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ORIGINAL PAPER

The Binding of Donepezil with External Mouth of K^+-Channels of Molluscan Neurons

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Abstract Earlier, we have shown a strong inhibitory effect of donepezil on K^+-current of molluscan neurons (Solntseva et al., Comp Biochem Physiol 144, 319–326, 2007). In the present work, a possible interaction of donepezil with the external mouth of the channel was examined using, as a tool, tetraethylammonium (TEA), a classical antagonist of potassium channels. Experiments were conducted in isolated neurons of snail Helix aspersa using the two-microelectrode voltage-clamp technique. A high-threshold slow-inactivating K^+-current involving Ca^{2+}-dependent (I_C) and Ca^{2+}-independent (I_K) components was recorded. The I_C was estimated at 30 mV, and I_K at 100 mV. The IC_{50} values for blocking effect of donepezil on I_C varied from 5.0 to 8.9 μM in different cells. Corresponding values for I_K varied from 4.9 to 9.9 μM. The IC_{50} values for blocking effect of TEA on I_C lied in the range of 200 to 910 μM, and on I_K lied in the range of 100 to 990 μM. The comparison of the effects of donepezil and TEA on the same cells revealed significant correlation between IC_{50} values of these effects. The value of Spearman coefficient of correlation (r) was 0.77 for I_C ($P < 0.05$), and 0.82 for I_K ($P < 0.05$). In the presence of TEA, the effect of donepezil, both on I_C and I_K, appears significantly weaker than in control solution. Dose–response curves of donepezil effect both on I_C and I_K were shifted right along horizontal axis when donepezil was applied in combination with TEA. Results suggest that TEA interferes with donepezil and precludes the occupation by donepezil of its own site. We suppose that the site for donepezil is situated near the TEA site with possible overlap.

Keywords Donepezil · Tetraethylammonium · Voltage-gated potassium current · Molluscan neurons

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Introduction

Donepezil is a potent acetylcholinesterase (AChE) inhibitor used for treatment of Alzheimer's disease (AD) (Miller 2007; Seltzer 2007). It is possible, however, that other mechanisms of its actions exist besides AChE inhibition. Donepezil was demonstrated to protect neurons against beta-amyloid (Abeta)-induced apoptosis (Arias et al. 2005; Kimura et al. 2005), as well as to ameliorate memory impairment caused by cholinergic synapse dysfunction unrelated to apoptosis (Watanabe et al. 2008).

Compelling evidence indicates that excessive K^+-efflux and intracellular K^+-depletion are key steps in early phase of apoptosis (Yu 2003). The involvement of K^+-channels in the mechanisms of Abeta-induced neurodegeneration is well documented. The enhancement of voltage-gated K^+-current followed by drop

in intracellular K^+-concentration and cell death was observed in various types of nervous cells as affected by Abeta (Colom et al. 1998; Yu et al. 1998; Chung et al. 2001; Pan et al. 2004; Pannaccione et al. 2007). Reduction of K^+-current by K^+-channel blocker tetraethylammonium attenuated Abeta-induced neuronal death (Yu et al. 1998). This data raises the possibility that manipulation aimed at reduction of K^+-current may help to reduce neuronal degeneration in patients with AD. In this light, a study of interaction of donepezil with voltage-gated K^+-currents looks promising.

The blocking effects of donepezil on voltage-activated K^+-current were shown in neurons of rat (Yu and Hu 2005) and snail *Helix pomatia* (Solntseva et al. 2007). The mechanism(s) of K^+-current suppression by donepezil is unclear. One can suggest both direct and indirect action of the drug on channel protein. Donepezil has been shown to be a potent agonist of the sigma1 receptor (Maurice et al. 2006), which is known to govern various biochemical and physiological processes (Hayashi and Su 2005), including intracellular calcium homeostasis (Vilner and Bowen 2000) and functioning of ion channels (Zhang and Cuevas 2002). This way, donepezil can interfere with cellular metabolism and, hence, may indirectly influence channel protein. However, direct interaction of donepezil with the latter looks more likely because of the rapid onset and rapid washing of its effect. A simple explanation of the effect of donepezil is a blockade of the external mouth of K^+-channel. In the present work, this suggestion was examined on molluscan neurons using, as a tool, tetraethylammonium (TEA), a classical antagonist of K^+-channels (Hagiwara and Saito 1959; Gutman et al. 2005; Wei et al. 2005).

TEA is a cationic K^+-channel pore blocker that acts from both sides of the membrane. The amino acids residue at the position equivalent to *Shaker* 449 located externally next to the selectivity filter was demonstrated to comprise the primary component of the TEA binding site. All four channel subunits contribute more or less equally to TEA blocking potency (Andalib et al. 2004; Korn and Trapani 2005).

In the present work, we investigated the competition between donepezil and TEA for a possible common binding site in K^+-channels of molluscan neurons. If our suggestion of donepezil interaction with the external mouth of K^+ -channel is correct, extracellular TEA would decrease the blocking effect of donepezil on K^+-current.

Methods

The experiments were performed on isolated neurons of the land snail *Helix aspersa*. Neurons were isolated without any pretreatment of the ganglia with proteolitic enzymes. The recording chamber with a neuron was continuously perfused with a Ringer solution containing (in mM): NaCl 100, KCl 4, $CaCl_2$ 5, $MgCl_2$ 4, $NaHCO_3$ 3, 4-aminpyridine (4-AP) 1, Tris–Cl 5 (pH = 7.6). A two-microelectrode voltage-clamp technique was used. The microelectrodes were filled with potassium citrate solution (2 M) and had tip resistances of 12–14 MΩ. The experiments were performed using a MEZ 7101 microelectrode amplifier and a CEZ 1100 voltage clamp amplifier (Nihon Kohden, Japan). Voltages and currents were recorded using a RJG 4024 four-channel pen-recorder with a bandwidth of up to 40 kHz. High threshold K^+-currents were triggered by depolarizing test pulses from the holding potential of −50 mV. In tracing the *I–V* curves, the current responses to equivalent hyperpolarizing pulses were added to cancel linear leakage. Donepezil solutions were prepared by dissolving tablets of *Aricept* (Pfizer) containing 5 mg of donepezil hydrochloride in a Ringer solution. The liquid was filtered, and the drug solution was introduced to the working chamber at stopped flow. More detailed description of preparation of donepezil solution can be found in our previous work (Solntseva et al. 2007). All the other chemicals were purchased from Sigma. Statistical analysis was performed using the Prism 3.0 (GraphPad) software. Group data is presented as mean ± S.E.M. A nonparametric test was used to analyse the correlation between the effects of donepezil and TEA.

Results

High-threshold K^+-currents were evoked by depolarizing test pulses of 150 to 500 ms applied from the holding potential of −50 mV. Test pulses varied from −30 to +100 mV with increments of 10 mV. The threshold of K^+-current activation was near

−20 mV. In most of the cells examined, an outward current had slow gate kinetics and demonstrated the N-shaped *I–V* curve indicating summation of Ca^{2+}-dependent (I_C) and Ca^{2+}-independent (I_K) components. To exclude possible contamination of slow-inactivating K^+-current with high-threshold fast-inactivating K^+-current (I_{Adepol}) (Bal et al. 2001), 1 mM 4-AP was added to the control solution.

Donepezil caused a fast, reversible and dose-dependent reduction of the amplitude of K^+-current recorded at all test potentials ($n = 9/9$). This effect observed in the neurons of snail *Helix aspersa* was similar to that described in our previous work on neurons of another snail species, *Helix pomatia* (Solntseva et al. 2007). In the present study, IC_{50} values for blocking effect of donepezil varied from 5.0 to 8.9 μM in different cells for current registered at 30 mV (I_C), and varied from 4.9 to 9.9 μM for current registered at 100 mV (I_K). In the same cells, TEA also caused a fast, reversible and dose-dependent suppression of the K^+-current recorded at all test potentials ($n = 9/9$). In contrast to the effect of donepezil, the strength of the TEA effect varied significantly from cell to cell. The IC_{50} values for blocking effect of TEA lied in the range of 200 to 910 μM for I_C, and within 100 to 990 μM for I_K. The comparison of the effects of donepezil and TEA on the same cells revealed significant correlation between IC_{50} values of these effects. The value of Spearman coefficient of correlation (r) was 0.77 for I_C ($P < 0.05$, Fig. 1(left)), and 0.82 for I_K ($P < 0.05$, Fig. 1(right)).

Further, we compared the effect of donepezil on K^+-current in the presence and absence of TEA. In these experiments, the concentrations of donepezil and TEA were chosen to cause an approximate half-reduction of the amplitude of K^+-current. Figure 2 illustrates the time course of one of the experiments where 6 μM donepezil was applied alone and in combination with 200 μM TEA. Each symbol shows the peak of outward current recorded at 30 mV (I_C). It is noted that both donepezil and TEA rapidly and reversibly suppress K^+-current roughly by half, and their effects are not additive. The current reduction by donepezil in the presence of TEA was much weaker than in control solution, indicating that

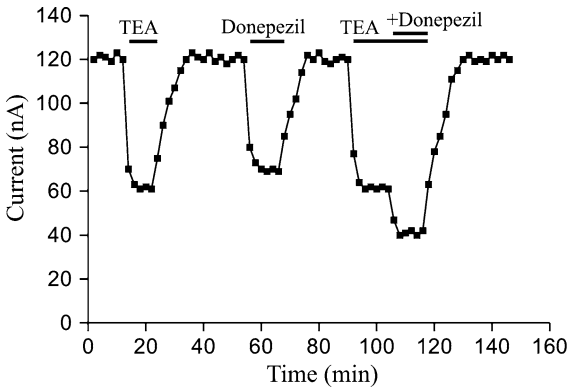

Fig. 2 Time course of changes in the peak of K^+-current during the experiment. The outward current was evoked by test pulses from −50 to +30 mV with 2-min intervals. TEA and donepezil applications are indicated by bars

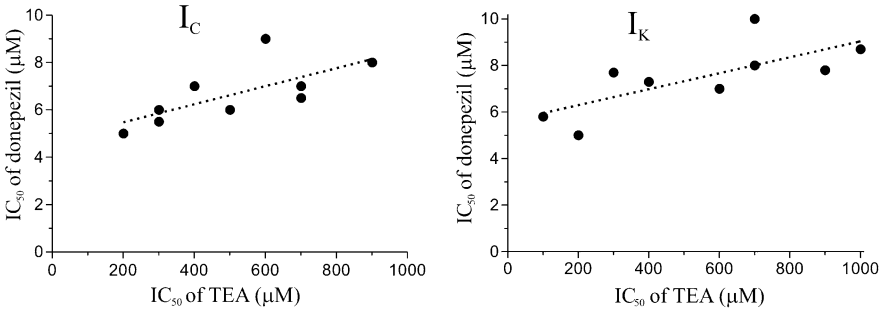

Fig. 1 The correlation between IC_{50} values of inhibitory effects of TEA and donepezil on I_C (left) and I_K (right). Nine cells were examined. Each symbol shows the result obtained from one cell. IC_{50} values for blocking effect of donepezil on current registered at 30 mV (I_C) varied from 5.0 to 8.9 μM in different cells, and for current registered at 100 mV (I_K) from 4.9 to 9.9 μM. The IC_{50} values for blocking effect of TEA lied in the range of 200 to 910 μM for I_C, and within 100 to 990 μM for I_K. The value of Spearman coefficient of correlation (r) was 0.77 for I_C ($P < 0.05$), and 0.82 for I_K ($P < 0.05$)

external TEA might prevent donepezil from binding with its own site.

Figure 3a and b shows the *I–V* curves of K^+-current of the same cell. The outward current within the potential range from −20 to 70 mV was provided essentially by I_C with little contamination of I_K, while in the range of 80 to 100 mV, it was composed solely of I_K. Maximal I_C was observed at +30 mV, while maximal I_{DR} was recorded at the highest test potential applied (+100 mV). The *I–V* curve constructed in the presence of 6 μM donepezil (Fig. 3a) indicates that both components of K^+-current are decreased. TEA also potently suppressed K^+-current both at 30 mV and at 100 mV (Fig. 3b). In the presence of TEA, the effect of donepezil became weaker on both I_C and I_K (Fig. 3b). At 30 mV, donepezil reduced peak outward current by 50 nA in the absence of TEA, and by 21 nA in the presence of TEA. At 100 mV, the corresponding values were 41 nA and 20 nA. The changes in the peak amplitude of K^+-currents were not accompanied by a change of kinetics of activation/inactivation as seen from the current traces shown on Fig. 3c and d.

Figure 4 shows averaged dose–response curves for donepezil effects on I_C (Fig. 4(left)) and I_K (Fig. 4(-right)) ($n = 4$). The concentration of TEA was different on different cells and was chosen to cause an approximate half-reduction of the amplitude of K^+-current. The blocking effect of 5 μM donepezil on I_C was 34.8 ± 10.8% in control solution, and 13.8 ± 6.8% in TEA-containing solution. This difference is statistically significant (paired *t*-test, $P < 0.05$). The corresponding values for 10 μM donepezil were 70.1 ± 10.2% vs. 42.5 ± 13.1 ($P < 0.05$). Similar results were obtained on I_K. In control solution 5 μM donepezil suppressed the I_K by 30.1 ± 9.1%, but in the presence of TEA, the effect was only 13.8 ± 5.5% ($P < 0.05$). 10 μM donepezil suppressed the I_K by 59.9 ± 10.8%, and with combination with TEA by 36.3 ± 9.4% ($P < 0.01$). Continuous lines show best fits with the Boltzmann equation. The IC_{50} values for blocking effect of donepezil on I_C and I_K were calculated as 6.9 μM and 7.8 μM, correspondingly, and, in the presence of TEA, these values increased up to 10.9 μM and 11.1 μM, correspondingly.

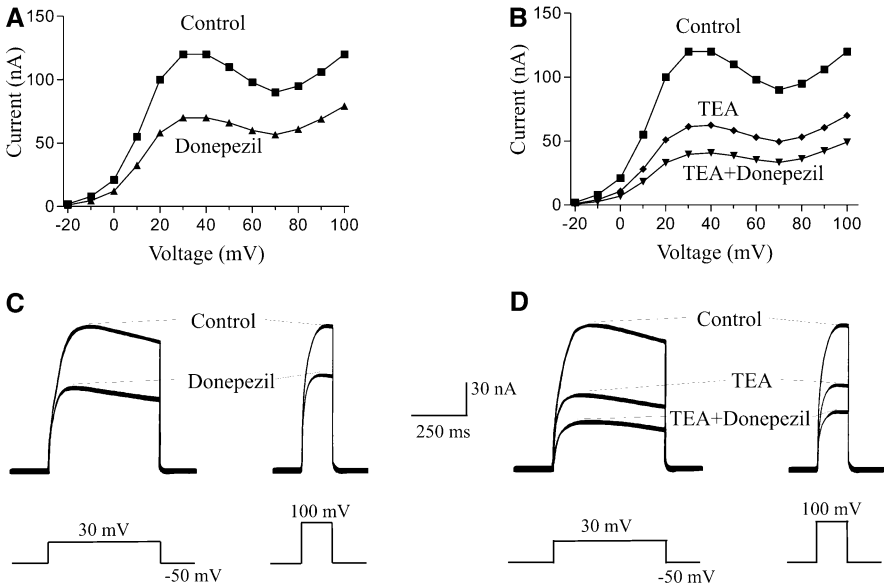

Fig. 3 The decrease of donepezil's effect on K^+-currents in the presence of TEA. (**a**) and (**b**): Current-voltage relationships for peak outward current, constructed in normal solution (Control), in the presence of 6 μM donepezil (Donepezil), 200 μM TEA (TEA), and 200 μM TEA in combination with 6 μM donepezil (TEA + Donepezil). (**c**) and (**d**): Original current recordings obtained in control solution and after application of donepezil, or TEA, or TEA in combination with donepezil. The recordings were obtained from the same neuron as in (**a**) and (**b**). The neuron was held at −50 mV. The currents were elicited with a 500 ms depolarizing step to 30 mV and with 150 ms depolarizing step to 100 mV

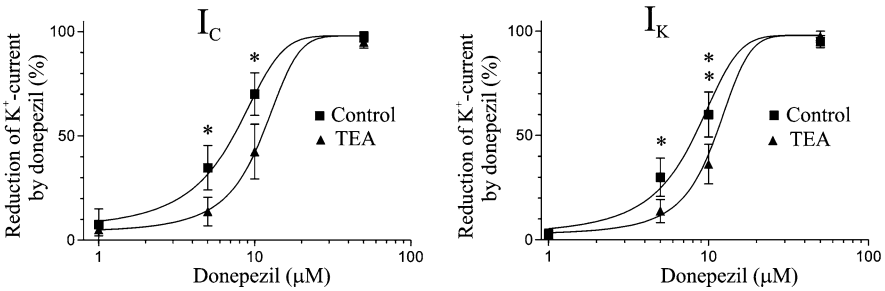

Fig. 4 Dose–response curves of blocking effect of donepezil on the peak amplitude of outward current recorded at 30 mV (I_C) (left) and 100 mV (I_K) (right) in control solution and in the presence of TEA. The normalized effect of donepezil is plotted vs logarithmic concentration of donepezil. Each value represents the mean ± S.E.M. of four cells. Date was fitted with Boltzmann function. One asterisk indicates P values of <0.05 and two asterisks indicate that of <0.01 between donepezil and donepezil + TEA effects

Discussion

In the present work, we show that both donepezil and TEA cause a suppression of voltage-gated K^+-current in molluscan neurons. The variability of TEA effects among the cells tested was much more pronounced in comparison with donepezil effects. This observation is in line with literature data indicating that TEA sensitivity ranges from 0.1 to >100 mM across the voltage-gated K^+-channels (Gutman et al. 2005; Wei et al. 2005). Additionally, potassium channels are known to have two different outer vestibule conformations with different sensitivity to TEA (Consiglio and Korn 2004; Trapani et al. 2006). In contrast, little variability in donepezil potency to suppress K^+-current was described in rat neurons (Yu and Hu 2005). Such a difference between ranges in TEA and donepezil effective concentrations assumes that their binding sites are not identical. At the same time, our other results indicate that TEA and donepezil can interfere in the outer vestibule of K^+-channel. First of all, significant correlations were observed between the effects of TEA and donepezil both on I_C and on I_K. Secondly, it was found that current reduction induced by donepezil became significantly weaker in the presence of TEA. The attenuation of donepezil effect in the presence of TEA results in a right shift of dose–response curves of donepezil inhibition of both I_C and I_K along the horizontal axis. Taken together, our data suggest that sites for TEA and donepezil are located side by side and, possibly, overlap.

The relevance of K^+-channel blockade by donepezil to the Alzheimer's disease treatment looks questionable. The potency of donepezil to suppress K^+-currents is 2–3 orders of magnitude lower than that to inhibit acetylcholinesterase (Cheng et al. 1996; Snape et al. 1999), and plasma concentration of donepezil in the patients receiving effective doses of the drug was reported to be much lower than that required for K^+-channels blockade (Rogers et al. 1998). Nevertheless, our study seems to be relevant to the pharmacology of K^+-channels since this paper is a first report on the interaction of donepezil, applied in low micromole concentrations, with the external mouth of K^+-channels.

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