Available online at www.sciencedirect.com
SCIENCE DIRECT
Neuroscience Letters 402 (2006) 159–163
Neuroscience Letters
www.elsevier.com/locate/neulet

3-Benzidino-6(4-chlorophenyl) pyridazine blocks delayed rectifier and transient outward potassium current in acutely isolated rat hippocampal pyramidal neurons

Hui-Zhi Du, Chao-Feng Zhang, Miao-Yu Li, Pin Yang *

Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education, Institute of Molecular Science, Shanxi University, Taiyuan 030006, PR China

Received 29 November 2005; received in revised form 8 March 2006; accepted 29 March 2006

Abstract

3-[(β-morpholinoethyl)amino]-4-methyl-6-phenylpyridazine (minaprine) is an acetylcholinesterase (AChE) inhibitor. 3-Benzidino-6(4-chlorophenyl) pyridazine (BCP) and minaprine have a central pyridazine ring in common. In this study, we investigated the effects of BCP on delayed rectifier potassium current ($I_{K(DR)}$) and transient outward potassium current ($I_{K(A)}$) in acutely isolated rat hippocampal pyramidal neurons by using whole-cell patch-clamp technique. $I_{K(DR)}$ and $I_{K(A)}$ were inhibited by BCP (0.01–500 μM) in a concentration-dependent and voltage-dependent manner. The IC_{50} value for the blocking action of BCP on $I_{K(DR)}$ and $I_{K(A)}$ was calculated as 7.13 ± 0.18 μM and 0.55 ± 0.11 μM, respectively. At the concentration of 10 μM, BCP shifted the activation curve of $I_{K(DR)}$ to positive potential by 29.09 mV. Meanwhile, at the concentration of 10 μM, BCP also shifted the activation and inactivation curve of $I_{K(A)}$ to positive potential by 34.18 and 22.47 mV, respectively. In conclusion, BCP potently inhibits $I_{K(DR)}$ and $I_{K(A)}$ in rat hippocampal pyramidal neurons.

Keywords: Whole-cell patch-clamp technique; Hippocampus pyramidal neurons; 3-Benzidino-6(4-chlorophenyl) pyridazine; Transient outward potassium current; Delayed rectifier potassium current

It was hypothesized that the cognitive loss associated with Alzheimer's disease (AD) was related to reduction of acetylcholine (ACh) and central cholinergic deficit. Thus, increasing ACh by acetylcholinesterase (AChE) inhibitors might enhance cognitive function in AD patients [1,10]. Furthermore, central potassium channels play important roles in regulation of learning and memory [6,8]. Several AChE inhibitors have been found effective on central outward potassium currents. Tetrahydroaminoacridine (tacrine) inhibits delayed rectifier potassium current ($I_{K(DR)}$) [7,19] and transient outward potassium current ($I_{K(A)}$) [14]. Metrifonate inhibits slow component of the after-hyperpolarization tail current (sI_{AHP}) [12]. Galantamine blocks $I_{K(DR)}$, but not $I_{K(A)}$ in rat dissociated hippocampal pyramidal neurons [11]. Donepezil blocks $I_{K(DR)}$ in pyramidal neurons of rat hippocampus and neocortex [20].

3-[(β-morpholinoethyl)amino]-4-methyl-6-phenylpyridazine (minaprine, Fig. 1) has selective affinity for muscarinc M_1 receptors and possesses related memory-enhancing properties [16,18]. Also, it enhances short-term retention in rats in the social memory test [13]. A classical structure–activity relationship exploration suggests that the critical elements for high AChE inhibition be as follows: (i) presence of a central pyridazine ring, (ii) necessity of a lipophilic cationic head, (iii) change from a 2- to a 4-5-carbon units distance between the pyridazine ring and the cationic head [3]. Using pyridazine and benzidine as building blocks, we synthesized 3-benzidino-6(4-chlorophenyl) pyridazine (BCP, Fig. 1), which has $IC_{50} = 0.30$ μM on electric eel AChE. In the present study, we investigated the effects of BCP on two main voltage-activated outward potassium currents including $I_{K(A)}$ and $I_{K(DR)}$ in acutely isolated rat hippocampal pyramidal neurons.

Wistar rats of 7–10 days were purchased from the Experimental Animal Center of Shanxi Medical University. Single rat hippocampal pyramidal neurons were acutely isolated by enzymatic digestion and mechanical dispersion [15]. Briefly, 400–600 μm thick brain slices were cut from hippocampal region in ice-cold artificial cerebrospinal solution (ACS) (pH 7.4) containing: NaCl 124 mM, KCl 5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM,

* Corresponding author. Tel.: +86 351 7011022; fax: +86 351 7011022.
E-mail address: yangpin@sxu.edu.cn (P. Yang).

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.03.067

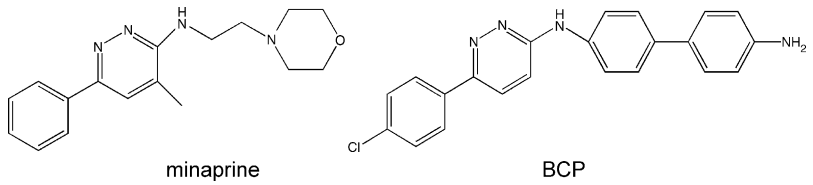

Fig. 1. Structrues of minaprine and BCP.

$CaCl_2$ 2.4 mM, Glucose 10 mM and $NaHCO_3$ 26 mM. These tissue pieces were incubated for at least 30 min at 32 °C in ACS, and then transferred into ACS containing 0.5 mg/ml protease at 32 °C for 35 min. Throughout the entire procedure the media were continuously saturated with a 95% O_2–5% CO_2 gas to maintain a pH value as 7.4. After digestion, the tissue pieces were washed three times with ACS. Through a series of Pasteur pipettes with decreasing tip diameter, neurons were isolated by triturating the brain fragments. Then, the cell suspension was transferred into a culture dish which is filled with 1 ml extracellular solution (pH 7.4) containing: NaCl 150 mM, KCl 5 mM, $MgCl_2$ 1.1 mM, $CaCl_2$ 2.6 mM, Glucose 10 mM, *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) 10 mM and tetrodotoxin (TTX) 0.001 mM. After 20 min, pyramidal cells settled on the bottom of the chamber. Neurons with bright, smooth appearance and apical dendrites were selected for recording.

A patch-clamp amplifier (Axon patch 200B, Axon Instruments, USA) was used to record whole-cell currents. A micropipette puller (PP 830, Narishige, Japan) was used to pull the electrodes. Patch pipettes had a tip resistance of 7–12 MΩ when filled with pipette solution (pH 7.3) containing: KCl 65 mM, KOH 5 mM, KF 80 mM, $MgCl_2$ 2 mM, HEPES 10 mM, Ethylene glycol-bic[2-aminoethylether]*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 10 mM and adenosine triphosphate disodium salt (Na_2ATP) 2 mM. Followed by the formation of a giga-ohm seal, the patch was disrupted by suction to obtain the whole-cell clamp configuration. Capacity transients were cancelled and series resistance was compensated (>70%). The leakage currents were subtracted. The currents were stored in a PC486 computer using an interface (Digidata 1200B, Axon Instrument, USA) and pCLAMP version 6.0.4 software (Axon Instrument, USA). All data were analyzed with pCLAMP CLAMPFIT6.0 (Axon Instrument, USA) and Origin 5.0 software (Microcal software, USA). All processed data were presented as mean ± S.D., and statistical comparisons were made using the paired Student's *t*-test, and the probabilities less than 0.05 were considered significant.

Protease, Na_2ATP and HEPES were purchased from Sigma, and other reagents were of AR grade.

In whole-cell patch-clamp recording, the total potassium currents were recorded with 150 ms depolarizing pulses from −50 to +60 mV in 10 mV steps following a hyperpolarizing prepulse of 400 ms to −110 mV (Fig. 2A). The delayed rectifier potassium currents ($I_{K(DR)}$) were elicited by a similar protocol in which a 50 ms interval at −50 mV was inserted after the prepulse. Currents at the end of the depolarizing pulse were referred to as $I_{K(DR)}$ (Fig. 2B). Subtraction of Fig. 2B from Fig. 2A revealed the fast transient potassium currents. The peak of the subtracted currents was referred to as $I_{K(A)}$ (Fig. 2C). Effects of BCP on $I_{K(DR)}$ or $I_{K(A)}$ were observed at +60 mV when depolarized from −50 mV. In the control test without BCP, $I_{K(DR)}$ and $I_{K(A)}$ decreased by 3.5 ± 2.1% and 4.2 ± 3.0% ($n = 8$) in 15 min current-recording, respectively. Upon the application of BCP (50 μM), the inhibitory effect of BCP on $I_{K(DR)}$ occurred in a few minutes until reached a maximum and steady value in about 12 min (Fig. 3). With different concentration of BCP, it took 12 ± 2 min for inhibitory effects on $I_{K(DR)}$ and $I_{K(A)}$ to reach the steady value. Therefore, in the present study, signals were firstly recorded in natural states, and then recorded in 12 min after addition of BCP into external solution.

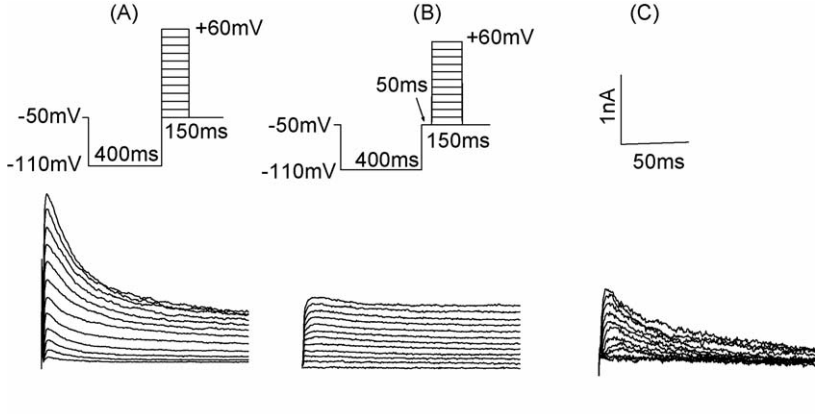

Fig. 2. Outward potassium currents in a hippocampal pyramidal neuron. (A) Total outward potassium current stimulated with 150 ms depolarizing pulses from –50 to +60 mV in 10 mV steps following a hyperpolarizing prepulse of 400 ms to −110 mV (inset). (B) $I_{K(DR)}$ stimulated with similar protocol as in (A), except for a 50 ms interval at −50 mV was inserted after the prepulse (inset). (C) Isolated $I_{K(A)}$ by subtracting current traces of (B) from those of (A).

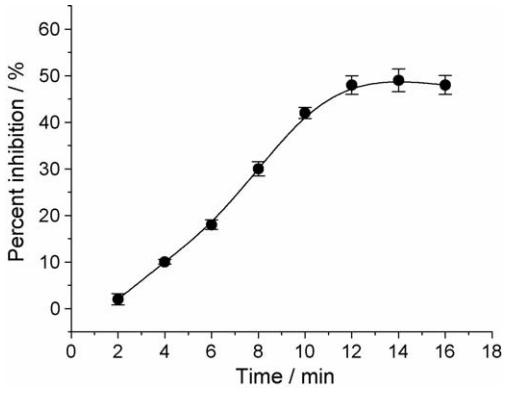

Fig. 3. Time curves of the blocking effect of BCP on $I_{K(DR)}$. The inhibitory effect of BCP (50 μM) on $I_{K(DR)}$ occurred in a few minutes until reached a maximum and steady value in about 12 min. Each point represents mean ± S.D. ($n = 8$).

With application of 0.01, 0.1, 0.2, 0.5, 1, 5, 10, 50, 100, 500 μM BCP, the amplitudes of $I_{K(DR)}$ and $I_{K(A)}$ decreased in a concentration-dependent manner. Concentration–response data were fitted with the Hill function: $I = I_{max}/[1 + (IC_{50}/C)^n]$, where I is the percent inhibition, I_{max} is the maximal percent inhibition, IC_{50} is 50% of maximum inhibition, C is the concentration of BCP, and n is the Hill coefficient. The IC_{50} value of BCP for blocking $I_{K(DR)}$ was calculated as 7.13 ± 0.18 μM with n of 0.75 ± 0.21, and the IC_{50} value of BCP for blocking $I_{K(A)}$ was calculated as 0.55 ± 0.11 μM with n of 0.15 ± 0.10 (Fig. 4A). Fig. 4B shows the effects of 10 μM BCP on the current–voltage (I–V) curves of $I_{K(DR)}$ and $I_{K(A)}$. I–V curves were obtained by plotting the currents evoked by 150 ms depolarizing from a HP of −50 to +60 mV at 10 mV steps against membrane potentials. As shown in Fig. 4B, the amplitudes of $I_{K(DR)}$ and $I_{K(A)}$ decrease differently at different membrane potentials. The relative reduction of $I_{K(DR)}$ and $I_{K(A)}$ by 10 μM BCP is illustrated in Fig. 4C at membrane potential between −20 and +60 mV. Inhibitory effects of BCP on $I_{K(DR)}$ and $I_{K(A)}$ become weaker and weaker with changes of membrane potential from −20 to +60 mV. The results indicate that the amplitudes of $I_{K(DR)}$ and $I_{K(A)}$ decrease in a voltage-dependent manner in the presence of BCP at the concentration of 10 μM. Effects of BCP on $I_{K(DR)}$ and $I_{K(A)}$ activation curves were detected by conductance–voltage relationship. The activation curves for $I_{K(DR)}$ and $I_{K(A)}$ in the absence and presence of 10 μM BCP were shown in Fig. 4D. Conductance–voltage curves were constructed by plotting G/G_{max} versus membrane potentials, and the curves were fitted by a 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, and k is the slope factor. In the absence and presence of 10 μM BCP, the value of $V_{1/2}$ was −6.86 ± 0.81 mV and 22.23 ± 1.00 mV ($n = 5$, $P < 0.01$), with k of 15.99 ± 0.80 mV and 16.63 ± 0.90 mV ($n = 5$, $P > 0.05$) for $I_{K(DR)}$; and the value of $V_{1/2}$ was −0.31 ± 0.67 mV and 33.87 ± 4.68 mV ($n = 5$, $P < 0.01$), with k of 18.79 ± 0.77 mV and 23.12 ± 4.19 mV ($n = 5$, $0.05 > P > 0.01$) for $I_{K(A)}$. Fig. 5 shows the inactivation curves for $I_{K(A)}$ in the absence and presence of 10 μM BCP. $I_{K(A)}$ was plotted versus membrane potentials. The curves were fitted with Boltzmann equation: $I/I_{max} = 1\{1 + \exp[(V - V_{1/2})/k]\}$, where I/I_{max} is the normalized data, V is membrane potential, $V_{1/2}$ is the potential for half-maximal inactivation, and k is the slope factor. In the absence and presence of 10 μM BCP, the value of $V_{1/2}$ was −81.24 ± 0.67 and −58.77 ± 0.37 mV ($n = 5$, $P < 0.01$), with k of −6.94 ± 0.58 mV and −8.28 ± 0.33 mV ($n = 5$, $P > 0.05$).

Minaprine, besides its original antidepressive properties, has cholinomimetic activities that could be, at least in part, mediated by their selective affinity for M_1 muscarinic receptors. An in vivo administration of minaprine (30 mg/kg) to rats significantly increases ACh levels in the hippocampus (38%). While minaprine presents a very weak in vitro activity on electric eel AChE (IC_{50} = 600 μM) [3,5]. Thus, minaprine could be developed as a potential applicable drug for the treatment of senile dementias and cognitive impairments occurring in elderly people [18]. BCP and minaprine have a central pyridazine ring in common. In this study, we found that BCP inhibited $I_{K(DR)}$ and $I_{K(A)}$ in acutely isolated rat hippocampal pyramidal neurons. Therefore, the presence of other groups associated with the pyridazine ring might make the compound more specific for either $I_{K(DR)}$ or $I_{K(A)}$. In addition, although tacrine is structurally related to the potassium channel blocker 4-aminopyridine (4-AP), the blockade of potassium channel by tacrine cannot be only attributed to the 4-AP-sensitive $I_{K(A)}$ [7]. Further studies involving other pharmacological compounds structurally related to 4-AP and minaprine, etc. are needed to understand the role of various groups and structure–activity relationship of AChE inhibitors in the blockade of the potassium current [17].

Under physiological conditions, potassium currents are important for the regulation of neuronal excitability and the maintenance of baseline membrane potential. Potassium currents control action potential duration and repolarization, release of neurotransmitters and hormones, Ca^{2+}-dependent synaptic plasticity. Since $I_{K(A)}$ is transient, repolarization is mainly related to $I_{K(DR)}$. As in other neurons, $I_{K(A)}$ in hippocampal neurons was thought to modulate the timing of repetitive action potential generation and the time required to reach the threshold to fire an action potential [4]. Enhancement of potassium currents leads to a reduction in $[K^+]_i$, involving the pathogenesis of neuronal death [2]. In this study, BCP (0.01–500 μM) inhibited $I_{K(DR)}$ and $I_{K(A)}$ in a concentration-dependent and voltage-dependent manner. We also notice that the maximum inhibition of BCP on $I_{K(DR)}$ and $I_{K(A)}$ only reaches about 50% even at the concentration of 500 μM of BCP. Because voltage-activated K^+ channels in rat pyramidal neurons are composed of various K^+ channel subtypes such as Kv1, Kv2, Kv3 and Kv4, BCP is probably selective to some K^+ channel subtypes. It needs further studies to understand the channel selectivity of BCP [11].

In this study, BCP (10 μM) remarkably decreased the amplitudes of $I_{K(DR)}$ and $I_{K(A)}$ in voltage-dependent manner. Therefore, charge screening effects at the membrane play a major role and could explain the reduction of the current through the potassium channels. Our results also demonstrated that 10 μM BCP shifted the activation and inactivation curves of $I_{K(A)}$ and the activation curves of $I_{K(DR)}$ to more positive potentials. However, the positive shift in the activation of $I_{K(A)}$ (about 34.18 mV) is

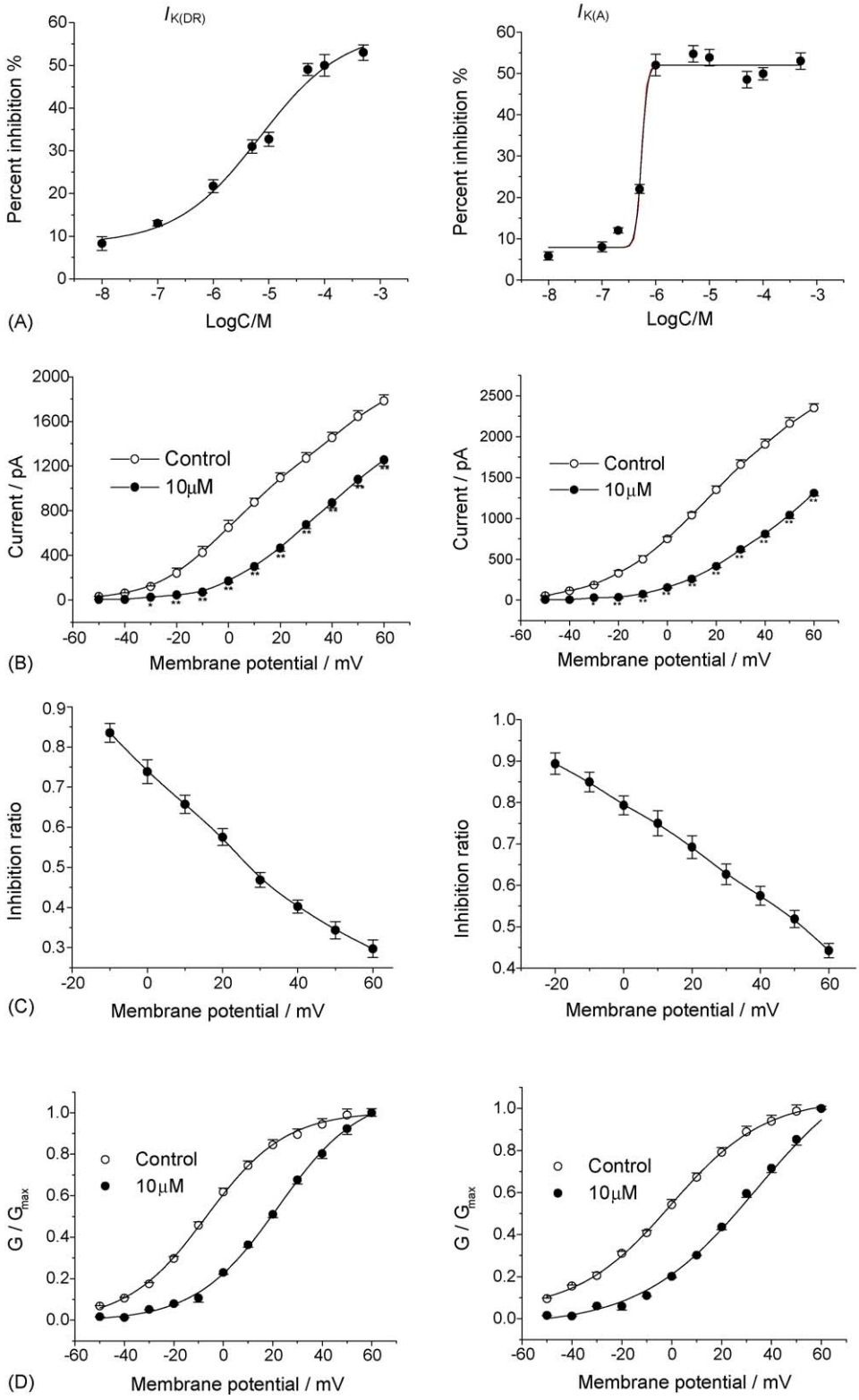

Fig. 4. Effects of 10 μM BCP on $I_{K(DR)}$ (left) and $I_{K(A)}$ (right). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. (A) Concentration–response curves for the blockade of BCP on $I_{K(DR)}$ and $I_{K(A)}$. (B) Effects of 10 μM BCP on the I–V curves of $I_{K(DR)}$ and $I_{K(A)}$. Each point represents mean of five cells. (C) The relative reduction of $I_{K(DR)}$ and $I_{K(A)}$ by 10 μM BCP, calculated for membrane potential between −20 and +60 mV. (D) The activation curve of $I_{K(DR)}$ and $I_{K(A)}$ in the absence and presence of 10 μM BCP.

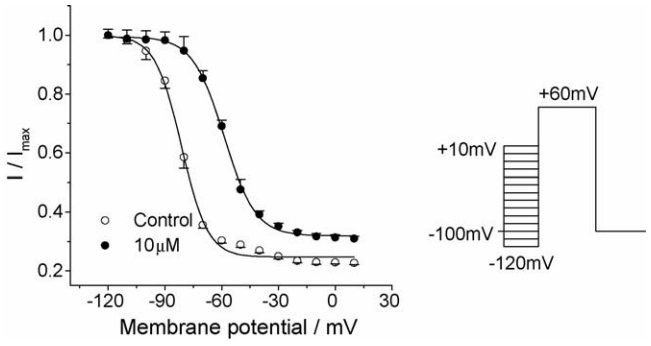

Fig. 5. Effect of 10 μM BCP on the inactivation curve of $I_{K(A)}$. Currents were elicited with a 120 ms test pulse to +60 mV preceded by 80 ms prepulses to potentials between –120 and −10 mV, and holding potential is −100 mV (see right). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

slightly more than that of $I_{K(DR)}$ (about 29.09 mV). These results suggest that although BCP exhibit comparable blocking effects on these two kinds of currents, the blocking potency of BCP on $I_{K(A)}$ is slightly stronger than that of $I_{K(DR)}$. In addition, a slightly more depolarizing shift of the steady-state activation curve of $I_{K(A)}$ by BCP than that of $I_{K(DR)}$ by BCP would account for the inhibition of BCP to these two kinds of currents. Similar shift of the activation curve has been used to explain the Pb^{+}-induced inhibition to the delayed rectifier K^{+} current in hippocampal neurons [9].

Our results support the proposition that the action of AChE inhibitors may be related to potassium channels [6]. The IC_{50} value of BCP towards $I_{K(DR)}$ and $I_{K(A)}$ in this study is about 7.13 and 0.55 μM, respectively, slightly higher than that of BCP towards AChE on electric eel. It suggests that BCP appear to be more sensitive to AChE than to these two kinds of currents and AChE could be major action site of BCP. However, potassium channels might still be other new targets for AChE inhibitors besides AChE. Also, it would be more significant once research targets are expanded to other mammalian neurons.

Acknowledgement

This work was supported by National Nature Science Foundation of China (No. 30470408).

References

[1] G. Benzi, A. Moretti, Is there a rationale for the use of acetylcholinesterase inhibitors in the therapy of Alzheimer's disease? Eur. J. Pharmacol. 346 (1998) 1–13.

[2] C.D. Bortner, F.M. Hughes Jr., J.A. Cidlowski, A primary role for K^{+} and Na^{+} efflux in the activation of apoptosis, J. Biol. Chem. 272 (1997) 32436–32442.

[3] J.M. Contreras, Y.M. Rival, S. Chayer, J.J. Bourguignon, C.G. Wermuth, Aminopyridazines as acetylcholinesterase inhibitors, J. Med. Chem. 42 (1999) 730–741.

[4] B.X. Gao, L. Ziskind-Conhaim, Development of ionic currents underlying changes in action potential waveforms in rat spinal motoneurons, J. Neurophysiol. 80 (1998) 3047–3061.

[5] S. Garattini, G.L. Forloni, S. Tirelli, H. Ladinsky, S. Consolo, Neurochemical effects of minaprine, a novel psychotropic drug, on the central cholinergic system of the rat, Psychopharmacology 82 (1984) 210–214.

[6] A.L. Harvey, E.G. Rowan, Effects of tacrine, aminopyridines, and physostigmine on acetylcholinesterase, acetylcholine release, and potassium currents, Adv. Neurol. 51 (1990) 227–233.

[7] D. Kraliz, S. Singh, Selective blockade of the delayed rectifier potassium current by tacrine in *Drosophila*, J. Neurobiol. 32 (1997) 1–10.

[8] P.W. Landifield, T.A. Pilter, Prolonged Ca^{2+}-dependent after hyperpolarizations in hippocampal neurons of aged rats, Science 226 (1984) 1089–1095.

[9] M. Madeja, U. Mubho, N. Binding, U. Witting, E.-J. Speckmann, Effects of Pb^{+} on delayed-rectifier potassium channels in acutely isolated hippocampal neurons, J. Neurophysiol. 78 (1997) 2649–2654.

[10] M.S. Mega, The cholinergic deficit in Alzheimer's disease: impact on cognition, behaviour and function, Int. J. Neuropsychopharmacol. 3 (2000) 3–12.

[11] Y.P. Pan, X.H. Xu, X.L. Wang, Galantamine blocks delayed rectifier, but not transient outward potassium current in rat dissociated hippocampal pyramidal neurons, Neurosci. Lett. 336 (2002) 37–40.

[12] J.M. Power, M.M. Oh, J.F. Disterhoft, Metrifonate decreases sI_{AHP} in CA1 pyramidal neurons in vitro, J. Neurophysiol. 85 (2001) 319–322.

[13] S. Puglisi-Allegra, S. Cabib, V. Cestari, C. Castellano, Post-training minaprine enhances memory storage in mice: involvement of D1 and D2 dopamine receptors, Psychopharmacology 113 (1994) 476–480.

[14] M.A. Rogawski, Tetrahydroaminoacridine blocks voltage-dependent ion channels in hippocampal neurons, Eur. J. Pharmacol. 142 (1987) 169–172.

[15] N. Sang, Z.Q. Meng, Blockade by magnesium of sodium currents in acutely isolated hippocampal CA1 neurons of rat, Brain Res. 952 (2002) 218–221.

[16] M. Sansone, M. Battaglia, J. Vetulani, Minaprine, but not oxiracetam, prevents desipramine-induced impairment of avoidance learning in mice, Pol. J. Pharmacol. 47 (1995) 69–73.

[17] H. Sugimoto, Structure-activity relationships of acetylcholinesterase inhibitors: Donepezil hydrochloride for the treatment of Alzheimer's disease, Pure Appl. Chem. 71 (1999) 2031–2037.

[18] P. Worms, J.P. Kan, R. Steinberg, J.P. Terranova, A. Perio, K. Biziere, Cholinomimetic activities of minaprine, Naunyn Schmiedebergs Arch. Pharmacol. 3401 (1989) 411–418.

[19] W. Zhang, H.W. Jin, S.F. Xu, L.T. Qu, X.L. Wang, Inhibition of tacrine on delayed rectifier and transient outward potassium currents in cultured rat hippocampal neurons, Acta Pharm. Sin. 39 (2004) 93–96.

[20] C.B. Zhong, W. Zhang, X.L. Wang, Effects of donepezil on the delayed rectifier-like potassium current in pyramidal neurons of rat hippocampus and neocortex, Acta Pharm. Sin. 37 (2002) 415–418.