Single voltage-activated Na^+ and K^+ channels in the somata of rat motoneurones

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1. Voltage-activated Na^+ and K^+ channels were investigated in the soma membrane of motoneurones using the patch-clamp technique applied to thin slices of neonatal rat spinal cord.

2. One type of TTX-sensitive Na^+ channel, with a conductance of 14·0 pS, was found to underlie the macroscopic Na^+ conductance in the somata of motoneurones. These channels activated within a potential range between −60 and −20 mV with a potential of half-maximal activation (E_{50}) of −38·9 mV and steepness factor (k) of 6·1 mV.

3. Kinetics of Na^+ channel inactivation could be fitted with a single exponential function at all potentials investigated. The curve of the steady-state inactivation had the following parameters: a half-maximal potential ($E_{h,50}$) of −81·6 mV and k of −10·2 mV.

4. Kinetics of recovery of Na^+ channels from inactivation at a potential of −80 mV were double exponential with fast and slow components of 16·2 (76%) and 153·7 ms (24%), respectively. It is suggested that the recovery of Na^+ channels from inactivation plays a major role in defining the limiting firing frequency of action potentials in motoneurones.

5. Whole-cell K^+ currents consisted of transient (A)- and delayed-rectifier (DR)-components. The A-component activated between −60 and +20 mV with an E_{50} of −33·3 mV and k of 15·7 mV. The curve of steady-state inactivation was best fitted with an $E_{h,50}$ of −82·5 mV and k of −10·2 mV. The DR-component of K^+ current activated smoothly at more positive potentials. E_{50} and k for DR-currents were +1·4 and 16·9 mV, respectively.

6. The most frequent single K^+ channel found in the somata of motoneurones was the fast inactivating A-channel with a conductance of 19·2 pS in external Ringer solution. In symmetrical high-K^+ solutions the conductance was 50·9 and 39·6 pS for inward and outward currents, respectively. The channel activation took place between −60 and +20 mV. The curve of steady-state inactivation of single A-channels had an $E_{h,50}$ of −87·1 mV and k of −12·8 mV. In high-K^+_o solution A-channels demonstrated a rapid deactivation at potentials between −110 and −60 mV. The time constant of the channel deactivation depended on the membrane potential and changed from 1·5 ms at −110 mV to 6·3 ms at −60 mV.

7. Delayed-rectifier K^+ channels were found in the soma membrane at a moderate density. The channel conductance in Ringer solution was 10·2 pS and in symmetrical high-K^+ solutions was 31·1 and 22·5 pS for inward and outward currents, respectively. The activation of the channels took place at −60 to 0 mV with an E_{50} of −43·8 mV and k of 8·5 mV. In external high-K^+_o solution DR-channels showed a slow deactivation with a time constant of 5·9 ms at −110 mV and 60·0 ms at −60 mV.

8. Tetraethylammonium suppressed both A- and DR-components of whole-cell K^+ conductance and reduced the amplitudes of the single-channel currents. The concentration giving 50% inhibition (IC_{50}) values was 14·8 and 0·8 mM, respectively.

9. TTX-sensitive Na^+ channels, together with A- and DR-types of K^+ channels, form the basis of voltage-activated conductance in the soma membrane of rat motoneurones.

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Until now, voltage-activated ionic channels in the soma membrane of motoneurones have not been studied at the single-channel level. Numerous investigations carried out during the last 40 years by means of conventional intra- and extracellular electrodes provided knowledge about the main macroscopic conductances underlying membrane excitability in motoneurones (Brock, Coombs & Eccles, 1953; Coombs, Eccles & Fatt, 1955; Barrett & Crill, 1980; Barrett, Barrett & Crill, 1980; Fulton & Walton, 1986). Application of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) to thin slices of rat brain and spinal cord (Edwards, Konnerth, Sakmann & Takahashi, 1989) has enabled the study of voltage-activated ionic currents in visually identified motoneurones under conditions of whole-cell voltage clamp and intracellular perfusion (Takahashi, 1990). It was shown that the activation of inward Na^+ currents during membrane depolarization was followed by the development of outwardly directed transient (A-type) and delayed-rectifier (DR) K^+ currents. However, because of insufficient space clamp of membrane of motoneurones within the slices and problems with a complete pharmacological or electrophysiological separation of the current components in a broad range of physiological voltages, no one current component could be described in detail.

The main purpose of the present study was to apply the patch-clamp technique to thin slices of neonatal rat spinal cord, in order to characterize single Na^+ and K^+ channels in excised membrane patches obtained from the soma membrane of motoneurones.

Additionally, one type of Na^+ and several types of K^+ voltage-gated ion channels have recently been described in enzymatically demyelinated toad, rat and human axons (Jonas, Bräu, Hermsteiner & Vogel, 1989; Safronov, Kampe & Vogel, 1993; Scholz, Reid, Vogel & Bostock, 1993; for review see Vogel & Schwarz, 1995). It was of special interest, therefore, to extend our knowledge about peripheral ion channels to those in the cell soma and to compare properties of axonal and somatic channels in rat motoneurones.

Some of these results have been presented to the German Physiological Society (Safronov & Vogel, 1994).

METHODS

Preparation

Experiments were performed on 200 μm thin slices, cut from the lumbar enlargement (L3–6) of the spinal cord of 2- to 8-day-old rats, by means of the patch-clamp technique (Hamill *et al.* 1981; Edwards *et al.* 1989). Rats were rapidly decapitated and the spinal cords were carefully cut out. The slices were prepared and kept according to a procedure given by Takahashi (1990). Under microscopic control the membranes of motoneurones were cleaned from connective tissue by repetitive blowing and suction of Ringer solution through a broken patch pipette.

Solutions

Ringer solution for preparation and maintaining the slices contained (mM): NaCl, 115; KCl, 5·6; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11; NaH_2PO_4, 1; $NaHCO_3$, 25 (pH 7·4 when bubbled with 95% O_2–5% CO_2). During all the experiments the slices were perfused with low Ca^{2+}–high Mg^{2+} Ringer solution, in order to reduce synaptic activity in motoneurones. This solution was obtained from the one above by setting the concentration of Ca^{2+} and Mg^{2+} to 0·1 and 5 mM, respectively. This solution is referred to below as Ringer solution. A tetraethylammonium (TEA)-containing solution used for investigation of Na^+ channels (Ringer–TEA solution) contained (mM): NaCl, 95; KCl, 5·6; $CaCl_2$, 0·1; $MgCl_2$, 5; glucose, 11; NaH_2PO_4, 1; $NaHCO_3$, 25; TEA-Cl, 20 (pH 7·4 when bubbled with O_2–CO_2).

The study of K^+ channels was mainly performed with inside-out membrane patches for reasons given below. The pH of tetrodotoxin (TTX)-containing Ringer solution used for filling the pipettes was buffered with Hepes–NaOH buffer (Ringer–TTX solution) and contained (mM): NaCl, 136·4; KCl, 5·6; $CaCl_2$, 0·1; $MgCl_2$, 5; glucose, 11; Hepes 10; and 0·1–0·5 μM TTX. The pH was adjusted to 7·4 with 4·6 mM NaOH. This solution was also used for recording whole-cell K^+ currents in Fig. 6. Ca^{2+}-free Ringer–TTX solution was obtained from Ringer–TTX solution by omitting calcium ions. High-K^+_o solution (155 mM K^+), used on the external side of the membrane, contained (mM): NaCl, 5; KCl, 152·5; $CaCl_2$, 2·2; $MgCl_2$, 1; Hepes, 5 (pH adjusted to 7·4 with 2·5 mM KOH). TTX (0·1 μM) was added to block voltage-activated Na^+ channels. All blocking agents studied were added directly to the external solutions. Bovine serum albumin (0·05%) was added to all dendrotoxin (DTX)-containing solutions to prevent non-specific binding of the blocker molecules to the surface of the experimental chamber.

The solution used on the internal side of the membrane for the study of Na^+ channels (high-Cs^+_i) contained (mM): NaCl, 5·8; CsCl, 134; $MgCl_2$, 1; EGTA, 3; Hepes, 10 (pH adjusted to 7·3 with

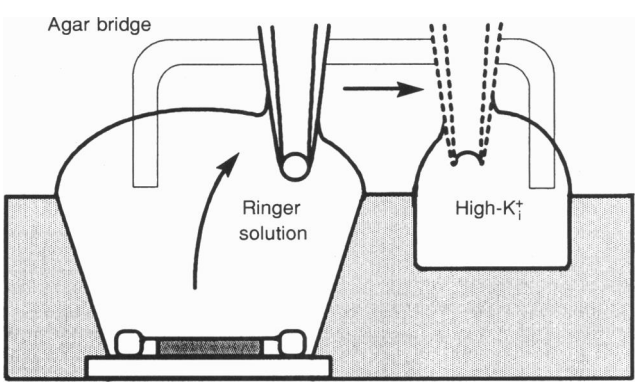

Figure 1
Scheme of Teflon chamber with two baths used for study of inside-out patches.

9·2 mM NaOH). The standard internal solution for investigation of K^+ channels (high-K_i^+) contained (mM): NaCl, 5; KCl, 144·4; $MgCl_2$, 1; EGTA, 3; Hepes, 10 (pH adjusted to 7·3 with 10·6 mM KOH).

Experimental chamber for investigation of inside-out patches

The Teflon chamber used in our experiments contained two compartments (Fig. 1). The slice was fixed with a grid of nylon threads glued to a stainless-steel frame in the main glass-bottomed 0·6 ml bath which was continuously perfused with Ringer–TEA solution (3 ml min^{-1}). Whole cells, cell-attached and outside-out membrane patches were studied in the main bath. An investigation of inside-out patches in the main bath was, however, complicated, since the perfusion of the slice with internal high-K_i^+ solution produced a strong depolarization of the neuronal membranes and, as a consequence, the destruction of the cells in the slice. Therefore, the investigation of the inside-out patches was carried out in an additional small 0·2 ml bath located approximately 0·5 mm away from the main bath (Fig. 1). The small bath filled with internal high-K_i^+ solution was electrically connected with the main bath through an agar bridge. The hydrophobicity of Teflon allowed us to form high menisci with the solutions in both baths by appropriate positioning of the perfusing and sucking needles. In order to obtain an inside-out patch in the small bath, we first established the cell-attached patch configuration in the main bath. Then the pipette was carefully pulled away to obtain a membrane vesicle. Finally, the tip of the pipette with the vesicle was moved through the air from the main bath into the small bath. The external membrane of the vesicle was destroyed during such a transition that resulted in the formation of an inside-out patch in the small bath. This simple method allowed us to obtain several inside-out patches from the same slice. If the cell membrane was in a good state, the success rate of the transition through the air was about 90%. If, after the transition through the air the vesicle remained unbroken, then the exposure of the pipette tip to the air was repeated, sometimes several times, until the external membrane of the vesicle was destroyed. Some stable inside-out patches, in turn, could survive three to ten transitions between air and bath solution.

Current recordings

The patch pipettes were pulled in two stages from borosilicate glass tubing (GC 150; Clark Electromedical Instruments, Pangbourne, UK). Those which were used for single-channel recordings were coated with Sylgard 184 (Dow Corning, USA). The pipettes used for whole-cell recordings had a resistance of 3–6 MΩ and those for excised patches 4–35 MΩ. All the pipettes were fire polished directly before the experiments. The patch-clamp amplifier was a List EPC-7 (Darmstadt, Germany). The effective corner frequency of the low-pass filter, unless otherwise mentioned, was 2 kHz in the experiments with Na^+ channels and 1 kHz during the study of K^+ channels. The frequency of digitization was at least twice as high as that of the filter. The data were stored and analysed by using commercially available software (pCLAMP, Axon Instruments, Foster City, CA, USA). Capacity and leakage currents were digitally subtracted in all recordings by using records with either negative pulses or positive pulses which activated no channels. The current amplitudes and durations of the channel openings in tail events were measured directly with two cursors. Offset potentials were nulled directly before formation of a seal. Mistakes in the clamped potential evoked by the series resistance of the electrode were not corrected. All experiments were carried out at a room temperature of 21–24 °C.

Identification of motoneurones

The motoneurones were identified in spinal cord slices as the largest visible cells in the ventrolateral region of the ventral horn. The mean lengths of the long and the short diameters of the investigated cells were 22·3 ± 1·0 μm (11 cells) at 2 days; 25·3 ± 0·1 μm (23 cells) at 3 days; 24·1 ± 0·8 μm (45 cells) at 4–5 days and 24·7 ± 0·6 μm (15 cells) at 6–7 days. Values are quoted as means ± S.E.M. The cell diameters given here correspond closely to those reported for neonatal rat motoneurones (Fulton & Walton, 1986; Takahashi, 1990). In current-clamp recording mode, the motoneurones spontaneously fired short action potentials which were followed by a typically prolonged after-hyperpolarization. The mean resting potential was −68·8 ± 1·3 mV (65 cells). In several experiments the motoneurones were identified through retrograde labelling with the fluorescent dye Evans Blue (Takahashi, 1990). In general, all motoneurones investigated showed similar membrane currents and only the proportion between the current components varied slightly from cell to cell.

The present study is based on recordings from 56 whole cells and 129 membrane patches.

RESULTS

Na^+ channels in the somata of rat motoneurones

Activation of voltage-gated Na^+ channels could not be studied with conventional microelectrodes or with patch pipettes in whole-cell recording mode because the long processes and complicated geometry of the motoneurones did not allow us to achieve a sufficient space clamp of the cell membrane. Na^+ currents recorded from whole cells usually activated abruptly at potentials around −50 mV (Takahashi, 1990). In order to avoid the problem connected with insufficient voltage clamp, we studied Na^+ channels in outside-out membrane patches obtained with 4–10 MΩ pipettes. The patches from the somata of motoneurones usually contained three to ten Na^+ channels. A typical example of Na^+ channel activity recorded in external Ringer–TEA solution and internal high-Cs_i^+ solution is shown in Fig. 2*A*. The patch membrane held at −80 mV was first hyperpolarized to −120 mV for 50 ms to remove the steady-state inactivation and then depolarizing steps to different test potentials (E_t) were applied. Transient inwardly directed single-channel currents observed during membrane depolarization were reversibly abolished by addition of 0·5 μM TTX (3 outside-out patches) and almost disappeared in external low-Na^+ solution (3 outside-out patches) in which all sodium ions except those of the buffer were substituted with caesium ions. TTX reduced the number of open channels whereas the single-channel conductance was not affected.

The amplitude of the single Na^+ channel as a function of membrane potential (i–E curve) is given in Fig. 2*B*. The data were fitted with a straight line crossing the voltage axis at E_{Na} = +52 mV (the theoretical reversal potential for

Na^+ ions under present experimental conditions) with a slope conductance of 14·0 pS (9 outside-out patches). The i–E curve demonstrates the high selectivity of Na^+ channels for sodium ions.

Fifteen single-channel recordings similar to those shown in Fig. 2*A* were averaged to obtain Na^+ currents (Fig. 3*A*). The currents activated rapidly within 1 ms at all potentials investigated. The kinetics of Na^+ current inactivation could always be fitted with a one exponential function (dotted lines in Fig. 3*A*). The peak amplitude of the averaged Na^+ current as a function of membrane potential (I–E curve) is shown in Fig. 3*B*. The threshold for Na^+ channel activation was around −60 mV. The peak amplitude of the Na^+ current was divided by the driving force (E_{Na}–E_t) in order to obtain the value of the Na^+ conductance (Fig. 3*C*). The potential of half-maximum activation of the channels (E_{50}) was −38·9 mV and the steepness factor (k) was 6·1 mV (9 outside-out patches). The channels reached full activation at potentials of about −20 mV.

The steady-state inactivation of Na^+ channels was also investigated with averaged currents obtained from outside-out membrane patches. The test 50 ms prepulses to different potentials were followed by a control voltage step to −30 mV (Fig. 4*A*). Each trace in Fig. 4*A* is the average of thirteen to fourteen single-channel recordings. Complete steady-state inactivation of the currents was observed at E_t of −50 mV. The potential of half-maximum steady-state inactivation ($E_{h,50}$) was −81·6 mV and k was −10·2 mV (Fig. 4*B*, 10 outside-out patches).

The time course of Na^+ current inactivation could be fitted with a monoexponential function at all potentials investigated (Fig. 3*A*). The time constant of the channel

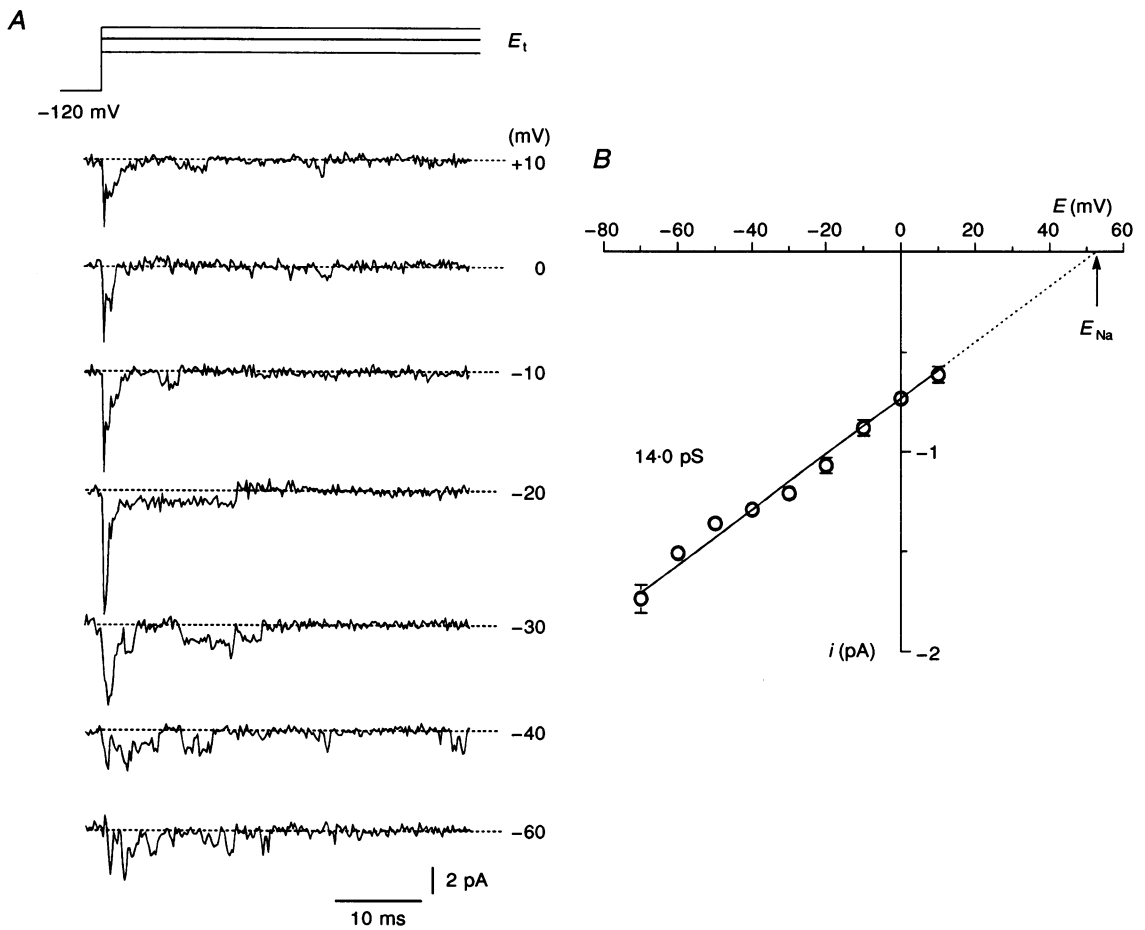

Figure 2. Ionic currents through single Na^+ channels in the soma membrane of rat motoneurones

A, unitary Na^+ currents activated in an outside-out patch by a voltage step to different test potentials (E_t). The holding potential was −80 mV. Steady-state inactivation of the channels was removed by a 50 ms prepulse to −120 mV. Low-pass filter frequency was 2 kHz. *B*, current–voltage (i–E) relationship for single Na^+ channels. The channel conductance was 14·0 pS (9 outside-out patches). In all figures, error bars denote ± S.E.M. if it exceeds the size of the circles.

inactivation (τ_{in}) depended strongly on membrane potential (Fig. 4*C*, data from 9 outside-out patches). The value of τ_{in} changed gradually from about 4 ms at a potential of −50 mV to less than 1 ms at potentials positive to −10 mV.

The kinetics of the recovery of Na^+ channels from inactivation is always of special interest since it can define the limiting firing frequency of action potentials in motoneurones. In the present study, channel recovery from inactivation at a potential of −80 mV, which is close to the resting membrane potential, was investigated. The double-pulse protocol used in these experiments is shown in Fig. 5*A*. The channels were activated and subsequently inactivated, by a 60 ms voltage pulse from −80 to −40 mV. The second test voltage pulse of the same amplitude was applied to the membrane with a time delay. This time delay was varied over a broad range, from several milliseconds to 0·3 s. Each cycle of stimulation contained episodes with the time delay increasing progressively from the shortest to the

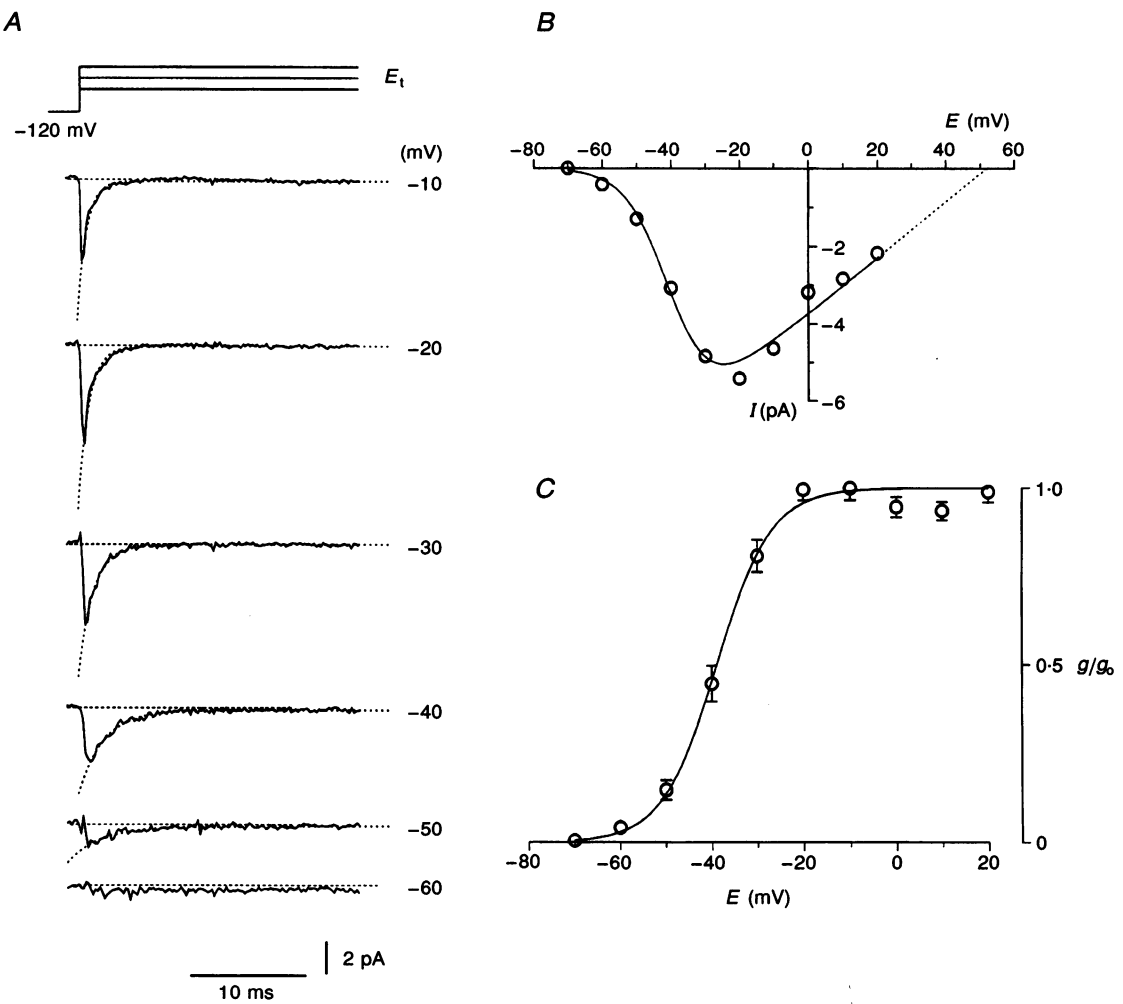

Figure 3. Activation of Na^+ channels

A, averaged Na^+ currents at different membrane potentials. Each trace is the average of 15 recordings similar to those shown in Fig. 2*A*. The time course of the channel inactivation was fitted with one exponential (superimposed dotted lines). Outside-out patch. *B*, current–voltage (*I–E*) relationship for averaged peak Na^+ currents. The points were fitted with equation:

$$g_o/(1 + \exp(-(E - E_{50})/k))(E - E_{Na}),$$

where g_o is the maximum conductance. *C*, activation curve for Na^+ channels. The points were fitted with the Boltzmann equation:

$$g/g_o = 1/(1 + \exp(-(E - E_{50})/k)),$$

where g is Na^+ conductance, E_{50} is −38·9 mV and k is 6·1 mV (9 outside-out patches). Conductance was obtained as peak-current amplitude divided by driving force.

longest or vice versa. Such cycles of patch stimulation were repeated several times. Four single-channel recordings obtained for the time delays of 30 (*A*) and 6 ms (*B*) are shown in Fig. 5. Each of the lowermost traces is the result of averaging fifty-two single-channel recordings.

The ratio between the peak amplitudes of the currents evoked by the second (I_t) and the first (I_{contr}) voltage pulses was plotted against the time delay between the pulses (Fig. 5*C*). The time course of Na^+ channel recovery from inactivation at a potential of −80 mV was fitted with two exponentials with time constants of 16·2 ms (76%) and 153·7 ms (24% of the amplitude, data from 11 outside-out patches).

K^+ channels in the somata of rat motoneurones

Whole-cell K^+ currents were investigated in external Ringer–TTX solution. Patch pipettes, 3–6 MΩ, used for the macroscopic current recordings were filled with high-K^+_i solution. The currents activated by depolarizing voltage steps consisted of two components (Fig. 6): the first activated rapidly and inactivated within 100 ms which is typical for the A-current described for motoneurones (Takahashi, 1990), the second formed a steady-state current which decayed slowly with a time constant of about 20 s. This slowly activating current component appears to correspond mainly to delayed rectifier (DR)-current. Progressive depolarization of the 150 ms prepulse reduced the amplitude of the A-current activated by a voltage pulse to 0 mV (Fig. 6*A*). Depolarization to −60 to −50 mV completely prevented the activation of transient A-currents. In contrast, varying the prepulse between −130 and −50 mV did not influence the amplitude of the DR-current. By subtracting the recording obtained with a prepulse to −50 mV from those observed after prepulses to different potentials, one could construct the steady-state inactivation curve for A-currents (Fig. 6*C*). The curve decayed between −120 and −60 mV with an $E_{h,50}$ of −82·5 mV and k of −10·2 mV (4 cells). In order to separate A- and DR-currents (I_A and I_{DR}) and to obtain the activation curves for both components, we activated K^+ currents by progressively increasing depolarizing pulses either after 150 ms hyperpolarization to −120 mV (I_{A+DR} current) or after 150 ms depolarization to −60 mV (I_{DR} current, Fig. 6*B*). The time constant of 50% activation of I_{DR} was 11·0 ± 2·9 ms at −20 mV and 5·1 ± 1·5 ms at +10 mV (4 cells). The amplitude of I_{DR} was measured at

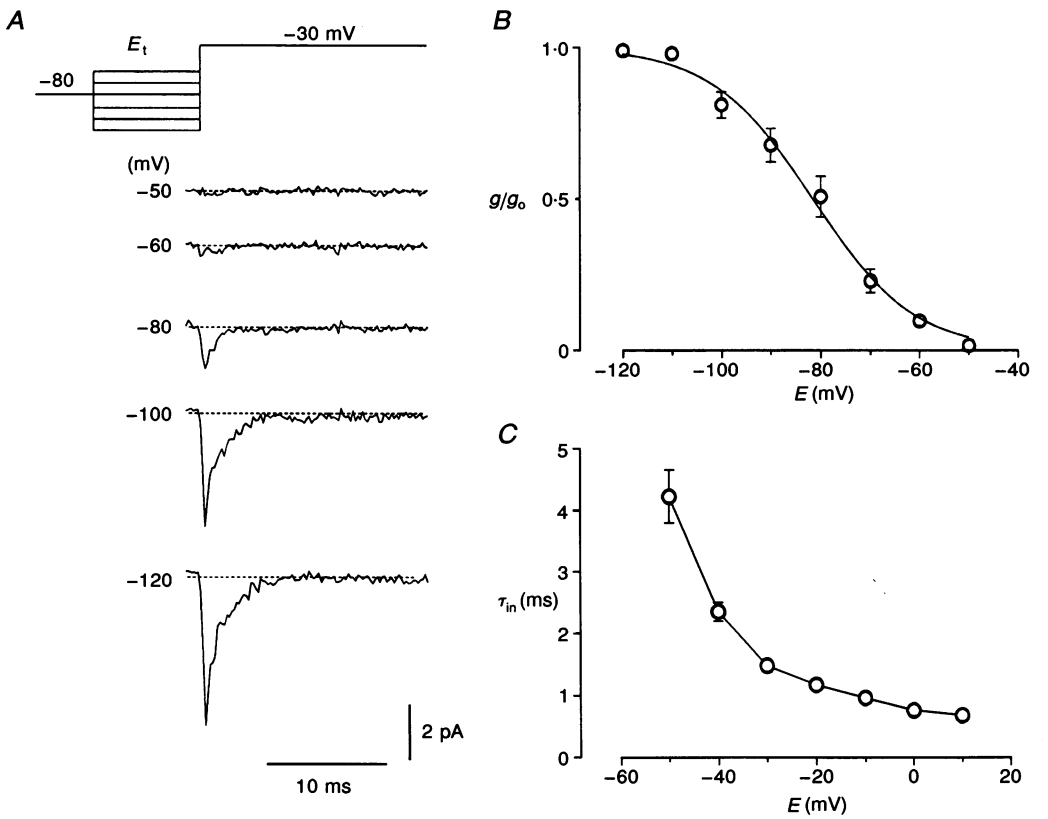

Figure 4. Steady-state inactivation of Na^+ channels

A, averaged Na^+ currents (each from 14 recordings) activated by a voltage step to −30 mV which followed a 50 ms prepulse to different test potentials (E_t). Outside-out patch. *B*, steady-state inactivation of Na^+ channels as a function of membrane potential. $E_{h,50}$ was −81·6 mV and k was −10·2 mV (10 outside-out patches). *C*, potential dependence of the time constant of Na^+ channel inactivation (data from 9 outside-out patches).

the end of depolarizing pulses. A-current was obtained as the difference between the corresponding I_{A+DR} and I_{DR} traces. The time constant of I_A inactivation was 5–60 ms. It was voltage dependent and changed from cell to cell. More precise investigation of the fast inactivation kinetics of whole-cell A-currents was not possible because of insufficient space clamp of the neuronal membrane under present experimental conditions. The amplitude of the A-current was obtained as a peak value of I_A. The activation curves for I_A (open circles, continuous line) and I_{DR} (filled circles) are presented in Fig. 6*C*. The conductance values were obtained as the current amplitudes divided by the driving force $E_t - E_K$, where $E_K = -84$ mV, was the calculated equilibrium potential for K^+ ions. Activation of I_A took place at more negative potentials than that of I_{DR}. The E_{50} values were −33·3 and 1·4 mV and k values were 15·7 and 16·9 mV for I_A (3 cells) and I_{DR} (5 cells), respectively. One possible reason for such a smooth activation of the delayed rectifier K^+ current in a very broad potential range could be an involvement of some other channel types, for instance, Ca^{2+}-activated K^+ channels, since in all our experiments external Ca^{2+} concentration was kept at 0·1 mM and intracellularly applied EGTA is known to be a slow Ca^{2+} buffer. In several experiments in which the slices were perfused with Ca^{2+}-free Ringer–TTX solution the activation of the DR-channels was found to be the same as in Ringer–TTX with 0·1 mM Ca^{2+}. Thus, Ca^{2+}-activated K^+ channels do not

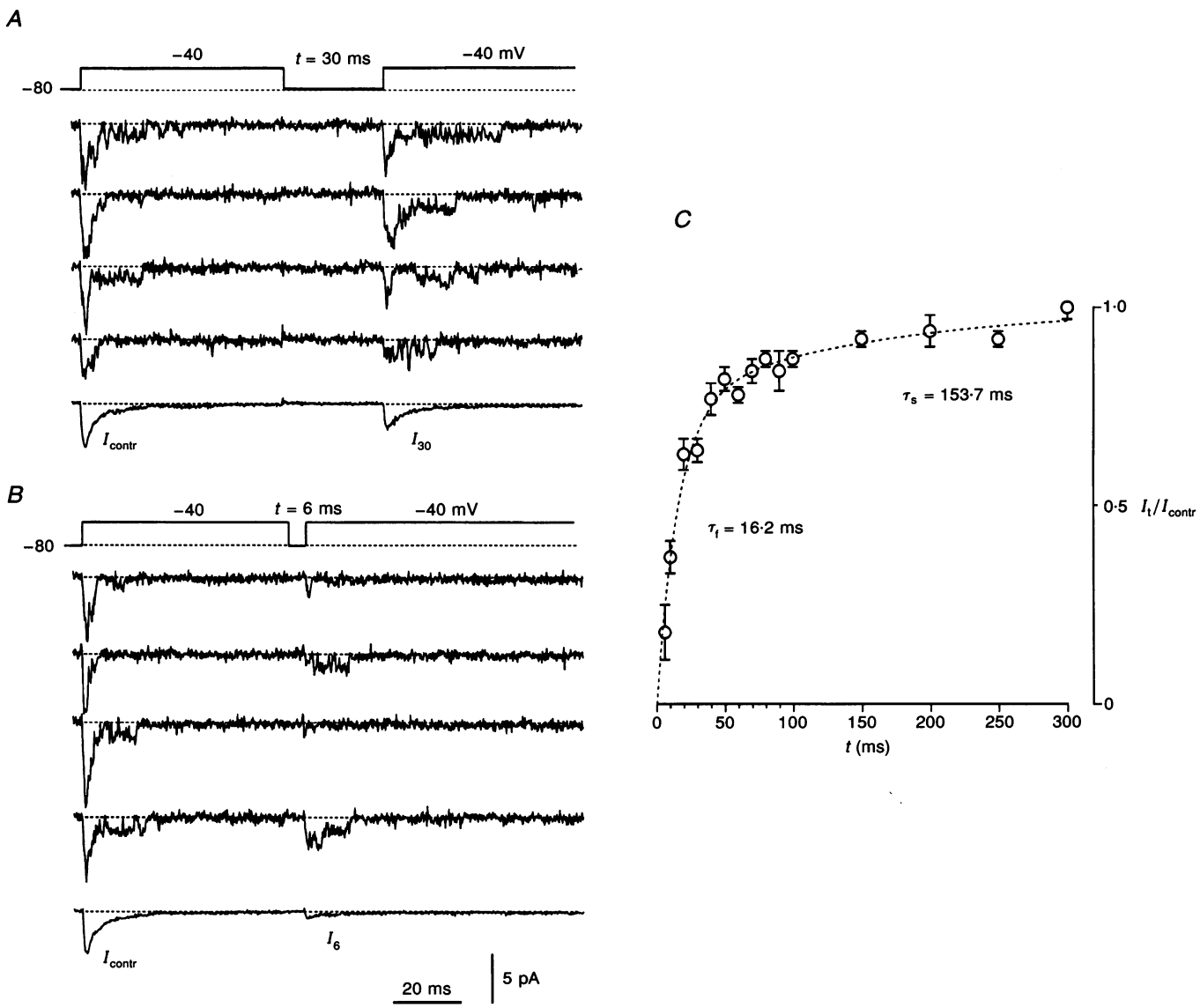

Figure 5. Kinetics of recovery of Na^+ channels from inactivation at a potential of −80 mV

Na^+ currents activated by two consecutive impulses from −80 to −40 mV with a time interval of 30 (*A*) and 6 ms (*B*). Each of the lowest traces in *A* and *B* is the average of 52 recordings. Outside-out patch. *C*, time course of Na^+ channel recovery from inactivation. The points were fitted with two exponentials with time constants of 16·2 and 153·7 ms (11 outside-out patches).

appear to contribute significantly to the DR-current component.

This electrophysiological separation of the current components by means of different prepulses provided an estimate of the ranges of the channel activation. The transient I_A current seems to consist of a homogeneous population of A-channels, since the inactivation kinetics were usually monoexponential, and keeping the membrane at −60 to −50 mV, completely prevented the channel activation. However, slowly activating over a broad potential range I_{DR} current could consist of several components of potential activated K^+ currents. To characterize the molecular basis of A- and DR-current components, a further investigation was performed at the single-channel level.

Outside-out membrane patches usually contained five to ten K^+ channels even if narrow pipettes with a resistance

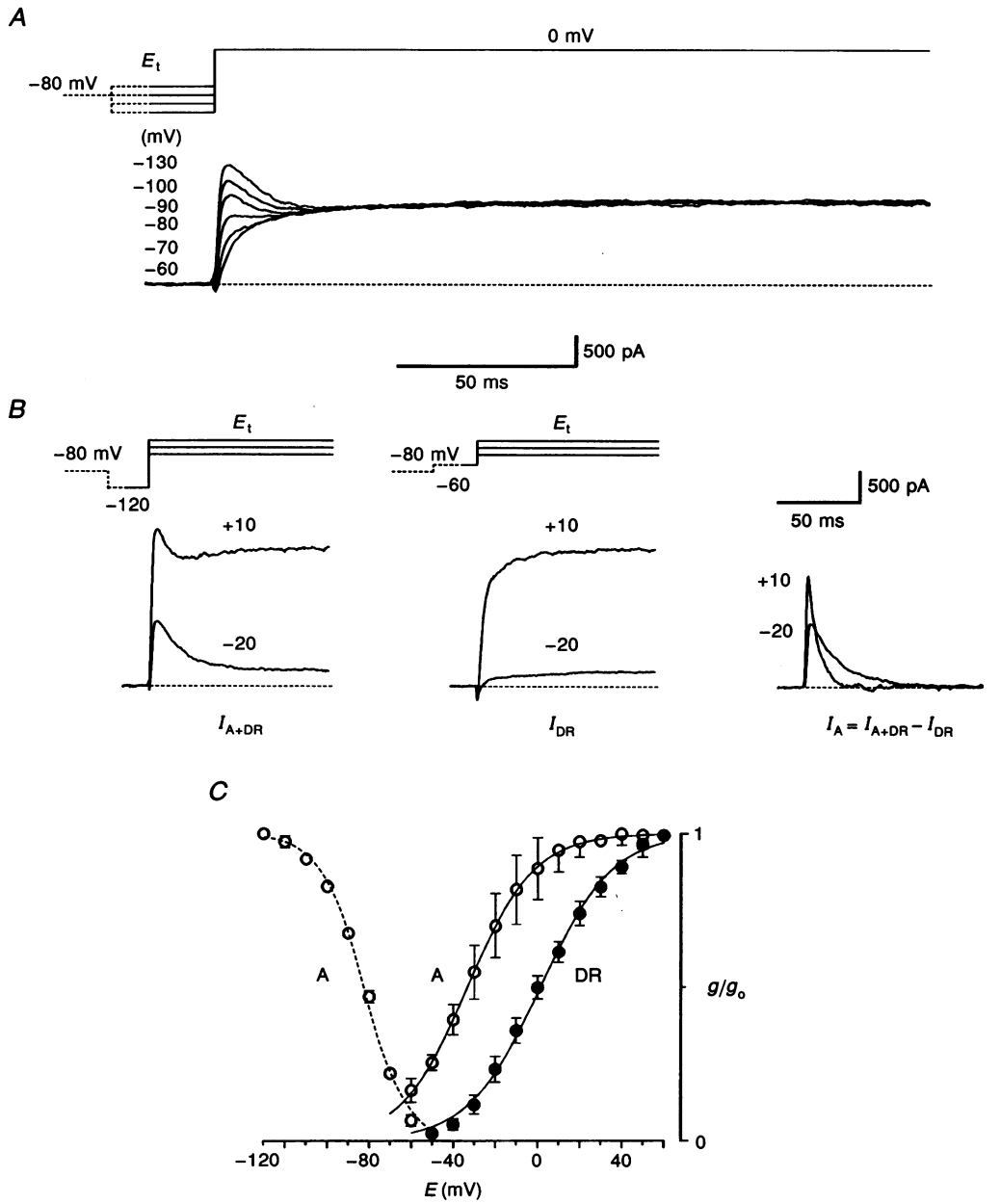

Figure 6. Whole-cell K^+ currents in membrane of rat motoneurones

A, K^+ currents activated by voltage step to 0 mV which followed 150 ms prepulses to different voltage levels (indicated near the corresponding traces). Holding potential was −80 mV. *B*, separation of A- and DR-current components. K^+ currents activated by progressively increasing depolarizing voltage steps after 150 ms prepulses to −120 (left) and −60 mV (middle). The difference between the corresponding traces was considered as A-current (right). Holding potential was −80 mV. *C*, steady-state inactivation curve for I_A (○, dashed line, $E_{h,50} = -82{\cdot}5$ mV and $k = -10{\cdot}2$ mV) and activation curves for I_A (○, continuous line, $E_{50} = -33{\cdot}3$ and $k = 15{\cdot}7$ mV) and I_{DR} (●, $E_{50} = 1{\cdot}4$ and $k = 16{\cdot}9$ mV).

as high as 35 MΩ were used. Two main groups of channels could be distinguished on the basis of their inactivation behaviour during a long depolarizing step from −80 to 0 mV in Ringer solution. The bigger channels with a conductance of about 20 pS inactivated within several hundreds of milliseconds, whereas the smaller ones with a conductance of about 10 pS could be clearly seen after inactivation of the main portion of the bigger channels. Since the population of the channels was not homogeneous in all the patches obtained, the single-channel properties could not be investigated in outside-out recording mode. In inside-out patch configuration the clamped membrane area was considerably smaller so that with 9–33 MΩ pipettes we could obtain patches which usually contained not more than three K^+ channels. Our attempts to characterize the channels in external Ringer–TTX solution ($E_K = -84$ mV) have failed, since the long openings of the smaller channels during 500 ms depolarizing pulses to potentials negative to −20 mV could often not be distinguished from insufficiently compensated leakage currents. In contrast, in symmetrical high-K^+ solution ($E_K = 0$ mV) the unitary currents were considerably bigger at potentials negative to −20 mV and, additionally, pronounced tail currents observed after voltage return to a holding level of −80 mV clearly indicated the channel opening at the end of the pulse. For these reasons the channel identification was mainly performed with inside-out patches in symmetrical high-K^+ solutions and only several important parameters were also studied in Ringer solution.

A-channels

A-channels were the most frequent K^+ channels in the soma membrane of motoneurones. In whole-cell currents the A-channel component could easily be identified according to its fast inactivation with τ_{in} of 15–60 ms. In approximately 50% of the outside-out multi-channel patches the fast inactivation kinetics of A-channels were also preserved. However, in the remaining 50% of outside-out patches the time course of inactivation was several times slower (τ_{in}, 50–150 ms) and some A-channels did not inactivate completely within some seconds. In inside-out patches A-channels always demonstrated the slow type of inactivation. In order to test whether a loss of intracellular factors after excision of the patch could be the reason for the slowing down of the inactivation kinetics, we studied the channel inactivation in cell-attached patches. In eleven experiments the kinetics of A-channel inactivation were as slow as in inside-out membrane patches. Addition of the reducing agent glutathione (1–3 mM) to the intracellular solution did not accelerate the inactivation kinetics of A-channels in inside-out patches in our experiments. This is in contrast to the reported effect of this substance on

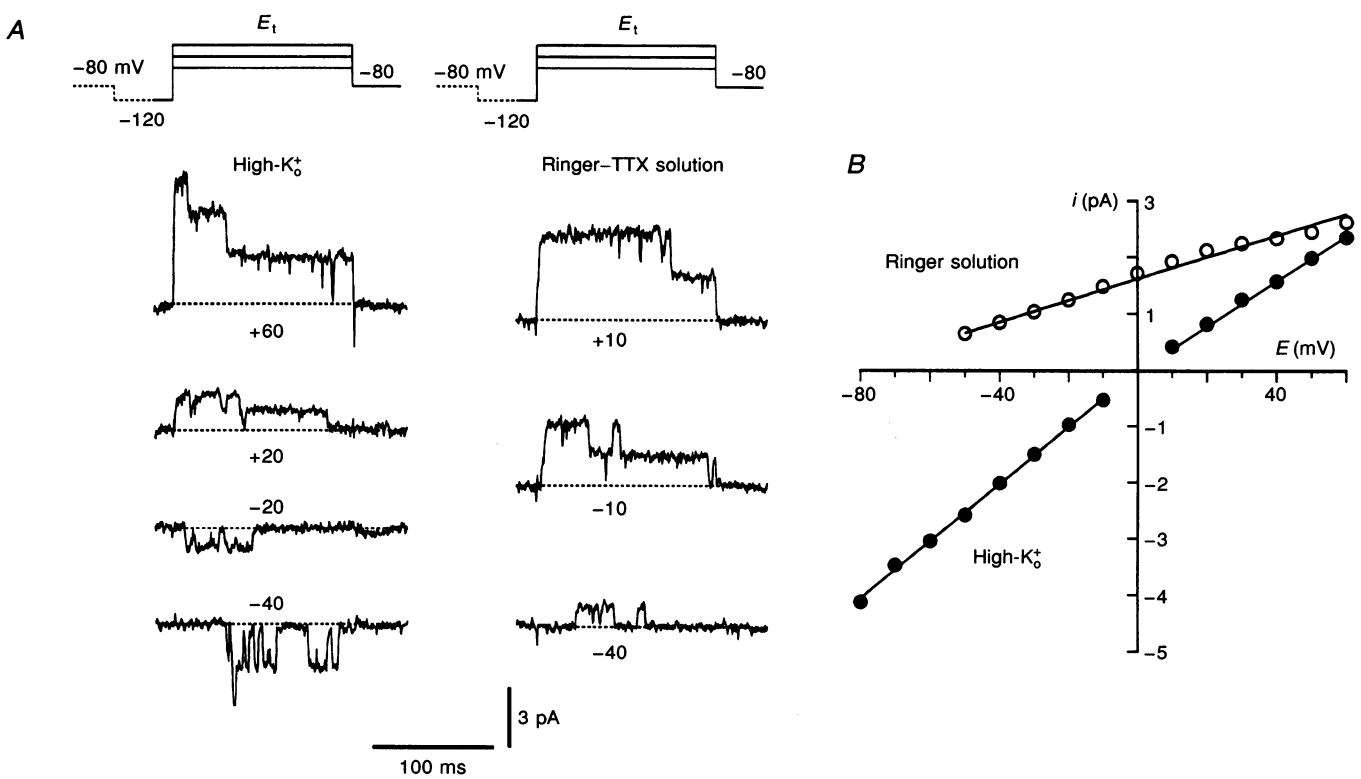

Figure 7. Potassium A-channels in the somata of rat motoneurones

A, recordings of unitary A-currents from inside-out membrane patches in external high-K^+_o and Ringer–TTX solutions. The pulse protocols are shown over the traces. Low-pass filter frequency was 1 kHz. *B*, *i–E* relationships for the A-channels obtained in Ringer and Ringer–TTX solution (○, 12 outside-out and inside-out patches) and high-K^+_o solutions (●, 8 patches).

cloned mammalian A-channels (Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991).

Figure 7*A* shows typical A-channel activity recorded in inside-out patches in high-K_o^+ and Ringer–TTX solutions. The channel inactivation was first removed with a 150 ms prepulse to −120 mV which was followed by 150 ms test pulses to different potentials. The channel conductance was 50·9 pS for inward currents and 39·6 pS for outward currents in high-K_o^+ solution (8 patches) and 19·2 pS in Ringer–TTX solution (12 patches, Fig. 7*B*). The averaged A-channel currents obtained in high-K_o^+ solution at different membrane potentials are presented in Fig. 8*A*. Such recordings were used for the construction of activation curves for A-channels. If the patch contained a homogeneous population of A-channels only, then the peak amplitudes of the averaged currents were divided by the driving force, to obtain an increase in conductance as a function of membrane potential. Such an increase in conductance is directly proportional to the change in the number of open A-channels. When the patch contained a mixture of several A- and DR-channels and recordings of A-channel activity were contaminated by relatively infrequent openings of DR-channels another method of evaluation was used. It was assumed that DR-channels activated as a steady-state current and that the peak of the averaged current reflects the moment in time when most of the A-channels are open. The corresponding traces were first averaged as mentioned above and the moment at which the current reached the peak value was defined. Each single episode was analysed further and the number of active A-channels at this

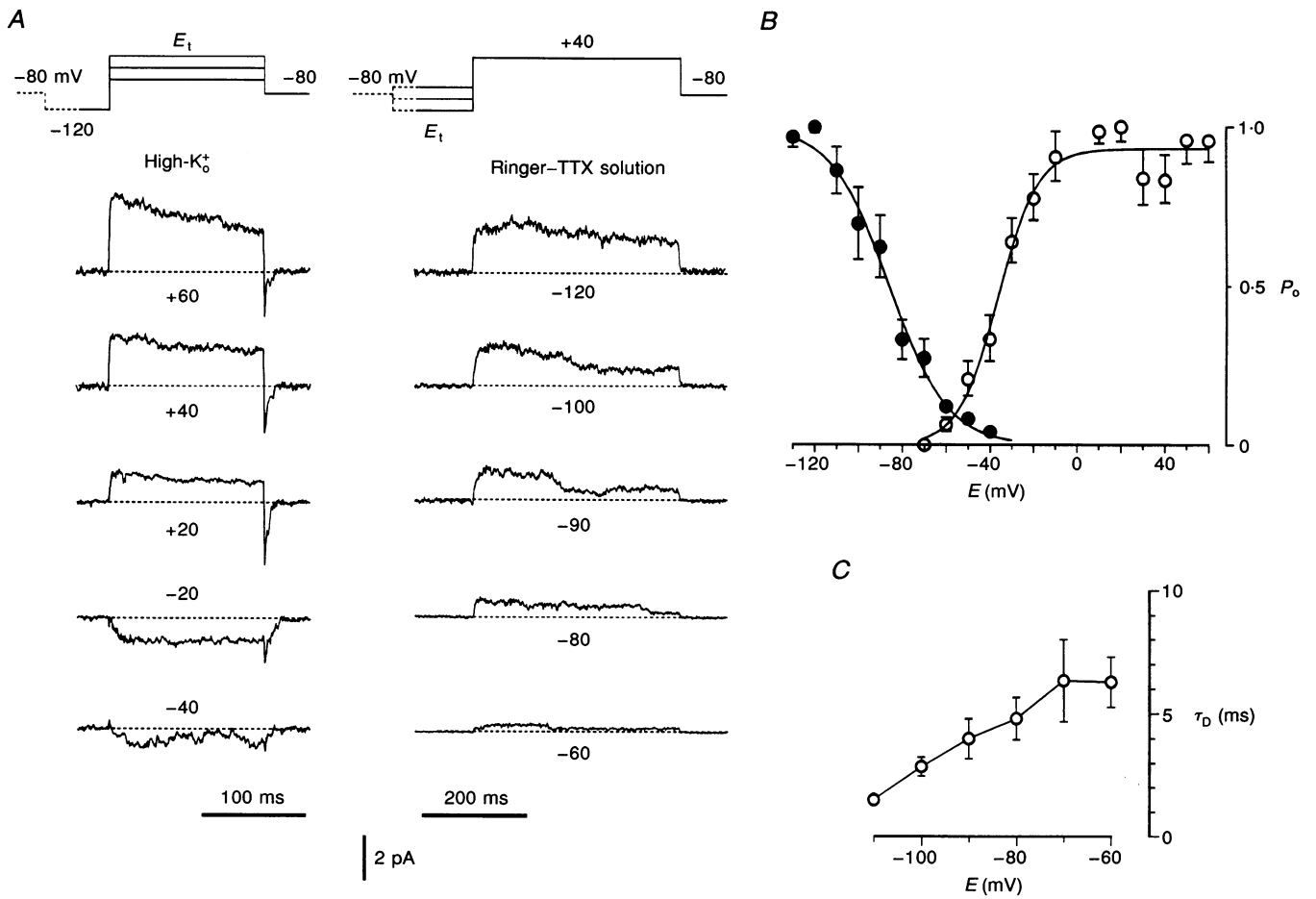

Figure 8. Activation and steady-state inactivation of A-channels

A, left panel: averaged A-currents at different membrane potentials in high-K_o^+ solution. Each trace is the average of 17 episodes similar to those presented in Fig. 7*A*. Right panel: averaged A-currents (each from 23 episodes) activated in Ringer–TTX solution by a voltage step to +40 mV which followed a test prepulse (E_t) to different potentials. Holding potential was −80 mV. The pulse protocols are given above the traces. *B*, activation (○, $E_{50} = -36{\cdot}2$ and $k = 9{\cdot}1$ mV, data from 8 inside-out patches, high-K_o^+ solution) and steady-state inactivation characteristics (●, $E_{h,50} = -85{\cdot}8$ and $k = -13{\cdot}2$ mV). P_o, open probability. Data are from 3 inside-out patches obtained in Ringer–TTX solution, one inside-out patch in external high-K_o^+ and from one cell-attached membrane patch with Ringer–TTX solution on the external side of the membrane. *C*, the time constant of the A-channel deactivation as a function of membrane potential, 3 inside-out patches in external high-K_o^+ solutions.

moment was defined. Then the total number of channel openings in all episodes was divided by the number of the episodes. This procedure was repeated at each membrane potential and then the values obtained were standardized against the highest value. The activation curve of A-channels is presented in Fig. 8*B* (open circles). A-channels activated between −60 and 0 mV, with an E_{50} of −36·2 mV and k of 9·1 mV (8 patches).

The steady-state inactivation of A-channels was investigated in inside-out and cell-attached patches with pipettes filled either with high-K_o^+ or with Ringer–TTX solutions (Fig. 8*A*, right side). The membrane was always held at a potential of −80 mV. Prepulses of 150 ms duration to different test potentials were followed by a control pulse to +40 mV (in some patches to 0 mV). The two methods of counting the single-channel events used for the construction of the activation curve were also used in the study of the channel inactivation. Recordings from patches containing only A-channels were simply averaged as shown in Fig. 8*A* (right) and the peak amplitudes were measured. No difference in the steady-state inactivation was revealed for A-channels in Ringer–TTX and high-K_o^+ solutions. The steady-state inactivation relationship in Fig. 8*B* (filled circles, $E_{50} = -85{\cdot}8$ mV and $k = -13{\cdot}2$ mV, 5 patches) is based on three inside-out and one cell-attached patch obtained in Ringer–TTX solution and on one inside-out patch in high-K_o^+ solution on the external side of the membrane.

Deactivation kinetics of A-channels were studied from the tail events observed in high-K_o^+ solution after returning the potential to the holding level (see Fig. 7*A*, left, uppermost trace). The mean lifetime of the channel was measured

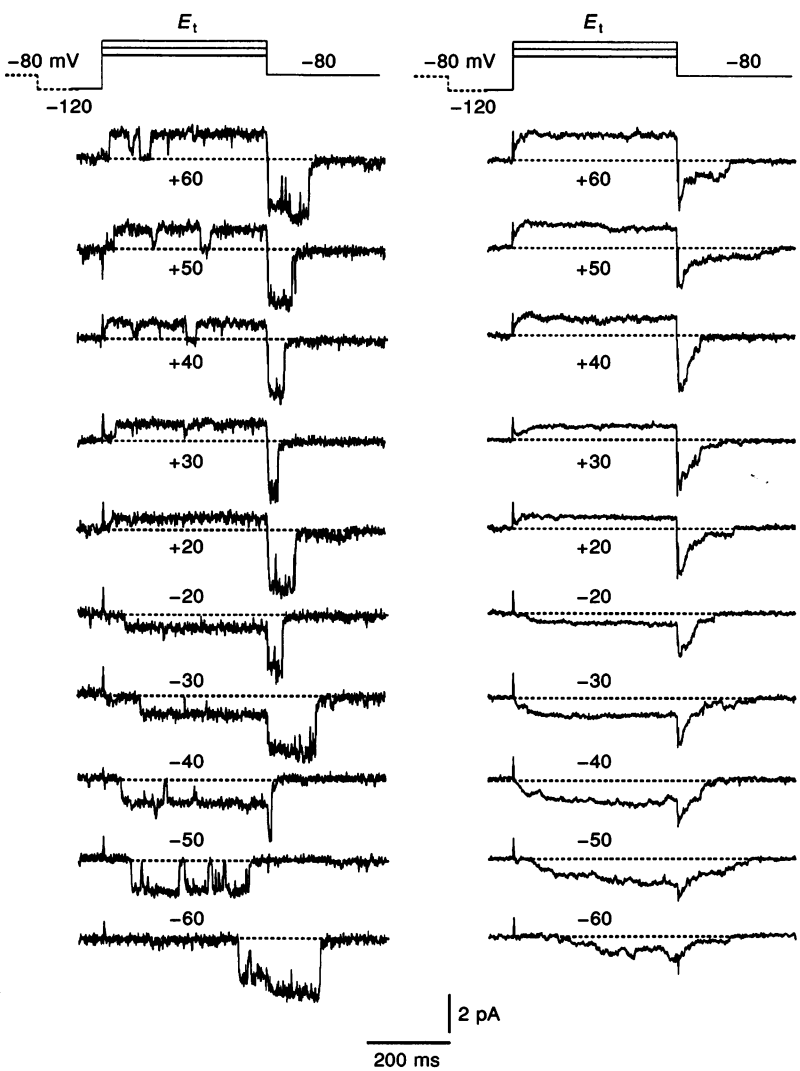

Figure 9. Potassium DR-channels in the somata of rat motoneurones

A, unitary currents through DR-channels at different membrane potentials recorded in external high-K_o^+ solution. Inside-out patch. The low-pass filter frequency was 0·5 kHz. *B*, averaged DR-currents (each from 6 to 14 episodes) obtained from the same inside-out patch as in *A*. The pulse protocols are shown above the corresponding traces.

directly at different holding potentials. The time constant of deactivation changed from 1·5 ± 0·2 ms at −110 mV to 6·3 ± 1·0 ms at −60 mV (Fig. 8*C*, 3 inside-out patches).

DR-channels

In comparison with A-channels, DR-channels were found at a lower density in the soma membrane. DR-channels usually appeared in the patch together with A-channels and only twice did we achieve inside-out patches containing solely DR-channels. However, in several patches with both A- and DR-channel types it was possible to select episodes containing only DR-channel events (Fig. 9, left panel). The first activity of the channels was observed at potentials of about −60 mV. DR-channels demonstrated long openings during depolarizing pulses and long tail currents after returning the potential to −80 mV. Only episodes containing unitary currents through DR-channels were selected at each potential, in order to obtain averaged DR-currents (Fig. 9, right panel, each trace is the average of 6–14 episodes). The kinetics of averaged DR-currents corresponded to those of whole-cell DR-currents.

Single-channel conductance of DR-channels was 31·1 pS for inward and 22·5 pS for outward currents in high-K_o^+ solution (6 patches) and 10·2 pS (5 patches) in Ringer solution (Fig. 10*A*). The activation curve for DR-channels in high-K_o^+ solution was constructed from the probability that the channel was open at the end of the depolarizing pulses. Such an opening was usually confirmed by the characteristic tail currents and DR-channels could be clearly separated from A-channels. DR-channels activated between −70 and 0 mV with an E_{50} of −43·8 mV and k of 8·5 mV (Fig. 10*B*, 5 patches). The time constant of DR-channel deactivation measured directly from the tail events changed from 5·9 ± 1·9 at −110 mV to 60·3 ± 25·1 ms at −60 mV (Fig. 10*C*, 5 patches).

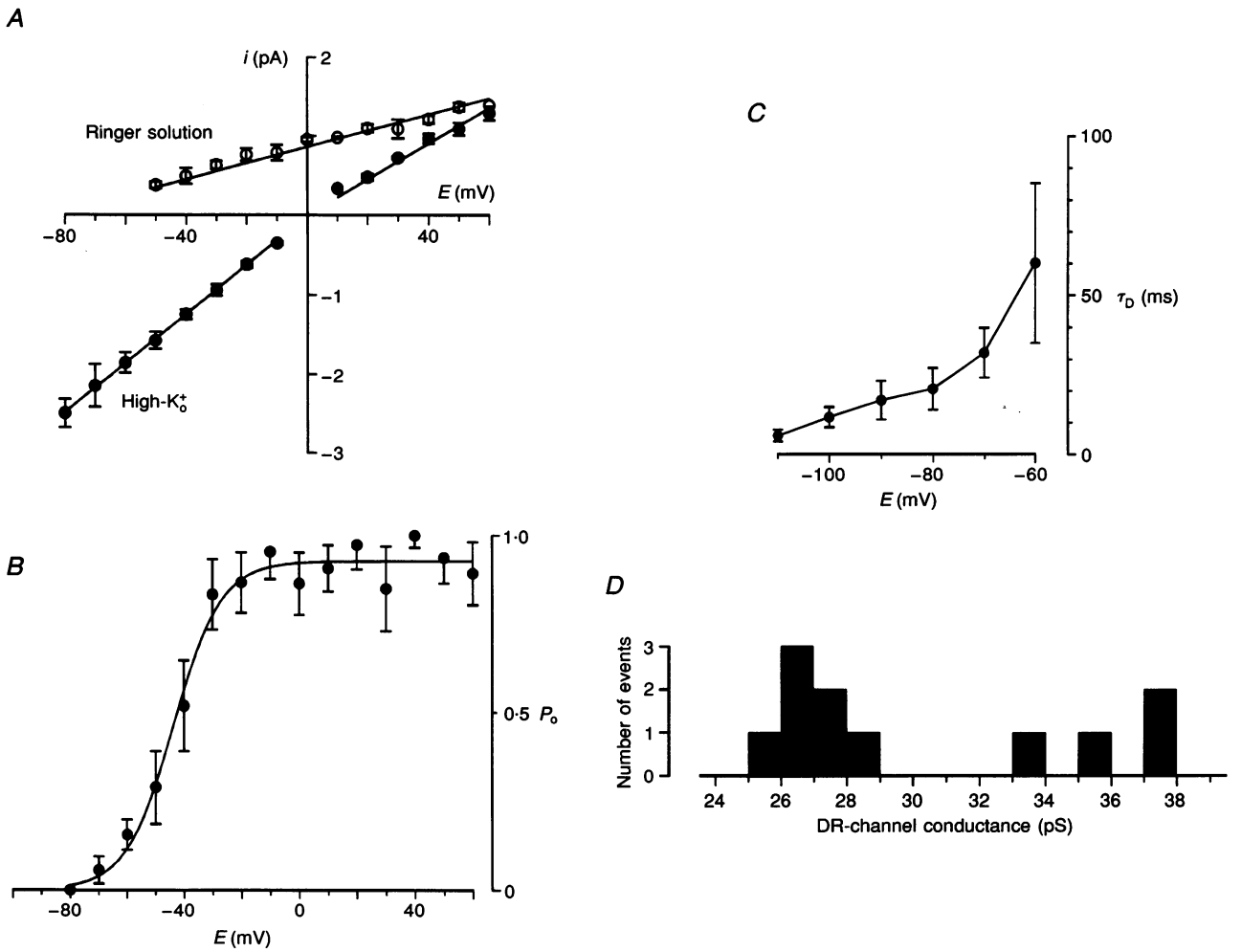

Figure 10. Single-channel conductances and activation properties of DR-channels

A, *i–E* curves for DR-channels obtained in external Ringer solution (open circles, 5 outside-out patches) and high-K_o^+ solutions (filled circles, 6 inside-out patches). *B*, activation curve for DR-channels. E_{50} was −43·8 mV and k was 8·5 mV. Data are from 5 inside-out patches obtained in high-K_o^+ solution. *C*, the time constant of DR-channel deactivation at different membrane potentials. 5 inside-out patches in high-K_o^+ solution. *D*, histogram of conductances of DR-channels in external high-K_o^+ solution measured for inward currents (11 inside-out patches).

In contrast to that of A-channels, the single DR-channel conductances demonstrated a strong scattering from the mean values reported above. Conductances of DR-channels measured for inwardly directed currents in high-K^+_o solutions in eleven inside-out patches are presented as a histogram in Fig. 10*D*. The first subpopulation of the channels had a conductance from 25 to 29 pS and the second was distributed between 33 and 38 pS. The channels from the first subpopulation were usually more noisy in the open state and seemed to present another gating mode of the channels from the second subpopulation. In the present study both these subpopulations of the channels were considered as one DR-channel class, since no further difference in the main electrophysiological properties of these channels was found.

Two of thirty-four patches contained voltage-activated channels of a very small conductance of approximately 10 pS in high-K^+_o solution. Unfortunately, the low density of these small channels did not allow us to perform a systematic study of their properties.

Pharmacology of K^+ channels

The action of K^+ channel blocking agents TEA, 4-aminopyridine (4-AP) and dendrotoxin (DTX) on macroscopic K^+ currents was studied in a whole-cell recording mode. The blocking effect on the unitary currents was investigated in multi-channel outside-out patches where the single-channel events could be observed after inactivation of the major part of the channels during membrane depolarization. No blocker was found to be highly specific in the suppression of only one of the two K^+ current components over the whole potential range investigated. TEA, at 1 and 20 mM, reduced the steady-state component of the whole-cell K^+ current at 0 mV by 42 and 88% (5 cells), respectively. The peak amplitude of the current was also considerably diminished. The amplitudes of the single DR- and A-channels were reversibly reduced in a concentration-dependent manner with an IC_{50} of 0·8 and 14·7 mM, respectively. In the present study DTX was tested at concentrations of 0·15–1 μM. A slight decrease in both components of whole-cell current by 0–30% (15 cells) did not depend on the blocker concentration and could not be clearly distinguished from the natural K^+ current run-down. The current recovery from the DTX block could not be observed since the reaction of DTX dissociation from the channels, lasting normally tens of minutes (Bräu, Dreyer, Jonas, Repp & Vogel, 1990), was considerably longer for the cell membrane within the slice. The same lack of a convincing blocking effect was observed for excised outside-out membrane patches (3 patches). During sustained membrane depolarization in the presence of 0·75 μM DTX, activity of both A- and DR-channels was still observed. Thus, in the spinal motoneurones DTX appears to be neither a potent nor a specific blocker of K^+ A- and DR-channels. 4-Aminopyridine was tested at concentrations between 0·3 and 6 mM. It reversibly reduced the amplitudes of both peak and steady-state whole-cell currents at a potential of 0 mV (6 cells). The peak current was slightly more sensitive to 4-AP, although it was not completely suppressed even at the highest blocker concentrations tested. The amplitudes of the single-channel currents were not changed in the presence of 4-AP although the channel open probability was reduced (2 outside-out patches).

DISCUSSION

Na^+ channels

Na^+ channels are known to play a major role in the fast membrane depolarization during firing of action potentials. Insufficient space clamp of the membrane of motoneurones has always been the main obstacle in the investigation of Na^+ channel activation. In the present study we avoided this problem by recording Na^+ currents from excised membrane patches. The activation of the channels in the soma took place within the same voltage range (between −60 and −20 mV) as described for axonal channels (Scholz *et al.* 1993). The single-channel conductance of 14 pS obtained in the present study is in good agreement with the values reported for Na^+ channel in myelinated axons (Jonas *et al.* 1989; Scholz *et al.* 1993) and seems to correspond to the conductance of TTX-sensitive Na^+ channel in the somata of vertebrate sensory neurones (Campbell, 1993). Since the inactivation kinetics of Na^+ channels in the somata of motoneurones was always fitted with one exponential it could be concluded that Na^+ conductance in the somata of spinal motoneurones is based on one type of completely inactivating Na^+ channel. The same complete inactivation of Na^+ currents was observed in our whole-cell experiments and was previously shown for the same preparation by Takahashi (1990). In contrast, the Na^+ channel in pyramidal neurones from rat sensorimotor cortex can change its modal gating and provide inactivating as well as persistent components of Na^+ conductance (Alzheimer, Schwindt & Crill, 1993). One type of Na^+ channel found in the myelinated axon always inactivated with biexponential kinetics. Na^+ conductances in the somata of motoneurones and in myelinated axons differ, in turn, from those in the somata of dorsal root ganglion (DRG) cells where two components of inactivation kinetics are formed by two different types of Na^+ channels (Elliott & Elliott, 1993; Ogata & Tatebayashi, 1993). The Na^+ channel in the somata of motoneurones described here is completely blocked by 0·1–0·5 μM TTX and its fast activation and inactivation kinetics closely resemble those reported for the TTX-sensitive component of Na^+ current in DRG cells.

In our experiments the time course of Na^+ channel recovery from inactivation was double-exponential with time constants of 16·2 ms (76%) and 153·7 ms (24%). These parameters are of special interest since the time course of the channel recovery from inactivation can define the limiting firing frequency of action potentials in motoneurones. The fast time constant of the channel

recovery of 16·2 ms obtained at a room temperature of 22–24 °C would correspond to a firing frequency of 62 Hz. Assuming a Q_{10} of around 3 for inactivation processes (Frankenhaeuser & Moore, 1963), one can estimate the frequency at normal body temperature of 36–37 °C as approximately 200 Hz. A limiting firing frequency of 180 Hz was reported for rat motoneurones (Granit, Kernell & Shortess, 1963). Thus, although the firing rate of action potentials in motoneurones is normally determined by conductances contributing to after-hyperpolarization (Granit *et al.* 1963), the limiting firing frequency of action potentials seems to be defined by the time of the fast recovery of Na^+ channels from inactivation. Recovery from inactivation of the TTX-sensitive component of Na^+ current in DRG cells also followed a double-exponential time course; however, the fast time constant was approximately three times faster (5 ms; Ogata & Tatebayashi, 1993) than that obtained in the present study. This difference in recovery of TTX-sensitive Na^+ channels from inactivation appears to underlie considerably higher limiting firing rate of action potentials observed in sensory neurones (Harper & Lawson, 1985).

Potassium A-channels

In membranes of motoneurones A-channels are supposed to contribute to fast repolarization of action potentials (Takahashi, 1990). Macroscopic A-currents recorded in the present study from motoneurones in whole-cell mode activated for 1–3 ms and inactivated rapidly with τ_{in} = 15–60 ms. Single channels, with a conductance of 19·2 pS in Ringer solution, investigated in inside-out or cell-attached membrane patches, inactivated several times slower with a τ_{in} of 50–150 ms. We conclude, however, that these channels are A-channels and form the basis of the macroscopic A-currents for four main reasons: (1) A-channels were activated in the same potential range as macroscopic A-currents, (2) the steady-state inactivation curve of A-channels corresponded closely to that obtained for whole-cell A-currents, (3) the A-channel with τ_{in} between 50 and 150 ms was the most frequent K^+ channel in membrane patches, but the whole-cell currents did not demonstrate a 50–150 ms component in inactivation kinetics. Thus, relatively slow, 50–150 ms, inactivation kinetics in isolated patches may be a modified form of the fast kinetics recorded in whole-cells, and (4) fast inactivating channels observed sometimes in multi-channel outside-out patches had a unitary conductance equal to that reported here for A-channels. Similar modifications of the channel inactivation after patch excision are known for other A-channels (Ruppersberg *et al.* 1991). The mechanism of this modification of A-channels in the somata of motoneurones, however, seems to be different since application of glutathione did not accelerate the channel inactivation. More surprisingly, the inactivation kinetics in outside-out patches were faster than those in cell-attached patches. It could be supposed that the contact of the patch pipette with the membrane and modification of the cytoskeleton play a crucial role in slowing down the A-channel inactivation. The membrane area of outside-out patches was always several times larger than that of inside-out or cell-attached patches and could probably contain some parts of the patch membrane which were not affected by mechanical contact with the pipette. In contrast to the fast inactivation kinetics, the steady-state inactivation curve for the channels was not changed. Thus, only the fast N-type inactivation of A-channels was subjected to modification.

The channel conductance of 19·2 pS in external Ringer solution obtained in the present study is in good agreement with the data reported for A-channels in sensory neurones (Kasai, Kameyama, Yamaguchi & Fukuda, 1986; McFarlane & Cooper, 1991). According to the observed difference in their inactivation kinetics A-channels of the same conductance were divided into fast and slow A-channel types in cultured sensory neurones (McFarlane & Cooper, 1991). In our experiments the scatter of inactivation time constants for A-channels was also large, but this most probably resulted from the modification of one channel type rather than from existence of two different types of A-channels, since the whole-cell K^+ currents had only one fast component in the inactivation kinetics.

Potassium DR-channels

Delayed-rectifier K^+ channels are involved in the repolarization phase of the action potential in the membrane of rat motoneurones (Takahashi, 1990). The macroscopic DR-current was measured in the present study as a K^+ current component which was not inactivated by a prepulse to −60 mV (Fig. 6). Activation of DR-current was slower than that demonstrated for A-currents and took place at potentials positive to −50 mV in external Ringer solution. The activation curve of this current component did not reach saturation at potentials as positive as +60 mV. The DR-channel had a conductance of 31·1 pS in high-K^+_o solution (inward current) and 10·2 pS in Ringer solution. Unfortunately, the activation curve for single DR-channels in external Ringer solution could not be obtained, because DR-channel openings could not always be distinguished from A-channel events at potentials positive to −20 mV. In contrast, DR-channel openings were clearly identified from the tail events in high-K^+_o solution. DR-channels activated at potentials between −70 and −10 mV. Such a difference in activation curves obtained for whole-cell and single-channel currents could be explained in several ways. Firstly, non-saturating activation of whole-cell DR-currents at high positive potentials can result from insufficient space clamp of the cell membrane when the real membrane potential was essentially lower than the clamped one. Such a mistake in voltage clamp would be more pronounced if DR-channels are not evenly distributed in the soma membrane but are predominantly clustered near axons and dendrites. Secondly, the activation curve for the single DR-channels was shifted to more negative potentials due to known 'shift

effect' of external K^+ on activation of delayed-rectifier K^+ channels observed in amphibian and squid axons (Matteson & Swenson, 1986; Safronov & Vogel, 1995). This effect was more pronounced in K^+ channels in isolated lamprey spinal neurones (unpublished observation of B. V. Safronov). Unfortunately, application of high-K^+_o solution to the spinal cord slices produced strong membrane depolarization and led to the destruction of the cell so that the shift effect could not be determined quantitatively in the whole-cell mode. Thirdly, DR-current recorded in whole-cell mode could further consist of several current components or strong depolarization could activate a non-specific conductance. Involvement of Ca^{2+}-dependent K^+ conductance could be excluded, because the currents recorded in Ca^{2+}-free Ringer–TTX solution and 3 mM EGTA-containing internal solution had the same activation range as those recorded in Ringer–TTX solution.

The properties of DR-channels correspond closely to those of cloned RCK1 (Kv1.1) K^+ channels (Stühmer *et al.* 1988). This is in good agreement with a high level of expressed mRNA from RCK1 in the rat spinal cord (Beckh & Pongs, 1990).

Comparison of the channels in the soma with axonal channels

It is of special interest to compare the properties of single channels found in the somata of rat motoneurones with those described for myelinated axons, since these two parts of the same cell are equipped with clearly different types of ionic channels. Na^+ channels in soma and axon exhibited a very high similarity in their single-channel conductances, potential range of activation and sensitivity to TTX, but differed in the kinetics of inactivation. Monoexponential inactivation kinetics of the somatic Na^+ channels corresponds to the fast component in double-exponential inactivation kinetics of the axonal channels (Kniffki, Siemen & Vogel, 1981).

Voltage-activated K^+ conductance in the axons of rat motoneurones is presented by three types of delayed-rectifier currents (Röper & Schwarz, 1989). Delayed-rectifier channels of F- (fast), I- (intermediate) and S- (slow) types were found to underlie the macroscopic conductance (Safronov *et al.* 1993). The axonal F-channels appear not to be expressed in the soma membrane, since a typical 200 ms component of the F-channel inactivation (Schwarz & Vogel, 1971) was not observed in whole-cell K^+ currents. Although the axonal F-channels have similar conductance and deactivation kinetics as the A-channels reported here, they could be clearly distinguished from one another. Activation of the axonal F-channels took place over a broader potential range (between −50 and +50 mV, Safronov *et al.* 1993) than that of A-channels in the soma. IC_{50} for suppression of A-channels by TEA was 14·7 mM, whereas the axonal F-channels were more sensitive by an order of magnitude, to the blocker ($IC_{50} = 1{\cdot}2$ mM, Safronov *et al.* 1993). Further, 1 μM DTX did not suppress A-current in the present study, but the component of the macroscopic K^+ current formed by F-channels was strongly blocked by 140 nM DTX in rat axon (Corrette, Repp, Dreyer & Schwarz, 1991).

DR-channels reported here resemble the axonal I-channels and have similar unitary conductance, deactivation kinetics and sensitivity to TEA (see Safronov *et al.* 1993). However, DR-channels in the soma were usually activated at more positive potentials and over a broader voltage range. Inactivation kinetics of DR-channels were about three times slower than those of axonal I-channels. DR-channels in the somata of motoneurones were found to exhibit only a very low, if at all, sensitivity to DTX, in contrast to the axonal I-channels which were suppressed by nanomolar concentrations of the blocker. It is interesting to note that delayed-rectifier K^+ currents in the somata of DRG cells had a DTX-sensitive component activated at potentials between −80 and −30 mV and a DTX-resistant component apparent at potentials positive to −40 mV (Stansfeld & Feltz, 1988). The channels underlying the DTX-sensitive component seem to correspond to axonal I-channels and the channels forming the DTX-resistant component appear to be similar to the DR-channels reported here for the soma membrane of rat motoneurones.

The axonal S-channels had a low conductance of 10 pS in high-K^+_o solution and are known to be active around the membrane resting potential (Safronov *et al.* 1993). Although in the present study we could sometimes observe activity of low conductance K^+ channels, they appeared to play no significant role in the soma membrane, since no component of delayed-rectifier K^+ current was found to activate at potentials close to the resting potential.

In contrast to the delayed-rectifier K^+ channels which are present, although in different forms, in both soma and axon, the fast inactivating A-channels are expressed in the soma membrane only and were not found in the myelinated axon. Similar A-channels were reported for the somata of DRG cells (Kasai *et al.* 1986) and sensory neurones (McFarlane & Cooper, 1991). Such channels are probably typical attributes of the soma membrane.

Investigation of the single channels by means of the patch-clamp technique applied to thin slices of neonatal rat spinal cord can provide further important information about the functioning of ionic channels underlying excitability in the somata of motoneurones and other cell types.

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Note added in proof

Since this paper was submitted the following report has been published: UMEMIYA, M. & BERGER, A. (1995). Single-channel properties of four calcium channel types in rat motoneurons. *Journal of Neuroscience* **15**, 2218–2224.

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