Tetrahydroacridine inhibits voltage-dependent Na^+ current in guinea-pig ventricular myocytes[1]

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KEY WORDS Alzheimer disease; cholinesterase inhibitors; sodium channels; tetrahydroacridine (tacrine); myocardium; patch-clamp techniques

ABSTRACT

AIM: To study the effects of tetrahydroacridine (tacrine) on voltage-gated Na^+ channels in cardiac tissues. **METHODS:** Single ventricular myocytes were enzymatically dissociated from adult guinea-pig heart. Voltage-dependent Na^+ current was recorded using whole cell voltage-clamp technique. **RESULTS:** (1) Tacrine reversibly inhibited Na^+ current with an IC_{50} value of 120 μmol/L (95 % confidence range: 108-133 μmol/L). (2) The inhibitory effects of tacrine on Na^+ current exhibited both a tonic nature and use-dependence. (3) Tacrine at 100 μmol/L caused a negative shift (about 10 mV) in the voltage-dependence of steady-state inactivation of Na^+ current, and retarded its recovery from inactivation, but did not affect its activation curve. (4) Intracellular application of tacrine significantly inhibited Na^+ current. **CONCLUSION:** In addition to blocking other voltage-gated ion channels, tacrine blocked Na^+ channels in guinea-pig ventricular myocytes. Tacrine acted as inactivation stabilizer of Na^+ channels in cardiac tissues.

INTRODUCTION

Cholinesterase (ChE) inhibitors (tacrine, donepezil, rivastigmine, huperzine A, *etc*) are the most efficacious drugs thus far to ameliorate the cognitive and mental deficits in Alzheimer's disease (AD)[1,2]. With increasing number of AD patients treated with ChE inhibitors, efforts have been made to understand how the drugs work and what side effects they may cause. The actions of ChE inhibitors on other targets were extensively studied. As the first drug approved by the US FDA in 1993 for the treatment of AD, tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) has been found to have a sophisticated pharmacological profile: it affects numerous types of neurotransmitter receptors, ion channels, as well as enzymes other than ChE[3]. Due to its structural similarity to 9-aminoacridine, a Na^+ channel blocker[4] and to 4-aminopyridine, a K^+ channel blocker[5], attention has been focused on its interactions with different ion channels. A large number of studies showed an inhibitory action of tacrine on voltage-gated K^+ channels both in nerve tissues (rat hippocampal neuron[6,7], the giant axon of *Myxicola*[8] and the myelinated axons of *Xenopus laevis*[9]) and in heart and muscles (guinea-pig ventricular myocyte[10], rabbit sino-atrial node[11,12], and the larval muscle of *Drosophila*[5], *etc*). In contrast, the effects of tacrine on voltage-gated Na^+ channels were only demonstrated in nerve tissues[7-9]. Furthermore, tacrine has been proposed to act as open-

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channel blocker in Na^+ channels of *Myxicola* giant axon[8], it is unknown whether tacrine may act as inactivation stabilizer of Na^+ channels. In the present study we first examined the effects of tacrine on voltage-dependent Na^+ current (I_{Na}) in guinea-pig ventricular myocytes, then conducted a further investigation on the mechanisms underlying the blocking effect of tacrine on cardiac Na^+ channels.

MATERIALS AND METHODS

Drugs and animals Tacrine was purchased from Sigma Co (St Louis, MO, USA). All other chemicals were commercial products of reagent grade. Male or female guinea-pigs weighing 300±40 g were supplied by Shanghai Laboratory Animal Center, Chinese Academy of Sciences [SCXK (Shanghai) 2002-0011].

Preparation of ventricular myocytes Single ventricular myocytes were prepared from adult guinea-pig heart using the enzymatic dissociation method described by Farmer *et al*[13]. Briefly, the heart was mounted in a Langendorff apparatus for retrograde aorta perfusion with Ca^{2+}-free Tyrode's solution for 10 min. Then it was perfused with Ca^{2+}-free Tyrode's solution containing 0.02 % collagenase I and 1 % bovine serum albumin for 2-4 min. The solution contained (in mmol/L): NaCl 135, KCl 5.4, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 5, and glucose 5, and was adjusted to pH 7.4 with NaOH, bubbled with 100 % O_2, and warmed at 37 ºC. After washing-out the enzyme, the ventricles were minced and gently triturated for 10 min in KB (Kraft-Bruhe) solution containing (in mmol/L): *L*-glutamic acid 50, KOH 80, KCl 40, $MgSO_4$ 3, KH_2PO_4 25, HEPES 10, egtazic acid 1, tacrine 20, and glucose 10, adjusted to pH 7.4 with KOH. The dissociated cells were filtered through 200 μm nylon mesh and stored in KB solution at 21-23 ºC. Only the cells with rod shape and clear cross striation were used for experiments.

Whole cell voltage-clamp recording A small aliquot of ventricular myocytes was placed into a 3-mL chamber mounted on the stage of a microscope (Optiphot-2; Nikon, Japan), and superfused with an external solution at a rate of 3 mL/min. The solution contained (in mmol/L): choline chloride 100, NaCl 40, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1, $CdCl_2$ 0.1, NaH_2PO_4 0.33, HEPES 10, and glucose 10, adjusted to pH 7.4 with NaOH.

Whole cell voltage-clamp recording was made from single myocyte using an Axopatch-1D amplifier (Axon instruments, USA) at 21-23 ºC. Voltage command protocols were provided by pClamp 6.0.4 software package (Axon Instruments, USA) via a DigiData-1200 interface. Patch pipettes (tip resistances 1-2 MΩ) were pulled on a P-97 microelectrode puller (Sutter Instruments Co, USA), and filled with a pipette solution containing (in mmol/L): CsCl 120, $MgCl_2$ 5, egtazic acid 11, HEPES 10, Na_2ATP 5, and brought to pH 7.3 with CsOH. With the given external and pipette solution, the transmembrane Na^+ gradient was reduced to 4:1, thus greatly improving the voltage control during activation of I_{Na}. After a gigaseal (seal resistance >1 GΩ) formation, the membrane was ruptured with gentle suction to obtain whole cell voltage-clamp configuration. Capacitance compensation was routinely optimized and series resistance was compensated by 40 %-80 %. Linear leak were subtracted digitally online. Signals were filtered at 5 kHz, digitized at 20 kHz, and stored in an IBM-compatible computer. The mean cell capacitance was 84.0±4.4 pF (n=19 from 9 animals).

For extracellular application, tacrine was dissolved in the external solution, and delivered to the recorded myocyte using a PBS-8 solution exchange system (ALA Ltd, USA). For intracellular application, tacrine was dissolved in the pipette solution, and diffused from the pipette into the myocyte after patch membrane was ruptured[14].

Data analysis and statistics The IC_{50} value was determined via fitting the concentrations-response relationship using computer software 'Prism 3.0'. Activation and inactivation plots were fitted to the Boltzmann equation: $Y=1/\{1+\exp[(V_c-V_{1/2})/k]\}$, where V_c is the conditioning potential; $V_{1/2}$ is the voltage at half-maximal activation or inactivation; k is the slope factor. The time course of recovery from inactivation was fitted with an exponential equation: $Y=A*\exp(-t/\tau)$, where A is the amplitude and τ is the time constant. Data are presented as mean±SEM. Paired or unpaired two-tailed t-test, as appropriate, was used for statistical analysis, and $P<0.05$ was judged to be significant.

RESULTS

Inhibition of Na^+ current by extracellular application of tacrine in ventricular myocytes Voltage-dependent Ca^{2+} currents were blocked by addition of Cd^{2+} 100 μmol/L to the external solution whereas voltage-dependent K^+ currents were eliminated by replacing K^+ with Cs^+ in the pipette solution. Under the conditions, the transient inward currents elicited by depolarizing steps represented the fast I_{Na}, which could

be blocked by tetrodotoxin 30 μmol/L (data not shown). Superfusion of the recorded myocyte with tacrine reduced the peak amplitude of I_{Na} in a concentration-dependent manner (Fig 1A). The steady-state inhibition was observed within 2 min, and the current partially recovered after washout for 5 min. The fit of the concentration-response curve (Fig 1B) resulted in an IC_{50} value of 120 μmol/L (95 % confidence range: 108-133 μmol/L) and a Hill coefficient of 1.15 (95 % confidence range: 1.04-1.26). The effect of tacrine on current-voltage (*I/V*) relationship of I_{Na} was shown in Fig 1C. In the control group, the threshold for I_{Na} activation was between -60 mV to -50 mV, maximal activation reached at -35 mV, and the reversal potential was 20.0±2.1 mV (n=11). Tacrine did not change the threshold, the potential of maximal activation, and the reversal potential, but caused a marked upward shift of the *I/V* curve: the maximal current elicited at -35 mV was reduced to 44 %±3 % of the control level (n=11, P< 0.01). The current returned to 84 %±5 % after washout for 5 min (n=7).

Properties of extracellular tacrine-induced inhibition in ventricular myocytes The action of extracellular tacrine on I_{Na} exhibited both a tonic block and use (frequency)-dependence. The tonic block was assessed with a train of 10 depolarizing pulses at a low frequency of 0.1 Hz. In the control group, the amplitude of I_{Na} was hardly changed during the train of pulses. However, it was instantaneously reduced by about 30 % during extracellular application of tacrine 100 μmol/L (Fig 2A, the top row). The data pooled from 4 individual observations confirmed that tacrine exerted a tonic block on Na^+ channels (Fig 2B).

The use-dependent inhibition was observed when a train of 20 depolarizing pulses was delivered at frequencies of 2.5 or 5 Hz. In the control group, the am-

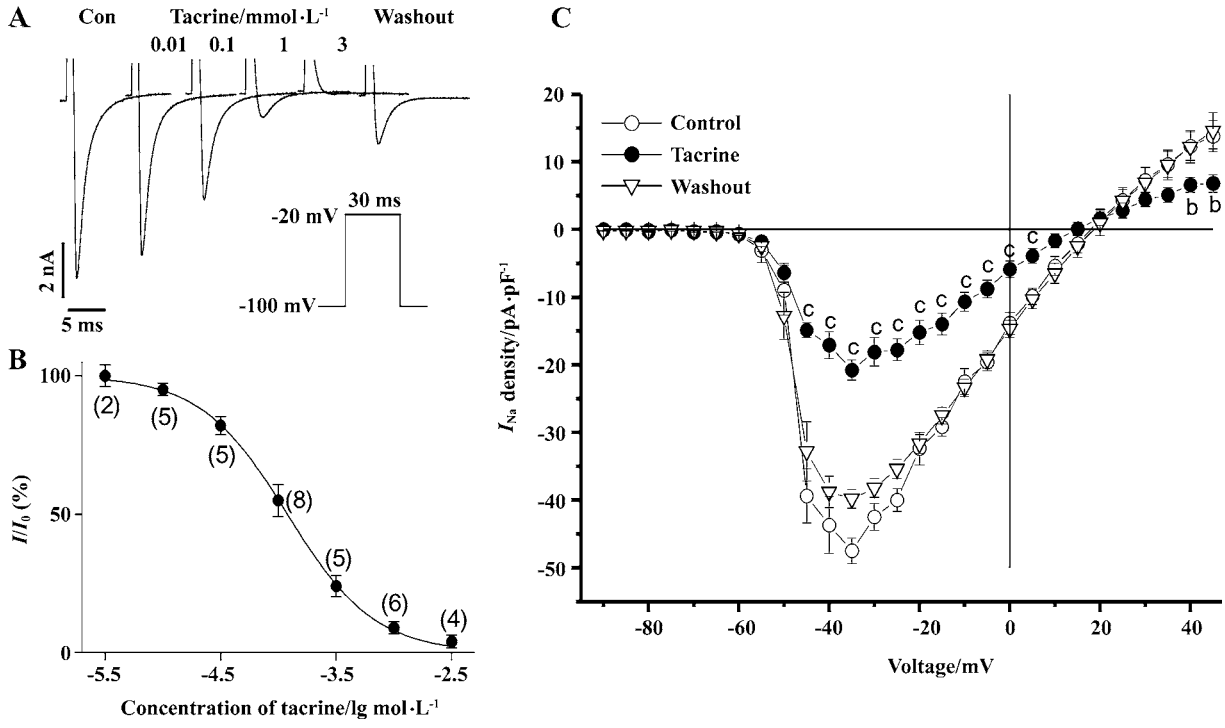

Fig 1. Inhibition of tacrine on Na^+ current in guinea-pig ventricular myocytes. (A) Representative records of Na^+ current elicited by 30 ms depolarizing pulses to -20 mV from a holding potential of -100 mV at a frequency of 0.1 Hz before, during application of tacrine, and after washout of the drug for 5 min. The records were taken from a single myocyte superfused with cumulative concentrations of tacrine. (B) Concentration-response curve of tacrine in inhibition of Na^+ current. Data were from 35 myocytes of 7 guinea-pigs. The number of myocytes tested at each concentration was indicated in parentheses next to the relevant data point. (C) Current-voltage (*I/V*) curves in control and in the presence of tacrine 100 μmol/L (n=11), and after washout of the drug (n=7). ^bP<0.05, ^cP<0.01 *vs* the control. The curves were constructed using a pulse protocol with a series of 30 ms depolarizing pulses delivered from a holding potential of -100 mV to different membrane potentials (-90 mV to +45 mV) with 5 mV increments at 0.1 Hz.

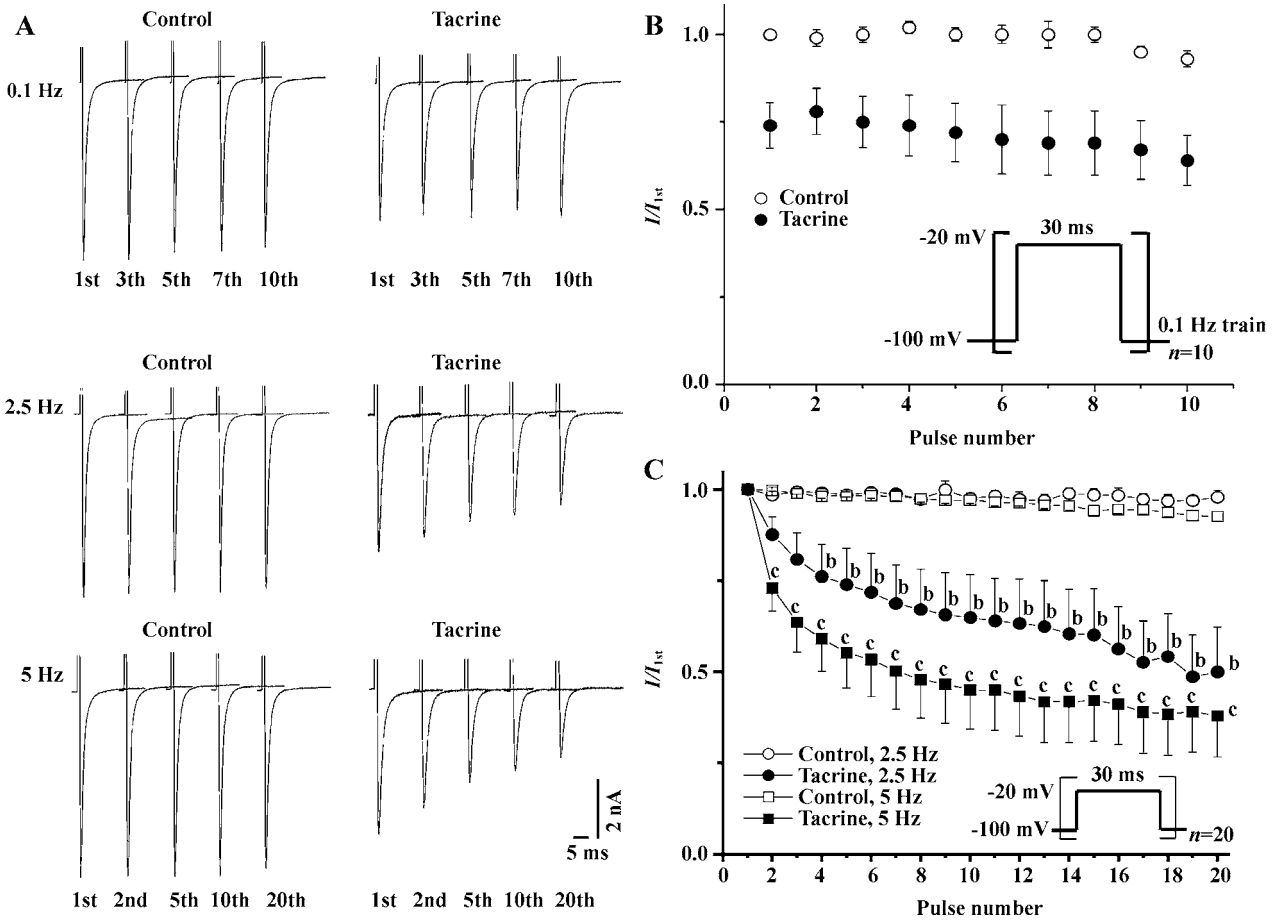

Fig 2. Tonic and use-dependent block of Na^+ current by tacrine. (A) Each row shows the representative current traces elicited by the indicated pulses in a train of 30 ms depolarizing pulses from a holding potential of -100 mV to -20 mV in control (the left panel) and in the presence of tacrine 100 μmol/L (the right panel). In the top row the train consisted of 10 pulses at 0.1 Hz, whereas in the middle and bottom row it contained 20 pulses at 2.5 and 5 Hz, respectively. (B) Tonic block of Na^+ current by tacrine 100 μmol/L. The relative current (I/I_{1st}) elicited by a train of 10 depolarizing pulses at 0.1 Hz was plotted (n=4). The inset shows the parameters of a single pulse in the train. (C) Use-dependent block of Na^+ current by tacrine 100 μmol/L. The relative Na^+ current (I/I_{1st}) elicited by a train of 20 depolarizing pulses at 2.5 Hz (n=8) and 5 Hz (n=8) was plotted. The inset shows the parameters of a single pulse in the train. $^bP<0.05$, $^cP<0.01$ *vs* the respective control.

plitude of I_{Na} showed a minor (2 %-3 %) reduction during the train of pulses at 2.5 Hz, whereas the current was progressively reduced during extracellular application of tacrine 100 μmol/L (Fig 2A, the middle row), a phenomenon consistent with use-dependent inhibition. The relative current (I/I_{1st}) elicited by the 2nd, 5th, 10th, and 20th pulse in the train was reduced by 12 %±5 %, 26 %±10 %, 35 %±12 %, and 46 %±12 %, respectively (n=8, Fig 2C). The use-dependent inhibition became more profound at 5 Hz (Fig 2A, the bottom row). During the application of tacrine, the amplitude of I_{Na} was rapidly suppressed, then reached a steady-state level. The relative current (I/I_{1st}) elicited by the 2nd, 5th, 10th, and 20th pulse was reduced by 27 %±6 %, 47 %±10 %, 55 %±12 %, and 62 %±11 %, respectively (n=8, Fig 2C).

Effect of extracellular application of tacrine on kinetic behaviors of Na^+ current in ventricular myocytes Extracellular application of tacrine did not affect the activation curve of I_{Na} (Fig 3A). In control and under tacrine 100 μmol/L, the voltage at half-maximal activation ($V_{1/2}$) was -46.4±1.8 mV and -46.0±3.2 mV (n=8, P>0.05), respectively, while the slope factor (k) was 3.2±0.5 mV and 4.2±0.4 mV (P>0.05).

Extracellular application of tacrine caused a negative shift in the voltage-dependence of the steady-state inactivation of I_{Na} (Fig 3B). In control and under tacrine 100 μmol/L, the voltage at half-maximal inactivation

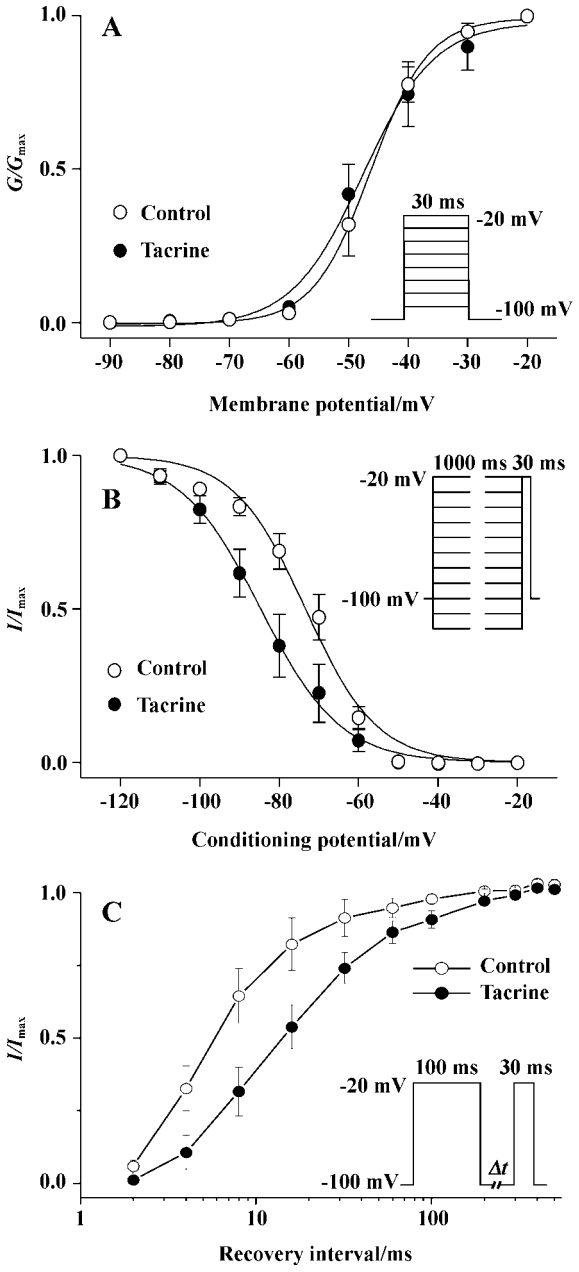

Fig 3. Effect of tacrine on kinetic behaviors of Na^+ current in ventricular myocytes. (A) Activation curves of Na^+ current in control and in tacrine 100 μmol/L (*n*=8). The pulse protocol: a series of 30 ms depolarizing pulses to different membrane potentials (-90 mV to -20 mV, with 10 mV increments) delivered from a holding potential of -100 mV at 1 Hz. (B) Steady-state inactivation curves of Na^+ current in control and in tacrine 100 μmol/L (*n*=10). The pulse protocol: 1000 ms prepulses at different membrane potentials (-120 mV to -20 mV, with 10 mV increments) were followed by a 30 ms depolarizing pulse to -20 mV at 0.33 Hz. (C) Recovery from fast inactivation in control and in tacrine 100 μmol/L (*n*=7). Double-pulse protocol (the inset): intervals with variable duration (from 2 to 500 ms) at -100 mV were inserted between a 100 ms conditioning pulse to -20 mV and a 30 ms test pulse to -20 mV, delivered at 1 Hz.

($V_{1/2}$) was -73.0±1.8 mV and -83.0±3.7 mV (*n*=10, *P*<0.05), respectively, whereas the slope factor (*k*) had no significant change (-8.7±1.1 mV *vs* -7.3±0.7 mV, *P*>0.05).

In ventricular myocytes, the time course of recovery of $I_{Na}$from inactivation was well fitted with a single exponential function (Fig 3C). In the control group, the time constants (τ) was 13.8±5.8 ms (*n*=7). Extracellular application of tacrine 100 μmol/L caused a marked prolongation in the time constant (τ=26.7± 4.8 ms, *P*<0.05).

Inhibition of Na^+ current by intracellular tacrine in ventricular myocytes Extracellularly applied drugs, like local anesthetics, could diffuse through the membrane and block Na^+ channels at an internal binding site. To determine the drug acts at an external or internal site of Na^+ channels, the effects of intracellular application of tacrine were investigated (Fig 4). In the control group with 7 myocytes dialyzed with normal pipette solution, I_{Na} ran down slowly. The relative current (I/I_{1st}) at 12 min was reduced by 17 %±3 %, as compared with the first current trace recorded immediately after patch membrane was ruptured. In 6 myocytes dialyzed with tacrine 100 μmol/L, a fast decline of I_{Na} appeared at 4 min, and reached a steady-state at 10 min. The relative current at 12 min was reduced by 44 %±8 % (*P*<0.05 *vs* the control group).

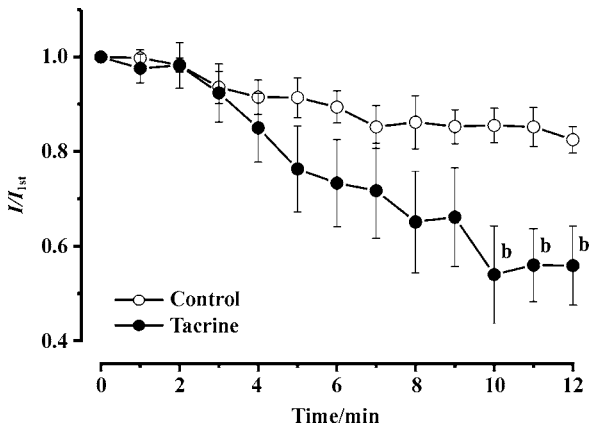

Fig 4. Inhibition of Na^+ current by intracellular application of tacrine in ventricular myocytes. The recording pipettes were filled with either the normal pipette solution or the pipette solution containing tacrine 100 μmol/L. Na^+ currents were elicited by 30 ms depolarizing pulses to -20 mV from a holding potential of -100 mV, delivered every minute. The relative current (I/I_{1st}) is plotted against the recording time. Time zero (*t*=0) indicates the time when patch membrane ruptured. ^bP<0.05 *vs* the control.

DISCUSSION

In the present study we demonstrated that tacrine caused reversible blockade of I_{Na} in guinea-pig ventricular myocytes. The finding was in accordance with those obtained from nerve tissues, such as rat hippocampal neurons[7], the giant axon of *Myxicola*[8], and the myelinated axon of *Xenopus laevis*[9]. As concerns to the mechanisms underlying the Na^+ channel-blocking action, we demonstrated that intracellular application of tacrine was almost as potent as extracellular one in blocking I_{Na}. Because the myocytes dialyzed with tacrine were continuously superfused with a drug-free external solution, it was impossible for intracellularly applied tacrine to block Na^+ channels from the external side. On the contrary, extracellularly applied tacrine may diffuse through the membrane and bind to an internal site of the channels. We found two line of evidence supporting the speculation: (1) The drug caused a negative shift (about 10 mV) in the voltage-dependence of steady-state inactivation of I_{Na}, without affecting its activation curve. Such a shift suggests that tacrine has a higher affinity for the inactivated state of cardiac Na^+ channels, thus causing a tonic block at 0.1 Hz. (2) Tacrine markedly slowed the recovery of I_{Na} from inactivation, a mechanism underlying the use-dependent block at higher frequencies. Therefore, tacrine, like the local anesthetic lidocaine, acts as inactivation stabilizer of Na^+ channels in ventricular myocytes[15]. In the myelinated axons of *Xenopus laevis*, tacrine also caused negative shift of the steady-state inactivation curve of I_{Na}[9]. In the giant axons of *Myxicola*, however, the drug did not affect the steady-state inactivation of I_{Na} and its recovery from inactivation[8]. The discrepancy may be attributed to the molecular and functional diversities of voltage-gated Na^+ channels in different tissues, as well as in different species[16].

Clinical benefits of tacrine in the treatment of AD were observed at plasma levels ranged from 5 to 70 μg/L (0.02-0.3 μmol/L)[17], which was far below the IC_{50} value of tacrine for blocking Na^+ channels (120 μmol/L). Similar concentrations of tacrine (30-100 μmol/L) were necessary to block other voltage-activated currents in cardiac tissues, such as slowly inactivating Ca^{2+} current[10], different K^+ currents (I_K, I_{K1})[10-12], and the cardiac pacemaker current (I_f)[18]. Due to the high concentrations used, the significance of channel-blocking effects of tacrine is unclear. It is evident that the drug should not directly affect the cardiac ion channels at the therapeutic dosage in the treatment of AD. Tacrine caused significant cardiovascular effects (increase of blood pressure and reduction of heart rate) in whole animals[19,20]. But the actions were found mediated via central and peripheral muscarinic mechanism, respectively. On the other hand, the multichannel-blocking effects of tacrine has led to a notion that it might be beneficial in the treatment of ventricular fibrillation[10]. Antiarrhythmic action of tacrine was indeed demonstrated in the ischemia and reperfusion-induced arrhythmias of rat. In this model, tacrine effectively suppressed the ventricular fibrillation via a mechanism of QT widening, which was partly due to blocking I_{K1} in ventricular myocytes, and partly due to the drug-induced bradycardia[21]. Clinical investigations have shown that the multichannel-blocking agents, such as dronedarone and tedisamil, are effective antiarrhythmics with a number of advantages over the existing agents, particularly in terms of safety[22]. Tacrine may provide a useful structural clue for design of novel antiarrhythmics with a more favorable therapeutic index.

In summary, our results showed that tacrine blocked Na^+ channels in guinea-pig ventricular myocytes in addition to blockade of other cardiac ion channels previously reported. Furthermore, we demonstrated that tacrine acted as inactivation stabilizer of cardiac Na^+ channels.

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