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On the Similar Effects of Chemical Reduction and Electrical Stimulation in Walking Leg Nerve Bundles of the Spider Crab

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SUMMARY

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Fluorescence techniques have been used to measure the binding of Mercurochrome to crustacean leg nerve bundles and reversal of binding by 2-mercaptoethanol. It was demonstrated that both parameters are increased by electrical stimulation of the nerve bundle. In this study it is shown that prior treatment of the nerve preparation with dithiothreitol or hydrazine sulfate increases the binding and the conduction-blocking activity of Mercurochrome to about the same extent as that induced by electrical stimulation. Following chemical reduction, electrical stimulation produces no further increase in binding or conduction block by the mercurial over and above the effect of reduction alone. Unlike electrical stimulation, however, reducing agents do not increase the release of bound Mercurochrome or reversal of the conduction block by 2-mercaptoethanol. The data are compatible with the possibility that electrolytic reduction of disulfides may be involved in a conformational change in membrane proteins during electrical excitation.

INTRODUCTION

It has long been postulated that, during the conduction of an impulse in electrically excitable membranes, conformational changes occur which are essential to changes in cation permeability (1). In recent years ample evidence has been obtained that such conformational changes do, in fact, take place and can be correlated with conduction of the nerve impulse (2, 3). It has also been postulated that protein conformational changes play an essential role in other electron transport systems such as oxidative phosphorylation (4).

Investigation of the proteins of axonal membranes in our laboratory revealed that conduction block by thiol-reactive compounds is potentiated by electrical stimulation of the nerve fibers (5, 6). In addition, fluorescence measurements demonstrated that the binding of Mercurochrome to nerve bundles and the release of bound Mercurochrome by 2-mercaptoethanol are both significantly increased by electrical stimulation (7). Possible explanations of these effects of electrical stimulation include (a) altered permeability of the nerve membrane, (b) a conformational change

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exposing previously inaccessible thiol groups, and (c) electrolytic reduction of disulfide groups with the transient formation of thiols, possibly followed by disulfide rearrangement (6). Only with the third of these possibilities would it be expected that chemical reduction of disulfides should mimic the effects of electrical stimulation in altering the interaction of thiol reagents with the axonal membranes.

We have compared the effects of treating nerve bundles with strong reducing agents with the effects of electrical stimulation described previously. The conduction-blocking activity of Mercurochrome and the mercurial-binding capacity of the nerve bundles were determined following incubation with a reducing agent. In addition, the recovery of excitability and release of bound Mercurochrome by 2-mercaptoethanol were determined in these treated fibers.

MATERIALS AND METHODS

All experiments were carried out as described previously (7). Spider crabs (*Libinia emarginata*) were obtained from the Marine Biological Laboratory, Woods Hole, Mass. Walking leg nerve bundles were dissected just prior to each experiment and were placed in a glass dish containing physiological saline (416 mM NaCl, 10 mM KCl, 11 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 55 mM $MgCl_2$, and 2.5 mM glucose, pH 7.8–8.0). All experiments were performed at room temperature (20–25°). After each experiment the nerve bundles were blotted, weighed, and frozen for protein assay (8).

The binding of a wide range of bath concentrations of Mercurochrome (1–500 μM) was measured as the difference in the relative fluorescence intensity of the test solution before and after incubation with a nerve bundle. Similarly, reversal of binding was measured as the appearance of fluorescence in a solution of 20 mM 2-mercaptoethanol after incubation with a mercurial-blocked nerve bundle. Controls, described previously (3), demonstrated the usefulness of this technique. In addition, the amount of bound Mercurochrome which may be released by 2-mercaptoethanol was determined by homogenization of the mercurial-treated nerve bundle in 2 ml of 50 mM 2-mercaptoethanol followed by centrifugation of the homogenate (20 min at 27,000 × *g*) and assay of the fluorescence intensity of the supernatant.

For the experiments to determine the effect of electrical stimulation, the nerve was stimulated with a 2-msec square pulse of threshold intensity at 9/sec throughout the incubation with Mercurochrome. The duration of the incubation was 30 min for all concentrations of Mercurochrome tested. For most experiments with reducing agents, each nerve bundle was incubated for 10 min in 10 mM dithiothreitol (Pierce Chemical Company) or 1 mM hydrazine sulfate (Mallinckrodt Chemical Works) and washed thoroughly with physiological saline prior to incubation with Mercurochrome. In order to estimate the approximate rate of reaction of DTT[1] with the nerve bundles, experiments were also performed using 1-, 15-, and 30-min initial treatment times. All electrophysiological recordings were made with extracellular Ag-AgCl electrodes as described previously (6). In order to increase the sensitivity to small changes in fluorescence, the external bath volume of the nerve chamber was limited to 5 ml, and large nerve bundles (1-mm external diameter) were selected.

Fluorescence measurements were made using an Aminco-Bowman spectrophotofluorometer equipped with an ellipsoidal mirror condensing system. A stable reference solution of anthracene in cyclohexane was used to standardize each set of readings. All reagents were of analytical grade.

RESULTS

The fluorescence of Mercurochrome in artificial sea water (Instant Ocean, Aquarium Systems, Inc.) is directly proportional to concentration up to 10 μM, above which inner filter effects appear. Samples in the concentration range above 10 μM were diluted to be read in the linear portion of the standard curve expressing the relationship between concentration of Mercurochrome and fluorescence intensity (9).

Using external electrophysiological recordings, it was observed that the ability of

[1] The abbreviation used is: DTT, dithiothreitol.

TABLE 1

Effect of electrical stimulation on conduction-blocking activity of Mercurochrome and its reversal by 2-mercaptoethanol following treatment with dithiothreitol

Nerve bundles were incubated for 10 min in 10 mM DTT and washed thoroughly in physiological saline prior to 30 min of incubation in Mercurochrome. Conduction block was measured as the reduction in peak amplitude of the action potential. Recovery of peak action potential amplitude after 20 min of incubation in 20 mM 2-mercaptoethanol was taken as a measure of reversal of conduction block. Each value represents the mean ± standard error of three to five experiments.

Mercurochrome	Electrical stimulation	Conduction block	Reversal by 2-mercaptoethanol
μM		%	%
1	No	77 ± 3	34 ± 8
	Yes	73 ± 5	33 ± 5
5	No	100	26 ± 3
	Yes	90 ± 5	33 ± 6
10	No	100 (35 ± 7)[a]	41 ± 5
	Yes	100 (55 ± 5)	40 ± 1
50	No	100	30 ± 2
	Yes	100	46 ± 8
100	No	100 (46 ± 17)	43 ± 14
	Yes	100 (54 ± 13)	35 ± 10
500	No	100 (90 ± 10)	35 ± 11
	Yes	100 (75 ± 8)	35 ± 2

[a] The figures in parentheses are the conduction block measured after only 10 min of incubation.

nerve bundles to conduct a normal action potential was not significantly altered by DTT or hydrazine sulfate. Intracellular microelectrode analysis of the effects of disulfide reducing agents and thiol reagents on single axons from the circumesophageal connectives of *Homarus americanus* has demonstrated that DTT alone has no measurable effect on nerve conduction, but that prior treatment with DTT significantly alters the nature and the time course of the effects of thiol reagents such as Mercurochrome.[2]

The data in Table 1 demonstrate that incubation of spider crab nerve bundles for 10 min in 10 mM DTT abolished or markedly reduced the effect of electrical stimulation on the conduction-blocking activity of Mercurochrome and on the recovery of excitability by 2-mercaptoethanol. This is further illustrated in the bar graph in Fig. 1 for a single concentration of Mercurochrome (10 μM) with reference to earlier data obtained without DTT treatment (7). Following DTT, electrical stimulation was seen to increase the effect of 10 μM Mercurochrome from 35 ± 7% to 55 ± 5%, whereas in the absence of prior DTT treatment conduction block was potentiated to 100% by electrical stimulation.

Treatment with 1 mM hydrazine sulfate prior to incubation with Mercurochrome also eliminated the potentiating effect of electrical stimulation which was described previously (7). The conduction-blocking activity of 50 μM Mercurochrome, after incubation with 1 mM hydrazine sulfate, was 86 + 7% without stimulation and 95 ± 5% with stimulation. Reversal of block by 2-mercaptoethanol was measured as 32 ±

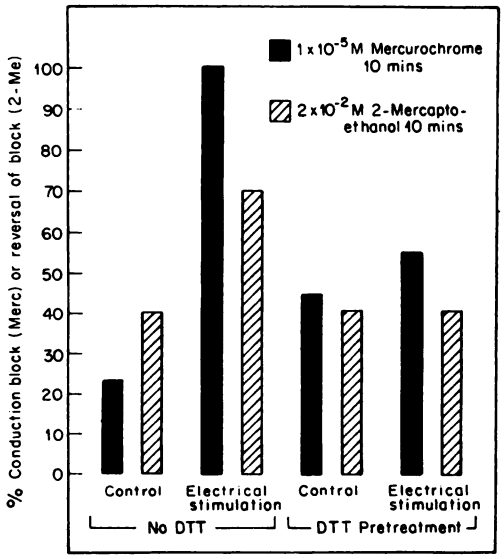

FIG. 1. *Percentage conduction block (solid bars) by 10 μM Mercurochrome (Merc) and percentage reversal of block (hatched bars) by 20 mM 2-mercaptoethanol (2-Me) for experiments without prior incubation in dithiothreitol and for experiments with 10-min treatment in 10 mM DTT*

In each case the "control" refers to measurements in the absence of electrical stimulation. All bars are drawn to the upper limit of the mean ± the standard error.

[2] J. K. Marquis and J. G. Perry, unpublished observations.

8% without stimulation and 33 ± 10% with stimulation.

Figure 2 is a plot of the amount of Mercurochrome bound to nerve bundles as a function of Mercurochrome concentration in the external bath solution. Prior treatment with DTT produced a measurable increase (as large as 1000%, see Table 2) in the amount of Mercurochrome bound at all concentrations tested except 1 μM. Electrical stimulation during the incubation with Mercurochrome produced little or no additional increase in binding. A comparable increase in the amount of Mercurochrome bound was obtained with 1-, 15-, and 30-min DTT treatment periods followed by prolonged washing to eliminate the possible interaction between reducing agent and mercurial. Thus the site of action of these compounds is relatively accessible to the external bathing solution.

Similar data with 50 μM Mercurochrome were obtained after treatment with another reducing agent, hydrazine sulfate. After 20

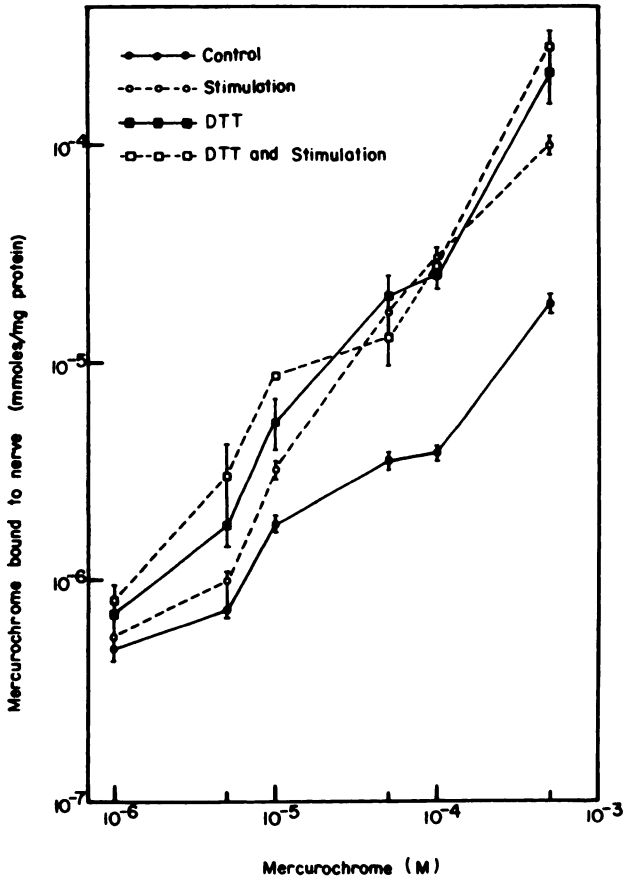

FIG. 2. *Mercurochrome bound after 30 min of incubation, with and without electrical stimulation and in both untreated and DTT-treated nerve bundles, plotted as a function of molar concentration of Mercurochrome in bath solution*

For the experiments with electrical stimulation, the nerve was stimulated with a 2-msec square pulse of threshold intensity at 9/sec throughout the incubation with Mercurochrome. For the experiments with DTT, each nerve bundle was incubated for 20 min in 10 mM DTT and washed thoroughly with physiological saline prior to incubation with Mercurochrome. Each point represents the mean of *n* experiments (see Tables 2 and 3). Vertical bars are used to indicate the range of the mean ± standard error except where the values lie within the area of the symbol used to denote a point.

TABLE 2

Effect of DTT treatment on binding of Mercurochrome to nerve bundles, and release of bound Mercurochrome by 2-mercaptoethanol in the absence of electrical stimulation

These data are the control figures referred to in Table 3.

Mercurochrome	DTT treatment	n	Mercurochrome bound	Effect (within experimental error)	Mercurochrome released	Effect (within experimental error)
μM			nmoles/mg protein	%	nmoles/mg protein	%
1	No	4	0.49 ± 0.07		0.16 ± 0.02	
	Yes	3	0.68 ± 0.2	None	0.11 ± 0.03	None
5	No	9	0.74 ± 0.07		0.26 ± 0.04 (0.20 ± 0.05)[a]	
	Yes	3	1.8 ± 0.4	+143	0.24 ± 0.04	None
10	No	8	1.9 ± 0.2		1 ± 0.2 (0.6 ± 0.4)	
	Yes	3	5.3 ± 1.6	+179	0.6 ± 0.2	None
50	No	9	3.5 ± 0.3		1.6 ± 0.2	
	Yes	3	20 ± 5	+471	3.1 ± 1.0	+94
100	No	5	3.8 ± 0.3		1.2 ± 0.1	
	Yes	3	25 ± 3	+558	3.4 ± 0.7	+183
500	No	4	19 ± 2		6 ± 0.9	
	Yes	3	210 ± 60	+1005	7.6 ± 0.4	+27

[a] Values in parentheses were obtained from homogenized nerve bundles as described under MATERIALS AND METHODS.

min of treatment in 1 mM hydrazine, the amount of Mercurochrome bound was increased from 3.5 to 13 nmoles/mg of protein in the absence of electrical stimulation, an increase of nearly 400%. Electrical stimulation during incubation with Mercurochrome following hydrazine treatment produced no measurable additional effect.

Figure 3 is a plot of the amount of bound Mercurochrome released from the nerve bundles after 15 min of incubation in 2-mercaptoethanol. It is evident that DTT treatment abolishes the increase in Mercurochrome release which was observed previously in the presence of electrical stimulation (3). Unlike electrical stimulation, however, DTT itself produces no significant increase in the amount of bound Mercurochrome released (see also Table 2).

Similar data with 50 μM Mercurochrome were obtained following treatment in 1 mM hydrazine sulfate. In this case, the amount of bound Mercurochrome released in the presence of 2-mercaptoethanol increased from 1.6 to 2.6 nmoles/mg of protein, a total increase of only 111% (including standard errors), compared to an increase of 660% observed with electrical stimulation (7). Electrical stimulation following hydrazine treatment produced no additional effect on Mercurochrome release.

The absence of any effect of electrical stimulation on either Mercurochrome binding or release of bound Mercurochrome following treatment with disulfide reducing agents is evident from the data in Table 3.

DISCUSSION

The present study demonstrates the similar effects of chemical reduction and electrical stimulation on the excitability and mercurial-binding capacity of crustacean nerve membranes. Reducing agents, such

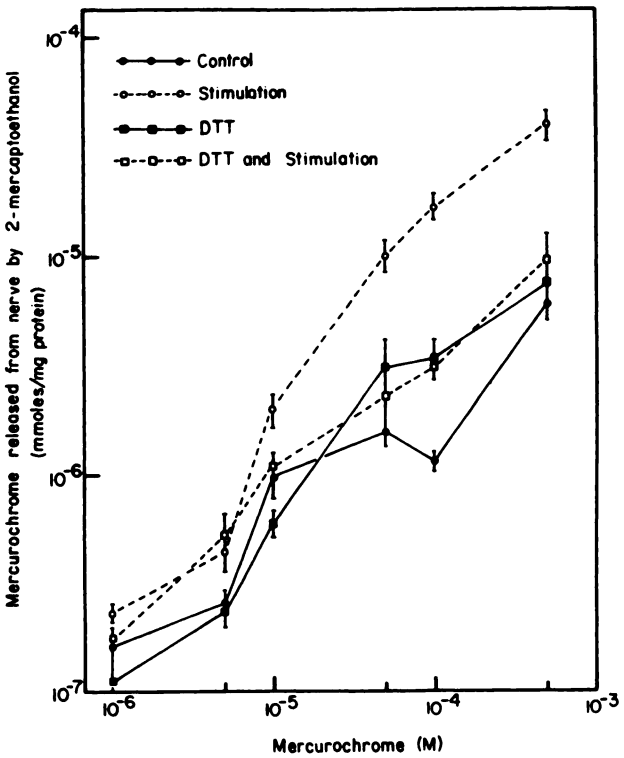

FIG. 3. *Bound Mercurochrome released from a nerve bundle after 15 min of incubation with 20 mM 2-mercaptoethanol, with and without electrical stimulation and in untreated and DTT-treated nerve bundles, plotted as a function of molar concentration of Mercurochrome in initial bathing solution*

Data points are indicated as in Fig. 2.

as DTT and hydrazine sulfate, and electrical stimulation both augment the binding of the thiol reagent Mercurochrome to nerve bundles and potentiate the conduction-blocking activity of this compound. With prior reduction by DTT or hydrazine sulfate the effect of electrical stimulation on all parameters measured was abolished.

Unlike electrical stimulation, however, reduction by DTT or hydrazine sulfate produced no significant increase in the amount of bound Mercurochrome released from the nerve membrane in the presence of 2-mercaptoethanol. Following treatment with reducing agents, there was also no measurable increase in the reversal of conduction block by 2-mercaptoethanol, in either the absence or presence of electrical stimulation.

Thus, it would seem that the additional Mercurochrome bound after DTT treatment is more tightly bound, or less accessible to 2-mercaptoethanol, than that which is bound by electrical stimulation. This suggests that while stimulation and chemical reduction both increase the binding of the mercury derivative, this binding does not occur at equivalent sites. It would appear that electrical stimulation affects sites of importance to electrical conduction more selectively than does treatment with reducing agents. Only with electrical stimulation are all parameters of the effect of mercurials on nerve membranes altered, that is, binding of thiol reagent, conduction block by the mercurial, release of thiol reagent, and reversal of conduction block by 2-mercaptoethanol.

It is evident from these data and those presented previously (7) that less than half of the Mercurochrome bound to the nerve bundles can be removed by 2-mercaptoeth-

TABLE 3

Effect of electrical stimulation on binding of Mercurochrome to untreated and DTT-treated nerve bundles

Mercurochrome	DTT treatment	n	Mercurochrome bound	Increase over control[a] (within experimental error)	Mercurochrome released[b]	Increase over control[a] (within experimental error)
μM			nmoles/mg protein	%	nmoles/mg protein	%
1	No	4	0.55 ± 0.1	12	0.23 ± 0.02	43
	Yes	3	0.8 ± 0.15	None	0.18 ± 0.02	None
5	No	6	1 ± 0.1	35	0.45 ± 0.08 (0.38 ± 0.1)[b]	72
	Yes	3	1.9 ± 0.6	None	0.53 ± 0.14	None
10	No	8	3.3 ± 0.3	74	2 ± 0.3 (1.2 ± 0.4)	100
	Yes	3	8.7 ± 0.1	64	1.1 ± 0.2	83
50	No	9	17 ± 2	386	10 ± 2	523
	Yes	3	13 ± 4	None	1.7 ± 0.8	None
100	No	5	31 ± 3	716	17 ± 3	1316
	Yes	3	27 ± 4	None	3.1 ± 0.4	None
500	No	4	99 ± 10	421	40 ± 6	567
	Yes	3	270 ± 60	None	9.6 ± 3	None

[a] Control figures are the data presented in Table 2 for untreated and DTT-treated nerve bundles.

[b] Values in parentheses were obtained from homogenized nerve bundles as described under MATERIALS AND METHODS.

anol, whether by simple incubation or by elution from homogenized bundles. It seems likely that Mercurochrome is held to the membrane by nonspecific hydrophobic interactions in addition to its ability to act as a thiol reagent. The possibility of reaction with other functional groups cannot be excluded.

Karlin and Bartels (10) demonstrated that, although DTT has no direct effect on the resting potential, action potential, or active Na^+ transport of the electroplax, prior treatment with DTT altered the response of the postsynaptic membrane to both excitatory (e.g., carbamylcholine) and inhibitory (e.g., hexamethonium) molecules. These authors concluded that reduction of a disulfide bond in the vicinity of the postsynaptic cholinergic receptor alters the specificity of that receptor. More recently, Ben-Haim *et al.* (11) located a reactive disulfide bond, which is acted upon by DTT, within a few Angstroms from the anionic site of the acetylcholine receptor in the postsynaptic membrane of the frog neuromuscular junction. Although synaptic and conducting membranes each possess unique characteristics, they represent two classes of electrogenic membranes and, as such, may have certain functional elements in common, including the involvement of protein sulfhydryl groups and disulfides in the control of ion permeability changes.

The results described here are compatible with the postulate that electrolytic reduction of disulfides, with the formation of thiol groups, might take place during the conduction of the nerve impulse. It should be noted that, in the photosynthetic apparatus of spinach chloroplasts, a similar increase in the binding of thiol reagents is induced by exposure to light (12, 13). There too, the possibility exists that light-

dependent electron flow might be able to reduce disulfides.

If disulfide-thiol oxidation-reduction reactions should be of importance in nerve conduction or in other membrane phenomena, it would be very important for this process to be relatively reversible. As pointed out by Green (14), with respect to energy-transducing systems in which oxidation-reduction energy is involved in the transformation of a nonenergized to an energized state, it would be useful for the energized state to be metastable. In nerve membranes this metastable state, expressed by the resting membrane potential, could be postulated as involving "high-energy" disulfides, which, through electron-induced electrolytic reduction, transiently form thiol groups that, in turn, rearrange to form disulfides of lower potential energy, thus altering the cation permeability of the membrane. As postulated previously (6), such rearrangements could be a self-propagating process which might be associated with the action potential. Rearrangement to the initial "high-energy" disulfides would, of course, have to proceed by a process different from that involved in the conduction of the nerve impulse, most likely through *an ATP-requiring mechanism.*

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