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Effects of neostigmine and physostigmine on the acetylcholine receptor-ionophore complex in frog isolated sympathetic neurones

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1 The effects of neostigmine and physostigmine, reversible carbamate acetylcholinesterase (AChE)-inhibitors, on nicotinic acetylcholine-induced inward currents (I_{ACh}) were investigated in enzymatically isolated single sympathetic ganglion cells from the bullfrog. The 'concentration clamp' technique which combines intracellular perfusion with a rapid external solution change under single electrode voltage-clamp conditions was used.

2 Pretreatment with neostigmine and physostigmine did not enhance I_{ACh} at any concentrations, suggesting that AChE activity had already disappeared during the enzymatic treatment of the preparation.

3 Both neostigmine and physostigmine inhibited I_{ACh} in a dose-dependent manner with IC_{50} values of 7.0×10^{-4} M and 1.1×10^{-4} M, respectively. The blockade by neostigmine was competitive, while that by physostigmine was non-competitive.

4 The inhibition of I_{ACh} by neostigmine and physostigmine showed no apparent voltage dependency.

5 Neostigmine did not cause obvious changes of the kinetics of I_{ACh}. However, physostigmine reduced both the fast and slow time constants of inactivation of I_{ACh}, thus facilitating the rate of inactivation without affecting the activation kinetics of I_{ACh}.

6 These results suggest that neostigmine and physostigmine have different direct actions on the ACh receptor-ionophore complex. Neostigmine may act on the ACh-receptor (the binding site of ACh) while physostigmine may interact with the ACh-gated cation channels.

Introduction

To date, several lines of evidence suggest that, in addition to their action on acetylcholinesterase (AChE), AChE-inhibitors antagonize responses to acetylcholine (ACh) (Eccles & MacFarlane, 1949; Magleby & Stevens, 1972; Kuba *et al.*, 1973; Pascuzzo *et al.*, 1984; Fiekers, 1985; Slater *et al.*, 1986). However, the precise mechanisms of the interaction of these drugs with the ACh receptor-ionophore complex remain to be elucidated. One of the reasons is that under normal conditions the inhibition of AChE *per se* enhances the ACh response (Slater *et al.*, 1986) and the kinetics of the ACh-induced current may be affected by the rate of hydrolysis of ACh (Kuba & Nishi, 1979; MacDermott *et al.*, 1980; Fiekers, 1985). In the present experiments, the abolition of AChE activity secondary to enzymatic treatment of frog sympathetic ganglia, for the isolation of single neurones, made it possible to examine the direct effect of AChE-inhibitors on the ACh receptor-ionophore complex. Using the concentration jump (termed 'concentration clamp') technique (Akaike *et al.*, 1986), which combines internal perfusion and rapid change of external solution within a few ms under single electrode voltage-clamp conditions, we examined the effects of the carbamate AChE inhibitors, neostigmine and physostigmine, on the nicotinic ACh-induced inward currents.

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Methods

Preparation

The 9–10th ganglia of the paravertebral sympathetic chain of the bullfrog (*Rana catesbiana*) were excised and the connective tissue surrounding the ganglia removed by careful dissection. The capsule enveloping the ganglia was digested in frog Ringer solution containing 0.3% collagenase (Sigma, type I) for 25 min at 37°C. Thereafter single neurones were isolated mechanically from the ganglia with fine pins and left overnight at 10°C (Sanyo Incubator) in a culture medium consisting of equal volumes of Eagle's MEM (Nissui) and normal frog Ringer solution.

Electrical measurements

The suction pipette technique was used for intracellular perfusion and voltage-clamping (Hattori *et al.*, 1984; Ishizuka *et al.*, 1984; Akaike *et al.*, 1986). The inner diameter of the electrode was between 5 and 7 μm and the electrode resistance was less than 300 kΩ. The membrane potential was controlled with a single electrode voltage-clamp sample-and-hold amplifier (Ishizuka *et al.*, 1984). Both voltage and current were monitored on a digital storage oscilloscope (National, type VP-5730A) and simultaneously stored on FM data recorder. The currents recorded were filtered at 0.5 kHz, using a low pass filter (NF Circuit Design Block, FV-665A) and sampled by an A/D converter at 1.5 kHz for kinetic analysis using a computer (NEC PC9801XL).

Solution

The bathing medium contained (mM): NaCl 112, KCl 2, $CaCl_2$ 2 and glucose 5, HEPES-Tris-base (pH 7.4). The internal solution contained (mM): K-aspartate 100, KCl 20, NaCl 10 and EGTA 2.5, HEPES-Tris-base (pH 7.25). Drugs were applied using the 'concentration clamp' technique (Akaike *et al.*, 1986).

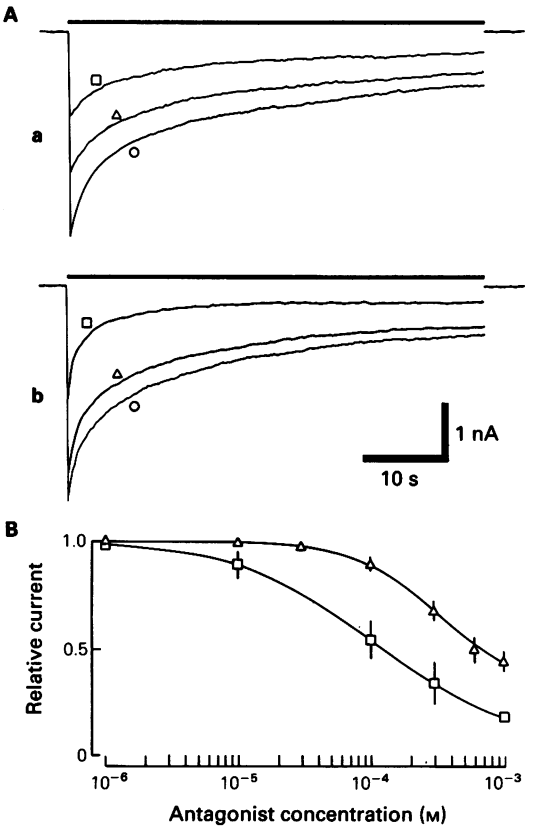

Figure 1 Effects of neostigmine and physostigmine on acetylcholine (ACh)-induced inward currents of bullfrog isolated sympathetic neurone at a holding potential (V_H) of -50 mV. (A) Responses to 6×10^{-6} M ACh in the absence (○) and presence of (a) neostigmine 3×10^{-4} M (△) or 10^{-3} M (□), or (b) physostigmine 10^{-5} M (△) or 10^{-4} M (□). Drugs were applied for the periods indicated by the horizontal bar above each response. Each trace in (a) and (b) was obtained from the same cell. (B) Relationship between the dose of the acetylcholinesterase inhibitor, (△) neostigmine or (□) physostigmine, and the ACh (6×10^{-6} M)-induced peak inward current. Data are mean values obtained in 6 to 9 experiments; vertical lines indicate s.e. mean.

Results

In the enzymatically isolated sympathetic neurones of the frog, both ACh- and nicotine-induced inward currents were effectively suppressed by curare and hexamethonium (C_6) while muscarine did not induce any response, indicating that ACh-induced currents were mediated by nicotinic ACh receptor-ionophore complexes. Effects of neostigmine and physostigmine on the nicotinic ACh-induced inward currents (I_{ACh}) in the isolated neurones at a holding potential (V_H) of -50 mV are shown in Figure 1A. It is evident that the pretreatment with either neostigmine or physostigmine did not produce potentiation of I_{ACh} at any concentration. Rather, both drugs depressed I_{ACh} in a dose-dependent manner. Figure 1B shows the relationship between dose of AChE-inhibitor and the relative amplitude of peak I_{ACh}. Physostigmine was much more potent than neostigmine in inhibiting I_{ACh}. The concentrations causing 50% inhibition (IC_{50}s) of neostigmine and physostigmine were 7.0×10^{-4} M and 1.1×10^{-4} M, respectively.

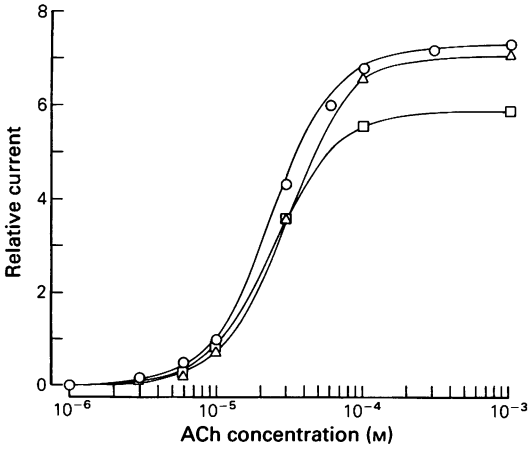

Figure 2 Dose-response curves to acetylcholine (ACh). Control (○), neostigmine 6×10^{-4} M + ACh (△) and physostigmine 10^{-4} M + ACh (□). The holding potential (V_H) was −50 mV. Data are mean values obtained in 4 to 6 experiments.

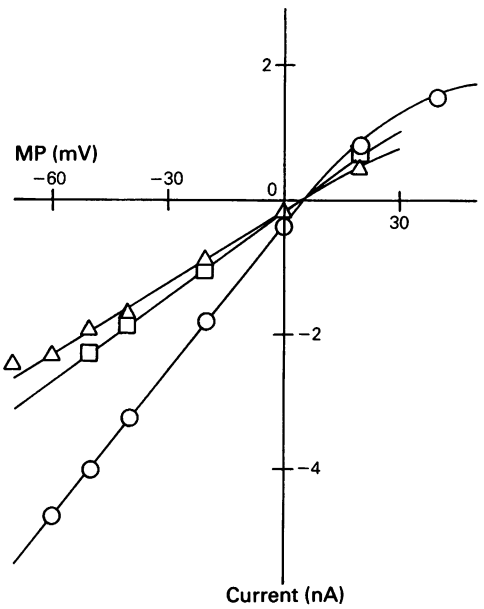

Figure 3 Current-voltage (I-V) relationships between the peak amplitude of currents induced by 6×10^{-6} M acetylcholine (ACh) alone (○), 6×10^{-4} M neostigmine + ACh (△) or 10^{-4} M physostigmine + ACh (□) and membrane potential (MP). All data were obtained from the same cell. The results are typical of reproducible observations obtained from 6 experiments.

Figure 2 shows ACh dose-response curves with and without AChE-inhibitors. The response to ACh showed a sigmoidal increase with increasing concentration with a half-maximal value (K_D) of 2.2×10^{-5} M at a V_H of −50 mV. Neostigmine (6×10^{-4} M) induced a slight parallel shift to the right of the dose-response curve for ACh without altering the maximal response, while physostigmine (10^{-6} M) depressed the maximal response without shifting K_D.

The current-voltage (I-V) relationships between I_{ACh} peak amplitude and membrane potential with and without neostigmine or physostigmine are shown in Figure 3. Neither neostigmine nor physostigmine altered the voltage-dependence of the I-V relationship over the voltage range −70 mV to +20 mV.

Figure 4 shows the effects of neostigmine (6×10^{-4} M) and physostigmine (10^{-4} M) on the kinetics of I_{ACh}. The amplitude of I_{ACh} with and without neostigmine or physostigmine was normalized to the same size. Neostigmine did not cause any change in activation or inactivation kinetics (Figure 4Aa). Physostigmine accelerated the inactivation phase without affecting activation. The time constants of the inactivation phase were quantitatively determined with and without physostigmine. Figure 4B shows a typical case. The time course of inactivation of I_{ACh} consists of double exponentials (Andreev *et al.*, 1984; Slater *et al.*, 1984). Physostigmine reduced both the fast and slow time constants in all cases.

Discussion

The present study shows a direct interaction of AChE-inhibitors with the nicotinic ACh receptor-ionophore complex. An advantage of this preparation is that there is no AChE activity after the enzymatic treatment used for isolating the cells and this fact means that the effect of the inhibitors is unrelated to hydrolysis of ACh. The disappearance of AChE activity by proteolysis has been demonstrated in other preparations (Albuquerque *et al.*, 1968; Fiekers, 1985). In addition, the 'concentration clamp' technique (Akaike *et al.*, 1986) enables us to change the extracellular ACh concentration within a few ms. Thus, we can discount the time-dependent change of ACh concentration at the cell membrane (MacDermott *et al.*, 1980; Fiekers, 1985), and analyse the activation and inactivation kinetics of I_{ACh} precisely.

Two representative carbamate AChE-inhibitors, neostigmine and physostigmine, block I_{ACh} through different mechanisms. The blockade of I_{ACh} by neostigmine is characteristic of competitive inhibition in that the dose-response curve is shifted to the right without depression of the maximum response. The

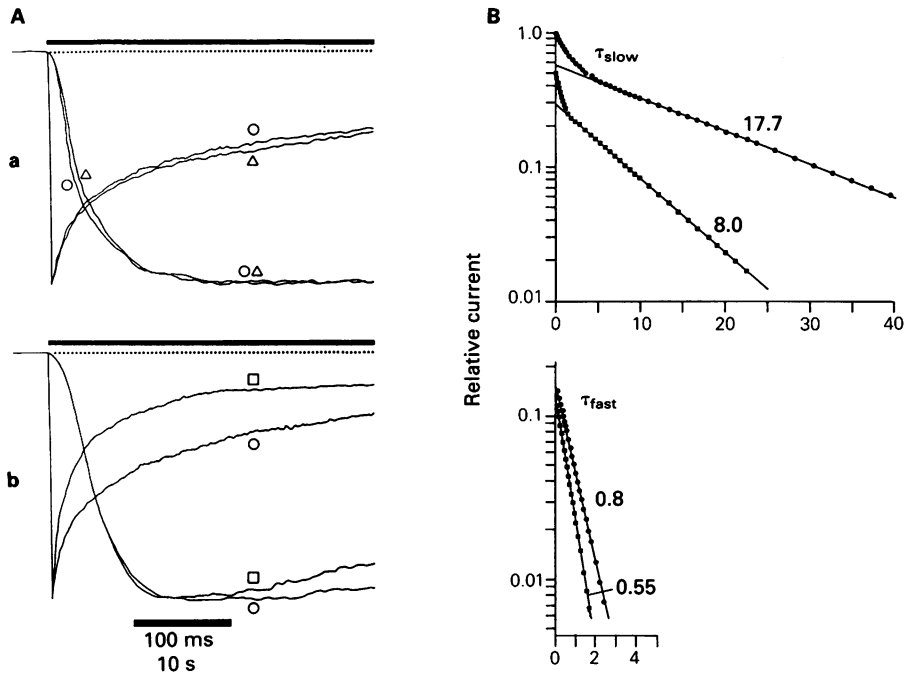

Figure 4 (A) Effects of (a) neostigmine (6×10^{-4} M) and (b) physostigmine (10^{-4} M) on the kinetics of acetylcholine-induced inward currents (I_{ACh}) at a V_H of -50 mV: ACh 6×10^{-6} M alone (○), neostigmine 6×10^{-4} M + ACh (△) and physostigmine 10^{-4} M + ACh (□). Drugs were applied for the periods indicated by the horizontal bars above each trace. The amplitude of each current was normalized. Note the different time scales for the traces of activation (100 ms scale) and inactivation (10 s scale). (B) Semilogarithmic plot of the inactivation phase of I_{ACh} shown in A(b): ACh 6×10^{-6} M alone (●) and physostigmine 10^{-4} M + ACh (■). Ordinate scales: corrected amplitude on a log scale calculated by subtracting the amplitude of the plateau component from the recorded amplitude of I_{ACh}. Abscissa scales: time after the peak I_{ACh} in ms. The slow component is shown by straight lines in the upper panel. The fast component was obtained by subtracting the slow component from the currents shown in the upper trace and is depicted in the lower graph. Figures indicate time constants (ms). These results are typical of reproducible observations obtained from 8 other experiments.

blockade did not show a voltage-dependency. Neostigmine also caused little change of the inactivating kinetics of I_{ACh}. This evidence suggests that neostigmine acts directly on the nicotinic ACh receptor, consistent with its blockade of α-bungarotoxin binding to homogenates of molluscan ganglia (Shain *et al.*, 1974; Carpenter *et al.*, 1976; Ono & Salvaterra, 1981) or *Torpedo* electric organ (Pascuzzo *et al.*, 1984). On the other hand, the blockade of I_{ACh} by physostigmine is characteristic of non-competitive inhibition in that the maximum response was depressed without changing K_D. In addition, physostigmine augmented inactivation of I_{ACh}, as is characteristic of an open channel blocker. This evidence suggests that physostigmine has a direct action on ACh-gated cation channels rather than the ACh receptor. The irreversible AChE-inhibitor, diisopropylfluorophosphate has also been known to have an action on the ACh receptor-ionophore complex (Kuba *et al.*, 1973).

It has been found that neostigmine (10^{-5} M) slows inactivation of the fast excitatory postsynaptic current (e.p.c.) in bullfrog sympathetic ganglion cells (Kuba & Nishi, 1979; MacDermott *et al.*, 1980). However, in our preparation, neostigmine did not slow the inactivation of I_{ACh}. One of the differences between our preparations and those used in previous studies is that in ours AChE activity was removed. This suggests that the rate of hydrolysis of ACh by AChE is one determinant of the decay of e.p.c. In *Aplysia* neurones (Slater *et al.*, 1986) and skeletal muscle endplate (Fiekers, 1985), neostigmine both slowed inactivation of I_{ACh} and split the inactivation of miniature endplate current into two components. Despite the presence of AChE in these preparations, these effects of neostigmine were still observed when

carbachol, which is not hydrolysed by AChE, was used instead of ACh or when collagenase was used to destroy the AChE activity, suggesting that neostigmine slows the inactivation of I_{ACh} but not through inhibition of AChE. Therefore, the variations in the effects of the drug on kinetics might be due to differences between the preparations used.

In summary, the studies described above demonstrate that neostigmine and physostigmine have different direct actions on the ACh receptor-ionophore complex, the former acting on the ACh binding site and the latter on the channel.

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