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KINETICS OF AGONIST CONDUCTANCE CHANGES DURING HYPERPOLARIZATION AT FROG ENDPLATES

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The effect of rapid hyperpolarization on acetylcholine- or carbachol-induced currents was studied at voltage clamped frog endplates. Following a hyperpolarizing step the agonist-induced conductance increased approximately exponentially to a new level. The rate constant for this process was smaller during hyperpolarization or lowered temperature, and was also smaller for acetylcholine than for carbachol. The results are interpreted in terms of a potential-dependent conformational change of the receptor.

Introduction The conductance change produced by agonists acting at the endplate membrane can be defined as $\Delta I/(V - E)$, where ΔI is the additional current produced by the agonist, V is the membrane potential and E is the equilibrium potential for the agonist action. Over a range of potentials from +50 mV to −180 mV the equilibrium conductance change produced by iontophoretic or bath application of various agonists increases exponentially with membrane hyperpolarization, an *e*-fold change being obtained with hyperpolarizations of the order of 100 mV (unpublished observations). Furthermore, the time constant for decay of endplate currents also increases exponentially with hyperpolarization (Magleby & Stevens, 1972). A simple model which accounts for these observations is to suppose that in the presence of an agonist the receptors distribute between inactive (R) and active (R*) forms, which interconvert with voltage-dependent rate constants f and b.

$$R \underset{b}{\overset{f}{\rightleftharpoons}} R^*$$

Under these circumstances following a step change in membrane potential the new agonist conductance should develop exponentially with a rate constant (f + b).

Methods The experiments were performed on isolated sartorius muscles of *Rana temporaria*. Acetylcholine or carbachol was applied iontophoretically to sensitive endplate regions from single or twin pipettes. A conventional two electrode voltage clamp technique was used. However, to obtain sufficient temporal resolution several improvements were necessary. Firstly, in order to change the membrane potential as rapidly as possible the output amplifier of the feedback system was an Analog Devices 171J giving an output swing of ±140 V. Aluminium foil wrapped around the intracellular voltage recording electrode was connected to the output of its unity gain voltage follower. Although the total clamp current was monitored in the usual way, this was not considered to give a perfectly reliable measure of the extra agonist current at early times following steps, partly because the area reached by agonist may not be perfectly isopotential, but mainly because this record is contaminated by current flowing to charge distant parts of the fibre. If the length constant of the endplate changes during agonist action it may not then be valid to subtract this current to obtain the agonist-induced current. Therefore the membrane current in the region acted upon by the agonist was taken to be proportional to the voltage drop in the Ringer solution just outside this region. This voltage drop was measured with an extracellular NaCl-filled microelectrode placed just outside the membrane as close to the tip of the iontophoretic pipette as was practical. This electrode was also 'cathodally screened' as above. Finally, most of the experiments were conducted at low temperatures (2°-6° C) achieved by the use of two Peltier elements (Mectron Frigistor) controlled by feedback from a bath thermistor. The bath temperature was separately monitored with an electronic thermometer (Cormark 1601). The records were photographed with simultaneous fast and slow time bases. All four microelectrodes were placed with their tips within a length equal to 1-2 fibre diameters. The iontophoretic pipette tip was positioned close to the endplate membrane so as to limit the area activated. The Ringer solution contained (mM): Na, 112; Cl 118.1; K 2.5; Ca 1.8; Tris 5; the pH was 7.4.

Results With the present system, step hyperpolarizations of 100 mV could be achieved in 50 μs (checked by inserting the extracellular microelectrode). However, the extracellular field (taken to be proportional to membrane current) did not 'settle' until 100-200 μs following the

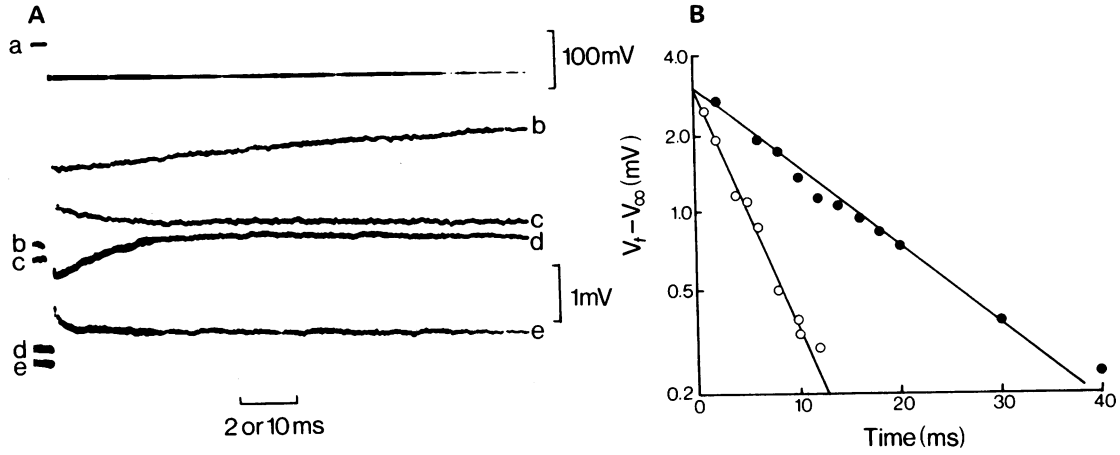

Figure 1 (A) Effect of sudden hyperpolarization on extracellular field potentials produced by iontophoretic acetylcholine application. (a) Shows the membrane potential; (e) and (d) show the extracellular field potential produced by the step hyperpolarization in the absence (e) and presence (d) of acetylcholine; (c) and (b) are the same as in (e) and (d) photographed simultaneously with a faster time base. (B) The onset of the increase in agonist-induced conductance produced by hyperpolarization. V_t is the difference in the extracellular field potential established by a step hyperpolarization from −80 to −155 mV in the presence and absence of agonist at various times following the step. V_∞ is the steady difference in field potential reached at t = 100 ms. $(V_t - V_\infty)$ is plotted semilogarithmically on the ordinate scale, and the abscissa scale is time elapsed from the step. V_t is assumed proportional to I_t (see text). Acetylcholine (•) or carbachol (○) were applied iontophoretically to the same endplate spot from separate barrels of a twin pipette. The solid lines have slopes of 74 s^{-1} (acetylcholine) and 216 s^{-1} (carbachol).

command, which therefore constituted a 'dead time' during which no measurement was available. In the absence of agonist the 'current' record then remained almost steady for the duration of the step (100 ms), though there was often an early dip. During the action of either acetylcholine or carbachol the membrane current was at all times larger, and consisted of a step that was apparently instantaneous followed by a further slow increase (Figure 1A). The agonist-induced current was obtained by measurement and subtraction of currents obtained in the presence and absence of agonist. The slow increase in current was apparently exponential, since if log $(V_\infty - V_t)$, where V_t is the extra field potential at time t and V_∞ the final steady potential, was plotted against t a straight line was obtained (Figure 1B). The slope of this line gave an apparent rate constant k, which decreased with increasing hyperpolarization. For example in one experiment with acetylcholine the values of k following steps to 88, 130, 157 and 213 mV were 96, 70, 41 and 21 s^{-1} respectively, at 4° C. After correction to −100 mV assuming a voltage dependence of e-fold per 70 mV (the overall value seen in three experiments), the mean value of k obtained in 15 experiments with acetylcholine (2°-6° C) was 160 ± 21 $^{-1}$ (± s.e. mean). At room temperature (20° C) k was much larger (700 s^{-1}).

Finally, in three experiments acetylcholine and carbachol were compared at the same endplate patch. The carbachol-induced conductance increase following hyperpolarization occurred faster than that obtained with acetylcholine. In these experiments the ratio k_{carb}/k_{ACh} was 2.38 ± 0.15 (mean ± s.e. mean). In one experiment at 20° C the values for k were 3.1 ms^{-1} (carbachol) and 0.7 ms^{-1} (acetylcholine) at −100 mV, giving a ratio of 4.4.

Discussion For a single site receptor the rate constant for increase in conductance is given by

$$k = f\frac{a}{K+a} + b$$

where a is the agonist concentration and K the equilibrium dissociation constant. It is assumed that in the present experiments the receptors are largely saturated ($a \gg K$) so that $k \approx (f + b)$. (f + b) for acetylcholine determined in the present experiments is very similar to the value of b determined from decay of the endplate current (Magleby & Stevens, 1972) or from analysis of endplate noise (Katz & Miledi, 1972; Anderson &

Stevens, 1973). This implies that b is considerably greater than f, so that even when all the receptors are saturated by acetylcholine only a small fraction are in the active form. This finding agrees with indirect evidence from analysis of endplate current decay following neostigmine treatment (Katz & Miledi, 1973).

If the receptor were composed of a number of subunits each of which independently underwent a conformational change and more than one subunit had to be in the active conformation for the channel to be open, then the conductance increase following a step hyperpolarization should follow a sigmoidal path (Hodgkin & Huxley, 1952). On the other hand if there were a concerted transition of all subunits the conductance increase should be simply exponential; however in this case the rate constant would depend on a power of the agonist concentration. Although in a number of the present experiments in which acetylcholine alone was tested the scatter in the measurements was much less than shown in Figure 1B, no consistent deviation from exponentiality (at least in the direction expected for independence) has yet been observed. However, any deviation produced by independence of subunits would be very small in the present experiments, in which the fraction of receptors activated before applying the hyperpolarization was already significant compared to the fraction finally activated. Experiments with steps from much more positive holding potentials should resolve this question.

The observation that conductance increases much faster when carbachol is used rather than acetylcholine is interesting for two reasons. Firstly, it lessens the possibility that the responses studied were artefacts. Secondly, the observed ratio of rate constants for carbachol and acetylcholine is similar to the ratio of channel lifetimes obtained by comparison of noise spectra (Katz & Miledi, 1972), implying that both methods probe essentially the same molecular event.

The present technique should be capable of considerable extension. For example a close comparison of b and (f + b) at a single endplate might permit an accurate estimate of f. Also, if binding is assumed very rapid compared to conformation changes, various possible models for receptor activation lead to simple, and testable, predictions for the dependence of the conductance change kinetics on agonist concentration.

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