Biochimica et Biophysica Acta, 774 (1984) 288–292
Elsevier

**BBA Report**

BBA 70166

# EFFECT OF WATER-SOLUBLE CARBODIIMIDE ON GATING IN SODIUM CHANNELS

G.N. MOZHAYEVA, A.P. NAUMOV and E.D. NOSYREVA

*Academy of Sciences of the U.S.S.R., Institute of Cytology, 194064 Leningrad (U.S.S.R.)*

(Received January 6th, 1984)

*Key words: Ion channel; $Na^+$ channel; Nerve membrane; Carbodiimide; Gating charge; Voltage clamp; (R. riribunda)*

**The treatment of nodal membrane with water-soluble carbodiimide results in a deceleration of the time-course of activation and inactivation of $Na^+$ channels and in a decrease in the steepness of the voltage-dependence of the channel gating. The effective charge of activation decreases from about 5 to 3 electronic charges. Carbodiimide is effective only at low (4.3–4.9) pH, which means that it reacts most probably with carboxyl groups. The data are consistent with the proposal that carboxyl groups may serve as 'gating particles'.**

The operation of gating machinery in electrosensitive ionic channels underlies generation and propagation of excitation in nerve and muscle cells. The electrosensitivity of the channels suggests the existence of a system of mobile charges in their structure. At present, practically nothing is known about chemical nature of constituents of the gating machinery, although the functional properties of this mechanism for a number of channels have been described in detail. In present work, we examine the effect of one of the water-soluble carbodiimides on the $Na^+$ channels. These compounds are known to react specifically (under certain conditions) with carboxyl groups [1–3].

The work was done on single nerve fibre from the frog *Rana ridibunda* under voltage-clamp conditions. Membrane potential ($E$) was defined as inside minus outside. The elimination of linear component of membrane current was accomplished with $P/2$ or $P/4$ procedures. Both positive (test) and negative (control) steps started from holding potential. Depending on the size of the channel currents, this program was repeated from 2 to 32 times. The average data were reconverted to analogue form and reproduced on d.c. pen-recorder. The control solution contained (in mM): 110 NaCl, 2 $CaCl_2$, 10 Tris-HCl, 8 tetraethylammonium chloride (pH 7.6). Water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl) (Peptide Inst. Corp., Japan) was applied either alone or with 50 mM hydroxylamine or glycine methyl ether HCl. These solutions contained, in addition, 20 mM KCl and 5 mM $CaCl_2$. The pH of these solutions was adjusted to 4.3–4.9 by addition of small amounts of HCl. Carbodiimide solutions were prepared just before application. All measurements were carried out in control solution at 8–10°C.

The time of exposure of the membrane to carbodiimide varied from 10 to 30 min and in every experiment was chosen so that the parameters of activation reached the new steady-state level. For this purpose, outward currents were measured every minute during carbodiimide treatment and the carbodiimide solutions was substituted by a control one in the 1–2 min after current kinetics had stopped changing. In six experiments, carbodiimide was applied twice and activation parameters were determined both after the first and after the second exposure.

The carbodiimide treatment resulted in considerable inhibition of sodium conductance ($g_{Na}$). After exposure to carbodiimide for long enough to reach maximum changes in gating function, $g_{Na}$

ranged from 2% to 15% of the initial value. It should be noted that, in the presence of carbodiimide, $g_{Na}$ continued to decrease up to the point of complete disappearance, even after the gating parameters had stopped changing. In order to rule out the possibility of misrepresentation of results due to some kind of current-dependent artefact, currents before the treatment were measured with a prepulse both to $-100$ mV and to potentials from $-70$ to $-50$ mV, so that current size decreased by a factor of 10–50. For example, in the experiment presented in Fig. 1, currents before the carbodiimide treatment were measured with prepulse to $-66$ mV, so they were reduced 10-fold as compared with currents measured with prepulse to $-100$ mV. In several experiments, tetrodotoxin (Sankyo, Japan) was used to decrease currents in untreated node. In addition, the compensation of series resistance was used in all experiments [4].

It can be seen from Fig. 1 that carbodiimide treatment at low pH considerably slows the activation kinetics of $g_{Na}$. The time-course of activation was approximated by a simplified equation $(1-\exp t/\tau_a)I_p$, where $I_p$ is the peak current value and $\tau_a$ is the time constant of activation. The voltage-dependence of $\tau_a$ tends to have a maximum. Following the carbodiimide treatment, the maximal value of $\tau_a$ increased by a factor of $1.8 \pm 0.4$ and $\tau_a$ at $E = \text{zero}$ by a factor of $3.0 \pm 0.2$ ($n = 8$).

The fraction of channels open at the current peak ($n_p$) as a function of $E$ was determined in three ways. (1) 'Instantaneous' currents for a constant number of open channels [5] were measured and $n_p$ for particular $E$ was calculated as normalized ratio of peak current to corresponding value of 'instantaneous' current [6]. (2) $n_p$ was calculated as normalized peak chord conductance. Inasmuch as 'instantaneous' current-voltage relations for normal [7] and for carbodiimide-treated (this work, not illustrated) membrane are essentially linear over the voltage range from $-50$ to $+40$ mV, second method of calculation gives the same result as the first one. (3) 'Tail' currents in response to a post-pulse of fixed level were measured and $n_p$ was determined as normalized 'tail' current amplitude [8]. Such a measurement is shown in Fig. 1a. It can be seen from Fig. 1c that the $n_p(E)$-curve from 'tail' current measurements coincides with chord conductance curve. The number of channels open at the peak of the current depends generally on parameters both of activation and inactivation. However, as evidenced by our experiments, the prepulses inactivating up to 98% of $g_{Na}$ did not change the shape of $n_p(E)$ curve (if, of course, the influence of series resistance and other current-dependent artefacts was negligible). So, the $n_p(E)$ curve is a good measure of the steady-state voltage-dependence of channel activation.

On a fairly general assumption concerning the mechanism of activation, the theoretical voltage-dependence of the number of open channels at potentials where channels just begin to open is given by an exponential function of $E$, that is, by a straight line on a semilogarithmic plot (see, for example, Ref. 9). The slope of this line is proportional to effective charge of activation ($Z_{ef}$) the displacement of which is required for transition of the channel between the open and, energetically most distant from it, closed state (this closed state is sometimes named the 'fully' closed one). Fig. 1 shows that after carbodiimide treatment, the $n_p(E)$ curve becomes flatter over all potential range tested. In 14 experiments with 100 mM carbodiimide, $Z_{ef}$ was estimated for normal and treated membrane to be $5.16 \pm 0.18$ and $3.00 \pm 0.09$ electronic charges ($e$), respectively. With 50 mM carbodiimide plus 50 mm hydroxylamine, analogous values were $5.05 \pm 0.14$ and $2.79 \pm 0.17$ $e$ ($n = 16$). Thus, the two sets of experiments yielded quite identical results: $Z_{ef}$ decreased by a factor of 1.8. Essentially the same result was obtained in five experiments with carbodiimide + glycine methyl ether.

At normal pH, carbodiimide proved to be absolutely noneffective in reducing $Z_{ef}$. Fig. 2 shows an experiment in which measurements were made firstly on an unaffected node, then after application of carbodiimide at pH 7.5 and lastly after carbodiimide treatment at pH 4.8. It can be seen that the steepness of $n_p$ voltage-dependence remained unchanged after carbodiimide at pH 7.5 but was significantly reduced after carbodiimide treatment at pH 4.8. On average, from four such experiments, $Z_{ef}$ was $5.3 \pm 0.3$ before and $5.6 \pm 0.2$ after carbodiimide treatment at pH 7.5–7.6. As to some other properties of $Na^+$ conductance, carbodiimide at pH 7.6 was not without effect. Thus, following the application of carbodiimide at normal pH, currents were decreased though to lesser extent than after carbodiimide treatment at low pH, and voltage dependence of $n_p$ was shifted to the left by 5–10 mV. Both of these effects seem not to be specific to carbodiimide; for example, similar changes in $g_{Na}$ have occurred after more-or-less prolonged exposure of the membrane to solutions with low pH (unpublished observations). Low pH itself had no after effect on the steepness of $n_p(E)$ relation/

The carbodiimide treatment produced also considerable slowing and decreasing of the steepness of voltage-dependence of channel inactivation: thus, currents in treated membrane inactivated only partially, even after prolonged (50–100 ms) depolarizations.

It might be proposed that there preexist two populations of $Na^+$ channels: one with normal kinetics and potential-dependence and another, much less numerous, with 'unusual' kinetics and potential dependence, the latter being resistant to carbodiimide. Then, apparent changes in kinetics and potential-dependence of $g_{Na}$ would result simply from inhibition of the conductance in channels with normal gating. This hypothesis seems to be inconsistent with the following facts. (1) Experiments with prolonged exposure of the membrane to carbodiimide (see above) show that there are no channels resistant to carbodiimide. (2) If this proposal held, the rate of current rise in normal membrane should depend on a prepulse, because the contribution of both proposed populations to the total current should differ for different prepulses due to the difference in inactivation parameters. However, this is not the case. (3) In a number of experiments, absolute current sizes with prepulses to potentials more positive than −60 mV were larger in the carbodiimide-treated membrane than those in the intact one.

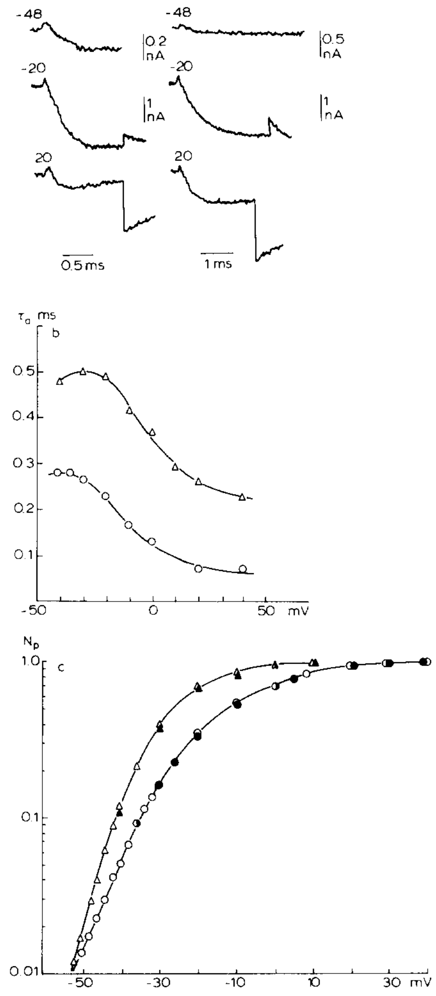

Fig. 1. Effect of water-soluble carbodiimide treatment on kinetics and steady-state properties of activation of sodium channels. (a) Currents through the $Na^+$ channels before (left) and after 11 min treatment with 100 mM carbodiimide at pH 4.8 (right). All measurements were carried out in the control solution at pH 7.6. Numbers of the left of each record show membrane potential during the test pulse ($E_t$), in mV. Holding potential was −80 mV. Currents were measured with prepulses (50 ms in duration) to −66 mV before and to −100 mV after the treatment. Test pulses to −20 and to +20 mV were followed by the post-pulse ($E_p$) to −10 mV before and to zero potential after carbodiimide treatment. Note the difference in time-scales for currents before and after the carbodiimide treatment. (b) Time constant of activation ($\tau_a$) as a function of $E$ before (○) and after (△) carbodiimide treatment. (c) Semi-logarithmic plot of the fraction of open $Na^+$ channels ($n_p$) as a function of potential before (triangles) and after (circles) carbodiimide treatment. Open symbols indicate a normalized chord peak $g_{Na}$. Filled symbols indicate the normalized amplitude of 'tail' currents in response to fixed $E_p$ to −10 mV before and to zero potential after the carbodiimide treatment.

---

In order to take into account the sodium inactivation within the interval from peak current to the end of the pulse, the 'tail' current value was multiplied by the ratio of peak current amplitude to current value at the end of $E_t$ [8]. The logarithmic limiting slope of these curves correspondes to effective charge of activation ($Z_{ef}$) equal to 5.14 electronic charges ($e$) for normal and 3.13 $e$ for carbodiimide-treated membrane, respectively. Node 221-83, $t = 10\,°C$.

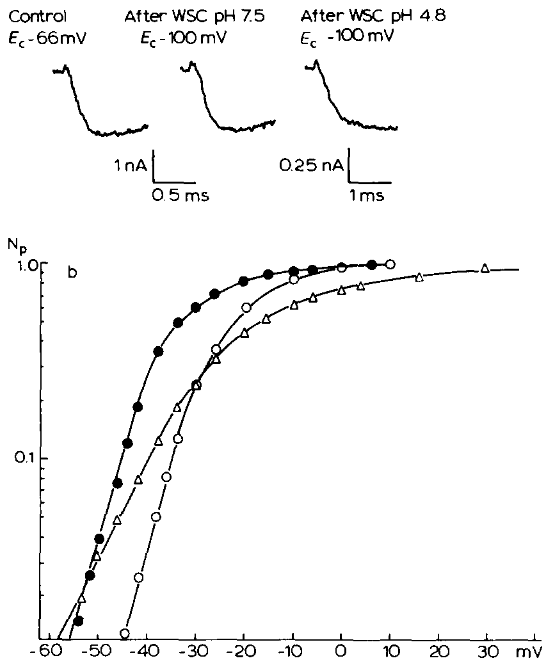

Fig. 2. Carbodiimide is effective only at low pH. (a) Currents at $E_t$ = zero. Left-hand trace, untreated membrane; middle trace, after 10 min treatment with 100 mM carbodiimide at pH 7.5; right-hand trace, after 10 min treatment with 100 mM carbodiimide at pH 4.8 in the same experiment. Note the difference in time-scales. (b) Voltage dependence of the fraction of open channels in the untreated node (○), after application of water-soluble carbodiimide (WSC) at pH 7.5 (●), and after subsequent carbodiimide treatment at pH 4.8 (△). $Z_{ef}$ values are: 5.30 *e* in control; 5.30 *e* after carbodiimide treatment at pH 7.5; and 2.94 2*e* after carbodiimide treatment at pH 4.8 Node 154-83, $t = 9.5\,°$C.

Thus, it is reasonable to assume that carbodiimide-induced changes in kinetics and potential-dependence of $g_{Na}$ resulted from modification of the gating machinery in each channel.

Previously, carboxyl group reagents (carbodiimide and trialkyloxonium salts) have been examined as $Na^+$ channel modifiers with emphasis on tetrodotoxin binding [7,10–13]. Our experiments allowed to test tetrodotoxin binding to channels which preserved their conductivity. It turned out that the population of carbodiimide-modified channels is nonhomogeneous by its sensitivity to tetrodotoxin blockage. Most of them had a dissociation constant ($K_d$) of about $2.1 \cdot 10^{-8}$ M and 10–15% of these channels left unblocked at a tetrodotoxin concentration as high as $1 \cdot 10^{-5}$ M. The $K_d$ for normal channels was estimated in the same series of experiments to be $5 \cdot 10^{-9}$ M. On the other hand, tetrodotoxin did not influence the kinetics and voltage-dependence of the currents, which suggests that the properties of the gating machinery of all carbodiimide-modified channels are the same.

All effects of carbodiimide are essentially irreversible.

Carbodiimide molecules are hydrophilic and positively charged at the pH employed; therefore they cannot cross the membrane. Thus, they can attack the targets only on the external surface of the membrane.

The fact that carbodiimide exerts its effect only at low pH suggests that the observed effects of the carbodiimide are due to interaction of carbodiimide with carboxyl groups [1–3], though reaction with oxy groups of tyrosine [14] and phosphodiester groups [15] cannot be ruled out. The first stage of reaction of carbodiimide with carboxylic acid is the formation of unstable acylisourea [1]. This compound reacts with water to regenerate carboxylic acid, but with excess of carbodiimide (as in our experiments) acylisourea will be formed again and again. Several ways of transformation of acylisourea into stable compounds are known [1–3,16–17]. (1) Rearrangement into *N*-acylurea. (2) Interaction with a nucleophile. In our experiments the presence of nucleophiles such as hydroxylamine or glycine methyl ether did not influence significantly the channel modification. The possibility remains that such a reaction occurs with some nucleophilic group, e.g., an amino group, properly situated on the membrane surface. (3) Intramolecular acylation of a peptide bond with formation of a cyclic imide if the carboxyl group belongs to an aspartic acid chain. (4) The formation of azlactone for the C-terminal group. (5) In the presence of hydroxylamine, the sequence of reactions is possible which leads to substitution of a carboxyl group by an amino group. In the study by Riem and Sheraga [16], the modification of carboxyl groups of ribonuclease with carbodiimide was performed without addition of nucleophiles. Those authors assumed that the most probable products of modification were acylurea and cyclic imide. In that work, the concentration of carbodiimide was 1–2 mM and the time of the treatment was 6 h. Therefore, one can expect that at a carbodiimide concentration of 100 mM, the rate of modification will be comparable with that in our work.

The value of $Z_{ef}$ is determined by the number of mobile charged groups and by fractional distance travelled by each of them in the electric field. It is natural to explain our data on the assumption that part of mobile gating charge is represented by carboxyl groups. This is consistent with the assumption by Gilly and Armstrong [8] that the mobile gating charge is completely or partially composed of negatively charge particles. At potentials negative enough, they reside on the external surface of the membrane, which corresponds to the closed state of the channel. When depolarizing the membrane, they move towards the internal surface of the membrane and the channel opens. In the 'closed' position, these particles can interact with components of the external solution. Carbodiimide modification leads either to loss (or change) of the charge and/or to restriction of the motion of these groups. In any case, the steepness of $n_p(E)$ curve should decrease.

It is conceivable that, along with mobile acid groups, there are acid groups 'fixed' on the membrane surface and they influence gating process in a way suggested by the surface charge hypothesis [18–20].

## References

1 Hoare, D.G. and Koshland, D.E. (1967) J. Biol. Chem. 242, 289–305

2 Means, G.E. and Feeny, R.E. (1971) Chemical Modification of Proteins, p. 254, Holden Day, San Francisco

3 Stepanov, V.M. and Matyash, L.F. (1973) Uspekhi Biol. Nauk (Russ.) 75, 163–182

4 Sigworth, F.J. (1980) J. Physiol. 307, 97–129

5 Hodgkin, A.L. and Huxley, A.F. (1952) J. Physiol. 117, 500–544

6 Chiu, S.Y. (1980) J. Physiol. 309, 499–519

7 Mozhayeva, G.N., Naumov, A.P. and Negulyaev, Yu.A. (1982) Gen. Physiol. Biophys. 1, 5–19

8 Gilly, W.F. and Armstrong, C.M. (1982) J. Gen. Physiol. 79, 935–964

9 Almers, W. (1978) Rev. Physiol. Biochem. Pharmacol. 82, 96–190

10 Shrager, D. and Profera, C. (1973) Biochim. Biophys. Acta 318, 141–147

11 Reed, J.K. and Raftery, U.A. (1976) Biochemistry 15, 944–953

12 Baker, D.F. and Robinson, K.A. (1977) J. Physiol. 266, 3–4 P

13 Spalding, B. (1980) J. Physiol. 305, 485–500

14 Carraway, K.L. and Koshland, D.E. (1968) Biochim. Biophys. Acta 160, 272–274

15 Smith, M., Moffatt, J.O., Khorana, H.G. (1958) J. Am. Chem. Soc. 80, 6204–6212

16 Riem, J.P. and Sheraga, H.A. (1966) Biochemistry 5, 99–115

17 Hoare, D.G., Olson, A. and Koshland, D.E. (1968) J. Am. Chem. Soc. 90, 1638–1643

18 Frankenhaeuser, B. and Hodgkin, A.L. (1957) J. Physiol. 137, 218–244

19 Mozhayeva, G.N. and Naumov, A.P. (1970) Nature 228, 164–165

20 Hille, B., Woodhull, A.M. and Shapiro, B.I. (1975) Philos. Trans. R. Soc. Ser. B. 270, 301–318