Novel Wasp Toxin Discriminates between Neuronal and Cardiac Sodium Channels

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Received August 21, 2000; accepted February 27, 2001

ABSTRACT

Pompilidotoxins (PMTXs), derived from the venom of solitary wasp has been known to facilitate synaptic transmission in the lobster neuromuscular junction, and a recent further study from rat trigeminal neurons revealed that the toxin slows Na⁺ channel inactivation without modifying activation process. Here we report that β-PMTX modifies rat brain type II Na⁺ channel α-subunit (rBII) expressed in human embryonic kidney cells but fails to act on the rat heart α-subunit (rH1) at similar concentration. We constructed a series of chimeric mutants of rBII and rH1 Na⁺ channels and compared modification of the steady-state Na⁺ currents by β-PMTX. We found that a difference in a single amino acid between Glu-1616 in rBII and Gln-1615 in rH1 at the extracellular loop of D4S3-S4 is crucial for the action of β-PMTX. PMTXs, which are small peptides with 13 amino acids, would be a potential tool for exploring a new functional moiety of Na⁺ channels.

Because voltage-dependent Na⁺ channels are a main component for the generation of the rapid depolarization during the initial phase of action potential, many natural toxins are designed to modify their functions so to capture a prey and to defend itself from predators. These Na⁺ channel-specific natural toxins are very useful tools for understanding and correlating the structure and function of Na⁺ channel (Catterall, 1980, 1995, 2000; Strichartz et al., 1987). For example, a group of natural toxins, such as α-scorpion toxin and sea anemone toxin has been found to eliminate specifically the fast inactivation process of Na⁺ channels from the outer side of cell membrane without modifying activation process. By using the site-directed mutagenesis and the tracer binding methods, Rogers et al. (1996) were able to localize their sites of action in the extracellular loop of D4S3-S4 of the Na⁺ channel α-subunit. Furthermore, slowing the inactivation process and reducing the steepness of voltage-dependence of steady state inactivation curve by these toxins leads them to implicate possible involvement of their binding sites in coupling channel activation to fast inactivation.

Recently, β-PMTX has been purified from the venom of the spider wasp and identified as a novel polypeptide neurotoxin with 13 amino acid residues and molecular mass of approximately 1530 Da (Konno et al., 1998). Sahara et al. (2000) have reported that β-PMTX causes a slowing of inactivation process in TTX-sensitive Na⁺ channels from rat trigeminal neurons. Consistent with this report, α-PMTX in which lysine at position 12 of β-PMTX is replaced by arginine, was found to induce a facilitation of both excitatory and inhibitory postsynaptic potentials, suggesting a possibility of increase in the firing frequency of Na⁺ channels (Konno et al., 1997, 1998; Harsch et al., 1998).

In this report, we attempted to identify the residue in Na⁺ channels involved in the suppression of inactivation process by β-PMTX, and we discuss molecular differences responsible for pharmacological actions among α-scorpion toxin, sea anemone toxin, and β-PMTX. This information will increase our insight into the mechanism of activation-inactivation coupling of Na⁺ channel localized on the extracellular face of Na⁺ channels.

Materials and Methods

Construction of Chimeras and Point Mutation of Na⁺ Channels. Chimeras and point-mutated Na⁺ channels were constructed using two cDNA clones coding the α-subunits of rBII (Stühmer et al.,...
1989) and rH1 (Rogart et al., 1989). To construct the chimeras by substitutions of D1 and D4, BsiWI and ClaI sites were created in the cDNA clones, respectively, as described previously (Ishii et al., 1999; Kimura et al., 2000). The sites of ligation (sequence numbers based on the rBII amino acid sequence) for the chimeras were 427 (BHHH) and 1474 (BBBH). We also studied the reverse chimeras, HHBB and HHHH. To substitute segments within D4, MfeI, ClaI, and BsiWI sites were created in the cDNA clones. The ligation sites (sequence numbers based on the rBII amino acid sequence) for the chimeras were 1474 and 1605 for substitution of the D4S1-S2 region, and 1648 and C-terminal end for substitution of the D4S4-S6 region. For substitution and point mutations of the region from the transmembrane segment D4S3 to the extracellular D4S3-S4 loop, a polymerase chain reaction-based mutagenesis method was employed using appropriately designed primers. All of the resulting chimeras and point mutants were confirmed with restriction mapping and sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Transient Transfection and Cell Culture.** The constructed chimeras and point-mutated cDNA clones were inserted into a mammalian expression vector pcI-neo (Promega, Madison, WI) or pcDNA3.1 (Invitrogen, Carlsbad, CA), and were then transiently cotransfected with CD8 cDNA into HEK cells using the SuperFect transfection reagent (QIAGEN, Hilden, Germany). The cells were grown to 50% confluence in Dulbecco’s modified Eagle’s medium (Life Technologies, Grand Island, NY), containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 30 U/ml penicillin G (Life Technologies), and 30 μg/ml streptomycin (Life Technologies), in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The transfected cells were used for electrophysiological experiments as late as 3 to 4 days after being replated on 35-mm tissue culture dishes. Transfection-positive cells were identified using CD8-Dynabeads (Dynal, Oslo, Norway) before the Na⁺ current recording.

**Electrophysiological Recording.** Macroscopic sodium currents from the transfected cells were measured using a whole-cell, patch clamp method. Leakage subtraction was carried out using the P/4 procedure (Armstrong and Bezanilla, 1974). The bath solution contained 70 mM NaCl, 67 mM N-methyl-d-glucamine, 1 mM CaCl₂, 1.5 mM MgCl₂, 10 mM glucose, and 5 mM HEPES, pH 7.4. The pipette solution contained 70 mM CsF, 60 mM CsCl, 12 mM NaF, 5 mM ethylene-bis (oxonitrito) tetraacetic acid and 5 mM HEPES, pH 7.4.

A bolus application of a stock solution of 10 mM β-PMTX (final concentration, 100 μM) was employed. Volume of perfusion chamber was 1 ml. In the case of the dose-response curve, data for 1, 3, and 10 μM β-PMTX were collected from cells soaked for 3 to 10 min in the corresponding solutions.

Data were presented as mean ± S.D. (number of observations), unless otherwise stated.

### Results

**Difference in the Sensitivity to β-PMTX between rBII and rH1.** A family of superimposed rBII Na⁺ currents expressed in a HEK cell is shown in Fig. 1Aa. The decay phase of rBII Na⁺ currents was greatly prolonged by an application of 100 μM β-PMTX (Fig. 1Ab, b and c). The current at 15 ms from the beginning of the voltage step was almost zero at all membrane potentials in the control, whereas it was increased by β-PMTX in a voltage-dependent manner with its maximum value near –20 mV (Fig. 1Aa, d). Because the relationship of the peak Na⁺ currents was not affected by β-PMTX, the toxin is considered to selectively interfere with Na⁺ channel inactivation process without affecting the activation. In contrast, the current passing through rH1 Na⁺ channels was entirely unaffected by β-PMTX (Fig. 1B). To further analyze the action of β-PMTX on rBII Na⁺ currents we employed a two-pulse protocol in which a 50-ms prepulse at various conditioning potentials was followed by a test pulse at –20 mV, and normalized peak Na⁺ currents (n = 6) were plotted against the conditioning prepulse potentials (Fig. 1C). In the presence of β-PMTX, a significant fraction of the Na⁺ channels remained active despite being subjected to long and large depolarizations (–30 to +60 mV for 50 ms). To measure the magnitude of modification of Na⁺ channel by β-PMTX, we used the ratio of the peak to the current at 15 ms in the presence of toxin at the membrane potential of –20 mV (Fig. 1D). Because the effect of β-PMTX reached a saturation level within 2 min, we calculated the modification ratio at 3 min after application of the toxin.

**Slowing the Inactivation Process by β-PMTX without Change in Peak I₁Na and Time to Peak of the Current.** As shown in Fig. 1, β-PMTX slowed decay of the current in Na⁺ channels of rBII without changing peak current as well as time to peak of the current. This occurs only if activation is much faster than inactivation. However, especially for neuronal Na⁺ channels, this has been considered not to be the case, because removing inactivation produced several changes in activation parameters. One line of evidence is that Gonoji and Hille (1987) exhibited a substantial increase in I₁Na, of neuroblastoma cells after removing inactivation by papain. Therefore, we tested the effect of papain on the expressed rBII Na⁺ channels as shown in Fig. 2: papain...
(1 mg/ml) was included in the whole-cell patch pipette and we waited at least 5 min to observe a square-like current. Contrary to native neuronal Na\(^+\) channels, both of peak \(I_{\text{Na}}\) and time to peak in rBII Na\(^+\) channels were not altered by papain treatment. In addition, the current-voltage relationship of papain-treated Na\(^+\) channels was comparable with that of nontreated Na\(^+\) channels (Fig. 2): a large current at negative potentials would be expected if the inactivation were predominant over the activation process after removal of inactivation. Thus, our data did not support the slow activation and fast inactivation processes. Similar results are obtained in rH1 (Fig. 2B). These results indicate that activation processes of both Na\(^+\) channels examined are fast enough not to be affected by change in the inactivation process. Thus, it is reasonable to assume that activation is not altered by \(\beta\)-PMTX in both species of Na\(^+\) channels, and the modification ratio used in this study is a good measure of change in inactivation.

The Origin of D4S3-S4 Loop Is a Determinant Factor for the Difference in Sensitivity. To determine the structural basis of the marked difference in the sensitivity to \(\beta\)-PMTX between rBII and rH1, we constructed chimeric Na\(^+\) channels in which the amino acid residues in rBII were converted their cardiac isoforms. First, four types of chimeric mutants of Na\(^+\) channels were constructed and assayed for the modification ratio by \(\beta\)-PMTX. Each chimera is referred to by the name of the isoform from the N terminus to the C terminus in sequence, in which domains from the heart are named H and those from the brain are named B. Chimeras HBBB and HHHB were modified by \(\beta\)-PMTX and yielded modification ratios of 0.43 and 0.82, respectively (Table 1). In contrast, the reverse chimeras BHBB and BHHB were insensitive to the toxin. These findings indicate that the source of D4 in rBII is important for the effect of \(\beta\)-PMTX. We then constructed chimeric mutants of Na\(^+\) channels in which a part of the segments in D4 from rH1 was replaced by a part from rBII isoforms (Table 1). Of the three mutant channels, rH1 chimera 1, in which five amino acid residues in D4S3 and two residues in the extracellular loop were replaced, was largely modified by \(\beta\)-PMTX, yielding the modification ratio of 0.73. However, the other two mutants in which amino acid residues in D4S1-S2 or residues in D4S4-S6 of rH1 were converted to the rBII isoform (rH1 chimera 2, or rH1 chimera 3) were entirely insensitive to \(\beta\)-PMTX. The results indicate that the amino acid residues in the transmembrane segment S3 and the extracellular loop between S3 and S4 in D4

Fig. 2. Effect of removing inactivation by papain on time to peaks of expressed sodium channels of rBII and rH1. A, left, a family of current records (top) of rBII sodium channels elicited by voltage steps between −110 mV and +40 mV from a holding potential of −100 mV are compared with that (bottom) subjected to papain (1 mg/ml) digestion applied from intracellular side through a whole-cell patch pipette. Removal of inactivation did not affect time to peaks of rBII Na\(^+\) currents at all potentials examined (right). I-V curves were taken around at 6 min from the beginning of the whole-cell patch clamp both for controls and papains (\(n = 3\)), and constructed by normalizing to peak values at −20 mV (−30 mV for rH1). Therefore, the control values were taken from another set of nontreated cells. I-V curves are essentially not altered by papain (left, insets). B, The same procedure was applied to rH1 sodium channels. Data are displayed as in A. Similarly to rBII channels, removal of inactivation had little influence on time to peaks and I-V curves of rH1 Na\(^+\) channels (\(n = 4\)).
(D4S3-S4) of rBII channels may be responsible for the action of $\beta$-PMTX.

We next attempted to further narrow the responsible area for the $\beta$-PMTX effect by constructing a series of chimeric mutants in which various amino acid residues in D4S3 and the extracellular loop of D4S3-S4 of rBII were individually replaced with the corresponding amino acids of the rH1 (Table 2). rBII chimera 1, in which seven amino acid residues in this region of rBII were replaced by the corresponding amino acids in rH1, was insensitive to $\beta$-PMTX. Moreover, replacement of five amino acid residues in S3 of rH1 with those of rBII (rH1 chimera 4) rendered the chimera insensitive to $\beta$-PMTX. In contrast, a chimera made by replacement of two amino acids in the extracellular loop of D4S3-S4 of rH1 with those of rBII (rH1 chimera 5) was found to be more sensitive to the toxin than the wild-type rBII. These results strongly suggest that two amino acid residues (Glu-1616 and Val-1619) in the structure of the chimeric Na$^+$ channel reconstructed by introducing a small part of rBII into rH1, could affect $\beta$-PMTX binding. Involvement of other sites in the $\beta$-PMTX binding was observed with the mutant of rBII-E1613R. This mutant had a reduced sensitivity to $\beta$-PMTX, whereas rBII-E1613D showed sensitivity similar to that of wild-type channels. Unexpectedly, rH1-F1619V, which has Glu at position 1615, was sensitive to the toxin. Because this mutant’s sensitivity disappeared by substituting Asp at 1612 (equivalent to 1613 in rBII) with Asn, it is reasonable to assume that the toxin may be able to bind to Asp at 1612 close to 1615 in a-helix conformation of rH1-based chimeric Na$^+$ channels. The double mutant (rH1-D1612N&F1619V) restored the sensitivity to $\beta$-PMTX by introducing Glu into position 1615 (rH1 chimera 5&D1612N), equivalent to the critical site in rBII for $\beta$-PMTX.

Another single mutant rBII-V1620F was as sensitive to $\beta$-PMTX as wild-type rBII. These results demonstrate that the major molecular determinant for the action of $\beta$-PMTX is probably Glu-1616 in the extracellular loop of D4S3-S4 of rBII. Interestingly, conversion of Glu into positively charged Lys (rBII-E1616K) dramatically reduced the sensitivity. Presumably, the positive charge in the toxin molecule may bind with the negatively charged Glu by electrostatic interaction at the outer mouth of the D4S3-S4 loop in rBII. Moreover, we found that Glu-1613 in rBII plays an important role in $\beta$-PMTX binding. Mutant of rBII-E1613R yielded a modification ratio of 0.15, less than 0.26 of the wild-type, suggesting that introduction of a positive charge into 1613 may electrically influence the negative charge in the nearby critical site, 1616.

Another interesting feature is that chimeric Na$^+$ channels, in which parts of rBII are introduced into rH1, were more susceptible to $\beta$-PMTX than the wild-type rBII. HHHB chimera gave the modification ratio of 0.82 (Table 1). Also, introduction of Val at 1619 together with Glu at 1615 (rH1 chimera 5) or in addition to these mutations replacement of S3 with that of rBII (rH1 chimera 1) yielded modification ratios of 0.75 and 0.73, respectively (Table 2). This unique nature of chimeric channels strongly suggests that not only the extracellular S3-S4 loop of D4 but also the quaternary structure of the chimeric Na$^+$ channel reconstructed by introducing a small part of rBII into rH1, could affect $\beta$-PMTX binding. Involvement of other sites in the $\beta$-PMTX binding was observed with the mutant of rBII-E1613R. This mutant had a reduced sensitivity to $\beta$-PMTX, whereas rBII-E1613D showed sensitivity similar to that of wild-type channels. Unexpectedly, rH1-F1619V, which has Glu at position 1615, was sensitive to the toxin. Because this mutant’s sensitivity disappeared by substituting Asp at 1612 (equivalent to 1613 in rBII) with Asn, it is reasonable to assume that the toxin may be able to bind to Asp at 1612 close to 1615 in a-helix conformation of rH1-based chimeric Na$^+$ channels. The double mutant (rH1-D1612N&F1619V) restored the sensitivity to $\beta$-PMTX by introducing Glu into position 1615 (rH1 chimera 5&D1612N), equivalent to the critical site in rBII for $\beta$-PMTX.

### TABLE 1

<table>
<thead>
<tr>
<th>Wild-Type/Chimera</th>
<th>Amino Acid Sequence</th>
<th>Modification Ratio [\text{Mean} \pm \text{S.D. (Number of Observations)}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type rBII</td>
<td>![Amino Acid Diagram]</td>
<td>0.26 ± 0.03 (n = 5)</td>
</tr>
<tr>
<td>wild type rH1</td>
<td>![Amino Acid Diagram]</td>
<td>0 (n = 4)</td>
</tr>
<tr>
<td>BHHH</td>
<td>![Amino Acid Diagram]</td>
<td>0 (n = 3)</td>
</tr>
<tr>
<td>HBBB</td>
<td>![Amino Acid Diagram]</td>
<td>0.43 ± 0.06 (n = 4)</td>
</tr>
<tr>
<td>HHBB</td>
<td>![Amino Acid Diagram]</td>
<td>0.82 ± 0.06 (n = 4)</td>
</tr>
<tr>
<td>BBBH</td>
<td>![Amino Acid Diagram]</td>
<td>0 (n = 3)</td>
</tr>
<tr>
<td>rH1 chimera 1</td>
<td>![Amino Acid Diagram]</td>
<td>0.73 ± 0.11 (n = 4)</td>
</tr>
<tr>
<td>rH1 chimera 2</td>
<td>![Amino Acid Diagram]</td>
<td>0 (n = 3)</td>
</tr>
<tr>
<td>rH1 chimera 3</td>
<td>![Amino Acid Diagram]</td>
<td>0 (n = 4)</td>
</tr>
</tbody>
</table>

The modification ratio $\frac{Y_{\text{max}}}{1 + (EC_{50}/\beta - \text{PMTX})^{n_H}}$ is given by the equation, where $Y_{\text{max}}$ indicates the maximum response for $\beta$-PMTX action, $EC_{50}$ denotes the half-maximal concentration, $n_H$ is the Hill coefficient, and $\beta - \text{PMTX}$ represents the $\beta$-PMTX concentration.

A higher sensitivity to $\beta$-PMTX of HHHB than rBII is explained by an increase in $Y_{\text{max}}$ (from 0.29 to 1) and reduction in $n_H$ (from 1.0 to 0.48), whereas $EC_{50}$ values were relatively constant ($EC_{50}$ values for rBII and HHHB are 11 $\mu$M and 5.6 $\mu$M, respectively). Thus, amino acid changes
between these two Na\textsuperscript{+} channel isoforms can be considered to cause a large change in efficacy and a relatively small change in affinity to \(\beta\)-PMTX.

**Other Kinetic Parameters That Indicate the Effect of \(\beta\)-PMTX.** Modification by \(\beta\)-PMTX can be measured by several indices of inactivation kinetics other than the modification ratio, such as time constants of the current decay or relative contribution of each component to the total current. Na\textsuperscript{+} currents measured in this study from several mutants or chimeras constructed from rBII and rH1 including wild-type channels exhibited double exponential decay [fast (\(\tau_f\)) and slow (\(\tau_s\)) components] of the inactivation phase. In Fig. 4, the effect of \(\beta\)-PMTX on these kinetic parameters (\(A_1, A_2, \tau_s,\) and \(C\)) for each mutant are compared, when current decay is fitted by the equation of

\[
f(t) = A_1 \times e^{-\frac{t}{\tau_f}} + A_2 \times e^{-\frac{t}{\tau_s}} + C
\]

Because \(\tau_f\) was not essentially altered by \(\beta\)-PMTX similarly to those by long-chain \(\alpha\)-scorpion toxin (Possani et al., 1999), fitting was performed by fixing \(\tau_f\) values of PMTX data to those of control data. Thus, data for \(\tau_f\) is not given in Fig. 4, but control \(\tau_f\) values are provided in the figure legend. The most prominent feature shown in Fig. 4 is a large increase in constant component, which reasonably corresponds to the modification ratio; i.e., any mutants or rBII Na\textsuperscript{+} channels that exhibited effective modification ratio also increased the constant component from nearly 0 to 20 to 60% of the total current. Moreover, consistent changes in other parameters were also observed (Fig. 4).

**Discussion**

In the present study, we have found that \(\beta\)-PMTX exhibits an isoform-specific sensitivity to Na\textsuperscript{+} channels depending on the presence or absence of an anionic residue at the binding site. The anionic residue, Glu-1616 in D4S3-S4 loop of rBII is the site responsible for \(\beta\)-PMTX-binding, although the homologous site in rH1, Gln-1615, is not. To confirm an importance of negative charge in this residue, a mutant with replacement of Glu-1616 with Lys (rBII-E1616K) was tested.

![Figure 3](https://example.com/fig3.png)
This mutation completely abolished β-PMTX effect. Moreover, structural analysis of PMTXs revealed that some cationic residues (Arg-1, Lys-3, and Lys/Arg-12) were found critical for exhibiting toxic activity (Konno et al., 2000). Therefore, it is most likely that the positively charged amino acid residues in β-PMTX may bind with this site on D4S3-S4 loop by electrostatic interaction. 

Because both α-scorpion and sea anemone toxins exert an inhibitory action to inactivation mechanism in different Na⁺ channel isoforms (Romey et al., 1976; Catterall, 1980, 1995; Eitan et al., 1990; Possani et al., 1999), although to different extents, it is quite reasonable to find out that the sites of action of both toxins retain common chemical property, anionic amino acids, Glu-1613 of rat brain type IIA Na⁺ channel α-subunit (rBIIA) (Rogers et al., 1996) and Asp-1612 of rH1 (Benzinger et al., 1998). Using site-directed mutagenesis, it was found that negative charge on these residues and positive charge on these toxins play an important role in their binding with Na⁺ channels. Rogers et al. (1996) have reported that α-scorpion toxin and sea anemone toxin bindings occur at Glu-1613 in transmembrane segment D4S3 of rBIIA, because E1613R mutant significantly decreases not only binding affinity but also their toxin potency. Along with this report, Benzinger et al. (1998) have provided the information on an importance of D1612 in rH1 Na⁺ channels and K37 in sea anemone toxin, anthopleurin B (ApB) for binding. The substitution of acidic aspartic acid residue with neutral asparagine and basic arginine residues, respectively, increases affinity constants (Kᵣ) for ApB from 0.5 nM for wild-type rH1 to 30 nM for D1612N and to 480 nM for D1612R; Kᵣ for ApB K37D also increased to 200 nM for wild-type Na⁺ channel. Similarly, many reports pointed out that positive charges in various sites in α-scorpion and sea anemone toxins are involved in binding with Na⁺ channels through electrostatic interaction (Gallagher and Blumenthal, 1994; Loret et al., 1994; Khra et al., 1995; Benzinger et al., 1997; Zilberberg et al., 1997; Froy et al., 1999). Considering the results obtained for α-scorpion and sea anemone toxins, it is reasonable to speculate that the isoform specific difference in the sensitivity to β-PMTX is due to the presence or absence of anionic residue in the binding site. Actually, the site in rBII sensitive to this toxin is anionic residue, E1616 and the site homologous in insensitive rH1 neutral Q1615. 

Interestingly, the chimeric E1616Q mutant, which was insensitive to β-PMTX, is known to be as sensitive to α-scorpion toxin (LqTx) as the wild-type (Rogers et al., 1996). Moreover, sea anemone toxins (ATX II, ApA, and ApB) generally affect the inactivation process of cardiac Na⁺ channel isoform (Chahine et al., 1996; Benzinger et al., 1997, 1998). Accumulating evidences have shown that Na⁺ channels in different tissues exhibit differential functional properties, possibly because of expression of diverse Na⁺ channel genes (Trimmer and Agnew, 1989; Stühmer and Parekh, 1992; Eggen and Mandel, 1997; Raman and Bean, 1997; Mantegazzza et al., 1998). In our study, β-PMTX discriminates between neuronal and cardiac sodium channels by recognizing the difference in a single amino acid residue. Because the toxin, with only 13 amino acids, is very small compared with other Na⁺ channel-specific polypeptide toxins, such as α-scorpion toxin (60 to 65 amino acids) and sea anemone toxin (46 to 49 amino acids), the receptor site may be limited to the critical single amino acid in the wild-type Na⁺ channel protein molecule. On the contrary, the receptor sites for α-scorpion and sea anemone toxins, containing many disulfide bonds, have been identified at the wide variety of regions, D1S5-S6, D4S5-S6, and D4S3-S4 loops in Na⁺ channel (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Rogers et al., 1996). Taking into account that PMTXs lack disulfide bonds, with which it might otherwise have taken a complicated configuration similar to those of the α-scorpion and sea anemone toxins, the small and simple structure of PMTXs would offer a special advantage to classify and characterize various Na⁺ channel isoforms.
Acknowledgments

We would like to express our sincere gratitude to Dr. K. Imoto (National Institute for Physiological Sciences, Okazaki, Japan) for providing HEK cells, and to Dr. M. Noda (National Institute for Basic Biology, Okazaki, Japan) for the generous gift of α-subunit of rat brain type II Na⁺ channel, rIIIC cDNA (pRII-2A).

References


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