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Differences in open state of NBA-modified cardiac Na^+ channels

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Abstract. Patch clamp recordings from neonatal cardiac Na^+ channels treated with *N*-bromoacetamide (NBA, $5-50 \times 10^{-}$ mol/l) showed modified Na^+ channel activity. By chemical removal of inactivation, repetitive openings with an increased life time and burst-like activity occurred. NBA-modified Na^+ channels differ in life time and may attain either a slightly (mean open time 3.1 ± 0.2 ms) or a strongly (mean open time 15.2 ± 1.4 ms) prolonged open state. This strongly suggests a heterogeneous population of NBA-modified Na^+ channels in newborn rat cardiocytes.

Key words: Patch clamp, cardiac Na^+ channels, channel modification, heterogeneous population

Introduction

Cardiac Na^+ channels are of outstanding significance for excitation and impulse conduction in heart muscle. Patch clamp recordings in embryonic, neonatal and adult cardiocytes of various species have provided evidence for a non-uniform channel population. Differences in the unitary current size (Cachelin et al. 1983; Kunze et al. 1985; Kohlhardt et al. 1986a), differences in the TTX-sensitivity (Ten Eick et al. 1984), late openings which underly the slowly inactivating component of Na^+ current (Patlak and Ortiz 1985; Kunze et al. 1985) and spontaneous failure of inactivation (Kohlhardt et al. 1986a) indicate divergent channel properties. Such divergent properties reflect hetero- or tautochannels (Benoit et al. 1985), i.e. several classes or a channel which may switch between different configurations. Their functional importance for action potential generation are not yet completely understood. The present patch clamp experiments with the chemical *N*-bromoacetamide (NBA), proven to eliminate Na^+ inactivation irreversibly (Oxford et al. 1978), demonstrate another example for a heterogeneous population, namely different open state kinetics in NBA-modified cardiac Na^+ channels.

Methods

Single Na^+ channel currents were recorded at 19 °C with an L/M-EPC 5 amplifier (List Electronic, Darmstadt) in inside-out patches excised from neonatal cardiocytes by employing the standard patch clamp technique (Hamill et al. 1981). The cells were disaggregated from 2 to 4-day-old rats and cultured for 15–20 h before use. Before dissecting a patch, the seal formation between the patch pipette and the cell membrane developed under conditions where the cardiocytes were kept in a physiological saline containing glucose and pyruvate as substrates. The patch clamp records were filtered at 1 kHz using an eight-pole Bessel filter, digitized with a sampling rate of 5 kHz, and stored on floppy discs. Details of the methods including data acquisition and analysis were essentially the same as previously described (Kohlhardt et al. 1986b).

Solutions (*composition in* mmol · l^{-1})

A. Pipette solution (extracellular solution): NaCl 137; $CaCl_2$ 0.2; $MgCl_2$ 10; Hepes 10, pH 7.4.
B. Intracellular solution (facing the cytoplasmic side of the inside-out patches): CsCl 130; NaCl 5; KCl 5.4; MgCl 2.5; glucose 10; EGTA 2; Hepes 10; pH 7.4.
N-bromoacetamide was purchased from Sigma Chemie, Munich. To avoid degradation, NBA-containing solutions were protected from light.

Results and discussion

Modification of cardiac Na^+ channels resulting from treatment with the peptide bond-cleaving reagent NBA required the exposure of the cytoplasmic surface of the membrane to the chemical. Other than in myelinated nerve fibres (Wang 1984), treating the external face proved ineffective. Consistent with results in other excitable membranes (Patlak and Horn 1982), the

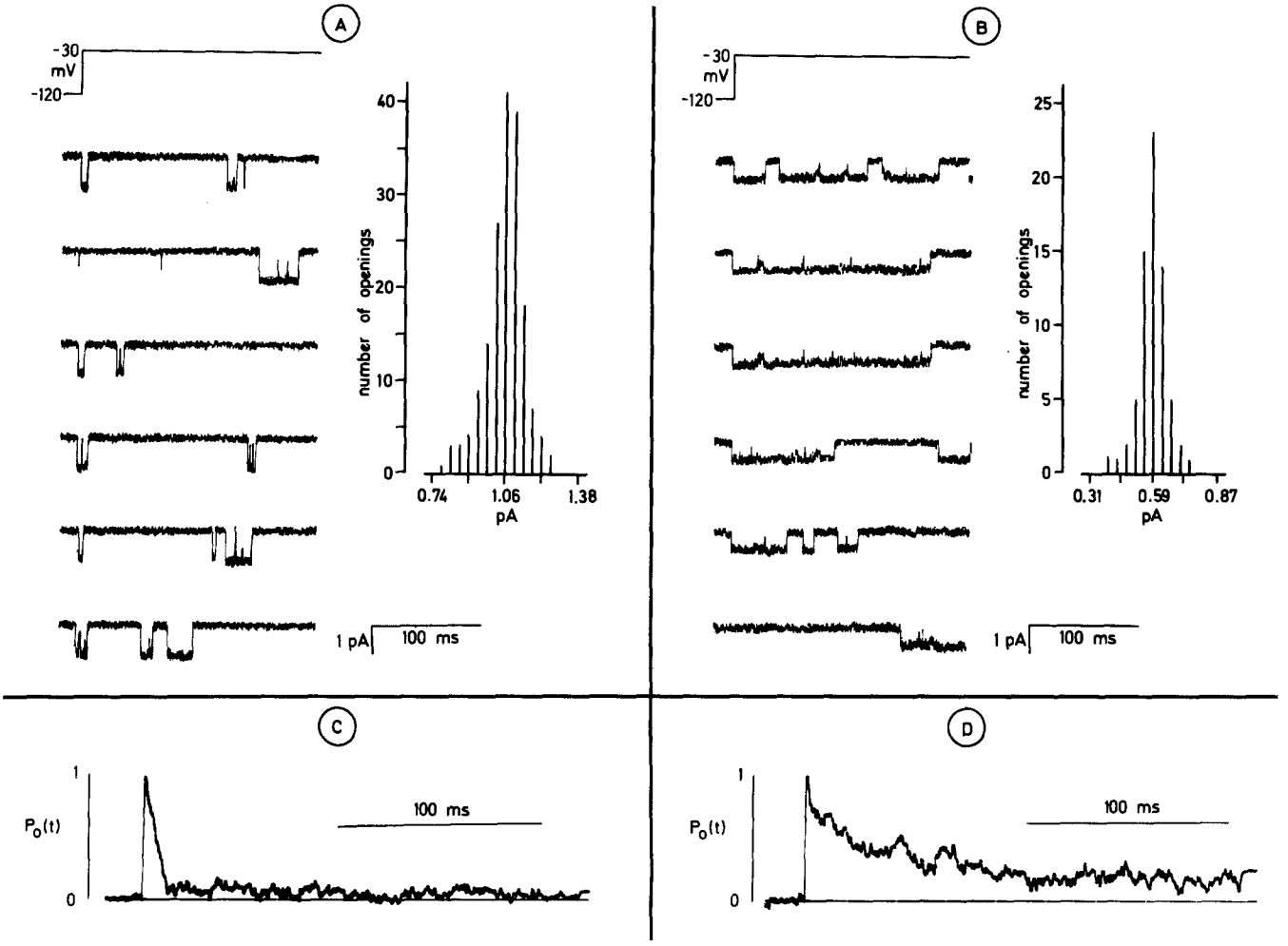

Fig. 1 A–D. NBA-modified cardiac Na^+ channels. *Upper part*: **A** and **B:** Consecutive records of single Na^+ channel currents from two inside-out patches (Patch 245 and 244) exposed to the same NBA concentration (5×10^{-5} mol/l) at the cytoplasmic face and kept under identical conditions (holding potential -120 mV; step potential -30 mV). The samples are corrected for capacity and leakage currents and were low-pass filtered at 1kHz. The insets demonstrate the amplitude distributions in both patches. The Gaussian function yielded a peak value of 1.02 ± 0.008 pA (patch 245, part **A**) and of 0.59 ± 0.07 pA (patch 244, part **B**). *Lower part:* **C** and **D:** Ensemble averages of the single Na^+ channels currents recorded from the patches shown in **A** and **B**. For comparison, both averages are normalized and presented as $P_0(t)$ which may vary between 0 and 1. The *solid line* indicates zero current

NBA concentration needed for removal of Na^+ inactivation varied considerably from one patch to another and ranged from 5 to 50×10^{-5} mol/l. Bathing the cytoplasmic face of the sarcolemma in an effective concentration instantaneously induces modified Na^+ channel activity. All patches contained 2–3 functioning Na^+ channels and were equilibrated with NBA for 3 min before the recording period began. They were stepped from a holding potential close to -120 mV to -30 mV for 220 ms at a rate of 0.33 Hz.

NBA-modified Na^+ channel activity is demonstrated in Fig. 1. Consistent with results in rat myotubes (Patlak and Horn 1982) or in GH_3 cells (Horn et al. 1984), membrane depolarization triggered long-lasting elementary Na^+ currents. Channel activity continued to appear until the membrane was repolarized to the holding potential. Short gaps in the 1 ms range chopped most of the openings but the gaps between these grouped openings lasted several tens of milliseconds. Despite the application of very different NBA concentrations, uniform channel life time was found (Table 1). This apparent concentration independence suggests that the chemical modifies Na^+ channels in an all-or-none fashion.

In comparing both patches illustrated in Fig. 1 it becomes evident that NBA-modified cardiac Na^+ channels may differ from each other in their mean open time, $\bar{t}_0$, and in the time-dependent open probability, $P_0(t)$. As a result from 5 inside-out patches, two different responses can be distinguished. Compared with the life time in normal Na^+ channels found to be 1.50 ± 0.14 ms at -30 mV in 4 control inside-out patches, $\bar{t}_0$ can be prolonged by a factor of about 2 or of almost 10 (see Table 1). Moreover, $P_0(t)$ declined

Table 1. Open state characteristics and unitary current amplitude of NBA-treated cardiac Na^+ channels at -30 mV. Compared with mean open time of normal Na^+ channels in control patches at -30 mV (1.50 ± 0.14 ms) the data obtained in the presence of NBA were grouped into a slightly (upper part) and into a strongly (lower part) increased open time. The two resultant mean values differed significantly ($p < 0.01$) from each other. Mean open time was calculated from $\bar{t}_0 = \sum_i T_i n_i / n$ (Fenwick et al. 1982) where T_i is the duration of n_i channels and n means a count of events. The unitary current was calculated from the Gaussian function of the elementary current amplitude distribution

Patch	Mean open time [ms]	i_{unit} [pA]	NBA concentration [mol/l]
245	3.5	1.02	5×10^{-5}
248	3.2	1.13	1×10^{-4}
249	2.7	1.25	5×10^{-5}
Mean ± SEM	3.1 ± 0.2 ms	1.13 ± 0.07 pA	
244	18.0	0.59	5×10^{-5}
253	14.0	0.66	1×10^{-4}
248/2	13.6	0.74	5×10^{-4}
Mean ± SEM	15.2 ± 1.4 ms	0.66 ± 0.04 pA	

much slower during depolarization and approached a larger steady state value in the patches showing elementary Na^+ currents with the larger life time (compare Fig. 1 C and 1 D). One reason for this unexpected difference in $\bar{t}_0$ could be that the Na^+ channels present in an individual patch do not possess the same NBA vulnerability. That is, some of them might be resistant to a given NBA concentration while the other fraction responds to the chemical.

This possibility may be tested by a biased open time analysis based on the time-dependent open probability of normal Na^+ channels. As found in reconstructed macroscopic Na^+ currents averaged from control patches at -30 mV, $P_0(t)$ declines rapidly with a time constant of 1.1 ± 0.1 ms ($n = 4$) and reaches 0.5% of $P_0(t)_{maximal}$ within the first 10 ms of membrane depolarization. Openings in the NBA-treated patches occurring during this early phase were, therefore, suspected to be potentially normal events arising from the activity of a NBA-resistant Na^+ channel. In fact, some of them showed a life time close to the normal value in control patches, 1.5 ms. Nevertheless, disregarding these openings and calculating $\bar{t}_0$ from events occurring after the initial 10 ms period yielded a value which is only slightly larger than that obtained from the unbiased analysis (4.3 ms instead of 3.5 ms in patch 245). This seems to justify the conclusion that two types of NBA-modified Na^+ channels exist. NBA treatment usually induced in an individual patch either the long (15 ms) or the short (3 ms) channel life time. This is noteworthy in that all patches studied contained more than one functioning Na^+ channel.

It was a consistent finding that the unitary current size was larger in short-lasting than in long-lasting Na^+ channel openings. It amounted to 1.13 ± 0.07 pA in the former which is close to the current size found in normal Na^+ channels at the same membrane potential (-30 mV), but 0.66 ± 0.04 pA in the latter. The small current might be due to a reduced channel conductance or could result from a loss of the channel selectivity for Na^+ and might be another consequence of the NBA treatment. Then, the effect of the chemical might be more complex and not restricted to the gating process. This clearly disagrees with NBA-treated Na^+ channels in rat myotube membranes (Patlak and Horn 1982) showing similarly long-lasting, but exclusively fully sized openings. Whether this discrepancy might be related to the presence of Mg^{++} ions at the cytoplasmic membrane surface in our experiments remains to be elucidated. On the other hand, neonatal cardiac Na^+ channels might, analoguous to the differential TTX-sensitivity of embryonic and adult skeletal muscle Na^+ channels (Weiss and Horn 1986), not share with adult channels all properties and, therefore, respond specifically to NBA.

Fully sized, short-lasting and small sized, long-lasting channel openings were found to coexist occasionally during depolarization (Fig. 2), a phenomenon which might indicate that an individual Na^+ channel once activated may switch between the two states. This idea could not be supported by a likelihood analysis according to

$$L_{(N)} = \left(\frac{1}{1 + \left(\frac{N-1}{N} \right) \cdot \frac{MOT}{MC}} \right)^n$$

(Colquhoun and Hawkes 1983). N means the number of channels whose likelihood to open sequentially is tested, in this case 2; MOT and MC refer to mean open and mean closed time in the sample and n is the number of events in the sample. Fully sized, short-lasting and small sized, long-lasting openings are estimated to originate with a likelihood of 70% from two Na^+ channels.

NBA reacts with several amino acids including methionine, cysteine and cystine. The chemical cleaves peptides by binding with tryptophane, tyrosine and histidine (Means and Feeney 1971). The critical reaction responsible for the irreversible channel modification has yet to be identified. The resultant removal of Na^+ inactivation may be expected to develop in an all-or-none fashion, i.e. the covalent binding of the NBA molecule with its target destroys the process which terminates the open state under normal conditions. Consequently, two channel populations may be assumed in order to explain the observed differences in the open state kinetics of neonatal cardiac Na^+ channels. As Na^+ channels are supposed to leave their

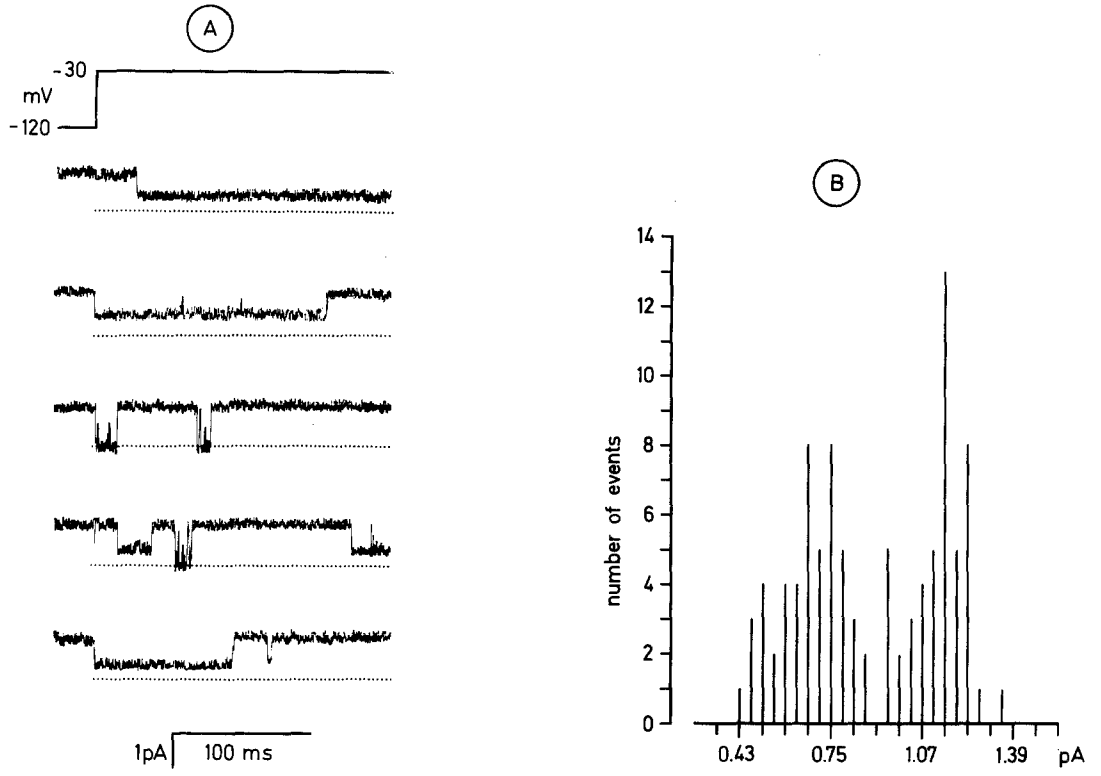

Fig. 2. A. Consecutive single Na^+ channel recordings from an ensemble of 80 samples collected in a NBA-treated (5×10^{-5} mol/l) inside-out patch from a cultured cardiocyte. The patch was stepped from -120 mV to -30 mV at a rate of 0.33 Hz. The samples (corrected for capacity and leakage currents and filtered at 1 kHz) show two unitary current levels. Taking the fully sized current (1.14 pA) and indicated by the dotted lines as a guide, a value of 3.1 ms was obtained for $\bar{t}_0$. The life time in the small sized openings (0.70 pA) amounted to 13.9 ms. **B**. Histogram of elementary Na^+ current amplitudes as recorded in the same patch. A Gaussian analysis yielded a bimodal frequency distribution with a maximum at 0.70 pA and at 1.14 pA

open state, after NBA treatment, by a transition into a closed state (Horn et al. 1984), this exit rate might distinguish the both populations.

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