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Voltage-Induced Activation of Mechanosensitive Cation Channels of Leech Neurons

M.C. Menconi[1], **M. Pellegrini**[2], **M. Pellegrino**[1]

[1]Dipartimento di Fisiologia e Biochimica "G. Moruzzi", Università di Pisa, Via S. Zeno 31, 56127, Pisa, Italy
[2]Scuola Normale Superiore piazza Cavalieri 7, 56126, Pisa, Italy

Abstract. The voltage dependence of stretch-activated cation channels in leech central neurons was studied in cell-free configurations of the patch-clamp technique. We established that stretch-activated channels excised from identified cell bodies of desheathed ganglia, as well as from neurons in culture, were slowly and reversibly activated by depolarizing membrane potentials.

Negative pressure stimuli, applied to the patch pipette during a slow periodical modulation of membrane potential, enhanced channel activity, whereas positive pressures depressed it.

Voltage-induced channel activation was observed, with soft glass pipettes, both in inside-out and outside-out membrane patches, at negative and positive reference potentials, respectively.

The results presented in this study demonstrate that membrane depolarization induces slow activation of stretch-activated channels of leech central neurons. This phenomenon is similar to that found in *Xenopus* oocytes, however, some peculiar features of the voltage dependence in leech stretch-activated channels indicate that specific membrane-glass interactions might not necessarily be involved. Moreover, following depolarization, stretch-activated channels in membrane patches from neurons in culture exhibited significantly shorter delay to activation (sec) than their counterparts from neurons of freshly isolated ganglia (hundreds of sec).

Key words: Leech — Neurons — Voltage-sensitivity — Stretch-activated channels — Growth cone

Introduction

Mechanosensitive ion channels are expressed in most plant and animal cells. They play a clear-cut role in cells that are specialized in hearing, touch and osmoreception, (Guharay & Sachs, 1984; Morris, 1990; Sachs, 1992; Sackin, 1995; Bourque & Oliet, 1997). On the other hand, the functional role of these channels in cells that are not mechanosensors remains unclear and the role of mechanosensitive channels as mechanotransducers is still debated (Hamill & McBride, 1997; Sachs & Morris, 1998; Wan, Juranka & Morris, 1999; Zhang & Hamill, 2000*b*). It is well established that these channels have intrinsic stretch-sensitivity because they can be activated in the plasma membrane that lacks cytoskeleton structures, like blebs (Zhang et al., 2000), in liposomes (Sukharev et al., 1993) and by polyunsaturated fatty acids (Maingret et al., 1999). However, it has been also clearly demonstrated that extrinsic factors such as membrane infolding (Zhang & Hamill, 2000*a*) as well as the condition of the cortical cytoskeleton can regulate cytoprotective responses to stretching (Ko & McCulloch, 2000).

In most preparations the experimental activation of stretch-activated channels (SACs) has been induced by pressure applied to the pipette or by hypotonic swelling. In addition, it has been recently shown that membrane depolarization slowly activates mechanogated channels in *Xenopus* oocytes, through membrane movements that resulted in tension changes (Gil, Silberberg & Magleby, 1999). Intriguing aspects of this type of activation are its dependence on the type of glass used for pipette fabrication and the absence of activation in the outside-out configuration (Gil, Magleby & Silberberg, 1999).

Leech central neurons express stretch-activated cation channels in all membrane domains. SACs, which are distributed in the growth cone membrane, admit calcium and exhibit typical pharmacological features of mechanotransducer ion channels. Their blockage enhances neurite outgrowth in cultured AP neurons (Calabrese et al., 1999). Since studying what affects the activity of these channels is important for understanding their role in growth cones, we attempted to analyze the voltage-dependence of SAC activity in the AP neurons. Here we show that the voltage-induced SAC activation can be obtained with soft glass, in both inside-out and outside-

Correspondence to: M. Pellegrino

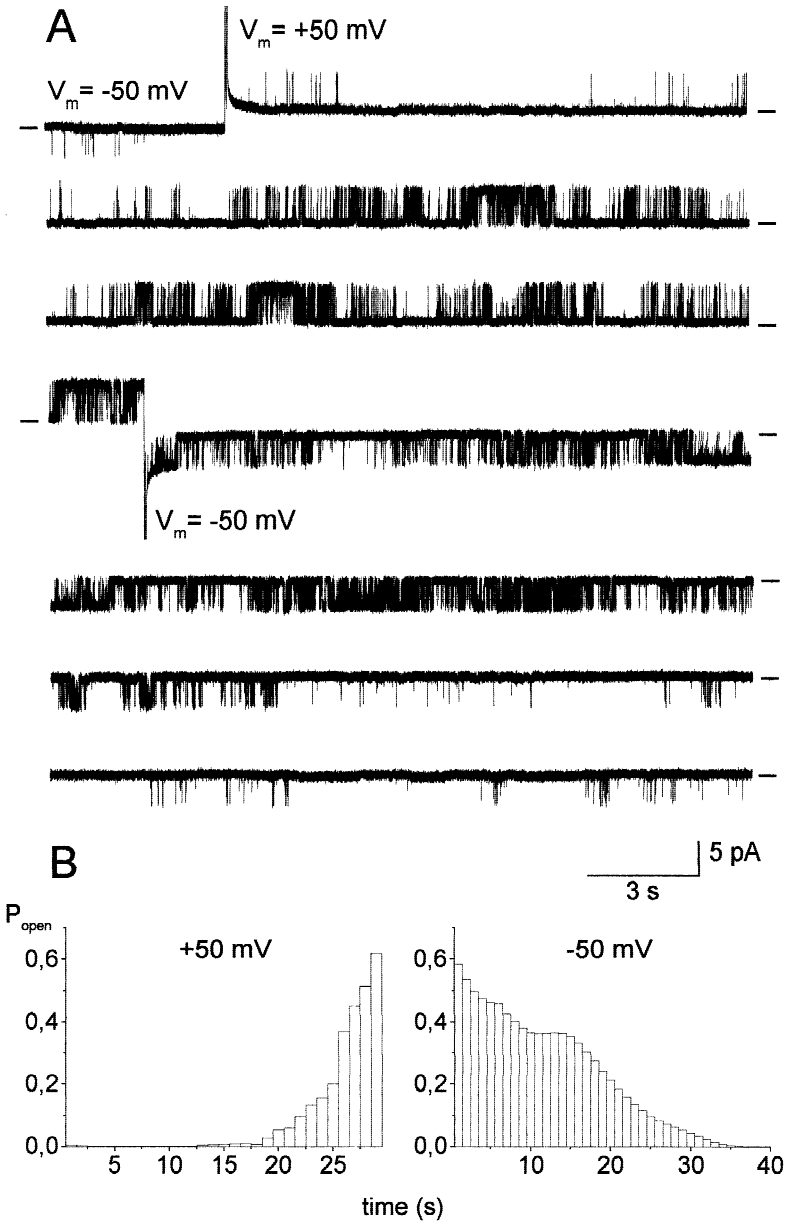

Fig. 1. (*A*) Continuous current recordings from an inside-out patch containing a single SAC. The membrane potential was initially held at −50 mV and changed two times to induce slow channel activation and deactivation. Outward currents, entering the pipette, are indicated as upward deflections. The mark next to each record corresponds to the current with the channel closed. (*B*) Plots illustrate probability of being open during activation (left graph) and deactivation (right graph).

out configurations. Moreover, we report that SACs excised from cultured AP neurons exhibit a significantly shorter delay to activation by depolarization than their counterparts from neurons of freshly isolated ganglia.

Materials and Methods

Adult specimens of *Hirudo medicinalis* L., obtained from a commercial supplier (Ricarimpex, Eysines France), were used in this study. According to the method of Fuchs, Nicholls and Ready (1981), segmental ganglia were removed from the leech central nervous system and neurons identified as Anterior Pagoda (AP) were isolated by pipette suction, after 1 hr of enzyme treatment (2 mg/ml collagenase/dispase, Boehringer, Mannheim, Milan, Italy). Neurons were plated onto Concanavalin A (Sigma, Milan, Italy) coated culture Petri dishes (Falcon 3001, Becton Dickinson, Le Pont De Claix, France) in Leibowitz-15 medium (Sigma, Milan, Italy), with 0.6% glucose, 100 μg/ml gentamicin (Sigma, Milan, Italy) and 2% fetal bovine serum (Sigma, Milan, Italy) and maintained at 20–22°C for 3–7 days.

ELECTROPHYSIOLOGY

The patch-clamp technique (Hamill et al., 1981) in the inside-out and outside-out configurations was used. Single channel currents were recorded from naked cell bodies in desheathed ganglia or from both soma and growth cone membranes of cultured AP cells. Patch electrodes, pulled in two stages from 1.5 mm o.d. glass capillary tubes 7087 (Blaubrand, Wertheim, Germany), had resistances of 4–5 MΩ, a "bubble number" of about 4 (Corey & Stevens, 1983) and were coated

with Sigmacote (Sigma, Milan, Italy). A 'gentle' sealing protocol (Hamill & McBride, 1997) was routinely applied. In most recordings we obtained tight seal just by releasing a positive pressure of 10 mm Hg, without applying negative pressure. Currents were recorded with a patch-clamp amplifier (Axopatch 1D, Axon Instruments, Foster City, CA), by low pass filtering at 1 kHz. Data were displayed on an oscilloscope and stored on the hard disk of a PC for off-line analysis as Axotape (Axon Instruments) files, after AD conversion at 5 kHz with a Labmaster TL1 interface (Axon Instruments). Analysis was carried out using both pClamp software (Axon Instruments) and software developed for the purpose in our laboratory. To obtain the depolarization-induced delay to activation, in each record the time interval between the onset of depolarization and the attainment of the first opening lasting at least 1 sec (t_1) was measured. After the determination of the maximal number of active channels, the mean P_{open}, was extracted from mean NP_{open} calculated in at least 60-sec-long data segments, starting from t_1. The mechanical stimulation of SACs was induced applying negative or positive pressure to the back of the patch pipette through the sidearm of the holder.

Solutions

Symmetrical pipette and bath Na^+ solutions contained (in mM): 155 NaCl, 1 $MgCl_2$, 10 HEPES (NaOH), 5 KCl, 1 $CaCl_2$, pH 7.2. Symmetrical K^+ solutions contained (in mM): 120 KCl, 1 $MgCl_2$, 10 HEPES (KOH), 0.01 $CaCl_2$, pH 7.2. In some experiments, to block SACs in outside-out configuration, gentamicin sulfate (Sigma, Milan, Italy) was added to the bath solution at a final concentration of 200 μM.

Statistical Analysis

Mathematical transformations and statistical analysis were performed using Sigma Plot 3.0 and Sigma Stat 2.0 (Jandel Scientific). The comparisons between different groups were carried out by the Mann-Whitney test. Mean values were expressed as mean ± SEM. Differences were considered significant when $P < 0.01$.

Results

Depolarization Induced Activation of SACs in Inside-Out Patches Formed with Soft Glass Pipettes

Membrane patches just excised after 'gentle' sealing in symmetrical Na^+ solutions and held at a depolarizing membrane potential displayed only occasional brief openings. In the absence of applied pressure a progressive activation of SACs spontaneously developed within 20 to 375 sec (12 patches at $V_m = +80$ mV). The steady-state open probability reached values that were variable from patch to patch. Applying alternate hyperpolarizing and depolarizing steps of tens of seconds induced channel deactivation and activation, respectively, with extremely slow kinetics. Figure 1*A* illustrates a typical current record obtained in symmetrical 155 mM NaCl solutions. This inside-out membrane patch, excised from an AP cell body of a freshly desheathed ganglion, contained a single channel, previously identified as a stretch-activated cation channel by its conductance value, the typical outward rectification of current and the increase of opening frequency and mean channel open time in response to stretch (Pellegrino et al., 1990; Calabrese et al., 1999). The membrane potential, initially held at −50 mV, was stepped to +50 mV and then stepped back to −50 mV. Single channel P_{open}, initially low, increased 40-fold over the initial value about 25 sec after the depolarizing step, (Fig. 1*B*, left graph) and decreased to one 40th of the initial value about 22 sec after repolarization (Fig. 1*B*, right graph). The delay of voltage-induced activation was widely variable from patch to patch and displayed a dependence on the patch history in the same recording. Mean values of delay to activation in different patches, for voltage steps between −80 and +80 mV, were in the range 1–300 sec. Although variable, the activation delay was inversely related to the mean level of single channel activity. In Fig. 2 the relationship between the mean P_{open} and the activation delay time, measured at a V_m of +80 mV in 18 separate patches, is plotted. Similar depolarization-induced slow channel activation was also observed when the experiments were repeated in symmetrical 120 mM KCl solutions.

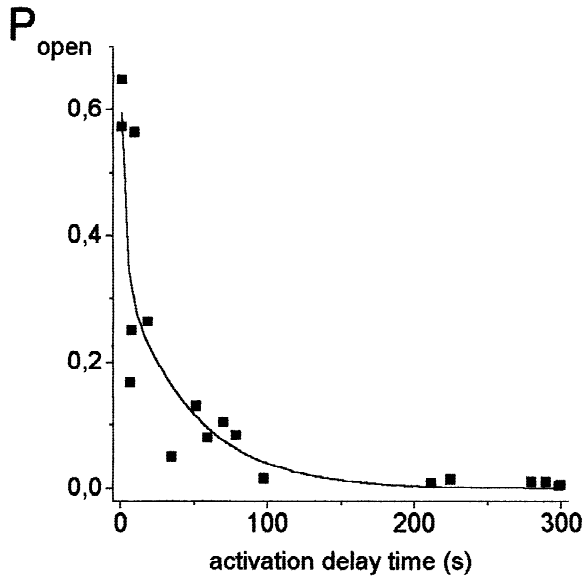

Fig. 2. Graph illustrating the relationship between channel activity, expressed as probability of being open at a V_m of +80 mV, and activation delay, in 18 separate patches. The double-exponential curve fitted to the points has $\tau_1 = 2.7$ sec and $\tau_2 = 46.2$ sec.

Modifying Basal Membrane Tension to Inside-Out Patches Affected The Concurrent Voltage-Induced Channel Activation

The application of negative pressure to the pipette, during voltage-induced activation, enhanced, whereas posi-

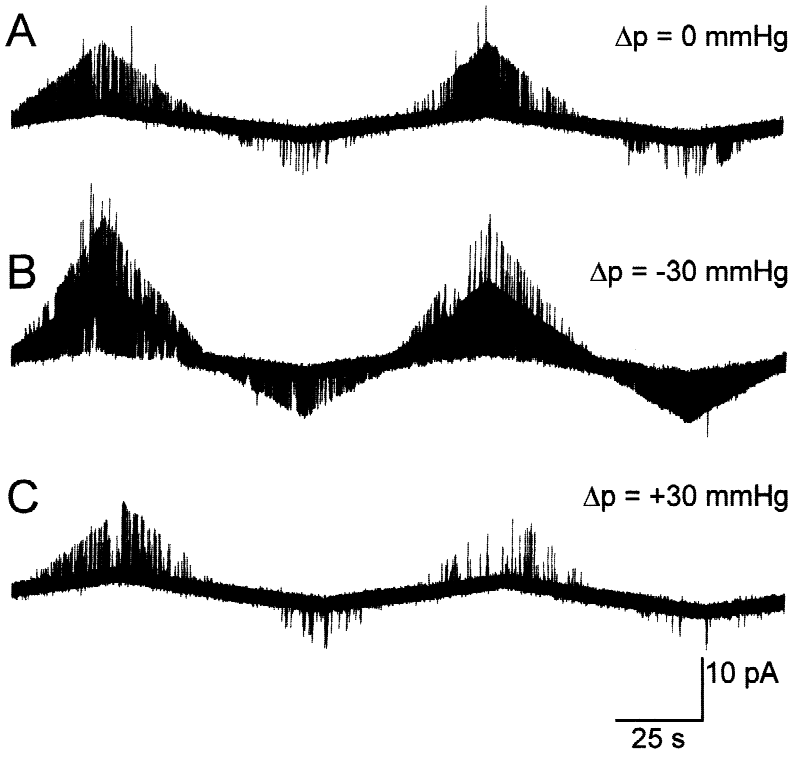

Fig. 3. Consecutive records displaying channel activity induced by modulation of command potential with a 0.01 Hz triangular wave varying between −80 and +80 mV. Periodical forcing was applied (*A*) alone and in combination with a pipette pressure of (*B*) −30 mm Hg or (*C*) +30 mm Hg. The inside-out membrane patch excised from a freshly naked cell body contained three channels, whose openings are displayed upward at depolarizing potentials.

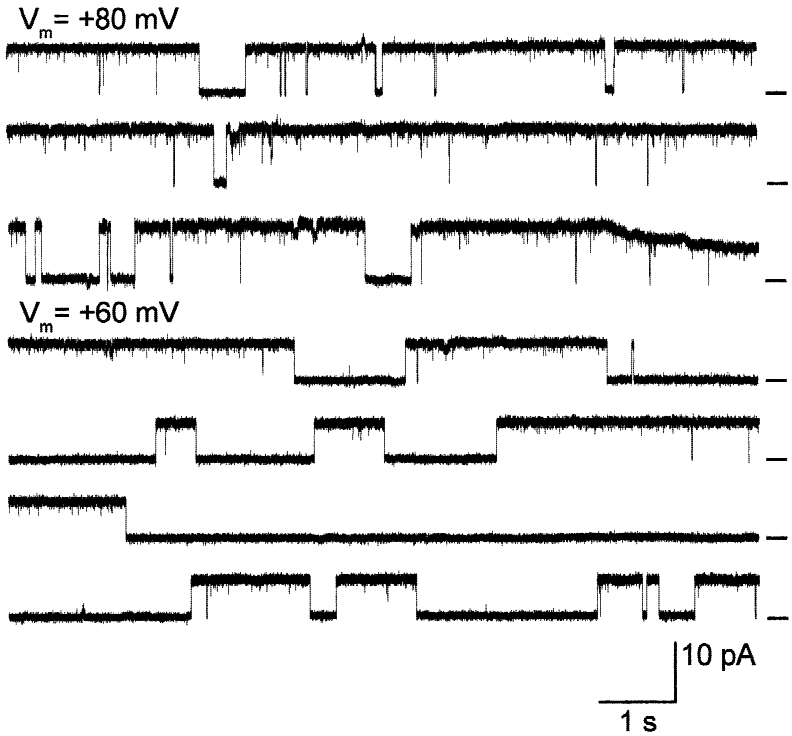

Fig. 4. Continuous current records from an inside-out patch, illustrating the susceptibility of single channel open probability to a small reduction of positive membrane potential. The mark next to each record corresponds to the current with the channel closed.

tive pressure reduced, channel activity. Figure 3 illustrates these effects in a record on a slow time scale. The membrane patch contained at least three SACs. Membrane potential was periodically modulated by a 0.01 Hz triangular wave, varying between −80 and +80 mV. In the absence of applied pressure, channel activation occurred during the depolarizing half of the cycle, whereas deactivation followed during repolarization (Fig. 3*A*).

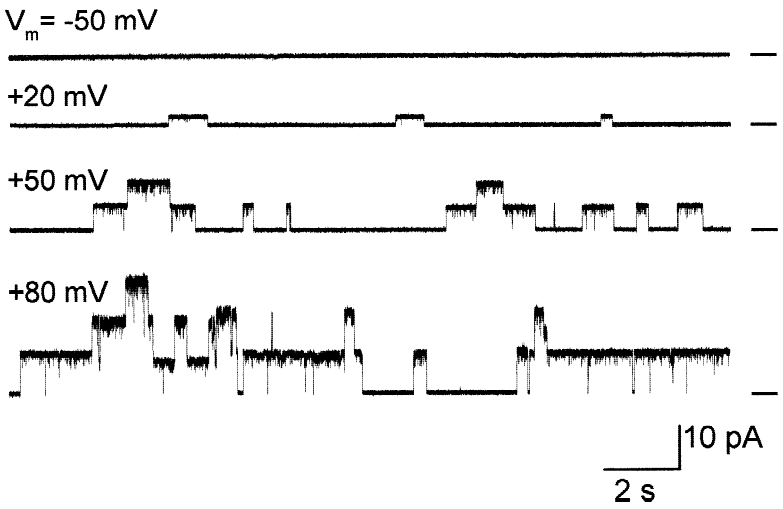

Fig. 5. Samples of channel activity in an inside-out membrane patch containing at least three SACs, maintained at the different membrane potentials indicated at the left of each trace. Single channel open probability, calculated from 180 sec-long data segments including the illustrated traces, was <0.001, 0.01, 011, 0.19, at −50, +20, +50 and +80 mV, respectively. The mark next to each record corresponds to the current with all channels closed.

Applying a constant pressure of −30 mm Hg during the depolarizing sections of the following two cycles of voltage modulation increased the open probability of channels at all membrane potentials (Fig. 3*B*). The opposite effect was observed when setting the pressure at +30 mm Hg (Fig. 3*C*). The additive effects of membrane stretch and membrane depolarization were also confirmed by applying brief stretches during steady state voltage-induced activation by command steps. The observed effects were transient increases of channel activity, associated with increased opening frequency and mean open time, during suction (*not shown*).

Membrane Patches from Cultured Neurons Exhibited Distinctive Susceptibility to Changes of Membrane Potential

Our investigations were extended to the study of membrane patches isolated from identified AP neurons that had been in culture for 3–7 days and were sprouting on Concanavalin A. The application of the same stimulation protocol as that used in freshly desheathed ganglia revealed clear-cut differences in voltage sensitivity, as expressed by various indicators. First, the delay to voltage-induced activation was significantly shorter in patches from cultured neurons than in those from ganglia. Since the steady channel activity was dependent on the patch history, a comparison of the mean delay to activation was carried out in well-defined experimental conditions to minimize the range of measured values. Measurements of the mean delay in response to a step from −80 to +80 mV were taken in 10 patches from cultured neurons and in 12 from freshly desheathed ganglia, just after excision (Fig. 6). Second, SACs appeared sensitive to small changes of positive membrane potential, as shown in the continuous current record from an inside-out patch isolated from a cultured cell body, in

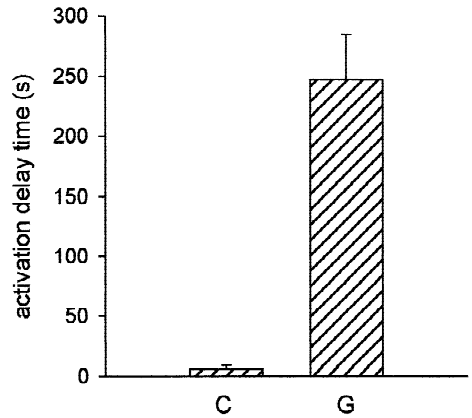

Fig. 6. Plot summarizing the delays to the first activation of SACs in inside-out configuration. The mean values, which refer to 12 patches from freshly desheathed ganglia (G) and 10 from cultured neurons (C), are significantly different ($P < 0.01$). The mean values ± SEM were 247.0 ± 37.9 sec (minimal value 20 sec) for G and 6.2 ± 2.8 sec (minimal value 0.09 sec) for C.

Fig. 4. It can be observed that the percentage of time open was reduced from about 80 to less than 50% with the occurrence of long closures and a decrease in mean channel open time, when the membrane potential, initially held at +80 mV, was slowly reduced by 20 mV. Third, the steady-state open probability of SACs appeared highly dependent on membrane potential in these patches. Figure 5 illustrates representative samples of currents recorded in an inside-out patch excised from the membrane of a growth cone, at the four indicated membrane potentials in the absence of applied pressure.

SACs Were Activated by Membrane Depolarization in Outside-Out Patches

The routine occurrence of voltage-induced SAC activation in our experiments revealed a surprising difference

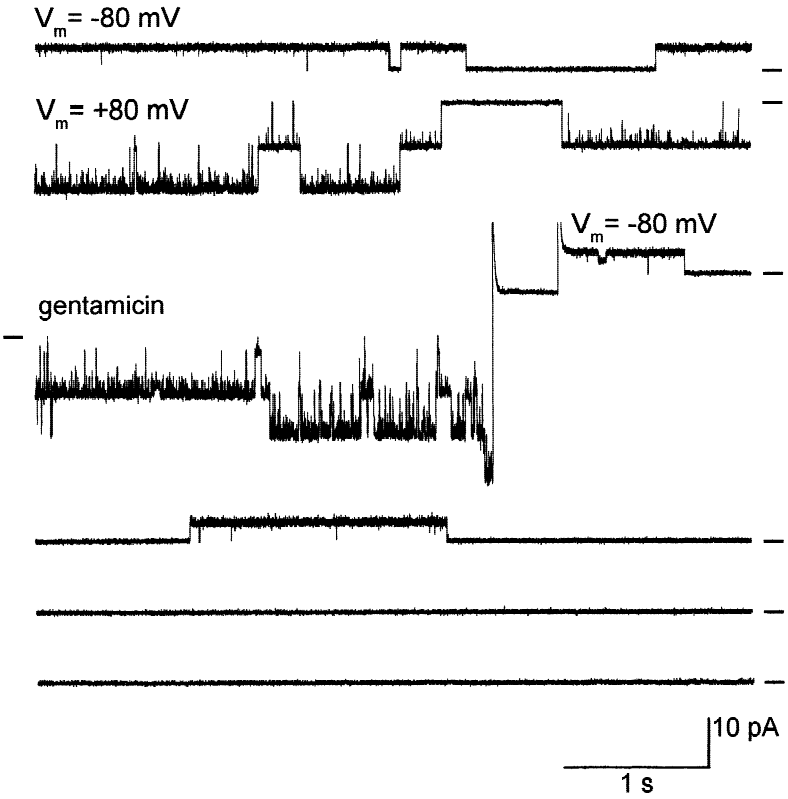

Fig. 7. Voltage dependent block of gentamicin (200 μM) applied in bath solution in an outside-out patch containing at least three channels, from a ganglion. The first (nonconsecutive) two traces from the top show channel activity at membrane potentials of −80 and +80 mV, in the absence of gentamicin. The third trace shows the effect of the inversion from +80 to −80 mV, during drug application. The last three consecutive traces show the block of inward currents. The mark next to each record corresponds to the current with all channels closed.

between mechanosensitive channels in *Xenopus* oocytes and those in leech neurons which prompted us to investigate the possibility of inducing voltage activation in leech neuron patches oriented in the outside-out configuration. The polarity of the membrane in the outside-out configuration was assessed according to two criteria. First, because of the typical outward rectification, in inside-out, the channel current flowing into the pipette should be larger than that flowing out. Second, gentamicin is known to block only inward currents in the inside-out configuration, when applied in the pipette, because it acts from the outside in a voltage-dependent mode (Hamill & McBride, 1996; Calabrese et al., 1999). Thus, the outside-out configuration was unambiguously identified because current rectification was reversed and gentamicin blocked inward currents when applied in the bath. Figure 7 illustrates the voltage-dependent block of inward currents by bath-applied gentamicin, in a patch from a desheathed ganglion. In both Fig. 7 and Fig. 8 it can be noted that inward currents, discriminable for their smaller amplitude, are displayed upward because they flow into the patch pipette as expected in the outside-out configuration.

We found that membrane potential steps induced SAC activation and deactivation in the outside-out configuration. Figure 8 shows two examples of such effects. In the first patch from the membrane of a cultured cell body (Fig. 8*A*), the effect of a depolarizing step of −80 to +80 mV is shown. In the second example (Fig. 8*B*), the response to a deactivating membrane potential step of +80 to −80 mV was recorded in a patch from a growth cone.

Discussion

In excised patches of membrane from leech AP neurons, formed with soft glass pipettes, depolarizing membrane potentials consistently induced a slow and reversible activation of SACs. This activation was observed both in Na^+ and in K^+ symmetrical solutions of pipette and bath. The voltage dependence observed in SACs of leech neurons is similar to that of mechanosensitive channels in *Xenopus* oocytes, reported by Silberberg and Magleby (1997). In the latter case, the unusually slow voltage-dependent activation of channels was shown to be due to voltage-induced membrane displacement in the patch pipette, associated with increased membrane tension, rather than to the intrinsic voltage dependence of channels (Gil, Silberberg & Magleby, 1999). The main features of the voltage dependence that we observed in SACs of leech neurons indicate that the phenomenon is probably associated with voltage-induced changes in membrane tension. This finding is consistent with the observation that suction and depolarization added to their effects, while positive pressure applied to the pipette inhibited the voltage-induced channel activation. Furthermore, assuming that basal channel activity gives a mea-

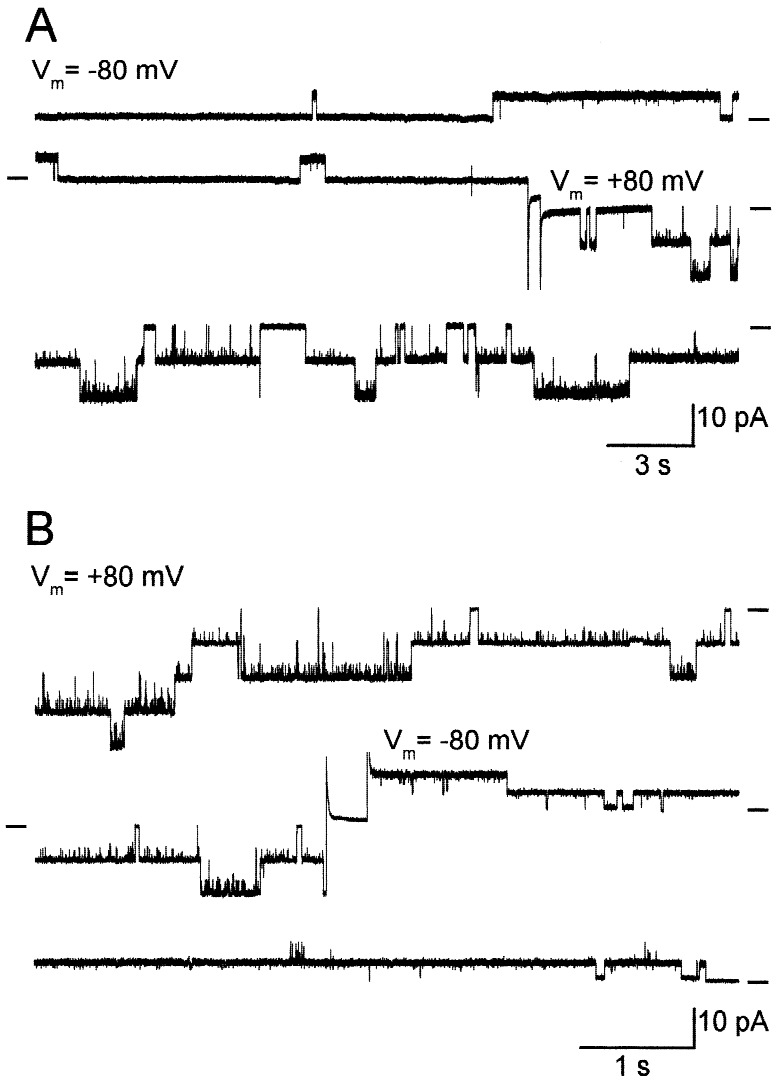

Fig. 8. (*A*) Channel activation stepping the membrane potential from −80 to +80 mV, in an outside-out patch from the cell body of a cultured neuron. (*B*) Deactivating effect of a membrane potential step from +80 to −80 mV in an outside-out patch from a growth cone. The mark next to each record corresponds to the current with all channels closed.

sure of the resting tension in the membrane patch, as expected, the delay to voltage-induced activation was inversely related to the level of channel activation.

Gil et al. have suggested that the mechanism underlying the slow voltage-induced activation of mechanosensitive channels in *Xenopus* oocytes requires a glass-specific interaction between membrane patch and pipette (Gil, Magleby & Silberberg, 1999). They cite two main points to support this hypothesis: firstly, voltage-induced channel activation was not observed with soft glass pipettes, but only with borosilicate glass pipettes and secondly, it was not observed in outside-out patches.

Our findings on leech neurons differ in these two relevant aspects from the results obtained in *Xenopus* oocytes. With pipettes fabricated with soft glass, we found voltage-induced SAC activation and we reproduced the channel activation in the outside-out configuration, applying the proper reference potential. The outside-out configuration was checked by the polarity of the channel current rectification as well as by the voltage-dependent block from outside by gentamicin.

Our current level of knowledge does not permit us to account for these substantial differences. However, in our experience, the elasticity of the membrane in *Xenopus* oocytes is higher than that in leech neurons, as evidently suggested by the wider withdrawal of pipette necessary to get a slow patch excision. Thus, differences in the mean extension of membrane patch, in its housing in the recording pipette, in the pipette radius and membrane elasticity might all be responsible for the differences between the two preparations (*see* Sachs & Morris, 1998). How these factors can affect voltage-induced channel activation remains to be determined.

Since the soft glass we used is different from that used by Gil et al. (1999), the possibility that the observed differences might be due in part to the different composition of the glass remains open.

As a matter of fact, our findings indicate that soft

glass probably allows voltage-induced changes in membrane tension. This has to be taken into account when studying the voltage dependence of ion channels, to avoid artifactual contributions due to changes in membrane tension, as already pointed out for borosilicate glass pipettes (Gil et al., 1999).

The nature of the slow voltage-dependent activation and decay of SACs is still unclear and among possible mechanisms we cannot rule out a direct effect of voltage on the channel or on structures associated with it.

An exciting outcome of this study is that SACs in membrane patches from neurons in culture exhibited significantly shorter delay to voltage-induced activation than their counterparts from neurons of freshly isolated ganglia. This enhanced voltage sensitivity may be indicative of a high mechanosusceptibility of SACs in cultured growing neurons and in particular from growth cones, similar to that reported for molluscan neurons (Small & Morris, 1994). Thus, these channels might operate as mechanotransducers in this membrane domain. These results are also in keeping with recent findings demonstrating that SACs in AP growth cones exhibit distinctive kinetic and conductance properties, probably associated with a specific cytoskeleton assembly (Pellegrini et al., 2001). However, since all the available data concerning SACs of leech have been obtained with single-channel recording techniques and the tight seal formation could make the patch hypermechanosensitive (Hamill & McBride, 1997; Sachs & Morris, 1998; Wan et al., 1999), it remains to be determined how the environment surrounding SACs in situ enable them to exhibit voltage-induced activation and/or mechanosensitivity.

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