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Effects of sulphydryl inhibitors on frog sartorius muscle: N-ethylmaleimide

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Summary

1. The characteristic action of the —SH inhibitor, N-ethylmaleimide (NEM), is muscle rigour. The dose-response curve indicates a biphasic effect with maximum rigour tension produced by 1·0 mM NEM; beyond 1·0 mM there was an inverse relationship between dose and response.

2. NEM produces a membrane depolarization unrelated to rigour development.

3. NEM causes a sustained increase in ^{45}Ca efflux from whole muscle. Pretreatment of a muscle with ethylenediamine tetra-acetic acid (EDTA, 5 mM) to remove membrane calcium does not alter the NEM induced ^{45}Ca efflux.

4. It is suggested that the primary site of NEM action is inhibition of calcium uptake by the sarcoplasmic reticulum thereby producing rigour. At concentrations above 1·0 mM, NEM may affect the myofilaments.

Introduction

In a previous report (Kirsten & Kuperman, 1970) the effects of two organomercurial-SH inhibitors on frog skeletal muscle were described; these were *p*-chloromercuribenzoic acid (PCMB) and *p*-chloromercuribenzenesulphonic acid (PCMBS). The two agents were shown to have many effects in common, as would be expected if they are acting as –SH inhibitors, but there was one important qualitative difference and many differences in kinetics of action. The differences were thought to be caused by different cellular sites of –SH attack and different rates of transport to the same sites. By studying several –SH inhibitors with varying molecular structures and physicochemical properties, a variety of pharmacological actions on skeletal muscle may be revealed, each one caused by –SH inhibition at a specific cellular site. With this end in view, we chose to study the effects of N-ethylmaleimide (NEM). This compound departs from the organomercurial structure and blocks –SH groups through an alkylating mechanism. Furthermore, NEM has greater selectivity for accessible –SH groups on protein molecules and passes easily through cell membranes (Jacob & Jandl, 1962; Webb, 1966). For these reasons, the pharmacological effects of NEM were expected to differ from those of PCMB or PCMBS, thus revealing additional roles of –SH groups in skeletal muscle function.

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Methods

Isolation of the preparation, together with the procedures and instrumentation used in the mechanical, radiocalcium and intracellular recording studies, have been described previously (Kirsten & Kuperman, 1970).

Results

Mechanical effects

In a preliminary study (Okamoto & Kuperman, 1966) two effects of NEM on the unstimulated skeletal muscle were observed; these were contraction and rigour. Sandow & Isaacson (1966) also reported a contractile effect of NEM. In the present experiments, however, the contractile effect was seen infrequently and was of very variable amplitude. We have no explanation for this discrepancy at present. Nevertheless, the rigour effect is reproducible and has now been studied in greater detail.

Rigour was produced by concentrations of NEM ranging from 0·1 to 20 mM. The effect of each concentration was the same whether or not the muscle was stimulated by electrical pulses. Examples of NEM rigour in the stimulated muscle are shown in Fig. 1.

Potentiation of the evoked maximal twitch by 0·1–20 mM NEM was never observed. At 0·1–1·0 mM, the evoked twitch tension decreased gradually to zero during the maintenance of rigour (Fig. 1, top section). At concentrations greater than 1·0 mM, the depression of evoked twitch tension occurred prior to onset of rigour (Fig. 1, bottom section).

From Fig. 1, it is obvious that no simple relationship exists between NEM concentration and rigour tension. A dose-response study for NEM concentrations between 0·1 and 20 mM revealed a biphasic effect (Fig. 2). Threshold concentration for rigour was approximately 0·1 mM; maximum rigour tension was produced by 1·0 mM; beyond 1·0 mM there was an inverse relationship between dose and

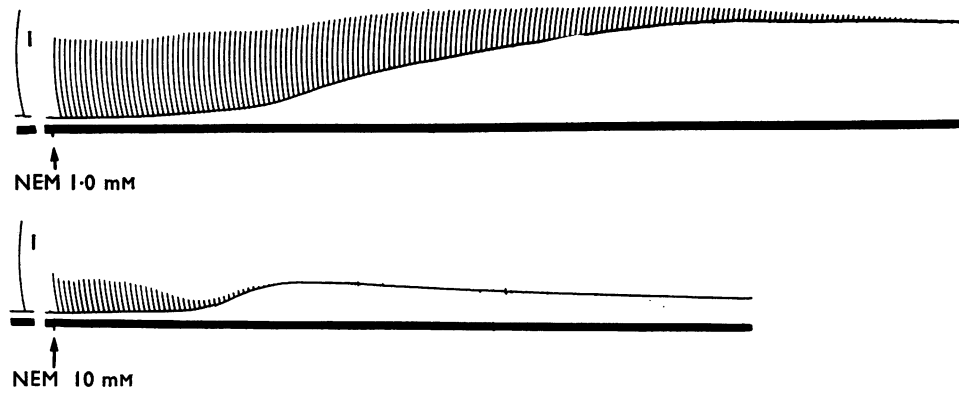

FIG. 1. Effect of 1·0 and 10·0 mM NEM on the electrically evoked twitch of isolated curarized frog sartorius muscle. Evoked twitch tension diminishes as rigour tension increases until at about maximum rigour tension the muscles become inexcitable. A 200 ms electrically evoked control tetanus is shown to the left of the arrows. The time markers indicate 5 seconds. Paired muscles. Calibration bar: 2·5 g. Stimulation frequency: 1 pulse/15 seconds.

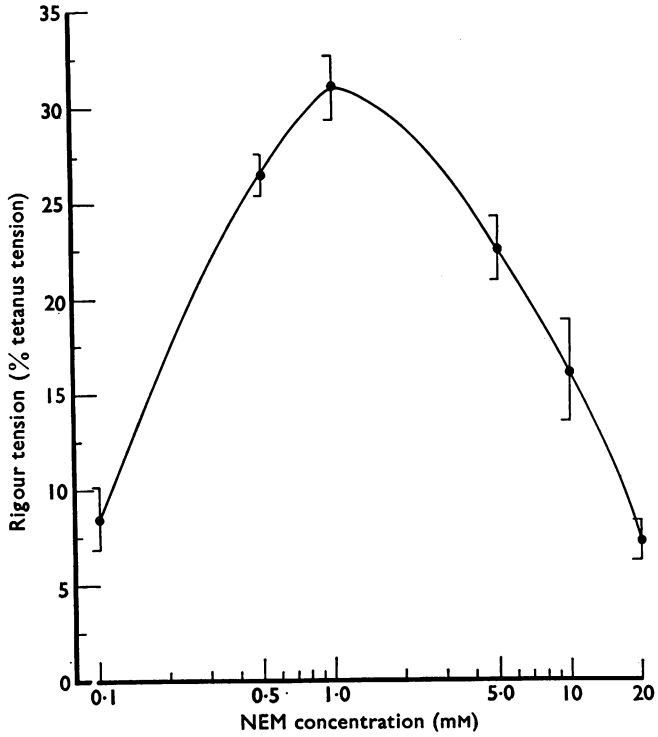

FIG. 2. Dosage-response relationship for NEM on frog sartorius muscle. The rigour tension is expressed as a percentage of control tetanus tension before the addition of NEM. The muscle was not stimulated after the addition of NEM. Values given are means for six muscles, and the standard error is indicated ($n=6$; ±S.E.).

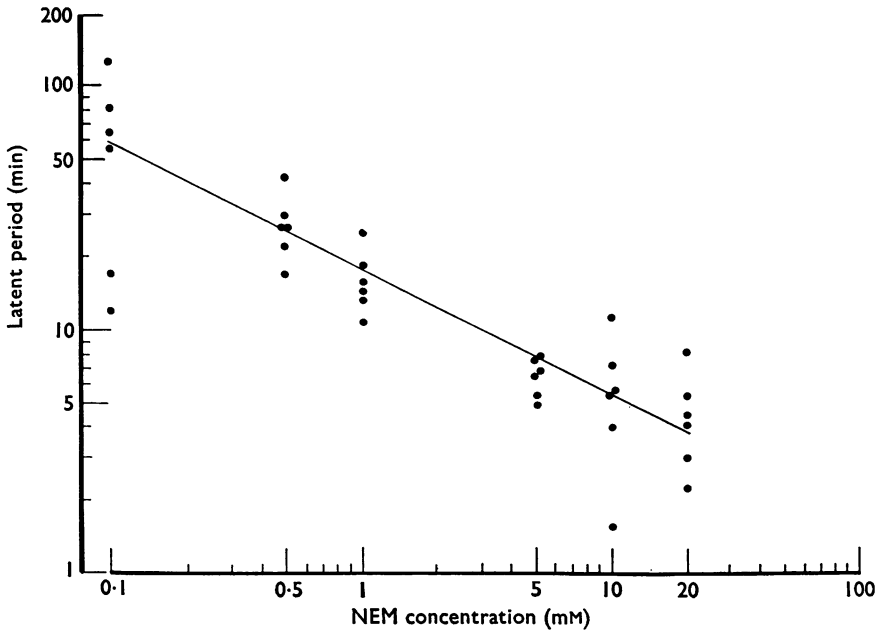

FIG. 3. Latent period to onset of NEM induced rigour tension. Each point represents a single experiment. The muscle was not stimulated after the addition of NEM. The indicated regression line is highly significant ($P<0{\cdot}001$).

response. On the other hand, the time to onset of rigour was a linear function of dose and varied in a reciprocal manner (Fig. 3).

If NEM was added to the muscle after a 30 min pretreatment with 5.0 mM EDTA, rigour still occurred, albeit at a somewhat reduced amplitude ; too few experiments were done at each NEM concentration to test for significance of the amplitude difference. Similarly, NEM action was not seriously impaired by a 10 min pretreatment with isotonic KCl. If 10–20 mM caffeine was added to a muscle already in NEM rigour, no additional rigour tension was produced. The application of equimolar concentrations of NEM and cysteine had no effect on the resting or electrically stimulated muscle. However, cysteine could not reverse NEM rigour.

Effects on ^{45}Ca efflux

An increase in rate of ^{45}Ca efflux from skeletal muscle is produced by the rigour-producing agents caffeine (Isaacson & Sandow, 1967), quinine (Isaacson & Sandow,

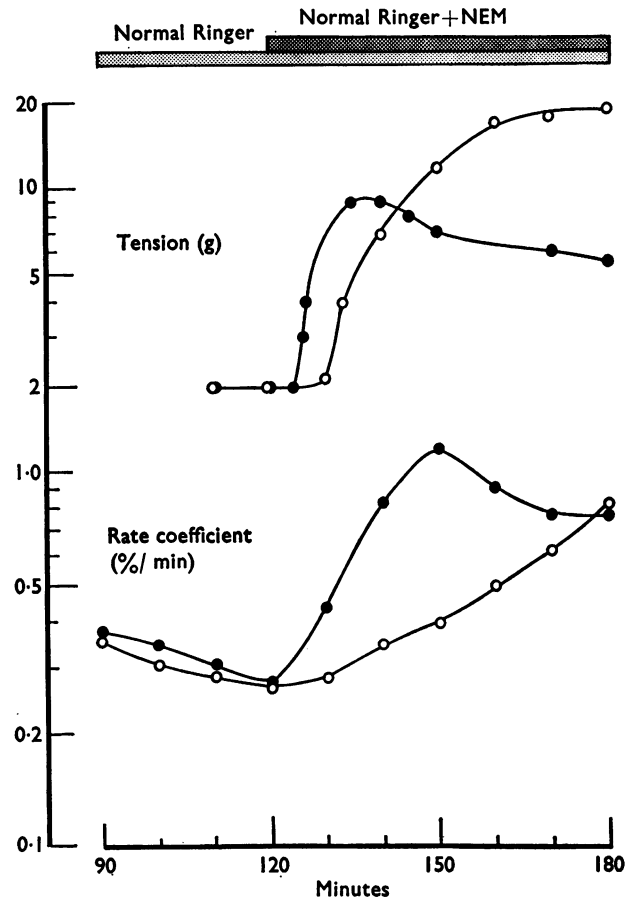

FIG. 4. The development of rigour tension for 1·0 (○——○) and 10·0 (●——●) mM NEM (top curve) compared with the rate coefficient for ^{45}Ca release (bottom curve). The top curve was recorded from a single pair of sartorius muscles and replotted with tension on a logarithmic scale. The muscles were held at an initial tension of 2 g. The bottom curve gives the changes in ^{45}Ca efflux after the addition of NEM to the Ringer solution at 120 minutes. Each point is the mean of five separate experiments ($n=5$). Notation at top of graph indicates addition of NEM.

1967; Isaacson, Yamaji & Sandow, 1970) and iodoacetic acid (Bianchi, 1963), and in each case it is thought that the calcium efflux derives primarily from the sarcoplasmic reticulum. In the present experiments, we found that rigour-producing concentrations of NEM also increase the rate of ^{45}Ca efflux. The effects of 1·0 and 10 mM NEM on both the ^{45}Ca rate coefficient and rigour tension are shown in Fig. 4. Note that 10 mM causes a faster and greater increase in ^{45}Ca rate coefficient than 1 mM NEM although the lower concentration produces the greatest rigour. Thus, there appears to be no relationship between the NEM effect on rigour and the effect on rate of efflux of ^{45}Ca.

The effect of NEM on ^{45}Ca efflux was also determined in the presence of and after 30 min pretreatment with 5 mM EDTA. There was no influence of EDTA on the NEM-induced ^{45}Ca efflux, and this is illustrated for 10 mM NEM in Fig. 5. If cysteine and NEM were applied in equimolar concentration, no effect on ^{45}Ca efflux was produced.

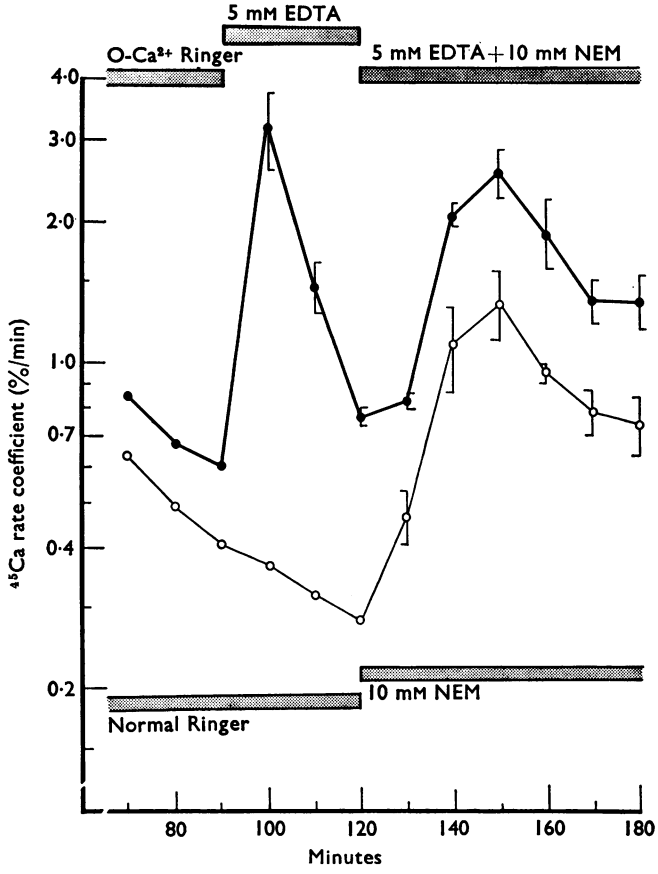

FIG. 5. Effect of NEM, in the presence and absence of EDTA, on the rate coefficient for ^{45}Ca release from isolated paired sartorius muscles. The experimental muscles (●——●) were washed out in calcium-free Ringer to which 5·0 mM EDTA was added at 90 min and 10·0 mM NEM at 120 min. The transient EDTA-induced ^{45}Ca efflux is shown. The control muscles (○——○) were washed out in normal Ringer solution for 120 min after which 10·0 mM NEM was added to the washout medium. Each point is the mean of three muscles ($n=3$). The standard error (±S.E.) is given for the later portion of the curve. Notations at the top and bottom of the graph refer to top and bottom curves, respectively.

Effects on resting and action potentials

The effects on resting transmembrane potential were determined after application of 1·0 and 10 mM NEM. As shown in Fig. 6, both concentrations produced the same amount of depolarization (about 60 mV) but at different rates. It is important to note that although 1·0 mM produced a slower rate of depolarization, it produces much greater rigour tension (Fig. 2); in fact, the peak rigour is produced before any effect of 1·0 mM NEM on resting potential was recorded. Spontaneous, low-amplitude fluctuations in resting potential were never observed after NEM application, and this is in contrast to the effect of organomercurials (Kirsten & Kuperman, 1970).

The effects of NEM on the electrically evoked action potential were similar to those produced by the organomercurials (Kirsten & Kuperman, 1970). As seen in Fig. 7, the amplitude and rate of rise and fall of the action potential were severely depressed at a time when resting membrane potential was at or close to normal.

All effects of NEM on membrane potential were prevented if cysteine was added simultaneously and in equimolar concentration.

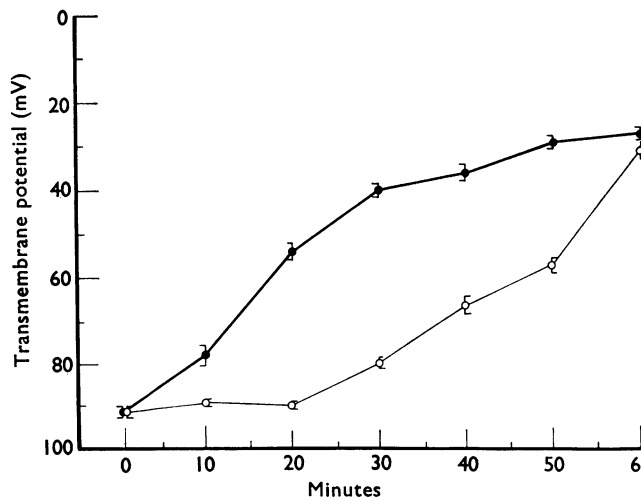

FIG. 6. Effect of 1·0 mM NEM (○) and 10·0 mM NEM (●) on the kinetics of depolarization of frog sartorius muscle. The average control resting potential is given at zero time. The values shown are pooled averages (±S.E.) of ±5 min from the time indicated. Each point is the average of twenty-five or more impalements made on three muscles.

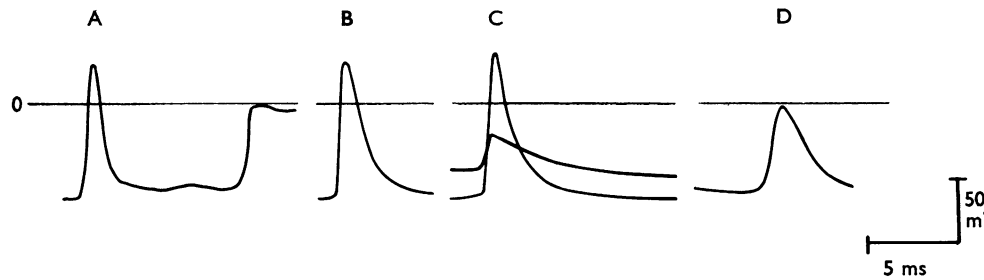

FIG. 7. Effect of 1·0 mM NEM on evoked action potentials in surface muscle fibres. The zero potential line is shown. (A) Control action potential in Ringer solution followed by loss of membrane potential due to movement. (B) Action potential after 10 min in 1·0 mM NEM. (C) Action potentials from the same fibre after 20 and 25 min (small spike) in 1·0 mM NEM. (D) Action potential after 25 min in 1·0 mM NEM. Calibration bars are shown.

Discussion

In contrast to the effects of the organomercurial –SH inhibitors described in a preceding paper (Kirsten & Kuperman, 1970), the primary effect of NEM on frog sartorius muscle is rigour. In speculating about the mechanism of NEM rigour, we suggest that it is associated with –SH group inhibition because NEM has a high specificity for –SH groups, and cysteine completely prevents the rigour action. We are still left with the problem of the cellular site of NEM action. The NEM-induced depolarization and depression of excitability are indicative of a surface membrane site of action but these effects are probably more related to depression of the electrically evoked twitch than to rigour. Evidence against the surface membrane as a site of NEM rigour is: (1) the rigour begins and reaches a peak before depolarization or depression of spike amplitude occur; (2) rigour is produced by NEM, albeit at a somewhat reduced level, after prolonged treatment of the muscle depolarizing concentrations of EDTA or KCl.

An important clue to the site of NEM rigour is that NEM causes a sustained increase in ^{45}Ca efflux from the whole muscle, an effect also caused by other rigour-producing drugs like caffeine and quinine (Isaacson & Sandow, 1967). The kinetics of rigour development by NEM and its effect on ^{45}Ca efflux were similar, suggesting a relationship between the two effects. In these experiments, NEM was added to the muscle bath during a period of time when ^{45}Ca is assumed to be emerging primarily from the sarcoplasmic reticulum (Bianchi, 1961; Isaacson & Sandow, 1967). If this assumption is valid, then a sustained increase in ^{45}Ca efflux from the muscle reflects an increase in the amount of free intracellular calcium released from the sarcoplasmic reticulum. In the context of present ideas concerning the control of free myoplasmic calcium concentration (compare Sandow, 1970), it is possible that NEM causes release of calcium from the sarcoplasmic reticulum or blocks the active re-uptake of calcium; either effect would lead to a rise of free myoplasmic calcium above the threshold level required for contraction. Hasselbach (1966) found that NEM inhibits uptake of calcium by isolated vesicles of the sarcoplasmic reticulum and –SH groups located on the outer surface of these vesicles were implicated in active calcium transport (Hasselbach, 1966; Hasselbach & Seraydarian, 1966). It is therefore, most likely that the primary effect of NEM on intact muscle is to inhibit calcium re-uptake by the sarcoplasmic reticulum, an effect leading to both the rigour and increased calcium efflux.

We showed that EDTA did not antagonize the NEM induced increase in ^{45}Ca efflux. Assuming that the chelating action of EDTA is exerted only at the surface membrane of the muscle cell (Bianchi, 1965), this finding provides additional support for an intracellular site of NEM action. On the basis of the relatively high permeability of biological membranes to NEM (Webb, 1966), a rapid penetration of this compound to sites in the sarcoplasmic reticulum would be expected. Nevertheless, NEM rigour has a long latent period that is inversely related to concentration; even at the optimum rigour-producing concentration of 1·0 mM, the latent period was about 20 min. This delay to onset of rigour probably depends on the time required for the myoplasmic concentration of free calcium to attain threshold level rather than on the time needed for penetration to intracellular sites of action.

Above 1·0 mM concentration of NEM, we observed an inverse relationship between NEM concentration and rigour tension. NEM also produced a dose-dependent reduction of electrically evoked twitch at all concentrations tested. Thus,

NEM has two opposing actions on the muscle cell, one leading to rigour and the other leading to depression of contractile tension, and each has its own dose-response relationship. It can be assumed that the concentration needed for rigour development is considerably lower than that needed for depression of contraction. Myosin ATPase activity (Blum, 1962), the F-actin/myosin interaction (Bailin & Bárány, 1967) and the troponin-tropomyosin interaction (Yasui, Fuchs & Briggs, 1968) are all influenced by –SH inhibitors and are possible molecular sites of action of NEM associated with contractile depression.

Another interesting property of NEM is depression of muscle action potential amplitude in the absence of significant alteration of resting potential. We previously described this same effect for organomercurial –SH inhibitors (Kirsten & Kuperman, 1970). Dissociation of effects on excitability from effects on resting potential is usually interpreted to indicate specific effects on molecular mechanisms underlying excitation. Huneeus-Cox, Fernandez & Smith (1966), on the basis of similar results on the squid giant axon, proposed that protein –SH groups are involved in ion-gating during excitation.

Comparing the effects of NEM on the frog sartorius muscle with those of PCMB and PCMBS (Kirsten & Kuperman, 1970), we see that the three –SH inhibitors produce a number of effects in common, for example membrane depolarization, depression of excitability, increase in rate of ^{45}Ca efflux, depression of contraction. However, qualitative differences exist; NEM and PCMBS produce rigour but PCMB does not; only PCMB produces twitch fractionation; both organomercurials cause spontaneous contractions and fluctuations in membrane potential but NEM does not. In some cases, these qualitative differences can be explained by assuming different cellular sites of –SH attack based, in turn, on different solubility properties and patterns of distribution in the muscle cell. It is also possible that the functional —SH groups on different structural and enzyme proteins vary widely in their sensitivity to attack by different —SH inhibitors.

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