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Sodium-dependent Potassium Channels in Leech P Neurons

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Abstract. In leech P neurons the inhibition of the Na^+-K^+ pump by ouabain or omission of bath K^+ leaves the membrane potential unaffected for a prolonged period or even induces a marked membrane hyperpolarization, although the concentration gradients for K^+ and Na^+ are attenuated substantially. As shown previously, this stabilization of the membrane potential is caused by an increase in the K^+ conductance of the plasma membrane, which compensates for the reduction of the K^+ gradient. The data presented here strongly suggest that the increased K^+ conductance is due to Na^+-activated K^+ (K_{Na}) channels. Specifically, an increase in the cytosolic Na^+ concentration ($[Na^+]_i$) was paralleled by a membrane hyperpolarization, a decrease in the input resistance (R_{in}) of the cells, and by the occurrence of an outwardly directed membrane current. The relationship between R_{in} and $[Na^+]_i$ followed a simple model in which the R_{in} decrease was attributed to K^+ channels that are activated by the binding of three Na^+ ions, with half-maximal activation at $[Na^+]_i$ between 45 and 70 mM. At maximum channel activation, R_{in} was reduced by more than 90%, suggesting a significant contribution of the K_{Na} channels to the physiological functioning of the cells, although evidence for such a contribution is still lacking. Injection experiments showed that the K_{Na} channels in leech P neurons are also activated by Li^+.

Key words: K_{Na} channels — Na^+-K^+ pump — Leech — P neuron — Retzius neuron — Ion-sensitive microelectrodes

Introduction

One important factor controlling neuronal excitability is the activity of K^+ channels in the plasma membrane, which determines the membrane potential (E_m) at rest, the depolarizing effect of excitatory input, as well as the duration and frequency of action potentials. The diversity of K^+ channels is large, and the activity of the different channels is affected by numerous parameters, such as membrane potential, cytosolic Ca^{2+}, ATP, endogenous polyamines, phosphorylation, and/or protein interactions (*see* Hille, 2001). In addition, in mammalian heart muscle cells, various neurons from both vertebrates and invertebrates, as well as in *Xenopus* oocytes, K^+ channels have been found that are activated by cytosolic Na^+ (K_{Na} channels; Martin & Dryer, 1989; Egan et al., 1992; Dryer, 1994). As neuronal excitation is primarily based on Na^+ influx, K_{Na} channels may provide a simple negative feedback mechanism which, besides accelerating the decline of single action potentials, could generally reduce cellular excitability after a period of enhanced excitatory activity (*see* Dryer, 2003; Bhattacharjee & Kaczmarek, 2005). In any case, K_{Na} channels should be activated under pathophysiological conditions, such as hypoxia and ischemia, and help to stabilize E_m, thereby prolonging the survival time of the cell.

K_{Na} channels have a high unitary conductance, show multiple subconductance states, and appear to occur in clusters (Dryer, 1994; 2003). K_{Na} channel activity is strongly dependent on $[Na^+]_i$, with Hill coefficients varying between 2.7 and 4.6, and in most preparations channel activity is half maximal at $[Na^+]_i$ between 30 and 80 mM. Recently, K_{Na} channels have been identified molecularly as being encoded by certain 'slowpoke' genes: *slo*2.1 and *slo*2.2 (Bhattacharjee et al., 2003; 2005; Yuan et al., 2003; *see* Elkins, Ganetzky & Wu, 1986). The functional characterization of the Slo2.1 ("Slick") channels showed a remarkable dependence of channel activity on cytosolic Cl^-, and moreover, these channels contain an inhibitory ATP-binding site, suggesting that they 'integrate multiple indicators of the metabolic state of a cell' (Bhattacharjee et al., 2003). In contrast, Slo2.2

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("Slack") channels have no ATP-binding site and their activity is only weakly dependent on cytosolic Cl^-.

In leech P neurons, the continuous rundown of the concentration gradients for Na^+ and K^+ after inhibition of the Na^+-K^+ pump is, over a prolonged period, not paralleled by a membrane depolarization. In the presence of ouabain, a specific Na^+-K^+ pump inhibitor, E_m remains stable or is even slightly shifted to the negative direction (*see* Schlue, 1991), and pump inhibition by omitting bath K^+ even causes a temporary membrane hyperpolarization by up to 40 mV (Schlue & Deitmer, 1984). The stabilization of E_m or, respectively, the membrane hyperpolarization, were found to be primarily due to a large increase in the K^+ permeability of the plasma membrane, which might be mediated by K_{Na} channels, as the ouabain-induced increase in K^+ permeability was strongly suppressed in the absence of bath Na^+ (Schlue, 1991). The results presented in this article confirm this suggestion by demonstrating that in P neurons an increase in $[Na^+]_i$ was paralleled by an E_m shift to the negative direction, a drop in the input resistance (R_{in}) of the cells, and by the occurrence of an outwardly directed membrane current. By using a simple model, the drop in R_{in} could be attributed to K^+ channels that are activated by the binding of three Na^+ ions, with half-maximal activation occurring at $[Na^+]_i$ between 45 and 70 mM. Injection experiments showed that the K_{Na} channels in leech P neurons are also activated by Li^+.

Material and Methods

Preparation

The experiments were done on the mechanosensory P (pressure) neurons in intact segmental ganglia of the leech *Hirudo medicinalis*, which were dissected from the ventral nerve cord of adult leeches as described by Schlue & Deitmer (1980). For comparison, some experiments were also made on the 'multifunctional' Retzius neurons. The ganglia were pinned ventral side up in an experimental chamber by piercing the connectives with fine steel needles and continuously superfused at room temperature (20–25°C) with a rate of 2.7 ml min^{-1}, which exchanged the chamber volume about 14 times per min. All segmental ganglia were used for the experiments, except for those in segments 5 and 6, which show structural and functional peculiarities (Macagno, 1980).

Ion-sensitive Microelectrodes

The cytosolic concentrations of Na^+ and K^+ ($[Na^+]_i$, $[K^+]_i$) were measured by double-barrelled microelectrodes inserted into the cell soma, which allowed for the simultaneous recording of one ion species together with E_m (*see* Ammann, 1986). The ion-sensitive barrel was either filled with a K^+ sensor based on the neutral carrier valinomycin (cocktail Ib; Fluka, Buchs, Switzerland), backfilled with 100 mM KCl, or a Na^+ sensor based on the neutral carrier ETH227 (cocktail Ia; Fluka), backfilled with 100 mM NaCl. The reference barrel was always filled with 0.5 M K_2SO_4/8 mM KCl. The electrode potentials were recorded by voltmeters with an input resistance of 10^{15} Ω (FD223; World Precision Instruments, Sarasota, FL) and referred to the potential of the bath electrode (agar bridge with chlorinated silver wire). The output signals were AD-converted and recorded on a PC with an acquisition rate of 20 Hz.

The electrodes were calibrated by using solutions with the following compositions. K^+ calibration: 0–100 mM KCl or NaCl, whereby [NaCl] + [KCl] = 100 mM; Na^+ calibration: 0–50 mM NaCl, 80 mM KCl, 0.73 mM $CaCl_2$ plus 1 mM EGTA (ethylene glycol-bis[β-aminoethylether]; Sigma, Deisenhofen, Germany); free $[Ca^{2+}] \sim 10^{-7}$ M. The calibration solutions contained 0.5 mM $MgCl_2$ and were buffered to pH = 7.30 by 10 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; AppliChem, Darmstadt, Germany). The pH of the K^+ calibration solutions was adjusted with NaOH, that of the Na^+ calibration solutions, with KOH.

Before calibration, the electrode was dipped into physiological solution (*see* Bathing Solutions), and all electrode potentials were set to 0 mV. The ion potentials in the various calibration solutions were obtained by subtracting the potential of the reference barrel from the potentials of the ion-sensitive barrels, and the resulting calibration curves were fitted by applying the Nikolsky-Eisenman equation (*see* Ammann, 1986). Electrodes were only used if their detection limit was below the concentrations recorded experimentally, and if the concentration dependence of the ion potential corresponded to at least 70% of the Nernstian slope.

Current and Ion Injection

To monitor the input resistance (R_{in}) of the cells, a single-barrelled microelectrode filled with 0.5 M K_2SO_4/8 mM KCl was inserted into the cell soma, in addition to the ion-sensitive microelectrode. Via this electrode, hyperpolarizing current pulses of −1 nA amplitude and 1 s duration were applied every 10 s, and R_{in} was calculated from the amplitude of the membrane hyperpolarization induced by these current pulses. The current-induced hyperpolarization usually showed a marked 'depolarizing sag' (*see* Figs. 3*B*, 4*C*; *see* McCormick & Pape, 1990), which reflects the activation of hyperpolarization-activated cation (I_h) channels (Gerard, Hochstrate & Schlue, 1999; *compare* Jansen & Nicholls, 1973). For the calculation of R_{in} we used the initial, maximum hyperpolarization, which occurred before the I_h channels were significantly activated.

To investigate the relation between $[Na^+]_i$, E_m, and R_{in} in more detail, a double-barrelled Na^+-sensitive microelectrode and a double-barrelled electrolyte-filled microelectrode were inserted into a single cell, the barrels of the latter electrode being filled with 0.5 M Na_2SO_4/8 mM KCl or, respectively, 0.5 M K_2SO_4/8 mM KCl. The Na^+-sensitive electrode was used to monitor $[Na^+]_i$ and E_m and the electrolyte-filled electrode to apply hyperpolarizing current pulses to determine R_{in} as well as depolarizing direct current for the iontophoretic injection of Na^+ or K^+. In the course of a Na^+ injection experiment, the hyperpolarizing current pulses were applied via the K_2SO_4-filled barrel and, vice versa, in a K^+ injection experiment via the Na_2SO_4-filled barrel, whereby the effect of the current pulses on E_m was independent of which barrel was used (*see* Fig. 4). The insertion of the electrolyte-filled electrode had no persistent effect on $[Na^+]_i$ or E_m, suggesting that Na^+ diffusion out of the Na_2SO_4-filled barrel can be neglected (*compare* Jansen & Nicholls, 1973).

The effect of injecting Na^+ or other alkali ions on the membrane current was investigated by applying a technically less demanding experimental configuration. The cells were impaled with a single-barrelled, K_2SO_4/KCl-filled microelectrode for the recording of E_m and by a double-barrelled microelectrode, one barrel being also filled with K_2SO_4/KCl for current injection. The other barrel was used for the iontophoretic injection and contained 0.5 M Na^+, Li^+, K^+, or Rb^+, either as SO_4^{2-} or Cl^- salt. The membrane current was recorded by applying a voltage-ramp pro-

tocol (*see* Fig. 7*A*): E_m was first clamped to −50 mV, i.e., close to its resting value, for a few seconds. After an abrupt shift to −100 mV, it was then linearly changed to −30 mV within 7 s and finally again shifted to −50 mV.

Electrode impalement often caused a significant injury of the cells: R_{in} and $[K^+]_i$ were low, and $[Na^+]i$ was high (*see* Fig. 3). Except for the experiments in which the effect of electrode insertion was investigated (Figs. 3, 5), all other experiments were started after recovery from the injury, i. e., after the recorded parameters had reached stable values.

Measurement of $[Ca^{2+}]_i$

The cytosolic free calcium concentration ($[Ca^{2+}]_i$) was measured by using the fluorescent Ca^{2+} indicator Fura-2 (Molecular Probes, Eugene, OR), as described previously in detail (*see* Hochstrate & Schlue, 1994). Fura-2 was iontophoretically injected into the cells by using conventional microelectrodes the tip of which was filled with an aqueous solution of the pentapotassium salt of the dye (100 mM; −20 nA for 20 s). Afterwards, the preparation was transferred into a chamber that was continuously perfused with the same rate as in the electrophysiological experiments. The Fura-2 fluorescence was alternately excited by light of 340 and 380 nm wavelength, and the emitted fluorescence (F_{340}, F_{380}) was measured by a photon-counting photomultiplier tube with a data acquisition rate of 1 Hz. After correction for the autofluorescence of the preparation, $[Ca^{2+}]_i$ was calculated from the ratio $R = F_{340}/F_{380}$, according to the equation given by Grynkiewicz et al. (1985). All experimental data are given as mean ± standard deviation (SD).

Bathing Solutions

The physiological solution had the following composition (in mM): 85 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to pH 7.40 with NaOH, which increased the Na^+ concentration by 4 mM. Ouabain (Sigma) was added to the solutions in solid form shortly before use. In the K^+-free solution, KCl was replaced by NaCl.

Model Calculations

The results presented in Fig. 5 show that in leech P neurons there is a close relationship between R_{in} and $[Na^+]_i$. To describe this relationship, a simple model was applied, which is based on the assumption that the decrease in R_{in} is due to K^+ (K_{Na}) channels that are activated by the binding of Na^+ ions at the cytosolic channel face. Using the Hill formalism (*see* Weiss, 1997) the following equilibrium was assumed, with n being the number of Na^+ ions necessary for channel activation:

$$K_{Na} \cdot Na_n^+ \leftrightarrow K_{Na} + n \cdot Na^+ \tag{1}$$

With the dissociation constant:

$$K_{diss} = \frac{[K_{Na}] \cdot [Na^+]_i^n}{[K_{Na} \cdot Na_n^+]} \tag{2}$$

and the balance:

$$[K_{Na}]_{tot} = [K_{Na} \cdot Na_n^+] + [K_{Na}] \tag{3}$$

it is obtained:

$$[K_{Na} \cdot Na_n^+] = [K_{Na}]_{tot} \cdot \frac{1}{1 + \frac{K_{diss}}{[Na^+]_i^n}} \tag{4}$$

It is assumed that $[K_{Na} \cdot Na^+{}_n]$ is proportional (γ) to the membrane conductance (g_{KNa}) contributed by the K_{Na} channels to the total membrane conductance (g):

$$g = g_B + g_{KNa} \tag{5}$$

with g_B being the 'basal' membrane conductance without K_{Na} channel activation. The maximal g_{KNa} at saturating $[Na^+]_i$ is given by $\gamma \cdot [K_{Na}]_{tot}$, and with the assumption that the maximal g_{KNa} is f-times g_B, it is obtained:

$$g = g_B \cdot \left[1 + f \cdot \frac{1}{1 + \frac{K_{diss}}{[Na^+]_i^n}}\right] \tag{6}$$

Since the ratio g_B/g is identical with 'rel. R_{in}' as used in Fig. 5*B*, equation 6 can be applied to describe the relation between rel. R_{in} and $[Na^+]_i$.

As in our experiments the K_{Na} channels were usually not fully activated, a rigorous fit with the variation of all parameters (K_{diss}, n, and f) was not feasible. Therefore, we first estimated f, and then calculated K_{diss} by using the $[Na^+]_i$ at which rel. R_{in} was reduced to 50% of its resting value. With these data we computed the relation between rel. R_{in} and $[Na^+]_i$ with different n, in order to estimate the number of Na^+ ions necessary to achieve K_{Na} channel activation.

Results

Inhibition of the Na^+-K^+ Pump in Leech P Neurons: Temporary Membrane Hyperpolarization Despite Breakdown of the K^+ Concentration Gradient

The effect of inhibiting the Na^+-K^+ pump in leech P neurons by the cardiac glycoside ouabain on the cytosolic concentrations of Na^+ and K^+ ($[Na^+]_i$, $[K^+]_i$) and on the membrane potential (E_m) is shown in Fig. 1*A*, *B* (*compare* Schlue, 1991). The resting values of these parameters were: $[Na^+]_i$ = 12 ± 8 mM (n = 17), $[K^+]_i$ = 83 ± 14 mM (n = 27), and E_m = −44.2 ± 7.4 mV (n = 44). Immediately after the beginning of the 10 min ouabain application, the cells depolarized by a few mV, and $[Na^+]_i$ began to increase and $[K^+]_i$ to decrease, whereby speed and extent of these concentration changes varied considerably from experiment to experiment. Nevertheless, despite the breakdown of the transmembrane concentration gradients, E_m remained stable or was even shifted to the negative direction. A stabilization of E_m, followed later by a slight and constant hyperpolarization, was usually observed when the changes in $[Na^+]_i$ and $[K^+]_i$ were moderate and slow, but when the changes were strong and fast, a distinct temporary hyperpolarization by maximally 7 to 15 mV occurred, which, after several minutes, turned to a pronounced depolarization. Before E_m became more positive than at rest, $[K^+]_o$ decreased to ~30 mM. The effects of ouabain were reversible, but recovery of the various parameters was slow and took about 30 min to complete (*see* Schlue & Deitmer, 1984; Schlue, 1991).

A 10 min ouabain application had no effect on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$; resting value 54 ± 18 nM, n = 18), as measured by

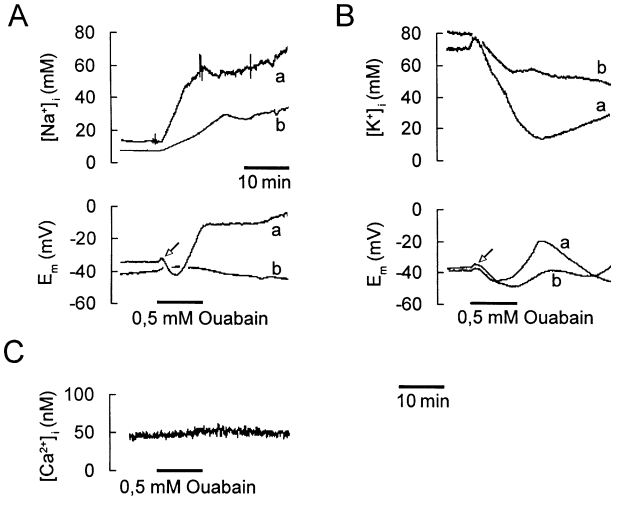

Fig. 1. Effect of ouabain on $[Na^+]_i$, $[K^+]_i$, and E_m, as well as on $[Ca^{2+}]_i$. (*A*) Simultaneous recordings of $[Na^+]_i$ and E_m in two different P neurons (*a, b*) by using double-barrelled ion-sensitive microelectrodes. The duration of the ouabain application was 10 min. (*B*) Simultaneous recordings of $[K^+]_i$ and E_m in two further cells (*a, b*). Note that the cells in which ouabain evoked pronounced changes in $[Na^+]_i$ and $[K^+]_i$ initially depolarized (*arrows*) and then temporarily hyperpolarized, before E_m was shifted to the positive direction. In contrast, cells with moderate changes in $[Na^+]_i$ and $[K^+]_i$ tended to hyperpolarize for a prolonged period. (*C*) A 10 min ouabain application had no effect on $[Ca^{2+}]_i$.

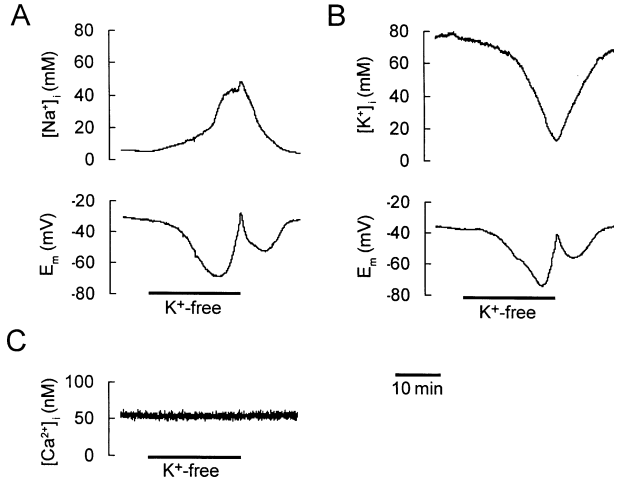

Fig. 2. Effect of omitting bath K^+ on $[Na^+]_i$, $[K^+]_i$, and E_m, as well as on $[Ca^{2+}]_i$. (*A*) Simultaneous recording of $[Na^+]_i$ and E_m in a P neuron by using a double-barrelled ion-sensitive microelectrode. K^+ was omitted from the bath for 20 min. (*B*) Simultaneous recording of $[K^+]_i$ and E_m in a different cell. Note the marked membrane hyperpolarization that occurs despite the continuous decrease in $[K^+]_i$. (*C*) In 6 of 7 cells a 20 min omission of bath K^+ had no effect on $[Ca^{2+}]_i$.

Fura-2, neither during the exposure nor after ouabain washout (Fig. 1*C*; $n = 7$ experiments). In contrast, a relatively fast oubain-induced $[Ca^{2+}]_i$ increase has been found by using Ca^{2+}-sensitive microelectrodes (Schlue, 1991). We suspect that this increase was due to an augmented membrane depolarization and hence activation, of voltage-dependent Ca^{2+} channels caused by the impalement of the relatively large Ca^{2+}-sensitive electrodes (*see* Kennedy & Thomas, 1996). When measured with Na^+- or K^+-sensitive microelectrodes, or with single electrolyte-filled electrodes, the ouabain-induced membrane depolarization occurred more slowly or was even absent (*see* Fig. 1*A*, *B*). However, the effects of ouabain may also be augmented by the impalement of relatively fine electrodes. In P neurons, a $[Ca^{2+}]_i$ increase is detectable when E_m becomes more positive than −25 mV (*see* Dierkes et al., 1997), and this threshold was exceeded in some of the experiments presented here and by Schlue (1991).

The inhibition of the Na^+-K^+ pump by omitting bath K^+ also led to an increase in $[Na^+]_i$ and to a decrease in $[K^+]_i$, which both proceeded more slowly than upon ouabain application (Fig. 2*A, B*; *see* Schlue & Deitmer, 1984). The concentration changes were accompanied by a slowly developing, marked membrane hyperpolarization by up to 45 mV, which later turned abruptly to a depolarization. In K^+-free solution, an initial depolarization, such as upon ouabain application, did not occur, and $[K^+]_i$ even decreased to ~10 mM, before E_m became more positive than at rest. In 6 of 7 experiments, the 20 min omission of bath K^+ had no effect on $[Ca^{2+}]_i$ (Fig. 2*C*). In one experiment, after 19 min in K^+-free solution, $[Ca^{2+}]_i$ suddenly increased to 450 nM, but it recovered rapidly after restoration of bath K^+.

$[Na^+]_i$ and R_{in} Recovery After Electrode Impalement

The temporary membrane hyperpolarization after inhibition of the Na^+-K^+ pump is primarily due to an increase in the K^+ permeability of the plasma membrane, which might be caused by the increase in $[Na^+]_i$, as the K^+ permeability was hardly affected when the pump was inhibited in Na^+-free solution (Schlue, 1991). This presumption is supported by the relationship between $[Na^+]_i$, E_m, and R_{in} that was found after impaling microelectrodes into P neurons. In the experiment shown in Fig. 3, the cell was first impaled by a double-barrelled Na^+-sensitive microelectrode that was used to measure E_m and $[Na^+]_i$, and about 40 s later by a conventional, electrolyte-filled microelectrode that was utilized for the application of hyperpolarizing current pulses in order to determine R_{in}. The injury of the cell caused by the insertion of the Na^+-sensitive microelectrode appeared to be small, as the recorded $[Na^+]_i$ was low and E_m close to its final value. However, the subsequent insertion of the conventional microelectrode induced a transient membrane depolarization and a large $[Na^+]_i$ increase, suggesting a significant but

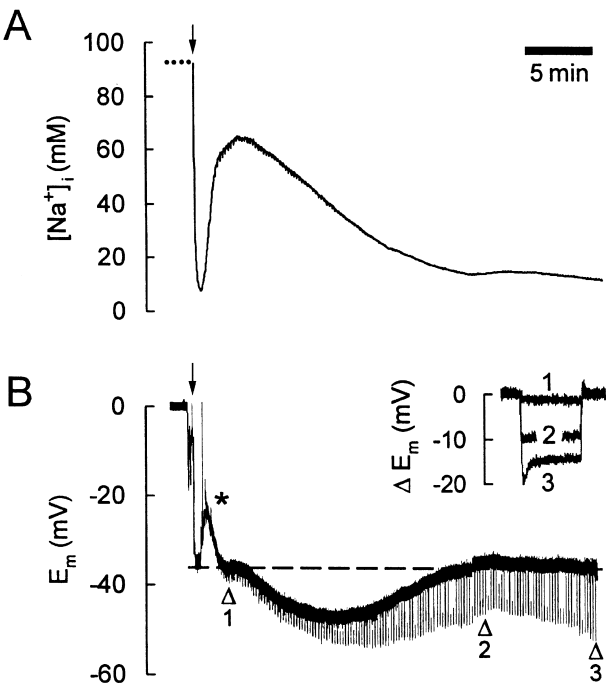

Fig. 3. E_m, $[Na^+]_i$, and R_{in} after electrode impalement. Simultaneous recording of $[Na^+]_i$ (*A*) and E_m (*B*) by means of a double-barrelled Na^+-sensitive microelectrode the impalement of which is marked by the arrows. R_{in} was determined from the shift in E_m upon injection of hyperpolarizing current pulses (−1 nA, 1 s duration, 10 s interval) by means of a conventional microelectrode that was impaled ~40 s later, discernible from the onset of the temporary depolarization (marked by asterisk) and the large increase in $[Na^+]_i$. Figures in *B* identify single current pulse-induced E_m shifts that are shown in the inset at an expanded time scale. For superimposition the traces were vertically shifted to the mean E_m before the onset of the current pulse. Note the 'depolarizing sag' at the strongest hyperpolarization (*3*), which is due to activation of I_h channels (*see* Gerard, Hochstrate & Schlue, 1999).

reversible cell damage. The $[Na^+]_i$ increase reached its maximum of ~55 mM at a moment when E_m had already returned near to the resting value. At the same time, R_{in} was only 1.6 MΩ, which is less than 10% of the resting value (21 ± 7 MΩ, $n = 16$; *see* Fig. 5). Subsequently, $[Na^+]_i$ continuously decreased, and in parallel R_{in} increased, while E_m was temporarily shifted to the negative direction. The three parameters reached their final values 20 to 30 min after electrode insertion. Corresponding experiments with K^+-sensitive microelectrodes showed that $[K^+]_i$ and R_{in} were usually small after electrode insertion and then both increased with a similar time course (*not shown*).

Na^+ Injection Evokes a Membrane Hyperpolarization and a Decrease in R_{in}

The close relationship between $[Na^+]_i$, E_m, and R_{in} was also evident in experiments in which Na^+ was iontophoretically injected into P neurons. In the experiment shown in Fig. 4, during the 3 min Na^+ injection $[Na^+]_i$ gradually increased from a basal level of 8 mM to almost 40 mM. Upon the onset of the Na^+ injection, R_{in} immediately dropped, which is probably due to voltage-dependent ion channels that are activated by the concomitant membrane depolarization. During the injection, R_{in} remained substantially decreased, although E_m gradually recovered and almost reached its initial value at the end of the injection.

At the beginning of the Na^+ injection, the E_m shifts induced by the hyperpolarizing current pulses used to determine R_{in} showed an initial hyperpolarizing overshoot, and furthermore, after switching off the current pulse, the cells generated a burst of action potentials (Fig. 4*C*, response *2*). The cause of the overshoot is not known, nor is that for the generation of the action potentials, but the two effects might be coupled. Thus, during the Na^+ injection the overshoot became smaller and the generation of the action potentials ceased (Fig. 4*C*, response *3*), and the same, albeit less pronounced, relationship was also observed during the injection of K^+ (responses *6* and *7*). The steady-state hyperpolarization during the current pulses slightly increased with ongoing Na^+ injection, which probably reflects the deactivation of voltage-dependent ion channels due to the membrane repolarization.

After switching off the Na^+ injection current, $[Na^+]_i$, immediately began to recover, and E_m jumped to a value that was by −14 mV more negative than the resting E_m recorded before the Na^+ injection. Furthermore, R_{in} increased instantly, probably due to the further deactivation of voltage-dependent ion channels, but initially it was clearly smaller than the resting value. The subsequent recovery of $[Na^+]_i$, E_m, and R_{in} proceeded in parallel and was finished within about 5 min.

The hyperpolarization after switching off the injection current should increase the activity of hyperpolarization-activated cation (I_h) channels, which are also expressed in P neurons (Gerard, Hochstrate & Schlue, 1999). However, in the presence of 2 mM Cs^+, a relatively specific I_h channel blocker (*see* Pape, 1996), the changes in E_m and R_{in} during and after the injection of Na^+ were virtually unchanged (*see* Fig. 5), which excludes a significant contribution of I_h channels to the decrease in R_{in}.

Upon the iontophoretic injection of K^+ both E_m and R_{in} were changed by virtually the same degree as at the beginning of the Na^+ injection, but except for the slight reduction of the hyperpolarizing overshoot and the attenuated generation of action potentials after switching off the hyperpolarizing current pulses, the changes persisted over the injection period (Fig. 4*B, C*). After cessation of the K^+ injection, E_m and R_{in} immediately returned close to their resting

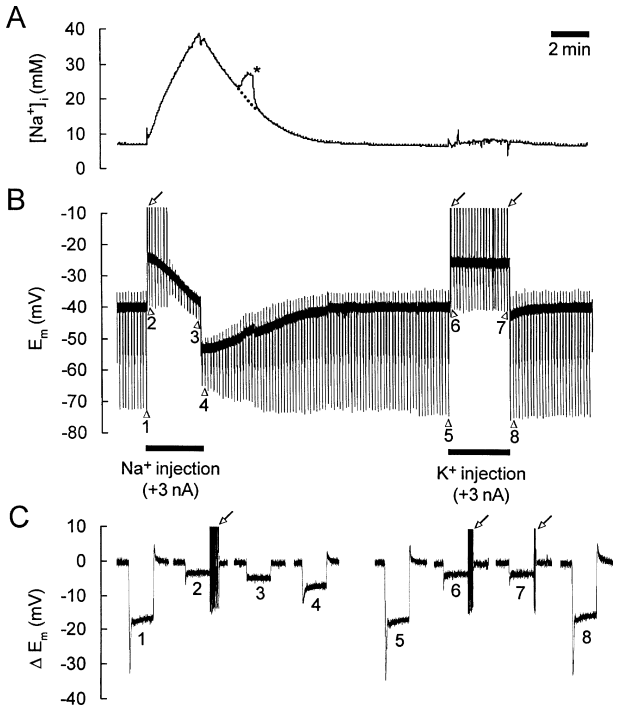

Fig. 4. Effect of iontophoretic injection of Na^+ or K^+ on $[Na^+]_i$, E_m, and R_{in}. Simultaneous recording of $[Na^+]_i$ (*A*) and E_m (*B*) by using a double-barrelled Na^+-sensitive microelectrode. The injection of Na^+ or, respectively, K^+ was performed by means of a further double-barrelled microelectrode, one barrel containing 0.5 M Na_2SO_4/8 mM KCl and the other 0.5 M K_2SO_4/8 mM KCl. This latter electrode was also used to apply hyperpolarizing current pulses (-1 nA, 1 s duration, 10 s interval) in order to determine R_{in}. During the first half of the experiment the current pulses were given via the K_2SO_4-filled barrel and during the second half, via the Na_2SO_4-filled barrel. Figures in *B* identify single current-induced E_m shifts that are shown in *C* at an expanded time scale. Arrows in *B* and *C* mark bursts of action potentials generated after switching off the hyperpolarizing current pulses, when the cell was significantly depolarized due to the injection of Na^+ or K^+. The 'depolarizing sag' seen in the current pulse responses *1*, *4*, *5*, and *8* reflects the activation of I_h channels (*compare* Fig. 3). The cause of the hyperpolarizing overshoot in the responses *2*, *6*, and *7* is unknown; an activation of I_h channels should not occur, as the E_m reached during the responses was not sufficiently negative. The temporary deflection of the $[Na^+]_i$ signal marked by the asterisk in *A* is probably an artefact.

values. As expected, the injection of K^+ had no effect on $[Na^+]_i$ (Fig. 4*A*).

It is noted that Na^+ injection experiments similar to those described here have been performed by Jansen & Nicholls (1973), in order to investigate the mechanism of the long-lasting afterhyperpolarization (AHP), which occurs in P neurons following periods of enhanced action potential activity. These authors also observed a hyperpolarization after Na^+ injection (which they attributed to an enhanced activity of the Na^+-K^+ pump), but they did not detect an appreciable change in R_{in}. We suspect that in the experiments of Jansen & Nicholls (1973) the $[Na^+]_i$ increase was smaller than in our experiments. In their experiments the time integral of the injection current varied between ~2 and ~8 nA × min, while in ours it was 9 nA × min. Furthermore, Jansen & Nicholls (1973) usually injected Na^+ by means of a single electrode, which should strain the cells less than the two double-barrelled electrodes used by us, so that in their experiments the transport capacity of the Na^+-K^+ pump might be higher and the injected Na^+ is extruded more effectively.

Relation Between $[Na^+]_i$ and R_{in}

The relation between $[Na^+]_i$ and R_{in} as derived from the recovery from electrode impalement (Fig. 3) or, respectively, Na^+ injection experiments (Fig. 4), is presented in Fig. 5. Altogether, the data show that R_{in} was low when $[Na^+]_i$ was high. On average, the relation between R_{in} and $[Na^+]_i$ obtained from electrode impalement data was shifted to lower $[Na^+]_i$ as referred to that derived from Na^+ injection experiments (Fig. 5*B*). Thus, after electrode impalement a 50% reduction of R_{in} or, more precisely, a recovery to 50% of the final R_{in} value took place at $[Na^+]_i$ = 23 ± 6 mM (n = 8), while after Na^+ injection the same effect occurred at $[Na^+]_i$ = 33 ± 4 mM (n = 6; P = 0.003).

In the Na^+ injection experiments, $[Na^+]_i$ was maximally raised to 40 mM and up to this concentration R_{in} had not reached a minimum that was independent of $[Na^+]_i$. Such a minimum was evident only in two electrode impalement experiments in which R_{in} was reduced to ~7% of its resting value at $[Na^+]_i$ > 50 mM (*see* Fig. 5). In any case, the relation between R_{in} and $[Na^+]_i$ was rather steep, suggesting a high extent of cooperativity with respect to the channel-activating mechanism. This expectation is supported by the finding that the pooled data shown in Fig. 5*B* could be described well by a simple model, which presupposes that the drop in R_{in} is due to K^+ channels that are activated by the binding of 3 Na^+ ions (*see* Model Calculations), with channel activity being half-maximal at $[Na^+]_i$ = 45 mM after electrode impalement and at 70 mM after Na^+ injection. It is noted that ouabain application was found to cause an R_{in} decrease by ~90% and a $[Na^+]_i$ increase to ~60 mM (Schlue, 1991; *compare* Figs. 1, 2), which fits well with the data shown in Fig. 5.

In Retzius Neurons R_{in} is Hardly Affected by Cytosolic Na^+

In contrast to P neurons, leech Retzius neurons depolarize upon ouabain application, but after omission of bath K^+ their E_m is only slightly affected, although $[K^+]_i$ substantially decreases and $[Na^+]_i$ increases (Deitmer & Schlue, 1981; 1983; Kilb &

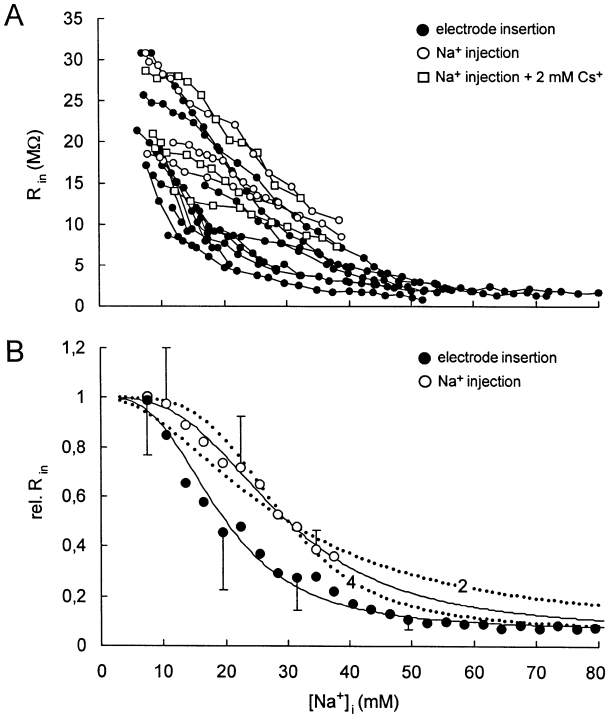

Fig. 5. Relationship between R_{in} and $[Na^+]_i$. (*A*) Raw data from experiments in which the recovery of R_{in} and $[Na^+]_i$ after electrode impalement (*see* Fig. 3; $n = 8$) or after Na^+ injection was monitored (*see* Fig. 4; $n = 6$). Three of the Na^+ injection experiments were performed in the presence of 2 mM Cs^+ to exclude a possible contribution of I_h channels to the drop in R_{in}. The R_{in} values were determined at $[Na^+]_i$ increments of ~2 mM. (*B*) Normalized and averaged data. The R_{in} values determined at different $[Na^+]_i$ in the single experiments shown in *A* were normalized to the maximum R_{in} after recovery from electrode impalement or, respectively, to the R_{in} before Na^+ injection (rel. R_{in}). Then all data points within a $[Na^+]_i$ range of 3 mM were averaged, whereby the Na^+ injection experiments performed in the presence or absence of Cs^+ were pooled. Solid lines were calculated under the assumption that the decrease in R_{in} is due to K_{Na} channels that are activated by the binding of 3 Na^+ ions (*see* Model Calculations). The half-maximal activation of the channels was taken to occur at $[Na^+]_i = 45$ mM (electrode insertion) or 70 mM (Na^+ injection), and at maximum channel activation R_{in} was assumed to be reduced to 7% of its resting value ($f = 13.3$). For comparison, the relation between rel. R_{in} and $[Na^+]_i$ that is expected if channel activation needs the binding of 2 or 4 Na^+ ions is also shown for the Na^+ injection data (*dotted lines*).

Schlue, 1999; Dierkes et al., 2005). This stabilization of E_m seems not to be due to an increase in the K^+ permeability of the plasma membrane, as R_{in} was found to be unaffected by the removal of bath K^+ (Deitmer & Schlue, 1983). Na^+ injection experiments as shown in Fig. 6 specifically exclude a significant relationship between $[Na^+]_i$ and R_{in}. In Retzius neurons, when using the same injection parameters as in P neurons, the $[Na^+]_i$ increase was small, and moreover, $[Na^+]_i$ reached a steady state, indicating an equilibrium between Na^+ injection and Na^+ extrusion.

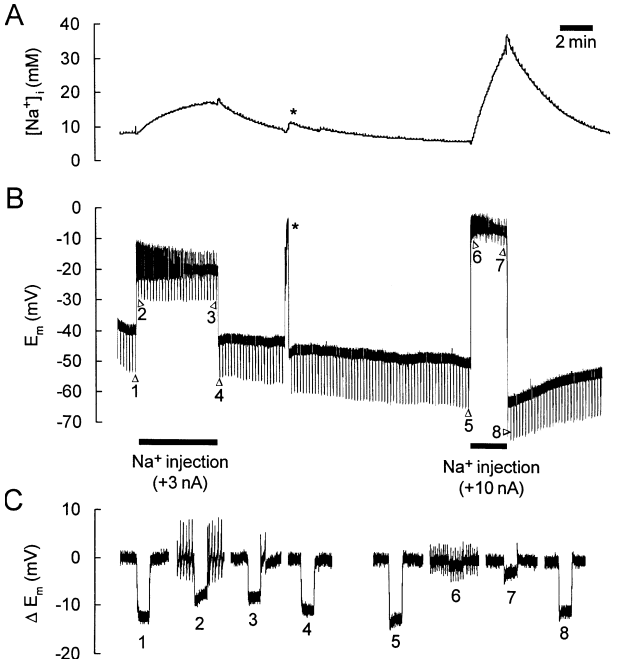

Fig. 6. Effect of iontophoretic injection of Na^+ on $[Na^+]_i$, E_m, and R_{in} in Retzius neurons. The experimental procedure was the same as described in Fig. 4. Asterisks mark changes in $[Na^+]_i$ and E_m caused by a 15 s Na^+ injection performed to readjust the injection current. The broadening of the E_m trace at the beginning of the Na^+ injections is due to the generation of action potentials (*see C*, traces *2* and *6*). After cessation of the 10 nA injection current the cells hyperpolarized by ~−12 mV.

During the injection, the cells were constantly depolarized and R_{in} was moderately reduced. The two parameters instantaneously recovered after switching off the injection current, but R_{in} recovery was not complete (~−10%) and took about 5 min (Fig. 6*B, C*). A $[Na^+]_i$ increase comparable to that in P neurons was achieved by raising the injection current, which also enhanced the membrane depolarization and the reduction of R_{in}. However, despite the strong increase in $[Na^+]_i$, the current-induced membrane depolarization was constant. After switching off the injection current, E_m was initially more negative than before the injection and then returned to its resting value within a few minutes. The recovery of R_{in} was again incomplete, but the discrepancy from the resting value was small (~−15%).

Effect of Injecting Na^+ and Other Alkali Ions on the Membrane Current

Together with the results published by Schlue & Deitmer (1984) and Schlue (1991) the data presented so far strongly suggest that leech P neurons, but not Retzius neurons express Na^+-dependent K^+ (K_{Na}) channels (*see* Dryer, 1994; 2003; Bhattacharjee & Kaczmarek, 2005). This conclusion is supported by the effect of injecting Na^+ into P neurons on the

membrane current, which was measured upon clamping E_m to values between −100 and −30 mV by using a linear voltage ramp (Fig. 7*A*). Under control conditions, the inwardly directed membrane current between −100 and −50 mV was almost linearly dependent on E_m, while the outwardly directed current at more positive E_m increased overproportionally. After the Na^+ injection, the membrane current was virtually unchanged when E_m was more negative than ∼−70 mV, but at more positive E_m the current was increasingly enlarged. Correspondingly, the Na^+-induced current component, i. e., the difference between the currents recorded after and before the Na^+ injection, was always outwardly directed and showed considerable rectification. A reversal of the current direction could not be detected. After the injection, the Na^+-induced membrane current decreased within 5–10 min, in close correspondence to the recovery of R_{in} (*see* Fig. 4*B*).

The injection of Li^+ into P neurons changed the membrane current in a similar way as the Na^+ injection, but the effect was more pronounced and hardly reversible (Fig. 7*B*). On average, the Li^+-induced current was about five times as large as the Na^+-induced current. It is emphasized that after the insertion of a Li^+-containing electrode a normal R_{in} near 20 MΩ (*see* Fig. 5*A*) was only measured when the electrode resistance was high (>80 MΩ), but these electrodes tended to become clogged after switching on the injection current. When using electrodes with a lower resistance, R_{in} was usually small (3–10 MΩ) and mostly decreased further without applying an injection current, whereby E_m remained stable or even hyperpolarized for a prolonged period.

The injection of K^+ into P neurons had virtually no effect on the membrane current (*see* Fig. 4). After the injection of Rb^+ a small inward current at negative E_m was detected (Fig. 7*B*), the origin of which was not investigated. In Retzius neurons, the injection of the various alkali ions had virtually no effect on the membrane current, although the injection current was increased to account for the larger volume of the Retzius cell soma (Fig. 7*C*).

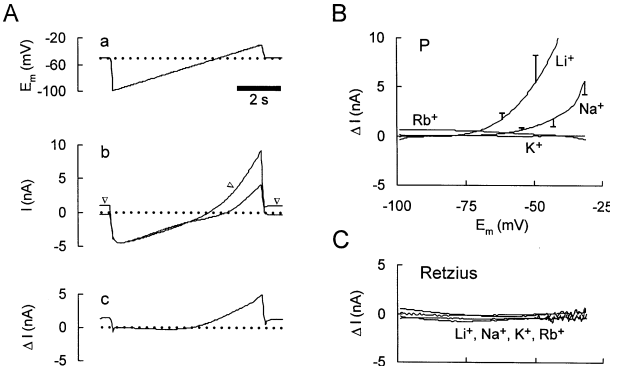

Fig. 7. Effect of injecting Na^+ and other alkali ions on the membrane current in P and Retzius neurons. The cells were impaled with a single-barrelled microelectrode for the recording of E_m and by a double-barrelled microelectrode for current injection and for the iontophoretic ion injection. (*A*) Effect of Na^+ injection in a P neuron. (*a*) Voltage protocol: E_m was first clamped to −50 mV for a few seconds, and after an abrupt shift to −100 mV it was then linearly changed to −30 mV within 7 s and finally again shifted to −50 mV. (*b*) Superimposed membrane currents before and ∼10 s after the Na^+ injection (3 nA for 3 min; *see* Fig. 4), the latter being marked by arrowheads. (*c*) Na^+-induced membrane current: difference between the currents after and before the Na^+ injection. (*B*) Membrane currents in P neurons as induced by the injection of Na^+, Li^+, K^+, or Rb^+ in dependence on E_m. The currents were determined as illustrated in *A*. (*C*) Membrane currents in Retzius neurons. The experimental procedure was the same as for P neurons, except that the injection current was increased to 10 nA to account for the larger volume of the Retzius cell body (*compare* Fig. 6). Data in *B* and *C* are the mean of $n = 3$ to 6 experiments. Standard deviations of the Na^+- and Li^+-induced membrane currents in P neurons are exemplified in *B*.

Discussion

In leech P neurons the inhibition of the Na^+-K^+ pump leaves E_m virtually unaffected over a long period, or even temporarily shifts it to the negative direction, although the concentration gradients for Na^+ and K^+ across the plasma membrane are substantially attenuated (*see* Figs. 1, 2). This E_m stabilization or, respectively, membrane hyperpolarization, is due to the activation of K^+ channels, which compensates for, or even overcompensates the attenuation of the K^+ concentration gradient (Schlue & Deitmer, 1984; Schlue, 1991). The activity of these K^+ channels is reflected by a strong decrease in R_{in} and, in particular, by a marked increase in the changes of E_m caused by moderate changes in the K^+ concentration of the bath solution.

Functional Link Between $[Na^+]_i$ Increase and K^+ Channel Activation

In Na^+-free solution the inhibition of the Na^+-K^+ pump by ouabain caused only a small increase in the sensitivity of E_m to bath K^+ (Schlue, 1991), suggesting a functional link between $[Na^+]_i$ increase and K^+ channel activation. This presumption is supported by the general observation in P neurons that when $[Na^+]_i$ was high, R_{in} was low, and E_m tended to the negative direction: 1) After electrode insertion $[Na^+]_i$ was often strongly increased and R_{in} reduced, while E_m was close to its resting value, although the concentration gradient for K^+ should be attenuated to a similar degree as the Na^+ gradient (Fig. 3). The recovery of $[Na^+]_i$ and R_{in} occurred in parallel and was accompanied by a temporary membrane hyperpolarization, which probably reflects the restoration

of the K^+ gradient at a still increased but gradually decreasing K^+ selectivity of the plasma membrane. 2) During the iontophoretic injection of Na^+, i.e., with increasing $[Na^+]_i$, the concomitant membrane depolarization became continuously smaller, indicating the activation of an outward current. This outward current is mediated by ion channels, because R_{in} remained small, although the cell repolarized and voltage-dependent ion channels should be deactivated (Fig. 4). 3) After cessation of the Na^+ injection, i.e., when $[Na^+]_i$ was maximally increased, the cells hyperpolarized and R_{in} remained substantially reduced, which indicates the persistence of the outward current. Subsequently, E_m and R_{in} recovered in parallel with $[Na^+]_i$. 4) The activation of an outward current by the injection of Na^+ was demonstrated directly by voltage-clamp experiments (Fig. 7*A*, *B*).

It is noted that the contribution of an enhanced Na^+-K^+ pump activity to the membrane hyperpolarization after raising $[Na^+]_i$ is probably small. For example, the electrogenic pump current coupled to the recovery from the Na^+ load shown in Fig. 4*A* was estimated to ~230 pA. This estimation is based on the rate of the $[Na^+]_i$ increase after pump inhibition (3.8 mM/min; *see* below), which reflects the Na^+ leak current, the rate of the $[Na^+]_i$ recovery after the Na^+ load (−6.9 mM/min), which reflects the enhanced pump activity, as well as from the volume of the P neuron cell soma (ϕ ~50 μm; 70% cytosol), which allows to convert concentration changes into ion movements. With R_{in} ~10 MΩ (*see* Fig. 5*A*) the pump current should shift E_m by ~−2.3 mV into the negative direction, which is less than 20 % of the experimentally observed hyperpolarization (−14 mV; Fig. 4*B*).

The K^+ Channels are Directly Activated by Cytosolic Na^+

The relationship between R_{in} and $[Na^+]_i$ in P neurons could be well described by a simple model in which the K^+ channels were assumed to be activated by the binding of three Na^+ ions at their cytosolic face, with half-maximal channel activation occurring at $[Na^+]_i$ = 45 or 70 mM (Fig. 5). These parameters closely correspond to those found for Na^+-activated K^+ (K_{Na}) channels in various other preparations (Dryer, 1994; 2003), which strongly suggests that the K^+ channels activated in P neurons upon inhibition of the Na^+-K^+ pump, electrode insertion, or Na^+ injection also belong to this family of ion channels. This view is supported indirectly by various observations that argue against an alternative activation mechanism. Thus, although channel activity appears to be modulated by E_m (*see* below; Fig. 7*A, B*), an activation of the K^+ channels by an E_m shift can be excluded. Channel activation occurred when the cells were depolarized due to the iontophoretic injection of Na^+ but not K^+ (Fig. 4), when E_m was fairly stable, as upon ouabain application (Fig. 1; Schlue, 1991) or after electrode insertion (Fig. 3), and also when the cells were hyperpolarized after the omission of bath K^+ (Fig. 2; Schlue & Deitmer, 1984). An activation by Ca^{2+} is excluded, because ouabain application or omission of bath K^+ had no effect on the cytosolic free Ca^{2+} concentration over the period of ~20 min in which K^+ channel activation occurred (Figs. 1*C*, 2*C*). Furthermore, at least in cultured P neurons, Ca^{2+}-dependent K^+ channels appear to be virtually absent (Stewart, Nicholls & Adams, 1989). An activation by a decrease in the cytosolic ATP concentration seems improbable, since the inhibition of the Na^+-K^+ pump implies the omission of a major energy-consuming process so that the ATP concentration is more likely to increase rather than to decrease. A decrease in the cytosolic ATP concentration might occur if the Na^+-K^+ pump was strongly activated by a large $[Na^+]_i$ increase, but the recovery from such an increase always proceeded monophasically, giving no hint for an ATP depletion (*see* Fig. 4*A*; *see* also Fig. 1 in Dierkes & Schlue, 2005). Finally, an acidification of the cytosol, which should occur upon an increase in $[Na^+]_i$ (*see* Kilb & Schlue, 1999), is unlikely to be involved in K^+ channel activation, as the marked drop in the cytosolic pH caused by an extracellular application of ammonium was paralleled by a depolarization and not by a hyperpolarization (Deitmer & Schlue, 1987).

The conclusion that the K^+ channels activated after inhibition of the Na^+-K^+ pump, electrode insertion, or Na^+ injection are primarily controlled by $[Na^+]_i$ does not exclude a modulatory effect of other cellular parameters. One factor affecting channel activity seems to be E_m, as the Na^+-induced membrane current showed significant outward rectification (Fig. 7*A, B*). This voltage dependence may contribute to the discrepancy between the $[Na^+]_i$-R_{in} relationships after Na^+ injection or, respectively, electrode insertion (Fig. 5). Thus, after Na^+ injection the cells were always hyperpolarized (*see* Fig. 4*B*), and hence channel activity and the concomitant R_{in} drop should be relatively small. Vice versa, after electrode impalement, E_m was often close to its resting value (*see* Fig. 3*B*), and therefore channel activity and R_{in} drop should be comparatively large. Furthermore, an augmentation of channel activity by cytosolic Cl^- and/or a reduction by ATP has been found in heterologously expressed K_{Na} channels of the Slo2.1 ("Slick") type (Bhattachajee et al., 2003; 2005; Yuan et al., 2003), but it is unclear whether in our experiments these parameters were sufficiently changed to exert a significant effect. However, even significant changes in these parameters must not have a marked effect, as K_{Na} channels

of the slo2.2 (''Slack'') type are hardly affected by Cl^- and ATP.

The K_{Na} Channels in P Neurons are also Activated by Li^+

The effect of injecting Li^+ on the membrane current and R_{in} indicates that the K_{Na} channels of leech P neurons are also activated by this ion (Fig. 7), and moreover, the channels seem to be even more sensitive to Li^+ than to Na^+. Assuming that the K_{Na} channels are half-maximally activated at $[Na^+]_i = 70$ mM and that three Na^+ ions must bind for activation (*see* Fig. 5), the $[Na^+]_i$ increase to ~40 mM caused by the Na^+ injection should activate about 16% of the channels. In contrast, the channels seem to be almost fully activated after the Li^+ injection, as the Li^+-induced membrane current was about five times larger than the Na^+-induced current (Fig. 7B). This difference is only partly due to the fact that the Na^+ injection is counteracted by the Na^+-K^+ pump. At the beginning of the Na^+ injection the pump effect is small, as deduced from the continuous slowing down of $[Na^+]_i$ recovery after the injection, and therefore, the $[Na^+]_i$ increase without pump counteraction will be given by the linear extrapolation of the initial, injection-induced $[Na^+]_i$ increase. According to this extrapolation, $[Na^+]_i$ would increase to about 60 mM, corresponding to a channel activation of only 40%. Furthermore, the mobility of Li^+ is smaller than that of Na^+, and therefore, the iontophoretic injection of Li^+ should be relatively less effective than the injection of Na^+.

The Li^+ sensitivity of the K_{Na} channels in leech P neurons contrasts with most observations in vertebrates according to which channel activation is Na^+-specific (e.g. Dryer, 2003; but *see* Liu, Schaffner & Barker, 1998). However, experiments in invertebrate neurons demonstrate or at least suggest that these cells also possess Li^+-sensitive K_{Na} channels (snail: Partridge & Thomas, 1974; 1976; Kiss et al., 2000; crayfish: Hartung, 1985), and to our knowledge there is no report on K_{Na} channels in invertebrates in which an activation by Li^+ is excluded. Furthermore, also in snail neurons the K_{Na} channels appear to be more sensitive to Li^+ than to Na^+ (Partridge & Thomas, 1976). Thus, the available data suggest that the K_{Na} channels in invertebrate neurons, but not in vertebrate neurons, are in general sensitive to Li^+ (*see* Martin & Dryer, 1989).

Pump Inhibition by Ouabain and Omission of Bath K^+

The effects of inhibiting the Na^+-K^+ pump by ouabain or omission of bath K^+ differed in several aspects (Figs. 1, 2). Thus, ouabain application induced an initial membrane depolarization, which was not observed after K^+ omission. Furthermore, the hyperpolarization after K^+ omission was much larger than that upon ouabain application, and $[K^+]_i$ decreased more before the cells depolarized. It seems unlikely that theses differences are primarily due to a different extent of Na^+-K^+ pump inhibition. In the presence of ouabain the initial changes in $[Na^+]_i$ and $[K^+]_i$ occurred slightly faster than in the absence of bath K^+, which may suggest a more efficient pump inhibition, but the difference was not significant (ouabain: 3.8 ± 2.4 mM /min, $n = 12$; K^+-free: 3.1 ± 1.8 mM /min, $n = 9$; $p = 0.44$; *see* Figs. 1, 2; compare Dierkes et al., 2005). So far the different effects of ouabain and omission of bath K^+ appear to be predominantly due to different changes in the K^+ equilibrium potential (E_K).

At rest the extracellular K^+ concentration ($[K^+]_o$) near to the cell bodies (5.8 mM) and in the neuropile (6.3 mM) was found to be slightly higher than the K^+ concentration in the bath (4.0 mM; Schlue & Deitmer, 1980). Ouabain application induces a slight temporary $[K^+]_o$ increase by 1.2 mM in the cell body region and by 3.8 mM in the neuropile, which shifts E_K by +5 mV or +12 mV into the positive direction. In contrast, omission of bath K^+ causes a $[K^+]_o$ decrease to 1.6 mM in the cell body region and to 2.7 mM in the neuropile, corresponding to E_K shifts of −33 mV or −22 mV into the negative direction. Consequently, the depolarizing effect of suppressing the electrogenic pump current should be enhanced when the Na^+-K^+ pump was inhibited by ouabain but attenuated when pump inhibition was performed by omitting bath K^+, which is in line with the experimental data. The different shifts of E_K also explain why the membrane hyperpolarization is so much larger after K^+ omission than upon ouabain application and why $[K^+]_i$ dropped more before the cells depolarized. We emphasize that the negative E_K shift after omission of bath K^+ significantly affects E_m only if the K^+ selectivity of the plasma membrane is increased due to the activity of the K_{Na} channels, as evident from the slow development of the membrane hyperpolarization (*see* Schlue & Deitmer, 1984). Finally, we note that the decrease in $[K^+]_o$ upon omission of bath K^+ might be larger than found experimentally, because a $[K^+]_o$ of ~2 mM should be sufficient to maintain a significant activity of the Na^+-K^+ pump (*see* Glitsch, 2001).

No Significant K_{Na} Expression in Retzius Neurons

In Retzius neurons, K_{Na} channels appear to be virtually absent, since R_{in} was hardly dependent on $[Na^+]_i$ and the injection of Na^+ or Li^+ had no effect on the membrane current (Figs. 6, 7*C*). During the Na^+ injection Retzius neurons were constantly depolarized, but after cessation of the

injection current the cells also hyperpolarized temporarily, which could be explained by an enhanced activity of the Na^+-K^+ pump. The same calculation as for P neurons (*see* above) gave a pump current of 1.4 nA, which should cause a hyperpolarization by 14 mV, in close correspondence to the experimental value (12 mV; Fig. 6*B*). The calculation is based on the following parameters. Initial rate of the ouabain-induced $[Na^+]_i$, increase ("leak current"): 2.4 mM / min (Dierkes et al., 2005), $[Na^+]_i$ recovery after the Na^+ load: −12.8 mM /min (Fig. 6*A*), diameter of Retzius cell soma: 80 μM, R_{in} after Na^+ injection: 10 MΩ. In addition, as R_{in} was slightly reduced, Ca^{2+}-dependent K^+ channels might be involved, which are strongly expressed in these cells (Stewart, Nicholls & Adams, 1989). Leech Retzius neurons have been shown to possess ATP-dependent K^+ (K_{ATP}) channels that are activated upon a drop in the cytosolic ATP concentration (Frey, Hanke & Schlue, 1993; Frey, Lucht & Schlue, 1998), and which may fulfill similar functions as the K_{Na} channels in P neurons.

Physiological Significance

There is growing evidence that K_{Na} channels contribute to normal neuronal functioning, such as action potential recovery and/or control of excitability (Bhattacharjee & Kaczmarek, 2005). In particular, it has been demonstrated that in active neurons $[Na^+]_i$ increases sufficiently in certain locations to evoke a significant channel activation (*see* Rose, 2002). Furthermore, in most cells K_{Na} channels seem to be highly expressed, so that the activation of a small channel fraction may be functionally significant. Moreover, channel activity may be enhanced by an increase in the cytosolic Cl^- concentration and/or a drop in the cytosolic ATP concentration (Bhattacharjee et al., 2003). Under pathophysiological conditions, such as hypoxia or ischemia, K_{Na} channels will protect neurons in that they stabilize E_m, thereby preventing the activation of voltage-dependent Na^+ or Ca^{2+} channels.

Leech P neurons respond to a period of enhanced action potential activity with a prominent afterhyperpolarization (AHP; Baylor & Nicholls, 1969; Jansen & Nicholls, 1973). Various observations showed that the AHP is due to an enhanced activity of the Na^+-K^+ pump and to an increase in the K^+ conductance of the plasma membrane (*see* Scuri, Mozzachiodi & Brunelli 2002). However, a participation of K_{Na} channels appears unlikely, because the AHP was hardly affected by reducing the extracellular Na^+ concentration by 90%. Thus, the available data give no evidence that the K_{Na} channels are involved in the physiological functioning of leech P neurons, even after periods of high action potential activity. Nevertheless, in view of their high expression it is hard to believe that the K_{Na} channels are activated only under extreme, more or less pathophysiological conditions.

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