A VOLTAGE- AND ACTIVITY-DEPENDENT CHLORIDE CONDUCTANCE CONTROLS THE RESTING STATUS OF THE INTACT RAT SYMPATHETIC NEURON.

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Running head: Chloride conductance in the rat sympathetic neuron

SUMMARY

Remarkable activity-dependence was uncovered in the chloride conductance that operates in the subthreshold region of membrane potential, by using the twomicroelectrode voltage-clamp technique in the mature and intact rat sympathetic neuron. Both direct and synaptic neuron tetanization (15 Hz, 10 s duration to saturate the response) resulted in a long-lasting (not less than 15 min) increase of cell input conductance (+70-150% 10 min after tetanus), accompanied by the onset of an inward current with the same time course. Both processes developed with similar properties in the postganglionic neuron when presynaptic stimulation was performed under current- or voltage-clamp conditions, and were unaffected by external calcium upon direct stimulation. The posttetanic effects were sustained by gCl increase since both conductance and current modifications were blocked by 0.5 mM 9AC (a chloride channel blocker) but were unaffected by TEACl or cesium chloride treatments. The chloride channel properties were modified by stimulation: their voltage sensitivity and rate of closure in response to hyperpolarization strongly increased. The voltage dependence of the three major conductances governing the cell subthreshold status (gCl, gK and gL) was evaluated over the -40/-110 mV membrane potential range in unstimulated neurons and compared with previous results in stimulated neurons. A drastic difference between the voltage-conductance profiles was observed, exclusively sustained by gCl increase. The chloride channel thus hosts an intrinsic mechanism, a memory of previous neuron activity, which makes the chloride current a likely candidate for natural controller of the balance between opposite resting currents, and thus of membrane potential level.

INTRODUCTION

Most of the voltage-dependent variables controlling excitable membrane properties exhibit their peak sensitivity to voltage in the membrane potential range which is indicated, more or less appropriately, as ‘resting’. We in fact recently demonstrated (Sacchi et al. 1999) that the subthreshold behavior of the rat sympathetic neuron is actually determined by a mix of at least three ionic components: a potassium conductance (most likely the sum of two distinct conductance fractions) sensitive to TEA and Cs treatments; a chloride component blocked by typical chloride channels blockers and cancelled by substituting isethionate for chloride ions, and a leakage component. The first two conductances exhibit clear-cut voltage-dependent properties over the membrane potential range commonly considered as passive; a stable passive behavior is actually obtained only with very high internal negativity (about -120 mV) when the active conductances vanish and only the voltage-independent leakage conductance survives. Moreover, the chloride conductance generates a current that depends not only on the momentary chloride conductance value but also on transmembrane chloride ion distribution, which continuously varies as the membrane potential of the neuron is artificially displaced. It was suggested that if voltage steps are able to modify chloride conductance and internal chloride ion distribution, the opposite might also hold true, namely that modifications in chloride conductance will potentially influence membrane potential values and thus neuronal excitability and responsiveness. The dynamic interplay, in the subthreshold potential range, between voltage-dependent conductances and ion distribution would provide the neuron with an intrinsic mechanism capable of continuously controlling its resting membrane potential. Thus, the latter would no longer represent a predetermined electrical status, set by basal and constant ionic and leakage conductances, and their respective stationary equilibrium potentials (as in common models of neuronal resting potential: Forsythe and Redman 1988; Jones 1989; Xu and Adams 1992; Lamas et al. 2002); the neuron would instead be able to adjust its resting and active properties to the continuously modifying conditions under which it is expected to operate.

In the present study we demonstrate that this mechanism does exist in the intact and mature rat sympathetic neuron, when the internal ionic medium is not clamped but is free to readjust to the new voltage gradients. The chloride conductance exhibits not only the previously demonstrated voltage dependence over the potential range -40/-120 mV, but also a novel activity...
dependence, as it strongly increases following even moderate neuronal activity, while the potassium conductance(s) is virtually unaffected. The cumulative conductance profiles over voltage of the neuron at rest or after activity are, therefore, profoundly different, and these differences are sustained by chloride conductance modifications. We are still unable to define the actual trigger for this chloride conductance increase, but its involvement in neuronal behavior is expected to be crucial, as indicated by the unbalance in the holding current under voltage-clamp conditions, and by the ensuing modifications in neuronal membrane potential when the latter is free to move. On the other hand, sympathetic ganglia have represented a useful tool for the analysis of posttetanic effects under current-clamp conditions, although confusing and conflicting results were sometimes obtained; see, for example, reviews by Katayama and Nishi, 1986, and Tokimasa and Akasu, 1995.

The present study represents the first attempt to quantitatively evaluate and dissect the individual conductances underlying the 'resting' status of the rat sympathetic neuron, and the respective modifications following physiological activity. It partially answers the starting observation of how small changes in membrane potential can be amplified by the steepness of the conductance-voltage relationship of the different currents activatable in the neuron, and how this mechanism might be of physiological interest. In fact, they help appreciating the functional significance of previous recurrent observations, highlighting the importance of the basal membrane potential level as a crucial parameter in defining the mix of voltage-dependent and synaptic channel types used by the neuron in its firing strategy (Belluzzi and Sacchi 1988; Sacchi et al. 1998).

METHODS

Experiments were performed on superior cervical ganglia freshly dissected from young rats (120-150 g body weight; either sex) during urethan anesthesia (1 - 1.5 g kg⁻¹ ip) and maintained in vitro at 37°C. The body weight; either sex) during urethan anesthesia (1 - 0.7 g kg⁻¹ ip) and maintained in vitro at 37°C. Occasionally, when the stability of the long-lasting impalement proved not to be compromised, the more physiological 2.2 mM Ca²⁺ concentration was applied. The bath was grounded through an agar-3 M KCl bridge.

Synaptic stimulation of the neuron was obtained by applying single current pulses (0.3 ms duration) of variable strength to the cervical sympathetic trunk, which contains the entire preganglionic input to the neuronal population. Stimuli were also applied in trains of 15 Hz, 10 s duration. Direct stimulation under current-clamp conditions was obtained by applying current pulses of 3-5 ms duration and 2-7 nA intensity to the neuron through the current electrode, making minor intensity adjustments during tetanus to maintain them suprathreshold.

When TEACl (tetrathylammonium chloride, Sigma) was used, appropriate amounts of NaCl were removed from the standard saline composition to maintain isoosmolarity. 9AC (anthracene-9-carboxylic acid, Sigma) was dissolved in ethanol at a final 0.8% concentration and bath applied by means of a continuous and rapid superfusion system. When Cd²⁺ was used, the initial bathing medium was switched to a phosphate- and bicarbonate-free solution, buffered with 15 mM Tris-HCl. The instantaneous cell input conductance was evaluated by applying voltage steps of ±40 mV amplitude and 10 ms duration to the neuron held under voltage-clamp conditions at membrane potential levels in the -40/-120 mV voltage range. Alternatively, voltage ramp commands -50/+40 mV (relative to the different holding potentials) and 200 ms duration were used with similar results. It was previously reported that the membrane chord conductance in the sympathetic neuron displays neither fast voltage sensitivity nor instantaneous rectification at membrane potential values negative to -50 mV, and the overall stability of the preparation under two-microelectrode impalement has been proved (Sacchi et al. 1999).

Attention was systematically paid to avoid any spike discharge from each neuron tested before tetanus application (see below). This was relevant in the case of synaptic stimulation, which is obligatorily applied to the preganglionic sympathetic trunk and thus involves activation of the whole neuron population. A practical consequence was that only one single neuron could be tested per ganglion.

Long-lasting recordings 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.

RESULTS

The effect of activity on cell input conductance

More than one hundred rat sympathetic neurons were analyzed over periods of at least 12 min, utilizing the two-microelectrode voltage-clamp technique to measure
their cell input conductance. Figure 1 shows the effects of a conditioning tetanus (15 Hz, 10 s duration) on neurons stimulated either directly through the current microelectrode (A; n = 8) or synaptically via the preganglionic fibers (B; n = 7). Tetanization was performed under current-clamp conditions; spikes were discharged by each of the synaptically applied stimuli, and in 70-100% of the trials when direct stimulation was used. In the same neurons the accompanying modifications in the holding current values (relative to the prestimulus condition, dashed lines) were evaluated and pooled in C. Bars indicate the SE of mean conductance and current values. It is evident that stimulation is followed by a relevant posttetanic increase in the overall cell input conductance (a mean increase of +71.2% after 10 min in the directly stimulated neurons, n = 8, vs. +115.8 in the case of synaptic stimulation, n = 7), paralleled by the onset of an inward current (see, for example, the initial part of Fig. 5, A-B). Despite the large variability from neuron to neuron, we present the absolute conductance and current values without any attempt to correct them for cell dimension. However, this general description qualitatively applies to each single neuron tested here. The effects were long-lasting; they were followed systematically up to 10 min, and occasionally even longer. In isolated observations the conductance increase vanished within 15 min after the tetanus, while in other neurons it was still evident after 30 min. The mode of stimulation apparently was irrelevant in determining the time course of the conductance increase and current modifications (current data have been pooled). The magnitude of conductance change was slightly greater after synaptic stimulation; in addition, an early and transient conductance increase was observed in some neurons (prominent in 3/7 neurons) during the first minute following synaptic - but not direct- stimulation. This effect is most likely related to the short-lived activation of a postsynaptic chloride conductance, exclusively mediated by stimulation of the neuronal nicotinic receptors (gADP, see Sacchi et al. 2000). In preliminary tests, direct stimulation at 15 Hz for 400 ms (7 spikes discharged) was able to evoke a slight increase in conductance in 2/4 neurons, while synaptic tetanus of the same intensity was ineffective in two other neurons. Ten spikes elicited by direct stimulation at 1 Hz were similarly sufficient to induce a detectable raise in conductance. The threshold for conductance increase was thus far below the standard intensity used in these experiments and the final effect on conductance obtained with increasing neuron activation was similarly graded. The standard 10 s tetanization was supramaximal to evoke peak conductance activation, since any subsequent tetanization was unable to generate additional effects (see below). Therefore, we preferred to saturate the response in order to minimize any stimulation pattern-related variability.

The role of spikes and calcium entry on cell input conductance increase

In the preceding experiments neurons discharged spikes throughout the entire stimulation time. This raises the question of whether spiking is a prerequisite for generating the observed input conductance increase. In a group of experiments, illustrated in Fig. 2A, the synaptic stimulation was applied under voltage-clamp conditions at a constant -50 mV holding potential. Preganglionic stimulation evoked in the neurons EPSCs of 20-32 nA initial current amplitude, which gradually declined during the train. The ensuing posttetanic effects on cell input conductance were absolutely similar to those observed when synaptic stimulation was
performed under current-clamp conditions, both as concerns the short-lived initial transient increase and the final value at 10 min (a mean increase of +107.3%, n = 5). In one of these experiments the +74.1% posttetanic conductance increase at 10 min vanished within another 5 min, with a rebound to -19% of the pretetanic value at 25 min after stimulation.

Calcium ions enter the sympathetic neuron very effectively through voltage-dependent channels during each spike and less efficiently through the nicotinic synaptic channels during the synaptic current. From simulations in the ideal rat sympathetic neuron, in fact, the amount of calcium charge entering the neuron during a single spike arising from a -70 mV basal membrane potential is about 11 pC, while the amount of calcium contaminating a typical ganglionic EPSC, evoked under voltage-clamp conditions at the same holding potential, is estimated to be 2-3 pC (Sacchi et al. 2000). The possible involvement of internal calcium increase in sustaining the rise in posttetanic input conductance was examined in experiments in which the transmembrane calcium movements during spikes evoked by standard direct stimulation were blocked by 10µM nifedipine + 0.5mM CdCl₂ (Fig. 2B). In further experiments, the cell conductance increase, once induced in the neuron, proved to be insensitive to subsequent modifications in external calcium concentration in the 2-5 mM range (not shown). These results suggest that the process is external calcium-independent.

In these voltage-clamp experiments trains of negative voltage commands (-40 mV of 10 ms duration) were unable to generate any conductance modification, and similarly ineffective were hyperpolarizing current pulses of the same intensity and frequency as those used to directly stimulate neurons under current-clamp conditions. These observations would rule out any effect on cell conductance due per se to artificial current application.

The effects of stimulation persist in the ganglion and do not summate

The two very different stimulation modalities, both resulting in cell input conductance increase, most likely converge on a common cellular mechanism, since their effects do not summate when successively applied to the same neuron with supramaximal protocols. Different stimulation patterns were used in single neurons, by applying direct or synaptic stimulation under either current- or voltage-clamp conditions in variable sequences (an example is illustrated in Fig. 3A); systematically, the rise in conductance, once fully developed, was completely insensitive to any form of additional stimulation.

In principle, the possibility exists that the observed conductance increase might be mediated by experimental handling of the neuron, which hosts two microelectrodes and is exposed to unphysiological current applications. A second point is relevant from a functional point of view, namely whether the synchronous massive synaptic stimulation of a group of neurons is actually able to induce widespread modifications in the membrane properties of all the involved neurons, sufficiently stable and long-lasting to be ascertained at random in time after stimulation. This was verified by applying a preliminary standard tetanus to the sympathetic trunk (the unique input which feeds synaptically the whole ganglion neuronal population); thereafter, single neurons were tested by repeating the synaptic stimulation procedure within a few minutes. The results of these experiments are shown in Fig. 3, B-C, in which the time course of input conductance and holding current modifications due to the second tetanus is illustrated. It is evident that the second synaptic stimulation becomes ineffective even in neurons not exposed to preliminary manipulations; in these it was actually possible to ascertain the persistence of the effects of the first tetanus on conductance, at various time intervals after its application. The time lapse...
between the first (neuron not impaled) and second stimulation (neuron impaled) was obviously crucial, since the conductance increase is long-lasting but reversible. In a fortunate example, entirely performed in normal 2 mM \([\text{Ca}^{2+}]_o\), the recovery from the first stimulation effects could be followed by analysing the response to direct stimulation in three distinct neurons at increasing times from the conditioning tetanus. In the first neuron the second stimulation was ineffective 18 min after the first tetanus; the second displayed a modest conductance increase (a maximum of +21%) at 30 min, while the third exhibited a strong recovery of the response (+67.1%) after an hour.

The chloride conductance sustains the cell input conductance increase

Dissection of the conductances underlying the resting status of the sympathetic neuron is based on pharmacological and ion substitution experiments (Sacchi et al. 1999). Treatment with K channel blockers (20 - 50 mM TEACl + 5 mM CsCl) is expected to leave the chloride conductance (plus the constant leakage

![Figure 3](image)

**Figure 3.** A, effect of successive standard tetani (first arrow: synaptic stimulation under voltage-clamp conditions; second arrow: synaptic stimulation under current-clamp conditions; third arrow: direct stimulation under current-clamp conditions) on cell input conductance of the same neuron held at -50 mV holding potential under voltage-clamp conditions. B, effect of a second synaptic tetanic stimulation (current-clamp conditions, \(n = 6\); arrow) on input conductance of neurons which had been previously conditioned by a first synaptic tetanus while not impaled by the two microelectrodes. Holding potential was -50 mV, under voltage-clamp conditions. C, the accompanying modifications of the holding current, relative to the prestimulus level, are illustrated for the same neurons. Bars indicate the SE of mean conductance and current values.

![Figure 4](image)

**Figure 4.** A, effect of the standard tetanus (arrow) on input conductance of neurons exposed to 0.5 mM 9AC under voltage-clamp conditions (pooled data from directly, \(n = 4\), or synaptically stimulated neurons, \(n = 4\)). B, modifications in the holding current values, relative to the prestimulus level, observed in the same neuron pool as in A. C, effect of direct stimulation (20-25 current pulses successively applied under current-clamp conditions and evoking spikes of 20-120 ms duration, \(n = 8\); arrow) on cell input conductance measured in neurons exposed to 50 mM TEACl + 5 mM CsCl. Voltage-clamp conditions; holding potential was -50 mV. Bars indicate the SE of mean conductance and current values.
conductance) unaffected, while treatment with chloride channel blockers, or substitution of isethionate for chloride ions, isolates the compound potassium component. This protocol was used to evaluate the relative participation of the active conductances in the posttetanic effects. Fig. 4, A–B, illustrates the effects of 0.5 mM 9AC, a chloride channel blocker, on the posttetanic conductance and holding current response in neurons stimulated either directly (n = 4) or synaptically (n = 4). The results in the two subgroups were similar so that conductance and current data were pooled. It is evident that the large posttetanic conductance increase, typical of the normal neuron, and the associated inward shift of the holding current, were replaced by a small decrease in conductance and the onset of an outward current component (compare with Fig. 1 A and C). This suggests that the posttetanic effects are sustained in the untreated neuron by a chloride conductance increase (but does not still rule out the involvement of the potassium conductance; see however the next section).

Treatment with 9AC was able not only to prevent the posttetanic conductance increase, but also to reverse it once it had developed in normal saline. This was systematically observed when cell conductance was activated by neuron stimulation; an example is illustrated in Fig. 5, A–B, in which both the stimulation-related raise in cell conductance and the holding current inward shifts are not only efficiently cancelled by 9AC application, but also reversed to decreased conductance, compared to the initial value in control saline, and outward current.

The complementary demonstration that the posttetanic conductance increase is actually sustained by independent components, and thus develops also when potassium channels are blocked (TEA + Cs), was complicated by the fact that under these conditions the neuronal spike duration is 30-120 ms and synaptic stimulation proves unpractical. The standard tetanization was therefore replaced by the discharge of 15-20 long-lasting, directly evoked spikes that proved to generate a conductance increase very similar in time course and relative magnitude (the potassium component is now cancelled) to that of normal neurons (Fig. 4 C; n = 8).

The effect of stimulation on conductance voltage dependence

The conductance values discussed so far were measured at constant membrane potential levels. As chloride distribution across the membrane depends on membrane potential, in order to assess the voltage-dependence of chloride conductance, the latter must be measured at steady-state, after the cell has adapted its chloride content to the new membrane potential level (see below).

We previously demonstrated that the steady-state values of potassium and chloride conductances, and the ratios between them, are continuously modified by membrane potential as it moves in the subthreshold voltage range, and only vanish at membrane potential levels negative to -120 mV. A continuous description of the active and leakage conductances over the -40/-120 mV potential range was thus provided (Sacchi et al. 1999). In those experiments, there was no suspicion that any of these ganglionic conductances might also be influenced by previous activity, so that no care was taken to avoid spike discharge in the neurons. They were actually stimulated at 1 Hz throughout, and several neurons were successively tested in the same stimulated ganglion. The data presented in the preceding sections urged reconsideration of the problem: we therefore repeated the old protocol in new experiments, testing only neurons that had not been stimulated before.

The selective contribution of Cl⁻ and K⁺ conductances were pharmacologically determined by applying appropriate antagonists. The dashed lines in Fig. 6

![Figure 5.](image)

Figure 5. Holding current (A) and neuron input conductance (B) modifications following standard tetanization (first arrow), and the effect of 0.5 mM 9AC bath immission (second arrow). Total cell input conductance was measured by applying 40-mV negative steps of 10 ms duration. Three examples of these current recordings, taken in control solution at -50 mV holding potential (with activation of I_A on returning to -50 mV) before tetanization (a), at the peak of the stimulation-evoked conductance raise (b), and 5 min after 9AC application (c), are shown in C. Dashed lines indicate the initial holding current value. Note the shifts in holding current level following tetanization and 9AC application, and the same time course of the accompanying conductance modifications.
reproduce the conductance values previously reported (Sacchi et al. 1999), that actually pertain to stimulated neurons and have been confirmed by new observations in stimulated neurons (n = 3). New data obtained in resting neurons are displayed in the same panels, and clearly illustrate that the overall conductance-voltage profile is profoundly different in the resting and stimulated preparations (A; n = 9); following stimulation, the increase in cell input conductance, described above for the unique -50 mV membrane potential level (Fig. 1), has now been verified over a wide membrane potential range. It is worth noting that the stimulation-related increase in cell conductance at -50 mV (+66.7%) in these independent experiments is comparable with the peak posttetanic increase observed in the directly stimulated neurons of Fig. 1A (+71.2%).

As regards cumulative potassium conductance (9AC treatment, n = 8; Fig. 6B), despite the great variability in neurons and experimental conditions, stimulated or resting neurons exhibited identical voltage dependence in the -40/-70 mV membrane potential range, with minor, statistically not significant, differences (see also Table 1) for the -80 and -90 mV values. In two experiments, conductance measurements at rest were repeated after substituting 136 mM Na isethionate for an isoosmotic amount of NaCl (final [Cl-] = 18 mM), and similar conductance-voltage profiles were obtained.

On the contrary, large differences were revealed between the resting and stimulated chloride conductance profiles (TEA + Cs treatment, n = 7; Fig. 6C). The shapes of the curves are totally different; the resting chloride conductance exhibits a mild voltage dependence, whereas in stimulated neurons the conductance drastically increases in the -40/-70 mV range. This confirms and extends the results of Fig. 4C.

The virtually complete voltage independence of the leakage conductance component, and its constant low value, are confirmed by the new experiments in the unstimulated neurons (TEA+Cs+9AC treatment, n = 7; Fig. 6D). In this regard, input conductances as low as 5-7 nS were occasionally measured with the present two-microelectrode technique at -120 mV. These figures favorably compare with the resistance values measured with patch-clamp techniques in mechanically and enzymatically treated neurons of the same origin. Thus the leakage conductance might well be affected by impalement damage, but the usually larger membrane conductance values observed in the intact neuron at less negative membrane potential levels might actually reflect the presence of active conductances, that become unavailable during manipulations required to isolate neurons.

The curves of Fig. 6 were obtained from different neuron groups, not readily comparable in dimensions and properties. However, the individual ionic conductances, recovered as summated mean resting gK (Fig. 6B) and gCl (Fig. 6C) values, individually corrected for the mean leakage conductance value (19.3 nS, Fig. 6D), yield a good recovery of the average total input conductance of the untreated resting neurons illustrated in Fig. 6A (recovery of 104.2% at -50 mV; 112.4 at -70 mV; 127.3 at -90 mV; 117.2 at -110 mV), suggesting that the analysis is reasonably accurate. The systematic excess in the recovery of the summated conductances might be due to an incomplete blockade of the individual

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**Figure 6.** Input conductance of resting, unstimulated neurons under control conditions and after selective blockade of individual ionic components. Cell input conductance was measured in voltage-clamp experiments in which a ‘staircase’ voltage command protocol was applied. Holding potential was varied in 10 mV steps over the -40/-110 mV membrane potential range, and the cell input conductance was evaluated 90 s after imposing the new voltage level. A, control neurons (n = 9); B, different neurons exposed to 0.5 mM 9AC to isolate cumulative gK, plus the leakage component (n = 8); C, neurons exposed to 20 - 50 mM TEA+5 mM CsCl to isolate gCl (n = 7); D, neurons exposed to 50 mM TEA+5 mM CsCl + 0.5 mM 9AC to isolate leakage conductance (n = 9). Bars indicate the SE of the mean conductance values obtained under steady-state conditions. Dashed lines reported in each panel illustrate the conductance-voltage curves evaluated under the same experimental conditions in neurons which had been continuously synaptically stimulated at 1 Hz (data from Sacchi et al. 1999) to show the stimulation-related modifications of individual conductance profiles. In each panel typical current tracings recorded in the indicated ionic media during -50/-70/-90 mV staircase voltage commands are illustrated.
components, which moreover might not be equally affected at all membrane potentials levels, and to the presence of minor conductances, insensitive to the pharmacological treatments employed here. For example, the A-current is mildly inhibited by 10-50 mM TEACl and CsCl (Belluzzi et al. 1985), while 9AC blockade of ClC-1 channels, the channel type presumably sustaining gCl in this neuron, is strong (≥95%) but incomplete at the concentration used here (Astill et al. 1996). In the experiments illustrated in Fig. 6 a ‘staircase’ voltage-clamp protocol was used, in which the cell input conductance was measured at command voltages increasing in successive 10 mV steps; the neuron remained at each potential level for a period of at least 90 s to allow chloride ion redistribution and the progressive slow closure of the chloride channels (Sacchi et al. 1999). These relatively slow voltage shifts are probably similar to those encountered by the neuron during its physiological activity. However, two crucial issues remained to be clarified, namely the sensitivity of posttetanic increase in gCl to previous history of membrane potential and the actual lack of posttetanic effects on the isolated gK, even when tested with large instantaneous voltage steps. Two -50/-90/-50 mV voltage sequences (the sojourn at -90 mV was now 150 s in duration), separated by the standard direct tetanus under current-clamp conditions, were successively applied to the same neuron in normal saline or after 0.5 mM 9AC treatment (Table 1). The isolated potassium conductance proved to be insensitive to stimulation, as expected, and the relatively close numerical values at -50 and -90 mV are in line with the results of Fig. 6B. The strong voltage dependence of cell conductance after activity was similarly confirmed in control neurons (Table 1). The -50 mV values of posttetanic cell input conductance in control solution, reported in Table 1, are relatively low because they were measured 90 sec after the tetanus, before the posttetanic effects had fully developed.

Data reported in Table 1 raise an interesting point: the pretetanus -90 mV values are significantly smaller than those measured at the same potential during the staircase voltage sequences in Fig. 6A (24.3±3.4 vs. 54.8±9.3 nS; P<0.01, Student’s t test). This observation was drawn from different neurons so that the figures may not be comparable. However, the mean initial -50 mV conductance values in the two groups were similar, so the differences might be meaningful. This would suggest that the simple staircase protocol might be sufficient to elicit changes in chloride conductance: thus, gCl might not only depend on voltage and previous suprathreshold activity, but also keep track of the mode in which the membrane voltage potential varies. However, this intriguing point was not further investigated. In these experiments a long-lasting command step of -40 mV amplitude was instantaneously applied. This markedly displaces chloride from its equilibrium and makes the chloride currents at the new membrane potential level more evident. Membrane potential migrations, in fact, generate in the sympathetic neuron measurable chloride currents, significant chloride redistribution, changes in intracellular chloride content and cell input conductance. Hyperpolarizing steps produce inward chloride currents that rapidly peak and decay thereafter with long-lasting time constant, accompanied by a parallel decrease in the overall cell input conductance. Examples of these transient chloride currents during -50/-70/-90 mV command cycles, recorded in different external media, are illustrated in panels of Fig. 6. We previously dissected the contribution of channel gating and chloride redistribution in determining the relaxation currents, by measuring instantaneous cell input conductance during the transients (by continuously applying voltage steps of -40 mV amplitude and 10 ms duration to the neuron held under voltage-clamp), and showed that the two processes occur on similar time scales, but channel closing in response to the hyperpolarizing step is faster than chloride redistribution and therefore dominates the kinetics of the current transients. We suggested that this behavior is sustained by specialized chloride channels that are open at the moment in which the negative voltage step is applied, and then close during maintained hyperpolarization according to a voltage-dependent rate constant (Sacchi et al. 1999). These processes, however, develop phenomenologically over a long time course and become faster with increasing membrane polarization: the mean time constants measured in previous experiments proved to be of 31 – 10 s in the -50/-130 mV voltage range. This requires that the correct conductance value pertinent to each membrane potential level has to be measured only when the new steady-state is reached, usually 90 – 150 s after imposing the new voltage command. The currents generated by the large and long-lasting voltage steps used in the experiments of Table 1 are particularly suited to evaluate kinetic aspects of the readjustments occurring in the neuron. We report here that gCl voltage dependence is low in the unstimulated neuron (Fig. 6C), and markedly increases after stimulation. The additional pertinent observation is that the decay time constant of 1Cl is slow in the resting

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**Table 1. Input conductance during repeated voltage-clamp command cycles in control and 0.5 mM 9AC treated neurons before and after tetanization**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>-50</th>
<th>-90</th>
<th>-50</th>
<th>-50</th>
<th>-90</th>
<th>-50</th>
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<tbody>
<tr>
<td>Control</td>
<td>61.5</td>
<td>24.3</td>
<td>67.8</td>
<td>78.0</td>
<td>29.7</td>
<td>65.9</td>
</tr>
<tr>
<td>(n=5)</td>
<td>± 5.9</td>
<td>± 3.4</td>
<td>± 5.2</td>
<td>± 5.7</td>
<td>± 5.1</td>
<td>± 3.6</td>
</tr>
<tr>
<td>9AC</td>
<td>30.5</td>
<td>28.4</td>
<td>31.2</td>
<td>31.1</td>
<td>34.1</td>
<td>33.1</td>
</tr>
<tr>
<td>(n=3)</td>
<td>± 6.7</td>
<td>± 8.7</td>
<td>± 5.4</td>
<td>± 3.5</td>
<td>± 9.6</td>
<td>± 0.8</td>
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| Cell input conductance measured under voltage-clamp conditions during two successive -50/ -90/-50 mV voltage sequences, separated by the standard direct tetanus, applied to two neuron groups: maintained in normal saline or after 0.5 mM 9AC treatment. The sojourn at -90 mV was of 150 s duration; the first -50 mV conductance value of the second cycle was taken at 90 s after the tetanus. Number of observations in parentheses; mean values ± SE are indicated. |  |  |  |  |  |  |

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neurons (38.4 ± 4.0 s, n = 8) and becomes significantly faster in the stimulated neurons (22.9 ± 2.1 s; n = 8. P<0.01). This indicates that stimulation affects not only the final gCl magnitude, but also its kinetic properties.

Posttetanic effects simulated in a neuron model
Conductances and ionic gradients are mutually linked in the neuron at rest. All the conductances operating in the ganglion neuron have been previously characterized experimentally, by defining their magnitude and the voltage- and time dependence of their activation and inactivation properties (Belluzzi and Sacchi 1991). The resulting set of continuous equations, over a wide membrane potential range, was included in a comprehensive mathematical model (Sacchi et al. 1998). The voltage-dependent chloride conductance here addressed was similarly characterized in neurons continuously stimulated at 1 Hz (Sacchi et al. 1999), and its contribution to neuron behavior was added to the model. Over the membrane potential range here considered (-40/-120 mV), the main features of the resulting model can be simplified as illustrated in the electrical equivalent of Fig. 7B: this scheme has been presented in a previous paper (Sacchi et al. 1999) and is reported here for clarity. Briefly, the new findings are represented by a voltage-dependent compound potassium conductance fed by a constant potassium battery, and the novel voltage-dependent chloride conductance fed by a voltage-dependent chloride battery, which continuously varies with membrane potential migrations. This model does not take into account additional minor current components insensitive to the blockers used and electrogenic pump contributions, which have been considered to remain constant over time and membrane potential level; it provides however an useful tool for the understanding of the mode of interaction of the three conductances analysed in the present study in the subthreshold region. All the variables depicted have been experimentally estimated (neuron stimulated) and mathematically characterized by continuous equations over a wide membrane potential range; they have now been complemented with new data concerning the truly resting neuron (see legend to Fig. 7B). Simulations depicted in the initial part of Fig. 7A predict the values of resting chloride and potassium currents, and chloride equilibrium potential, for resting potentials of either -50 or -70 mV. The remaining part of the curves in Fig. 7A shows the effects of an instantaneous increase in gCl.

Figure 7. A, simulations, according to the circuit in B performing iterative computation of different variables, show the effects of an instantaneous gCl increase in a resting sympathetic neuron at a basal membrane potential level of -50 (continuous lines) or -70 mV (dashed lines). The amount of gCl increase is that predicted by Fig. 6C for the unstimulated vs. stimulated neuron. An additional steady current of +0.73 nA (not measured experimentally and due to imprecise leakage evaluation, minor or residual active currents, electrogenic pump contributions,...) was applied to maintain the initial stationary level of -70 mV (+0.01 nA at -50 mV) under current-clamp conditions. The time courses of the ensuing modifications in the neuron membrane potential (V_m), chloride ion equilibrium potential (E_{Cl}), chloride current (I_{Cl}), cumulative potassium current (I_K), cell input conductance (g_{cell}) and threshold inward charge required to fire the neuron (C_{thr}) are shown. C_{thr} has been calculated, before and at different times after gCl increase, according to the ideal neuron model illustrated in previous papers (Belluzzi and Sacchi 1988, 1991). B, electrical model of the rat sympathetic neuron in the subthreshold membrane potential region (from Sacchi et al. 1999; repeated for clarity). Equations describing cumulative gK voltage dependence, steady state E_{Cl} voltage dependence and voltage sensitivity of I_{Cl} gating time constant are considered to be unaffected by stimulation and are the same as those reported in the indicated paper. The new equation describing gCl conductance-voltage relationship adapted to the unstimulated resting neuron is (V = membrane potential in mV): g_{Cl} = 828.12 
\left\{ \exp\left[-0.64 \cdot (63.68 - V) \cdot e/kT\right] + \exp\left[0.64 \cdot (63.68 - V) \cdot e/kT\right] \right\}^{-1} \cdot 128.89 \cdot \left[1 + \exp\left[2.43 \cdot (-34.23 - V) \cdot e/kT\right]\right]^{-1} \text{nS}
elicted by ongoing 1 Hz spike discharge in an ideal, initially silent sympathetic neuron (according to data in Fig. 6C). This simulation dissects the complicated mutual adjustments of the multiple variables that contribute to accommodate the neuron to varying voltage-dependent conductances and subthreshold membrane potential levels. Some of the effects are not readily predictable: for example, the final steady-state increase in cell input conductance at -70 mV is lower than expected from the gCl amount initially imposed (100.3 vs. 109.3 nS), due to a decrease in gK following membrane depolarization from -70 to -61.4 mV (see Fig. 6B).

Raised conductance and neuron depolarization are expected to engender potentially conflicting results, since the first effect acts to stabilize the membrane, while the second moves the neuron towards its firing threshold. Simulations help to clarify this point. The resulting changes in neuronal excitability, expressed in terms of the threshold inward charge required to fire the neuron, are illustrated in the lowest panel of Fig. 7. In this example, membrane potential (depolarized by the increased gCl from a basal -70 mV level to -63 mV) is the major controller of neuron excitability, whereas input conductance produces appreciable but short-lived effects only in the moment in which it is abruptly raised. A different picture is seen when simulation is repeated at a more positive membrane potential. The increase in gCl is large (and cell input conductance) in the neuron stimulated at -50 mV and is stable over time, but it generates a membrane depolarization of 3.0 mV, which is only accompanied by a minor drop in threshold charge. The starting membrane potential level thus exerts a crucial role in conditioning not only resting excitability, but also the final post-stimulation effects, which vary in a complex, non linear manner and are difficult to test experimentally.

**DISCUSSION**

The intact and mature rat sympathetic neuron hosts a cellular mechanism devoted to controlling membrane chloride conductance, which is active over a membrane potential region where neuron behavior is usually considered passive. This mechanism is not obligatorily linked to synaptic activation and operates in parallel to a considered passive. This mechanism is not obligatorily linked to synaptic activation and operates in parallel to a companion compound potassium conductance. Both processes are clearly voltage-dependent at membrane potentials negative to -40 mV, and silenced with very high internal negativity (about -120 mV); only here does the neuron exhibit truly passive behaviour. The voltage dependence of the ganglionic potassium and chloride conductances was quantitatively characterized in previous work (Sacchi et al. 1999). We describe here an additional crucial property, exclusive of the chloride conductance, namely its dependence on previous neuronal activity. Activation of the neuron results in a consistent, long-term increase in cell input conductance, specifically sustained by the chloride channel, that also acquires marked voltage dependence. It may be of interest to observe that in the absence of previous stimulation chloride conductance only varies by about 40% over the -90 to -40 mV membrane potential range, and this may help explaining why the existence of an active chloride conductance in the subthreshold region of membrane potential had so far escaped experimental detection. The mode of neuron activation appears relatively unimportant as concerns the recording mode (current- or voltage-clamp), the activation mode (direct or synaptic stimulation), the stimulation sequences or frequencies applied. In the present experiments the neuron was usually activated by high frequency tetanization, but a maintained preganglionic stimulation frequency as low as 1 Hz, well compatible with the ongoing discharge frequencies operating physiologically in the autonomic nervous system (reviewed by Jäning 1995), is suitable for its activation. We suspect that mere membrane potential shifts in the subthreshold voltage region might be sufficient to elicit a detectable response. The effect is long-lasting (it was followed occasionally for up to 30 min and more), but transient and may revert to decreased conductance, suggesting that the momentary chloride conductance value represents a balance between activation and turn-off processes. The neuron input conductance is expected to vary in the subthreshold range within the physiological limits outlined by the curves in Fig. 6A: between the curve pertinent to the unstimulated neuron and that of the neuron synaptically activated at 1 Hz. Such limits could possibly be even broader in the case of strong synaptic tetanization, especially at membrane potential levels negative to -70 mV, where 1 Hz stimulation appears less effective.

The main question is what exactly does resting mean in this context. The absence of spike discharge during the experiment makes it implicit that the neuron has not been stimulated, but this gives no insight into its previous history. We have actually no information about the real memory of this process in the operating neuron, or of the modalities for cancelling any previous level of gCl activation. Experiments are being planned on denervated neurons, in which synaptic stimulation and firing will be abolished for a controlled extent of time.

The cellular mechanism underlying this behavior is unidentified; we prove that neither membrane potential migrations associated with spike discharge, nor synaptic activity by itself, or external calcium entry into the cell, represent the required trigger for its induction and development. The molecular bases of the signaling pathway, and the reasons for the increased voltage dependence of the resting conductance and the acceleration of its kinetic properties following stimulation, however, remain to be elucidated. It will be noted that a chloride-mediated posttetanic control may bear some relevance to the neuronal functioning: two independent chloride-related mechanisms actually operate in sequence in the sympathetic neuron. The first is sustained by an early chloride conductance increase (of about 20 nS)
mediated exclusively through activation of the nicotinic channels by native acetylcholine. It generates a relatively long-lasting current (it decays with a mean time constant of about 370 ms), $I_{ADPS_{syn}}$ that exhibits fast onset, is calcium-independent and insensitive to specific chloride channel blockers (Sacchi et al. 2000). The second mechanism described here partially overlaps the first (see points at 1 min after tetanization in Fig. 1B and 2A) but then prolongs the effects initially brought about by pure nicotinic channel activation.

The presence of a persistent chloride current governing the resting status of the sympathetic neuron has been largely overlooked: it is of limited magnitude and mixed with other currents; it requires that its driving force be artificially increased by voltage steps before it becomes discernible; the internal chloride ion concentration must not be clamped but free to readjust according to the momentary membrane potential level; the two alternative $gCl$ conditions, unstimulated-stimulated, have never been taken into account before. The importance of the chloride current arises from the equilibrium that persists in the resting neuron between out- and inward currents; one of the inward components is actually the chloride current, that is kept constantly inward by the internal chloride concentration, that is in turn maintained higher than its electrochemical equilibrium by active transport mechanisms. The potassium conductance is potentially voltage-dependent, but will remain stable until an external event modifies its value; this trigger is not represented by any of the neuron activation procedures tested here. At steady state, any constant membrane potential level will thus generate a constant potassium current, being fed by a constant potassium battery. On the contrary, the chloride channel hosts an intrinsic mechanism, its activity dependence, which makes the chloride current a candidate for natural controller of the balance between the opposite resting currents, and thus of the final membrane potential level. It might represent an intrinsic mechanism to continuously adjust neuron basal status to its activation degree.

Previous isolated observations are in line with this view. GABA depolarizes the rat sympathetic neuron (Adams and Brown 1975) by inducing an inward current, when applied at membrane potentials negative to the chloride equilibrium potential (Sacchi et al. 1999). It has been reported that isethionate substitution for chloride ions reduces cell input conductance, as measured in current-clamp (Adams and Brown 1975) or voltage-clamp conditions (Sacchi et al. 1999), and hyperpolarizes the neuron (Adams and Brown 1975); a similar result has been obtained here by pharmacologically blocking the chloride channels (not shown). Moreover, a distinct inward current accompanying $gCl$ increase has been recorded in the present experiments, under voltage-clamp conditions. Less recent observations, under current-clamp conditions, have demonstrated that ganglionic tetanization was followed by a sequela of membrane potentials shifts; one of these, the ‘late slow excitatory postsynaptic potential’ exhibited a slow onset, an extremely prolonged time-course and a sufficient magnitude to induce a long-lasting late afterdischarge in bullfrog neurons (Nishi and Koketsu 1968). The existence of an analogous response in sympathetic ganglia of the guinea-pig and rabbit has been demonstrated by Ashe and Libet (1981). This latter description is phenomenologically reminiscent of the present findings: the late depolarization was resistant to nicotinic, muscarinic or adrenergic antagonists; it appeared with latencies in the range of seconds, rise times in the minute time scale, duration of up to 20 min or more; even a 1 Hz stimulation frequency applied in a sufficiently large number of pulses was almost as effective as higher frequency tetanization. These results have not actually been linked to chloride participation. The new findings presented in this and the preceding paper (Sacchi et al. 1999) would suggest that the envisaged mechanism associated with $gCl$ represents one of the physiological controllers of the neuronal excitability machinery, in which activity would control further activity by modifying the cell input conductance and the basal membrane potential level.

The active chloride movements, and their control, are a homeostatic process not completely understood (Misgeld et al. 1986; Jentsch 1996; Rohrbough and Spitzer 1996; Delpire 2000; Russell 2000). Chloride conductance, on the other hand, plays important roles in regulating excitability and the equilibrium between excitation and inhibition. In muscle, the high resting chloride conductance and the passive distribution of chloride ions suppress depolarizing inputs and stabilize the membrane potential at its negative level (Palade and Barchi 1977). In neurons, active chloride distribution represents an important controller of interneuronal communication mediated by GABA synapses: the developmental shifts in the activity of chloride-related cotransporters control the distribution of chloride ions; the ensuing equilibrium potential may vary widely and lead to relevant changes in neuronal response to synaptic activation. During development, responses to GABA switch in fact from depolarization to hyperpolarization (reviewed by Ben-Ari 2002). If an activity-dependent $gCl$ increase were a general, widespread mechanism in other neurons as well, the posttetanic conductance increase would generate similar effects in all of them by affecting their passive properties; the final physiological result, however, would be conditioned by chloride transmembrane distribution. In the sympathetic neuron, chloride ions are constantly maintained at an internal concentration higher than predicted by the nernstian equilibrium, by active mechanisms which move chloride ions into the cell. The opposite holds true for motoneurons and CNS neurons, in which active chloride extrusion frequently occurs. Under these conditions, $gCl$ modifications will generate membrane potential shifts in opposite directions, from the resting status, according to the momentary chloride driving force. This principle is well documented for the short-lived synaptic conductance modifications, occurring for example in motoneurons (Coombs et al.
1955), in hippocampal pyramidal neurons (Staley 1994), in spinal neurons (Rohrbough and Spitzer 1996) and rat auditory neurons (Ehrlich et al. 1999). It would also be applicable when modifications of the mechanisms controlling chloride distribution develop with a long time course, as in the present case, with widespread and long-term effects on neuronal firing strategy. Interestingly, the presence of anion channels has been demonstrated in rat brain synaptosomal membranes, and their possible role in mediating transmitter release by controlling the polarization of the presynaptic terminal membrane has been suggested (Nomura and Sokabe 1991; Yuto et al. 1997). These data open a series of questions of general interest concerning the role of chloride in synaptic signal processing, the mechanisms sustaining the chloride gradients in different conditions, the relationship between chloride conductance and activity. They would stress, anyway, the relevance of the processes governing chloride ion movements in distinct compartments and during different aspects of neuron functioning; they would also explain apparently conflicting posttetanic effects, that have been observed in the absence of a detailed description of the actual chloride ion dynamics and knowledge of the history of previous activity.

ACKNOWLEDGEMENTS
This paper was supported by grants from the Ministero della Università e della Ricerca Scientifica e Tecnologica within the national research projects ‘Cofin 1999’ and ‘Cofin 2001’.

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