Participation of a Chloride Conductance in the Subthreshold Behavior of the Rat Sympathetic Neuron

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Sacchi, Oscar, Maria Lisa Rossi, Rita Canella, and Riccardo Fesce. Participation of a chloride conductance in the subthreshold behavior of the rat sympathetic neuron. J. Neurophysiol. 82: 1662–1675. 1999. The presence of a novel voltage-dependent chloride current, active in the subthreshold range of membrane potential, was detected in the mature and intact rat sympathetic neuron in vitro by using the two-microelectrode voltage-clamp technique. Hyperpolarizing voltage steps applied to a neuron held at −40 to −50 mV elicited inward currents, whose initial magnitude displayed a linear instantaneous current-voltage (I-V) relationship; afterward, the currents decayed exponentially with a single voltage-dependent time constant (63.5 s at −40 mV; 10.8 s at −130 mV). The cell input conductance decreased during the command step with the same time course as the current. On returning to the holding potential, the ensuing outward currents were accompanied by a slow increase in input conductance toward the initial values; the inward charge movement during the transient response (a mean of 76 nC in 8 neurons stepped from −50 to −90 mV) was completely balanced by outward charge displacement during the off response. The chloride movements accompanying voltage modifications were studied by estimating the chloride equilibrium potential (ECl) at different holding potentials from the reversal of GABA evoked currents. [Cl−], was strongly affected by membrane potential, and at steady state it was systematically higher than expected from passive ion distribution. The transient current was blocked by substitution of isethionate for chloride and by Cl− channel blockers (9AC and DIDS). It proved insensitive to K+ channel blockers, external Ca2+ intracellular Ca2+ chelators [bis-(o-aminophenoxy)-N,N,N′,N′-tetracetic acid (BAPTA)] and reduction of [Na+]i. It is concluded that membrane potential shifts elicit a chloride current that reflects readjustment of [Cl−]. The cell input conductance was measured over the −40 to −120-mV voltage range, in control medium, and under conditions in which either the chloride or the potassium current was blocked. A mix of chloride, potassium, and leakage conductances was detected at all potentials. The leakage conductance was voltage independent and constant at −14 mV. Conversely, gCl decreased with hyperpolarization (80 nS at −40 mV, undetectable below −110 mV), whereas gK displayed a maximum at −80 mV (55.3 nS). Thus the ratio gCl/gK continuously varied with membrane polarization (2.72 at −50 mV; 0.33 at −110 mV). These data were forced in a model of the three current components here described, which accurately simulates the behavior observed in the “resting” neuron during membrane migrations in the subthreshold potential range, thereby confirming that active K and Cl conductances contribute to the genesis of membrane potential and possibly to the control of neuronal excitability.

I N T R O D U C T I O N

Below the threshold potential for spike generation, the neuronal membrane is generally considered to exhibit a virtually passive electrical behavior: the concept itself of “resting” potential implies that a relatively fixed equilibrium value for membrane potential is set by the relative magnitudes of basal potassium and leakage conductances, and their respective equilibrium potentials.

We have recently shown that the momentary (and past) value of membrane potential markedly affects neuronal response to artificially imposed voltage steps as well as to physiological synaptic activation, mostly via a modification of the inactivation state of IA potassium conductance. Although voltage-dependent inactivation of IA does not directly contribute to determining the membrane potential, it considerably modifies the neuronal response to synaptic activation and the shape of the action potential (Sacchi et al. 1998).

In this paper we examine in detail the electrical properties of the intact and mature rat sympathetic neuron “at rest” and demonstrate the presence of relevant active chloride conductances in the under-threshold range of membrane potential values. This novel chloride current can be readily demonstrated over a wide voltage range provided that the membrane potential is moved, even by a few millivolts, and that internal chloride concentration is not clamped, as in whole cell patch-clamp experiments, but remains free to readjust according to the new voltage level.

Detailed information on membrane chloride conductance and channels has been obtained primarily from nonmammalian and nonneuronal systems; in most cases, the chloride permeability proved to be the dominant resting ion conductance and to play a special role in stabilizing the membrane potential and thereby determining the passive characteristics of the membrane. Additional functions have been attributed to chloride channels activity in cell volume regulation and transepithelial transport (for review, see Strange et al. 1996). The relationships among molecular identity, gene superfamilies, and functional expression have been partly elucidated (reviewed by Jentsch 1996; Pusch and Jentsch 1994). Comparable information on gCl has been similarly obtained in neurons, but it remains in doubt which channel types are ubiquitously present and which are expressed in a highly specific manner, and little is known about their mechanisms of activation and their specific physiological function.

The macroscopic chloride conductance has been characterized in some detail by several investigators. A hyperpolarization-activated chloride current, which exhibits inwardly rectifying properties, has been described in Aplysia neurons (Chesney-Marchais 1983), in hippocampal pyramidal neurons (Madison et al. 1986; Staley 1994), and in the dissociated rat...
sympathetic neuron (Clark et al. 1998; see, however, Lamas 1998; Selyanko 1984). A calcium-dependent chloride current, responsible for a slow afterdepolarization following spike firing, has been described in rat sympathetic neurons after axotomy (Sánchez-Vives and Gallego 1994) and as a normal complement of the mouse sympathetic ganglion cell (De Castro et al. 1997). On the other hand, chloride currents activated by injected calcium have been described in several neuron types and produce slow depolarizing aftereffects (reviewed by Mayer et al. 1990; Scott et al. 1995). Single voltage-dependent chloride channels have been dissected and kinetically characterized in rat hippocampal neurons in culture (Franciolini and Nonner 1987), in acutely dissociated rat cerebral cortical neurons (Blatz 1991), and in Aplysia neurons (Chesnoy-Marchais and Evans 1986).

The particular chloride conductance described here in the mature sympathetic neuron originates physiologically relevant currents and chloride ion redistribution, and its activation state slowly changes when the membrane potential of the neuron is displaced. The resulting modifications in chloride conductance and equilibrium potential modify, in turn, the resting potential (and input impedance) of the neuron. Thus the idea arises that the dynamic result of its electrical property but rather the dynamic result of its previous history (both in terms of electrical changes and chloride regulation), and in turn influences neuronal excitability and responsiveness.

METHODS

All experiments were performed on superior cervical ganglia isolated from young rats (5–6 wk old; 120–150 g body weight) during urethane anesthesia (1–1.5 g kg$^{-1}$ ip) and maintained in vitro at 37°C. After surgery, the animals were killed with an overdose of anesthetic. The ganglion was desheathed and pinned to the bottom of a chamber mounted on the stage of a compound microscope; individual neurons were identified at a magnification of $\times 500$ by using diffraction interference optics and impaled with two independent glass micro-electrodes filled with neutralized 4 M potassium acetate (30–40 MΩ resistance). Recordings were obtained under two-electrode voltage-clamp conditions as described previously (Belluzzi et al. 1985). The preparation was continuously superfused with a medium (in mM: 136 NaCl, 5.6 KCl, 5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 Na$_2$HPO$_4$, 14.3 NaHCO$_3$, and 5.5 glucose) pregassed with 95% O$_2$–5% CO$_2$ to a final pH 7.3. Atropine sulfate 10$^{-5}$ M was systematically added to the saline. The bath was grounded through an agar-3 M KCl bridge. The usual protocol was to hold the potential at −40 or −50 mV, and to jump to the test potential in the −40/−130-mV range. Long-lasting current tracings were filtered at 5 kHz and digitized continuously on tape (Biologic, DTR-1200; 0–10 kHz). Data were analyzed on Pentium personal computers (AST) with pCLAMP (Axon Instruments) and MATLAB 386 (The MathWorks, Natick, MA) software packages.

The low chloride solutions were made by replacing 136 mM NaCl with isoosmolar amounts of sodium isethionate or methanesulfonate and were applied when both microelectrodes were inside the cell. Potentials arising between the bath and the reference agar-bridge electrode were measured by comparing the potential of the 3 M KCl agar bridge with that of a broken-tip microelectrode filled with 3 M KCl (Alvarez-Leefmans et al. 1988); values around +1–2 mV were measured and were not taken into account.

γ-Aminobutyric acid (GABA) was focally applied to the soma of identified neurons by 0.1-s pulses of pressure (20 psi; PDES 2.1, NPI, Tamm, Germany) to the back of micropipettes of 2–4 μm internal diameter, filled with 1 mM GABA dissolved in the same solution present in the bath. The pipette was positioned as close as possible to the neuron and maintained in place, in some experiments, or removed and repositioned during subsequent applications to avoid the development of receptor desensitization due to agonist leakage from the pipette; no differences were apparent between these procedures. GABA applications were repeated at 30-s intervals over an appropriate range of voltage commands, while maintaining the neuron under voltage-clamp conditions at different holding potentials over the −40/−120-mV range.

When TEA-Cl (tetraethylammonium chloride, Sigma) was used, appropriate amounts of NaCl were removed from the control solution composition to maintain isoosmolarity. 9AC (anthracene-9-carboxylic acid, Sigma) and DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid, Sigma) were bath applied by exchanging the normal medium with drug-containing medium by means of a continuous rapid perfusion system.

RESULTS

Neuronal general behavior in the subthreshold range

Representative examples of current recordings under two-electrode voltage-clamp conditions from a rat sympathetic neuron in response to hyperpolarizing voltage steps are displayed in Fig. 1A. The neuron was stepped from a holding potential of −50 mV to a series of command potentials between −60 and −130 mV, in 10-mV increments. The current was probed at different potentials for a period of 150 s, and the initial holding potential was thereafter restored and maintained for further 210 s, before starting a new cycle. An inward current developed with hyperpolarization and instantaneously reached its maximum value. This value displayed a linear current-voltage ($I-V$) relationship (Fig. 1B) as expected for leakage currents, but was not maintained (Fig. 1, A and C). The currents decayed toward a new steady-state value with a time course systematically well fit by a single exponential. The unusually long-lasting traces are differently illustrated with appropriate time resolution and acquisition bandwidth in Fig. 1, A–C (in A the superimposed tracings are heavily filtered for clarity); the figure also shows the stability of the recordings, which is a must for subsequent analysis. In spite of the linear $I-V$ relationship observed for the initial values of the currents, the final steady-state components were scaled by variable factors with respect to the starting values and were not proportional to the voltage step, suggesting that the decaying currents were sustained by (active) conductances that changed during the sojourn at the new imposed voltage levels. In other words, these currents should be interpreted as tail currents sustained by a conductance already active at the holding potential of −50 mV. This was an unexpected finding, because the electrical properties of the sympathetic neuron are generally considered to be exclusively “passive” over the membrane potential range tested (the inward rectifier current of the IQ or IH type is absent or negligible).

On termination of the command steps, the neuron generated large outward transient currents (Figs. 1A and 2, trace a), which also decayed exponentially.

The time courses of all currents became faster with increasing membrane polarization; the mean values of the decay time constants in our experiments (neurons held at −50 or −40 mV) are illustrated in Fig. 1D: they were calculated from the on responses in the −60/−130-mV voltage range or from the off
FIG. 1. Transient currents in the subthreshold voltage region. A: family of currents evoked in a sympathetic neuron by long-lasting hyperpolarizing commands in the −60/−130-mV membrane potential range, repeated in 10-mV steps. Holding potential was −50 mV and was maintained for 210 s before starting a new cycle. B and C: currents observed in different neurons with appropriate time resolution to show the early pattern of current development. In C the neuron was successively stepped to −70, −80, and −90 mV from a holding potential of −50 mV. D: time constant of decay of the transient current component measured at different membrane potentials during ON responses (○, n = 4–8) or OFF responses on returning to the holding level (●, n = 4–13). Bars indicate SE.

FIG. 2. Current and input conductance measured during voltage and [Cl]$_e$ modifications. Currents recorded during a −50/−100/−50-mV cycle (top panel) in normal saline (trace a) or after substituting 136 mM Na-isethionate for NaCl (final [Cl]$_e$ = 18 mM; trace b) are illustrated. Total cell input conductance was continuously measured by applying 40-mV hyperpolarizing steps of 10 ms duration at 1 Hz. Three examples of these recordings, taken in control solution at −50 mV (with activation of $I_A$ on returning to −50 mV), at the onset and late during the −100-mV voltage jump, are shown. Note the shift in the holding current level in the presence of low external chloride, and the same time course of current and conductance modifications.
responses in the −40/−50-mV region (Fig. 2 illustrates an example of the whole time course).

The instantaneous cell input conductance at different membrane potentials was estimated by measuring the currents induced by short (10 ms) hyperpolarizing voltage pulses of −30/−40 mV amplitude, superimposed on the longer voltage commands. The membrane chord conductance was measured assuming that the single conductances at hyperpolarized potentials showed neither fast voltage sensitivity (the current level during the short step was constant, after the capacity transient) nor instantaneous rectification (the total instantaneous I-V relationship was linear down to −130 mV). Figure 2 shows an example of these tests, during a 3-min −50/−100/−50-mV cycle (the current response is illustrated in trace a, middle panel). The input conductance at −50 mV was constant (58 nS) during the application of the test pulses at 1 Hz, indicating that the procedure did not perturb the resting state (trace a in the bottom panel). At −100 mV the cell input conductance values were initially confirmed but thereafter slowly decreased with the same time course of the current decay, reaching a final steady-state value of 31 nS. The opposite was observed during the OFF response: the cell input conductance smoothly increased, reaching within a few minutes the values originally measured at −50 mV at the beginning of the cycle.

Among the possible current carriers, the chloride ion appeared to be the most likely candidate when Na-isethionate was isosmotically substituted for the external NaCl (136 mM). This is illustrated in Fig. 2 (trace b, same neuron as a): the holding inward current at −50 mV was reduced by 0.68 nA, and both the transient inward current during the command at −100 mV and the outward transient on returning to −50 mV were completely canceled with a general behavior now constantly passive during the long-lasting pulse. Consistently, the cell input conductance proved to be reduced to 24 nS at rest and displayed very limited excursions during the voltage migrations imposed thereafter.

It immediately appeared evident that the excess electric charge inwardly displaced during the transient phase of the currents (relative to the final steady state) was balanced out by the outward charge that left the neuron on returning to the initial membrane potential level. This was tested in eight neurons during −50/−90/−50-mV commands: despite the different time courses of the currents, the mean charge entering the neuron during the transient response at −90 mV was 76 nC and was balanced out by a symmetrical outward displacement of 77 nC at −50 mV.

**Chloride distribution in the sympathetic neuron**

The possible role for chloride suggested by the present observations urged a precise understanding of its movements at rest and during voltage pulses. Previous work in the rat sympathetic neuron demonstrated that intracellular chloride activity at rest, as measured with ion-sensitive microelectrodes, is higher than predicted from a passive distribution of the ion (aCl = 29.9 mM) (Ballanyi and Grafe 1985). The same conclusion was reached by directly measuring intracellular chloride concentration with an electron microprobe ([Cl]i = 32 mM) (Galvan et al. 1984). This unbalance ([Cl]i = 23.3 mM is the expected Nernst’s value at −50 mV resting potential) is maintained by inward pumping of chloride through an active process, which involves an electroneutral K⁺:Cl⁻ co-transport and possibly a Na⁺:K⁺:Cl⁻ co-transport (as suggested for the amphibian dorsal root ganglion neurons by Alvarez-Leefmans et al. 1988).

The presence of GABA-sensitive chloride channels in rat sympathetic neurons has been long demonstrated (Adams and Brown 1975). There is no evidence for a role of GABA as a ganglionic neurotransmitter; when applied via the bathing medium, however, it produced a large fall in cell input resistance accompanied by a clear-cut membrane depolarization, which reversed between −40 and −50 mV. We have repeated here this type of experiment, using the voltage-clamp technique and perisomatic 1 mM GABA-CI application, to indirectly determine the ECl from reversal potential of the currents evoked over a range of command potentials. Neurons were maintained at variable holding levels for at least 210 s (long enough to reach a steady-state condition) before the first application of GABA; successive applications were performed every 30 s to minimize receptor desensitization (Dominguez-Perrot et al. 1996). Large currents were recorded with repeated pulse application of constant amounts of GABA, allowing the desired ECl to be evaluated. Moreover, a drastic effect of the holding potential appeared evident both on the intensity and the direction of flow of the currents, as illustrated in the typical recordings of Fig. 3A: when GABA was applied at −40 mV, for example, the response to GABA was an inward chloride current of relatively small intensity if the holding potential was also −40 mV, whereas a large and outward current followed the sojourn at −90 mV. This behavior was systematically observed. The results of these tests in three different neurons are summarized in Fig. 3B, in which ECl and the corresponding [Cl]i, are plotted against the steady-state holding potential over the −40/−120 mV range. The internal chloride concentration was confirmed to be higher than predicted by a passive distribution of Cl⁻, and this occurred at all voltage levels tested.

Figure 3 reflects the static chloride distribution in the sympathetic neuron, when voltage and ionic gradients are presumably unvarying over time. The complementary dynamic information is illustrated in Fig. 4, in which the effects of chloride readjustment during and after a voltage pulse are probed. If the transient currents of Fig. 1A were actually related to chloride leaving the cell during hyperpolarization and entering the cell during depolarization, as indicated by the data of Fig. 3B, then the GABA currents at a constant voltage should be affected by the chloride driving force smoothly changing between the initial and final values. This was actually detected when GABA was applied at different times (small letters of Fig. 4) during repeated −50/−90/−50-mV cycles. During the hyperpolarizing step the chloride currents evoked by GABA were inward and displayed a major decrease in amplitude while ECl was changing (traces a–d); conversely, on returning to −50 mV, Cl currents were initially outward, decreased in amplitude, and eventually reversed when [Cl]ᵢ had fully readjusted (traces e–h). From the data presented in Figs. 2, 3A, and 4, it would appear that the transient inward currents, the loss of internal chloride, and the shifts of ECl in the negative direction (vice versa during neuronal depolarization) are parallel aspects of the same process, namely the redistribution of chloride according
FIG. 3. Steady-state chloride distribution in the sympathetic neuron. A: responses to 0.1-s perisomatic pressure ejection of 1 mM GABA (■) to the same neuron maintained at −40, −70, and −90 mV holding potentials. The pulses are repeatedly applied every 30 s to the cell during de- and hyperpolarizing voltage jumps adequate to determine the GABA reversal potential pertinent to the actual holding level. Note the different intensity and flow direction of the chloride current at the same membrane potential as a function of the holding level. B: the $E_{Cl}$ values determined as shown in A in 3 different neurons are plotted against the holding level at which the GABA-current reversal potential was measured. Dashed line illustrates the [Cl$^-$]$_i$ steady-state values expected on the basis of Nernst’s equation.

FIG. 4. Chloride movements during voltage jumps. A −50/−90/−50-mV cycle was repeatedly applied to a neuron. During the current development, isolated GABA pulses (see Fig. 3A) were applied at different times in the course of the ON response (small letters a–d) or the OFF response (letters e–h). The corresponding GABA-evoked currents are shown in the panels labeled by the same letters. The variable intensity of the GABA currents, and their reversal during the OFF response at constant membrane potential, reflect the chloride redistribution during the voltage steps.
to the transmembrane potential, and are accompanied by time-dependent changes in input conductance.

Ionic and pharmacological properties of the transient currents

Discernible chloride currents are also evoked by depolarizing membrane shifts, even of small amplitude, and in the presence of other voltage-dependent ionic currents. This is illustrated in Fig. 5A, which shows the response recorded during a −50/−40/−50-mV cycle: the currents are virtually symmetrical and coherent with the direction of chloride flow. In this experiment, 170 mM bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA)-4K was dissolved in the K-acetate solution, which filled both intracellular microelectrodes, and diffused into the neuron, reaching an internal concentration sufficient to cancel the spike afterhyperpolarization. The fast Ca²⁺ chelator had no apparent effects on the transient ON-OFF currents. A more complex command sequence generated the tracing of Fig. 5Ab. A very long-lasting pulse to −30 mV was applied to a neuron from a holding potential of −50 mV: at this potential all the voltage-dependent currents described in this neuron start to be activated (Belluzzi and Sacchi 1991), and a large and persistent outward potassium current (I_KV plus the residual I_KCa) was actually recorded. At the command onset, however, the transient outward chloride-related component (96.5 nC total outward charge displacement, measured relative to the noninactivating fraction of potassium current) systematically appeared superimposed on the delayed current. The chloride OFF current was recorded in isolation on returning to

FIG. 5. Ionic properties of transient currents during depolarizing voltage steps. A: currents evoked by a −50/−40/−50-mV cycle (a) and by a −50/−30-mV voltage command with the return to the initial level through a sojourn at −40 mV (b). A transient current component is recognized, superimposed onto the noninactivating outward current evoked at −40 and −30 mV (arrows in b indicate the level of the leakage current, as evaluated from −50/−70-mV voltage pulses). In a, 170 mM bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA)-4K was diffusing intracellularly from the current and voltage microelectrodes. The transient charge displacement during depolarization (96.5 nC, measured in trace b relative to the steady-state current level) is balanced out by the inward charge during the 1st repolarization to −50 mV (64.5 nC); the same amount is returned later by repolarizing the cell through an intermediate step at −40 mV (48.8 nC recovered at −40 mV, plus additional 43.9 nC at the final −50 mV level). B: pure chloride currents are obtained as difference of currents generated by ramp commands (−50/+20 mV, relative to the holding potential; 200 ms duration) applied in control saline and after blocking the chloride channels with 0.5 mM 9AC (traces a–d) or after substituting 136 mM Na-methanesulfonate for an isoosmolar amount of NaCl (final [Cl⁻] = 18 mM; traces e–f). In a–c the holding level was −40 mV; the difference current tracing indicates a chloride reversal potential at −34 mV. With a holding potential at −110 mV (trace d), virtually no chloride current is observed during the ramp (the chloride channels are closed, see Fig. 9C). In a different neuron, the same ionic manipulation revealed an E_Cl value close to −44 mV (holding potential at −50 mV), becoming about −68 mV after shifting the steady-state membrane potential to −70 mV.
−50 mV (the potassium channels are rapidly closed by repolarization); at this potential 84.5 nC were returned as inward charge. A second long-lasting command to −30 mV was applied thereafter, and an intermediate step at −40 mV was applied before returning to the starting level. Chloride OFF currents were recorded at both levels; the charge that had moved during the ON response was recovered in a first fraction of 48.8 nC at −40 mV and a second fraction of 43.9 nC at the final level of −50 mV. As in the case of Fig. 2, the substitution of isethionate for chloride virtually canceled any transient ON-OFF response within this voltage range.

In further experiments the chloride current was isolated as the difference current between measurements performed during a voltage ramp (from −50 to +20 mV, relative to the holding level; 200 ms duration) in control solution and after specifically blocking the chloride channels or reducing the external chloride complement. In Fig. 5B, traces a–c, 0.5 mM 9AC was used as a chloride channel blocker in a neuron held at −40 mV; the dissected chloride current (trace c) exhibited a linear I-V relationship and reversed at about −34 mV, in agreement with the GABA experiments of Fig. 3B. In the same neuron, the chloride current was virtually canceled when the holding potential was −110 mV (the chloride channels are closed at this membrane potential; see Fig. 9C). In a different neuron, the isolation procedure was applied at −50 (trace e) and −70 mV (trace f) before and after substituting 136 mM Na-methanesulfonate for NaCl, thus reducing [Cl\(^−\)], to ~18 mM. The reversal potential of chloride currents displayed a strong dependence on the holding potential but was systematically a few millivolts less negative than the holding potential (+6 mV in trace e; +2 mV in f), in line with previous observations.

ICI was insensitive to modifications of [Ca\(^{2+}\)]\(_{e}\) in the 2- to 5-mM range, to external 0.5 mM cadmium-Cl\(_2\) (and to intracellular BAPTA application, see the first paragraph of this section) and to reduction in potassium or sodium concentration of the bathing medium. Similarly, it was unaffected by 5 mM external caesium-Cl and potassium channel blockers; this is illustrated in Fig. 7 (see also Fig. 8C), which shows the cumulative effects of the latter treatments: the chloride-related inward charge displacement within successive 10-mV steps of long duration from −40 to −80 mV was unaffected by reducing external sodium to 60% in the presence of 5 mM caesium and 50 mM TEA-Cl.

The chloride current in the sympathetic neuron was quite efficiently blocked by 0.5 mM 9AC (see Figs. 5B and 8B) or 0.3 mM DIDS. The block, as evaluated by the reduction in amplitude of the transient current, was usually complete with 9AC; in two of three neurons tested with DIDS, the block was complete, whereas in the third, \(I_{Cl}\) was reduced to 14.3%. Other drugs, such as 4 × 10\(^{−5}\) M ouabain, 20 μM bicusculine, and a benzodiazepine (2 × 10\(^{−7}\) M flunitrazepam-HCl), were devoid of any detectable effect.

Current variance during membrane potential migrations

Although the holding current had to be increased to hyperpolarize the neuron, the current variance was systematically reduced, and conversely the variance increased in depolarized conditions. This finding is consistent with the conductance measurements, indicating that voltage-dependent channels, opened by depolarization, sustain the recorded current.

When the neuron was progressively hyperpolarized from −50 to −100 mV through steps of 2-min duration, a decrease in current variance accompanied each hyperpolarizing step (from 0.075 nA\(^2\) at −50 mV to 0.014 nA\(^2\) at −100 mV), and small further reductions of the variance were often observed during each step, suggesting that channels were slowly deactivating during the procedure (Fig. 6).

In response to larger hyperpolarizing voltage steps (e.g., −50 to −90 mV) current variance systematically declined during the command; this was true even after correcting for the extra variance added by the slow drift in mean current (especially at early times during the command). Conversely, on depolarization the current variance slowly increased. Again, the observations are consistent with the conductance measurements and confirm that channel activation/deactivation contributes to the currents. As expected, isethionate substitution for chloride or the addition of DIDS markedly reduced current variance and abolished its dependence on voltage (typically, in a neuron exposed to isethionate, 0.0047 nA\(^2\) at −40 mV vs. a stable value of 0.0035 nA\(^2\) at −90 mV).

The current variance was in general too low to permit noise analysis to characterize single-channel behavior; indeed, the very low single-channel conductance reported for chloride channels (typically, 1 pS for skeletal muscle chloride channels, CIC-1) (Pusch et al. 1994) is known to heavily hamper such an approach.
**Ionic conductances active in the subthreshold membrane potential range**

The present data suggest that in the voltage range in which the neuron exhibits a behavior conventionally indicated as "passive," active conductances do exist, and these are controlled by membrane potential. The contribution by potassium, chloride, and leakage currents was thus systematically evaluated by isolating the individual components at different holding levels within the $-40/-120$-mV range. The neuron membrane potential was progressively moved in 10-mV steps, each level was maintained for 120–180 s before imposing an additional negative voltage gradient, and during this period the cell input conductance was measured as illustrated in Fig. 2 (the application rate of the test pulses was actually minimized, because this procedure might have been damaging to the neuron with high internal negativity). The results of these experiments provide the continuous tracings of the transmembrane current at different levels, represented in Fig. 8, and the voltage dependence of the steady-state values of cell input conductance, illustrated in Fig. 9. Control tracings reproduce a common pattern at each step (Fig. 8A), namely the expected inward transient current and the input conductance slowly decaying to the steady-state values pertinent to the imposed potential. The current transients were small because they now reflected limited fractional changes in $[\text{Cl}^-]_i$, but they were systematically evoked even at $-120$ mV. The total input conductance (mean values from 10 neurons; Fig. 9A) showed an unexpected behavior in that it progressively decreased with increasing negativity, after a small increase around $-60$ mV, and was down to 37% of its maximum value at $-120$ mV. Treatment with 9AC abolished the chloride current, leaving only the potassium plus leakage fractions in the tracings. A typical recording is shown in Fig. 8B: the squared behavior of the trace indicates that any transient component was actually canceled throughout. The mean steady-state conductance values ($n = 6$) exhibit a complex voltage dependence, consistently observed in each cell, namely a slight decrease at $-50$ mV, a peak value around $-80$ mV and a progressive decrease for higher polarizations with a minimum of 22 nS at $-120$ mV (Fig. 9B, ●). The biphasic nature of the curve is most likely due to the sum of at least two different conductances, because the addition of 5 mM

**FIG. 7. Chloride nature of the transient current component.**

A: currents recorded during hyperpolarizing voltage commands in successive 10-mV steps starting from an initial membrane potential of $-40$ mV; the neuron is bathed in normal saline (dotted trace) and in the presence of reduced external sodium (to 60%), 5 mM caesium-Cl and 50 mM TEA-Cl (solid line). B: the transient inward charge displacements measured in the 2 different media (from the integral of the current tracings relative to the late steady-state levels) are compared for each single voltage step.

**FIG. 8. Transient currents in different media.** Neurons are jumped to membrane potential levels of increasing negativity, in successive 10-mV steps, and membrane currents are continuously recorded in control saline (A), in the presence of 0.5 mM 9AC to block the chloride current component (B) and in the presence of 50 mM TEA-Cl plus 5 mM caesium-Cl to block the potassium current fraction (C); the previous treatments, 9AC + TEA + Cs, are cumulatively applied in D.
caesium to the bath generated a monotonic decay of steady-state conductance, by apparently subtracting a consistent fraction of the residual potassium conductance in the $-50/-120$-mV region (Fig. 9B, ○). Caesium is usually considered to be a specific blocker of the inward rectifier current in this range of membrane potential, but this current is hardly detected in the rat sympathetic neuron (and is unknown to inactivate with increasing negativity). Contributions to the total potassium conductance could arise from $g_{KV}$, $g_{KCa}$, and $g_A$. In simulations from a five-conductance model of the rat sympathetic neuron (Belluzzi and Sacchi 1991), a cumulative potassium conductance of 9.1 nS was calculated at $-50$ mV; it is questionable, however, whether such computations, based on extrapolation of curves fitting experimental data obtained at less negative potentials, might reflect physiological behavior.

Single calcium-activated potassium channels have been shown in cultured rat sympathetic neurons to be active at negative potentials ($-45/-67$ mV), although with a low $P_{open}$ (Smart 1987). Similarly, M-type channels may open, but only at membrane voltages positive to $-60$ mV (Stansfeld et al. 1993). The precise characterization of K currents at negative potentials, however, was not the aim of this study and was not further pursued.

To block all potassium conductances, 50 mM TEA plus 5 mM caesium were applied to the bath (Fig. 8C). The current tracing consistently showed the typical transient chloride currents at each voltage step, whereas the cell input conductance smoothly decayed with voltage toward a final value of 20 nS at $-120$ mV (Fig. 9C; $n = 5$). Finally, the blockers of both potassium and chloride channels were cumulatively applied,
with the result that the current amplitude became virtually insensitive to the membrane potential (Fig. 8D); the cell input conductance also remained clamped around a constant value of 11–15 nS (Fig. 9D; \( n = 3 \)). The latter values thus represent a pedestal, independent of membrane potential, which reflects the true passive behavior of the neuron, onto which voltage-dependent conductances are superimposed. Once this background is subtracted, the values of Fig. 9B apply to the voltage-dependent potassium conductance, whereas those of Fig. 9C describe the chloride conductance. Both conductances exhibit a strong and selective voltage dependence, so that their ratio continuously varies with membrane potential (\( g_{\text{Cl}}/g_{\text{K}} = 2.72 \) at \(-50\) mV, 0.75 at \(-70\) mV and 0.35 at \(-90\) mV); they tend, however, to decrease with increasing internal negativity, vanishing at \(-120\) mV, where total conductance virtually coincides with that of the leakage component. This conclusion is confirmed by difference currents recorded during voltage ramps. The well-defined chloride current dissected in Fig. 5Bc at \(-40\) mV in the presence and absence of 9AC is actually canceled when the procedure is repeated at \(-110\) mV (Fig. 5Bd).

The application of Goldman’s equation to the relation \([\text{Cl}^-]_i\) versus membrane potential (see Fig. 5B) indicates that steady-state \([\text{Cl}^-]_i\), only accounts for part of the change in steady-state chloride conductance, \(g_{\text{Cl}}\) (Fig. 9C), over the \(-40/-120\) mV range, and does not predict the observed shape for the \(g_{\text{Cl}}\) versus membrane potential relation. Thus channel activation-deactivation must also be involved.

The curves of Fig. 9, A–C, were obtained independently from different cell groups, so that the values are not readily comparable; nevertheless, the summated \(g_{\text{K}}\) and \(g_{\text{Cl}}\) from Fig. 9B and C account for 92–99% of the average total input conductance in control neurons (A) in the voltage range \(-50/-90\) mV; the recovery is less accurate above and below this region. The chloride conductance-voltage relationship in the subthreshold region was described by the following Boltzmann-type equation (\(V = \) membrane potential in mV)

\[
g_{\text{Cl}} = 102.7 \left[1 + \exp\left[1.47 \cdot (-63.65 - V) \cdot (kT)^{-1}\right]\right]^{-1} \text{nS} \tag{1}
\]

whereas the compound potassium conductance-voltage relationship was fitted by the empirical equation

\[
g_{\text{K}} = 113.17 \left[\exp\left[-1.61 \cdot (-79.41 - V) \cdot (kT)^{-1}\right]\right. + \exp\left[1.61 \cdot (-79.41 - V) \cdot (kT)^{-1}\right]^{-1} + 612.42 \left[1 + \exp\left[3.35 \cdot (-18.20 - V) \cdot (kT)^{-1}\right]\right]^{-1} \text{nS} \tag{2}
\]

The block of a conductance actively contributing to the neuron steady state is expected to modify the holding current by an amount proportional to the canceled conductance. This was verified in the experiments: when isethionate was substituted for chloride, a mean outward current of 0.43 nA (\( n = 11 \)) was measured in neurons held at \(-50\) mV, suggesting that a current of equal intensity and opposite sign was being blocked by the treatment. This would correspond, with the uncertainties related to a precise evaluation of \(E_{\text{Cl}}\), to the subtraction to the system of \(-86\) nS of chloride conductance. In the 9AC experiments (\( n = 8 \)) the mean current at \(-50\) mV was \(+0.47\) nA, in good agreement with previous data and the \(g_{\text{Cl}}\) values of Fig. 9C. When the potassium channel blockers were used, an inward current of \(1.52\) nA (mean value of 4 observations) was conversely observed at a holding potential of \(-40\) mV, suggesting that the suppressed \(g_{\text{K}}\) was \(-29\) nS.

Cell conductances were measured also in three neurons maintained at \(22^\circ\)C. The cell input conductance values proved to be scaled by a constant factor over the whole membrane potential region tested. The temperature sensitivity coefficient \(Q_{10}\) was 1.55, a value typical of diffusive processes, suggesting that open channel probabilities are temperature independent.

**Model for the neuronal subthreshold behavior**

The conductances here characterized in the subthreshold range of membrane potential were forced in a mathematical model of the simplified electrical circuit shown in Fig. 10A. Cell capacitance and steady current contributions by active ionic pumps were neglected, in simulating the time courses of the slowly evolving currents of interest.

The leakage conductance value, \(g_{\text{L}}\), was considered to be voltage independent and constant at \(14\) nS, whereas the values of potassium and chloride conductances are voltage dependent. Potassium channel kinetics are sufficiently fast to make their precise evaluation irrelevant to the present purposes (activation and deactivation of potassium currents are not discernible in Figs. 1B and 2, for example), so a conventional, voltage-independent value of \(\tau = 4\) ms was used in the computations for potassium channel gating time constant, in line with the \(\tau_{\text{K}}\) of \(I_{\text{K}}\) at the membrane potentials here considered (Belluzzi and Sacchi 1991). Thus the compound potassium conductance, \(g_{\text{K}}\), is assumed to rapidly settle to the values predicted by Eq. 2. Chloride conductance, \(g_{\text{Cl}}\), is considered to relax toward its steady-state value, predicted by Eq. 1, with a time constant, \(\tau_{\text{Cl}}\), influenced by voltage according to the equation best fitting the data of Fig. 1D, which presumably describes the voltage sensitivity of \(I_{\text{Cl}}\) gating time constant: \(\tau = 253.55 \exp(0.04 V) + 11.47\) ms (V in mV).

The two batteries in the circuit represent the electromotive forces generated by the Nernstian equilibrium potentials for potassium (\(E_{\text{K}}\), assumed constant at \(-90\) mV) and chloride (\(E_{\text{Cl}}\)). The steady-state value of \(E_{\text{Cl}}\) is expected to vary with voltage according to the linear equation best fitting data points of Fig. 3B: \(E_{\text{Cl}} = 0.6 - V_{\text{h}} - 13.82\) mV (\(V_{\text{h}}\) = holding potential in mV). However, its detailed time course must be computed from time-dependent changes in \([\text{Cl}^-]_i\), during chloride redistribution. At the beginning of transients, \([\text{Cl}^-]_i\), can be estimated from the steady-state values of \(E_{\text{Cl}}\); subsequently, changes in \([\text{Cl}^-]_i\), are determined by \(\text{Cl}^-\) movements and the cell volume; thus the latter can be estimated from the ratio between total \(\text{Cl}^-\) charge transfer and the change in steady-state \([\text{Cl}^-]_i\), on displacing membrane potential. Once the cell volume is known, then \([\text{Cl}^-]_i\), can be updated in the mathematical model during the transient, based on the momentary chloride current, and employed to update \(E_{\text{Cl}}\) in turn. Total charge transfers from the various experiments illustrated in Figs. 8 and 9 point to cell volumes ranging between 30 and 60 pl (i.e., the volume of spherical neurons with diameter between 38 and 48 \(\mu\)m). Such values for cell volume, which are in reasonable agreement with the size of the neurons, as seen under the microscope, would result in time constants in the order of 30–100 s (i.e., within a range very similar to the time constants of \(g_{\text{Cl}}\) changes) for the chloride redistribution process, depend-
ing on chloride conductance and the amplitude of the voltage step.

A noninactivating chloride current is generated, in this model, at membrane potentials positive to $-120$ mV; its transient behavior during voltage jumps is not related to inactivation, but simply reflects the slow process of channel activation/deactivation and the similarly slow driving-force shifts that accompany chloride redistribution. Figure 10Ba illustrates the responses of the model neuron to commands in the subthreshold voltage range typically used in Figs. 1A and 5A. The amplitude and time course of the computed current tracings, as well as the nonlinear voltage dependence of the late component, favorably compare with the experimental data, suggesting that the equivalent circuit in Fig. 10A is adequate to provide a correct description of the ganglion cell membrane in the subthreshold region, and that the simulation of chloride redistribution yields valid time-varying estimates of $E_{Cl}$ and $[Cl^-]_i$, during the simulation of current transients (Fig. 10B, trace b). Figure 10C shows a plot of the mean values of the steady-state potassium and chloride currents operating at different membrane potentials, together with their standard errors, as estimated from the variability of experimental measurements of the single conductances. Although these are not the only currents operating in the neuron “at rest” (at least leakage and pump current must be considered in addition), they suggest that the potassium outward current component dominates for membrane potential values positive to $-80$ mV, at steady state. The crucial aspect, however, is that when the membrane potential is displaced (by sustained activation of the synaptic input, for example), the currents will depart from this static picture for up to 1–2 min, and chloride conductance and redistribution will determine the momentary values of membrane potential and net current flow.

The charge transfers predicted by the model in response to successive 10-mV voltage steps from $-40$ to $-120$ mV, for a cell volume of 30 pl, are compared in Fig. 10D (●) to the values measured using the same protocol in the experiment illustrated in Fig. 8C. The good fit between the two sets of data
is further evidence in support of the present mode of correlating observed transient currents and transmembrane chloride movements.

**Discussion**

The central findings presented in this paper describe the transmembrane movements of chloride during membrane potential excursions in the subthreshold range, and the participation of a chloride conductance in the genesis and control of the actual membrane potential of the neuron at rest. We demonstrate that discernible transient currents are associated with membrane potential modifications in either directions within the \(-30/-130\) mV voltage range and that the direction and size of these currents are coherent with the chloride movements during the voltage migrations. Further arguments in favor of the chloride nature of the current transients include the following: 1) the current reversal potential is coincident with the momentary chloride equilibrium potential and is influenced by the \(E_{\text{Cl}}\) shifts related to membrane potential; 2) the current is blocked by impermanent ions substituting for external chloride and by treatments, such as 9AC and DIDS, known to block chloride channels in other systems; 3) it is unaffected by reduced external sodium concentration and blockers of the potassium channels; moreover, 4) there is a good correlation between measured charge movements and the accompanying modifications of chloride concentration.

Previous work has strongly suggested that the intracellular chloride concentration in the mammalian sympathetic neuron is higher than that predicted by a passive Nernst distribution, and evidence has been provided for the existence of an active electroneutral mechanism sustaining the inward flux of chloride that maintains the ion unbalanced; in the present paper we demonstrate that this holds over a wide range of membrane potential, so that at steady-state the chloride battery sustains an inward current at any membrane potential level. Membrane potential proves to be the main controller of the intracellular inward current at any membrane potential level. Membrane potential, but not the total amount of charge transfer during a voltage step, controls the duration of the transients: \(I_{\text{Cl}}\) time course becomes faster with increasing internal negativity, suggesting that, although the kinetics of the channel are very slow, channel closure rate constant depends on membrane potential. The simple model discussed above suggests that the shifts of the actual intracellular chloride concentration follow time courses similar to the changes in conductance and that the kinetics of the two processes fully account for the time course of the transient currents.

The basic properties of the chloride current here described markedly differ from those of the currents referred to in the introduction as hyperpolarization-activated inward rectifier current and calcium-activated current. The conductance underlying the former is activated, and not shut off, by increased membrane negativity; the charge movements are not symmetrical, and the pharmacological profiles are also different. As regards the calcium-activated current, calcium transmembrane movements are expected to be negligible, if ever present, in the membrane potential range here considered; moreover, cadmium treatment and the addition of BAPTA to the solution filling the microelectrodes proved ineffective in our experiments. Some functional similarities do exist, on the contrary, with the well-known chloride current present in skeletal muscle (Palade and Barchi 1977). The high resting chloride conductance, its activation with depolarization, and, conversely, its decrease on hyperpolarization are in fact reminiscent of the behavior of the ClC-1 channels. The chloride conductance that has been observed in hippocampal pyramidal neurons shares the properties of the cloned and functionally expressed chloride channel ClC-2 (Smith et al. 1995). Recently, mRNA for ClC-2 has been shown to be present in rat superior cervical ganglia as well and may be functionally expressed (Clark et al. 1998); the chloride current described in that paper, coherently, exhibited the properties of a typical hyperpolarization-activated current. ClC-1 is considered to be muscle specific; its presence also in ganglia, if it were detected, would be consistent with the properties of the chloride current determined here by electrophysiological techniques.

The resting condition of the mammalian sympathetic neuron is often interpreted as the result of potassium and leakage
current flow, each of them displaying a defined equilibrium potential and remaining sufficiently constant over time and independent of membrane potential to confer stable passive properties to the neuronal behavior at membrane potentials negative to some $-50 \text{ mV}$. The nature of both conductances, however, is ill-defined because the contribution of each of at least five different potassium conductances coexisting in the soma ($g_{KV}$, $g_{KCa}$, $g_{AHP}$, $g_{M}$) are not specified, and similarly the physical nature of the leakage current is poorly understood in terms of its ionic carrier$(s)$ and equilibrium potential. In an accurate study on frog sympathetic neurons, Jones (1989) demonstrated that the resting potential primarily takes origin from a voltage-insensitive potassium current, not $I_M$ or $I_O$ (the inwardly rectifying current), whereas in cultured parasympathetic neurons from rat intracardiac ganglia the resting potential appeared to be set by a voltage-insensitive leakage current and a voltage-dependent potassium current of the delayed rectifier type (Xu and Adams 1992). These data provide a rather rigid view of the resting potential, which would appear to be virtually a constant among the multiple neuronal electrical variables and would be predetermined by factors insensitive to voltage and time, and potentially modified only by shifts in the potassium equilibrium potential.

A different picture arises from the present findings in which the individual potassium, chloride, and leakage conductances have been isolated and measured over a wide membrane potential range. The values of each conductance and the ratios between them are continuously modified by membrane potential, and active conductances only vanish at membrane potential levels negative to $-120 \text{ mV}$; here only will the neuron exhibit a truly passive behavior. At any membrane potential level positive to $E_K$, the holding current in the experiments is built up by the sum of three currents: one of them (the leakage) possibly reflects the only true passive properties of the neuronal geometry; the second is represented by the outward voltage-dependent potassium current, fed by a virtually constant potassium battery; the third component is the chloride current, which is obligatorily inward at steady-state, as the consequence of the chloride internal concentration being maintained higher than at equilibrium, and is generated by a voltage-dependent chloride battery that produces a constantly small driving force. When the holding potential moves, the system is perturbed with the result that the potassium conductance attains, presumably within milliseconds, the value appropriate to the new potential, whereas the chloride current relaxes to the new steady-state value with a time constant of tens of seconds. Thus in addition to leakage current and potassium conductances, the electrical properties of the neuron at “rest” are modified by previously undetected voltage-dependent and slowly time-varying chloride conductances and currents, at membrane potentials positive to $-120 \text{ mV}$.

As opposed to the classical view of a voltage-insensitive potassium conductance and a stable potassium battery, which are expected to clamp the membrane at a fixed level, much more flexible is the situation in which both chloride conductance and chloride battery are voltage dependent. The previous activity of the neuron will influence the momentary status of the chloride battery and this, like any event acting on the chloride ionic gradient, is expected to modify in turn the resting potential of the neuron and its excitability. The chloride conductance may also be specifically regulated; in cortical astrocytes, for example, it has been shown to depend on intracellular calcium and ATP concentration and more generally on the metabolic activity of the brain (Lascola and Kraig 1996). Membrane potential thus appears to constitute a dynamic link between previous history (and chloride regulation) and momentary excitability of the neuron, rather than a predetermined electrical status. In this perspective, the demonstration of a mechanism capable of sustaining a variable resting potential in the sympathetic neuron helps appreciating the functional significance of many previous findings. In fact, in earlier studies we recurrently observed that the initial membrane potential level is a crucial parameter in the cell firing strategy. It appears that membrane potential controls the mix of different channel types available on the cell to generate different firing patterns, and that this effect is so strong, for example, to completely subvert the repolarization of the action potential: this process, in fact, depends on the delayed rectifier current, $I_{KV}$, and the calcium-dependent current, $I_{KCa}$, when the neuron is depolarized at rest, but these conductances are completely substituted for by the transient potassium current, $I_{AHP}$, when the $I_{AHP}$ inactivation is removed by increased internal negativity ($\approx 50 \text{ mV}$) (Belluzzi and Sacchi 1991). Even more relevant under a functional perspective, the spike discharge following synaptic activation is controlled by the underlying $I_A$, which antagonizes the synaptic current flow in the subthreshold range. The practical result is that the threshold synaptic conductance (and thus the degree of activation of the synaptic input which is necessary to elicit postsynaptic firing) increases with increasing membrane negativity, mirroring the voltage dependence of $I_A$ inactivation removal (Sacchi et al. 1998). The identification of an active ionic mechanism, such as the chloride conductance here characterized, capable of controlling the basal membrane potential of the active neuron, yields new intriguing hints about how the neuron may modify its excitability properties under different functional conditions.

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