

EFFECT OF IONOPHORE X-537A ON DESENSITIZATION RATE AND TENSION DEVELOP- MENT IN POTASSIUM-DEPOLARIZED MUSCLE FIBRES

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1 The effects of the ionophore X-537A were studied on carbamylcholine (carbachol)-induced desensitization and on tension development in relaxed potassium-depolarized frog sartorius muscles.

2 X-537A accelerated carbachol-induced desensitization in Ca^{2+} -deficient solutions without having any effect on the conductance of the membrane in the absence of carbachol or on the extent of the carbachol-induced increase in conductance.

3 In Ca^{2+} -deficient solution, the acceleration of desensitization by the ionophore was concentration-dependent. No effect was observed with concentrations less than $5 \mu\text{M}$ and maximal acceleration was evident with $10 \mu\text{M}$.

4 The influence of X-537A on desensitization was time-dependent. At $20 \mu\text{M}$ X-537A, there was a marked acceleration of desensitization by the end of 5 min exposure. An additional gradual acceleration occurred during a 5 to 30 min treatment. No acceleration of desensitization was evident when X-537A was simultaneously applied with carbachol to the end-plate region without prior exposure to the ionophore.

5 Desensitization also was accelerated by 30 min exposure to $20 \mu\text{M}$ X-537A in solutions containing Ca^{2+} or deficient in both Mg^{2+} and Ca^{2+} ; the rate being increased 2.8-fold in Ca^{2+} -containing solutions, 2.9-fold in Ca^{2+} -deficient solutions containing Mg^{2+} , and 2.5-fold in divalent cation-deficient solutions.

6 Tension development gradually occurred in relaxed potassium-depolarized muscle preparations exposed to $20 \mu\text{M}$ X-537A. The onset of tension development occurred only after approximately 25 min of exposure both in preparations kept in Ca^{2+} -deficient or Ca^{2+} -containing solutions. By the end of 90 min in the ionophore, the tension developed was approximately 12% and 23% of the initial potassium contracture in those preparations maintained in the Ca^{2+} -deficient or Ca^{2+} -containing solutions, respectively.

7 We assume that the increase in desensitization rate following exposure to X-537A results from an elevation of the intracellular Ca^{2+} concentration. That muscle tension gradually increased during exposure to the ionophore supports this conclusion. The acceleration of desensitization by X-537A in the absence of external Ca^{2+} supports the view that the site of calcium acceleration is not on the external surface of the end-plate membrane either at or near the agonist-recognition site but rather on the inner surface.

Introduction

Application of carbachol activates end-plate receptors at the neuromuscular junction of skeletal muscle fibres producing a rapid increase in ionic conductance of the end-plate membrane. In the continued presence of agonist, the conductance gradually returns toward the pre-activation state as 'desensitization' occurs (Thesleff, 1955). Manthey (1966) has shown that raising the external calcium concentration accelerates

the rate of carbamylcholine (carbachol)-induced desensitization. Although the site of calcium action in desensitization is not established, it has been suggested recently that this site is located on the internal surface of the postjunctional membrane and therefore is distinct from the agonist-recognition site on the external surface of this membrane (Nastuk & Parsons, 1970; Cochrane & Parsons, 1972; Parsons, Schnitzler & Cochrane, 1974). This view would be greatly strengthened by the demonstration that the rate of desensitization was accelerated by raising the level of

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intracellular ionized calcium in a muscle preparation maintained in a calcium-deficient solution.

The most direct approach is to inject Ca^{2+} by electrically-controlled iontophoresis as has been done by Miledi (1973) and Kanno, Cochrane & Douglas (1973) to demonstrate that elevation of internal calcium initiates secretion. Our attempts to use this approach have been unsuccessful. Consequently, our desensitization experiments have concentrated on the influence of agents which increase the internal level of ionized calcium. In our initial studies, described in this paper, we used the ionophore, X-537A, which is thought to transport Ca^{2+} across the cell membrane as well as to mobilize cell Ca^{2+} from sarcoplasmic reticulum and mitochondria (Entman, Gillette, Wallick, Pressman & Schwartz, 1972; Scarpa & Inesi, 1972; Scarpa, Baldassare & Inesi, 1972; Pressman, 1972, 1973; Levy, Cohen & Inesi, 1973; Lin & Kun, 1973).

To test the hypothesis that the site of Ca^{2+} action in desensitization is located on the inner surface of the endplate membrane we have used the ability of X-537A to raise intracellular Ca^{2+} by mobilizing cell calcium even in the absence of external calcium. In addition, we have examined the influence of elevating internal Ca^{2+} by X-537A on development of tension in relaxed potassium-depolarized muscle preparations.

A preliminary account of some of these results has been published (Schnitzler, DeBassio & Parsons, 1975).

Methods

General methods

All experiments were performed *in vitro* on potassium-depolarized sartorius muscle preparations of the frog (*Rana pipiens*) at room temperature (18–20°C) during the period of November to May. The potassium-depolarized preparation was used because it eliminates the change in membrane potential and contraction associated with end-plate activation in polarized fibres. In addition, X-537A rapidly depolarizes muscle fibres maintained in normal

sodium Ringer solution presumably because this agent transports monovalent as well as divalent cations (Pressman, 1973; Devore & Nastuk, 1975; Schnitzler & Parsons, unpublished observations). As desensitization rate is influenced both by membrane potential (Magazanik & Vyskocil, 1970) and by intracellular sodium (Manthey, 1966; Parsons *et al.*, 1974), other experimental conditions used previously to minimize muscle movement such as hypertonic Na-sucrose solutions were considered inappropriate for these studies.

Most of the experiments were done with muscles kept in a Ca^{2+} -deficient, isotonic potassium propionate solution (Solution C). A few other experiments were done in an isotonic potassium solution containing Ca^{2+} (Solution B) or in a solution devoid of both Ca^{2+} and Mg^{2+} (Solution D). The composition of these test solutions is summarized in Table 1. In the Ca^{2+} -deficient solution (Solution C) and in the divalent cation-deficient solution (Solution D), 1 mM ethylene-(oxyethylenenitrilo)-tetraacetic acid (EGTA) was added to facilitate the removal of residual calcium. Dimethylsulphoxide (DMSO), the solvent for the ionophore was included in all the isotonic potassium solutions at a final concentration of 0.5%. In all experiments the duration of DMSO exposure was kept at 30 min regardless of the ionophore exposure time. In preliminary experiments, we determined that 0.5% DMSO did not influence the extent of carbachol-induced activation or the rate of desensitization. The ionophore, X-537A, (Hoffman-La Roche Inc., Nutley, N.J.) was prepared as a stock solution in DMSO, diluted to a desired concentration, and introduced by adding it to the solution bathing the muscle and by microperfusing it along with carbachol at the junctional region of individual fibres.

Carbachol (1 mM) (Sigma Chemical Co., St. Louis, Mo.), an analogue of acetylcholine which is resistant to hydrolysis by acetylcholinesterase, was used to activate the postjunctional membrane receptors. The carbachol was microperfused from a 50–100 μm diameter pipette onto the end-plate region of an individual muscle fibre by hydrostatic pressure (Manthey, 1966; Johnson & Parsons, 1972). The junctional region of individual muscle fibres was

Table 1 Composition of Ringer solutions

Solution*	NaCl (mM)	KCl (mM)	K propionate (mM)	CaCl ₂ (mM)	Ca propionate (mM)	MgCl ₂ (mM)	EGTA (mM)	Tris (mM)	DMSO %
A	120	2.5	—	1.8	—	—	—	1	—
B	—	—	122.5	1.3	0.5	2.0	—	1	0.5
C	—	—	122.5	—	—	2.0	1	1	0.5
D	—	—	122.5	—	—	—	1	1	0.5

* pH = 7.2–7.3

located visually by following nerve twigs to their termination under a magnification of 300 \times using a Bausch and Lomb dynoptic microscope with a Leitz long working distance objective (\sim 1.2 cm). No more than one end-plate was used per muscle.

Standard electrophysiological recording techniques were used to measure the resting membrane potential and effective membrane conductance of individual muscle fibres (Nastuk & Parsons, 1970; Manthey, 1972). The micropipettes used in this study were filled with 3 M KCl and had resistances ranging from 5–8 M Ω .

Results are reported as mean values \pm the standard error of the mean. Tests of significance between different treatment groups were made by the nonpaired Student's *t* test. Values of *P* less than 0.05 were regarded as significant.

Measurement of receptor activation and desensitization rate

The extent of end-plate activation was estimated from the increase in end-plate conductance produced by the local application of carbachol. For these experiments, the maximum acceptable rise time of activation was 4 s and minimum extent of conductance increase was 3-fold.

The time course of end-plate desensitization during sustained carbachol perfusion was estimated from the

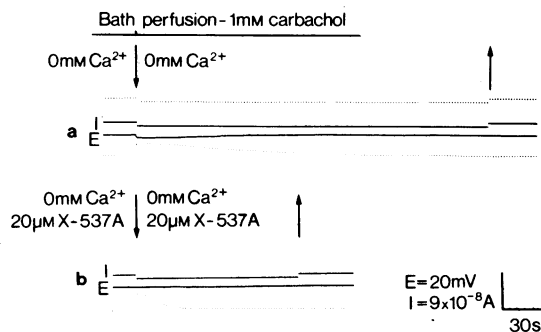


Figure 1 Effect of the ionophore X-537A on the time course of the carbachol-induced conductance change. (a) Response from a control fibre maintained in the Ca²⁺-deficient solution (Solution C) for 30 min before 1 mM carbachol perfusion. (b) Response from another fibre exposed for 30 min to Solution C plus 20 μM X-537A. In both examples, the dots above the I trace denote transient hyperpolarizing current pulses applied every other second. The dots below the E trace indicate the shift in membrane potential produced by the current pulses. The arrows indicate the start and end of carbachol application. The breaks in example (a) indicate 1 min intervals when the recording camera was turned off. The half-time of desensitization was 63 s in (a) and 18 s in (b).

rate of decline of the effective membrane conductance, after the initial increase. The half-time of conductance return was used as an index of desensitization rate (Manthey, 1966; 1972). In all experiments when desensitization was complete the conductance returned to within 0.5 μmho of its pre-carbachol value.

Measurement of tension development

Muscles were mounted vertically at rest length in a 30 ml chamber and equilibrated in Solution A for at least 30 min before exposure to either Solution B or C. Rest length was determined either *in vitro* from the length-tension relationship for indirectly elicited twitches or from a measurement of *in situ* rest length before dissection. During the initial moments of exposure to the isotonic potassium solution, muscles contracted and then relaxed (Hodgkin & Horowitz, 1960). The X-537A containing solution was added after the potassium contractions had subsided. Isometric tension development was measured by a Grass FT03 Strain gauge.

Results

Influence of X-537A on carbachol-induced desensitization

Effect in Ca²⁺-deficient solutions. When muscle preparations maintained in the Ca²⁺-deficient isotonic potassium propionate solution (Solution C) were exposed to 20 μM X-537A for 30 min, the rate of 1 mM carbachol-induced desensitization was increased. Ionophore treatment had no effect on the conductance of the end-plate membrane in the absence of carbachol nor on the extent of the carbachol-induced increase in conductance. A record which illustrates the acceleration of 1 mM carbachol-induced desensitization following treatment with 20 μM X-537A is shown in Figure 1. Example (a) is taken from a control muscle fibre equilibrated for 30 min in the Ca²⁺-deficient, isotonic potassium propionate solution, containing 2.0 mM Mg²⁺ and 1 mM EGTA (Solution C). Application of carbachol, indicated by the first arrow, produced a rapid increase in input conductance which persisted for a brief period and then returned toward the pre-perfusion level even though the carbachol application continued until the second arrow. The half-time of conductance return, from the maximum value, was 63 s in this fibre. Example (b) is taken from a muscle treated for 30 min with Solution C containing 20 μM X-537A. In this case, the rate of conductance return is much faster; the half-time value being 18 s in this fibre. Results from many experiments following a 30 min treatment with 20 μM X-537A on fibre input conductance and rate of 1 mM carbamylcholine-induced desensitization in muscles

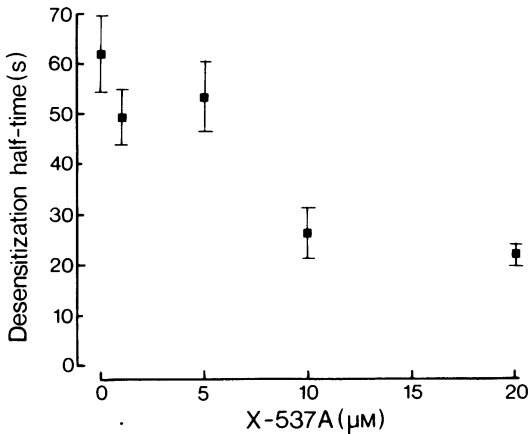


Figure 2 Influence of X-537A concentration on 1 mM carbachol desensitization half-time in muscles maintained in the Ca^{2+} -deficient solution (Solution C). The duration of ionophore exposure was 30 min for all concentrations. Each point represents mean of the half-time from at least 6 fibres. Vertical lines show s.e. mean.

maintained in the Ca^{2+} -deficient solution are summarized in Table 2.

The dose-response relationship between X-537A concentration and desensitization half-time for muscles maintained in the Ca^{2+} -deficient solution is shown in Figure 2. At all concentrations, the duration of ionophore exposure was 30 minutes. In low concentration (1 or 5 μM), the ionophore did not influence desensitization rate (Figure 2). However, in the presence of 10 μM X-537A, the rate of desensitization was significantly increased and doubling the ionophore concentration to 20 μM produced no additional acceleration of desensitization.

The time course of the acceleration of desensitization by ionophore treatment in muscles maintained in the Ca^{2+} -deficient solution is shown in Figure 3. For these experiments, the concentration of X-537A was kept at 20 μM and duration of ionophore exposure

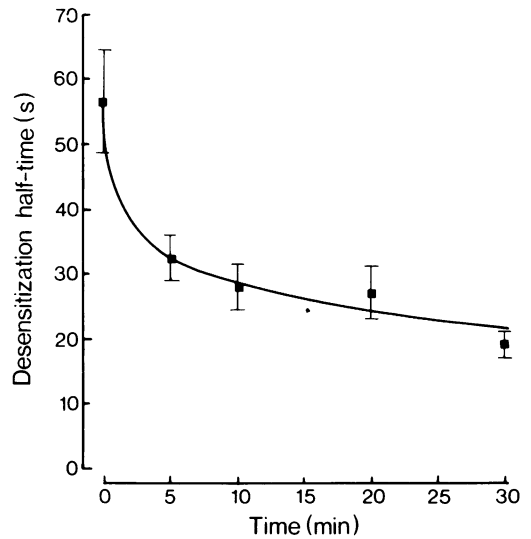


Figure 3 Time course of X-537A acceleration of 1 mM carbachol-induced desensitization. Muscles maintained in the Ca^{2+} -deficient solution (Solution C) and exposed to 20 μM X-537A for various periods of time. Exposure to 0.5% dimethylsulphoxide was 30 min in all experiments. Each point represents the mean value from at least 7 fibres. Vertical lines show s.e. mean. The curve was fitted using the equation,

$$T/2(t) = 21.6 e^{-t/1.17} + 35 e^{-t/58.1},$$

with t in minutes.

varied. In all instances, the DMSO exposure was 30 minutes. X-537A produced a marked acceleration of desensitization by the end of 5 min treatment. An additional gradual acceleration occurred during a 5 to 30 min treatment. The curve in Figure 3 is a computer fit to the data points and represents the sum of the two exponential expressions shown below,

$$T/2(t) = 21.6 e^{-t/1.17} + 35 e^{-t/58.1}$$

where t = minutes.

Table 2 Influence of 20 μM X-537A on fibre input conductance and rate of 1 mM carbachol-induced desensitization in muscles equilibrated in Ca^{2+} -free, 2 mM Mg^{2+} , 1 mM EGTA, K propionate solution

Condition	Input conductance prior to carbachol ($\text{mho} \times 10^{-6}$)	Maximum input conductance during carbachol perfusion ($\text{mho} \times 10^{-6}$)	Desensitization half-time (s)	No. Fibres
Control	$4.4 \pm 0.4^*$	17.2 ± 1.4	58.8 ± 5.5^a	16
X-537A for 30 min	4.1 ± 0.4	15.9 ± 2.1	20.5 ± 1.4^a	13

* Mean \pm s.e. mean

^a Significant $P < 0.05$

Caffeine, Ca^{2+} , and La^{3+} have been found to accelerate desensitization without equilibration prior to carbachol application (Manthey, 1970; Parsons, Johnson & Lambert, 1971; Cochrane & Parsons, 1972). In these instances, simultaneous perfusion of these agents with carbachol is sufficient to influence desensitization rate. Since most of the influence of X-537A treatment on desensitization occurs within the first 5 min, the question of an immediate effect of X-537A had to be investigated. However, the inclusion of the ionophore along with carbachol in the perfusion fluid without pre-equilibration did not influence the desensitization rate. In 10 fibres perfused with 1 mM carbachol alone, the half-time of conductance return was 56.8 ± 7.7 s whereas in 8 other fibres perfused simultaneously with 1 mM carbachol and $20 \mu\text{M}$ X-537A, the half-time of conductance return was 50.3 ± 5.5 seconds.

Effects in divalent cation deficient solution. X-537A is also an ionophore for Mg^{2+} (Pressman, 1972). Although Mg^{2+} is an ineffective substitute for Ca^{2+} in desensitization under other experimental conditions (Nastuk & Parsons, 1970; Parsons *et al.*, 1973), experiments were done to insure that the acceleration of desensitization by ionophore treatment was not related to Mg^{2+} . The influence of X-537A on desensitization was studied on muscles maintained in a divalent cation-deficient, potassium propionate solution containing 1 mM EGTA (Solution D).

Previously, Lambert & Parsons (1970) found that endplate activation by carbachol is reduced when no divalent cations are present. A reduced end-plate responsiveness to 1 mM carbachol also was observed in the present experiments regardless of whether the ionophore was present or not. A significant acceleration of desensitization was produced by 30 min treatment with $20 \mu\text{M}$ X-537A. In 8 fibres maintained in the divalent cation-deficient solution for 30 min, the half-time of 1 mM carbachol-induced desensitization was 82.3 ± 15.0 seconds. In another 8 fibres, following a 30 min exposure to $20 \mu\text{M}$ X-537A, the half-time of 1 mM carbachol-induced desensitization was 32.4 ± 4.6 seconds.

Effect in calcium containing solution. X-537A is thought to raise internal Ca^{2+} by transporting extracellular Ca^{2+} across the cell membrane as well as by mobilizing intracellular Ca^{2+} (Entman *et al.*, 1972; Scarpa & Inesi, 1972; Scarpa *et al.*, 1972; Pressman, 1972; 1973; Levy *et al.*, 1973; Lin & Kun, 1973). Exposure to X-537A in the presence of external calcium should therefore accelerate desensitization to a greater extent than when no external calcium is present. Consequently, experiments were done in which muscles were maintained for 30 min either in Solution B or Solution B containing $20 \mu\text{M}$ X-537A before the application of 1 mM carbachol. In the

presence of calcium, the rate of desensitization was faster for both the control and ionophore-treated fibres than in those respective fibres maintained in the Ca^{2+} -deficient solution (cf. Table 2). The half-time of desensitization was 44.3 ± 9.7 s for 6 fibres maintained in Solution B without ionophore and was 16.0 ± 0.8 s for 4 fibres exposed for 30 min to Solution B containing $20 \mu\text{M}$ X-537A. However, the increase in desensitization rate indicated by the ratio of control to test half-time values, was similar. The rate was increased 2.8-fold when Ca^{2+} was present (Solution B) and 2.9-fold when calcium was omitted (Solution C).

Effect of $20 \mu\text{M}$ X-537A on tension development in relaxed potassium-depolarized muscle preparations

It is generally believed that most of the pharmacological actions of X-537A are related to its ability to act as an ionophore for divalent or monovalent cations (See Cochrane, Douglas, Mouri & Nakazato, 1975). In the present study we assumed that the acceleration of desensitization was due to the ability of X-537A to increase the level of internal calcium. If this assumption is valid, then tension development should occur during X-537A treatment as the internal Ca^{2+} concentration rises. Consequently, experiments were undertaken to measure tension output from relaxed potassium-depolarized fibres exposed to $20 \mu\text{M}$ X-537A.

Both sartorii were utilized; one muscle served as a control and the other as a test preparation. All muscles initially were equilibrated for at least 30 min in a Tris-buffered sodium Ringer solution at rest length (Solution A). The muscles were then transferred to an isotonic potassium solution (either Solution B or C). The muscles contracted during the initial moments of exposure to the elevated potassium solution and then relaxed (Hodgkin & Horowitz, 1960). One muscle of the pair was maintained in the isotonic potassium propionate solution without ionophore (although 0.5% DMSO was present) for the duration of the experiment. The test muscle was transferred to the isotonic potassium propionate solution (either Solution B or C) containing $20 \mu\text{M}$ X-537A immediately after the potassium contracture had subsided. Three of these 'paired muscle' experiments were done in Solution B and Solution C, respectively. In the present study no attempt was made to quantitate these results in terms of tension development per cross sectional area or unit of muscle mass. The change in muscle tension with ionophore exposure was expressed as a percentage of the tension developed during the initial potassium contracture produced in an individual preparation.

No tension development occurred in the relaxed potassium-depolarized preparations exposed to $20 \mu\text{M}$ X-537A during an initial 25 min period. However, after 25–40 min in X-537A, muscle tension gradually

increased. The onset of tension development was similar in muscles exposed to the ionophore in either the presence or absence of external calcium (Solution B or C, respectively). The first measurable increase in tension occurred at 26, 42 and 45 min in those preparations exposed to the ionophore in Solution B and at 25, 32 and 45 min in those preparations exposed to the ionophore in Solution C. By the end of a 90 min exposure to 20 μM X-537A (a time arbitrarily chosen to terminate these experiments) the tension had increased to $23 \pm 9\%$ and $12 \pm 3\%$ of the initial potassium contracture in the Ca^{2+} -containing (Solution B) or Ca^{2+} -deficient solution (Solution C), respectively. In the absence of the ionophore, muscle tension gradually declined in those preparations kept in Solution C so that by the end of 90 min the tension decreased by approximately 4%. No change in tension was noted after the initial contracture-relaxation cycle had occurred in those 3 preparations maintained in Solution B and not exposed to the ionophore.

Discussion

The results presented here show that exposure of potassium-depolarized muscles to X-537A (1) accelerated carbachol-induced desensitization without changing the extent of end-plate activation and (2) caused tension development in relaxed, potassium-depolarized muscles. These effects occurred in the presence or absence of external calcium and, we assume, reflect a gradual increase in the level of internal ionized calcium.

It has generally been accepted that most of the effects of X-537A are related to its ability to act as a Ca^{2+} ionophore (Pressman, 1972; 1973). We anticipated a more pronounced acceleration of desensitization and a more rapid onset of tension development when calcium was present in the bathing solution. This was not observed. X-537A releases calcium from sarcoplasmic reticulum (Entman *et al.*, 1972; Pressman, 1972; Scarpa & Inesi, 1972; Scarpa *et al.*, 1972; Levy *et al.*, 1973) and from mitochondria (Lin & Kun, 1973). That the magnitude of the change in desensitization rate and onset of tension development was similar in the presence and absence of external calcium suggests that the increase in internal Ca^{2+} concentration occurred primarily from the mobilization of cell calcium rather than from calcium transport across the sarcolemma. However, that the magnitude of tension development at 90 min was greater in Ca^{2+} solutions may indicate that some additional Ca^{2+} was transported by the ionophore.

The time course of 20 μM X-537A-induced change in desensitization rate is described by the sum of two exponential expressions; the first having a fast decay and the second a much longer time constant. Scarpa *et al.* (1972) and Levy *et al.* (1973) found that 20 μM X-

537A produced an initial rapid release of Ca^{2+} from sarcoplasmic reticulum vesicles followed by a much slower phase of Ca^{2+} release. This complex time course of X-537A-induced change in desensitization half-time may be a reflection of these two phases of Ca^{2+} mobilization by the ionophore.

End-plate membrane activation by carbachol was not altered by X-537A exposure at a time when the rate of desensitization was markedly enhanced. It follows then that this amount of elevation of the internal Ca^{2+} concentration has no effect on the responsiveness of the agonist-recognition site or of any subsequent components in the permeability-activation system (cf. Lapa, Albuquerque & Daly, 1974) prior to carbachol activation.

We assumed that the increase in desensitization rate following exposure to X-537A resulted from an elevation of the intracellular Ca^{2+} concentration. That muscle tension gradually increased during exposure to the ionophore supports this conclusion. However, tension increased after a much longer delay suggesting that the change in internal Ca^{2+} concentration required to affect desensitization rate is less than that for the initiation of contraction. The concentration of Ca^{2+} in muscle at rest is $\sim 0.1 \mu\text{M}$ with tension development occurring when the Ca^{2+} concentration exceeds $\sim 1 \mu\text{M}$ (Godt, 1974). After 5 min in X-537A, the rate of desensitization was increased dramatically. With longer durations of exposure, the rate increased less markedly, suggesting the influence of the ionophore on desensitization was approaching some maximum effect by the end of 30 minutes. It appears then that when the internal Ca^{2+} concentration reaches $\sim 1 \mu\text{M}$ and tension development begins, the Ca^{2+} effect on desensitization is almost complete. A limit to the influence of calcium on desensitization rate also has been observed under other conditions (Nastuk & Parsons, 1970; Cochrane & Parsons, 1972).

It was suggested that the site of Ca^{2+} action in desensitization is on the inner surface of the end-plate membrane (Nastuk & Parsons, 1970; Cochrane & Parsons, 1972). In the experiments described here, desensitization increased during exposure to X-537A in the absence of external calcium. This effect was not related to Mg^{2+} because ionophore exposure in a divalent cation-deficient solution also increased desensitization. The acceleration of desensitization during exposure to X-537A under these conditions supports the view that the site of calcium acceleration is not on the external surface of the end-plate membrane either at or near the agonist-recognition site but rather on the inner surface.

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