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Short- and Long-Term Amiodarone Treatments Regulate $Ca_v3.2$ Low-Voltage-Activated T-type Ca^{2+} Channel through Distinct Mechanisms

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ABSTRACT

Low-voltage-activated T-type Ca^{2+} channels have been recognized recently in the mechanisms underlying atrial arrhythmias. However, the pharmacological effects of amiodarone on the T-type Ca^{2+} channel remain unclear. We investigated short- and long-term effects of amiodarone on the T-type ($Ca_v3.2$) Ca^{2+} channel. The $Ca_v3.2$ (α_{1H}) subunit derived from human heart was stably transfected into cells [human embryonic kidney (HEK)-$Ca_v3.2$] cultured with or without 5 μM amiodarone. Patch-clamp recordings in the conventional whole-cell configuration were used to evaluate the actions of amiodarone on the T-type Ca^{2+} channel current ($I_{Ca.T}$). Amiodarone blockade of $I_{Ca.T}$ occurred in a dose- and holding potential-dependent manner, shifting the activation and the steady-state inactivation curves in the hyperpolarization direction, when amiodarone was applied immediately to the bath solution. However, when the HEK-$Ca_v3.2$ cells were incubated with 5 μM amiodarone for 72 h, $I_{Ca.T}$ density was significantly decreased by 31.7 $\pm$ 2.3% for control, -93.1 ± 4.3 pA/pF ($n = 8$), versus amiodarone, -56.5 ± 3.2 pA/pF ($n = 13$), $P < 0.001$. After the prolonged administration of amiodarone, the activation and the steady-state inactivation curves were shifted in the depolarization direction by -7.1 ($n = 41$) and -5.5 mV ($n = 37$), respectively, and current inactivation was significantly delayed [time constant (τ): control, 13.3 $\pm$ 1.1 ms ($n = 6$) versus amiodarone, 39.6 $\pm$ 5.5 ms ($n = 6$) at -30 mV, $P < 0.001$)]. Nevertheless, short-term inhibitory effects of amiodarone on the modified T-type $Ca_v3.2$ Ca^{2+} channel created by long-term amiodarone treatment were functionally maintained. We conclude that amiodarone exerts its short- and long-term inhibitory actions on $I_{Ca.T}$ via distinct blocking mechanisms.

Amiodarone is widely accepted as an effective drug for the treatment of supraventricular and life-threatening ventricular tachyarrhythmias (Kodama et al., 1997, 1999). Amiodarone has been referred to as a class III antiarrhythmic agent; however, its pharmacological actions are, in fact, very complex (Amiodarone Trial Meta-Analysis Investigators, 1997), exerting class I, II, and IV antiarrhythmic properties according to the Vaughan Williams classification. In electrophysiological experiments, reported short-term effects of amiodarone have included the blocking of various types of ion channels, such as Na^+ channels (Follmer et al., 1987), L-type Ca^{2+} channels ($I_{Ca.L}$) (Nishimura et al., 1989), and K^+ channels (Kodama et al., 1999). Reported long-term effects of amiodarone on cardiac myocytes and papillary muscles include prolonging the action potential durations and refractory periods (Kodama et al., 1997, 1999). However, short- and long-term effects of amiodarone on the T-type Ca^{2+} channel ($I_{Ca.T}$) have not been fully examined.

$I_{Ca.T}$ is characterized by its activation at low voltage, rapid inactivation, and slow deactivation (Matteson and Armstrong, 1986) and may function in cardiac pacemaker activity under physiological conditions (Kawano and Dehaan, 1990). $I_{Ca.T}$ seems to play a particularly important role in pathophysiological or remodeled cardiovascular tissue (Ertel et al., 1997): atrial $I_{Ca.L}$ was down-regulated by sustained rapid atrial pacing in dogs, whereas $I_{Ca.T}$ was not reduced (Yue et al., 1997). Furthermore, there is evidence that T-type Ca^{2+} channel blockade may attenuate the functional remodeling caused by atrial fibrillation (Fereh et al., 1999). $I_{Ca.T}$ may provide a continuing leak of Ca^{2+} into the cell, and $I_{Ca.T}$ inhibition may therefore be beneficial for preventing electrical remodeling caused by atrial tachycardia.

Cohen et al. (1992) demonstrated that amiodarone inhibits

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ABBREVIATIONS: $I_{Ca.L}$, L-type Ca^{2+} channel current; $I_{Ca.T}$, T-type Ca^{2+} channel current; HEK, human embryonic kidney; Ami, amiodarone; DMSO, dimethyl sulfoxide; T_3, tri-iodothyronine; I-V, current-voltage.

$I_{Ca.T}$ and shifts the steady-state inactivation curve in the hyperpolarization direction in guinea pig atrial myocytes. However, because the activation voltage range and the inactivation voltage range of $I_{Ca.L}$ and $I_{Ca.T}$ overlap, distinct isolation of one current from another contains possible errors. For example, other Ca^{2+} channel currents in the heterogeneous Ca^{2+} channel expression system, such as in cardiac myocytes, may interfere with pharmacological evaluations of $I_{Ca.T}$ using a wide variety of experimental protocols. The aim of this study was to examine the detailed short- and long-term effects of amiodarone on $I_{Ca.T}$ by the use of the conventional whole-cell patch-clamp technique and the heterologous expression system of the human α_{1H} T-type ($Ca_v3.2$) Ca^{2+} channel.

Materials and Methods

Expression of Ca^{2+} Channel Proteins and Cell Culture. The $\alpha 1$ subunit of $Ca_v3.2$ T-type Ca^{2+} channel (α_{1H}) subunit derived from human heart, which forms cardiac $I_{Ca.T}$, was stably expressed in human embryonic kidney (HEK) 293 cells with no auxiliary subunits (HEK-$Ca_v3.2$ cells). A profile and a procedure for channel expression were described in detail in a previous report (Cribbs et al., 1998). In brief, cDNA encoding the human cardiac α_{1H} subunit (CACNA1H) was inserted into the transfection vector pcDNA3, and HEK 293 cells were transfected with 2 μg of this vector by using calcium phosphate precipitation. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/l streptomycin in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. This medium was supplemented with 300 mg/l G418 (neomycin analog) for the selection of recombinant HEK-$Ca_v3.2$ cells.

Whole-Cell Current Recordings. HEK-$Ca_v3.2$ cells were seeded onto glass-bottomed dishes and were incubated in culture medium for 12 to 24 h without amiodarone before electrophysiological measurements. Macroscopic $I_{Ca.T}$ was recorded in the conventional whole-cell patch-clamp technique using an EPC-9 amplifier (HEKA Electronik, Lambrecht, Germany), at room temperature (22–25°C). Patch pipettes were pulled from 75-mm plain capillary tubes (Drummond Scientific Company, Broomall, PA) with a micropipette puller (model P-97; Sutter Instrument Company, Novato, CA) and fire-polished subsequently. The electrode had a resistance of 1.0 to 5.0 MΩ when the pipette was filled with the pipette solution (see below). Capacitance was minimized, and series resistance was compensated electrically as much as possible without oscillation (60–75%). Current signals were eight-pole Bessel-filtered at 3.3 kHz, digitally sampled at 10 kHz, and stored on a 400-MHz Intel Pentium II-based computer using Microsoft Windows 98 operating system under control of a data acquisition program, Pulse/PulseFit (version 8.11; HEKA Electronik). To investigate the channel availability (steady-state inactivation), conventional double-pulse protocol was applied every 5 s: test pulses of 400 ms at −10 mV after prepulses of 1000 ms from −100 to 0 mV (increment = 5 mV) were applied. Electrophysiological data were collected 3 min or later after application of amiodarone for the evaluation of its short-term effects. To examine the long-term effects of amiodarone, we cultured HEK-$Ca_v3.2$ cells with or without 5 μM amiodarone using 0.05% dimethyl sulfoxide (DMSO) as vehicle for 72 h on glass-bottomed dishes, followed by amiodarone-free culture medium (Dulbecco's modified Eagle's medium) for 12 to 24 h. Voltage-dependent effects of amiodarone on $I_{Ca.T}$ were appreciated in the conductance transforms. Relative conductance (activation) values were calculated as follows: Fractional conductance = $I_{Ca.T}/G_{max}$ $(V_t - V_{rev})$ (A), where $I_{Ca.T}$ represents the current amplitude at the test potential (V_t), and G_{max} is the maximal conductance value obtained from linear regression line of each current-voltage (I-V) relation extrapolated through the estimated reversal potential (V_{rev}). Voltage-dependent activation and availability (steady-state inactivation) were evaluated by a Boltzmann equation fit to the normalized data to peak currents measured in protocols as follows: $Fraction = 1/\{1 + \exp[(V - V_{1/2})/k]\}$ (B), where the normalized data (*Fraction*) were expressed as a function of voltage (V), the test potential in the case of conductance, and the conditioning potential in the case of voltage-dependent availability. Parameters estimated by the fit were the half-point of the relationship ($V_{1/2}$) expressed in millivolts, and k is the slope factor. Recovery from inactivation was evaluated by depolarizing pulse pairs; the prepulses at 0 mV for the duration of 500 ms followed by a test pulse to 0 mV for 200 ms at intervals varying from 5 ms to 3 s. The time course of recovery was fitted by a single exponential equation. Dose-response curves were obtained by the following Hill equation as follows: $Fraction = 1/\{1 + (IC_{50}/[C]^h)\}$ (C), where [C] is the concentration of amiodarone, IC_{50} is the half-maximal blocking concentration of $I_{Ca.T}$ by amiodarone, and h is the Hill coefficient.

Solutions. The recording chamber was filled with bath solution of the following composition: 120 mM tetraethylammonium chloride, 6 mM CsCl, 5 mM 4-aminopyridine, 0.5 mM $MgCl_2$, 0.5 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid disodium salt hydrate, 10 mM HEPES, 1.8 mM $CaCl_2$, and 10 mM glucose (pH was adjusted to 7.4 with 1 N tetraethylammonium hydroxide solution). The patch-clamp electrode was filled with pipette solution of 130 mM CsCl, 2 mM $MgCl_2$, 2 mM ATP, 0.5 mM GTP, 5 mM EGTA, and 5 mM HEPES (pH was adjusted to 7.3 with 1 N CsOH). Amiodarone hydrochloride was dissolved in DMSO to prepare a stock solution. On the day of the experiment, aliquots of the stock solution were diluted with the bath solution. Data were acquired by using computer software (Pulse/PulseFit, version 8.11; HEKA Electronik), and all curve fittings and figures were made on SigmaPlot (version 6.1; SPSS, Inc., Chicago, IL).

Drugs. Amiodarone hydrochloride was a gift of Taisyo Pharmaceutical Co. (Tokyo, Japan). Tri-iodothyronine (T_3) was purchased from Wako Pure Chemical Ind. (Osaka, Japan), and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Statistics. Group data are shown as mean ± S.E. Analysis of variance and Tukey-Kramer procedure were used for multiple comparisons, and Student's t test was used for comparison between two means. Differences were considered significant when p values were <0.05.

Results

In conventional whole-cell patch-clamp experiments using HEK-$Ca_v3.2$ cells, T-type Ca^{2+} currents ($I_{Ca.T}$) were recorded. Figure 1A shows representative examples of $I_{Ca.T}$ change with (○) or without (□) acute amiodarone application. Peak current was rapidly decreased by the application of 2 μM amiodarone and was partially reversed upon washout. Although these currents were obtained with the conventional, whole-cell configuration, that is, with the intracellular milieu dialyzed in pipette solution, currents did not exhibit significant "run-down". For example, current peak amplitude was −1.12 nA at 1 min and −1.10 nA at 11 min after obtaining the whole-cell mode in this patch (Fig. 1A, d and e), and the percentage amplitude of $I_{Ca.T}$ at 11 min was 98.2 ± 1.0% when $I_{Ca.T}$ at 1 min after obtaining the whole-cell mode was assigned as 100% ($n = 5$, $P > 0.01$). Figure 1, B and C, illustrates the short-term effects of 1 μM amiodarone on representative whole-cell current traces elicited by a depolarization pulse of 300-ms duration, ranging from −80 to +50 mV in 5-mV steps, applied from the holding potential of −100 mV, and an I-V relationship constructed using group data, indicating a reduction of the maximum current by 25.4 ± 8.0% ($n = 23$). To analyze the dose-dependent inhibitory

effect of amiodarone, the activation and the steady-state inactivation (availability) curves were compared (Fig. 1D). Both the activation and the steady-state inactivation curves were shifted by amiodarone in the hyperpolarization direction in a dose-dependent manner. The average values for potentials of half-activation ($V_{1/2}$, activation) and half-inactivation ($V_{1/2}$, inactivation) under control condition and the presence of amiodarone are summarized in Table 1. Dose-dependent significant shifts of activation and steady-state inactivation curves by amiodarone were observed; that is, 2 and 5 μM amiodarone shifted the activation and the steady-state inactivation curves in the hyperpolarization direction by 4.1 $\pm$ 1.4 mV (n = 19) and 9.5 $\pm$ 1.9 mV (n = 6), respectively (paired test, $P < 0.001$). DMSO as a vehicle (control) at the concentration of 0.1% had no significant effect on $I_{Ca.T}$ (data not shown).

To evaluate the tonic and use-dependent blocking properties of amiodarone on $I_{Ca.T}$, a train of pulses was applied (Fig. 1E). Repetitive voltage-clamp pulses without amiodarone resulted in a decrease of $I_{Ca.T}$ by 24.3 $\pm$ 2.6% (n = 6), indicating that the interval between pulses is too short compared with the time constant for the recovery from inactivation. Amiodarone caused a marked tonic block (35.7 $\pm$ 3.2%, n = 6), which is evident as a reduced $I_{Ca.T}$ during the first pulse of the train, followed by a further decrease during repetitive pulses (apparent use-dependent block) (35.4 $\pm$ 5.4%, n = 6), which indicates that the pulse interval is too short compared with the unblocking rate of amiodarone (Table 2). Because a use-dependent block (24.3%) occurs without amiodarone at a stimulation frequency of 1 Hz, amiodarone-delimited use-dependent block ratio was calculated (17.2%).

Because amiodarone reportedly exerts short- and long-term effects on various cardiac ion channels, we examined the long-term effects of amiodarone on $I_{Ca.T}$. $I_{Ca.T}$ recorded from HEK-Ca_v3.2 cells treated with 2 μM amiodarone for 72 h revealed a significant delay in recovery from inactivation in the absence of amiodarone in the bath solution [τ: 2 μM long-term effects of amiodarone (n = 6), 618 $\pm$ 71 ms]. Figure 2A shows the representative $I_{Ca.T}$ from HEK-Ca_v3.2 cells with and without amiodarone treatment for 72 h. HEK-Ca_v3.2 cells undergoing long-term treatment with amiodarone expressed significantly smaller $I_{Ca.T}$ than those cells not treated with amiodarone (DMSO control cells), indicating a reduction of the maximum peak $I_{Ca.T}$ by 31.7 $\pm$ 2.3%, although amiodarone was excluded from both the culture medium and the bath solution for more than 12 h (Fig. 2, A and B). The activation and steady-state inactivation curves obtained from HEK-Ca_v3.2 cells cultured with 5 μM amiodarone were shifted in the depolarization direction by $-$7.1 and $-$5.5 mV, respectively (Fig. 2C and Table 1). Because there are a number of studies on the interaction of amiodarone and thyroid hormones in clinical and experimental studies (Guo et al., 1997; Bosch et al., 1999; Shahrara and Drvota, 1999), we conducted experiments for the evaluation of long-term

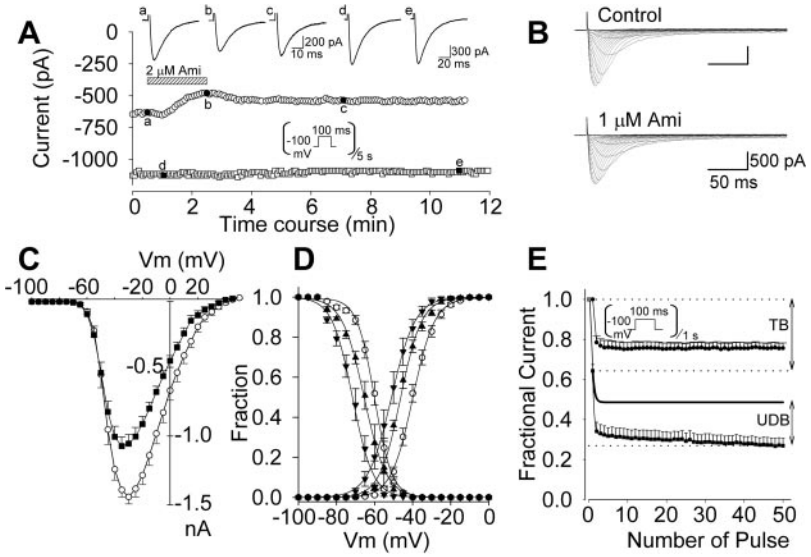

Fig. 1. Short-term amiodarone effects on $I_{Ca.T}$. A, typical time course of the peak $I_{Ca.T}$ recorded from HEK-Ca_v3.2 cells with (○) or without 2 μM amiodarone (□). Amiodarone (2 μM) was immediately applied to the bath solution for 2 min. Five different current traces of $I_{Ca.T}$ at $-$10 mV obtained from a pair of different patches (●, ■) are shown before (a), during application of 2 μM amiodarone (b), during washing-out period (c), and at 1 (d) and 11 min (e) after obtaining the whole-cell mode in the absence of amiodarone. $I_{Ca.T}$ was elicited from the holding potential of $-$100 mV at the test potential of $-$10 mV with the stimulation frequency of 0.2 Hz. B, representative whole-cell current families before (top traces) and after (bottom traces) application of 1 μM amiodarone (Ami) obtained from an HEK-Ca_v3.2 cell. Currents were elicited from a holding potential of $-$100 mV at the stimulation frequency of 0.2 Hz. C, I-V relationship with (■, n = 23) or without 1 μM amiodarone (○, n = 78). Amiodarone (1 μM) decreased the maximum peak $I_{Ca.T}$ by 25.4 $\pm$ 8.0% (paired comparison with n = 23). D, dose-dependent shifts of activation and steady-state inactivation curves caused by amiodarone. Curves were fitted by the Boltzmann equation in each data group. Control (○, n = 78 for activation and n = 55 for inactivation), 2 μM amiodarone (▲, n = 19 for activation and n = 14 for inactivation), and 5 μM amiodarone (▼, n = 6 for activation and n = 9 for inactivation). E, time course for development of use-dependent block of $I_{Ca.T}$ by 2.5 μM amiodarone. $I_{Ca.T}$ was recorded by a train of 50 pulses consisting of 100-ms duration at a test potential $-$10 mV from the holding potential of $-$100 mV with the stimulation frequency of 1 Hz. Shown are data for controls (●, n = 6) and for short-term 2.5 μM amiodarone application (■, n = 6). Each point in the presence of amiodarone indicates fractional peak currents normalized by the value for the first pulse of the train (□, pulse 0), which was obtained before drug application. A train of 50 pulses, beginning with pulse 1, was applied 3 min after the application of amiodarone, during which no electrical stimulation was applied. The fitting curve for control data (●) was reconstructed (boldface line), in which the point for the control current number 1 (●, 1.0) was reassigned to the current number 1 during short-term amiodarone application (■, 0.643), yielding a drug-independent use-dependent block component during short-term amiodarone application. Ratios for tonic block (TB) and drug-delimited use-dependent block (UDB) during short-term amiodarone application are identified.

amiodarone effects on $I_{Ca.T}$ with or without thyroid hormone T_3. As shown in Fig. 2, exposure of HEK-Ca_v3.2 cells to T_3 for 72 h did not modify the long-term effect of amiodarone on $I_{Ca.T}$, in terms of current amplitude and voltage dependence of activation and steady-state inactivation curves.

The short-term application of amiodarone shifted the activation and steady-state inactivation curves in the hyperpolarization direction, and long-term treatment of HEK-Ca_v3.2 cells by amiodarone shifted the same curves in the depolarization direction. Therefore, we found that $I_{Ca.T}$ in HEK-Ca_v3.2 cells subjected to long-term amiodarone treatment was diminished because of a mechanism that is different compared with those in short-term treatment.

To study the affinity of amiodarone for the distinct channel states (i.e., activation, inactivation, and resting states), we examined whether the time course for recovery from inactivation of $I_{Ca.T}$ was modulated by short- and long-term amiodarone treatment. Thus, we examined the recovery time course from inactivation by using the pulse protocol illustrated in Fig. 3. Recovery from inactivation was significantly delayed by immediately applied amiodarone at a concentration of 2 μM [time constant τ: control ($n = 6$), 490 ± 35 ms; 2 μM amiodarone ($n = 6$), 1081 ± 95 ms] ($p < 0.001$), which is consistent with the findings shown in Fig. 1E. Recovery from inactivation recorded in HEK-Ca_v3.2 cells subjected to long-term amiodarone treatment for 72 h was significantly delayed in the absence of amiodarone in the bath solution, which indicates that gating properties of $I_{Ca.T}$ might be modified by long-term amiodarone treatment.

Long-term treatment of HEK-Ca_v3.2 cells by amiodarone for 72 h not only decreased the current density and retarded the recovery from inactivation but also modified the channel kinetics (i.e., amiodarone delayed the channel relaxation). When the peaks of the current traces were matched at the same test potential of −30 mV, the $I_{Ca.T}$ treated with long-term amiodarone showed a significantly delayed relaxation (Fig. 4, inset a). $I_{Ca.T}$ relaxation was delayed as well when the peaks of the current traces were matched at the test potentials that displayed the maximum current (Fig. 4, inset b). The $I_{Ca.T}$ relaxation process at each test potential was well-fitted by a single exponential function yielding τ for $I_{Ca.T}$ relaxation (Fig. 4). After the short-term application of amiodarone, $I_{Ca.T}$ relaxation was unaffected, regardless of the significant shift of the activation curve in the hyperpolarized direction. On the other hand, during long-term amiodarone treatment, τ was significantly larger than that observed without amiodarone treatment, indicating retarded inactivation processes of channel gating produced by long-term amiodarone treatment.

We examined whether $I_{Ca.T}$ subjected to long-term amiodarone treatment has sensitivity to a short-term application of amiodarone. Short-term application of 1 μM amiodarone lessened $I_{Ca.T}$ that received long-term 5 μM amiodarone treatment for 72 h in the representative $I_{Ca.T}$ traces by 28.1%, as shown in Fig. 5A, and by 31.8 ± 17.1% ($n = 9$) in group data (Fig. 5B). The I-V relationship of $I_{Ca.T}$ was shifted in the hyperpolarization direction by the action of short-term amiodarone administration on $I_{Ca.T}$ that had undergone

TABLE 1

Short- and long-term effects of amiodarone on activation and inactivation parameters

Activation and inactivation parameters were determined by protocols described under *Materials and Methods* and in Figs. 1, 2, and 5. Data from each cell were collected before and in the presence of 1, 2, and 5 μM amiodarone for the evaluation of short-term effects of the drug. For the evaluation of long-term effects of the drug, cells were incubated with [Ami(C)] or without 5 μM amiodarone (DMSO) for 72 h, followed by amiodarone-free culture medium for 12 to 24 h. Experiments for the evaluation of Ami(C) were performed under control condition of the bath solution without amiodarone.

	$V_{1/2}$	
	Activation	Inactivation
	mV	
Short-Term Effects on $I_{Ca.T}$		
Control	−42.5 ± 0.4 ($n = 78$)	−59.8 ± 0.5 ($n = 78$)
1 μM Ami	−44.4 ± 1.0 ($n = 23$)	−62.5 ± 0.9* ($n = 30$)
2 μM Ami	−46.1 ± 1.0** ($n = 19$)	−66.1 ± 1.5** ($n = 14$)
5 μM Ami	−51.2 ± 4.9† ($n = 6$)	−70.4 ± 2.1†† ($n = 9$)
Long-Term Effects on $I_{Ca.T}$		
Control (DMSO)	−43.3 ± 0.3 ($n = 66$)	−60.0 ± 0.4 ($n = 76$)
Ami(C)	−36.2 ± 0.7‡ ($n = 41$)	−54.5 ± 0.6‡ ($n = 37$)
Ami(C) + 1 μM Ami	−43.9 ± 0.7$^{\blacktriangle}$ ($n = 9$)	−63.2 ± 0.9$^{\blacktriangle}$ ($n = 11$)

* $P < 0.01$ vs. control (paired).
** $P < 0.001$ vs. control (paired).
† $P < 0.01$ vs. 1 μM Ami (paired).
†† $P < 0.001$ vs. 1 μM Ami (paired).
‡ $P < 0.001$ vs. control (DMSO) (nonpaired).
$^{\blacktriangle}$ $P < 0.001$ vs. Ami(C) (paired).

TABLE 2

Tonic and use-dependent block of $I_{Ca.T}$ by amiodarone

Ami(C), HEK-Ca_v3.2 cells were treated with 5 μM amiodarone for 72 h, followed by amiodarone-free culture medium for 12 to 24 h.

	Tonic (Resting) Block	Use-Dependent Block	Amiodarone-Delimited Use-Dependent Block
		%	
Control		24.3 ± 2.6 ($n = 6$)	
Control + 2.5 μM Ami	35.7 ± 3.2 ($n = 6$)	35.4 ± 5.4* ($n = 6$)	17.2 ± 3.4 ($n = 6$)
Ami(C)		30.1 ± 3.3 ($n = 6$)	
Ami(C) + 2.5 μM Ami	39.5 ± 3.1 ($n = 5$)	30.6 ± 3.7 ($n = 5$)	15.0 ± 3.3 ($n = 5$)

* $P < 0.05$ vs. control (paired).

long-term treatment previously (Fig. 5B). Both the activation and the steady-state inactivation curves were shifted in the hyperpolarization direction by the short-term application of amiodarone, regardless of the long-term amiodarone treatment (Fig. 5C). The average values of $V_{1/2}$ for activation and $V_{1/2}$ for inactivation obtained from HEK-Ca_v3.2 cells that had undergone long-term amiodarone treatment, and the short-term effects of amiodarone on them, are summarized in Table 1. To identify the blocking properties of $I_{Ca.T}$ by long-term amiodarone treatment and their modulation by short-term amiodarone application, a train of pulses was applied (Fig. 5D). Short-term amiodarone application caused marked tonic block and use-dependent block of $I_{Ca.T}$ nearly identical with those observed without long-term amiodarone treatment (Fig. 1E): short-term 2.5 μM amiodarone treatment inhibited $I_{Ca.T}$ by 35.7 ± 3.2% (n = 6) in tonic block and by 17.2 ± 3.4% (n = 6) in amiodarone-delimited use-dependent block (Fig. 1E, Table 2), whereas short-term 2.5 μM amiodarone inhibited $I_{Ca.T}$ in cells that received long-term treatment with amiodarone by 39.5 ± 3.1% (n = 5) in tonic block and by 15.0 ± 3.3% (n = 5) in amiodarone-delimited use-dependent block (Fig. 5D, Table 2).

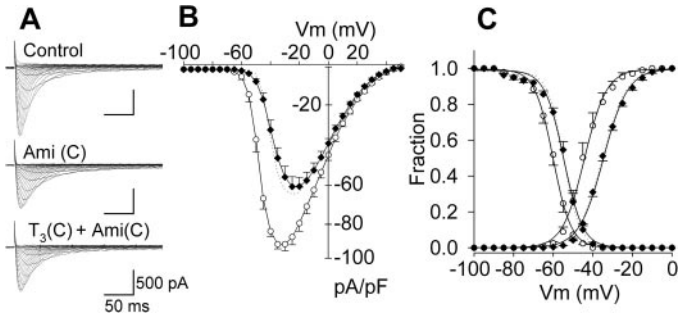

Fig. 2. Comparison of short- and long-term effects of amiodarone. A, representative whole-cell current traces obtained from an HEK-Ca_v3.2 cell cultured for 72 h without amiodarone (control, DMSO), with 5 μM amiodarone [Ami(C)], and with 5 μM amiodarone plus 0.12 nM T_3 [Ami(C) + T_3(C)]. B, I-V relationships for $I_{Ca.T}$ with or without long-term amiodarone treatment corrected by cell capacitance. Control (DMSO) (○, n = 66), long-term treatment of 5 μM amiodarone [Ami(C)] (♦, n = 41), long-term treatment of 5 μM amiodarone with 0.12 nM T_3 [Ami(C)+ T_3(C)] (broken line without symbols, n = 6). C, shifts of activation and steady-state inactivation curves by long-term amiodarone treatment. Curves were fitted by the Boltzmann equation in each data group. Control (DMSO) (○, n = 66), Ami(C) (♦, n = 41), and Ami(C) + T_3(C) (broken line without symbols, n = 6).

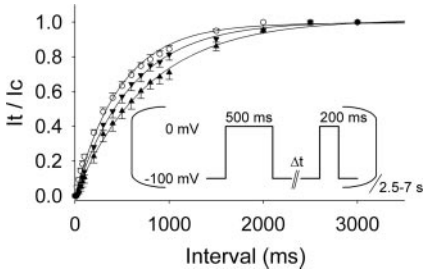

Fig. 3. Time course of recovery from inactivation of $I_{Ca.T}$. Curves were fitted to the data using a single exponential equation yielding time constant (τ). control (○, n = 6), 490 ± 35 ms; short-term 2 μM amiodarone (▲, n = 5), 1081 ± 95 ms; long-term 2 μM amiodarone (▼, n = 5), 618 ± 71 ms. Inset, the pulse protocol.

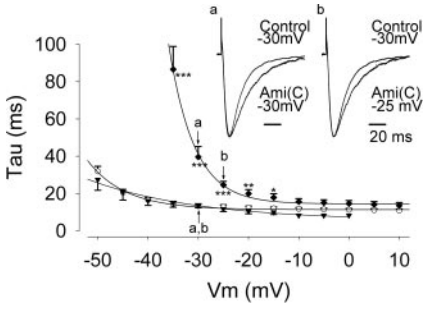

Fig. 4. $I_{Ca.T}$ relaxation (inactivation) with or without long-term amiodarone treatment. The decay of the current was fitted by a single exponential equation. Inactivation time constant (τ) was plotted against the test potential. Each plot was constructed for three groups of data: the control (DMSO) condition (○, n = 6), short-term treatment with 5 μM amiodarone (▼, n = 6), and long-term treatment with amiodarone [Ami(C)] (♦, n = 6). a, representative current traces at −30 mV (indicated by arrows) with or without long-term amiodarone treatment that were normalized to the peak current. b, representative current traces with or without long-term amiodarone treatment at the maximum current [control at −30 mV, Ami(C) at −25 mV, indicated by arrows) normalized to the peak current. *, $P < 0.05$ versus control (nonpaired): **, $P < 0.01$ versus control (nonpaired); ***, $P < 0.001$ versus control (nonpaired).

Finally, we obtained IC_{50} values for amiodarone in short-term application on $I_{Ca.T}$, long-term application on $I_{Ca.T}$, and short-term application on $I_{Ca.T}$ from the HEK-Ca_v3.2 cells that received long-term treatment with amiodarone. As a tonic-blocking action, short-term amiodarone application inhibited $I_{Ca.T}$ with an IC_{50} value of 2.4 μM, whereas application of amiodarone for 72 h lessened $I_{Ca.T}$ with an IC_{50} value of 9.4 μM (Fig. 6). The short-term effect of amiodarone on $I_{Ca.T}$ in HEK-Ca_v3.2 cells that received long-term treatment with amiodarone exhibited an IC_{50} value of 2.8 μM (Fig. 6).

Discussion

Short- and long-term effects of amiodarone on Ca_v3.2 T-type Ca^{2+} channel current ($I_{Ca.T}$) were studied using the conventional patch-clamp technique. Short-term amiodarone effects were the following: 1) blocking of $I_{Ca.T}$ occurred in a dose-dependent manner; 2) activation and steady-state inactivation curves of $I_{Ca.T}$ were shifted in the hyperpolarization direction; 3) delayed recovery from inactivation of $I_{Ca.T}$; and 4) blocking of $I_{Ca.T}$ in a tonic and use-dependent manner, regardless of whether long-term amiodarone treatment was applied or not. Long-term amiodarone effects were the following: 1) $I_{Ca.T}$ density was significantly decreased; 2) activation and the steady-state inactivation curves of $I_{Ca.T}$ were shifted in the opposite direction (i.e., the depolarization potentials); 3) fast current inactivation (relaxation) was significantly delayed; 4) short-term inhibitory effects of amiodarone on $I_{Ca.T}$ that received long-term treatment with amiodarone were functionally maintained; and 5) amiodarone caused both tonic and use-dependent block of $I_{Ca.T}$ corresponding to its action on the recovery from inactivation. In conclusion, the short- and long-term effects of amiodarone on $I_{Ca.T}$ were distinct in their activities, which indicate the complicated pharmacological actions of amiodarone when administered for the treatment of $I_{Ca.T}$-related arrhythmias in clinical usage.

Short-Term Effects of Amiodarone on Cardiac Ca^{2+} Channels. Although short-term application of amiodarone promptly decreased $I_{Ca.T}$, we could not reverse $I_{Ca.T}$ completely upon washout of the drug (Fig. 1A). As already pointed out, amiodarone is a highly lipophilic drug (oil/water partition coefficient, log P = 5.95, and pK_a = 8.7 at 37°C) (Chatelain et al., 1986). Short-term effects of amiodarone

cannot be consistently and completely reversed during short-term experiments in the literature (Raatikainen et al., 1996; Kodama et al., 1997).

In 1992, using guinea pig atrial cells, Cohen et al. demonstrated that amiodarone at a concentration of 10 μM decreased $I_{Ca.T}$, shifting the steady-state inactivation curve in the hyperpolarization direction. As far as we know, this is the only published report showing an inhibitory effect of amiodarone on $I_{Ca.T}$. However, in their report, no blocking mechanism or the inhibitory potency of amiodarone on $I_{Ca.T}$ was observed. In our study, we used a heterologous expression system of α_{1H} ($Ca_v3.2$) in HEK 293 cells to examine the detailed pharmacological effects of amiodarone on homogenous $I_{Ca.T}$. It has been shown that some calcium antagonists demonstrate a general affinity for blocking both $I_{Ca.L}$ and $I_{Ca.T}$. For example, bepridil (Yatani et al., 1986; Uchino et al., 2005), mibefradil (Mehrke et al., 1994), and efonidipine (Masumiya et al., 1998, 2000) block $I_{Ca.L}$ and $I_{Ca.T}$ with different efficacies and affinities. Lubic et al. (1994) studied the interaction of amiodarone with receptors for the 1,4-dihydropyridine in rat and rabbit myocardial membranes and found that amiodarone completely inhibited the specific binding to myocardial membrane receptors by 1,4-dihydropyridine Ca^{2+} channel blockers. These results provide evidence that amiodarone modifies current kinetics by directly interacting with the Ca^{2+} channel and/or its immediate lipidic environment.

Short-term effects of amiodarone on blocking $I_{Ca.L}$ have been reported as follows: 1) amiodarone decreased $I_{Ca.L}$ in a concentration-dependent manner, with an IC_{50} value of 0.36 to 5.8 μM; 2) amiodarone (5–16 μM) shifted the steady-state inactivation curve of $I_{Ca.L}$ in the hyperpolarizing direction by 9 to 10 mV; and 3) amiodarone caused both tonic and use-dependent reduction of $I_{Ca.L}$ (Nishimura et al., 1989; Valenzuela and Bennett, 1991; Varró et al., 1996). Short-term inhibitory actions of amiodarone might be attributed to binding to a single receptor. The block depends on whether the membrane is held at rest or is hyperpolarized electrically for longer or shorter periods, and it depends on the frequency and type of stimulation (Hille, 1977). Short-term application of 2.5 μM amiodarone caused both tonic and use-dependent block of $I_{Ca.T}$ (Fig. 1E), in which tonic block primarily reflects rested-state block. The fractional ratio of (tonic block)/(use-dependent block) was approximately 2.1 for short-term 2.5 μM amiodarone application on $I_{Ca.T}$ (Table 2). The lower the

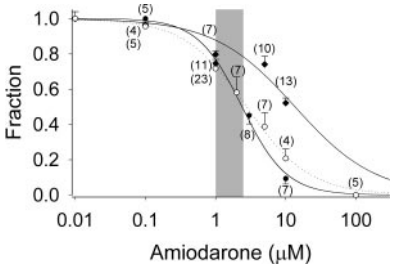

Fig. 6. Dose-response relationship of short- and long-term actions of amiodarone on $I_{Ca.T}$. Short- and long-term effects of amiodarone were evaluated by a pulse protocol composed of a holding potential of −100 mV to a test potential for the maximum current (−25 to −35 mV) at the stimulation frequency of 0.2 Hz. Short-term effect (●, n = 5–23), long-term effect (♦, n = 5–13), and short-term effect on cells treated with 5 μM long-term amiodarone (○, n = 4–11) on $I_{Ca.T}$. Data were fitted by the Hill equation yielding IC_{50} values of 2.4, 9.4, and 2.8 μM, respectively. Therapeutic plasma concentration (1–3 μM) is indicated by shading (Singh et al., 1989).

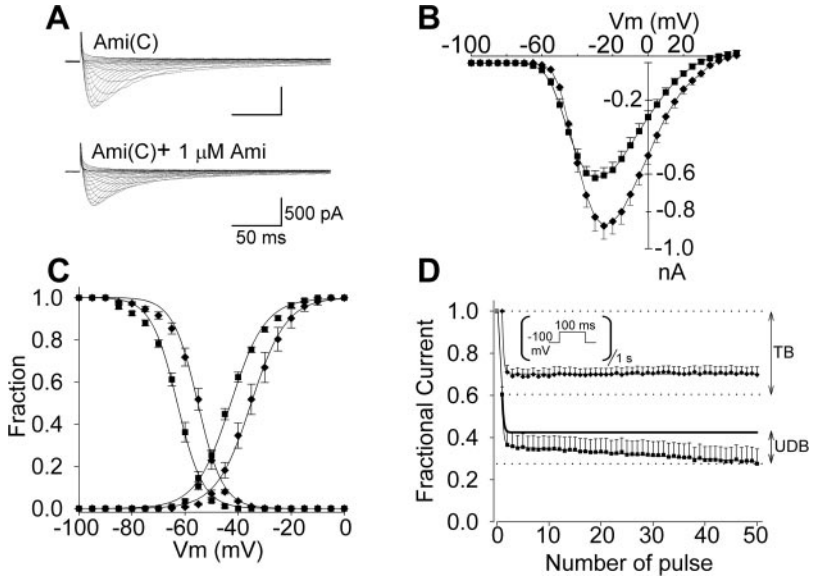

Fig. 5. Effects of short-term amiodarone application on $I_{Ca.T}$ with long-term amiodarone treatment for 72 h. A, representative $I_{Ca.T}$ families obtained from an HEK-$Ca_v3.2$ cell with long-term amiodarone treatment [Ami(C)] and short-term effects of 1 μM amiodarone [Ami(C) + 1 μM Ami]. B, I-V relationship of $I_{Ca.T}$ treated with 5 μM amiodarone [Ami(C)] (♦, n = 9) and short-term 1 μM amiodarone application [Ami(C) + 1 μM Ami] (■, n = 9). C, voltage dependence of activation and steady-state inactivation curves of long-term amiodarone treatment (♦, n = 9), and short-term amiodarone treatment (1 μM) and effects on continually treated $I_{Ca.T}$ (■, n = 9). Note that the activation and steady-state inactivation curves were significantly shifted, with short-term application of 1 μM amiodarone, in the hyperpolarization direction by 8.6 ± 1.1 mV (n = 9) and 8.0 ± 1.5 mV (n = 11), respectively ($P < 0.001$). D, time course for the development of use-dependent block of $I_{Ca.T}$ recorded from HEK-$Ca_v3.2$ cells treated with long-term 5 μM amiodarone with or without short-term 2.5 μM amiodarone application. The same pulse protocol consisting of a train of 50 pulses described in Fig. 1E was applied. Shown are data for long-term 5 μM amiodarone [Ami(C)] (♦, n = 6) and short-term 2.5 μM amiodarone application on $I_{Ca.T}$ recorded from HEK-$Ca_v3.2$ cells treated with long-term 5 μM amiodarone [Ami(C) + 2.5 μM Ami] (■, n = 6). The fitting curve for long-term amiodarone data (♦) was reconstructed (boldface line), in which the point for the current number 1 (♦, 1.0) was reassigned to the current number 1 during short-term amiodarone application (■, 0.605), yielding a drug-independent use-dependent component during short-term amiodarone application. Ratios for tonic block (TB) and drug-delimited use-dependent block (UDB) during short-term amiodarone application are identified.

ratio, the stronger the gain of potency obtained during the high frequency of stimulation. Fractional tonic block by short-term 2.5 μM amiodarone on $I_{Ca.T}$ (35.7%) and inhibition in cells that underwent long-term treatment with amiodarone on $I_{Ca.T}$ (39.5%) were statistically identical (P = 0.45). Moreover, drug-delimited use-dependent block of short-term 2.5 μM amiodarone on $I_{Ca.T}$ (17.2%) and inhibition in cells that underwent long-term treatment with amiodarone on $I_{Ca.T}$ (15.0%) were also statistically identical (P = 0.66). These phenomena can be qualitatively explained by assuming that amiodarone preferentially binds to the resting or activated state rather than to the inactivated state of the α_{1H} T-type Ca^{2+} channel, regardless whether the channel received long-term amiodarone treatment or not. Taken together, these data and ours indicate that the short-term blocking effects of amiodarone on $I_{Ca.L}$ and $I_{Ca.T}$ are alike, suggesting that amiodarone blocks these two types of Ca^{2+} channels via the same mechanism.

Long-Term Effects of Amiodarone on Cardiac Ion Channels. The most striking difference in the effects produced by short- versus long-term administration of amiodarone can be found on the shifts in activation and steady-state inactivation curves (Figs. 1D and 2C) and on current relaxation (Fig. 4). Desensitization of $I_{Ca.T}$ by long-term amiodarone treatment is unlikely, because the potency of short-term amiodarone on $I_{Ca.T}$ (IC_{50} = 2.4 μM) and the potency of short-term amiodarone on $I_{Ca.T}$ continually treated with amiodarone (IC_{50} = 2.8 μM) are nearly identical (Fig. 6). It is widely recognized that the most prominent long-term effects of amiodarone include prolongation of the refractory period and action potential durations (Kodama et al., 1997, 1999), suggesting the inhibition of K^+ currents (Kamiya et al., 2001). Their studies suggest that decreases in I_{Kr}, I_{Ks}, and I_{to} are due to down-regulation of channel proteins primarily caused by gene regulations for ion channel expression. A comprehensive study of cDNA microarrays of ion channels by long-term amiodarone treatment was reported by Le Bouter et al. (2004). They found that Na^+ channel α- and β-subunits; L-type Ca^{2+} channel α_1, β_1, and β_2 subunits; T-type Ca^{2+} channel α_{1G} subunit; Kv2.1, Kv1.5, and Kv4.2, ATP-sensitive K^+ channels; Kir6.2; and SUR2 were down-regulated, and that other K^+ channel α- and β-subunits, such as KNCA4, KCNK1, KCNAB1, and KCNE3, were up-regulated. However, possible remodeling of the T-type Ca^{2+} channel α_{1H} subunit by long-term amiodarone treatment has not yet been studied. The cardiac T-type Ca^{2+} channel is composed of two subtypes of α subunits, α_{1H} and α_{1G}. The α_{1H} subunit differs from the α_{1G} subunit in many respects (Perez-Reyes, 2003). Hagelüken et al. (1995) reported that amiodarone is a direct activator of G_i and G_o proteins, activating nonselective cation channels in HL-60 cells via these proteins. Lledo et al. (1992) and Lu et al. (1996) proposed that $I_{Ca.T}$ could be modulated by hormones and neurotransmitters coupled to the G_q or G_i/G_o families. It is therefore speculated that amiodarone may exert its long-term effects on the $Ca_v3.2$ T-type Ca^{2+} via G-protein(s). It is more interesting that α_{1H} T-type Ca^{2+} channel kinetics were drastically modified by long-term amiodarone treatment, particularly on the current relaxation phase. Although no proteins or signals that modulate the kinetics of α_{1H} have yet been identified, our results suggest the presence of functional auxiliary protein(s) regulated by amiodarone. Because the T-type Ca^{2+} channels are postulated to play important roles in pathological conditions of the heart, long-term amiodarone treatment may reverse the remodeling of $I_{Ca.T}$. Further study is needed to clarify the interaction of G_i/G_o, transcription factors for $Ca_v3.2$ channel expression, modulators for the promoter regions of the channel gene, and/or subsidiary proteins with the $Ca_v3.2$ proteins in the presence of amiodarone.

Thyroid Hormone-Independent Effects of Amiodarone on $I_{Ca.T}$. It is known that amiodarone can cause either hypothyroidism (Martino et al., 1984) or hyperthyroidism (Martino et al., 1987). Cardiac electrophysiologic changes induced by long-term treatment with amiodarone closely resemble those induced by hypothyroidism (Kodama et al., 1997). However, hypothyroidism does not mimic all of the long-term effects of amiodarone (Lambert et al., 1987; Bosch et al., 1999). Guo et al. (1997) have demonstrated that 72-h exposure to 1 μM amiodarone significantly decreased the current density of I_{to} and I_K in the cardiac myocytes only when the cells were cocultured with T_3. In our study, down-regulation of $I_{Ca.T}$ in long-term treatment with amiodarone was mediated independently of thyroid hormone action. It is noteworthy that exposure of HEK-$Ca_v3.2$ cells to amiodarone in the presence of T_3 resulted in changes of channel kinetics and density of $I_{Ca.T}$ that were identical with those observed in the absence of T_3 (Fig. 2). Based on these findings, we conclude that the inhibition and regulation of $I_{Ca.T}$ as the result of long-term amiodarone treatment are exerted independently of its action through the modulation of thyroid hormone secretion.

In conclusion, as short-term effects, amiodarone binds directly to the α_{1H} channel, decreases the current, and shifts activation and steady-state inactivation curves in the hyperpolarization direction. On the other hand, as long-term effects, amiodarone regulates the expression of the α_{1H}, probably through unknown protein(s) and/or signaling molecules. The short- and long-term effects of amiodarone on $I_{Ca.T}$ are completely different in their pharmacological actions, which indicate the complicated therapeutic activity of amiodarone on the regulation of T-type Ca^{2+} channel-related arrhythmias in clinical application.

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