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High Conductance Anion Channel in Schwann Cell Vesicles From Rat Spinal Roots

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ABSTRACT Potassium uptake, possibly together with chloride, is one of the presumed functions of Schwann cells in the peripheral nervous system. However, the presence of chloride channels has not been demonstrated in adult Schwann cells. We present here a new method which allows single channel recordings to be made from Schwann cells in situ without enzymatic treatment. Isolated rat spinal roots were split mechanically into several bundles. Within about 30 min after this procedure small bleb-like vesicles (~20–30 μm in diameter) with a clean surface appeared at the edges of the fibre bundles. Immunofluorescence microscopy with a surface marker for Schwann cell membranes (monoclonal antibody O4) revealed that the vesicles originate from Schwann cells. In standard patch clamp recordings with symmetrical bath and pipette solutions (excised inside-out configuration) an anion channel with the following characteristics was mainly observed: 1) single channel slope conductance of 337 ± 5 pS in 125 mM KCl and 209 ± 6 pS in 125 mM K^+ methylsulphate; 2) ion permeability ratio: $P_{Cl}/P_K/P_{gluconate}$ = 1/0.12/0.06; 3) linear current–voltage relationship (range ± 60 mV); and 4) voltage- and time-dependent inactivation (the channel was most active at potentials ± 20 mV). Pharmacologically, the channel was completely blocked with zinc (1 mM) and barium (10 mM). A similar anion channel, showing characteristics 1 – 4), has been described in cultured Schwann cells of newborn rats (Gray et al., 1984). We now demonstrate that this channel is also present in adult Schwann cells in situ.

INTRODUCTION

Potassium uptake is one of the presumed functions of Schwann cells in the peripheral nervous system. One of the mechanisms currently under consideration is passive KCl uptake. Thereby, potassium entering glia via ion channels is balanced by the entry of chloride and water (Barres et al., 1990a, b; Boyle and Conway, 1941; Grafe and Ballanyi, 1987; Gray and Ritchie, 1985). Inwardly rectifying K^+ channels, as one of the prerequisites for passive KCl uptake, have been detected recently on paranodal Schwann cell membrane (Wilson and Chiu, 1990). The presence of chloride channels, on the other hand, has not been demonstrated in adult Schwann cells, although such channels have been observed in cultured Schwann cells of newborn rats (Gray et al., 1984). The main reason for the missing evidence of Cl^- channels seems to be the lack of an adequate method for the electrophysiological study of Schwann cell membrane in situ. In the present study we describe a new method by which, without preceding enzymatic treatment, single anion channel currents were recorded from Schwann cells in adult rat spinal roots.

The method is based on our observation that Schwann cells form vesicles in isolated, mechanically *split rat* spinal roots. Vesicle formation on isolated cells has been long known (Hogue, 1919; Zollinger, 1948). In the last few years such vesicles from skeletal muscle were used for single channel recordings with patch clamp tech-

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niques (Burton et al., 1988; Quasthoff et al., 1990; Standen et al., 1984; Stein and Palade, 1988). One important advantage of this method is that no enzymatic treatments, which might produce artefacts in single channel recordings (Franke and Dudel, 1987), are necessary to obtain a clean membrane surface. We have now explored whether the vesicle method is applicable for single channel recordings from another in vitro preparation—the isolated rat spinal root. Here we describe single channel currents recorded from a high conductance anion channel in Schwann cell vesicles.

MATERIALS AND METHODS

Wistar rats (250–400 g) were used. The animals were anaesthetized with urethane (1.25 g/kg i. p., supplemented as required) for a laminectomy to expose the cauda equina. Subsequently, five to ten ventral and dorsal roots were removed (length around 30 mm). One isolated spinal root at a time was transferred into a culture dish with the following bathing solutions (in mM): *Solution A* ("125 KCl"): KCl 120, $MgCl_2$ 3, EGTA 1, HEPES 5, KOH 5; *Solution B* ("125 NaCl"): NaCl 120, $MgCl_2$ 3, EGTA 1, HEPES 5, NaOH 5; *Solution C* ("125 KMS"): K^+ methylsulphate 120, $MgCl_2$ 3, EGTA 1, HEPES 5, KOH 5. The pH of the solutions was 7.2; experiments were performed at room temperature. In the culture dish, the spinal roots were split mechanically into several fibre bundles. In doing so, the bundles were always kept below the surface of the solution. Mechanical stabilization of the roots was achieved by adhesive tape. Within about 30 min after this procedure small bleb-like vesicles occurred at the edges of the fibre bundles. The number of blebs varied between none and up to 15 per spinal root. Sometimes aggregates of vesicles were seen. Figure 1 illustrates the way the spinal roots were split before the vesicles appeared.

Two-step indirect immunofluorescence staining was performed to determine the cellular origin of the blebs. Teased fibres of spinal roots, prepared as for patch clamp experiments, were labeled with the monoclonal antibody O4, a surface marker for Schwann cell membranes (Mirsky et al., 1990). The primary mouse monoclonal antibody O4 (supernatant fluid) and the secondary anti-mouse antibody conjugated with fluorescein isothiocyanate (FITC) were generously provided by Dr. H. Rohrer (MPI Martinsried, F.R.G.). The antibody O4 was used in a dilution of 1:200 in phosphate buffer solution (PBS). The fibres were incubated for 12 h. Subsequently, they were washed three times and then fixed in 4% paraformaldehyde in PBS for 15 min. After washing the fibre bundles were incubated in the anti-mouse antibody conjugated with FITC in a dilution of 1:100 (in PBS) for 20 min. The preparations were examined with a Nikon fluorescence microscope (Microphot-FX).

Standard patch clamp techniques (Hamill et al., 1981) were used to measure single channel activity from excised inside-out patches. These were obtained after the formation of a gigaseal by abruptly moving the patch pipette away from the vesicle. Patch pipettes were pulled (DMZ puller; Zeitz, Augsburg, F.R.G.) from borosilicate glass tubes (GC 150 TF-10, Clark Electromedical Instruments, Pangbourne, England), coated with Sylgard, and fire-polished immediately before the experiment. Single channel currents were recorded with pipettes of 3–10 MΩ resistance. Pipettes were filled with either solution A, B, or C (see above). The patch clamp amplifier was a List EPC-7. Data were low pass filtered at 3 kHz, digitized (CED 1401, CED Cambridge, England; sampling rate 8 kHz), and stored on a personal computer. Data were analyzed and plotted by means of a commercial patch clamp programme (EPC, CED Cambridge, England). Voltages given in the text and figures indicate pipette potential.

Drugs and test solutions were applied via a multibarrelled pipette perfusion system. After obtaining an

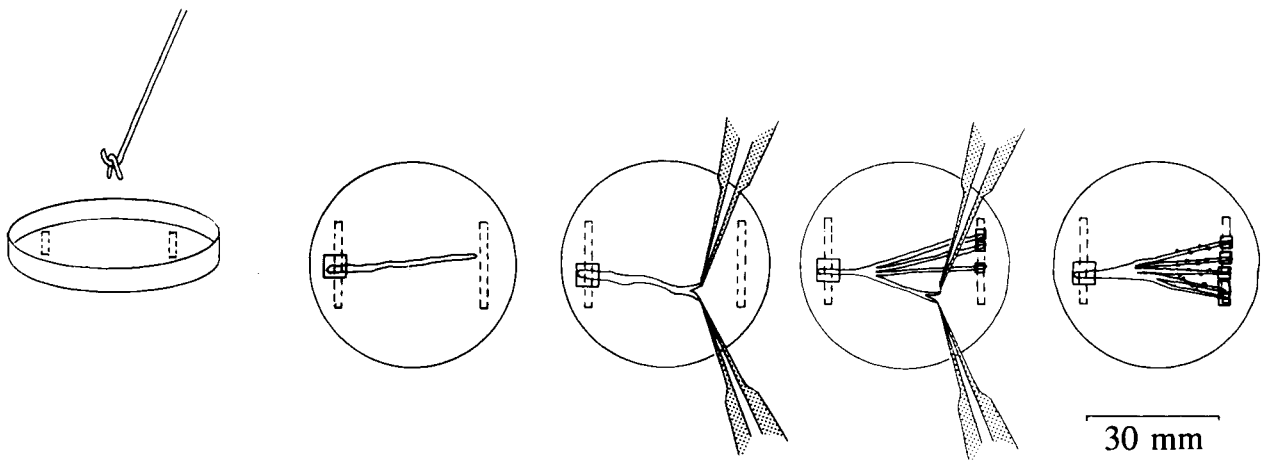

Fig. 1. Mechanical splitting of a rat spinal root in a culture dish. The spinal root was teased into several bundles by means of fine forceps. The bundles were stabilized by adhesive tape. Small bleb-like vesicles appeared at the edges of the fibres within 30 min.

inside-out patch, the patch was consecutively moved in one of the parallel streams ejected from the pipettes. The ground electrode was electrically connected to the bath solution by a 125 mM KCl agar bridge. The agar bridge was not exposed to the test solutions. Test solutions for the determination of permeability ratios contained (in mM): *Solution D*: solution A plus 125 mM KCl; *Solution E*: solution A plus 125 mM K^+ gluconate.

RESULTS

Morphology and Immunofluorescence Microscopy of Schwann Cell Blebs

Within 30 min after splitting of the isolated rat spinal roots, small bleb-like vesicles occurred at the edges of the fibre bundles. These vesicles, which had a diameter of 20–30 μm, showed a clean surface in phase-contrast microscopy (Fig. 2, left). In order to characterize the origin of the vesicles we used the monoclonal antibody O4, a surface marker for Schwann cell membrane (Mirsky et al., 1990). Using immunofluorescence microscopy, all vesicles (we examined about 50), as well as Schwann cells, were found to be O4 positive; an example is shown on the right in Figure 2. No immunoreactivity was observed when the primary antibody was omitted from the staining procedure.

High Conductance Anion Channel

Gigaseals were obtained with a high rate of success on the vesicles. One third of excised patches in the inside-out configuration displayed single channel current activity. A regular observation was currents from up to four high conductance anion channels. Figure 3 illustrates a typical observation obtained from a membrane patch bathed in identical, 125 mM NaCl-containing solutions in bath and pipette (solution B, see Materials and Methods). The holding potential was 0 mV; from this level, pipette voltage was changed from −50 to +50 mV in steps of 10 mV (pulse duration 1,000 ms). The larger voltage steps induced clearly visible current fluctuations between an open and a closed level. At the voltages around zero, however, channel activity was not prominent since these potentials were close to the chloride equilibrium potential (symmetrical solutions) and the channel spent most of the time in the open configuration. Current fluctuations at pipette potentials outside ± 20 mV occurred with variable duration: after being active for a short time (several hundred milliseconds up to seconds) the channel shut to a closed state. The openings during the active phase were interrupted by closures of varying length within the millisecond range.

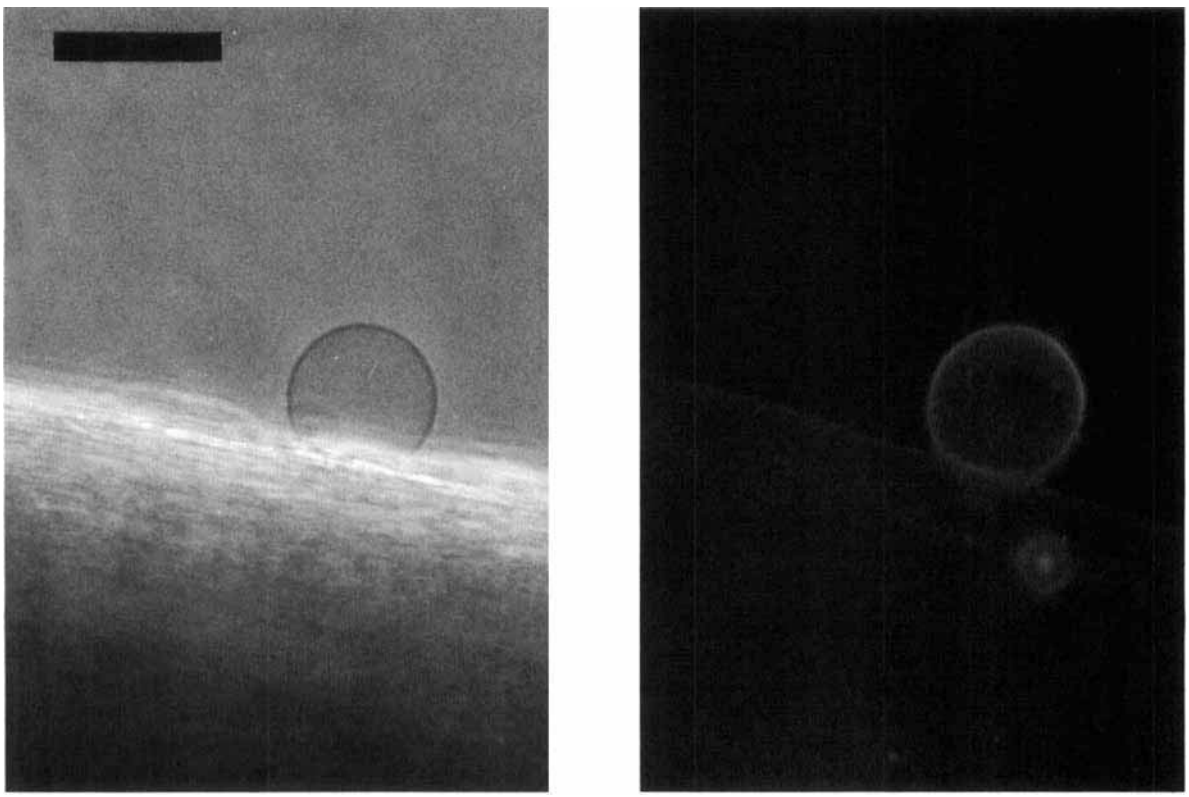

Fig. 2. Phase-contrast (on the left) and immunofluorescence micrographs (on the right) of the same vesicle which occurred at the edge of a teased rat spinal root. Two-step indirect immunofluorescence staining was performed with the monoclonal antibody O4, a surface marker for Schwann cell membrane. The vesicle (as well as Schwann cells) was found to be O4 positive. Scale bar = 30 μm.

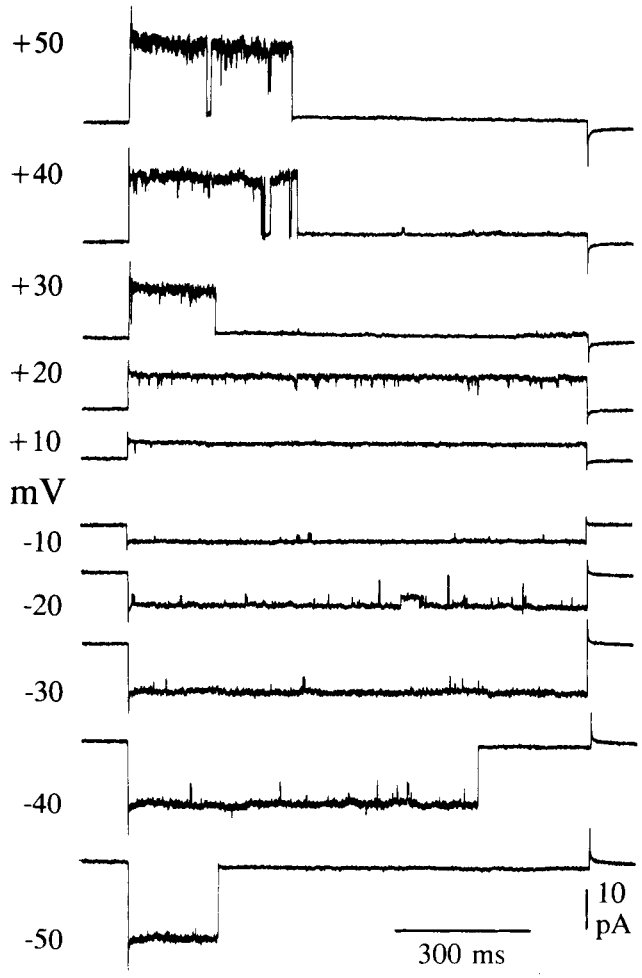

Fig. 3. Single channel recordings of the anion channel of excised, inside-out patches of Schwann cell vesicles with 125 mM NaCl (solution B; see Materials and Methods) on both sides of the membrane. The voltage was held at 0 mV and then stepped to each new holding potential for 1,000 ms. (Voltages given indicate pipette potential.) The open duration of the anion channel was voltage dependent with a maximum in the range of ± 20 mV.

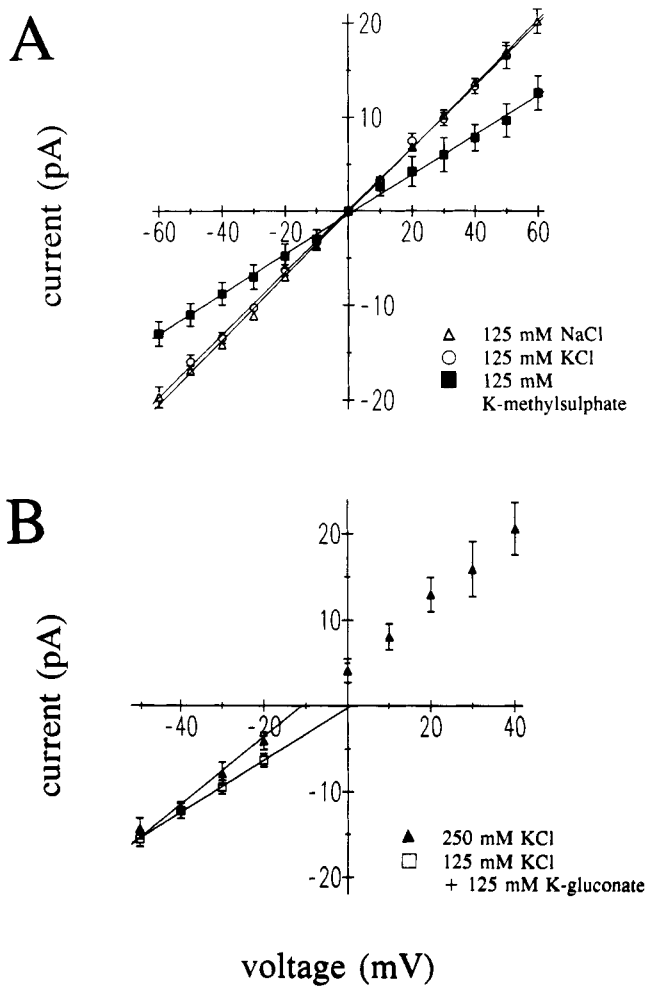

Fig. 4. Single channel current–voltage relationship of the anion channel. Illustrated are averaged data (mean ± SD) from patches in symmetrical (A) and asymmetrical solutions (B). A: Symmetrical solutions: The slope conductance was 337 ± 5 pS (n = 11) in 125 mM KCl (open circle), 339 ± 4 pS (n = 7) in 125 mM NaCl (open triangle), and 209 ± 6 pS (n = 9) in 125 mM K^+ methylsulphate (filled square). B: Asymmetrical solutions: Filled triangles show the current–voltage relationship with 125 mM KCl in the pipette solution and 250 mM KCl in the bath solution (n = 11); note the shift in the reversal potential of −11.5 mV. Unfilled squares show the current–voltage relationship with 125 mM KCl in the pipette solution and 125 mM KCl plus 125 mM K^+ gluconate in the bath solution (n = 7); note the shift in the reversal potential of 1.5 mV. For calculation of permeability ratios see text.

Selectivity

Channel conductance and selectivity were determined in a first series of experiments. Figure 4 illustrates current–voltage (I-V) relationships of single ion channels with electrophysiological characteristics as described above. In symmetrical solutions of different ionic composition a linear I-V relationship with a reversal potential of 0 mV was always observed (Fig. 4A). The calculated slope conductances were: 337 ± 5 pS (mean ± SD, n = 11) in 125 mM KCl (solution A), 339 ± 4 pS (n = 7) in 125 mM NaCl (solution B), and 209 ± 6 pS (n = 9) in 125 mM K^+ methylsulphate (solution C).

Further experiments were performed to determine the permeability ratios for cations and anions (Fig. 4B). In these experiments the pipette was always filled with 125 mM KCl (solution A). The bath contained either an identical 125 mM KCl solution or test solutions with 250 mM KCl (solution D) or 125 mM KCl plus 125 mM K^+ gluconate (solution E). The shift of the reversal potential of the I-V relationship in the test solutions was measured and used to calculate permeability ratios by means of the Goldman-Hodgkin-Katz equation. Ion activities were calculated according an equation given by Ammann (1986, p. 76). Thereby, the reversal potential shift of −11.5 mV observed in the solution with 250 mM KCl revealed a permeability ratio of $P_K/P_{Cl} = 0.12$. Using this ratio, $P_{gluconate}/P_{Cl}$ was then calculated. In this case, the shift of 1.5 mV observed in the solution with 125 mM KCl plus 125 mM K^+ gluconate revealed a

ratio of 0.06. In the gluconate-containing solution outward current recordings at positive pipette potentials (inwardly directed anion currents) showed rapid changes between open and closed states. Therefore, exact measurements of current amplitudes were not possible.

Ensemble Current

A voltage- and time-dependent inactivation was a typical observation made on the high conductance anion channels. To illustrate this electrophysiological characteristic, recordings of ensemble currents were made. Figure 5 was obtained from an excised, inside-out patch by repeating the voltage commands described in Figure 3 20 times. Afterwards, the membrane currents of identical voltage pulses were averaged. The result of this computation clearly shows that the inactivation of the channel was voltage dependent with a high open probability centered around 0 mV. As the potential increased in either directions the periods of channel activity became shorter and the channel entered long-lasting closed states. The time constant of inactivation was in the range of several hundreds of milliseconds, e.g., at + 50 mV the single exponential time constant was about 300 ms.

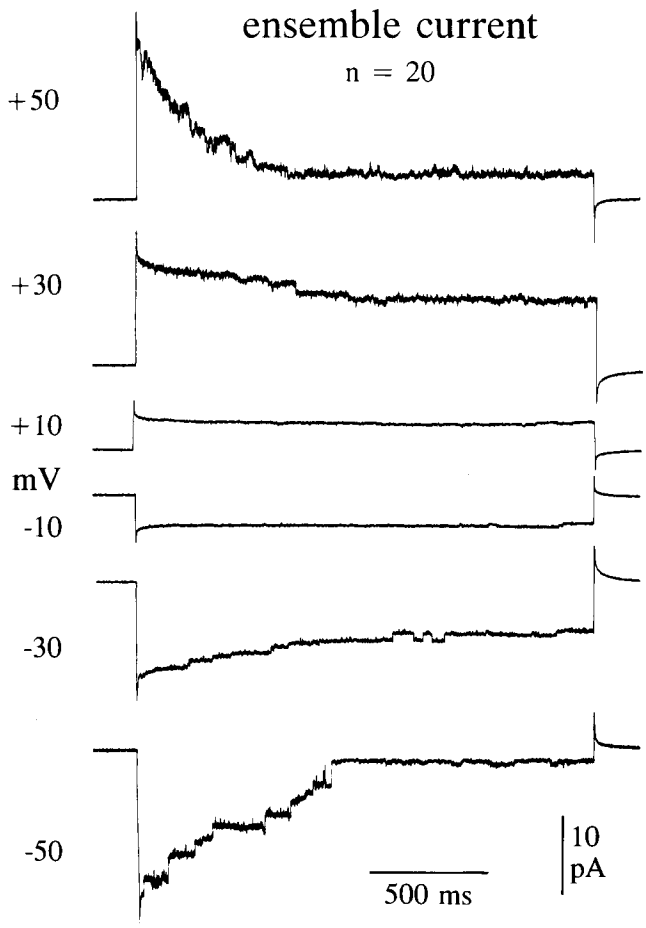

Fig. 5. Average (ensemble current) of the openings of a single anion channel from a series of voltage steps from a holding potential of 0 mV to the (pipette) potentials indicated. Each voltage step was repeated 20 times with an interval of 5 s. The duration of the open state was voltage dependent with a maximum in the range of ± 20 mV (solution B, see Materials and Methods).

Pharmacological Observations

In a last series of experiment, the effects of zinc and barium were explored. Figure 6 illustrates the effect of zinc, a divalent cation which completely blocked the high conductance anion channel. Figure 6A shows channel activity during voltage commands to ± 30mV. The recording was made with the patch pipette (inside-out configuration) exposed to the control solution (solution C). The channel spent most of the time in the open state. Only short-lasting periods of closures were observed. The patch pipette was then shifted to the adjacent fluid stream of the multi-barrelled application pipette, which contained the control solution plus 1 mM Zn^{2+}. Now, the electrophysiological characteristics of channel activity changed. After about 30 s exposure to Zn^{2+}, the channel opened for brief periods only, a behaviour which can be described as a flickering block (Fig. 6B). After about another 30 s, channel activity completely disappeared —indicating that Zn^{2+} had completely blocked the ability of the channel to pass anions (Fig. 6C). The patch pipette was then withdrawn and exposed to the control solution. After a few minutes, a complete recovery from the blocking action of Zn^{2+} was observed (Fig. 6D).

We also made the observation that barium ions interfered with the current activity of the high conductance anion channel. The experiment illustrated in Figure 7 was made similar to the experimental protocol just described for Zn^{2+}. Ba^{2+} in a concentration of 1 mM produced a flickering channel block (Fig. 7A–C), whereas 10 mM Ba^{2+} completely blocked the channel (Fig. 7D–F).

DISCUSSION

The vesicles observed in the isolated rat spinal roots were stained with the antibody O4, which recognizes a lipid (probably sulfatide) antigen of the membrane in Schwann cells and oligodendrocytes (Mirsky et al., 1990). Fibroblasts have never been found to be O4 positive (Mirsky et al., 1990). This indicates that the high conductance channels recorded on O4 positive vesicles are normal constituents of Schwann cell membrane in situ. However, it is not possible to differentiate between myelinating and non-myelinating Schwann cells. The microscopic appearance and the ease by which gigaseals were obtained indicate that the membrane of the vesicles might be a rather clean lipid bilayer with no or only few elements of the extracellular matrix and/or the cytoskeleton. Therefore, ion channels in the vesicle membrane which normally are regulated by the cy-

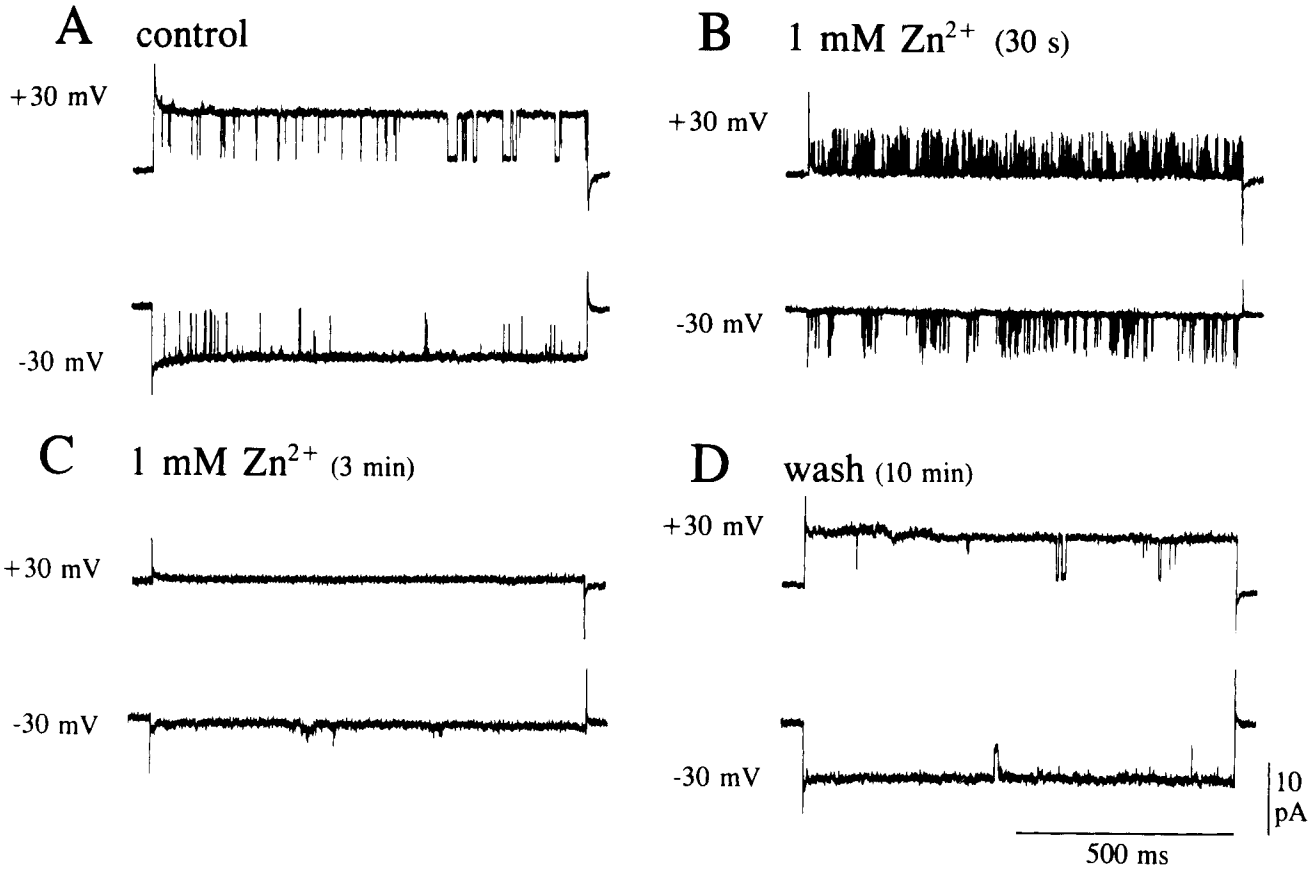

Fig. 6. Reversible block of the anion channel by Zn^{2+}. The voltage was held at 0 mV and then stepped to the pipette potentials indicated. A: Control in symmetrical solutions with 125 mM K^+ methylsulphate. B: 1 mM Zn^{2+} applied for 30 s caused rapid current fluctations between open and closed states. After about 3 min a complete block of the anion channel was observed (C). This effect was reversible after 10 min in the control solution (D).

toskeleton, other membrane proteins (e.g., G-proteins), cytoplasmic factors, and/or components of the extracellular matrix may display current characteristics very different from "physiological" activity. However, it is doubtful whether conventional patch clamp seals reveal single channel activity closer to the "physiological" situation. In fact, it was shown recently by Milton and Caldwell (1990) that lipid blebs, similar to the ones used in the present study, readily form when suction is applied to patch clamp electrodes in conventional experiments.

The main electrophysiological characteristics of the channel described in the present study are: 1) high conductance; 2) ion selectivity sequence: chloride < methylsulphate << potassium < gluconate; 3) long-lasting active periods of the channel only at membrane potentials between around ± 20 mV in symmetrical Cl^- or methylsulphate concentrations; and 4) complete inactivation after voltage steps from 0 mV to the potential range above +30 or below −30 mV. The inactivation is slow (several hundred milliseconds up to seconds).

Single anion channel currents with such properties have been previously found in glial cells (in Schwann cells: Gray et al., 1984; McLarnon and Kim, 1990; in astrocytes: Jalonen et al., 1989; Nowak et al., 1987; Sonnhof, 1987). So far, the discussion about the physiological function of these channels was hindered by the fact that all studies mentioned used cultured cells. Therefore, the possibility remained that the expression of such Cl^- channels in glial cells might have been a consequence of deprivation of a neuronal surrounding. However, in our experiments, adult Schwann cells were used in contact to axons. Therefore, we assume that high conductance anion channels are normal proteins in the membrane of adult Schwann cells in situ.

The physiological function, however, of the high conductance anion channels remains unclear. One of the problems is activity around 0 mV. It seems obvious, therefore, that they do not contribute to the membrane conductance at resting potential. However, the relationship between open probability and resting potential was observed in symmetrical anion concentrations. It is possible that this relationship changes when, in accordance to the physiological situation (see measurements of intracellular Cl^- concentrations in glial cells: Ballanyi et al., 1987; Bührle and Sonnhof, 1983; Coles et al., 1989), asymmetric Cl^- concentrations would be used. In fact, it was shown recently that anion channels on

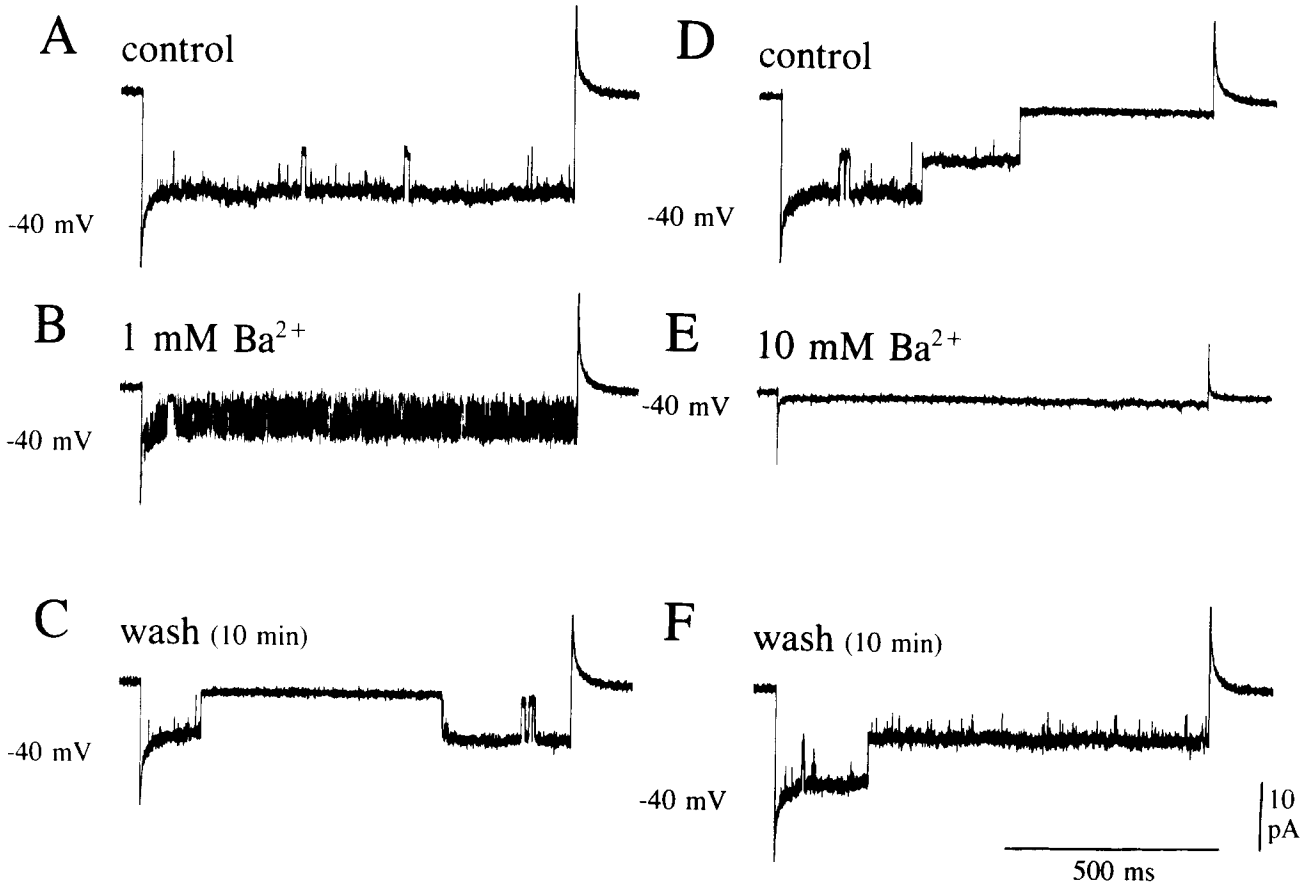

Fig. 7. Concentration dependent block of the anion channel by Ba^{2+} in the bathing solution. Single channel recordings of excised, inside-out patches (for pulse protocol see Fig. 6). A, D: Control in symmetrical solutions with 125 mM K^+ methylsulphate (solution C, see Materials and Methods). B: 1 mM Ba^{2+} caused a "flickering" block of the anion channel. E: 10 mM Ba^{2+} blocked the channel completely. These effects were reversible (C, F).

vesicles from sarcoplasmic reticulum (Hals and Palade, 1990) and *Torpedo* electroplax (Richard and Miller, 1990) are gated not only by membrane potential but also by the permeant ion itself. This issue was not further explored in the present study.

The blocking effect of zinc is in accordance with the pharmacological activity of this ion on high conductance anion channels in other tissues (Schlichter et al., 1990; Woll et al., 1987). Therefore, zinc might be a pharmacological tool to study these channels. However, the effect of zinc should also be discussed in context with an intriguing previous finding. Gray et al. (1984) noted that the high conductance anion channels in cultured Schwann cells of 1–2 day old rats did not become active until several minutes after the cytoplasmic surface had been exposed to the bathing medium. The authors suggested that these channels may normally be kept in an inactive state by some unknown internal factor. No such delay between patch exposure and channel activity was found in our experiments. This may indicate that the inhibitory factor is not present in the fluid filling the vesicles. Zinc is a physiological trace element in the mammalian brain and it is not completely unlikely that this ion might be the internal factor proposed by Gray et al. (1984). In the mammalian central nervous system, for example, a zinc-induced antagonism to the action of N-methyl-D-aspartate on cortical and hippocampal neurons has been described (Peters et al., 1987; Westbrook and Mayer, 1987). For a definitive conclusion, however, quantitative information about the free intracellular Zn^{2+} concentration in Schwann cells and the exact dose–response relationship between Zn^{2+} and the anion channel is necessary.

A new finding is the strongly reduced open probability of the channel induced by the cytoplasmic application of Ba^{2+}. Barium ions are well known as potent blockers of certain potassium channels (Hille, 1984). However, many of the data obtained by using Ba^{2+} as a "selective" K^+ channel blocker need a reinterpretation in view of the possibility, raised by the present study, that Ba^{2+} might have blocked anion channels as well.

One of the possible functions of the high conductance anion channel in the Schwann cell membrane might be promotion of passive KCl uptake (see Introduction). Our data are not sufficient to support or to exclude this hypothesis. However, the pharmacological properties

described above could be used to design experiments with the aim of exploring this question. Potassium uptake in glial cells or activity-dependent K^+ accumulation in the extracellular space of the nervous system should be tested by using either zinc ions as blockers of the channel or by exploring differences between solutions containing either chloride or methylsulphate as permeant anions or gluconate as a nonpermeant anion.

In conclusion, the present paper describes a new method for the study of single channel currents of Schwann cell membrane in situ. The main advantages of the technique are 1) that enzymatic treatment is not necessary and 2) the ease by which gigaseals can be obtained. Furthermore, this technique can give access to Schwann cell membrane regions outside the cell body. The method was used to characterize a high conductance anion channel in Schwann cell membrane.

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