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Changes in Intracellular Ca^{2+} Produced in the Mouse Diaphragm by Neuromuscular Blocking Drugs

I. KIMURA, T. KONDOH AND M. KIMURA

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

Abstract—Differences between the effects of depolarizing and competitive neuromuscular blocking drugs on Ca^{2+}-aequorin luminescences (Ca^{2+} transients) emitted during twitches were compared in indirectly-stimulated diaphragm muscles of mice. Succinylcholine enhanced intracellular Ca^{2+} transients at concentrations of 1·3–2·5 μM and inhibited them at concentrations of 5·0–50 μM, demonstrating a biphasic response as in the case of twitch tensions. However, the response to Ca^{2+} transients was two to three times more sensitive than the response to twitch tension. Decamethonium 2·4–96 μM and carbachol 5·5–109 μM produced similar results. In contrast, pancuronium and (+)-tubocurarine inhibited them in the same concentration ranges. The relation between Ca^{2+} transients and twitch tensions was hyperbolic and was computer-simulated by the Hill equation in the case of succinylcholine, decamethonium and carbachol, whereas it was represented by a single exponential equation in the case of pancuronium, (+)-tubocurarine, and submaximal nerve-stimulation voltage. Spontaneous Ca^{2+} transients, on the other hand, were generated only in response to depolarizing drugs at concentrations having a neuromuscular blocking effect. These results suggest that depolarizing and competitive neuromuscular blocking drugs affect intracellular Ca^{2+} mobilization by different routes mediated by acetylcholine receptors.

Neuromuscular blocking drugs are usually classified into two groups, i.e., depolarizing drugs such as succinylcholine (SuCh) and decamethonium (C_{10}), and competitive blocking drugs such as pancuronium and (+)-tubocurarine ((+)-TC). The properties of the competitive blocking drugs are well understood, but the mechanism of depolarizing drugs at postsynaptic cholinergic receptors is controversial. Burns & Paton (1951) reported that C_{10} may increase the firing threshold around the endplate by causing sustained depolarization. Thesleff (1955a, b), however, demonstrated that the neuromuscular block induced by acetylcholine (ACh), C_{10}, and SuCh is not due to a persistent depolarization of the skeletal muscle endplate, but to a decrease in the sensitivity of the endplates to the transmitter. Although not completely understood, it is now generally accepted that the block by depolarizing agents does not entirely parallel their depolarizing effect. Desensitization of the ACh receptor has been demonstrated to be closely correlated with intracellular Ca^{2+} (Miledi 1980). The intracellular Ca^{2+} mechanisms involved in neuromuscular block, however, have not yet been investigated.

Direct measurement of intracellular Ca^{2+} concentrations in skeletal muscle has mainly been reported in amphibia (Blinks et al 1978; Eusebi et al 1983; Konishi & Kurihara 1987), but few such studies have been reported in mammals (Eusebi et al 1980). We have previously reported Ca^{2+}-aequorin luminescences (Ca^{2+} transients) in directly-stimulated mouse diaphragm (Kimura et al 1985, 1987, 1990). Recently, we have succeeded in measuring Ca^{2+} transients in indirectly-stimulated phrenic nerve-diaphragm muscles of mice (Kimura et al 1989). Using improved techniques, the present study investigates the effects of neuromuscular blocking drugs on nicotinic acetylcholine (n-ACh) receptor-mediated intracellular Ca^{2+} mobilization. In this study we have attempted to distinguish between the mechanisms of depolarizing and competitive blocking drugs based on their effects on Ca^{2+} transients and twitch tension.

Correspondence to: I. Kimura, Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan.

Materials and Methods

Mechanical measurement

Male ddY mice (7–9 weeks old, 28–42 g) were decapitated and exsanguinated. Segments of the right phrenic nerve-diaphragm with tendon attached were removed and cut into strips 10 mm wide. The tendon was tied with a silk thread and connected to an isometric transducer. The four corners of a muscle strip were carefully pinned to rubber plates in a chamber, and resting tension was adjusted to 200 mg. This loading enabled us to obtain constant twitch responses repeatedly during indirect stimulation. A modified Krebs solution of the following composition (mM) was used for the bath: NaCl 122, KCl 5·9, $CaCl_2$ 2·5, $MgCl_2$ 1·2, $NaHCO_3$ 15·5, glucose 11·5. High $[K^+]_o$ solutions (7·1–16·5 mM $[K^+]_o$) were prepared by replacing Na^+ ion with K^+ ion, and SO_4^{2-} ion was substituted for Cl^- to keep the product of $[K^+]_o[Cl^-]_o$ constant (Hodgkin & Horowicz 1959). The bath was maintained at 36°C by means of a heated copper plate under the chamber, regulated by a thermo-module (Komatsu Electronics). The solution was equilibrated with 95% O_2 and 5% CO_2 and was perfused through the chamber at a rate of 1·5 mL min^{-1}. The phrenic nerve was stimulated at 0·1 Hz using supramaximal square pulses (0·4–0·8 V) of 0·1 ms duration through a pair of platinum wire electrodes 1 mm apart. In some experiments, another pair of platinum electrodes was positioned at either side of the costal margin to stimulate the muscle directly. Isometric tension was recorded on a polygraph (type 8K20, San-ei) coupled to a carrier amplifier (RP-3, Nihon Kohden) via an isometric transducer (model SB-1T-H, Nihon, Kohden).

Simultaneous measurement of Ca^{2+}-related aequorin luminescence and twitch tension

The procedures for measuring Ca^{2+}-aequorin luminescences (Ca^{2+} transients) were those of Kimura et al (1985), except that the technique was improved as follows. The aequorin solution (1 mg mL^{-1}) was pressure-injected at 5–6 atm for 2 s through the micropipette into the myoplasm once or twice per fibre in an endplate-rich area of muscle 1–1·5 mm in diameter. Usually, 30–50 fibres of a diaphragm muscle segment were loaded with aequorin. Ca^{2+} transients emitted from the injected area were measured using a photon counter (slightly modified model C767, Hamamatsu Photonics) and a photomultiplier tube (model R464, Hamamatsu Photonics) attached to an acrylic optical fibre (Ryo-mi Plastics). The optical fibre used as a light guide was shorter (14 mm) and wider (3 mm in diameter) than used in our previous report (20 and 2 mm, respectively) in order to decrease photon loss. The end of this optical fibre was immersed in nutrient solution and placed near the aequorin-injected area, 0·5 mm apart from the surface of the muscle. Without aequorin loading, nerve-stimulation alone did not produce light. Light signals and tension were simultaneously recorded on a polygraph. Analog outputs were analysed every 10 s using an FFT analyzer (SM-2701, Iwatsuu) synchronized by trigger pulses from an electric stimulator (MSE-3, Nihon Kohden). In some cases, signals were averaged 30 times by a signal processor (7T07A, San-ei) in order to improve signal-to-noise ratio. Data analysis was performed using a microcomputer (PC-9801, NEC) and printed out on an X-Y plotter (MP3200, Graphtec) or a printer (NM-9950, NEC). Ca^{2+} transients were constantly monitored for at least 30 min, and all of the responses to the drugs used were reversible after washout. The sensitivity of the luminescence measurements in the above systems is considered to be greater than 0·1 μM Ca^{2+} (Blinks et al 1982).

Reagents

Aequorin (Mayo Clinic), acetylcholine chloride (Daiichi), carbachol chloride (CCh, Sigma), decamethonium bromide (C_{10}, Sigma), pancuronium bromide (Sankyo), succinylcholine chloride $2H_2O$ (SuCh, Nakarai), and (+)-tubocurarine chloride $3{\cdot}5\ H_2O$ ((+)-TC, Nakarai) were used.

Statistical treatment and analysis of the aequorin signal-tension curve

Data are expressed as means ± s.e.m. The paired *t*-test was performed at $P=0{\cdot}05$ and 0·01 levels of significance. Aequorin signal-tension curves were analysed using a non-linear regression program and the following equation,

$$T = A + B \log L \qquad \text{(eqn 1)}$$

as well as by a non-linear equation based on the Gauss-Newton and Berman algorithm (Berman et al 1962) using the Hill equation,

$$T = VL^r/(K^r + L^r) \qquad \text{(eqn 2)}$$

where T is % twitch tension, L is % luminescence, A and B are coefficients, V is estimated maximal T, r is the Hill coefficient, and K is the dissociation constant.

Results

A comparison was made between Ca^{2+} transients and twitch tension in the indirectly (via the phrenic nerve) and directly-stimulated diaphragm muscles of mouse (Fig. 1, Table 1). In the case of direct stimulation, time-to-peak amplitude for Ca^{2+} transients was 7·7 ms, i.e. faster than in frog skeletal muscle (40 ms, Blinks et al 1978). Moreover, duration at half

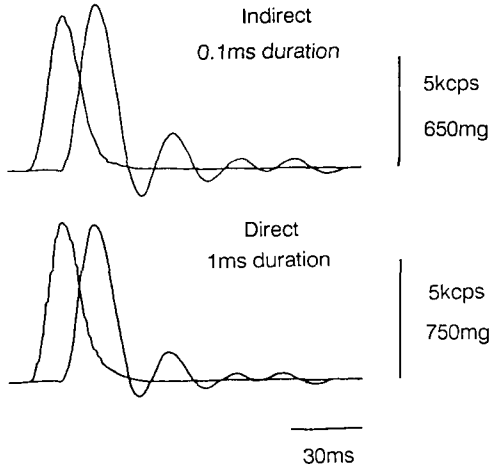

FIG. 1. Typical fast-sweep records of aequorin luminescence (left peak trace) and twitch tension (right peak trace) from the same mouse phrenic nerve-diaphragm preparation stimulated indirectly (indirect, 0·1 ms duration, above) and directly (direct, 1 ms duration, below). Averages of thirty responses are superimposed. The same results were consistently obtained in 6 separate experiments. kcps: kilocounts s^{-1}.

Table 1. Aequorin luminescence and twitch tension compared in indirectly- and directly-stimulated mouse muscles.

	Aequorin luminescence		Twitch tension	
Method of Stimulation	Time to peak (ms)	Duration at half-amplitude (ms)	Time to onset (ms)	Time to peak (ms)
Indirect	9·0 ± 0·2	14·1 ± 0·2	7·9 ± 0·1	21·8 ± 0·2
Direct	7·7 ± 0·2**	14·4 ± 0·3	7·1 ± 0·1**	21·1 ± 0·1**

Diaphragmatic muscle was stimulated with 0·1 Hz square pulses (supramaximal voltage) of 0·1 ms duration in the case of indirect stimulation and 1 ms duration in the case of direct stimulation. All data were obtained from fast traces of averaged records of thirty signals of aequorin luminescence and twitch tension during 5 min from the same preparation. The data given are means from 6 separate experiments ± s.e.m. ** $P < 0{\cdot}01$ significantly different from the corresponding value obtained in response to indirect stimulation (paired *t*-test).

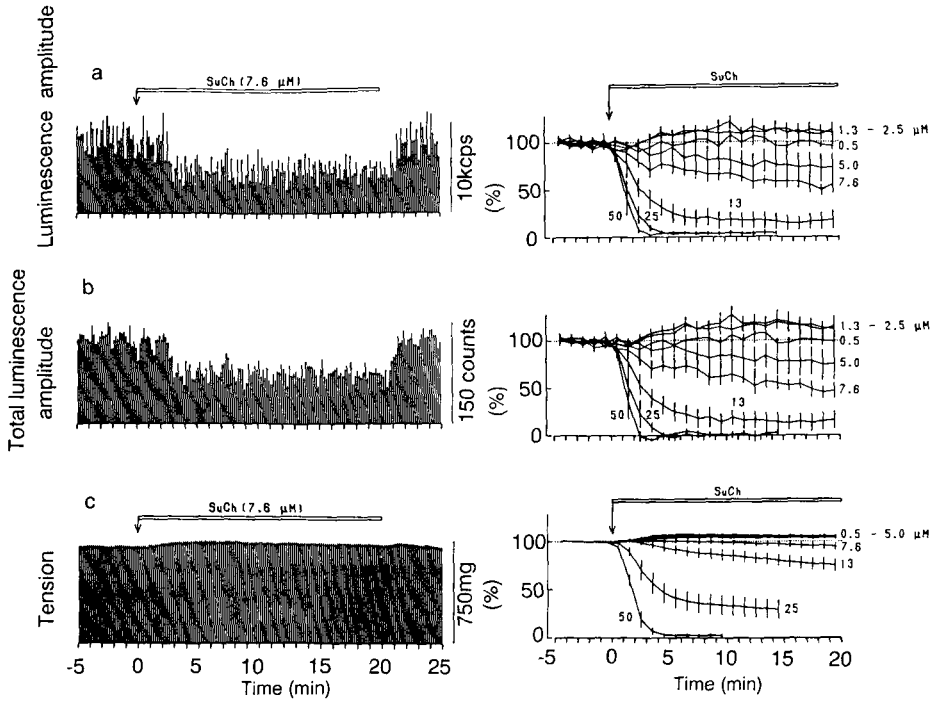

FIG. 2. Typical results (a) showing the effects of 7·6 μM SuCh on peak amplitude of aequorin luminescence (upper trace), total 10 s luminescence level (middle trace) and twitch tension (lower trace) in indirectly-stimulated diaphragm muscles of mice. Data are plotted against time (min). The light signals (Ca^{2+} transients) and twitch tensions were transformed using a microcomputer. (b) Effect of SuCh (0·5–50 μM) on peak amplitude of luminescence (upper), total amount of luminescence (middle) and twitch tension (lower). Averaged values of six points measured at 10 s intervals within the course of a single minute are expressed as a percentage of the control value before drug administration. Further averaged values ± s.e.m. from 5–9 separate experiments were plotted against time (min). The significance of differences as determined on the basis of the *t*-test are not shown.

amplitude was 14·5 ms, i.e. briefer than in the extensor digitorum longus (244 ms) and soleus muscles (468 ms) of the rat (Eusebi et al 1980). Thus, the increase in intracellular Ca^{2+} in intact mouse diaphragm muscle has been shown to be faster and briefer than in other skeletal muscles reported. In the case of indirect stimulation, time-to-peak amplitude was 9 ms with respect to the light signal and 22 ms in the case of twitch tension. A 0·5– 1 ms slower onset was obtained in response to indirect stimulation than in response to direct stimulation, which may represent synaptic delay in indirect stimulation (Katz & Miledi 1965).

The time-dependent effects of SuCh were compared with respect to Ca^{2+} transients and twitch tension. At 7·6 μM, SuCh inhibited Ca^{2+} transients by 50% 5 min after application, while it slightly enhanced twitch tension (Fig. 2a). The effects of SuCh were investigated quantitatively at various concentrations (Fig. 2b). SuCh did not affect either peak amplitude or total level (time integral) of Ca^{2+} transients at 0·5 μM but did enhance them by 10–20% above the control at 1·3–2·5 μM and slightly inhibited them at 5 μM. At higher concentrations (5–50 μM), SuCh inhibited Ca^{2+} transients within 5–10 min after application. The shape of Ca^{2+} transients was unaffected by SuCh, since the peak amplitude and total amount of Ca^{2+} transients were affected by SuCh to a similar extent. Ca^{2+} transients were inhibited to a greater extent than twitch tension in this range of concentrations. Both effects were reversible. The above results indicate that Ca^{2+} transients are more susceptible to SuCh inhibition than twitch tension.

The effect of SuCh on both Ca^{2+} transients and twitch tension was biphasic (Fig. 3a). SuCh enhanced twitch tension at 0·5–5 μM and inhibited it at 13–50 μM; the same percentage augmentation or percentage block of Ca^{2+} transients occurred with concentrations that were two to three times smaller. CCh and C_{10} produced similar effects, although the difference between Ca^{2+} transients and twitch tension was somewhat less than in the case of SuCh (Fig. 3a). Differences in effects on the two parameters were more marked in reponse to high $[K^+]_o$ which is a non-specific membrane-depolarizing agent and was used as a positive control for the depolarizing effect. Twitch tension was increased at 7·1–11·8 mM and reduced at 13·0 mM. At 16·5 mM high $[K^+]_o$ suppressed twitch tension completely. In contrast, the Ca^{2+} transients showed only a concentration-dependent suppression over the entire range of concentrations (data not shown). The effect of pancuronium and (+)-TC on both parameters was monophasic and concentration-dependent (Fig. 3b) in marked contrast to the effect of depolarizing agents. For the same drug concentration, the percentage depression of twitch tension was less than the percentage depression of Ca^{2+} transients, but the difference was not statistically significant. ACh applied to the bath had only a slight effect on both parameters. It slightly increased them at 0·1 mM, and at 1 mM significantly enhanced twitch tension (5·3 ± 1·9%, n = 5) but slightly decreased Ca^{2+} transients (9·8 ± 7·7%, n = 5).

The effects of SuCh, C_{10}, CCh, pancuronium and (+)-TC at 4–8 different concentrations and submaximal electrical

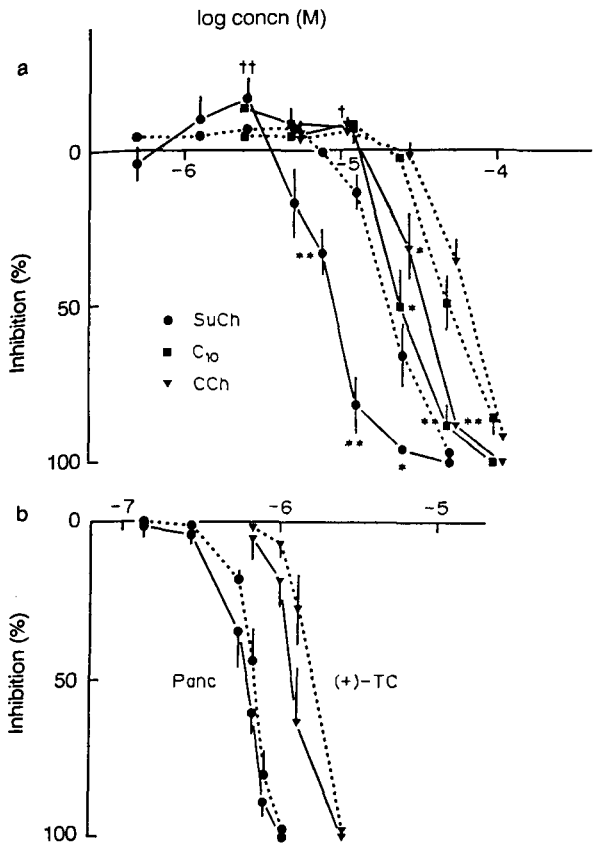

FIG. 3. Log-concentration-inhibition curves for (a) SuCh (●, 0·5–50 μM), C_{10} (■, 2·4–96 μM), and CCh (▼, 5·5–109 μM), and (b) pancuronium (Panc) (●, 0·14–1·1 μM) and (+)-TC (▼, 0·7–2·7 μM) for twitch tensions (dotted lines) and peak amplitude of luminescence (solid lines). Negative inhibition on the ordinate indicates enhancement. Data are mean values ± s.e.m. from 5–9 separate experiments taken 10 min after drug application when a steady-state response had been attained $*P<0.05$ and $**P<0.01$, % luminescence amplitudes significantly different from twitch tensions based on the paired t-test. $\dagger P<0.05$ and $\dagger\dagger P<0.01$, significantly different from 0% inhibition based on the paired t-test only when the luminescence was enhanced.

nerve-stimulation on Ca^{2+} transients and twitch tension were compared (Fig. 4). When equation 1 was employed to fit the data for an exponential relationship, the coefficients for correlations between Ca^{2+} transients and twitch tension were found to be 0·98 in the case of SuCh, 0·96 for C_{10} and CCh, 0·98 for (+)-TC and pancuronium, and 0·89 in the case of electrical nerve-stimulation. All coefficients were statistically significant ($P<0.01$). Nevertheless, the data for SuCh were distributed to one side of the stimulated curve in the inhibitory portion and to the other side in the potentiating portion of the Ca^{2+} transients, because SuCh strongly affected Ca^{2+} transients when twitch tension alterations were slight, e.g. when twitch tensions were inhibited by 15%, Ca^{2+} transients were suppressed by 80%. In addition, the upper part of the curves tended to exceed 100%. We, therefore, attempted to fit the curve using equation 2 for a hyperbolic relationship. The relationship for SuCh (0·5–50 μM) was more hyperbolic than exponential between the percent maximum twitch tension and the percent peak amplitude of Ca^{2+} transients. C_{10} (2·4–96 μM) and CCh (5·5–109 μM) yielded similar results. Hill coefficients were estimated to be 1·2 in the case of SuCh, 0·92 for C_{10} and 0·98 for CCh. In contrast, the data for (+)-TC were distributed to the opposite side of the curve using equation 2 between the upper and lower inhibitory part for half-maximal inhibition of Ca^{2+} transients. The data for submaximal electrical nerve-stimulation yielded similar results. In the case of pancuronium, the curve also fitted equation 1 better, although the curves for both equations gave similar results. Regression coefficients (B) were estimated to be 91·6 in the case of pancuronium, 57·3 for (+)-TC and 30·4 for submaximal electrical nerve-stimulation. The correlation curves for the competitive blocking drugs, therefore, were different from the curves for the depolarizing drugs. The best-fit curves are plotted in Fig. 4 (dotted lines).

Spontaneous weak Ca^{2+} transients were generated repeatedly by SuCh (Fig. 5a). The peak amplitude of this response was up to 5% for nerve-stimulated Ca^{2+} transients before application of SuCh. The threshold concentration to initiate the response corresponded to the concentration necessary to initiate the neuromuscular blocking action. These agonistic responses were prolonged for at least 20 min during blockade of neuromuscular transmission by 7·6–50 μM SuCh, although a shorter prolongation was observed at 100–3000 μM. The same tendency was seen with C_{10} (2·4–96 μM) and CCh (5·5–109 μM), but not with high $[K^+]_o$ (7·1–16·5 mM). These spontaneous Ca^{2+} transients were increased by increasing concentrations of $[Ca^{2+}]_o$ from 2·5 mM in normal nutrient solution to 5–10 mM (Fig. 5b). They disappeared in Ca^{2+}-free nutrient solution and were inhibited by (+)-TC (6·7 μM). These results indicate that the resting intracellular $[Ca^{2+}]$ level was elevated during the blockade of neuromuscular transmission by the depolarizing drugs.

Discussion

The competitive blocking drugs (pancuronium and (+)-TC) affected Ca^{2+} transients and twitch tension almost equally. The slight difference in sensitivity may be due to the fact that twitch tension was recorded from the whole muscle, while Ca^{2+} transients were measured in the relatively few, superficial fibres loaded with aequorin. The relation between Ca^{2+} transients and twitch tension was exponential. In contrast, the depolarizing agents (SuCh, C_{10}, and CCh) inhibited Ca^{2+} transients at two to three times lower concentrations than twitch tension. The relation between Ca^{2+} transients and twitch tension was hyperbolic and computer-fitted to the Hill equation. These differences are based on the finding that Ca^{2+} transients are inhibited to a greater extent than twitch tension by depolarizing drugs but not by competitive blocking drugs. This cannot therefore be explained on the basis of an artifact caused by aequorin injection or by blocking the binding sites for ACh, as in the case of pancuronium and (+)-TC. It is uncertain, however, that depolarizing drugs act directly on aequorin thereby decreasing its sensitivity for Ca^{2+}.

SuCh enhances Ca^{2+} transients and twitch tension at low concentrations. These effects cannot be explained by depolarization of pre- and post-synaptic membranes, because high $[K^+]_o$ did not increase Ca^{2+} transients. It is likely that low concentrations of SuCh may act on presynaptic regions to

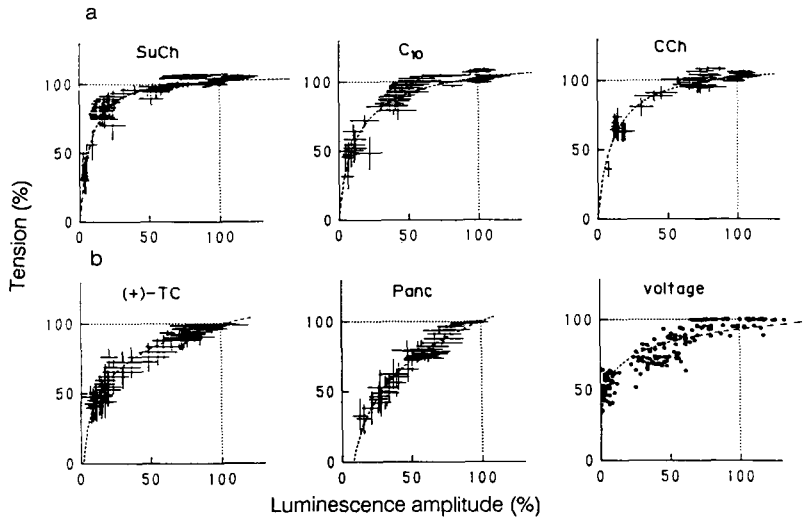

FIG. 4. The relationships between percentage twitch tension and percentage luminescence amplitude for (a) SuCh (0·5–50 μM), C_{10} (2·4–96 μM), CCh (5·5–109 μM), and (b) (+)-TC (0·7–2·7 μM), pancuronium (Panc) (0·14–1·1 μM), and changes in stimulation voltage. The protocol as shown in Fig. 2b was performed at four to eight concentrations for each drug. All data for both parameters every min are plotted together as the means ± s.e.m. from five to nine separate experiments. In the case of changes in stimulation voltage, all data for both parameters are plotted every 10 s. The data are simulated by the nonlinear least square method using equations 1 or 2 (see Methods). The best-fit equation was equation 2 in the case of SuCh, C_{10} and CCh, and equation 1 in the case of (+)-TC, pancuronium (Panc) and changes in stimulation voltage. Equation 1 was further converted to the following equation,

$$L = a\, e^{bT} \qquad \text{(eqn 3)}$$

Where a and b are coefficients. The best-fit curve is plotted using dotted lines.

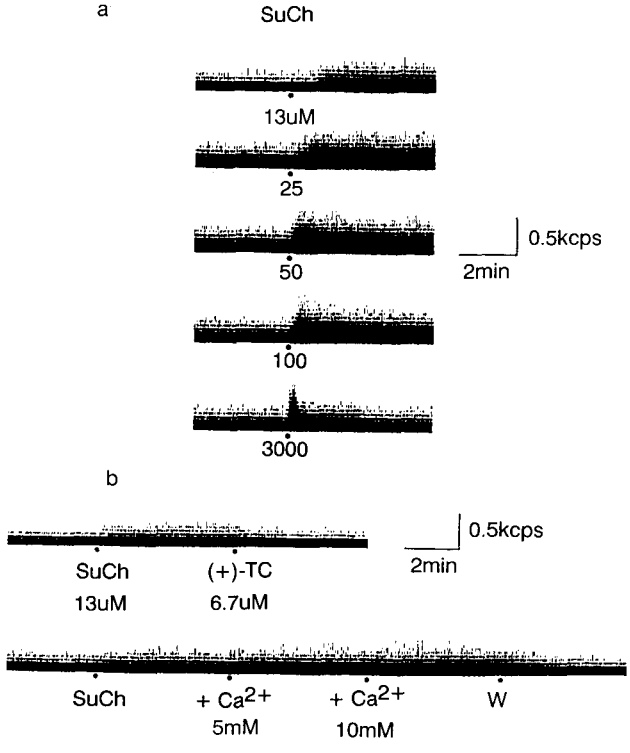

FIG. 5. Typical data showing increased spontaneous Ca^{2+}-transients induced by SuCh in mouse phrenic nerve-diaphragm preparations. (a) Concentration-dependence of SuCh on increases in spontaneous Ca^{2+} transients. SuCh was applied at the time indicated by the closed circles. The data shown were obtained from the same preparation. (b) Effect of (+)-TC and $[Ca^{2+}]_o$ on SuCh (13 μM)-induced increases in spontaneous Ca^{2+} transients. (+)-TC (6·7 μM) was added 5 min after application of SuCh (upper). $[Ca^{2+}]_o$ was increased cumulatively from 2·5 mM (normal Krebs solution) to 5–10 mM at 5 min intervals and then washed away (W; lower). Similar results were obtained consistently in 4–9 separate experiments.

potentiate the release of ACh (Standaert & Adams 1965; Blaber & Christ 1967) so that some fibres are presumably firing repetitively.

An increase in spontaneous Ca^{2+} transients was observed during the blockade of neuromuscular transmission by SuCh, C_{10}, and CCh. Spontaneous Ca^{2+} transients were dependent on $[Ca^{2+}]_o$ and appeared to be governed by the n-ACh receptor, because they were blocked by (+)-TC. These agonistic responses may reflect Ca^{2+} influx through n-ACh receptor channels (Miledi et al 1980) or Ca^{2+} release from the n-ACh receptor itself (Chang & Neumann 1976). In patch-clamp studies, these depolarizing agents were classified as agonists or partial agonists rather than as antagonists (Albuquerque et al 1988). Transient depolarization by CCh was converted to sustained depolarization in the presence of ouabain (Creese et al 1976), which suggests that the receptor channel may repeatedly be activated as long as the drug is present.

Ca^{2+} transients have usually been found not to parallel tension entirely (Blinks et al 1978; Konishi & Kurihara 1987). Two different mechanisms for Ca^{2+} transients induced by ACh may exist, i.e. one related and the other not related to, twitch tension; the Ca^{2+} transients not related to twitch tension may be more sensitive to depolarizing agents, while both Ca^{2+} transients may be equally inhibited by the competitive drugs. Previously (Kimura et al 1989), we demonstrated two distinct Ca^{2+} transients in diaphragm muscles of mice. One is fast Ca^{2+} mobilization (first phase) which is linked to the generation of twitch tension, and the other is slow Ca^{2+} mobilization (second phase), which is observed in the presence of an anticholinesterase agent and is blocked by lower concentrations of (+)-TC. The second phase of Ca^{2+} transients and the spontaneous Ca^{2+} transients discussed herein are similar in that they were not

accompanied by twitch tensions. It is unlikely, however, that a single type of ACh receptor mediates both Ca^{2+} transients because the second phase was blocked at 0·01–0·1 μM of (+)-TC, whereas the spontaneous Ca^{2+} transients were blocked by (+)-TC only at concentrations higher than 5 μM.

The agonistic properties may be attributable to the inhibition of nerve-stimulated responses through an increase in resting $[Ca^{2+}]_i$ and/or activation of other signal transduction systems. Desensitization of ACh receptor seems incompatible with the repeated increase in spontaneous Ca^{2+} transients. The possibility, however, remains that desensitization-resistant ACh receptor channels (Dionne 1989) contribute to the spontaneous Ca^{2+} transients. Alternatively, depolarizing blockers produced sustained depolarization during perfusion (Head 1974), although iontophoretic application produced transient depolarization (Thesleff 1955a, b, 1958). The release of intracellular Ca^{2+} is prevented during prolonged depolarization (Eusebi et al 1983). Our results, therefore, may be partly explained by sustained depolarization during perfusion.

In conclusion, depolarizing and competitive neuromuscular blocking drugs affect intracellular Ca^{2+} mobilization differently. Ca^{2+} transients are more sensitive to inhibition by depolarizing neuromuscular blocking drugs than is twitch tension. In contrast, Ca^{2+} transients and twitch tension are equally susceptible to competitive neuromuscular blocking drugs. Depolarizing drugs may block twitch tension by inhibiting the by-passed route of Ca^{2+} mobilization accompanied by a local increase in resting $[Ca^{2+}]_i$.

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