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Abolition with chloramine-T of inactivation in barnacle muscle fibers results in stimulation of the ouabain-insensitive sodium efflux

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The hypothesis that chloramine-T stimulates the basal Na^+ efflux in barnacle fibers as the result of the entry of trigger Ca^{2+} into the myoplasm from the bathing medium was examined in this study. Two reasons for doing so can be given. One is that the oxidant is known to abolish inactivation in sodium and potassium channels. The other is that L-type Ca^{2+} channels are present in barnacle fibers, and an increase in internal free Ca^{2+} in these fibers is known to stimulate the Na^+ efflux, particularly in ouabain-poisoned fibers. The results of the experiments are as follows: (i) Chloramine-T exerts a biphasic effect on the Na^+ efflux: inhibition is followed by stimulation, the threshold concentration being 10^{-5} M. This is also found to be the threshold concentration for shortening of these fibers. (ii) The kinetics of the inhibitory effect resemble those of ouabain. (iii) Ouabain is without effect on the stimulatory phase caused by chloramine-T. (iv) Application of chloramine-T after the full effect of 10^{-4} M-ouabain is reached elicits solely a stimulatory response. (v) The dose–response curves for the stimulatory action of chloramine-T in unpoisoned and ouabain-poisoned fibers are alike except that the threshold concentration is less than 10^{-5} M in poisoned fibers. (vi) Basal light emission from unpoisoned and ouabain-poisoned fibers loaded with the photoprotein, aequorin, some 60 min beforehand increases as soon as they are exposed to 10^{-4} M chloramine-T. The response recorded in unpoisoned fibers is monophasic and usually transitory, whereas it is multiphasic and usually sustained in ouabain-poisoned fibers. (vii) The dose–response curve for chloramine-T shows a shift to the left in poisoned fibers. (viii) The magnitude of the rise in light emission depends on the external Ca^{2+} concentration. A rise fails to take place in the nominal absence of external Ca^{2+}. Taken together, these results support the above hypothesis that chloramine-T causes the entry of trigger Ca^{2+} into the myoplasm from the outside and provide evidence that stimulation of the Na^+ efflux is associated not only with this event but also with a reduced Na^+ gradient resulting from inhibition of the membrane Na^+/K^+-ATPase system by the oxidant. Thus, the suggestion put forward is that this oxidant promotes reverse Na^+/Ca^{2+} exchange and is able to exert multiple effects on membrane transport.

Introduction

Chloramine-T is an oxidant which acts to modify sulfhydryl amino acids such as methionine to form sulfoxides, and then methionine sulfones [1,2]. Several workers have found it to slow or abolish inactivation of voltage-dependent Na^+ channels [3–5]. But subsequent work has disclosed that this action is not confined to Na^+ channels; for example, the oxidant alters inactivation of K^+ channels [6]. These observations have raised the question whether chloramine-T can also alter inactivation in other types of channels such as Ca^{2+} channels. To address this question, barnacle muscle fibers were chosen as a model system for at least four reasons: first, upon isolation and cannulation, these fibers are known to be somewhat depolarized [7,8]. Second, they possess DHP-sensitive Ca^{2+} channels [9]. Third, as a rule, a fall in myoplasmic pCa stimulates the resting Na efflux [8]. And fourth, they are ideal for experiments involving the injection of the Ca^{2+} indicator aequorin [10]. The following communication describes experiments that were designed to explore the possibility that if chloramine-T has the ability of abolishing inactivation in the L-type Ca^{2+} channels present in these fibers (largely in the T-system), one would then expect stimulation of the Na^+ efflux in unpoisoned and ouabain-poisoned fibers to

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occur as the result of the entry of trigger Ca^{2+} into the myoplasm from the bathing medium.

Materials and Methods

(i) The species of barnacles, the methods of dissection, cannulation, microinjection of these fibers, and counting of the Na^+ activity in the effluent and the fibers were the same as those described by Bittar [8]. The artificial seawater (ASW) used had the following composition (mM): NaCl, 465; KCl, 10; $MgCl_2$, 10; $CaCl_2$, 10; $NaHCO_3$, 10 and pH 7.8. Solutions containing Ca^{2+} in varying concentration were prepared by reducing or increasing NaCl in an osmotically equivalent amount. Solutions of 2.5 and $5 \cdot 10^{-4}$ M aequorin at pH 7.5 were used for injection into fibers freshly dissected from the same muscle bundle. This was done with the aid of an 0.5 μl SGE microsyringe onto which a fine capillary with a tip diameter of approximately 100 μM was mounted. Ouabain (10^{-4} M) was applied to some of these fibers about 30 min. after loading with aequorin. Thus, at least 60 min were allowed for equilibration of aequorin in the myoplasm before transfer of the fiber for the monitoring of light emission (vidé infra). All experiments were carried out at an environmental temperature of 22° to 24°C. (ii) The method used for recording luminescence from an aequorin reaction mixture lying in the myoplasm of unpoisoned and ouabain-poisoned fibers was a modification of that described by Bittar and Keh [10] for the measurement of firefly luminescence. The light-measuring apparatus, housed in a light-tight black Perspex box, consisted of: (1) a microinjector mounted on a Prior micromanipulator which was driven by an Aminco motor unit under remote control; (2) a platform anchored to a Palmer screw stand on the side of which a cannulated aequorin-loaded fiber could be suspended in a Perspex bathing chamber (covered wherever possible with aluminum foil) that was emptied and filled simultaneously via an outlet-inlet system which was also under outside control. This chamber lay adjacent to (3): an end-window RCA photomultiplier tube (6342) whose dynode chain was connected to a DC power supply unit (Hewlett-Packard 6515A) located outside the box. When applying a voltage of 900 or 1000 V the dark current at 22 ± 1°C was ≤ 0.5 nA; and (4) the anode current was measured with a Keithley 485 picoammeter and converted to an analogue voltage signal, which was recorded on a Watanabe chart recorder, the response time of which was about 0.3 s for a full-scale deflection (2 V). The digit representation of the picoammeter was simultaneously fed to an IBM Personal Computer through a MetraByte MBC-488 interface card. Reading, display and analysis of the picoammeter data was carried out with the aid of two software programs that were written in this laboratory by Dr. Patrick Hamilton. The results given in this paper are expressed as the mean ± S.E. Student's t-test was employed to compare the data statistically. Values for $P < 0.05$ were considered as being significant. Estimates of the size of the observed effects on the Na^+ efflux and basal light output from fibers loaded with aequorin were calculated on the basis of the rate constant plots and peak flash, respectively.

All reagents used were analytical grade. Ouabain was obtained from Sigma, St. Louis, MO. Chloramine-T was purchased from Aldrich, Milwaukee, WI. Aequorin samples were a gift from Dr. O. Shimomura of the Marine Biological Laboratory, Woods Hole, MA.

Results

Preliminary trials with chloramine-T revealed that it exerts a biphasic action on the basal Na^+ efflux in unpoisoned fibers. That is, inhibition is followed by stimulation and that the threshold concentration for such action lies in the region of 10^{-5} M.

Employing chloramine-T in higher concentrations e.g. 10^{-3} M, the fall in basal Na^+ efflux is found to average 42 ± 4% ($n = 7$). This type of experiment was repeated with the aim of comparing the magnitude of this inhibition with that produced by ouabain, e.g.,

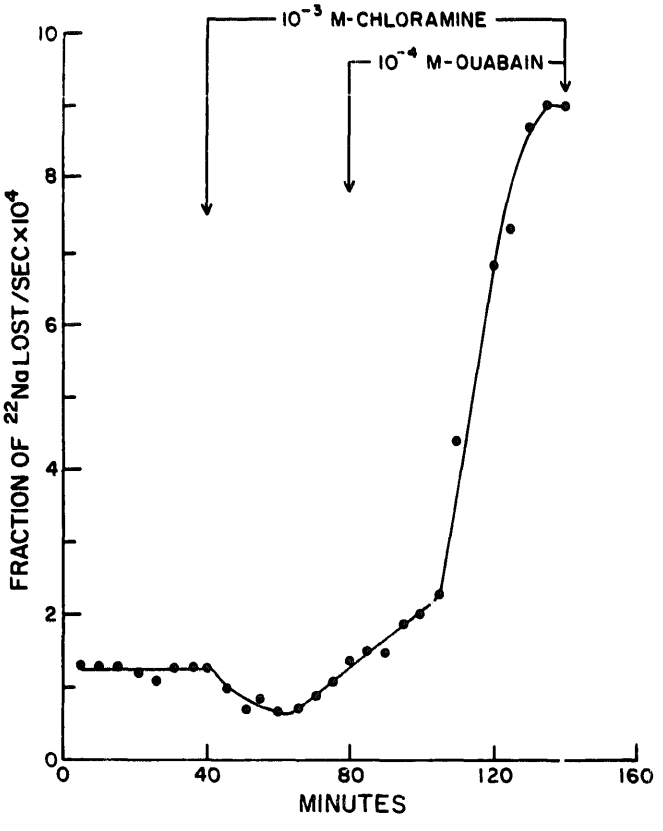

Fig. 1. The biphasic behavior of the basal Na^+ efflux toward the external application of 10^{-3} M chloramine-T and the failure of subsequent application of 10^{-4} M ouabain to alter the course of the stimulatory phase of this response (rate constant plot).

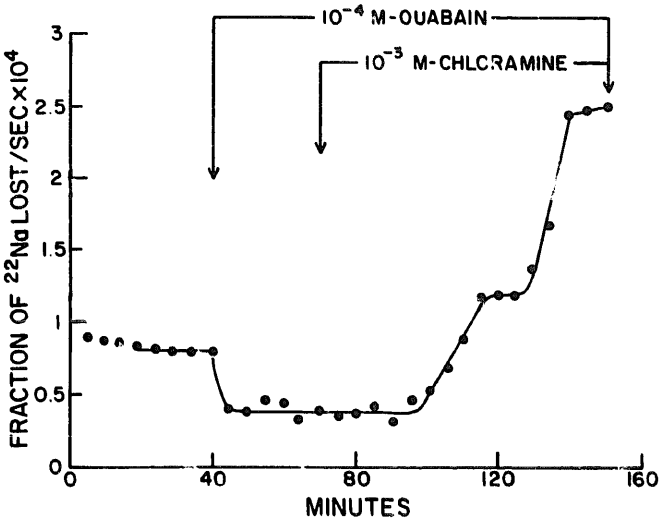

Fig. 2. Occurrence of the response of the ouabain-insensitive Na^+ efflux to external application of 10^{-3} M chloramine-T in two distinct phases.

10^{-4} M, a maximally inhibitory concentration [11]. The results of these experiments show that 10^{-3} M chloramine-T reduces the basal Na efflux by 52 ± 3% (n = 4) and 10^{-4} M ouabain by 50 ± 7% (n = 4).

These studies have also revealed that the delayed stimulatory effect caused by chloramine-T consists of two phases, as illustrated in Fig. 1. It can be seen that external application of 10^{-4} M ouabain during the period of the first phase is without effect. Though brief, the second phase is characterized by a very sharp rise in the Na^+ efflux. In experiments of this type the last few points were not plotted, since the fiber counts were regarded as far too low for the computation of reliable rate constants for $^{22}Na^+$ loss.

Presented in Fig. 2 is a representative experiment indicating that inhibition of the remaining Na^+ efflux by 10^{-3} M chloramine-T fails to take place in a fiber pretreated with 10^{-4} M ouabain. This result strongly suggests that the prompt inhibitory effect of chloramine-T at this concentration involves abolition of membrane Na^+/K^+-ATPase activity. Additionally, it can be seen that the two stimulatory phases are in this instance quite distinct. Estimates of maximal stimulation provide a value of 670 ± 101% (n = 4) which is significantly less than 1364 ± 125% (n = 4) obtained by applying 10^{-3} M chloramine-T before 10^{-4} M ouabain (P being < 0.01).

The preceding observations raised the possibility that the sensitivity of unpoisoned and ouabain-poisoned fibers toward chloramine-T are not the same. Hence experiments were done to rule in or out this possibility. The results obtained are summarized in Figs. 3A and B. In both situations, 50% stimulation occurs with approx. $5 \cdot 10^{-4}$ M chloramine-T. Notice that with ouabain-poisoned fibers, the threshold concentration is less than 10^{-5} M.

In order to verify the idea that stimulation of the Na^+ efflux by chloramine-T is the result of the entry of trigger Ca^{2+} from the bathing medium, internal free Ca^{2+} levels in unpoisoned and ouabain-poisoned fibers were monitored before and after external application of chloramine-T by employing the aequorin method (e.g., Ref. 12). Because the oxidant elicits contraction in these fibers, the concentration used in experiments of this type was intermediate e.g., 10^{-4} M. The results thus obtained can be summarized as follows: (i) In the first group of experiments, both unpoisoned and ouabain-poisoned fibers were suddenly exposed to 10^{-4} M chloramine. Comparisons were made only between fibers showing relatively identical basal levels of light emission. As illustrated in Figs. 4A and B, the rise in light output is prompt in both test fibers but the signal recorded in the unpoisoned one is monophasic and transient, whilst in the poisoned fiber it is multiphasic and sustained over a similar time-period. (ii) To determine the minimal concentration of chloramine-T required to increase light emission, unpoisoned fibers were used. These experiments show a response with as

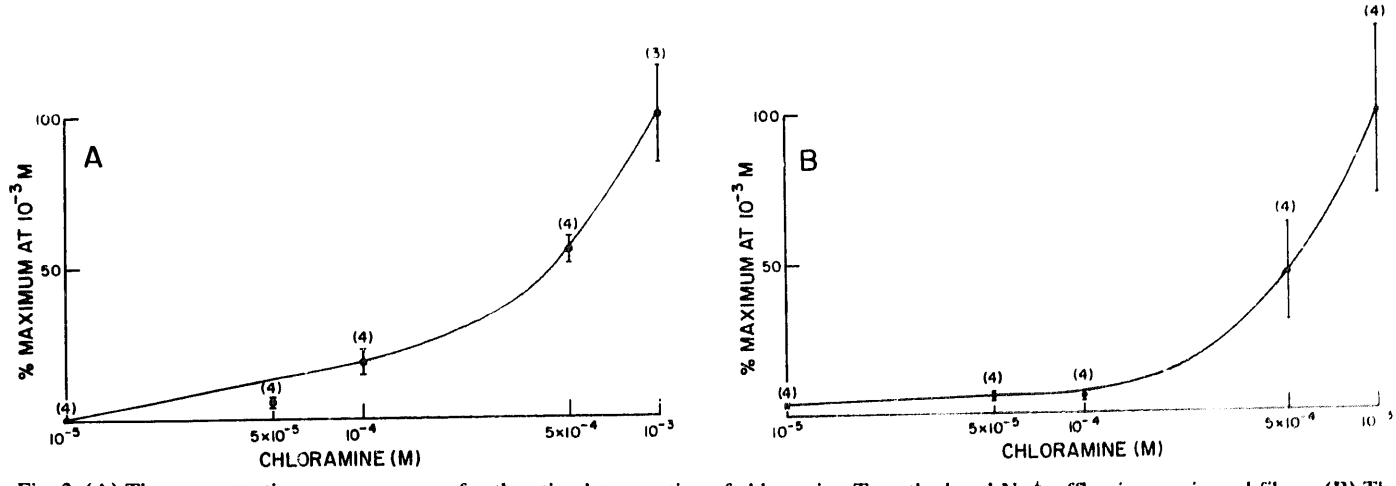

Fig. 3. (A) The concentration-response curve for the stimulatory action of chloramine-T on the basal Na^+ efflux in unpoisoned fibers. (B) The concentration–response curve for the stimulatory action of chloramine-T on the ouabain-insensitive Na^+ efflux.

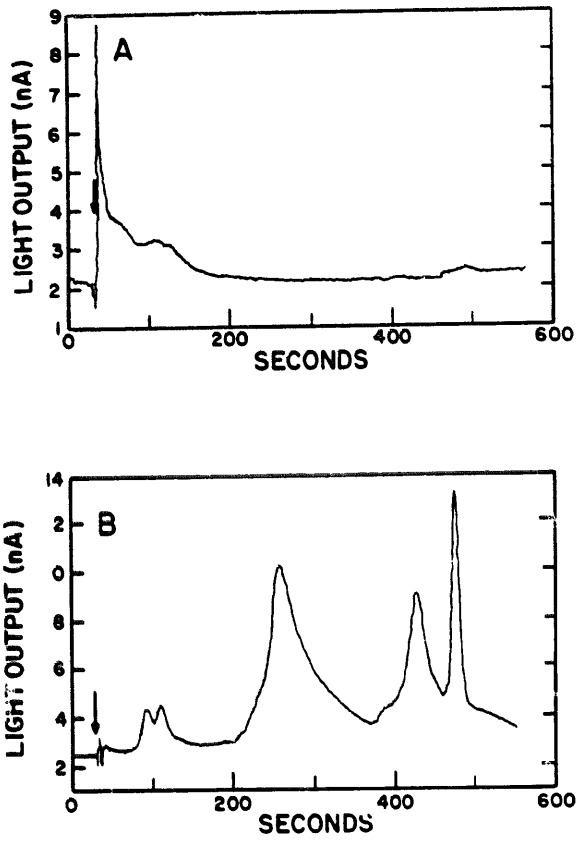

Fig. 4. (A) The monophasic rise and decay in light emission from an unpoisoned fiber following external application of 10^{-4} M chloramine-T (arrow indicates time at which oxidant was added). (B) The sustained, multiphasic rise in light emission from a fiber pretreated with 10^{-4} M ouabain, followed by 10^{-4} M chloramine-T (at the time indicated by the arrow).

little as 10^{-6} M chloramine-T which is prompt in onset, monophasic and transient. This is illustrated in Fig. 5. Notice that the signal in this case fails to decay completely. On occasion, as little as 10^{-7} M chloramine-T is found to increase light emission in unpoisoned fibers. Put together, such results are interpreted to mean that the putative sarcolemmal Ca^{2+}/Mg^{2+}-

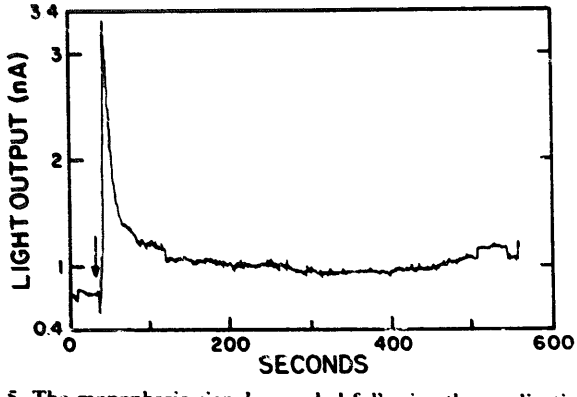

Fig. 5. The monophasic signal recorded following the application of 10^{-6} M chloramine-T to a unpoisoned fiber. Decay of this signal is incomplete.

ATPase is involved as a second site of action of chloramine-T and that by inhibiting this enzyme myoplasmic pCa falls sufficiently to elicit quick shortening of these fibers. (iii) Next, it seemed necessary to compare light emission from unpoisoned and ouabain-poisoned fibers exposed to chloramine-T in varying concentration. Shown in Figs. 6A and B is that there is a shift to the left of the dose–response curve pertaining to poisoned fibers. The large variation seen with 10^{-4} M and 10^{-3} M-chloramine-T is a consistent finding that is attributable to the severe shortening that these fibers

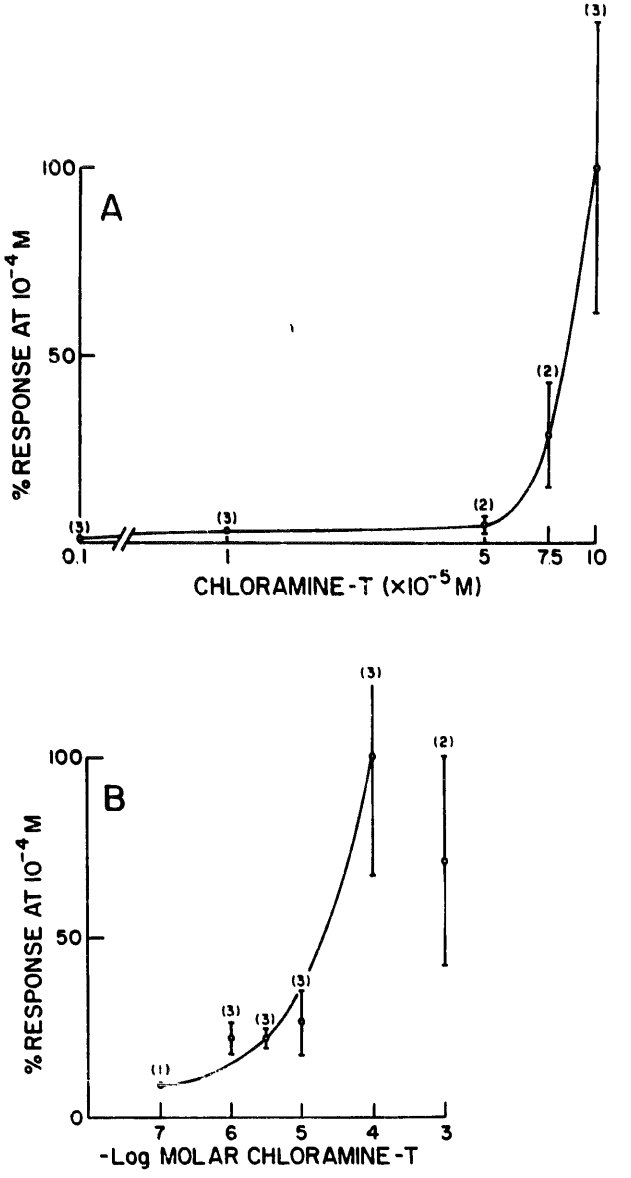

Fig. 6. (A) Concentration-response curve for the increase in basal light emission caused by chloramine-T in unpoisoned fibers. Vertical bars span ± S.E. The absence of a bar indicates a S.E. within the symbol, ○. The number of measurements done is recorded in parentheses. The fibers used were isolated from the same barnacle specimen. (B) Concentration-response curve for the increase in basal light emission caused by chloramine-T in ouabain-poisoned fibers. Vertical bars span ± S.E. The number of measurements done is recorded in parentheses. The fibers used were isolated from the same barnacle specimen.

undergo upon exposure to the oxidant in concentrations approaching the millimolar range. (iv) In the last group of experiments, light emission was monitored in unpoisoned fibers suspended in ASW containing Ca^{2+} in varying concentration, and then suddenly exposed to 10^{-4} M chloramine-T. Shown in Figs. 7A and B are the kinetics of light emission from a fiber suspended in 5 mM Ca^{2+}-ASW and 20 mM Ca^{2+}-ASW before and after applying chloramine-T. Though both signals are monophasic and prompt in onset, the amplitude of the signal recorded in the second fiber is considerably greater. Notice, too, that light emission fails to return to its original base-line level. The inference thus drawn from these experiments was that the entry of external Ca^{2+} leading to a raised internal free Ca^{2+} rather than the transiently raised internal free Ca^{2+} resulting from inhibition of the SL Ca^{2+}/Mg^{2+}-ATPase plays the predominant role in the occurrence of increased light emission which chloramine-T elicits. This line of thinking led to the performance of experiments with ouabain-poisoned fibers in which the external Ca^{2+} concentration lay in the range of 0 to 20 mM. The results of these experiments are summarized in Fig. 8. It is thus apparent that increments in light output are a function of external Ca^{2+} and that a rise in light output does not occur in the nominal absence of external Ca^{2+}. Failure to see a rise may seem puzzling but not if one assumes that myoplasmic pCa in fibers suspended in Ca^{2+}-free ASW may lie in the region of 8 rather than 7.

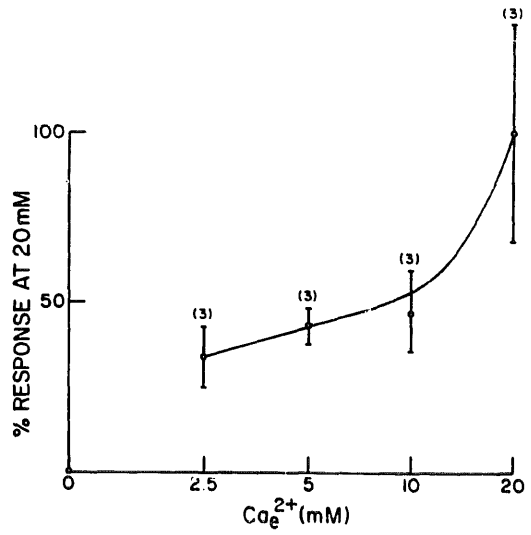

Fig. 8. Dependency on the external Ca^{2+} concentration of the rise in basal light emission caused by chloramine-T. Vertical bars span ± S.E. The number of measurements done is recorded in parentheses. The number of measurements using nominally Ca^{2+}-free-ASW is three. The fibers used were isolated from the same barnacle specimen.

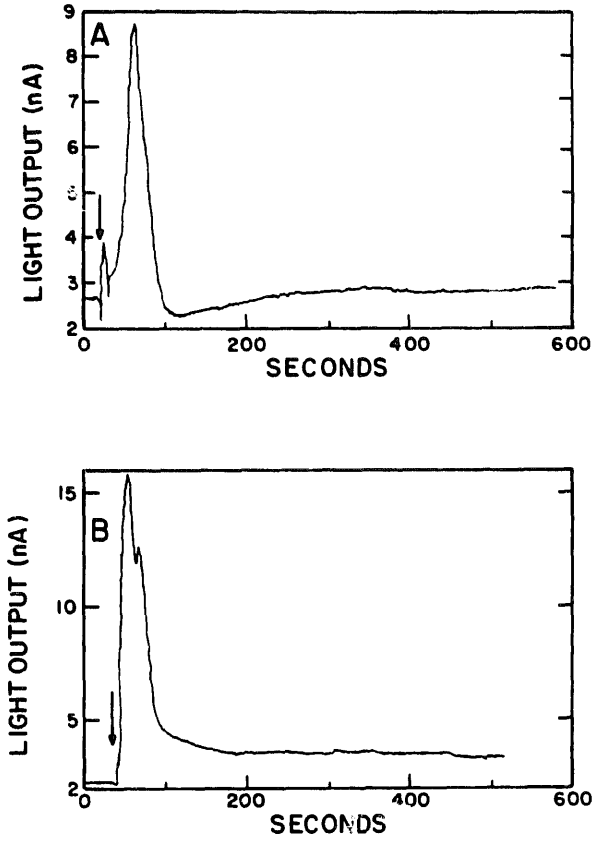

Fig. 7. (A) The transient, monophasic signal recorded following the application of 10^{-4} M chloramine-T to a unpoisoned fiber suspended in 5 mM Ca^{2+}-ASW. Note the occurrence of a motion artifact while the bathing medium was being replaced by 10^{-4} M chloramine-T/5 mM Ca^{2+}-ASW. Additionally note that a temporary drop in basal light output takes place following complete decay of the monophasic signal. This is thought to be due to temporary shortening of the fiber. (B) Incomplete decay of the monophasic signal recorded following the application of 10^{-4} M chloramine-T to a fiber suspended in 20 mM Ca^{2+}-ASW. Note that the amplitude of the rise is almost double that recorded in the preceding experiment.

Discussion

Several findings are notable. In the first place, chloramine-T is able to reduce the basal Na efflux in unpoisoned fibers fairly promptly and markedly. The kinetics of this result bear a close resemblance to those obtained with ouabain The fact that the onset of the effect is prompt strengthens the argument that the membrane Na^+/K^+-ATPase is the immediate point of action of chloramine-T. Further supporting evidence is provided by the experiments carried out with ouabain showing that the glycoside is ineffective when applied after the oxidant. By the same token, application of chloramine-T after ouabain fails to reduce the remaining Na^+ efflux (vidé Fig. 2). However, the possibility still exists that a raised internal free Ca^{2+} concentration resulting from the action of chloramine-T on the sarcolemmal Ca^{2+}/Mg^{2+}-ATPase and/or any L-type Ca^{2+} channels lying in the sarcolemma may reduce the activity of the membrane Na^+/K^+-ATPase system. This point has been touched upon by Yingst [13] and Bittar,

Nwoga and Huang [14] but in the present situation it looks as if a transient rise (e.g., in unpoisoned fibers) in myoplasmic free Ca^{2+} is not a sufficiently significant factor contributing to the observed fall in the resting Na^+ efflux.

Secondly, it is a characteristic of unpoisoned and ouabain-poisoned fibers not only to promptly shorten upon exposure to chloramine-T (e.g., 10^{-3} M) but also to show stimulation of the Na^+ efflux. Importantly, however, there is a lag period of about 20 min preceding the onset of stimulation in both unpoisoned and ouabain-poisoned fibers. This lag is thought to be due to diffusional delays in chloramine-T reaching its second site of action, namely the putative L-type Ca^{2+} channels lying in the T-system. Since there are two distinct time to peak phases, it is tempting to speculate that the second diffusional delay through the cleft and T-system is associated with the second phase and that this second phase has to do with the sarcoplasmic reticulum Ca^{2+} release channel as a likely site of action of chloramine-T.

Thirdly, the experiments with aequorin indicate quite clearly that whereas the signal recorded in unpoisoned fibers is monophasic, the signal recorded in fibers poisoned with ouabain is sustained. Moreover, these experiments indicate that the omission of external Ca^{2+} prior to the application of chloramine-T prevents the multiphasic signal from occurring. Because the aequorin experiments were short in duration, e.g., less than 10 min, nothing in particular can be said about myoplasmic pCa in terms of the two 'time to peak' phases of the Na^+ efflux.

Fourthly, the finding of a high Na^+ efflux is not altogether surprising, particularly since the Na^+/Ca^{2+} exchanger is known to be driven in the reverse mode by the Ca^{2+} electrochemical gradient. Conditions such as a raised internal free Ca^{2+}, however, are not a pre-requisite, as pointed out by Bittar and Huang [15]. Whether a persistent condition of open Ca^{2+} channels created by chloramine-T is accompanied by an inward passage of Na^+, thus causing a greater narrowing of the Na gradient is a question that obviously merits investigation. Equally important is the question whether external Mg^{2+} which is known to competitively inhibit I_{Ca}, and which is present in the bathing medium in a concentration of 10 mM rather than 50 mM, as in seawater, promotes reverse Na^+/Ca^{2+} exchange on account of reduced entry into the myoplasm via the open Ca^{2+} channels and hence, a raised internal pMg. As will be recalled, Bittar and Huang [15] and more recently, Bittar, Xiang and Huang [16] have obtained evidence that a raised myoplasmic pMg is a key regulator of Na^+/Ca^{2+} exchange in the reverse mode.

In summary, therefore, it appears that chloramine-T acts on several membrane proteins, notably the membrane Na^+/K^+-ATPase and the putative L-type Ca^{2+} channel. Whilst its untoward effect on the transport enzyme is immediate, its effect on the bulk of the Ca^{2+} channel protein appears to be preceded by a lag period presumably because of diffusional delays.

Acknowledgements

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