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Decamethonium both opens and blocks endplate channels

(endplate kinetics/partial agonist/local anesthetics/ion channel)

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ABSTRACT **Miniature endplate currents, endplate current fluctuations ("membrane noise"), and voltage-jump current relaxations were studied in voltage-clamped frog muscle fibers during decamethonium action. All three types of experiments revealed two kinetic processes controlling the opening of endplate channels, one that reflects agonist action and another that reflects local anesthetic-like blocking activity. The kinetic constants for these two steps were evaluated from measurements of the fast and slow time constants as a function of decamethonium concentration. At −130 mV membrane potential and 13°, the mean open time of decamethonium-activated channels is 2.8 msec. The forward and backward rate constants for channel blocking are 1.7×10^7 M^{-1} sec^{-1} and 10^3 sec^{-1}. The voltage dependencies of the channel lifetime and of the blocking equilibrium are similar to those seen with pure agonists and local anesthetics, respectively.**

The transmitter at the nerve–muscle synapse of vertebrate skeletal muscle, acetylcholine (AcCho), acts by combining with postjunctional receptors and inducing the opening of discrete ion channels (1–3). A number of other quaternary ammonium compounds mimic this action of acetylcholine, although for some of them the maximum effect is quite feeble. Compounds that produce only small maximum potential or conductance changes are termed partial agonists. It has been generally supposed that these agonists are partial because they are relatively ineffective in triggering the conformational change of the receptor necessary for channel opening, either because the rate constants for this conformational change are such that the resting state is favored (4) or because of low selectivity in binding to the active form (5). However, recently we have found (6) that the current–voltage relation of endplate membrane activated by high concentrations of the typical partial agonist decamethonium (DECA) resembles that obtained with a mixture of a full agonist and a local anesthetic (7). If decamethonium possessed hybrid agonist and local anesthetic activity, it would no longer be necessary to postulate that it is particularly ineffective in triggering the opening of endplate channels.

In order to test this idea further, we have performed kinetic experiments similar to those that have been successful in elucidating local anesthetic action at the neuromuscular junction (7–11) and *Aplysia* neurons (12). These experiments have shown that local anesthetics reversibly bind to and block the open endplate channels. Blocked open channels cannot close until they have been vacated by anesthetic. This can be represented by the scheme:

$$X \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} X^* \underset{b}{\overset{d \cdot f}{\rightleftharpoons}} X^*D \qquad [1]$$

in which X represents the closed, X* the open, and X*D the open but blocked channel of zero conductance.

The processes leading to channel opening are lumped together as a single isomerization. This is justified by the finding that fluctuation and relaxation experiments reveal only one major kinetic process when indubitably full agonists are used (1, 2, 13–15). The blocking reaction is represented as a pseudo-first-order step, because it can be assumed that the concentration of free blocker is approximately constant. Thus the blocking rate constant is equal to $d \cdot f$, in which d is the DECA concentration.

The two kinetic processes predicted by this model could be detected in three separate ways: by using miniature endplate currents (m.e.p.c.s) which are due to spontaneous rapid transients of AcCho concentration, by statistical analysis of DECA-induced current fluctuations ("membrane noise"), and by inducing rapid perturbations of the open–closed channel equilibrium with stepwise changes in membrane potential (voltage jumps). The concentration and voltage dependencies revealed in these experiments show that DECA action can be quantitatively accounted for by scheme 1.

METHODS

All the experiments reported here were done on voltage-clamped endplates of frog (*Rana pipiens*) sartorius muscles at 11°–13° in rapidly flowing (1 ml/sec) Ringer solution of the following composition (mM): Na^+ 116; K^+ 2.7; Mg^{2+} 10; Cl^- 138.7; $H_2PO_4^-$ 0.2; HCO_3^- 1; pH ~ 7.4. In Ringer solution containing 1.8 mM Ca^{2+} and no Mg^{2+} the results were qualitatively similar, except that both the agonist and blocking potency of DECA increased about 2-fold.

The two-microelectrode clamp method (16) gives only local control of membrane potential, so the current flow through a small well-clamped patch of synaptic membrane was measured separately using a fire-polished Ringer solution-filled micropipette (tip diameter: 2.5–4 μm) placed on the nerve terminal. The extracellular potential change measured between this "focal" micropipette and a reference electrode was taken to be proportional to the agonist-induced current through the endplate patch under the pipette tip. For voltage jump experiments the reference electrode was a similar micropipette placed on nearby (within 10 μm) nonjunctional membrane, though for most of the noise and m.e.p.c. work a simple gross Ringer solution/agar bridge was used. Details of the techniques used are being published elsewhere (6).

Focal current recordings were stored on magnetic tape. The autocorrelation function (a.c.f.) of focal current fluctuations was calculated using a Saicor 42A 100-point correlator after bandpass filtering (0.5–2500 Hz) and digitization at 50- to 200-μsec sample intervals. Data points of the a.c.f.s and digi-

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Abbreviations: DECA, decamethonium; AcCho, acetylcholine; m.e.p.c., miniature endplate current; a.c.f., autocorrelation function.

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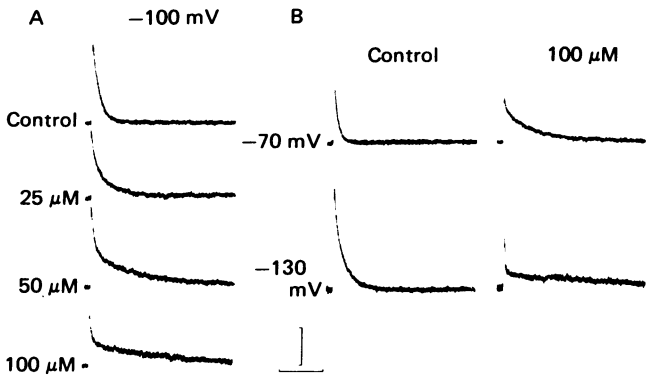

FIG. 1. Effect of DECA on focally recorded m.e.p.c.s in voltage-clamped muscle fibers. Negative shift in extracellular potential is upwards and corresponds to inward membrane current. Calibration marks indicate 500 μV shift in extracellular potential and 30 msec. (*A*) Effect of DECA concentration on m.e.p.c. decay at a holding potential of −100 mV. Oscilloscope traces of single m.e.p.c.s are shown. The top record represents control m.e.p.c. in the absence of DECA. Concentration of DECA applied is shown at the left of each trace. M.e.p.c.s were selected so that their amplitudes were representative. (*B*) Effect of membrane potential on m.e.p.c. decay during application of 100 μM DECA. Holding potential is marked at the left of control m.e.p.c.s.

tized records of m.e.p.c.s or voltage-jump experiments were stored in a PDP 11/40 computer. Time constants were determined by eye fitting of one or two machine-generated exponentials to the data points displayed simultaneously on an oscilloscope screen. Several samples of DECA-induced focal current fluctuations were also subjected to power spectral analysis (2). The high-frequency (50–2500 Hz) spectral distribution was Lorentzian; the time constants calculated from the corner frequency of this Lorentzian agreed closely with those estimated from the a.c.f.s of the same samples.

RESULTS

Decamethonium Induces Biphasic Decay of Miniature Endplate Currents. An m.e.p.c. is due to a brief local action of a packet of AcCho released from the nerve terminal. Because the falling phase of an m.e.p.c. is normally a single exponential whose time constants equals the AcCho-activated channel lifetime, it seems likely that the m.e.p.c. tail is due to the channel-closing step labeled α in scheme 1 (2). If DECA has a local anesthetic action, one would expect that in its presence the m.e.p.c. tail would split up into two components. The fast initial component would represent mainly blocking of open channels by DECA, while the slow component would be due to the intermittent closing of open channels as they are released from the blocked state. Fig. 1 shows that DECA does indeed produce the expected modification of the m.e.p.c. decay. Furthermore, if the DECA concentration or the membrane potential is increased, the initial component gets faster and relatively larger, whereas the slow component is prolonged. These effects are very similar to what is found with conventional local anesthetics (8–10, 17–20). Data from several m.e.p.c. experiments are summarized in Tables 1 and 2.

Decamethonium-Induced Endplate Current Fluctuations Show Two Decay Processes. The m.e.p.c. experiments suggest that DECA possesses local anesthetic-like activity, so that in principle it might block the channels that it itself opens by a separate agonist activity. This was confirmed by examining the statistical fluctuations in the endplate current evoked by DECA acting alone. Bath perfusion of DECA at concentrations that modify the m.e.p.c. decay led to the development of an inward

Table 1. Effect of DECA concentration on m.e.p.c. decay parameters at −100 mV holding potential

[DECA], μM	τ_{fast}, msec	τ_{slow}, msec	A_{fast}/A_{slow}
25	2.4	9	0.4
50	1.9	18	0.6
100	1.3	30	0.9

Mean values from four fibers are given. At least five individual m.e.p.c.s were averaged in each fiber. The time constant of control m.e.p.c.s at −100 mV in the absence of DECA was 4.8 msec. A_{fast}/A_{slow} represents amplitude ratio of fast and slow component.

clamp current of between −10 and −100 nA, together with a steady negative shift of the focal extracellular potential. This shift was accompanied by an increase in noise level of the recording, which could be seen to be at rather higher frequencies than that induced by AcCho or carbachol. Similar high-frequency focal current fluctuations have been recorded by Katz and Miledi (4). The a.c.f. of the fluctuations (Fig. 2) revealed, however, that in addition to a fast component with a time constant τ_{fast} in the 100- to 600-μsec range, a slower component with a time constant of several milliseconds is also present. These two-component fluctuations resemble those seen when full agonists are applied in the presence of local anesthetics (9, 10). Application of carbachol at the same endplate induced fluctuations whose a.c.f. showed only a single component with the time constant expected from previous work (1, 14, 21). The likelihood that these two-component a.c.f.s are due to combined agonist–local anesthetic properties of DECA was strengthened when the influences of membrane potential and DECA concentrations were studied (Figs. 2*B* and 4*A*). Hyperpolarizing the membrane or raising the DECA concentration accelerated the fast component and slowed and reduced the relative amplitude of the slow component, just as is seen with local anesthetics (10). In terms of the channel-blocking hypothesis, the first component is mainly due to channels fluctuating rapidly between open–unblocked (X*) and open–blocked (X*D) states, while the slow component is mainly due to fluctuation between closed (X) and open (X* or X*D) states.

Voltage-Jump Current-Relaxation Experiments Reveal Channel Blocking. For low DECA concentrations the membrane currents flowing during rectangular changes in potential resembled qualitatively those seen using carbachol at the same endplate patch (Fig. 3*A*). However, the rapid initial steps at the make or break of the potential change were smaller for DECA than for carbachol, even though both agonists produced similar currents at the holding potential. This suggests that in the case of DECA an additional fast process of opposite polarity intervenes following a hyperpolarizing step, which acts to reduce the observed current below the expected ohmic level. The subsequent major slow increase in current was slower for DECA than for carbachol. With higher concentrations of DECA the responses resembled strikingly those seen using a mixture of

Table 2. Effect of membrane potential on m.e.p.c. decay parameters during application of 100 μM DECA

Membrane potential, mV	$\tau_{control}$, msec	τ_{fast}, msec	τ_{slow}, msec	A_{fast}/A_{slow}
−70	3.4	2.2	12	0.4
−130	6.3	0.7	40	1.6

Mean values from five fibers are shown. At least five individual m.e.p.c.s were averaged in each fiber. A_{fast}/A_{slow} represents amplitude ratio of fast and slow component.

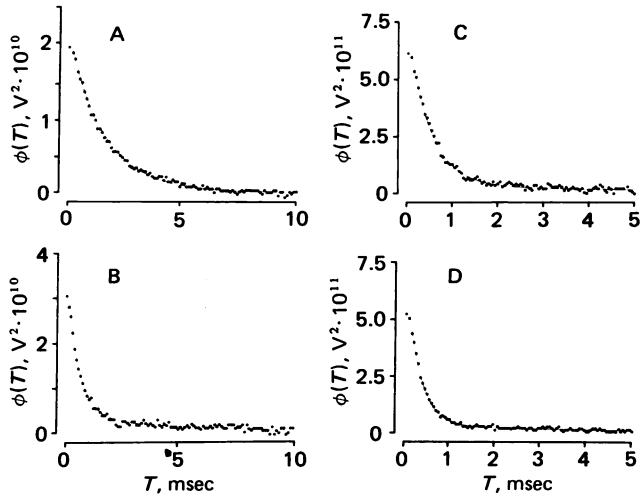

FIG. 2. Autocorrelation functions of focally recorded, agonist-induced endplate current fluctuations. Control a.c.f. calculated from current samples taken in the absence of agonist has been subtracted. Data points represent values of the autocorrelation function $\phi(T)$ for relative time displacement T. Continuous lines are single exponentials or the sum of two exponentials. (*A* and *B*) Comparison of a.c.f.s of current fluctuations induced by low concentrations of carbachol (10 μM) and DECA (50 μM) acting at the same membrane patch. Holding potential: −130 mV. The carbachol a.c.f., shown in *A*, is fitted by a single exponential with a time constant τ = 1.6 msec. The a.c.f. for DECA, shown in *B*, is fitted by the sum of a fast and a slow exponential with the time constants τ_{fast} = 0.52 msec and τ_{slow} = 5.8 msec. The amplitude ratio of the fast and slow component was A_{fast}/A_{slow} = 13. (*C* and *D*) Comparison of a.c.f.s of current fluctuations induced by 50 μM DECA, shown in *C*, and 100 μM, shown in *D*. Same membrane patch. Holding potential: −130 mV. The time constants and amplitude ratios of the fitted exponentials were in *C*: τ_{fast} = 0.45 msec; τ_{slow} = 5.3 msec; A_{fast}/A_{slow} = 15; and in *D*: τ_{fast} = 0.30 msec; τ_{slow} = 6.9 msec; A_{fast}/A_{slow} = 21. The slow time constants were obtained by analyzing the current sample on a 4-fold slower time base. By using power spectral analysis it was ascertained that the slow tail in the a.c.f.s corresponded to a smooth distribution of power in the low frequency range (not shown).

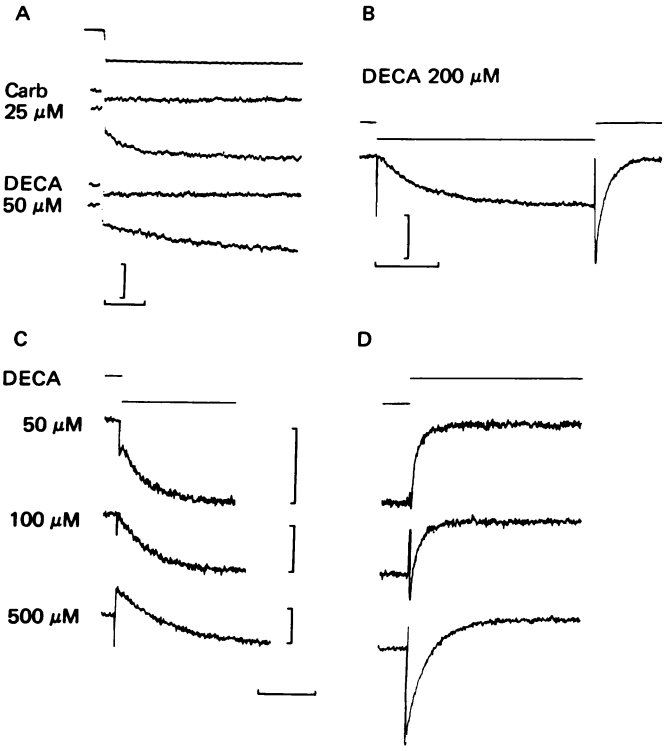

FIG. 3. Voltage jump relaxations of DECA-induced endplate current. Current was recorded as the extracellular potential difference between a microelectrode placed onto a nerve terminal and a nearby reference microelectrode. Negative shift in extracellular potential, corresponding to inward membrane current, is downwards. (*A*) Oscilloscope traces of single responses to hyperpolarizing voltage jumps recorded from the same patch of membrane in the presence of *equiactive* concentrations of carbachol and DECA. Time course of membrane potential change (from −70 mV to −130 mV) is shown in top trace. In each pair the upper trace shows the response in the absence, the lower in the presence, of agonist. The size of the rapid initial increase in membrane current following the voltage jump appears somewhat smaller in the presence of DECA. Initial fast relaxation is not resolved. Calibration marks indicate 400 μV and 4 msec. (*B*) Current relaxation following hyperpolarizing rectangular change in membrane potential. The membrane potential is stepped from a holding potential of −70 mV to −130 mV for 70 msec and then is stepped back to −70 mV. Time course of change in membrane potential is shown in upper trace. Lower trace shows time course of current relaxation. Five responses were averaged on a digital computer and the residual current flowing in the absence of DECA was subtracted. Sample interval 150 μsec. Calibration marks represent 250 μV and 20 msec. Following the hyperpolarizing jump, membrane current *decreases* rapidly after the ohmic increase and then slowly relaxes to the new equilibrium value. Following the depolarizing potential jump, a rapid *increase* in membrane current is observed after the ohmic decrease, followed by a slow decrease in current. (*C*) Relaxations following voltage jump from −70 mV to −130 mV in the presence of the DECA concentrations marked left of the traces. Time course of potential change is shown in uppermost trace. At each concentration the duration of the step was adjusted to allow the current to reach its new steady-state value. In order to facilitate comparison of time constants the relaxation amplitudes were scaled to the same value. Calibration bars shown beside each trace indicate 150 μV. Time calibration: 20 msec. (*D*) Relaxations following potential jump from −130 mV to −70 mV. Same experiment as that shown in *C*. Note transient *increase* in membrane current following a depolarizing voltage step in the presence of high DECA concentrations. Same calibration as in *C*.

suberyldicholine and procaine (7), the major feature in both cases being a large surge of inward current at the termination of the hyperpolarizing step (Fig. 3*B*). The slow relaxations were monoexponential. Their time constants τ_{slow} were voltage dependent and increased with DECA concentration (Fig. 3*C*). This concentration dependence is the reverse of what is found for AcCho-induced relaxations (15, 22).

Although the fast initial relaxation could not be resolved, it was clearly not instantaneous, because in most experiments tails of decreasing current immediately following hyperpolarizing steps could be detected (Fig. 3*C*). This would be expected if the time constant of the fast relaxation corresponds to that of the major, fast component of the DECA-induced noise a.c.f. Time constants derived from the slow relaxations could be used successfully to fit the slow component of the a.c.f. of DECA-induced membrane noise obtained under identical experimental conditions. Thus it can be concluded that the same two processes appear in both relaxation and noise experiments, in agreement with the fluctuation–dissipation theorem (23, 24). However, fluctuation experiments are best suited to measure the fast process, whereas relaxation experiments are preferable for measurement of the slow process. Qualitatively, the interpretation of the relaxation experiments in terms of scheme 1 is straightforward. At the holding potential the channels distribute between the three states: closed, open, and open-blocked. A sudden hyperpolarization drives DECA molecules into those channels that were open just before the step was applied, resulting in a fast decrease in conductance. A slower re-equilibration between open channels, which can be either open-blocked (X*D) or open-unblocked (X*), and closed channels

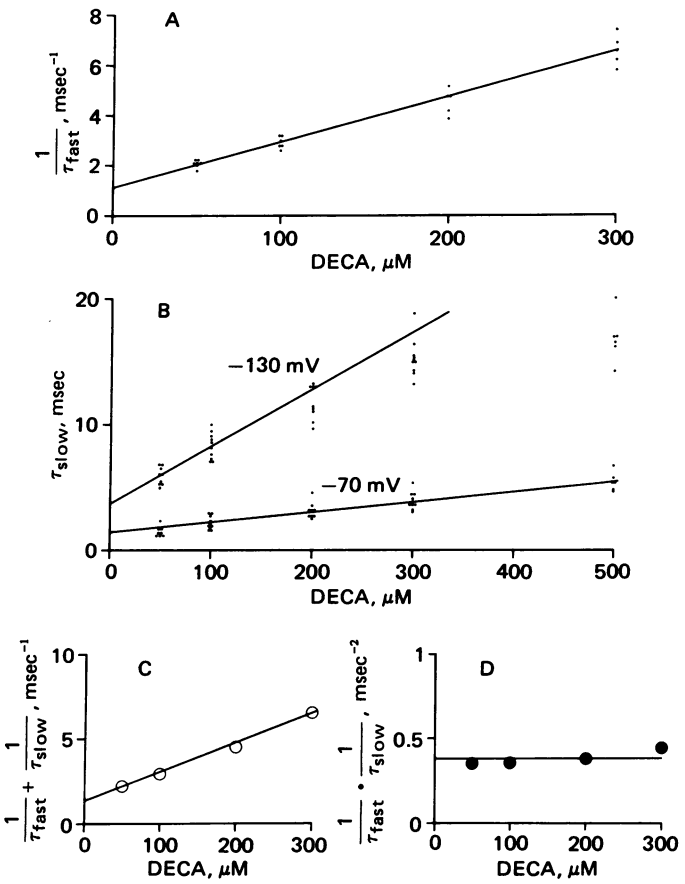

FIG. 4. Concentration dependence of fast (τ_{fast}) and slow (τ_{slow}) time constants obtained from fluctuation and relaxation analysis. In all graphs the abscissas show DECA concentration. In *A* and *B* each point at a given concentration represents a separate experiment. (*A*) Ordinate plots $1/\tau_{fast}$. The values of τ_{fast} represent the fast component of a.c.f.s calculated from DECA-induced current fluctuations at −130 mV membrane potential. The regression line has an ordinal intercept of 1.12 $msec^{-1}$ and an abscissal intercept (not shown) at −60 μM DECA. (*B*) Ordinate plots τ_{slow}. Values of τ_{slow} are obtained from relaxation experiments. The upper regression line was fitted to data points at −130 mV using only the values obtained at 50 and 100 μM. It has an ordinal intercept of 3.7 msec and an abscissal intercept (not shown) at −80 μM DECA. The lower regression line was calculated using all data points from 50 to 500 μM measured at −70 mV membrane potential. Its ordinal and abscissal intercepts are 1.3 msec and −130 μM DECA. (*C*) Ordinate plots sum of reciprocal fast and slow time constants (*S*). The mean values of τ_{fast} and τ_{slow} of the experiments shown in *A* and *B* were used. The regression line drawn has an ordinal intercept of $(\alpha + b)$ = 1.4 $msec^{-1}$ and a slope (f) of 1.7×10^7 M^{-1} sec^{-1}. (*D*) Ordinate plots products of fast and slow time constants (*P*). A line parallel to the abscissa was fitted using least squares. It has an ordinal intercept of (αb) = 0.38 $msec^{-2}$. The reaction rates α and b were calculated by solving the two simultaneous equations in $(\alpha + b)$ and (αb).

then supervenes; only open–unblocked channels can close. This process is retarded because the closing rate α is multiplied by the steady-state probability p that an open channel is unblocked.

Rate Constants of DECA-Induced Receptor Activation and Channel Blocking. At −130 mV both the slow and fast time constants could be measured accurately over a considerable range of DECA concentrations (50–300 μM), by combining the noise and voltage jump data (Fig. 4 *A* and *B*). Preliminary analysis showed that both $1/\tau_{fast}$ and τ_{slow} showed roughly linear variation with DECA concentration. This is the result expected if β in scheme 1 is very small and the blocking reaction occurs very much faster than the open–close transition (7, 11). However, comparison of the ordinal intercepts, which should yield b and $1/\alpha$, shows that the assumption of very different kinetics for blocking and opening steps is not exact. The fast and slow time constants thus are not simply related to the blocking step and to the open–close transition because of coupling between both reactions. Therefore, the data for DECA concentrations from 50 to 300 μM were analyzed, assuming only that β was negligible, by using the procedure suggested by Eigen and de Maeyer (25). According to scheme 1 the sums (S) and products (P) of the reciprocal fast and slow time constants are given by $S = d{\cdot}f + \alpha + b$ and $P = \alpha b$. The data showed the expected linear variation of S (Fig. 4*C*) and lack of significant variation of P with d (Fig. 4*D*). The slight upturn in the P plot may represent a minor contribution of β. From the slopes and intercepts in the two plots the following values for the kinetic constants were obtained: α = 0.36 $msec^{-1}$; b = 1.01 $msec^{-1}$; f = 1.71×10^7 M^{-1} sec^{-1}. These blocking rate constants are similar to those for procaine and QX-222 (7, 11). The apparent dissociation constant for binding of DECA to open channels is thus 59 μM at −130 mV.

Insufficient measurements of the fast time constant were available at −70 mV to allow this approach to be used. Therefore, b/f and α at −70 mV were obtained from the abscissal and ordinal intercepts in the plot τ_{slow} versus d, which showed excellent linearity (Fig. 4*B*). The values obtained were b/f = 147 μM and α = 0.81 $msec^{-1}$. If it is assumed that both b/f and α vary exponentially with membrane potential (2, 7, 11), it can be calculated that the blocking equilibrium constant decreases e-fold for 66-mV hyperpolarization, and the channel closing rate constant decreases e-fold for 75-mV hyperpolarization. Both these voltage sensitivities agree approximately with previous estimates for conventional local anesthetics (7, 11) and full agonists (2, 13–15).

We have no direct evidence that β is indeed negligible for DECA concentrations below 300 μM. However, this seems plausible for three separate reasons. (*i*) For the three pure agonists we have examined, AcCho, carbachol, and suberyldicholine, the relation $(\beta + \alpha)$ versus agonist concentration shows a low concentration plateau in which β is negligible. (*ii*) The P and S plots show little deviation from linearity. (*iii*) DECA concentrations below 300 μM evoke total endplate currents less than 100 nA; this is very much less than expected for opening of most of the channels (2) and shows either that β is negligible or that these DECA concentrations are already much in excess of the dissociation constant for binding to open channels. This latter possibility can be excluded by comparison with the determined value of b/f, which, even if β were non-negligible, should be roughly correct. It seems likely, though, that the curving down in the plot of τ_{slow} versus d at −130 mV is due to a significant contribution of β at concentrations above 300 μM.

DISCUSSION

The above data show that DECA can both open and block endplate channels. Taken separately, the agonist and anesthetic actions may appear quite ordinary. However, in the light of the present results the lifetime originally suggested for DECA-induced channels (4, 21) can be revised. The mean channel lifetime $\tau_{DECA} = 1/\alpha$ lies between that of AcCho- and carbachol-induced channels. The voltage dependence calculated for α is found to be similar to that reported for other (full) agonists. The channel-blocking action shows only minor, quantitative, differences from pure local anesthetics. The voltage sensitivity of the block is not greater than that seen with procaine (7) or

QX-222 (11). This suggests that only one of the cationic heads of DECA enters the channel, in agreement with the expectations of an elementary electrostatic mechanism (26). It is unlikely that the other head is within easy reach of the anionic binding site on the receptor, because then one would expect nearly zero-order behavior of $1/\tau_{fast}$.

At first sight our results and analyses may seem to overemphasize the importance of the actions of a rather feeble nicotinic agonist. However, our approach may have more general significance. First, kinetic analyses of any agonist action must consider the possible importance of parasitic local anesthetic activity. Indeed, Cohen *et al.* (27) have already suggested from biochemical evidence that dansylcholine (28) may have combined local anesthetic and agonist properties.

Second, the molecular basis proposed here for the weak (partial) action of DECA may apply equally well to other partial agonists. It cannot, of course, be expected that the blocking kinetics would, in each case, fall into a favorable time range, so that individual verification may prove difficult and in certain cases partial agonism could reflect ineffective channel opening. Furthermore, it seems possible that even full agonists have a local anesthetic action when applied at high enough concentrations. In preliminary experiments we found that in the presence of high (>5 μM) concentrations of suberyldicholine the equilibrium current–voltage curve has a sigmoid shape similar to that found for DECA (6), and following a hyperpolarizing voltage jump a *slow* decrease in conductance is observed after the initial fast conductance increase.

Third, although effects that resemble channel blocking seem to be very widespread, and in certain cases are most simply explained by this concrete molecular theory, it has been stressed (7, 11, 29) that so far there is no conclusive evidence against a type of allosteric action, involving stabilization of a hypothetical, third, nonconducting receptor state. In the case of DECA, however, a direct interaction with the channel seems to be proven on quite different grounds. DECA penetrates the endplate membrane rather well, and the conditions required for this penetration are exactly those needed to open the endplate channels (30). Furthermore, this DECA uptake saturates with concentrations that according to our data would block about half of the open channels in depolarized muscles (30). This evidence indicates that DECA can enter and traverse the channel, at concentrations comparable to those needed for the blocking action. It seems probable that channel permeation and channel blockage are related facets of the same phenomenon (31). The present study thus would constitute a time-resolved description of the initial step in the permeation of an organic cation through a biological channel.

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