J. Membrane Biol. 82, 41–48 (1984)

The Journal of
Membrane Biology

Effects of Chemical Modification of Carboxyl Groups on the Voltage-Clamped Nerve Fiber of the Frog

Michael Rack† and Karl-Heinz Woll‡

†Institut für Physiologische Chemie, Universität des Saarlandes, D-6650 Homburg/Saar,
and ‡I. Physiologisches Institut, Universität des Saarlandes, D-6650 Homburg/Saar, Federal Republic of Germany

Summary. Voltage-clamped single nerve fibers of the frog *Rana esculenta* were treated with the carboxyl group activating reagent N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in the presence of different primary amines and without added amine. Carboxyl groups form stable amide bonds with primary amines in the presence of EEDQ. EEDQ treatment reduced the sodium current considerably and irreversibly, regardless of the presence of a primary amine in the Ringer's solution. The potassium current was also reduced. After modification the reduced sodium currents inactivated slowly and incompletely. The descending branch of the sodium current-voltage relation, $I_{Na}(E)$, was shifted along the voltage axis in the depolarizing direction. The size of the shift was strongly dependent on the amine present during modification with EEDQ. The voltage-dependence of sodium inactivation, $h_\infty(E)$, was shifted to more positive values of membrane potential by EEDQ in the presence of ethylenediamine (11 mV) and glucosamine (3 mV). In contrast, a small shift to more negative potentials occurred in the presence of taurine (−3 mV) or without the addition of an amine (−2 mV). A tenfold increase of the calcium concentration still shifted the $I_{Na}(E)$ and $h_\infty(E)$ curves of the chemically modified fibers. However, these shifts were smaller than those observed on untreated fibers. The currents remaining after the modification were completely blocked by tetrodotoxin; no change of the reversal potential occurred.

Key Words Voltage clamp · node of Ranvier · Na current · chemical modification · carboxyl groups

Introduction

The marked effect of divalent ions, hydrogen ions and other monovalent ions on the voltage dependence of the sodium and potassium permeability strongly suggests that anionic sites of the membrane contribute to the electrical field across the nerve membrane. Most of the external surface potential, set up by fixed charges, is created by negatively charged groups. These fixed charges on the outside of the membrane affect the voltage dependence of the excitability parameters (for example: Frankenhaeuser & Hodgkin, 1957; Blaustein & Goldman, 1968; Hille, 1968; Brismar, 1973; Drouin & Neumcke, 1974; Vogel, 1974; Begenisich, 1975; Hille, Woodhull & Shapiro, 1975).

The most likely candidates for anionic sites on a membrane are carboxyl or phosphate groups. From experiments with divalent uranyl ions it was concluded that many of the surface acidic groups are phosphate groups (D'Arrigo, 1975).

Chemical modification of sodium channels with reagents known to modify carboxyl groups resulted in a reduced binding of the specific sodium channel blocker tetrodotoxin (TTX) (Shrager & Profera, 1973; Reed & Raftery, 1976). Sodium channels, still generating action potentials, even in extremely high concentrations of TTX could be demonstrated (Baker & Rubinson, 1975, 1977). Sodium currents were measurable in the presence of high concentrations of TTX or saxitoxin (STX) after treatment of nerve and muscle fibers with a carboxyl group modifying reagent (Sigworth & Spalding, 1980; Spalding, 1980). Furthermore, a carboxyl group was assumed to be part of the selectivity filter and probably identical with the TTX-binding acidic group (Hille, 1975*a*,*b*). However, treatment of muscle fibers with a carboxyl group modifying reagent resulted in TTX-resistant sodium channels with no significant change in their selectivity (Spalding, 1980).

In order to investigate the chemical nature and functional importance of the negative charges, we have modified the carboxyl groups of the nerve membrane covalently and studied the effects on sodium currents. Interpretation of data obtained from chemical modification studies is not without difficulty. The most important problems are the specificity of the reagent applied and the question to what extent the modification reaction has proceeded. Problems that have to be considered are extensively reviewed by Means and Feeney (1971).

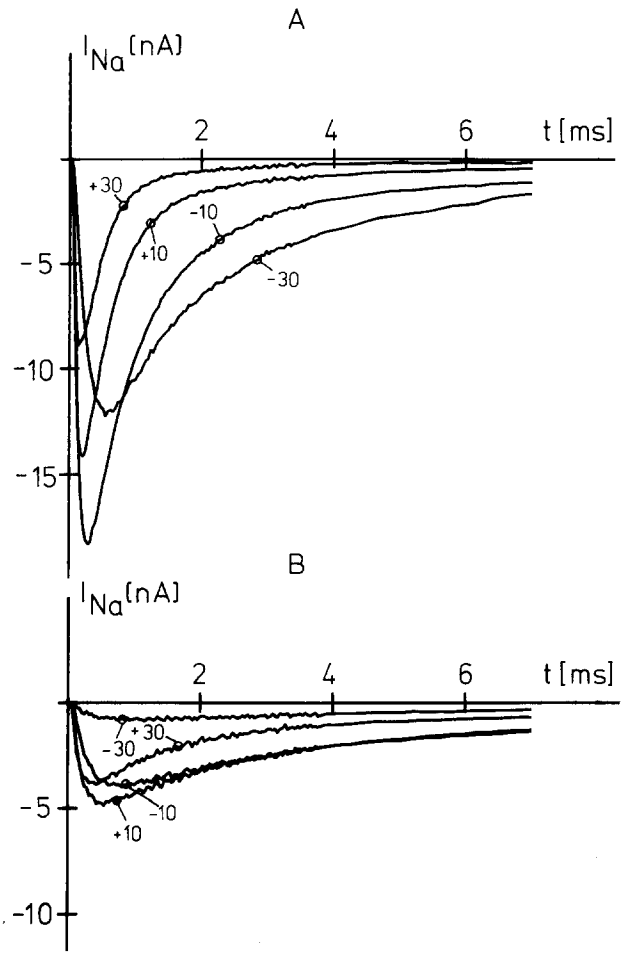

Fig. 1. Na currents associated with 6.6-msec depolarizing pulses to potentials between −30 and +30 mV before (*A*) and after (*B*) treatment with EEDQ and ethylenediamine for 10 min. K currents were blocked by internal Cs and 12 mM TEA from outside

For chemical modification we used the highly specific carboxyl group activating reagent EEDQ. This reagent is known to convert carboxyl groups into highly reactive esters. These active esters react readily with nucleophiles available and form stable amide bonds in the presence of primary amines (Belleau & Malek, 1968; Belleau, DiTullio & Godin, 1969). This mechanism of action makes it possible to introduce amines with different properties into the membrane. We have studied the action of EEDQ in the presence of a diamine (ethylenediamine), a monoamine (glucosamine) and a monoamine bearing an additional negative charge (taurine). The action of EEDQ without primary amine was also investigated. In addition, we studied the effects of increased calcium concentration in fibers that have been chemically modified with the carboxyl group reactive reagent. Part of the results have been presented in abstract form (Rack, 1983; Rack & Woll, 1984).

ABBREVIATIONS: N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), Morpholinopropanesulfonic acid (MOPS), Morpholinoethanesulfonic acid (MES), Dimethylformamide (DMF), Tetrodotoxin (TTX), Tetraethylammonium (TEA), Trimethyloxonium (TMO).

Materials and Methods

Single nerve fibers were dissected from the sciatic nerve of the frog *Rana esculenta* (Stämpfli, 1969). A node of Ranvier was voltage clamped at 15°C by the method of Nonner (1969). The fiber was cut on both sides of the node at a distance of about 0.75 mm. The ends of the fiber were in 117 mM CsCl, 5 mM NaCl, 2 mM MOPS, pH 7.3; this solution was used for blocking K currents. The potential at which 30% of the Na channels are inactivated was taken as the normal resting potential and defined as $E = -70$ mV. The fibers were held at $E = -70$ mV. The command voltage pulses were generated by a 10-bit D/A converter under computer control. Membrane currents were filtered by a 10-kHz low-pass filter and sampled in 37 and 185-μsec intervals by means of a 12-bit A/D converter, also operating under computer control. Absolute membrane currents were calculated by assuming a longitudinal axoplasmic resistance of 10 MΩ, corresponding to a value of 140 MΩ/cm for the resistance per unit length of a frog nerve fiber of 14 μm diameter (*see* Stämpfli & Hille, 1976). An analogue circuit was used to compensate the symmetrical component of the capacitive and leakage currents. All potentials are given as absolute potentials.

To measure the $h_\infty(E)$ curve, 40-msec conditioning pulses to varying potentials followed by a constant test pulse to +10 mV were used. Normalized test pulse current was plotted against membrane potential during the conditioning pulse. The equation

$$h_\infty = \frac{1}{1 + \exp\dfrac{E - E_h}{k}} + C \qquad (1)$$

was fitted to the experimental points. In this equation, k is the slope parameter and E_h is the potential at which $h_\infty = 0.5 + C$. C is used for the description of a noninactivating component of the sodium permeability.

Solutions

The node was superfused continuously with Ringer's solution with or without a chemical reagent. The control Ringer's solution contained (mM): 110 NaCl, 2.5 KCl, 2 $CaCl_2$, 12 TEA, and 4 MOPS. The pH was adjusted to 7.2 with 1 N NaOH.

Several test solutions have been used. Ringer's with increased Ca concentration contained 20 mM $CaCl_2$ instead of 2 mM, with no change in the concentration of the other salts or pH. The Ringer's solution with reduced NaCl contained $\frac{2}{3}$ choline chloride and $\frac{1}{3}$ NaCl or $\frac{4}{5}$ RbCl and $\frac{1}{5}$ NaCl. EEDQ was dissolved in dimethylformamide (DMF). The dissolved reagent was slowly added to stirred Ringer's solution (mM: 110 NaCl, 2.5 KCl, 2 $CaCl_2$, 12 TEA, and 4 MES) and the pH adjusted to 5.5 with 1 N NaOH. Alternatively, the NaCl was replaced by RbCl and the

pH adjusted to 5.5 with tetramethylammoniumhydroxyde instead of NaOH. This second solution was used in several experiments in order to prevent shifts of the reversal potential that may occur simply by Na ion influx during the relatively long time of modification and washing. Rubidium was used as the permeability of the Na channel for this ion is below 3% of that for sodium ions (Hille, 1972). Quaternary amines may be used for the same purpose; to exclude a possible contamination with primary amines, that may be present in such a preparation, we favored the use of RbCl. A 2 mM solution of EEDQ in Ringer's was prepared, the final concentration of DMF was 2%. If desired, an appropriate amine was added to give a final concentration of 10 mM. EEDQ is slowly hydrolyzed by water. As revealed by the change of absorption at 262 nm the $t_{1/2}$ of EEDQ hydrolysis in Ringer's solution, pH 5.5, is approx. 75 min. Therefore the individual Ringer's solution was freshly prepared for each experiment and applied to the nerve fiber immediately after preparation. After application the preparation was washed with reagent-free Ringer's solution for at least 10 min before measurements were continued.

Materials

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) and tetramethylammonium hydroxide were obtained from Sigma, München, F.R.G. Ethylenediamine, taurine, rubidium chloride, and tetraethylammonium chloride (TEA) were from Fluka, Neu-Ulm, F.R.G. Morpholinopropanesulfonic acid (MOPS), morpholinoethanesulfonic acid (MES) and glucosamine were from Serva, Heidelberg. All other chemicals were analytical grade and were purchased from Merck, Darmstadt, F.R.G.

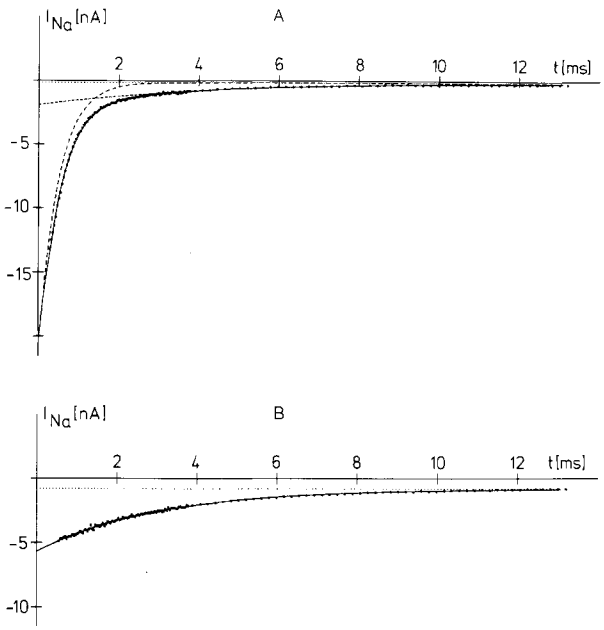

Fig. 2. Fit of the decaying phase of the Na current associated with a 14-msec pulse to +10 mV before (*A*) and after (*B*) treatment with EEDQ and ethylenediamine. The currents in *A* and *B* were fitted with the equations: $I_{Na} = -21.28 \exp(-t/0.49) - 1.76 \exp(-t/3.9) - 0.16$ [nA], and $I_{Na} = -4.89 \exp(-t/2.96) - 0.81$ [nA], respectively

Results

EEDQ and Ethylenediamine

Two mM EEDQ were applied to the nerve fiber in the presence of 10 mM ethylenediamine for 10 min and then washed out with reagent-free Ringer's solution. During the application of the chemicals at low pH, I_{Na} and I_K decreased quickly and completely. A strong, irreversible decrease of the currents was observed after washing with normal Ringer's.

As the modification procedure lasts a relatively long time an increase of the sodium concentration of the axoplasmic side of the fiber may occur, thereby causing a shift of the reversal potential (E_{Na}). In order to prevent such an effect, NaCl-free Ringer's solutions were used during the chemical procedure and washout when the reversal potential was examined while the currents were measured in full NaCl before and after modification (*see* Materials and Methods).

Figure 1 compares Na currents before (*A*) and after (*B*) treatment with EEDQ and ethylenediamine. The size of the Na inward currents in *A* and *B* shows that the Na permeability is about four times smaller in *B* than in *A*. The time to peak was increased by a factor of 1.54 and the decay of the Na current was considerably slower after treatment with the carboxyl group reactive reagent in the presence of ethylenediamine. For a quantitative analysis, the decaying phase of the Na inward current associated with a 14-msec pulse was fitted by the sum of one or two exponentials and a time-independent term I_∞ (Fig. 2). The normal Na current was best fitted with two exponentials ($\tau_{h1} = 0.49$ msec, $\tau_{h2} = 3.93$ msec) and $I_\infty = -0.16$ nA, whereas the Na current recorded after treatment could be described by a single exponential with a relatively large time constant ($\tau_h = 2.96$ msec) and $I_\infty = -0.81$ nA. Even during a 3-sec pulse to 0 mV inactivation of the Na current in fibers treated with EEDQ and ethylenediamine was not complete.

Plotting I_{Na} versus pulse potential revealed that treatment with 2 mM EEDQ and 10 mM ethylenediamine for 10 min shifted the descending branch of the $I_{Na}(E)$ curve by 12 mV in the depolarizing direction, but did not alter the reversal potential (Fig. 3). Part of the 12-mV shift could be due to an artefact of series resistance caused by the considerable reduction of I_{Na} during the chemical treatment. However,

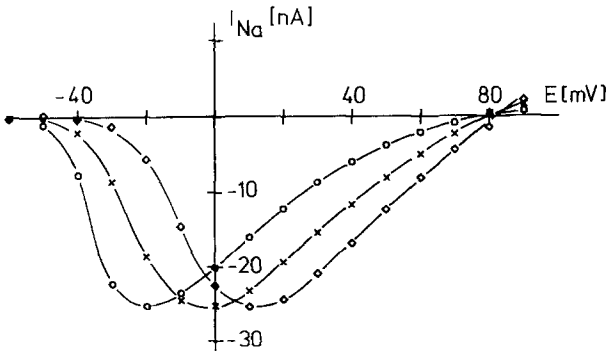

Fig. 3. Peak sodium current-voltage curve, $I_{Na}(E)$, before (o) and after treatment with 2 mM EEDQ and 10 mM ethylenediamine for 10 min, (x) in 2 mM Ca, (◇) in 20 mM Ca. Currents after treatment multiplied by 2.7 (x) or 3.68 (◇) to facilitate comparison of the $I_{Na}(E)$ curves

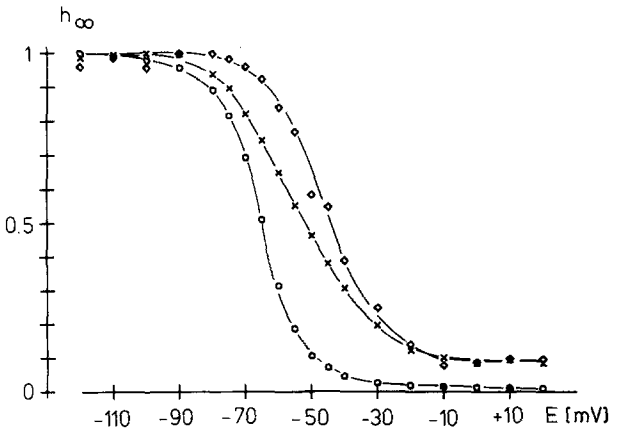

Fig. 4. Steady-state inactivation curve, $h_\infty(E)$, of the sodium system before (o) and after (x) treatment with 2 mM EEDQ and 10 mM ethylenediamine for 10 min, both measured in 2 mM Ca. The curve (◇) is the $h_\infty(E)$ curve with 20 mM Ca after treatment. Points were fitted by Eq. (1) with $E_h = -64.8$ mV, $k = 6.5$ mV and $C = 0.018$ for o, $E_h = -54.5$ mV, $k = 11.9$ mV and $C = 0.083$ for x, and $E_h = -46.1$ mV, $k = 9.0$ mV and $C = 0.089$ for ◇. At −100 mV, the current after EEDQ/ethylenediamine treatment in 2 mM Ca was 29.2% of the current measured before in the same Ringer's solution

a similar shift (13.5 mV) was observed when the currents before and after modification were of equal size (*see* Table); this was achieved by recording the currents before modification in $\frac{1}{5}$ Na ($\frac{4}{5}$ Na replaced by Rb) and the currents after modification in full Na. To facilitate comparison, the currents in Fig. 3 are scaled so that the maxima of the $I_{Na}(E)$ curves are equal.

Subsequent exposure of the chemically modified fiber to 20 mM $CaCl_2$ (instead of 2 mM) caused a further shift of the descending branch of the $I_{Na}(E)$ curve by 14 mV (◇ in Fig. 3). Average values for the shifts caused by modification and for the shifts produced by 20 mM Ca are given in the Table.

On the same fibers we measured the steady-state inactivation curve, $h_\infty(E)$. The modification procedure had three marked effects: it shifted the potential for half inactivation (E_h) to more positive values of membrane potential, decreased the slope of the curve, and induced a noninactivating component (Fig. 4). To account for this noninactivating component the constant C was introduced into Eq. (1) (*see* Materials and Methods). In the experiment of Fig. 4 the 10-min treatment with EEDQ and ethylenediamine resulted in a shift of E_h from −64.8 to −54.5 mV. The slope factor k was increased from 6.5 to 11.9 mV. In a total of five experiments the average shift of E_h was 11.3 (± 1.6) mV (mean ± SD), i.e. similar to the shift of the descending branch of the $I_{Na}(E)$ curve (*see* Table).

Subsequent exposure of the modified fiber to 20 mM Ca induced a further shift of the $h_\infty(E)$ curve (◇ in Fig. 4). E_h was shifted from −54.5 to −46.1 mV and k decreased from 11.9 to 9.0 mV. Thus, the high Ca concentration partially re-established the normal slope of the $h_\infty(E)$ curve. As shown in the Table, the effect of 20 mM Ca on the $h_\infty(E)$ curve, expressed as change in the half potential E_h, was smaller after modification than before.

In order to investigate how far the reaction has proceeded during our 10-min treatment protocol, we superfused a fiber for 20 min with EEDQ and ethylenediamine. The shift of the descending branch of the $I_{Na}(E)$ curve measured in 2 mM Ca^{++} was 19.6 mV instead of 12.1 mV and that of E_h of the $h_\infty(E)$ curve 13.3 mV instead of 11.3 mV when compared with the average shifts after 10 min; in addition the slope factor k increased by 6.4 mV instead of 4.7 mV during the prolonged treatment. Subsequent exposure of the modified fiber to 20 mM Ca^{++} produced a shift of the descending branch of the $I_{Na}(E)$ curve by 12.2 mV, and of the $h_\infty(E)$ curve by 12.1 mV, i.e. resulted in the same shifts as after our standard treatment protocol of 10 min (*see* Table). Thus, all carboxyl groups that can be affected by an increase of Ca ion concentration have been modified during our 10-min treatment.

EEDQ and Glucosamine

The same procedure as described above was used to test the effects of glucosamine in the presence of EEDQ. When the single charged amine was present instead of the double charged ethylenediamine, the descending branch of the $I_{Na}(E)$ curve was shifted by only 5.8 to 6.5 mV and the E_h of the $h_\infty(E)$ curve by only 2.7 mV while k increased by 2.9 ± 0.6 mV.

Table. Average shifts of the descending branch of the $I_{Na}(E)$ curve and of the half potential E_h of the $h_\infty(E)$ curve produced by modification of the carboxyl groups and by increasing the Ca concentration from 2 to 20 mM. In addition, the increase of the slope factor k of the $h_\infty(E)$ curve is given. Data are given in mV as means ± SD

Effect of modification					Effect of 20 mM Ca			
					Before modification	After modification	Before modification	After modification
EEDQ and:	$I_{Na}(E)$ curve (1)[a]	(2)[b]	h_∞ curve, (E_h) (3)	h_∞ curve, (k) (4)	$I_{Na}(E)$ curve (5)	$I_{Na}(E)$ curve (6)	h_∞ curve, (E_h) (7)	h_∞ curve, (E_h) (8)
Ethylenediamine: (n = 5)	12.1 ± 1.2	13.5 ± 2.2	11.3 ± 1.6	4.7 ± 0.6		13.6 ± 0.6		11.0 ± 1.4
Glucosamine: (n = 3)	5.8 ± 0.6	6.5 ± 1.1	2.7 ± 1.7	2.9 ± 0.6		14.0 ± 1.0		15.3 ± 1.0
Taurine: (n = 3)	4.5 ± 2.5	5.4 ± 2.7	−2.9 ± 2.2	5.0 ± 1.8	16.0 ± 1.3 (n = 16)	14.7 ± 7.3	16.8 ± 1.6 (n = 15)	16.4 ± 6.7
No amine added (n = 3)	5.0 ± 1.5	6.6 ± 1.5	−1.8 ± 2.2	2.9 ± 0.4		13.2 ± 1.7		15.7 ± 1.5

[a] Currents before and after modification recorded in full Na_o.
[b] Currents before modification recorded in $\frac{1}{3}$ and $\frac{1}{9}$ Na_o (*see* Materials and Methods), currents after modification in full Na_o.

The data are summarized in the Table. The reduction of the Na currents and the slow and incomplete inactivation were essentially the same as described above.

EEDQ and Taurine

Besides the positively charged amino group, taurine contains a negatively charged sulfonic acid group. Thus, no change of the net charge will occur by this modification reaction. Using the 10-min treatment protocol, we found a small shift of the E_h of the $h_\infty(E)$ curve to more negative values of membrane potential (−2.9 mV), an increase of k by 5.0 ± 1.8 mV, while the descending branch of the $I_{Na}(E)$ curve was shifted by 4.5 to 5.4 mV in the depolarizing direction (*see* Table). The other effects were the same as in the presence of ethylenediamine or glucosamine (*see above*).

EEDQ Without Added Primary Amine

As described above, the reduction of the Na permeability, the slow and incomplete inactivation and the increase of the slope factor k occurred regardless of the properties of the added amine. The same effects could be observed after EEDQ treatment without externally added primary amine. Presumably, the highly active esters formed by EEDQ react with membrane-bound nucleophilic groups in the immediate proximity (e.g. a lysine residue). The shift of the $I_{Na}(E)$ curve produced by EEDQ treatment without added amine was similar to that caused by EEDQ in the presence of taurine (*see* Table). Figure 5 shows the effect of a 10-min EEDQ treatment on the $h_\infty(E)$ curve. This modification procedure had two marked effects: it induced a noninactivating component and decreased the slope of the curve, while the potential for half inactivation

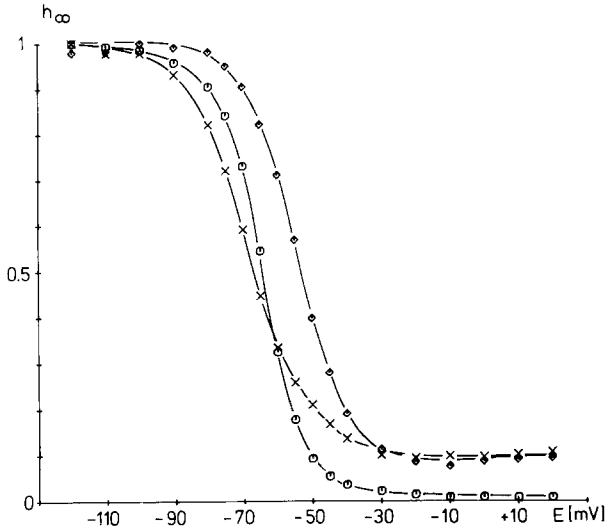

Fig. 5. Steady-state inactivation curve, $h_\infty(E)$, of the sodium system before (o) and after (x) treatment with 2 mM EEDQ for 10 min without added primary amine, both measured in 2 mM Ca. The curve (◇) is the $h_\infty(E)$ curve with 20 mM Ca after treatment. Points were fitted by Eq. (1) with E_h = −64.2 mV, k = 6.0 mV and C = 0.012 for o; E_h = −68.4 mV, k = 8.5 mV and C = 0.101 for x, and E_h = −54.3 mV, k = 7.0 mV and C = 0.085 for ◇. At −100 mV, the current after EEDQ treatment without added primary amine in 2 mM Ca was 27.2% of the current measured before in the same Ringer's solution

(E_h) was little affected. In the experiment of Fig. 5 the 10-min treatment resulted in a shift of E_h from −64.2 to −68.4 mV and the slope factor k was increased from 6.0 to 8.5 mV. The average shifts are summarized in the Table. These effects did not markedly change when the fiber was superfused for 20 min with the reagent and no added primary amine.

Leakage Current, K Current and Tetrodotoxin Sensitivity

None of the modification reactions caused a significant change of the leakage current. The fibers remained stable, no "run-down" of the fiber was induced. With KCl, instead of CsCl, applied to the cut ends of the fiber, and TEA-free Ringer's solution the potassium currents measured after the modification reaction were found to be considerably and irreversibly reduced. No detailed studies concerning the modification of K currents were made. As in untreated fibers, no sodium inward current was measurable when 300 nM TTX were applied to fibers chemically modified with the carboxyl group reactive reagent. Half of the sodium current, left after treatment with EEDQ and ethylenediamine, was blocked by 3 nM TTX.

Discussion

Chemical modification of the nerve fibers with EEDQ in the presence or absence of differently charged amines caused a considerable reduction of Na currents; the inactivation became slow and incomplete; furthermore, the slope factor k of the $h_\infty(E)$ curve increased. These effects occurred regardless of the presence or absence of an external amine. In addition, shifts of the $I_{Na}(E)$ and the $h_\infty(E)$ curve were observed which strongly depended on the amine added.

The mechanism of action of EEDQ has been described in detail by Belleau et al. (1969). The reagent reacts with carboxyl groups to form highly activated esters. Subsequently, these esters react readily with amines, to form stable amide bonds; other nucleophiles, like SH-groups, may also react with such activated esters. Furthermore, these esters are hydrolyzed by water, and the carboxyl groups originally present are recovered. Reagents, that react in a manner like EEDQ, are known to introduce intra- or intermolecular crosslinks (Hoare, Olson & Koshland, 1968). A membrane-bound nucleophile, e.g. a lysine residue, will react with a carboxyl group esterified by EEDQ if it is in the immediate proximity. Thus, if a membrane-bound nucleophile is available near a membrane-bound carboxyl group a reaction depends only on the activation by EEDQ. If such a reaction occurs, it is a favored one. Thus, EEDQ without external amine may cause reactions that will also occur in the presence of external amines. (In high concentrations of external amine a competition between membrane-bound and external amine may become important.) Thus, the effects that occur regardless of the presence or absence of an external amine are probably due to crosslink reactions.

In contrast, the shifts of the $h_\infty(E)$ and the $I_{Na}(E)$ curves strongly depended on the primary amine given from outside. The two-step mechanism of action with EEDQ and amines allowed the introduction of amines with different properties, i.e. different charges (*see* Fig. 6) causing different effects on the $h_\infty(E)$ and the $I_{Na}(E)$ curves. Treatment of nerve fibers with EEDQ and ethylenediamine shifted the descending branch of the $I_{Na}(E)$ curve and the half potential E_h of the $h_\infty(E)$ curve to more positive potentials, i.e. acted like an increase of $[Ca]_o$. As this chemical procedure may have removed negatively charged groups, it is clear that this might cause an effect similar to that occurring as a result of binding or neutralization of negative charges by Ca^{++} ions. However, there was still a strong effect of increased Ca concentration on the modified fibers, although somewhat smaller than on untreated fibers (*see* Table). As the prolonged modification experiment demonstrates, a 10-min treatment of the nerve fiber (our standard procedure) leads to a complete modification of carboxyl groups that interact with Ca ions; the shifts of the $I_{Na}(E)$ and the $h_\infty(E)$ curves by an increase of the Ca concentration were the same after a 10- and a 20-min treatment with EEDQ and ethylenediamine. Therefore, one can conclude that most of the negatively charged groups affected by Ca^{++} ions are *not* carboxyl groups. The most likely candidates left for interaction with Ca^{++} ions on a biological membrane are phosphate groups. This is in accordance with previous findings of D'Arrigo (1975), who concluded (from experiments with divalent uranyl ions) that most of the surface charges at the outside of the membrane are due to the presence of phosphate groups.

It should be noted that the chemical treatment with EEDQ in the presence of ethylenediamine or glucosamine resulted in a shift of the $I_{Na}(E)$ curve and the half potential E_h of the $h_\infty(E)$ curve to more positive potentials, i.e. acted like an increase of $[Ca]_o$. However, in the experiments with taurine and without added amine the $h_\infty(E)$ curves are somewhat shifted to more negative values of mem-

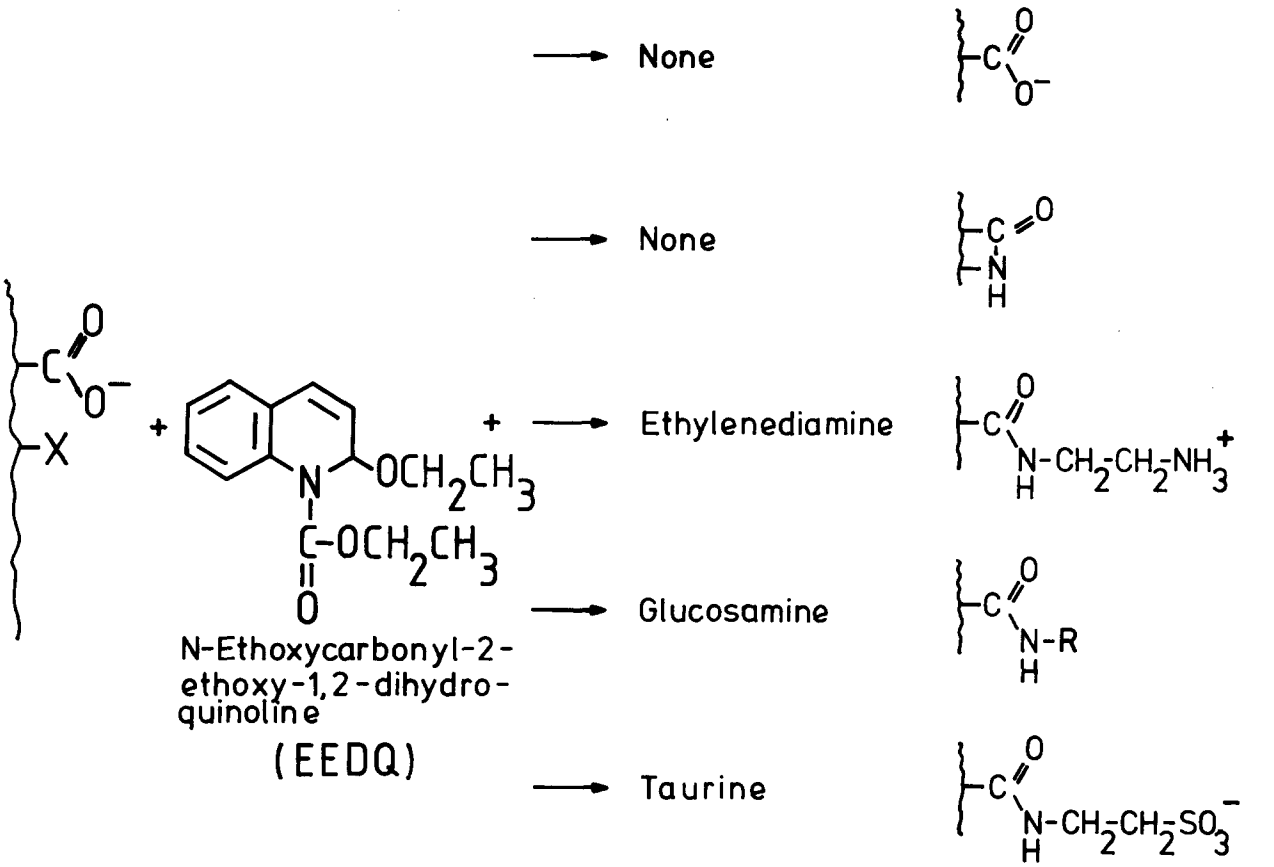

Fig. 6. Reaction scheme of carboxyl groups with EEDQ and amines. On the left, the membrane with a carboxyl group and the structure of EEDQ are shown. X represents a nucleophile located near the carboxyl group. On the right, the resulting derivatives of carboxyl groups after treatment with different amines in the presence of EEDQ are illustrated. As can be seen, differently charged derivatives are obtained, depending on the amine present during the EEDQ application

brane potential; the $I_{Na}(E)$ curves are slightly shifted to more positive potentials. Thus, in these cases, and in contrast to the well-known Ca effects, the carboxyl group modification can cause shifts into opposite directions. This might be interpreted as a modification of different carboxyl groups, having a separate influence on the position of the $I_{Na}(E)$ and $h_\infty(E)$ curves. However, it is also conceivable that steric alteration of the Na channel at a single site slightly facilitates inactivation and, by the same time, inhibits activation.

It is interesting to compare the effects of EEDQ with the effects of carbodiimide and trimethyloxonium (TMO), reagents that also react with carboxyl groups (and other groups). Carbodiimide and TMO make sodium channels insensitive to tetrodotoxin (TTX) and saxitoxin (STX). TTX-resistant action potentials are observed after treatment of crab nerves with 100 mM water-soluble carbodiimide in the presence of glycinemethylester (Baker & Rubinson, 1975). Sodium currents are measurable in the presence of high concentrations of TTX and STX after treatment of muscle and nerve fibers with 50 mM TMO (Sigworth & Spalding, 1980; Spalding, 1980). EEDQ treatment, however, did not alter the TTX sensitivity of the nerve fiber. The difference is probably due to the different specificity of the reagents. At present EEDQ is not known to activate groups other than carboxyl groups. Carbodiimides may cause crosslink reactions like EEDQ (*see above*) and react further with phosphate, tyrosine and sulfhydryl groups (Kurzer & Douraghi-Zadeh, 1967; Carraway & Koshland, 1968; Carraway & Triplett, 1970); additionally, a stable adduct of carbodiimides with carboxyl groups by internal rearrangement has been described (Smith, Moffatt & Khorana, 1958). TMO was shown to react with carboxyl groups and to alkylate a large number of other groups when used in organic phase (Curphey, 1971). In aqueous solution TMO is much more selective due to competition with water (Parsons et al., 1969; Reed & Raftery, 1976).

The inactivation time constant is essentially unchanged after TMO treatment (Sigworth & Spalding, 1980). This is in contrast to the effect of EEDQ, regardless of the presence of an external primary amine. However, TMO is simply an alkylating reagent and cannot cause crosslink reactions (*see above*). The voltage dependence of activation of Na current in untreated nerve fibers corresponds within 4 mV to that of fibers after treatment with the reagent TMO (Sigworth & Spalding, 1980). This result is comparable to the small shifts of the descending branch of the $I_{Na}(E)$ curve caused by EEDQ without amine or in the presence of taurine. Neither TMO nor EEDQ cause a significant change in the reversal potential of the sodium current, indicating that they do not significantly alter the selectivity of the sodium channel.

This investigation was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 38 "Membranforschung." We wish to thank Professor H. Meves for his support and his advice. The excellent technical assistance of C. Waschow is gratefully acknowledged.

References

Baker, P.F., Rubinson, K.A. 1975. Chemical modification of crab nerves can make them insensitive to the local anaesthetics tetrodotoxin and saxitoxin. *Nature (London)* **257**:412–414

Baker, P.F., Rubinson, K.A. 1977. TTX-resistant action potentials in crab nerve after treatment with Meerwein's Reagent. *J. Physiol. (London)* **266**:3P

Begenisich, T. 1975. Magnitude and location of surface charges on *Myxicola* giant axons. *J. Gen. Physiol.* **66**:47–65

Belleau, B., Malek, G. 1968. A new convenient reagent for peptide syntheses. *J. Am. Chem. Soc.* **90**:1651–1652

Belleau, B., DiTullio, V., Godin, D. 1969. The mechanism of irreversible adrenergic blockade of N-carbethoxydihydroquinolines-Model studies with typical serine hydrolases. *Biochem. Pharmacol.* **18**:1039–1044

Blaustein, M.P., Goldman, D.E. 1968. The action of certain polyvalent cations on the voltage-clamped lobster axons. *J. Gen. Physiol.* **51**:279–291

Brismar, T. 1973. Effects of ionic concentration on permeability properties of nodal membrane in myelinated nerve fibres of *Xenopus laevis*. Potential clamp experiments. *Acta Physiol. Scand.* **87**:474–484

Carraway, K.L., Koshland, D.E., Jr. 1968. Reaction of tyrosine residues in proteins with carbodiimide reagents. *Biochim. Biophys. Acta* **160**:272–274

Carraway, K.L., Triplett, R.B. 1970. Reaction of carbodiimides with protein sulfhydryl groups. *Biochim. Biophys. Acta* **200**:564–566

Curphey, T.J. 1971. Trimethyloxonium tetrafluoroborate. *Org. Synth.* **51**:142–147

D'Arrigo, J.S. 1975. Axonal surface charges: Evidence for phosphate structure. *J. Membrane Biol.* **22**:255–263

Drouin, H., Neumcke, B. 1974. Specific and unspecific charges at the sodium channels of the nerve membrane. *Pfluegers Arch.* **351**:207–229

Frankenhaeuser, B., Hodgkin, A.L. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (London)* **137**:218–244

Hille, B. 1968. Charges and potentials at the nerve surface. Divalent ions and pH. *J. Gen. Physiol.* **51**:221–236

Hille, B. 1972. The permeability of the sodium channel to metal cations in myelinated nerve. *J. Gen. Physiol.* **59**:637–658

Hille, B. 1975*a*. An essential ionized acid group in sodium channels. *Fed. Proc.* **34**:1318–1321

Hille, B. 1975*b*. The receptor for tetrodotoxin and saxitoxin. *Biophys. J.* **15**:615–619

Hille, B., Woodhull, A.M., Shapiro, B.I. 1975. Negative surface charge near sodium channels of nerve: Divalent ions, monovalent ions, and pH. *Phil. Trans. R. Soc. London B.* **270**:301–318

Hoare, D.G., Olson, A., Koshland, D.E., Jr. 1968. The reaction of hydroxamic acids with water-soluble carbodiimides. A Lossen rearrangement. *J. Am. Chem. Soc.* **90**:1638–1643

Kurzer, F., Douraghi-Zadeh, K. 1967. Advances in the chemistry of carbodiimides. *Chem. Rev.* **67**:107–152

Means, G.E., Feeney, R.E. 1971. Chemical Modification of Proteins. Holden-Day, San Francisco

Nonner, W. 1969. A new voltage clamp method for Ranvier nodes. *Pfluegers Arch.* **309**:176–192

Parsons, S.M., Jao, L., Dahlquist, F.W., Borders, C.L., Jr., Groff, T., Racs, J., Raftery, M.A. 1969. The nature of amino acid side chains which are critical for the activity of lysozyme. *Biochemistry* **8**:700–712

Rack, M. 1983. Membrane-bound carboxyl groups have an essential influence on the voltage-sensitivity of the sodium channel of nerve. *Hoppe-Seyler's Z. Physiol. Chem.* **364**:1197–1198

Rack, M., Woll, K.-H. 1984. Carboxyl group modification in myelinated nerve fibres affects sodium currents. *Pfluegers Arch.* **400**:R38

Reed, J.K., Raftery, M.A. 1976. Properties of the tetrodotoxin binding component in plasma membranes isolated from *Electrophorus electricus*. *Biochemistry* **15**:944–953

Shrager, P., Profera, C. 1973. Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents modifying carboxyl groups. *Biochim. Biophys. Acta* **318**:141–146

Sigworth, F.J., Spalding, B.C. 1980. Chemical modification reduces the conductance of sodium channels in nerve. *Nature (London)* **283**:293–295

Smith, M., Moffatt, J.G., Khorana, H.G. 1958. Carbodiimides. VIII. Observations on the reactions of carbodiimides with acids and some new applications in the synthesis of phosphoric acid esters. *J. Am. Chem. Soc.* **80**:6204–6212

Spalding, B.C. 1980. Properties of toxin-resistant sodium channels produced by chemical modification in frog skeletal muscle. *J. Physiol. (London)* **305**:485–500

Stämpfli, R. 1969. Dissection of single nerve fibres and measurement of membrane potential changes of Ranvier nodes by means of the double air gap method. *In:* Laboratory Techniques in Membrane Biophysics. H. Passow and R. Stämpfli, editors. pp. 157–166. Springer Berlin-Heidelberg-New York

Stämpfli, R., Hille, B. 1976. Electrophysiology of the peripheral myelinated nerve. *In:* Frog Neurobiology. R. Llinas and W. Precht, editors. pp. 3–32. Springer, Berlin-Heidelberg

Vogel, W. 1974. Calcium and lanthanum effects at the nodal membrane. *Pfluegers Arch.* **350**:25–39

Received 25 January 1984; revised 7 June 1984