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A COMPARISON OF THE TIME COURSE OF EXCITATION AND INHIBITION BY IONTOPHORETIC DECAMETHONIUM IN FROG ENDPLATE

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1 The depolarization of frog endplate by a brief iontophoretic application of decamethonium was slower in time course than the inhibition of a long carbachol response produced by the same decamethonium pulse, or than the excitation produced by a brief equipotent carbachol pulse.

2 The delay between the peak inhibition and peak excitation produced by decamethonium, about 50 ms, is too great to be explained by slow receptor activation kinetics, since Katz & Miledi (1973) have shown that the lifetime of decamethonium-activated receptors is only 0.25 milliseconds.

3 If doses of carbachol and decamethonium are adjusted to give response amplitudes in the ratio corresponding to the ratio of their presumed maximum responses, then there is little difference in the time courses of the responses.

4 This observation, together with the finding that increasing the dose applied slows the decamethonium response much more than the carbachol response, suggests that a decamethonium response contains contributions from a much wider area of receptive membrane than does a carbachol response of equal amplitude.

5 Simulation shows that these geometrical effects are sufficient to account for the rapidity of inhibition compared to excitation without postulating slow receptor kinetics.

6 It is pointed out that similar effects may account for certain results obtained in iontophoretic studies of desensitization.

Introduction

In 1957 del Castillo & Katz (1957a, b) suggested that agonists activate receptors in a two-stage process: $A + R \rightleftharpoons AR \rightleftharpoons AR^*$, where AR is an inactive intermediate complex, which then isomerises to give the active complex AR*. Their main evidence for this was that certain agonists would inhibit the action of others which they took to indicate that the agonist could combine with the receptor without activating it. Perhaps the strongest evidence for a sequential mechanism was that depolarizations produced by iontophoretic decamethonium pulses were much slower than those produced by carbachol, and that this difference seemed too great to be explained by diffusion alone. Furthermore, an effective inhibition of acetylcholine existed very early in the decamethonium response, suggesting that the decamethonium had combined with receptors, but only subsequently activated them.

The present work was undertaken initially to check, extend and quantitate these effects. However it then became clear that although the phenomenon certainly existed, its explanation was to be sought in terms of diffusional delays and the geometrical complexities of the iontophoretic method, rather than a kinetic receptor model. An abstract describing this work has already appeared (Adams, 1974).

Methods

Recordings from superficial endplates of sartorius muscles of *Rana temporaria* or *R. pipiens* were made with conventional intracellular techniques. The experiments were performed at room temperature (18–22°C) using a Ringer of the following composition (mM): Na 113.5, K 1.9, Cl 114.1, HCO_3 2.4, H_2PO_4 0.064 and Ca 1.2. Two or three barrelled pipettes for iontophoresis contained decamethonium iodide (Koch-Light), carbachol chloride (Koch-Light) and 0.9% w/v NaCl solution (saline). The retaining currents on both drug barrels were initially set to high values. When a sensitive endplate region was located, the carbachol retaining current was then reduced until small pulses gave carbachol responses which showed no signs of desensitization. Finally the decamethonium barrel retaining current was reduced almost to the

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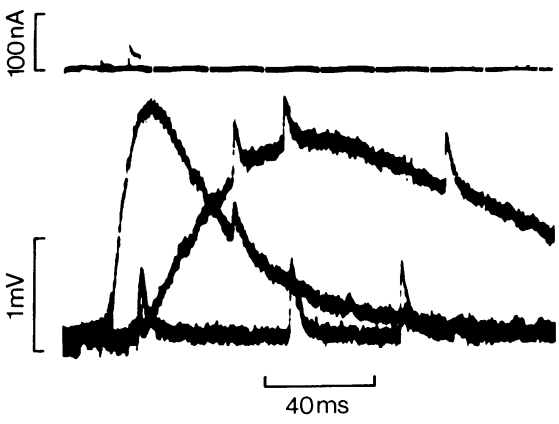

Figure 1 Comparison of time courses of depolarization by brief carbachol or decamethonium pulses. Upper beam: pipette current. Lower beam: membrane potential. Three records were superimposed: a response to carbachol (fast depolarization), a response to decamethonium (slow depolarization) and a base line. Miniature endplate potentials are present on all records. Carbachol and decamethonium were applied from separate barrels of a twin pipette.

point where a modification of the carbachol response became detectable. In order to obtain responses to long (0.5–1 s) pulses of carbachol which superimposed exactly on successive trials (see Figures 2 and 4 and also Adams, 1974) the repetition rate had to be very slow (<0.03 Hz). The arrangements for voltage-clamp have already been described (Adams, 1975a). The iontophoretic currents were monitored in all experiments although they are not always shown in the records.

Results

Inhibition precedes excitation

Figure 1 shows an experiment which simply reproduces the original observation of del Castillo & Katz (1957a), that depolarizations produced by a short pulse of decamethonium from one barrel of a pipette are considerably slower than depolarizations produced by a pulse of carbachol from another barrel. In these experiments the pipette could always be manipulated to give carbachol responses with times to peak <20 milliseconds. However in these positions decamethonium responses of similar amplitude had times to peak of ~80 milliseconds. It was also noted, in agreement with del Castillo & Katz, that even very large decamethonium pulses would only give small (<10 mV) depolarizations, while there seemed to be no such limit on the size of carbachol responses. This agrees with the quantitative observations on the dose-response curves (Adams, 1975b).

Attempts were made to measure the inhibition existing at various times in the decamethonium response by eliciting carbachol responses at various times. However this technique proved unsatisfactory since it requires carbachol responses which are very short compared to the rise-time of the decamethonium response, and though the carbachol responses alone met this requirement, in the presence of decamethonium they became very rounded.

Therefore the following method was adopted. Having positioned the pipette tip close to the receptors (as judged by carbachol responses with <20 ms rise-times), a short pulse was then applied to the decamethonium barrel and the resulting slow depolarization recorded. A long (500 ms–1 s) pulse was then applied to the carbachol barrel. Because of the nature of diffusion from a point source (Waud, 1967) the resulting depolarization rose very slowly, and did not always reach a plateau before the end of the expelling pulse. However it was judged that even longer pulses increased the likelihood of desensitization, which would affect the closest receptors most, slow up the responses, and mask the effect being sought. Finally, the long carbachol pulse was reapplied, and the decamethonium pulse also applied toward the end of the carbachol response. Provided that the response to decamethonium alone was rapid, a rapid inhibition of carbachol depolarization occurred, which sometimes passed over into an excitation. This method, and the results obtained, are shown in Figure 2 (see also Adams, 1974).

'Inhibition' and 'excitation' are taken to refer to responses to decamethonium plus carbachol relative to the original carbachol trace. It was usually quite clear that the decamethonium inhibition peaked earlier than the original decamethonium depolarization. In 12 experiments the time to peak of the control decamethonium depolarization was 88 ± 9.6 ms (mean ± s.e.) and the time to peak of the inhibition was 38 ± 4.5 ms (the times being measured in both cases from the start of the decamethonium pulse). The ratio of the times to peak for excitation and inhibition was 2.4 ± 0.3.

Is the delay artefactual?

The simplest artefact liable to occur in this type of experiment is interbarrel coupling, leading to iontophoresis of one compound when another was intended. Although a spacer was not used, two observations indicated that this effect was not important in the present experiments. First, in some experiments a triple barrelled pipette was used, one barrel being filled with saline. Pulses to this barrel did not expel carbachol or decamethonium from the other barrels. Second, the depolarization produced by a

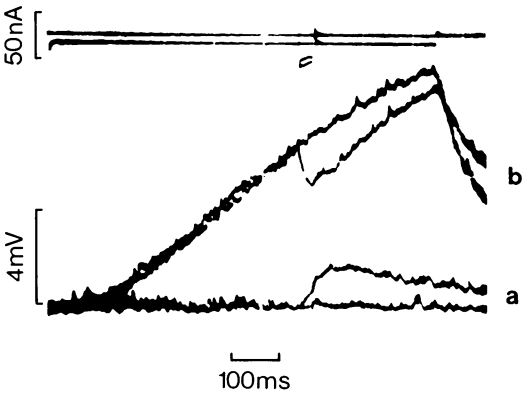

Figure 2 Comparison of time courses of inhibition and excitation by iontophoretic decamethonium. The traces marked (a) show a base line and a depolarization produced by a decamethonium pulse (current monitored in top beam). The traces marked (b) show 2 successive depolarizations produced by a long carbachol application, during one of which the decamethonium pulse was also applied. Note that the carbachol + decamethonium trace falls more slowly than the carbachol alone trace at the end of the pulse.

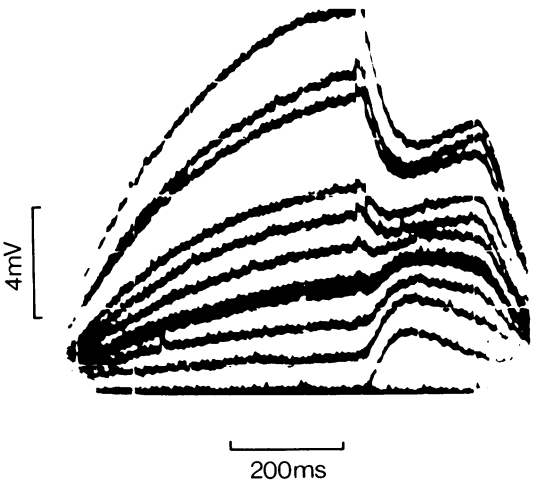

Figure 3 Decamethonium responses during varying carbachol backgrounds. The records show a base line, a response to decamethonium alone, and then decamethonium responses to the same dose during increasing carbachol backgrounds. Note the short upward deflections preceding the decamethonium inhibitions. The base line appears half wave rectified because of occlusion by the oscilloscope mask.

pulse to the decamethonium barrel was reduced or eliminated by increasing the backing voltage applied to the decamethonium barrel, but was unaltered by increasing the backing voltage applied to the carbachol barrel, or *vice versa*. Thus it seemed unlikely that the effects ascribed to one drug were due to the other.

A second possible problem was mentioned above, that the long carbachol application might desensitize the receptors immediately under the pipette tip. However this effect would tend to slow the decamethonium responses (whether excitatory or inhibitory) and could not explain the above results. Some evidence that this process could occur was obtained when comparing the decamethonium inhibitions at different strengths of carbachol application (Figure 3), since the inhibitions were slowed by increasing the carbachol background. Another possible explanation for this is considered below.

A third artefact was the occasional unexpected appearance of triphasic decamethonium responses during background carbachol. This effect is visible in Figure 3, where it will be noted that during intense carbachol action the decamethonium inhibition is preceded by a depolarization. On close examination it became apparent that this early 'excitation' coincided with the decamethonium expelling pulse itself and had the form of a very short electrotonic potential. However, it was quite clear that the electrode was not 'semi-intracellular' (del Castillo & Katz, 1955) since even a large pulse to the decamethonium barrel alone produced no such effect. This initial depolarizing response showed the following additional features. Its amplitude was proportional to the intensity of carbachol action. It was still present when the decamethonium barrel backing voltage was increased sufficiently to prevent completely subsequent decamethonium responses. It could also be seen when a pulse to the saline barrel was applied during intense carbachol action. It was concluded the local carbachol action lowered the resistance of the membrane under the pipette tip to allow access of the decamethonium expelling current to the interior of the fibre, producing a reversible 'pharmacological' impalement.

A fourth artefact was anticipated that might theoretically account for the main result noted above. This was that the membrane resistance might be sufficiently lowered by carbachol action to reduce significantly the fibre time constant. This would tend to speed up responses to concurrently applied decamethonium (whether excitatory or inhibitory). However in voltage clamp experiments the inhibition still clearly preceded excitation (Figure 4). In 2 experiments the mean times to peak of inhibition and excitation were 12 and 26 ms respectively, so although this factor might operate to some extent in the potential recording experiments, at least part of the delay remains to be accounted for.

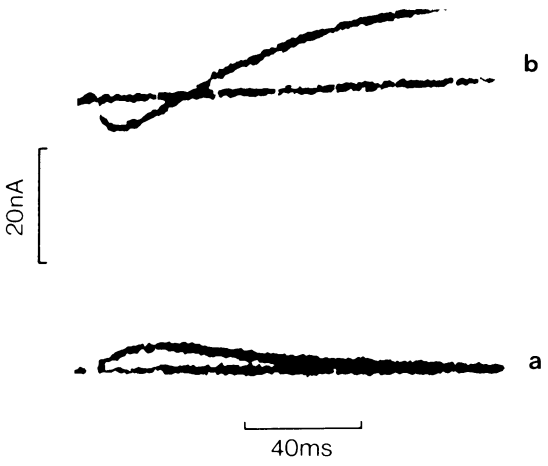

Figure 4 Decamethonium inhibition and excitation under voltage clamp. The clamp current was recorded separately from the iontophoretic current across a resistor in the feedback circuit. Decamethonium alone (a) produced a monophasic response, whereas superimposed on carbachol (b) it is diphasic. (For further explanations see Figure 2).

Considerations mitigating against a 'kinetic' interpretation

One can idealise the present experiments in terms of the 2 step model of receptor activation outlined in the Introduction. Suppose that a very short pulse of decamethonium is applied to the receptors, which then binds very rapidly, so that immediately after the pulse the intermediate AR concentration is raised instantaneously to a finite value, while both [AR*] and free [A] are negligible. This situation can be represented

$$A + R \xleftarrow{k} AR \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} AR^*$$

(This situation will not occur during the pulse, but it can be taken to obtain from the peak of the inhibition, which is taken as $t=0$).

An expression for [AR*] as a function of t is obtained by solving the equations in $d[AR]/dt$ and $d[AR^*]/dt$. The time at which [AR*] reaches a peak, t_{max}, is obtained by setting the derivative of this expression equal to zero and solving for t. One obtains $t_{max} = 1/(r_1-r_2) \ln (r_2/r_1)$, where r_1 and r_2 refer to the positive and negative roots respectively of the equation

$$r_1, r_2 = [-(\beta + k + \alpha) \pm \sqrt{(\beta + k + \alpha)^2 - 4k\alpha}]/2.$$

Since [AR] is maximal at $t=0$ and [AR*] at $t = t_{max}$, t_{max} gives the delay between peak inhibition and excitation. Since the maximum decamethonium response is small $\beta \ll \alpha$. Also, Katz & Miledi (1973) have shown that for decamethonium $\alpha \sim 0.25$ ms^{-1}. Clearly t_{max} will not at all reflect the numerical value of $1/\beta$.

These considerations suggest that an alternative explanation of the observed delay should be sought. The simplest assumption would be that true equilibrium exists throughout the responses, and that the form of the responses is determined by access factors.

An equilibrium interpretation

Since the efficacy of decamethonium is much less than that of carbachol, to obtain decamethonium and carbachol responses of equal amplitude many more receptors must combine with decamethonium than need to combine with carbachol. The main way in which additional receptors are recruited by increasing the 'dose' is probably not by increasing the local concentration of decamethonium around a fixed area, but by spreading the area of receptors activated. If the amplitude of responses to decamethonium and carbachol are adjusted to be in the ratio of their extrapolated maximum responses in bath application experiments (Adams, 1975b), then the responses differ very little in their time courses (Figure 5a). Any residual difference can probably be explained by a slower diffusion of decamethonium. Whereas increasing doses of carbachol evoke responses of approximately similar time-course, increasing doses of decamethonium produce progressively slower responses, as though successively wider areas of receptors were being activated (Figures 5 & 6).

So one can suggest that in the interaction experiments described above the decamethonium excitation would be slow, because the receptors involved are distributed over a wide area, whereas the inhibition of carbachol is fast, since carbachol action is confined to the receptors immediately under the pipette tip, and decamethonium can only inhibit carbachol where carbachol is present.

To test this idea a simple computer simulation of the postulated situation was set up. The receptors were considered as an infinite uniform plane located a vertical distance λ under the pipette tip. The receptor plane was divided into a series of concentric annuli, the nth annulus having a radius of n microns. Decamethonium and carbachol concentrations, normalized with respect to their respective receptor binding constants, were assumed for the central annulus (D_o and A_o). The average decamethonium concentration at the nth annulus was calculated assuming that the expelling pulse was very short compared to the responses. The appropriate diffusion equation (del Castillo & Katz, 1955) is then

$$D_r = \frac{Q}{8(\pi kt)^{1.5}} \exp(-r^2/4kt)$$

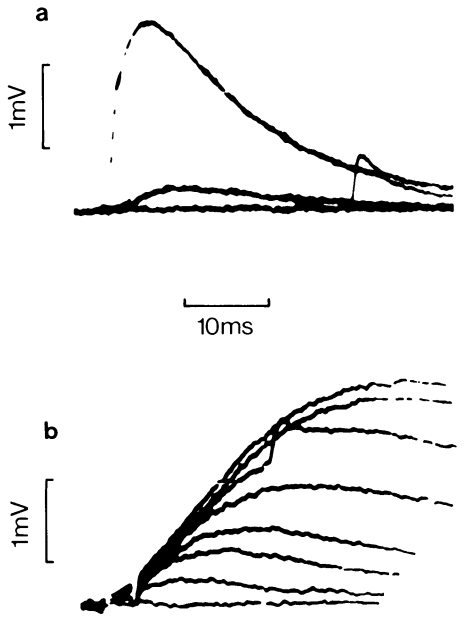

Figure 5 (a) Comparison of time courses of depolarizations produced by carbachol or decamethonium whose amplitudes were in the ratio 7 to 1. One carbachol and two decamethonium responses (during one of which a miniature endplate potential occurred) superimposed on a base line. (b) Effects of increasing decamethonium dose at the junctional spot shown in (a).

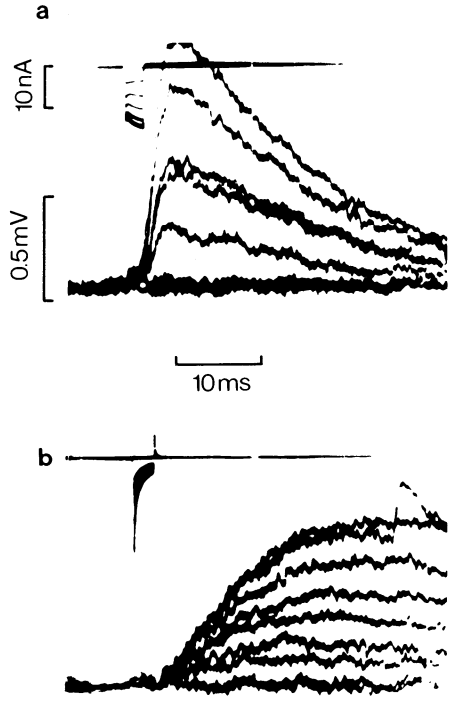

Figure 6 Comparison of effect of increasing carbachol (a) or decamethonium (b) doses. Note that the rise time of the miniature endplate potential occurring in (b) is only slightly faster than the smallest carbachol response shown in (a).

where k is the diffusion coefficient of decamethonium, Q is the quantity expelled, D_r is the concentration at a distance r from the point source, and t is time. This can be reworked to give the concentration at the nth annulus in terms of the peak concentration at the *zero*th annulus, $D_{o,max}$

$$D_n = \frac{\lambda^3 \exp(-X_n^2/4kt) \exp 1.5}{(6kt)^{1.5}} D_{o,max}$$

where X_n is the average distance of the nth annulus from the pipette and λ is the vertical distance of the source from the receptive plane. An approximate expression for the carbachol concentration at the nth annulus, A_n, was obtained assuming that the expelling pulse was sufficiently long for a steady state to be achieved. Then

$$A_n = \left(\frac{\lambda}{X_n}\right)^2 A_o$$

This simplifies the calculation although even for very long pulses a true steady state may not be achieved (Waud, 1967). Y_n, the fraction of the receptors at the nth annulus activated by the combination $A_n + D_n$, was obtained assuming

$$Y_n = \left[\frac{A_n + D_n/\sqrt{Z}}{A_n + D_n + 1}\right]^2$$

where Z is the ratio of the maximum responses to carbachol and decamethonium (Adams, 1975c). The relative response of the nth annulus, R_n, was then calculated using the relation $R_n = \pi(2n-1)(Y_n)$.

The programme caused n to be incremented from zero, and corresponding values of R_n to be calculated and summed to give a total response R_s, until adding a further value of R_n caused R_s to change by less than some criterion (usually 0.01 or 0.05), when the calculation terminated and the final value of R_s was presented. This process was then repeated for other suitable values of t. Figure 7 shows a typical set of calculations for a fixed value of $D_{o,max}$ and increasing values of A_o (corresponding to the experiment shown in Figure 3). The model predicts quite successfully the slow excitation, the initial early inhibitions, and the rightward shift of the inhibition peak with increasing carbachol applications. The main defect is that the tails of the decamethonium responses tend to be rather

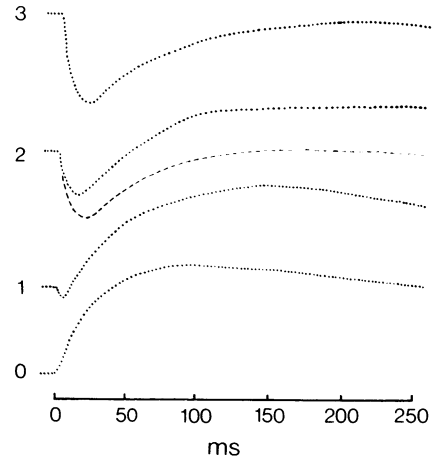

Figure 7 Simulation of interaction of iontophoretic carbachol and decamethonium. A double barrelled pipette a vertical distance λ above the receptive plane delivers a short pulse of decamethonium and a long pulse of carbachol. The total endplate response is computed as the sum of the responses of concentric annuli of inner radius n μm. The curves show responses to a fixed dose of decamethonium in the presence of increasing background doses of carbachol. The ordinate is the relative response (arbitrary units). The following parameter values were inserted in the text equations. $D_{o,max} = 2$; $A_o = 0, 1, 2, 3$; $\lambda = 5$ μm; $k = 0.6$ μm^2 ms^{-1}; $Z = 10$; criterion = 0.05. The dashed curve shows the effect of slightly increasing Z (to 13.5). See text for further explanations.

prolonged, but this is probably merely due to the (in retrospect) rather poor assumption of infinite plane geometry.

Discussion

It seems quite clear that the experimental observations can be explained without postulating special kinetic behaviour merely by differences in the spatial distribution of carbachol and decamethonium, arising mainly from the greater relative efficacy of carbachol. In principle it might be possible to repeat the experiments using bath application of carbachol to avoid the problem of spatial inhomogeneity, but probably any delay due to slow receptor activation would be undetectably small in any case. An alternative would be to use a very small isolated patch of receptors, perhaps by blocking off all the surrounding receptors by bungarotoxin treatment. It certainly seems plausible that some of the rate constants for conformational change of the receptor are slow compared to the potential rapidity of iontophoresis. But unfortunately most experiments will tend to yield overall rate constants in which the contribution of 'slow' steps is masked by that of 'fast' steps.

Considerable caution must be exercised in placing kinetic interpretations on the results of iontophoretic experiments (see for example, Steinbach, 1971). As a further example of the possible distortions that complex geometry may introduce, it is interesting to consider the desensitization experiments of Katz & Thesleff (1957), which share with the present experiments the feature of superimposing short test pulses on long conditioning pulses. Katz & Thesleff found, rather unexpectedly, that the rate of desensitization by acetylcholine or carbachol first decreased with increasing agonist concentration and then reached a minimum and started to increase. In bath application experiments only increasing desensitization rate with increasing agonist concentrations is seen (Rang & Ritter, 1970; Adams, 1975a). In the iontophoretic experiments the desensitization rate was estimated as the rate constant for decline in amplitude of repetitive agonist responses superimposed on a prolonged conditioning application. The test agonist pulses will produce responses which contain components from receptors located at various distances, the more distant receptors contributing slower, but in the absence of a conditioning dose, smaller responses. The conditioning dose produces 2 effects: (a) desensitization of receptors, the more distant receptors desensitizing more slowly; (b) modification of the responses of undesensitized receptors to the test dose. This second effect, arising from the sigmoidicity of the dose-response curve (Katz & Thesleff, 1957; Adams, 1975b) consists qualitatively of a reduction of the contribution of near receptors (because these receptors are activated by agonist concentrations which are near the top of the dose-response curve) and an increase in the contribution of distant receptors (since here the agonist concentration is near the bottom of the dose-response curve). The validity of this analysis is confirmed by the finding that the response to a test dose is slowed by the conditioning dose and that this effect, unlike the desensitization, appears immediately on applying the conditioning dose (Katz & Thesleff, 1957; Adams, unpublished observations). Combining (a) and (b), one can see that while increasing the conditioning dose will increase the rate of desensitization at any fixed site, there will be an additional tendency for the apparent overall rate to decrease, because increasing the conditioning dose increases the relative importance of contributions to the test response from more distant receptors which are desensitizing at slower rates. It is difficult to predict which of these effects might predominate for any given range of conditioning doses. However this discussion shows that the decrease in the desensitization rate

observed by Katz & Thesleff might be due to the geometrical complexities of the iontophoretic situation rather than to unusual features of desensitization kinetics. Other differences between iontophoresis and bath application desensitization findings, for example the different relationships between the rate of desensitization and the final desensitization achieved (compare Katz & Thesleff, 1957, Figure 2 and Adams, 1975a, Figure 4) can be explained in the same way.

Note added in proof. The simulations shown in Figure 7 have been repeated assuming a finite line receptor distribution and a linear decrease of agonist concentration with distance in the steady state. Under these conditions qualitatively identical results were obtained.

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