

# Distinction among Neuronal Subtypes of Voltage-Activated Sodium Channels by $\mu$ -Conotoxin PIIIA

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The functional properties of most sodium channels are too similar to permit identification of specific sodium channel types underlying macroscopic current. Such discrimination would be particularly advantageous in the nervous system in which different sodium channel family isoforms are coexpressed in the same cell. To test whether members of the  $\mu$ -conotoxin family can discriminate among known neuronal sodium channel types, we examined six toxins for their ability to block different types of heterologously expressed sodium channels. PIIIA  $\mu$ -conotoxin blocked rat brain type II/IIA (rBII/IIA) and skeletal muscle sodium current at concentrations that resulted in only slight inhibition of rat peripheral nerve (rPN1) sodium current. Recordings from variant lines of PC12 cells, which selectively express either rBII/IIA or rPN1 channel subtypes, verified that

the differential block by PIIIA also applied to native sodium current. The sensitivity to block by PIIIA toxin was then used to discriminate between rBII/IIA and rPN1 sodium currents in NGF-treated PC12 cells in which both mRNAs are induced. During the first 24 hr of NGF-treatment, PN1 sodium channels accounted for over 90% of the sodium current. However, over the ensuing 48 hr period, a sharp rise in the proportion of rBII/IIA sodium current occurred, confirming the idea, based on previous mRNA measurements, that two distinct sodium channel types appear sequentially during neuronal differentiation of PC12 cells.

**Key words:** PC12 cells; ion channel; sodium current; growth factor; CNS; PNS

Toxins represent potent tools for distinguishing among the various isoforms of voltage-dependent ion channels. In particular, conotoxins have been instrumental in identifying individual subtypes of voltage-dependent calcium channels (McCleskey et al., 1987). Far less progress has been made in identifying toxins that can be used in an analogous manner to identify sodium channel subtypes. As is the case for calcium channels, the macroscopic sodium current in individual neurons often involves the activation of a complex mixture of sodium channel subtypes. However, unlike calcium channels, the functional properties of most sodium channel subtypes are similar at the level of macroscopic current, precluding discrimination on the basis of functional differences. Tetrodotoxin sensitivity has served as a standard tool for identifying certain sodium channel subtypes in both nerve and muscle, and this toxin has provided important but limited distinction among neuronal sodium channel types (Campbell, 1992).

Early studies on  $\mu$ -conotoxin GIIIA offered the promise of further discrimination between two different sodium channel subtypes that were both TTX-sensitive (Cruz et al., 1985). GIIIA blocked sodium current recorded from skeletal muscle (SkM1) at concentrations that had no discernible effects on neuronal sodium channels. Subsequent studies of GIIIA have been helpful in

understanding the interactions between toxin- and pore-forming regions of the skeletal muscle SkM1 channel type (French and Dudley, 1999), but unfortunately, little advance has been made in identifying conotoxins that serve as discriminators between neuronal sodium channel types. The recent report that PIIIA blocked brain type sodium channels but spared the sodium channel responsible for action potential propagation suggested that this conotoxin might serve such a purpose (Shon et al., 1998). However, the PIIIA-insensitive sodium channel responsible for motoneuron action potential was not known. Our studies took advantage of the fact that the  $\alpha$  subunit of central and peripheral neuron (PN1) sodium channel types can be heterologously expressed in *Xenopus* oocytes to test a battery of conotoxins on skeletal muscle, brain, and peripheral nerve isoforms. Our findings identify PIIIA as the first conotoxin that can discriminate between known neuronal sodium channel subtypes. The application of PIIIA conotoxin to NGF-treated PC12 cells also provides the first discrimination between PN1 and brain type II/IIA (rBII/IIA) components of macroscopic sodium current. Moreover, the time course of appearance of these two channel isoforms is markedly different and mirrors the differential time course of induction of the corresponding mRNAs.

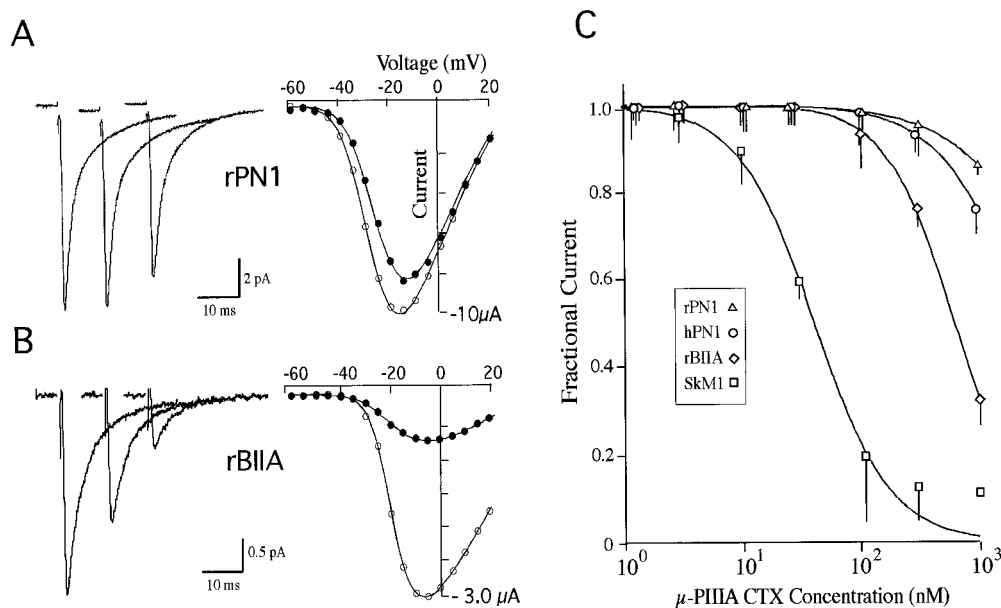
## MATERIALS AND METHODS

Complementary DNAs encoding each of four different sodium channel  $\alpha$  subunits [skeletal muscle SkM1 (Trimmer et al., 1989), rat BII/IIA (rBII/IIA) (provided by A. Goldin, Irvine, CA) (Auld et al., 1988), human PN1 (hPN1) (provided by F. Hoffman, Munich, Germany) (Klugbauer et al., 1995), and rPN1 (Toledo-Aral et al., 1997)] were used to transcribe RNAs for injection into *Xenopus* oocytes. Oocytes were surgically isolated from anesthetized *Xenopus* frogs (Nasco, Fort Atkinson, WI) and enzymatically treated with 10 mg/ml collagenase (Life Tech-

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**Figure 1.** Determination of the sensitivity of heterologously expressed sodium channel isoforms to the conotoxin PIIIA. *A, B, Left panels.* The time-dependent block of inward rPN1 (top 3 traces) and rBIIA (bottom 3 traces) sodium current in *Xenopus* oocytes after application of 1  $\mu$ M PIIIA conotoxin. The individual sodium currents were elicited by a voltage step to  $-10$  mV from a holding potential of  $-100$  mV and acquired at 10 sec intervals. *Right panels.* The current–voltage relationships for individual rPN1 (top) and rBIIA (bottom) sodium current before (open circles) and after (filled circles) equilibrium block by 1  $\mu$ M PIIIA conotoxin. *C.* The concentration-dependent equilibrium block of sodium current measured for four different sodium channel isoforms. All data points reflect the mean  $\pm$  SD from three different oocytes (except for the 1  $\mu$ M SkM1 data point, which reflected a single measurement). The magnitude of the block was determined on the basis of the decrease in current measured during a step to  $-10$  mV.

nologies, Grand Island, NY) for 20 min before mechanical removal of the follicle cell layer. Oocytes were maintained in a nutrient OR-3 medium composed of 50% L-15 medium, 100  $\mu$ g/ml gentamycin, 4 mM glutamine, and 30 mM Na-HEPES (all from Life Technologies) with the pH adjusted to 7.6 with NaOH. Injected oocytes were maintained at 18°C in OR-3 medium before recording.

Sodium current from oocytes was measured using a two-microelectrode oocyte voltage clamp (TEV200; Dagan Instruments, Minneapolis, MN). The recording solution contained (in mM): sodium methanesulfonate 100, NaCl 10, Na HEPES 10, and CaCl<sub>2</sub> 2, with the pH adjusted to 7.0. To minimize voltage errors during the recordings, the microelectrode resistance was  $<1$  M $\Omega$ , and in some recordings the size of the sodium current was reduced by substituting 90% of the sodium with *N*-methyl-D-glucamine. Typically, the cells were held at  $-100$  mV to remove all inactivation, and the sodium currents were elicited by 20 msec positive-going pulses. Toxins were applied at specified concentrations by use of a continuous flow system. The speed of flow was adjusted to provide complete exchange of solution within 5 sec. Each test concentration of toxin was continuously applied until no further block of sodium current was observed. At such time, the equilibrium block of sodium current was determined on the basis of the reduction of peak inward current. Equilibrium block by the toxins was generally achieved within 1 min of application. It is unlikely that the vitelline membrane posed any significant block to the peptide toxins because a complete and rapid block of current was observed after application of TTX.

To induce sodium channel synthesis in PC12 cells, three lines (wild-type PC12, FR3IIb, and PN1-1) were treated with the appropriate growth factors. The FR3IIb cell line has been described previously (Lin et al., 1998) and expresses primarily rBII/IIA mRNA. The PN1-1 line was generated by transfection of PC12 cells with pRSVneo. Among the neomycin-resistant clones, one clone (PN1-1) exhibited unusually high levels of PN1 and extremely low levels of rBII/IIA mRNAs, as assessed by Northern blot analysis. To induce sodium channels, wild-type and FR3IIb lines were treated with 10 ng/ml FGF, and the PN1-1 line was treated with 100 ng/ml NGF, 2 d before recording. The cells were plated and grown on 35 mm plastic tissue culture dishes. Northern blot analyses were performed as described previously (D'Arcangelo et al., 1993). Hybridizing mRNA was detected using a Molecular Dynamics (Sunnyvale, CA) Phosphorimager.

Whole-cell recordings of PC12 sodium current were made by means of an Axopatch 200A amplifier (Axon Instruments, Burlingame, CA). The recording solution contained (in mM): NaMES 140, MgCl<sub>2</sub> 0.2, CaCl<sub>2</sub> 0.2, and Na HEPES 10, adjusted to pH 7.2. The pipette solution contained (in mM): CsMES 140, CsEGTA 10, and Cs HEPES 10, adjusted to pH 7.2. To record sodium currents, the cells were held at  $-120$  mV and stepped to positive potentials for 20 msec. Both PC12 cell and oocyte sodium currents were digitized at 50 kHz and analyzed off-line using HEKA Pulse and Pulse Fit software (Instrutech, Great Neck, NY).

Capacitive transients were compensated using a combination of manual compensation on the amplifier and further processing using either a P/4 or P/10 leak subtraction protocol.

## RESULTS

### Screening of conotoxins on sodium channel $\alpha$ subunits expressed in *Xenopus* oocytes

Four different sodium channel  $\alpha$  subunit types were expressed individually in *Xenopus* oocytes. All four  $\alpha$  subunits exhibited qualitatively similar function in terms of both voltage-dependence and characteristic slow inactivation of inward current caused in large part by lack of  $\beta$ -1 sodium channel subunit in oocytes. To test for block of inward current by specific conotoxin types, oocytes expressing a single type of  $\alpha$  subunit were treated for 5 min at each concentration, after which time the peak sodium current was determined from the current–voltage relationships (Fig. 1*A, B*). Generally, at least seven concentrations were tested for block on each of three oocytes. The highest concentrations used were, in some cases, still too low to obtain more than a partial block because of the insensitivity of the  $\alpha$  subunit to the particular toxin. Higher concentrations could not be tested because of the limited availability of these toxins.

The blocking ability of each toxin was determined by fitting the relationship between peak sodium current versus toxin concentration, according to the equation  $F = IC_{50}/([Tx] + IC_{50})$ , where the equilibrium half-blocking concentration is represented by  $IC_{50}$ . Tx represents the toxin concentration, and  $F$  is the fraction of unblocked peak sodium current (Fig. 1*C*). The data fit by this relationship represented an average of three cells for each toxin concentration. The  $IC_{50}$  for each toxin was then determined from the midpoint of the fitted curve to the average data or on the basis of the extrapolated midpoint value. Table 1 indicates the mean  $IC_{50}$  values determined for individual toxin block of the SkM1, rBII/IIA, hPN1, and rPN1  $\alpha$  subunit types. As expected, all four  $\alpha$  subunit types were sensitive to TTX in the nanomolar range (Table 1). PIIIA and GIIIA both inhibited SkM1 sodium current in the nanomolar range but contrasted in their ability to block the neuronal isoforms of sodium channels. GIIIA required high concentrations to block both rBII/IIA and rPN1, indicating a general lack of sensitivity on the part of neuronal isoforms to this toxin.

**Table 1. IC<sub>50</sub> values for toxin block of sodium channel  $\alpha$  subunit types**

	SkM1	rBII/IIA	hPN1	rPN1
TTX	45 nM	12 nM	38 nM	4 nM
PIIIA	41 nM	690 nM	3.1 $\mu$ M*	6.2 $\mu$ M*
GIIIA	19 nM	2.7 $\mu$ M*	NT	6.0 $\mu$ M*
GmVIA	4.8 $\mu$ M*	2.5 $\mu$ M*	NB	NT
PVIA	5.2 $\mu$ M*	2.9 $\mu$ M*	1.9 $\mu$ M*	6.4 $\mu$ M*
MrVIA	438 nM	532 nM	NT	345 nM

An asterisk indicates those midpoint values that were estimated on the bases of extrapolated curves. NT and NB indicate not tested and no block, respectively.

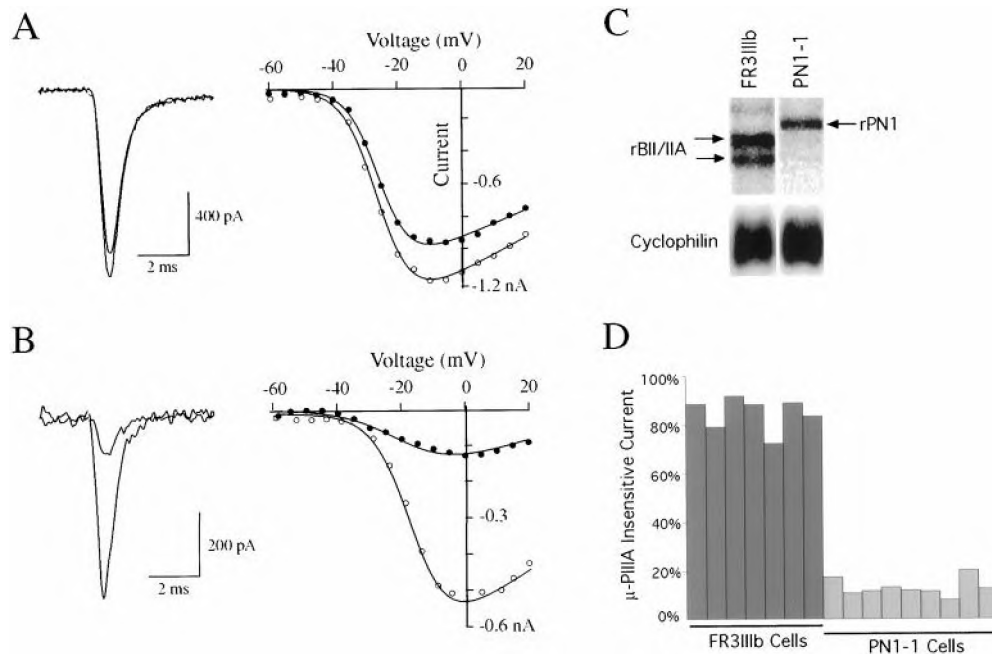
In contrast, PIIIA blocked rBII/IIA at concentrations intermediate to those of rPN1 and SkM1 (Fig. 1*A,B*). The IC<sub>50</sub> for PIIIA block of rBII/IIA was 690 nM compared with an extrapolated value of 6.2  $\mu$ M for block of rPN1 sodium current. MrVIA, GmVIA, and PVIA each inhibited sodium current, but no differences in sensitivity were observed among the neuronal sodium channel types (Table 1).

### PIIIA conotoxin distinguishes CNS and PNS components of sodium current in PC12 cells

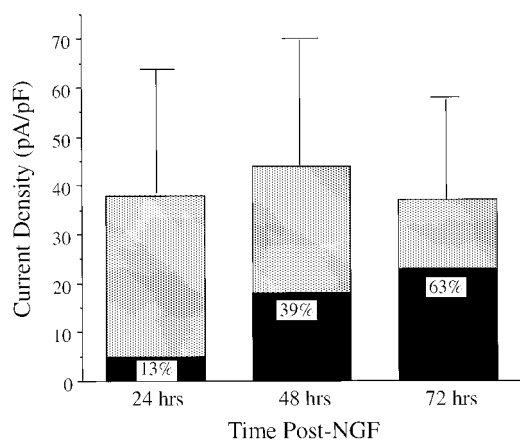
Rat PC12 cells, a cell line established from a pheochromocytoma, acquire the ability to generate sodium-based action potentials after treatment with NGF, concomitant with the elaboration of other neuronal phenotypic traits (for review, see Halegoua, 1991). PC12 cells coexpress two distinct sodium channels in response to treatment with NGF (Mandel et al., 1988; D'Arcangelo et al., 1993), but the time course of induction of the two mRNAs is distinct. NGF treatment leads to a transient rise in mRNA en-

coding rPN1 channels within the first 24 hr, followed by the appearance of mRNA coding for rBII/IIA channels. Electrophysiological analyses at times subsequent to the mRNA increases reveals that induction of sodium current is not associated with changes in sodium current kinetics (Toledo-Aral et al., 1995).

Our results from *Xenopus* oocytes expressing either rPN1 or rBII/IIA sodium channels suggested that PIIIA could provide a tool for distinguishing between these components of native sodium current in PC12 cells. To test this idea, variant lines of PC12 cells were established that expressed, based on mRNA measurements, either rBII/IIA (FR3IIIb line) or rPN1 (PN1-1) sodium channel types (Fig. 2*C*). Whole-cell patch-clamp recordings of FR3IIIb and PN1-1 cells were performed within 48 hr of treatment with growth factor. The ability of PIIIA conotoxin to block sodium current was quantitated by the fractional decrease in peak sodium current in response to application of 1  $\mu$ M toxin. Based on the findings from channels expressed in *Xenopus* oocytes, this concentration was expected to block <10% of rPN1 compared with >75% block of rBII/IIA sodium current. The results from representative FR3IIIb and PN1-1 cells are shown in Figure 2, *A* and *B*. PIIIA blocked only 13% of peak sodium current in the PN1-1 cells, consistent with expression of the rPN1 sodium channel type. In the FR3IIIb cells, 77% block was observed, consistent with expression of rBII/IIA sodium channels (Fig. 2*A,B*). The current-voltage relationships for both cell lines indicated no shift in voltage-dependent properties of sodium current associated with the block of inward current (Fig. 2*A,B*). The fractional blocks for seven individual FR3IIIb cells and for nine different PN1-1 cells are shown in Figure 2*D*. The overall average



**Figure 2.** Determination of the sensitivity of native rPN1 and rBII/IIA sodium channels to inhibition by conotoxin PIIIA. *A*, *Left panels*, Sodium currents recorded PN1-1 and FR3IIIb cells in the absence and presence of 1  $\mu$ M PIIIA conotoxin. Small block of rPN1 current (*top*) contrasts with the large block of rBII/IIA current (*bottom*). The individual sodium currents were elicited by a voltage step to  $-10$  mV from a holding potential of  $-100$  mV and were acquired at 10 sec intervals. *Right panels*, The current-voltage relationships for individual rPN1 (*top*) and rBII/IIA (*bottom*) sodium currents before (*open circles*) and after (*filled circles*) equilibrium block by 1  $\mu$ M PIIIA conotoxin. *C*, Northern blot analyses of the FR3IIIb versus PN1-1 cell lines using a sodium channel isoform-specific probe. The measurements of mRNA were made after 5 hr of NGF treatment for the PN1-1 cells and after 72 hr of FGF treatment for the FR3IIIb cells. *D*, A histogram comparing the amount of PIIIA conotoxin block for individual PN1-1 and FR3IIIb cells. The percentage inhibition was determined on the basis of block of inward current by 1  $\mu$ M PIIIA conotoxin during a step depolarization to  $-10$  mV from a holding potential of  $-100$  mV.



**Figure 3.** Time-dependent changes in the fractional inhibition of PC12 sodium current by PIIIA conotoxin after growth factor induction. The sodium current density (mean  $\pm$  SD pA/pF) is shown for 24, 48, and 72 hr after growth factor treatment. The shaded area represents the current that is PIIIA-insensitive, and the solid area represents the amount of current that is PIIIA-sensitive. The fractional sensitivity of overall sodium current to PIIIA toxin is indicated as a percentage at each time point (see Results for SD). Each time point represents the average of nine cells.

for FRIIb cells corresponded to 87% compared with an average value of 13% for PN1–1 cells.

PIIIA conotoxin was then applied, at a fixed 1  $\mu$ M concentration, to wild-type PC12 cells to quantitate the relative contributions of rBII/IIA and rPN1 sodium channels to the macroscopic current. Previous measurements of mRNA indicated that rPN1 mRNA peaked during the first 12 hr after NGF treatment, whereas rBII/IIA mRNA was induced after 48–72 hr. Therefore, we quantitated the fractional block of sodium current by PIIIA at 24 hr time points after NGF treatment. Based on the findings from the variant lines, 1  $\mu$ M conotoxin was expected to block an average of 13% of rPN1 current and an average of 87% rBII/IIA sodium current. During the initial 24 hr, an average of 13  $\pm$  12% of the NGF-induced sodium current in wild-type PC12 cells was blocked by PIIIA, pointing to rPN1 sodium current (Fig. 3). The amount of PIIIA-sensitive current increased to an average of 39  $\pm$  15% at 48 hr and to 63  $\pm$  16% at 72 hr, supporting the idea that the relative proportion of rBII/IIA current was increasing over time. The increase in proportion of PIIIA-sensitive sodium current was not associated with any significant increase in sodium current density (Fig. 3). In a separate experiment, the PIIIA-sensitive current measured 13% at 24 hr and 51% at 48 hr (data not shown).

## DISCUSSION

Our findings indicate that PIIIA  $\mu$ -conotoxin permits distinction between two major sodium channel types whose origins are predominantly the CNS (rBII/IIA) or PNS (rPN1). Although the difference in sensitivities between the rPN1 and rBII/IIA channel types was only an order of magnitude, the difference was sufficient to reveal the distinct current contributions in PC12 cells undergoing differentiation by NGF. One question that arises is whether the conotoxin sensitivity measured for channels expressed in oocytes is the same as that for native sodium channels in mammalian cells. We addressed this question through quantitative comparisons of conotoxin sensitivity for both rPN1 and rBII/IIA  $\alpha$  subunit types. Comparisons of the sensitivities to PIIIA conotoxin show excellent agreement between oocytes and PC12 cells.

A fixed 1  $\mu$ M concentration of PIIIA led to a 10% block of rPN1 in oocytes compared with 13% in PC12 cells. Similar agreement was indicated by the 75 and 87% block of rBII/IIA in oocytes and PC12 cells, respectively.

Our findings provide the first direct evidence that two structurally distinct sodium channel types are coexpressed in PC12 cells. To date, the idea that two sodium channel isoforms underlie the NGF-induced sodium current in PC12 cells has been based solely on inference from a variety of indirect measurements. Assignment of sodium current to either rPN1 or rBII/IIA channels in PC12 cells relied on the differences in time courses for the appearance of respective mRNA species. For example, because a brief treatment with NGF selectively triggers upregulation of rPN1 mRNA, but not rBII/IIA mRNA, it was possible to record sodium currents at times when only PN1 sodium channels should be expressed (Toledo-Aral et al., 1995). However, precedent also existed for induction of sodium channel mRNA in PC12 cells without expression of functional sodium channels (Ginty et al., 1992). The existence of intracellular pools of  $\alpha$  subunits in neurons (Schmidt et al., 1985) further prevented unequivocal assignment of induction of a specific sodium channel mRNA with macroscopic current. Thus, the ability to discriminate between functional channels on the cell surface provides a more direct measurement of signaling effects on specific sodium channel types.

In addition to discriminating between rPN1 and rBII/IIA functional channels in PC12 cells, the differential toxin sensitivity provided an estimation of the proportions of the two channel types after NGF treatment. The proportions, based on functional estimates, compared favorably with the estimates based on mRNA measurements. Previous mRNA measurements after pulsed NGF treatment of PC12 cells indicated that mRNA coding for rPN1 first appeared at  $\sim$ 1 hr, peaked at 5 hr, and declined to near zero values during the first 24 hr period. In contrast, in the continued presence of NGF, both sodium channel mRNAs are induced, but mRNA coding for rBII/IIA mRNA appears over a much slower time course than rPN1, peaking after several days instead of after 5 hr. Consequently, our first functional estimate corresponded to a time when the dominant mRNA species corresponded to rPN1. At 24 hr, only 13% of the sodium current was PIIIA-sensitive in each of two experiments. This value agreed with the predicted amount of block based on a pure rPN1 type sodium current as determined by the PN1–1 variant cell line. Therefore, it is likely that the current observed during the first 24 hr is, in fact, contributed almost exclusively by the rPN1 channel type. During the subsequent 48 hr period, we observed a steep increase in the proportion of PIIIA-sensitive current, once again consistent with the observed increase in mRNA encoding rBII/IIA sodium channels. Furthermore, in these experiments, the increase in proportion occurred without significant changes in sodium channel density. Thus, the switch to predominantly rBII/IIA sodium channel function likely reflects a decrease in rPN1 mRNA in addition to accumulation of rBII/IIA mRNA.

In conclusion, PIIIA has provided unequivocal evidence for the contributions of two distinct sodium channel types to TTX-sensitive macroscopic sodium current in PC12 cells. More importantly, the time course studies indicate that the functional appearance of the peripheral nerve and brain type sodium currents are well predicted by the time course of the individual mRNA species. The functional significance of two channel types exhibiting different time courses of expression during differentiation is not known. However, further studies using PIIIA  $\mu$ -conotoxin to

dissect the individual contribution by each channel type *in vivo* should help provide answers to this important question.

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