these ETs (Fig. 4A). The inhibition of <i>I<sub>Kach</sub></i> by ET-1 and ET-3 in the presence of staurosporine was 57.0 ± 5.8% (n = 7) and 53.7 ± 5.5% (n = 8), respectively, not significantly different from the control condition. PMA (100 nM), a PKC activator, could not mimic the inhibitory effects of ETs in five cells (data not shown). To test the involvement of IP<sub>3</sub> in the ET-induced <i>I<sub>Kach</sub></i> inhibition, we used a pipette solution containing a high concentration (20 µM) of IP<sub>3</sub>. We thought that the preactivation of the IP<sub>3</sub> pathway may damp the ET-induced <i>I<sub>Kach</sub></i> inhibition if IP<sub>3</sub> production is a prerequisite for the <i>I<sub>Kach</sub></i> inhibition. Intracellular perfusion of cells with IP<sub>3</sub> abolished the ET-3-induced inhibition of <i>I<sub>Kach</sub></i> but not the ET-1-induced inhibition (Fig. 4B). The inhibition of <i>I<sub>Kach</sub></i> by ET-1 and ET-3 was 52.1 ± 7.6% (n = 5) and 2.4 ± 1.5% (n = 5), respectively. The ET-3-induced inhibition of <i>I<sub>Kach</sub></i> with this pipette solution was significantly smaller than that in the control condition. However, inclusion of 100 µM IP<sub>3</sub> also failed to inhibit the ET-1-induced <i>I<sub>Kach</sub></i> inhibition (53.1 ± 7.2%, n = 3). In addition, the <i>I<sub>Kach</sub></i> density recorded with IP<sub>3</sub>-containing pipette solution (16.6 ± 3.0 pA/pF, n = 6) was not significantly different from that with normal pipette solution (19.3 ± 2.1 pA/pF, n = 6) during
the activation with 10 µM carbachol. Therefore, IP3 inclusion might indirectly interfere with the inhibitory pathway on $I_{K_{ACh}}$ by ET-3 but not by ET-1. IP3 is known to produce a Ca$^{2+}$ release from the sarcoplasmic reticulum, and the increased Ca$^{2+}$ might activate calmodulin. Accordingly, we examined the influence of KN-62, a Ca$^{2+}$-calmodulin-dependent protein kinase II inhibitor (32), on the inhibitory effects of ETs on $I_{K_{ACh}}$. However, KN-62 failed to affect the inhibition of $I_{K_{ACh}}$ by ETs (Fig. 4C). The inhibition of $I_{K_{ACh}}$ by ET-1 and ET-3 in the presence KN-62 was 71.9 ± 6.4% (n = 6) and 40.3 ± 6.0% (n = 8), respectively. Therefore, the IP3-calmodulin pathway does not seem to be involved in the $I_{K_{ACh}}$ inhibition by ET-1 or ET-3.

Recently, ET$_A$ receptors were shown to couple to PTX-sensitive G protein (14, 25, 35). The activation of $I_{K_{ACh}}$ in response to 10 µM carbachol was completely abolished in atrial cells preincubated with PTX. Intracellular loading of 100 µM GTP$_\gamma$S was still capable of activating $I_{K_{ACh}}$ through direct activation of $G_\text{i}$ proteins. ET-1 (30 nM) and ET-3 (30 nM) inhibited the GTP$_\gamma$S-induced $I_{K_{ACh}}$, even in the PTX-treated cells (Fig. 4D), indicating that the inhibitory effects of ETs on $I_{K_{ACh}}$ are not mediated by PTX-sensitive G proteins.

Effects of ETs on the Single $K_{ACh}$ Channel Current

The unitary $K_{ACh}$ channel current was recorded from cell-attached patches using patch pipettes containing 1 µM carbachol. In these experiments, bath solution and pipette solution contained 140 mM K$^+$, and the pipette potential was clamped at various potentials. When pipette potential was positive, the unitary $I_{K_{ACh}}$ was recorded as an inward current, which is shown as a downward deflection (Fig. 5). The current-voltage relationship for the single channel current was determined
ETs were sustained, and NPo scarcely recovered after the washout. These findings suggest that ETs inhibit I_{KACH} via production of soluble intracellular second messenger(s).

Single channel activity was also recorded from cell-attached patches with patch pipettes containing 30 nM ET-1 or 30 nM ET-3. The patch pipettes also included 10 µM atropine and 100 µM theophylline to block muscarinic and adenosine receptors. In this condition the channel openings were observed. The mean slope conductance with patch pipettes containing ET-1 and ET-3 were 46.6 ± 0.5 (n = 9) and 46.9 ± 0.4 pS (n = 7), respectively, and showed inward rectification. The channel conductance was not significantly different from that recorded with patch pipettes containing 1 µM carbachol, suggesting K_{ACH} channel activity. The NPo values of the channel with ET-1- and ET-3-containing pipettes were 0.156 ± 0.027 (n = 9) and 0.144 ± 0.034 (n = 7), respectively. These NPo values were slightly greater than the NPo of the basal spontaneous opening of K_{ACH}, channels recorded using pipette solution containing atropine and theophylline (0.057 ± 0.015, n = 9), indicating K_{ACH} channel activation by ET-1 and ET-3.

Effects of ETs on Action Potential

Action potential of guinea pig atrial cells stimulated at a rate of 0.2 Hz was recorded in the whole cell current-clamp mode. The baseline characteristics of action potentials were as follows: resting membrane potential (RMP) was −73.4 ± 1.2 mV, action potential amplitude was 131.1 ± 1.6 mV, APD at 50% repolarization (APD_{50}) was 72.9 ± 7.5 ms, and APD at 90% repolarization (APD_{90}) was 107.1 ± 8.0 ms (n = 21). ET-1 (30 nM) shortened APD_{90} by 87.0 and 80.1% with a slight increase in RMP in two cells. ET-3 (30 nM) also shortened APD_{90} by 59.8 and 71.2% in two cells. When the APD was shortened by 1 µM carbachol, ET-1 and ET-3 transiently shortened APD further and then partly restored APD toward control (Fig. 6A). After the application of carbachol, APD_{50} was 16.1 ± 3.8% of the control and was prolonged to 36.3 ± 8.6% by 30 nM ET-1 (n = 8, P < 0.05) and to 29.4 ± 4.0% by 30 nM ET-3 (n = 9, P < 0.05; Fig. 6B). Carbachol (1 µM) significantly increased RMP from −74.3 ± 1.3 to −77.3 ± 1.1 mV (n = 17). Addition of 30 nM ET-1 and 30 nM ET-3 significantly decreased RMP to −73.3 ± 2.3 (n = 8) and −72.8 ± 1.7 mV (n = 9), respectively. Thus ET-1 and ET-3 functionally antagonized the muscarinic receptor-mediated action potential shortening.

DISCUSSION

Since the discovery of ETs, it has been demonstrated that they produce positive inotropic and chronotropic responses in cardiac tissues of various mammalian species (6, 11, 12, 15, 18, 23). However, underlying mechanism(s) responsible for these effects of ETs have not been fully understood. Effects of ETs on the membrane current system of cardiac cells have been examined in several studies. In terms of effects of ET-1 on the Ca^{2+} current (I_{Ca}), inconsistent results have been re-
current in rat (16) and guinea pig atrial cells (25). Although Kim (16) showed that not only ET-1 but also ET-3 activated the outward current in rat atrial cells, Ono et al. (25) reported that ET-3 failed to elicit the outward current in guinea pig atrial cells. Consistent with the former study, 30 nM ET-3 also activated the PTX-sensitive outward current in atrial cells, although the activation of the current was somewhat smaller and slower than that by ET-1 in this study. In addition, ET-1 and ET-3 activated the outward current in the presence of the ATP-sensitive K⁺ channel blocker glibenclamide. Furthermore, ET-3 as well as ET-1 shortened APD in single atrial cells. These results suggest that the ETs commonly activate I_{KACCH}.

This study has demonstrated that ET-1 and ET-3 produced dual effects on I_{KACCH}, i.e., enhancement followed by inhibition in the absence and presence of glibenclamide, when the I_{KACCH} was preactivated by the muscarinic agonist carbachol. When the I_{KACCH} was maximally activated by a higher concentration of carbachol, ETs produced only an inhibition of I_{KACCH}, which was only partially reversed by the washout of the peptide. The inhibitory effects of ET-1 and ET-3 were concentration dependent, and their EC₅₀ values for inhibiting the carbachol (10 µM)-induced I_{KACCH} were 0.31 and 1.13 nM, respectively. The reduction of I_{KACCH} after ETs was reflected by changes in action potential configuration. ET-1 and ET-3 partially reversed the carbachol-induced action potential shortening.

In the present study ET-1 and ET-3 inhibited the carbachol-induced I_{KACCH} within the same order of magnitude; the EC₅₀ for ET-1 was approximately three times smaller than that for ET-3. The concentration-response curve for the inhibitory effect of ET-1 was shifted by BQ-485 but not by BQ-788, suggesting that the ET-1 effect is mediated by ETₐ receptors. However, it cannot be concluded that the ET-3 effect is also mediated by ETₐ receptors, because ET-3 is supposed to be a relatively specific ETₐ agonist and to show ~100 times less affinity for ETₐ receptors than ET-1 (27). In addition, the concentration-response curve for the inhibitory effect of ET-3 was shifted by BQ-788 and more effectively by BQ-485. Moreover, sarafotoxin S6c, which specifically recognizes ETₐ receptors but not ETₐ receptors (29), failed to inhibit the I_{KACCH}. Therefore, the ET-3 effect may be mediated by “atypical” ET receptors, i.e., BQ-788- and BQ-485-sensitive and S6c-insensitive receptors. Thus ET receptors mediating the ET-3 effect on I_{KACCH} could not be readily classified into ETₐ or ETₐ subtypes. Similar atypical ET receptors have been described in rat atrial muscles (26). There may be an additional type of ET receptor that has not been discriminated on the basis of molecular biologic techniques.

According to Kim (16) and Ono et al. (25), the activation of I_{KACCH} by ETs is mediated by the PTX-sensitive G protein. However, the inhibitory effect of ETs on I_{KACCH} that was induced by GTPγS was not influenced by PTX. Because it is known that ET-1 and ET-3 produce phosphoinositide hydrolysis (6, 15), we tested the involvement of two downstream messengers,
PKC and IP3, in the inhibitory effects of ET-1 and ET-3 on I_{K,ACH}. PKC is unlikely to be involved in the inhibitory effects of ETs on I_{K,ACH}, because staurosporine failed to modify the inhibitory effects of ETs and PMA could not mimic the effects of ETs.

In the terms of the experiments using the pipette solution containing IP3, unexpected results were obtained. Originally, we considered that intracellular loading of excessive IP3 might d apt the ET-induced I_{K,ACH} inhibition if IP3 inhibits K_{ACH} channels by acting as a ligand or by releasing Ca^{2+} from the sarcoplasmic reticulum. In fact, the inclusion of IP3 in the pipette solution almost abolished the inhibitory effect of ET-3 on I_{K,ACH} but not that of ET-1. However, the inhibition of the Ca^{2+}-calmodulin pathway, which might be activated by IP3-induced Ca^{2+} release from the sarcoplasmic reticulum of cardiac cells (2), failed to affect the I_{K,ACH} inhibition by ET-1 and ET-3. In addition, the density of I_{K,ACH} recorded with IP3-containing pipette solution was not significantly different from that recorded with normal pipette solution. Therefore, a reasonable interpretation would be that ET-3 may inhibit I_{K,ACH} by some mechanism(s) that can be antagonized by intracellular IP3 overload. ET-1 might inhibit I_{K,ACH} by some intracellular mechanism(s) that is clearly different from those of ET-3.

Both ETs inhibited the single K_{ACH} channel activity recorded in the cell-attached mode using a carbachol-containing pipette solution. These findings suggest that both ETs appear to inhibit I_{K,ACH} via soluble second messenger(s) and not via a membrane-delimited process. However, we obtained smaller effects of ETs on the single K_{ACH} channel activity, although we employed a higher concentration (100 nM) of ETs than those used in the whole cell experiments. One possible explanation might be that access of some soluble messenger(s) to the K+ channels in the patch membrane might be limited. Further experimentation is needed to clarify the precise mechanism(s) of the I_{K,ACH} inhibition by ETs.

This study has demonstrated that ET-1 and ET-3 inhibit the preactivated I_{K,ACH}, although the receptors and mechanisms involved in the I_{K,ACH} inhibition could not be fully clarified. Previously, it was reported that ET-1 and ET-3 produce positive chronotropic and inotropic responses in guinea pig atrial preparations (6, 12, 24). It was reported that ET-1 prolonged the APD in isolated guinea pig atria (6, 12). Therefore, inhibition of I_{K,ACH} might in part contribute to the electromechanical responses to ETs in atrial preparations. However, ETs are known to elicit marked positive inotropic responses without affecting APD in a ventricular preparation in which I_{K,ACH} does not contribute to the repolarization of the action potential (15, 17). An increase in I_{Ca} and/or the Ca^{2+} sensitivity of cardiac myofilament resulting from the activation of the Na+\textquoteright H+ exchanger (19) may be also important for the electromechanical responses to ETs.

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