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Existence of two fast inactivation states in cardiac Na channels confirmed by two-stage action of proteolytic enzymes

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Fast inactivation of Na channels in neonatal cardiac cells was removed by the action of proteolytic enzymes trypsin or papain. Two stages were apparent in the time course of this process. During the first one, both number of channel reopenings and the mean open time increased markedly even though fast inactivation remained complete. The second stage was manifested by the disappearance of all signs of fast inactivation without further noticeable changes in channel mean open time. At the same time the nonrandom clustering of blank response (response without channel openings) trials became prominent. The data obtained support the interpretation of two separate fast inactivation states in cardiac Na channels as suggested in our previous papers (Zilberter et al. (1989) in Neuromuscular Junction (Sellin, L.C., Libelius, R. and Thesleff, S., eds.), pp. 43–50, Elsevier, Amsterdam, and Zilberter et al. (1991) J. Mol. Cell. Cardiol. 23, (Suppl.) 61–72).

Introduction

The present paper continues our studies of cardiac Na channel inactivating states [1,2]. In these previous publications the properties of a Ca-dependent slow inactivation state were described and the results suggested the existence of a second, fast inactivation state between resting state(s) and the above mentioned slow inactivation state (see kinetic scheme in Fig. 3A). Assuming that different fragments of a channel protein are responsible for the ordinary and the new fast inactivation states, one could expect their consecutive (nonsimultaneous) removal under the action of appropriate chemicals. Demonstration of this result would be an additional argument in favour of the presence of two fast inactivation processes. We used trypsin and, in some experiments, papain which are known to remove fast inactivation by specifically affecting arginine and lysine residues in the Na channel molecule [3]. In addition, they are less detrimental to the cell membrane compared to pronase, NBA and other chemicals [4,5]. The action of trypsin and papain was studied previously on squid axon [6], GH_3 cells [7] and neuroblastoma cells [8], however, the transition from normal to fully modified Na channels was not described in detail.

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Materials and Methods

Cardiomyocytes from neonatal rats were cultured as described elsewhere [9] and routinely used within 24–48 h. The single channel recordings were performed by a standard path-clamp technique (inside-out patch configuration) [10]. The extracellular solution contained (mM): 140 NaCl; 5.4 KCl; 1 $CaCl_2$; 1.2 $MgCl_2$; 10 glucose; 5 Hepes/Na^+ buffered to pH 7.3. The solution on the cytoplasmic side of the membrane was composed of (mM): 140 KF; 2 ATP; 5 EGTA; 5 Hepes/K^+ buffered to pH 7.3. All experiments were carried out at room temperature (22–24° C). The single-channel currents were recorded on FM tape (Teac R400C), then filtered to 1–3 kHz by a four-pole Bessel filter, digitized using a 12-bit ADC and entered into a computer (ATARI-1040ST^F). Single-channel analysis was done using the modified algorithm described elsewhere [11].

Trypsin (TPCK-treated) and papain were obtained from Sigma.

Results

Unlike previous investigations of the action of trypsin on other cell types [7,12], the complete removal of fast inactivation in our experiments took no less than 40–60 min. In the course of this comparatively long-lasting process two stages of trypsin action were clearly distinguishable, as illustrated in Fig. 1. An example of con-

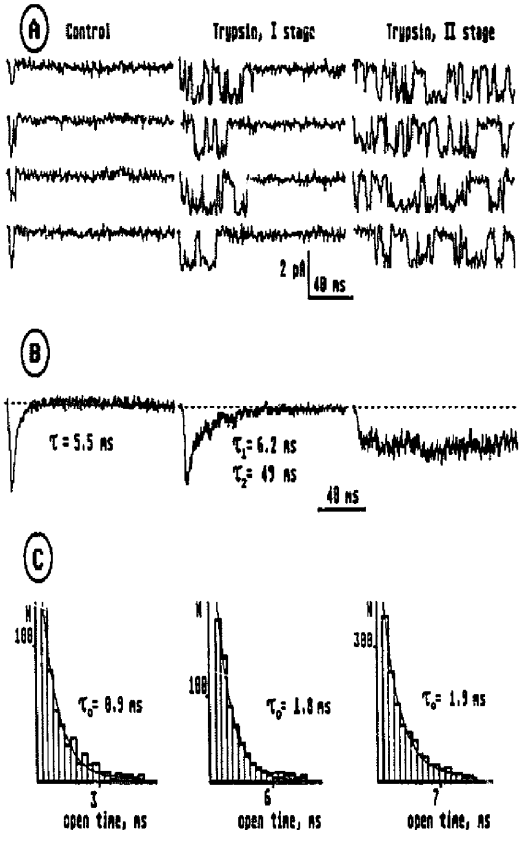

Fig. 1. Two stages of 200 μg/ml trypsin action on Na channels. (A) The original current traces in response to 150-ms depolarizing pulses to −60 mV from −140 mV holding potential. (B) The Na currents obtained by averaging 100–200 traces of single-channel responses. (C) Na channel open time distributions.

trol current traces in response to 150 ms pulse from −140 mV holding potential to −60 mV is shown in Fig. 1A (left). 10–15 min after trypsin application to the cytoplasmic face of the membrane the period of channel activity became longer, manifesting the beginning of the first stage of trypsin action (Fig. 1A, middle). The mean channel open time in this stage increased significantly compared to the control conditions (1.8 ms and 0.9 ms, respectively, see channel open time distributions in Fig. 1C). Both these processes led to a slowing of the decay of macroscopic currents obtained by averaging of 100–200 patch responses. Thus, in the control the inactivation phase of the averaged current was fitted satisfactorily with a single exponential function with the time constant, $\tau = 5.5$ ms (Fig. 1B, left), whereas in the first stage of trypsin action a biexponential function was required with time constants $\tau_1 = 6.2$ ms and $\tau_2 = 49$ ms (Fig. 1B, middle) Note that despite the obvious modification in channel behaviour produced by trypsin, current inactivation remained complete. Only after about 50 min of trypsin action (40–60 min in other experiments) did all signs of fast inactivation disappear (Fig. 1A, right) and the averaged current became steady-state after an initial activation phase (Fig. 1B, right). Interestingly, the mean channel open time at this second stage of trypsin action did not change significantly compared to the first one (1.9 ms and 1.8 ms, respectively, see Fig. 1C). An important characteristic of channel activity is the probability of a blank response (response without channel openings) because it reflects the probability of channel transition from the rest state to one of the inactivating ones [13]. In the experiment under consideration this probability, $P(0)$, did not change significantly in the first stage comparing with the control and was 0.14 in control (number of trials, $N = 203$, at least three channels in the patch), 0.17 ($N = 259$), 0.18 ($N = 140$) and 0.21 ($N = 230$) in 20 min, 35 min and 45 min of trypsin action, respectively. Unfortunately, in the first stage the 'run analysis' [14] could not give any reliable results in this and other experiments owing to the presence of at least three channels in the patch. Thus it was not

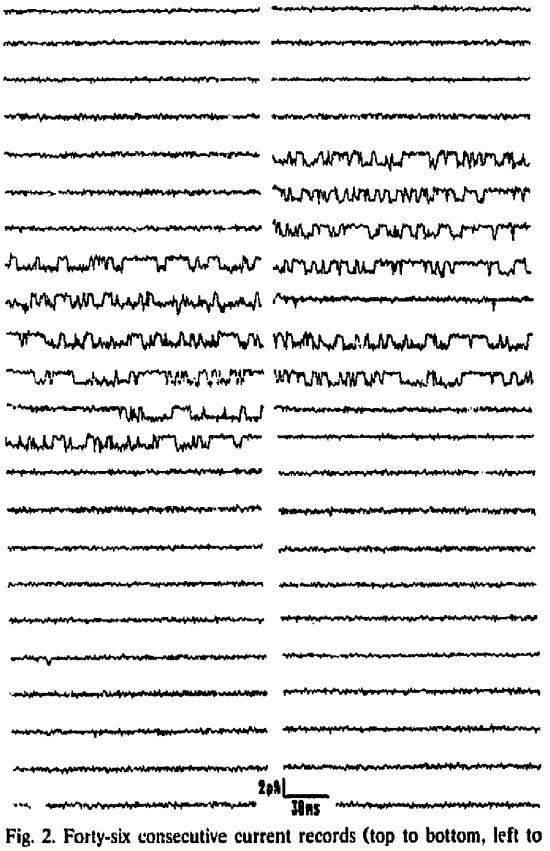

Fig. 2. Forty-six consecutive current records (top to bottom, left to right) obtained during the second stage of trypsin action. 190-ms depolarization to −60 mV from −140 mV holding potential at 2 Hz stimulation frequency.

clear whether there was a trend of blanks to nonrandom clustering. In the second stage of trypsin action, however, the $P(0)$ increased significantly (0.9 in the experiment considered) and tendency of blanks to be clustered together became prominent. Thus the 'run analysis' [14] of 418 trials gave $Z = 9.8$ ($p < 0.01$). The example of 46 sequential current traces recorded during the second stage of trypsin action is shown in Fig. 2. The clusters of blank and traces with channel openings are clearly seen. Qualitatively similar results were obtained in two other experiments with trypsin and two experiments with papain. In six experiments only part of the Na channels in the patch was fully modified by trypsin judging from the incomplete inactivation of the averaged current. In two experiments only the first stage was achieved despite more than 90 min of exposure to trypsin.

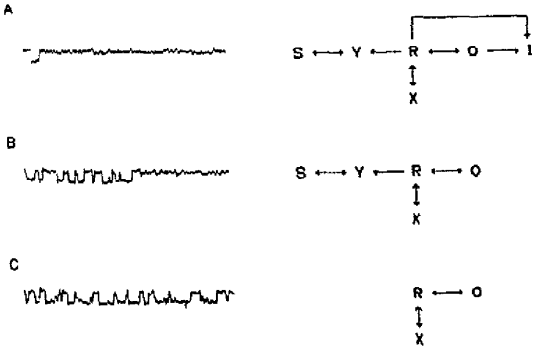

Fig. 3. Simplified kinetic schemes of Na channel conformational transitions at normal conditions (A), at the first (B) and at the second (C) stage of trypsin action. R, the resting state(s); O, the open state; I Y, the fast inactivation states; S, the Ca^{2+}-sensitive slow inactivation state; X, the very slow nonconductive state.

Discussion

Previous studies using proteolytic enzymes [3,6] or other chemicals [4,5] for removing Na channel fast inactivation, attributed the effects of these substances to cleavage of certain channel protein fragments. Furthermore, inactivation was assumed to be destroyed in an 'all or none' manner. However, several publications, [8,12,14], describing the effects of such substances on different cell types, reported the appearance of an additional slow component in the decay of macroscopic current during the first minutes of drug action. This fact was not explained and probably fast removal of inactivation (within several minutes) hampered studies of this effect in more detail. In our experiments no less than 40 min was necessary to destroy inactivation completely. The reason for this discrepancy is not clear. However, long-lasting removal of inactivation allowed us to investigate this process.

The interpretation of our results is based on the simplified kinetic scheme of Na channel conformational transitions shown in Fig. 3A. The existence of two fast inactivating states, I and Y, have been suggested previously [1,2]. The very slow nonconducting state X characterized by 10-s order kinetics, was apparent earlier in our experiments [2] and was also described by Huang et al. [15] in similar cells. The Ca-dependent slow inactivation S, possessing 100-ms order kinetics, is an intrinsic property of cardiac Na channels [1,2,16]. If we hypothesize that trypsin initially destroys the ordinary fast inactivation, I, the scheme is reduced to the one shown in Fig. 3B. What changes in channel parameters would be expected in this case? An increase of the channel reopening number and an increase of the channel mean open time. Passage through the remaining fast inactivation state Y would be the only way in which channel inactivation could occur during each trial. Subsequently, if state Y were removed (Fig. 3C) all signs of fast inactivation would disappear. The channel would then cycle between resting and open states until it 'stuck' for a long time in the nonconducting state X. This situation would lead to a nonrandom clustering of blank traces and traces with channel openings. The mean channel open time in this stage would not differ significantly from that in the previous one because of its independence on the R → Y transition. The probability of blanks, $P(0)$, may change towards an increase or decrease depending on the rate constants between state R and state X. The existence of state X may explain the difference between our results and those of Gonoi and Hille [4] who reported an increase in peak Na current after removal of fast inactivation in neuroblastoma cells. From the preceding discussion one can see that the observed trypsin effects on channel properties are consitent with the proposed channel state scheme in Fig. 3A. The situation with the slow state X is not, however, quite clear. We cannot reject the possibility that another similar state appears as a result of trypsin action on the channel protein or that under trypsin action the probability of channel transition to the normal existing X state becomes progressively larger. A similar effect was described by Horn R. et al. [14] concerning the action of NBA on Na channels in pituitary cells. In conclusion, trypsin and papain effects on cardiac Na channels are well described by assuming consecutive removal of two fast inactivation states.

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