Chronic Administration of Amiodarone Does Not Affect Na\(^+\)/Ca\(^{2+}\) Exchange Current in Guinea Pig Cardiac Ventricular Myocytes

Yasuhide Watanabe\(^1\),*, Isao Matsuoka\(^2\) and Junko Kimura\(^2\)

\(^1\)Department of Ecology and Clinical Therapeutics, School of Nursing, and \(^2\)Department of Pharmacology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan

Received March 13, 2002 Accepted June 3, 2002

ABSTRACT—We investigated chronic effects of amiodarone on Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{NCX}\)) and on the level of Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) mRNA in guinea pig ventricular myocytes using the whole-cell clamp technique and RT-PCR analysis, respectively. Guinea pigs were intraperitoneally injected with 80 mg/kg per day of amiodarone or the vehicle (saline) for 1 or 4 weeks. Single ventricular cells were isolated from the hearts of both groups of animals. Action potential duration at 90% repolarization level was prolonged to 143% and 165% of the control values by treatment with amiodarone for 1 and 4 weeks, respectively. I\(_{NCX}\) density and the level of NCX1 mRNA were not significantly changed by chronic treatment with amiodarone. The level of thyroid hormone (T\(_4\)) within the blood was not changed by the treatments. These results suggest that chronic treatment with amiodarone does not affect the Na\(^+\)/Ca\(^{2+}\) exchanger, with respect to the level of its mRNA and current density in guinea pig ventricular myocytes.

Keywords: Amiodarone, Cardiac myocyte (guinea pig), Chronic effect, Na\(^+\)/Ca\(^{2+}\) exchange current
School of Medicine, Fukushima Medical University. Male guinea pigs weighing 250 – 300 g were used. Guinea pigs in the amiodarone treatment group were injected intraperitoneally with 80 mg/kg per day of amiodarone every day for 1 or 4 weeks, and those in the saline group, with saline daily for 1 or 4 weeks. The average body weight of animals in the saline group was 354 ± 17 g (n = 6) after 1 week and 483 ± 44 g (n = 6) after 4 week, while that of those in the amiodarone group was 336 ± 20 g (n = 6) after 1 week and 415 ± 28 g (n = 6) after 4 weeks of treatments. Thus, the increase in body weight between 1 and 4 weeks was significantly disturbed by the amiodarone treatments.

Isolation of cells

Guinea pigs were anesthetized by intraperitoneal injection of pentobarbitone. The chest was opened under artificial ventilation. The aorta was cannulated in situ, and the heart was removed. After washing out the blood with Tyrode solution, the heart was mounted in a Langendorff perfusion system and the perfusate was changed to a nominally Ca\(^{2+}\)-free Tyrode solution and then to the one containing 0.01% w/v collagenase (Nagase, Tokyo) and 0.002% w/v alkaline protease (Nagase, Tokyo). After digestion for about 20 min, the heart was perfused with a high K\(^+\), low Cl\(^-\) solution [modified KB solution (28)]. Cardiac ventricular tissue was cut into small pieces in modified KB solution and gently shaken to isolate ventricular cells. The cell suspension was stored in a refrigerator (4°C). During perfusion, the temperature of the bath solution was maintained at 36 ± 0.5°C with a water jacket. Tyrode solution contained 140 mM NaCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.33 mM NaH\(_2\)PO\(_4\), 5.5 mM glucose and 5 mM HEPES-NaOH (pH 7.4). Modified KB solution contained 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH\(_2\)PO\(_4\), 3 mM MgCl\(_2\), 10 mM glucose, 0.2 mM EGTA and 10 mM HEPES (pH 7.2 with KOH).

Measurement of thyroid stimulating hormone (TSH) and T\(_4\)

To assess thyroid gland function, 3 – 5 ml blood samples were taken from the left ventricle before extracting the heart. TSH and thyroxine (T\(_4\)) were measured at BML, Inc. (Koriyama).

Patch-clamp recording

Membrane currents and action potentials were recorded by the whole-cell voltage clamp and the current-clamp method, respectively (29). Single ventricular cells were placed in a recording chamber on the stage of an inverted microscope (Nikon, Tokyo) and superfused with Tyrode’s 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 glass capillaries having diameters of 1.5 mm with a microelectrode puller (model pp-83; Narishige, Tokyo). The pipette resistance was 2 – 3 M\(\Omega\) when filled with the pipette solution. Patch-clamp amplifiers for the whole cell voltage clamp were TM-1000 (Act ME, Tokyo) and EPC-7 (List, Dermstadt, Germany), and the latter was also used for recording action potentials with the current clamp method. Current signals were filtered at 2.5-kHz bandwidth. The signals were stored on-line and analyzed with a computer (PC-9801RX; NEC, Tokyo).

The action potential was recorded in the current clamp mode from single cardiac ventricular myocytes stimulated at 0.1 Hz with Tyrode’s extracellular solution and a pipette solution containing 120 mM KOH, 20 mM KCl, 50 mM DL-aspartic acid, 5 mM MgATP, 3 mM MgCl\(_2\), 20 mM BAPTA and 20 mM HEPES (pH 7.2 with KOH). The current-voltage (I-V) relationships were obtained by ramp pulses stimulated 0.1 Hz from a holding potential of −60 mV, initially depolarized to 60 mV, then hyperpolarized to −110 mV, and depolarized back to −60 mV at a speed of 640 mVs\(^{-1}\). The descending limb current (from 60 to −110 mV) was used to plot I\(_{\text{NCX}}\) (25, 26). For recording I\(_{\text{NCX}}\), the pipette solution contained 20 mM NaCl, 20 mM BAPTA, 13 mM CaCl\(_2\) (free Ca\(^{2+}\) concentration, 433 nM), 120 mM CsCl, 8 mM MgCl\(_2\), 50 mM Aspartic acid, 5 mM Na\(_2\)ATP and 10 mM HEPES (pH 7.2 with CsOH). The extracellular solution contained 140 mM NaCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.2 mM ouabain, 0.01 mM nifedipine, 0.005 mM ryanodine and 5 mM HEPES-CsOH (pH 7.2).

RT-PCR

Total RNA was extracted from ventricular myocytes using the acid-guanidinium thiocyanate phenol chloroform method (30). The first strand cDNA was prepared from 1 \(\mu\)g of total RNA with random primers using moloney murine leukemia virus reverse transcriptase in a final volume of 20 \(\mu\)l. The cDNA was then diluted with 80 \(\mu\)l of sterile water and used as the template in PCR. DNA amplification was carried out in 10 \(\mu\)l of solution containing 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 1.5 mM MgCl\(_2\), 125 \(\mu\)M deoxynucleotide triphosphates, the cDNA template (1 \(\mu\)l), 1 \(\mu\)M primer mix and 25 units/ml Taq polymerase (Pharmacia, Tokyo). The thermocycle consisted of an initial 2-min incubation at 94°C, followed by 20 – 26 cycles of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C. The incubation at 72°C in the final cycle was extended to 10 min. Sequences of PCR primers for NCX cDNA were 5’-CTTGGTCCCCACCTACAGAAT-3’ (sense strand, corresponding to bases 2384-2403) and 5’-CGAGGAGATGG-3’ (antisense strand, corresponding to bases 2892-2673). As a control for RT-PCR, 500 bp \(\beta\)-actin cDNA fragments were amplified with sense primer (5’-GAAGCGAGGGTGGATGTCTTA-3’) and antisense
primer (5'-ACCATCTACCGTCTCTAC-3'). PCR products were separated by electrophoresis in 1.5% agarose gels. After staining with ethidium bromide, PCR products were quantitated by NIH Image 1.62 software.

Drugs
Amiodarone, ouabain, nifedipine, and ryanodine were purchased from Sigma Chemical Co., St. Louis, MO, USA. KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate) was a kind gift from Kanebo Co., Ltd. (Osaka). Amiodarone was dissolved in physiological saline (15, 31). Nifedipine and KB-R7943 were first dissolved in dimethylsulfoxide (DMSO) and added to extracellular solutions so that the final concentration of DMSO was ≤0.1%, which did not affect $I_{\text{NCX}}$. All the chemicals were the highest grade available.

Data analyses
All values are presented as means ± S.E.M. (number of experiments). Student’s t-test and analysis of variance were used for statistical analyses. $P$ values less than 0.05 were considered significant.

RESULTS

Chronic effect of amiodarone on action potentials
It has been shown that chronic administration of amiodarone prolongs the APD in guinea pig, rabbit and canine cardiac myocytes (9, 17, 32). Therefore, APD prolongation can be used as an indicator of amiodarone exerting electrophysiological effects. Using the current-clamp method, we first examined the action potentials in guinea pig ventricular cells treated with saline or amiodarone for 1 and 4 weeks. Figure 1 shows typical action potentials recorded from cells under these conditions. APDs were measured at 90% repolarization (APD$_{90}$) and the values are summarized in the right panels of Fig. 1, A and B. APDs of amiodarone-treated cells were significantly longer than those of saline cells and they were longer in cells treated for 4 weeks than in cells treated for 1 week. The average resting potentials were about −72 mV and were not significantly different in amiodarone-treated and non-treated cells or in those treated for 1 and 4 weeks (Fig. 1).

![Figure 1](image-url)

**Fig. 1.** Typical action potentials recorded in ventricular cells from saline (left) and amiodarone (AM) treated (middle) guinea pigs. A: 1 week of treatment with amiodarone. B: 4 weeks of treatment with amiodarone. Summarized data (right) of action potential duration measured at 90% repolarization (APD$_{90}$). The values are expressed as means ± S.E.M. (18–29 cells from 6 guinea pigs). *$P$<0.05, based on unpaired t-test.
Chronic effect of amiodarone on $I_{\text{NCX}}$

$I_{\text{NCX}}$ was induced with 1 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 with a holding potential of −60 mV. KB-R7943 at 100 μM was added to the external solution to inhibit $I_{\text{NCX}}$ completely (29, 33). Figure 2 shows typical pairs of I-V relationships in saline and cells with KB-R7943 from guinea pigs treated with saline for 1 week (Fig. 2A, left), with amiodarone for 1 week (Fig. 2A, middle), with saline for 4 weeks (Fig. 2B, left) and with amiodarone for 4 weeks (Fig. 2B, middle). Net $I_{\text{NCX}}$ densities measured at 50 mV are summarized in the right panels of Fig. 1, A and B. $I_{\text{NCX}}$ density was not significantly different in the saline and amiodarone groups for both 1- and 4-week treatments, indicating that chronic treatment with amiodarone does not affect $I_{\text{NCX}}$.

RT-PCR analysis

Although there was no differences in $I_{\text{NCX}}$ between the saline and amiodarone-treated groups, there might be a change in mRNA level of NCX. Therefore, we examined whether the level of NCX1 mRNA was affected by amiodarone-treatment using RT-PCR with oligonucleotide primers specific for the guinea pig cardiac NCX1 (34). Figure 3 (upper panels) shows a single 309-bp band corresponding to NCX1 mRNA and a 500-bp band corresponding to β-actin. RT-PCR products of NCX1 and β-actin were the same in the saline and amiodarone groups treated for 1 and 4 weeks. Figure 3 (lower panels) show the average ratios of NCX1 and β-actin RT-PCR products in 1- and 4-week-treated guinea pigs. These results indicate that chronic administration of amiodarone does not affect NCX1 mRNA levels in guinea pig ventricular myocytes.

Chronic effects of amiodarone on thyroid function

Since it has been reported that chronic treatment with amiodarone could affect thyroid function, we measured T$_4$ and TSH in the blood of the guinea pigs. As shown in Fig. 4, the level of T$_4$ was the same in the saline and 4 weeks amiodarone treated groups. TSH was too low to detect in both groups.

DISCUSSION

In this study, chronic treatment of amiodarone for 1 and 4 weeks prolonged APD$_{90}$ in guinea pig ventricular cells, indicating that amiodarone exerted its electrophysiological effects on the cardiac myocyte. However, $I_{\text{NCX}}$ and mRNA of NCX were not changed. The prolongation of APD has been attributed to suppression of various K$^{+}$ channels, including $I_{\text{Kr}}$, $I_{\text{Ks}}$, and $I_{\text{to}}$ (15, 17, 32). Kv1.5 mRNA was decreased in cardiac myocytes (17, 32). Therefore, the
Chronic Effect of Amiodarone on $I_{\text{NCX}}$

The synthesis of $K^+$ channel mRNAs may be impaired by amiodarone. This indicates that the mechanism regulating NCX mRNA expression is different from those of $K^+$ channels.

One of the well-known side effects of amiodarone is alteration of thyroid function. Amiodarone is a benzofuran derivative structurally similar to the thyroid hormones. Each amiodarone molecule contains two iodines, and 10% of the iodine becomes free iodine (20). Chronic treatment with amiodarone has been shown to decrease plasma levels of 3,5,3'-triiodothyronine ($T_3$) and to increase serum $T_4$ and 3,3',5'-triiodothyronine reverse T (rT3) (35). For the mechanism of the decreasing effect of $K^+$ current by amiodarone, it has been suggested that long-term treatment with amiodarone may antagonize $T_3$ at a cellular or subcellular level and thereby counteract its hormonal effects on $K^+$ channels (35). In this study, chronic administration of amiodarone did not change serum $T_4$. This does not necessarily mean that thyroid function was not altered in our preparation, because we did not measure serum $T_3$. Since APD was prolonged, amiodarone likely affected thyroid function, possibly by interacting with $T_3$ receptors in the ventricular cells, as was suggested by Guo et al. (35). However, this mechanism does not seem to apply NCX.

**Fig. 3.** Effects of 1 and 4 weeks of amiodarone treatments on NCX mRNA levels in ventricular cells. Upper panels: PCR products of NCX (309 bp) and $\beta$-actin (500 bp) are shown as indicated. Lower panels: Quantitative comparison of the ratios of RT-PCR products of NCX and $\beta$-actin primers in guinea pig hearts after 1 (left) and 4 (right) weeks of amiodarone treatments. The values are expressed as means $\pm$ S.E.M. from 3 or 4 guinea pig hearts.

**Fig. 4.** Comparison of thyroid hormone ($T_4$) between saline and 4-week amiodarone-treated groups. The values are expressed as means $\pm$ S.E.M. from 4 guinea pig hearts.
Hojo et al. (36) showed that thyroid hormone stimulated NCX in cultured neonatal rat cardiocytes. In contrast, Boerth and Artman (37) showed that NCX mRNA levels were decreased by hyperthyroidism and increased by hypothyroidism in adult and neonate rabbit hearts. Cernohorsky et al. (38) also showed that hyperthyroidism reduced NCX, while hypothyroidism increased NCX mRNA and protein in developing rat heart. The level of cardiac NCX protein was elevated in rat by chronic treatment with amiodarone (40 mM/kg body weight intraperitoneally for 6 weeks) (31). Therefore, we expected that amiodarone would modulate thyroid function and affect the Na\(^+\)/Ca\(^2+\) exchanger. However, in our hands, the serum level of T\(_3\) did not change from either 1 or 4 weeks of treatment with amiodarone, and In\(_{\text{NCX}}\) and the level of NCX mRNA were not affected. One possibility was that the level of alteration of thyroid function was undetectable by T\(_3\) and yet it was sufficient for K\(^+\) channel modulation but not enough for NCX mRNA. Further study is required for the unanswered relation among amiodarone, NCX and the thyroid function.

Acknowledgments
We thank Ms. Sanae Sato and Dr. Tomoyuki Ono for their technical assistance. This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (09670098, 11670096, 11357020).

REFERENCES

18 Gray FD, Mihailidou SA, Hansen SP, Buhtagiar AK, Bevk LN, Rasmussen HH and Whallay WD: Amiodarone inhibits the Na\(^+\)/K\(^+\) pump in rabbit cardiac myocytes after acute and chronic treatment. J Pharmacol Exp Ther 284, 75 – 82 (1997)
26 Watanabe Y and Kimura J: Blocking effect of bepridil on Na\(^+\)/Ca\(^2+\) exchange current in guinea pig cardiac myocytes.
Chronic Effect of Amiodarone on \( \text{I}_{\text{NCX}} \)


28 Isenberg G and Klockner U: Calcium tolerant ventricular myocytes prepared by preincubation in a “KB medium”. Pfloggers Arch 395, 6 – 18 (1982)


