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Bis(3)-Tacrine Inhibits the Sustained Potassium Current in Cultured Rat Hippocampal Neurons

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Summary

Bis(3)-tacrine is a dimeric AChE inhibitor derived from tacrine with a potential to treat Alzheimer's disease. It was recently been reported to act as a fast off-rate antagonist of NMDA receptors with moderate affinity. In the present study, we aimed to explore whether bis(3)-tacrine could modulate the function of native sustained potassium current in cultured rat hippocampal neurons using whole-cell patch-clamp technique. We found that bis(3)-tacrine inhibited the amplitude of sustained potassium current in a reversible and concentration-dependent manner, with a potency two orders of magnitude higher than that of tacrine. The inhibition was voltage-independent between 0 to +60 mV. The IC_{50} values for bis(3)-tacrine and tacrine inhibition of sustained potassium current were 0.45±0.07 and 50.5±4.8 μM, respectively. *I-V* curves showed a more potent inhibition of sustained potassium current by bis(3)-tacrine (1 μM) compared to tacrine at the same concentration. Bis(3)-tacrine hyperpolarized the activation curve of the current by 11.2 mV, albeit leaving the steady-state inactivation of the current unaffected.

Key words

Bis(3)-tacrine • Sustained potassium current • Hippocampus • Alzheimer's disease

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Introduction

Bis(3)-tacrine, (1,3-*N*-heptylene-bis-9,9'-amino-1,2,3,4-tetrahydroacridine), a novel dimeric acetylcholineesterase (AChE) inhibitor derived from tacrine, has been demonstrated to inhibit AChE and γ-aminobutyric acid subtype A ($GABA_A$) receptors (Carlier *et al*. 1999, Li *et al.* 2007), and it was recently reported to act as uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist with fast off-rate (UFO) and moderate affinity, which interacts with its targets only during pathological states with no physiological activation (Luo *et al.* 2010). It can reverse cognitive impairment resulted from scopolamine in both water maze and object recognition tasks (Han *et al.* 2012), might represent a promising drug candidate for various neurodegenerative disorders, including Alzheimer's disease (AD). To date, no data about its actions on potassium currents in central nervous system neurons have been reported.

This work aimed to explore whether bis(3)-tacrine was able to modulate voltage-gated potassium channels as reported by many authors for the modulation of rat hippocampal neurons by tacrine (Wooltorton and Mathie 1993), huperzine A (Li and Hu 2002a, Li and Hu 2002b); of DRG neurons and Kv1.2 encoded potassium channels expressed in oocytes by bis(7)-tacrine (Nie *et al.* 2007). In the present study, using whole-cell patch-clamp recording, we aimed at investigating the effects of bis(3)-tacrine (compared to

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tacrine) on sustained potassium current in cultured rat hippocampal neurons.

Methods

Cell culture

Primary cultures of hippocampal neurons from Sprague-Dawley rats at embryonic day 18 were prepared as previously described with modifications (Liu *et al.* 2008a, Liu *et al.* 2008b). In brief, hippocampi were dissected and incubated with 0.25 % trypsin for 15 min at 37 °C, and then mechanically dissociated using a fire-polished Pasteur pipette. The resulting cell suspension was diluted at a density of 1×10^5 cells/ml^{-1} with high glucose Dulbecco's modified Eagle's medium solution containing 10 % fetal bovine serum, and plated in 35-mm dishes coated with poly-L-lysine (20 μg/ml^{-1}). Cells were incubated at 37 °C in a humidified incubator with 5 % CO_2. After approximately 24 h, the medium was replaced with a serum-free Neurobasal medium containing B_{27} supplement and 0.5 mM L-glutamine to inhibit the growth of glia cells. This medium was subsequently given half-changes twice weekly, and neurons were cultured for 7-14 days before use in experiments. All animal experimental procedures were reviewed and approved by the Animal Care and Use Committee at Jianghan University, and were performed in accordance with the National Institutes of Health guidelines on animal care.

Whole-cell electrophysiological recordings

Whole-cell patch-clamp recordings were performed at room temperature (22-25 °C) using an Axon 200B amplifier (Molecular Devices Co., Union City, CA, USA), a Digidata 1320A A/D converter (Molecular Devices Co.) and pCLAMP 10.2 software (Molecular Devices Co.). Current traces were low-pass filtered at 1-5 kHz and sampled at frequencies of 5-20 kHz. Series resistance was compensated by 75-85 %. Linear leak and residual capacitance currents were subtracted online using a P/6 protocol. The liquid junction potential measured to be less than 3 mV was not compensated. The membrane potential was held at -50 mV, unless noted otherwise. Neurons were placed in an extracellular medium containing (in mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 HEPES and 0.001 tetrodotoxin (pH was adjusted to 7.3 with NaOH, and the osmolality was adjusted to ~340 mosmol/kg with sucrose). The patch-pipettes were filled with an intracellular solution containing (in mM): 125 potassium gluconate, 20 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, and 5 MgATP with pH 7.3 and ~315 mosmol/kg in osmolality. Drug solutions were prepared in extracellular medium and applied to neurons by using a rapid solution exchange system (SF-77B Perfusion Fast-Step, Warner Instruments, Hamden, CT). With this system, the 10-90 % rise time of the junction potential at an open pipette tip was <2 ms (Liu *et al.* 2008a, Liu *et al.* 2008b). Neurons were bathed constantly in extracellular medium flowing from one barrel between drug applications.

Chemicals and applications

All drugs and chemicals used were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA), except Alkylene-linked tacrine dimer bis(3)-tacrine•2 HCl, that was kindly provided by Prof. Yifan Han (The Hong Kong Polytechnic University, Hong Kong, China). Unless indicated, all media and supplements used for cell culture were purchased from Gibco (Carlsbad, CA, USA).

Data analysis

Average values are expressed as mean ± S.E.M, with *n* equal to the number of cells studied. Statistical significance of results was assessed using Student's *t*-test or analysis of variance (ANOVA), as noted. Statistical analysis of concentration-response data was performed using the nonlinear curve-fitting program ALLFIT (DeLean *et al*. 1978), which uses an ANOVA procedure. Values reported for concentration-response analysis are those obtained by fitting the data to the equation:

$$Y = E_{max} / [1 + (EC_{50} / X)^n]$$

where X and Y are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the concentration yielding 50 percent of maximal effect (EC_{50} for activation, IC_{50} for inhibition), and *n* is the slope factor.

The steady-state activation/inactivation curves were fitted with the Boltzmann equation:

$$G/G_{max} = 1/\{1+\exp[-(V-V_{1/2})/k]\}$$

where G/G_{max} is the normalized conductance, V is membrane potential, $V_{1/2}$ is the potential for half-maximal activation or inactivation, and k is the slope factor. Mono- or bi-exponential functions were used to fit current decay and recovery from inactivation data.

Results

Bis(3)-tacrine inhibited the sustained potassium current

The sustained potassium current was eliminated by 20 mM tetraethylammonium (TEA) (n=5, data not shown). In the present study we found that the sustained potassium current could also be reversibly inhibited by bis(3)-tacrine (Fig. 1A), in a concentration-dependent manner (10^{-9}-10^{-4} M) (Fig. 1B). In contrast, the inhibition by tacrine of sustained potassium current is similar, but the potency is about two orders of magnitude below that of bis(3)-tacrine (IC_{50} values of 50.5±4.8 μM compared to 0.45±0.07 μM, p<0.05, n=9, Student's t test).

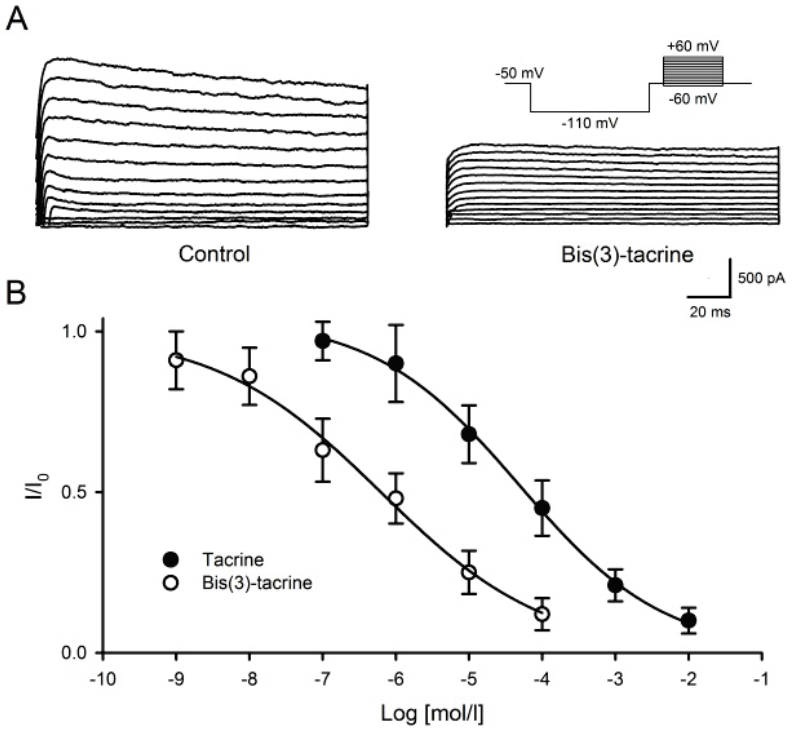

Fig. 1. Bis(3)-tacrine inhibited the sustained potassium current. (**A**) Family of currents evoked in control conditions and with 10^{-7} M bis(3)-tacrine by depolarizing command pulses from -60 mV to +60 mV (10 mV steps) following a 400 ms hyperpolarizing prepulse at -110 mV with a 50 ms interval at -50 mV to inactivate fast transient potassium current. (**B**) The concentration-response curves of bis(3)-tacrine (n=9) and tacrine (n=9).

Effect of membrane potential on inhibition of sustained potassium current

As illustrated in Figure 2A, bis(3)-tacrine/tacrine suppressed the sustained potassium current. They both depressed the I-V curves relative to control at all different holding potentials. At the depolarizing voltage of +60 mV, the amplitude of sustained potassium current was reduced by 16.8 % and 52.6 % compared to control values by tacrine (1 μM) and bis(3)-tacrine (1 μM), respectively.

To explore whether the change in percentage current in the presence of drugs is voltage-dependent or not, the bar chart graph in Figure 2B, was drawn by plotting of percentage current (I/I_0) (ordinate) against the membrane potential (abscissa). It is evident that in the presence of bis(3)-tacrine (1 μM), the change in percentage current is independent of membrane potential from 0 to +60 mV (ANOVA, p>0.05; n=5).

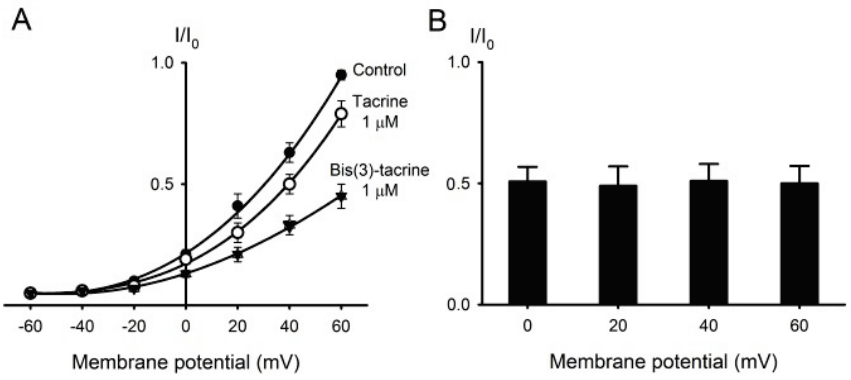

Fig. 2. Effect of membrane potential on inhibition of sustained potassium current by bis(3)-tacrine/tacrine. (**A**) Current/voltage (*I-V*) curves showed a more potent inhibition of sustained potassium current by bis(3)-tacrine (1 μM) compared to tacrine at the same concentration. (**B**) The percentage current (I/I_0) at 0, +20, +40 and +60 mV in the presence of bis(3)-tacrine (1 μM) (n=5, p>0.05 *versus* 0 mV).

Effect of bis(3)-tacrine on the steady-state activation and inactivation of sustained potassium current

We further examined the effect of bis(3)-tacrine on the steady-state activation of sustained potassium current. The conductance-voltage (*G-V*) curves in Figure 3A show a leftward shift in the presence of bis(3)-tacrine (1 μM). As a result, the steady-state activation curve became steeper. The half-maximal activation voltage was shifted to the hyperpolarizing direction by 11.2 mV (from -7.8±1.1 to -19.0±3.2 mV) (*n*=6) by bis(3)-tacrine.

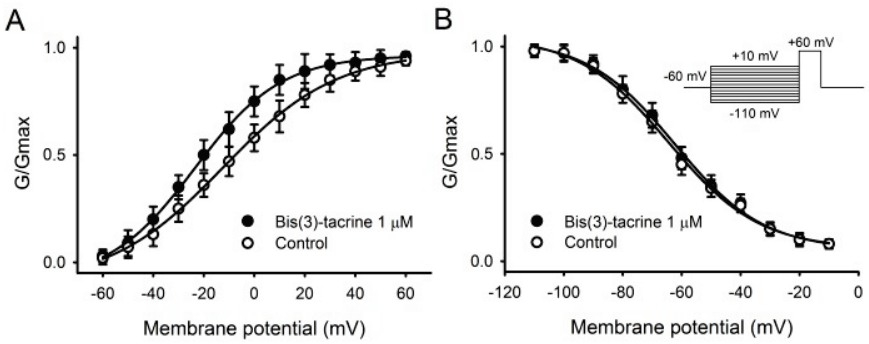

Fig. 3. Effect of bis(3)-tacrine on the steady-state activation (**A**) and inactivation (**B**) of sustained potassium current. Pulse protocols similar to those in Figure 1 were used to study the steady-state activation. Steady-state inactivation was studied using pulse protocols with hyperpolarizing prepulses from -110 to 10 mV (10 mV steps) followed by a step to a fixed voltage (+60 mV).

To measure the steady-state inactivation, depolarizing steps to +60 mV for 500 ms were elicited after a series of 2,000 ms prepulses from -110 to +10 mV in 10 mV increments. The *G-V* curves in Figure 3B show that bis(3)-tacrine had no effect on the inactivation of sustained potassium current. The voltage values at half-maximal inactivation conductance for sustained potassium current in the absence and presence of bis(3)-tacrine (1 μM) are -60.9±5.2 and -59.5±5.0 mV (Student's *t* test, $p>0.05$; $n=7$), respectively.

Discussion

Sustained potassium current is one of the main voltage-activated potassium currents, and plays a critical role in maintaining neuronal excitability (Pongs 1999). In the present study, it is the first time that we report bis(3)-tacrine can inhibit sustained potassium current in cultured rat hippocampal pyramidal neurons.

Our results show that the inhibition by bis(3)-tacrine and tacrine are both concentration-dependent, the extent of inhibition by bis(3)-tacrine of sustained potassium current shows a potency two orders of magnitude higher compared to tacrine (Fig. 1B). This has also been noted for other AChE inhibitors, such as huperzine A (Li and Hu 2002a, Li and Hu 2002b), galantamine (Pan *et al.* 2003b), rivastigmine (Pan *et al.* 2003a) and bis(7)-tacrine (Nie *et al.* 2007, Li *et al.* 2010) suppress potassium currents in both central and peripheral neurons.

Furthermore, we analyzed the possible mechanisms underlying these inhibitions. It had been reported that both cholinesterase inhibitors, huperzine A and bis(7)-tacrine, inhibited sustained potassium current in voltage-dependent and voltage-independent way, respectively (Li and Hu 2002a, Li and Hu 2002b, Nie *et al.* 2007). It can be seen from our result shown in Figure 2B that there was no significant change in percentage current (I/I_0) during the alteration of membrane potentials in the presence of bis(3)-tacrine as compared with control, that means the inhibition of sustained potassium current by bis(3)-tacrine is voltage-independent. Therefore, it is unlikely for bis(3)-tacrine to inhibit the currents by blocking the pore of potassium channel.

The kinetic properties of sustained potassium current were also significantly affected by bis(3)-tacrine. It was interesting that in the presence of bis(3)-tacrine the activation curve of sustained potassium current shifted leftwards. Earlier activation may indicate an increase in current amplitude; however, a decrease in sustained potassium current was clearly observed in the present study. One possible explanation could be that although the activation curve was slightly shifted by about 11 mV, bis(3)-tacrine is binding with its acting sites in the potassium channel in the whole process, letting less current leak through, thus decreasing the amplitude of the potassium current. Besides, bis(3)-tacrine did not significantly affect the inactivation curve of sustained potassium current. These phenomena coincided with those of inhibition of sustained potassium current by huperzine A in hippocampal neurons (Li and Hu 2002b);

but in some degree differ from those of inhibition by bis(7)-tacrine in DRG neurons and Kv 1.2 encoded potassium channels expressed in oocytes (Nie *et al.* 2007), which shows that bis(7)-tacrine shifts the activation/inactivation curves of sustained potassium current leftwards in both preparations, the effect being more pronounced for the latter. The difference was unlikely induced by using different species of neurons coming from central and peripheral nervous system, respectively, but probably suggest the different degree of protonized structures among these three compounds may lead to their binding to different depths of acting sites in the potassium channels.

It is generally accepted that the massive neuronal death which occurs in AD is due to apoptosis (Zhu *et al.* 2006). Growing evidence has shown that in the process of neuronal apoptosis and neurodegeneration induced by up-regulating potassium channel activities, neuronal potassium homeostasis is seriously disrupted (Yu 2003). The finding that TEA, and other potassium channel blockers attenuate cell apoptosis in cultured cortical neurons (Yu *et al.* 1997, Yu *et al.* 1999, Wang *et al.* 2000) support this speculation. Many AChE inhibitors are able to inhibit potassium currents, such as tacrine (Rogawski 1987, Kraliz and Singh 1997), huperzine A (Li and Hu 2002a, Li and Hu 2002b), donepezil (Zhang *et al.* 2004), galantamine (Pan *et al.* 2003b), bis(7)-tacrine (Nie *et al.* 2007, Li *et al.* 2010), etc. One possible pharmacological implication of our finding is that the blockade of voltage-activated potassium current by bis(3)-tacrine might lead to suppression of apoptosis and a substantial increase in cell survival.

Bis(3)-tacrine has been demonstrated to inhibit AChE and γ-aminobutyric acid subtype A ($GABA_A$) receptors (Carlier *et al.* 1999, Li *et al.* 2007). Recently, Luo *et al.* (2010) reported that bis-(propyl)-cognitin, which is the same compound as bis(3)-tacrine in our report, is an uncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist with fast off-rate (UFO) and may represent a promising drug candidate for various neurodegenerative disorders. Further studies need to answer the question that if bis(3)-tacrine influence GABA-activated current, as we studied before (Zhou *et al.* 2009). Han *et al.* (2012) reported that it can reverse cognitive impairment resulted from scopolamine in both water maze and object recognition tasks; and under the same condition, the relative potency to improve cognitive capacity was 5-20 folds over that of tacrine. This compound could be referred as a multi-target, potential effective drug for treatment of AD and thus may be favourable for suppressing the over-activation of potassium channels that emerges in the pathogenesis of AD.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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