Aconitine-induced Modification of Single Sodium Channels in Neuroblastoma Cell Membrane

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Abstract. Aconitine-modified sodium channels in the neuroblastoma cell membrane were investigated with patch-clamp technique in outside-out configuration. When aconitine (0.1 mmol/l) was present in the pipette solution two types of modified single sodium channels were observed. The first type showed openings with normal amplitude (slope conductance 15.5 pS) and bursting behaviour. The second type of modified channel openings was characterized with low amplitude (slope conductance 2.8 pS) and longer open time as comparing to unmodified channels. The low-amplitude channels were shown to have altered ion selectivity: they were permeable to NH_4^+. Both populations of aconitine-modified channels could be blocked by tetrodotoxin. In contrast to macroscopic current experiments (Mozhayeva et al. 1977) the development of aconitine modification was not affected by repetitive stimulation and external application of the agent had no effect on single sodium channels in outside-out membrane patch.

Key words: Neuroblastoma membrane — Patch clamp — Single sodium channels — Aconitine — Selectivity

Introduction

Modification of ionic channels of excitable cells can provide information about specific structures involved in its functions. Among the drugs widely used for the treatment of TTX-sensitive sodium channels is the naturally occurring alkaloid aconitine. Aconitine has for many years been known to change gating functioning and selectivity properties of sodium channels (Mozhayeva et al. 1977; Campbell 1982; Grishchenko et al. 1983). The patch clamp technique gives an opportunity to study how the effects of aconitine seen at the integral current level are reflected in the properties of modified single sodium currents.

The purpose of our study was to identify and characterize single channel currents through aconitine-modified sodium channels. Outside-out patch clamp experiments were performed on neuroblastoma cells; this model was used earlier to study changes of integral sodium currents after treatment by aconitine (Grishchenko et al. 1983).

Materials and Methods

Experiments were carried out on cultured mouse neuroblastoma cells C1300, clone N18Al (Konobasova et al. 1981). The cells were grown in Eagle's medium supplemented with 10% bovine serum inactivated at 56 °C. They were plated out on coverslips in glass Petri dishes 3—5 days before experiments.

Patch pipettes (resistance 5—10 mOhm) were fabricated from Pyrex or soft glass tube and Sylgard coated. The pipettes were filled with the solution containing (mmol/l): 130 KF, 1 $CaCl_2$, 2 $MgCl_2$, 10 EGTA-TrisOH, 10 HEPES-TrisOH, pH 7.2.

For single sodium current measurements coverslips with cells were placed in 1.5 ml capacity bath. Giga-seal formation was performed in a bath solution containing (mmol/l): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-TrisOH, 10 glucose, pH 7.2.

After excising an outside-out membrane patch the pipette was moved to the small volume compartment (0.1—0.2 ml glass tube) of the experimental chamber to improve the signal to noise ratio. Control external solution contained (mmol/l): 160 NaCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-TrisOH, pH 7.2. Where appropriate, the external solution contained 160 mmol/l NH_4^+ instead of Na^+. Aconitine (SERVA) in stock ethanol solution was added to either external or internal solution to obtain final concentration of 0.1—0.2 mmol/l. All experiments were carried out at room temperature (20—22 °C).

Single-channel currents were measured with an EPC-7 patch-clamp amplifier (List Electronic, FRG). Current records were filtered at 3 kHz with a Bessel-type low-pass filter, digitized at 10 kHz with 13 bit conversion accuracy by a signal averager NTA 1024 (Metrimpex, Hungary) which was connected to a 15VUMS-025-28 minicomputer (USSR). Pulse generation were also controlled by the computer. At each potential 200 records were recorded and stored on floppy discs. In some experiments currents were recorded on an analog FM-tape recorder NO67 (USSR) and later digitized in the same manner. Removal of capacity and leakage currents was performed by subtraction of an average of traces containing no channel openings from traces containing channel events. Additional 2 kHz-filtration was performed by the computer using digital Gaussian filter subroutine (Colquhoun and Sigworth 1983).

For the amplitude histogram computations the original records were dis-

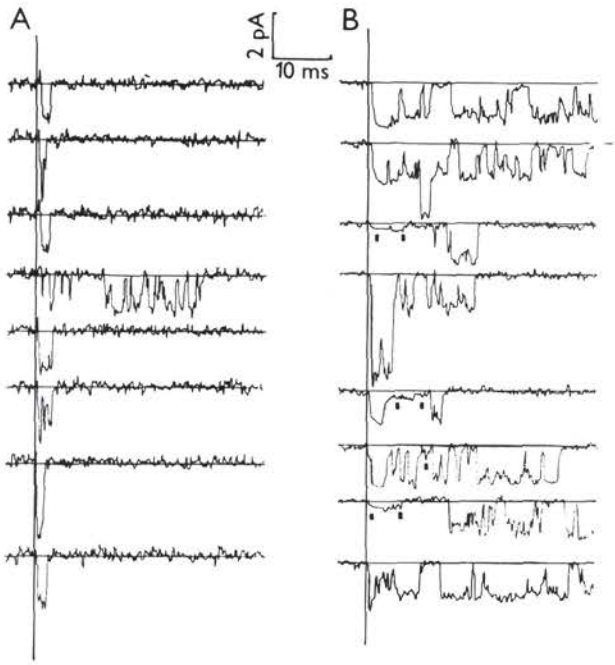

Fig. 1. The effect of aconitine on single sodium channel currents in neuroblastoma outside-out patch. *A*. Normal single channel openings from 8 consecutive sweeps. *B*. Single channel recordings in the presence of 0.1 mmol/l aconitine in the pipette solution. Holding potential was set at −80 mV, conditioning pulse was −100 mV, 60 ms; test pulse was −30 mV, 40 ms. The vertical line indicates start of the test pulse, the horizontal line indicates zero current level. Filled squares indicate low amplitude modified openings.

played and parts of records were selected. Then the aplitude of every sampled point from the selected parts of the records was used as histogram entry. When there was no opening in the selected parts, the resulting histogram was well fitted by a single Gaussian curve with a mean current value of 0 pA (baseline). When openings were present, additional peak(s) appeared in the histogram, corresponding to the level of current through open single channel(s). Fitting of the amplitude histograms was performed by the maximum likelihood method with Mann's computational algorithm (Mann et al. 1983). Mean open times were calculated by averaging the duration of single channel events.

Results

Fig. 1 shows single sodium channel current records at 40 ms depolarizing step to −30 mV in normal neuroblastoma cell membrane (*A*) and in the presence of

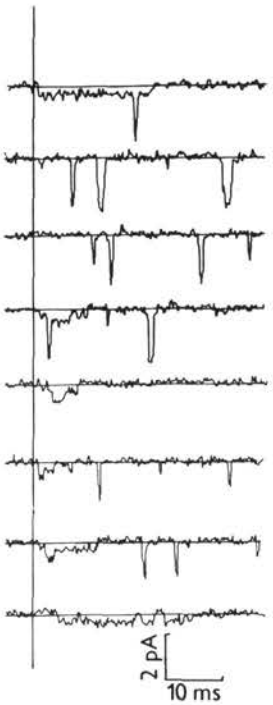

Fig. 2. Low amplitude modified openings are easier to detect at more negative test potentials. The same outside-out patch as in Fig.1*B* (0.1 mmol/l aconitine in the pipette). Test pulse −60 mV.

0.1 mmol/l aconitine in the pipette solution (*B*). Holding potential was at −80 mV, conditioning pulses were usually −100 mV, 60—100 ms. Fig. 1*A* shows 8 sweeps exhibiting normal-looking Na^+ channel openings with a mean amplitude of -1.39 ± 0.21 pA and a mean open time of 0.82 ms. It can be seen in all traces except trace 4 that brief opening(s) appeared during the first few milliseconds of the depolarizing voltage step. As in the experiment illustrated in Fig. 1*A* we usually worked with patches containing 2—4 channels. Nevertheless, in most records the openings concentrated around the start of the pulse, and reopenings were negligible in untreated neuroblastoma membrane patches. The record containing repetitive openings shown in Fig. 1*A*, trace 4, was the only one of 200 sweeps recorded in this experiment.

When aconitine was present in the pipette solution (Fig. 1*B*) the single channel behaviour was modified in two ways. Firstly, openings with nearly normal amplitude (NA, mean -1.42 ± 0.16 pA for this experiment) showed the

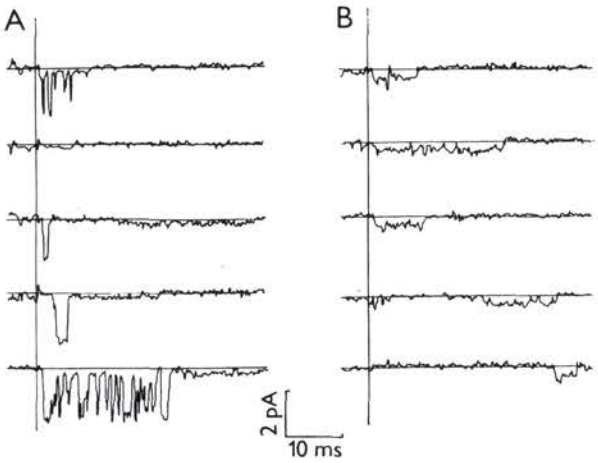

Fig.3. Modification of single sodium channels by aconitine leads to alteration of selectivite properties. Single current records through aconitine-modified sodium channels in normal (160 Na^+) external solution (*A*) and in the external solution containing 160 NH_4^+ instead of Na^+ (*B*).

bursting behaviour in 80% of sweeps. Secondly, in addition to the NA modified events openings with low amplitudes (LA, mean -0.24 ± 0.13 pA for this experiment) and longer open times were observed. The mean open time for NA modified channels was 1.2 ± 0.5 ms ($n = 5$) that is close to normal values; for LA channels it was 2.4 ± 0.3 ms ($n = 3$).

In our experiments with untreated membranes the appearance of single channel events at test potentials more negative than -50 mV was rare. Fig. 2 shows aconitine-modified single sodium channel current records at -60 mV depolarizing step. At this test potential it was easier to observe LA modified openings characterized by a mean amplitude of 0.32 ± 0.13 pA and a mean open time of 3.7 ms. In neuroblastoma membrane aconitine caused openings to occur at more negative test potentials as compared to unmodified sodium channels. It should be noted that the percentage of repetitive NA-openings (mean amplitude -1.98 ± 0.29 pA, mean open time 0.41 ms) was low at -60 mV. The mean open time of LA modified openings at -60 mV test pulse was prolonged whereas it was shortened for NA type as compared with the corresponding values at -30 mV. Obviously, it was due to the above conditions that we could observe distinctly the existence of the other low-amplitude type, of aconitine-modified sodium channels at more negative potentials.

The channel slope conductance was calculated from the amplitude histo-

grams which were collected at 3—5 test potentials from sets of 100—200 traces. For the experiment illustrated in Fig. 1*A* (unmodified channels) single sodium channel conductance was 14.5 pS, with a mean value of 14.8 ± 0.8 pS (3 experiments). For aconitine modified channels from the experiment shown in Fig. 1*B* the slope conductance for NA openings was 14.1 pS, and for LA openings it was 2.8 pS; means for 4 experiments were 15.5 ± 0.9 pS and 2.7 ± 0.1 pS respectively.

Both types (NA and LA) of aconitine-modified single sodium channels were blocked by 10^{-7} mol/l tetrodotoxin (not shown).

In normal membrane the replacement of sodium by ammonium external solution led to complete disappearance of any single channel events distinct from noise level during depolarizing test pulses within -60 mV and 10 mV. The results of one of the experiments with equimolar substitution of external Na^+ by NH_4^+ ions with aconitine present in the pipette solution are illustrated in Fig. 3. Fig. 3 shows single currents through the modified channels in sodium (A) and ammonium (B) external solution at -40 mV test potential. In normal (Na^+) solution a mixture of two types, NA and LA, of modified channel events was observed. In ammonium solution only well resolved openings with small amplitudes were recorded. Mean open time for single channel events in ammonium solution was 2.3 ± 0.4 ms; for LA fraction of modified channels in sodium solution it was 2.4 ± 0.3 ms (averages from 4 experiments at -40 mV test pulses). Within the experimental error, the mean open times proved to be the same for the openings observed in ammonium external solution and for LA-openings in sodium solution. Thus, the range of activation and the kinetic behaviour of channel openings in the presence of aconitine in the pipette were quite similar in the ammonium external solution to those of LA modified openings in sodium solution. It is natural to consider single channel events in ammonium solution as representing LA type of aconitine modified channels. For the experiment presented in Fig. 3 (test pulse -40 mV) the mean amplitude of LA modified openings in sodium and ammonium solution was 0.23 ± 0.13 pA and 0.43 ± 0.13 pA, respectively. Mean amplitude values (3 experiments) for -30 mV test potential were 0.32 ± 0.13 pA in sodium solution and 0.33 ± 0.09 pA in ammonium solution. The average slope conductance of modified channels in ammonium solutions was 2.6 ± 0.3 pS ($n = 4$); this value is close to the conductance of LA openings in sodium solution.

The experiments with ionic substitution allowed us to conclude that LA aconitine-modified sodium channels have altered selectivity as compared to normal sodium channels: in particular, they are permeable to NH_4^+. Selective properties of NA modified channels were apparently unchanged.

The effects of aconitine on single sodium channel currents described above were observed only when aconitine was added to the internal solution on outside-out membrane patch. Addition of aconitine 0.1—0.2 mmol/l to the

external solution had no effect on single currents in patch despite repetitive (10 Hz) stimulation by depolarizing pulses during 5—10 minutes. As shown earlier (Grishchenko et al. 1983) in whole-cell experiments aconitine acted identically on the properties of sodium channels upon both external and internal application. The modification of integral sodium currents was found to be enhanced by repetitive depolarization (Mozhayeva et al. 1977). In the present work macroscopic sodium currents were measured in whole-cell mode on neuroblastoma cells and similar results were obtained (not shown). In whole-cell configuration typical aconitine modification occurred after external application of the agent and was markedly enhanced by depolarizing stimulation. These control measurements confirmed that there was an obvious difference in aconitine effects between excised patch and whole cell: after excision the agent became ineffective from the outside. In our single channel experiments (see Fig. 1—3) some peculiarities concerning the development of the aconitine effect were noted. When aconitine was present in pipette solution the modification could be observed immediately after the patch excision. NA to LA openings ratio remained unchanged during experiments although some of them were long lasting with thousand depolarizing pulses at different potentials. The application of depolarizing pulse series at 10 Hz to outside-out patch with aconitine inside the pipette did not results in any increase of the LA-fraction openings. Hence, unlike in whole-cell experiments, in outside-out experiments the level of the selectivity-connected aconitine modification of single sodium channels was not influenced by repetitive depolarization.

Discussion

The data obtained showed that in the presence of aconitine in the internal solution there were two types (NA and LA) of modified single sodium channels in outside-out membrane patch which differed by their kinetic and conductance properties. TTX-sensitivity of both types of modified openings confirmed that both types of channel events represented single currents through sodium channels. The first (NA) type of modified channels displayed an increased (in comparison with unmodified channels) percentage of burst-like openings characterized with amplitude and mean open time close to values observed for normal single sodium channels. This type of aconitine modification is similar to that observed in inside-out mode experiments with single sodium channels from adult cardiac myocytes (Nilius et al. 1986). The other (LA) type of openings observed in our experiments with aconitine were low amplitude events with longer open time. The slope conductance of LA channels was near 1/6 of that for normal events. LA-openings were not recorded in aconitine experiments

with cardiomyocyte membrane patches (Nilius et al. 1986). A modification similar to the LA-type was produced by veratridine treatment of single sodium channels translated by oocytes (Sigel 1987) and in neuroblastoma cells (Bernes and Hille 1988). In contrast to veratridine-modified single sodium channels (Barnes and Hille 1988), after aconitine treatment NA and LA openings appeared to be independent events (see e. g. Fig. 2).

As in the presence of aconitine two populations of modified channels characterized with different conductance levels were found in the membrane patch the following possibility should be considered. Nagy and coworkers (Nagy et al. 1983; Nagy 1988) suggested that there were more than one conductance state of normal sodium channel in neuroblastoma cells. It could not be excluded that in our experiments normal sodium channels in the untreated membrane might have different levels of conductivity. From this point of view aconitine modification may consist simply of some change of kinetic parameters of sodium channels. The prolongation of open time of the low-conductance state would result in recording of two types (NA and LA) of modified channel events with different amplitudes in a patch. However, such an explanation of the aconitine effect seems quite insufficient because a) under normal conditions no openings with amplitude similar to that of LA-openings were actually observed, and b) after the modification a difference in selectivity between modified LA and NA (and also normal) sodium channels occurred. LA modified openings are most likely due to actual alterations of sodium channel conductivity and selectivity mechanisms induced by aconitine. In this respect our data support the general conclusions concerning aconitine action obtained earlier in integral current studies (Mozhayeva et al. 1977; Grischenko et al. 1983).

An unexpected observation concerns the discrepancy between ways of development of the aconitine effect in whole cell and in excised patch. As it was pointed out above, repetitive depolarization pulses enhanced the modification of sodium channels by aconitine in macroscopic current experiments. This means that the time course of the developing aconitine effect can be observed during whole-cell experiments.

Similar observations were reported for other alkaloids, such as batrachotoxin (Khodorov and Revenko 1979) and grayanotoxin (Seyama and Narahashi 1981). In our experiments modification of single sodium channels occurred at the same time when channel openings could be seen in outside-out mode (with aconitine present in the pipette solution). Attempts to enhance the level of modification (or induce it when aconitine was added to the external solution only) were unsuccesfull. It can be speculated that the interaction of aconitine with sodium channel is promoted by some unknown cytoplasmic factor(s) and that this factor is absent in outside-out patch preparation. In our experiments such a „promotor" had to be present during aconitine treatment in whole-cell

stage which preceded the formation of the outside-out patch preparation. After gigaseal formation and disruption of a part of the membrane, the solution is exchanged through the pipette hole between the pipette interior and the cell interior. This exactly is the condition suitable for aconitine interaction with sodium channels in the neighbouring compartments of the membrane. Excision of membrane patch leads to the loss of promotor and accordingly to the breaking off of the modification process. Therefore it may be assumed that some cytoplasmic components distributed near the inner surface of the membrane are involved in regulatory processes of sodium channel activity. There is some evidence in favour of the above speculation. In electrophysiological experiments the relevance of the undercoat structure and cytoskeleton in squid giant axon to generation of Na^+ currents was shown (Matsumoto 1984; Matsumoto et al. 1984). Data confirming sodium channel interaction with cellular proteins were obtained in biochemical experiments (Edelstein et al. 1988). The possible role of phosphorylation-dephosphorylation processes in neurotoxin-induced modification of channels could not be excluded (Catteral 1984). Our assumption concerning the existence of a promoting intracellular factor involved in aconitine (or other alkaloids) interaction with sodium channel does not contradict experiments with veratridine-induced modification (Sigel 1987; Barnes and Hille 1988); the latter experiments were carried out in cell-attached mode, providing conditions for interaction of the alkaloid with sodium channel similarly as in the integral current experiments. It may be assumed that a better knowledge of mechanisms underlying the interaction of aconitine with sodium channels in neuroblastoma cells can be derived from inside-out and cell-attached patch experiments.

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