British Journal of Pharmacology (2000) 129, 1113–1120

www.nature.com/bjp

Both α_{1A}- and α_{1B}-adrenergic receptor subtypes couple to the transient outward current (I_{To}) in rat ventricular myocytes

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1 Regulation of transient outward current (I_{To}) by α_1-adrenergic (α_1AR) plays a key role in cardiac repolarization. α_1ARs comprise a heterogeneous family; two natively expressed subtypes (α_{1A} and α_{1B}) and three cloned subtypes (α_{1a}, α_{1b} and α_{1d}) can be distinguished. We have examined the electrophysiological role of each α_1AR subtype in regulating I_{To} in isolated rat ventricular myocytes.

2 Reverse transcription-PCR study revealed the presence of three subtype mRNAs (α_{1a}, α_{1b} and α_{1d}) in rat myocytes.

3 Radioligand binding assay using [^{125}I]-HEAT showed that the inhibition curves for α_{1A}AR-selective antagonists (WB4101, 5-methylurapidil, (+)-niguldipine and KMD-3213) in rat ventricles best fit a two-site model, with 30% high and 70% low affinity binding sites. The high affinity sites were resistant to 100 μM chloroethylclonidine (CEC), while the low affinity sites were highly inactivated by CEC.

4 Whole cell voltage clamp study revealed that methoxamine reduced a 4-aminopyridine(4-AP)-sensitive component of I_{To} in the isolated rat ventricle myocytes. Lower concentrations of KMD-3213 (1 nM) or 5-MU (10 nM) did not affect the methoxamine-induced reduction of I_{To}. On the other hand, CEC treatment (100 μM) of isolated myocytes reduced the methoxamine-induced reduction of I_{To} by 46%, and the remaining response was abolished by lower concentrations of KMD-3213 or 5-MU.

5 The results indicate that rat ventricular myocytes express transcripts of the three α_1AR subtypes (α_{1a}, α_{1b} and α_{1d}); however, two pharmacologically distinct α_1AR subtypes (α_{1A} and α_{1B}) are predominating in receptor populations, with approximately 30% α_{1A}AR and 70% α_{1B}AR. Although both α_{1A} and α_{1B}AR subtypes are coupled to the cardiac I_{To}, α_{1B}ARs predominantly mediate α_1AR-induced effect.

British Journal of Pharmacology (2000) **129,** 1113–1120

Keywords: α_1-adrenergic receptor subtype; CEC, KMD-3213; 5-methyl urapidil; rat; transient outward current

Abbreviations: 4-AP, 4-aminopyridine; AR, adrenergic receptor; CEC, chloroethylclonidine; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; [^{125}I]-HEAT, 2-{β-(4-hydroxy-3 [^{125}I]-iodophenyl) ethylamino methyl} tetralone; I_{To}, transient outward current; KB, kraftbrühe; KMD-3213, (−)-R-1-(3-Hydroxyproryl)-5-[2-[2-[2-(2,2,2-trifluoroethoxy) phenoxy] ethylamino] propyl] indoline-7-carboxamide dihydrobromide; 5-MU, 5-methylurapidil; RT–PCR, reverse transcription-polymerase chain reaction; WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl)-1,4 benzodioxane

Introduction

Activation of cardiac α_1-adrenergic receptors (α_1ARs) leads to rapid changes in contractility, metabolic responses, and electrophysiologic properties of the myocardium (Endoh & Blinks, 1988; Terzic *et al*., 1992; Shah *et al*., 1988; Apkon & Nerbonne, 1988; Braun *et al*., 1990; Tohse *et al*., 1990). α_1-Adrenergic agonists induce positive inotropic effects which are related either to an increase in free cytosolic Ca^{2+} levels or to an increase in the responsiveness of myofilaments to Ca^{2+} (Endoh & Blinks, 1988). Further, α_1AR-stimulated increase in free cytosolic Ca^{2+} levels may be closely related to the prolongation of the plateau phase of action potentials in the mammalian myocardium, for which several subcellular mechanisms have been proposed. Patch-clamp studies in single cardiac myocytes showed that mechanisms other than the direct L-type Ca^{2+} channel regulation, including an inhibition of transient outward current (I_{To}) (Apkon & Nerbonne, 1988; Braun *et al*., 1990; Ertl *et al*., 1991; Tohse *et al*., 1990; Ravens *et al*., 1989; Fedida *et al*., 1990; Wang *et al*., 1991), promotion of Na^+-H^+ exchange (Terzic *et al*., 1992), activation of delayed rectifier K^+ current (I_K) (Dirksen & Sheu, 1990), ACh-activated K^+ channel ($I_{K,ACh}$) (Kurachi *et al*., 1989), or activation of Na^+, K^+-ATPase (Shah *et al*., 1988), all seems to be closely related to the cardiac α_1AR stimulation. Among the membrane ionic currents, I_{To} is a major determinant of the plateau duration, and can be strongly inhibited through the activation of α_1AR, indicating that the prolongation of action potentials by α_1-adrenergic agonists may be explained by reduction of I_{To} in rat.

Recently, it was found that α_1ARs comprise a heterogeneous family. Heterogeneity of α_1ARs (α_{1A} and α_{1B}) was first suggested by pharmacological studies based on differential affinity of a variety of agents such as the agonists methoxamine and oxymetazoline, and the antagonists WB4101, (+)-niguldipine and 5-methylurapidil (5-MU), differential sensitivity to the alkylating agent chloroethylclonidine (CEC), and differing requirements for extracellular calcium in signal transduction (Morrow *et al*., 1986; Han *et al*., 1987; Gross *et al*., 1988; Tsujimoto *et al*., 1989). More recently, the cloning of three distinct cDNA encoding α_1AR subtypes (α_{1a}, α_{1b}, α_{1d}) has

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been reported (Cotecchia *et al*., 1988; Schwinn & Lomasney, 1992; Perez *et al*., 1991; Lomasney *et al*., 1991; Hirasawa *et al*., 1993). The uncertain relationship between the cloned and native subtypes has been the source of much confusion; however, very recent studies provide evidence supporting the idea that the α_{1a}AR (formerly α_{1c}AR) cDNA encodes the pharmacological α_{1A}AR subtype and that the α_{1b}AR cDNA clone appears to encode the natively expressed, pharmacologically defined α_{1B}AR subtype (Laz *et al*., 1994; Perez *et al*., 1994). The functional role of the native α_{1D}AR still remains to be defined.

The functional role of each α_1AR subtype in the regulation of cardiac ionic channels is still uncertain. In the present study, we examined the role of each α_1AR subtype in the regulation of I_{To} in rat ventricular myocytes. We have identified the subtypes of α_1AR expressing in the adult rat myocytes, and pharmacologically characterized the role of each α_1AR subtype in the regulation of I_{To}. The results obtained show that α_1ARs in the rat ventricular myocytes consist of about 30% of α_{1A}AR and 70% of α_{1B}AR, and that both subtypes are involved in the regulation of I_{To}.

Methods

Isolation of myocytes

Rat single ventricular myocytes were enzymatically isolated using standard procedures (Isenberg & Klockner, 1982). Briefly, rats weighing 150–200 g were anaesthetized by ether, and the hearts were quickly removed and rinsed in Ca^{2+} free Tyrode solution. Using a Langrendorff technique, collagenase (Yakult, Tokyo) of 50 units ml^{-1} was perfused approximately 15–20 min at 37°C. Ventricular muscles were minced by scissors, dispersed with gentle agitation in KB (kraftbrühe) medium and filtered through 200 μm nylon mesh. Cells were stored in KB medium. More than 80% of myocytes were rod shaped, viable cells. Sixty-five per cent of total isolated myocytes were tolerant to 1.8 mM of extracellular Ca^{2+}. The Tyrode solution contained (in mM); NaCl 135.0, KCl 5.4, NaH_2PO_4 0.3, HEPES 5.0, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.6. The pH was adjusted to 7.4 with NaOH. KB medium contained (in mM); L-Glutamic mono K 50.0, Taurine 10.0, KCl 25.0, KH_2PO_4 10.0, EGTA 0.5, HEPES 10.0, $MgCl_2 \cdot 6H_2O$ 3.0, glucose 10.0. The pH was adjusted to 7.2 with KOH.

Reverse transcription (RT)–PCR assay

RT–PCR analysis was performed as described previously (Hirasawa *et al*., 1993; Horie *et al*., 1993). Briefly, total RNA extracted from enzymatically isolated ventricular myocytes was treated with RNase-free DNase I (Stratagene, La Jolla, CA, U.S.A.), and 10 μg of each sample was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (GIBCO–BRL, Gaithersburg, MD, U.S.A.). After heating at 94°C for 5 min to terminate the reactions, samples were stored at −20°C. Oligonucleotide primers were constructed from the published cDNA sequences.

The sequences of the α_{1a}AR primers were, 5′-CATCGTGGTCGGCTGCTTCGTCCTCTGCTG-3′ (coding sense) corresponding to bases 1261–1290 of the cloned full-length sequence, 5′-TCCCAC(CG)GG(AG)ATGCG(AC)AC-CA(GT)GTCCTTGTG-3′ (anticoding sense) which anneals to bases 1576–1547 (21).

The sequences of the α_{1b}AR primers were, 5′-TATGTGC-CATCTCCATTGACCGCTAC-3′ (coding sense) corresponding to bases 645–672 of the cloned full-length sequence, 5′-ATGAAGAAGGGGAGCCAACATAAGATGAA-3′ (anticoding sense) which anneals to bases 1175–1147 (Cotecchia *et al*., 1988).

The sequences of the α_{1d}AR primers were, 5′-GGCAAGGCCTCCGAGGTGGT-3′ (coding sense) corresponding to bases 1286–1305 of the cloned full-length sequence, 5′-ACGACGATGGCCAACGTCTTGGCA-3′ (anticoding sense) which anneals to bases 1458–1435 (Lomasney *et al*., 1991).

The sequences of the GAPDH primers was 5′-TCCCTCAAGATTGTCAGCAA-3′ (coding sense) corresponding to bases 506–525 of the cloned full-length sequence, 5′-AGATCCACAACGGATACATT-3′ (anticoding sense) corresponding to bases 795–814 (Ford *et al*., 1994).

Each reverse transcription mixture was diluted 1 : 5 in RNase-free water and 2 μl were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 mM of each primer) spanning the given sequence for amplification, 200 μM of each dNTP (dATP, dCTP, dGTP, dTTP), KCl 50 mM, Tris-HCl 10 mM (pH 8.3), $MgCl_2$, 10 mM, 0.01% (w v^{-1}) gelatin and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus Corporation, Norwalk, CT, U.S.A.) in a final volume of 25 μl. The reaction mixture was then overlaid with 3 drops ($\sim$50 μl) of mineral oil and amplified for 30 cycles in a Perkin Elmer Cetus thermal cycler (Norwalk, CT, U.S.A.). The amplification profiles consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. cDNA or α_{1a}AR inserted into pBluescript II KS(+) were positive controls for α_{1a}AR, while genomic DNA fragments from isolated rat ventricular myocytes were amplified as positive controls for α_{1b}AR, α_{1d}AR and GAPDH. Negative control reactions without template were routinely included in PCR amplifications with both primer sets. The predicted sizes of the amplified PCR products for α_{1a}AR, α_{1b}AR, α_{1d}AR and GAPDH were 316, 531, 173 and 309 bp, respectively. PCR products were analysed by polyacrylamide gel electrophoresis and visualized by ethidium bromide.

Sequencing

Each PCR product was purified after the agarose gel electrophoresis by the SUPREC-01 (TaKaRa, Kyoto, Japan), blunted by T4 polymerase and subcloned into the *Eco*RV site of Bluescript II KS(+) plasmid. They were sequenced by the Genesis 2000 DNA analysis system (Du Pont Medical Products, Wilmington, DE, U.S.A.) to confirm the PCR products obtained be the same as the published cDNA clone.

[^{125}I]-HEAT binding

Crude particulate fractions were prepared from rat ventricles by the following procedure. The ventricles were washed twice with phosphate-buffered saline, minced by scissors in buffer A (in mM: sucrose 250, EDTA 5, $MgCl_2$ 1, pH 7.6), and homogenized. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4°C to remove the nuclei. Then, the pellet was resuspended and centrifuged at $20{,}000 \times g$ for 10 min at 4°C. The supernatant was discarded. The pellet, the membrane fraction, was resuspended to the final protein concentration of 0.9 mg ml^{-1} in 50% glycerol incubation solution (in mM: Tris-HCl 50.0, EGTA 5.0, $MgCl_2$ 12.5, pH 7.4). It was frozen at −80°C until binding assay. The protein concentration was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, U.S.A.).

2-{β-(4-hydroxy-3 [^{125}I]-iodophenyl) ethylaminomethyl} tetralone ([^{125}I]-HEAT) binding assay was carried out, using the incubation solution, Buffer B, which contained (in mM); Tris-HCl 50.0, EGTA 5.0, $MgCl_2$ 12.5, pH 7.4 at 25°C. Measurement of specific [^{125}I]-HEAT binding was performed by incubating 100 μl of membrane preparation with [^{125}I]-HEAT (2200 Ci mmol^{-1}) in a final volume of 250 μl buffer B for 60 min at 25°C in the presence or absence of competing drugs. The reaction was terminated by adding ice-cold washing buffer (in mM: Tris-HCl 50.0, $MgCl_2$ 12.5, pH 7.4 at 4°C) and immediately filtering through Whatmann GF/C glass fibre filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, U.S.A.). The radioactivity of the wet filters was determined in a gamma-counter at an efficiency of 85%. 10 μM phentolamine was used to define non specific binding. All assays were conducted in duplicate.

CEC treatment

Isolated myocytes were suspended to the density of 5×10^5 cells ml^{-1} in KB medium, and incubated at 30°C for 30 min with or without 100 μM CEC. After the incubation, the myocytes were washed three times with KB medium, and centrifuged at $1000 \times g$ for 3 min. The myocytes were re-suspended and used for electrophysiological recordings. For radioligand binding assay the myocytes were pelleted by centrifugation at $1000 \times g$ for 3 min and the pellet was homogenized in 2 ml of ice-cold buffer A and centrifuged at $1000 \times g$ at 4°C for 10 min to remove nuclei. The supernatant was then centrifuged at $35{,}000 \times g$ for 20 min at 4°C and the pellet was homogenized, and was frozen at −80°C until assay.

Electrophysiological recordings

Voltage clamp experiments were carried out with single patch electrode in the whole cell clamp mode (Hamil *et al*., 1981). Pipettes with an 1-2 MΩ resistance were pulled from aluminosilicate capillary glass using a programmable multi-step puller (Model P-87, Sutter Instrument, U.S.A.). The recording electrodes were filled with the solution containing (mM), KCl 150, $MgCl_2$ 1, ATP 4, EGTA 5, HEPES 5. Solution for superfusion contained (in mM): NaCl 135.0, KCl 5.4, NaH_2PO_4 0.3, HEPES 5.0, $MgCl_2$ 0.5, $CaCl_2$ 1.8, $CdCl_2$ 0.2, glucose 5.6. A T-2000 voltage clamp amplifier (Act ME, Tokyo, Japan) was used (Narahashi *et al*., 1987). Pulse generation and data acquisition were controlled by a Compaq PC computer running pCLAMP software Ver. 5.6 (Axon Instruments, Foster City, CA, U.S.A.), which was interfaced through a 100-kHz Labmaster board (Scientific Solutions, Cleveland, OH, U.S.A.). All experiments were performed at room temperature (22°C) under the perfusion of 1 μM propranolol (Sigma) and 10–20 μM tetrodotoxin (Wako, Osaka, Japan).

We defined '4-AP sensitive component of I_{To}' as the difference after 4-AP insensitive current was subtracted from control or reference current (Apkon & Nerbonne, 1991). We defined 'peak amplitude of I_{To}' or 'I_{To} peak' as an absolute value at the peak of outward current. We also defined 'peak amplitude of 4-AP sensitive component of I_{To}' as the maximum value of 4-AP sensitive component of I_{To}. Effects of α_1AR agonists and antagonists on the I_{To} were expressed by the '% reduction of I_{To}' against 4-aminopyridine (4-AP)-sensitive component. The α_1AR-mediated per cent reduction of I_{To} was calculated as $p/q \times 100$ (%), where p represents the reduced amplitude of I_{To} by α_1AR agonist methoxamine, while q represents the reduced amplitude of I_{To} by 4 mM 4-AP, i.e. the amplitude of 4-AP sensitive component of I_{To}.

Data analysis

Analysis of competition date were performed by LIGAND (Munson & Rodbard, 1980), a nonlinear curve-fitting program. The presence of one, two, or three different binding sites was assessed by using *F*-test in the program. The model adopted was that which provided the significantly best fit ($P < 0.05$). All results are shown as the means ± s.e.mean of the given numbers (n) of experiments. Significance of differences was determined with ANOVA following *post hoc* tests.

Chemicals

The following drugs were used: [^{125}I]-HEAT (2-[β-[4-hydroxy-3-[^{125}I]-iodo-4-hydroxyphenyl]-ethyl-aminomethyl] tetralone) (specific activity 2200 Ci mmol^{-1}) (NEN, Boston, MA, U.S.A.); KMD-3213 dihydrobromide, ((−)-(R)-1-(3-hydroxypropyl)-5-[2-[2-[2-(2,2,2-trifluoroethoxy) phenoxy]ethylamino]propyl] indoline-7-carboxamide dihydrobromide) (Kissei Pharmaceutical Co., Matsumoto, Japan); phentolamine hydrochloride (CIBA-Geigy, Summit, NJ, U.S.A.); prazosin hydrochloride (Pfizer, Groton, CT, U.S.A.); CEC, 5-MU and WB4101 (2-[2,6-dimethoxyphenoxyethyl]-aminomethyl-1,4 benzodioxan) (Research Biochemicals, Natick, MA, U.S.A.); methoxamine (Sigma, St. Louis, MO, U.S.A.); (+)-niguldipine hydrochloride (Byk Gulden, Konstanz, Germany). All other chemicals were of reagent grade.

Results

Reverse transcription–PCR (RT–PCR)

Expression of α_1AR subtype mRNA in isolated rat ventricular myocytes was examined with RT–PCR. As shown in Figure 1, PCR products of α_1ARs were identical to the predicted sizes. In all samples examined, GAPDH–PCR products was observed. Also, we confirmed that the sequences of the PCR products obtained were found to be identical to nucleotide positions 1261–1576 of rat α_{1a}AR cDNA, 645–1175 of rat α_{1b}AR cDNA and 1286–1458 of rat α_{1d}AR cDNA, respectively (data not shown). To ensure that the amplified products were originated from mRNA rather than from contaminating genomic DNA, we compared PCR products with or without the reverse transcription reaction, and found that the PCR products of each α_1AR was not detected without the reverse transcription reaction (data not shown).

Radioligand binding study

Inhibition of specific [^{125}I]-HEAT binding by several α_1AR antagonists was examined in membrane preparation from rat ventricles (Table 1). A competitive binding curve for a non-selective α_1AR antagonist, prazosin, was best fitted by a one-site model (Table 1). On the other hand, nonlinear regression analysis by Ligand showed that inhibition curves for α_1AR subtype-selective antagonists including a potent α_1AR selective antagonist KMD-3213 (Shibata *et al*., 1995) in rat ventricles best fit a two-site model ($P < 0.05$, vs a one-site model), with approximately ~24–32% high and ~68–76% low affinity sites (Table 1).

To further characterize these affinity sites, binding studies were performed in isolated ventricular myocytes treated with

and without 100 μM CEC before preparing membrane. Without CEC treatment (control), the inhibition of specific [^{125}I]-HEAT binding by KMD-3213 in membrane preparations from isolated myocytes best fitted to a two-site model ($P<0.05$, vs a one-site model), with $22\pm3\%$ high and $78\pm3\%$ low affinity sites ($n=3$) (Table 2, which is similar to that obtained in membrane preparations from rat ventricular tissue (Table 1). Pretreatment of isolated myocytes with 100 μM CEC caused a 67.7% decrease ($n=2$) in the B_{max} of [^{125}I]-HEAT binding sites, and completely eliminated low affinity sites for KMD-3213 (Table 2).

Whole cell voltage clamp study

Figure 2D shows the current–voltage relationship between the 4-AP sensitive component of I_{To} and membrane potentials at depolarizing pulse. I_{To} is observed in the potential range positive to -30 mV. Methoxamine (100 μM) decreased the peak amplitude of the 4-AP sensitive component of I_{To} by 20–22%.

Figure 3 shows the effects of methoxamine on I_{To} in the absence (panel A and B) and presence (panel C and D) of 1 μM prazosin. As shown in Figure 3A,B, 100 μM methoxamine significantly reduced the peak amplitude of I_{To} (per cent reduction of I_{To} was $21\pm0.9\%$, $n=8$), which inhibitory effect was reversed after washout of methoxamine. As shown in Figure 3D,E,F, the reduction of I_{To} by methoxamine was completely inhibited by 1 μM prazosin (per cent reduction of I_{To} was $2.2\pm1.7\%$, $n=6$).

As shown in Figure 4, methoxamine decreased I_{To} in a concentration-dependent manner. As summarized in Figure 4C, however, 1 μM prazosin did not block the methoxamine-induced reduction of I_{To} above 300 μM methoxamine, suggesting that higher concentrations of methoxamine (above 300 μM) might cause a non-α_1AR-mediated effect on I_{To}. Thus, 100 μM of methoxamine was used in the following electrophysiological studies.

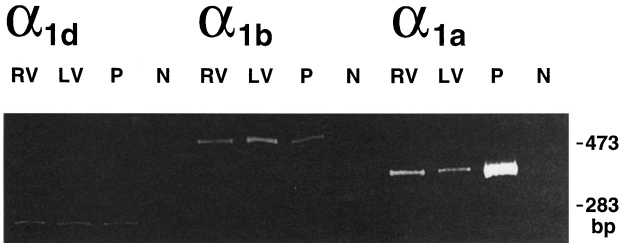

Figure 1 Reverse Transcription-PCR analysis of α_{1a}AR, α_{1b}AR, α_{1d}AR and GAPDH mRNA in isolated rat ventricular myocytes. mRNA samples from isolated right (RV) and left (LV) ventricular myocytes were reverse transcribed to cDNA. As positive controls (P), genomic DNA from isolated ventricular myocytes was served for α_{1b}AR, α_{1d}AR and GAPDH, and cDNA of α_{1a}AR inserted to plasmid was served for α_{1a}AR. Reaction mixture without template was served as negative controls (N). Samples were amplified by PCR, as described in Methods. PCR products were analysed on polyacrylamide gel and stained by ethidium bromide.

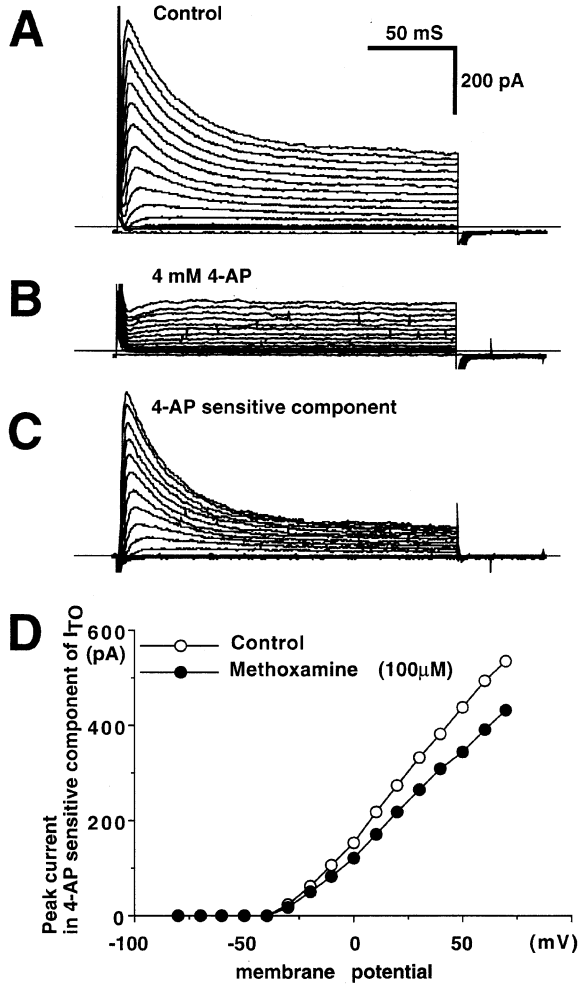

Figure 2 Effects of methoxamine on current–voltage relationship of 4-AP sensitive I_{To}. Myocytes were clamped at a holding potential of -80 mV and then 200 ms-depolarizing pulses were applied to various potential (from -70 to 70 mV) by 10 mV step. Panels A and B show current tracings in control (panel A) and in the presence of 4 mM 4-AP (panel B). The current tracings in panel C show 4-AP sensitive components of I_{To}, which were given after the currents in presence of 4-AP (panel B) were subtracted from the currents in control (panel A). Panel D shows the relationship between the peak amplitude of 4-AP sensitive components of I_{To} and membrane potentials at depolarizing pulses in the absence and presence of 100 μM methoxamine.

Table 1 Inhibition of [^{125}]-HEAT binding by α_1 antagonists in rat ventricular membrane

Compound	K_H (nM)	K_L (nM)	R_H %	R_L %	P *value*
WB 4101	0.038 ± 0.001	7.5 ± 1.1	24 ± 2	76 ± 2	<0.05
5-Methylurapidil	1.3 ± 0.55	120 ± 15	31 ± 2	69 ± 2	<0.05
(+) Niguldipine	6.7 ± 3.2	120 ± 27	26 ± 3	74 ± 3	<0.05
KMD-3213	0.52 ± 0.21	31 ± 2.9	32 ± 2	68 ± 2	<0.05
Prazosin	0.041 ± 0.016	–	100	–	N.S.

Inhibition of specific [^{125}]-HEAT binding by α_1AR antagonists was determined in membrane preperations from rat ventricles as described. Each value is the mean = standard error of 3–5 different experiments. N.S. = Not significant.

Table 2 Effects of CEC treatment on inhibition of [^{125}]-Heat binding by KMD-3213 in isolated rat ventricular myocytes

Compound	K_H (nM)	K_L (nM)	R_H %	R_L %	P *value*
Control	0.12 ± 0.03	13 ± 3	22 ± 3	78 ± 3	<0.05
CEC (100 μM)	0.21 ± 0.03	–	100	–	N.S.

Inhibition of specific [^{125}]-HEAT binding by KMD-3213 was determined in membrane preperations from isolated rat ventricular myocytes as described. The best two-site fit was determined by nonlinear regression analysis of the averaged curve, and R_H and R_L were determined as described. The *P* value for the best two-site fit compared with the best one-site fit is given. Each value is the mean $\pm$ s.e.mean of three different experiments. N.S. = Not significant.

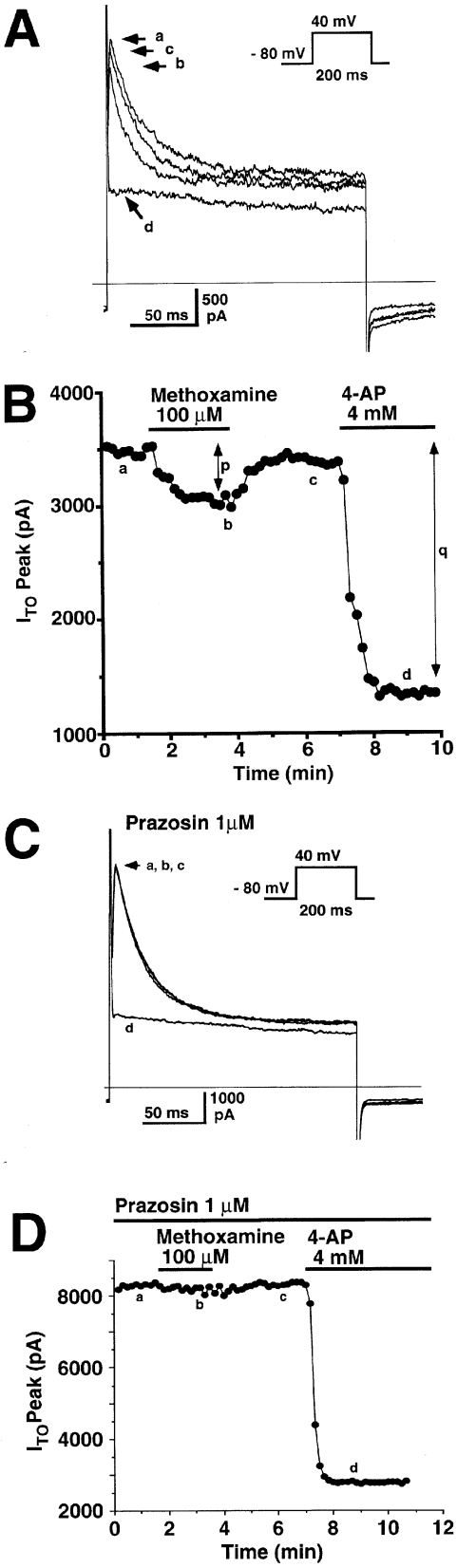

Figure 3 Effects of methoxamine on I_{To} in isolated rat ventricular myocytes. The effects of methoxamine on I_{To} in the absence (panels A and B) and presence (C and E) of 1 μM prazosin were shown, respectively. After control (a), 100 μM methoxamine was superfused (b), and then washed out (c). At the end of experiments, 4 mM 4-AP was superfused (d). Panel A: Current tracings of I_{To}. Membrane voltage was held at -80 mV and depolarized to 40 mV for 200 ms. Thin horizontal line was 0 pA level. Panel B: Time-course of the peak amplitude of I_{To}. An arrow p represents the reduced amplitude of I_{To} by methoxamine, while an arrow q represents the reduced amplitude of I_{To} by 4 mM 4-AP. Per cent reduction of I_{To} was calculated as p/q $\times$ 100 (per cent). Panel C: Current tracings of I_{To} in the presence of 1 μM prazosin. Voltage clamp protocol was the same as panel A. Panel D: Time-course of the peak amplitude of I_{To}. Prazosin (1 μM) abolished the reduction of I_{To} by 100 μM methoxamine. In panels B and D, vertical axis represents an absolute maximum value of outward current as 'I_{To} peak'.

α_1AR subtypes and I_{To} reduction

Next, we examined the effects of α_{1A}AR subtype-selective competitive antagonists of KMD-3213 and 5-MU, and α_{1B}AR-alkylating agent CEC on the methoxamine-induced reduction in I_{To} (Figures 5 and 6). One hundred μM decreased I_{To} ($21\pm0.9\%$ reduction of I_{To}, $n=6$) (Figure 5A). Low concentration (1 nM) of KMD-3213 did not affect the methoxamine-induced reduction of I_{To} (Figure 5B; $22\pm2.1\%$ reduction of I_{To}, $n=6$); however, higher concentration (1 μM) of KMD-3213 abolished the methoxamine-induced effect (Figure 5C; $0.2\pm0.6\%$ reduction of I_{To}, $n=6$).

The CEC pretreatment of myocytes (100 μM, 30 min) significantly ($P<0.05$) inhibited the methoxamine-induced

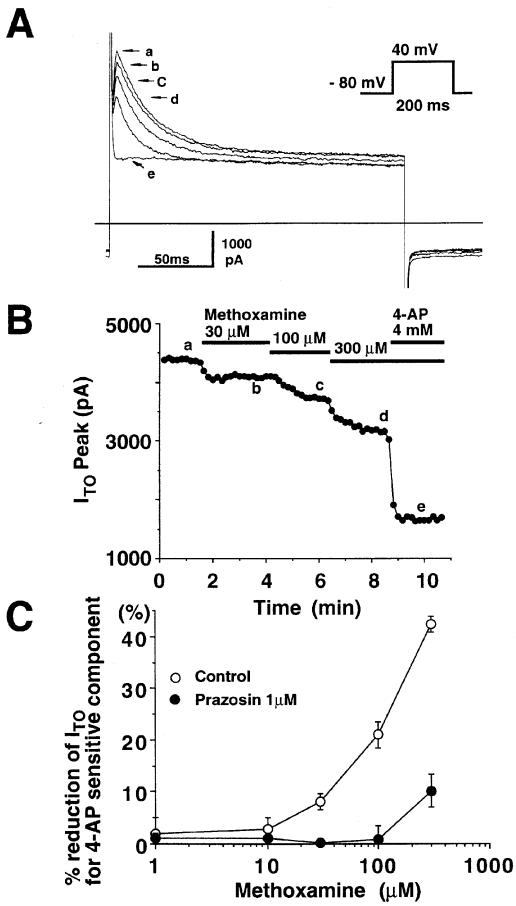

Figure 4 Concentration-dependent reduction of I_{To} by methoxamine. Panels A and B: After control (a), methoxamine was superfused in a cumulative fashion ((b) 30 μM, (c) 100 μM, (d) 300 μM), and at the end of experiment 4 mM 4-AP was superfused (e). Panel A: Current tracings of I_{To} ((a) control, (b) methoxamine 30 μM, (c) 100 μM, (d) 300 μM, (e) 4-AP 4 mM) were superimposed. Command pulse was applied from -80 to 40 mV for 200 ms. Panel B: Time-course of the effect of the methoxamine on the peak amplitude of I_{To}. The concentration of methoxamine was increased from 30 to 300 μM. Panel C: Concentration-dependent reduction of I_{To} by methoxamine in the absence and presence of 1 μM prazosin, respectively. Note that 1 μM prazosin did not completely inhibit the 300 μM methoxamine-induced reduction of I_{To}. Each value is the mean$\pm$s.e.mean of 4–6 different experiments.

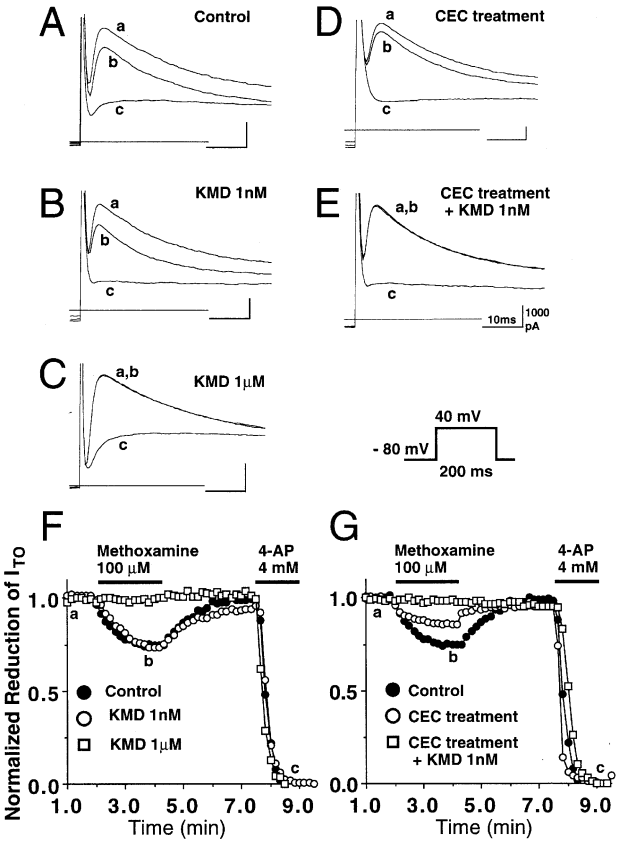

Figure 5 Effects of KMD-3213 and CEC treatment on the methoxamine-induced reduction of I_{To}. After control (a) 100 μM methoxamine (b) was superfused, and washed out. Then, 4 mM 4-AP (c) was superfused. Panels were obtained from myocytes without (A, B and C) and with (D and E) CEC pretreatment, respectively. Panels A, B and C show the superimposed tracings of I_{To} obtained in control (A), in the presence of 1 nM KMD-3213 (B), and in the presence of 1 μM KMD-3213 (C), while panels D and E show those obtained in CEC treatment alone (D), and CEC treatment plus 1 nM KMD-3213 (E), respectively. Voltage clamp pulse was applied from -80 to 40 mV for 200 ms. All calibration bars in panels A–E represent 10 ms and 1000 pA. Panel F: The time-course of the effect of methoxamine in control (filled circle) and in the presence of 1 nM KMD-3213 (unfilled circle) and 1 μM KMD-3213 (unfilled square). Amplitude of I_{To} was normalized with 4-AP-sensitive component as unity. Normalized reduction of I_{To} was plotted every 10 s. Panel G: The time-course of the effect of methoxamine in control (without CEC treatment; filled circle), and in myocytes pretreated by CEC in the absence (unfilled circle) and the presence of 1 nM KMD-3213 (unfilled square), respectively. Amplitude of I_{To} was normalized with 4-AP-sensitive component as unity. Normalized reduction of I_{To} was plotted every 10 s.

reduction of I_{To} (11 ± 0.7% reduction of I_{To}, $n=6$) (Figure 5D). Furthermore, the remaining response was completely inhibited by adding low concentration (1 nM) of KMD-3213 (1.2 ± 0.2% reduction of I_{To}, $n=6$). The effects of low and high concentrations of α_{1A}AR-selective antagonists of KMD-3213, 5-MU, and non-selective α_1AR antagonist prazosin, and CEC treatment on 100 μM methoxamine-induced per cent reduction of I_{To} are summarized in Figure 6.

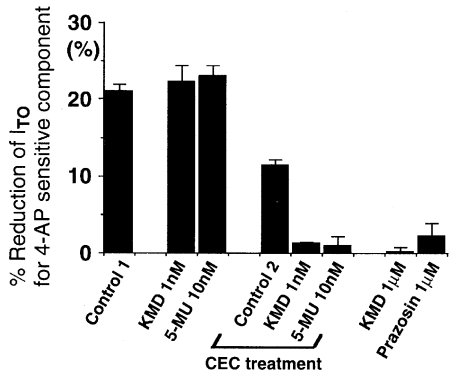

Figure 6 Effects of α_1AR antagonists and CEC treatment on the methoxamine-induced reduction of I_{To}. 100 μM methoxamine was superfused in the absence (Control 1), in the presence of antagonists (KMD-3213 1 nM and 1 μM, 5-MU 10 nM, prazosin 1 μM), with CEC treatment (Control 2), with CEC treatment and KMD-3213 1 nM (CEC + KMD-3213 1 nM) and with CEC treatment and 5-MU 10 nM (CEC + 5-MU 10 nM). The values were 21.0 ± 0.9 for Control 1, 22.1 ± 2.1 for 1 nM KMD-3212, 23.0 ± 1.3 for 10 nM 5-MU, 11.4 ± 0.7 for Control 2, 2.5 ± 0.2 for CEC treatment and 1 nM KMD-3213, 2.2 ± 1.2 for CEC treatment and 10 nM 5-MU, 0.2 ± 0.6 for 1 μM KMD-3213 and 2.2 ± 1.7 for 1 μM prazosin. Each value is the mean $\pm$ s.e.mean of 6–8 different experiments.

Discussion

Three α_1AR subtypes encoded by three distinct cDNAs (α_{1a}, α_{1b} and α_{1d}) are identified by molecular cloning studies (Cotecchia *et al*., 1988; Schwinn *et al*., 1992; Perez *et al*., 1991; Lomasney *et al*., 1991; Hirasawa *et al*., 1993) and two α_1AR subtypes (α_{1A} and α_{1B}) are defined based on pharmacological studies (Morrow *et al*., 1986; Han *et al*., 1987; Gross *et al*., 1988; Tsujimoto *et al*., 1989). Recent studies reveal that pharmacological α_{1A}AR and α_{1B}AR are encoded by α_{1a} and α_{1b}, respectively (Laz *et al*., 1994; Perez *et al*., 1994; Ford *et al*., 1994; Price *et al*., 1994), but the physiologic role of the receptor encoded by α_{1d} remains unresolved. In isolated rat ventricular myocytes, we identified mRNA of α_{1a}, α_{1b} and α_{1d} by a RT–PCR assay and distinguished two pharmacological α_1AR subtypes by a radioligand binding assay using α_1 subtype selective antagonists. The population of α_{1A}AR and α_{1B}AR are approximately 30% and 70% (Table 1). We used four α_{1A}AR selective antagonists and one α_{1B}AR selective antagonist. 5-MU, (+) niguldipine and WB4101 are α_{1A}AR selective antagonists which have been characterized by pharmacological studies and KMD-3213 is a recently developed α_{1A}AR selective antagonist which is potent to discriminate α_{1A}AR from α_{1B}AR (Shibata *et al*., 1995). CEC, an irreversible α_{1B}AR antagonist, alkylates α_{1B}AR and α_{1D}AR more than α_{1A}AR (Laz *et al*., 1994). When the K_i values of α_1 antagonists for rat cloned α_1ARs are compared, WB4101 could distinguish α_{1A}AR and α_{1D}AR from α_{1B}AR but could not differentiate α_{1A}AR from α_{1D}AR, and 5-MU and (+) niguldipine could distinguish α_{1A}AR from α_{1B}AR or α_{1D}ARs but could not differentiate α_{1D}AR from α_{1B}AR (Laz *et al*., 1994). In our results (Table 1), the population ratio of R_H:R_L for WB4101 is 24 : 76 (per cent), and the ratio for 5-MU and (+) niguldipine is 26–31 : 69–76 (per cent). KMD-3213 can differentiate α_{1A}AR from α_{1B}AR or α_{1D}AR because the high affinity site for KMD-3213 is resistant to the alkylation by CEC treatment and the low affinity site is sensitive to CEC as shown in Table 2. The population ratio of R_H:R_L for KMD-3213 is 22 : 78 (per cent). The R_H for WB4101, which may include both α_{1A}AR and α_{1D}AR, is nearly equal to or lower than the R_H for 5-MU, (+) niguldipine and KMD-3213, which may include only α_{1A}AR. This result suggests that the population of α_{1D}AR is very small in protein level. There are two reports in which α_1AR mRNA is relatively quantified in rat heart by RNAse protection assay. Price *et al*. (1994) reported that each expression of α_1AR mRNA is in the following order, α_{1b} $(++++)>>\alpha_{1a}$

(++)>α_{1d} (+). Stewart *et al.* (1994) reported the ratio of α_{1a}:α_{1b}:α_{1d} in mRNA was 1.4:1:0.6. These studies revealed that α_{1d} is the smallest in mRNA level. Although protein expression may not be proportional to mRNA expression, the population of α_{1D}AR would be too small to be discriminated from α_{1B}AR by radioligand binding assay, though α_{1d} mRNA could be detected by RT–PCR assay. Thus, taken together with the results of RT–PCR and radioligand binding studies, pharmacological α_{1A}AR and α_{1B}AR detected by radioligand binding study in rat ventricular myocytes may reflect the expression of α_{1a}AR and α_{1b}AR with a small population of α_{1D}AR, respectively. Thus, the K_i values of α_1 antagonists (K_H and K_L) obtained from radioligand binding assay are considered to represent the K_i for α_{1A}- and α_{1B}-ARs, respectively. In fact, the estimated affinity of KMD-3213 at the two sites in rat heart (K_H=0.52 nM and K_L=32 nM) are in good agreement with those obtained in rat submaxillary gland (α_{1A}AR-expressing tissue) and liver (α_{1B}AR-expressing tissue), respectively (Shibata *et al.*, 1995). Thus, the K_i values of α_1 antagonists (K_H and K_L) obtained from radioligand binding assay may represent the K_i for α_{1A}- and α_{1B}-ARs, respectively, and we used the concentration of each drug which can be considered to block each α_1AR subtype selectively in the following electrophysiological studies to determine the effect of each α_1AR subtype on I_{To}.

Depolarization-activated K currents in rat ventricular myocytes are classified into two kinetically distinct voltage dependent K currents (Apkon & Nerbonne, 1991): one that activates and inactivates rapidly, and can be preferentially attenuated by 4-AP, referred to I_{To}. Another K current, referred to I_K, activates and inactivates slowly, and can be preferentially attenuated by tetraethyl ammonium (TEA). First, we confirmed their observation (Figure 2); thus, we observed that depolarization-activated K current in rat ventricular myocytes consisted of a time-dependent, rapidly inactivating, 4-AP-sensitive component (Figure 2C) and also a non-inactivating component on which 4-AP had little effect (Figure 2B). As a 4-AP sensitive component mainly contributes to the peak amplitudes of I_{To}, and 4-AP attenuate the prolongation of action potential duration by phenylephrine in rat papillary muscles (Tohse *et al.*, 1990), we examined 4-AP-sensitive (rather not TEA-sensitive) component to estimate accurately the effect of α_1AR stimulation on I_{To} in this study.

The degree of I_{To} reduction by α_{1A}AR stimulation is extremely changed by CEC treatment, selective inactivation of α_{1B}AR . Before CEC treatment the lower concentrations of KMD-3213 (1 nM) and 5-MU (10 nM), which can selectively block α_{1A}AR, did not affect the reduction of I_{To} by methoxamine; however, after CEC treatment the same concentrations of these antagonists abolished the reduction of I_{To}. Hence, the role of α_{1A}AR can be manifested when α_{1B}ARs are inactivated. The α_{1A}AR certainly couples to the I_{To} reduction and the contribution of α_{1A}AR is approximately 54% of α_{1B}AR in our experimental condition. These findings suggest that the effect mediated by α_{1B}AR can apparently predominate when both α_{1A} and α_{1B}ARs are stimulated. In fact, previous reports showed that the role of α_{1A}AR is minor (Fedida *et al.*, 1990; Wenge *et al.*, 1994).

Our results indicate that stimulation of either α_{1A}AR (α_{1A}AR or α_{1B}AR) reduces I_{To} and that α_{1B}AR plays a predominant role in reducing I_{To}. The contribution of α_1AR subtypes to I_{To} is still in discussion (Wang *et al.*, 1991; Nagashima *et al.*, 1996). The different interpretation seems to rise from the condition in which α_1AR subtype selective antagonists were used. Wang *et al.* (1991) concluded that simultaneous stimulation of both α_{1A}AR and α_{1B}AR is required for the reduction in peak amplitude of I_{To}. In our study, the K_i values of α_{1A}AR and α_{1B}AR in rat ventricular myocytes were 1.3 nM and 120 nM for 5-MU, 6.7 nM and 120 nM for (+) niguldipine and 0.12–0.52 nM and 13–31 nM for KMD-3213 (Tables 1 and 2). The occupancy of either subtype at the antagonist concentration used can be calculated using the formula:

$$R = 100/(1 + K_i/L),$$

where R is the occupancy (per cent) of receptor, K_i is the dissociation constant and L is the concentration of antagonist (Chang *et al.*, 1975). The low concentration of KMD-3213 (1 nM) and 5-MU (10 nM), used in our electrophysiological study, yields approximately 90% occupancy at α_{1A}AR and less than 10% occupancy at α_{1B}AR. The concentrations of 5-MU (100 nM) and (+)niguldipine (100 nM), which Wang *et al.* (1991) used to block α_{1A}AR stimulation, yields more than 90% occupancy at α_{1A}AR and approximately 45% occupancy at α_{1B}AR. Since about half of α_{1B}ARs can be blocked in their experimental condition, the effects of α_{1B}AR stimulation on the I_{To} reduction might be underestimated due to the undesirable occupancy at α_{1B}AR by α_{1A}AR selective antagonists.

In rat and rabbit papillary muscles, the α_1ARs have been shown to cause the prolongation of action potential duration and increase Ca^{2+} influx as a result of inhibition of I_{To}, leading to positive inotropic action. Previous studies examining the contribution of each α_1AR subtype in developing I_{To} showed inconsistent observations: thus, Nagashima *et al.* (1996) reported that the α_{1A}AR subtype, rather than α_{1B}AR, caused inhibition of I_{To} in rat and rabbit papillary muscles, while Wang *et al.* (1991) showed that stimulation of both α_{1A}AR and α_{1B}AR subtypes contributed to the α_1AR-mediated reduction in I_{To} of isolated rat myocytes. In this study, on the other hand, we observed that α_{1B}ARs predominantly mediated α_1AR-induced effect, although both α_{1A}AR and α_{1B}AR subtypes are coupled to the cardiac I_{To}. One of major reasons for these apparently discrepant observations would be due to the lack of highly subtype-selective ligands. Using highly α_{1A} AR-selective antagonists including KMD-3213, we could successfully separate the two subtypes in the present study. Additionally, we measured the 4-AP-sensitive component of I_{To}, not the whole depolarizing outward current, in this experiment. To investigate the possible effect of any drug or ligand on I_{To}, the current must be separated adequately. In this regard, we viewed that this has not been performed properly in previous studies, which may lead to the apparently discrepant conclusions.

By performing radioligand binding assay and electrophysiologic study in isolated myocytes, our present work demonstrates that both α_{1A}- and α_{1B}ARs are co-expressing in rat ventricular myocytes in population ratio of approximately 30:70, and that both α_{1A}AR and α_{1B}AR are involved in the reduction of I_{To}. The interaction between α_1AR subtypes and the signal transduction mechanism(s) from each subtype receptor to I_{To} requires further study.

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(Received November 1, 1999
Accepted December 21, 1999)