Axotomy-Induced Expression of Calcium-Activated Chloride Current in Subpopulations of Mouse Dorsal Root Ganglion Neurons

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Axotomy-induced expression of calcium-activated chloride current in subpopulations of mouse dorsal root ganglion neurons. J Neurophysiol 90: 3764–3773, 2003. First published August 27, 2003; 10.1152/jn.00449.2003. Whole cell patch-clamp recordings of calcium-activated chloride current \( I_{\text{Cl(Ca)}} \) were made from adult sensory neurons of naive and axotomized mouse L4-L6 lumbar dorsal root ganglia after 1 day of culture in vitro. A basal \( I_{\text{Cl(Ca)}} \) was specifically expressed in a subset of naive medium-diameter neurons (30–40 μm). Prior nerve injury, induced by sciatic nerve transection 5 days before experiments, increased both \( I_{\text{Cl(Ca)}} \) amplitude and its expression in medium-diameter neurons. Moreover, nerve injury also induced \( I_{\text{Cl(Ca)}} \) expression in a new subpopulation of neurons, the large-diameter neurons (40–50 μm). Small-diameter neurons (inferior to 30 μm) never expressed \( I_{\text{Cl(Ca)}} \). Regulated \( I_{\text{Cl(Ca)}} \) expression was strongly correlated with injury-induced regenerative growth of sensory neurons in vitro and nerve regeneration in vivo. Cell culture on a substrate not permissive for growth, d,l-polyornithine, prevented both elongation growth and \( I_{\text{Cl(Ca)}} \) expression in axotomized neurons. Regenerative growth and the induction of \( I_{\text{Cl(Ca)}} \) expression take place 2 days after injury, peak after 5 days of conditioning in vivo, slowly declining thereafter to control values. The selective expression of \( I_{\text{Cl(Ca)}} \) within medium- and large-diameter neurons conditioned for rapid, efficient growth suggests that these channels play a specific role in postinjury behavior of sensory neuron subpopulations such as neuropathic pain and/or axonal regeneration.

INTRODUCTION

Axotomy of adult peripheral neurons induces both rapid axon regeneration and neuropathic pain. It has been demonstrated in vivo (Jacob and McQuarrie 1993; Tanaka et al. 1992) and in vitro (Lankford et al. 1998; Smith and Skene 1997) that conditioning lesions increase the ability of the associated primary afferent neurons to regenerate successfully. In contrast, unsuccessful regeneration after nerve injury is thought to be associated with persistent spontaneous electrical activity, which plays an important role in the occurrence and maintenance of pain-related behavior in both animal models and humans (Devor and Seltzer 1999; Gracely et al. 1992; Lindenlaub and Sommer 2000; Ochs et al. 1989). It is well established that axotomy induced alterations in somata membrane current properties such as changes in sodium channel expression and calcium and potassium current amplitude (Abdulla and Smith 2001a; Andre et al. 2003; Baccei and Kocsis 2000; Everill and Kocsis 1999; Sleeper et al. 2000). Although the subpopulations of afferent neurons that develop subthreshold oscillations and ectopic impulse activity after nerve injury include both medium to large cutaneous and muscle myelinated A afferents (Liu et al. 2000b, 2002; Michaels et al. 2000; Tal et al. 1999), no specificity of axotomy-induced ionic channel alteration is reported relative to either the myelinated A neurons or the small unmyelinated C neurons except for sodium channel expression in C neurons (Waxman et al. 1999). Moreover, recent studies demonstrate that neighboring uninjured neuron could also develop ongoing discharges probably related to the products issued from the Wallerian degeneration that first occurs after axotomy (Ma et al. 2003; Michaels et al. 2000; Obata et al. 2003; Wu et al. 2001). From these results it appears that some of the electrophysiological modifications induced by nerve injury could be unrelated to pain behavior but rather be assimilated to adaptive responses to this environmental stress. Presently, no electrophysiological data are available concerning possible correlation with the physiological process that does occur after nerve injury, namely the axonal regeneration.

Calcium ions through their numerous effectors are probably involved both in electrical hyperexcitability (Ayar et al. 1999; Xing and Hu 1999) and in axonal growth (Fields 1998) after nerve injury. Among the ionic effectors of calcium, chloride channels are the less studied. Calcium-activated chloride channels are activated by an increase in intracellular calcium concentration after either external calcium entry or internal calcium release (Scott et al. 1995). In peripheral sensory neurons, calcium-activated chloride currents are developmentally regulated with a peak in expression during peripheral synaptogenesis (Bernheim et al. 1989). They persist under physiological conditions, in a subset of postnatal and adult dorsal root ganglion (DRG) neurons (Abdulla and Smith 1999; Currie and Scott 1992; Lovinger and White 1989). In naive DRG neurons, \( I_{\text{Cl(Ca)}} \) activation promotes afterdepolarization after spike firing (De Castro et al. 1997; Mayer 1985), and in adult sympathetic and nodose ganglion neurons, \( I_{\text{Cl(Ca)}} \) expression is upregulated by axotomy (Lancaster et al. 2002; Sanchez-Vives and Gallego 1994). Besides a potential role in electrical activity, \( I_{\text{Cl(Ca)}} \) has been shown to be involved in other cellular functions such as...

In this study, we performed a detailed analysis of $I_{\text{Cl(Ca)}}$ expression in adult lumbar DRG neurons in naive mice and at various time points after sciatic nerve transection. We used size criteria to identify different functional DRG neuronal subpopulations (Harper and Lawson 1985a,b) and the elongating mode of axonal growth to select regenerating axotomized neurons in vitro as previously demonstrated (Smith and Skene 1997). In naive mice, $I_{\text{Cl(Ca)}}$ is expressed in a subset of medium-diameter neurons. Nerve injury strongly and specifically upregulated $I_{\text{Cl(Ca)}}$ in the population of medium-diameter neurons and induced its expression in large-diameter neurons.

**METHODS**

**Animals and surgery**

Adult female Swiss mice (6–12 wk old, CERJ, Le Genest St Isle, France) were housed in cages with a 12/12 h light/dark cycle and fed food and water ad libitum. The care and use of mice conformed to institutional policies and guidelines. Mice were deeply anesthetized by intraperitoneal injection of equithesin (0.6% pentobarbital sodium and chloral hydrate (0.4 ml/100 g body wt)). The left sciatic nerve was exposed at the mid-thigh level and sectioned, and a 3- to 5-mm fragment of nerve was removed. For retrograde neuronal tracing, surrounding tissues were treated with gauze and each proximal sciatic stump was inserted into a plastic capsule filled with 1 μl of 5% true blue chloride (Molecular Probes) diluted with sterile distilled water. The stumps were kept in position and protected from light for 30 min. When time was up, the capsules were removed and proximal stumps were rinsed with a 9 g/l NaCl solution and wiped clean. Mice were kept alive for 5–7 days unless otherwise stated. During the first 3 days, a penicillin (20 μg/ml)/streptomycin (0.2 mg/ml) mixture was added preventively to the water supply. Mice were killed by CO$_2$ inhalation followed by cervical dislocation, and their dorsal root ganglia were then removed.

**Cell dissociation**

Neuron cultures were established from lumbar (L$_4$–L$_6$) dorsal root ganglia. Ganglia were successively treated by two incubations with collagenase A (1 mg/ml, Roche Diagnostic, France) for 45 min each (37°C) and then with trypsin-EDTA (0.25%, Sigma, St Quentin Fallavier, France) for 30 min. A two-step collagenase incubation allowed removal of collagen debris and thus improvement of the overall efficiency of enzymatic action. They were mechanically dissociated by passing 8–10 times through the tip of a fire-polished Pasteur pipette in neurobasal (Life Technologies, Cergy Pontoise, France) culture medium supplemented with 10% fetal bovine serum and DNase (50 U/ml, Sigma). Isolated cells were collected by centrifugation and suspended in neurobasal culture medium supplemented with 2% B27 (Life Technologies), 2 mM glutamine, penicillin/streptomycin (20 U/ml, 0.2 mg/ml). Dissociated neurons were counted with a Malassese cell and plated on dL-polynornithine (0.5 mg/ml)-laminin (5 μg/ml)-coated glass cover slips at a density of 2,500 neurons per cover slip and were incubated in an incubator with a humidified 95% air-5% CO$_2$ atmosphere. In preliminary experiments, we checked that neuronal density (from 500 to 3,000 neurons per cover slip) did not affect cell morphology nor channel expression. Two hours after plating, the culture medium was carefully removed and replaced to eliminate dead cells and tissue debris. For survival experiments, cells were counted 2 and 24 h after plating, using phase contrast under a Zeiss photonic microscope. The cells were maintained in culture at 37°C.

**Electrophysiology**

Calcium current, $I_{\text{Ca}}$, and calcium-activated chloride current, $I_{\text{Cl(Ca)}}$, in DRG neurons were recorded after 1 day in vitro (DIV). Whole cell patch-clamp recordings were made at 20–22°C under conditions optimized for the isolation of $I_{\text{Ca}}$ and $I_{\text{Cl(Ca)}}$ separately from other voltage-activated currents. The bathing solution contained (in mM) 130 tetraethylammonium chloride, 2 CaCl$_2$, 1.5 MgCl$_2$, 10 HEPES, and 10 glucose, and the pH was adjusted to 7.4 with CsOH. Recording pipettes were filled with the following solution (in mM): 145 CsCl, 10 HEPES, 2 Mg-ATP, 0.5 and Na$_2$-GTP, pH 7.35, adjusted with CsOH. In most experiments, no calcium buffer was added to allow intracellular calcium variations. To verify whether any calcium contamination in the intracellular medium could occur and modify the results, in a series of experiments on axotomized neurons, we added 0.1 mM EGTA. Under this condition, the number of neurons expressing $I_{\text{Cl(Ca)}}$, and their amplitudes were unchanged (n = 10). Under our experimental conditions, chloride reversal potential was close to 0 mV, and step depolarization to 0 mV was used to record a pure $I_{\text{Cl}}$, whereas repolarization to −80 mV was used to visualize an inward calcium-activated chloride current when present. Ramp protocols from −80 to +40 mV for 500 ms followed by repolarization to −80 mV were routinely used for $I_{\text{Ca}}$ and $I_{\text{Cl(Ca)}}$ recordings. Comparison with current-voltage relationships obtained with rectangular steps showed a 5–15% underestimation of maximum calcium current amplitudes when recorded with ramp protocols and no differences between $I_{\text{Cl(Ca)}}$ amplitudes. Whole cell currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The experimental parameters were controlled with a computer equipped with a Digidata 1200 analogue interface (Axon Instrument). We used pClamp software (Clampex 8.01; Axon Instruments) for data acquisition and analysis. Signals were filtered at 2 kHz and sampled at 4 kHz. Glass electrodes (3–4 MΩ) were made from capillary glass, using a Narishige puller, and coated with paraffin wax to minimize pipette capacitance. The average access resistance was 5.8 ± 3.3 MΩ, n = 180. Measurements of cell capacitance was determined by fitting a single-exponential curve to the uncompensated current trace resulting from a voltage-clamp step from −80 to −70 mV. As far as possible, series resistance compensation (40–70%) was used to reduce voltage errors. However, as some cells had large dendritic or axonal processes at 1 DIV, capacitance compensation was not always efficient due to the appearance of a second slower capacitative time constant giving values of capacitance >100 pF. We did not carry our current-voltage protocols in such neurons. Instead, we simply scored the presence of a chloride current and estimated from a ramp protocol its maximum amplitude on repolarization to −80 mV. For each experiment, cell size was estimated by means of an eyepiece micrometer scale.

**Visual and quantitative analysis of neurite outgrowth**

Cells were fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4, treated for 5 min in phosphate-buffered saline (PBS) containing 0.3% Triton X100, and incubated for 30 min in 10% normal goat serum in PBS. They were then incubated overnight at 4°C with the monoclonal anti-neurofilament 200 (phosphorylated and nonphosphorylated), clone NS2 (1:400; Sigma). The cells were then incubated for 2 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (1:200; Jackson Labs, Interchim France) and were mounted in FluorSave (Calbiochem, La Jolla, CA). Neurite-length analysis was carried out on fixed cultures displayed on a video monitor. We classified neurons as described by (Smith and Skene 1997): neurons with processes with >1.5 branches/100 μm were classified as arborizing, whereas those with <1.5 branches/100 μm were classified as elongated. Neurons with neurites shorter than one cell diameter were classified as having no neurites. The percentages of neurons with various types of mor-

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morphology were determined by counting, using microscopy at ×20 magnification.

Statistics

Data are expressed as means ± SE. Statistical evaluations are based on ANOVA, *t*-test, or $\chi^2$ test as appropriate. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Biophysical properties of $I_{\text{Cl(Ca)}}$

Whole cell patch-clamp recordings performed on lumbar L₄–L₆ DRG neurons from control animals (naive neurons) showed that following a step depolarization to 0 mV to activate a calcium current, $I_{\text{Ca}}$, an inward tail current was activated on repolarization to −80 mV holding potential. The inward tail current was abolished if step depolarization was set to +80 mV to decrease calcium current strongly (Fig. 1A). The activation kinetics of tail current was dependent on the duration of the depolarizing pulse to 0 mV. Increasing the duration of voltage command from 15 to 400 ms to activate longer $I_{\text{Ca}}$ induced a progressive increase in inward tail current amplitude. Half time of activation was 62 ± 18 ms, and maximal activation was achieved within 250 ± 50 ms, $n = 11$ (not shown). In ~10% of experiments, an inward tail current could be observed on repolarization but did not display the calcium dependence as defined in the preceding text. These experiments were discarded. Finally, blocking $I_{\text{Ca}}$ with 100 μM CdCl₂ and 50 μM NiCl₂ resulted in inhibition of the inward tail current, confirming that it was indeed calcium dependent ($n = 4$, not shown).

For pharmacological characterization of the tail current, we used ramp protocols facilitating the rapid evaluation of currents activated between −80 and +40 mV and accurate estimation of the maximum amplitude of $I_{\text{Ca}}$ and inward tail current. Bath application of 100 μM niflumic acid, a chloride channel inhibitor, rapidly induced a decrease (74 ± 12%, $n = 8$) in tail current, which in most cases was followed by a gradual decrease in $I_{\text{Ca}}$ amplitude (Fig. 1B). 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB), an open-chloride channel blocker, at 100 μM induced a decrease (30.8 ± 4.2%, $n = 4$) in inward tail current amplitude, followed by a decrease in $I_{\text{Ca}}$ amplitude. Furosemide, an inhibitor of the chloride transport-
ers, at a concentration of 200 μM, had no effect \((n = 3)\). The reversal potential \(E_{\text{rev}}\) of the tail current, obtained by extrapolating the data recorded between \(-120\) and \(-40\) mV, was \(1.5 \pm 6.6\) mV, \(n = 6\) with internal and external solutions both containing 145 mM chloride ions (Fig. 1C). Under partial substitution of methane sulfonate (30 mM CsCl /15 mM Cs-methane sulfonate) for intracellular chloride ions, \(E_{\text{rev}}\) was \(-36 \pm 9\) mV, \(n = 5\), indicative of chloride conductance responsible for the tail current. Thus the inward tail current is a calcium-activated chloride current, \(I_{\text{Cl(Ca)}}\).

**Cell expression of \(I_{\text{Cl(Ca)}}\) in naive cultures is specific to a subset of medium-diameter neurons**

In naive cultures, \(I_{\text{Cl(Ca)}}\) was not recorded in all DRG neurons. Instead, it was exclusively expressed in a medium-diameter neuronal population with a somatic diameter of 30–40 μm and a membrane capacitance of 40–70 pF (55 ± 12 pF, \(n = 68\)). We detected no \(I_{\text{Cl(Ca)}}\) in small neurons (diameter <30 μm, \(n = 20\)) or in large neurons (diameter >40 μm, \(n = 20\)). Based on the level of expression of low-voltage-gated \(I_{\text{Ca}}\) with respect to high-voltage-gated \(I_{\text{Ca}}\), two typical electrophysiological profiles were identified in the medium-diameter neuron population. In the type 1 population, the low-voltage-gated \(I_{\text{Ca}}\) was either absent or of small amplitude (\(-0.30 \pm 0.05\) nA, \(n = 15\)), whereas the high-voltage-gated \(I_{\text{Ca}}\) was always present. This population accounted for 81% of the medium-diameter neurons (86/106 medium-diameter neurons investigated). Within this type 1 population, \(I_{\text{Cl(Ca)}}\) was recorded in 26 neurons classified either as type 1a [17 neurons expressed a small amplitude \(I_{\text{Cl(Ca)}}\)] or as type 1b [10 neurons expressed a large amplitude \(I_{\text{Cl(Ca)}}\) activated by a smaller amplitude high-voltage-gated \(I_{\text{Ca}}\) (Fig. 2A and Table 1)]. However, in the remaining 59 type 1 neurons, the opening of a high-voltage-gated \(I_{\text{Ca}}\) (\(-6.3 \pm 0.5\) nA, \(n = 59\)) did not activate \(I_{\text{Cl(Ca)}}\). The type 2 population was characterized by a large low-voltage-gated \(I_{\text{Ca}}\) (\(-3.8 \pm 0.4\) nA, \(n = 20\); Fig. 2A). This population accounted for 19% of the medium-diameter neurons (20/106). In 40% (8/20) of this subset of neurons, calcium entry through voltage-gated \(I_{\text{Ca}}\) activated \(I_{\text{Cl(Ca)}}\). Thus in naive cultures, 33% of medium-diameter DRG neurons expressed \(I_{\text{Cl(Ca)}}\) (Fig. 2B).

**Nerve injury-induced \(I_{\text{Cl(Ca)}}\) expression in medium- and large-diameter neurons**

Five days after nerve injury, \(L_{\text{C}}\)-\(L_{\text{L}}\) ganglia from the operated and nonoperated sides were removed for dissociation. We counted neurons at the end of dissociation, before plating and found that there were no significant differences among naive (2,900 ± 280 neurons per ganglion, \(n = 10\)), contralateral (2,950 ± 350 neurons per ganglion, \(n = 7\)), and operated ganglia (2,750 ± 220 neurons per ganglion, \(n = 7\); \(P > 0.05\), \(t\)-test). Cell counting 2 h after plating and after 1 DIV showed that no significant cell death occurred in naive (90 ± 5% cell survival, \(n = 10\)), contralateral (86 ± 10% cell survival, \(n = 7\)), and axotomized cultures, (i.e., operated ganglia, 87 ± 7% cell survival, \(n = 7\); \(P > 0.05\), \(t\)-test). Experiments performed on neurons from contralateral cultures gave similar results to those performed on neurons from naive cultures in terms of

\[\text{Fig. 2.} \quad I_{\text{Cl(Ca)}}\] is selectively expressed in a subset of medium-diameter neurons from adult naive cell cultures. A: current traces were obtained with voltage-ramp protocols, and maximal current amplitudes were measured (see METHODS). Neurons expressing \(I_{\text{Cl(Ca)}}\) had 2 major electrophysiological profiles based on the ratio of low-voltage-gated \(I_{\text{Ca}}\) to high-voltage-gated \(I_{\text{Ca}}\), peak amplitudes. Type 1 has a ratio \(<1\) and type 2 a ratio \(>1\). Note that \(-10% of type 1 neurons expressed an \(I_{\text{Cl(Ca)}}\) of particular large amplitude associated with a weak-amplitude, high-voltage-gated \(I_{\text{Ca}}\). B: not all adult DRG neurons express \(I_{\text{Cl(Ca)}}\) which was detected in only 33% of medium-diameter neurons. No \(I_{\text{Cl(Ca)}}\) was detected in small- and large-diameter populations.

\(I_{\text{Cl(Ca)}}\) expression [19% of medium neurons; 4/21; \(\chi^2\) test \(P = 0.5\) with respect to naive neurons; no expression in small (\(n = 10\)) or large (\(n = 10\)) neurons]. The distribution of medium-diameter neurons with respect to \(I_{\text{Ca}}\) was similar in naive, contralateral, and axotomized cultures. Type 1 and 2 subpopulations accounted for 85 and 15% of axotomized medium-diameter neurons, respectively (Fig. 3A). However, \(I_{\text{Cl(Ca)}}\) expression was significantly stronger in axotomized cultures than in naive and contralateral cultures. In medium-diameter neurons, the percentage of cells expressing \(I_{\text{Cl(Ca)}}\) increased from 33 to 63% after prior nerve injury (\(\chi^2\) test \(P < 0.01\) relative to naive neurons). In addition to the medium-diameter population, 56% of large neurons expressed \(I_{\text{Cl(Ca)}}\) under these conditions whereas small neurons did not express \(I_{\text{Cl(Ca)}}\) (\(n = 10\); Fig. 3B). Furthermore, axotomy significantly increased the amplitude of \(I_{\text{Cl(Ca)}}\) in type 1a medium-diameter neurons (Table 1). As previously reported, axotomy also differentially affected the amplitude of high-voltage-gated \(I_{\text{Ca}}\) depending on the cell population (Table 1) and decreased the amplitude of low-voltage-gated \(I_{\text{Ca}}\) in medium type 2 population (see Andre et al. 2003). The use of 100 μM niflumic acid (\(n = 10\)) and estimations of reversal potential (\(n = 5\) and calcium dependence confirmed that the tail currents induced by nerve injury were calcium-activated chloride currents.
Nerve injury-induced $I_{\text{Cl(Ca)}}$ expression is specific to neurons with elongation growth

Depending on experimental conditions, various modes of cell growth were observed after 1 DIV (Fig. 4A). In naive and contralateral cultures, 100% of sensory neurons had either no neurites, or displayed an “arborizing” mode of growth with extensive branching and modest neurite length (Fig. 4B, a and b). The expression of $I_{\text{Cl(Ca)}}$ in naive and contralateral cultures did not depend on the cell growth mode and, as reported in the preceding text, such currents were detected in 30% of naive medium-diameter neurons (Fig. 4C).

In cultures from axotomized ganglia, 40% of all neurons grew similarly to naive or contralateral cultures (no neurites or “arborized”) with a pattern of $I_{\text{Cl(Ca)}}$ expression similar to that of naive neurons (30% of medium-diameter neurons; 8/27 Fig. 4C). The remaining 60% of DRG neurons had long, sparsely branched axons typical of the “regenerating” mode of growth stimulated by prior nerve injury (Smith and Skene 1997) (Fig. 4, Ac and Bc). In a series of experiments, axotomized neurons were retrogradely labeled with a vital tracer to further correlate axotomy with the elongating growth. Cell counting show that 71 ± 6%, n = 2 of neurons were labeled 5 days after nerve injury. Elongated neurons were all labeled and accounted for 76 ± 2% (n = 2) of labeled neurons (54% of total population). Thus the overall increase in $I_{\text{Cl(Ca)}}$ in these cultures was due to its induction in neurons displaying “regenerative” growth, both in the medium (46/59; 78%)- and in the large (14/18; 78%)-diameter neurons (Fig. 4C). In the medium population, $I_{\text{Cl(Ca)}}$ was expressed in 76% (38/50) of type 1 neurons and in 89% (8/9) of type 2 neurons. Therefore our results demonstrate that the increase in $I_{\text{Cl(Ca)}}$ expression in axotomized neurons is a feature of neurons displaying elongation growth.

$I_{\text{Cl(Ca)}}$ expression is dependent on regenerative growth

As the elongation growth has been demonstrated to be characteristic of the process of regeneration (Smith and Skene 1997), we further investigated the correlation between $I_{\text{Cl(Ca)}}$ expression and nerve regeneration. In experiments performed

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\begin{array}{|c|c|c|c|c|}
\hline
\text{Neuron type} & \text{HVA }I_{\text{Ca}}, \text{nA} & \text{A} & \text{B} & \text{C}
\\hline
\text{Medium, type 1a} & -6.0 \pm 0.6 (17) & -0.9 \pm 0.2 (17) & -2.6 \pm 0.3 (38)** & -2.8 \pm 0.3 (38)**
\\hline
\text{Medium, type 1b} & -1.1 \pm 0.3 (10) & -5.2 \pm 1.0 (10) & -2.7 \pm 0.5 (8)** & -6.7 \pm 0.6 (8)
\\hline
\text{Medium, type 2} & -1.9 \pm 1.0 (8) & -1.9 \pm 0.4 (8) & -2.3 \pm 0.3 (8) & -1.8 \pm 0.5 (8)
\\hline
\text{Large} & -8.5 \pm 0.5 (10) & \text{---} & -3.1 \pm 0.5 (14)** & -2.4 \pm 0.4 (14)
\\hline
\end{array}
\]

Data show the effects of axotomy on high-voltage-gated (HVA) $I_{\text{Ca}}$ and $I_{\text{Cl(Ca)}}$ amplitudes in $I_{\text{Cl(Ca)}}$-expressing neurons. Values are means ± SE with the number of neurons in parentheses. ** P < 0.01 Student’s $t$-test.

FIG. 3. Nerve injury induces the upregulation of $I_{\text{Cl(Ca)}}$ expression in medium-diameter neurons and the appearance of $I_{\text{Cl(Ca)}}$ in large-diameter neurons. A: current traces obtained with voltage-ramp protocols showed that axotomy did not affect the electrophysiological profiles of medium-diameter neurons as defined in naive cultures. B: axotomy induced the expression of $I_{\text{Cl(Ca)}}$ in large-diameter neurons. C: nerve injury induced a significant increase in the number of medium-diameter neurons expressing $I_{\text{Ca}}$ (63%; $P < 0.01$ with respect to naive neurons, $\chi^2$ test) and strong expression in a new population of DRG neurons, the large-diameter neurons (56%).

\[I_{\text{Cl(Ca)}}\]
from naive or axotomized animals on a nonpermissive substrate for growth, d,l-polyornithine (poly-O) alone instead of the usual double coating (poly-O plus laminin), and analyzed cell viability and $I_{\text{Cl(Ca)}}$ expression after 24 h. No difference in cell viability was observed between substrates (90 ± 5 and 89 ± 4% of neurons, $n = 4$, on poly-O and on poly-O plus laminin, respectively $P > 0.05$, $t$-test). Control experiments performed in naive cultures show that poly-O prevented arborizing growth but not basal $I_{\text{Cl(Ca)}}$ expression after 24 h (3 of 15 neurons, 20% of the total population). In cultures from axotomized ganglia, single coating with poly-O totally prevented neurite extension (Fig. 5Aa), and $I_{\text{Cl(Ca)}}$ was recorded in 26.3 ± 18.3% of neurons with diameters of 30–50 µm (5 batches, $n = 16$; Fig. 5B). $I_{\text{Cl(Ca)}}$ amplitude under this condition was $-0.98 ± 0.36 \text{nA}$ ($n = 5$). When neurons from the same culture were grown on a permissive substrate (poly-O plus laminin, Fig. 5Ab), 68.1 ± 14.5% of neurons with diameters of 30–50 µm (4 batches, $n = 19$, $P < 0.01$, $t$-test) expressed $I_{\text{Cl(Ca)}}$; mean amplitude was $3.5 ± 0.9 \text{nA}$ ($n = 11$; $P < 0.01$, $t$-test; Fig. 5B). Thus like elongation growth increased functional expression of $I_{\text{Cl(Ca)}}$ in axotomized neurons takes place after 1 DIV and requires the presence of an appropriate growth substrate.

FIG. 4. Nerve-injury-induced $I_{\text{Cl(Ca)}}$ expression is specific to regenerating neurons. A: phase-contrast microscopy of naive (a), contralateral, (b) and axotomized (c) cultures of adult mouse DRG neurons at 1 day in vitro (DIV). Scale bar is 50 µm. B: representative light micrograph of neurofilament-stained neurons showing the 3 types of growth identified among these cultures: no growth (a), arborized growth (b), and elongation growth (c). Scale bar = 25 µm. C: in naive and contralateral cultures, neurons had either no neurites or an arborized mode of growth. In axotomized cultures, elongation growth was observed in 60% of the neuronal cell population. The graph shows the prevalence of $I_{\text{Cl(Ca)}}$ expression in elongated medium-diameter neurons ($P < 0.01$, $\chi^2$ test with respect to arborized neurons from axotomized cultures) and elongated large-diameter neurons ($P < 0.01$, $\chi^2$ test with respect to arborized neurons from axotomized cultures). The basal expression of $I_{\text{Cl(Ca)}}$ in naive, contralateral and axotomized cultures is specific to arborized neurons and neurons with no neurites, regardless of experimental conditions. These neurons account for ~30% of all medium-diameter neurons.

2–6 h after dissociation, when neurons have not yet extended their neurites, $I_{\text{Cl(Ca)}}$ expression was very low and not significantly different between naive and axotomized cultures (1/6 and 0/6, respectively). We then cultured dissociated neurons

FIG. 5. D,L-polyornithine, a nonpermissive substrate for growth, prevents growth of axotomized neurons and reduces the expression of $I_{\text{Cl(Ca)}}$. A: representative light micrograph of neurofilament-stained neurons from an axotomized culture on d,l-polyornithine substrate (a) and on d,l-polyornithine plus laminin substrate (b). B: random recordings in medium- and large-diameter neurons cultured on d,l-polyornithine demonstrate weak $I_{\text{Cl(Ca)}}$ expression. Experiments performed on neurons from the same cultures grown on d,l-polyornithine plus laminin confirmed the overall increase in $I_{\text{Cl(Ca)}}$ expression in axotomized cultures. Scale bar in A, 50 µm.
Reinnervation in vivo leads to the downregulation of $I_{\text{Cl(Ca)}}$ expression in vitro

We investigated the pattern of $I_{\text{Cl(Ca)}}$ expression over time after axotomy. One day after nerve section, neurons had no neurites or an arborizing mode of growth. Forty-eight hours after injury, 25 ± 4% neurons displayed elongation growth, increasing to 60 ± 7% 1 wk after injury. Thereafter, the number of elongating neurons slowly decreased, reaching 20 ± 2% 30 days after nerve injury and 6 ± 2% 40 days after injury (Fig. 6A). Cell counts after lumbar ganglion dissociation showed that no significant neuronal death had occurred 4 and 6 wk after injury (2,300 ± 100 neurons per ganglion, $n = 2$ and 2,500 ± 100 neurons per ganglion, $n = 2$, respectively). An increase in $I_{\text{Cl(Ca)}}$ expression was observed provided that the neurons displayed elongation growth, (i.e., from 2 days after nerve injury until reinnervation), and the level of expression was constant within this population (14/17; 6/7; 3/4 at 2, 30, and 40 days after injury; Fig. 6A). We also determined levels of expression of $I_{\text{Cl(Ca)}}$ characteristic of neurons that were not elongating, which were also constant at each time point (4/10; 1/5; 1/6; 4/11 at 1, 2, 30, and 40 days after injury, respectively, Fig. 6B).

DISCUSSION

Our study shows that in adult mouse DRG neurons, the weak, basal cellular expression of calcium-activated chloride channels is confined to a subset of medium-diameter sensory neurons. Nerve injury induces both the upregulation of $I_{\text{Cl(Ca)}}$ expression in medium-diameter neurons and its appearance in large-diameter sensory neurons. This $I_{\text{Cl(Ca)}}$ upregulation is correlated with the process of nerve regeneration, both in vitro and in vivo, and involves a part of neuronal subpopulations implicated in abnormal electrical activity.

$I_{\text{Cl(Ca)}}$ expression in naive sensory neurons

We have analyzed the biophysical and pharmacological properties of $I_{\text{Cl(Ca)}}$ recorded in adult mouse L 4–L 6 lumbar DRG neurons. Our results are basically in agreement with those of Mayer (1985) and Currie and Scott (1992) in their initial characterization of $I_{\text{Cl(Ca)}}$ in rat post natal DRG neurons. $I_{\text{Cl(Ca)}}$ was fully activated with 200-ms depolarization and displayed a greater sensitivity to niflumic acid than NPPB. Unlike calcium-activated potassium channels, $I_{\text{Cl(Ca)}}$ was not closely related to a calcium channel type but seemed to depend on the overall amount of calcium entering into the cell, including the large T-type $I_{\text{Ca}}$ characteristic of type 2 population (unpublished observations).

DRG neurons form a morphologically and functionally heterogeneous population (Harper and Lawson 1985a,b), and as in most electrophysiological studies, we used size criteria to identify the neuronal population. As previously reported by others, for different preparations and different developmental stages, not all sensory neurons expressed $I_{\text{Cl(Ca)}}$ (Bernheim et al. 1989; Currie and Scott 1992; Mayer 1985). We found that in adults, ∼30% of medium-diameter neurons expressed $I_{\text{Cl(Ca)}}$. Within this restricted subset of medium-diameter neurons (30–40 μm), we identified two subpopulations on the basis of typical electrophysiological profiles based on T-type calcium current expression. Such heterogeneous expression of T-type calcium currents has been observed in previous studies and is thought to be a key determinant of the electrophysiological behavior of sensory neuron subpopulations (Schroeder et al. 1990). Although a significant $I_{\text{Cl(Ca)}}$ expression required a 24-h delay, it was independent of the growth status of the
neurons because it was recorded in neurons that were not extending neurites and in arborizing neurons as well as in naive neurons cultured on a nonpermissive substrate, such as d,l-polyornithine, which prevented neurite outgrowth. This delay necessary for \( I_{\text{Cl(Ca)}} \) expression could be a consequence of the dissociation procedure that by itself induced an injury. We hypothesized that independently of growth, there are other cellular rearrangements, such as cell volume regulation, that do occur just after such an injury and could lead to this basal expression.

**Inducible \( I_{\text{Cl(Ca)}} \) expression in axotomized DRG neurons**

This study shows that nerve injury induces an increase in \( I_{\text{Cl(Ca)}} \) expression and amplitude. In agreement with previous experiments in vivo (Mayeux and Valmier 1995), we confirmed that sciatic nerve section also did not lead to cell death in vitro. Thus the increase in \( I_{\text{Cl(Ca)}} \) expression cannot be attributed to a selective death of nonexpressing \( I_{\text{Cl(Ca)}} \) neurons. The increase in \( I_{\text{Cl(Ca)}} \) amplitude, its expression in a new population, and its recovery to basal levels after regeneration further confirm that the main effect of nerve injury on \( I_{\text{Cl(Ca)}} \) currents is to increase channel expression. A change in cell size after axotomy may also have an effect, given the specificity of cell expression. In a rat model of peripheral injury, the shrinkage of neuronal cell bodies leading to a shift to smaller sizes after axotomy has been reported (Lankford et al. 1998). However, in our study, the increase in \( I_{\text{Cl(Ca)}} \) expression is exclusive to larger cells, demonstrating that the small-diameter nociceptive population is definitively not involved. Therefore the expression of \( I_{\text{Cl(Ca)}} \) in axotomized DRG neurons identifies this channel protein as an objective electrophysiological marker of most medium- and large-diameter neurons, which are likely to be mechanosensitive (\( \alpha \beta \delta \) fibers) and proprioceptive (\( \alpha \alpha \beta \) fibers).

The increase in \( I_{\text{Cl(Ca)}} \) amplitude of type 1a medium-diameter neurons (85% of the medium-diameter population) is likely to result from an increase in the number of functional channels expressed per neuron. We have also to consider the possibility of regulation at the protein level leading to an increase in the opening probability of \( I_{\text{Cl(Ca)}} \), and changes in calcium homeostasis after axotomy may be involved in such \( I_{\text{Cl(Ca)}} \) regulation (Lancaster et al. 2002; Sanchez-Vives et al. 1994). Although the biophysical and pharmacological properties of \( I_{\text{Cl(Ca)}} \) induced by conditioning lesions were similar to those of basal \( I_{\text{Cl(Ca)}} \), basal and regulated \( I_{\text{Cl(Ca)}} \) could arise from different molecular identities. Indeed, the colocalization of at least two types of calcium-activated chloride channels has been demonstrated in various preparations (Hartzell 1996; Hussy 1992) that could account for the growth-dependent and -independent regulations of \( I_{\text{Cl(Ca)}} \) expression observed in the present work. Consistent with these studies, several genes encoding calcium-activated chloride channels have been cloned, but the molecular basis of neuronal \( I_{\text{Cl(Ca)}} \) remains unknown (Jentsch and Gunther 1997).

**Inducible \( I_{\text{Cl(Ca)}} \) and regeneration**

As previously reported in other studies (Hu-Tsai et al. 1994; Lankford et al. 1998; Smith and Skene 1997), our results show that a conditioning nerve injury induces a new mode of neuronal growth in vitro together with enhanced regeneration. This regenerating mode of growth is characterized by the rapid extension of long, sparsely branched axons. In our sciatic nerve transection model, elongating neurons accounted for 60% of the neuronal population. This value is consistent with the percentage of neurons in L4-L6 DRGs that extend through the sciatic nerve and are thus disrupted by nerve section [60–70%, (Himes and Tessler 1989)]. Retrograde labeling of axotomized neurons shows that 70% of neurons present in our cultures were axotomized and confirms that elongating neurons correspond to axotomized neurons. Interestingly, ~17% of axotomized neurons did not display the regenerating mode of growth. Because recordings from nonelongating neurons that comprise both uninjured and injured non-regenerating neurons (46% of total population) showed a basal \( I_{\text{Cl(Ca)}} \) expression, our data demonstrate that the increase in \( I_{\text{Cl(Ca)}} \) expression induced by nerve injury was specific to axotomized neurons displaying regenerating growth. Unlike some modifications relevant to neuropathic pain (Ma et al. 2003; Obata et al. 2003), the electrophysiological and phenotypical modifications we observed were specifically displayed on injured neurons and not on adjacent uninjured neurons. Thus our results suggest that regulation of \( I_{\text{Cl(Ca)}} \) expression is not associated with Wallerian degeneration but with the regeneration following this process. The observation that a 24-h delay in vitro is necessary for functional protein expression could be related to a preferential localization of the channels on the growing axons in vivo. We cannot rule out an effect of our culture conditions because we demonstrate that the culture substrate is not only essential but also specific for neuronal growth and \( I_{\text{Cl(Ca)}} \) channel expression (see also Guan et al. 2003). Although our experiments do not demonstrate an increased \( I_{\text{Cl(Ca)}} \) expression in vivo, this close relationships between growth competence and \( I_{\text{Cl(Ca)}} \) expression was further confirmed by demonstrating a temporal correlation between the two processes. Competence for elongation growth required 48 h after injury, which is consistent with the results of Smith et al. (Smith and Skene 1997) and is related to the distal injury. \( I_{\text{Cl(Ca)}} \) expression was strictly correlated with the appearance of elongation growth. Several weeks after nerve injury, cell numbers close to those in naive animals confirmed that there was no massive loss of injured neurons due to nerve degeneration but instead, efficient regeneration. After this regeneration in vivo, both the mode of growth and the level of \( I_{\text{Cl(Ca)}} \) expression were similar to those in naive culture. Furthermore, using in vitro experiments, with a substrate nonpermissive for neurite growth, we clearly demonstrated that effective, functional expression of the channel protein after nerve injury is possible provided that the neuron is allowed to extend elongated neurites in vitro. A direct effect of poly-O on \( I_{\text{Cl(Ca)}} \) is unlikely because this substrate did not prevent its basal expression. This growth dependence is unique among ion channels known to be regulated by peripheral lesions. Indeed, unlike \( I_{\text{Cl(Ca)}} \) expression, the decrease in calcium and potassium current amplitudes usually reported after nerve injury are independent of growth (Abdulla and Smith 2001a; Baccelli and Kocsis 2000), and it now appears that some of these electrophysiological changes are not specific to injured neurons (Ma et al. 2003). Thus these results indicate that \( I_{\text{Cl(Ca)}} \) expression is correlated with the process of nerve regeneration both in vitro and in vivo. Altogether, these data suggest that inducible \( I_{\text{Cl(Ca)}} \) may be regulated by factors allowing regeneration.
ICl(Ca) and electrical activity

Besides an enhanced regeneration, many studies have reported that after nerve injury, medium- and large-diameter neurons can develop an increased excitability and a propensity to generate spontaneous ectopic discharges in vivo and in vitro (Abdulla and Smith 2001b; Liu et al. 2000a, 2002). It is striking that under our experimental conditions (distal injury, time after injury, and culture conditions), the neuronal subpopulations expressing ICl(Ca) are those involved in the genesis of abnormal electrical activity: medium- and large-diameter neurons. This result is particularly relevant given that specific ionic modifications induced by nerve injury have been demonstrated exclusively within the small nociceptive population (Cummins and Waxman 1997). In naïve sensory and axotomized sympathetic neurons, ICl(Ca) was shown to promote afterdepolarization after spike firing (De Castro et al. 1997; Mayer 1985), and its increased expression in axotomized DRG neurons could thus contribute to their enhanced excitability. In future studies, determination of the chloride homeostasis as well as of the physiological activator of ICl(Ca) should provide clues to understand ICl(Ca) contribution to electrical activity.

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DISCLOSURES

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REFERENCES


Hartell HC. Activation of different Cl currents in Xenopus oocytes by Ca liberated from stores and by capacitative Ca influx. J Gen Physiol 108: 157–175, 1996.


Xing JI and Hu SJ. Relationship between calcium-dependent potassium channel and ectopic spontaneous discharges of injured dorsal root ganglion neurons in the rat. *Brain Res* 838: 218–221, 1999.