Electrophysiological estimation of the actions of acetylcholinesterase inhibitors on acetylcholine receptor and cholinesterase in physically isolated *Aplysia* neurones

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1 The actions of representative cholinesterase inhibitors on the acetylcholine responses of physically isolated single neurones from the pedal ganglion of *Aplysia californica* were studied, using electrophysiological techniques and rapid agonist application to analyse both the inhibitory actions on the acetylcholine receptor-channel complex and the degree of inhibition of acetylcholinesterase activity on the same neurone. The inhibitors used were physostigmine, edrophonium and diisopropylfluorophosphate (DFP).

2 When selected neurones were suddenly exposed to 50 μM acetylcholine by a 'concentration clamp' technique a large Na-dependent inward current was initiated, and decayed in the continued presence of acetylcholine without external perfusion. However, if perfusion of the acetylcholine solution was reinitiated the current increased somewhat, indicating that the decay of current was due to some combination of receptor desensitization and local depletion of acetylcholine at the membrane by acetylcholinesterase.

3 With simultaneous application of acetylcholine (50 μM) and physostigmine (0.1 to 100 μM) there was a dose-dependent reduction of peak amplitude of the acetylcholine response. However, physostigmine at low concentrations (0.1 to 10 μM) caused a time-dependent increase in the current amplitude alone with a time- and dose-dependent inhibition of acetylcholinesterase activity. At the highest concentration of physostigmine (100 μM) acetylcholinesterase activity was abolished but the current peak was very depressed. After removal of physostigmine from the bathing solution, the current amplitude decreased toward the control at the two lower concentrations as the inhibitory actions on acetylcholinesterase activity were almost reversible, while at the two higher concentrations (10 and 100 μM) the current increased and the inhibition of acetylcholinesterase remained.

4 When acetylcholine (50 μM) and edrophonium (0.1 to 10 μM) were applied simultaneously, edrophonium caused a dose-dependent increase in the peak amplitude that was correlated with a dose-dependent inhibition of acetylcholinesterase activity. Prolonged exposure to edrophonium did not change the peak amplitude and there was no time-dependent change in the inhibition of acetylcholinesterase activity. At the highest concentration of edrophonium used (100 μM), simultaneous application with acetylcholine augmented the peak amplitude relative to control, but to a lesser extent than 10 μM . Prolonged exposure to the highest concentration of edrophonium caused a time-dependent reduction in the peak amplitude. The effects of edrophonium were quickly reversible after the removal of the drug from the bathing solution.

5 DFP (1 and 10 mM), similar to 100 μM physostigmine, caused a dramatic reduction of the peak current on simultaneous application with acetylcholine. During exposure to DFP the current amplitude and acetylcholinesterase activity were very depressed. After removing DFP from the bathing solution the current amplitude increased to more than the control level after 1 mM DFP, while it did not recover to the control level after 10 mM DFP. The inhibition of acetylcholinesterase activity remained at both concentrations.

6 These results indicate that all three cholinesterase inhibitors have dose-dependent actions both at the acetylcholine receptor-channel complex and at acetylcholinesterase. The methods we have developed may be useful in the evaluation of various cholinesterase inhibitors.

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Introduction

Acetylcholinesterase is an enzyme that degrades acetylcholine and is widely distributed in the nervous system. It is located extracellularly on both neurones and glia (Rotundo, 1987). Because it lowers the concentration of acetylcholine this enzyme has functional effects both on synaptic transmission and on the response of receptive neurones to exogenously applied acetylcholine. In the presence of low concentrations of cholinesterase inhibitors, the peak amplitude of the acetylcholine response is increased (Dreyer *et al.*, 1978), reflecting the inhibition of acetylcholine hydrolysis. However, some anticholinesterases have, in addition to their inhibitory action on the enzyme, direct actions on the acetylcholine receptor-channel complex (Gage & McBurney, 1975; Kordas *et al.*, 1975; Kuba & Tomita, 1975; Magleby & Terrar, 1975).

Recently we have developed an electrophysiological technique that allows the direct estimation of acetylcholinesterase activity on physically (non-enzymatically) isolated single neurones of *Aplysia californica* and *kurodai* (Oyama *et al.*, 1988a). When the solution around a single isolated neurone was rapidly changed from sea water to sea water containing acetylcholine, an inward current was elicited, which peaked and decayed in the continued presence of acetylcholine. A part of the decay is due to receptor desensitization (Katz & Thesleff, 1957). However, restarting the flow of solution around the isolated neurone was found to cause an increase in current but to a value less than the initial peak. If carbachol, an acetylcholine agonist not hydrolyzed by acetylcholinesterase, was used, there was no increase in current when perfusion of the carbachol solution was reinitiated. Thus both receptor desensitization and acetylcholinesterase appear to contribute to the current decay in the presence of acetylcholine. In the absence of flow of the acetylcholine solution, the esterase activity appears to be sufficient to cause a local reduction of the acetylcholine concentration at the membrane. The rise of the current when flow is reinitiated is thus a convenient and rapid electrophysiological indicator of acetylcholinesterase activity, and can be used to monitor the inhibition of the esterase.

In this study, we have examined the actions of representative drugs from three classes of anticholinesterase. Physostigmine (eserine) is a carbamate inhibitor that is reversible, edrophonium is a quaternary ammonium compound that is rapidly active and reversible, while diisopropylfluorophosphate (DFP) is an irreversible alkyl fluorophosphate inhibitor. We have used an electrophysiological monitor of acetylcholine receptor activation and of acetylcholinesterase activity to investigate the relative

actions and differences in time course of action of these anticholinesterases.

Methods

The experimental method (internal perfusion with a concentration-clamp technique) was a slight modification of that previously described (Akaike *et al.*, 1986). Experiments were performed on single neurones physically isolated from the pedal ganglia of *Aplysia californica* (Oyama *et al.*, 1988c). Physically isolated neurones were used because the enzymatic treatment for dissociation of neurones removes acetylcholinesterase from their membrane (Kostenko *et al.*, 1974). The ganglion was incubated for 30 min in normal extracellular solution (NES, Table 1) to which 250 g l^{-1} sucrose was added. This treatment caused the neurones to shrink and facilitated the removal of the connective tissue and the top of the capsule, which were carefully removed with fine forceps and a pair of iris scissors. The ganglion was then returned to NES, and the exposed neuronal cell bodies were repeatedly washed by hand pipetting of NES. Individual cell bodies of 120 to 200 μm diameter which were freed from the ganglion were sucked into a pipette with a tip of about 400 μm in diameter, and the axon was cut. These isolated neurones stored in NES remained viable for experiments for up to 2 days after dissociation at room temperature (23 to 24°C).

The suction-pipette technique was employed for internal perfusion and voltage clamping (Lee *et al.*, 1980; Ishizuka *et al.*, 1984). The suction pipette was made from glass capillary of 3 mm inside diameter. The capillary was pulled to a shank length of about 3 mm, the tip of the pipette was cut at an outer diameter of 140 to 160 μm and then fire-polished to give inner and outer diameters of about 30 μm and 100 μm , respectively. The membrane potential was measured through an Ag-AgCl wire in a 3 M KCl-agar plug mounted on the suction pipette holder. The reference electrode (also an Ag-AgCl wire) was placed in a polyethylene catheter which was used for rapid solution changes (Krishtal *et al.*, 1983; Akaike *et al.*, 1986). The resistance between the Ag-AgCl wires was less than 10 k Ω when the suction pipette was filled with normal internal solution (NIS; Table 1) and the measurement made in NES. Both electrodes were led to a voltage-clamp circuit (DAGAN 8100). The cell body was drawn on to the suction pipette and held on the tip of the pipette by negative pressure of 300 to 450 mmHg. The membrane inside the pipette either spontaneously ruptured or was broken by hand with a thin Pt-Ir wire. The membrane potential was controlled by a single-electrode voltage-clamp system switching at 10 to

Table 1 Ionic compositions of external and internal solutions (in mM)

Normal external solution (NES)		Normal internal solution (NIS)	
NaCl	450	K aspartate	305
KCl	10	KCl	55
CaCl ₂	10	Mannitol	300
MgCl ₂	50	EGTA	0.1
HEPES	10	HEPES	20
pH	7.7 to 7.75	pH	7.3

The pH of NES and NIS was adjusted by appropriate amounts of NaOH and Tris base, respectively.

30 kHz and passing current for 25 or 50% of the cycle (Ishizuka *et al.*, 1984). Both current and voltage outputs were monitored on a storage oscilloscope, a digital memory oscilloscope and X-Y recorder.

In order to change the solution around the neurone rapidly, the tip of the suction pipette with the cell body attached was inserted into the polyethylene catheter (a diameter of less than 2 mm) through a pin hole of 1 mm diameter (see Figure 1 of Akaike *et al.*, 1986). The lower end of this catheter was then submerged in fluid in a tissue culture dish (diameter 2.5 cm, depth 1 cm). Negative pressure was applied to the upper end of the catheter to suck the solution from the dish through the catheter and past the neurone. The negative pressure was adjusted to make the rate of flow inside the catheter at least 8 cm per second. The suction was controlled by an electromagnetic valve operated by d.c. The external solution around the cell could be completely changed within 20 ms with this technique, and the usual procedure was to stop the perfusion after 400 or 800 ms of flow. For the purposes of changing solution, the various solutions were put in different dishes, and the dishes were mounted on a circular turntable fixed to a micromanipulator stage. To switch from one solution to another, the dish containing the desired solution was positioned at the end of the catheter by lowering the stage and rotating the turntable, then raising the stage until the tip of the catheter, still containing the previous solution, was again submerged. Suction was then applied to replace the solution in the catheter. The efficacy of the rapid external perfusion was tested in our previous study (Oyama *et al.*, 1988b).

Since the acetylcholinesterase activity of neurones stored in NES seemed to decrease gradually 2 days after isolation, neurones were used within 1 day after dissociation. Only neurones having action potentials of more than 80 mV in amplitude were studied. The neurones isolated from the pedal ganglia have various ionic currents in response to acetylcholine. A population of dark yellow neurones located on the caudal half of the dorsal surface of the pedal ganglion between the pedal connective and pedal nerve

showed a large Na-dependent inward current when the cell was voltage-clamped at -60 mV and were used in these studies. Because the neurone was perfused with NES and NIS to make Cl and K equilibrium potentials about -60 and -90 mV, respectively, any K current was outward and any contamination of the inward current carried by Cl was minimized. In the neurones selected for study the inward current elicited by acetylcholine was almost totally eliminated in Na-free solution (NaCl replaced with equimolar Tris Cl). For this study, only neurones that showed a constant amplitude response to $50 \mu\text{M}$ acetylcholine applied at intervals of 10 min were employed. The efficacy of the internal perfusion for *Aplysia* neurones has also been tested in a previous study (Oyama *et al.*, 1988b).

Figure 1 illustrates the experimental protocol in diagrammatic form. Each acetylcholine application consisted of two periods of solution flow (each lasting 400–800 ms) separated by 15 s. The first solution application led to an inward current that rapidly peaked and then decayed. The second flow 15 s later always gave a second increase of current, but never reached the value of the original peak. After the preparation was stable, a standard protocol was followed in which seven of the above paired flows were given, at 10 min intervals. After the initial acetylcholine control (a), the preparation was washed with NES for 10 min. It was then given paired perfusions with acetylcholine plus inhibitor at one concentration (b), after which it was washed with inhibitor in NES for 10 min. Two more tests with acetylcholine plus inhibitor were given while washing with inhibitor (c, d), after which acetylcholine without inhibitor was tested at the onset of the wash (e) and at 10 (f) and 20 (g) min thereafter. The effects of the anticholinesterases were analysed in terms of the ratio of peak amplitudes of the test as compared to control for the first perfusion (R_p) and the ratio of relative increase in amplitude of the current elicited by a second perfusion after 15 s in the presence of the stationary acetylcholine solution (R_s). The inhibition of acetylcholinesterase should be reflected in an increase in the first peak and a

Figure 1 (A) Method for estimating the action of acetylcholinesterase inhibitors on the response elicited by two rapid and time-fixed perfusions of $50 \mu\text{M}$ acetylcholine. Two arrows over the dotted line (holding current level) indicate the initiation of perfusion, and are separated by 15 s. The perfusion in each case lasted 400 or 800 ms, after which flow was stopped. In this study, we examined the action of acetylcholinesterase inhibitors on the peak amplitude of the first response (h_c and h_t) in control and test responses, and the effect of the inhibitor on the amplitude of response obtained on a second perfusion. The ratio of peaks of the first response, R_p , should increase due to inhibition of acetylcholinesterase, but decrease if the inhibitor has a dominant effect on the acetylcholine receptor or associated ion channel. The second response is quantified by measurement of the maintained current 15 s after the first perfusion (m_c) and the new current generated by the second perfusion (n_c). This new current is an indicator of the remaining cholinesterase activity. Thus the ratios (R_e) of the new to maintained currents, test over control, should become very small as cholinesterase activity is abolished. (B) Experimental protocol. Seven measurements (a) to (g) were performed during each experiment. The interval between each application was 10 min and the period of continuous presence of acetylcholine in each application was 25 s during which time two perfusions were made (a) control in normal external solution (NES; normal). (b) Simultaneous application of acetylcholine with acetylcholinesterase inhibitor (inhibitor). At this time, NES was switched to NES containing acetylcholinesterase inhibitor. After a period of 25 s, the external solution was switched to NES containing inhibitor. This was designed to obtain information concerning the competition at the receptor between acetylcholine and acetylcholinesterase inhibitors. (c) and (d) NES containing acetylcholine and acetylcholinesterase inhibitor was applied to the neurone 10 min after the application of acetylcholine. These procedures were followed to observe the time-dependent actions of the inhibitor. (e) The external solution was switched to the NES containing acetylcholine, and the inhibitor in the external solution was removed from the external solution within 30 ms. After this procedure, the neurone was washed with NES every 30 s. In (f) and (g) acetylcholine was applied to follow recovery from the drug.

decrease in the second, while actions of the inhibitor at the level of the receptor or channel should result in a reduction of the current amplitude, most obvious in the first peak.

All cholinesterase inhibitors were purchased from Sigma. Physostigmine and edrophonium were dissolved in NES just before use. DFP was dissolved in propylene glycol and used as previously described (Kuba *et al.*, 1974). Control applications of the appropriate dilution of propylene glycol in NES had no effect on the R_p and R_e of responses to $50 \mu\text{M}$ acetylcholine.

Results

Effect of cholinesterase inhibitors at low concentrations

Figure 2 shows the effect of $1 \mu\text{M}$ physostigmine on the acetylcholine ($50 \mu\text{M}$) response using the protocol of Figure 1. Simultaneous application of acetylcholine with physostigmine (b) resulted in a slightly reduced peak amplitude, but there was practically no change in the time course of the response. However, after 10 min of treatment with physostigmine (c) the amplitude of the first response was increased, while the ratio (R_e) of increase induced by the second perfusion was decreased. A further small reduction of the second response occurred in the next 10 min (d).

Figure 2 Effect of $1 \mu\text{M}$ physostigmine on the response elicited by two rapid and time-fixed (400 ms) perfusions of $50 \mu\text{M}$ acetylcholine. The triangle and arrow indicate the first and second perfusion, respectively. (a) to (g) over each current trace indicate those manipulations described in Figure 1B.

Figure 3 Effect of $1\ \mu\text{M}$ edrophonium on the response elicited by two perfusions of acetylcholine as indicated by the triangle and arrow. (a) and (g) over the current trace indicate those manipulations described in Figure 1B.

After 20 min of washing the response almost reached control values (f and g).

Figure 3 shows the same experiment but with $1\ \mu\text{M}$ edrophonium. Simultaneous application of $50\ \mu\text{M}$ acetylcholine with edrophonium (b) caused an

increase in the peak amplitude of the first response and a decrease in the ratio of the second response. Both effects appeared to reach a steady state immediately after the application, as the time course of responses (c) and (d) was very similar to that elicited by simultaneous application (b). Removal of edrophonium from the external solution resulted in quick recovery of the response toward the control (e) and repeated washing of the neurone gave complete recovery of the response (f and g).

Effect of cholinesterase inhibitors at high concentrations

The highest acetylcholinesterase inhibitor concentration employed in this study was $100\ \mu\text{M}$. Figure 4A shows the effect of $100\ \mu\text{M}$ physostigmine. Simultaneous application of acetylcholine with this concentration of physostigmine decreased the peak amplitude of the first response and diminished the second response (b). Longer exposure further reduced the peak amplitude (c and d). On removal of

Figure 4 (A) Effect of $100\ \mu\text{M}$ physostigmine on the acetylcholine response. The triangle and black arrow indicate the perfusion of the solution containing acetylcholine. The arrows joined by a bar indicate the period of the presence of physostigmine. The current trace under the current trace of (b) shows the current elicited by $100\ \mu\text{M}$ physostigmine in this neurone. (B) Effect of $100\ \mu\text{M}$ edrophonium on the acetylcholine response. Symbols are the same as in (A). The current trace under the current trace of (b) shows the current elicited by $100\ \mu\text{M}$ edrophonium. The properties of this current have been previously characterized (Oyama *et al.*, 1988b,c).

physostigmine there was a slight recovery (e). After repeated washing the peak amplitude of the first response increased to more than control level but the ratio of the second response was still suppressed (f and g). Figure 4B shows the effect of $100\ \mu\text{M}$ edrophonium. With simultaneous application the peak amplitude of the first response increased and the second response diminished (b). In continued presence of edrophonium the first response was slightly reduced but was still similar in amplitude to the control (c and d). On washing, the first response increased in amplitude and the second recovered slightly (e). With repeated washing there was an almost complete return to control (f and g). Internal perfusion of either edrophonium or physostigmine at $100\ \mu\text{M}$ had no effect on the second response.

Dose-dependency and time course of the action on the first peak current

Figure 5A shows the dose-dependence and the time course of the action on physostigmine (0.1 to $100\ \mu\text{M}$) on the peak amplitude of the first response. With simultaneous application there was a dose-dependent reduction of peak amplitude of the first response (b). However, 10 min after the start of treatment with 0.1 to $10\ \mu\text{M}$ physostigmine, the response amplitude increased in a dose-dependent fashion. The highest concentration ($100\ \mu\text{M}$) caused a time-dependent decrease in amplitude of the peak of the first response (c and d). Removal of physostigmine from the external solution did not result in a significant immediate change in the amplitude of the response irrespective of the concentration (e). After repeated washing the amplitude of the response decreased at the two lower concentrations (0.1 and $1\ \mu\text{M}$) while it increased at the two higher concentrations (f and g).

Figure 5B shows the same experiment but with edrophonium (0.1 to $100\ \mu\text{M}$). Simultaneous application with acetylcholine gave a dose-dependent increase in peak amplitude at concentrations of 0.1 to $10\ \mu\text{M}$, but the increase in amplitude with $100\ \mu\text{M}$ edrophonium was less than that with $100\ \mu\text{M}$ edrophonium (b). For the three lower concentrations (0.1 to $10\ \mu\text{M}$) the amplitude of the response did not decrease as compared to that with the initial simultaneous application. However, at the highest concentration ($100\ \mu\text{M}$) the peak amplitude gradually decreased (c and d). With washing, the amplitude of the response at the three lower concentrations was reduced but it increased at the highest concentration (e). These results suggest that at the lower concentrations the primary action of edrophonium is to block acetylcholinesterase while at $100\ \mu\text{M}$ there are also actions on the receptor-channel complex. Repeated

Figure 5 The dose-dependency and time course of the action of acetylcholinesterase inhibitors on the peak amplitude of the first response. (A) The effect of physostigmine (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). The ratio was calculated as R_p of Figure 1A. Each point indicates the mean value and vertical lines s.e. of five experiments. (a) to (g) are those manipulations indicated in Figure 1B. (B) The effect of edrophonium (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). Each point indicates the mean value of ratio calculated with R_p of Figure 1A, and vertical lines show s.e. of five experiments. (a) to (g) are those manipulations indicated in Figure 1B.

washing with NES produced complete recovery with all concentrations (f and g).

Dose-dependency and time course of the acetylcholinesterase activity

Figure 6A shows the dose-dependence and time course of action of physostigmine (0.1 to $100\ \mu\text{M}$) on the ratio (R_e of Figure 1A) of increase in the response on a second perfusion, which is an indication of remaining acetylcholinesterase activity. Simultaneous application of acetylcholine and physostigmine reduced the ratio of increase in the response

Figure 6 Dose-dependency and time course of the action of acetylcholinesterase inhibitors on the ratio of increase in the second response. Each point indicates the mean value of the ratio calculated as R_2 (Figure 1A), and vertical lines show s.e., of five experiments. (a) to (g) are similar to those in Figure 1B. (A) The effect of physostigmine (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM). (B) The effect of edrophonium (0.1 (▲), 1.0 (△), 10 (●) and 100 (○) μM).

in a dose-dependent fashion (b). There was a further decrease in the response in the continued presence of physostigmine in a time- and dose-dependent manner (c and d). The time course of action appeared more rapid at higher concentrations. Washing did not immediately affect the second response at all concentrations (e). At the two lower concentrations the effects were reversible with time but at the higher concentration this was not the case (f and g).

Figure 6B shows the same study with edrophonium (0.1 to 100 μM). The action of edrophonium on acetylcholinesterase appeared to reach a steady state immediately after application at all concentrations (b, c and d) and its effect was very dependent on the dose. Upon washout of edrophonium there was a quick recovery at the three lower concentrations but not at the highest concentration (e). Repeated washing of the neurone produced a complete recovery to the control level within 20 min (f and g).

Figure 7 The effect of diisopropylfluorophosphate (DFP) on the response elicited by two rapid and time-fixed (800ms) perfusions of 50 μM acetylcholine. The concentration of the drug was 1 mM. (a) Control. The triangle and arrow indicate the perfusion. (b) Simultaneous application of acetylcholine with DFP. (c) and (d) 20 and 40 min after the start of DFP application. (e) 20 min after the removal of DFP from the external solution and repeated washing of the neurone. (f) After washing DFP for 40 min, 1 mM edrophonium was applied and reduced both peak amplitude and response to second perfusion.

Effect of DFP, an irreversible anticholinesterase

Figure 7 shows the action of 1 mM DFP on the response elicited by two perfusions of 50 μM acetylcholine. The peak amplitude of the first response was greatly reduced by the simultaneous application of DFP and acetylcholine, and the second response was decreased (b). The mean percentage inhibition of the peak amplitude of the first response was $57.3 \pm 4.4\%$ (mean $\pm$ s.e. of four experiments). Further exposure to DFP (20 min and 40 min, c and d respectively) did not further decrease the amplitude of the first response; however, the amplitude was still lower than that of the control. During exposure to DFP, the amplitude of the second response was slightly reduced (c and d). The peak amplitude of the first response increased to more than the control level 20 min after removal of DFP from the external solution and repeated washing of the neurone (e). Further increases in the dose of DFP (10 mM) greatly decreased both responses, but did not completely inhibit the response to the second application in two experiments. At this concentration the response did not recover to the control level after washing the neurone for 60 min. Methylsulphonylfluoride, another irreversible cholinesterase inhibitor, had actions similar to those of DFP.

The effect of a high concentration of edrophonium (1 mM) was tested after washing out the DFP for 40 min (f). The peak was suppressed and the small response to the second perfusion was blocked. This

Figure 8 Comparison of the biochemical estimation of inhibitory action of physostigmine on acetylcholinesterase activity with the electrophysiological method described here. (●) Replotted from a previous paper by Carpenter *et al.* (1976). Data from the present electrophysiological studies of cholinesterase activity (△ and □) replotted from (c) and (d) of Figure 6A, respectively.

observation indicates that high concentrations of edrophonium act on the receptor-channel complex.

Discussion

It is reasonable to question whether electrophysiological methods can adequately estimate acetylcholinesterase activity of single neurones. Although we did not conduct biochemical studies on single isolated neurones in parallel with this study, a comparison was made with a previous study of *Aplysia* acetylcholinesterase activity. Figure 8 shows the dose-response plots for the inhibitory action of physostigmine on acetylcholinesterase activity. Biochemical measurement of acetylcholinesterase activity was conducted in ganglionic homogenates of *Aplysia dactylomela* 15 min after the application of physostigmine (filled circles of Figure 8 replotted from Carpenter *et al.*, 1976). In the present study, the activity was estimated 10 and 20 min after the application of physostigmine to single isolated neurones of *Aplysia californica*. In spite of the differences in the method and preparation, the dose-response relation for acetylcholinesterase blockade is similar. Thus it can be concluded that our electrophysiological estimation is adequate. It has the major advan-

tages of being applicable to intact single neurones, which enables the interactions between receptor activation and acetylcholinesterase activity to be studied and quantified. The electrophysiological technique also allows the time course of actions on the same single neurone to be studied.

Effect of physostigmine

The inhibitory action of physostigmine on acetylcholinesterase activity was time- and dose-dependent. While the recovery from the drug action was slight at the two higher concentrations, responses at the two lower concentrations tended to recover within 20 min toward the control level, but never completely. With regard to the action on the peak amplitude of the first response, in spite of complete inhibition of acetylcholinesterase activity with 100 μ M physostigmine, the response was greatly suppressed. The simultaneous application of acetylcholine and physostigmine induced a dose-dependent decrease in the peak amplitude of the first response. These results suggest that physostigmine has a rapid and strong inhibitory action on the acetylcholine receptor-channel complex. Some inhibitory effects were previously suggested for this and other carbamate acetylcholinesterase inhibitors (Carpenter *et al.*, 1976; Pascuzzo *et al.*, 1984; Fiekers, 1985; 1986; Slater *et al.*, 1986). Therefore, the amplitude of the response in the presence of physostigmine was modified by the inhibitory action both on acetylcholinesterase activity, resulting in an increase in the response, and on the receptor-channel complex, resulting in a decrease in the response. The onset and recovery from the inhibitory action on the receptor-channel complex was quicker than that on acetylcholinesterase activity. At these concentrations, in spite of repeated washing of the neurone, the inhibition of acetylcholinesterase activity still remained (Figure 5A).

Effect of edrophonium

The increase in amplitude of the first response was well correlated with the inhibitory action of edrophonium (0.1 to 10 μ M) on acetylcholinesterase activity (Figures 5B and 6B). This inhibition of acetylcholinesterase reached a steady state immediately after the application and the level of inhibition was dose-dependent (Figure 6B). Thus, edrophonium at less than 10 μ M does not appear to affect the acetylcholine receptor-channel complex. At 100 μ M edrophonium augmented the peak amplitude, but less than at 10 μ M, suggesting that this is the threshold concentration for interaction with the receptor-channel complex. The indicators of edrophonium action on acetylcholinesterase (increase of

the first peak and decrease of the second) were rapidly reversible at the lower concentrations. The effect of edrophonium on the acetylcholine receptor-channel complex was less robust and much less rapid as compared with its inhibitory action on acetylcholinesterase activity.

Differences in the action between the cholinesterase inhibitors

The dose-dependence of the actions of cholinesterase inhibitors was systematically examined only for the two reversible agents, physostigmine and edrophonium. The differences in the action of these inhibitors can be summarized as follows: (1) physostigmine has a prominent inhibitory action on the acetylcholine receptor-channel complex even at low concentrations, while that of edrophonium is very weak and is seen only at concentrations higher than those required for almost complete inhibition of acetylcholinesterase activity. (2) The time course of the inhibitory action of physostigmine on acetylcholinesterase activity is much slower than that of edrophonium and the recovery from the effect of physostigmine is also very slow and incomplete during the 20 min wash, while that of edrophonium is complete within 20 min of wash. (3) The action of DFP is most similar to that of physostigmine both in terms of relative actions at the acetylcholine-channel complex and in terms of time course. However, the actions on the acetylcholine receptor-channel complex were partly reversible, while, as expected, the blockade of acetylcholinesterase was much less so.

Previous studies comparing the inhibitory action of cholinesterase inhibitors on acetylcholinesterase

activity have been performed primarily using biochemical methods. Such studies cannot relate the degree of inhibition to the functional acetylcholine response recorded electrophysiologically. Our results show that the electrophysiological estimation of acetylcholinesterase activity can provide useful information about the action of the drugs both on this enzyme and on the acetylcholine receptor-channel complex. They also demonstrate that of the three drugs we studied all affect the acetylcholine receptor-channel complex, in addition to their actions on acetylcholinesterase.

Acetylcholinesterase inhibitors are drugs that have clinically important actions, and are useful in treatment of such diseases as myasthenia gravis and Alzheimer's Disease. A variety of inhibitors of cholinesterase have been used in these treatments, including physostigmine (Christie *et al.*, 1981) and tetrahydroaminoacridine (Summers *et al.*, 1986) for treatment of Alzheimer's patients. Since in these diseases the therapeutic aim is to increase the action of acetylcholine it might be counterproductive to use an acetylcholinesterase inhibitor which also depresses acetylcholine action at the level of the nicotinic cholinergic receptor, although the importance of nicotinic receptor activation in the central effects of acetylcholinesterase inhibitors is unclear. Effects of these drugs on muscarinic receptors remain as yet unknown. The methods we have developed may have a utility in the evaluation of various anticholinesterase drugs.

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