

Short communication

Glutathione accelerates sodium channel inactivation in excised rat axonal membrane patches

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Abstract. The effects of glutathione were studied on the gating behaviour of sodium channels in membrane patches of rat axons. Depolarizing pulses from -120 to -40 mV elicited sodium currents of up to 500 pA, indicating the simultaneous activation of up to 250 sodium channels. Inactivation of these channels in the excised, inside-out configuration was fitted by two time constants ($\tau_{h1} = 0.81$ ms; $\tau_{h2} = 5.03$ ms) and open time histograms at 0 mV revealed a biexponential distribution of channel openings ($\tau_{short} = 0.28$ ms; $\tau_{long} = 3.68$ ms). Both, the slow time constant of inactivation and the long lasting single channel openings disappeared after addition of the reducing agent glutathione (2–5 mM) to the bathing solution. Sodium channels of excised patches with glutathione present on the cytoplasmatic face of the membrane had inactivation kinetics similar to channels recorded in the cell-attached configuration. These observations indicate that redox processes may contribute to the gating of axonal sodium channels.

Key words: sodium channel, axon, glutathione, metabolism, reducing agent, cysteine, disulfide bridges

Introduction

Multiple time constants have been described for inactivation of voltage-dependent neuronal Na^+ channels. Different mechanisms were discussed to explain this observation: (a) inactivation via a multi step process, (b) different populations of Na^+ channels and (c) switching of individual channels between two principal gating modes (for review see [5,7]). A further possibility was raised by recent data obtained from K^+ channels. Ruppertsberg et al. [9] observed that inactivation of cloned K^+ channels is regulated by the reducing agent glutathione. These authors proposed a model in which disulfide bridges alter the mobility of the inactivation "ball". In the present study we explored the effects of glutathione on Na^+ channels from rat axons.

Materials and Methods

Male Wistar rats (250–350 g) were anaesthetized with urethane (1.25 g/kg, i.p.). After laminectomy, 5–10 dorsal spinal roots were removed and transferred into a culture dish. Enzymatic dissociation and paranodal demyelination were performed in two steps according to the procedures described by Jonas et al. [2] with some modifications. Spinal roots were first treated at 37°C with collagenase ("Worthington" type CLS II, 135 U/mg; Biochrom, Berlin; 3 mg/ml, diluted in normal Ringer solution) for 80 min and subsequently incubated for 30 min in protease (type XXIV; 8 U/mg; Sigma, Deisenhofen, Germany; 1 mg/ml, diluted in calcium-free Ringer solution). After this procedure the spinal roots were dissociated by gentle shaking and transferred into dishes precoated with poly-L-lysine (1 mg/ml; Sigma). Single channel recordings were performed in a bathing solution of the following composition (in mM): CsCl 145, CaCl_2 0.46, MgCl_2 1.18, EGTA 1.0, HEPES 10, pH 7.2 (adjusted with NaOH). The pipette solution contained (in mM): Na gluconate 150, CaCl_2 2.2, MgCl_2 1.2, HEPES 10, pH 7.4. Glutathione was added to the bathing solution via a multi pipette array positioned close to the patch pipette. Experiments were performed at room temperature by means of standard patch clamp techniques. Patch pipettes were drawn by a DMZ puller (Zeitz, Augsburg, Germany) from borosilicate glass tubings (GC 150 TF-10, Clark Electromedical Instruments, Pangbourne, U.K.), coated with Sylgard and fire-polished immediately before the experiment. The pipettes had resistances between 10–20 M Ω . Recordings were made with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, U.S.A.). The current signals were low pass filtered at 2 kHz and digitized at a sampling rate of 10 kHz. They were stored and analyzed using a Tandon 386/33 computer and pClamp 5.5 software (Axon Instruments). Averaged data are expressed with mean \pm SEM.

Results

Sodium currents were elicited in about 20% of the nodal/paranodal membrane patches. These currents had variable amplitudes between a few and up to 500 pA indicating the activation of one or up to 250 single sodium channels. Voltage steps from -120 to -40 mV

in the cell-attached configuration resulted in sodium channel inactivation which was fitted with a single exponential of 0.83 ± 0.14 ms in 69 different axon patches from 15 rats. However, a striking alteration in inactivation was observed within a few minutes after excision of the membrane patches to an inside-out configuration. Fitting of the inactivation kinetics in this situation required two time constants: an unchanged fast component ($\tau_{h1} = 0.81 \pm 0.26$ ms) and a new slow component ($\tau_{h2} = 5.03 \pm 3.1$ ms). These are averaged data from 51 patches with successful transition from the cell-attached to the inside-out configuration.

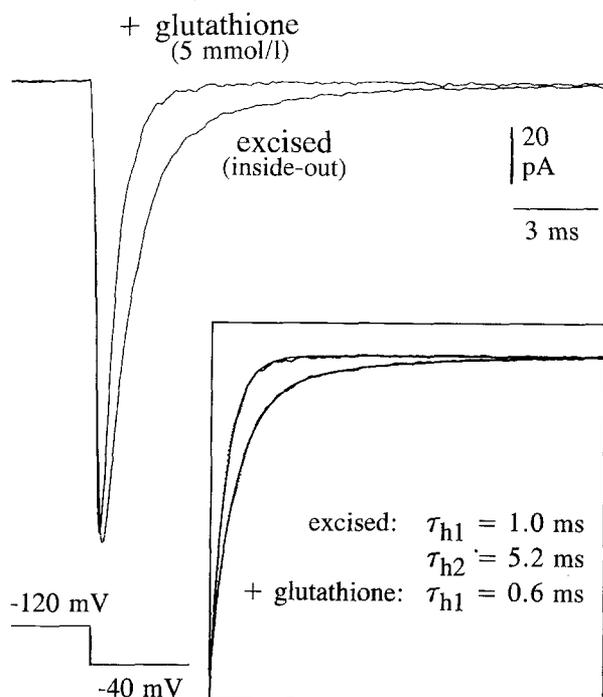


Fig. 1. Glutathione accelerates inactivation of excised axonal sodium channels. The figure shows a multi channel recording obtained with a patch pipette from a paranodally demyelinated rat dorsal root axon. Sodium currents were induced by changing the membrane potential from -120 to -40 mV (averaged currents from 10 identical voltage steps; automatic subtraction of leakage and capacitive currents by means of a prepulse protocol). Such voltage steps were performed on an excised membrane patch (inside-out configuration) and after the application of glutathione (5 mmol/l) to the bathing solution around the patch pipette (cytoplasmic face of the membrane). The time constant(s) of inactivation were determined as illustrated in the inset (11% of the current amplitude declined with slow kinetics).

We tested the effects of the reducing agent glutathione in view of the possibility that slowing of inactivation might be due to cysteine oxidation as described for potassium channels [9]. Such an experiment is illustrated in Fig. 1. The figure shows a multi channel recording obtained with a patch pipette from a rat dorsal root axon. Illustrated are the

averaged current traces of 10 voltage steps from -120 to -40 mV. Such voltage steps were performed on an excised membrane patch (inside-out configuration) and 2 min after the application of glutathione (5 mM) to the cytoplasmic face of the membrane. Glutathione accelerated sodium channel inactivation by changing the kinetics from a process with two time constants to a current decline with single exponential decay. Averaged data from 14 inside-out membrane patches exposed to glutathione (2-5 mM) show an inactivation time constant of 0.80 ± 0.10 ms.

Another difference between the kinetics of sodium channels in the cell-attached and excised, inside-out configuration was revealed in open time histograms. Open times were calculated from single channel currents recorded for several seconds following a voltage step from the holding potential of -120 mV to either -30 or 0 mV (late or background currents in the terminology of Patlak & Ortiz [8]). Only openings longer than 0.1 ms were included in the analysis. A uniform distribution of voltage-independent openings was found in cell-attached recordings ($\tau_{open} = 0.24 \pm 0.02$ ms at -30 mV, $n=14$; 0.24 ± 0.06 ms at 0 mV, $n=14$). Open time histograms from excised, inside-out patches, on the other hand, revealed a biexponential distribution of short and long lasting open times ($\tau_{short} = 0.22 \pm 0.02$ ms, $\tau_{long} = 4.40 \pm 2.3$ ms at -30 mV, $n=16$; $\tau_{short} = 0.28 \pm 0.2$ ms, $\tau_{long} = 3.68 \pm 2.5$ ms at 0 mV, $n=30$). Addition of glutathione to the bathing solution suppressed the long-lasting single channel openings seen in excised membrane patches. This is illustrated in Fig. 2. Open time histograms calculated from 12 experiments with glutathione (5 mM) present in the bathing solution revealed a single time constant of 0.15 ± 0.01 ms at -30 mV ($n=8$) and of 0.21 ± 0.03 ms at 0 mV ($n=7$).

Discussion

Alterations in inactivation of single axonal sodium channels caused by glutathione may indicate that cysteine-based disulfide bridges contribute to the kinetics of this process as suggested by Ruppertsberg et al. [9]. However, in contrast to potassium channels with an established "ball and chain" model of inactivation, the possible contribution of cysteine residues to inactivation of sodium channels is unclear. Stühmer et al. [11] and Vassilev et al. [12] have shown that the region between repeats III and IV is important for the inactivation of sodium channels. However, the responsible peptide segment in this region of the protein does not contain cysteine [12]. On the other hand, cysteine is found in several other peptide segments and modifications of these cysteines may be indirectly involved in the inactivation process. Furthermore, it is interesting to note that the oxidizing agent iodate, which is known to promote disulfide bridges (for example in insulin) inhibits inactivation of nodal sodium channels [10].

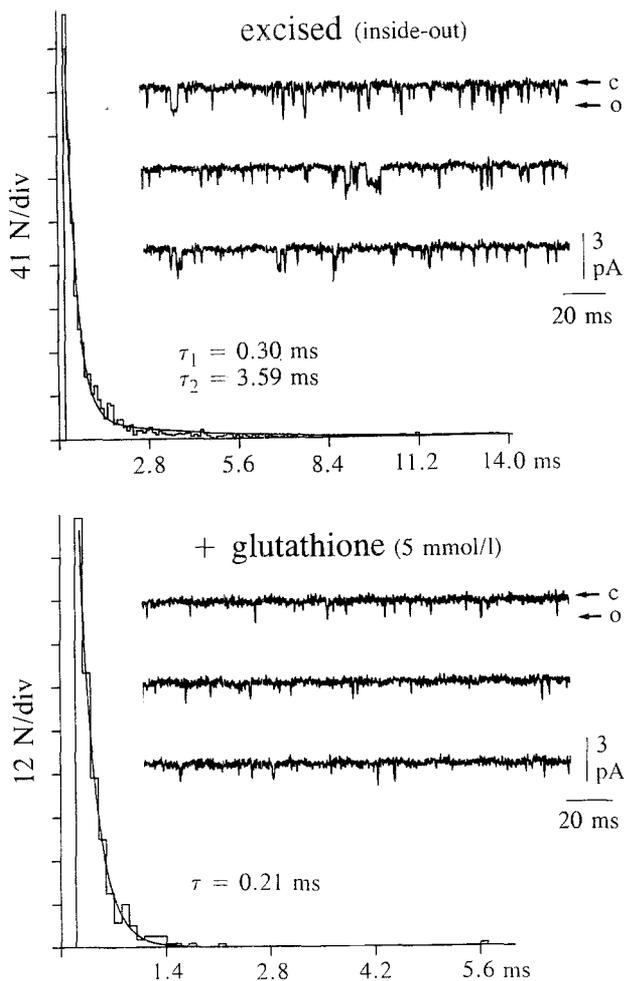


Fig. 2. Effects of glutathione on the open time histogram of excised axonal sodium channels. Single sodium channel current activity was observed for several seconds following a change in the membrane potential from -120 to 0 mV (late or background currents in the terminology of Patlak & Ortiz [8]). This experimental protocol was performed on an excised membrane patch (inside-out configuration) before and after the application of glutathione (5 mmol/l) to the bathing solution around the patch pipette. The open time histograms were determined as illustrated in the insets.

Alterations in gating of sodium channels caused by membrane excision have been described by many authors. A general finding is a negative shift in the voltage dependence of permeability parameters, first described by Fenwick et al. [1]. Changes in current decay and single channel openings similar to our findings were seen in cardiac myocytes [6]. On the other hand, it was concluded that sodium channels in excised membrane patches largely preserve their intrinsic kinetic properties when they face a "physiological" cytoplasmic environment [3]. One reason for such different experimental results might be that the usual way of analysis of single channel inactivation kinetics is based on averaged ensemble currents of just a few channels. The nodal axonal membrane, however, is unique with respect to the high density of sodium channels. For example, in

some of our recordings, patches contained more than 250 channel proteins. As a consequence, changes in gating of only a few percent of the channels can be detected.

Sodium channels of excised patches with glutathione present on the cytoplasmic face of the membrane had inactivation kinetics similar to channels recorded in the cell-attached configuration. This observation indicates that redox processes contribute to changes in gating of axonal sodium channels caused by membrane excision.

It has been a general view that the kinetics of sodium channels are mainly regulated by membrane potential. However, the observed effects of glutathione are another example of a possible direct link between cellular metabolism and functional activity of sodium channels. Other known factors in this respect are phosphorylation, G protein interaction (for review see [7]), and metabolites of the glycolytic pathway [4].

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