Biochimica et Biophysica Acta, 648 (1981) 93–99
Elsevier/North-Holland Biomedical Press

BBA 79416

FLUCTUATION ANALYSIS OF Na^+ CHANNELS MODIFIED BY BATRACHOTOXIN IN MYELINATED NERVE

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(Received February 23rd, 1981)

Key words: Na^+ channel; Fluctuation analysis; Batrachotoxin; Nerve membrane

(1) Single myelinated nerve fibres of *Rana esculenta* were treated with the steroidal alkaloid batrachotoxin, and Na^+ currents and Na^+-current fluctuations were measured near the resting potential under voltage-clamp conditions. Between test pulses the fibres were held at hyperpolarizing membrane potentials. (2) The spectral density of Na^+-current fluctuations was fitted by the sum of a $1/f$ component and a Lorentzian function. The time constant $\tau_c = 1/(2\pi f_c)$ obtained from the corner frequency f_c of the Lorentzian function approximately agreed with the activation time constant τ_m of the macroscopic currents. (3) The conductance γ of a single Na^+ channel modified by batrachotoxin was calculated from the integral of the Lorentzian function and the steady-state Na^+ current. At the resting potential $V = 0$ we obtained $\gamma = 1.6$ pS, higher γ-values of 3.2 and 3.45 pS were found at $V = -8$ and -16 mV, respectively. (4) The conductance of a modified Na^+ channel is significantly lower than the values 6.4 to 8.85 pS reported in the literature for normal Na^+ channels. Hence, our experiments are in agreement with the view that batrachotoxin acts in an 'all-or-none' manner on Na^+ channels and creates a distinct population of modified channels.

Introduction

Na^+ channels in axon membranes can be modified by a large number of neurotoxins [1]. Among the toxins a class of lipid-soluble compounds has very pronounced effects on the gating processes of Na^+ activation and inactivation. Of these, batrachotoxin has been studied in experiments on squid giant axons [2] and in great detail on myelinated nerve fibres (compare Ref. 3 for a summary of the experiments). In these investigations it was demonstrated that treatment with batrachotoxin creates a new component of Na^+ currents which does not inactivate, exhibits different activation properties and is resistant to several local anesthetics, and it has been suggested that these alterations are due to a fraction of Na^+ channels modified by batrachotoxin.

The aim of the present investigation was the determination of properties of single modified Na^+ channels. To this end Na^+ currents and Na^+-current fluctuations were recorded in myelinated nerve fibres which had been treated by batrachotoxin. From the analysis the conductance of a single modified Na^+ channel could be obtained and was found to be significantly lower than that of a normal Na^+ channel. This result supports the idea that batrachotoxin creates a distinct population of modified Na^+ channels.

Methods

Single motor and sensory fibres were dissected from the tibial nerve of the frog, *Rana esculenta* [4], and a node of Ranvier was voltage-clamped at 15°C [5]. Displacements of the membrane potential from its resting value (where 30% of the normal Na^+ channels were inactivated) are denoted by V. Where necessary for calculations, the absolute membrane poten-

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tial $E = V + E_0$ was obtained from the clamp potential E_0 at $V = 0$. Membrane currents were calibrated using a longitudinal axoplasm resistance determined from electrical measurements by a method similar to the one described recently [6].

To block K^+ channels from inside, the ends of the fibre were cut in an 'artificial' axoplasm solution composed of 113 mM CsCl, 7 mM NaCl and 4 mM morpholinopropanesulphonic acid buffer adjusted to pH 7.2 with NaOH. In some experiments an axoplasm solution of 113 mM tetraethylammonium chloride and 7 mM NaCl was applied. To block K^+ channels also from outside all extracellular solutions contained 10 mM tetraethylammonium chloride.

Na^+ currents and Na^+-current fluctuations were recorded in K^+-free Ringer's solution (test solution) containing 110.5 mM NaCl, 2 mM $CaCl_2$, 10 mM tetraethylammonium chloride and 4 mM morpholinopropanesulphonic acid/NaOH buffer at pH 7.2. At the end of an experiment current fluctuation measurements were repeated in test solution with 300 nM tetrodotoxin to block all Na^+ channels.

Batrachotoxin is a steroidal alkaloid of molecular weight 538. It was a gift of Drs. J. Daly and B. Witkop [7] and was kept at –20°C in ethanol stock solution (100 μg batrachotoxin in 1 ml absolute ethanol). Nerve fibres were treated with batrachotoxin during stopped flow of test solution by perfusing some millilitres of test solution plus 1.6 μM batrachotoxin into the fluid pool surrounding the test node. The fibre was then stimulated at a holding potential of $V = 0$ for approximately 2 min at a frequency of 3 Hz with a 50 ms hyperpolarizing prepulse to $V = -50$ mV followed by a 5 ms depolarizing pulse to $V = 50$ mV. During the stimulation period irreversible effects of batrachotoxin on Na^+ channels developed. Hence, the flow of test solution could be resumed in the following course of the experiment.

After the treatment with batrachotoxin the holding potential was set to $V_H = -32$ mV. In the first series of experiments the holding level was taken as reference potential for measurement of membrane currents and for adjusting the analogue circuit to compensate the leakage and capacity currents during a hyperpolarizing potential step. Later, we realized that a small Na^+ influx through batrachotoxin-modified Na^+ channels occurred even at –32 mV. Therefore, we chose in the following experiments a reference potential of –48 mV at which all modified Na^+ channels are closed. The two types of experiment, however, did not yield different values for the parameters of modified Na^+ channels.

After compensating linear components of the leakage and capacity currents, values of steady-state currents were read off the oscilloscope at voltages V between –30 and 140 mV to obtain current-voltage relations. Current relaxations elicited by voltage steps from the reference level to the test potentials ($V =$ –16, –8 and 0 mV) were then recorded on film. Next, fluctuation measurements were performed under computer control between 145 and 473 ms after the onset of the test pulse [8]. 60 test pulses at 3-s intervals were applied to average the spectral density of current fluctuations and the mean current. For each test potential the pulse sequence was first performed in test solution and, again, in test solution with 300 nM tetrodotoxin. In a few experiments current-voltage curves were taken in the presence of tetrodotoxin. No significant deviations from linearity were found.

The recorded steady-state currents mainly arise from the Na^+ ion flux through batrachotoxin-modified Na^+ channels which do not inactivate [3]. Thus, the stationary permeabilities, P, of these channels can be calculated from the Goldman-Hodgkin-Katz equation [9,10]

$$P = I_s \frac{RT}{EF^2 [\mathrm{Na}]_0} \frac{\exp(EF/RT) - 1}{\exp((E - E_r) F/RT) - 1} \tag{1}$$

where I_s is the steady-state current at the potential E, $[Na^+]_0$ is the external Na^+ concentration, E_r the reversal potential of the steady-state currents, R the gas constant, T the absolute temperature, F the Faraday constant and $RT/F = 24.83$ mV at 15°C. By normalization with respect to the maximum P value the steady-state probabilities p of open modified Na^+ channels were obtained: $p = P/P_{max}$. Such $p(V)$ curves were calculated for all experiments in which the reference potential was set to –48 mV. We checked that subtraction of the small tetrodotoxin-resistant current from I_s gave p values which were indistinguishable from those calculated without this correction for currents not flowing through Na^+ channels.

Batrachotoxin-modified Na^+ channels open and close with first-order kinetics [3]. Therefore, the time constant τ_m of activation at a given test potential was obtained by fitting a single exponential function to the current relaxation occurring within 2–3 ms after a voltage step from the reference to the test potential (see Fig. 2).

The differences between one-sided spectral densities and mean currents obtained from fluctuation measurements in test solution and in test solution with tetrodotoxin were considered as the respective quantities $S(f)$ and I arising from Na^+ channels. The following relation was fitted to the values of $S(f)$:

$$S(f) = \frac{S_0}{1 + (2\pi f \tau_c)^2} + \frac{S_1}{f} \tag{2}$$

where the first term describes a Lorentzian spectrum of amplitude S_0 and the second term S_1/f an additional contribution inversely proportional to the frequency f. The variance of conductance fluctuations was obtained by integration of the first term: $var = S_0/(4\tau_c)$ and was used to calculate the current i through a single channel and the number N of modified Na^+ channels per node:

$$i = var/(I(1 - p)) \tag{3}$$

$$N = I/(ip) \tag{4}$$

In these equations p denotes the steady-state probability at the test potential E. From i the chord conductance γ of a modified Na^+ channel was obtained through $\gamma = i/(E - E_r)$ and its permeability π by replacing in Eqn. 1 the mean current I by the single-channel current i.

Results

The calculation of the single-channel current i and the number N of channels requires the value of the probability p of the open-channel state (compare Eqns. 3, 4). We have determined these probabilities by normalizing stationary permeability values as calculated from Eqn. 1. Fig. 1 shows values of $p(V)$ obtained by averaging the results from experiments in which the reference potential was set to −48 mV. At $V = 0$ and −16 mV the mean probabilities are 0.75 and 0.27, respectively. For $V = -8$ mV, $p = 0.55$ is obtained from the solid curve through the points. These p values were used in the analysis of the first series of experiments with a −32 mV reference potential for which $p(V)$ curves had not been determined.

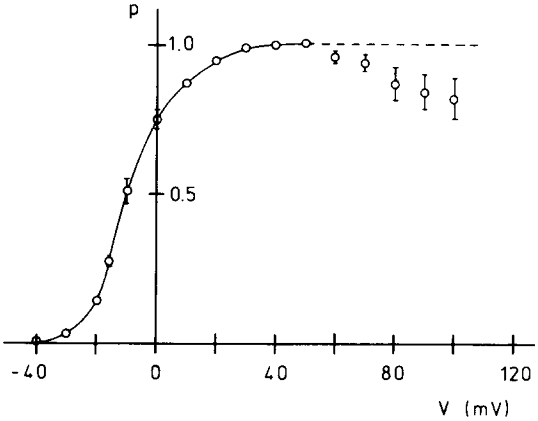

Fig. 1. Normalized steady-state Na^+ permeability p as function of voltage V. Symbols and bars represent mean values ± S.E. from 4–9 experiments. For data without bar S.E. is smaller than size of symbol. The curve through the points was drawn by eye. In the nine experiments used for analysis the resting potential was $E_0 = -71.6 \pm 1.2$ mV and the reversal potential of the steady-state current $E_r = 24.0 \pm 5.3$ mV (mean values ± S.E.).

In Figure 2 transient and steady-state currents are illustrated for two test potentials of 0 and −8 mV. The current relaxations at the beginning of the test pulse were obtained from photographs and the steady-state values between 145 and 473 ms from the analysis of current fluctuations. Note the faster rise of currents at 0 mV compared with the activation at −8 mV and the perfect horizontal time course of the steady-state currents. Similar results were found in all other experiments. The mean values of the activation time constant τ_m and of the steady-state currents I from all fibres are listed in Table I.

Fig. 3 shows one-sided spectral densities of Na^+-current fluctuations from the same fibre for which currents have been presented in Fig. 2. The curves through the measured points are fits corresponding to Eqn. 2, and the arrows give the locations of the corner frequencies $f_c = 1/(2\pi\tau_c)$ of the Lorentzian components. The values of the parameters S_0, τ_c and S_1 for this particular fibre are listed in the legend to Fig. 3. Similar to τ_m the time constant τ_c is smaller at 0 mV compared to the one at −8 mV. A summary of the fit parameters derived from all experiments is contained in Table I.

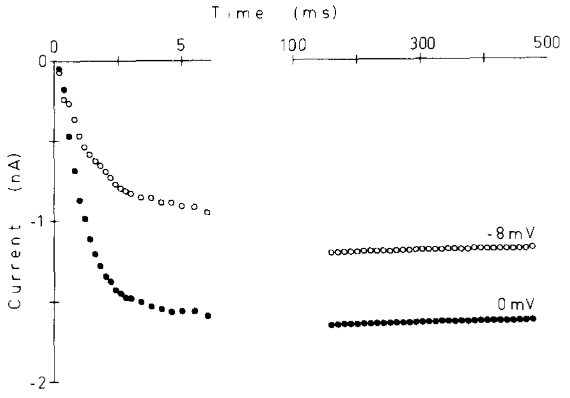

Fig. 2. Current relaxation and steady-state Na$^+$ currents at V = −8 mV (open symbols) and 0 mV (filled symbols). Note different time scaling during relations and in the steady-state. The activation time constants, τ_m, and the steady-state Na$^+$ currents, I, are 1.60 ms, −1.19 nA (−8 mV) and 0.85 ms, −1.64 nA (0 mV). Reference potential = holding potential = −32 mV. Experiment B2, motor fibre. Temperature 15°C.

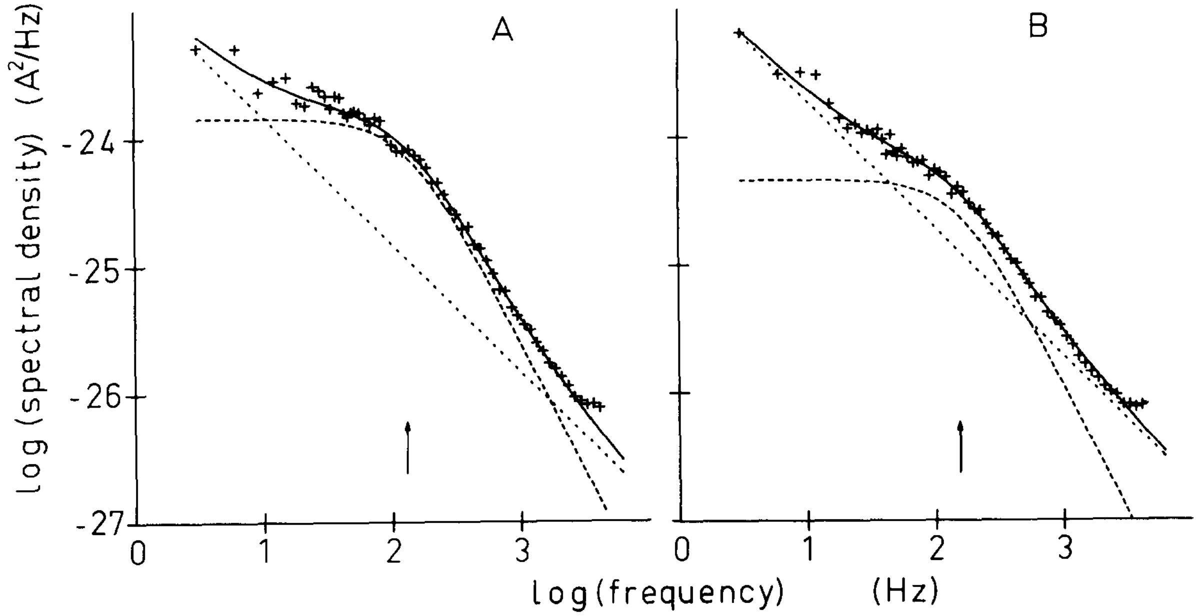

Fig. 3. Spectral densities of Na$^+$-current fluctuations at V = −8 mV (A) and 0 mV (B). Symbols represent data calculated from current fluctuations, solid lines are fits corresponding to Eqn. 2. The interrupted curves give the contributions of the Lorentzian functions and of the $1/f$ components (lines of slope −1). Arrows indicate the locations of the corner frequencies f_c. The values of the fitted parameters are: (A) S_0 = 1.43 pA^2/Hz, τ_c = 1.21 ms, S_1 = 14.8 pA^2/Hz and (B) S_0 = 0.46 pA^2/Hz, τ_c = 1.00 ms, S_1 = 19.4 pA^2/Hz. Experiment B2, motor fibre. Temperature 15°C.

TABLE I

PARAMETERS OF Na$^+$ CURRENT AND SPECTRAL DENSITY OF Na$^+$-CURRENT FLUCTUATIONS

Values are mean ± S.E., number of measurements in parenthesis.

V (mV)	I (nA)	τ_m (ms)	S_0 (pA^2/Hz)	τ_c (ms)	S_1 (pA^2)
0	1.59 ± 0.34 (6)	0.77 ± 0.05 (7)	0.24 ± 0.06 (7)	0.77 ± 0.06 (6)	16.0 ± 3.1 (6)
−8	1.53 ± 0.21 (7)	1.15 ± 0.09 (7)	0.73 ± 0.22 (7)	0.81 ± 0.17 (7)	20.6 ± 4.7 (7)
−16	0.78 ± 0.10 (13)	1.11 ± 0.08 (11)	0.82 ± 0.16 (14)	0.96 ± 0.06 (14)	12.3 ± 4.7 (13)

TABLE II

PARAMETERS OF MODIFIED Na^+ CHANNELS

Values are mean ± S.E., number of averaged values in parenthesis.

V (mV)	p	i (pA)	γ (pS)	π (10^{-15} cm^3 · s^{-1})	N ($\times 10^3$)
0	0.75 ± 0.03 (8)	0.18 ± 0.03 (5)	1.61 ± 0.23 (5)	5.67 ± 1.03 (5)	15.9 ± 6.3 (5)
−8	0.55	0.32 ± 0.05 (7)	3.21 ± 0.47 (7)	9.16 ± 1.43 (7)	11.4 ± 2.1 (7)
−16	0.27 ± 0.02 (9)	0.40 ± 0.04 (11)	3.45 ± 0.36 (11)	10.55 ± 0.95 (11)	10.7 ± 2.8 (11)

The calculation of the parameters i, γ, π and N of Na^+ channels modified by batrachotoxin was performed as described in Methods. Since no significant differences between motor and sensory fibres have been found, the results from both types of fibres have been averaged in Table II.

Discussion

After treatment with batrachotoxin the steady-state Na^+ current and the spectra density of Na^+-current fluctuations are orders of magnitude higher than under control conditions: Whilst without toxin a stationary Na^+ inward current of approximately 0.02 nA and a Lorentz amplitude $S_0 \approx 10^{-3}$ pA^2/Hz in the spectral density could be detected at the resting potential [11], the corresponding quantities at $V = 0$ after the application of batrachotoxin are 1.6 nA and 0.24 pA^2/Hz, respectively (compare Table I and Figs. 2,3B). This comparison confirms that the currents and current fluctuations recorded in the present investigation mainly originate from Na^+ channels modified by batrachotoxin and that our analysis yields the parameters of these channels.

At increasing depolarizations the steady-state currents rectify more than predicted by the Goldman-Hodgkin-Katz equation (Eqn. 1). This becomes apparent from the decline of the normalized permeabilities $p(V)$ in Fig. 1 at potentials $V > 50$ mV. In contrast, the peak Na^+ currents under control conditions can be described satisfactorily by permeabilities reaching a plateau value at about 80 mV [12]. Nevertheless, we have used permeabilities as calculated from Eqn. 1 to determine the probability of the open state of Na^+ channels modified by batrachotoxin. The alternative formulation of current-voltage curves $I_s(E)$ in terms of a chord conductance $g = I_s/(E - E_r)$ would produce g values declining even more strongly than the permeabilities at increasing depolarizations.

Our recorded spectral densities exhibit significant excess fluctuations between 3 and 50 Hz (see Fig. 3), though slow systematic variations of the mean current producing low-frequency contributions were compensated as described recently [8]. Therefore, we had to add the term S_1/f to the right-hand side of Eqn. 2 to obtain a decent fit of the spectral densities in the whole frequency range between 3 Hz and 5 kHz. Because of the perfect horizontal time course of the mean currents during the fluctuation measurements, illustrated in Fig. 2 and found in all other experiments, this '$1/f$ noise' cannot be due to some trends in the membrane current which had not been eliminated. Hence it should originate from the Na^+ ion flux through open channels.

Since at present no satisfactory theory on $1/f$ noise in membranes exists [13], we tried to apply the following empirical relation [14] to our fitted S_1/f components:

$$S(f) = \alpha \frac{I^2}{n} \cdot \frac{1}{f} \qquad (5)$$

In this equation n denotes the total number of mobile charge carriers in the system, and the dimensionless parameter α is approximately 10 for 1 M salt solutions and is proportional to the concentration [14]. The empirical relation predicts a proportionality between the amplitude S_1 and the square of the mean current, I, which is roughly fulfilled in our experiments. However, unreasonably high numbers n of mobile ions are derived if $\alpha = 1$ is taken for 0.1 M solutions. Referred to the number N of modified Na^+

channels it is $n/N = I^2/(S_1N) = 11.5 \pm 1.4$ (mean ± S.E., 23 measurements at $V = 0, -8, -16$ mV). A still higher number of $n/(pN) = 28.2 \pm 3.4$ follows if I^2/S_1 is divided by the number $p \cdot N$ of open modified channels. Thus the empirical relation (Eqn. 5) does not yield a meaningful number of mobile sodium ions per modified Na^+ channel.

The first component of amplitude S_0 in Eqn. 2 gives the contribution from the statistical opening and closing of modified Na^+ channels to the spectral density of Na^+-current fluctuations. This identification follows from the approximate agreement between the parameter τ_c obtained in the fit of the spectra and the activation time constant τ_m derived from the relaxations of the macroscopic currents (compare Table I). Since modified Na^+ channels open and close with first-order kinetics, only one Lorentzian component appears in Eqn. 2, whereas the sum of three Lorentzian functions was used to describe the m^3 activation kinetics of normal Na^+ channels [11]. The variance of the Lorentzian component was then used to calculate the single-channel current i and the number N of modified Na^+ channels with Eqns. 3 and 4. For the derivation of the equations it must be assumed that all channels are identical and non-interacting, and have only one conducting state [6]. The validity of these assumptions cannot be tested directly from our experiments, but indirect arguments in favour of these channel properties will be presented below.

The number N of modified channels is not significantly dependent on the test voltage and is about $12 \cdot 10^3$ per node (see Table II). Since approximately 10^5 Na^+ channels are located in the nodal membrane of the same frog species at a hyperpolarizing holding potential [15], approximately 12% of all Na^+ channels have been modified by batrachotoxin. This fraction could be increased by applying higher toxin doses or by stimulating the fibre longer in the presence of batrachotoxin. But the number of modified channels employed in our experiments was already sufficient to record clean mean currents and spectral densities needed for an accurate determination of single-channel parameters. For a nodal area of 35–60 μm^2 [16] the average distance between modified Na^+ channels in our experiments would be 54–71 nm. Since no indications for interactions between the more densely distributed normal Na^+ channels have been detected [17], the modified Na^+ channels should also operate independently of each other.

The current i through a single open modified Na^+ channel is strongly voltage-dependent (0.18 and 0.40 pA at 0 and −16 mV, respectively). Part of this rise of i with increasing hyperpolarization is due to an altered driving force $E - E_r$ between the test potential E and the reversal potential E_r. But the single-channel conductance $\gamma = i/(E - E_r)$ exhibits the same voltage dependence as i (compare Table II). However, the use of chord conductances may not be appropriate, since the calculation of γ assumes a linear current-voltage relation whereas the probability p needed to determine i was derived from the non-linear Goldman-Hodgkin-Katz equation (Eqn. 1). To avoid this inconsistency, we have calculated the permeability π of a single modified Na^+ channel, but this quantity also assumes higher values at increasing hyperpolarizations (see Table II). For normal Na^+ channels a reverse voltage dependence of γ has been found and interpreted by an incomplete closure of Na^+-channels by the activation-gating subunits [11]. Since such a multistate model yields a conductance increase with increasing depolarizations, it cannot explain our observed anomalous voltage dependence of γ and π for modified Na^+ channels. A possible reason for this behaviour could be the use of permeabilities in our analysis which do not saturate but decline at strong depolarizations (see Fig. 1). Hence, we could have overestimated the p value at −16 mV or underestimated it at 0 mV. Both possibilities would weaken our observed voltage dependence of the γ and π values.

For the conductance of normal Na^+ channels in myelinated nerve values of 7.9 pS [11], 6.4 pS [6] and 8.85 pS [8] have been reported. These results are significantly higher than the conductances between 1.61 and 3.45 pS found for modified Na^+ channels. This difference is surprising since Na^+ channels modified by batrachotoxin are permeable to several relatively big cations (e.g. methylammonium) which cannot pass through normal Na^+ channels [3]. Therefore, we had expected a higher conductance of modified channels compared to the one of normal Na^+ channels. The opposite result might be explained by the simultaneous movement of ions of different kinds through a modified Na^+ channel, thereby reducing its conductance.

We have tried to test this hypothesis by investigating the effects of several possible permeable ions on the steady-state currents and the spectral density of current fluctuations. Since K^+ is not present in our extra- and intracellular solutions, this cation cannot be responsible for the observed small conductance of modified Na^+ channels. Therefore, we began to study the effects of tetraethylammonium ions, 10 mM of which were added to all extracellular solutions to block K^+ channels (see Methods). If this cation would be permeable through modified Na^+ channels, drastic changes in the reversal potential E_r of the steady-state currents and in the single-channel parameters should occur after inverting the concentration gradient of this ion. However, in four experiments with 113 mM tetraethylammonium chloride instead of 113 mM CsCl in the axoplasm solution no changes of E_r or of the single-channel conductance could be detected. This excludes the possibilities that tetraethylammonium or Cs^+ interfere with the Na^+ flux through modified Na^+ channels. Next, we performed experiments with extracellular test solutions in which Cl^- had been replaced by acetate or methylsulfate anions. Again, no clear shifts of E_r after an exchange of anions were found. This confirms an earlier observation that the permeability of modified Na^+ channels to Cl^- is negligible [3]. Finally, we tested the effects of Ca^{2+} in the extracellular solution, which is known to produce a measurable inward current through modified Na^+ channels [3]. We have found (unpublished data) that raising the extracellular Ca^{2+} concentration from 2 to 10 mM shifts the peak and steady-state Na^+ permeability curves by 15 mV into the depolarizing direction but reduces the peak permeability $P_{Na^+}^{peak}$ less than the steady-state permeability $P_{Na^+}^{ss}$ (reduction factors 0.76 and 0.63 for $P_{Na^+}^{peak}$ and $P_{Na^+}^{ss}$, respectively). The shift and reduction of $P_{Na^+}^{peak}$ with increasing Ca^{2+} concentration can be explained by a decrease of the number of negative outer surface charges around normal Na^+ channels at the nodal membrane and is in agreement with the results of a previous investigation [18]. The stronger reduction of the steady-state permeability $P_{Na^+}^{ss}$ implies that the decrease of the stationary Na^+ currents through modified channels cannot be explained by surface-charge effects alone. This indicates a specific action of Ca^{2+} on the Na^+ flux through the modified channels and could be the origin of the small conductance of these channels.

In conclusion, this investigation has shown that the two components of Na^+ currents in myelinated nerve observed after treatment with batrachotoxin originate from two types of Na^+ channel with different single-channel conductances. This supports the idea that batrachotoxin acts in an 'all-or-none' manner on Na^+ channels and creates a distinct population of modified channels in the nodal membrane.

Acknowledgements

This research was supported by the Deutsche Forschungsgemeinschaft, SFB 38 'Membranforschung', Bonn-Bad Godesberg. We thank Mrs. M. Sinnwell for help with the manuscript.

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