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**Acute and Chronic Amiodarone Treatments Regulate $Ca_v3.2$ Low-Voltage-Activated T-type $Ca^{2+}$ Channel Through Distinct Mechanisms**

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**Running title:** Amiodarone Affects T-Type $Ca^{2+}$ Channel Kinetics.

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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; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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**Abstract**

Low-voltage-activated T-type $Ca^{2+}$ channels have recently been recognized in the mechanisms underlying atrial arrhythmias. However, the pharmacological effects of amiodarone on the T-type $Ca^{2+}$ channel remain unclear. We investigated acute and chronic effects of amiodarone on the T-type ($Ca_v3.2$) $Ca^{2+}$ channel. The $Ca_v3.2$ ($\alpha_{1H}$) subunit derived from human heart was stably transfected into cells (HEK-$Ca_v3.2$) cultured with or without 5 μM amiodarone. Patch clamp recordings in the conventional whole-cell configuration were employed to evaluate 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 acutely to the bath solution. However, when the HEK-$Ca_v3.2$ cells were incubated with 5 μM amiodarone for 72 hours, $I_{Ca.T}$ density was significantly decreased, by 31.7 ± 2.3%, control, -93.1 ± 4.3 pA/pF (n = 8) vs. amiodarone, -56.5 ± 3.2 pA/pF (n = 13), $P<0.001$. Following the chronic administration of amiodarone, the activation and the steady-state inactivation curves were shifted in the depolarization direction by -7.1 mV (n = 41) and -5.5 mV (n = 37), respectively, and current inactivation was significantly delayed (time constant ($\tau$): control, 13.3 ± 1.1 ms (n = 6) vs. amiodarone, 39.6 ± 5.5 ms (n = 6) @-30 mV, $P<0.001$). Nevertheless, acute 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 acute and chronic inhibitory actions on $I_{Ca.T}$ via distinct blocking mechanisms.



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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 per the Vaughan Williams classification. In electrophysiological experiments, reported acute 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 chronic effects of amiodarone on cardiac myocytes and papillary muscles include prolonging the action potential durations and refractory periods (Kodama et al., 1997, 1999). However, acute and chronic 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 et al., 1990). $I_{Ca.T}$ appears to play a particularly important role in pathophysiological or remodeled cardiovascular tissue (Ertel et al., 1997): atrial $I_{Ca.L}$ was downregulated 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.



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Cohen et al. (1992) demonstrated that amiodarone inhibits $I_{Ca.T}$ and shifts the steady-state inactivation curve in the hyperpolarization direction in guinea pig atrial myocytes. However, since 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 acute and chronic effects of amiodarone on $I_{Ca.T}$ by use of the conventional whole cell patch clamp technique and the heterologous expression system of the human $\alpha_{1H}$ T-type ($Ca_v3.2$) $Ca^{2+}$ channel.

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## Materials and Methods

**Expression of $Ca^{2+}$ channel proteins and cell culture.** The α1 subunit of $Ca_v3.2$ T-type $Ca^{2+}$ channel ($\alpha_{1H}$) 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 $\alpha_{1H}$ subunit (CACNA1H) was inserted into the transfection vector pc DNA3, 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 (DMEM), 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-bottom dishes and incubated in culture medium for 12-24 hours without amiodarone prior to 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, U.S.A) with a micropipette puller, Model P-97 (Sutter Instruments Company, Novato, CA, U.S.A), and fire-polished subsequently. The electrode had a resistance of 1.0-5.0 MΩ when the pipette was filled with the pipette solution (see below). Capacitance was minimized and series resistance was



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compensated electrically as much as possible without oscillation (60-75%). Current signals were 8-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 (V. 8.11, HEKA Electronik). To investigate the channel availability (steady-state inactivation), conventional double-pulse protocol was applied at every 5 seconds (s): test pulses of 400 ms at -10 mV following 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 acute effects. To examine chronic 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 hours on glass-bottom dishes, followed by amiodarone-free culture medium (DMEM) for 12-24 hours. Voltage-dependent effects of amiodarone on $I_{Ca.T}$ were appreciated in the conductance transforms. Relative conductance (activation) values were calculated as follows:

$$\textit{Fractional conductance} = I_{Ca.T} / G_{max} (V_t - V_{rev}), \qquad \text{(A)}$$

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 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:

$$\textit{Fraction} = 1 / \{1 + \exp [(V - V_{1/2}) / k]\}, \qquad \text{(B)}$$

where the normalized data (Fraction) were expressed as a function of voltage (V), the test



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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 mV, 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:

$$Fraction = 1 / \{1 + (IC_{50} / [C]^{h})\}, \qquad \text{(C)}$$

where [C] is the concentration of amiodarone, $IC_{50}$ is the half-maximal blocking concentration of $I_{Ca.T}$ by amiodarone, and h is Hill coefficient.

**Solutions.** The recording chamber was filled with bath solution of the following composition (mM): Tetraethylammonium chloride120, CsCl 6, 4-aminopyridine 5, $MgCl_2$ 0.5, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate 0.5, HEPES 10, $CaCl_2$ 1.8, and glucose 10 (pH was adjusted to 7.4 with 1 N Tetraethylammonium hydroxide solution). The patch-clamp electrode was filled with pipette solution of (mM): CsCl 130, $MgCl_2$ 2, Adenosine 5'-Triphosphate (ATP) 2, Guanosine 5'-Triphosphate 0.5, EGTA 5, and HEPES 5 (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 experiment, aliquot of the stock solution were diluted with the bath solution. Data were acquired by using computer software (Pulse/PulseFit, V. 8.11, HEKA Electronik), and all curve fittings and figures were made on SigmaPlot (version6.1; SPSS, Inc., Chicago, IL, U.S.A).



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**Drugs.** Amiodarone hydrochloride was a gift of Taisyo Pharmaceutical Co. (Tokyo, Japan). Triiodothyronine ($T_3$) was purchased from Wako Pure Chemical Ind. (Osaka, Japan), and all other chemicals were from Sigma Chemical Co. (St Louis, MO, U.S.A).

**Statistics.** Group data show as mean ± standard error (SE). Analysis of variance, Turkey's HSD procedure, was used for multiple comparisons, and Student's t test was used for comparison between two means. Differences were considered significant when p value were<0.05.

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**Results**

In conventional whole cell patch clamp experiments using HEK-$Ca_v3.2$ cells, T-type $Ca^{2+}$ currents ($I_{Ca.T}$) were recorded. Fig. 1A shows representative examples of $I_{Ca.T}$ change with (○) or without (□) acute amiodarone application. Peak current was rapidly decreased by application of 2 μM amiodarone, and 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, e), and the percent 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). Figs. 1B and 1C illustrate the acute 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 a current-voltage (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



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by amiodarone were observed, i.e., 2 μM and 5 μM amiodarone shifted the activation and the steady-state inactivation curves in the hyperpolarization direction by 4.1 ± 1.4 mV (n = 19) and 9.5 ± 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 ± 2.6% (n = 6), indicating that the interval between pulses is too short as compared with the time constant for the recovery from inactivation. Amiodarone caused a marked tonic block (35.7 ± 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 ± 5.4%, n = 6), which indicates that the pulse interval is too short as 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 acute and chronic effects on various cardiac ion channels, we examined the chronic effects of amiodarone on $I_{Ca.T}$. $I_{Ca.T}$ recorded from HEK-$Ca_v3.2$ cells treated with 2 μM amiodarone for 72 hours revealed a significant delay in recovery from inactivation in the absence of amiodarone in the bath solution (τ: 2 μM chronic amiodarone (n = 6), 618 ± 71 ms). Fig. 2A shows the representative $I_{Ca.T}$ from HEK-$Ca_v3.2$ cells with and without amiodarone treatment for 72 hours. HEK-$Ca_v3.2$ cells chronically treated with amiodarone expressed significantly smaller $I_{Ca.T}$ than those cells not treated with



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amiodarone (DMSO control cells), indicating a reduction of the maximum peak $I_{Ca.T}$ by 31.7 ± 2.3%, although amiodarone was excluded from both the culture medium and the bath solution for more than 12 hours (Figs. 2A and 2B). 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 mV and -5.5 mV, respectively (Fig. 2C and TABLE 1). Because there is a number of studies on the interaction of amiodarone and thyroid hormones in clinical and experimental studies (Bosch et al., 1999, Guo et al., 1997, Shahrara et al., 1999), we have conducted experiments for the evaluation of long-term 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 hours did not modify the chronic effect of amiodarone on $I_{Ca.T}$, in terms of current amplitude and voltage dependency of activation and steady-state inactivation curves.

Interestingly, the acute application of amiodarone shifted the activation and steady-state inactivation curves in the hyperpolarization direction, and chronic treatment of HEK-$Ca_v3.2$ cells by amiodarone shifted the same curves in the depolarization direction. Accordingly, we found that $I_{Ca.T}$ in HEK-$Ca_v3.2$ cells subjected to chronic amiodarone treatment was diminished owing to a mechanism that is different compared to those in acute actions.

In order to study the affinity of amiodarone for the distinct channel states (i.e., activation, inactivation, and resting states), we examined whether time course for recovery from inactivation of $I_{Ca.T}$ was modulated by acute and chronic amiodarone treatment. Thus, we examined the recovery time course from inactivation by using the pulse protocol illustrated



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in Fig. 3. Recovery from inactivation was significantly delayed by acutely 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 chronic amiodarone treatment for 72 hours 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 chronic amiodarone treatment.

Chronic treatment of HEK-$Ca_v3.2$ cells by amiodarone for 72 hours 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 chronic 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). Following the acute 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 chronic 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 or not $I_{Ca.T}$ subjected to chronic amiodarone treatment has



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sensitivity to an acute application of amiodarone. Acute application of 1 μM amiodarone lessened $I_{Ca.T}$ that received chronic 5 μM amiodarone treatment for 72 hours 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 acute amiodarone administration on $I_{Ca.T}$ that had previously undergone chronic treatment (Fig. 5B). Both the activation and the steady-state inactivation curves were shifted in the hyperpolarization direction by the acute 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_v$3.2 cells that had undergone chronic amiodarone treatment, and the acute effects of amiodarone on them, are summarized in TABLE 1. In order to identify the blocking properties of $I_{Ca.T}$ by chronic amiodarone treatment and their modulation by acute amiodarone application, a train of pulses was applied (Fig. 5D). Acute amiodarone application caused marked tonic block and use-dependent block of $I_{Ca.T}$ nearly identical to those observed without chronic amiodarone treatment (Fig. 1E): acute 2.5 μM amiodarone 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 acute 2.5 μM amiodarone inhibited $I_{Ca.T}$ in cells that were chronically treated 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).

Finally, we obtained $IC_{50}$ values for amiodarone in acute application on $I_{Ca.T}$, chronic application on $I_{Ca.T}$, and acute application on $I_{Ca.T}$ from the HEK-$Ca_v$3.2 cells that were



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chronically treated by amiodarone. As a tonic blocking action, acute amiodarone application inhibited $I_{Ca.T}$ with an $IC_{50}$ of 2.4 μM, whereas application of amiodarone for 72 hours lessened $I_{Ca.T}$ with an $IC_{50}$ of 9.4 μM (Fig. 6). The acute effect of amiodarone on $I_{Ca.T}$ in HEK-$Ca_v$3.2 cells chronically treated with amiodarone exhibited an $IC_{50}$ of 2.8 μM (Fig. 6).

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## Discussion

Acute and chronic effects of amiodarone on $Ca_v3.2$ T-type $Ca^{2+}$ channel current ($I_{Ca.T}$) were studied using the conventional patch clamp technique. Acute amiodarone effects were: (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 whether chronic amiodarone treatment was applied or not. Chronic amiodarone effects were: (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) acute inhibitory effects of amiodarone on $I_{Ca.T}$ treated by chronically applied 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 acute and chronic 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.

**Acute effects of amiodarone on cardiac $Ca^{2+}$ channels.** Although an acute 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 pKa = 8.7 at 37°C) (Chatelain et al., 1986). Consistently, acute effects of amiodarone cannot be reversed completely during short-term



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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 employed a heterologous expression system of $\alpha_{1H}$ ($Ca_v3.2$) in HEK 293 cells in order 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 (Uchino et al., 2005; Yatani et al., 1986), mibefradil (Meherke 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 (DHP) in rat and rabbit myocardial membranes, and found that amiodarone completely inhibited the specific binding to myocardial membrane receptors by DHP $Ca^{2+}$ 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.

Acute 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 $IC_{50}$ of 0.36-5.8 μM, (2) amiodarone (5-16 μM) shifted the steady-state inactivation curve of $I_{Ca.L}$ in the hyperpolarizing direction by 9-10 mV, and (3) amiodarone caused both tonic and use-dependent reduction of $I_{Ca.L}$ (Nishimura et al., 1989; Valenzuela et al., 1991; Varró et al.,



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1996). Acute 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). Acute application of 2.5 μM amiodarone caused both tonic and use-dependent block of $I_{Ca.T}$ (Fig. 1E), where tonic block primarily reflects rested state block. The fractional ratio of (tonic block) / (use-dependent block) was approximately 2.1 for acute 2.5 μM amiodarone application on $I_{Ca.T}$ (TABLE 2). The lower the ratio, the stronger the gain of potency obtained during the high frequency of stimulation. Fractional tonic block by acute 2.5 μM amiodarone on $I_{Ca.T}$ (35.7%) and inhibition in cells chronically treated with amiodarone on $I_{Ca.T}$ (39.5%) were statistically identical ($P$ = 0.45). Moreover, drug-delimited use-dependent block of acute 2.5 μM amiodarone on $I_{Ca.T}$ (17.2%) and inhibition in cells chronically treated 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 the inactivated state of the $\alpha_{1H}$ T-type $Ca^{2+}$ channel regardless whether the channel received chronic amiodarone treatment or not. Taken together, these data and ours indicate that the acute 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.

**Chronic effects of amiodarone on cardiac ion channels.** The most striking difference in the effects produced by acute vs. chronic administration of amiodarone can be found on the shifts in activation and steady-state inactivation curves (Figs. 1D and 2C), and on



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current relaxation (Fig. 4). Desensitization of $I_{Ca.T}$ by chronic amiodarone treatment is unlikely because the potency of acute amiodarone on $I_{Ca.T}$ ($IC_{50}$, 2.4 μM) and the potency of acute amiodarone on $I_{Ca.T}$ chronically treated with amiodarone ($IC_{50}$, 2.8 μM) are nearly identical (Fig. 6). It is widely recognized that the most prominent chronic 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 chronic amiodarone treatment was reported by Le Bouter et al. (2005). They found that $Na^+$ channel α- and β-subunits, L-type $Ca^{2+}$ channel $\alpha_1$, $\beta_1$ and $\beta_2$ subunits, T-type $Ca^{2+}$ channel $\alpha_{1G}$ subunit, Kv2.1, Kv1.5, Kv4.2, ATP-sensitive $K^+$ channels, Kir6.2, and SUR2 were downregulated, and that other $K^+$ channel α- and β-subunits, such as KNCA4, KCNK1, KCNAB1, and KCNE3, were upregulated. However, possible remodeling of the T-type $Ca^{2+}$ channel $\alpha_{1H}$ subunit by chronic amiodarone treatment has not yet been studied. The cardiac T-type $Ca^{2+}$ channel is composed of two subtypes of α subunits, $\alpha_{1H}$ and $\alpha_{1G}$. The $\alpha_{1H}$ subunit differs from the $\alpha_{1G}$ subunit in many respects (Perez-Reyes, 2003). Intriguingly, Hagelüken et al. (1995) reported that amiodarone is a direct activator of $G_i$ and $G_{(0)}$ 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 chronic effects on the $Ca_v3.2$ T-type $Ca^{2+}$ via G-protein(s). More



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interestingly, $\alpha_{1H}$ T-type $Ca^{2+}$ channel kinetics were drastically modified by chronic amiodarone treatment particularly on the current relaxation phase. Although no proteins or signals that modulate kinetics of $\alpha_{1H}$ 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, chronic amiodarone treatment may reverse the remodeling of $I_{Ca.T}$. Obviously, 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 hours 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 co-cultured 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. Importantly, 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 to those observed in the absence of $T_3$ (Fig. 2). Based on these findings, we conclude that the inhibition and



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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 $\alpha_{1H}$ 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 $\alpha_{1H}$ probably through unknown protein(s) and/or signaling molecules. The acute and chronic 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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**Legends for Figures**

Fig. 1. Acute amiodarone effects on $I_{Ca.T}$. A. Typical time course of the peak $I_{Ca.T}$ recorded from HEK-$Ca_v$3.2 cells with (○) or without 2 μM amiodarone (□). Amiodarone (2 μM) was acutely 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 min (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 (upper traces) and after (lower traces) application of 1 μM amiodarone (Ami) obtained from an HEK-$Ca_v$3.2 cell. Currents were elicited from a holding potential of -100 mV at the stimulation frequency of 0.2 Hz. C. Current-voltage (I-V) relationship with (■, n = 23) or without 1 μM amiodarone (○, n = 78). 1 μM amiodarone decreased the maximum peak $I_{Ca.T}$ by 25.4 ± 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), 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 acute 2.5 μM amiodarone application (■,



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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 prior to 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 (bold line), where the point for the control current #1 (●, 1.0) was reassigned to the current #1 during acute amiodarone application (■, n = 0.643), yielding a drug-independent use-dependent block component during acute amiodarone application. Ratios for tonic block (TB) and drug-delimited use-dependent block (UDB) during acute amiodarone application are identified.

Fig. 2. Comparison of acute and chronic effects of amiodarone. A. Representative whole cell current traces obtained from an HEK-$Ca_v3.2$ cell cultured for 72 hours 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), chronic treatment of 5 μM amiodarone (Ami(C)) (♦, n = 41), chronic treatment of 5 μM amiodarone with 0.12 nM $T_3$ (Ami(C)+ $T_3$(C)) (dotted line without symbols, n = 6). C. Shifts of activation and steady-state inactivation curves by chronic 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) (dotted line without symbols, n = 6).



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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 (tau, τ). control (○, n = 6), 490 ± 35 ms; acute 2 μM amiodarone (▲, n = 5), 1081 ± 95 ms; chronic 2 μM amiodarone (▼, n = 5), 618 ± 71 ms. Inset illustrates 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 (tau, τ) was plotted against the test potential. Each plot was constructed for 3 groups data: the control (DMSO) condition (○, n = 6), acute 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 chronic amiodarone treatment that were normalized to the peak current. **b**: representative current traces with or without chronic amiodarone treatment at the maximum current (Control@-30mV, Ami (C)@-25mV, indicated by arrows) normalized to the peak current. $^{*}$, $P<0.05$ vs. control (non-paired): $^{**}$, $P<0.01$ vs. control (non-paired): $^{***}$, $P<0.001$ vs. control (non-paired).

Fig. 5. Effects of acute amiodarone application on $I_{Ca.T}$ with chronic amiodarone treatment for 72 hours. A. Representative $I_{Ca.T}$ families obtained from an HEK-$Ca_v$3.2 cell with chronic amiodarone treatment Ami(C) and acute effects of 1 μM amiodarone (Ami(C) + 1 μM Ami).



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B. I-V relationship of $I_{Ca.T}$ treated with 5 µM amiodarone (Ami(C)) (♦, n = 9) and acute 1 µM amiodarone application (Ami(C) + 1 µM Ami) (■, n = 9). C. Voltage dependence of activation and steady-state inactivation curves of chronic amiodarone treatment (♦, n = 9), and acute amiodarone treatment (1 µM) and effects on chronically treated $I_{Ca.T}$ (■, n = 9). Note that the activation and steady-state inactivation curves were significantly shifted, with acute 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 development of use-dependent block of $I_{Ca.T}$ recorded from HEK-$Ca_v$3.2 cells treated with chronic 5 µM amiodarone with or without acute 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 chronic 5 µM amiodarone (Ami(C)) (♦, n = 6) and acute 2.5 µM amiodarone application on $I_{Ca.T}$ recorded from HEK-$Ca_v$3.2 cells treated with chronic 5 µM amiodarone (Ami(C) + 2.5 µM Ami) (■, n = 6). The fitting curve for chronic amiodarone data (♦) was reconstructed (bold line), where the point for the current #1 (♦, 1.0) was reassigned to the current #1 during acute amiodarone application (■, 0.605), yielding a drug-independent use-dependent component during acute amiodarone application. Ratios for tonic block (TB) and drug-delimited use-dependent block (UDB) during acute amiodarone application are identified.

Fig 6. Dose-response relationship of acute and chronic actions of amiodarone on $I_{Ca.T}$. Acute and chronic effects of amiodarone were evaluated by a pulse protocol composed of a



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holding potential of -100 mV to a test potential for the maximum current (-25 mV~ -35 mV) at the stimulation frequency of 0.2 Hz. Acute effect (●, n = 5-23), chronic effect (♦, n = 5-13), and acute effect on cells treated with 5 μM chronic amiodarone (○, n = 4-11) on $I_{Ca.T}$. Data were fitted by the Hill equation yielding $IC_{50}$ values of 2.4 μM, 9.4 μM, and 2.8 μM, respectively. Therapeutic plasma concentration (1-3 μM) is indicated by shading (Singh et al., 1989).

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**TABLE 1**

**Acute and chronic effects of amiodarone on activation and inactivation parameters**

Activation and inactivation parameters were determined by protocols described in the Materials and Methods section and Figs. 1, 3, and 5. Data from each cell were collected before and in the presence of 1 μM, 2 μM, and 5 μM amiodarone for the evaluation of acute effects of the drug. For the evaluation of chronic effects of the drug, cells were incubated with (Ami(C)) or without 5 μM amiodarone (DMSO) for 72 hours, followed by amiodarone-free culture medium for 12-24 hours. Experiments for the evaluation of Ami(C) were performed under control condition of the bath solution without amiodarone.

| | Acute effects on $I_{Ca.T}$ | | Chronic effects on $I_{Ca.T}$ | |
|---|---|---|---|---|
| | $V_{1/2}$, activation (mV) | $V_{1/2}$, inactivation (mV) | $V_{1/2}$, activation) (mV) | $V_{1/2}$, inactivation) (mV) |
| 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) | | |
| 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▲ (n = 9) | -63.2 ± 0.9▲ (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) (non-paired): ▲, *P*<0.001 vs. Ami(C) (paired).



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**TABLE 2**

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

| | 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) |

Ami(C): HEK-$Ca_v$3.2 cells were treated by 5 μM amiodarone for 72 hours, followed by amiodarone-free culture medium for 12-24 hours.

*, $P$<0.05 vs. Control (paired):



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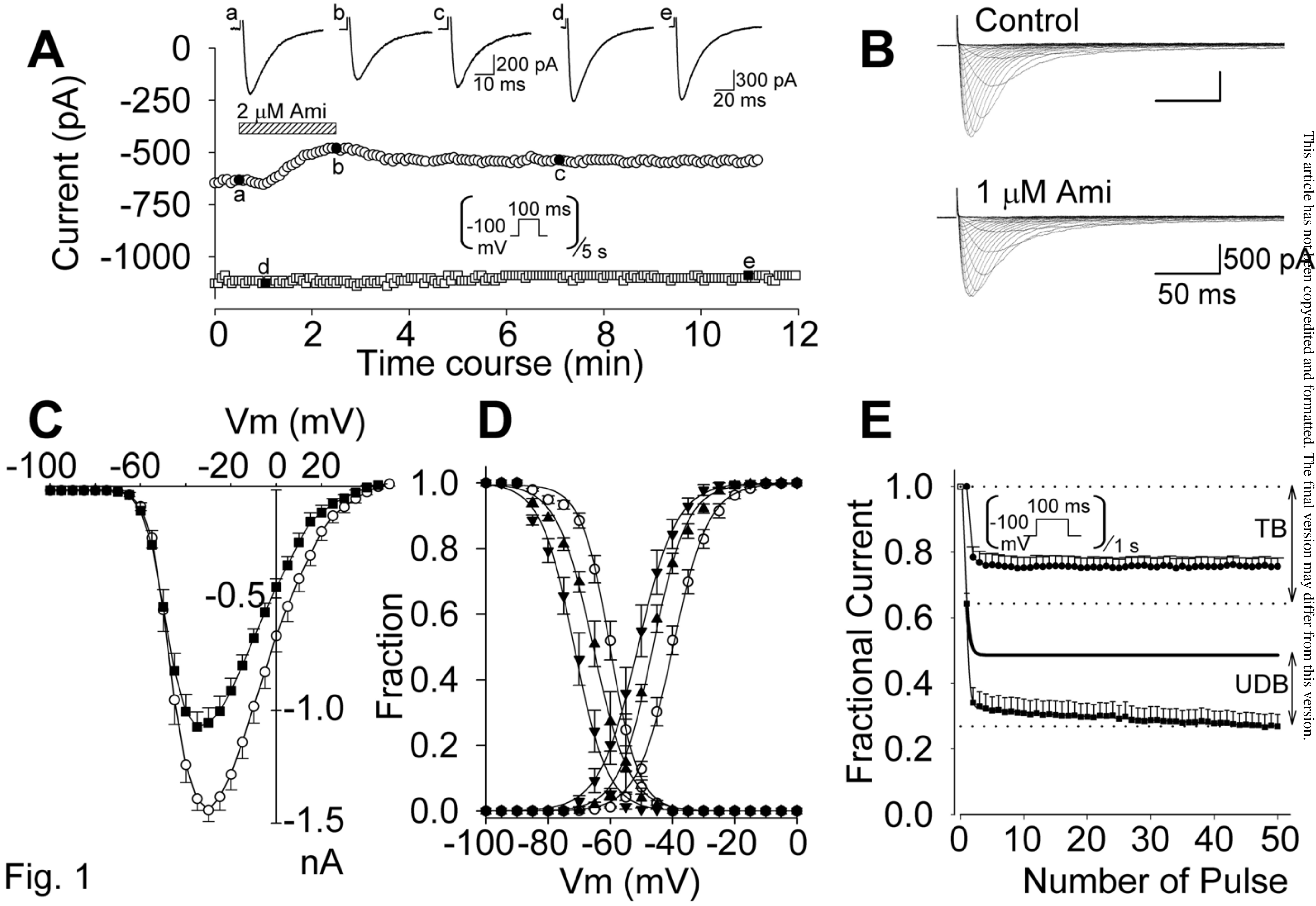


Fig. 1

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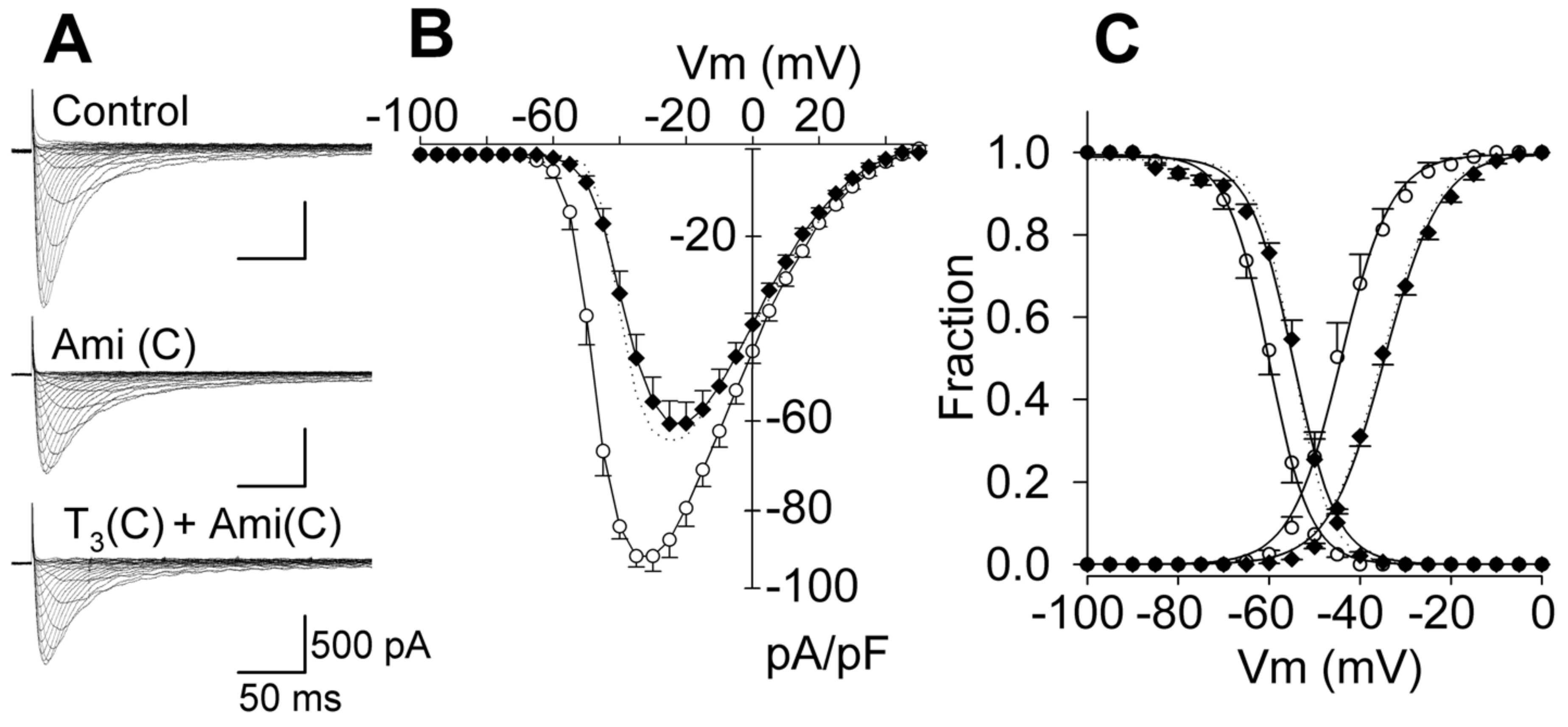


Fig. 2

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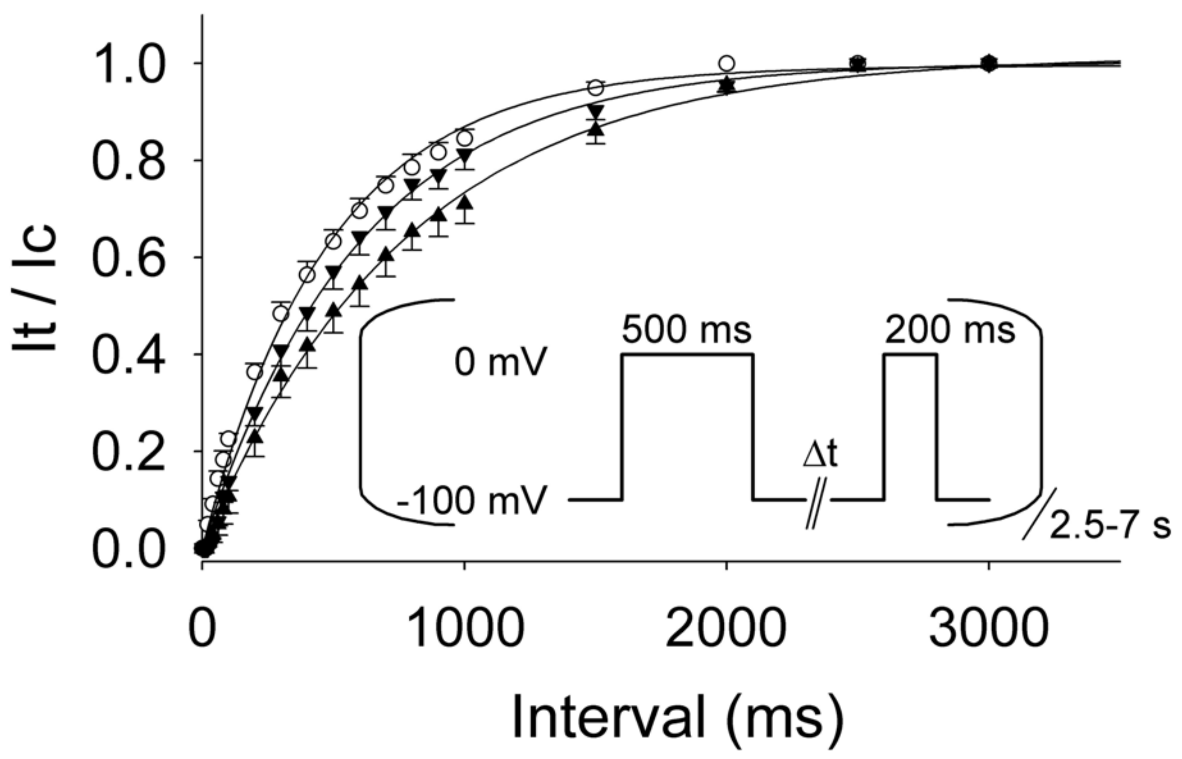


Fig. 3

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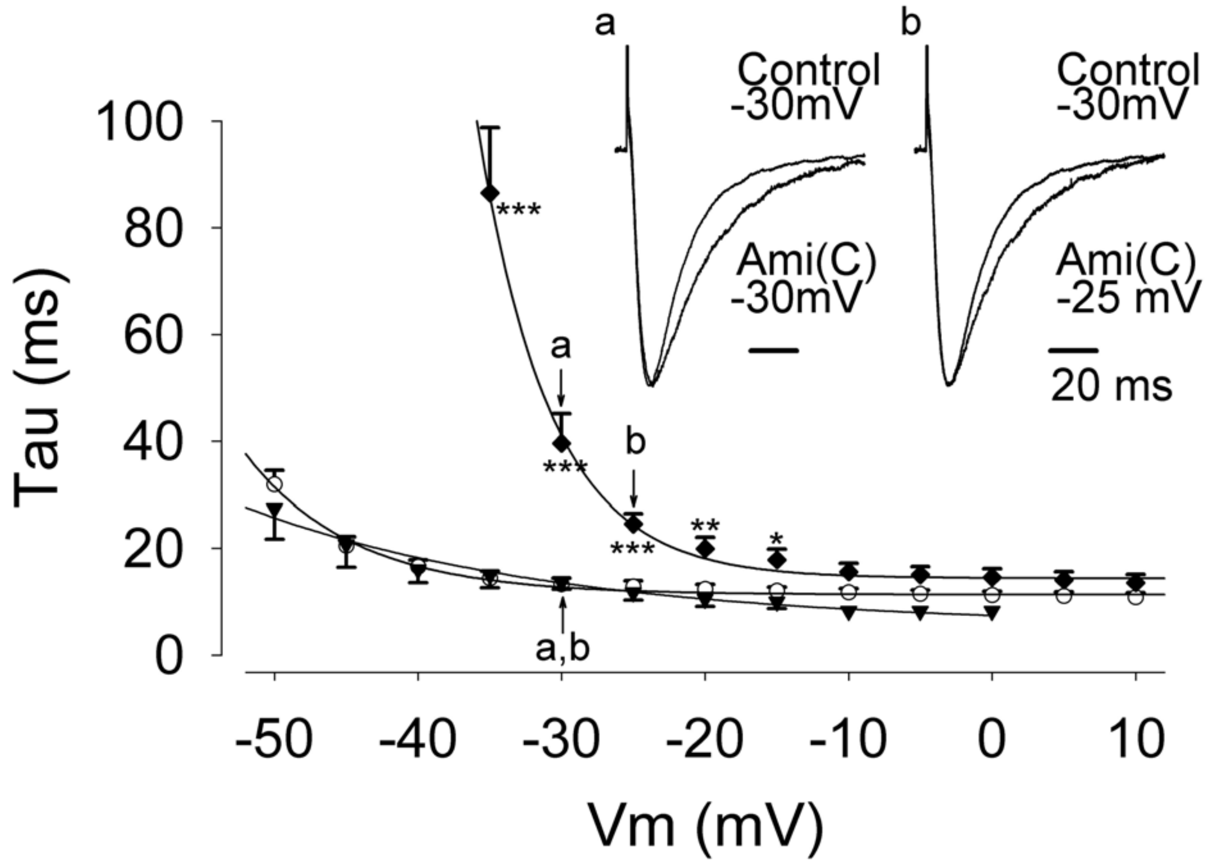


Fig. 4

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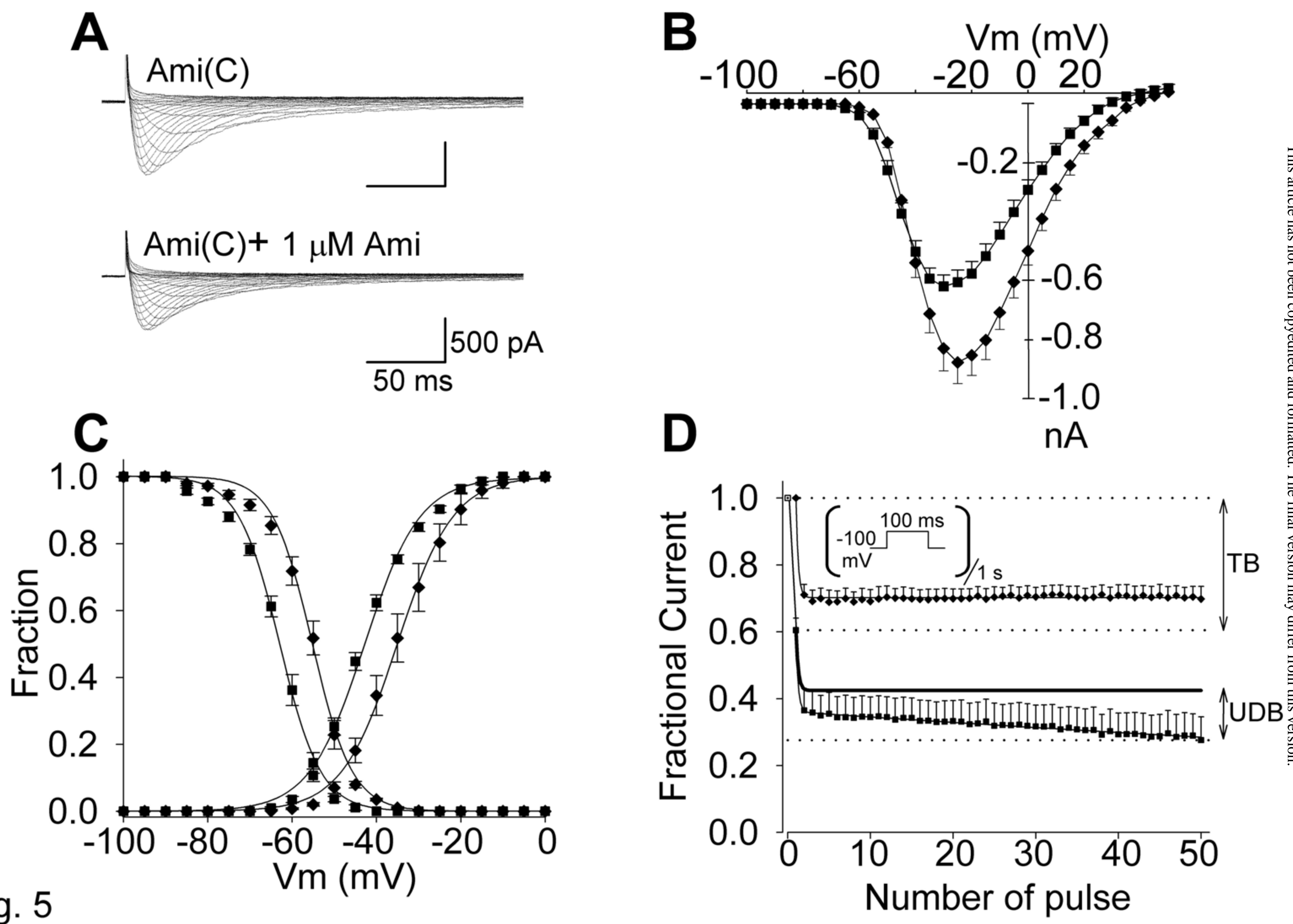


Fig. 5

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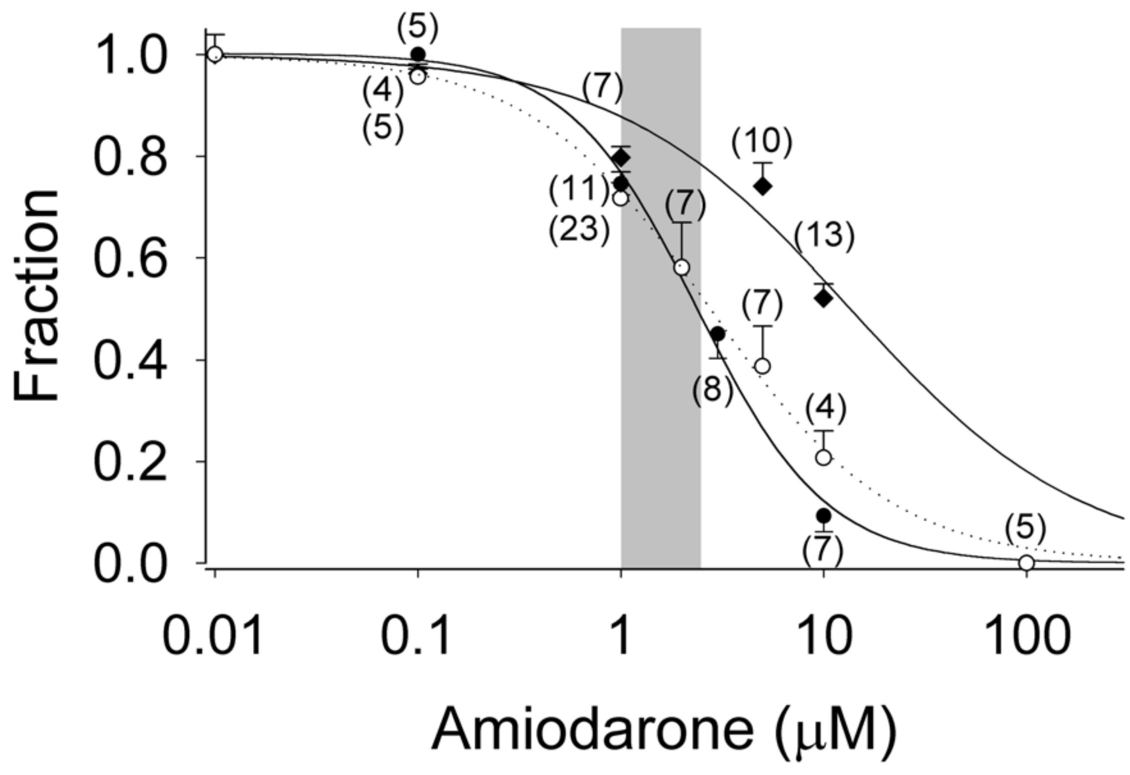


Fig. 6

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