MODIFICATION OF SODIUM AND GATING CURRENTS BY AMINO GROUP SPECIFIC CROSS-LINKING AND MONOFUNCTIONAL REAGENTS

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ABSTRACT To test the possible role of lysine residues in Na channel function the effects of several imidoesters on Na and gating currents were studied in voltage-clamped single frog nerve fibers. Mono- and bisimidoesters were used. These reagents modify amino groups exclusively and do not change the net charge. The three bisimidoesters used easily introduce cross-links between neighboring amino groups. Their structure is almost identical; only the length of the spacers between the two amino-reactive groups is different. An irreversible reduction of Na currents and gating currents was observed with the longest (dimethyl suberimidate [DMS]) and the shortest (dimethyl adipimidate [DMA]) of the cross-linkers used. Of the three cross-linking reagents only the shortest made Na current inactivation slow and incomplete. The steady-state inactivation curve, $h_\infty(E)$, was shifted by >25 mV in the hyperpolarizing direction by each of the reagents. The voltage dependence of activation, however, remained unchanged. Furthermore, the effects of two different monoimidoesters (ethyl acetimidate [EAI] and isethionyl acetimidate [IAI]) on gating currents were tested. EAI can penetrate a membrane, whereas IAI is membrane impermeant. IAI was almost without effect, whereas EAI caused a considerable reduction of the gating currents. EAI and DMS reduced the Q_{off}/Q_{on} ratio without affecting the decay of the Na currents. The results show that lysine residues are critically involved in Na channel gating.

INTRODUCTION

Chemical modification of excitable tissues can yield information about the specific structures essentially involved in the function of ionic channels (see Brodwick and Eaton, 1982). This approach has become more powerful since the amino acid sequence of Na channels has been determined recently (Noda et al., 1984; Noda et al., 1986). Functional changes produced by group-specific reagents can now be related to models of the molecular structure deduced from the analysis of the possible arrangement of the peptide chain (see Noda et al., 1984; Kosower, 1985; Greenblatt et al., 1985; Noda et al., 1986; Catterall, 1986; Guy and Seetharamulu, 1986). In each of four repeated units of homology a unique structural feature with many positive charges was found in a segment called S4. This segment is strikingly well conserved in the three types of sodium channels analyzed so far. It has been proposed that the positive charges in this segment represent the voltage sensor, possibly in conjunction with negatively charged residues clustered elsewhere (Noda et al., 1984). The positive charges are part of the side chains of only two amino acids: arginine and lysine.

To test the possible role of lysine residues we measured the change of sodium and gating currents caused by different amino group–specific reagents. Our electrophysiological measurements strongly support the view that lysine residues are critically involved in Na channel gating.

METHODS

The experiments were done on single motor or sensory nerve fibers dissected from the sciatic nerve of the frog *Rana esculenta* (Stämpfli, 1969). Na currents and gating currents were recorded at 12°C under voltage clamp conditions (Nonner, 1969). The fibers were cut on both sides of the node at a distance of ~0.75 mm. The ends of the fibers were bathed in 113 mM CsCl, 7 mM NaCl to block K channels from inside. The holding potential at which the Na current was 70% of the maximum Na current (measured with a prepulse of −40 mV and 50-ms duration) was taken as the normal resting potential ($E = -70$ mV). Unless stated otherwise, the holding potential was adjusted to $E = -100$ mV. All potentials are given as absolute potentials. The command voltage pulses were generated by a 12-bit D/A converter under computer control. Membrane currents were filtered at 25 kHz and sampled on-line at 10-μs or 100-μs intervals by a 12-bit A/D converter (Hof, 1986). 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 14-μm frog nerve fiber (Stämpfli and Hille, 1976).

Na currents were measured with depolarizing pulses of 14-ms duration which followed a 50-ms prepulse to −140 mV. Where indicated, the size of the Na currents was reduced by 6 nM tetrodotoxin (TTX) to minimize the error caused by the series resistance. To improve the signal-to-noise ratio, we averaged 3–10 Na current records; in gating current experiments we averaged 30 records. To correct the Na currents for capacitative and leakage currents, the current produced by a −30-mV pulse was scaled appropriately and subtracted from the currents produced by the depolarizing pulses.

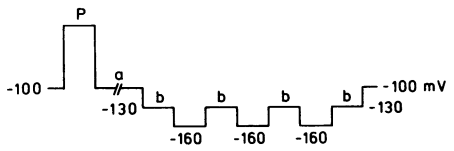

FIGURE 1 Pulse program for measuring gating currents. A depolarizing test pulse was followed by three hyperpolarizing pulses of fixed size −30 mV. The latter are superimposed on a reference potential which is 30 mV more negative than the holding potential. Time intervals: *a*, 500 ms; *b*, 40 ms. Gating current is current during test pulse *P* plus current during the three −30-mV pulses, the latter multiplied by the factor $P/90$.

To measure the $h_\infty(E)$ curve, we used 40-ms conditioning pulses to varying potentials followed by a constant test pulse to 0 mV. Normalized test pulse current was plotted against membrane potential during the conditioning pulse. The equation

$$h_\infty = [(1 - R)/\{1 + \exp[(E - E_h)/k_h]\}] + R \qquad (1)$$

was fitted to the experimental points. In this equation E_h is the potential at which $h_\infty = 0.5 + R/2$ and k_h is the slope parameter. R is used for the description of a noninactivating component of the sodium permeability (see Rack et al., 1986).

When measuring gating currents, most of the linear component of the capacitative and leakage current was compensated by an analogue circuit. The pulse program for measuring gating currents (see Fig. 1) consisted of a depolarizing test pulse and three hyperpolarizing pulses of −30 mV. The hyperpolarizing pulses had the same duration as the test pulse and served to measure the uncompensated remainder of the capacitative and leakage current. They were superimposed on a reference potential 30 mV more negative than the holding potential to minimize charge displacement during these negative pulses (see Nonner et al., 1978, page 83). The current during the −30-mV pulses was suitably scaled and subtracted from the current during the depolarizing test pulse.

Gating currents were corrected for the delays caused by the low pass filter (25 kHz) and the programmable amplifier. For this purpose, the time axis of the data points was shifted 13.76 μs with respect to that of the pulses (see Nonner et al., 1978). 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 integrated on- and off-responses were fitted with single exponential functions yielding the charge Q_{on} and Q_{off} and the time constants τ_{on} and τ_{off}.

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

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

where E_{mid} is the potential at which $Q_{on} = 0.5\ Q_{on\,max}$ and k is the number of millivolts required for an e-fold change of Q_{on}.

Solutions

The node was superfused continuously with Ringer solution with or without a chemical reagent. For the measurements of Na currents the node under investigation was superfused with Ringer solution (110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 4 mM morpholinopropanesulfonic acid [MOPS] buffer, pH 7.2). The Ringer solution also contained 12 mM tetraethylammonium chloride (TEA) to block K channels and, where indicated, 6 nM TTX. The gating current experiments were done with Na-free Ringer (110 mM NaCl replaced by 105 mM tetramethylammonium chloride [TMA]), containing 12 mM TEA and 300 nM TTX. The imidoesters were applied in 110 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 12 mM TEA, and 10 mM 2-(cyclohexylamino)-ethanesulfonic acid (CHES). The pH was adjusted to 9.0 with 1 N NaOH. The addition of imidoesters results in a strong acidic reaction. Therefore 1 N NaOH was added to the Ringer solution to bring the final pH back to 9.0 after addition of the desired amount of the chemical reagent. Because the reagents used are hydrolyzed by water, the individual Ringer solutions were prepared freshly for each experiment and applied to the nerve fiber immediately after preparation.

For modification the fibers were treated with the chemical reagents for 10 min and washed with reagent-free Ringer solution for at least 5 min before measurements were continued.

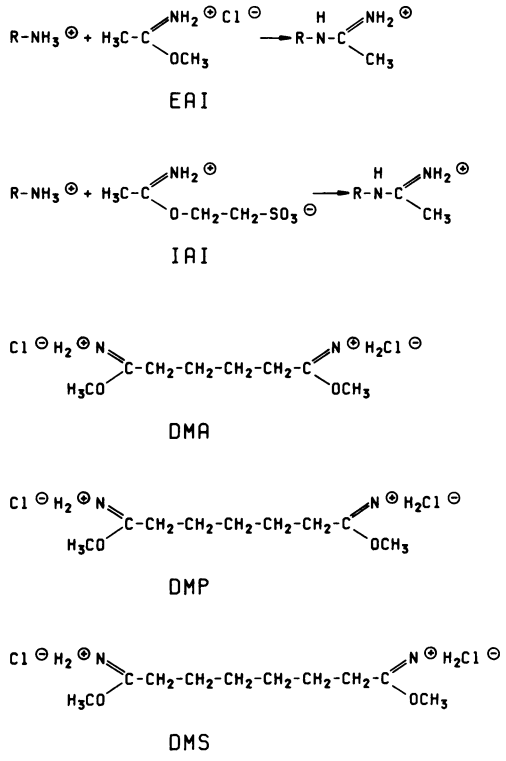

FIGURE 2 Comparison of the structure of the five amino group–modifying reagents used. Isethionyl acetimidate (IAI), ethyl acetimidate (EAI), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS). The reaction scheme of a primary amino group with an imidoester is illustrated for EAI as well as for IAI. A primary amino group (*left*) and the reaction products of EAI and IAI (*right*) are shown. As can be seen, the products resulting from EAI or IAI treatment are the same. In principle, the three cross-linkers react in the same way.

Materials

Ethyl acetimidate (EAI), isethionyl acetimidate (IAI), dimethyl adipimidate (DMA), and dimethyl pimelimidate (DMP) were obtained from Sigma Chemical GmbH München, FRG; dimethyl suberimidate (DMS) was from Fluka AG, Neu-Ulm, FRG; MOPS and CHES were from Serva Fine Biochemicals, Heidelberg, FRG. All other chemicals were analytical grade or in the purest form available and were purchased from E. Merck, Darmstadt, FRG.

RESULTS

Effects of Cross-Linking Reagents on Na Currents

Three amino group–reactive cross-linking reagents have been used: DMA, DMP, and DMS. Their chemical properties are almost identical. They differ only in one respect:

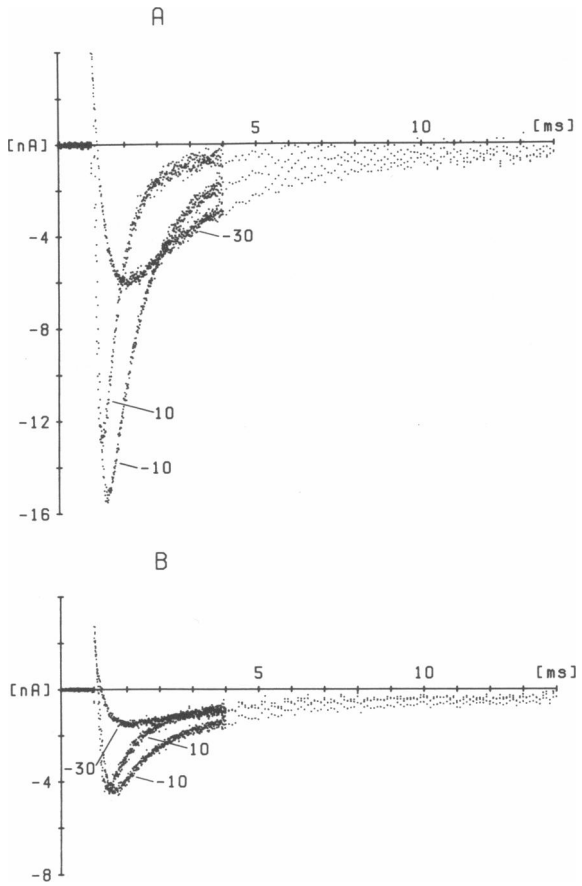

FIGURE 3 Na currents associated with 14-ms depolarizing pulses to −30, −10, and 10 mV after a 50-ms conditioning polarization to −140 mV before (*A*) and after (*B*) treatment with 10 mM DMA for 10 min. Holding potential, −100 mV. To reduce series resistance artefacts, we added 6 nM TTX to the Ringer solution.

the spacers between the two imidoester groups are of different length (see Fig. 2).

Fig. 3 compares Na currents before (*A*) and after (*B*) a 10-min treatment of a nerve fiber with 10 mM DMA. The size of the Na inward currents is about three times smaller after the modification procedure with the shortest of the cross-linkers used in this study. In a total of three experiments I_{Na} was reduced to 38 ± 3% of its original size. Furthermore the figure shows that the time to peak is not affected significantly, however, the decay of the currents appears to be slowed. To facilitate comparison of the decaying phase of the Na currents, we multiplied the Na inward current after chemical treatment with DMA by 3.4 (Fig. 4 *A*). This shows that the decay of the Na current is markedly slowed after DMA treatment.

The effects of DMA (and DMS) on the decay rate are described quantitatively in the legend of Fig. 4. The decaying phase of I_{Na} fits well to a single exponential (plus an additional term). The presence of only one time-dependent component of inactivation might be explained by the fact that TTX was present during our measurements (see Benoit et al., 1985).

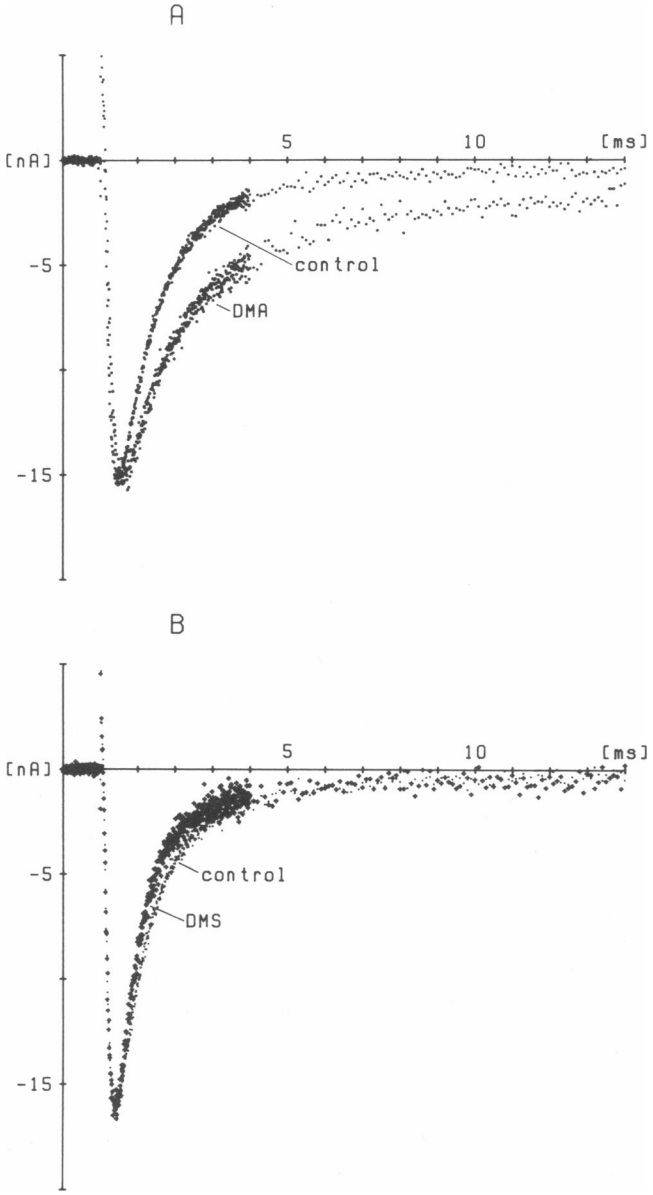

FIGURE 4 The decaying phase of the Na currents is slowed by DMA (*A*) but not by DMS (*B*). Equality of the peak currents before and after chemical modification was achieved by multiplying the Na current after DMA treatment by the factor 3.4 (*A*) and after DMS treatment by the factor 2.4 (*B*). Pulses to −10 mV. Holding potential, −100 mV. The Ringer solution contained 6 nM TTX. Without TTX, the reduction of peak I_{Na} was 30% instead of 31% after DMA treatment and 37% instead of 41% after DMS treatment. The decaying phase of I_{Na} associated with 14 ms depolarizing pulses was fitted by the equation $I_{Na} = A \exp(-t/\tau_h) + B$, where τ_h is the time constant of Na current inactivation. The values obtained are:

	A	*B*	τ_h		*A*	*B*	τ_h
	nA	*nA*	*ms*		*nA*	*nA*	*ms*
Control	−22.6	−0.7	1.3	Control	−23.9	−0.5	1.1
After DMA	−5.6	−0.6	2.2	After DMS	−11.1	−0.3	0.9

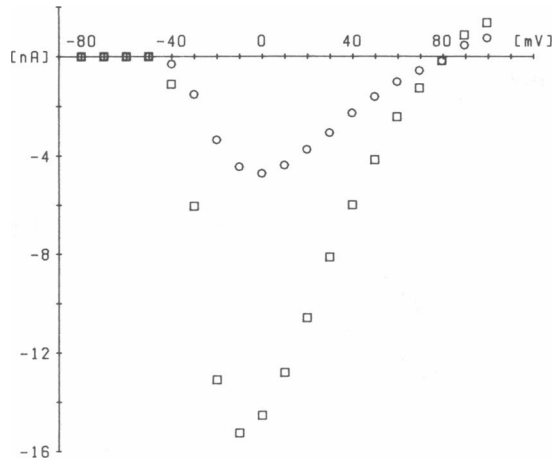

FIGURE 5 Peak sodium current-voltage curve, $I_{Na}(E)$, in 6 nM TTX before (*squares*) and after (*circles*) treatment with 10 mM DMA for 10 min. Holding potential, −100 mV.

Thus, the reduction of I_{Na} measured after DMA or DMS treatment might (at least in part) also be due to a change in the TTX sensitivity of the Na channel caused by the chemical modification procedure. This however, could be ruled out as the reduction of peak I_{Na} was not significantly different when the Ringer solution contained no TTX (see legend of Fig. 4).

Plotting I_{Na} versus pulse potential reveals that neither the descending branch of the I_{Na} (E) curve nor the reversal potential are shifted by the modification procedure (Fig. 5).

DMA treatment shifted the steady-state inactivation curve, $h_\infty(E)$, to more negative values of membrane potential and decreased its slope. In the experiment of Fig. 6 *A*, E_h shifted from −65.1 to −95.7 mV, while k_h increased from 6.9 to 13.1 mV. In a total of three experiments with 10 mM DMA the average shift of E_h was −29.3 ± 1.0 mV (mean ± SEM), while k_h increased by 6.4 ± 0.1 mV. As expected from a covalently binding chemical reagent none of these effects were reversible on washing.

The effects of the longest of the cross-linkers used, DMS, did not differ much from those of DMA except that DMS treatment did not cause a slowing of Na current inactivation (see Fig. 4 *B*). DMP, a cross-linker with a maximal effective length of 0.85 nm, intermediate between that of DMA (0.73 nm) and that of DMS (0.97 nm) (Hajdu et al., 1979), also did not affect the decaying phase of the Na current.

A 10-min treatment with 10 mM DMS reduced I_{Na} to 38 ± 3% (n = 3) of its original size. This reduction is equal to that obtained with DMA (see above) and is similar to the reduction of I_{Na} reported for the monofunctional imidoester ethyl acetimidate (EAI). With 10 and 20 mM EAI, I_{Na} was reduced to 54 and 44%, respectively (see Rack, 1985). Using 30 mM EAI, we observed a reduction to 42%. Thus, increasing the concentration of the reagent above 20 mM hardly affects the size of I_{Na}. As with DMA

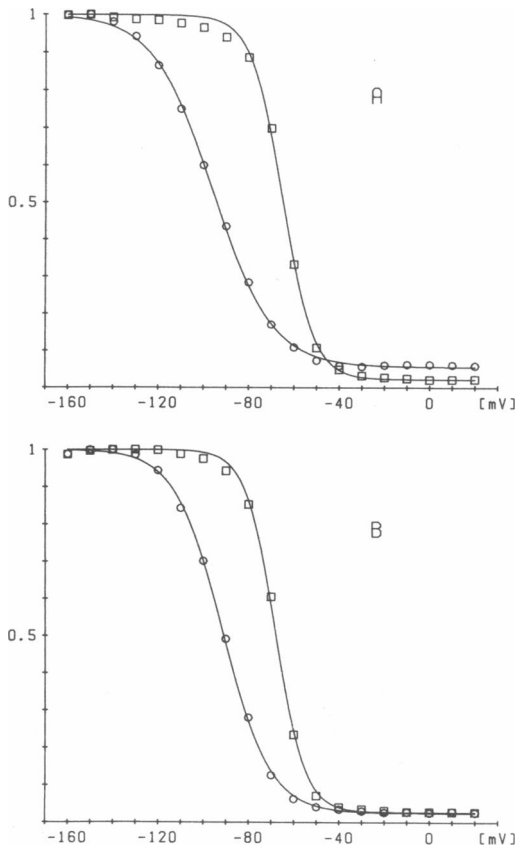

FIGURE 6 Steady-state inactivation curves, $h_\infty(E)$, of the sodium system before (*squares*) and after (*circles*) a 10-min treatment with 10 mM DMA (*A*) and 10 mM DMS (*B*). Points were fitted by Eq. 1 with the following parameters:

	E_h	k_h	R
	mV	*mV*	
Control (*A*)	−65.1	6.9	0.022
DMA-treated (*A*)	−95.7	13.1	0.056
Control (*B*)	−67.9	6.4	0.028
DMS-treated (*B*)	−91.3	10.5	0.025

or EAI, the $h_\infty(E)$ curve is strongly shifted to more negative values of membrane potential. In the experiment of Fig. 6 *B*, E_h shifted from −67.9 to −91.3 mV and k_h increased from 6.4 to 10.5 mV. In a total of three experiments with DMS E_h was shifted by −23.7 ± 1.5 mV, while k_h increased by 4.5 ± 0.3 mV.

Comparison of Fig. 6, *A* and *B*, also demonstrates the most striking difference between the effects of the two cross-linking reagents DMA and DMS. As shown by the points at $E > -40$ mV, DMA but not DMS makes Na current inactivation more incomplete (compare also Fig. 4, *A* and *B*).

Effects of Cross-Linking Reagents on Gating Currents

The inset of Fig. 7 shows gating current records at depolarizations to 0 mV before (*A*) and after (*B*) a 10-min

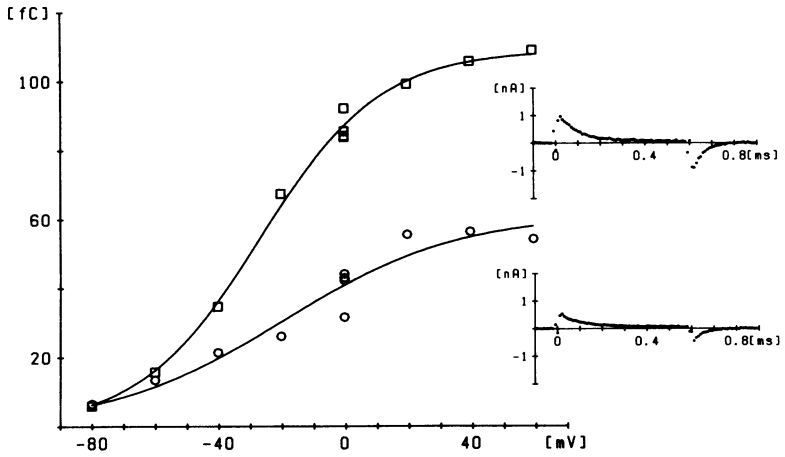

FIGURE 7 On-charge movement, Q_{on}, as a function of the test pulse potential, E, before (*squares*) and after (*circles*) DMA treatment. Pulse duration, 0.6 ms. Points fitted by Eq. 2 with $E_{mid} = -26.7$ mV, $k = 19.2$ mV, $Q_{on\,max} = 109$ fC for squares; $E_{mid} = -19.5$ mV, $k = 27.8$ mV, $Q_{on\,max} = 61$ fC for circles. Inset shows original records of the on- and off-charge movement measured with test pulses to 0 mV before (*top*) and after (*bottom*) DMA treatment. Holding potential, −100 mV.

treatment of the nerve fiber with 10 mM DMA. As can be seen, both the on- and off-charge movements are considerably reduced after the modification procedure. The on-charge movement at various pulse potentials, $Q_{on}(E)$, is plotted in Fig. 7. DMA treatment reduces $Q_{on\,max}$ from 109 to 61 fC. In a total of three experiments $Q_{on\,max}$ was reduced to 57 ± 2%. The midpoint potential, E_{mid}, was −27.7 ± 3.0 mV before and −14.2 ± 3.3 mV after modification, and the slope factor, k, increased from 19.8 ± 1.2 to 25.8 ± 1.1 mV (mean ± SEM, $n = 3$). The time constant of the on-response, τ_{on}, at 0 mV increased from 76.6 ± 3.4 to 100.2 ± 6.2 μs. The Q_{off}/Q_{on} ratio (for 0.6-ms test pulses to 0 mV) was 0.67 ± 0.02 in untreated and 0.67 ± 0.05 in DMA-treated fibers.

Furthermore, we have tested the effect of the longest of the cross-linkers used (DMS) on the gating currents. Similar to DMA, DMS reduced $Q_{on\,max}$ and increased the slope factor k of the $Q_{on}(E)$ curve. In three experiments $Q_{on\,max}$ decreased to 52 ± 6%, and k increased from 17.1 ± 1.4 to 21.9 ± 2.1 mV. τ_{on} at 0 mV increased from 72.3 ± 3.5 to 93.7 ± 4.2 μs. However, in two respects the action of DMS differed from that of DMA. The midpoint potential, E_{mid}, remained nearly unchanged (−31.3 ± 1.9 mV before and −30.4 ± 3.4 mV after DMS treatment). The Q_{off}/Q_{on} ratio (for 0.6-ms test pulses to 0 mV) decreased from 0.64 ± 0.01 before to 0.46 ± 0.06 after the DMS modification procedure.

Effects of Monofunctional Imidoesters IAI and EAI on Gating Currents

In further experiments, we studied the effect of the monoimidoesters IAI and EAI (see Fig. 2) on gating currents. The reaction of IAI and EAI with amino groups does not result in a change of the net charge because amino groups are converted to amidates, which are also positively charged. As previously shown (Rack, 1985), the lipid-soluble EAI, but not the lipid-insoluble IAI, irreversibly reduces the Na current.

In keeping with the observations on Na currents, IAI was nearly without effects on the gating currents. The $Q_{on}(E)$ curves before and after a 10-min treatment with 10 and 20 mM IAI were almost identical. By contrast, EAI had clear effects on gating currents. Fig. 8 shows original and integrated records of gating currents before and after a 10-min treatment of the nerve fiber with 20 mM EAI. The records show a clear reduction of the on- and off-responses at depolarizations to 0 mV. The on-response is reduced from 140 to 100 fC, whereas the off-response is affected more markedly and is reduced from 84 to 50 fC. Consequently, the Q_{off}/Q_{on} ratio is decreased. In a total of six experiments with 20 mM EAI the Q_{off}/Q_{on} ratio (for 0.6-ms test pulses to 0 mV) decreased from 0.65 ± 0.02 to 0.49 ± 0.01. This effect is very similar to that obtained with DMS but clearly different from that of DMA (see

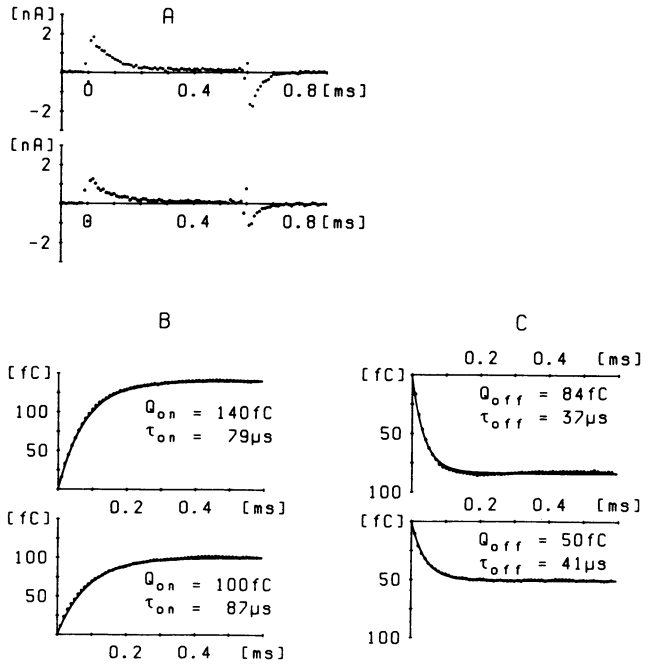

FIGURE 8 Original records of the on- and off-response measured with test pulses to 0 mV before (*top*) and after (*bottom*) EAI treatment (*A*). Integrated on- (*B*) and off-responses (*C*) of the same gating currents. Values for Q_{on}, τ_{on}, Q_{off}, τ_{off} are given next to the records. The Q_{off}/Q_{on} ratio decreased from 0.60 to 0.50 after EAI treatment. Holding potential, −100 mV.

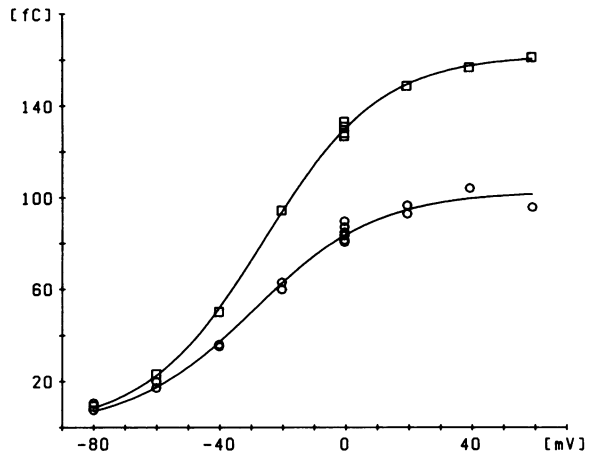

FIGURE 9 On charge movement, Q_{on}, as a function of the test pulse potential, E, before (*squares*) and after (*circles*) EAI treatment. Pulse duration, 0.6 ms. Points fitted by Eq. 2 with $E_{mid} = -25.8$ mV, $k = 18.8$ mV, $Q_{on\,max} = 162$ fC for squares; $E_{mid} = -28.8$ mV, $k = 19.7$ mV, $Q_{on\,max} = 103$ fC for circles. Holding potential, −100 mV.

above). The on time constant τ_{on} increases from 79 to 87 μs and the off time constant τ_{off} from 37 to 41 μs (see Fig. 8, *B* and *C*). That is, the velocity of the charge movement is only slightly slowed by EAI modification.

Fig. 9 presents the $Q_{on}(E)$ curve before and after EAI treatment. $Q_{on\,max}$ was reduced from 162 to 103 fC. In six experiments EAI caused a reduction of $Q_{on\,max}$ to 71 ± 3%. Thus, the reduction caused by EAI is less pronounced than that after DMA or DMS treatment. E_{mid} and k remained almost unchanged ($E_{mid} = -25.0 \pm 1.6$ mV, $k = 20.3 \pm 0.5$ mV before and $E_{mid} = -26.0 \pm 3.0$ mV, $k = 21.6 \pm 0.8$ mV after chemical treatment).

As shown in Fig. 3 of Rack (1985) EAI causes a strong shift of the $h_\infty(E)$ curve to more negative values of membrane potential. Consequently, part of the Na channels might be inactivated even at the rather negative holding potential of −100 mV, at which the experiments described above were carried out. In further experiments we measured gating currents before and after treatment with EAI at a holding potential of −120 mV. In a total of three experiments $Q_{on\,max}$ was reduced to 76 ± 2%, compared with 71 ± 3% at the less negative holding potential. Thus, the shift of the $h_\infty(E)$ curve cannot account for the major part of the reduction of $Q_{on\,max}$ by EAI treatment. While E_{mid} of the $Q_{on}(E)$ curve remained unchanged at either holding potential, k increased from 18.3 ± 1.1 to 22.4 ± 1.3 mV at a holding potential of −120 mV, an increase of 4.1 mV compared with 1.3 mV at a holding potential of −100 mV.

DISCUSSION

The experiments describe the effects of cross-linking and monofunctional amino group–reactive chemical reagents on Na currents and charge movement in the frog node of Ranvier. The results suggest that modification of lysine residues in the nerve membrane has drastic effects on the function of the Na channels. (A discussion of the possible role of lipids follows.) The data obtained are summarized in Table I.

Our major findings are: (*a*) with the exception of the membrane-impermeant reagent IAI the amino group–specific chemical reagents reduced charge movement and Na currents irreversibly. (*b*) Although the three cross-linkers used are chemically almost identical, only the shortest one of this series markedly slowed the decay of the Na currents. (*c*) The $h_\infty(E)$ curve was strongly shifted (≈25 mV) in the hyperpolarizing direction by each of the cross-linking reagents. The voltage dependence of activation, however, remained unchanged. (*d*) Treatment with imidoesters can enhance charge immobilization without affecting inactivation.

On the Chemistry of the Reagents Used

The discussion of the effects of the imidoesters can be based on a detailed knowledge of the chemical properties of these long and often-used reagents (see Han et al., 1984). Probably most important, imidoesters are among the few chemical reagents that can be regarded as truly specific. In proteins, only primary amino groups are modified (Hunter and Ludwig, 1962). Among the amino acids common in proteins, only lysine has a primary amine in its side chain. Another very important point is that the reaction product

TABLE I
EFFECTS OF AMINO GROUP REAGENTS ON GATING AND SODIUM CURRENTS

	Reduction of $Q_{on\,max}$ to	Estimated reduction of N to*	Shift of E_{mid}	Increase of k	Decrease of the Q_{off}/Q_{on} ratio‡	Decaying phase of I_{Na}
	%	%	*mV*	*mV*		
DMA	57	74	−27.7 to −14.2	19.8 to 25.8	None	Slowed
DMS	52	67	None	17.1 to 21.9	0.64 to 0.46	No effect
EAI§	71	76	None	20.3 to 21.6	0.65 to 0.49	No effect¶
EAI‖	76	93	None	18.3 to 22.4	0.79 to 0.66	No effect¶

Averages from *n* experiments; for details see text. *Calculated from Eqs. 4 and 5. ‡Measured with 0.6-ms pulses to 0 mV. §Holding potential, −100 mV. ‖Holding potential, −120 mV. ¶See Rack, 1985.

of imidoesters with amino groups retains the positive charge; that is, no change in net charge occurs (Hunter and Ludwig, 1962). The imidoester IAI, which contains a negatively charged sulfonic acid group has been shown to be membrane impermeant. Modification of amino groups at the outer surface of the membrane has been demonstrated with radioactively labeled IAI on erythrocytes (Whiteley and Berg, 1974). By contrast, EAI that contains the same reactive group but lacks the sulfonic acid (see Fig. 2) can modify proteins at the inner surface of a membrane when given from outside (Whiteley and Berg, 1974). EAI and IAI treatment results in the same amidines as reaction products. Therefore, by comparison of the effects of IAI and EAI, it is possible to discriminate between functionally important amino groups located at the outer surface or in the rest of the membrane.

It has to be mentioned that the intermediate product resulting during the reaction between an amino group and an imidoester is, in principle, able to react with another amino group located nearby (Browne and Kent, 1975; Siezen, 1979). Thus, cross-link reactions between amino groups, but only between amino groups, might be possible. This side reaction can be detected when the modification is carried out below or near pH 8 (Browne and Kent, 1975). Consequently, the modification procedures were carried out at pH 9.

The homobifunctional imidoesters used are designed to introduce cross-link reactions. Different effects of the cross-linkers tested can be attributed only to the different length of the spacers between the two identical reactive groups (see Fig. 2). If only one part of the cross-linker reacts (because no further amino group is available in appropriate proximity), the second imidoester of the reagent (see Fig. 2) will be hydrolyzed. Thus, modification by the cross-linkers might result in a reaction with a single amino group and introduce a more bulky residue than modification by, e.g., IAI or EAI.

Effects of IAI and EAI on Gating Currents

IAI treatment was almost without effect on gating currents. Thus, from the known chemistry of this reagent it is clear that modification of amino groups present in proteins or lipids at the outer side of the axonal membrane does not affect charge movement. Moreover, this result shows that a change of, for example, the pH value or the buffer substance during the modification procedure has no irreversible influence on the gating currents. By contrast, the membrane-permeant imidoester EAI irreversibly reduces the maximum on charge movement $Q_{\text{on max}}$ · $Q_{\text{on max}}$ and k are defined by the equations

$$Q_{\text{on max}} = Nz' \tag{3}$$

$$k = \frac{RT}{z'F} \tag{4}$$

$$z' = \alpha z, \tag{5}$$

where N is the number of gating particles, z' their effective valence, z their valence, and α the fraction of the membrane field that the particles traverse.

The reduction of $Q_{\text{on max}}$ could thus be caused by a change in N or z'. After EAI treatment a slight increase in k was observed, that is, z' is reduced. This change in z' can in principle be due to a decrease of α or z. However, from the chemistry of EAI we can exclude a change in the valence of the gating particles as the positive charge of an amino group is retained by the modification with imidoesters. Then, the observed small change in k can only be due to a small decrease in α. The increase of k, however, cannot fully explain the reduction of $Q_{\text{on max}}$. In consequence, N has to be reduced also by EAI treatment (see Table I). The reduction of N can be calculated using Eqs. 4 and 5 with the values for $Q_{\text{on max}}$ and k, derived from a fit using Eq. 2. Thus, even very small variations of k lead to a relatively large alteration of N. Therefore, the reduction of N should only be regarded as a rough estimate.

Effects of Cross-Linking Reagents on Gating Currents

The cross-linking reagents DMA and DMS caused a stronger reduction of $Q_{\text{on max}}$ than treatment with the monofunctional reagent EAI. The increase in k can only partly account for the strong decrease in $Q_{\text{on max}}$ after treatment with both cross-linkers. In consequence, N, the number of gating charges, is more markedly reduced by the cross-linking reagents than after treatment with the monofunctional imidoester (see Table I). As the charges themselves cannot be destroyed by either of the reagents used, the results suggest that part of the gating charges are immobilized by treatment with the cross-linking reagents.

The effects of DMA and DMS resemble in some respects the effects of EEDQ (Meves and Rubly, 1987). Of the five reagents used, only DMA, the cross-linker with the shortest span, produced a shift of the $Q_{\text{on}}(E)$ curve to more positive values of membrane potential (by 13.5 mV). A similar shift of E_{mid} (by 16.5 mV) was observed by Meves and Rubly (1987) after treatment of nerve fibers with 2 mM EEDQ. It should be noted that the shift of E_{mid} of the $Q_{\text{on}}(E)$ curve after DMA treatment is not accompanied by any shift of the $I_{\text{Na}}(E)$ curve. The situation is similar to that after EEDQ treatment, where the descending branch of the $I_{\text{Na}}(E)$ curve is shifted by only 5 mV (Rack and Woll, 1984). EEDQ is a "zero length" cross-linker (see Kunkel et al., 1981) that can introduce covalent bonds between carboxyl and amino groups directly. It is possible that the short cross-linker DMA and EEDQ produce their nearly identical shift of the $Q_{\text{on}}(E)$ curve by fixation of the same amino group to a further reactive part of the peptide chain located nearby. Moreover, as pointed out by Meves and Rubly (1987), the shift of E_{mid} by EEDQ is not due to a change in surface potential (see Rack and Woll, 1984). The same is clear for DMA treatment.

With 2 mM EEDQ, k increased by 6.8 mV similar to the

4.8 and 6.0 mV increase observed with DMS and DMA, respectively. There was also a reduction of $Q_{on\,max}$ by EEDQ which, however, was less pronounced than that produced by DMA or DMS.

Effects on Q_{off}/Q_{on} Ratio

EAI and DMS (but not DMA) treatment decreases the Q_{off}/Q_{on} ratio, i.e., enhances charge immobilization (see Table I). This process is normally closely related to inactivation of the Na channels (Armstrong and Bezanilla, 1977; Khodorov, 1981). It is therefore interesting to note that neither EAI (see Rack, 1985) nor DMS (see Fig. 4 *B*) affect the time constant of inactivation. The same phenomenon has been observed with the local anesthetic benzocaine by Neumcke et al. (1981). They discussed two explanations: (*a*) a special effect of TTX that is present in high concentration (300 nM) during the gating current measurements and in small concentration during the Na current measurements, and (*b*) a special state of the Na channel in which immobilization of some of the charges has occurred while the channel is still open. As a further explanation we can add (*c*) an effect of TMA which replaces external Na in the gating current experiments (see Methods). Our experiments show that also the reverse situation occurs: DMA slows the decaying phase of the Na current (Fig. 4 *A*) but does not affect Q_{off}/Q_{on}. Clearly, the apparent dissociation between charge immobilization and Na current inactivation deserves further investigation.

Effects of the Cross-Linking Reagents on Na Currents

The cross-linkers reduced I_{Na} and $Q_{on\,max}$ more than EAI. This additional effect may be due to the formation of intra- or intermolecular cross-links. Alternatively, a residue bulkier than that after EAI treatment may have been introduced by the reaction of the cross-linkers with a single amino group.

The reduction of the Na current caused by the imidoesters (except IAI) may in principle be due to a reduction of the single-channel conductance or a reduction of the number of functional channels. In the latter case one would expect that an increase of the concentration of the reagent reduces I_{Na} further. The first possibility seems more likely, as 10 mM EAI, 20 mM EAI (see Rack, 1985), and 30 mM EAI reduce I_{Na} to 54, 44, and 42% and cause a shift of the $h_\infty(E)$ curve by -13.1, -17.3, and -20.6 mV, respectively. An alternative explanation that cannot be ruled out, however, is that the population of Na channels in the frog node is not homogeneous.

Only the shortest of the cross-linkers used, DMA, slowed the decaying phase of the Na currents. This suggests that this effect of DMA is caused by a cross-link reaction as modification of only one amino group, e.g., by EAI or the possible reaction of a longer bisimidoester with a single amino group, did not alter the decay of I_{Na}. The absence of an effect of DMS and DMP on the decay of the Na currents suggests that the two amino groups linked by DMA have a distance not appropriate for these slightly longer reagents (see Fig. 2). Two other reagents that can introduce cross-link reactions, glutaraldehyde (Schmidtmayer, 1985; Mozhayeva et al., 1986) and EEDQ (Rack and Woll, 1984), are also able to inhibit Na current inactivation partially. It is likely that amino group modification occurs in the formation of cross-links by either of these reagents. However, the modification of further amino side chains has to be considered. Interestingly, the cross-links introduced by these reagents are also very short, even shorter than those after DMA treatment.

The steady-state inactivation curve, $h_\infty(E)$, is strongly shifted in the hyperpolarizing direction by DMA and DMS treatment. Again this shift is clearly stronger than that observed after treatment with the monofunctional imidoester EAI (Rack, 1985) or that seen after treatment with a less amino group–specific succinimide ester (Rack et al., 1984). A shift of the $h_\infty(E)$ curve by, for example, a change in Ca concentration or pH is usually accompanied by a similar shift of the descending branch of the $I_{Na}(E)$ curve (see Stämpfli and Hille, 1976). With the imidoesters, however, no shift of the $I_{Na}(E)$ curve is observed. A selective shift of the $h_\infty(E)$ curve has also been reported after chemical modification with reagents able to react with histidine residues (Rack et al., 1986).

From the points discussed above, it appears that the functional changes observed are due to the modification of one (or more) lysine residue(s) of the Na channel protein. Two other possibilities have to be mentioned, however. (*a*) The free *N*-terminal amino group of the channel protein chain might be strongly involved in Na channel gating. (*b*) Alternatively, a lipid containing a primary amine located on the inner surface of the membrane may have been modified. This, however, would mean that one (or more) lipids have to be regarded as an essential part of the gating "machinery" of the voltage-dependent Na channel. We cannot rule out a functional role of the lipids closely associated with the channel protein; it seems possible that the process of inactivation is strongly influenced by the modification of membrane lipids (see Rack et al., 1986).

The results show that even a slight structural change of amino groups (without destruction of charge) has a profound effect on charge movement of the axonal membrane. This suggests that amino groups are a functionally important part of the gating "machinery" or part of the gate itself.

The functional changes reported here are in accordance with the view that at least some of the numerous lysine residues as present in the unique structural features called S4 (Noda et al., 1984 and 1986) or elsewhere in the protein are functionally important for the gating mechanism of the Na channel.

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