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Effects of Reagents Modifying Carboxyl Groups on the Gating Current of the Myelinated Nerve Fiber

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Summary. The effect of the carboxyl group activating reagent N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) on the gating current of the frog node of Ranvier was investigated. A 10-min treatment with 2 mM EEDQ (in the presence or absence of 10 mM ethylenediamine) irreversibly reduced the slope of the on charge-voltage relation $Q_{on}(E)$, shifted its midpoint potential E_{mid} in the positive direction and reduced the maximum charge $Q_{on\,max}$ measured with strong depolarizing pulses. In six experiments, 2 mM EEDQ + 10 mM ethylenediamine increased the factor k (a reciprocal measure of the slope of the $Q_{on}(E)$ curve) from 16 to 22 mV. In five experiments, 2 mM EEDQ alone increased k from 16 to 23 mV. In a single experiment, 5 mM EEDQ + 10 mM ethylene diamine increased k from 17 to 31 mV. The reduction in slope suggests that EEDQ decreases the valence of the gating particles or reduces the fraction of the membrane field that they traverse. In addition, EEDQ (which inhibits inactivation of the sodium current, *see* M. Rack and K.H. Woll, *J. Membrane Biol.* **82**:41–48, 1984) caused a small increase of the off charge Q_{off}, and a marked increase of the Q_{off}/Q_{on} ratio, i.e. inhibited charge immobilization. Since the effects of EEDQ occurred regardless of the presence or absence of ethylenediamine, they are probably due to crosslinking reactions. The effects of EEDQ were compared with those of the water-soluble carbodiimide EDC. Treatment with 10 or 50 mM EDC (plus 10 or 50 mM ethylenediamine) caused a smaller increase of k than treatment with 2 mM EEDQ but reduced $Q_{on\,max}$ by the same amount.

Key Words voltage clamp · node of Ranvier · gating current · chemical modification · carboxyl groups

Introduction

Reagents which react with carboxyl groups have attracted the attention of electrophysiologists because they strikingly modify the properties of ionic channels. Mostly, trimethyloxonium (TMO) and carbodiimide have been used. Their effect on the sodium channels is threefold: a) the channels become less sensitive or insensitive to tetrodotoxin (TTX) or saxitoxin (STX), b) the current is reduced, c) its inactivation is slowed. The first publication was by Shrager and Profera (1973). The subsequent publications are quoted in a recent paper by Worley, French and Krueger (1986) which describes the effect of TMO on the single-channel level.

Rack and Woll (1984) were the first to use N-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) for chemical modification of nerve fibers. The reagent converts carboxyl groups into highly reactive esters which react readily with nucleophiles such as externally added ethylenediamine or membrane-bound lysine. EEDQ reduces the sodium current; its inactivation becomes slow and incomplete; depending on the nucleophiles added, a shift of the voltage dependence of activation and inactivation occurs; surprisingly, no change in TTX-sensitivity is seen.

We have studied the effect of EEDQ (with or without ethylenediamine) on the intramembrane charge movement ("gating currents") which is responsible for the opening and closing of the sodium channels. This seemed interesting because Mozhayeva, Naumov and Nosyreva (1984*a*,*b*, 1986) from their work with water-soluble carbodiimide concluded that part of the gating charges are carboxyl groups which, at the normal resting potential, are located at the external surface of the channel molecule. Unlike water-soluble carbodiimide, EEDQ is soluble in organic solvents and can probably permeate into the membrane. With the averaging technique used for measuring gating currents we have investigated the question whether EEDQ has really no effect at all on the TTX sensitivity.

Part of the results have been published in abstract form (Meves & Rubly, 1987).

Materials and Methods

The experiments were done on single motor or sensory nerve fibers from the tibial or peroneal nerve of the frog *Rana esculenta*. A node of Ranvier was voltage clamped at 10°C by the

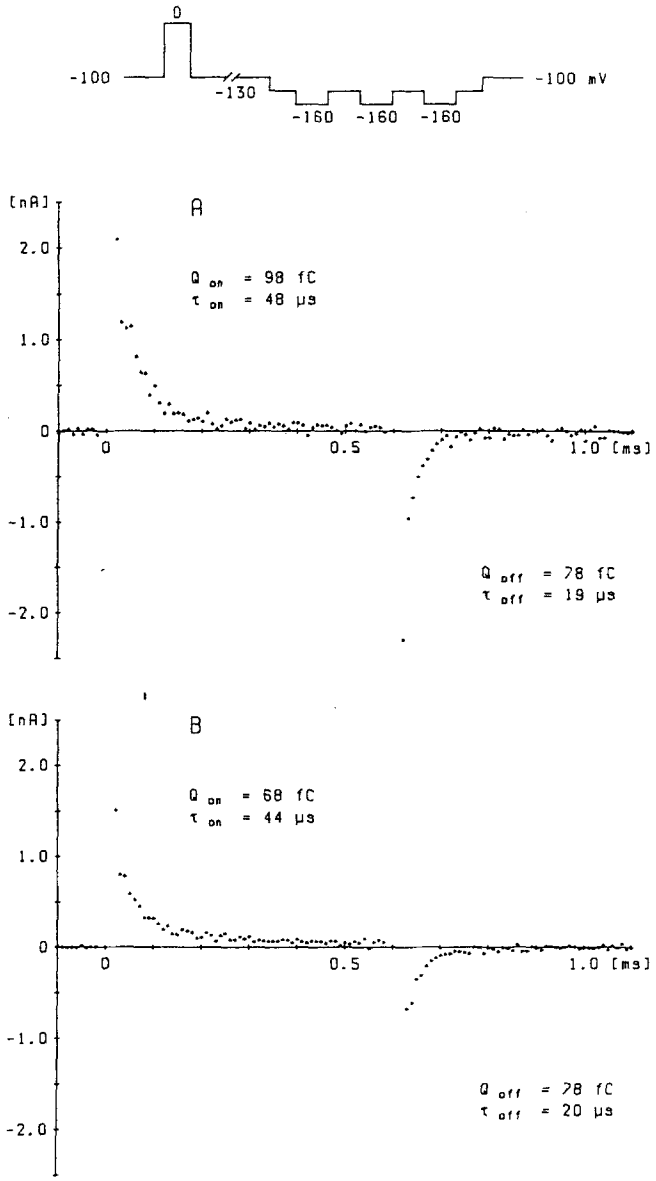

Fig. 1. Examples of gating current recorded before (*A*) and after (*B*) treatment with 2 mM EEDQ + 10 mM ethylenediamine at pH 5.5. Pulse program on top shows depolarizing test pulse from −100 mV (holding potential) to 0 mV followed by pulse to −130 mV (reference potential) and three hyperpolarizing pulses from −130 to −160 mV. (*A*) 24 times averaged; (*B*) 64 times averaged. The charges Q_{on}, Q_{off} and the time constants τ_{on}, τ_{off} are indicated

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 bathed in 113 mM CsCl + 7 mM NaCl to block K channels from the inside. The potential at which the Na current was 70% of the maximum Na current (measured with a prepulse of −40 mV and 50-msec duration) was taken as the normal resting potential (E = −70 mV). The holding potential was adjusted to E = −100 mV, unless otherwise mentioned. Membrane currents were filtered at 25 kHz and sampled on-line at 10-µsec intervals by a 12-bit A/D converter (Hof, 1986). An analog circuit was used to compensate most of the linear component of the capacitative and leakage current. To obtain absolute values of membrane current, the recording resistance was assumed to be 10 MΩ; measurements of the recording resistance gave values close to 10 mΩ (*see* Meves, Rubly & Watt, 1987).

The pulse program for measuring gating currents is shown in Fig. 1. It consisted of a depolarizing test pulse of variable amplitude P and three hyperpolarizing pulses of fixed size (−30 mV), superimposed on a reference potential of −130 mV. The −30 mV pulses helped to compensate residual capacitative and leakage current. The current during the −30 mV pulses was suitably scaled, multiplied by −1 and subtracted from the current during the test pulse. In the experiment of Fig. 6, the three −30-mV pulses were replaced by two pulses of size $-P/2$, also superimposed on a reference potential of −130 mV. Either 24 or 64 records were averaged with a repetition rate of 1 Hz.

Gating currents were corrected for the effect of the low-pass filter (25 kHz) and for the additional delay caused by the programmable amplifier. For this purpose, the time axis of the data points was shifted 15 µsec with respect to that of the pulses (*see* Nonner, Rojas & Stämpfli, 1978). Examples of the gating current are shown in Fig. 1. Integration of the on- and off-response of the gating current gave records like those in Fig. 5. Before integrating the on-response the small time-independent current flowing during the pulse was measured (by taking the average of the last 20 current points) and subtracted. The gating currents themselves or the integrated on- and off-responses were fitted with single- or double-exponential functions using the equation

$$y(t) = A \exp(-t/\tau_1) + B \exp(-t/\tau_2) + C. \quad (1)$$

The relation between steady-state values of charge movement during the on-response Q_{on}, and test pulse potential E was fitted by the equation

$$Q_{on}(E) = Q_{on\,max}/[1 + \exp((E_{mid} - E)/k)] \quad (2)$$

where E_{mid} is the potential at which $Q_{on} = 0.5\ Q_{on\,max}$ and k is the number of mV required to change Q_{on} e-fold. For the relation between the time constant τ_{on} and E the equation

$$\tau_{on}(E) = 2\ \tau^*_{on}/[\exp(\eta(E - E_{mid})/k) + \exp((1 - \eta)(E_{mid} - E)/k)] \quad (3)$$

was used where τ^*_{on} is τ_{on} at E_{mid} and η determines the symmetry of the curve (*see* Dubois & Schneider, 1982). The time constant τ_{on} reaches a maximum at a potential

$$E^* = E_{mid} - k \ln(\eta/(1 - \eta)). \quad (4)$$

Solutions

For the measurement of Na currents, the node was superfused with Ringer's solution containing (mM): 110 NaCl, 2.5 KCl, 1.8 $CaCl_2$ and 4 morpholinopropane sulfonic acid (MOPS). The pH was adjusted to 7.2 with 1 N NaOH. The Ringer's solution also contained 12 mM tetraethylammonium chloride (TEA) to block K channels from the outside. For the measurement of gating currents, Na-free Ringer's solution (105 mM tetramethylammonium chloride (TMA), 1.8 mM $CaCl_2$, 10 mM MOPS, pH 7.2) + 12 mM TEA + 300 nM TTX was used. EEDQ was applied in RbCl Ringer's rather than in NaCl Ringer's in order to avoid an influx of Na^+ ions during the EEDQ treatment. First, EEDQ was

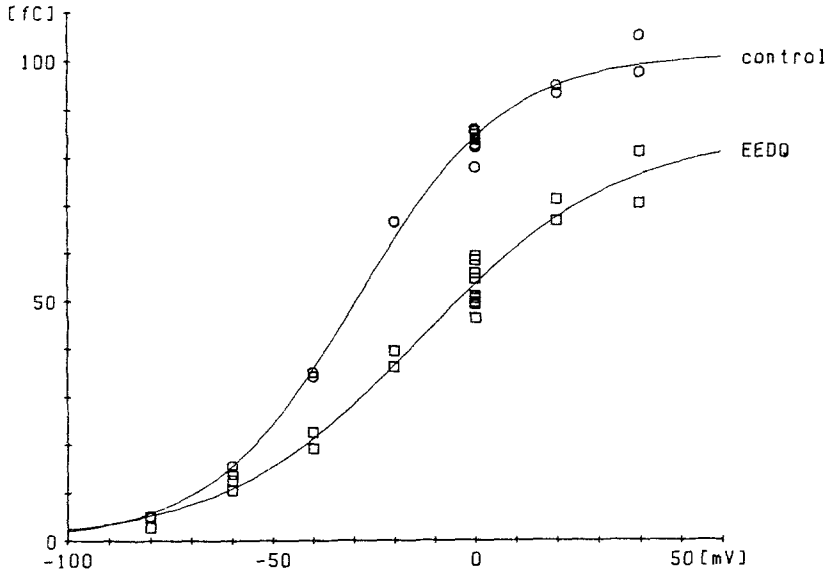

Fig. 2. Q_{on} (E) curve before (○) and after (□) treatment with 2 mM EEDQ and 10 mM ethylenediamine at pH 5.5. The points were fitted by Eq. (2) with the parameters

	$Q_{on\,max}$	E_{mid}	k
control	101 fC	−29 mV	18 mV
after treatment	85	−13	25

dissolved in dimethylformamide (DMF). An appropriate amount of this solution, was slowly added to stirred RbCl-Ringer's [110 mM RbCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 12 mM TEA and 4 mM morpholinoethane sulfonic acid (MES)]. In some experiments, ethylenediamine was added. Finally, the pH was adjusted to 5.5 with 1 N TMA-OH. The final concentration of DMF was 2%. The treatment always lasted 10 min. Afterwards the node was washed with reagent-free solution for several minutes before measurements were made. For comparison, experiments with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) and ethylenediamine were done. The two substances were dissolved in RbCl-Ringer's buffered with 4 mM 2,6-dimethylpyridine-3-sulfonic acid (instead of 4 mM MES) and the pH was adjusted to 4.75. The Ringer's solutions with EEDQ or EDC were freshly prepared for each experiment since both substances are hydrolyzed by water.

Results

Effect of EEDQ on the $Q_{on}(E)$ curve

Figure 1 compares gating currents at a pulse potential of 0 mV before (A) and after (B) treatment with 2 mM EEDQ and 10 mM ethylenediamine. The treatment lasted for 10 min; afterwards the node was washed with reagent-free solution for 33 min. The treatment reduced the size of the on-response Q_{on} to 69% of the control value but had no effect on the off-response Q_{off} and on the time constants τ_{on} and τ_{off}.

Figure 2 shows a complete $Q_{on}(E)$ curve measured on another fiber before and after treatment with 2 mM EEDQ and 10 mM ethylenediamine. The treatment reduced Q_{on} at 0 mV from 83 to 53 fC (averages from 8 or 10 measurements), i.e., to 64%. It markedly decreased the slope of the curve (increasing k from 18 to 25 mV) and shifted the midpoint potential E_{mid} by 16 mV. In addition, $Q_{on\,max}$ became somewhat smaller. The Table gives average values from six experiments. On average, k was 16.0 mV before and 21.8 mV after treatment. The corresponding values for E_{mid} were −28.6 and −14.8 mV. The maximum charge $Q_{on\,max}$ decreased on average to 85.1% of the control value.

The effect of 2 mM EEDQ on the $Q_{on}(E)$ curve was independent of the presence or absence of ethylenediamine. The Table gives average values from five experiments with 2 mM EEDQ without ethylenediamine. The slope factor k was 16.2 mV before and 23.0 mV after treatment, very similar to the values obtained before and after 2 mM EEDQ + 10 mM ethylenediamine. The values for E_{mid} and for the percentage reduction of $Q_{on\,max}$ were also not significantly different from the values measured in the experiments with 2 mM EEDQ + 10 mM ethylenediamine (t-test, $P > 0.05$).

One out of three fibers survived the treatment with 5 mM EEDQ and 10 mM ethylenediamine. The $Q_{on}(E)$ curves measured in this experiment are shown in Fig. 3. The effect was similar to that of 2 mM EEDQ but stronger: the slope was drastically reduced (k increased from 17 to 31 mV) and E_{mid} shifted by 30 mV. None of three fibers survived the treatment with 10 mM EEDQ and 10 mM ethylenediamine.

Effect of EEDQ on the $\tau_{on}(E)$ curve

In the experiment of Fig. 1, the effect of EEDQ on the time constant τ_{on} measured at a pulse potential of 0 mV was negligible. However, when τ_{on} was

Table. Effect of EEDQ and EDC on the Q_{on} (E) and τ_{on} (E) curve[a]

	2 mM EEDQ + 10 mM ethylenediamine		2 mM EEDQ		10 mM EDC + 10 mM ethylenediamine	
	before treatment	after treatment	before treatment	after treatment	before treatment	after treatment
	$n = 6$	$n = 6$	$n = 5$	$n = 5$	$n = 4$	$n = 4$
$Q_{on\,max}$ [%]	100	85.1 ± 10.7 [73.4]	100	61.9 ± 7.4 [70.4]	100	71.1 ± 7.1 [89.3]
E_{mid} [mV]	−28.6 ± 1.9	−14.8 ± 2.9	−33.1 ± 1.5	−16.6 ± 3.9	−25.6 ± 1.5	−19.5 ± 1.0
k [mV]	16.0 ± 1.0	21.8 ± 1.4	16.2 ± 0.6	23.0 ± 1.0	18.4 ± 1.1	20.6 ± 1.0
	$n = 3$	$n = 3$	$n = 3$	$n = 3$		
E^* [mV]	−22.3 ± 0.8	−6.6 ± 3.8	−20.9 ± 1.2	−8.5 ± 0.1		
η	0.37 ± 0.03	0.41 ± 0.07	0.38 ± 0.00	0.43 ± 0.03		
k [mV]	17.6 ± 0.9	24.0 ± 0.8	20.3 ± 0.3	30.4 ± 1.8		

[a] Averages ± SEM from n experiments. The $Q_{on\,max}$ value expected from the increase of k (*see* page 68) is given in brackets.

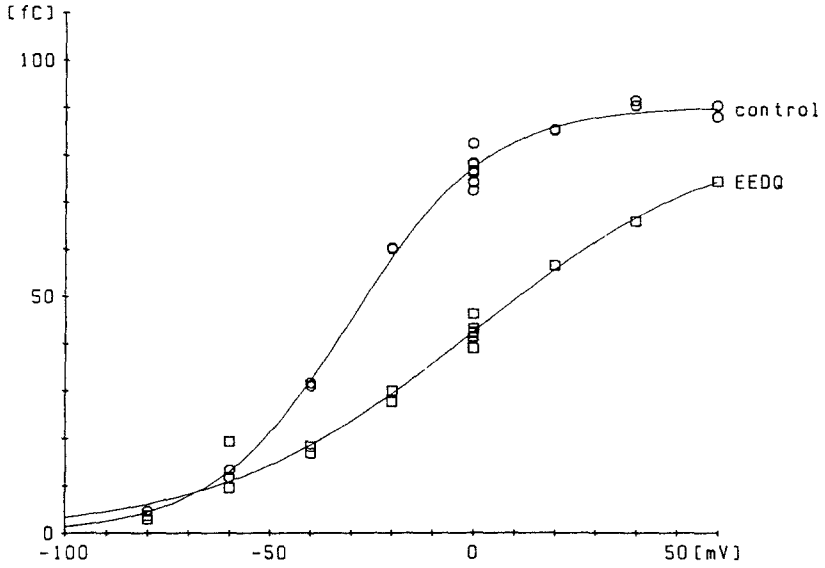

Fig. 3. Q_{on} (E) curve before (○) and after (□) treatment with 5 mM EEDQ and 10 mM ethylenediamine at pH 5.5. The points were fitted by Eq. (2) with the parameters

	$Q_{on\,max}$	E_{mid}	k
control	90 fC	−30 mV	17 mV
after treatment	85	±0	31

measured over the entire potential range from −80 to 40 mV, a clear effect of EEDQ on the $\tau_{on}(E)$ curve was observed. As Fig. 4 shows, the curve was much flatter after EEDQ treatment than before, corresponding to an increase of k from 16 to 31 mV. Also, E^*, the potential at which τ_{on} reaches a maximum, was shifted from −23.5 to −3.7 mV.

Figure 4 is from the fiber which survived the treatment with 5 mM EEDQ and 10 mM ethylenediamine. The effect of 2 mM EEDQ and 10 mM ethylenediaamine was similar but weaker (*see* averages in the Table). Again, 2 mM EEDQ alone had the same effect as 2 mM EEDQ + 10 mM ethylenediamine (*also see* Table).

Effect of EEDQ on Charge Immobilization

EEDQ reduces Q_{on} but not Q_{off} (Fig. 1). This observation is confirmed by the experiment of Fig. 5 which shows integrated on- and off-responses (measured with 0.6-msec pulses to 20 mV) before and after treatment with 2 mM EEDQ and 10 mM ethylenediamine. It is clear that the treatment reduces Q_{on} but not Q_{off}; the latter even increases slightly. The ratio Q_{off}/Q_{on} is 51/96 = 0.54 before and 62/72 = 0.86 after treatment. Average values ± SEM were 0.61 ± 0.02 before treatment ($n = 4$) and 0.79 ± 0.03 after treatment ($n = 5$). Four experiments with 2 mM EEDQ alone gave similar results, namely 0.51 ± 0.05 before treatment and 0.68 ± 0.09 after treatment.

The increase of the Q_{off}/Q_{on} ratio suggests inhibition of charge immobilization. This phenomenon was studied by measuring Q_{off} as a function of pulse duration (Fig. 6). Before treatment, Q_{off} decreased exponentially with increasing pulse duration and reached a constant value for pulse durations longer than 3 msec; this confirms previous observations on

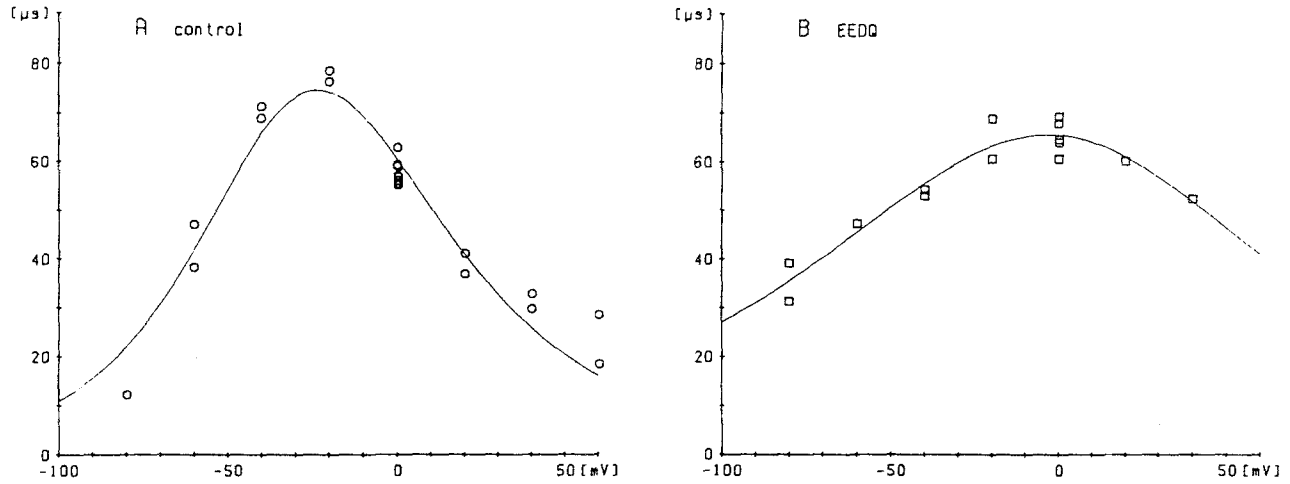

Fig. 4. τ_{on} (E) curve before (○) and after (□) treatment with 5 mM EEDQ and 10 mM ethylenediamine at pH 5.5. Same experiment as Fig. 3. The points were fitted by Eq. (3) with E_{mid} as determined in Fig. 3 and the parameters

	τ^*_{on}	η	k
control	73 μsec	0.40	16 mV
after treatment	66	0.53	31

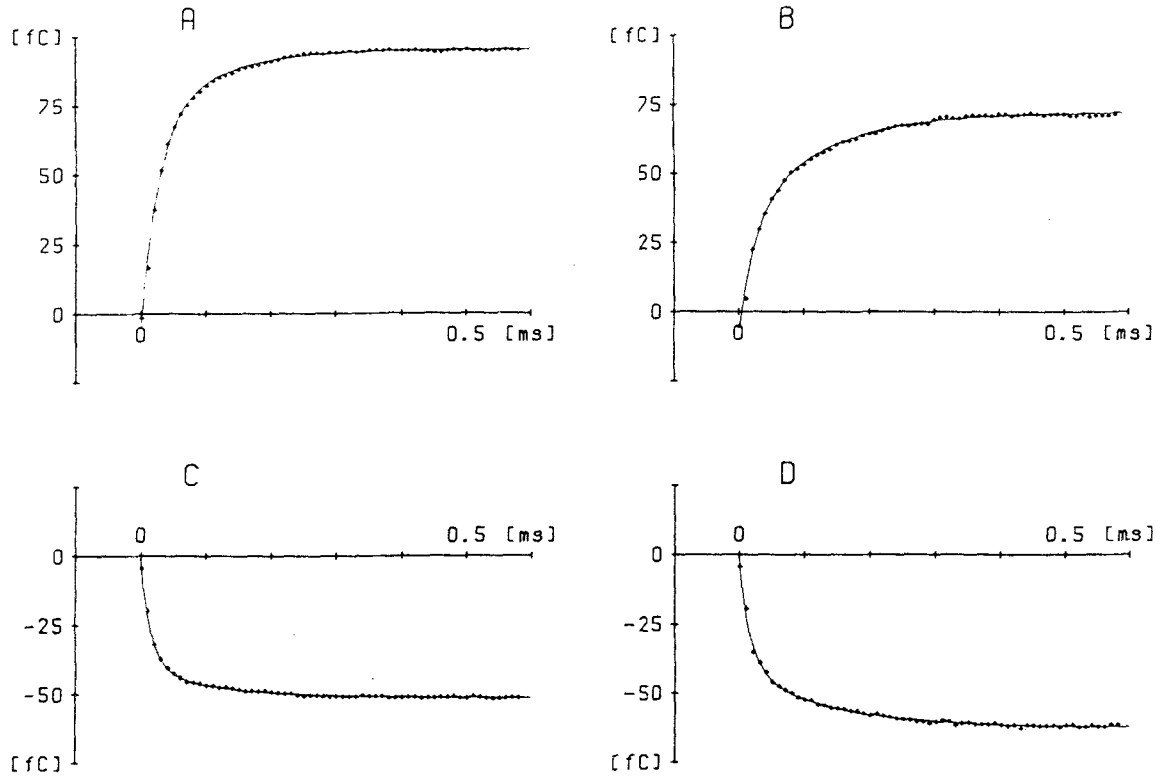

Fig. 5. Integrated on- and off-responses before (A, C) and after (B, D) treatment with 2 mM EEDQ and 10 mM ethylenediamine at pH 5.5. Pulse potential 20 mV, pulse duration 0.6 msec. The responses were fitted by Eq. (1) with the following parameters:

		A	B	C	τ_1	τ_2
on-response	control	−71 fC	−31 fC	96 fC	27 μsec	98 μsec
	after treatment	−38	−43	72	23	114
off-response	control	41	11	−51	17	120
	after treatment	40	22	−62	16	124

The Q_{off}/Q_{on} ratio was 0.54 before and 0.86 after treatment

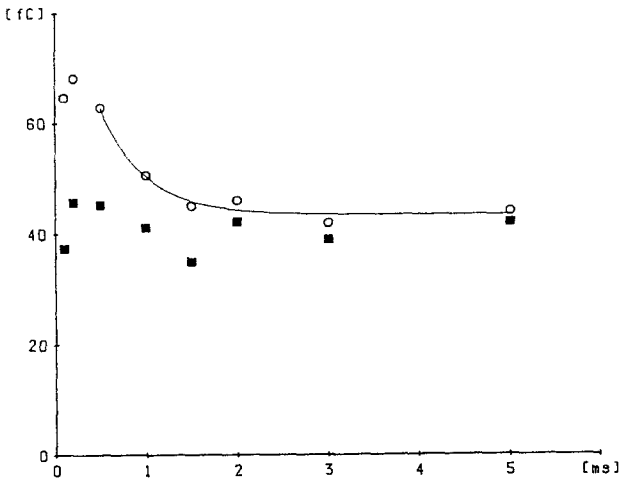

Fig. 6. Off-response Q_{off} plotted against pulse duration for the same fiber before (○) and after (■) treatment with 2 mM EEDQ and 10 mM ethylenediamine at pH 5.5. Pulse potential 0 mV. Points ○ for pulse durations > 0.5 msec fitted by an exponential function with τ = 0.50 msec.

the node of Ranvier by Nonner (1980), Dubois and Schneider (1982), Neumcke, Schwarz and Stämpfli (1985) and Drews (1987). After treatment, Q_{off} was almost independent of pulse duration. Averaging the results of several experiments (before treatment n = 5, after treatment n = 3) and taking Q_{off} measured with 0.2-msec pulses as 100%, Q_{off} at 3-msec pulse duration was 58.5 ± 5.5% before and 87.4 ± 1.7% after treatment (average ± SEM).

Before treatment, the decrease of Q_{off} with increasing pulse duration (Fig. 6) was accompanied by a decrease of the time constant τ_{off}. This supports the idea that Q_{off} and τ_{off} are correlated (Dubois & Schneider, 1982, 1985; Drews, 1987). After treatment, τ_{off} was almost independent of pulse duration as was Q_{off}.

Experiments with EDC

The effect of EDC on the $Q_{on}(E)$ curve was weaker than that of EEDQ. As shown in Fig. 7, treatment with 10 mM EDC and 10 mM ethylenediamine caused only a slight decrease of the slope (increasing k from 20 to 24 mV) and a small positive shift of E_{mid}. However, it reduced $Q_{on\ max}$ considerably. The Table gives average values from four experiments. The percentage reduction of $Q_{on\ max}$ by 10 mM EDC + 10 mM ethylenediamine was not significantly different from that by 2 mM EEDQ + 10 mM ethylenediamine or 2 mM EEDQ alone (t-test, $P > 0.05$).

Treatment with 10 mM EDC and 10 mM ethylenediamine had only a weak effect on the $\tau_{on}(E)$ curve. The time constant τ_{on} increased slightly at all pulse potentials; at 0 and 20 mV the increase was by a factor of 1.14 ± 0.05 and 1.21 ± 0.07, respectively (averages ± SEM, n = 4).

Treatment with 10 mM EDC and 10 mM ethylenediamine increased the Q_{off}/Q_{on} ratio (measured with 0.6-msec pulses to 20 mV) from 0.60 ± 0.05 to 0.86 ± 0.08 (averages ± SEM from four experiments), i.e. caused inhibition of charge immobilization similar to that caused by 2 mM EEDQ. After treatment with 10 mM EDC and 10 mM ethylenediamine Q_{off} was almost independent of pulse duration. In two experiments, Q_{off} at 3-msec pulse duration was 90.6 and 90.3% of Q_{off} at 0.2-msec pulse duration. Thus, treatment with 10 mM EDC and 10 mM ethylenediamine inhibited charge immobilization as effectively as treatment with 2 mM EEDQ and 10 mM ethylenediamine.

In four experiments, the fibers were treated with 50 mM EDC and 50 mM ethylenediamine, concentrations still only half as high as those used by Mozhayeva et al. (1984*a*,*b*, 1986). Only one fiber survived the treatment. The effect of the $Q_{on}(E)$ curve was similar to that in Fig. 7 but the shift of E_{mid} was stronger (15 mV instead of 3 mV). The effect on the slope remained negligible (k = 20 mV before and k = 19 mV after treatment). The time constant τ_{on} at 0 and 20 mV increased by a factor of 1.57 and 1.30, respectively, i.e. more than after treatment with the lower concentration.

Expected and Observed Decrease of $Q_{on\ max}$

The maximum charge $Q_{on\ max}$ and the slope factor k are defined by the equations

$$Q_{on\ max} = Nz'$$
$$k = RT/z'$$

where N is the number of gating charges, z' their effective valence and R and T have their usual meaning. Consequently, an increase of k by the factor x must lead to a decrease of $Q_{on\ max}$ by the factor $1/x$, provided N does not change. In the Table, the $Q_{on\ max}$ values expected from the increase of k are given in brackets. In two cases the observed $Q_{on\ max}$ is smaller than the expected $Q_{on\ max}$, suggesting a decrease of N. In one case the observed $Q_{on\ max}$ is larger than the expected $Q_{on\ max}$, but the difference is small. In the single experiment with 5 mM EEDQ + 10 mM ethylenediamine (Fig. 3), the observed $Q_{on\ max}$ (85 fC) is much larger than the expected $Q_{on\ max}$ (90 × 17/31 = 49 fC). If this difference is real, it could reflect an increase of N due to recruitment of mobile charges (*cf.* Neumcke, Schwarz & Stämpfli, 1980) or dissociation of aggregates of gat-

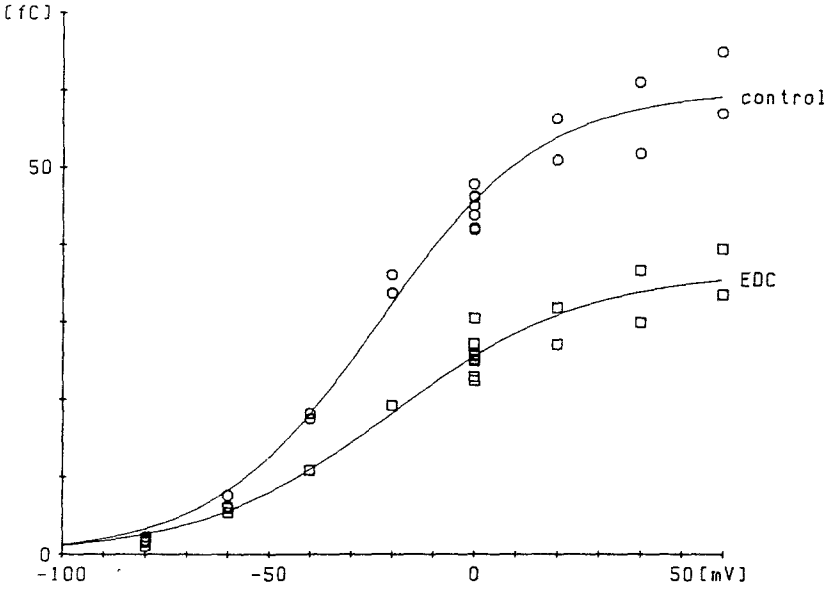

Fig. 7. Q_{on} (E) curve before (○) and after (□) treatment with 10 mM EDC and 10 mM ethylenediamine at pH 4.75. The points were fitted by Eq. (2) with the parameters

	$Q_{on\,max}$	E_{mid}	k
control	60 fC	−23 mV	20 mV
after treatment	37	−20	24

ing particles (*cf.* Dubois, Schneider & Khodorov, 1983).

Effects on Na Currents and TTX Sensitivity

We confirmed the observations of previous authors about the effects of EEDQ and EDC on the Na currents. In agreement with Rack and Woll (1984), treatment with 2 mM EEDQ and 10 mM ethylenediamine reduced peak I_{Na} to 35.0 ± 1.5% of the control value (average ± SEM, n = 3); 2 mM EEDQ without ethylenediamine decreased peak I_{Na} to 30.4 ± 6.3% (n = 3). Treatment with 10 mM EDC and 10 mM ethylenediamine or with 10 mM EDC alone reduced peak I_{Na} to 70.2 ± 2.7% (n = 3) or 63.1 ± 4.2% (n = 3), respectively, confirming previous work by Mozhayeva et al. (1984*a*,*b*, 1986). With either treatment the decaying phase of the Na currents was markedly slowed. A shift of E_{half}, the half potential of the descending branch of the $I_{Na}(E)$ curve, occurred more regularly when EEDQ or EDC were given together with 10 mM ethylenediamine and less regularly when they were applied alone.

In untreated fibers, the Na inward current in the presence of 300 nM or 30 μM TTX was 0.33 ± 0.03 nA and 0 nA, respectively (n = 3, pulse potential 0 mV). This is to be expected if K_D = 3.6 nM (Schwarz, Ulbricht & Wagner, 1973) and the normal I_{Na} = 30 nA. After treatment with 2 mM EEDQ without ethylenediamine, the Na inward current in 300 nM TTX was reduced to 0.15 ± 0.03 nA (n = 2). In 30 μM TTX a small Na inward current was seen after the treatment ($I_{Na\,peak}$ = 0.054 ± 0.007 nA, n = 5). This is illustrated by Fig. 8. Before the treatment only gating current is visible, after the treatment a small, slowly inactivating Na inward current ($I_{Na\,peak}$ = −0.06 nA) appears in addition to the (reduced) gating current. A Na inward current of similar size (−0.04 nA) recorded in 35 μM TTX after treatment with 50 mM EDC and 50 mM hydroxylamine is shown in Fig. 6*a* of Mozhayeva et al. (1984*b*).

Discussion

The major effect of EEDQ is a strong irreversible reduction of the slope of the $Q_{on}(E)$ curve. This is reflected in a marked increase of the slope factor k; concomitantly, the half potential E_{mid} is shifted in the positive direction.

The slope factor k equals RT/z' with $z' = \alpha z$ where z is the valence of the gating particles and α is the fraction of the membrane field that the particles traverse. Thus, the increase of k could be either due to a decrease of α or to a decrease of z.

The mechanism of action of EEDQ has been described in detail by Belleau et al. (1969) (*see also* Rack & Woll, 1984). The reagent reacts with carboxyl groups to form highly activated esters. In a second step, the esters react either with amines (e.g. ethylenediamine) to form stable amide bonds or with membrane-bound nucleophiles (e.g. lysine residues or SH groups). The latter reactions are crosslinking reactions. Since EEDQ increases k both in the presence and in the absence of ethylenediamine, the effect is probably due to crosslinking reactions. It seems plausible that crosslinking reac-

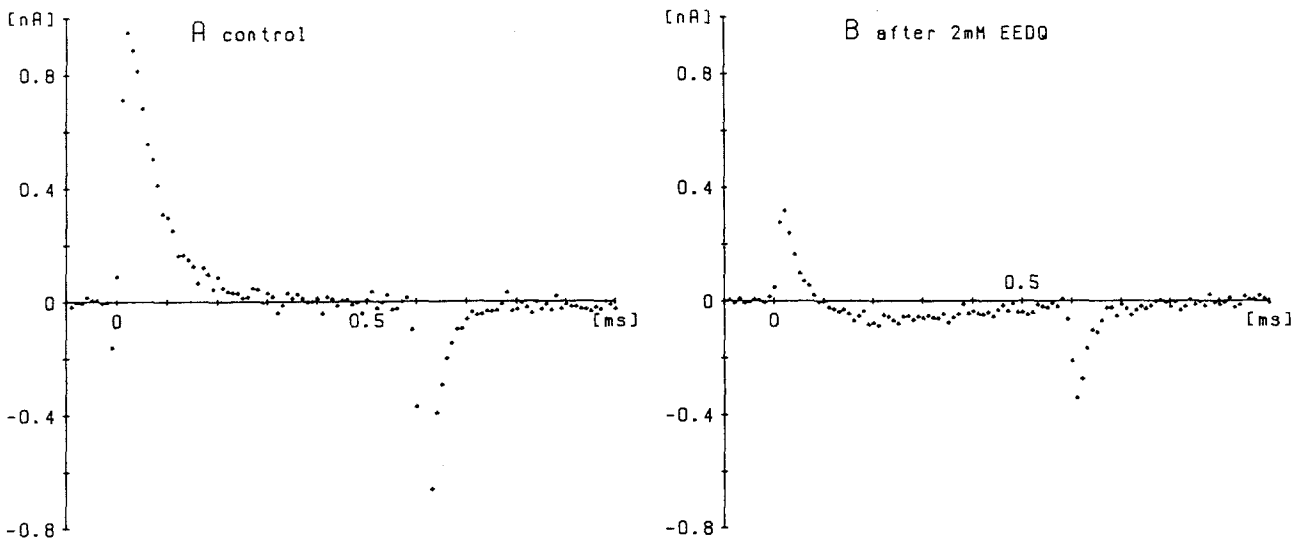

Fig. 8. Current associated with pulses to 0 mV in the presence of 30 μM TTX and 110 mM Na before (*A*) and after (*B*) treatment with 2 mM EEDQ without ethylenediamine at pH 5.5. Holding potential and reference potential −90 mV, 64 times averaged

tions hinder the movement of the gating particles, i.e. decrease α. Specifically, it could be assumed that EEDQ causes crosslinking between carboxyl groups and lysine residues. The work of Noda et al. (1984) suggests lysine and arginine residues as positive gating charges, acting possibly in conjunction with negative charges. However, our data do not exclude the alternative explanation that EEDQ reduces z. The latter explanation would imply that the negative gating charges are carboxyl groups, a view expressed by Mozhayeva et al. (1984*a*,*b*, 1986).

It is interesting to compare the effect of EEDQ with that of other carboxyl group reagents and other crosslinking reagents. The water-soluble carbodiimide EDC in a concentration of 10 or 50 mM caused a smaller increase of k than 2 or 5 mM EEDQ. Also, the shift of E_{mid} by 50 mM EDC was only half as large as the shift by 5 mM EEDQ. The stronger effect of EEDQ could be explained by its lipid solubility; it might act on regions of the sodium channel marcromolecule to which EDC has no access. In two other respects the two substances act very similar: they inhibit charge immobilization [as they inhibit inactivation of the sodium current, *see* Rack and Woll (1984) and Mozhayeva et al. (1984*a*,*b*)] and they reduce the TTX-sensitivity so that a very small Na inward current persists in the presence of 30 μM TTX. TMO, another substance reacting with carboxyl groups, also inhibits sodium inactivation (*see* Gülden & Vogel, 1985) and also reduces the TTX-sensitivity. The Na inward current of TMO-treated nodes in the presence of 1 μM TTX amounts to 37% of that in toxin-free solution (Gülden & Vogel, 1985), suggesting that TMO reduces the TTX-sensitivity more effectively than EEDQ or EDC.

From other crosslinking reagents, glutaraldehyde has been most frequently studied (*see* review by Brodwick & Eaton, 1982). In the squid axon, 30 mM glutaraldehyde applied internally substantially reduce the size of the gating current (Meves, 1974). In the node of Ranvier, 10 mM glutaraldehyde considerably decrease the sodium current and slow its inactivation (Schmidtmayer, 1985). It would be interesting to see whether the effect of glutaraldehyde on the $Q_{on}(E)$ curve of the node of Ranvier, in particular on the slope of the curve, is similar to that of EEDQ.

The slope of the $Q_{on}(E)$ curve is also strongly diminished by a depolarizing prepulse (Nonner, 1980; Khodorov, 1981) or by the local anaesthetics QX-314 (Cahalan & Almers, 1979) or QX-572 (Khodorov, 1981). By contrast, benzocaine and oenanthotoxin which also reduce the gating current do not appreciably change the slope of the $Q_{on}(E)$ curve or its midpoint potential E_{mid} (Khodorov, 1981; Neumcke, Schwarz & Stämpfli, 1981). Particularly striking is the close resemblance between our Fig. 5 and Fig. 12 of Cahalan and Almers (1979) which show that Q_{on}, but not Q_{off}, is markedly reduced by EEDQ and QX-314, respectively. To explain the preferential block of the on gating current by QX-314, Cahalan and Almers (1979) assume that "the drug attacks the immobilizable, but not the immobilization-resistant component of charge movements." In other words, the charge movement remaining after voltage-dependent block by QX-314 is

thought to be immobilization-resistant. The situation after EEDQ treatment is similar but somewhat different: the remaining charge is "slowly immobilizable" rather than "immobilization-resistant." Immobilization is so much slowed that it cannot be detected with 0.6-msec pulses (Fig. 5). A slight reduction (namely to 87.4%, *see* page 68) is seen with 3-msec pulses. It is difficult to use much longer pulses in gating current experiments. However, 40-msec conditioning pulses were used by Rack and Woll (1984) for studying inactivation of the sodium current, a process closely related to charge immobilization. Their Figs. 4 and 5 clearly demonstrate inactivation in EEDQ-treated nodes.

A further resemblance between our results with EEDQ and those of Cahalan and Almers (1979) with QX-314 is the positive shift of the midpoint potential E_{mid}. The 16.5-mV shift by 2 mM EEDQ (*see* Table) is not due to changes of the surface potential at the inner or outer side of the membrane, because Rack and Woll (1984) showed that the shift of the activation curve (indicative of a change of the surface potential) by 2 mM EEDQ alone is only 5 mV. More likely, the various components of the charge movement—for instance the components resistant to charge immobilization (*see* Fig. 7 of Khodorov, 1981) or to local anaesthetic (*see* Fig. 16 of Khodorov, 1981) or to EEDQ (present paper)—have a midpoint potential different from the E_{mid} of the total charge movement, i.e. *see* different fractions of the electrical field across the membrane.

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