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N-Ethylmaleimide-induced conductance changes in the giant neuron of *Aplysia*

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Reagents which bind to or oxidize sulfhydryl groups have been found to have widespread effects on excitable membranes, suggesting that such groups may have some function in the gating of ionic conductances in such membranes. In certain cases the specificity of action of sulfhydryl reagents has been demonstrated by competition with appropriate pharmacological agents. Thus curare inhibits the depolarizing action of such reagents on frog endplate[17], and specific odorants can competitively block the effects of sulfhydryl reagents on olfactory receptors[6]. Indeed, Karlin and co-workers have used a cholinomimetic SH-binding compound to demonstrate a functionally important SH group in the cholinergic receptor of *Electrophorus* electroplax[9].

The action of these reagents is not restricted to chemoreceptors, however, as they can cause conduction block in frog nerve and lobster axon[16], as well as squid axon[8]. Since loss of excitability preceded any reduction of the resting potential in the latter case, it has been argued that the reagents might be acting directly on electrically-excitable conductance mechanisms[8]. This idea has received support from experiments of Marquis and Mautner[12] showing that mercurial binding to crustacean nerve is increased by electrical excitation. However, since membrane resistance has never been monitored in these studies, and since SH-reagents can increase K^+ permeability in erythrocyte membrane[11], it is important to exclude non-specific permeability increases as a cause of conduction failure.

We have attempted to investigate this point by determining the effect of N-ethylmaleimide (NEM) on the membrane properties of the giant neuron of the abdominal ganglion of *Aplysia californica*. This cell (R2 of Frazier, *et al.*[4]) was chosen because its large size simplifies conductance measurements, its somatic membrane is electrically excitable[5], and a considerable amount of information is available about its membrane conductances[3,5,13,14]. NEM was the sulfhydryl reagent of choice because of its relative specificity for SH groups, the fact that it forms a covalent, and

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hence irreversible, bond with them, and because of its widespread use in earlier studies.

Ganglia were pinned to the Silastic bottom of a 3 ml recording chamber containing artificial seawater (ASW) of the following composition (m*M*): NaCl, 451; KCl, 10; $CaCl_2$, 13; $MgSO_4$, 28; $MgCl_2$, 21; Tris base, 10; adjusted to pH 7.7–7.8 with HCl. Intracellular recordings from R2 neurons were made in a conventional manner using 3 *M* KCl- or 0.6 *M* K_2SO_4-filled microelectrodes of 10–20 MΩ minimum resistance. Resting potentials were measured both by reference to an external low-resistance microelectrode and by disimpalement. When the neuron's input resistance was to be measured, two electrodes were inserted into the cell. One was used for injecting hyperpolarizing triangular current waves of 15 sec duration (from a constant voltage source via a 10^9 Ω resistor) while the other recorded the resultant change in membrane potential. Current was monitored by an inverting operational amplifier connected to bath ground and current–voltage traces were monitored directly on the oscilloscope as described by Strumwasser[18].

Control data were taken for a period of at least an hour in normal seawater at room temperature. If the experiment involved an alteration in seawater composition, the new solution was introduced and potential *vs.* current data were taken until equilibrium values were reached. NEM was then made up in a concentrated solution from frozen dry aliquots and added to the chamber to give a final concentration of 5 m*M*.

The resting potential of R2 in our artificial seawater averaged —47 mV (Table I) which agrees well with the —47.8 mV reported by Russell and Brown[13,14]. When NEM is applied to the solution bathing the ganglion, a characteristic series of events takes place in R2 within 10–20 min. Firstly, the threshold for synaptic activation of the cell by stimuli applied to the left connective nerve rises concomitantly with a decrease in the size of the EPSP evoked for a given value of stimulus voltage. Secondly, it is noted that during this period the threshold for activation of R2 by transmembrane current injection increases beyond the capacity of our electrodes to deliver stable current (about 1 μA). Underlying these events is a 5–10-fold decrease in the cell's input resistance (Table I), so that neither method of excitation can lower the resting potential to threshold, which is normally —35 to —40 mV. The resting potential remains relatively unchanged during the first 20–40 min of NEM action, by which time the resistance change has developed fully (Table I). Subsequently there is a

TABLE I

EFFECT OF NEM ON MEMBRANE POTENTIAL AND RESISTANCE

All results are reported as mean ± S.D. (number of cells).

Treatment	*Membrane potential (mV)*	*Input resistance (MΩ)*
Normal ASW	—47 ± 7 (47)	0.9 ± 0.2 (17)
+5 m*M* NEM, 20 min	—47 ± 7 (15)	0.1 ± 0.0 (4)
low Ca^{2+}–high Mg^{2+}	—47 ± 8 (4)	0.7 ± 0.3 (4)
+5 m*M* NEM, 20 min	—44 ± 5 (4)	0.1 ± 0.0 (4)

slow decline in membrane potential, with values on the order of —35 mV being attained after 60 min.

Since the NEM-SH bond is essentially irreversible, the subsequent addition of a strong reducing agent would not be expected to reverse the effect of NEM on membrane conductance if that effect were in fact due to the formation of such a bond. We could not cause reversal of the NEM effect by treatment of previously-reacted cells with 10 m*M* dithiothreitol for one hour. By the same token, reaction of NEM with cysteine prior to cell treatment should protect against any NEM effect. By following the optical absorption of NEM at 302 nm[7], we found that it reacted completely with our cysteine essentially immediately at a ratio of 5 m*M* NEM–10 m*M* cysteine. (Lack of equivalence in this reaction probably indicates partial oxidation of the cysteine used.) Treatment of R2 with this combined solution resulted in no change in input resistance (0.8 ± 0.1 (3) MΩ) after one hour. NEM never brought the pH of our seawater below 7.6 at the concentrations used, and we were unable to reverse the effect of NEM on membrane conductance by treatment for up to one hour with pH 9.1 artificial seawater. Thus it appears that NEM is affecting membrane conductance solely by its capacity to bind covalently to SH groups.

It also seemed possible that the observed effects of NEM might be an indirect result of massive activation of other neurons in the ganglion which might have inhibitory synapses onto R2, especially since IPSPs are very close to the resting potential in R2. We therefore repeated the NEM experiments in a low Ca^{2+} (1 m*M*)–high Mg^{2+} (100 m*M*) solution. This solution blocks chemical synaptic transmission in *Aplysia*[1], and abolishes synaptic input to R2 (our unpublished observations). As shown in Table I, however, it does not modify the effect of NEM. Thus it appears that NEM affects R2 directly.

If the drastic fall in membrane resistance induced by NEM were due to a nonspecific increase in permeability to all ions, then one would expect a concomitant drop in membrane potential. Since the potential decrease in NEM-treated cells occurred much more slowly than the resistance change, it seemed likely that NEM was inducing a selective conductance increase to an ion or set of ions such that the membrane was clamped near the normal resting potential. We therefore checked for conductance increases to Na^+, K^+, and Cl^- by altering their concentrations in the extracellular medium and comparing the resulting membrane potentials in the presence and absence of NEM.

Reduction of the concentration gradient for Na^+ by total replacement with Tris in the medium reduced the action potential overshoot in R2 from + 40 ± 5 mV (4) to + 6 ± 8 mV (4), in general agreement with the findings of Geduldig and Junge[5]. Table II shows that in the absence of NEM the membrane was hyperpolarized by this treatment, but that it had no effect on the membrane potential of NEM-treated cells, from which we infer that NEM does not significantly increase sodium permeability. On the other hand, increasing the extracellular K^+ concentration to 100 m*M* following NEM treatment depolarized the cell to —28 ± 2 mV (3). Thus part of the increase in conductance caused by NEM is due to an increase in K^+ permeability.

We also bathed the ganglion in chloride-free artificial seawater (Cl^- replaced

TABLE II

EFFECT OF ION REPLACEMENTS ON THE NEM POTENTIAL

Membrane potential (mV)			
Test solution	*Normal ASW*	*Test solution*	*+5 mM NEM, 20 min*
Na^+-free	−55 ± 10 (4)	−70 ± 11 (4)	−45 ± 4 (4)
Cl^--free	−47 ± 6 (8)	−59 ± 5 (8)	−23 ± 4 (4)
Cl^--free + 10^{-4} *M* ouabain		−43 ± 2 (3)	−26 ± 4 (3)

by acetate, acetate-buffered) to look for NEM-induced changes in Cl^- permeability. As expected from the results of Brown *et al.*[2], this medium hyperpolarized the cell. Interestingly, ouabain blocked this effect (Table II), suggesting the involvement of an electrogenic Na^+ pump. Nonetheless, NEM acts to depolarize the cell in Cl^--free solutions, whether or not ouabain is present (Table II). The magnitude and polarity of this shift in membrane potential indicates a substantial increase in Cl^- permeability following NEM action.

Since the somata of *Aplysia* neurons contain cholinergic receptors[3], it is necessary to ask whether NEM might be acting via such receptors. Treatment with 1.3×10^{-4} *M* D-tubocurarine was sufficient to block the prominent cholinergic synaptic input to cell R15 of the ganglion, while prior and continuing treatment with this agent did not affect NEM's ability to block excitability in R2 without changing its resting potential. While NEM action via excitatory receptors is apparently excluded by its failure to increase sodium permeability, we have not been able to entirely exclude the possibility that some portion of its action is on non-cholinergic inhibitory receptors. Potassium-dependent 'slow' inhibitory receptors which are not blocked by curare have been described in *Aplysia* neurons[10], but not in cell R2, whose inhibitory synaptic input[4] appears to be of the 'rapid', or chloride-dependent[10,15], cholinergic type.

Our results indicate that the reaction of NEM with membrane constituents brings about a large increase in conductance to chloride and potassium ions, without significantly affecting sodium conductance, and without producing a significant change in resting potential,

This is paradoxical, however, in that the equilibrium potentials for chloride and potassium are normally more negative than the resting potential in R2 by 7 and 30 mV, respectively[13,14]. Thus an increase in permeability to these two ions alone would be expected to hyperpolarize the cell. Leakage of these ions through their increased permeabilities could eventually set up new, more positive, equilibrium potentials for these ions, but one should still see a transient hyperpolarization during this process, and none was observed. Our results cannot be explained by invoking a positively electrogenic sodium pump, since ouabain does not hyperpolarize NEM-treated cells (Table II). It seems unlikely that NEM might induce a permeability increase to some other ion with a depolarizing equilibrium potential in sufficient

degree to exactly balance the untreated membrane potential, but we have not tested this possibility.

Regardless of the mechanism of NEM action, our results are phenomenologically similar to those obtained from squid axon[8], in that the generation of action potentials was abolished without significant reduction of the resting potential. Although it is possible that NEM does react with excitable conductance channels, the drastic fall in membrane resistance alone would be sufficient to abolish excitability. Previous workers[8,16] used 2.5-fold lower NEM concentrations on axons which are presumably devoid of chemical receptors, so that our results from *Aplysia* somata may not be directly comparable. However they do provide clear evidence, in this system at least, that loss of excitability without membrane potential change is not in itself a sufficient criterion for concluding that NEM reacts with electrically-excitable conductance channels.

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1 AUDESIRK, G., Spontaneous and light-induced compound action potentials in the isolated eye of *Aplysia*: initiation and synchronization, *Brain Research*, 59 (1973) 229–242.

2 BROWN, A. M., WALKER, J. L., AND SUTTON, R. B., Increased chloride conductance as the proximate cause of hydrogen ion concentration effects in *Aplysia* neurons, *J. gen. Physiol.*, 56 (1970) 559–582.

FRANK, K., AND TAUC, L., Voltage clamp studies of molluscan neuron membrane properties. In J. F. HOFFMAN (Ed.), *Cellular Functions of Membrane Transport*, Prentice-Hall, Englewood Cliffs, N. J., 1964, pp. 113–135.

4 FRAZIER, W. T., KANDEL, E. R., KUPFERMANN, I., WAZIRI, R., AND COGGESHALL, R. E., Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*, *J. Neurophysiol.*, 30 (1967) 1288–1351.

5 GEDULDIG, D., AND JUNGE, D., Sodium and calcium components of action potentials in the *Aplysia* giant neurone, *J. Physiol. (Lond.)*, 199 (1968) 347–365.

6 GETCHELL, M. L., AND GESTELAND, R. C., The chemistry of olfactory reception: stimulus-specific protection from sulfhydryl reagent inhibition, *Proc. nat. Acad. Sci. (Wash.)*, 69 (1972) 1494–1498.

7 GREGORY, J. D., The stability of N-ethylmaleimide and its reaction with sulfhydryl groups, *J. Amer. chem. Soc.*, 77 (1955) 3922–3923.

8 HUNEEUS-COX, F., FERNANDEZ, H. L., AND SMITH, B. H., Effects of redox and sulfhydryl reagents on the bioelectric properties of the giant axon of the squid, *Biophys. J.*, 6 (1966) 675–689.

9 KARLIN, A., PRIVES, J., DEAL, W., AND WINNIK, M., Affinity-labeling of the acetylcholine receptor in the electroplax, *J. molec. Biol.*, 61 (1971) 175–188.

10 KEHOE, J., Ionic mechanisms of a two-component cholinergic inhibition in *Aplysia*, *J. Physiol. (Lond.)*, 225 (1972) 85–114.

11 KNAUF, P. A., AND ROTHSTEIN, A., Chemical modification of membranes. I. Effects of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red blood cell, *J. gen. Physiol.*, 58 (1971) 190–210.

12 MARQUIS, J. K., AND MAUTNER, H. G., The binding of thiol reagents to axonal membranes: the effect of electrical stimulation, *Biochem. biophys. Res. Commun.*, 57 (1974) 154–161.

13 RUSSELL, J. H., AND BROWN, A. M., Active transport of K^+ by the giant neuron of the *Aplysia* abdominal ganglion, *J. gen. Physiol.*, 60 (1972) 519–533.

14 RUSSELL, J. M., AND BROWN, A. M., Active transport of chloride by the giant neuron of the *Aplysia* abdominal ganglion, *J. gen. Physiol.*, 60 (1972) 499–518.
15 SATO, M., AUSTIN, G., YAI, H., AND MARUHASHI, J., The ionic permeability changes during acetylcholine-induced responses of *Aplysia* ganglion cells, *J. gen. Physiol.*, 51 (1968) 321–345.
16 SMITH, H. M., Effects of sulfhydryl blockade on axonal function, *J. cell. comp. Physiol.*, 51 (1958) 161–171.
17 SOBRINO, V. A., AND DEL CASTILLO, J., Activation of the cholinergic endplate receptors by oxidizing reagents, *Int. J. Neurosci.*, 3 (1972) 251–258.
18 STRUMWASSER, F., Neural and humoral factors in the temporal organization of behavior, *The Physiologist*, 16 (1973) 9–42.