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

HIROSHI YAMAGUCHI, NAOYA SAKAMOTO, YASUHIDE WATANABE, TOSHIHIRO SAITO, YOSHIKI MASUDA, AND HARUAKI NAKAYA
Department of Pharmacology and Third Department of Internal Medicine, Chiba University School of Medicine, Chiba 260, Japan

Yamaguchi, Hiroshi, Naoya Sakamoto, Yusuhide 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 (I_{K,ACH}) were examined in isolated guinea pig atrial cells using patch-clamp techniques. ET-1 or ET-3 produced a transient activation of I_{K,ACH} in atrial cells held at ~40 mV. When I_{K,ACH} was preactivated by 1 µM carbachol, however, both ETs produced a transient potentiation followed by a sustained inhibition of the current. When I_{K,ACH} was maximally activated by 10 µM carbachol or 100 µM adenosine, these ETs produced only a sustained inhibition of the I_{K,ACH}. Their inhibitory effects on the preactivated I_{K,ACH} 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 I_{K,ACH} may contribute to the positive isotropic and chronotropic effects of ETs in atrial tissues.

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 a modified 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.
buffer (pH 7.4). GTP (sodium salt, 100 µM) or guanosine 5’-O-(thiotriphosphate) (GTPγS; lithium salt, 100 µM) was also added to the pipette solution. The free Ca2+ 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 CaCl2 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 a 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) and superfused with the HEPES-Tyrode solution instead of carbachol. 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, Japan). Command pulse signals were generated by a patch-clamp amplifier at room temperature (20–25°C) and stored on a video cassette recorder (model NV-H6, Panasonic, Osaka, J apan) through a pulse code modulator (model VR-10B, Instrutech, New York, NY). Later, the data were transferred to the hard disk of the computer at a sampling rate of 10 kHz, filtered at 1.5 kHz through a digital Gaussian filter, and analyzed by pCLAMP software. Briefly, the amplitude of the single channel current was obtained by constructing an amplitude histogram or by measuring the distance of two horizontal lines set by eye at the closed and open levels. There was no significant difference in the unitary amplitude determined by the two methods. To obtain the single channel conductance, measurement of the single channel current amplitude was repeated at different test potentials in some of the experiments. Channel openings were identified by an algorithm that used amplitude information and measured with an interactive threshold for detecting events that was set at 50% of the expected amplitude. Channel activity is represented by the product Np_o, i.e., the number of channels in the patch (N) times the ratio of the channel open time to the total sampled time (P_o).

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, phosphol 12-myristate 13-acetate (PMA), and 1,5-isouquinolinesulfonyl)-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62) from Selkagaku (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, and KN-62 were dissolved in dimethyl sulfoxide. PTX was dissolved in dimethyl sulfoxide. PTX was dissolved in distilled water.

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

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 (EC50) 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 µM) to the bath (Fig. 1B). ET-1 (30 nM) transiently increased the carbachol (1 µM)-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). 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 µg/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 µM glibenclamide. In addition, the ETs produced a transient potentiation followed by a sustained inhibition of the outward current induced by 1 µM 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 µM)-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 µM) 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., M₂-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 µM)-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 GTPγS (100 µM), 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 GTPγS-induced outward current by 87.5 and 90.0% without transient activation in two cells. ET-3 (30 nM) also inhibited the GTPγS-induced current by 88.9 and 76.9% in two cells without activation of the outward current. Because ETs commonly inhibited carbachol-, adenosine-, and GTPγS-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<sub>KACH</sub> (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<sub>KACH</sub> 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<sub>KACH</sub>. ET-1 and ET-3 inhibited I<sub>KACH</sub> in a concentration-dependent fashion, and the maximal inhibition of I<sub>KACH</sub> (~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 (10 µM)-induced I<sub>KACH</sub> 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<sub>KACH</sub> 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<sub>KACH</sub> through the activation of ET<sub>A</sub> receptors, whereas ET-3 inhibits I<sub>KACH</sub> through the activation of BQ-485- and BQ-788-sensitive atypical ET receptors.

To elucidate the subcellular mechanism(s) by which ETs inhibit I<sub>KACH</sub>, we evaluated influences of various compounds modulating the signal transduction system on ET-induced I<sub>KACH</sub> 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<sub>KACH</sub>. ET-1 (3 nM) and ET-3 (3 nM) inhibited the carbachol (10 µM)-induced I<sub>KACH</sub> 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<sub>KACH</sub> by these ETs (Fig. 4A). The inhibition of I<sub>KACH</sub> 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<sub>KACH</sub> 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<sub>KACH</sub> inhibition if IP<sub>3</sub> production is a prerequisite for the I<sub>KACH</sub> inhibition. Intracellular perfusion of cells with IP<sub>3</sub> abolished the ET-3-induced inhibition of I<sub>KACH</sub>, but not the ET-1-induced inhibition (Fig. 4B). The inhibition of I<sub>KACH</sub> 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<sub>KACH</sub> 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<sub>KACH</sub> inhibition (53.1 ± 7.2%, n = 3). In addition, the I<sub>KACH</sub> 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 IKACH by ET-3 but not by ET-1. IP3 is known to produce a Ca2+ release from the sarcoplasmic reticulum, and the increased Ca2+ might activate calmodulin. Accordingly, we examined the influence of KN-62, a Ca2+-calmodulin-dependent protein kinase II inhibitor (32), on the inhibitory effects of ETs on IKACH. However, KN-62 failed to affect the inhibition of IKACH by ETs (Fig. 4C). The inhibition of IKACH 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 IKACH inhibition by ET-1 or ET-3.

Recently, ETα receptors were shown to couple to PTX-sensitive G protein (14, 25, 35). The activation of IKACH in response to 10 µM carbachol was completely abolished in atrial cells preincubated with PTX. Intracellular loading of 100 µM GTPγS was still capable of activating IKACH through direct activation of Gi proteins. ET-1 (30 nM) and ET-3 (30 nM) inhibited the GTPγS-induced IKACH, even in the PTX-treated cells (Fig. 4D), indicating that the inhibitory effects of ETs on IKACH are not mediated by PTX-sensitive G proteins.

Effects of ETs on the Single KACH Channel Current

The unitary KACH 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 IKACH 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...
in four cells, and the mean slope conductance was $47.6 \pm 1.6\, \text{pS} \ (n = 4)$ and displayed inward rectification. To minimize the influence of spontaneous decrease in the channel activity due to the desensitization, ETs were used to shorten the time course of the effects. Application of 100 nM ET-1 or 100 nM ET-3 inhibited the channel activity through the reduction of the open probability without affecting the amplitude of unitary events (Fig. 5, A and B). Channel activity can be expressed as $NP_o$, where $N$ is the number of $K_{ACH}$ channels in the patch membrane and $P_o$ is the open probability. ET-1 (100 nM) decreased $NP_o$ from $0.461 \pm 0.064$ to $0.259 \pm 0.056 \ (n = 6, \ P < 0.05)$, and ET-3 (100 nM) decreased it from $0.344 \pm 0.101$ to $0.158 \pm 0.051 \ (n = 5, \ P < 0.05; \ Fig. 5, C and D). Inhibitory effects of ETs were sustained, and $NP_o$ scarcely recovered after the washout. These findings suggest that ETs inhibit $K_{ACH}$ 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 conductances with patch pipettes containing ET-1 and ET-3 were $46.6 \pm 0.5 \ (n = 9)$ and $46.9 \pm 0.4\, \text{pS} \ (n = 7)$, 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 $NP_o$ values of the channel with ET-1- and ET-3-containing pipettes were $0.156 \pm 0.027 \ (n = 9)$ and $0.144 \pm 0.034 \ (n = 7)$, respectively. These $NP_o$ values were slightly greater than the $NP_o$ of the basal spontaneous opening of $K_{ACH}$ channels recorded using pipette solution containing atropine and theophylline $(0.057 \pm 0.015, \ n = 9)$, 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 $-73.4 \pm 1.2\, \text{mV}$, action potential amplitude was $131.1 \pm 1.6\, \text{mV}$, APD at 50% repolarization ($APD_{50}$) was $72.9 \pm 7.5\, \text{ms}$, and APD at 90% repolarization ($APD_{90}$) was $107.1 \pm 8.0\, \text{ms} \ (n = 21)$. ET-1 (30 nM) shortened $APD_{90}$ by $87.0$ and $80.1\%$ 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, $APD_{90}$ was $16.1 \pm 3.8\%$ of the control and was prolonged to $36.3 \pm 8.6\%$ by 30 nM ET-1 ($n = 8, \ P < 0.05$) and to $29.4 \pm 4.0\%$ by 30 nM ET-3 ($n = 9, \ P < 0.05; \ Fig. 6B$). Carbachol (1 µM) significantly increased RMP from $-74.3 \pm 1.3\, \text{to -77.3 \pm 1.1\, \text{mV}} \ (n = 17)$. Addition of 30 nM ET-1 and 30 nM ET-3 significantly decreased RMP to $-73.3 \pm 2.3\% \ (n = 8)$ and $-72.8 \pm 1.7\, \text{mV} \ (n = 9)$, 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 Ca²⁺ 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_{KACH}.

This study has demonstrated that ET-1 and ET-3 produced dual effects on I_{KACH}, i.e., enhancement followed by inhibition in the absence and presence of glibenclamide, when the I_{KACH} was preactivated by the muscarinic agonist carbachol. When the I_{KACH} was maximally activated by a higher concentration of carbachol, ETs produced only an inhibition of I_{KACH}, 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 EC_{50} values for inhibiting the carbachol (10 µM)-induced I_{KACH} were 0.31 and 1.13 nM, respectively. The reduction of I_{KACH} 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_{KACH} within the same order of magnitude; the EC_{50} 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 ET_{A} receptors. However, it cannot be concluded that the ET-3 effect is also mediated by ET_{A} receptors, because ET-3 is supposed to be a relatively specific ET_{B} agonist and to show ~100 times less affinity for ET_{A} 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 ET_{B} receptors but not ET_{A} receptors (29), failed to inhibit the I_{KACH}. 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_{KACH} could not be readily classified into ET_{A} or ET_{B} 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_{KACH} by ETs is mediated by the PTX-sensitive G protein. However, the inhibitory effect of ETs on I_{KACH} 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 IₖCAC. PKC is unlikely to be involved in the inhibitory effects of ETs on IₖCAC, because staurosporine failed to modify the inhibitory effects of ETs and PMA could not mimic the effects of ETs.

In terms of the experiments using the pipette solution containing IP₃, unexpected results were obtained. Originally, we considered that intracellular loading of excessive IP₃ may damp the ET-induced IₖCAC inhibition if IP₃ inhibits KₐCH channels by acting as a ligand or by releasing Ca²⁺ from the sarcoplasmic reticulum. In fact, the inclusion of IP₃ in the pipette solution almost abolished the inhibitory effect of ET-3 on IₖCAC but not that of ET-1. However, the inhibition of the Ca²⁺-calmodulin pathway, which might be activated by IP₃-induced Ca²⁺ release from the sarcoplasmic reticulum of cardiac cells (2), failed to affect the IₖCAC inhibition by ET-1 and ET-3. In addition, the density of IₖCAC 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 IₖCAC by some mechanism(s) that can be antagonized by intracellular IP₃ overload. ET-1 might inhibit IₖCAC by some intracellular mechanism(s) that is clearly different from those of ET-3.

Both ETs inhibited the single KₐCH channel activity recorded in the cell-attached mode using a carbachol-containing pipette solution. These findings suggest that both ETs appear to inhibit IₖCAC via soluble second messenger(s) and not via a membrane-delimited process. However, we obtained smaller effects of ETs on the single KₐCH 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 IₖCAC inhibition by ETs.

This study has demonstrated that ET-1 and ET-3 inhibit the preactivated IₖCAC, although the receptors and mechanisms involved in the IₖCAC 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 IₖCAC 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 IₖCAC does not contribute to the repolarization of the action potential (15, 17). An increase in Iₖ and/or the Ca²⁺ sensitivity of cardiac myofilament resulting from the activation of the Na⁺/H⁺ exchanger (19) may be also important for the electromechanical responses to ETs.

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