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EFFECT OF SEVERAL "SPECIFIC" CHEMICAL REAGENTS ON THE Na^+, K^+ AND LEAKAGE CURRENTS IN VOLTAGE-CLAMPED SINGLE NODES OF RANVIER

JOHN F. W. KEANA[a] and ROBERT STÄMPFLI[b]

[a]*Department of Chemistry, University of Oregon, Eugene, Oreg. 97403 (U.S.A.) and* [b]*Physiologisches Institut der Universität des Saarlandes, 665 Homburg, Saar (G. F. R.)*

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SUMMARY

The magnitude of the Na^+, K^+ and leakage currents through voltage-clamped single nodes of Ranvier of the frog versus time were monitored with the aid of a computer as the node was perfused with one of the following "specific" chemical reagents in an appropriate Ringer's solution: six enzymes, *N*-bromosuccinimide, *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide methiodide, tetranitromethane, *N*-ethylmaleimide, $NaIO_4$, $NaIO_3$, and sodium cyanoborohydride.

While the enzymes examined showed little effect on the ionic currents several of the other reagents permanently altered selectively the K^+ current, leading to the tentative conclusion that accessible SH groups are more important to the operation of the K^+ mechanisms than to the Na^+ mechanisms. With some reagents the leakage current suddenly increased dramatically while the Na^+ and K^+ mechanisms were still functioning "normally", though at reduced levels. Accessible iminium or enamine linkages or 1,2-dihydroxy groupings may be modified if present, without profound effects on the ionic currents. Some experimental basis is provided for the possibility that not all "channels" of a particular ion are chemically alike.

INTRODUCTION

Despite the impressive gains which have been made in recent years in the electrophysiological description of ion permeability changes associated with the passage of an action potential, progress has been slow in coming toward providing a detailed picture of those events on a molecular level [1]. Available evidence seems to strongly favor the notion that Na^+ and K^+ follow separate and distinct ionic pathways as they transverse the membrane during passage of an action potential [2]. If so, these ionic channels* must differ chemically from each other in order to explain,

* For convenience we use the term "channel" to denote the pathway(s) taken by the various ions as they cross the membrane. No structural implication is intended.

for example, ion selectivity differences and differences in their interaction with highly specific pharmacological agents such as tetrodotoxin and tetraethylammonium ion. One approach to probe the chemical makeup of nerve has been through a study of the effects of enzymes and chemical reagents on the excitability, the action potential and the resting potential (refs 4–6 and ref. 3 for a review). Thus far, the voltage-clamp technique, which permits observation of the action of the chemical on the individual ionic currents, has not been employed in these kinds of experiments. In order to look for differences in the chemical makeup of the Na^+, K^+ and leakage channels in frog sciatic nerve, we have treated some 90 voltage-clamped single nodes of Ranvier with thirteen organic functional group or peptidyl amino acid "specific" reagents while monitoring with the aid of a computer the magnitudes of the respective ionic currents.

EXPERIMENTAL METHODS

A single myelinated motor or sensory fiber was dissected [7] from the sciatic nerve of the frog, *Rana esculenta*. A node of Ranvier was voltage-clamped using the method and apparatus described by Nonner [8]. A pulse train consisting of a 40-ms hyperpolarizing pulse of −40 mV (removes resting sodium inactivation and provides a value for the leakage current), followed by a 2-ms depolarizing pulse of +60 mV (optimized peak inward I_{Na^+}), followed by a 10-ms pulse of +120 mV (provides steady state outward I_{K^+} of convenient magnitude) was delivered by an on-line computer every 2–6 s during an experiment. Leakage-corrected values for I_{Na^+} (max) and I_{K^+} (steady state) versus time were immediately available for plotting.

The node was continuously superfused at 15 °C with Ringer's solution followed by a test solution. All test solutions contained, in addition to the chemical reagent and buffer, 2.0 mM $CaCl_2$, and quantities of NaCl and KCl were adjusted so that the final concentration of Na^+ was about 110 mM and K^+ was about 2.5 mM. The choice of an appropriate buffer was dictated by the chemical reagent since buffers containing amino groups will chemically react with many of the reagents. Tris buffer, 2.0 mM, pH 7.4, was used where possible, otherwise bicarbonate buffer, 10 mM, pH 7.3–7.7, or phthalate buffer [11], 5 mM, pH 4.7–5.3, was used.

The choice of the chemical reagent [9, 10] was dictated by the obvious requirements that the composition of each test solution had to be compatible with both the functioning node of Ranvier and the reagent itself. Owing to the desirability of holding the osmolarity of each test solution reasonably close to that of normal Ringer's solution, the reagents had to perform well at concentrations no greater than about 50 mM, i.e. optimally, interesting events should have occurred within 1–5 min after application of the reagent so that normal run-down of the fiber was not an important factor in subsequent data analysis. The reagent had to be effective in the pH range 4.5–8.5, be soluble to a useful extent in aqueous solutions containing 2 mM Ca^{2+} and at most 1 % (v/v) of methanol or acetone as cosolvent, and not react with the aqueous solvent system to a large extent over a several-minute period. The functioning node and the physical set-up required that the temperature be within the range of 4–25 °C, thus excluding reagents which were likely to be effective (for chemical or solubility reasons) at other temperatures.

All chemicals were reagent grade and used without further purification. *N*-Bromosuccinimide and *N*-ethylmaleimide, were purchased from Schuchardt, Munich,

Germany; Sodium cyanoborohydride ($NaBH_3CN$), tetranitromethane, and *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide methiodide were purchased from EGA, Steinheim/Albuch, Germany; $NaIO_3$ and $NaIO_4$ were purchased from Riedel-DeHaen, Seelze-Hannover, Germany; chymotrypsin A_4, trypsin, lysozyme, phospholipase D, collagenase and hyaluronidase were purchased from Boehringer, Mannheim, Germany.

RESULTS AND DISCUSSION

The effects of several chemical reagents on the Na^+ and K^+ currents in a voltage-clamped single node of Ranvier are summarized in Tables I–IV. For each run a plot of log I_{Na^+} (max) and log I_{K^+} (steady state) versus time was prepared. Approximately linear regions were discerned from the plots and the best straight line through the data points within these regions was determined by a least-squares calculation which also gave for each line, the associated correlation coefficient, r, and the slope, $k_{Na^+}(s^{-1})$ or $k_{K^+}(s^{-1})$. The number of points used for each least-squares calculation and the time interval over which the points were collected (approximately evenly spaced) are also given. Normally, the linear region(s) of each plot constituted at least 50 % of the time period of application of the reagent. Since the node was continuously bathed in fresh test solution, the concentration of the reagent did not change with time. Thus, as a first approximation, one might refer to k_{Na^+} and k_{K^+} as apparent pseudo first-order rate constants corresponding to the detectable chemical reaction of the reagent with the nodal membrane components.

We observed at times considerable variation between the results of two runs with the same test solution(s) on two different fibers. For this reason we have reported data taken from individual fibers rather than attempt to calculate some "average" response. While every effort was made to assure uniform animal care, dissection and mounting procedures, occasionally, seemingly good preparations would function poorly or not at all owing presumably to some unsuspected mechanical injury at some point in the operation. Thus, even excellent fibers could have suffered some degree of mechanical injury which could have affected the magnitude of the observed currents and the "resistance" of the fiber toward a given reagent. Also differing amounts of connective tissue adhering to the nodes could affect the accessibility of the reagent to the nodal membrane surface. We therefore wish to emphasize that the absolute magnitude of a given rate constant is not as important as the general trend of reactivity of fibers toward a chemical reagent.

Normal run-down

The apparent pseudo first-order rate constants associated with the normal run-down of the Na^+ and K^+ currents of two representative fibers, 15 and 16 (Fig. 1) superfused with Ringer's solution are given in Table I. Leakage currents remained essentially unchanged throughout the run-down experiments. Since a first-order rate constant of $1.49 \cdot 10^{-4}\ s^{-1}$, for example, corresponds to a half-life of 4650 s or 1.3 h, normal run-down of a good fiber can be safely ignored when significant changes in current magnitude due to the effects of a chemical reagent occur within a period of a few minutes.

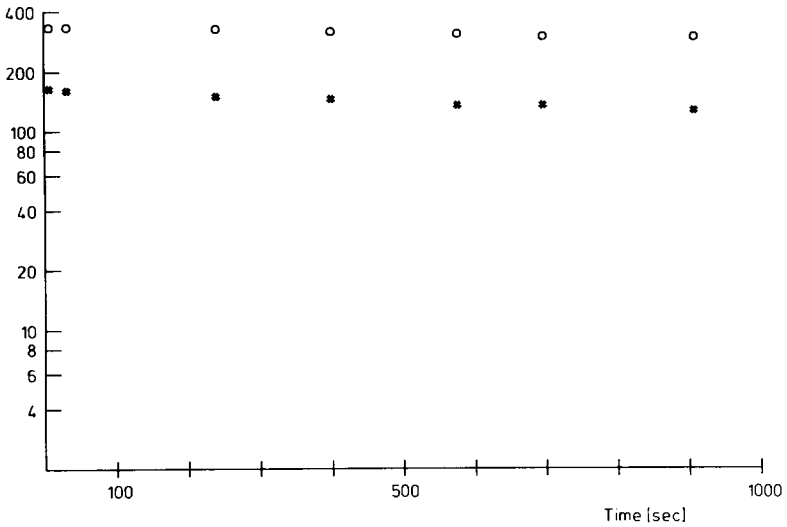


Fig. 1. A normal run-down experiment on a motor fiber (fiber 16) showing the Na^+ (○) and K^+ (*) currents versus time. Ordinate: Ionic current (log scale, arbitrary units); Abscissa: time in s.

Enzyme experiments

Several studies of the effect of various enzymes (e.g. collagenase, phospholipases, chymotrypsin, trypsin, hyaluronidase, papain and ribonuclease) applied externally to frog myelinated fibers, lobster, squid and crayfish axons and internally to the latter three have been reported (see ref. 3 for review). Externally applied (1 mg/ml) these agents, except for phospholipases A and C [12] show little effect on either the resting potential, the excitability or the action potential although Strickholm and Clark [13] have data which suggest that the passive Cl^- permeability of the cell surface is reduced by the action of chymotrypsin and trypsin on crayfish axon. Pronounced morphological alterations, however, have been observed in other studies as a result of treatment of lobster axons and frog myelinated fibers with trypsin [3].

We describe here the action of six enzymes (1–2 mg/ml in Ringer's solution, pH 7.3) on the ionic currents through voltage-clamped single nodes of Ranvier. In general, exposure (several 30-s periods or continuously for 15–30 min) of nodes of motor and sensory fibers to either lysozyme (see below, however), collagenase, hyaluronidase, chymotrypsin A_4 or trypsin led to a 2–3-fold increase in the ratio k_{Na^+}/k_{K^+}, without greatly altering the magnitude of either rate constant (Table I). These effects were even less noticeable with phospholipase D. The similarity in the action of those enzymes used suggests that the small changes observed quite possibly stem from interactions between the enzymes and the nodal membrane surface which have little to do with the activity of the enzymes.

Additionally, lysozyme curiously caused a rapid and reversible reduction in the magnitude of both the Na^+ and K^+ currents, not unlike a pH effect. Thus,

I_{Na^+} (lysozyme) = (0.86–0.92)I_{Na^+} (Ringer) while I_{K^+} (lysozyme) = (0.66–0.80)I_{K^+} (Ringer)

(3 fibers). The reversibility of the action of lysozyme suggests that the observed

TABLE I

REPRESENTATIVE RUN-DOWN AND ENZYME EXPERIMENTS

Fiber No. (M, S, U)*		Composition of test solution**	Duration*** (s) (number of points)	$-k_{Na^+} \cdot 10^4\ s^{-1\dagger}$ $(r)^{\dagger\dagger}$	$-k_{K^+} \cdot 10^4\ s^{-1\dagger}$ $(r)^{\dagger\dagger}$	k_{Na^+}/k_{K^+}	Breakdown‡ time (A)
A. Normal run-down experiments							
15	(U)	R	1431 (13)	1.49 (0.989)	2.26 (0.979)	0.66	
16	(M)	R	907 (7)	1.31 (0.982)	2.72 (0.993)	0.48	—
B. Enzyme experiments							
6	(S)	2.1 mg/ml lysozyme in R	1988 (19)	2.72 (0.994)	1.68 (0.993)	1.6	—
17′	(M)	2.0 mg/ml collagenase in R	1711 (12) (incl 330-s appl; 13-min appl)	2.47 (0.994)	1.41 (0.968)	1.8	
7′	(S)	2.0 mg/ml hyaluronidase in R	2130 (18) (incl 330-s appl; 110-min appl)	1.75 (0.967)	0.65 (0.899)	2.7	—
8′	(M)	2.0 mg/ml trypsin in R	2540 (21) (incl 330-s appl; 117-min appl)	4.72 (0.973)	3.90 (0.965)	1.2	—
11′	(M)	2.0 mg/ml chymotrypsin A_4 in R	2255 (17) (incl 330-s appl; 110-min appl)	2.97 (0.985)	2.06 (0.951)	1.4	—
8a	(M)	1.9 mg/ml phospholipase D	430 (3)	1.80 (0.825)	2.47 (0.960)	0.73	—

* M, motor fiber; S, sensory fiber; U, uncertain.

** R refers to normal Ringer's solution, BR to bicarbonate-buffered Ringer's solution, PR to phthalate-buffered Ringer's solution. When two entries appear for a fiber, the second test solution was applied immediately after the first one.

*** Time over which data points were taken for the calculation of k_{Na^+} and k_{K^+}. Unless otherwise stated the reagent was applied continuously.

† Apparent pseudo first-order rate constant.

†† Correlation coefficient.

‡ Total time of exposure to a reagent before fiber breakdown occurred.

effects are not due to covalent bond cleavages, but rather perhaps to electrostatic interactions between the positively charged lysozyme molecule ($pK \sim 11$) and the negatively charged membrane surface or to extraneous anions associated with the commercial sample of lysozyme. Leakage currents were not significantly affected by either lysozyme or any of the other enzymes studied.

A pronounced difference between sensory and motor fibers became apparent from an examination of the magnitude of the Na^+, K^+ and leakage currents at the beginning of each experiment (i.e. before chemical treatment of the fiber). Although there was considerable variation among individual fibers, the ratio I_{Na^+}/I_{K^+} averaged 2.54 for 29 motor fibers whereas this ratio was 1.56 for 13 sensory fibers. Sensory fibers in general appeared to show slightly less leakage current as well.

N-Bromosuccinimide experiments

Aqueous *N*-bromosuccinimide has been used extensively for the selective modification (and cleavage sometimes) of tryptophane residues within a polypeptide chain, although, with lysozyme as substrate, the tryptophane, histidine and tyrosine residues are all oxidized at pH 4 and 5 at nearly the same rates [14]. *N*-Bromosuccinimide oxidizes SH groups more rapidly than tryptophane residues and methionine and cystine residues [10] as well as carbon–carbon double bonds [9] can be attacked.

Table II shows that when the concentration of *N*-bromosuccinimide in the bicarbonate-buffered Ringer's superfusing solution was only 0.10 mM the apparent pseudo first-order rate constants k_{Na^+} and k_{K^+} (fiber 1) were respectively 27 and 14 times the corresponding rate constants associated with normal run-down of a fiber (fiber 16). The reagent showed no marked selectivity between the Na^+ and K^+ currents, the ratio k_{Na^+}/k_{K^+} being about that of normal run-down. Comparable results were obtained with fiber 2.

The action of *N*-bromosuccinimide on the leakage current was most interesting. During the first 300 s of application of 0.10 mM reagent the leakage current gradually increased after which time it increased dramatically and within a few seconds the trans-membrane potential dropped to near 0 mV (current clamp measurement). It would thus appear that *N*-bromosuccinimide is readily able to generate "holes" in the nodal membrane through which ions may easily pass.

Several fibers were also pretreated with phospholipase D or lysozyme for several minutes prior to exposure to 0.1 M *N*-bromosuccinimide. In general, the useful lifetime of the fiber (e.g. fibers 12–14) was prolonged somewhat in the presence of *N*-bromosuccinimide, and, while both k_{Na^+} and k_{K^+} were diminished by a factor of 2–4 over the corresponding values of fiber 2, the K^+ current appeared to enjoy more protection than the Na^+ current.

N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide methiodide (I) experiments

N-Ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide methiodide is one of several water-soluble carbodiimides which have enjoyed recent application as reagents for the selective modification of accessible carboxylic acid groups in a protein under very mild conditions (see refs 15 and 16 for reviews). Shrager and Profera [17] have recently used a similar water-soluble carbodiimide *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide (II) (see below) to study the effect of possible carboxyl group modification on tetrodotoxin binding to crab nerve. Their results would appear to be

TABLE II

REPRESENTATIVE *N*-BROMOSUCCINIMIDE AND *N*-ETHYL-*N'*-(3-DIMETHYLAMINOPROPYL)-CARBODIIMIDE METHIODIDE EXPERIMENTS

Fiber No. (M, S, U)*		Composition of test solution**	Duration*** (s) (number of points)	$-k_{Na+} \cdot 10^4\ s^{-1}$ † (r)††	$-k_{K+} \cdot 10^4\ s^{-1}$ † (r)††	k_{Na+}/k_{K+}	Breakdown‡ time (s)
A. *N*-Bromosuccinimide experiments							
1	(M)	0.10 mM *N*-bromosuccinimide in BR	218 (13)	35.5 (0.995)	37.5 (0.978)	0.95	300
2	(U)	same as 1	250 (15)	27.2 (0.997)	40.3 (0.980)	0.68	300
12	(M)	1.9 mg phospholipase D in R	189 (0)	No effect on currents			
		0.10 mM *N*-bromosuccinimide in BR	402 (12)	12.0 (0.923)	18.2 (0.958)	0.67	360
13	(S)	2.0 mg/ml lysozyme in R	327 (4)	12.9 (0.968)	10.7 (0.960)	1.2	—
		0.10 mM *N*-bromosuccinimide in BR	402 (8)	21.0 (0.988)	10.1 (0.892)	2.1	480
14	(M)	same as 13	360 (2)	1.76	4.60	3.8	—
			1065 latter 956 (11)	6.49 (0.995)	4.07 (0.969)	1.6	
B. *N*-Ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide methiodide (I) experiments							
21	(U)	10 mM (I) in R	18 (4)	163 (0.990)	1416 (0.983)	0.12	
22	(M)	2.2 mM (I) in R	51 (7)	41.6 (0.980)	276 (0.994)	0.15	—
23	(M)	0.41 mM (I) in R	126 (4)	11.0 (0.987)	59.4 (0.955)	0.19	—

Footnotes: see Table I.

consistent (see below, however) with the idea [2] that a carboxyl group is likely present at or near the Na^+ channel. Bass and Moore [18] have suggested that the ionizable groups [11, 19] showing a pK_a of 5.15 (Na^+ current) and 4.63 (K^+ current) may be attributable to carboxylic acid moieties of glutamic and aspartic acid residues.

It was therefore of interest to attempt to modify any accessible carboxylic acid residues on the nodal surface using the carbodiimide technique. To our surprise, upon performing the "control" experiments with Ringer's solutions containing carbodiimide (I) (10, 2.2 and 0.41 mM) but no added nucleophile other than 2 mM Tris buffer, we observed a remarkable selective and essentially irreversible reduction in magnitude of the K^+ current (see Table II, fibers 21–23 and Fig. 2). Thus, after 10 mM carbodiimide was applied to one fiber for a period of 210 s, the resulting K^+ current was 18 % of the original value. The leakage current remained unaffected until 330 s after the initial application of reagent at which time the leakage current increased dramatically, as was the case with 0.10 mM *N*-bromosuccinimide (see above). With lower concentrations of the carbodiimide fiber breakdown did not occur.

The semi-log plots of I_{K^+} versus time at all concentrations of (I) employed tended to level off (see Fig. 2, for example), suggesting that a t_∞ correction must be made in order to achieve at all reasonable first-order kinetics. If we assume that once a K^+ channel reacts with the carbodiimide it is completely blocked, then the t_∞ correction can be associated with either a population of K^+ channels essentially unaffected by (I) or else there are at least two (and quite possibly several) apparent pseudo first-order rate constants operating, one with a half life considerably longer than the other. In this event at least two populations of somehow structurally different

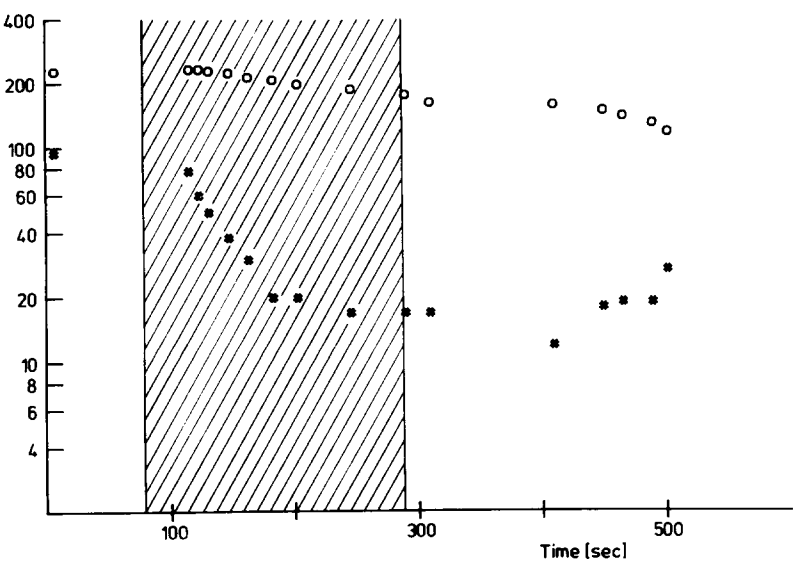


Fig. 2. Effect of 10 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide methiodide on the Na^+ (○) and K^+ (*) currents in a motor fiber (fiber 20, not presented in Table II). Note the leveling off in the K^+ current during continued exposure and the relatively minor overall effect on the Na^+ current. Fiber breakdown occurred some 330 s after initial application of the carbodiimide, thus the last five points are of dubious value owing to the relatively large leakage correction necessary (4- to 6-fold greater than that associated with the earlier points).

TABLE III

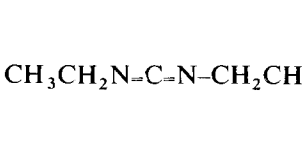
REPRESENTATIVE TETRANITROMETHANE AND *N*-ETHYLMALEIMIDE EXPERIMENTS

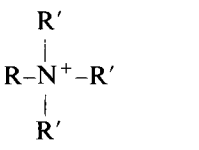
Fiber No. (M, S, U)*		Composition of test solution**	Duration*** (s) (number of points)	$-k_{Na+} \cdot 10^4\ s^{-1}$ † (r)††	$-k_{K+} \cdot 10^4\ s^{-1}$ † (r)††	k_{Na+}/k_{K+}	Breakdown‡ time (s)
A. Tetranitromethane experiment							
26	(U)	1 % methanol in R	116 (6)	26.5 (0.995)	25.8 (0.973)	1.0	—
		5.6 mM tetranitromethane, 1 % methanol in R, pH = 8.09	144 (5)	43.4 (0.996)	111 (0.999)	0.39	—
B. *N*-Ethylmaleimide experiments							
31	(M)	Run-down control, BR	90 (5)	28.0 (0.977)	29.5 (0.969)	0.95	—
		41 mM *N*-ethylmaleimide in BR	186 (5) (after initial rapid changes)	34.0 (0.947)	14.9 (0.980)	2.3	192
32	(M)	Run-down control, BR same as 31	110 (2)	4.33	9.76	0.44	—
			141				
			the first 8 (3)	775 (0.999)	205 (0.999)	3.8	—
			then the next 133 (6)	25.9 (0.895)	25.4 (0.789)	1.0	150
33	(M)	10.6 mM *N*-ethylmaleimide in BR	246				
			the first 8 (3)	298 (0.999)	120 (0.999)	2.5	—
			then the next 238 (10)	18.5 (0.991)	26.9 (0.980)	0.69	330

Footnotes: see Table I.

K^+ channels are required which differ in their reactivity toward carbodiimide (I). While other interpretations are possible, a reasonable alternative to the above would be that the t_∞ correction is associated with a residual K^+ conductance remaining after a channel reacts. Such a modified K^+ channel might be experimentally distinguishable from unmodified channels, however, we have as yet not pursued this interesting possibility.

The observed selectivity of carbodiimide (I) toward the K^+ current likely stems from the structural similarity to the well known K^+ channel blocker, tetraethylammonium ion (III) and several derivatives (e.g. "C_5" and "C_9") used recently by Armstrong [20] in a study of the nature of the K^+ channel in squid axons. Interestingly, however, Armstrong observed that octyltrimethyl ammonium ion "O" was ineffective at concentrations of 0.3 and 1.2 mM, suggesting that this substance cannot enter blocking sites (in squid axon).





carbodiimide (I): $R=CH_3$; $A^-=I^-$

carbodiimide (II): $R=H$; $A^-=Cl^-$

(III): $R=R'=CH_3CH_2$

"C_5": $R=CH_3(CH_2)_4$; $R'=CH_3CH_2$

"C_9": $R=CH_3(CH_2)_8$; $R'=CH_3CH_2$

"O": $R=CH_3(CH_2)_7$; $R'=CH_3$

Our results with carbodiimide (I), however, prompt a call for caution in the interpretation of the effect of carbodiimide (II) on the tetrodotoxin binding to crab nerve [17] cited above. Because of the structural similarity between the two carbodiimides, it is quite possible that carbodiimide (II) (100 mM) could have caused a non-specific breakdown of the crab nerve (analogous to our results with 10 mM carbodiimide (I) on frog nerve) with concomitant effects on tetrodotoxin binding having little to do with the "intact" Na^+ channel. It is pertinent to note that the crab nerves did not conduct an action potential after treatment for 40 min with carbodiimide (II) (100 mM) [17].

Tetranitromethane

The use of tetranitromethane as a specific reagent in protein chemistry has been recently reviewed [10, 21]. In aqueous solution at pH > 8 containing ethyl alcohol as a cosolvent tetranitromethane selectively modifies tyrosine residues, converting them to 3-nitrotyrosine residues. The reagent is normally present in substantial excess. Rapid oxidation of SH groups also occurs, however, and on occasion the reagent has attacked methionine residues and tryptophane residues, and caused apparent formation of cross-linkages when applied to insulin, lysozyme and other proteins [10, 21].

Because of the rather low solubility of tetranitromethane in water we found it necessary to use methanol as a cosolvent in our experiments. Control experiments indicated that fibers did not tolerate the presence of 1 % methanol in Ringer's at

pH 8.09 well, k_{Na+} and k_{K+} being increased (fiber 26) by factors of 20 and 9.5, respectively, over values for normal run-down (fiber 16). Nevertheless, a second pronounced effect was observed when the bathing solution was switched to one containing 5.6 mM tetranitromethane. In fiber 26, for example, k_{Na+} approximately doubled whereas k_{K+} increased by a factor of about 4. When, however, the bathing medium was switched once again to the "control" solution, the Na^+ current reversibly returned to its original level after allowing for the enhanced run-down due to the 1 % methanol present, whereas the K^+ current continued to decline. Thus tetranitromethane reversibly diminished the Na^+ current while irreversibly diminishing the K^+ current.

N-Ethylmaleimide experiments

N-Ethylmaleimide has been widely used in protein chemistry owing to its rather highly selective reaction with available SH groups [15]. The effect of the reagent on the resting and action potential of frog sciatic nerve bundles [4] and squid giant axon [5, 6] have been reported. Externally applied to squid axon, *N*-ethylmaleimide (2 mM) led to irreversible blocking of the action potential in 4–45 min whereas with desheathed frog nerve irreversible blockage occurred in 21±9 min.

In our experiments the action of *N*-ethylmaleimide (41 mM in bicarbonate-buffered Ringer's solution, pH 7.0) on the node (Fig. 3) resembled closely in many respects the action of tetranitromethane described above. During the first few seconds of application the Na^+ current fell more rapidly than the K^+ current ($k_{Na+} = 3.8$ k_{K+} for fiber 32). Continued application of the reagent caused a smooth decrease in both currents at rates 3–6-fold greater than expected run-down. A change back to

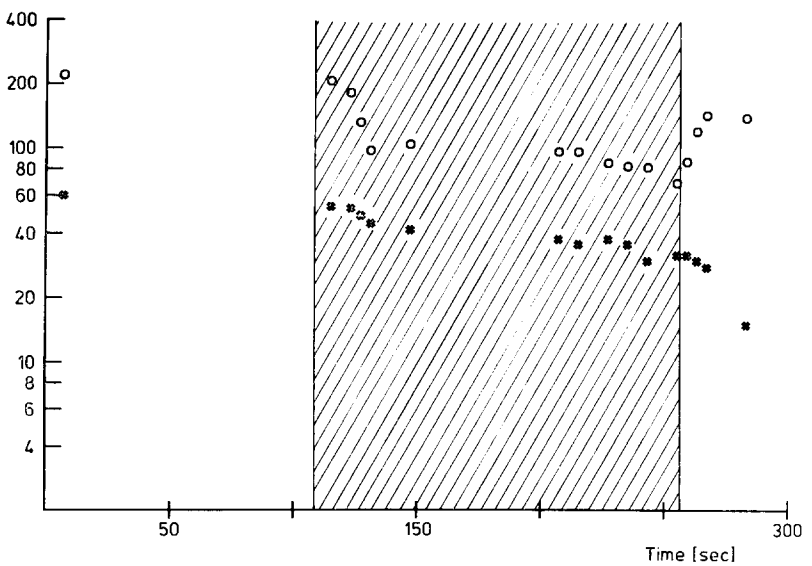


Fig. 3. Effect of 41 mM *N*-ethylmaleimide on the Na^+ (○) and K^+ (*) currents in a motor fiber (fiber 32). Note that while the Na^+ current is diminished more than the K^+ current, this effect is essentially reversible with the Na^+ current while irreversible with the K^+ current. The leakage current increased dramatically at about the time the last point was recorded.

bicarbonate-buffered Ringer's solution, however, led to nearly complete recovery of the Na^+ current (corrected for expected run-down), whereas the K^+ current, like with tetranitromethane, continued to decline. When lower concentrations of reagent was used, the above changes were much attenuated, with the K^+ current response being at least partially reversible.

Our studies with *N*-ethylmaleimide illustrate the difficulty of drawing meaningful conclusions about the molecular nature of the individual ionic channels in nerve based only on the ability of a reagent to block the action potential or alter the resting potential, though other useful information has come from such studies [4, 5]. Taking fiber 32 as an example, a first order rate constant of $25.9 \cdot 10^{-4}\ s^{-1}$ for the Na^+ current ($k_{K+} = 25.4 \cdot 10^{-4}\ s^{-1}$) during exposure corresponds to a half life of 267 s. Fiber breakdown occurred after only 150 s, however. Thus, the action potential would be "blocked" after 150 s due to a massive presumably non-ion selective increase in the leakage current caused by reagent at a time when the Na^+ and K^+ mechanisms were still functioning well, albeit at reduced current levels.

Two major differences between the action of *N*-ethylmaleimide and tetranitromethane are the fact that the former required a concentration 7 times that of the latter to show its effects clearly, and that tetranitromethane did not cause fiber breakdown whereas *N*-ethylmaleimide in every case caused fiber breakdown within 5 min after the beginning of its application.

Since the reaction of *N*-ethylmaleimide with SH groups is irreversible it is likely that the effect on the Na^+ current does not involve formation of covalent bonds, and might arise by simple reversible dissolution in the membrane. The effect of *N*-ethylmaleimide on the K^+ current probably does originate from formation of a covalent bond between the reagent and the nodal membrane. These results, when coupled with those of tetranitromethane and *N*-bromosuccinimide begin to suggest that accessible SH groups of nodal membrane protein are somehow important to K^+ conductance changes. Additional experiments with other SH reagents are in progress and may or may not support this very tentative view.

Sodium metaperiodate ($NaIO_4$) and sodium iodate ($NaIO_3$).

Periodate oxidation has been extensively used, particularly in sugar chemistry, for the selective oxidative cleavage of 1,2-dihydroxy groups to dialdehydes [22, 23]. Periodate, unfortunately, is also known to react with tryptophane, tyrosine, cystine, cysteine, methionine and histidine residues within a peptide chain, and serine and threonine are likely to be attacked as *N*-terminal residues [24].

Since periodate is converted to iodate during an oxidation it was also of interest to learn the effects of iodate on the ionic currents. Few uses of $NaIO_3$ as an oxidizing agent in biochemistry have appeared other than its reported [25] oxidation of cystine to two molecules of the corresponding sulfonic acid and its oxidative cleavage of insulin in which two disulfide bonds are oxidized. We examined the action of IO_4^- on the node at pH 4.9, since experiments at pH 7.4 were precluded by formation of an insoluble precipitate, probable $Ca(IO_4)_2$. Progress of the reaction was followed by periodically switching back to normal Ringer's since at pH 4.9 nearly all the Na^+ and K^+ currents had vanished due to the well known [11, 19] effect of low pH. Table IV shows that even with 20 mM IO_4^- k_{Na+} and k_{K+} were only 4-fold greater than normal run-down. IO_3^- (20 mM) was less effective than periodate at pH 4.9 (not

TABLE IV

REPRESENTATIVE $NaIO_4$, $NaIO_3$ AND SODIUM CYANOBOROHYDRIDE EXPERIMENTS

Fiber No. (M, S, U)*		Composition of test solution**	Duration ***(s) (number of points)	$-k_{Na^+} \cdot 10^4\ s^{-1}$† (r)††	$k_{K^+} \cdot 10^4\ s^{-1}$† (r)††	k_{Na^+}/k_{K^+}	Breakdown‡ time (s)
A. $NaIO_4$ experiment							
7	(U)	20 mM $NaIO_4$ in PR, pH = 4.89	400 (5)	9.27 (0.986)	12.3 (0.982)	0.75	420
B. $NaIO_3$ experiment							
43	(M)	20 mM $NaIO_3$ in BR	1185				
			latter 1125 (8)	3.61 (0.988)	5.84 (0.992)	0.62	—
C. Sodium cyanoborohydride experiments							
38	(M)	103 mM sodium cyanoborohydride in PR, pH = 4.7–5.3 (addition of HCl required)	865 (7)	12.0 (0.989)	17.0 (0.989)	0.71	—
52	(M)	101 mM sodium cyanoborohydride in BR	900 (16)	11.6 (0.906)	8.21 (0.808)	1.4	—
				39.8 (0.986) corr. for t_∞ value	35.4 (0.918) corr. for t_∞ value	1.1	—
53	(M)	same as 52	643 (16)	14.0 (0.981)	13.2 (0.942)	1.1	—
			the last 568 (12)	12.1 (0.987)	9.52 (0.976)	1.3	

Footnotes: see Table I.

shown). IO_3^- could be used also at pH 7.4 (fiber 43) where k_{Na+} and k_{K+} were slightly greater than normal run-down.

Considering the several functional groups attacked by IO_4^-, one might have expected the ionic currents to be rather more vulnerable to the presence of IO_4^-. The effects of IO_4^- could be diminished by the inherently slow nature of its reaction with "normal" substrates. Since IO_4^- is itself an anion, its approach to the membrane surface may well be impeded by the layer of negative surface charges thought to be present on the membrane, and therefore, substrates may be present and yet not react. In direct contrast, profound effects are observed when IO_3^- and IO_4^- are applied to the inside of the fiber [26]

Sodium cyanoborohydride experiments

Sodium cyanoborohydride [27] is closely related to the better known sodium borohydride [14]. The latter, however, is unstable at pH 7 or less while the former can be used even at pH 3. At pH 6–7, sodium cyanoborohydride rapidly reduces enamine linkages and iminium linkages, $\rangle C{=}N^+\langle^{R}_{R}$, to the corresponding saturated amines [27]. The reagent thus is able to effect reductive aminations (i.e. reductive alkylations) under more favorable conditions than does sodium borohydride.

The experiment on fiber 38 (Table IV) with pH 4.7–5.3 and 103 mM sodium cyanoborohydride showed that at this relatively high concentration the reagent increased k_{Na+} and k_{K+} less than a factor of ten of over those of normal run-down. The effects of 101 mM reagent at pH 7.4 (fibers 52 and 53) were comparable. In all instances the semi-log plots tended to level off, indicative of a progressive decrease in vulnerability of the fiber toward the reagent. The high concentrations required for observable effects suggest that there are probably no accessible aldehyde, ketone or iminium groupings or disulfide bonds which are essential to the maintenance of the ionic currents, a conclusion in keeping with the studies of Hille [2] where action potentials were observed with frog nodes when hydrazinium ion was used in place of Na^+. Hydrazone formation would have been expected in Hille's experiments had accessible iminium, ketone or aldehyde functional groups been present, though those reactions could have gone undetected.

CONCLUSIONS

We have shown that chemical modification of the nodal membrane by reaction with *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide methiodide, tetranitromethane and *N*-ethylmaleimide leads to a marked irreversible decrease in the K^+ current while not permanently altering the Na^+ current significantly. *N*-Bromosuccinimide, in contrast, dramatically reduces both currents. The common denominator among the latter three reagents is the ability to modify accessible SH groups. Thus, it would appear that accessible SH groups are more crucial to the operation of the K^+ mechanism than the Na^+ mechanism. On the other hand, apparently accessible iminium, ketone, aldehyde or 1,2-dihydroxy groups can be modified, if present, without profound effects on either ionic current. Similarly, enzymatic treatment of the node was also without large effect.

Our work provides some experimental basis for the interesting idea that not all channels of a particular ion are chemically alike, an important consideration for

future biochemical isolation and purification work. We have shown that some reagents can cause fiber breakdown (and therefore conduction block) through a dramatic increase in the leakage current while the Na^+ and K^+ mechanisms are functioning more or less normally.

The nature of the functioning node precludes to a great extent the kind of rigorous [28] biochemical approach expected with studies of, for example, the modification of specific residues of an enzyme. The observed reduction of the Na^+ and K^+ currents in our experiments is likely a composite effect of both chemical modification of the macromolecules constituting the ionic channels and modification of the nodal membrane at sites remote from the ionic channels. However, when one of the ionic currents is selectively affected over the other, it is rather likely that the effect of chemical modification somewhere very near those channels is being observed.

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