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European Journal of Pharmacology 496 (2004) 49–54

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Effects of amiodarone on mutant Na^+/Ca^{2+} exchangers expressed in CCL 39 cells

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Received 1 April 2004; received in revised form 27 May 2004; accepted 8 June 2004

Abstract

Using the whole cell voltage clamp, we reported previously that amiodarone acutely inhibits Na^+/Ca^{2+} exchange current (I_{NCX}) in guinea pig cardiac ventricular myocytes. Intracellular application of trypsin via the patch pipette attenuated the blocking effect of amiodarone, suggesting that amiodarone affects the Na^+/Ca^{2+} exchanger (NCX) from the cytoplasmic side. Here, we attempted to detect the site of amiodarone inhibition using wild type NCX1, mutants, and NCX3 expressed in CCL39 fibroblasts. I_{NCX} was recorded by ramp pulses. Amiodarone at 30 μM inhibited I_{NCX} by 80% in cells expressing wild type NCX1. However, 30 μM amiodarone inhibited I_{NCX} by about 55% in cells expressing mutant NCX1 with amino acids 217-671 (ΔXIP) or 247-671 (Δ247-671) deleted in the long intracellular loop between the transmembrane segments (TM) 5 and 6. I_{NCX}s from NCX mutants deleted of cytoplasmic TM1-2, TM3-4 or the C-terminus were inhibited by amiodarone to a similar extent as the wild type. Amiodarone also inhibited I_{NCX} of NCX3 by 76%. These results suggest that a long intracellular loop may be involved in the inhibition of NCX1 by amiodarone, but that other intracellular loops, XIP region or C terminus are not involved in the amiodarone inhibition of NCX1.

Keywords: Antiarrhythmic drug; Amiodarone; Na^+/Ca^{2+} exchange current; NCX1; CCL39 fibroblast

1. Introduction

There is abundant evidence from large-scale clinical trials such as BASIS, CASCADE, GESICA, CHF-STAT, CAMIAT, and EMIAT (Burkart et al., 1990; The CASCADE investigators, 1993; Doval et al., 1994; Singh et al., 1995; Cairns et al., 1997; Julian et al., 1997) that amiodarone is effective for treating life-threatening supraventricular and ventricular tachyarrhthmias and for preventing sudden cardiac death. Amiodarone is a Class III antiarrhythmic drug in the Vaughan Williams classification; it not only blocks K^+ channels but also Na^+- and L-type Ca^{2+}-channels as well as α- and β-adrenoceptors (Singh, 1994; Kodama et al., 1997). The K^+ channels which are inhibited by acute administration of amiodarone are I_{Kr}, $I_{K.ACh}$, $I_{K.Na}$ and those inhibited by chronic administration are I_{Ks} and I_{to} (Kodama et al., 1999).

Recently, we found that acute administration of amiodarone in a therapeutic concentration range inhibits Na^+/Ca^{2+} exchange current (I_{NCX}) with an IC_{50} of 3.3 μM and a Hill coefficient of 1 in guinea pig cardiac ventricular myocytes (Watanabe and Kimura, 2000). We also studied the effect on I_{NCX} of chronic treatment with amiodarone for 1 or 4 weeks, but could not detect any change in the magnitude of I_{NCX} and NCX1 mRNA (Watanabe et al., 2002a,b). Because intracellular treatment with trypsin via the pipette solution attenuated the effect of acute administration of amiodarone, we concluded that the site of the inhibitory action of acutely administered amiodarone is most likely on the cytoplasmic side of Na^+/Ca^{2+} exchanger (NCX) (Watanabe and Kimura, 2000).

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doi:10.1016/j.ejphar.2004.06.020

The aim of this study was to detect the site of amiodarone inhibition on the NCX1 molecule. To do so, deletion mutants of each internal loop of NCX1 were expressed in CCL 39 fibroblasts, and the effect of amiodarone on each mutant was determined by measuring I_{NCX} with the whole cell voltage clamp.

2. Materials and methods

2.1. Construction and stable expression of NCX1, NCX3, and NCX1 mutants

cDNAs of dog heart NCX1.1 and rat brain NCX3.3 were subcloned into *Sac*II and *Hin*dIII sites in pCRII (designated pCRII-NCX1 and pCRII-NCX3, respectively) (Iwamoto et al., 1999; Iwamoto and Shigekawa, 1998). For construction of NCX1 mutants with amino acids 64-99, 161-166, 217-671, and 247-671 deleted (designated Δ64-99, Δ161-166, ΔXIP, and Δ247-671), sense and antisense primers (5′-ATATAT*AGATCT*AATCTAACCTTGATGGCCCTGGGAT-3′ and 5′-ATATAT*AGATCT*GTCAGCAATGATAGAGACTCCGAGA-3′ (*Bgl*II sites are italicized), 5′-ATATAT*ACGCGT*GTGTTCTTCGTGACAGCAGCCTGGA-3′ and 5′-ATATAT*ACGCGT*CTCTCCATCTGGGACCACATAAACA-3′ (*Mlu*I sites are italicized), 5′-ATATAT*CTCGAG*GGAGAACACACCAAGCTGGAAGTG-3′ and 5′-ATATAT*CTCGAG*CACCCAAGCGAACACAACACAGAT-3′ (*Xho*I sites are italicized), and 5′-ATATAT*CTCCGAG*GGAGAACACACCAAGCTGGAAGTG-3′ and 5′-ATATAT*CTCGAG*GTCTCCTTCGTGCTCGATGATCAT-3′ (*Xho*I sites are italicized), respectively) were used in combination with two outer primers corresponding to the 3′ (antisense) and 5′ noncoding (sense) regions, respectively. For construction of an NCX1 mutant with amino acids 927-938 deleted (designated ΔC), the outer sense primer (5′ noncoding region) and the antisense primer 5′-ATATAT*AAGCTT*TAGAAGAAAATGTACAAGAGCCA-3′ (the *Hin*dIII site is italicized) were used. Using these pairs of primers, DNA fragments were generated by polymerase chain reaction (PCR) with pCRII-NCX1 as a template, digested with restriction enzymes, and then inserted between the *Sac*II and *Hin*dIII restriction sites of the mammalian expression vector pKCRH (Iwamoto et al., 1999). The modified cDNAs were verified by sequencing (ABI PRISM, Perkin-Elmer). To stably express the exchangers, Lipofectin (GIBCO BRL) was used to transfect pKCRH plasmids into CCL39 cells. Clones exhibiting high NCX activity were selected by treating colonies with 500 μg/ml G418 for 10 days and then with 10 μM ionomycin for 30 min. The ionomycin treatment (“Ca^{2+}-killing”) effectively eliminates cells with low exchange activity (Iwamoto et al., 1998).

2.2. Patch-clamp recording

I_{NCX} was recorded by the whole cell patch-clamp method (Watanabe and Kimura, 2001; Watanabe et al., 2002a,b). CCL39 fibroblasts adhering on small pieces of a cover glass were placed in a recording chamber (1 ml volume) attached to an inverted microscope (Nikon, Tokyo, Japan) and were superfused with Tyrode solution at a rate of 5 ml/min. The temperature of the external solution was maintained at 36 ± 0.5 °C. Patch pipettes were forged from 1.5 mm diameter glass capillaries with a microelectrode puller (pp-83, Narishige, Tokyo, Japan). The pipette resistance was 2–3 MΩ. 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 with a computer (PC-9801RX, NEC, Tokyo, Japan) using a noncommercial software called RAM5.

Tyrode solution contained (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 modified KB solution contained (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). The pipette solution contained (in mM): NaCl 20, BAPTA 20, $CaCl_2$ 13 (free Ca^{2+} concentration 433 nM), $CsCl_2$ 120, $MgCl_2$ 3, aspartic acid 50, MgATP 5 and 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 and HEPES-CsOH 5 (pH 7.2). I_{NCX} was induced in CCL39 cells by 2 mM Ca^{2+} and 140 mM Na^+ in the external solution and 20 mM Na^+ and 433 nM free Ca^{2+} in the pipette solution. I_{NCX} was detected as a KB-R7943-sensitive current.

The current–voltage (I–V) relationships were obtained by ramp pulses as described previously (Watano et al., 1996). The membrane was initially depolarized from the holding potential of −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. The descending limb (from 60 to −110 mV) was plotted in the I–V relationship without a capacitance compensation. 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. The current magnitude was measured at 50 mV to calculate the current density and the percent inhibition. The percent inhibition was also measured at 0 mV, where there should be no contamination of nonselective cation current, and the values were not different from those calculated at 50 mV.

2.3. Drugs

Amiodarone, ouabain, ryanodine and nifedipine were purchased from Sigma (St Louis, USA). KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate) was a gift from Nippon Organon K.K. (Osaka, Japan). Amiodarone was first dissolved in ethanol and then

added to the Na^+-external solution containing bovine serum albumin (0.1–1.0%) as described by Polster and Broekhuysen (1976). The ethanol concentration in the Na^+-external solution was less than 0.1%. Nifedipine and KB-R7943 were dissolved in dimethylsulfoxide (DMSO) to make stock solutions which were added to the extracellular solution. The final concentration of DMSO was ≦0.1%, which did not affect I_{NCX}. All the chemicals used were the highest grade available.

2.4. Data analysis

All the data are presented as means ± S.E. (number of experiments). Student's *t*-test and analysis of variance

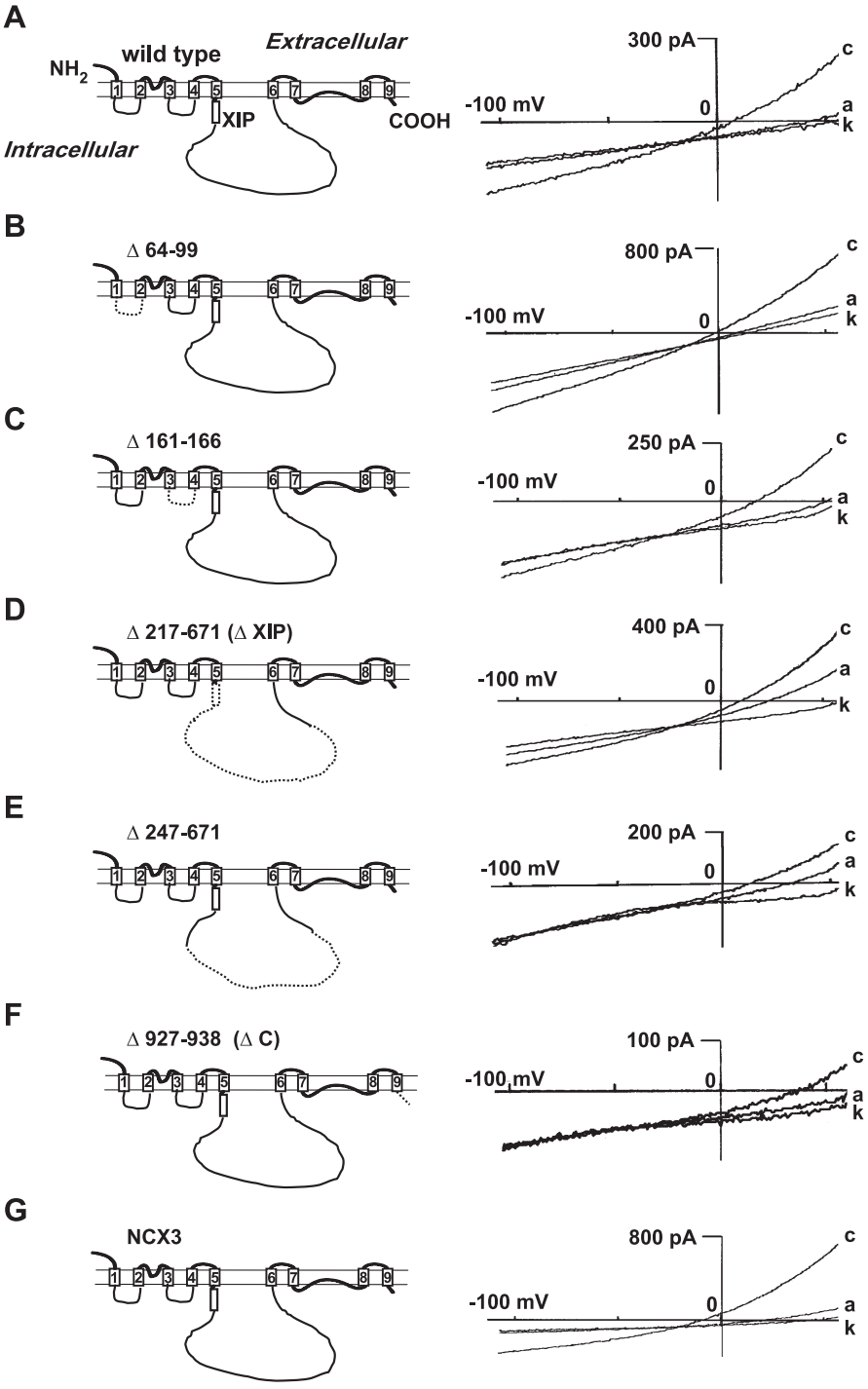

Fig 1. (Left) Topologies of NCX1 wild type, deletion mutants and NCX3. (Right) *I–V* curves obtained from CCL39 fibroblasts expressing the corresponding NCX. Control (c), in the presence of 30 μM amiodarone (a) and in the presence of 100 μM KB-R7943 (k). Deleted (Δ) amino acids are indicated.

(ANOVA) were used for statistical analyses. *P* values of less than 0.05 were considered significant.

3. Results

Fig. 1 shows the topologies of NCX1 (A), its mutants (B–F) and NCX3 (G) in the left panels and the corresponding *I–V* curves in the right panels. In all *I–V* curves, (c) is the control current, (a) is that in the presence of 30 μM amiodarone, and (k) that in the presence of 100 μM KB-R7943. After establishing the whole cell clamp mode, the Tyrode solution was changed to the control external solution. When I_{NCX} became stable, the control external solution was switched to one containing amiodarone. After the blocking effect of amiodarone reached a steady state, a high concentration (100 μM) of KB-R7943, a potent I_{NCX} inhibitor (Watano et al., 1996; Iwamoto et al., 1996a,b), was applied to completely block I_{NCX}. We confirmed that neither 30 μM amiodarone nor 100 μM KB-R7943 suppressed the control current in untransfected CCL39 cells (figure not shown), indicating that CCL39 cells do not express endogenous NCX. The *I–V* curves in Fig. 1A illustrate the effect of 30 μM amiodarone and 100 μM KB-R7943 on I_{NCX} of wild type NCX1. Amiodarone and KB-R7943 (k) inhibited the current with the same reversal potential, suggesting that the amiodarone-sensitive current was I_{NCX}. At 50 mV, 30 μM amiodarone inhibited I_{NCX} by 80 ± 4%. The same protocol was used with the cells expressing five different NCX1 mutants deleted of each intracellular domain and NCX3 (Fig. 1B–F). In Fig. 1E and F, inward I_{NCX} are not apparent. This appears to be due to the small magnitude of the current and not to functional alteration by the mutation, because a small fraction of inward current developed in several cells in both cases. Fig. 2 summarizes the I_{NCX} densities and percentages of inhibition by amiodarone in each case. I_{NCX} densities of wild type NCX1 and its mutants which were of canine heart origin, and rat brain NCX3, were compared with those of guinea pig cardiac ventricular myocytes. The I_{NCX} density calculated at 50 mV was not significantly different between the cardiac ventricular myocytes and the fibroblasts expressing wild type NCX1. The I_{NCX} densities tended to be smaller in the mutants than in the wild type and the smallest was in ΔC (Fig. 2A). The inhibitory effects of amiodarone on wild type NCX1 and NCX3 were similar to that on guinea pig ventricular myocytes. Amiodarone inhibition was significantly but not completely attenuated in two mutants, both of which had deletions of the third long internal loop excluding XIP (Δ247-671) or including XIP (ΔXIP or Δ217-671). We tested a mutant deletion of the total long loops (Δ216-765) in TM5-6, but we could not record I_{NCX} from this mutant.

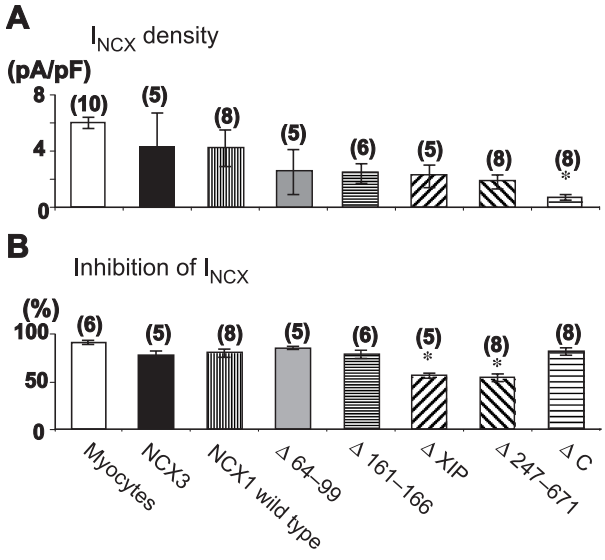

Fig 2. (A) Summarized data of I_{NCX} densities measured at 50 mV in A–G in Fig. 1. The I_{NCX} density was not significantly different in fibroblasts expressing wild type NCX1 and cardiac myocytes. Mutant I_{NCX} densities appear to be smaller than the wild type but only the ΔC mutant was significantly smaller than the wild type. (B) Inhibitory effects of 30 μM amiodarone on I_{NCX} from A to G in Fig. 1. Amiodarone inhibition was significantly greater in two mutants, ΔXIP and Δ247-671, than in ventricular myocytes. The values are expressed as means ± S.E% (number of cells). *$p < 0.05$.

4. Discussion

In this study using CCL39 fibroblasts transfected with various NCX1 mutants, we investigated the site in the NCX molecule where amiodarone binds and inhibits NCX1. Our previous study showed that amiodarone inhibition was sensitive to trypsin in the pipette with a Hill coefficient of 1, indicating that the amiodarone binding site is intracellular and that one amiodarone molecule is enough to block NCX (Watanabe and Kimura, 2000). Canine NCX1 consists of 938 amino acids with nine TMs with a large intracellular loop between TM5 and 6, which contains various modulating sites and a self-inhibitory region called XIP (Nicoll et al., 1990, 1999; Iwamoto et al., 1999). N- and C-termini are located on the external and internal sides, respectively (Nicoll et al., 1990, 1999; Iwamoto et al., 1999). Chen et al. (2000) reported that trypsin digestion of a scallop muscle membrane fraction released polypeptides derived from the large intracellular region of a scallop muscle NCX, since this peptide shows sequence homology to a corresponding region of the mammalian NCX1. That report (Chen et al., 2000) and our finding that amiodarone inhibition is trypsin-sensitive led us to hypothesize that the large internal loop of NCX1 may be involved in amiodarone block.

Amiodarone at 30 μM inhibited wild type I_{NCX} by 80 ± 4%, which was similar to its effect in cardiac myocytes, and NCX3. However, this was decreased significantly

to 54 ± 4% and 56 ± 4% in the two mutants deleted of the large intracellular loop including XIP (ΔXIP or Δ217-671) and excluding XIP (Δ247-671), respectively. In all the other mutants, I_{NCX} was inhibited by amiodarone to a similar extent as in the wild type. Therefore, the XIP region itself, the first (TM1-2) and the second (TM3-4) short intracellular loops and the C terminus as well of NCX do not seem to participate in the blocking effect of amiodarone. These results support our hypothesis that a long intracellular loop, TM5-6, is partly involved in the amiodarone inhibition of NCX1.

There are several possibilities for the reduction of amiodarone inhibition in the deletion mutants, ΔXIP (Δ217-671) and Δ247-671. One is a direct effect that the site of amiodarone inhibition is at least partly included in those sequences. Another possibility is an indirect effect that the binding site may not be in the internal loop but the configuration of NCX was changed by deletion of the long internal loop, resulting in the low affinity of amiodarone.

In guinea pig ventricular myocytes, the inhibitory effect of amiodarone on I_{NCX} was decreased but never completely abolished by trypsin applied intracellularly via the pipette solution. In contrast to amiodarone, the inhibitory effect of BDM (2,3-butanedione monoxime) was almost completely abolished by trypsin treatment (Watanabe et al., 2001). This may be because BDM is a small molecule with a molecular weight of 101, and the domain involved in BDM inhibition may be restricted in the long internal loop. In contrast, the site of amiodarone inhibition may consist of not only the long internal loop but also of some other domain of NCX1 or some cytosolic anchor protein which interact with the long internal loop. Alternatively, since only part of the long internal loop was deleted in this study (Δ217-671 out of the total amino acids of 217-738), amiodarone inhibition might have been completely abolished if the total long loops in TM5-6 were deleted. We could not test this possibility because the deletion mutant of the total long loop did not yield I_{NCX}.

The densities of I_{NCX} expressed by the NCX1 mutants were smaller than that of wild type. Possible reasons for this may be that (i) translation of mutant NCX mRNA is less efficient, (ii) translocation from cytoplasm to membrane is less efficient in the mutants, (iii) the function of NCX is impaired by mutation, (iv) proteolysis of mutant NCX is more rapid than that of wild type. Recently it was shown that NCX requires ankyrin-B as an anchor protein (Chen et al., 1997; Mohler et al., 2003). I_{NCX} density was the smallest with ΔC, which indicates that the C terminus is crucial for normal transport activity of NCX1.

In the present study, we could see attenuation of amiodarone block by the deletion mutation of the long internal loop. The XIP region, the first (TM1-2), and second short intracellular loop (TM3-4), and the C terminus of NCX do not seem to participate in the blocking effect of amiodarone. Further study is needed to elucidate the site and the mechanism of inhibition by amiodarone.

Acknowledgements

We thank Ms. Sanae Sato for her technical assistance. This work was supported by grants-in-aid from the Japan Foundation for Promotion of Science (11670096, 11357020, 13670092, 14570097).

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