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Inhibitory effect of amiodarone on Na^+/Ca^{2+} exchange current in guinea-pig cardiac myocytes

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1 The effect of amiodarone on Na^+/Ca^{2+} exchange current (I_{NCX}) was examined in single guinea-pig ventricular myocytes using the whole-cell voltage clamp technique.

2 I_{NCX} was recorded by ramp pulses from the holding potential of −60 mV in the presence of 140 mM Na^+ and 2 mM Ca^{2+} in the external solution, and 20 mM Na^+ and 398 nM free Ca^{2+} (19 mM Ca^{2+} and 30 mM BAPTA) in the internal solution.

3 External application of amiodarone suppressed I_{NCX} in a concentration-dependent manner. The IC_{50} value was 3.3 μM with a Hill coefficient of 1.

4 Intracellular application of trypsin *via* the micropipette attenuated the blocking effect of amiodarone, suggesting that amiodarone affects the cytoplasmic side of the molecule.

5 This inhibitory effect of amiodarone on the Na^+/Ca^{2+} exchanger may contribute to the cardioprotective action of the drug.

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Abbreviations: DMSO, dimethylsulphoxide; I_{NCX}, Na^+/Ca^{2+} exchange current; I-V curve, current-voltage relation curve; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulphonate

Introduction

Amiodarone is the most promising drug in the treatment of life-threatening supraventricular and ventricular tachyarrhythmias, and consequently the prevention of sudden cardiac death (reviews by Singh, 1994; Kodama *et al*., 1997; 1999). Amiodarone is a class III antiarrhythmic drug, but it also has class I, II and IV antiarrhythmic drug properties in the Vaughan Williams classification. In electrophysiological experiments on single cardiac myocytes, acute effects of amiodarone have been reported to block Na^+ channels (Follmer *et al*., 1987), L-type Ca^{2+} channels (Nishimura *et al*., 1989), two voltage-gated K^+ channels, i.e., the delayed rectifier K^+ current (Balser *et al*., 1991) and the inward rectifier K^+ current (Sato *et al*., 1994), and three ligand-gated K^+ channels i.e., the muscarinic acetylcholine receptor-operated K^+ current (Watanabe *et al*., 1996), the ATP-sensitive K^+ current (Takizawa & Nakaya, 1997) and the Na^+-activated K^+ current (Mori *et al*., 1996). However, effects of amiodarone on Na^+/Ca^{2+} exchange current (I_{NCX}) have not been examined.

Na^+/Ca^{2+} exchange is a major mechanism for extruding Ca^{2+} in cardiac myocytes (Blaustein & Lederer, 1999). The driving force of Na^+/Ca^{2+} exchange is the Na^+ concentration gradient. Therefore an increase in intracellular Na^+ concentration caused by digitalis, for example, impairs Ca^{2+} efflux by this transporter, accumulates Ca^{2+} under the membrane and subsequently leads to enhanced force of contraction of cardiac myocytes. Aomine & Fukui (1993) found that amiodarone decreased ouabain-induced developed tension in ventricular papillary muscles from guinea-pig, and predicted that amiodarone would inhibit Na^+/Ca^{2+} exchange. I_{NCX} can be recorded using the whole-cell patch clamp technique by loading Na^+ and Ca^{2+} in both internal and external solutions (Kimura *et al*., 1986; 1999). Recently a relatively selective Na^+/Ca^{2+} exchange inhibitor, KB-R7943, has been developed (Watano *et al*,. 1996; Iwamoto *et al*., 1996; Kimura *et al*., 1999), which enables identifying the exchange current as a KB-R7943 sensitive current. In the present study, we examined the effect of amiodarone on I_{NCX} in single guinea-pig ventricular cells studied under whole-cell voltage clamp conditions.

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Methods

Cell isolation

All experiments were performed under the regulation of the Animal Research Committee of the School of Medicine, Fukushima Medical University. Single ventricular cells were isolated from the guinea-pig heart by the following method. Guinea-pigs weighing 250–400 g were anaesthetized by intraperitoneal injection of pentobarbital. The chest was opened under artificial ventilation, the aorta was cannulated *in situ*, and the heart was mounted on a Langendorff perfusion system. After washing out the blood with Tyrode solution, the perfusate was changed to nominally Ca^{2+}-free Tyrode solution and then to one containing 0.01% w v^{-1} collagenase (Wako, Osaka, Japan) and 0.002% w v^{-1} alkaline protease (Nagase, Tokyo, Japan). After digestion for about 15–20 min, the enzymes were washed out by perfusing a high K^+, low Cl^- solution (modified KB solution; Isenberg & Klockner, 1982). The ventricular tissue was cut into the modified KB solution and gently shaken to isolate the cells. The cell suspension was stored in a refrigerator (4°C) for later use.

Patch-clamp recording

Whole-cell membrane currents were recorded by the patch-clamp method. Single cardiac ventricular cells were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Nikon, Tokyo, Japan) and superfused with the Tyrode solution at a rate of 5 ml min^{-1}. The temperature of the external solution was kept constant at 36 ± 0.5°C. The Tyrode solution had the following composition (in mM): NaCl 140, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.33, glucose 5.5 and HEPES–NaOH 5 (pH 7.4). The composition of the modified KB solution was (in mM): KOH 70, l-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 0.2 and HEPES–KOH buffer 10 (pH 7.2). Patch pipettes were made from glass capillaries with a diameter of 1.5 mm by a vertical microelectrode puller (pp-83, Narishige, Tokyo, Japan). The pipette resistance was $2 \sim 3$ MΩ, when filled with the pipette solution. The composition of the pipette solution was (in mM): NaCl 20, BAPTA 30, $CaCl_2$ 15–19 (free Ca^{2+} concentration 235–398 nM), $CsCl_2$ 120, $MgCl_2$ 3, Aspartic acid 50, MgATP 5, HEPES 10 (pH 7.2 with CsOH). The extracellular solution contained (in mM): NaCl 140, $CaCl_2$ 2, $MgCl_2$ 1, ouabain 0.02, nifedipine 0.01, ryanodine 0.01, HEPES–CsOH 5 (pH 7.2). The electrode was connected to a patch-clamp amplifier (TM-1000, Act ME, Tokyo, Japan). Recording signals were filtered at 2.5 kHz bandwidth, and the series resistance was compensated. Current signals were stored on-line and analysed by a computer (PC-9801RX, NEC, Tokyo, Japan) with the handmade software called RAM5.

The current-voltage (I-V) relationship was obtained by voltage clamp ramp pulses as described previously (Kimura *et al*., 1987; Watano *et al*., 1996). The holding potential was set at −60 mV. A double-ramp protocol (positive and negative slopes) was employed. The membrane was initially depolarized from −60 to +60 mV, then hyperpolarized from +60 to −110 mV and then depolarized back to −60 mV at a constant rate of 640 mV s^{-1}. The descending limb (from +60 to −110 mV) was plotted as the I-V relationship without capacitance compensation. The Ca^{2+} current (I_{Ca}), K^+ currents, Na^+-K^+ pump current and Ca^{2+} release channels of the sarcoplasmic reticulum were blocked by nifedipine, Cs^+, ouabain and ryanodine, respectively.

Drugs

Amiodarone, ouabain, ryanodine and nifedipine were purchased from Sigma Chemical Co., St Louis, U.S.A. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulphonate) was a gift from Kanebo Co. Ltd. (Osaka, Japan). Amiodarone was first dissolved in ethanol and was dissolved further in Na^+-external solution containing bovine serum albumin (1.0%) as described by Polster & Broekhuysen (1976). Ethanol concentration in the Na^+-external solution was less than 0.1%. Nifedipine and KB-R7943 were initially dissolved in dimethylsulphoxide (DMSO) as stock solutions and added to the extracellular solution. The final concentration of DMSO was ⩽0.1%, which did not affect the Na^+/Ca^{2+} exchange current. Trypsin (2.5 μg ml^{-1}) (Difco Laboratories, Detroit, MI, U.S.A.) was directly dissolved in the pipette solution. All the chemicals used were the highest grade available.

Data analysis

All the values were presented as mean ± s.e.mean (number of experiments). Student's *t*-test and analysis of variance were used for statistical analyses. A *P* value of less than 0.05 was considered significant. The concentration–response data were fitted and IC_{50} and Hill coefficient values were obtained by Delta Graph Professional (Polaroid Computing, Tokyo, Japan) on a Macintosh computer (Apple Computer, Mariani Avenue Cupertino, CA, U.S.A.). Per cent inhibition of the outward I_{NCX} at various concentrations of amiodarone was fitted by the following logistic equation:

$$\text{Per cent inhibition} = 100 \times 1/\{1 + (IC_{50}/[D])^{n_H}\}$$

Where [D] is the concentration of amiodarone, IC_{50} is the half-maximum concentration for inhibition of the drug and n_H is an empirical parameter describing the steepness of the fit and is equivalent to the Hill coefficient.

Results

Effects of amiodarone on I_{NCX}

I_{NCX} was induced by 2 mM Ca^{2+} and 140 mM Na^+ in the external solution and 20 mM Na^+ and 398 nM free Ca^{2+} in the pipette solution. Under these ionic conditions, the reversal potential of the exchange current at a 3Na:1Ca stoichiometry was calculated to be −73 mV. After establishing the whole-cell clamp mode, the external solution was changed from Tyrode solution to the control external solution while monitoring the increase in current until it reached a steady state. As shown in Figure 1A, when the current became stable, the control external solution was switched to one containing amiodarone. Amiodarone at 3 μM immediately suppressed the fast Na^+ current and then slowly suppressed the Na^+/Ca^{2+} exchange current. Suppression of the latter current was slow, taking more than 5 min before steady state was attained. Figure 1B illustrates the current-voltage (I-V) relationships obtained in control conditions (a) and after exposure to amiodarone (b). The I-V relationship recorded in the presence of amiodarone intersected with the control I-V curve at about −60 mV. After the effect of amiodarone reached a steady state, a high concentration (100 μM) of KB-R7943, a potent exchange current inhibitor, was applied to completely block I_{NCX}. Similar to the current recorded in the presence of amiodarone alone, the I-V curve obtained in the presence of KB-R7943 (c) also intersected with the control I-V curve at about −60 mV

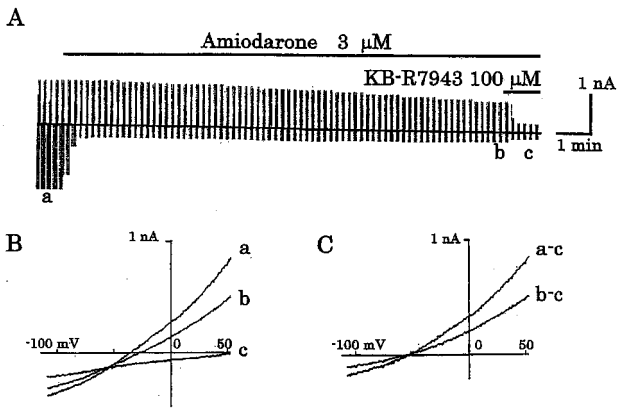

Figure 1 (A) Chart recording of membrane current. The horizontal bars above the current indicate when 3 μM amiodarone and 100 μM KB-R7943 were applied externally. (B) I-V curves obtained at the corresponding time points indicated in A. a is control, b in the presence of amiodarone and c in the presence of KB-R7943. (C) Difference I-V curves between a and c (a-c) and between b and c (b-c) in B.

(Figure 1B), suggesting that the amiodarone-sensitive current was I_{NCX}. Figure 1C illustrates the net I-V curves of I_{NCX} obtained by subtracting the I-V curve obtained with KB-R7943 (c) from those before (a) and after (b) amiodarone application, respectively.

We next tested different concentrations of amiodarone. Figure 2 illustrates representative net I-V curve obtained by subtracting the current in the presence of KB-R7943 (100 μM) from the current obtained under control conditions and following exposure to amiodarone. Each concentration of amiodarone was tested in a different cell. I-V curves obtained under control conditions and in the presence of amiodarone were superimposed in each panel. Amiodarone inhibited I_{NCX} in a dose-dependent manner. All the I-V curves crossed the voltage axis at around -60 mV, indicating that the amiodarone-sensitive currents were mainly produced by I_{NCX}. The current magnitude was measured at two different potentials; i.e. at $+50$ mV for the outward exchange current component and at -100 mV for the inward current component, and the per cent inhibition was calculated assuming that 100 μM KB-R7943 completely inhibited I_{NCX}. The concentration–response curves of amiodarone were plotted in Figure 3. Sigmoidal fitting of the curves yielded the IC_{50} values of amiodarone at 3.3 and 3.6 μM with the Hill coefficient of 1 for the outward ($n=27$) and inward ($n=27$) components, respectively. These results indicate that amiodarone inhibited both direction of the exchange current equipotently.

Effect of trypsin on the amiodarone inhibition of I_{NCX}

We examined whether amiodarone inhibited I_{NCX} from the inner side of the membrane by perfusing trypsin through the pipette solution. Trypsin (2.5 μg ml^{-1}) was included in the pipette solution and 10 μM amiodarone was applied in the external solution. As shown in Figure 4, amiodarone inhibited I_{NCX} by 37% in this cell. KB-R7943 at 100 μM still substantially blocked I_{NCX} even after the trypsin treatment. As summarized in Figure 5, amiodarone blocked I_{NCX} by $28\pm3\%$ ($n=4$) after trypsin treatment, while it blocked I_{NCX} by $75\pm4\%$ ($n=5$) without trypsin. Therefore, the blocking effect of amiodarone was significantly attenuated by trypsin. This result suggests that amiodarone affects the Na^+/Ca^{2+} exchanger from the intracellular side of the membrane.

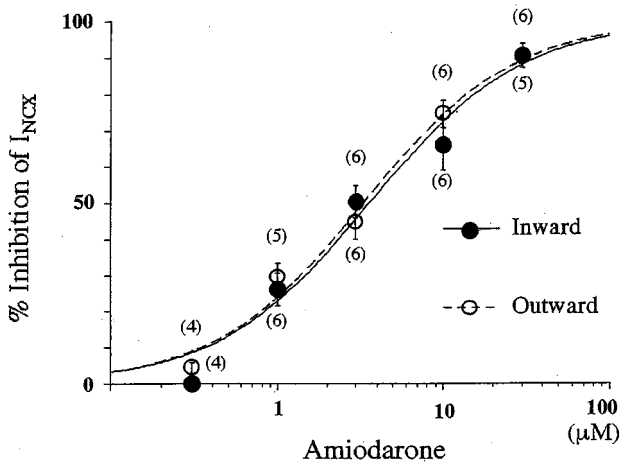

Figure 3 Concentration-inhibition curves of amiodarone. The average data obtained at 50 mV for the outward I_{NCX} and at -100 mV for the inward I_{NCX} were fitted. The IC_{50} values of amiodarone were 3.3 and 3.6 μM for the outward and inward I_{NCX}, respectively. The Hill coefficient was 1 for the both components.

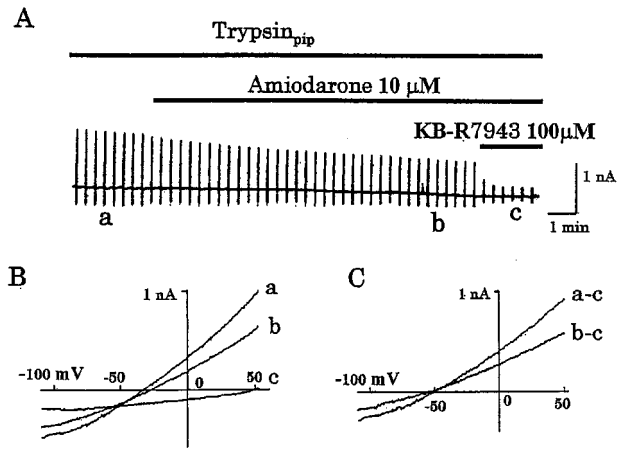

Figure 4 (A) Chart recording of the current observed when the pipette solution contained trypsin (2.5 μg ml^{-1}). The horizontal bars above the current indicate where trypsin, amiodarone 10 μM and KB-R7943 100 μM were applied. (B) I-V curves obtained at the corresponding labels in (A). a is control and b in presence of amiodarone and c in the presence of KB-R7943. (C) Difference I-V curves between a and c (a-c) and between b and c (b-c) in B.

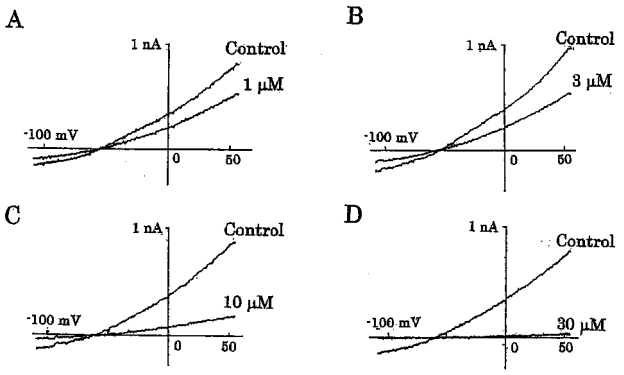

Figure 2 Difference I-V curves at different concentrations of amiodarone. (A) 1 μM, (B) 3 μM, (C) 10 μM, (D) 30 μM amiodarone. Each I-V curve was after subtraction of the current recorded in the presence of 100 μM KB-R7943. Control is before amiodarone application. The duration of application of amiodarone was 10 ~ 20 min.

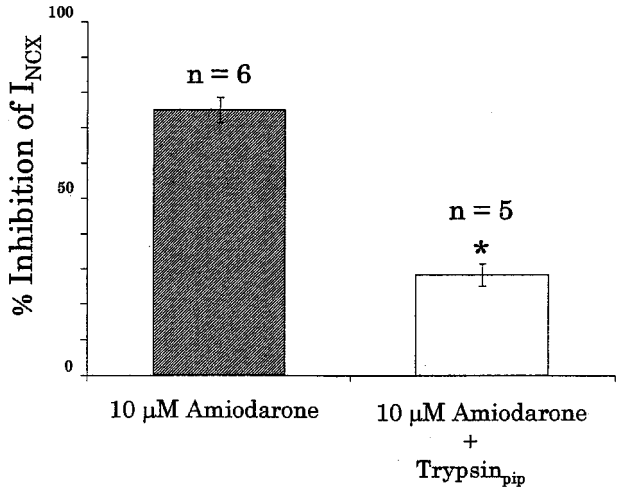

Figure 5 Comparison of the inhibitory effect of 10 μM amiodarone on I_{NCX} in the absence (left) and presence of trypsin (right) in the pipette solution. The data of trypsin were obtained by the experiments shown in Figure 4.

Discussion

Acute application of amiodarone has been reported to inhibit various ionic channels including Na^+-, Ca^{2+}- and K^+ channels (Kodama *et al*., 1997; 1999). In the present study, we found that amiodarone exerts an additional action by inhibiting I_{NCX} in a concentration-dependent manner in guinea-pig cardiac ventricular cells. The IC_{50} value was 3.3 ~ 3.6 μM and the Hill coefficient was 1.

The site of action of amiodarone is most likely on the cytoplasmic side of the exchanger, possibly in the internal loop, because intracellular treatment of a proteolytic enzyme, trypsin *via* the pipette solution attenuated the blocking effect of amiodarone. The irreversible and slow time-course of the suppression before reaching a steady state also supports this view. The Na^+/Ca^{2+} exchanger has a long intracellular loop containing various regulatory sites (Nicoll *et al*., 1990; 1999; Philipson *et al*., 1996; Iwamoto *et al*., 1999). 'De-regulation' of the exchanger by cytosolic trypsin or α-chymotrypsin treatment has been already demonstrated to affect activation by intracellular Ca^{2+} and ATP, inactivation by internal Na^+ (Hilgemann 1990; Matsuoka & Hilgemann, 1994), inhibition by H^+ (Doering & Lederer 1993), and inhibition by the calmodulin inhibitor, W-7 (Kimura, 1993). The site of action of amiodarone may be close to or overlap with the regulatory sites of internal Ca^{2+}, Na^+ and H^+ in the exchanger molecule. An equally plausible explanation, however, could be that a modification by trypsin of an internal site(s) on the exchanger protein or a regulatory protein alters the conformation of the protein in such a way that an amiodarone binding site within the membrane becomes partially occluded.

The following is a list of the ionic currents affected by amiodarone and the concentration range over which these effects are observed. I_{Na} was blocked by amiodarone (0.1 ~ 7.3 μM) use- and voltage-dependently in dog cardiac Purkinje myocytes and cat ventricular cells (Follmer *et al*., 1987). Amiodarone allosterically inhibits batrachotoxin-binding to the Na^+ channel, indicating that it binds preferentially to inactivated Na^+ channels (Sheldon *et al*., 1989). L-type Ca^+ channels were also blocked by amiodarone with an estimated dissociation constant of 5.8 μM in the resting state and 0.36 μM in the inactivated state (Nishimura *et al*., 1989). Amiodarone affects most types of K^+ channels present in cardiac ventricular cells. Amiodarone at 10 μM halved I_K tail current magnitude, but had no effect on I_{to} in rabbit ventricular cells (Kamiya *et al*., 1995; Varro *et al*., 1996). I_{to} of newborn rat ventricular myocytes was, however, inhibited with an IC_{50} 4.9 μM. Amiodarone at 10 ~ 20 μM suppressed I_{K1} by 12 – 14% in guinea-pig ventricular myocytes (Sato *et al*., 1994). The ligand-gated K^+ currents are also susceptible. Amiodarone inhibited $I_{K.Na}$ with an IC_{50} of ~1 μM in inside-out patches excised from guinea-pig ventricular myocytes (Mori *et al*., 1996). Amiodarone inhibited the carbachol-induced, adenosine-induced and GTPγS-induced $I_{K.Ach}$ of guinea-pig atrial cells (IC_{50} ~ 2 μM) (Watanabe *et al*., 1996). Amiodarone inhibited single K_{ATP} channel current of guinea-pig ventricular myocytes by inside-out patch with the IC_{50} value of 0.3 μM (Takizawa & Nakaya, 1997).

Acute and chronic clinical administration of amiodarone results in plasma levels in the range of 0.06 – 6.5 μg ml^{-1}, which corresponds to concentrations of 0.1 – 10 μM (Harris *et al*., 1983; Ikeda *et al*., 1984; Raeder *et al*., 1985). Therefore the concentrations of amiodarone that inhibit I_{NCX} are within the therapeutic range of the drug. Is this effect beneficial or harmful in the amiodarone therapy? The Na^+/Ca^{2+} exchanger is a bi-directional transporter and can operate both in the Ca^{2+} efflux and Ca^{2+} entry modes. Our results indicate that amiodarone inhibits the exchange current in both directions equally. When the cell depolarizes in the presence of an augmented intracellular Na^+ concentration, the Na^+/Ca^{2+} exchanger may reverse and induce Ca^{2+} entry. An intracellular Na^+ accumulation during anoxia or ischaemia precedes Ca^{2+} overload most likely *via* Na^+/Ca^{2+} exchange (Tani, 1990). In heart failure, Na^+/Ca^{2+} exchanger mRNA and protein levels increase (Studer *et al*., 1994). The Na^+/Ca^{2+} exchanger appears to contribute to the occurrence of delayed afterdepolarization and triggered activity (Lakatta, 1992). Under such pathological conditions, suppression of Na^+/Ca^{2+} exchanger may be beneficial and prevent Ca^{2+} overload. Indeed, Aomine & Fukui (1993) demonstrated that ouabain-induced increase in contraction was decreased by amiodarone. Therefore the inhibitory effect on the Na^+/Ca^{2+} exchanger may contribute to the cardioprotective effect of the drug. More work will be necessary to elucidate the molecular mechanism of the blocking effect of amiodarone on the Na^+/Ca^{2+} exchanger.

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