Effects of K⁺ Channel Blockers on Developing Rat Myelinated CNS Axons: Identification of Four Types of K⁺ Channels

JEROME DEVAUX, MAURICE GOLA, GUY JACQUET, AND MARCEL CREST

Laboratoire Intégration des Informations Sensorielles, Centre National de la Recherche Scientifique, 13402 Marseille Cedex 20, France

Received 6 August 2001; accepted in final form 12 November 2001

Devaux, Jerome, Maurice Gola, Guy Jacquet, and Marcel Crest. Effects of K⁺ channel blockers on developing rat myelinated CNS axons: identification of four types of K⁺ channels. J Neurophysiol 87: 1376–1385, 2002; 10.1152/jn.00646.2001. Four blockers of voltage-gated potassium channels (Kv channels) were tested on the compound action potentials (CAPs) of rat optic nerves in an attempt to determine the regulation of Kv channel expression during the process of myelination. Before myelination occurred, 4-aminopyridine (4-AP) increased the amplitude, duration, and refractory period of the CAPs. On the basis of their pharmacological sensitivity, 4-AP-sensitive channels were divided in two groups, the one sensitive to kaliotoxin (KTX), dendrotoxin-I (DTX-I), and 4-AP, and the other sensitive only to 4-AP. In addition, tetraethylammonium chloride (TEA) induced a more pronounced effect than KTX; this indicates that a fourth group of channels sensitive to 4-AP and DTX-I but insensitive to KTX had developed. The effects of KTX and DTX-I gradually disappeared during the period of myelination. Electron microscope findings showed that the disappearance of these effects was correlated with the ongoing process of myelination. This was confirmed by the fact that DTX-I and KTX enlarged the CAPs of demyelinated adult optic nerves. These results show that KTX- and DTX-sensitive channels are sequestered in paranodal regions. During the process of myelination, KTX had less pronounced effects than DTX-I on demyelinated nerves, which suggests that the density of the KTX-sensitive channels decreased during this process. By contrast, 4-AP increased the amplitude, duration, and refractory period of the CAPs at all the ages tested and to a greater extent than KTX and DTX-I. The effects of TEA alone also gradually disappeared during this period. However, effects of TEA on CAPs were observed when this substance was applied after 4-AP to the adult optic nerve; this shows that TEA-sensitive channels are not masked by the myelin sheath. In conclusion, the process of myelination seems to play an important part in the regulation and setting of Kv channels in optic nerve axons.

INTRODUCTION

Peripheral myelinated fibers have been found to contain three main voltage-gated potassium channels (Kv channels): a Kv channel sensitive to tetraethyl-ammonium chloride (TEA) that is present throughout the axons, and two channels sensitive to 4-aminopyridine (4-AP), that are distinguishable by their differential sensitivity to dendrotoxin and are located in internodal and paranodal regions (for review, see Vogel and Schwarz 1995). Although the molecular composition of channels sensitive to TEA or to 4-AP has not yet been determined, the channels sensitive to DTX are assumed to result from the association of the Kv1.1 and Kv1.2 α subunits (Reid et al. 1999; Stuhmer et al. 1989). These subunits have been detected in juxtaparanodal regions of peripheral myelinated fibers (Arroyo et al. 1999; Mi et al. 1995).

Little was known for a long time about the nature of the Kv channels present in central myelinated fibers. Myelinated and unmyelinated central fibers have a mean axonal diameter of 0.7 and 0.2 μm, respectively (Foster et al. 1982), which makes it impossible to tease the fibers and to perform voltage- or patch-clamp studies. Performing extracellular recordings on compound action potentials (CAPs) is therefore the only relevant method available for studying the pharmacology of K⁺ channels in myelinated central axons without damaging the nodes of Ranvier. Only two types of K⁺ channels have been pharmacologically identified so far to our knowledge in the adult rat optic nerve using this technique: one of these is sensitive to 4-AP and the other is sensitive to TEA (Gordon et al. 1988). Based on immunocytologic detection data, Baba et al. (1999) have reported that Kv1.1 and Kv1.2 α subunits are diffusely distributed in mouse optic nerves at P10 and are sequestered in juxtaparanodal regions during development. However, no electrophysiological studies were attempted to identify the variations in K⁺ expression during development and to compare the K⁺ channels present in CNS and PNS fibers.

In this study, we therefore performed CAPS recordings on normal rat optic nerves from birth to adulthood and on acutely demyelinated adult nerves. The effects of four Kv channel blockers were studied, namely those of the commonly used blockers TEA and 4-AP and two more specific blockers, dendrotoxin-I (DTX-I) and kaliotoxin (KTX). These toxins are known to block Kv channels containing the Kv1.1 and Kv1.2 α-subunits (Dolly and Parcej 1996) and the Kv1.1 and Kv1.3 α-subunits (Moure et al. 1999), respectively. Our results provide detailed information about the time-evolving patterns of expression and the localization of four Kv channel types in the optic nerve axons. One of them is sensitive only to TEA, whereas three of them are sensitive to 4-AP and are distinguishable by their differential sensitivity to DTX-I and to KTX.
METHODS

Wistar rats were anesthetized by halothane inhalation (Belamont) and decapitated with a guillotine in line with the European Community’s guiding principles on the care and use of animals (86/609/CEE) and the French decree No. 87/848. The optic nerves were dissected into artificial cerebrospinal fluid (ACSF), transferred to a perfusion chamber, and incubated for a 60-min equilibrium period. During this period and during the experiments, the optic nerves were kept at 37°C, oxygenated in a 95% O₂-5% CO₂ atmosphere and perfused at a flow rate of 1–2 ml/min with ACSF.

Adult optic nerve

The nerve was drawn into two suction ACSF-filled glass electrodes. Compound action potentials (CAPs) from optic nerves of rats older than P20 were evoked by applying a supramaximal stimulus (40-μs duration) at the distal nerve end. CAPs were recorded from the second electrode at the proximal nerve end using the method developed by Stys et al. (1991) that compensates for changes in the resistance of the recording electrode and makes it possible to remove and reposition the nerve in the electrode. The signals were amplified, digitized at 10 kHz, and stored on a hard disk. The area under the positive phase of the CAPs was subsequently calculated, and the ratio of the CAPs area and stored on a hard disk. The area under the positive phase of the CAPs was subsequently calculated, and the ratio of the CAPs area after versus before drug application was measured. When Kv channel blockers were tested, the electrodes were left in place throughout the experiment. The effects of the Kv channel blockers were measured 30–45 min after their application once the effects of the drug action had reached a steady state. For the refractory period measurements, paired stimuli were applied at various interpulse intervals, and the maximum amplitude of the second response was plotted as a function of the interpulse interval. To ensure that the amplitude of the second response was accurately assessed, the first response was subtracted from all the recordings.

Neonatal optic nerve

The CAPs from the optic nerves of rats younger than P20 were initially biphasic due to the fact that the action potential (AP) propagates down the nerve and invades the end of the nerve. Biphasic behavior corresponded to positive waves followed by negative waves and was observed only in neonatal optic nerves, which contain axons conducting at a uniform slow velocities. In adult nerves, which contain axons conducting at various velocities, the broad spectrum of the axonal diameter leads to the classical positive CAPs (for details, see Stys et al. 1991). To avoid obtaining biphasic patterns, CAPs were recorded with a suction electrode filled with ACSF containing tetradotoxin (TTX, 300 nm). After a 45- to 60-min equilibrium period, stable monophasic positive recordings of CAPs could subsequently be achieved. The electrodes were left in place throughout the experiment, and the procedure was similar in all other respects to that used on adult animals.

Electron microscopy

Rats of various ages were anesthetized with pentobarbital sodium (Nembutal) and perfused with 0.1 M phosphate buffer (PB) containing 2% glutaraldehyde (pH 7.4). The optic nerves were removed and postfixied with 2% osmium tetroxide in 0.1 M PB for 1 h and washed in distilled water. After being dehydrated in ethanol and propylene oxide, the nerves were infiltrated in Durcupan ACM resin (Fluka) and polymerized at 60°C for 48 h. Ultrathin sections (80 nm) were cut on an Ultracut (Leica), counterstained with uranyl acetate and lead citrate, and examined with a Zeiss electron microscope. At each stage, the proportions of unmyelinated, myelinated, and ensheathed axons were counted on electron micrographs of cross sections (7,000 times) in which 1,000–2,000 axons were examined in each case. Ensheathed axons corresponded here to fibers encircled by a single loop of noncompact myelin.

Demyelination

Optic nerves from rats aged between 30 and 50 days were incubated for 60 min in ACSF containing 0.05% 1-α-lysophosphatidylcholine (LPC). Control experiments were performed in ACSF prior to LPC treatment. CAPs were evoked and recorded as in the case of adult optic nerves. At the end of the electrophysiological experiments, control and LPC-treated optic nerves were fixed in 0.1 M PB containing 2% glutaraldehyde for 2 h and treated as described in the preceding text prior to electron microscopy.

Statistical analysis

All the data given here are means ± SE. The effects of drugs on the amplitude, area or refractory period of the CAPs were compared by performing a paired Student’s t-test. Morphometric values were compared using a nonpaired Student’s t-test.

Solutions and drugs

The ACSF contained (in mM) 126 NaCl, 3 KCl, 2 CaCl₂, 2 MgSO₄, 1.25 Na₂HPO₄, 26 NaHCO₃, and 10 dextrose, pH 7.4–7.5. Dendrotoxin-I (DTX-I), iberiotoxin, and apamine were purchased from Alomone Labs (Jerusalem, Israel), and kaliotoxin (KTX) was a gift from Dr. Sabatier (CNRS, Marseille, France). All the other compounds were from Sigma (St. Louis, MO).

RESULTS

TEA-sensitive K⁺ channels

TEA-sensitive K⁺ channels have been observed in adult rat optic nerves but have never been described so far during development. In adults, enlargement of CAPs in response to TEA were observed only when a preliminary increase in the duration of the CAPs was induced by blocking 4-AP-sensitive channels (Gordon et al. 1988). We also noticed a similar effect of TEA (10 mM) after applying 4-AP (500 μM) to adult nerves. More interestingly, we noted that TEA alone enhanced the amplitude and area of the CAPs in neonatal optic nerves from P1 to P16 (Fig. 1A, top). The effects of TEA were quantified by measuring the increase in the area of the CAPs and were investigated at concentrations of between 5 and 20 mM. At 10 and 20 mM, TEA induced a maximum increase in the area of the CAPs. The effects of TEA alone were transient during development and peaked at P11 when a 510 ± 126% increase in the area of the CAPs was recorded. These effects were associated with a lengthening of the refractory period (n = 4 in each age-group; P < 0.05; Fig. 1B). However, at P16, TEA increased weakly the area and refractory period of the CAPs (Fig. 1, A and B). TEA was subsequently effective only when a preliminary increase in the duration of the CAPs was induced by applying 4-AP. The effects of TEA decreased suddenly (Fig. 1A) because TEA induced a 217 ± 27% increase in the area of the CAPs at P14 and only a 37.87 ± 0.3% increase at P18. The changes in the effects of TEA were paralleled by a decrease in the duration, latency, and the refractory period of the CAPs (Fig. 1, A and B). After P16, TEA slightly increased the amplitude of the hyperpolarization occurring after the CAPs (Fig. 1A, * on the P21 and P42 traces). The latter effect decreased during maturation and disappeared in adulthood.
4-AP-sensitive K⁺/H11001 channels

4-AP-sensitive K⁺ channels have been described in the adult rat optic nerve by Gordon et al. (1988). Here we observed considerable effects of 4-AP (500 μM) on the amplitude and duration of the CAPs at all the ages tested (Fig. 2A). The effects of 4-AP were investigated at a concentration of 500 μM, which induced a 95% increase in the CAPs area in rat optic nerves. An increase of half this size in the CAPS area (IC50) was observed on applying 20 μM of 4-AP to P6 optic nerves (n = 4). The application of 4-AP also led to the occurrence of a posthyperpolarizing potential (Fig. 2A) that could be blocked by TEA (Fig. 5E). The effects of 4-AP were associated with a lengthening of the refractory period at all the ages tested (n = 5 in each age-group; P < 0.05; Fig. 2B). The increases in CAPs area induced by 4-AP were greater between P16 and P50, when a second and a third positive component appeared in the CAPs and led to a 1,302 ± 86% increase at P29. It was difficult, however, to discern the effect of 4-AP on the amplitude and duration of each individual component of the CAPs during this period. In adults, 4-AP tended to induce a broadening of each phase of the CAPs rather than an increase in their amplitudes (Fig. 2C). These effects were always associated with a lengthening of the refractory period (Fig. 2B).

Identification of two types of dendrotoxin-I-sensitive K⁺ channels with different kaliotoxin sensitivities

We first tested the effects of DTX-I, a 60-amino-acid peptide, which is known to block channels composed of Kv1.1 and Kv1.2 subunits (Dolly and Parcej 1996). DTX-I was applied at a concentration of 100 nM, which induced a 95–100% increase in the CAPs area. Dose-response curves were drawn up with DTX-I (0.1–500 nM) applied to six P6–P8 optic nerves, and IC50 values of 25 nM were obtained (data not shown).

As early as P1, DTX-I enhanced the amplitude and duration of the CAPs (Fig. 3A). Up to P8, the effects of DTX-I reached a steady state after 15 min. This delay was 30 min at P12, which suggests that the channels were less accessible to the toxin at this stage. From P1 to P12, the effects of DTX-I on the amplitude and the area of the CAPs gradually increased. The most conspicuous effect of DTX-I was observed at P12 (957 ± 167% increase in the area of the CAPs) and was paralleled by the emergence of a population of fast conducting fibers (Fig. 3A, →). After P12, the effects of DTX-I decreased slowly and reached a steady state after 45–60 min of application. Interestingly, between P12 and P29, DTX-I induced a more prominent increase in the amplitude of the second and third component of the CAPs than in the amplitude of the first fast component of the CAPs. By P29, DTX-I no longer had any effect, even after 60 min of drug application. The disappearance of the effects of DTX-I took place more slowly and belatedly than the disappearance of the effects of TEA.

To determine whether or not DTX-I-sensitive channels
might be composed of several K\(^+\) channel subtypes with different developmental patterns of expression, we investigated the action of KTX (100 nM; \(n = 25\)), a 38-amino-acid peptide, which blocks channels composed of Kv1.1 and Kv1.3 subunits at a concentration of 100 nM (Mourre et al. 1999). Up to P11, KTX (100 nM) exactly mimicked the effects of DTX-I. Neither DTX-I applied to KTX-treated nerves (Fig. 3A) nor KTX applied to DTX-I-treated nerves had any further effects (data not shown). Therefore at these ages, the channels sensitive to DTX-I were also sensitive to KTX. IC50 values of 10 nM were obtained with KTX.

After P11, DTX-I applied to KTX-treated nerves further enhanced the area of the CAPs. This result suggested the presence of a second type of Kv channels sensitive only to DTX-I. As in the case of DTX-I, the effects of KTX decreased during maturation, and a longer application time was required for a steady state to be obtained. Between P11 and P21, KTX also increased more prominently the late components of the CAPs. However, the effects of KTX decreased more sharply and more conspicuously than those of DTX-I (Figs. 3A, 3B, 3C, and 3D).

The presence of two DTX-I-sensitive K\(^+\) channels was confirmed by analyzing the CAPs refractory period (Fig. 4). From P1 to P8, we observed that KTX and DTX-I induced a similar increase in the duration of the refractory period (Fig. 4A, right). However after P8, DTX-I induced a greater increase in the refractory period than KTX. This additional effect of DTX-I was most striking at P14 (Fig. 4B, P14 in right panel). However, it was noted that from P21 onwards, even when both KTX and DTX-I broadened the CAPs (Fig. 3A), these toxins had no effect on the refractory period (Fig. 4B, P21 in right panel).

**Overlapping effects of the K\(^+\) channel blockers**

The combined effects of the blockers were tested to determine the pharmacological profile of the K\(^+\) channels. These experiments were carried out on P8 optic nerves because at this age, the optic nerve axons are rapidly accessible to the toxins. KTX was not tested here because at P8, its effects overlapped completely with those of DTX-I.

First, we observed that DTX-I had no effect when applied after 4-AP (\(n = 3\); Fig. 5A) but that this substance induced an additional broadening of the CAPs when applied after TEA (\(n = 4\); Fig. 5B). DTX-I-sensitive channels thus appeared to be also sensitive to 4-AP but insensitive to TEA. This was confirmed on applying TEA to DTX-I-treated nerves (\(n = 5\); Fig. 5C). Interestingly, the application of 4-AP to DTX-I-treated nerves induced an additional increase in amplitude at all the ages tested (\(n = 24\); Fig. 5D). It was concluded that DTX-I-sensitive channels did not constitute the whole 4-AP-sensitive channel population, which could thus be subdivided into a population of DTX-I-sensitive channels and a population of DTX-I-insensitive channels. At all the ages tested, 4-AP led to a broadening of the CAPs when applied after TEA, and TEA broadened the CAPs when applied after 4-AP (\(n = 20\) and \(n = 14\); Fig. 5, E and F). These results confirm that 4-AP- and TEA-sensitive channels form two distinct populations. TEA was also found to induce both a broadening and a decrease in the amplitude of the CAPs when applied after 4-AP or DTX-I.

Last, we investigated the effects of the Ca\(^{2+}\)-activated K\(^+\) channel blockers, iberiotoxin and apamine on P8 rat optic
Correlation between the process of myelination and the effects of DTX-I

To determine whether the disappearance of DTX-I effects resulted from a down-regulation of K\(^+\) channels or from the inaccessibility of the channels to the toxins due to the myelination, we first analyzed the time course of the process of myelination in the rat optic nerve using electron microscopy methods. From P1 to P6, all the axons in the optic nerve were unmyelinated and small in diameter (around 0.3 \(\mu\)M). The first axons to be ensheathed with oligodendro-glial processes were detected around P8 and were surrounded by one to three wraps of noncompact myelin (Fig. 6, A and C). The first myelinated axons were detected at P10 (Fig. 6B) concomitantly with the emergence in the CAPs of a population of fast conducting fibers (Fig. 3A, →). These fibers were surrounded by a thin layer of compact myelin and had a larger diameter than the unmyelinated and ensheathed axons. A few well-formed nodes of Ranvier were also detected at P10, but their number increased rapidly at later ages. Figure 6D shows a micrograph of one such node of Ranvier observed at P10.

We then compared the gradual changes occurring in the proportions of unmyelinated, ensheathed, and myelinated axons during development with the effects of DTX-I on the CAPs (Fig. 7). The transient increase in the effects of DTX-I observed on P12 coincided temporarily with the transient presence of ensheathed axons in the optic nerve (Fig. 7). The changes in the proportions of the myelinated axons were found to have a sigmoidal time course, starting around P10–P12 and plateauing at P25. These changes were reciprocal to the decrease in the effect of DTX-I, which suggests that the disappearance of the effects of DTX-I were correlated with the increasing number of myelinated axons.

Comparison between the effects of KTX and DTX-I

Because DTX-I further enhanced the area of the CAPs of KTX-treated nerves up to P11 (Fig. 4B), we compared the effects of KTX and DTX-I after the myelination process on LPC-treated adult optic nerves (\(n = 15\); Fig. 9A). No such effect was observed in the control nerves (Fig. 4A). Such a difference was observed in the control nerves (Fig. 9A), which indicates that under control conditions, myelin might render the DTX-I-sensitive channels inaccessible to the toxins.

To confirm this hypothesis, acute demyelination of P30–P50 optic nerves was induced using LPC (Chiu and Ritchie 1981; Low et al. 1983).

It was observed under electron microscopy that a 60 min treatment with 0.05% LPC led to a loosening of the paranodal myelin loops but no Wallerian degeneration or axonal damage. The length of the Ranvier’s nodes increased almost twofold (1.34 ± 0.11 \(\mu\)m in LPC-treated optic nerves as compared with 0.72 ± 0.04 \(\mu\)m in the control experiments; \(n = 102\); Fig. 8, A and C, top). Electrophysiological recordings showed that LPC induced a 51 ± 11.98 and 37 ± 12.59% decrease in the area and amplitude of the rat optic nerve CAPs, respectively (Fig. 8, B and C, bottom).

DTX-I applied to LPC-treated adult optic nerves (\(n = 15\)) led to a broadening of the CAPs, associated with an increase in the amplitude of the second slow peak (Fig. 9A) but not with an increase in the amplitude of the fast peak of the CAPs as in neonatal nerves (Fig. 4A). No such effect was observed in the control nerves (\(n = 6\); Fig. 9A), which indicates that under control conditions, myelin might render the DTX-I-sensitive channels inaccessible to the toxins.

To determine whether the second peak corresponded to an independent subset of fibers, we measured the refractory period of the fast and slow peaks after DTX-I application. It was observed under electron microscopy that a 60 min treatment with 0.05% LPC led to a loosening of the paranodal myelin loops but no Wallerian degeneration or axonal damage. The length of the Ranvier’s nodes increased almost twofold (1.34 ± 0.11 \(\mu\)m in LPC-treated optic nerves as compared with 0.72 ± 0.04 \(\mu\)m in the control experiments; \(n = 102\); Fig. 8, A and C, top). Electrophysiological recordings showed that LPC induced a 51 ± 11.98 and 37 ± 12.59% decrease in the area and amplitude of the rat optic nerve CAPs, respectively (Fig. 8, B and C, bottom).

DTX-I applied to LPC-treated adult optic nerves (\(n = 15\)) led to a broadening of the CAPs, associated with an increase in the amplitude of the second slow peak (Fig. 9A) but not with an increase in the amplitude of the fast peak of the CAPs as in neonatal nerves (Fig. 4A). No such effect was observed in the control nerves (Fig. 9A), which indicates that under control conditions, myelin might render the DTX-I-sensitive channels inaccessible to the toxins.

Comparison between the effects of KTX and DTX-I

Because DTX-I further enhanced the area of the CAPs of KTX-treated nerves up to P11 (Fig. 4B), we compared the effects of KTX and DTX-I after the myelination process on LPC-treated adult nerves (\(n = 8\); Fig. 10A at P29 and P51). Here it was observed that KTX also led to a broadening of the CAPs of LPC-treated adult nerves, associated with an increase in the amplitude of the second slow peak. However,
the effects of KTX were weaker than those of DTX-I and decreased further in the course of myelin maturation (Fig. 10A at P29 and P51). On examining the increase in the CAPs area induced either by KTX or by both KTX and DTX-I as a function of age, we observed that the effects of KTX peaked at P14 (Fig. 10A). The peak in the effects of DTX-I, which can be seen in Fig. 7, therefore resulted both from the development of channels sensitive only to DTX-I starting on P11, and from the increase in the effects of KTX.

### DISCUSSION

**Location of Kv channels in the optic nerve**

The results of our study show that DTX-I and KTX broadened the CAPs of neonatal optic nerve but not those of the optic nerves of rats older than P29. The disappearance of the effects of KTX and DTX-I was correlated with the increasing number of myelinated axons in the optic nerve. Furthermore, we observed that after P12, the decrease in the effect of KTX and DTX-I was detected first on the amplitude of the fast component of the CAPs and then on the overall CAPs. The fact that DTX-I and KTX broadened the CAPs in LPC-treated adult nerves demonstrates that the disappearance of these effects resulted from the inaccessibility of the channels to the toxins rather than from a downregulation of K⁺ channels. If DTX-I- and KTX-sensitive channels were nodal, their blockade would have resulted in a lengthening of the CAPs and refractory period in both LPC-treated and untreated nerves. Because this was not the case, we concluded that the DTX-I- and KTX-sensitive channels are paranodal and internodal and that they are inaccessible to these toxins in normal myelinated fibers.

The fact that 4-AP enlarged both the CAPs and the refractory period in adult nerves suggests that the channels sensitive to 4-AP, and insensitive to DTX-I, may have a nodal location. By contrast, the 4-AP-sensitive channels present in peripheral myelinated fibers are entirely paranodal (Waxman 1995). The location of some of the 4-AP sensitive channels in the node of Ranvier of optic nerve fibers may explain why CNS axons are much more sensitive to 4-AP. However, because 4-AP is able to diffuse through membranes (Choquet and Korn 1992), it may also block channels located in paranodal and internodal regions.

As far as TEA is concerned, we assumed that the disappearance of its effects was not due to a redistribution of the channels as occurs in the case of DTX-I- and KTX-sensitive channels for the following reasons: first, because the disappearance of TEA effects was not correlated with the increase in the number of myelinated fibers as it was with DTX-I but occurred earlier and more rapidly; and second, because TEA applied alone to adult LPC-treated nerves increased neither the area of the CAPs nor the refractory period (data not shown). The fact that TEA could have an effect after 4-AP application on adult nondemyelinated nerves suggests that TEA-sensitive

---

**FIG. 6.** Electron microscopic picture of the pattern of myelination in developing rat optic nerves. A and B: transverse sections of P10 and P12 optic nerves. Although a few ensheathed axons (EA) and myelinated fibers (M) were present at P10, most of the axons were still unmyelinated (UM). C and D: longitudinal P10 and P16 optic nerve sections, respectively. ↓, axonal contacts with a one-layer oligodendroglial process. *, the presence of a node of Ranvier. Scale bars: 0.5 μm.

**FIG. 7.** Developmental changes between P1 and P50 in the sensitivity of CAPs to DTX-I (●) and in the state of myelination (expressed in percentages): myelinated (X), unmyelinated (○), and ensheathed axons (□). The decrease in the effects of DTX-I was paralleled by an increase in the percentage of myelinated axons.
channels are nodal and accessible to TEA, like peripheral fibers (Waxman 1995), but require longer periods of depolarization to be activated.

Relationship between Kv channel setting and the myelination process

The results of several studies have indicated that myelin compaction and axo-glial junction formation are essential requirements for K⁺ channel clustering to occur in central and peripheral nervous systems (Arroyo et al. 1999; Rasband et al. 1998, 1999). In the present study, these results are extended using electrophysiological techniques. The decrease in the effects of DTX-I started precisely at the stage when the K⁺ channel clustering was observed by these authors, corroborat-

FIG. 8. Effects of l-α-lysophosphatidylcholine (LPC) treatment on optic nerve axons, A and B: LPC (0.05% for 60 min) induced a lengthening of the node of Ranvier. B and C: demyelination was associated with a decrease in the CAPs amplitude and area (n = 7). Scale bar: 0.5 μm. **, significantly different from control value, nonpaired t-test, P < 0.001; *, significantly different from control value, paired t-test, P < 0.05.

channels are nodal and accessible to TEA, like peripheral fibers (Waxman 1995), but require longer periods of depolarization to be activated.

Relationship between Kv channel setting and the myelination process

The results of several studies have indicated that myelin compaction and axo-glial junction formation are essential requirements for K⁺ channel clustering to occur in central and peripheral nervous systems (Arroyo et al. 1999; Rasband et al. 1998, 1999). In the present study, these results are extended using electrophysiological techniques. The decrease in the effects of DTX-I started precisely at the stage when the K⁺ channel clustering was observed by these authors, corroborat-

FIG. 8. Effects of l-α-lysophosphatidylcholine (LPC) treatment on optic nerve axons, A and B: LPC (0.05% for 60 min) induced a lengthening of the node of Ranvier. B and C: demyelination was associated with a decrease in the CAPs amplitude and area (n = 7). Scale bar: 0.5 μm. **, significantly different from control value, nonpaired t-test, P < 0.001; *, significantly different from control value, paired t-test, P < 0.05.

channels are nodal and accessible to TEA, like peripheral fibers (Waxman 1995), but require longer periods of depolarization to be activated.

Relationship between Kv channel setting and the myelination process

The results of several studies have indicated that myelin compaction and axo-glial junction formation are essential requirements for K⁺ channel clustering to occur in central and peripheral nervous systems (Arroyo et al. 1999; Rasband et al. 1998, 1999). In the present study, these results are extended using electrophysiological techniques. The decrease in the effects of DTX-I started precisely at the stage when the K⁺ channel clustering was observed by these authors, corroborat-

FIG. 9. Unmasking of paranodal DTX-I-sensitive K⁺ channels by LPC treatment. A: 0, 15, 30, and 45 min after the application DTX-I (100 nM, *), the CAPs of LPC-treated optic nerves were broadened (n = 15) but not that of control optic nerves (n = 6). This broadening was characterized by the emergence of a second slow peak. B: DTX-I had no effect on the refractory period of the initial fast peak of the CAPs in LPC-treated nerves (left) but enlarged the refractory period of the 2nd slow peak (right) (n = 15). C: when conditioned by applying another stimulus (interpulse interval, 10 ms), the slow peak in the DTX-I-treated nerves decreased, but the CAPs of the control LPC-treated nerves showed no change. *, the CAPs recorded 10 ms after the conditioning stimulus.

FIG. 10. Changes in the proportions of KTX- and DTX-I-sensitive K⁺ channels during development. Rat optic nerves were exposed first to KTX (100 nM) and then to KTX (100 nM) plus DTX-I (100 nM). The figure shows the percentage increase in the CAPs area induced by KTX (I, n = 26) and the additional increase induced by KTX plus DTX-I (C, n = 26). The data recorded between P29 and P50 were obtained on LPC-treated nerves.
ing the idea that K⁺ channels are redistributed under the myelin sheath as soon as the first nodes of Ranvier are formed.

Immunocytologic detection of Kv channels in the optic nerves has been performed so far only after P10–P14 (Baba et al. 1999; Rasband et al. 1998, 1999) when the K⁺ channels are densely packed in juxtaparanodal regions. The present pharmacological data made it possible to identify DTX-I-sensitive channels at earlier ages (as early as P1) and to establish that the effects of DTX-I and KTX peak at around P12.

We attributed this peak to both an increase in the number of channels sensitive to KTX and DTX-I and to the formation of channels sensitive only to DTX-I. The number of channels sensitive to KTX and DTX-I presumably increases from P1 to P8 because no important axonal morphologic changes were observed during this period (see Fig. 4) (Foster et al. 1982; Hildebrand and Waxman 1984) and because the full complement of optic nerve axons is present at birth and then decreases over the 2 wk following birth (Lam et al. 1982). Interestingly, the peak in the effects of KTX and the emergence of channels sensitive only to DTX-I were correlated with the increase in the number of ensheathed axons in the optic nerve. These results indicate that the first axo-glial contact is an important event in the regulation of the number of channels sensitive to KTX and DTX-I and in the setting of channels sensitive only to DTX-I.

In addition, we concluded that the number of channels sensitive to both KTX and DTX-I probably decreases after the onset of myelination and during the myelin maturation process because in P51 LPC-treated optic nerves, only 20% of the effect of DTX-I was due to the blockade of the channels sensitive to KTX.

According to the selectivity of KTX and DTX-I (Dolly and Parcej 1996; Mourre et al. 1999), we can suspect that the channels sensitive to KTX and DTX-I correspond to the expression of Kv1.1, Kv1.2, or Kv1.3 subunits. Kv1.3 subunit is weakly blocked by DTX-I, and its presence has never been described into the rat optic nerve. Thus Kv1.1 subunit might be the major component of the channels sensitive to KTX. In addition, our results suggest that the channels sensitive to DTX-I and insensitive to KTX may correspond to the expression of Kv1.2 subunits. Because Kv1.1 and Kv1.2 subunits could assemble into heteromeric channels, the emergence of channels insensitive to KTX could also be explained by a change in the Kv1.1/Kv1.2 mixture in heteromeric channels.

Role of Kv channels in the optic nerve

From P1 to P8, optic nerves contained almost entirely unmyelinated fibers. TEA, KTX, DTX-I, and 4-AP induced a broadening and an increase in the amplitude of the CAPs. CAPs correspond to the summation of all the action potentials (APs) generated by each fiber. Because virtually all fibers are recruited by the type of stimulation we used, the increase in the CAPs amplitude cannot have been due to the recruitment of unresponsive fibers. We therefore assumed that Kv channels sensitive to these blockers are involved in AP repolarization in unmyelinated axons. It has been reported elsewhere that 4-AP or TEA increase the amplitude and duration of the CAPs in adult unmyelinated CNS fibers (Bostock et al. 1982; Scholfield 1990; Sherratt et al. 1980).

In addition, it was observed that TEA associated with 4-AP or DTX-I induced a decrease in the amplitude of the CAPs. TEA and 4-AP combined have also been found to have similar effects on unmyelinated axons from mammalian CNS and from Lumbriacus terrestris (Radicheva and Kolev 1992; Scholfield 1990). These results suggest that the blockade of a large number of Kv channels induces the depolarization of the resting potential and thus decreases the electromotive force driving Na⁺ ions or inactivates the sodium channels, rendering some fibers unresponsive. A similar depolarization of the resting potential after application of TEA and 4-AP has been described in peripheral myelinated fibers (Baker et al. 1987).

After P29, the optic nerves contained almost entirely myelinated fibers. The toxins KTX and DTX-I increased the amplitude of the second slow peak in the CAPs of LPC-treated nerves. This second peak presumably reflected either the restoration of the axonal conduction in the slowly conducting demyelinated fibers, in which the toxins had broadened the AP and overcome the conduction block, or the occurrence of re-excitation following the initial CAPs. The first hypothesis was supported by the fact that DTX-I increased both the amplitude and the refractory period of the second slow peak as in unmyelinated fibers, suggesting that DTX-I-sensitive channels were involved in AP repolarization of demyelinated fibers. It has been reported on these lines that 4-AP increases the amplitude and duration of the CAPs of remyelinating peripheral fibers (Kocsis et al. 1982; Rasband et al. 1998) and that increases in the duration of the AP overcome conduction failure in demyelinated nerves (Bostock et al. 1978). The second hypothesis was supported by the fact that 4-AP is known to lead to the occurrence of re-excitations and delayed depolarizations in optic nerves and cutaneous afferent fibers (Gordon et al. 1988; Honmou et al. 1994). In the latter case, these re-excitations were found to have a longer recovery time than the initial action potential, and this might explain why the second slow peak of the CAPs had a longer refractory period than the initial CAP recorded in response to DTX-I application.

In any case, DTX-I-sensitive channels might play an important role consisting of preventing the occurrence of aberrant firing in developing optic nerves as in the CNS (Vabnick et al. 1999). The fact that the KTX effects peaked at P12 and that the channels sensitive only to DTX-I developed concomitantly with the increase in the number of ensheathed axons is in line with this hypothesis.

Interestingly, 4-AP was found here to have a more pronounced effect on immature than on mature fibers. This has also previously been found to be the case in white matter spinal tracts and peripheral nerves (Bowe et al. 1985; Eng et al. 1988; Kocsis 1985). However, in peripheral nerves, the effects of 4-AP have been shown to be very much attenuated during the course of maturation (Bowe et al. 1985; Eng et al. 1988). These results suggest that, in both CNS and peripheral nerves, 4-AP-sensitive channels may play a decisive role before and during the process of myelin formation. There are several possible reasons why 4-AP has greater effects on young animals. First, 4-AP-sensitive channels have been found to dampen the delayed depolarizations that occur after AP in peripheral nerves (David et al. 1995). These delayed depolarizations are known to have a greater amplitude in small diameter axons and those having a thin myelin sheath. Because optic nerve axons have a small diameter and acquire their mature structure rather slowly (Foster et al. 1982), these delayed depolarizations may occur frequently during myelin formation and maturation.
As far as the TEA-sensitive channels are concerned, we established that these channels are involved in AP repolarization from P1 to P16 but not later on. We observed here that the disappearance of the effects of TEA coincided with the decrease in the duration of the CAPs and in the length of their refractory period and that TEA increased the duration of the CAPs of 4-AP-treated nerves. These results suggested that TEA-sensitive channels have a slow activation rate like the S-type channels present in the peripheral nerves (Corrette et al. 1991; Safronov et al. 1993). TEA has also little effect on immature (3 wk old), mature (17 wk old), and demyelinated peripheral nerves (Eng et al. 1988; Rasband et al. 1998). Moreover, TEA blocks the 4-AP-induced postspike positivity in the PNS. It suggests that the TEA sensitive channels might have the same role in PNS and CNS. However, to our knowledge, it has never been described previously that TEA broadens the CAPs of peripheral neonatal nerves. The exact reason why TEA induced an increase in the amplitude of the posthypermolarization phase in immature nerves still remains to be elucidated. A such hyperpolarization has been described in rat optic nerve following repetitive stimulation, and have been attributed to activation of the electrogenic pump (Na+/K+)/ATPase (Gordon et al. 1990).

In conclusion, the data obtained in the present study show that there exists three populations of 4-AP-sensitive channels in the rat optic nerve: a population of paranodal channels sensitive only to DTX-I that may correspond to the I-type (Kf1) channels present in peripheral nerves (Corrette et al. 1991; Safronov et al. 1993); a population of nodal and paranodal/intermodal channels insensitive to DTX-I that may correspond to the F-type (Kf2) channels (Corrette et al. 1991; Safronov et al. 1993); and a population of paranodal channels sensitive to both KTX and DTX-I never described up to now. It was established here that the sequestration of all these channels and their time-evolving patterns of expression are closely correlated with the process of myelin formation and maturation. For instance, the channels sensitive to both KTX and DTX-I are set prior to myelination and have a transient expression during development. By contrast, the channel sensitive to DTX-I and insensitive to KTX is set concomitantly with axo-glial contact and forms the major component of the DTX-sensitive channels in adulthood.

We thank M. André and D. André for technical assistance, R. Fayolle for manufacturing the amplifier, stimulator, and digitizer, and H. Chagneux for technical assistance, R. Fayolle for for P. Shrag for comments and suggestions on the manuscript.

This study was supported by grants from the Association de Recherche contre la Scéleose En Plaques and the Ligue Française de recherche sur la Scéleose En Plaques.

REFERENCES


