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Modification by dantrolene, procaine and suxamethonium of caffeine-induced changes in aequorin luminescence transients and twitch tensions of directly-stimulated diaphragm muscle of mouse

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1 A convenient method is described for measuring simultaneously Ca^{2+}-related aequorin luminescence and twitch tension in the isolated diaphragm muscle of the mouse. Forty to fifty fibres were injected intracellularly with aequorin solution and the mechanical and luminescence responses to direct stimulation were recorded.

2 The replacement of Na^+ by K^+ (to obtain 59 or 143.4 mM K^+) in the nutrient solution decreased both aequorin luminescence and twitch tensions, but after a time lag, it produced a contracture.

3 Caffeine (5 or 10 mM) increased both aequorin luminescence and twitch tensions, and after a time lag, it also produced a contracture.

4 Dantrolene (1 and 30 μM) and procaine (10 μM, 300 μM and 1 mM) decreased aequorin luminescence transients and twitch tension. In addition procaine inhibited the caffeine-induced increase of aequorin luminescence, but dantrolene did not have this effect.

5 At concentrations causing neuromuscular block, suxamethonium (130 μM) decreased aequorin luminescence transients and twitch tension. By contrast, (+)-tubocurarine (6.5 μM) did not affect the aequorin luminescence in directly stimulated muscles.

6 These results suggest that Ca^+-related aequorin luminescence transients accompanied by twitch tensions reflect the intracellular fast mobilization of compartmentalized Ca^{2+} from plasma membrane or sarcoplasmic reticulum, and that the increase in resting luminescence caused by a K^+-or caffeine-induced contracture may be produced by the slow mobilization of Ca^{2+} from sarcoplasmic reticulum.

Introduction

The mechanisms of action of skeletal muscle relaxants such as dantrolene (Endo & Yagi, 1982), procaine (Ford & Podolsky, 1972; Thorens & Endo, 1975; Endo, 1975), and suxamethonium (SuCh; Thesleff, 1955) have been extensively studied from biochemical, electrophysiological, and mechanical aspects. Dantrolene was shown to reduce the amount of Ca^{2+} released from the intracellular Ca^{2+} store (Hainaut & Desmedt, 1974). Procaine inhibits the Ca^{2+}-induced Ca^{2+} release mechanism in skeletal muscle (Ford & Podolsky, 1972). SuCh produces neuromuscular block which is due to depolarization in the early phase and to desensitization in the late period (Thesleff, 1955; Rang & Ritter, 1969). The desensitization was shown to be closely related to intracellular Ca^{2+} (Miledi, 1980). Despite these previous studies, the effects of these drugs on intracellular Ca^{2+} concentration in intact skeletal muscles have not been studied directly. There is little information on the relationship between intracellular Ca^{2+} and force development in mammalian skeletal muscle under the action of these muscle relaxants. The development of techniques to measure aequorin luminescence (Shimomura *et al.*, 1962; Blinks *et al.*, 1978; Eusebi *et al.*, 1983) has made it possible to determine the intracellular Ca^{2+} concentration during a contraction, but these techniques have so far not been applied to mammalian skeletal muscle. Therefore, we developed a convenient method to determine Ca^{2+}-related aequorin luminescence and twitch tension simultaneously in mouse diaphragm muscle. Using this method we have investigated in diaphragm muscles the effects of dantrolene, procaine, and SuCh on intracellular Ca^{2+} levels, and their relation to tension development.

Methods

Mechanical

Male ddY mice were decapitated and bled. Diaphragm muscles with tendon attached were removed and cut into strips 5 mm wide. The tendon was tied with a silk thread. One end of the muscle was connected to an isometric transducer (SB-1T-H, Nihon Kohden) for mechanical recording. The other end was pinned to a rubber plate in a recording chamber (Figure 1). The resting tension was adjusted to 100 mg. A modified Krebs solution of the following composition (mM) was used: NaCl 122, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5. To prepare the high K^+ solutions (59 or 143.4 mM), the Na^+ in Krebs solution was replaced with K^+. The bath solution was maintained at 37 ± 1°C by a heated copper plate under the chamber. Temperature was regulated with a thermo-module (Komatsu Electronics), and the solution was equilibrated with 95% O_2 and 5% CO_2. The drug solutions perfused the preparation in the chamber.

Ca^{2+}-related aequorin luminescence

The method described by Blinks *et al.* (1978) was modified. Twice-distilled water was passed through a Chelex-100 (Bio. Rad. Laboratories; 50–100 mesh) column which had been previously washed with 0.1 N HCl and neutralized with 0.1 N KOH. Aequorin (Mayo Clinic) was dissolved in this prepared water, which contained 150 mM KCl, 5 mM HEPES (pH 7.45), and 10 μM EDTA. The aequorin solution (1 mg ml^{-1}) was then passed through a millipore filter (0.05 μm), and stored in a polyethylene vessel at 0°C.

Five % dimethylpolysiloxane (Fuji Systems) in Ca^{2+}-free distilled water was passed through a 0.5 μm millipore filter. In this silicone fluid, a glass capillary 2 mm in diameter with a fine glass filament core was immersed, and after being dried the capillary was drawn with a microelectrode puller. The advantage of silicone treatment of the glass microelectrodes was that air bubbles could be easily dislodged from the tip of the pipette. The micropipettes had a resistance of about 10–30 MΩ when filled with 3 M KCl. One microlitre of the aequorin solution was taken into an unfilled micropipette, which was then checked under a microscope for generation of aequorin luminescence during the injection of the aequorin solution into a 100 μM Ca^{2+} solution by applying a pressure of 3 atm from a nitrogen tank to the micropipette. Then a platinum wire was inserted into the micropipette to make electrical contact with the aequorin solution. The penetration of the capillary micropipette into muscle fibres was assessed by measuring membrane potential. The aequorin solution was then injected through the micropipette into the myoplasm once or twice per fibre. Usually, 40–50 fibres of a diaphragm segment were loaded with aequorin. Pressure pulses of 3 atm (nitrogen gas) with a duration of 1 s were applied using a pneumatic pump module (slightly modified PPS-2 and PPM-2; Medical System). The aequorin luminescence for the injected area (about 1 mm in diameter) was 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 (2 mm in diameter; Ryô-mi Plastics). The end of this optical fibre was immersed in the nutrient solution and placed near the aequorin-injected area (Figure 1). The distance between the end of the optical fibre and the preparation was 0.5 mm. The optical fibre had about twice the diameter of the injected area. Since it was placed very near to the preparation, light-gathering efficiency was sufficiently high. The open gate time of the photon counter was set at 10 ms and the closed gate time at 20 μs.

Electrical stimulation

The partition stimulating method described by Abe & Tomita (1968) was used to apply the stimulation (5 ms, 100 Hz for tetanic contraction; 5–50 ms, 0.1 Hz for twitch contraction). One end of a muscle segment was passed through holes in two Ag-AgCl plates placed

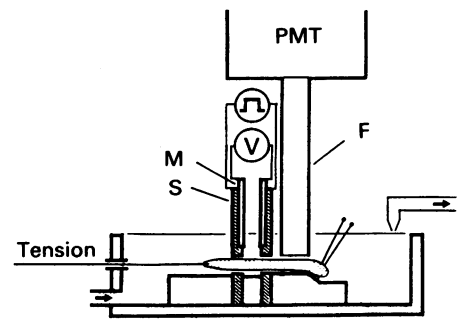

Figure 1 Apparatus used to measure tensions and aequorin signals. The nutrient solution perfused the preparation from left to right (see arrows). The right end of a muscle segment was pinned to a rubber plate; the other end was connected to an isometric transducer. The muscle was stimulated through 1 mm wide Ag-AgCl plates (S), where the muscle was passed through two holes. From two Ag-AgCl wires (M), the stimuli were checked simultaneously as the stimulation voltage per mm width of the muscle segment. Aequorin luminescence was directed through an optical fibre (F) onto a photomultiplier tube (PMT) whose output was led to a photon counter. Luminescence measurements were made from an area adjacent to the stimulated area of the muscle bundle.

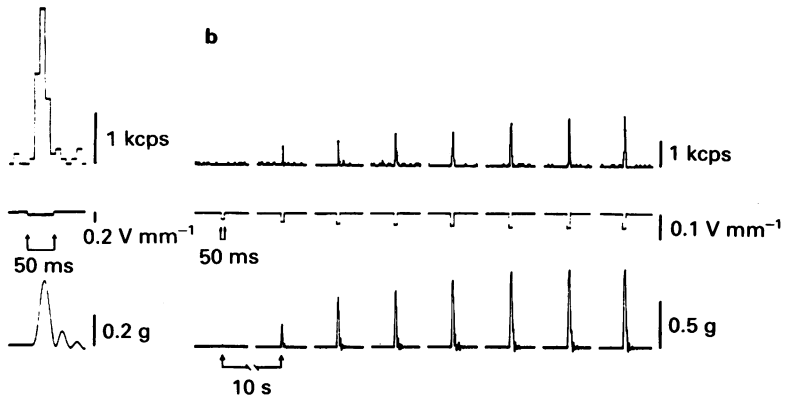

Figure 2 Typical recordings (b) of increasing aequorin luminescence (40 – 50 fibres were injected with aequorin, upper trace) and twitch tensions (lower trace) in mouse diaphragm muscle with increasing currents (middle trace) monitored as a single-shock stimulation voltage applied to the muscle bundle (50 ms, 0.1 Hz), and a typical fast trace (a). Stimuli shorter than 10 – 50 ms needed greater strength, and were not suitable for obtaining both an aequorin signal and a twitch tension. Rebound oscillations were seen after each twitch contraction. The effects were observed consistently in 3 experiments.

1 mm apart. The stimulation currents were applied to an area 1 mm wide in the muscle segment, and were monitored as stimulation voltage ($mV\,mm^{-1}$) by another pair of Ag-AgCl electrodes 1 mm apart. The apparatus used to measure both tension and aequorin signal is illustrated in Figure 1.

Reagents

Procaine HCl (Daiichi), dantrolene Na (Yamanouchi), SuCh 2 H_2O, (+)-tubocurarine Cl, caffeine H_2O and Triton X-100 (Nakarai) were used.

Results

Aequorin luminescence of directly stimulated muscle

The light emission from aequorin-injected muscle fibres and twitch tension were simultaneously measured at increasing single stimulation currents from threshold to supramaximal strength (duration, 50 ms; Figure 2). Figure 2a shows the photon counting of luminescence and twitch tension as seen in fast sweep records. Tension and light output increased with increasing stimulus strength. Aequorin signals of

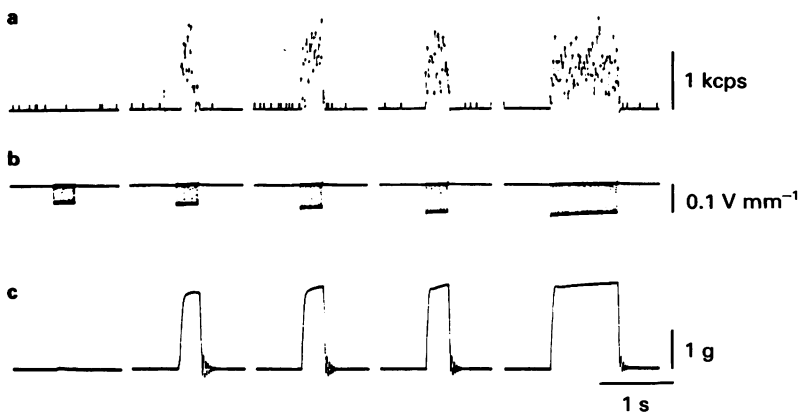

Figure 3 Typical recordings of increasing aequorin luminescence (a) and tetanic contractions (c) in mouse diaphragm muscle stimulated directly with trains of pulses (duration of each pulse 5 ms, frequency 100 Hz and duration 0.3 or 0.9 s) with increasing amplitudes and burst duration of stimulus currents (b). The effects were observed consistently in 3 experiments.

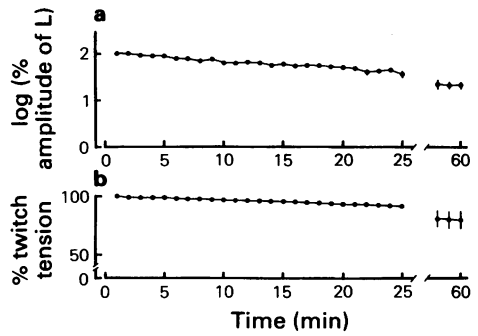

Figure 4 The gradual decrease in the aequorin luminescence (L) and twitch tensions occurring after the stabilization period of 10 min, following an injection of aequorin. Luminescence peak amplitude and twitch tension were expressed as a % of the control value at the end of the stabilization period. Then the logarithmic & luminescence peak amplitude (a) and % twitch tension (b) were plotted (every 1 min) against time (min) elapsed. Each point represents the mean value at a single time point ($n = 3$) and the vertical lines show s.e.mean.

smaller amplitudes were obtained by using shorter (10 or 5 ms) current pulses to stimulate the preparation (data not shown). Stimulation with a 50 ms current pulse occasionally evoked a train of 2–3 action potentials when examined with intracellular recording. However, current pulses shorter than 50 ms required a higher stimulation voltage to produce satisfactory responses (see Figure 3), and therefore 50 ms pulses were used in the present study. Rebound oscillations of twitch tension were seen after a peak twitch tension was induced by a single stimulation (see Figure 2).

Ca^{2+}-related aequorin luminescence signals were also recorded during tetanic contractions produced by bursts of high frequency stimuli applied to the muscle. Figure 3 shows the effect of increasing the amplitude of stimulus current and duration of the stimulus burst on light output and tension. Both the mechanical and luminescence responses showed the maximal amplitude when the stimulus currents were increased to more than 0.06 V mm^{-1} (Figure 3b).

During the first 10 min following aequorin injection the amplitude of aequorin luminescence in response to a constant supramaximal stimulus gradually increased. After this initial period the response stabilized for about 20 min. Experiments were, therefore, started only after the first 10 min period following aequorin injection. After this 10 min period of stabilization (as shown in Figure 4) the aequorin luminescence transient produced by single shock stimulation gradually declined. The gradual attenuation of aequorin signals may be partly due to the consumption of aequorin by the elevation of $[Ca^{2+}]_i$ during responses, and to a

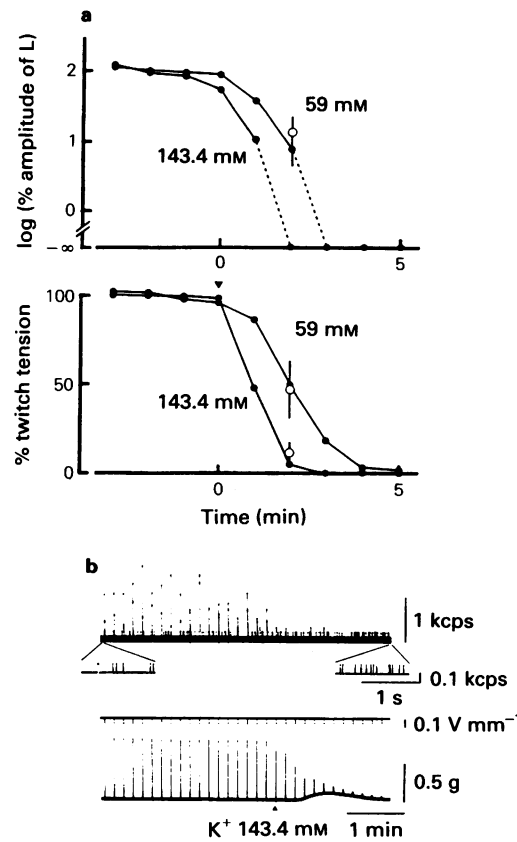

Figure 5 (a) Effects of 59 or 143.4 mM K^+-depolarizing solutions on log aequorin luminescence (upper) and twitch tensions (lower) in mouse diaphragm muscle stimulated directly (50 ms, 0.1 Hz). Logarithmic % luminescence (L) peak amplitude and % twitch tension (see legend to Figure 4) (●) are plotted against time (min) before and after K^+ application. The normal Krebs solution was completely replaced with that containing either 59 or 143.4 mM K^+ at '0' min (abscissa scale). Open circles represent the mean value at a single time point ($n = 3$) and vertical lines show s.e.mean. (b) Typical recording of aequorin luminescence (upper), current monitor (middle), and twitch tensions (lower) before and during the application of a 143.4 mM K^+-depolarizing solution. Note that the high K^+-solution caused aequorin luminescence and twitch tensions to decrease simultaneously, and a contracture was generated after a time lag. Resting luminescence was increased after K^+-application (insert upper right trace) compared to control level (insert upper left trace). (a and b) Derived from the same preparation.

smaller extent to the deterioration of the muscle fibres. As shown in Figure 4b, the decrease of twitch tension was slighter than that of the aequorin signal during a period of continuous stimulation for 1 h. Furthermore, the yield of the total photon counts

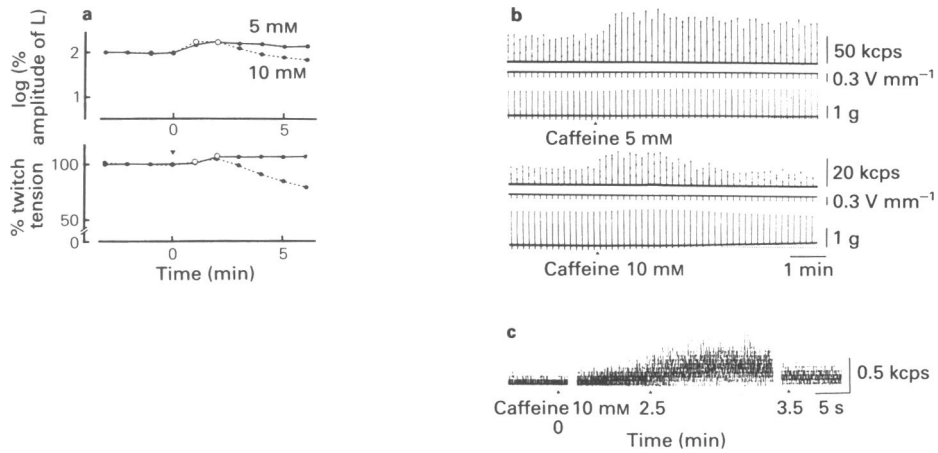

Figure 6 (a) Effects of 5 or 10 mM caffeine on log aequorin luninescence (upper) and twitch tension (lower) in mouse diaphragm muscle stimulated directly. Data were plotted as in Figure 5 against time (min) after caffeine application (completely replaced at 0 min). Open circles represent the mean values at single time points in different experiments ($n = 4$) and verticle lines show s.e.mean. (b) Typical recordings of aequorin luminescence (upper trace), current monitor (middle trace), and twitch tensions (lower trace) with 5 or 10 mM caffeine. Caffeine increased aequorin luminescence, and concomitantly increased twitch tensions, and then after a time lag of 3.5 min, a contracture was generated. (c) Increase in resting aequorin luminescence 2.5 min after the application of 10 mM caffeine. The effects were observed consistently in 3 experiments.

($2.8-4.7 \times 10^5$ kcps) checked by 1% Triton X-100 treatment of muscle was 10^5-10^6 times the photon emission (1.6 kcps) during one twitch response.

The partial or entire substitution of K^+ (59 or 143.4 mM) for Na^+ in the nutrient solution produced a decrease in the aequorin luminescence, and this was accompanied by a decrease in twitch tension (Figure 5a, b). The effects of 143.4 mM K^+ were greater than those of 59 mM K^+. Because the change in aequorin luminescence was sharper than that in twitch tension (Figure 5b), the logarithmic value of peak luminescence expressed as a percentage of the control value during the 3–4 min pretreatment period was plotted in Figure 5a, which shows a parallelism between aequorin light and twitch tension. In the following experiments, the logarithmic value of percentage peak luminescence was regarded as a measure of the magnitude of the aequorin luminescence. Treatment of the diaphragm with 143.4 mM K^+ solution produced, after a time lag, a contracture, which was associated with an increase in resting luminescence.

Effects of dantrolene and procaine on aequorin luminescence and caffeine-induced increase in aequorin luminescence

The effect of caffeine on intracellular aequorin luminescence was investigated before the application of dantrolene and procaine, because caffeine is thought to enhance Ca^{2+} release from the Ca^{2+} store site. Caffeine 5 mM increased aequorin luminescence, and concurrently twitch tensions (Figure 6a, b). Despite the continued presence of 10 mM caffeine the increases in aequorin luminescence did not persist for more than 2 min, but the caffeine-induced increase in twitch tension disappeared. This short-lasting increase in aequorin luminescence cannot be ascribed to aequorin consumption during caffeine treatment, because when the muscle was later treated with 1% Triton X-100 to release all the remaining aequorin, the total photon count was 10^5-10^6 times the photon emission during one twitch response.

Addition of 10 mM caffeine caused a slow, steady contracture after the light response was transiently potentiated. Caffeine 10 mM also increased resting luminescence slightly (Figure 6c), this being associated with an increase in the slow and prolonged development of force. In the present work, drug effects on resting glow were not investigated in detail.

Dantrolene (1 and 30 μM) decreased both aequorin luminescence and twitch tensions, but did not abolish the caffeine-induced increases in aequorin luminescence and twitch tensions (Figure 7a, b). The effects of higher concentrations of dantrolene could not be investigated because of its poor solubility.

Procaine (10 μM, 300 μM and 1 mM) decreased aequorin luminescence in a concentration-dependent manner and this was accompanied by a decrease in twitch tensions (Figure 8a, b). Procaine 300 μM markedly decreased, and 1 mM procaine eliminated, the

caffeine-induced increase in aequorin luminescence, whereas it did not sharply decrease the caffeine-induced increase in twitch tension. Procaine 10 μM slightly decreased the effect of 5 mM caffeine on aequorin luminescence (Figure 8a, b), but abolished the effect of 2.5 mM caffeine (data not shown). These results were quantitatively represented in concentration-response curves (Figure 9) and suggest that the caffeine-induced Ca^{2+} release was not completely blocked by dantrolene but was abolished by procaine.

Effects of suxamethonium and (+)-tubocurarine

Suxamethonium 130 μM, suppressed 80% of the indirectly stimulated muscle twitching (data not shown), slightly decreased aequorin luminescence of directly stimulated muscles, but had no effect on the caffeine-induced increase in aequorin luminescence (Figure 10a, b). A competitive neuromuscular blocker, (+)-tubocurarine, at 6.5 μM, induced the same degree of neuromuscular blocking effect as 130 μM SuCh, but did not decrease aequorin luminescence of directly stimulated muscles, nor was the caffeine-induced increase in aequorin luminescence affected by (+)-tubocurarine (6.5 μM).

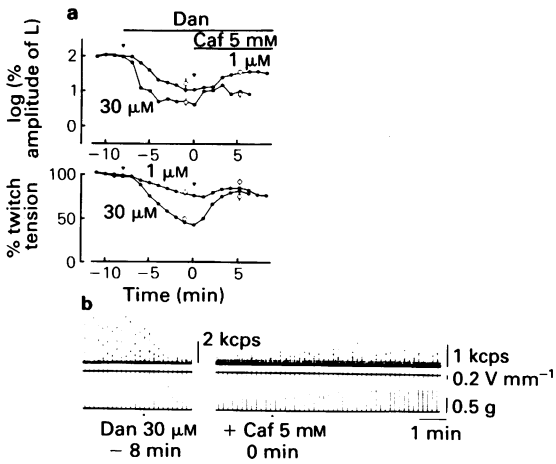

Figure 7 (a) Effects of 1 or 30 μM dantrolene (Dan) and 5 mM caffeine (Caf) on log aequorin luminescence (upper trace) and twitch tensions (lower trace) in mouse diaphragm muscle stimulated directly. Data were plotted as in Figure 5 against time (min) before and after the application of caffeine. Open circles represent the mean values at single time points in different experiments ($n = 3$) and the vertical lines show s.e.mean. (b) Typical recordings of aequorin luminescence (upper trace), current monitor (middle trace), and twitch tensions (lower trace) and the effects of 30 μM dantrolene and 5 mM caffeine in mouse diaphragm muscle stimulated directly. Dantrolene decreased aequorin luminescence and weakly suppressed the caffeine-induced increase in aequorin luminescence.

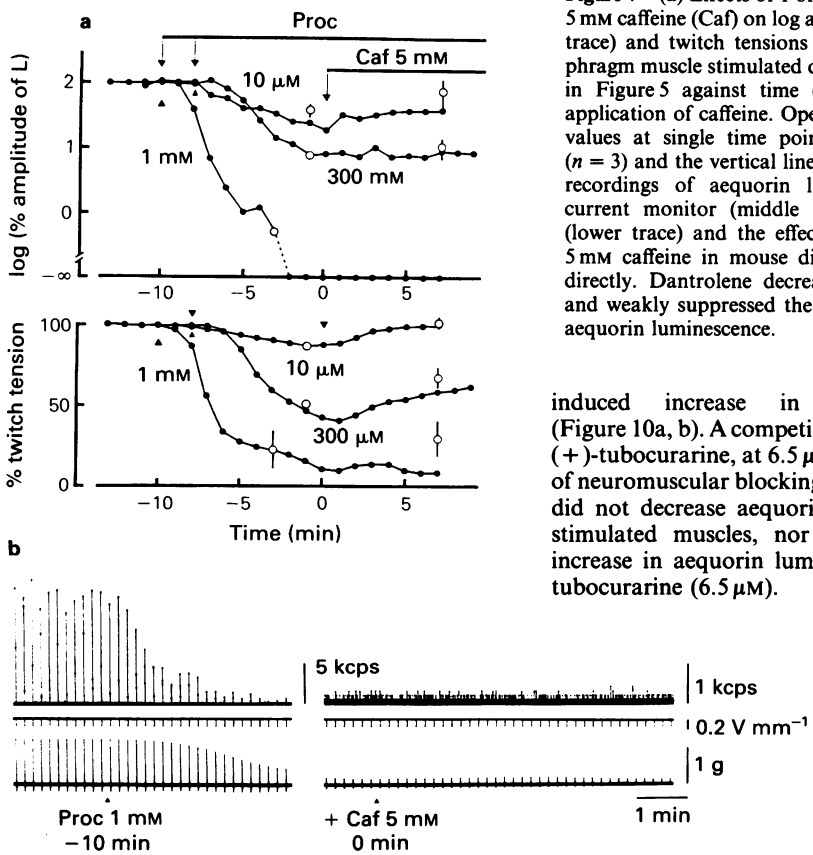

Figure 8 (a) Effects of 10 μM, 30 μM or 1 mM procaine (Proc) and 5 mM caffeine (Caf) on log aequorin luminescence (upper trace) and twitch tensions (lower trace) in mouse diaphragm muscle stimulated directly. Data were plotted as in Figure 5 against time (min) before and after the application of caffeine. Open circles represent the mean values at single time points in different experiments ($n = 3$) and the vertical lines show s.e.mean. (b) Typical recordings of aequorin luminescence (upper trace), current monitor (middle trace), and twitch tensions (lower trace) and the effects of 1 mM procaine and 5 mM caffeine in mouse diaphragm muscle stimulated directly.

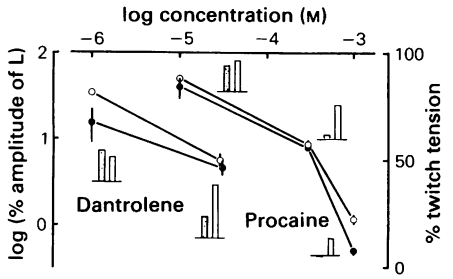

Figure 9 The effect of different concentrations of dantrolene (left trace), and procaine (right trace) on the aequorin luminescence (●) and twitch tension (○) of directly-stimulated diaphragm muscle of mouse. Columns show the increasing changes in log aequorin luminescence (stippled columns) or twitch tensions (open columns) caused by 5 mM caffeine in the presence of dantrolene or procaine. These data were replotted from Figures 7a and 8a.

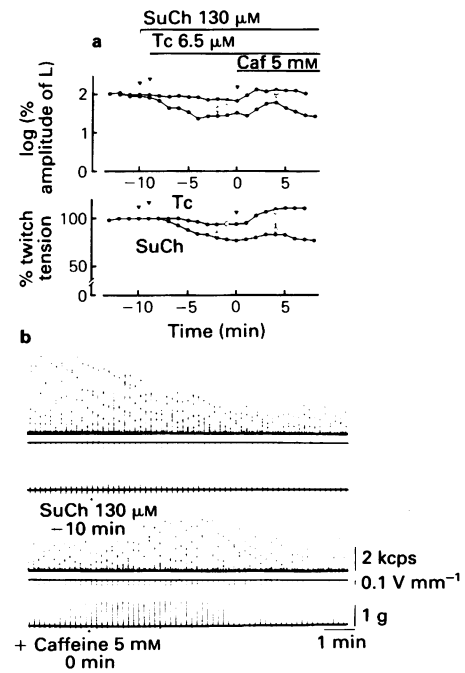

Figure 10 (a) Effects of 130 μM suxamethonium (SuCh) and 6.5 μM (+)-tubocurarine (Tc) and 5 mM caffeine (Caf) on aequorin luminescence transients (upper trace) and twitch tensions (lower trace) in mouse diaphragm muscles stimulated directly. Data (●) were plotted as in Figure 5 against time (min) before and after the application of caffeine. Open circles represent the mean values at a single time point in different experiments ($n = 3$) and the vertical lines show s.e.mean. (b) Typical recordings of aequorin luminescence (upper trace), current monitor (middle trace), and twitch tension (lower trace) with 130 μM SuCh alone or in the presence of 5 mM caffeine in mouse diaphragm muscle stimulated directly.

Discussion

A parallelism between aequorin luminescence and contractile tension was seen during direct (single shock) stimulation of mouse diaphragm muscles. Under the action of skeletal muscle relaxants, Ca^{2+}-related aequorin luminescence decreased more sharply than twitch tensions. Blinks *et al.* (1982) found that the maximum slope was approximately 2.5 on log (Ca^{2+} concentration) − log (L/L_{max}) plots for aequorin. In the present study, the aequorin luminescence as expressed by logarithmic relative peak luminescence showed a good correlation with % twitch tension except for a few effects of procaine on the caffeine-induced increase in twitch tension.

In order to obtain clear aequorin signals, it was more convenient to use 50 ms current pulses at low intensity than to use shorter current pulses. In practice, a stimulus shorter than 50 ms produced lower amplitudes of luminescence, but a stimulus of 50 ms induced a substantial amplitude of luminescence, even if such a long current pulse produced more than one action potential. Furthermore, single fibres were not used in the present study. It is possible that the results obtained contain contributions from fibres of different types with different time courses of changes in $[Ca^{2+}]_i$ and twitch tension.

In cardiac Purkinje fibres or ventricular muscles, an initial, transient positive inotropic effect of caffeine is accompanied by a transient increase in the aequorin signal (Hess & Wier, 1984; Konishi *et al.*, 1984). Frank (1982) demonstrated, in his study with isolated skeletal muscles, that K^+-induced contractures require extracellular Ca^{2+} ions, and suggested that twitch contraction can utilize membrane-bound 'trigger' Ca^{2+} ions. In our study, caffeine-induced twitch potentiation was associated with an increase in evoked aequorin luminescence, which seemed to be caused by triggering of the membrane-bound Ca^{2+}. On the other hand, neither the caffeine-induced contracture nor the K^+-induced contracture was associated with an increase in evoked aequorin luminescence. These contractures may instead be related to resting aequorin luminescence. The source of Ca^{2+} inducing the twitch contraction may be different from that inducing the contracture. The source for the latter is the sarcoplasmic reticulum. The source for the former may be membrane-bound Ca^{2+}, or some other site in the sarcoplasmic reticulum as suggested by Konishi *et al.* (1984).

Dantrolene sodium inhibits excitation-contraction coupling (Ellis & Carpenter, 1972; Ellis & Bryant, 1972; Putney & Bianchi, 1974), reduces the amount of Ca^{2+} released from the intracellular Ca^{2+} storage site (Hainaut & Desmedt, 1974), and weakly inhibits caffeine- and Ca^{2+}-induced Ca^{2+} release (Endo & Yagi, 1982). On the other hand, procaine inhibits the

Ca^{2+}-induced Ca^{2+} release mechanism (Ford & Podolsky, 1972), does not inhibit the 'depolarization'-induced release of Ca^{2+}, and blocks the caffeine-induced contracture of intact muscle fibres (Thorens & Endo, 1975). In the present study, dantrolene and procaine decreased aequorin luminescence transients. Dantrolene, however, weakly depressed and procaine abolished the caffeine-induced increase in evoked Ca^{2+} transients. From the experimental level of aequorin luminescence transients in skeletal muscle membranes, we also confirmed that dantrolene or procaine decrease the release of membrane-bound Ca^{2+}, and that procaine also inhibits caffeine-induced Ca^{2+} release.

Eusebi *et al.* (1983) suggested that the aequorin signal reflects intracellular Ca^{2+} release, and that this release is inactivated during prolonged depolarization. In fact, both aequorin luminescence and twitch tension were decreased during depolarization produced by high K^+ solution. The aequorin signal was depressed by SuCh, but was not affected by (+)-tubocurarine. In the present study, the depolarizing neuromuscular blocking agent SuCh decreased aequorin luminescence. These effects, therefore, do not seem to be caused only by the prolonged depolarization, and our results may be useful for clarifying the Ca^{2+}-related mechanism of depolarizing neuromuscular blocking actions in normal or diseased muscles.

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