THE BINDING OF THIOL REAGENTS TO AXONAL MEMBRANES: THE EFFECT OF ELECTRICAL STIMULATION

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Summary: Fluorescence techniques have been used to measure the binding of mercurochrome to walking leg nerve bundles of the spider crab. Reversibility of binding of mercurochrome by 2-mercaptoethanol was also measured. Both parameters were increased by electrical stimulation of the nerve bundle.

It has been demonstrated that the inhibitory action of certain thiol-reactive compounds on the process of nerve conduction is increased by electrical stimulation of the nerve fiber (1,2,3). Several postulates have been put forth to explain this "stimulation effect", including: altered permeability of the nerve membrane, unmasking of buried SH groups in the membrane, or electrolytic reduction of disulfides (2).

In earlier studies, it was also shown that with N-ethylmaleimide and mercurochrome there is a nonlinear relationship between dose of inhibitor and number of stimuli required to produce inexcitability (3).

Similarly, in the electroplax preparation (4), the blocking activity of N-ethylmaleimide varies with the level of the resting membrane potential. More recently, a "stimulation effect" was observed in the blocking activity of perhydrohistrionicotoxin, a poison isolated from the Columbian arrow frog (5).

In an effort to understand the quantitative aspects of the "stimulation effect" as well as the mode of action of sulfhydryl reagents on excitable membranes, we have conducted studies with mercurochrome using fluorescence techniques to measure the binding of this compound to walking leg nerve bundles of the spider crab, Libinia emarginata. Studies of the reversibility of the binding were carried out using 2-mercaptoethanol.

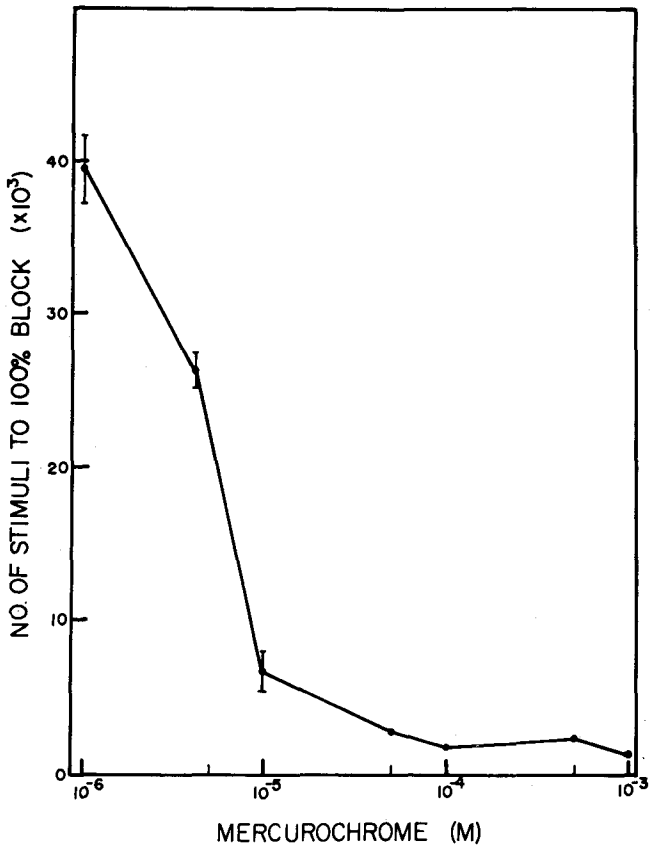

Figure 1. Relationship between mercurochrome concentration and number of stimuli required to induce complete block of conduction. Each point represents the mean of between 3 and 5 determinations obtained by extracellular recording. Vertical bars are used to indicate the range of the mean $\pm$ the standard error except where the values lie within the area of the symbol used to denote a point. The standard error is $\sqrt{\Sigma(X_i-\bar{X})^2/n(n-1)}$

Materials and Methods:

Walking leg nerve bundles of spider crabs obtained from the Marine Biological Laboratory, Woods Hole, Mass., were ligated at both ends and placed in a glass dish containing cold physiological saline (3). The pH of all bath solutions was maintained at 7.8-8.0, and all experiments were run at room temperature (20-25°C).

The binding of the fluorescent thiol reagent mercurochrome 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

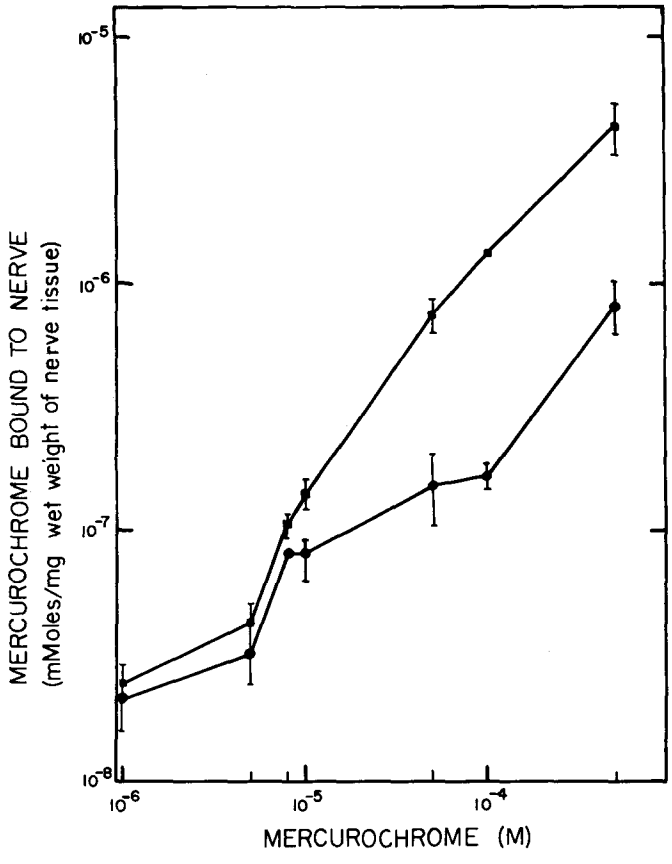

Figure 2. The amount in mMoles of mercurochrome bound per mg wet weight of nerve bundle after 30 mins. incubation, with and without electrical stimulation, is plotted as a function of the molar concentration of mercurochrome in the bath solution. For the experiments with electrical stimulation, the nerve was stimulated with a 2 msec square pulse of threshold intensity at 9/sec. Each point represents the mean of (n) experiments (see Table I). Vertical bars are used to indicate the range of the mean + the standard error except where the values lie within the area of the symbol used to denote a point.

appearance of fluorescence in a solution of 2×10^{-2}M 2-mercaptoethanol after incubation with a mercurial-blocked nerve bundle. All electrophysiological recordings were made with extracellular Ag-AgCl electrodes as described previously (3).

The effect of electrical stimulation on the binding or uptake of mercurochrome was determined by stimulating with a 2 msec square pulse of threshold intensity (9/sec) throughout the incubation period. The duration of the incubation was fixed at 30 mins. for all concentrations of mercurochrome. In order to increase the sensitivity to small

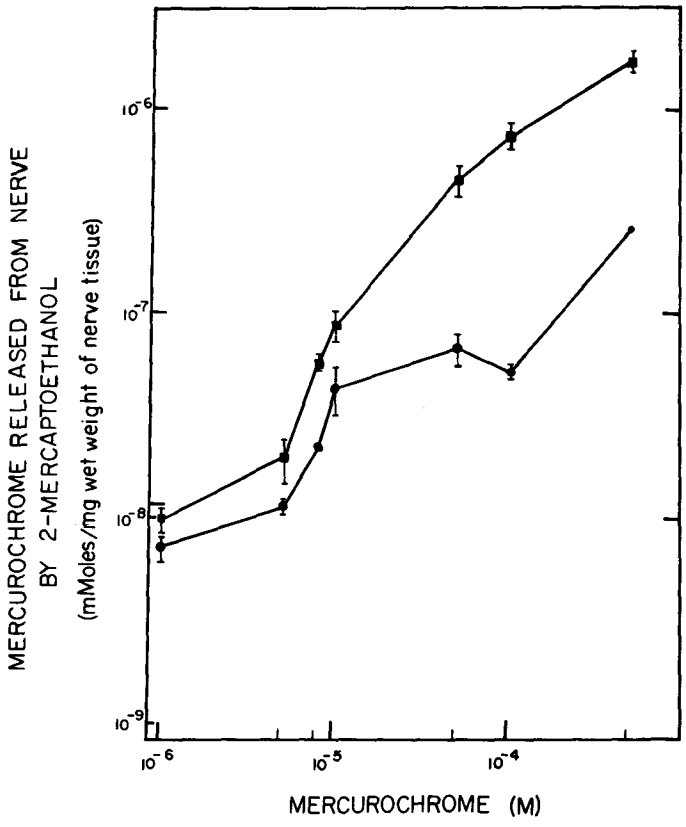

Figure 3. The amount in mMoles of bound mercurochrome released from a nerve bundle after 15 mins. incubation with 5×10^{-2}M 2-mercaptoethanol, with and without electrical stimulation, is plotted as a function of the molar concentration of mercurochrome in the initial bathing solution. Each point represents the mean of (n) experiments (see Table I). Vertical bars are used to indicate the range of the mean ± the standard error except where the values lie within the area of the symbol used to denote a point.

changes in fluorescence, the external bath volume was limited to 5 ml, and large nerve bundles (1mm external diameter) were selected.

Fluorescence measurements were made using an Aminco-Bowman spectrophotofluorometer equipped with an ellipsoidal mirror condensing system. An external reference standard, anthracene in cyclohexane, was used to standardize each day's experiments. All reagents were of analytical grade.

Results and Discussion:

The fluorescence of mercurochrome in artificial sea water followed the Beer-Lambert law up to a concentration of 10^{-5}M above which self-quenching occurred.

Samples in the concentration range above 10^{-5}M were diluted to be read in the linear portion of the standard curve expressing the relationship between concentration of mercurochrome and fluorescence intensity (6).

Controls demonstrated that an insignificant amount ($p. < 0.01$) of mercurochrome is adsorbed to the interior of the lucite chamber and to the electrodes. The use of 10^{-4}M 2',7'-dichlorofluorescein, an analogous molecule lacking the mercuri grouping, demonstrated a statistically-insignificant amount (0.02% of total bath amount per mg wet weight nerve) of nonspecific fluorophore adsorption to the nerve bundle with or without electrical stimulation.

Figure 1 expresses the relationship between the external bath concentration of mercurochrome and the number of stimuli required to produce 100% block of conduction.

Figure 2 demonstrates that electrical stimulation produces a significant increase in the binding of mercurochrome at bath concentrations above 5×10^{-6}M.

Figure 3 shows that electrical stimulation produces a measurable increase in the release of bound mercurochrome compared to the release without stimulation. In these experiments, electrical stimulation was applied during both the incubation with mercurochrome and with 2-mercaptoethanol, or only during the reversal phase. In either case, stimulation induces an increase in the release of bound mercurochrome.

In figures 2 and 3, the amount of mercurochrome measured is expressed as moles per mg wet weight of nerve tissue. Preliminary protein assays (7,8) demonstrate that a similar relationship is obtained when the bound mercurochrome is expressed in terms of moles per mg protein per mg wet weight of nerve tissue. A nerve bundle of 60 mg wet weight, for example, yields ca. 3 mg protein. Studies are presently in progress to separate the mercurial-binding complex of nerve membrane proteins.

The relative binding of 10^{-6}M - 5×10^{-4} M mercurochrome to nerve bundles with and without electrical stimulation and the reversal of binding by 2×10^{-2}M 2-mercaptoethanol under these conditions are presented in terms of overall %-effect in

TABLE I: THE EFFECT OF ELECTRICAL STIMULATION ON THE BINDING OF MERCUROCHROME AND THE REVERSAL OF BINDING BY 2-MERCAPTOETHANOL AS MEASURED IN THE WALKING LEG NERVE BUNDLE OF LIBINIA EMARGINATA.

Mercurochrome	Electrical Stimulation (6/sec)	n	%-B	%-Increased by stimulation	%-R	%-Increased by stimulation
1×10^{-6}	No	4	19±2		48 ±6	
	Yes	4	30±10	112	56± 8.3	143
5×10^{-6}	No	9	43± 7		26± 2	
	Yes	6	60± 5	135	59± 8	172
8×10^{-6}	No	9	48 ± 6		37 ± 4	
	Yes	6	100	130	72± 9	267
1×10^{-5}	No	8	60± 9		39± 11	
	Yes	8	100	177	71± 3	203
5×10^{-5}	No	9	69± 9		47± 9	
	Yes	9	100	481	71± 10	660
1×10^{-4}	No	5	75± 6		35 ±8	
	Yes	5	100	798	70± 5	148
5×10^{-4}	No	4	100		42 ±8	
	Yes	4	100	530	59± 8	667

%-B = % Conduction block
%-R = % Recovery from conduction block

Each figure represents the mean ± standard error of n experiments. In all experiments, a single nerve bundle was incubated for 30 minutes in 5 ml of mercurochrome in artificial sea water, pH 7.8-8.0, 18-20°C. Reversal of binding was tested by washing the nerve bundle for 15 minutes with 2×10^{-2} M 2-mercaptoethanol.

Table I. The %-conduction block produced by mercurochrome in electrically-stimulated nerve bundles was measured after 4 mins. of threshold intensity stimulation at 9/sec following the incubation period. The blocking effect of mercurochrome has been shown to depend upon concentration and time of incubation (3). Thus, %-conduction block varies directly with concentration in the absence of electrical stimulation, while the increase in conduction block in stimulated fibers varies with the number of stimuli as well.

Incubation of a nerve bundle for 30 mins. in a solution of 2×10^{-7}M mercurochrome produced no measurable electrophysiological effects and no significant loss of fluorescence in the solution, with or without electrical stimulation.

In order to know if functional integrity of the axons is essential for the observed binding of thiol reagents and the increase of binding by electrical stimulation, the nerve fibers were intentionally stretched or crushed to render them nonconducting. The nonconducting fibers were then incubated for the usual period, with and without electrical stimulation. Although there was no change in the amount of thiol reagent bound in the absence of electrical stimulation, there was no measurable increase in the amount of thiol reagent bound when the nonconducting fiber was electrically stimulated.

The observation that electrical stimulation increases both the binding of thiol reagents to axons and the release of bound thiol reagents from axons, strongly favors the hypothesis that a conformational change, involving membrane proteins and their thiol or disulfide groups, takes place during propagation of the nerve impulse (9,10,11).

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