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Mode of inhibitory actions of acute and chronic chloroquine administration on the electrically stimulated mouse diaphragm *in vitro*

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1 The effects of bath applied chloroquine (Chlo) and of acute and chronic Chlo administration on skeletal muscle reactivity to electrical stimulation and to drugs have been studied on mouse hemidiaphragm preparations *in vitro*.

2 Chlo (0.15–150 μM) produced a concentration-dependent inhibition of twitch and tetanic contractions due to direct muscle stimulation (MS). Acute and chronic administration of Chlo (45 mg kg^{-1}, i.p. daily, for 3–28 days) progressively shifted the concentration-response curve to bath-applied Chlo to the right, with maximum effect occurring from day 14 of Chlo pretreatment.

3 Acute and chronic administration of Chlo decreased the twitch and tetanus tension, raised the minimal fusion frequency (MFR) for tetanic contraction to occur and did not alter the twitch/tetanus tension ratio. Tetanus tension unlike twitch tension was not significantly decreased on day 3.

4 Caffeine (5–500 μM) – and isoprenaline (0.001–0.8 μM)-induced potentiations of twitch contraction were attenuated in a concentration-dependent manner by bath-applied Chlo and by acute and chronic administration of Chlo. Higher concentrations of caffeine (0.1–5 μM) and KCl (10 mM–130 mM) produced contracture of the muscle which was sensitive to inhibition by Chlo (50–150 μM). Moreover, the spike contractions superimposed on caffeine contracture were more sensitive to the inhibitory effect of Chlo than the contracture.

5 The inhibitory effects of dantrolene sodium and (+)-tubocurarine on MS and on indirectly stimulated hemidiaphragm respectively were not significantly altered by acute and chronic administration of Chlo. In contrast, the inhibitory concentration-response curve to procaine was shifted to the right.

6 The inhibitory effect of bath-appled Chlo, or acute and chronic pretreatment on twitch tension (MS) was not significantly antagonized by stepwise increase in extracellular Ca^{2+} (0.05–2.5 mM). Sodium thiocyanate (1–5 mM) reversed in a concentration-dependent manner the inhibitory effects of Chlo.

7 Complete recovery of twitch contractions occurred after 3 days of stopping daily Chlo administration, with partial recovery to tetanic tension after 28 days and no recovery of MFR. The reactivity of the diaphragm to bath applied Chlo was progressively restored, whereas the tension curve area to caffeine and KCl was still attenuated even 28 days after stopping Chlo pretreatment.

8 It is concluded that acute and chronic Chlo administration results in changes in reactivity of the hemidiaphragm muscle to electrical stimulation and to drugs such that there is a decrease in muscle strength and tolerance to Chlo *in vitro*. These effects are dependent on its direct inhibitory action on skeletal muscle and may result from interference with Ca^{2+} mobilization within the muscle.

Introduction

Chloroquine (Chlo) is a 4-aminoquinoline used for the treatment and recently for prophylaxis of malaria (Rollo, 1980). In addition it has been used extensively for decades in the treatment of rheumatoid arthritis, systemic and lupus erythematosus and other collagen diseases (McChesney & Rothfield, 1964; Hughes *et al.*, 1974; Magnussen & Olivarius, 1977; Stillman, 1981). In such pathological conditions in man, high daily doses (250–500 mg, or more) have been found necessary and for prolonged periods (Bagnall, 1957; Hughes *et al.*, 1974; Magnussen & Olivarious, 1977). Such doses are much larger than those necessary for therapy of acute malarial attacks (Rollo, 1980). These regimens have frequently resulted in important side effects, particularly changes in the cardiovascular function manifested as congestive heart failure and cardiovascular collapse (Thompson & Werbel, 1972; Hughes *et al.*, 1974; Magnussen & Olivarius, 1977), retinopathy (Thompson & Werbel, 1972) and myopathy of the skeletal muscle (Lüllman-Rauch, 1979).

Skeletal muscle myopathy associated with chronic Chlo administration has been confirmed by electron microscopic studies (Stauber *et al.*, 1981; Ette & Essien, 1986) and may be the cause of decreased muscle strength following Chlo administration (Rollo, 1980). It has been reported that Chlo can accumulate in lysosomes, smooth endoplasmic reticulum and sarcoplasmic reticulum. Such an effect usually results in ultrastructural changes in some cells which appear as swellings, autophagic vacuoles, vacuolar myopathy and degeneration of myofibrils (Smith & O'Grady, 1966; MacDonald & Engel, 1970; Oberc & Engel, 1977; Ayitey-Smith & Gbewonyo, 1977; Ette & Essien, 1986). The present experiments were designed to determine the effects on skeletal muscle of acute and chronic administration of Chlo with special emphasis on the following: (i) responsiveness of the diaphragm muscle to electrical stimulation and drugs; (ii) mechanism of action of Chlo-induced decrease in muscle tension; (iii) reversibility of the effect of chronic administration of Chlo and (iv) reactivity of some skeletal muscle relaxants following acute and chronic administration of Chlo.

Methods

Swiss Webster mice (30–40 g) of either sex were stunned and killed by exsanguination. The diaphragm with the phrenic nerve was dissected out according to the method of Bülbring (1946) and placed under an initial load of 2 g in an organ bath

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containing double glucose Tyrodes solution of the following composition (mM): Na^+ 149.2, K^+ 2.7, Ca^{2+} 3.6, Mg^{2+} 2.1, Cl^- 145.3, $H_2PO_4^-$ 0.4, HCO_3^- 11.9 and glucose 10. The bath was maintained at room temperature (approximately 20°C). The pH of the solution was 7.3–7.4. Contractions of the hemidiaphragm were elicited either indirectly via the phrenic nerve (NS) or directly (MS) with rectangular pulses of 0.5 ms at a frequency of 1 Hz and supramaximal voltage. A Grass S 88 stimulator connected to a Grass SIU5 stimulus isolation unit pulse generator was used to deliver the rectangular pulses. Tetanizing stimuli were delivered at 20–60 Hz for 5 s. Isometric contraction of the muscle to electrical stimulation or to drugs was recorded with a force-displacement transducer (Grass FT.03) and displayed on a Grass ink writing curvilinear polygraph (Model 7D, Grass Instruments, Quincy, U.S.A., MA). The minimal fusion frequency (MFR) was obtained in any one experiment by stimulating the muscle directly (MS) for 1 s. The muscles were stimulated with trains of pulses 2 ms duration, with 5 Hz increases in frequency starting at 10 Hz every 5 min with supramaximal voltage until the fusion frequency was identified. The muscles were pre-equilibrated for 60–90 min before use. During that time, the physiological solution in the organ bath was replaced every 20 min and the passive tension re-adjusted when necessary.

Concentration-response curves and activity profile for chloroquine

Concentration-response curves to Chlor were obtained by cumulative addition of graded concentrations. Since the response to Chlo developed slowly each concentration of Chlo was allowed to remain in contact with the preparation until a plateau response had been obtained before the preparation was challenged with an additional concentration of Chlo. In experiments in which the time course of effect of Chlo was investigated, a pair of phrenic nerve-hemidiaphragm preparations from the same animal was used, with one acting as control, while the other was treated with the appropriate concentration of Chlo. Chloroquine was then added to the tissue bath 1–2 min after the start of electrical stimulation and allowed to act for 30–90 min or until maximum inhibition had been obtained. The effectiveness of NS and MS was assessed in the presence of (+)-tubocurarine (5 μM). Twitch tension to NS was usually abolished while MS was only slightly reduced (5–10%) and (+)-tubocurarine was present in experiments in which the effect of MS was studied.

Concentration-response curves for dantrolene sodium, procaine, (+)-tubocurarine, caffeine and potassium chloride

Concentration-response curves to the inhibitory effect of dantrolene sodium, procaine and (+)-tubocurarine and to the stimulatory effect of caffeine were obtained by cumulative addition of graded concentrations of each agent. The concentration-response curve for potassium chloride was established by sequential addition of an appropriate concentration which was left in contact with the tissue until a maximum response for that concentration had been obtained. IC_{50} and IC_{100} are concentrations of agents producing 50 and 100% inhibition of twitch contractions, respectively.

Chloroquine pretreatment

Preliminary studies indicated that tolerated daily dose of chloroquine diphosphate in mouse ranged from 45–50 mg kg^{-1}, i.p. In studies in which mice were pretreated with Chlo, the animals were divided into two groups with one group receiving Chlo and the other acting as control. The chloroquine pretreated group was given daily i.p. injections of 45 mg kg^{-1} of Chlo in isotonic saline (NaCl, 0.9% w/v). Chlo was administered daily for up to 4 weeks and animals were killed on day 0 (control), 3, 7, 14, 21 and 28. They were allowed free access to water and mouse pellets throughout the period of drug administration. In another series of experiments to study the effect of recovery from acute and chronic Chlo administration, it was administered for 4 weeks. The animals were then allowed to recover from the effect of Chlo and killed 3, 7, 14 and 28 days after the last Chlo administration. Muscle tension measurement was calculated in units of tension g^{-1} tissue mass which was estimated at the conclusion of each experiment.

Results are expressed as mean values ± s.e.mean, based on at least four experiments. Significance was determined by the Student's t test for paired data. A probability level of $P < 0.05$ was considered significant.

Materials

The following drugs were used: chloroquine diphosphate (Sigma), caffeine, sodium thiocyanate (BDH), dantrolene sodium (Norwich Eaton Pharmaceuticals, New York, U.S.A.), procaine HCl (Sigma). All drugs were made up in 0.9% w/v sodium chloride solution and concentration expressed as the salt weight.

Results

Effect of chlorquine on twitch contraction to electrical stimulation of mouse diaphragm

Chlo reversibly inhibited twitches in response to both nerve stimulation (NS) and to direct muscle stimulation (MS) in a concentration-dependent manner (Figure 1). Twitch contraction to NS was more sensitive to the inhibitory effect of Chlo (0.15–4.53 μM) with an IC_{50} of 1.73 ± 0.12 μM ($n = 5$) as compared to MS (0.45–50 μM) with an IC_{50} of 5.10 ± 0.16 μM ($n = 6$). In another series of experiments the activity profile of the inhibitory effect of Chlo at the IC_{50} and IC_{100} and their

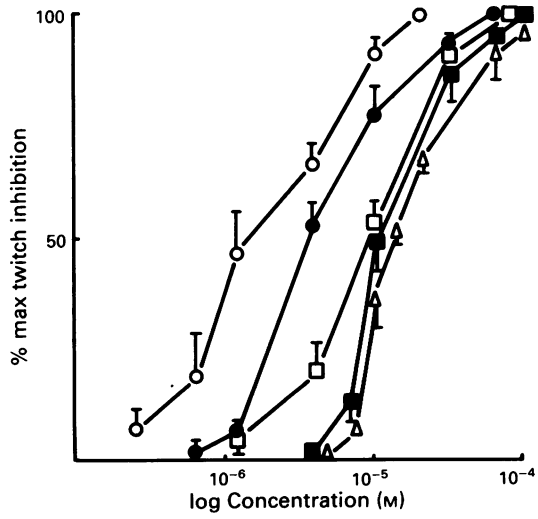

Figure 1 Effect of chlorquine (Chlo) on twitch tension due to direct muscle stimulation of the mouse diaphragm in normal non-treated and in acute and chronic Chlo pretreated preparations. Concentration-response curve to Chlo in non-treated diaphragm (day 0) (○); after pretreatment with Chlo for 3 days (●); 7 days (□); 14 days (■) and 28 days (△). The responses to Chlo are expressed as a percentage of maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines show s.e.mean.

reversal on washing were studied in order to identify any changes in muscle responsiveness to acute and chronic Chlo pretreatment. The response to MS was maximally inhibited at 2–3 min (IC_{50}) and 9–22 min (IC_{100}) while the response to NS was inhibited after 5–8 min (IC_{100}). Recovery from the effect of Chlo was faster for NS than MS and the data obtained (mS, day 0) from recovery of 5 muscle preparations following 10 min exposure to Chlo (50 μM) show that it required 25–30 min and 50–60 min to reverse the twitch inhibition to NS and MS to 100%, respectively, following washing with Chlo-free Tyrode solution.

Effect of acute and chronic chloroquine pretreatment on twitch response to electrical stimulation and on chloroquine concentration-response curve

Since Chlo is known to cause myopathy of the skeletal muscle which is associated with degenerative changes of the myofibrils, the effect of prolonged pretreatment with Chlo was studied to assess its effect on muscle strength. Chlo (0.75–100 μM) reversibly depressed the twitch height to NS and MS in preparations obtained from mice pretreated daily with Chlo (45 mg kg^{-1}, i.p.) for 3–28 days. Acute and chronic administration of Chlo shifted the concentration-response curve of Chlo to the right with maximum shift occurring from day 7 (Figure 1 and Table 1). Chlo pretreatment changed the concentration-response relations to Chlo, increasing the maximal, and minimal inhibitory concentrations for Chlo. The activity profile of Chlo was also altered being dependent on length of pretreatment with Chlo. The time to onset (Table 1) and maximum inhibition to MS was progressively increased with latent periods of 8.25 ± 2.70 and 62.4 ± 5.36 s on day 3 and day 28 pretreated diaphragms respectively, compared to control. Furthermore, the time to complete recovery of twitch amplitude (MS) after washout of Chlo was also dependent on the length of pretreatment taking 50–60 and 35–40 min for day 7 and day 28 pretreated diaphragms respectively. Recovery from the inhibitory effect of Chlo in these preparations was faster in NS than in MS. Moreover, recovery to NS and MS was faster in pretreated than in normal diaphragm.

Effect of acute and chronic chloroquine pretreatment on twitch and tetanus, and twitch/tetanus ratio

Chlo (3–15 μM) significantly depressed tetanus to NS and MS (Table 2) while higher concentrations (30–100 μM) abolished tetanic contractions. Chlo depressed to an equal extent the twitch and tetanic tension as indicated by the twitch/tetanus ratio of 0.10 ± 0.03 (Table 2). In diaphragms obtained from Chlo-pretreated animals, twitch tension and tetanus were significantly reduced by 15–70% and 20–65% respectively (Table 2), the effect being maximal from day 7 of pretreatment. Although Chlo-pretreatment depressed both twitch and tetanus tension to equal extent in day 7, 14 and 28 treated diaphragms, the decrease was larger in the twitch tension response in day 3 diaphragms as indicated by the decrease in the ratio of twitch/tension (Table 2).

Effect of acute and chronic chloroquine pretreatment on minimal fusion frequency

The minimal fusion frequency (MFR) was studied in non-Chlo-treated and in acute and chronic Chlo pretreated diaphragms. Throughout each experiment the muscles were stimulated with trains of pulses (15–18 Hz for 1 s) every 5 min so as to produce incomplete tetanic contraction (MFR). Chlo (5–10 μM) induced a concentration-dependent increase in the MFR of 40–50% from a mean control value of 18.2 ± 1.12 Hz in non-Chlo pretreated preparations. Acute and chronic Chlo pretreatment for 3–28 days also increased the MFR by 40–60%, an effect which was not dependent on the length of Chlo pretreatment (Table 2). In another series of experiments the effect of IC_{50} of Chlo (5.0 μM) on MFR was examined in muscles obtained from Chlo pretreated mice. Chlo did not increase further the MFR in these preparations (Table 2).

Effect of sodium thiocyanate and increase in extracellular calcium concentration on the inhibitory effect of chloroquine and acute and chronic chloroquine pretreatment on twitch tension

The depressant effect of Chlo (1.50–150 μM) on twitch tension (MS) was reversed in a concentration-dependent manner by

Table 1 Effect of chloroquine (IC_{50}) on twitch/tetanus tension ratio (Tw/Tt) and minimal fusion frequency (MFR) on chloroquine pretreated mouse diaphragm

Pretreatment day	*Tension (g)*				*In the presence of chloroquine (5.0 μM)* *Tension (g)*			
	Twitch	*Tetnus*	*Tw/Tt*	*MFR* (Hz)	*Twitch*	*Tetanus*	*Tw/Tt*	*MFR* (Hz)
Control	1.14 ± 0.62	13.5 ± 1.32	0.10 ± 0.03	18.5 ± 1.12	0.63 ± 0.17	5.33 ± 1.25	0.12 ± 0.03	26.7 ± 2.89
Day 3	0.77 ± 0.13*	11.7 ± 1.67	0.67 ± 0.01	27.2 ± 2.16*	0.77 ± 0.25	7.23 ± 2.54	0.11 ± 0.05	31.6 ± 1.92
Day 7	0.39 ± 0.12*	4.43 ± 0.44*	0.09 ± 0.03	28.5 ± 1.89*	0.42 ± 0.05	3.53 ± 1.66	0.12 ± 0.05	31.6 ± 2.01
Day 14	0.53 ± 0.06*	3.63 ± 0.73*	0.10 ± 0.04	27.1 ± 1.22*	0.55 ± 0.06	3.07 ± 1.28	0.14 ± 0.07	29.1 ± 1.86
Day 28	0.50 ± 0.11*	5.10 ± 0.12*	0.10 ± 0.02	26.4 ± 1.84*	0.62 ± 0.10	4.59 ± 1.44	0.14 ± 0.04	32.3 ± 2.12

* Significantly different from their respective controls $P < 0.05$.
Results are expressed as mean ± s.e.mean from at least 4 experiments.

Table 2 Chloroquine IC_{50} values and recovery profile on normal and chloroquine pretreated diaphragm

Pretreatment day	IC_{50} (μM)	*Time to inhibitory response* (s)	IC_{50} (μM)	*Recovery after 28 days pretreatment* *Tension (g)*			
				Twitch	*Tetanus*	*Tw/Tt*	*MFR* (Hz)
Control	5.10 ± 0.16	0.14 ± 0.05	25.8 ± 2.96	0.50 ± 0.11	5.10 ± 0.12	0.10 ± 0.03	26.4 ± 1.84
Day 3	8.13 ± 0.25	8.25 ± 2.70	26.3 ± 4.04	1.02 ± 0.07	8.99 ± 0.89	0.11 ± 0.01	26.7 ± 2.87
Day 7	10.3 ± 1.72	23.3 ± 1.92	18.7 ± 1.58	1.10 ± 0.08	8.67 ± 1.15*	0.12 ± 0.02	26.7 ± 2.35
Day 14	23.8 ± 2.82	27.8 ± 2.70	8.50 ± 0.62	1.01 ± 0.05*	8.73 ± 0.64*	0.12 ± 0.02	25.0 ± 3.01
Day 28	25.8 ± 2.96	62.4 ± 5.36	8.01 ± 0.30	1.06 ± 0.04*	9.01 ± 0.08*	0.11 ± 0.01	25.5 ± 29.6

* Significantly different from their respective controls $P < 0.05$;
Controls for recovery data are results obtained following Chlo pretreatment for 28 days;
Results are expressed as mean ± s.e.mean from at least 4 experiments. MFR – Minimal fusion frequency.

thiocyanate ions, SCN^{-1} (1–5 mM). The effects of Chlo (5–100 μM) were restored by 35–50% in the presence of 1 mM thiocyanate ion. The effect of SCN^- was rapid in onset (5–10 s) but tension returned to the original depressed level when the preparation was returned to Tyrode solution containing only Chlo. In Chlo pretreated diaphragm (day 28), the SCN^{-1} ion (1 mM) also restored the depressed twitch tension substantially to near control values of non-Chlo-treated diaphragms. When Chlo (100 μM) was added to Chlo pretreated preparations, the depressed twitch tension was also markedly reversed (30–40%) by the SCN^- ion (1 mM). In order to elucidate further the mechanism of the inhibitory action on the twitch tension, the effect of increase in $[Ca^{2+}]_o$ on the twitch tension was investigated. Stepwise increase in $[Ca^{2+}]_o$ of 0.05–2.5 mM did not affect the Chlo-induced inhibition in normal and Chlo (day 3 to day 28) pretreatment diaphragms. However, a Ca^{2+} concentration of 0.02 mM increased by 15–20% the twitch response to MS, the maximal effect occurring after 30 min of incubation.

Effect of chloroquine on caffeine- and isoprenaline-induced increase in twitch tension and on caffeine- and potassium-induced contracture

In order to elucidate further the possible effect of Chlo on Ca^{2+} sequestration, procedures known to interfere with Ca^{2+} mechanism were studied in normal and Chlo pretreated preparations. Caffeine (5–500 μM) and isoprenaline (0.001–0.8 μM) increased the twitch tension to direct muscle stimulation (MS) in a concentration-dependent manner (Figure 2). Chlo (2.5–150 μM) caused a concentration-dependent attenuation of the responses to caffeine (Figure 2a) and isoprenaline (Figure 2b). Propranolol (1.0 μM), reduced and/or abolished the twitch potentiation induced by isoprenaline but was without effect on the caffeine response. Caffeine (0.1–5 mM; Figure 3) and KCl (10 mM–130 mM) produced contracture of the mouse diaphragm in a concentration-dependent manner. The contracture produced by caffeine was superimposed with twitch contractions and Figure 4a shows the activity profile to caffeine. Potassium (20 mM–50 mM) induced a sustained contracture (lasting over 60 min) but higher concentrations (80 μM) produced a non-sustained contracture which usually declined to basal tension in 2–3 min in the presence of KCl (Figure 5a). Chlo (50–150 μM) produced a concentration-dependent attenuation of responses to caffeine and KCl. The twitch contraction to caffeine was more sensitive to inhibition by Chlo than was the contracture, being abolished at a Chlo concentration of 50 μM (Figure 4b). Chlo increased the latent period for caffeine- and KCl-induced contracture by 1–3 min, had no effect on the time for KCl contracture to fall to basal tension, but decreased that to caffeine by 10–20 min.

Effect of acute and chronic chloroquine pretreatment on caffeine, isoprenaline and KCl response

Chlo pretreatment daily for 3 to 28 days progressively shifted the concentration-response curve to caffeine and KCl to the right, the shift being maximum from day 14 (Figure 2a and b) but the maximum contraction to caffeine was not significantly depressed. Furthermore, the twitch contractions induced by caffeine (0.1–5 mM) were also attenuated and/or abolished by Chlo pretreatment. The decrease in the amplitude and frequency of twitch contraction was dependent on length of pretreatment with Chlo and was abolished from day 14 (Figure 4c–e). The twitch contraction was more sensitive to inhibition than was the contracture. The potentiation of twitch tension induced by caffeine (5–500 μM) was attenuated by pretreatment with Chlo for 7 to 28 days resulting in a significant shift ($P < 0.05$) to the right of the concentration-response curve. The shift was maximum after administration of Chlo for 14 days (Figure 2a). The effect of pretreatment with Chlo on isoprenaline-induced potentiation of twitch

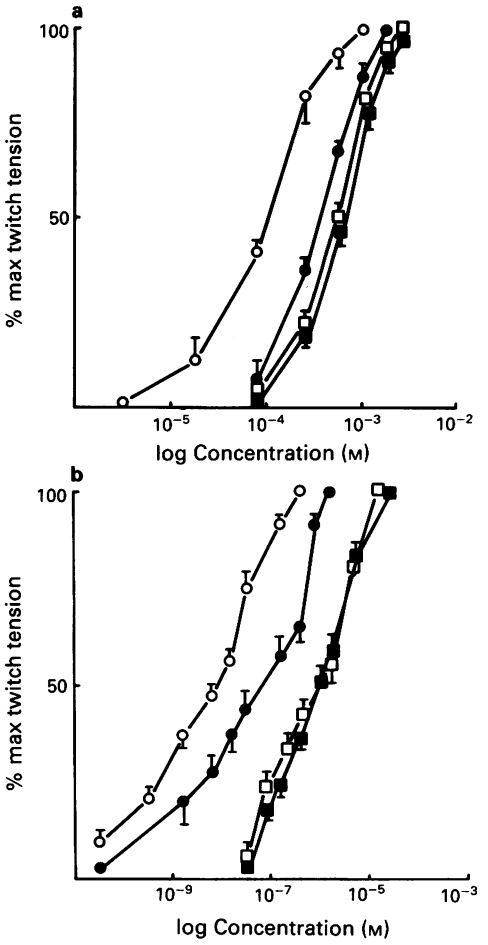

Figure 2 Effects of acute and chronic chloroquine (Chlo) administration on caffeine- and isoprenaline-induced potentiation of twitch contractions due to direct electrical stimulation of mouse diaphragm preparations. Concentration-response curve to caffeine (a) and isoprenaline (b) in non-treated diaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 7 days (●); 14 days (□) and 28 days (■). The responses to Chlo are expressed as a percentage of maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean. Note the progressive depression of the caffeine- and isoprenaline-induced twitch potentiation.

tension was qualitatively similar to that observed with caffeine (Figure 2b). The inhibitory effect of pretreatment with Chlo was dependent on the length of administration of Chlo with maximum shift of the concentration-curve occurring from day 14. Chlo pretreatment markedly altered the activity profile of KCl (130 mM; Figure 5a–d) on preparations obtained from animals treated with Chlo for 7–28 days. The duration of KCl contracture was prolonged, with the contracture being sustained for 8.5 ± 0.4 min compared to the control of 2.5 ± 0.2 min. Although the amplitude of the contracture was attenuated by $60 \pm 5.8\%$, the tension curve area (area under the contracture) was not significantly different from control of 550 ± 40.5 g s^{-1}.

Effect of acute and chronic chloroquine pretreatment on the inhibition of twitch tension by dantrolene sodium, procaine and (+)-tubocurarine

In order to evaluate the specificity of effect on Ca^{2+} sequestration of acute and chronic Chlo pretreatment on the diaphragm, drugs which are known to inhibit twitch and tetanus tension via different mechanisms on Ca^{2+} mobilization were examined in Chlo pretreated preparations. Dantrolene (0.1–10 μM) and procaine (1.0–100 μM; Figure 6) produced concentration-dependent inhibition of twitch tension due to

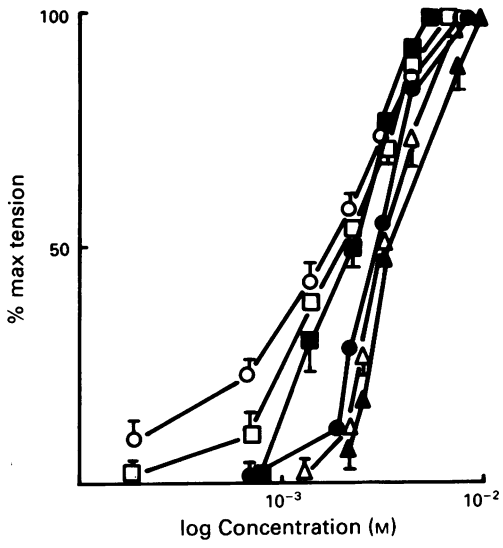

Figure 3 Effects of chloroquine added to the bath and of acute and chronic chloroquine (Chlo) administration on the contracture induced by caffeine in mouse diaphragm preparations. Concentration-response curves to caffeine in non-treated diaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 3 days (□); 7 days (■); 14 days (△) and 28 days (▲). The concentration-response curves to caffeine in the presence of Chlo (100 μM) are represented (●). The responses to caffeine are expressed as a percentage of maximal contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean.

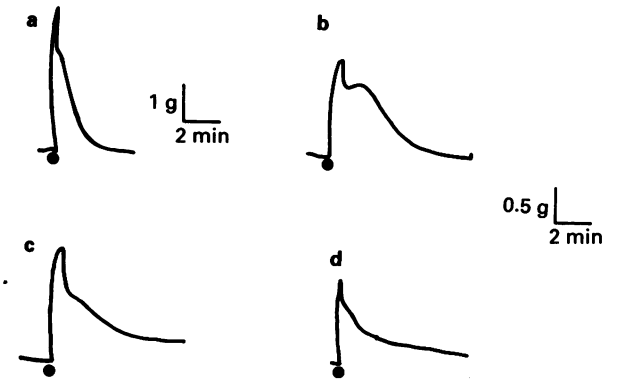

Figure 5 Effects of chloroquine (Chlo) added to the organ bath and of acute and chronic administration of Chlo on potassium-induced contraction in mouse hemidiaphragm preparation. Black dots indicate addition of 130 mM KCl. (a) Potassium contraction (130 mM) declined to basal tension in the presence of potassium in the bath. (b) Chlo (50 μM) decreased the contraction to potassium but prolonged the duration of the contraction. (c–d) Chlo administration (45 mg kg^{-1}, i.p.) daily for 7 days (c) and 28 days (d) on potassium-induced contraction. Chlo pretreatment decreased and prolonged the contraction to potassium.

MS. In Chlo pretreated preparations dantrolene inhibited the twitch contractions to MS in a concentration-dependent manner (Figure 6b) and pretreatment for 3–14 days did not alter the concentration-response relations i.e. the slope or the maximal or minimal effective concentrations for dantrolene. Although the IC_{50} values for dantrolene obtained before and after pretreatment with Chlo were not significantly different (day 3 to day 14), there was a shift to the right in day 28 pretreated diaphragms ($P < 0.05$). The time to onset and maximum inhibition of MS was not altered in any Chlo pretreated preprations (day 7 to day 28). Similarly procaine inhibited the twitch tension to MS following Chlo pretreatment for 3–28 days. However, the concentration-response curve to procaine was shifted significantly to the right ($P < 0.05$), an effect which was dependent on the length of pretreatment with Chlo (Figure 6a). Moreover, the rate of inhibition of twitch contraction by procaine was faster in the non-Chlo pretreated diaphragm (15–20 min) compared to pretreated preparations (30–40 min). (+)-Tubocurarine (0.1–5 μM) attenuated the twitch response to NS without significantly affecting that to MS. The concentration-effect relation was not altered by pretreatment with Chlo for 3–28 days (Figure 6c). Furthermore, pretreatment did not change the activity profile to (+)-tubocurarine; the time to maximum inhibition was not different from the control of 2.8 ± 0.35 min.

Effect of recovery from acute and chronic chloroquine pretreatment on tetanus, minimal fusion frequency, caffeine and KCl

In order to ascertain whether the effect of acute and chronic administration of Chlo observed in this study was reversible, a series of experiments was conducted on muscles obtained from

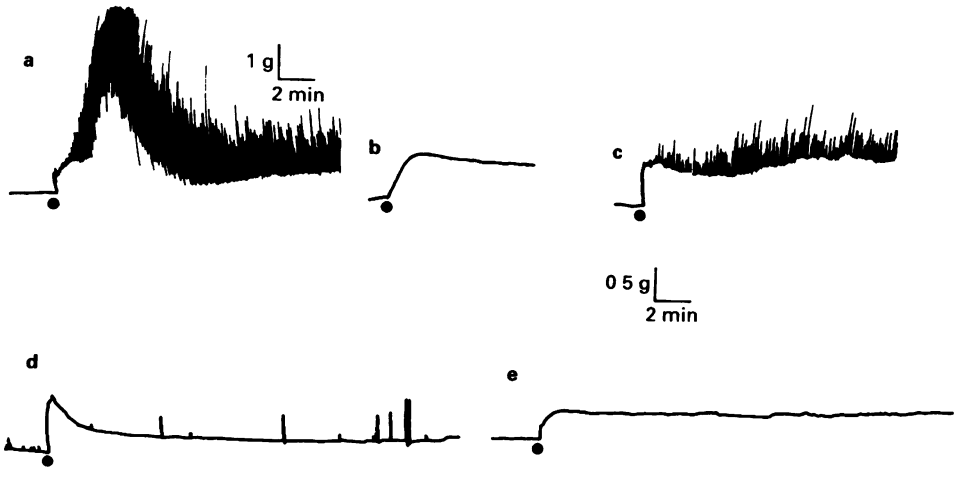

Figure 4 Effects of chloroquine (Chlo) added to the organ bath and of acute and chronic administration of chloroquine on caffeine-induced contracture in mouse hemidiaphragm preparation. (a) Caffeine-induced contracture (5 mM) was superimposed by spike contractions. (b) Chlo (50 μM) abolished the spike contractions and produced a sustained contraction to caffeine. (c–e) Chlo administration (45 mg kg^{-1}, i.p.) daily for 7 days (c), 14 days (d) and 28 days (e) on caffeine-induced contracture. Addition of 5 mM caffeine indicated by a black dot in each section. Chlo pretreatment produced a parallel decrease in the amplitude and frequency of spike contractions as well as a decrease in the contracture.

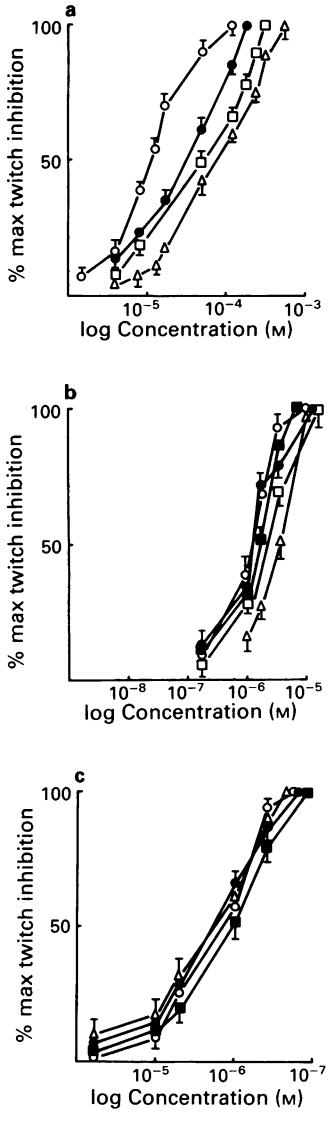

Figure 6 Effects of acute and chronic chloroquine (Chlo) administration on dantrolene sodium-, procaine- and (+)-tubocurarine-induced inhibition of twitch contraction due to electrical stimulation of mouse hemi-diaphragm preparations. Concentration-response curves to procaine (a), dantrolene (b) and (+)-tubocurarine (c) in non-treated hemidiaphragm (day 0, ○); after pretreatment with Chlo (45 mg kg^{-1}, i.p.) daily for 3 days (●); 7 days (□); 14 days (■) and 28 days (△). The responses to the inhibitory agonists are expressed as a percentage of the maximal depression of twitch contraction. Each symbol is the mean of at least four observations and vertical lines indicate s.e.mean. Experiments with (+)-tubocurarine were conducted in hemidiaphragm preparations stimulated indirectly via the phrenic nerve. Chlo pretreatment for 3–28 days selectively induced tolerance to the inhibitory effect of procaine.

mice pretreated with Chlo for 28 days and then allowed to recover from the effect of pretreatment. Reactivity of the muscle to the inhibitory effect of Chlo on twitch tension was gradually restored to normal as indicated by the progressive shift to the left of the concentration-response curve to Chlo (Table 2). Partial recovery was observed from day 7 with an IC_{50} of 18.7 ± 1.58 μM. The recovery profile to Chlo indicates that the time to onset and maximal inhibition of twitch contractions returned progressively to normal values with complete recovery occurring from day 14. Twitch and tetanus tension exhibited differential recovery. Twitch tension recovered to control tension after 3 days of stopping Chlo administration, whereas tetanus tension recovered only partially even after 28 days (Table 2). The MFR showed no recovery throughout the 28 days of observation (Table 2). The responsiveness of the diaphragm to the stimulatory effect of caffeine and KCl was partially restored with time after stopping the daily administration of Chlo. The contracture response to caffeine recovered faster than the twitch contractions with evidence of recovery being observed after 7 days of stopping pretreatment with Chlo. The tension curve area for caffeine after 14 and 28 days was 4.8 ± 0.25 and 5.2 ± 0.42 g min^{-1} respectively compared to a control of 24.5 ± 2.86 g min^{-1}. The tension curve area for KCl after 14 and 28 days was 6.34 ± 1.86 and 8.55 ± 1.98 g min^{-1} respectively compared to a control of 10.2 ± 1.58 g min^{-1}.

Discussion

The inhibitory effect of Chlo on twitch and tetanus tension and the increase in MFR is suggestive of an interference with Ca^{2+} sequestration normally necessary for excitation-contraction in skeletal muscle (Anderson, 1978; Frank, 1980). This effect of Chlo was not due to direct action on the contractile element e.g. troponin-tropomyosin complex and/or actomyosin adenosine triphosphatase since caffeine and KCl still produced contracture of the diaphragm in a concentration-dependent manner in the presence of Chlo and in pretreated preparations. Furthermore Na SCN which is known to potentiate twitch tension and K^+-induced contracture by increasing Ca^{2+} influx at the transverse tubular element and the terminal cisternal of the sarcoplasmic reticulum (Weiss & Bianchi, 1965; Bianchi & Bolton, 1966) also reversed Chlo-induced inhibition lending support to an intracellular site of action of Chlo. Since an increase in extracellular Ca^{2+} did not reverse Chlo-induced twitch inhibition, it seems unlikely that the main effect of Chlo is due to inhibition of extracellular Ca^{2+} influx.

The inhibitory effect of Chlo and Chlo-pretreatment (7–28 days) on K^+- and caffeine-induced contracture as well as on isoprenaline-induced twitch potentiation further support the contention that the site of action of Chlo is on intracellular Ca^{2+} sequestration. These agonists are known to produce their actions through interference at specific intracellular Ca^{2+} sites such as 'trigger' Ca^{2+} and SAR calcium (Bianchi, 1962; Weiss & Bianchi, 1965; Frank, 1980; Merican *et al.*, 1983). It is conceivable that this effect of Chlo is due to such intracellular Ca^{2+} inhibition.

In skeletal muscle, the MFR that cause a smooth tetanic contraction is considered to be a measure of the duration of the active state of the contractile elements which permit maximal tension development (Ritchie, 1954). In the present study, Chlo as well as acute and chronic Chlo administration increased the frequency required to fuse twitch response to a tetanus, suggesting that one of the results of Chlo treatment is a shortening in the duration of the active state. Since the intensity of the active state has been proposed to vary in proportion to a complex between Ca^{2+} and the contractile apparatus (Taylor, 1969), the duration of the active state would consequently correspond to the time course of the release and reuptake of Ca^{2+} within the muscle. The effect of Chlo on MFR is consistent therefore with an intracellular site of action, possibly Ca^{2+} stores of the SAR (Ayiteh-Smith & Vartanian, 1975).

The persistent reduction in muscle strength throughout the 4 weeks of Chlo administration is suggestive of the presence of Chlo in skeletal muscle contractile elements. It was to be expected that such muscle preparations would show greater reactivity to Chlo *in vitro*, but our results indicated a development of tolerance to Chlo. Since only the inhibitory effect of procaine on such skeletal muscle preparations was altered without any significant effect on (+)-tubocurarine and dantrolene sodium, it seems likely that tolerance to the effect of Chlo may in fact be a result of specific changes demonstrated to occur in muscles pretreated with Chlo (MacDonald & Engel, 1970; Oberc & Engel, 1977; Ayiteh-Smith & Gbewonyo, 1977; Lie & Schofield, 1978). Moreover, local anaes-

thetics such as procaine and dibucaine inhibit twitch and tetanic tension by an action on intracellular Ca^{2+} sequestration (Inoue & Frank, 1960; Franz & Perry, 1974). There is a structural similarity between Chlo, dibucaine and procaine (Chinyanga *et al.*, 1972; Kennedy & Knapp, 1982) which may explain the decreased responsiveness to Chlo and procaine reported in this study.

Acute and chronic administration of Chlo have been found to induce morphological changes in skeletal muscle (Ayiteh-Smith & Gbenwonyo, 1977; MacDonald & Engel, 1978; Lie & Scholfield, 1978). Since MFR contributes to the duration of active state during excitation-contraction coupling (Taylor, 1969; Anderson, 1978; Frank, 1980), the decreased muscle strength still observed after stopping Chlo pretreatment may be due in part to its effect on the active state in the contractile elements. In addition, reactivity of the muscle to caffeine, KCl and tetanus tension during recovery may be explained in part by these ultrastructural changes in response to Chlo reported by previous workers.

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References

ANDERSON, K.E. (1978). Effects of calcium and calcium antagonists on the excitation-contraction coupling in striated and smooth muscle. *Acta Pharmacol. Toxicol.*, **43**, Suppl. 1, 1–14.

AYITEY-SMITH, E. & GBEWONYO, A.J.K. (1977). Ultrastructural changes in the heart of rats treated with chloroquine. *West Afr. J. Pharmacol. Drug Res.*, **4**, 7–15.

AYITEY-SMITH, E. & VARTANIAN, G.A. (1975). Dual action of chloroquine on frog's skeletal muscle contraction. *Eur. J. Pharmacol.*, **30**, 29–35.

BAGNALL, A.W. (1957). The value of chloroquine in rheumatoid disease: a four year study of continuous therapy. *Can. Med. Assoc. J.*, **77**, 182–194.

BIANCHI, C.P. (1962). Kinetics of radiocaffeine uptake and release in frog sartorius. *J. Pharmacol. Exp. Ther.*, **138**, 41–47.

BIANCHI, C.P. & BOLTON, T.C. (1966). Effect of thiocyanate on radiocalcium uptake during potassium contracture of frog sartorius muscle. *J. Pharmacol. Exp. Ther.*, **151**, 456–463.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm of the rat. *Br. J. Pharmacol. Chemother.*, **1**, 38–61.

CHINYANGA, H.M., VARTANIAN, G.A., OKAI, E.A. & GREENBERGER, D.V. (1972). Chlorquine-induced depression of neuromuscular transmission. *Eur. J. Pharmacol.*, **18**, 256–260.

ETTE, E.I. & ESSIEN, E.E. (1986). Neuromuscular weakness and ultrastructural damage produced by desethylated metabolites of chloroquine and the reversal by calcium. *West Afr. J. Anat.*, **1**, 5–15.

FRANK, G.B. (1960). Effects of changes in extracellular calcium concentration on potassium-induced contracture of frog's skeletal muscle. *J. Physiol.*, **151**, 518–538.

FRANK, G.B. (1980). The current view of the source of trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. *Biochem. Pharmacol.*, **29**, 2399–2406.

FRANZ, D.N. & PERRY, R.S. (1974). Mechanisms for differential block among single myelinated and non-myelinated axons by procaine. *J. Physiol.*, **236**, 193–210.

HUGHES, J.T., ESIRI, M., OXBURY, J.M. & WHITTY, C.W. (1974). Chloroquine myopathy. *Q. J. Med.*, **40**, 85–90.

INOUE, F. & FRANK, G.B. (1962). Action of procaine on frog skeletal muscle. *J. Pharmacol. Exp. Ther.*, **136**, 190–196.

KENNEDY, R.L. & KNAPP, R.B. (1982). Local anaesthetics. In *Modern Pharmacology*. ed. Craig, C.R. & Stitzel, R.E. pp. 453–462. Boston: Little, Brown and Company.

LIE, S.O. & SCHOFIELD, B. (1978). Inactivation of lysosomal function in normal cultured fibroblasts by chloroquine. *Biochem. Pharmacol.*, **22**, 3109–3114.

LULLMAN-RAUCH, R. (1979). Drug-induced lysosomal storage disorders. In *Lysosomes in Applied Biology and Therapeutics*. ed. Dingle, J.T., Jacques, P.J. & Shaw, I.W. Vol. 6, pp. 49–58. Amsterdam: North-Holland.

McCHESNEY, E.W. & ROTHFIELD, N.F. (1964). Comparative metabolic studies of chloroquine and hydroxy-chloroquine. *Arthritis Rheuma*, **7**, 328–332.

MacDONALD, R.D. & ENGEL, A.G. (1970). Experimental chloroquine myopathy. *J. Neurosurg. Exp. Neurol.*, **29**, 479–499.

MAGNUSSEN, I. & OLIVARIUS, B. de F. (1977). Cardiomyopathy after chloroquine. *Acta Med. Scand.*, **202**, 429–433.

MERICAN, Z., NOTT, M.W. & SUNBHANITCH, M. (1983). Effects of increasing the frequency of twitches and of isoprenaline on maximal twitches and cyclic AMP levels in slow- and fast-contraction cat skeletal muscles. *Br. J. Pharmacol.*, **80**, 303–308.

OBERC, M.A. & ENGEL, W.K. (1977). Ultrastructural localization of calcium in normal and abnormal skeletal muscle. *Lab. Invest.*, **36**, 566–577.

RITCHIE, J.M. (1954). The duration of the plateau of the full activity in frog muscle. *J. Physiol.*, **124**, 605–612.

ROLLO, I.M. (1980). Drugs used in the chemotherapy of malaria. In *The Pharmacological Basis of Therapeutics*. ed. Goodman, L.S. & Gilman, A. pp. 1045–1068. New York – Toronto: Macmillan and Co.

SMITH, B. & O'GRADY, F. (1966). Experimental chloroquine myopathy. *J. Neurol. Neurosurg.*, **29**, 255.

STAUBER, W.T., HEDGE, A.M., TROUT, J.J. & SCHOTTELIUS, B.S. (1981). Inhibition of lysosome function in red and white muscles by chloroquine. *Exp. Neurol.*, **71**, 295–306.

STILLMAN, J.S. (1981). Antimalarials. In *Textbook of Rheumatology*. ed. Kelley, W.N., Harris, E.D., Ruddy, S. & Sledge, C.B. pp. 785–795. Philadelphia, London, Toronto: W.B. Saunders Company.

TAYLOR, C.P.S. (1969). Isometric muscle contraction and the active state on analogue computer study. *J. Biophys.*, **9**, 759–780.

THOMPSON, P.E. & WERBEL, L.M. (1972). *Antimalarial Agents: Chemistry and Pharmacology*. pp. 150–196. New York: Academic Press.

WEISS, G.B. & BIANCHI, C.P. (1965). The effect of potassium contraction on ^{45}Ca uptake in frog sartorius muscle. *J. Cell. Comp. Physiol.*, **65**, 385–392.

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