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Original Research

Comparison between huperzine A, tacrine, and E2020 on cholinergic transmission at mouse neuromuscular junction *in vitro*

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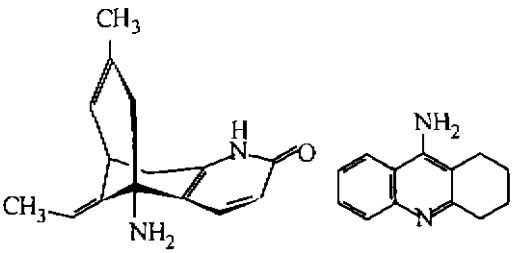
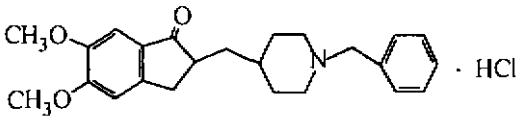
KEY WORDS cholinesterase inhibitors; huperzine A; tacrine; E2020; neuromuscular junction; diaphragm; phrenic nerve; electrophysiology

AIM: To compare the effects of huperzine A (Hup A), tacrine, and E2020 on cholinergic transmission at mouse neuromuscular junction *in vitro*. **METHODS**: The isolated mouse phrenic nerve-hemidiaphragm preparations were used with the conventional intracellular recording technique. The miniature end-plate potentials (MEPP), the mean quantal content of end-plate potentials (EPP), and the resting membrane potentials of muscle fiber were recorded. **RESULTS**: Hup A, tacrine, and E2020 at the concentration of 1.0 $\mu mol \cdot L^{-1}$ increased the amplitude, time-to-peak, and half-decay time of MEPP in the potencies of E2020 > Hup A > tacrine. Hup A did not significantly change the frequency of MEPP, the appearance of giant MEPP or slow MEPP, the resting membrane potentials, and the mean quantal content of EPP. **CONCLUSION**: Hup A is a selective and potent cholinesterase inhibitor, by which activity it facilitates the cholinergic transmission at mouse neuromuscular junction, and devoid of pre- and post-synaptic actions.

Acetylcholinesterase (AChE) inhibitors have been recently used in the treatment of Alzheimer's disease (AD) because they can improve symptoms in AD by prolonging the effects of endogenously released acetylcholine (ACh). Tacrine, an AChE inhibitor, is useful in the AD treatment. However, its clinical use has been limited by its liver toxicity[1]. Therefore a new generation of AChE inhibitors with a high therapeutic index and minimal toxicity, such as Hup A and E2020, have aroused our interest.



Huperzine A **Tacrine**



E2020

E2020, a piperidine-based AChE inhibitor, is an effective and relatively specific AChE inhibitor with a long duration of inhibitory action and devoid of unexpected toxicity in initial clinical studies[2]. Hup A, a novel alkaloid, is a potent and selective AChE inhibitor showing an increase in ACh level for several hours and fewer side effects than tacrine[3]. It made better the learning and memory in mice with higher efficacy than tacrine[4]. In phase II clinical trials, Hup A improved the memory quotient of AD patients with minimal side effects[5].

The ability of tacrine to alleviate AD symptoms is claimed not simply to relate with its anticholinesterase activity, but to combine with its potassium and sodium channel blocking properties[6]. However, few studies with Hup A and E2020 have been done on their possible mechanisms in the AD treatment except for their AChE inhibitory actions. Therefore, the present study was designed to further investigate the effects of Hup A and E2020 at the mouse neuromuscular junction.

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MATERIALS AND METHODS

Experiments were performed on the nerve-muscle hemidiaphragm preparations isolated from Kunming strain mice (18 – 22 g, Clean, ZKD-005) of either sex. The preparation attached with phrenic nerve was mounted in 40-mL recording chamber containing the Krebs' solution at 30 – 32 ℃ bubbled with 95 % O_2 + 5 % CO_2[7]. Before recordings the preparation was rested for at least 20 min. Drugs were prewarmed to 30 – 32 ℃. When the mean quantal content of EPP was studied, the preparation was firstly immersed in the high Mg^{2+}/low Ca^{2+} solution for at least 40 min for being stable, then the proportion of "failure" and "success" of EPP under same stimulation was calculated before and after the application of drug.

Intracellular recording technique was used with conventional glass microelectrode filled with KCl 3 mol·L^{-1} (tip resistance of 5 – 15 MΩ). The potential difference between the Ag/AgCl reference electrode in the bath and the recording electrode was measured by a high impedance amplifier (Axoclamp-2B, Axon Instruments Inc), displayed on a Nicolet 201 oscilloscope (Nicolet Instruments Inc), and stored on a DTR 1204 recorder (Biologic Instruments). The phrenic nerve was stimulated via a suction electrode by pulses of 50 μs duration and supramaximal voltage (SEN-7203 Electronic Stimulator, and SS-202J Isolator, Nihon Kohden). Analysis of signals was done by Axotape 2.0 Software (Axon Instruments Inc) and Poms Software with the computer (AST Bravo LP4/66d, AST Research Inc), and a DigiData 1200 Interface (Axon Instruments Inc).

Hup A (colorless powder, purity >98 %) was prepared by Department of Phytochemistry in this Institute. Tacrine was purchased from Sigma Chemical Co. E2020 (colorless powder, purity >98 %) was provided by Department of Organic Chemistry in this Institute. Other agents were AR.

Data in $\bar{x} \pm s$ were compared with ANOVA.

RESULTS

MEPP amplitude and time course The amplitude and time course of MEPP were modified by Hup A, tacrine, and E2020, at the concentration of 1.0 μmol·L^{-1}, with increased amplitude, prolonged time-to-peak and half-decay time.

The mean amplitude of MEPP in control was 0.64 ± 0.12 mV ($\bar{x} \pm s$, 430 MEPP, number of experiments ($n = 3$). Hup A increased this value to 1.15 ± 0.08 mV (413 MEPP, $n = 3$, $P < 0.05$), tacrine to 1.01 ± 0.04 mV (398 MEPP, $n = 3$, $P < 0.05$), and E2020 to 1.24 ± 0.02 mV (476 MEPP, $n = 3$, $P < 0.01$) (Tab 1). Their effects on MEPP amplitude were in the order of tacrine < Hup A < E2020.

Tab 1. MEPP amplitude in the presence of huperzine A, tacrine, and E2020. Their concentrations were 1.0 μmol·L^{-1}.

Amplitude /mV	Distribution/% Control	Huperzine A	Tacrine	E2020
0.2 – 0.4	11.8	2.5	3.1	2.7
0.4 – 0.6	43.3	11.1	11.1	10.1
0.6 – 0.8	25.1	21.7	24.6	18.6
0.8 – 1.0	7.8	20.1	24.9	14.4
1.0 – 1.2	5.4	15.7	9.7	10.6
1.2 – 1.4	3.6	12.7	8.0	8.8
1.4 – 1.6	2.1	8.6	4.5	7.4
1.6 – 1.8	0.9	3.5	4.5	5.6
1.8 – 2.0		2.5	3.5	5.1
2.0 – 2.2		1.6	1.4	5.3
2.2 – 2.4			1.6	4.2
2.4 – 2.6			1.5	2.6
2.6 – 2.8			1.6	1.1
2.8 – 3.0				1.3
3.0 – 3.2				1.0
3.2 – 3.4				1.2

Such relationship was also observed in the mean value of MEPP time-to-peak (Tab 2), and half-decay time.

Tab 2. MEPP time-to-peak in the presence of huperzine A, tacrine, and E2020. Their concentrations were 1.0 μmol·L^{-1}.

Time-to-peak /ms	Distribution/% Control	Huperzine A	Tacrine	E2020
0.10 – 0.14	8.8			
0.14 – 0.18	55.2	2.6	4.8	1.6
0.18 – 0.22	22.4	25.6	27.7	15.2
0.22 – 0.26	12.4	45.0	37.8	41.5
0.26 – 0.30	1.2	20.1	6.6	13.0
0.30 – 0.34		3.5	12.1	13.0
0.34 – 0.38		1.0	6.9	5.9
0.38 – 0.42		1.0	2.4	5.3
0.42 – 0.46		1.2	1.7	1.9
0.46 – 0.50				1.3
0.50 – 0.54				1.3

At the concentration of 1.0 μmol·L^{-1}, Hup A, tacrine, and E2020 had different effects on giant MEPP (gMEPP, those had amplitude two times larger than the model value for MEPP in control[8]) (Tab 1). gMEPP constituted 3.6 % of the total

recorded in control. Hup A did not increase the appearance of gMEPP significantly, which only numbered 6.7 % ($P > 0.05$ *vs* control), while tacrine and E2020 resulted in the big increase of gMEPP which represented 22.5 % and 32.8 % respectively ($P < 0.01$ *vs* control).

At the concentration of 1.0 $\mu mol \cdot L^{-1}$, Hup A, tacrine, and E2020 also had different effects on slow MEPP (sMEPP, those had time-to-peak two times larger than the model value for MEPP in control[8]) (Tab 2). In control, sMEPP only accounted for 0.3 %. Hup A changed little the incidence of sMEPP ($P > 0.05$ *vs* control), while tacrine and E2020 skewed the distribution to right and sMEPP occurred at 10.7 % and 15.7 % respectively ($P < 0.01$ *vs* control).

The scatter diagrams of MEPP amplitude *vs* time-to-peak showed that E2020 1.0 $\mu mol \cdot L^{-1}$ induced a population of slow-rising large amplitude MEPP with a positive correlation between these two parameters (Fig 1).

The cumulative plot[8] showed graphically that the distribution was changed by decreasing the number of larger events, while the smaller events remained the same after the application of E2020 1.0 $\mu mol \cdot L^{-1}$, suggesting that E2020 increase the larger subpopulation (Fig 2).

Frequency of MEPP At the concentrations of 0.05, 0.1, and 1.0 $\mu mol \cdot L^{-1}$, Hup A, tacrine, and E2020 had no significant effects on the frequencies of MEPP 20 min after their application ($P > 0.05$). Furthermore, Hup A and E2020 0.1 or 1.0 $\mu mol \cdot L^{-1}$, within the period of 0 – 60 min, did not change the MEPP frequencies ($P > 0.05$).

Mean quantal content of EPP Hup A, tacrine or E2020, at the concentrations of 0.05, 0.1, and 1.0 $\mu mol \cdot L^{-1}$, had no significant effects on the mean quantal content of EPP ($P > 0.05$). When time lasted to 100 min after Hup A 0.1 or 1.0 $\mu mol \cdot L^{-1}$ added, no significant changes of mean quantal content of EPP were observed ($P > 0.05$). As for E2020 1.0 or 5.0 $\mu mol \cdot L^{-1}$, no significant changes of mean quantal content of EPP were observed either when time lasted to 60 min ($P > 0.05$).

Resting membrane potentials of muscle fiber No significant effects of Hup A, tacrine, and E2020

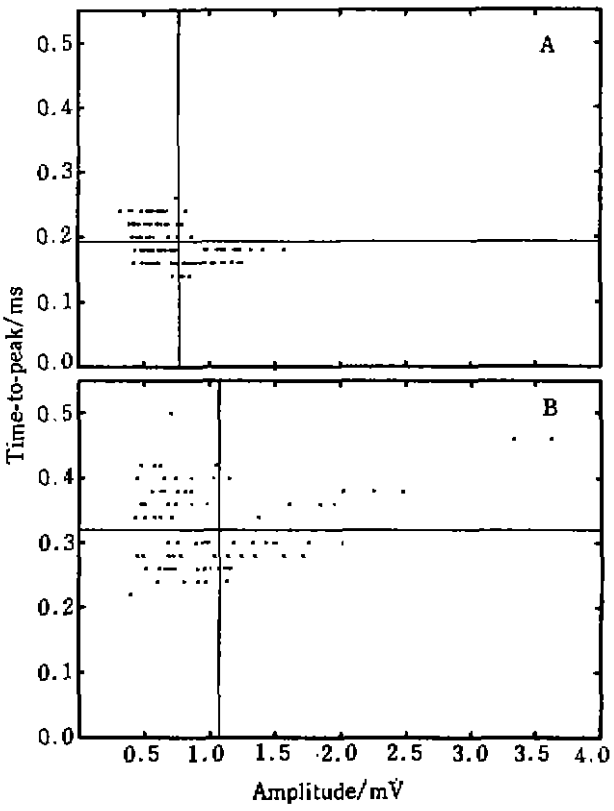

Fig 1. Relationship between MEPP amplitude and time-to-peak before (115 MEPP in A) and during E2020 1.0 $\mu mol \cdot L^{-1}$ (105 MEPP in B). Vertical and horizontal lines indicated the mean values.

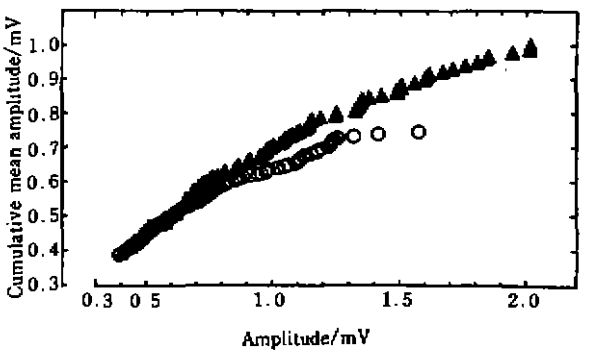

Fig 2. Cumulative mean amplitude *vs* MEPP amplitude before (○) and during E2020 1.0 $\mu mol \cdot L^{-1}$ (▲). Same preparations as Fig 1.

on resting membrane potentials of muscle fiber in mouse diaphragm were observed at concentrations of 0.1 – 100 $\mu mol \cdot L^{-1}$ 20 min after their treatment ($P > 0.05$). In the control, the resting membrane potential was – 77.9 ± 6.0 mV, Hup A 10

$\mu mol \cdot L^{-1}$ only increased it by 1.2 %, tacrine 10 $\mu mol \cdot L^{-1}$ increased it by 1.5 %, and E2020 10 $\mu mol \cdot L^{-1}$ decreased it only by 0.6 %.

DISCUSSION

The changes of the amplitude, time-do-peak, and half-decay time of MEPP can be explained by the anticholinesterase activities of the three compounds[9], therefore our results confirmed that Hup A, tacrine, and E2020 augmented neuromuscular cholinergic transmission, and their anticholinesterase potencies were E2020 > Hup A > tacrine, as previous demonstrated[10].

Hup A has no effects on resting membrane potentials of muscle fiber, indicating that the effects of Hup A may not be mediated through postsynaptic mechanism. Hup A did not increase the mean quantal content of EPP and the frequency of MEPP. Thus the possibility of presynaptic action can be excluded, complementing the studies that neither rate of ACh synthesis nor release of ACh might be influenced by Hup A[11]. Furthermore, in the presence of Hup A, neither the appearance of gMEPP nor sMEPP was changed, suggesting that non-specific promoting effects on the terminal ACh release be unlikely. These findings accorded with earlier observations in sympathetic ganglian preparation[12]. Therefore the facilitating effect of Hup A on neuromuscular ACh transmission was mediated by its mechanism of anti-AChE activity.

Tacrine did not change the EPP mean quantal content or MEPP frequency, or the resting membrane potentials of muscle fiber, which were agreed with earlier results[13]. For E2020 such observations were also seen. These findings indicated their lack of presynaptic or postsynaptic actions on neuromuscular junction at resting conditions.

Tacrine and E2020, in contrast to Hup A, increased the appearance of gMEPP and sMEPP. Similar results were obtained about tacrine by Canti *et al*[14]. However the exact mechanisms of gMEPP and sMEPP induced by tacrine have not yet been determined. There are several propositions: 1) tacrine may induced the fusion of dense-cored granules in the nerve endings[13]; 2) tacrine can increase the number of several vesicles cooperating for a quantum released concertedly[15]; 3) its potassium and sodium channel bolcking properties in addition to its anticholinesterase action may account for the appearance of gMEPP and sMEPP[16]. As for the mechanisms of gMEPP and sMEPP induced by E2020, it might share one of the mechanisms listed above, or it might have any mechanisms other than those listed above accounting for the gMEPP and sMEPP. The non-specific mechanism for E2020 in its facilitation of transmitter release, except for its anticholinesterase action, can not be excluded.

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石杉碱甲，他克林和 E2020 对离体小鼠神经肌肉接头胆碱能传递作用的比较

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关键词 胆碱酯酶抑制剂；石杉碱甲；他克林；E2020；神经肌肉接头；膈；膈神经；电生理学

目的：比较石杉碱甲，他克林和 E2020 对胆碱能传递的作用. **方法**：在小鼠的膈神经肌肉标本上用胞内记录的方法研究了石杉碱甲(Hup A)，他克林(tacrine)和 E2020 对自发释放的小终板电位(MEPP)，终板电位(EPP)的量子含量，以及肌细胞的静息膜电位的作用. **结果**：Hup A，tacrine 和 E2020 1.0 $\mu mol \cdot L^{-1}$ 可增强 MEPP 的振幅、上升相和半下降相，其作用强度为 E2020 > Hup A > tacrine. Hup A 对 MEPP 的频率，以及 gMEPP 和 sMEPP 的出现率无显著影响. 它对 EPP 的量子含量和肌细胞的静息膜电位亦无显著作用. **结论**：Hup A 是一种高选择性的 AChE 抑制剂，由此促进神经肌肉接头处的胆碱能传递.