Synaptic and Somatic Effects of Axotomy in the Intact, Innervated Rat Sympathetic Neuron

Oscar Sacchi, Maria Lisa Rossi, Rita Canella, and Riccardo Fesce

Introduction

The aim of the present study is to understand the progressive failure in transmission flow through the ganglionic synapse after axotomy of the principal sympathetic neuron. The analysis is based on the physical description of changes in the individual aspects of neuron functioning and on the eventual synthesis of these multiple components into an integrated view of neuron behavior.

The membrane of the rat sympathetic neuron hosts many ionic conductances ($g_{Na}$, $g_{Ca}$, $g_{A}$, $g_{Kv}$, $g_{Kc}$, $g_{AHP}$, $g_{Cl}$). Each of them has been previously isolated and kinetically characterized in the “normal” undamaged neuron and attributed a role in action potential electrogensis, development of afterpotentials, or control of the resting membrane potential. These analyses have provided a continuous mathematical description of each conductance over time and voltage, which represents a preliminary step for obtaining a complete molecular model of neuronal electrogenesis (Belluzzi and Sacchi 1991). In addition, a comprehensive mathematical model of the normal ganglionic synapse has been developed (Sacchi et al. 1998).

Here we have performed a similar description in the axotomized neuron in the intact ganglion. Neuron behavior has been characterized under voltage-clamp conditions at increasing times after sectioning the postganglionic trunks (resulting in axotomy of the principal sympathetic neurons). Several conductances have been isolated and characterized under these conditions and any modifications detected. Finally, the individual current components have been reassembled by simulating the current-clamp behavior of the neuron in a previously validated mathematical model, modified to include the experimentally observed changes to the elements of neuron electrogensis.

Modifications in the spike time course during fast repolarization, and the subsequent afterhyperpolarization (AHP), have been frequently described in other axotomized neurons under current-clamp conditions (for a review see Titmus and Faber 1990). To explain these observations, an analysis of the underlying potassium currents is required. Similarly, fast synaptic transmission in the axotomized neuron shows marked modifications related to the ensuing morphological damage (preganglionic synaptic terminals detach from their postsynaptic sites: Matthews and Nelson 1975; Purves 1975). The intrinsic electrophysiological correlate of these processes is unknown, as are the properties of the nicotinic receptor channel, whose sodium–potassium relative permeability might be modified by axotomy or by detachment of the synaptic inputs. This might lead to shifts in the acetylcholine (ACh) equilibrium potential as occurs within 1 day of denervation (Sacchi, unpublished observation).

Voltage-clamp techniques have been used to examine the effects of axotomy on the electrical properties of various types of neuron (dorsal root ganglion neurons: Abdulla and Smith 2001, 2002; André et al. 2003; Baceci and Kocsis 2000; Everill and Kocsis 1999; Yang et al. 2004; bullfrog sympathetic neurons: Jassar et al. 1993, 1994; vagal afferent neurons:...

Address for reprint requests and other correspondence: O. Sacchi, Department of Biology—Section of Physiology and Biophysics, Via Borsari, 46, I-44100 Ferrara, Italy (E-mail: sho@unife.it).

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Lancaster et al. 2002). In those studies, however, both sources of neuron damage—axotomy in vivo and subsequent neuron dissection from the tissue to study it in isolation—coexisted, preventing a clear-cut separation of single effects. Moreover, because of the methodologies used, none of these preparations permitted an integrated analysis of the synaptic and somatic effects that concurrently (and possibly independently) occur after axotomy. The present description is thus the first detailed voltage-clamp study of an axotomized sympathetic neuron in situ, in which a complete mathematical simulation of the whole synaptic transmission process at the ganglionic synapse is provided.

METHODS

Electrophysiological experiments were performed on superior cervical ganglia isolated from rats (120–250 g body weight) during urethane anesthesia (1–1.5 g kg⁻¹; intraperitoneal [ip] injection) and maintained in vitro at 37°C. After removal of the ganglion, 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 ×500 by using diffraction interference optics. The preparation was continuously superfused with a medium (in mM: 136 NaCl, 5.6 KCl, 5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 14.3 NaHCO₃, 5.5 glucose) pregassed with 95% O₂-5% CO₂ to a final pH 7.3. Choline chloride (10⁻⁵ M) was added to the saline. Neurons were impaled with one or two independent glass microelectrodes filled with neutralized 4 M potassium acetate (30–40 MΩ resistance). Recordings were obtained under two-electrode voltage-clamp conditions using a custom-made amplifier, as described previously (Belluzzi et al. 1985). The bath was grounded through an agar–3 M KCl bridge.

Axotomy was produced after exposing the ganglion at the bifurcation of the carotid artery using aseptic precautions, under ketamine (70 mg kg⁻¹ ip)–medetomidine (0.5 mg kg⁻¹ ip) anesthesia. The internal carotid nerve was identified at the cranial pole of the ganglion, and the external nerve at about 1 mm from the ganglion, and severed. Care was taken during dissection to preserve the ganglion’s vascular supply. Animals were maintained for 1–7 days after surgery before starting the electrophysiological experiments. For some animals, a “sham” procedure was performed, which consisted of exposing the ganglion and the postganglionic trunks, but not cutting them. Neurons in ganglia of sham-operated animals exhibited the same biophysical behavior as untreated controls; this rules out any persistent effect of anesthesia or surgery. Neurons from “normal-unoperated” and from “sham-operated” ganglia were thus usually pooled in a group of “control” neurons.

The use and handling of animals was approved by the Animal Care and Use Committee of the Ferrara University and authorized by the National Ministry of Health.

To activate the preganglionic input, single supramaximal current pulses of 0.3-ms duration were applied to the cervical sympathetic trunk through a fine suction electrode, positioned close to the caudal pole of the ganglion, either while the neuron was maintained at a constant holding potential (usually ~50 mV) or during the application of repetitive cycles in which the postsynaptic membrane potential was commanded to different voltages. In the latter case, the protocol included several cycles of stimulation; during each cycle (10-s duration) stimuli to the preganglionic trunk were applied 10 ms after stepping to a test potential in the ~20- to ~110-mV range, from a holding potential of ~50 mV, and the test potential was maintained for another 190 ms before returning to the holding potential. From the excitatory postsynaptic currents (EPSCs) recorded at the different command potentials a precise I–V relationship was derived, and the ACh reversal potential was estimated by extrapolating the EPSC peak amplitude I–V curve to zero current. Trains of supramaximal stimuli, 5, 10, and 15 Hz for 10 s, were also used at ~50-mV holding potential.

Large synaptic and ionic currents were recorded with good control of the membrane potential at any tested voltage (Sacchi et al. 1998); single currents were filtered at 5 kHz with an eight-pole Bessel filter, digitized at 10 kHz with a 12-bit A/D interface (Digidata 1200A operated by pCLAMP software; Axon Instruments, Union City, CA) and stored on disk for future analysis.

The neuron under current-clamp conditions was stimulated directly by applying current pulses of 3-ms duration and 2- to 7-nA intensity; intensity was adjusted to maintain the pulse just suprathreshold. Current was applied through the current electrode under two-electrode recording, and through the same electrode when single-electrode configuration was used (Sec1L amplifier, npi electronic, Tamm, Germany).

Data were analyzed on Pentium personal computers (AST) with pCLAMP (version 5.5; Axon Instruments) and MATLAB 386 (The MathWorks, Natick, MA) software packages. Voltage-dependent parameters were fitted throughout by Boltzmann equations of the form $B(V) = A [1 + exp(-b(V - V_c) 	imes F/R,T)]^{-1}$, where A is maximum amplitude, $b$ is the slope coefficient, and $V_c$ is the value of potential for which the equation has half its maximum value $B(V_c) = A/2$.

Statistical methods

The differences among various experimental conditions were examined by two-way ANOVA. Values of $F$ and $P$ are reported in the text for voltage dependency and treatment effect. In the figures, data are reported by pooling the results obtained from several cells under each experimental condition. Average values and SE are plotted for each condition. When analytical curves are also drawn (for single conductances/currents) they represent Boltzmann-type equations fitted to the pooled data. The effects of axotomy on single parameters were examined in different cell groups before/after surgery by Student’s t-test.

RESULTS

EPSC properties

Stimulation of the whole presynaptic input evokes in individual postganglionic neurons a synaptic macrocurrent (EPSC) that reflects the time course properties of the underlying elementary events if the release of transmitter quanta is made synchronous by minimizing differences in conduction time among the several preganglionic fibers innervating each cell. As reported previously (Sacchi et al. 1998), the ganglionic EPSC onset is described by a single exponential function (time constant: $\tau_v$, voltage insensitive and with a mean value of 0.57 ms) that reflects ACh binding and nicotinic channel opening rate constants, whereas EPSC decay is fitted by a second exponential function of time ($\tau_d$), voltage sensitive and reflecting the mean open time of the nicotinic channel at the different holding potentials. The mean time constants of spontaneous miniature synaptic currents (mEPSC) and EPSCs are mutually favorably comparable, so that, although some temporal dispersion occurs among the release times of individual quanta, release probability from distinct synapses as a function of time can be compressed, under appropriate stimulation conditions, to a very sharp Gaussian distribution, i.e., well approximated by an instantaneous pulse. In Fig. 1A, EPSCs are generated in a control neuron at different holding potentials in the ~100- to −30-mV range. A clear-cut reversal of the EPSC usually could not be achieved because of the large delayed potassium cur-
rents generated at membrane potentials positive to \(-30\) mV. It is clear, however, that the peak amplitude of the synaptic current \((I_{\text{max}})\) varied linearly with membrane potential; thus extrapolation of the regression line to zero current (Fig. 1D) represents a reliable measure of the ACh reversal potential \((E_{\text{ACh}})\). Similar conclusions were obtained when the synaptic inward charge (i.e., the area under the synaptic current curve) was plotted instead of the peak EPSC amplitude. The slope of the \(I-V\) relationship of the synaptic current provides an estimate of the maximum synaptic conductance—\(g_{\text{syn}} = I_{\text{max}}/(V_m - E_{\text{ACh}})\)—in each neuron tested. EPSC families similar to that shown in Fig. 1A have been evoked in normal, sham-operated (1 day after surgery) and axotomized neurons over the \(-100-\) to \(-20\) mV-membrane potential range. In normal neurons, mean \(g_{\text{syn}}\) was \(0.93 \pm 0.09\) \(\mu S\) per neuron, and \(E_{\text{ACh}}\) was \(-12.2 \pm 1.8\) mV \((n = 10)\); in sham-operated neurons \(g_{\text{syn}} = 0.92 \pm 0.06\) \(\mu S\) and \(E_{\text{ACh}} = -13.6 \pm 0.9\) mV \((n = 15)\). In axotomized neurons (a typical EPSC family after 1 day is illustrated in Fig. 1B) average \(g_{\text{syn}}\) values were \(0.27 \pm 0.06\) \(\mu S\) per neuron (after 1 day, \(n = 14\)), \(0.14 \pm 0.03\) \(\mu S\) (after 2 days; \(n = 4\)), and \(0.14 \pm 0.03\) \(\mu S\) (after 3 days; \(n = 6\)). Corresponding values of \(E_{\text{ACh}}\) were \(-13.3 \pm 1.3\), \(-13.3 \pm 3.7\), and \(-11.4 \pm 0.8\) mV. Effects of preganglionic stimulation in 4-day axotomized neurons were highly variable, and some neurons were completely silent. It should be noticed that neurons with EPSC properties similar to those of controls were occasionally observed; these, however, were considered to be intact neurons that had escaped axon injury and thus were discarded from the analysis. Actually, whereas sectioning the preganglionic sympathetic trunk denervates the entire population of superior cervical ganglia (SCG), entire postganglionic axotomy is impossible because postganglionic twigs are so numerous, besides the large internal and external carotid nerves, to prevent their complete identification.

In all normal neurons, the EPSC decay was well fitted by a single exponential function at any membrane potential level; the average time constant \((\tau_1)\) was in the 8.3- to 5.2-ms range \((at -100\) and \(-30\) mV, respectively) and exhibited limited voltage dependency in controls and after axotomy (Fig. 1E). The line in Fig. 1E shows the slope of \(\tau_1\) versus membrane potential in control neurons that corresponds to an \(e\)-fold change in 260 mV. Similar values were measured in 1- to 4-day axotomized neurons, despite the EPSC amplitude decrease; \(\tau_1\) values in axotomized neurons are compared in Fig. 1E with those of control neurons.
The calcium dependency of the ACh release process, evaluated by modifications of EPSC amplitude at low preganglionic stimulation rates (≤0.1 Hz), was similar in normal or axotomized neurons: decreasing extracellular Ca\(^{2+}\) concentration to 2 mM produced a mean decrease of −31.0 ± 3.8% in five normal neurons, which was not significantly different from a mean of −27.8 ± 4.0% in four axotomized neurons.

Spontaneous release of single quanta from the presynaptic terminals can be revealed in the postganglionic neuron in current- (Sacchi and Perri 1971) and voltage-clamp recordings (Sacchi et al. 1998). Despite their small size, mEPSCs were collected in six axotomized neurons (1-day) held between −60 and −100 mV. Miniatures were sufficiently numerous to grant a reliable estimate of their mean peak conductance per neuron that ranged from 2.8 to 5.2 nS (mean 3.7 ± 0.4 nS), which was not statistically different from previous observations in normal ganglia (range 2.3–5.4 nS, mean 4.0 ± 0.5 nS; n = 5; Sacchi et al. 1998). An unusually high frequency mEPSC sequence is illustrated in Fig. 1C. The frequency of mEPSCs, however, became appreciable only during low-frequency preganglionic stimulation. Comparison of the frequencies in control and axotomized neurons was not attempted because it was bisued by the large differences in the respective EPSC amplitude (and in the number of active synaptic boutons).

Presynaptic quantal dynamics

Data presented in Fig. 1 provide a steady-state description of the synaptic effects of axon injury on single EPSCs. For a complete description of the presynaptic effects, however, dynamics of ACh release from presynaptic terminals was also analyzed. Paired-pulse and trains of preganglionic stimulation were used. Tracings in Fig. 2, A and B show examples of short-term modulation of release at the ganglionic synapse in a normal (A) or axotomized neuron (B). Synaptic currents were evoked by two stimulus pulses (a1 and a2) of identical intensity, separated by increasing time intervals (5 ms to 10 s), applied to the cervical sympathetic trunk. The time course of paired-pulse modulation is illustrated in Fig. 2C, in normal (filled circles; n = 13) or 1-day axotomized (open circles; n = 7) neurons. In control neurons, the maximum decrease in synaptic strength (a mean decrease to 42% of the initial EPSC amplitude) was apparent at 5 ms after the conditioning stimulus (Fig. 2C). Thereafter, synaptic strength recovered in three kinetic phases. In a fast phase of about 50-ms-duration recovery to 78% of the starting value was achieved; during an intermediate phase of about 500-ms duration synaptic efficacy remained stable, and eventually a much slower recovery phase resulted in a complete return to the starting EPSC amplitude within some additional 9 s. Despite the strongly reduced EPSC amplitude in axotomized neurons, the major features of depression (and its complex kinetics), remained, although with some quantitative differences. The major effect was a marked reduction in the early phase of depression, followed by full recovery within 20 ms, and the appearance of a separate, delayed depression phase. Two-way ANOVA confirmed that synaptic depression was significantly different in control versus axotomized neurons (for interpulse intervals 5 ms to 1 s; F = 92.3, P < 0.01), and that depression degree varied with interpulse duration (F = 12.5; P < 0.01).

The capability to sustain ACh release was further explored using stimulus trains of 10-s duration at constant frequency (5, 10, and 15 Hz) in control or axotomized neurons. The behaviors of normal and sham-operated neurons are presented separately in Fig. 3, Aa and Ab to demonstrate the lack of effect of anesthesia or surgery. For 5 and 10 Hz (Fig. 3, B and C), normal and sham-operated neurons were pooled in a unique control group. The magnitude of the isolated EPSC varied from neuron to neuron; nevertheless, mean values proved to be reasonably coherent in the different experimental groups (19.0 ± 2.4 nA at −50 mV in normal, untreated neurons, n = 24; 18.2 ± 2.2 nA in sham-operated neurons, n = 23). Maintained stimulation invariably resulted in a progressive depression of synaptic strength, which developed over an initial, fast, and a late, slower phase (clearly recognizable at 10 and 15 Hz). Depression resulted in a final mean decrease to 37.5 ± 3.4% of the initial value in 15-Hz trains (n = 16), to 48.8 ± 2.7% in 10-Hz trains (n = 14), and 67.8 ± 3.6% in 5-Hz trains (n = 17).
Although the mean EPSC was reduced by a factor of 3 (in 1-day axotomized neurons; even more after 2 days; $P < 0.01$ in both cases), release capability was well maintained during repetitive stimulation (Fig. 3, A–C). This is easily perceived when EPSC values are normalized to the peak of the first EPSC in each train (Fig. 3, B and C). The final depression at the end of the 10-s train was systematically less in injured compared with control neurons. However, in 2-day axotomized neurons a clear-cut facilitation of transmitter release occurred during the first few seconds of stimulation, independent of frequency, and the overall depression was small (virtually absent at 10 Hz).

Two-way ANOVA confirmed that repetitive stimulation produced different effects on the amplitudes of synaptic currents, initially facilitating axotomized neurons but depressing control ones (for the first 4 s of the 10-Hz train, axotomized vs. control: $F = 128.6, P < 0.01$); it also confirmed that the EPSC amplitude varied during the train ($F = 4.5; P < 0.01$) and that the time-dependent changes were different in the two conditions ($F = 3.3, P < 0.01$). Facilitation in axotomized versus depression in controls was also observed at 5 Hz ($F = 4.5; P < 0.05$), but the changes in amplitude during the trains were small in this case (between $-15$ and $+9$%), so that time-dependent changes were not statistically significant ($F = 0.66$).

The possible relationship between initial EPSC amplitude and degree of depression was tested by plotting for each neuron the ratio between the EPSC amplitude at the end of the tetanus versus the initial one (a10s/a1 value) as a function of the first EPSC amplitude (Fig. 3, b panels). A significant, mild, and negative correlation ($P < 0.05$) between depression magnitude and initial current amplitude was detected only in control neurons, when stimulated at 5 and 10 Hz.

The observed differences prompted us to study the calcium dependency of the ACh release process during sustained stimulation. The decrease in the steady-state EPSC peak amplitude, brought about by changing from the usual 5 mM external Ca$^{2+}$...
to the more physiological 2 mM, was mentioned earlier. This same fractional reduction was maintained in control neurons throughout the whole 10-s train (in a typical neuron: EPSC mean decrease of $-32.7\%$ during the first 2 s, vs. $-29.3\%$ during the last 2 s of a 15-Hz train; $-33.6\%$ vs. $-30.6\%$ in another neuron stimulated at 10 Hz). The same behavior was noticed in 1-day axotomized neurons ($-33.1\%$, initial, vs. $-36.9\%$, final, in a train at 15 Hz, and $-20.6\%$ vs. $-19.5\%$ in a different neuron during a 10-Hz train).

**Potassium currents in axotomized neurons**

Depolarization evokes in rat sympathetic neurons different patterns of mixed potassium currents, depending on the initial holding potential. When the neuron is held at membrane potential levels positive to $-40$ to $-50$ mV, depolarization evokes long-lasting, maintained potassium currents that result from the summation of the delayed $I_{KV}$ and calcium-dependent $I_{KCa}$. When membrane polarization removes $I_A$ inactivation (fully removed by holding for 1 s at about $-110$ mV), depolarization to the same command levels evokes the $I_A$ current in addition to the other two components; this maneuver allows almost pure $I_A$ tracings, at any given membrane potential level, to be evaluated as the difference between currents in the absence or presence of $I_A$ inactivation (Fig. 4, C and F). All these individual potassium currents were isolated and kinetically characterized in previous studies (summarized in Belluzzi and Sacchi 1991).

Delayed currents were recorded 1 to 4 days after axotomy over the $-40$- to $+10$-mV membrane potential range, starting from a $-50$-mV holding potential, and compared with data from sham-operated controls. Typical current families in control and 1-day axotomized neurons are illustrated in Fig. 4. The differences between responses evoked with or without a 1-s prepulse at $-100$ mV to remove $I_A$ inactivation (Fig. 4, B and E, cf. Fig. 4, A and D) are apparent. An early, marked decrease in maximum delayed outward currents (Fig. 4, A and D) was observed 1 day after axotomy ($76.3 \pm 14.8$ nA vs. $129.7 \pm 14.9$ nA in control neurons at $+10$ mV; $n = 10$), followed by a slight additional decrease at 2 days ($62.3 \pm 8.6$ nA; $n = 7$) and thereafter remained virtually unchanged during the following 2 days ($62.2 \pm 5.4$ nA, $n = 4$ at 3 days; $57.8 \pm 13.2$ nA, $n = 5$ at 4 days). The mean I–V relationships for the corresponding groups are shown in Fig. 5A; the equations best fitting the underlying conductances are given in the legend to this figure.

No systematic differences were detected between sham-operated and axotomized animals in the kinetics of delayed outward currents. The total delayed outward current, $I_{KD}$, was accurately described by the sum of two exponential currents: a fast activating component followed by a much slower component. In all cases, after amplitude scaling, summed outward current ($I_{KD}$) of axotomized neurons proved kinetically very close to the $I_{KCa}$ fraction previously characterized in undamaged neurons (Belluzzi and Sacchi 1990), and particularly so during the early phase of current flow, which is involved in action potential development. This simplification was adopted in simulations (see following text), in which only the $I_{KD}$ current was considered, to account for both $I_{KV}$ and $I_{KCa}$ components, and the kinetics of the fast $I_{KCa}$ of the normal neuron was used.

**FIG. 4.** Representative potassium current tracings in normal (A and B) and 1-day axotomized (D and E) neurons. In A and D delayed $I_{KCa}$ current families are evoked at increasing positive voltage steps in the $-40$- to $+10$-mV range from a holding level of $-50$ mV ($I_A$ inactivated). In B and E delayed $I_{KD}$ and $I_A$ current components contribute to each tracing; they are evoked at the same command potentials as in A and D after a conditioning 1-s step at $-100$ mV to remove $I_A$ inactivation. Corresponding difference currents illustrated in C (B minus A) and F (E minus D) dissect, after leakage correction, the pure $I_A$ current families of the normal and axotomized neuron, respectively.
Families of almost pure $I_A$ currents obtained from the difference of tracings in Fig. 4, A and B and D and E, respectively, are shown in Fig. 4, C and F. Comparison of data from neurons of sham-operated and normal animals (Belluzzi et al. 1985) confirmed that the overall $I-V$ relationships of $I_A$ current were unaffected by the surgical manipulations ($I_A$ peak amplitude: 87.4 ± 13.6 nA at −10 mV, $n = 11$, vs. 78 ± 7.2 nA in normal animals, $n = 11$).

In all neurons ≤4 days postaxotomy, early decreases in $I_A$ peak current amplitude were observed at all command potentials (−75% at −10 mV, compared with controls). Peak current values were similar 1–3 days after surgery and were pooled to construct the single $I-V$ relationship illustrated in Fig. 5B (open circles; $n = 11$), and compared with sham-operated neurons (filled circles; $n = 11$). The equations shown in the legend to the figure describe the voltage dependency of the corresponding $A$ conductances.

Steady-state $I_A$ inactivation curves were obtained by a standard protocol: a 1-s prepulse to a membrane potential in the range −110 to −50 mV followed by a test step to +10 mV (see Fig. 5D). Significant shifts in steady-state inactivation curves were observed after axotomy: midpoints shifted by up to +17 mV (from −67.3 ± 1.6 to −55.7 ± 2.2 mV, after 2 days, $P < 0.005$; and to −50.3 ± 4.8 mV, after 4 days, $P < 0.01$) (Fig. 5C). It is evident that inactivation is progressively and smoothly removed with increasing negativity in the normally innervated control (the −90- to −100-mV step is still capable of removing some residual inactivation), whereas inactivation is abruptly and virtually completely removed within a restricted voltage range in the axotomized neuron (−50 to −70 mV; at −60 mV in the example illustrated in Fig. 5D). Slope coefficients of Boltzmann-type equations fitting the data were generally larger in controls than in axotomized neurons (−7.9 ± 0.8 mV in controls vs. −6.6 ± 0.5 mV at 2 days, $P < 0.05$, and highly variable after 4 days). No recovery toward normal values was noted after axotomy for 7 days.

**Observations under current-clamp conditions**

**SYNAPTIC TRANSMISSION SAFETY FACTOR AFTER AXOTOMY.** Synaptically evoked spikes are fired with difficulty in the axotomized sympathetic neuron; only subthreshold EPSPs are observed with increasing frequency and the neuron eventually becomes completely silent in response to supramaximal depolarizations. Patterns of cellular activity are thus progressively altered by the surgical trauma.}

![Graphs and diagrams](http://jn.physiology.org/)

**FIG. 5.** Potassium currents in axotomized neurons. A: $I-V$ relationships of delayed outward currents evoked over the −40- to +10-mV membrane potential range in control (filled circles) or axotomized neurons, 1 day (open circles; $n = 10$), 2 days (triangles; $n = 7$), 3 days (open squares; $n = 4$), and 4 days (diamonds; $n = 5$) after surgery. Bars indicate the SE of mean current values. Data points are fitted by Boltzmann-type equations with the following parameters: size, $g_{\text{Kmax}} = 1.46 \mu S$ (filled circles; control), 1.12 $\mu S$ (open circles; one day), 0.75 $\mu S$ (triangles; 2 days), 0.80 $\mu S$ (open squares; 3 days), 0.64 $\mu S$ (diamonds; 4 days); center voltage $V_c = −5.81 \text{mV}$ (control), −3.70 mV (1 day), −4.73 mV (2 days), −5.53 mV (3 days), −5.46 mV (4 days); slope factor $b = 8.41$ (control), 12.9 (1 day), 10.9 (2 days), 11.1 (3 days), 8.0 (4 days). B: $I-V$ curves of peak $I_A$ currents in control (filled circles, $n = 11$) or axotomized neurons (1–3 days; open circles, $n = 11$). Conductance values in axotomized neurons are fitted as above with $g_{\text{Amax}} = 0.50 \mu S$ (vs. 2.0 $\mu S$ in control); $V_c = −27.10 \text{mV}$ (vs. −25.31 mV); $b = 3.3$ (vs. 3.1 mV); C: steady-state $I_A$ inactivation curves in undamaged ($n = 9$) or axotomized neurons after 2 days (triangles; $n = 8$) and 4 days (diamonds; $n = 5$). Bars indicate SE of mean $h_A$ data. Normalized Boltzmann-type equations best-fitting data points have the following parameters: $V_c = −67.35$ (control), −55.71 (2 days), −50.28 (4 days); slope factor $b = 7.93$ (control), −6.66 (2 days), −6.07 (4 days). D: example of complete $I_A$ inactivation removal at −60 mV in a 2-day axotomized neuron.
preganglionic stimulation. The relation between threshold synaptic activation and membrane potential was examined by eliciting orthodromic responses during hyperpolarization produced by graded-current pulses. Reduced synaptic potential amplitude is expected to decrease the safety factor of synaptic transmission even in neurons that are still able to be discharged at the membrane potential level spontaneously verified in the experiment. This was systematically observed in axotomized neurons: membrane potential shifts of only a few millivolts, artificially imposed to the soma, were sufficient to block the action potential discharge (Fig. 6B), whereas in control neurons the synaptic input was able to fire the neuron held at a resting potential of −90 mV (Fig. 6A) or even more negative levels.

**I_A: Threshold Charge and Repolarization.** Spikes evoked under current clamp at different holding potentials were analyzed, in control and axotomized neurons, to examine the effects of I_A on spike genesis. Two properties of I_A are specifically important in this respect: 1) If holding voltage is sufficiently negative to remove steady-state I_A inactivation (negative to −50 mV), outward I_A is activated by depolarizing voltage steps in the subthreshold voltage range sufficiently to increase the inward charge required to fire the neuron, by shunting the excitatory drive (Sacchi et al. 1998). 2) Removal of I_A inactivation increases action potential depolarization rate; the latter therefore depends on the holding potential and increases with increasing membrane negativity (Belluzzi et al. 1985). The threshold inward charge needed to fire control or 1- or 2-day axotomized neurons has been determined to investigate the first point (Fig. 7A). The data show that the inward charge is similar in the three groups at −40 to −50 mV (with I_A virtually inactivated). However, when the holding potential is made more negative (progressive I_A inactivation removal) threshold inward charge increases in control neurons, although it becomes progressively less sensitive to hyperpolarization in axotomized neurons (where I_A is strongly reduced). Statistical analysis (two-way ANOVA) confirmed voltage dependency of the threshold inward charge in the −40- to −70-mV range (F = 63.4, P < 0.01) and its decrease after axotomy (F = 11.0, P < 0.01); the voltage dependency is also significantly attenuated in axotomized neurons (F = 5.18, P < 0.01).

With respect to the second point, the expected increase in repolarization rate with increasing membrane prestimulus negativity (evoked I_A becomes larger) was observed in control neurons. Conversely, the repolarization rate was reduced in 2-day axotomized neurons at all holding potentials, as a consequence of I_A depression and reduced total potassium currents (Fig. 7B). Statistical analysis (two-way ANOVA) confirmed the voltage dependency of the repolarization rate in the −40- to −70-mV range (F = 14.2, P < 0.01) and the marked decrease (about −30% at −70 mV) produced by axotomy (F = 27.5, P < 0.01).

**Spike Depolarization Rate and Overshoot Amplitude.** Changes in voltage-dependent Na+ conductance might also play a role in excitability of the axotomized neuron, as shown in bullfrog sympathetic neurons (Jassar et al. 1993) or rat dorsal root ganglion neurons (Abdulla and Smith 2002). In this respect, we have only indirect data from current-clamp observations. Spike depolarization rate, measured over the −30- to +0-mV voltage range, was significantly increased in 1- to 2-day axotomized neurons (175.7 ± 5.5 V/s, n = 19, vs. 147 ± 6.8 V/s, n = 16, in control neurons held at −60 mV; Student’s t-test, P < 0.01) and overshoot amplitude was similarly increased in the same neuron groups (26.8 ± 1.3 vs. 20.1 ± 1.6 mV in control; P < 0.01).

*Simulations of ganglionic synaptic transfer*

A multiconductance model of the somatic membrane and of the synaptic mechanisms operating in the mature rat sympathetic neuron was developed and discussed in previous papers (Belluzzi and Sacchi 1991; Sacchi et al. 1998, 1999). We refer to those papers for the detailed description of the seven separate types of voltage-dependent ionic conductances and of the fast synaptic nicotinic conductance. The complete electrical model of the sympathetic neuron is illustrated in Fig. 7C for clarity and a list of the constants and equations describing the voltage dependency of the variables used here for the normal
The sympathetic neuron is given in the appendix. The simplifications adopted are: 1) the delayed potassium current is modeled as a single lumped current, \( I_{KD} \), resulting from the summated \( I_{K(V)} \) and \( I_{K(Ca)} \); 2) the potassium ion battery is considered to be constant. The model has been updated with new equations describing the delayed potassium and \( I_A \) currents in axotomized neurons (see legend to Fig. 5 and appendix). The model, modified to account for changes brought about by axotomy, is used to simulate dynamically the synaptic activation of the neuron under current-clamp conditions revealing the participation of the individual components in information transfer through the ganglionic synapse.

The possible inward rectification of the nicotinic channel, described by Fieber and Adams (1991) and Mathie et al. (1990) in dissociated neurons and impairing outward current flow through the synaptic channel, has no practical influence on any part of the simulated action potential (Sacchi et al. 1998).

The threshold synaptic conductance for spike triggering was first evaluated in the mathematical model for control and 1- to 3-day axotomized neurons. Spikes were evoked by injecting just suprathreshold EPSCs in the ideal neuron, held in the \(-45\) to \(-100\)-mV membrane potential range. Computed EPSCs were generated by the model as a function of the synaptic conductance value tested. The results of these simulations are shown in Fig. 8A; they favorably compare with the companion data obtained from native neurons (Fig. 7), demonstrating the close relationship between membrane potential and synaptic power required to activate the neuron. The mean synaptic conductance measured in control neurons is always potentially capable of firing the neuron only when held at a membrane potential positive to \(-80\) mV; this value changes to \(-60\) mV in the 2-day axotomized neuron (Fig. 8A). The physiological level of membrane potential in the resting sympathetic neuron is not well defined; a range of potential more likely exists in each neuron, into which a variable membrane potential can fluctuate (Sacchi et al. 1999). In any case, synaptic transmission is expected to fade in the ganglion within the very first days after axotomy.

Computed action potentials, and the underlying ionic and synaptic currents, in the ideal normal or 1-day axotomized neuron are compared in Fig. 8, B and C. In this example, neurons were held at \(-80\) mV and the threshold synaptic conductance was first determined and then used in simulations. Simulations confirm the major experimental findings: 1) a lower amount of inward charge—either directly or synaptically applied—is required to fire the axotomized neuron; 2) despite the increased excitability, the safety factor for the synaptically evoked spike progressively decreases until transmission fails, within a couple of days after surgery; 3) the crucial role of the somatic membrane potential level in the interplay between the neuron excitability machinery and the efficacy of its synaptic drive is confirmed; 4) \( I_A \) contribution to spike electrogenesis is less prominent in the axotomized neuron, even though the reduced maximal \( g_A \) is partly compensated by the shift to the right of the steady-state \( I_A \) inactivation curve (Fig. 5C); 5) \( I_A \) impairment affects the spike-falling phase.

**Discussion**

Electrophysiological changes after axotomy have been widely studied in many neuron types under current-clamp conditions.
conditions (see, e.g., Titmus and Faber 1990). Functional studies on axotomized naïve sympathetic neurons, however, are not numerous (Gordon et al. 1987; Jassar et al. 1993, 1994; Sánchez-Vives and Gallego 1993, 1994) and very few are those in which the synaptic transmission process through the ganglionic synapse was considered as a whole (De Castro et al. 1995; Hunt and Riker 1966; Purves 1975). Previous observations led to a widely accepted description of the effects of axotomy, which can be summarized as follows: 1) synaptic transmission rapidly fades within a few days as a consequence of the progressive detachment of the presynaptic nerve endings from the injured neuron (this effect is well documented by electron microscopy: Matthews and Nelson 1975; Purves 1975); 2) neuronal excitability is increased, occasionally accompanied by incidence of spontaneous activity; 3) action potentials are preserved but their duration increases; 4) the electrotonic properties of the resting neuron are scarcely affected.

Successive voltage-clamp analyses clarified the basic mechanisms underlying some of these effects, by demonstrating that major targets of axotomy were the delayed potassium currents and the accompanying calcium current (Abdulla and Smith 2001; Everill and Kocsis 1999; Jassar et al. 1993, 1994; Lancaster et al. 2002; Yang et al. 2004) and, in some cells, the sodium current (Abdulla and Smith 2002; Jassar et al. 1993). The present results on delayed outward currents in axotomized sympathetic neurons parallel previous observations on dorsal root ganglion neurons (Abdulla and Smith 2001), bullfrog sympathetic neurons (Jassar et al. 1993, 1994), and vagal afferent neurons (Lancaster et al. 2002), in which attenuation in \( g_{Kc} \) and \( g_{KV} \) conductances have been demonstrated. In those studies, however, apart from an isolated observation by Everill and Kocsis (1999), who did not investigate functional consequences, \( I_A \) proved to be absent or not activatable; thus its direct involvement was never taken into account as a major determinant of neuron firing.
One of the most consistent findings reported in injured neurons is that axotomy reduces the amount of depolarizing current that is required to discharge an action potential. Activation of injured sympathetic neurons actually reveals clear-cut effects on threshold inward charge for firing and on spike repolarization; furthermore, both effects appear to depend substantially on the momentary membrane potential level onto which activity is raised. Reduced \(I_{KD}\) intensity per se justifies a decrease in repolarization power. The more complex voltage-dependent behavior of the axotomized neuron, however, can be explained only if effects of \(I_A\) flow are also considered because its main features arise from the voltage dependency of \(I_A\) inactivation removal and from overall \(I_A\) impairment. It will be noted that the major effects on spike repolarization and threshold inward charge are actually sustained by the qualitative current modifications in the excitability machinery \((I_A\) impairment) and to a lesser extent by the quantitative \(I_{KD}\) changes. Although strong, the decrease in \(I_{KD}\) by itself results in irrelevant changes in the current-clamp behavior of the axotomized neuron in the –40 to –50-mV holding potential range; differences arise only when voltage evokes \(I_A\) participation. Taken together, these data confirm the notion that \(I_A\) and the momentary membrane potential play central roles in controlling the excitability machinery and the electrical behavior not only of the normal, but also of the injured sympathetic neuron.

The reversible synaptic depression that follows postganglionic axon interruption is largely sustained by morphological changes. The severe decline of transganglionic synaptic transmission, in fact, is correlated with detachment of presynaptic nerve endings and loss of ultrastructurally identifiable postsynaptic sites. In the rat SCG the majority of synapses disappear within 1–3 days after section of the postganglionic nerves (Del Signore et al. 2004; Purves 1975), in parallel with synaptic depression measured in intracellular recordings (Purves 1975). Vacant but normal-looking presynaptic terminals were detected in axotomized ganglia, which provide direct evidence of separation of synapses from the postsynaptic site, without any major ultrastructural changes in the presynaptic element itself (Matthews and Nelson 1975). Moreover, the detached presynaptic terminals might still function normally because they have been shown to be able to sustain ACh release ≤3 wk after postganglionic axotomy (Brown and Pascoe 1954). The present functional evidence, that EPSC amplitude rapidly fades, is in line with the conclusion that the number of active synapses on the sympathetic neuron progressively decreases. Miniature EPSC amplitude, ACh null potential, and the overall capacity to release transmitter quanta during high-frequency preganglionic stimulation, however, are not compromised by neuron axotomy, suggesting that the active synaptic input onto the neuron reflects the number of surviving synapses, each of them exhibiting quasi-physiological pre- and postsynaptic properties. The progressive amplitude decrease of synaptic currents—that retain their normal shape—during development of postinjury effects would also suggest that the detachment process is ultimately a fast event, which has no clear electrophysiological correlates in its intermediate steps.

Transmitter release facilitation is revealed during preganglionic tetanization of axotomized neurons and early depression is less pronounced in paired-pulse experiments; facilitation, on the other hand, is unusual in normal boutons of intact sympathetic ganglia, which preferably exhibit synaptic depression at the frequencies experienced here. This is the only observation suggesting a possible defect at those synapses that remain on neurons after axon injury. Depression is also observed at the mammalian neuromuscular junction during repeated stimulation. However, when calcium ion concentration is lowered or when autoantibodies apparently target the voltage-gated Ca\(^{2+}\) channels that regulate ACh release at motor nerve terminals (the Lambert–Eaton myasthenic syndrome), reduced quantal content and facilitation of end-plate potential amplitudes during repetitive high-frequency stimulation typically occur. The mechanisms underlying this adaptive change are unclear, nor do we have evidence of a direct involvement of presynaptic calcium current in axotomized ganglia; this behavior may reflect a common compensatory process to sustain transmission in the face of an impairment of the nerve terminal function.

Denervation of rat SCG was recently studied in companion experiments to those reported here (Sacchi et al. 2005). With respect to the somatic biophysical aspects, denervation and axotomy both result in profound modifications of the neuron conductance complement, the major target being potassium current amplitude and kinetics. These effects are early and reversible in the case of denervation, but progressive and apparently irreversible in the case of axotomy. Direct neuron injury or deprivation of its normal connectivity presumably can act through different mechanisms, or at least with different time courses and reversibility (for axotomy-related neuron damage, see Ma et al. 2003; Stoll and Müller 1999; Waxman et al. 1999). The ultimate phenomenological results, however, are remarkably similar, as if the whole pre- and postsynaptic machinery of the neuron were equally sensitive to very different external agents. In this concern it will be noted that the presynaptic terminal and the postsynaptic neuron are mutually affected by injury of either element. Together with the somatic effects, relevant changes occur in the undamaged presynaptic terminals when the postsynaptic neuron is injured (present data); with the same time course as in axotomy, virtually identical postsynaptic modifications accompany degeneration of the presynaptic terminals in the denervated, but otherwise intact, sympathetic neuron (Sacchi et al. 2005).

The present results emphasize the advantage of studying a native neuron, maintained in the intact tissue under quasi-physiological conditions. Not only the natural contacts between preganglionic fibers and principal neurons, but the biophysical cell profile itself are fully preserved. The naïve sympathetic neuron (Belluzzi and Sacchi 1991; Sacchi et al. 1998) and the same cultured neuron (Malin and Nerbonne 2000; Marrion et al. 1987; Marsh and Brown 1991; McFarlane and Cooper 1993; Schofield and Ikeda 1988) have been intensively investigated. Data collected in the two experimental systems, however, cannot be mutually extrapolated from one system to the other because of the profound changes occurring after neuron dissociation: amplitudes of single macroconductances are decreased (each of them by at least one order of magnitude); the activation–deactivation–inactivation kinetics of the various ionic currents slow down; the list itself of the activatable conductances is modified: additional conductance components not present in the native neuron appear (such as a slow component of \(I_Na\)), whereas others are hardly detected [the slow component of \(I_Na\) (Belluzzi and Sacchi 1986) and, in many cultured cells, \(I_A\) itself].

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The results here reported suggest that axon injury and interruption of the physiological connectivity of the neuron do induce measurable changes in the somatic electrical properties, but the design of neuronal excitability is not profoundly altered, at a difference with the qualitative and functionally more relevant changes produced by isolation of the neuron and extraction from its natural environment.

**APPENDIX**

Lists of the constants (mean values) and of the equations (drawn from the Hodgkin–Huxley kinetic scheme), used in the numerical reconstruction of the action potential in the undamaged sympathetic neuron at 37°C, according to the model of Fig. 7C, are reported herein. For details see Belluzzi and Sacchi (1991) and Sacchi et al. (1995, 1998).

1) Equilibrium potentials (mV) and membrane capacitance (nF): $E_{Na} = +40; E_{Ca} = +145; E_{K} = -93; E_{Cl} = -73; E_{Cl}_{Ca} = -12.8; C_m = 0.24$. 

2) Peak conductances (µS) and permeability (cm/s): $g_{m} = 16.7; g_{h} = 2.1 \times 10^{-2}; g_{A} = 2.0; g_{K_{D}} = 1.46; g_{K_{D}} = 0.08; g_{syn} = 0.93$.

3) Sodium current, $I_{Na}$
- Steady-state activation: $m_a = 1/[1 + \exp(-(36.0 - V)/7.2)]$
- Steady-state inactivation: $h_a = 1/[1 + \exp((V + 53.2)/6.5)]$
- Activation–deactivation time constant of $m$ (ms): $\tau_m = 0.66 + 1/[430.0 \exp(0.089V) + 0.92 \exp(-0.033V)]$
- Inactivation time constant of $h$ (ms): $\tau_h = (0.0055V + 0.3 + 50.8)/(1 + \exp(-(59.5 - V)/7.9) + (1 + \exp((V + 40.9)/1.56))]$
- Calcium current, $I_{Ca}$
- Steady-state activation: $c_a = 1/[1 + \exp(-(8.1 - V)/9.8)]$
- Steady-state inactivation: $h_c = 1/[1 + \exp((V + 19.9)/4.5)]$
- Activation–deactivation time constant of $c$ (ms): $\tau_c = 0.1 + 1/[0.34 \exp(0.092V) + 1.88 \exp(-0.007V)]$
- Inactivation time constant of $h_c$ (ms): $\tau_{hc} = 17 + 1/[0.24 \exp(0.145V) + 0.04 \exp(-0.02V)]$
- Fast transient potassium current, $I_{K}$
- Steady-state activation: $a_w = 1/[1 + \exp(-43.0 - V)/13.2]$.
- Steady-state inactivation: $h_w = 1/[1 + \exp((V + 67.3)/7.9)]$
- Activation–deactivation time constant of $a$ (ms): $\tau_a = 0.2 + 1/[3.7 \exp(0.039V) + 0.05 \exp(-0.045V)]$
- Inactivation time constant of $h_a$ (ms): $\tau_{ha} = (0.04V + 6.0 + 87.0)/(1 + \exp(-(0.80 - V)/5.0)] + (1 + \exp((V + 55.0)/1.4))]$
- Delayed potassium current, $I_{KD}$
- Steady-state activation: $k_d = 1/[1 + \exp(-5.8 - V)/8.4)]$
- Activation–deactivation time constant of $kd$ (ms): $\tau_{kd} = 1.4 + 1/[1.292 \exp(0.274V) + 0.004 \exp(-0.126V)]$
- Synaptic current, $I_{syn}$
- EPSC onset time constant ($\tau_\alpha$, ms): 0.57
- EPSC decay time constant ($\tau_\beta$, ms): 5.56 $\exp(-0.004V)$

**Modifications of the model adapted to the axotomized neuron (3 days)**

1) Fast transient potassium current, $I_{K}$
- $\tilde{g}_A = 0.50 \mu S$
- Steady-state activation: $a_w = 1/[1 + \exp(-43.1 - V)/11.3]$
- Steady-state inactivation: $h_a = 1/[1 + \exp((V + 53.1)/6.4)]$

2) Delayed potassium current, $I_{KD}$
- $\tilde{g}_{KD} = 0.80 \mu S$
- Steady-state activation: $k_d = 1/[1 + \exp(+0.5 - V)/11.1)]$

**REFERENCES**


