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Effects of sulphydryl inhibitors on frog sartorius muscle: *p*-chloromercuribenzoic acid and *p*-chloromercuribenzenesulphonic acid

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Summary

1. Experiments were done on frog sartorius muscles to study the effects and mechanisms of action of the −SH inhibitors, *p*-chloromercuribenzoic acid (PCMB) and *p*-chloromercuribenzenesulphonic acid (PCMBS).

2. Both organomercurials produce a depolarization of the surface membrane which is associated with a period of asynchronous twitching and followed by inexcitability.

3. Only PCMB produces a unique fractionation of the electrically evoked twitch into an initial rapid and later slow phase.

4. PCMB and PCMBS increase the rate of ^{45}Ca efflux from whole muscle. Ethylenediamine tetraacetic acid (EDTA, 5 mM) causes only limited antagonism of the enhancement of ^{45}Ca efflux produced by PCMB whereas it completely antagonizes this same effect of PCMBS. EDTA selectively removes superficial calcium without penetrating into the intracellular space.

5. The results suggest that PCMB inhibits −SH groups in the terminal cisternae causing a fractionation of the twitch. PCMBS acts primarily at surface sites with limited access to the cisternae and sarcoplasmic reticulum.

Introduction

Many studies have been made of the effects of sulphydryl (−SH) inhibitors on enzymes, membrane fractions and model systems of skeletal muscle. For example, −SH inhibitors reduce calcium uptake by isolated sarcoplasmic reticulum (Carsten & Mommaerts, 1964; Hasselbach & Seraydarian, 1966); they induce the superprecipitation of actomyosin gel (Levy & Ryan, 1965); and they modify the interaction between calcium and tropinin (Yasui, Fuchs & Briggs, 1968). If the subcellular and molecular effects of −SH inhibitors could be correlated with their effects on the intact muscle, some useful additional clues to the role of −SH groups in the contraction-relaxation cycle may be revealed. With this view in mind, we determined the effects of −SH inhibitors on intact muscle. By selecting appropriate parameters of skeletal muscle function for measurement and analysis, it was hoped that the mechanism or at least the site of action would be elucidated.

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The results of experiments with two organomercurial —SH inhibitors are described in this paper; these are *p*-chloromercuribenzoic acid (PCMB) and *p*-chloromercuribenzenesulphonic acid (PCMBS). PCMB was selected as a test agent because of its previous extensive use in biochemical studies of muscle, and PCMBS was chosen because it has greater aqueous solubility and, accordingly, a slower rate of diffusion across biological membranes (Vansteveninck, Weed & Rothstein, 1965). This difference in hydrophilicity and membrane transport may be reflected in two different sites of —SH group attack, PCMB acting on internal structures and PCMBS acting primarily at the cell surface.

Methods

All experiments were performed on isolated sartorius muscles of *Rana pipiens* (Lemberger Co.) during all seasons of the year. The frogs were maintained in tap water at 5° C for 1–30 days before an experiment. After dissection the muscles were equilibrated at 22°–24° C in Ringer solution containing (mM): 110·8 NaCl, 2·0 KCl, 1·8 $CaCl_2$, 0·1 NaH_2PO_4 and 2·02 $NaHCO_3$. The pH was adjusted to 7·2 with HCl, Tris (hydroxymethyl) aminomethane, or NaOH. Calcium-free Ringer was prepared without the addition of calcium. In some experiments disodium ethylenediamine tetracetate (Na_2EDTA) was added to the calcium-free Ringer solution. All test agents were added to the normal, calcium-free, or EDTA-Ringer before use, and if necessary the pH readjusted to 7·2. Deionized water was used to prepare all solutions.

Drugs used. PCMB and PCMBS and Tris (hydroxymethyl) aminomethane were supplied by the Sigma Chemical Co. Na_2EDTA (Fisher Chemical), procaine HCl (Baker Chemical), (+)-tubocurarine (Mann Research) and cysteine (Calbiochem).

The two organomercurials differed in their solubilities in normal Ringer solution. The poor solubility of PCMB limited its use to concentrations of about 2·3 mM. The solubility of PCMBS was greater than 5·0 mM.

Mechanical response

In these experiments, the sartorius was excised with the pelvic bone and tibial tendon attached. This preparation was mounted in a 25 ml capacity paraffin muscle chamber. The pelvic bone was rigidly constrained with the chamber with the muscle resting horizontally on a massive platinum-iridium electrode under approximately 2 g tension. A fine metal chain was used to connect the tibial tendon to the force displacement transducer. During the 30 min equilibration in Ringer solution the muscle was subjected to twitch and tetanic stimulation. After the equilibration period, the muscle bath was changed for one containing the test agent. Tubocurarine chloride (5×10^{-5} g/ml) was routinely added to all solutions to block muscle excitation due to transmitter release from the electrically stimulated nerve branches.

Supramaximal electrical stimulation of the muscle was employed. Electrically evoked twitches were produced by rectangular pulses of 2 ms duration at a frequency of 1 pulse/15 s to 1 pulse/30 seconds. A 200 ms rectangular pulse chopped at a frequency of 500 shocks/s was used to produce a fused tetanus. Intervals between tetani were 1 min or longer. The isometric mechanical response to electrical stimula-

tion was measured by a force displacement transducer, amplified and displayed on an ink-writer.

Other mechanical response studies involved visual observation of asynchronous twitching under a stereomicroscope. In these experiments paired sartorius muscles were used. The control muscle was treated with the −SH inhibitor alone, while the experimental muscle was pretreated with a test agent before addition of the −SH inhibtor.

Radiocalcium efflux

^{45}Ca (New England Nuclear) was added to normal Ringer solution to make ^{45}Ca Ringer with a specific activity of 4 mCi/ml. Paired sartorius muscles (50–80 mg), with their tendons removed, were loaded with ^{45}Ca by soaking for 3 h at room temperature (22°–24° C) in ^{45}Ca Ringer. After loading, the muscles were rinsed with 20 ml of calcium-free Ringer solution and gently blotted (Whatman No. 42 filter paper) to remove solution adhering to the outer surface. The muscles were then placed in a chamber containing 3 ml of normal or calcium-free Ringer depending on the experiment. This collection fluid was changed for 3 ml of fresh solution at 10 min intervals through the entire washout period. At each 10 min interval 1 ml of the total 3 ml washout fluid was emptied into a polyethylene scintillation vial and 12 ml of scintillation fluor added. At certain times during the washout period (to be indicated for each experiment), the test agent was added to the collection fluid. The muscle chambers were shaken during the entire washout and at no time during the washout period were the muscles removed from the chamber.

At the completion of 180 min of washout the muscles were removed from the washout chamber, lightly blotted, and placed in silica crucibles. The muscles were dried overnight at 100° C, placed in a muffle furnace and ashed for 16 h at 500° C. Three ml of 0·1 N HCl was added to the muscle ash and the crucibles shaken for 3 h. One ml samples of the ashed muscle solution were then prepared for counting.

The samples obtained at each 10 min collection period and the acid-dissolved muscle ash were counted in a liquid scintillation spectrometer. After correction for decay and quenching the data were expressed as a rate coefficient curve. The rate coefficient is the percentage of the average radioactivity in the tissue during the time of collection that has emerged per minute during the collection period.

The scintillation fluor consisted of: 180 g naphthalene, 36 g 2,5-diphenyloxazole, 1·8 g 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)-benzene, and 600 ml 2-ethoxyethanol all dissolved in 3 l. of *p*-dioxane (spectroquality).

Membrane electrical properties

Sartorius muscles were mounted dorsal side upward in a plexiglass chamber (50 ml capacity). Muscles were mounted at rest length and allowed to equilibrate for 30–60 min in Ringer solution (22°–24° C).

Transmembrane potentials were recorded by means of glass microelectrodes filled with 3 M KCl. Only microelectrodes with resistance between 8–15 MΩ and tip potentials of 5 mV or less were used. If the resistance or tip potential changed during an experiment the microelectrode was discarded. The microelectrode was inserted into a 3% KCl-agar bridge. Both microelectrode and KCl-agar bridge were supported and positioned by a micromanipulator. A Ag–AgCl wire connected

the KCl-agar bridge to the electrometer amplifier. The output of the amplifier was displayed on an oscilloscope and electrometer amplifier and recorded on a chart recorder.

In order to minimize liquid junction potentials the indifferent (reference) electrode was devised to cancel the potentials arising at the microelectrode side. The Ringer solution surrounding the muscle was connected in series to a Ringer-agar and KCl-agar bridge. A Ag–AgCl wire connected the KCl-agar bridge to the ground of the amplifier. The junction potential of the entire system was measured (without the microelectrode in place) before each experiment and was normally below 2 mV. This potential along with the microelectrode tip potential was zeroed out before each experiment.

Action potentials were elicited by 0·1 ms duration rectangular pulses passed between two silver chlorided wires at opposite ends of the muscle. Action potentials were amplified as previously described and displayed on a storage oscilloscope.

Results

Contractor effects on unstimulated muscle

After a latent period of 2–3 min, 1·0 mM PCMB or PCMBS caused spontaneous, low amplitude, asynchronous contractions lasting for 10–15 min. With the muscle placed under a resting tension of 2 g, these contractions were just barely of sufficient amplitude to be detected and recorded by the measuring system. Consequently, direct microscopic visualization under a stereomicroscope was used (20 × magnification), and the marked temporal dispersion of contractile activity was evident. In a previous study in which the muscles were placed under a 0·5 g resting tension (Okamoto & Kuperman, 1966), these spontaneous contractions were capable of being recorded consistently and reached an amplitude of about 10–20% that of the maximal electrically evoked twitch.

Using the direct visualization technique, it was found that spontaneous contractions did not occur if the muscle bath contained, in addition to the —SH inhibitor, any of the following: 0·25 mM procaine, 1·0 mM cysteine, 116 mM KCl (in place of 2·0 mM), 110·88 mM choline Cl (in place of 110·88 mM NaCl).

Effects on electrically evoked twitch

A unique effect was produced by PCMB (0·25–2·3 mM) on the electrically stimulated muscle. This effect, as illustrated in Fig. 1, can be described as a fractionation of the twitch into two phases. The first phase appears like a normal contractor response ; the second phase begins during the relaxation period of the first and consists of a slow contraction. The amplitude of the second slow phase depended on how early it began during the relaxation period of the first, and this was both concentration and time dependent. Thus, the first noticeable effect of 0·5 mM PCMB (Fig. 1A) was merely a low amplitude ' tail ' on the relaxation period of the twitch. With the passage of time (Fig. 1A, 4·25 min), a distinct second phase began when the relaxation period of the first phase was only about 60% completed. After this peak effect, the slow phase began later and later during the relaxation period and, correspondingly, its amplitude decreased. It is worth noting in Fig. 1A that

the amplitude of the slow phase gradually develops to a peak with repeated stimulation and then gradually decreases until the slow phase disappears; meanwhile the first phase remains relatively constant. There is a slight potentiation of the first phase during the peak slow phase response but this potentiation did not occur in all experiments. The first phase usually began to decline at 12–15 min after application of 0·5 mM PCMB, and this continued until complete absence of response occurred after another 30–40 minutes.

Figure 1B illustrates the typical effect of 2·3 mM PCMB, the highest concentration tested. A maximal slow phase response is apparent after only 1 min of PCMB action, its amplitude being nearly equal to that of the first phase. Note also the relatively rapid decline of both the first and second phase of each electrically evoked muscle response, the second disappearing earlier.

In some experiments (not illustrated here) the second slow phase of contraction began immediately after complete relaxation of the initial contraction. In these cases the second phase was of very low amplitude, and this type of response occurred just before the complete disappearance of the fractionation effect. The most frequent type of response to all concentrations of PCMB was of the type

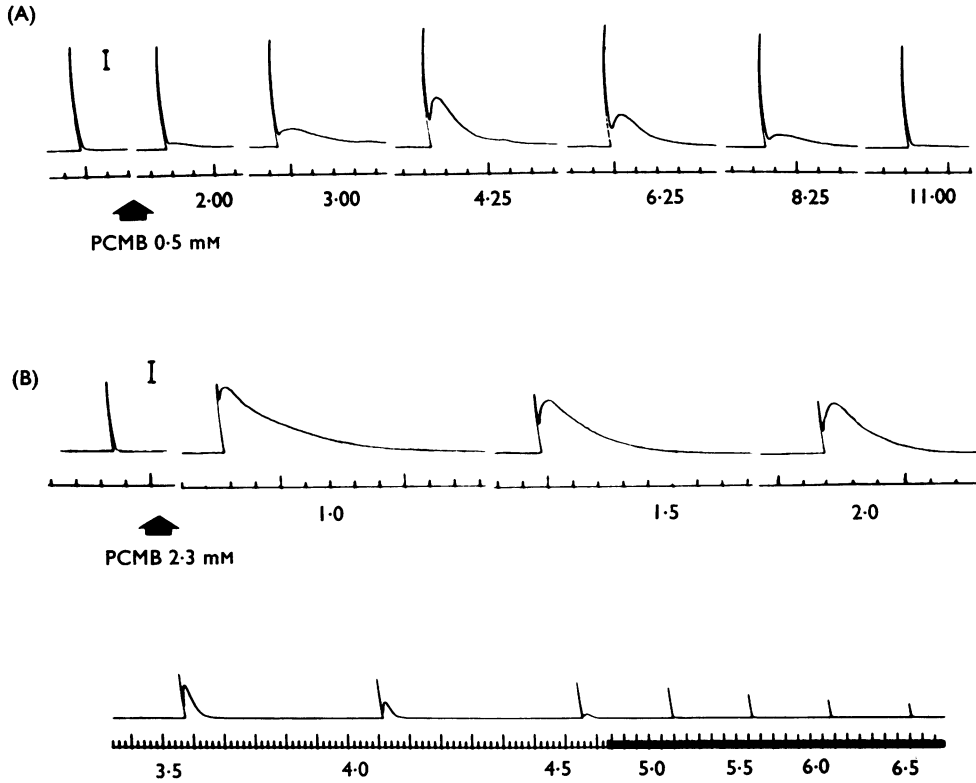

FIG. 1. Effect of 0·5 mM PCMB (A) and 2·3 mM PCMB (B) on the electrically evoked twitch of isolated curarized frog sartorius muscle. Low amplitude asynchronous twitching can be observed on the second phase of contraction and baseline. A maximum electrically evoked control twitch is shown to the left of the arrow. The time markers are in seconds, and the numbers below each twitch indicate the time in minutes after PCMB application. Calibration bars; 2·5 g. Stimulation frequency: 1 pulse/15 s (A); 1 pulse/30 s (B).

shown in Fig. 1—that is, the second phase begins some time during the relaxation period of the first phase.

If cysteine was added to the muscle bath in a molar concentration equal to that of PCMB, neither the twitch fractionation nor depression was produced. A concentration of procaine (0·25 mM) that did not affect muscle excitability antagonized the twitch fractionation but not the depression. The addition of 10 mM caffeine to the muscle bath after the electrically evoked twitch was completely abolished by PCMB, still caused rigour of about the same amplitude as in normal muscle.

The effect of PCMBS on the electrically evoked twitch was not the same as that of PCMB. At 0·25–5·0 mM, the sulphonated analogue caused twitch depression and did not produce the twitch fractionation seen with PCMB. The highest concentration of PCMBS tested (5·0 mM) also produced a low amplitude rigour equal to 2% of control tetanus tension. If 10 mM caffeine was added to a muscle that was in rigour and completely unresponsive to electrical stimulation, additional rigour tension developed. All effects of PCMBS were prevented by equimolar concentrations of cysteine but they were not affected by 0·25 mM procaine.

Effects on ^{45}Ca efflux

The firmly established role of calcium in excitation-contraction coupling and the fact that various drugs affect calcium movements in skeletal muscle (see Isaacson & Sandow, 1967), prompted us to determine the influence of the organomercurial agents on the rate of ^{45}Ca efflux.

Either PCMB or PCMBS was added to the muscle bath during the slow phase of ^{45}Ca washout, when it is presumed that the ^{45}Ca emerges primarily from the sarcoplasmic reticulum (Shanes & Bianchi, 1959; Isaacson & Sandow, 1967). The concentrations used were the same as those tested on the mechanical properties of the muscle, 0·25–2·3 mM PCMB and 0·25–5·0 mM PCMBS. Each concentration of either agent produced an increase in ^{45}Ca rate coefficient, and examples of this effect are shown in Figs. 2 and 3. In both cases the rate of ^{45}Ca efflux increases progresively after the addition of 1·0 mM of the compound until a peak effect is attained at 30 min for PCMB and 20 min for PCMBS. With PCMB the peak effect is maintained for at least another 30 min (Fig. 2) but the PCMBS effect slowly reverses during the same period of time (Fig. 3). The intensity of the peak effect is the same for both compounds, and is equal to a value about three times that before the —SH inhibitor is added.

No attempt was made to determine complete dose-response relationships but it was shown that concentrations of —SH inhibitor below 1·0 mM produced less intense effects on ^{45}Ca rate coefficient. Interestingly, even the lowest concentration of PCMB tested (0·25 mM) produced the same plateau-like effect on ^{45}Ca efflux as did 1·0 mM; but PCMBS produced a reversible increase in rate coefficient at all concentrations except 5·0 mM, the same concentration that causes rigour.

At equimolar concentrations, cysteine prevented the effects of PCMB or PCMBS on ^{45}Ca efflux but the antagonism was not complete. Procaine (0·25 mM) had no effect on PCMB or PCMBS induced ^{45}Ca efflux.

An important difference between the effects of the two —SH inhibitors on ^{45}Ca efflux is observed when the muscles are pretreated with EDTA (Figs. 4 and 5). The

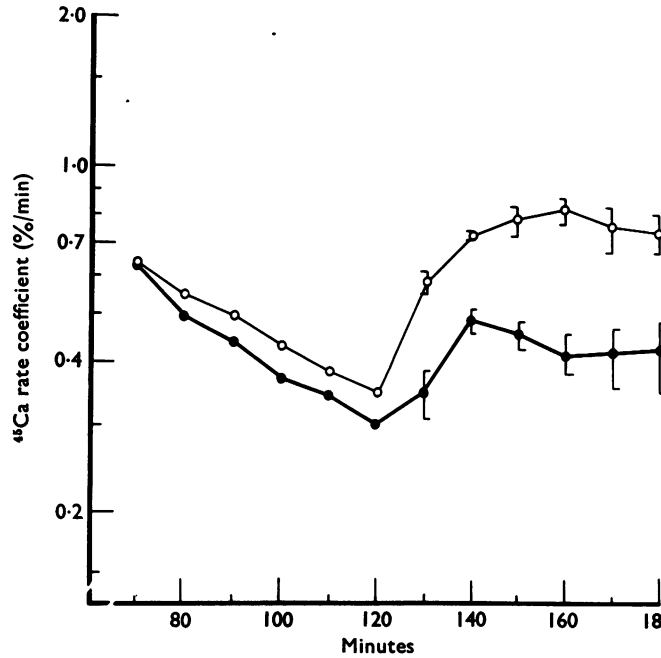

FIG. 2. Time course of the change in rate coefficient for ^{45}Ca release from paired sartorius muscles in normal Ringer solution. 1 mM PCMB (○) and 0·25 mM PCMB (●) were added to the Ringer solution from 120 min to the end of the washout. Each point is the mean of five separate experiments ($n=5$). The standard error (±S.E.) is given for the later portion of the curve.

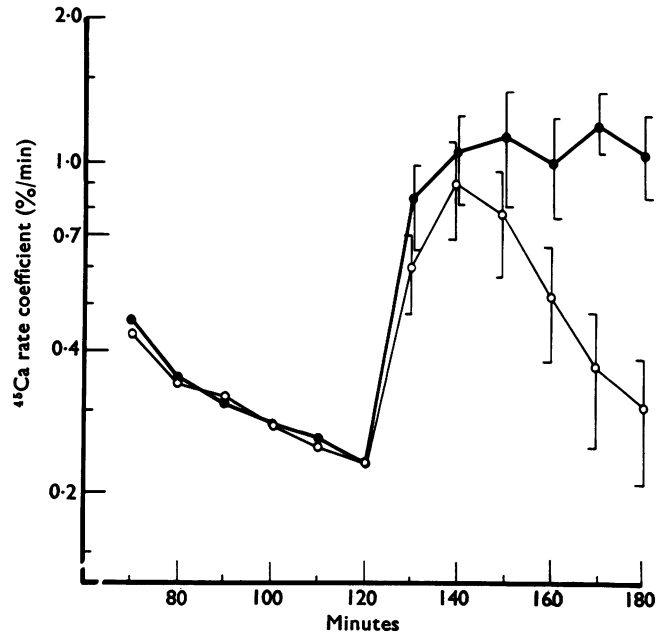

FIG. 3. Time course of the changes in rate coefficient for ^{45}Ca release from paired sartorius muscles in normal Ringer solution. 5 mM PCMBS (●) and 1 mM PCMBS (○) were added to the Ringer solution from 120 min to the end of washout. $n=5$; ±S.E.

effect of each agent was determined after the addition of 5 mM EDTA to the muscle bath. This concentration of EDTA caused by itself a prompt and transient increase in the ^{45}Ca rate coefficient. The application of 1·0 mM PCMB to the EDTA treated muscle resulted in a second increase in the rate of ^{45}Ca efflux but to a lesser degree than in the absence of EDTA (Fig. 4). EDTA can therefore be viewed as a partial antagonist of the stimulation of ^{45}Ca efflux by PCMB. On the other hand, as shown in Fig. 5, the same concentration of EDTA *completely* blocked the effect of 1·0 mM PCMBS on ^{45}Ca efflux. Even the effect of 5·0 mM PCMBS on ^{45}Ca efflux was almost completely antagonized by 5 mM EDTA.

Effects on resting and action potentials

The effects of 1·0 mM of each —SH inhibitor were determined on the intracellularly recorded membrane potential at rest and during electrical stimulation. The effects on resting transmembrane potential are shown in Fig. 6. Each agent caused a membrane depolarization but there was a marked difference in the time course, PCMB causing a faster rate of deporarization than PCMBS. After 10 min

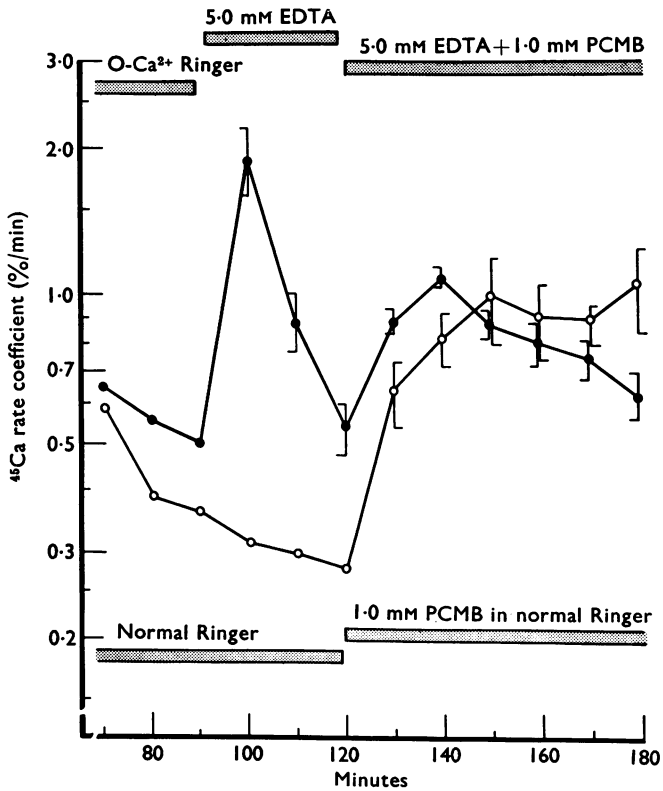

FIG. 4. Effect of PCMB, in the presence and absence of EDTA, on the rate coefficient for ^{45}Ca release from isolated paired sartorius muscles. The experimental muscles were washed out in calcium-free Ringer to which 5·0 mM EDTA was added at 90 min. The transient EDTA induced ^{45}Ca efflux is shown. The control muscles were washed out in normal Ringer solution for 120 min after which 1·0 mM PCMB was added to the washout medium. In the presence of EDTA the ^{45}Ca rate coefficient was approximately doubled by PCMB whereas it was increased at least three times if EDTA was absent. Notations at the top and bottom of the graph refer to top and bottom curves, respectively. $n=3$; ± S.E.

of treatment by PCMB, a maximum reduction in transmembrane potential of about 60 mV was noted. The same degree of depolarization by PCMBS took 30 min. The depolarization produced by each agent was prevented if an equimolar concentration of cysteine was present in the muscle bath, as is illustrated in Fig. 6. At a concentration of 0·25 mM, procaine did not alter the rate or intensity of depolarization produced by the —SH inhibitors. However, procaine did prevent the irregular fluctuations in transmembrane potential that were usually recorded during the first 5 min of treatment. These fluctuations could be associated temporally with the visible spontaneous muscle twitchings that were described earlier.

The typical effect of PCMB on the intracellularly recorded action potential is shown in Fig. 7. In the particular samples of muscle fibres that were impaled by the microelectrode, the resting potential was normal or almost normal at a time when spike amplitude, rate of rise and rate of fall were severely depressed. The effects of PCMBS were similar. With both agents, it appears that the mechanisms governing excitation are affected before those governing the resting membrane potential.

Discussion

The relatively high aqueous solubility of PCMBS has been used to explain its relatively slow diffusion across a biological membrane compared with PCMB

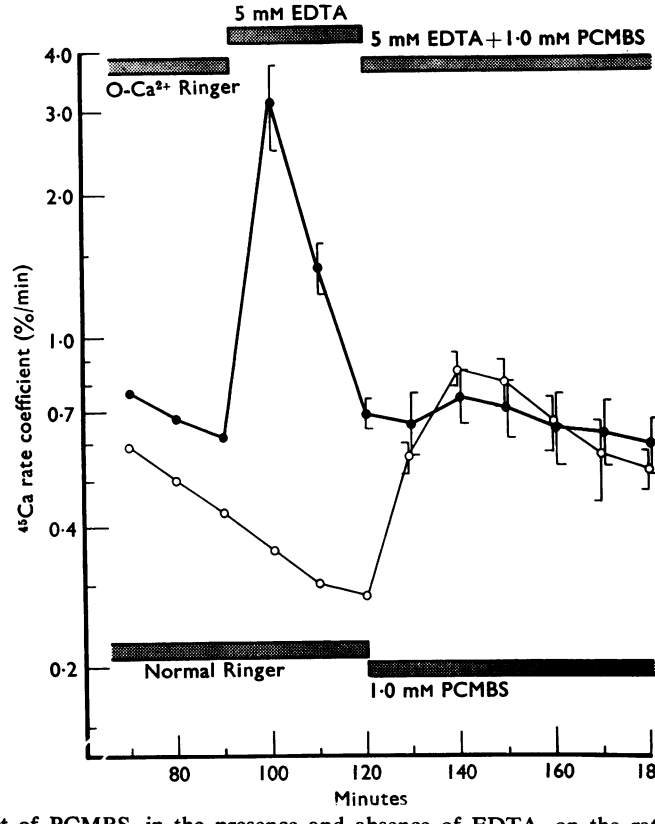

FIG. 5. Effect of PCMBS, in the presence and absence of EDTA, on the rate coefficient for ^{45}Ca release from isolated paired sartorius muscles. See legend to Fig. 4 for experimental details. Notations at top and bottom of graph refer to top and bottom curves, respectively. $n=3$; ±S.E.

(Vansteveninck *et al.*, 1965 ; Rega, Rothstein & Weed, 1967). Similarly, evidence from the present experiments indicates that PCMB penetrates to " deeper " sites of action in skeletal muscle fibres than PCMBS. This probably accounts for the only qualitative difference between pharmacological effects of the two agents: that is, PCMB produces fractionation of the evoked twitch into a fast and slow component whereas PCMBS lacks this effect. Otherwise, both organomercurials have a number of effects in common—for example, spontaneous contractions, membrane depolarization and depression of evoked twitch—although there are differences between the time action curves of these effects. If we assume that all effects of these compounds

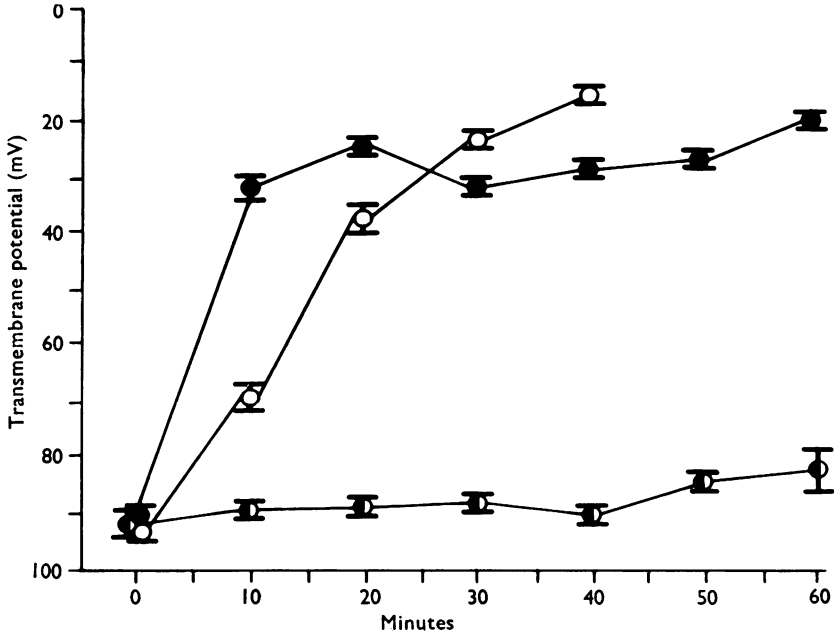

FIG. 6. Effects of 1·0 mM PCMB (●), 1·0 PCMB+1·0 mM cysteine (◐), and 1 mM PCMBS (○) on the kinetics of depolarization of frog sartorius muscle. The average control resting potential is given at zero time. The values shown are pooled averages (±S.E.) of ±5 min from the time indicated. Each open or closed circle is the average of twenty-five or more impalements made on four muscles. The half-open circles represent ten or more impalements on a single muscle. Procaine (0·25 mM) was added to the PCMB experiment and PCMBS experiment to block asynchronous contractions. In a single experiment without procaine (not shown) the results were the same as those above.

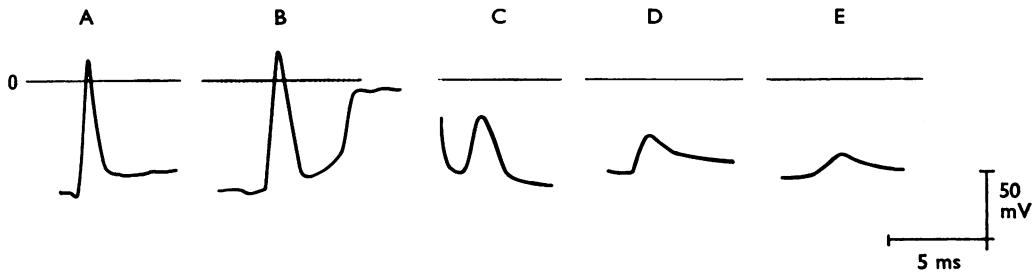

FIG. 7. Effects of 0·25 mM PCMB on the evoked action potential in surface muscle fibres. The zero potenialline is shown. (A) and (B), Control action potentials in Ringer solution followed by loss of membrane potential caused by movement (B). (C), (D) and (E), Action potentials recorded after about 10 min in 0·25 mM PCMB. Calibration bars are shown.

are caused by mechanisms involving −SH group inhibition, as is suggested by the antagonistic effects of cysteine, then qualitatively different effects may represent differences in the locus and/or function of the −SH groups attacked.

There are two lines of evidence suggesting that PCMB and PCMBS have different loci of action. One relates to the time course of the effect of −SH inhibitors on ^{45}Ca efflux; the other relates to the antagonism by EDTA of the action of −SH inhibitors on ^{45}Ca efflux. A concentration of PCMB that produced fractionation of evoked twitch, also caused a dose-related and sustained increase in rate of ^{45}Ca release from whole muscle. Although PCMBS also produced a dose-related increase in ^{45}Ca release, the response returned towards pretreatment level while PCMBS was present in the muscle bath. The loss of calcium from isolated membrane fragments of sarcoplasmic reticulum is stimulated by PCMB (Carsten & Mommaerts, 1964), and it is tempting to relate this effect to the sustained release of ^{45}Ca produced by PCMB in the whole muscle. Caffeine also promotes calcium loss from fragmented reticulum (Weber & Herz, 1968) and likewise produces sustained calcium release from whole muscle (Isaacson & Sandow, 1967); a similar relationship holds for quinine (Carvalho, 1968; Isaacson & Sandow, 1967). These results support the idea that drug induced stimulation of calcium efflux from sarcoplasmic reticulum leads to *sustained* increase in rate of loss of calcium from whole muscle. Following this line of reasoning, the inability of PCMBS to produce a sustained increase in ^{45}Ca efflux may be related to its inability to reach the sarcoplasmic reticulum in sufficient amounts, except when rigour-producing concentrations are applied. The kinetics of calcium efflux following PCMB indicates action at an internal compartment.

The results of experiments with EDTA also suggest that PCMB penetrates the sarcolemma membrane more readily than PCMBS. Previous work has shown that EDTA does not gain access to intracellular sites in skeletal muscle (Bianchi, 1965). Its prompt and transient release of ^{45}Ca is limited almost exclusively to surface membrane sites. Whereas the EDTA antagonism of PCMB action on ^{45}Ca was relatively weak, EDTA completely antagonized the release of sub-rigour producing concentrations of PCMBS. We interpret these results to mean that PCMBS releases the same component of surface calcium released by EDTA but PCMB releases primarily an internal store of calcium that is not accessible to EDTA.

We are still left with the problem of where PCMB acts to cause fractionation of the evoked muscle twitch. Recordings of action potentials reveal no mechanism whereby PCMB could be producing this effect by acting on the excitable membrane. On the other hand, there is evidence that PCMB stimulates calcium release from some component of the sarcoplasmic reticulum. The fact that PCMB did not cause muscle rigour indicates that the component of sarcoplasmic reticulum involved in the sequestering of calcium during relaxation—the longitudinal tubules—is *not* the site of PCMB action. Other components of the sarcoplasmic reticulum are directly implicated. On the basis of the experimental evidence presented here and of what is known about the mechanisms of excitation-contraction coupling, we propose that the terminal cisternae are primary sites of PCMB action associated with the twitch fractionation effect. Inhibition of −SH groups at these sites could result in a second and prolonged release of calcium in response to membrane depolarization, thus causing a second and prolonged contraction.

In further support of the terminal cisternae as a primary site of PCMB action is our finding that procaine prevents the twitch fractionation effect in concentrations below those needed to depress membrane excitability. Bianchi (1967) has presented evidence which suggests that the protonated form of procaine and other local anaesthetics inhibits calcium release by the terminal cisternae.

Other effects of both PCMB and its sulphonated analogue are spontaneous twitchings, spontaneous fluctuations in membrane potential and a steady membrane depolarization. These same effects are also observed when the muscle is bathed in a calcium-free Ringer solution (Koketsu & Noda, 1962). Perhaps −SH group inhibition in the excitable membrane leads to a labilization of the membrane similar to that caused by the absence of external calcium. In both cases the electrophysiological changes may be related to loss of calcium from anionic sites on membrane protein and consequent increases in membrane permeability. It is not unreasonable to suppose that modification of protein structure by −SH group inhibition leads to excessive loss of membrane calcium. Both organomercurials, especially PCMBS, release a component of calcium that is also affected by EDTA, and this is most likely a membrane calcium (see above).

Spontaneous muscle excitability was prevented by procaine in doses that did not antagonize effects of the organomercurials on depolarization or evoked action potential. Procaine interacts with membrane sites that exchange with extracellular calcium (Feinstein, 1963), and higher concentrations even cause calcium release (Kuperman, Altura & Chezar, 1968). This provides additional support for the proposed involvement of membrane calcium in at least some of the effects of −SH group inhibitors on the excitable membrane of skeletal muscle.

Membrane depolarization produced by the organomercurials was much greater than is caused by treatment with calcium-free Ringer with or without EDTA (Koketsu & Noda, 1962). Accordingly, the organomercurial induced depolarization is probably not caused solely by loss of membrane calcium. Prolonged treatment with PCMB or PCMBS may be expected to result in irreversible membrane damage and a non-selective increase in ion permeability. The significantly faster rate of depolarization produced by PCMB may again reflect the difference in solubility properties, the more hydrophilic PCMBS being a poor penetrator of lipid barriers in the membrane, thus reaching the crucial sites of −SH attack more slowly than PCMB.

It was interesting to observe that membrane excitability was depressed by the organomercurials before significant membrane depolarization occurred. A similar result was obtained by Smith (1958) in the PCMB treated squid giant axon. These findings suggest a specific role for −SH groups in the molecular processes underlying excitation.

In conclusion, we have shown that two organomercurial −SH inhibitors, with markedly different solubility properties, have several effects in common in the isolated frog sartorius muscle. However, there are differences in kinetics of action and at least one major qualitative difference. All effects are assumed to result from −SH group inhibition, and differences in kinetics or qualitative effect have been explained on the basis of different rates of penetration to sites of action or different cellular sites of −SH attack. Most effects of PCMBS can be accounted for by a surface membrane action whereas, in addition to effects on the cell surface, the less water soluble PCMB also penetrates to the sarcoplasmic reticulum. It is

reasonable to suppose that the molecules affected by the —SH inhibitors are proteins but the precise nature and localization of these molecules is a matter for speculation. The enzymatic activities of various ATPases, located in the excitable membrane and sarcoplasmic reticulum, are known to depend on functional —SH groups, and this is also true of myosin ATPase. At present, however, it is not possible to make a precise correlation between any effect of PCMB or PCMBS and the inhibition of any of these enzymes.

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REFERENCES

BIANCHI, C. P. (1965). The effect of EDTA and SCN on radiocalcium movement in frog *rectus abdominis* muscle during contractures induced by calcium removal. *J. Pharmac exp. Ther.*, **147**, 360–370.

BIANCHI, C. P. & BOLTON, T. C. (1967). Action of local anaesthetics on coupling systems in muscle. *J. Pharmac. exp. Ther.*, **157**, 388–405.

CARSTEN, M. E. & MOMMAERTS, W. F. H. M. (1964). The accumulation of calcium ions by sarcotubular vesicles. *J. gen. Physiol.*, **48**, 183–197.

CARVALHO, A. P. (1968). Calcium-binding properties of sarcoplasmic reticulum as influenced by ATP, caffeine, quinine, and local anaesthetics. *J. gen. Physiol.*, **52**, 622–642.

FEINSTEIN, M. B. (1963). Inhibition of caffeine rigor and radiocalcium movements by local anaesthetics in frog sartorius muscle. *J. gen. Physiol.*, **47**, 151–172.

HASSELBACH, W. & SERAYDARIAN, K. (1966). The role of sulfhydryl groups in calcium transport through the sarcoplasmic membranes of skeletal muscle. *Biochem. Z.*, **345**, 159–172.

ISAACSON, A. & SANDOW, A. (1967). Quinine and caffeine effects on Ca^{45} movements in frog sartorius muscle. *J. gen. Physiol.*, **50**, 2109–2128.

KOKETSU, K. & NODA, K. (1962). Membrane responses of frog skeletal muscle fibres in calcium-free media. *J. cell. comp. Physiol.*, **59**, 323–332.

KUPERMAN, A. S., ALTURA, B. T. & CHEZAR, J. A. (1968). Action of procaine on calcium efflux from frog nerve and muscle. *Nature, Lond.*, **217**, 673–675.

LEVY, H. M. & RYAN, E. (1965). Evidence that calcium activates the contraction of actomyosin by overcoming substrate inhibition. *Nature, Lond.*, **205**, 703–705.

OKAMOTO, M. & KUPERMAN, A. S. (1966). Muscle contraction produced by sulphydryl inhibitors. *Nature, Lond.*, **210**, 1062–1063.

REGA, A., ROTHSTEIN, A. & WEED, R. I. (1967). Erythrocyte membrane sulfhydryl groups and the active transport of cations. *J. cell. comp. Physiol.*, **70**, 45–52.

SHANES, A. M. & BIANCHI, C. P. (1959). The distribution and kinetics of release of radiocalcium in tendon and skeletal muscle. *J. gen. Physiol.*, **42**, 1123–1137.

SMITH, H. M. (1958). Effect of sulfhydryl blockade on axonal function. *J. cell. comp. Physiol.*, **51**-161–171.

VASTEVENINCK, J., WEED, R. I., and ROTHSTEIN, A. (1965). Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. gen. Physiol.*, **48**, 617–632.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. *J. gen. Phsyiol.*, **52**, 750–759.

WINEGRAD, S. (1968). Intracellular calcium movements of frog skeletal muscle during recovery from tetanus. *J. gen. Physiol.*, **51**, 65–83.

YASUI, B., FUCHS, F. & BRIGGS, F. N. (1968). The role of sulfhydryl groups of tropomyosin and tropinin in the calcium control of actomyosin contractility. *J. biol. Chem.*, **243**, 735–742.

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