Tamapin, a Venom Peptide from the Indian Red Scorpion *(Mesobuthus tamulus)* That Targets Small Conductance Ca^{2+}-activated K^{+} Channels and Afterhyperpolarization Currents in Central Neurons*

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The biophysical properties of small conductance Ca^{2+}-activated K^{+} (SK) channels are well suited to underlie afterhyperpolarizations (AHPs) shaping the firing patterns of a conspicuous number of central and peripheral neurons. We have identified a new scorpion toxin (tamapin) that binds to SK channels with high affinity and inhibits SK channel-mediated currents in pyramidal neurons of the hippocampus as well as in cell lines expressing distinct SK channel subunits. This toxin distinguished between the SK channels underlying the apamin-sensitive I_{AHP} and the Ca^{2+}-activated K^{+} channels mediating the slow I_{AHP} (sIAHP) in hippocampal neurons. Compared with related scorpion toxins, tamapin displayed a unique, remarkable selectivity for SK2 versus SK1 (−1750-fold) and SK3 (−70-fold) channels and is the most potent SK2 channel blocker characterized so far (IC_{50} for SK2 channels = 24 pm). Tamapin will facilitate the characterization of the subunit composition of native SK channels and help determine their involvement in electrical and biochemical signaling.

Potassium channels show an exceptional functional diversity, being implicated in neural and behavioral plasticity, secretion, and cell proliferation (1). In addition to regulating cell excitability, the channels themselves can be modulated in a cell-specific manner through second messengers, hormones, and neurotransmitters (2). The fact that >60 distinct K^{+} genes have now been cloned provides a molecular basis for this diversity (3).

The link between molecularly defined individual channel subunits and native currents is often difficult to establish. However, in the case of small conductance Ca^{2+}-activated K^{+} (SK) channels, the bee venom toxin apamin has proven to be a valuable tool for establishing correlations between cloned SK channels and native currents and for revealing the function of native SK channels in specific neuronal populations (4−7).

Apart from apamin, many scorpion venoms contain SK channel blockers, which have proved to be useful adjuncts to the classical bee venom toxin. These include scyllatoxin, isolated from the scorpion *Leiurus quinquestriatatus* (8−10), and PO5 from *Androctonus mauretanicus* (11). Both scorpions are found in desert regions of North Africa and the Eastern Mediterranean. Other more recently identified scorpion toxins that compete for apamin-binding sites with high affinity include κ toxin from the venom of the Central American scorpion *Tityus serrulatus* (12−14) and BmPO5 from the Asian scorpion *Buthus martensi Karsch*, widely distributed throughout China (15). Finally, toxins with greatly reduced binding affinities include PO1 and BmPO1 (15, 16). Two African scorpion toxins, mauen toxin from *Scorpio maurus* and Pi4 from *Pandinus imperator*, are less selective: they compete with apamin for binding to rat brain synaptosomes, but they also inhibit potassium currents generated by channels of the Kv1 family (17, 18).

The Indian red scorpion (*Mesobuthus tamulus*) causes annually a large number of deaths, especially among young children on the Indian subcontinent (19), and its venom has been a rich source for highly specific potassium channel blockers such as ibetoxin (20) and tamulustoxin (21). We have examined the venom for the presence of other selective ion channel blockers. Here we characterize the properties of a new toxin, termed tamapin, that blocks SK channels.

Based on sequence homology and disulfide bridges, tamapin can be assigned to toxin subfamily 5 (22). It displaces apamin from rat brain synaptosomes with high affinity and inhibits the afterhyperpolarizing current (I_{AHP}) mediated by native SK channels in hippocampal pyramidal neurons (4). Tamapin inhibits SK currents in heterologous expression systems and shows the highest potency for recombinant SK2 channels to date.

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1 The abbreviations used are: SK, small conductance Ca^{2+}-activated K^{+}; AHP, afterhyperpolarization; HPLC, high performance liquid chromatography; IK, intermediate conductance Ca^{2+}-activated K^{+}.
**Materials and Methods**

Toxins and Chemicals—Native apamin was purified from *Apis mellifera* bee venom, and [125I]monoiodoapamin (~2200 Ci/mmol) was prepared as described previously (23). Low pressure chromatography media were obtained from Amersham Biosciences. Protease inhibitors and bovine serum albumin fraction V, protease-free) were purchased from Sigma. All other chemicals were reagent-grade and obtained from Merck.

**Purification of Tamapin**—Indian red scorpions (*Mesob. tamulus*) were collected in the state of Maharashtra at the end of the rainy season (November through December) and kept at the Haffkine Institute. Scorpions were milked by electrical stimulation of their venom glands, and the venom was immediately lyophilized. Venom (220 mg) was resuspended in deionized water (50 ml) and adjusted to pH 3 with HCl. After gentle vortexing, the venom suspension was clarified by centrifugation (48,000 g, 60 min, 4 °C). The supernatant was removed from the mucous-like residue and passed through a 0.2-μm filter (Sartorius Corp., Göttingen, Germany).

The sample was applied in two batches to a Sephadex G-50 column (2.6 × 86 cm) equilibrated with 50 mM ammonium formate (pH 3.5). The column was eluted with the equilibration buffer (40 ml/h) and elution was monitored at 280 nm. 15-min fractions were collected, and those with apamin-like activity (determined by inhibitory binding assay) were pooled and lyophilized. Active fractions were dissolved in 20 ml of 50 mM sodium acetate buffer (pH 4.8) (buffer A) and applied to an SP 16/10 Hi-load cation-exchange column (Amersham Biosciences) equilibrated with buffer A. After washing, the column was eluted with a 500-ml linear gradient of 0–100% buffer B (buffer A containing 1 M NaCl) at a flow rate of 5 ml/min. Fractions were collected and monitored at 280 nm, and fractions with apamin-like activity were pooled. Active fractions (eluting at 350–450 ml NaCl) were rechromatographed on the same ion-exchange column using a shallower gradient (500 ml of 35–55% buffer B), affording the separation of two active peaks (corresponding to tamapin and tamapin-2). The fractions corresponding to each peak were pooled, desalted on a Sephadex G-10 column (2.6 × 94 cm) equilibrated with 10 mM ammonium formate (pH 4.8) and lyophilized.

Tamapin and tamapin-2 were independently purified to homogeneity by reversed-phase HPLC in two steps. In the first step, the sample was loaded onto a 300-A pore size C8 reversed-phase column (5 μm, 3.2 × 250 mm) equilibrated with 5% acetonitrile containing 0.05% trifluoroacetic acid (buffer C). The column was initially eluted with a linear gradient of 0–75% buffer D (buffer C containing 60% acetonitrile) for 10 min and subsequently with a linear gradient of 75–100% buffer D for 60 min. Fractions that eluted from the column (0.5 ml/min) were assayed for apamin-like activity. In the second step, active fractions were applied to the same column equilibrated with 5% acetonitrile and 100 mM sodium phosphate (pH 5.5) (buffer E). The column was eluted (0.5 ml/min) with a linear gradient of 0–100% buffer F (buffer E containing 80% acetonitrile). The purity of tamapin and tamapin-2 was checked by rerunning pooled active fractions from the second reversed-phase column on a 5-μm Jupiter C18 reversed-phase column (1 × 150 mm) equilibrated with 0.1% trifluoroacetic acid. Tamapin and tamapin-2 were judged to be 97–98% pure under these conditions.

**Electrospray Mass Spectrometry**—Purified tamapin and tamapin-2 were analyzed by electrospray ionization mass spectrometry using a triple quadrupole instrument equipped with an ionspray interface (PerkinElmer Life Sciences AFT 300 liquid chromatography/mass spectrometry/mass spectrometry). Samples (5 μl) were directly infused into the mass spectrometer at a flow rate of 50 μl/min. The ionspray voltage was set at 4600 V, the ring voltage at 350 V, and the orifice voltage at 30 V. Nitrogen was used as the curtain gas, and compressed air was used as the nebulizer gas.

**Amino Acid Sequencing**—Toxin samples were sequenced by automated Edman degradation using a PerkinElmer Life Sciences Model 494 pulsed liquid-phase sequencer (Procise). Phenylthiohydantoin (PTH) (29) derivatives were identified using an on-line reversed-phase PTH-C8 column in an Applied Biosystems 785A phenylthiohydantoin-derivative analyzer. The sequence of tamapin was confirmed by repeating the analysis four times on a total of three different samples. The sequence of tamapin-2 was confirmed by sequencing two different samples.

**Reduction and Pyridylethylation**—Lyophilized samples (~10–30 nmol) were reconstituted in 100 μl of denaturant buffer (6 M guanidine hydrochloride, 1 mM EDTA, and 0.15 mM Tris HCl (pH 8.0)). Following the addition of β-mercaptoethanol (2 μl), the solution was incubated under nitrogen (2 h, 37 °C). 4-Vinylpyridine (20 μl) was subsequently added, and the mixture was incubated under nitrogen at room temperature for a further 2 h. The reaction mixture was immediately desalted by reversed-phase HPLC on a C18 column.

**Apamin Binding Assay**—[125I]Apamin binding to rat brain synaptic plasma membranes was performed using a filtration binding assay essentially as described previously (23, 24). The incubation medium (1 ml) consisted of 10 mM KCl, 1 mM EDTA, and 25 mM Tris (pH 8.4) containing 0.1% bovine serum albumin. Aliquots of membranes (100 μg of protein) were incubated for 1 h on ice in the presence of 10 pM [125I]-apamin and crude Mesob. tamulus venom, individual chromatographic fractions, or purified toxins. The binding reaction was quenched by the addition of ice-cold incubation medium and rapid filtration (<20 s) through Whatman GF/B filters presoaked in 10% (v/v) polyethyleneimine. The computer program EBDA ( Biosoft, Cambridge, UK) was used to calculate the IC50 values (toxin concentration that inhibits 50% of the binding of [125I]-apamin) from the raw data. IC50 values were then used to calculate the true equilibrium dissociation constant of the inhibitory ligand (Kd) using the Cheng and Prusoff equation (55): IC50 = Kd (1 + L/IC50), where L is the free concentration of [125I]-apamin at half-displacement and Kd is the dissociation constant for [125I]-apamin. The experiments were designed to produce IC50 values in the range of 2–4 fold higher than the Kd. Replicate samples typically varied between 3 and 5%. Samples were counted in a calibrated γ-counter.

**Cell Culture and SK Channel Stable Transfections**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 supplemented with 2 mM t-glutamine, 1 mM of penicillin/streptomycin, and 10% fetal calf serum. Cells were grown in a humidified atmosphere (5% CO2 and 95% air) at 37 °C. For transfection, cells were grown to 50–60% confluency, and 6 μg of rat SK2 or rat SK3 cDNA (subcloned in the eukaryotic expression vector pcDNA3) were transfected using the CaPO4 method (25). Transfected cells were selected in medium supplemented with 0.4 mg/ml Geneticin (G418). To generate clonal cell lines, single cell clones were trypsinized and replated. This was performed at least twice before a cell line was established. For recordings, cells were cultured in the presence of 0.4 mg/ml G418. Expression of rat SK2 or rat SK3 was confirmed by immunofluorescence and by patch-clamp measurements.

The SK1-CHO-Flip-In cell line was generated using the Flip-In system ( Invitrogen). Briefly, human SK1 was cloned into the pcDNAs/FRT vector. For transfection, 0.9 μg of SK1-pcDNA/FRT were cotransfected with 10 μg of pOG44 into the CHO-Flip-In cells using LipofectAMINE together with the PlusTM reagent ( Invitrogen). Transfected cells were selected in medium containing 0.1 mg/ml hygromycin B. Expression of human SK1 was confirmed by patch-clamp recordings, where the half-maximal blocking concentrations of apamin and d-tubocurarine were applied. The cell line stably expressing human SK4/human IK1 channel was a kind gift of W. J. Joiner and L. K. Kaczmarek (26).

**Electrophysiology of Transfected Cells—Mesob. tamulus venom was performed in the whole-cell configuration of the patch-clamp technique.** HEK293 cells or CHO-Flip-In cells expressing SK1, SK2, SK3, or IK1 channels were grown on coverslips, placed in a recording chamber, and perfused at a rate of 4 ml/min. Currents were recorded using an EPC-9 amplifier (HEKA, Lambrecht, Germany), and data were acquired with Pulse/Pulsfit software (HERA) and analyzed with Igor Pro (WaveMetrics, Lake Oswego, OR). Pipettes were pulled from borosilicate glass (Kimble Glass Inc., Vineland, NY) with a vertical patch electrode puller (List Medical, Darmstadt, Germany). Pipettes had a resistance of 2–3 megohms when filled with intracellular solution (see below). After gigaseal formation, the capacitative transients were automatically compensated.

MgCl2 and CaCl2 concentrations were adjusted using EqCal (Biosoft) to obtain free calcium concentrations of 1 μM, 10 μM, and 1 mM. SK and IK channels were activated by whole-cell dialysis with an intracellular solution containing 130 mM KCl, 10 mM HEPES, 10 mM EGTA, and variable concentrations of MgCl2 and CaCl2 (see above) adjusted to pH 7.4 with KOH. Recordings were performed in a symmetrical potassium or potassium-free extracellular solution containing 144 mM KCl, 10 mM HEPES, 1 mM MgCl2, and 2 mM CaCl2 adjusted to pH 7.4 with KOH (symmetrical); 144 mM NaCl replaced KCl in the potassium-free solution. Currents were recorded upon application of voltage ramps from 100 to +40 mV with a duration of 400 ms, repeated every 5 s. Alternatively, 100- to 200-ms-long voltage steps from 100 to +40 mV with increments of 20 mV were applied. Given the large size of some currents, voltages were corrected off-line for the occurring voltage-clamp error.

**Electrophysiology of Hippocampal Slices—Wistar rats (19–30 days**
Tamapin, a Novel SK Channel Toxin

RESULTS

Purification and Characterization of Tamapin—Crude Mesob. tamulus venom inhibited the binding of [125I]monoiodoapamin to rat brain synaptic plasma membranes (Fig. 1A). Apamin binding (B) is expressed as a percent of total binding determined in the absence of any competing ligand (B0). The sigmoidal shape of the binding curve in this semilogarithmic plot suggests that there is only one molecule (or one class of molecules with similar affinity) that interacts with apamin-sensitive SK channels.

To purify the apamin-binding activity, crude venom was initially fractionated by gel-exclusion chromatography on Sephadex G-50. Several unresolved peaks absorbing at 280 nm were observed (Fig. 1B), the majority with molecular masses of ~10 kDa or less. Fractions active in the [125I]-apamin inhibitory binding assay (fractions 27–37) were pooled and chromatographed on an S-Sepharose ion-exchange column, eluting fractions with a salt gradient (0.05–1 M NaCl) (data not shown). Rechromatography of the most active fractions on the same column using a shallow salt gradient (0.05–0.55 M NaCl) yielded two peaks of inhibitory apamin-binding activity (peaks

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Figure 1. Purification of tamapin from Mesob. tamulus venom. A, [125I]-apamin binding to rat brain synaptosomes was inhibited in a concentration-dependent manner by crude Mesob. tamulus venom. Toxin binding (B) is expressed as fraction of total binding in the absence of venom (B0). B, shown are the results from Sephadex G-50 size-exclusion chromatography of venom after acid extraction. The gray box indicates the fractions that were pooled based on the competition assay performed with [125I]-apamin. C, shown are the results from the second S-Sepharose cation-exchange chromatography of active fractions obtained from the first ion-exchange chromatography step. Superimposed on the elution profile is the result of the competition assay performed with [125I]-apamin. The highest activities were found in fractions 10–30 (peak I) and 39–41 (peak II), corresponding to tamapin and tamapin-2, respectively. •, absorbance at 280 nm; ○, inhibitory binding activity. D, shown are the results from C2 reversed-phase HPLC of peak I. Biological activity (indicated as Tamapin) was identified by the [125I]-apamin competition assay. Inset, C2 reversed-phase HPLC of peak II. Tamapin in D. The ordinate scales indicate absorbance at 220 nm (left) and percent acetonitrile in elution buffer (right); the abscissa scale indicates time in minutes.
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The amino acids that are not conserved in the three toxin sequences are boxed in black. The RXCQ motif, conserved in PO5 (Swiss-Prot accession number P31719) and scyllatoxin (Swiss-Prot accession number P16341), is boxed in grey and is changed to RRCE in tamapin.

**TABLE I**

| Tamapin | Scyllatoxin | PO5 |
|---------|-------------|-----|
| C | N | L | L | S | C | K | L | G | L | G | E | C | T | G | C | E | C | Y | Y | 31 |
| C | N | L | L | S | C | K | L | G | L | G | E | C | T | G | C | E | C | Y | Y | 31 |
| C | N | L | L | S | C | K | L | G | L | G | E | C | T | G | C | E | C | Y | Y | 31 |

**TABLE II**

| Molecular masses of tamapin and tamapin-2 as determined by electrospray ionization mass spectrometry and amino acid sequencing |
|---------------------------------------------------------------|
| Mass spectrometry (± S.D.) | Amino acid sequence |
|-----------------------------|---------------------|
| Tamapin/2 | 3457.9 ± 0.2 | Da |
| Tamapin | 3459.1 | Da |
| Tamapin-2 | 3433.1 | Da |

I and II) (Fig. 1C). Tamapin (peak I) was finally purified to homogeneity by reversed-phase chromatography on a C8 column (Fig. 1D). Tamapin was judged to be >98% pure by chromatography on a reversed-phase C18 column using a shallow solvent gradient (20–40% acetonitrile in 0.1% trifluoroacetic acid) (Fig. 1D, inset). Homogeneity was confirmed by mass spectrometry and SDS-PAGE (data not shown). A unique N-terminal residue (alanine) was detected upon automated Edman degradation, which gave an initial yield of 56% and a repetitive yield of 93%.

The sequence of tamapin was determined after reduction and pyridylethylation of cysteine residues (Table I). The molecular mass of tamapin as determined by electrospray ionization mass spectrometry (3457.9 ± 0.2 Da) was 1 mass unit less than that calculated from the amino acid sequence (3459.1 Da) (Table II). Digestion of tamapin with either carboxypeptidase A or carboxypeptidase Y failed to detect any amino acids within the time that the ultimate and penultimate residues of dendrotoxin were hydrolyzed (data not shown). Taken together, the mass spectrometry data and the lack of digestion by carboxypeptidases strongly suggest that tamapin is amidated at its C-terminal tyrosine residue (see also Ref. 9). Tamapin shares 77% amino acid sequence similarity with scyllatoxin and 74% with PO5 (Table I), and all three toxins are 31 amino acids long. The six cysteine residues can be found at identical positions within the primary sequences of these toxins. The sequence similarity to other toxins competing with apamin in binding assays, such as maurotoxin, Pi1, PO1, and T. serrulatus κ toxin, is substantially lower (<30%).

A second active fraction (peak II) (Fig. 1C) was purified to homogeneity by reversed-phase chromatography (>97% pure by HPLC) (data not shown) in a manner analogous to tamapin. Upon sequencing, this second fraction was identified as an isoform of tamapin, differing by a single amino acid residue (His instead of Tyr) at its carboxyl terminus. The molecular mass of tamapin-2 determined by electrospray ionization mass spectrometry (3431.4 ± 0.2 Da) was 1 mass unit less than that calculated from the amino acid sequence (3433.1 Da) (Table II), suggesting that tamapin-2 might also be amidated.

Both tamapin and tamapin-2 inhibited the binding of [125I]monooiodoapamin to rat brain synaptic plasma membranes with equivalent high affinity (Kᵢ = 12 and 8 pm, respectively) (Fig. 2). Both toxins bound to a single class of binding sites. In comparison, tamapin inhibited neither the binding of [125I]dendrotoxin or [125I]-charybdotoxin to rat brain membranes nor charybdotoxin-sensitive 86Rb⁺ fluxes in C6 glioma cells (data not shown).

**Fig. 2. Tamapin binding to rat brain synaptosomes.** Shown are the results from the competition assay of purified tamapin (open circles) and tamapin-2 (filled squares) for 125I-apamin-binding sites on rat brain synaptosomes. For further details, see “Materials and Methods.” [125I]-Apanin binding (B) is expressed as percent of total binding in the absence of any competing ligand (Bₒ). The assay was performed in duplicate, and replicate samples typically varied between 3 and 5%.

Tamapin-2 was subsequently also tested on SK1 and SK3 have been cloned, SK1, SK2, and SK3 (26, 28), and they display different sensitivity to apamin, in part dependent on the expression systems utilized (28–31). An additional, less closely related member of the SK channel family is the intermediate conductance channel SK4, also known as IK1, which is not expressed in neuronal cells and is not sensitive to apamin (26, 32). Given their similar kinetic properties and Ca²⁺ dependence, the functional role of SK channels with different subunit compositions in different tissues and brain regions is hard to dissect. For this purpose, toxins capable of distinguishing different SK channels represent extremely valuable pharmacological tools. However, a number of toxins that have been isolated as putative SK channel modulators do not display any activity as blockers despite their capability of displacing apamin from its binding sites (12–14, 16, 18, 33, 34). It was therefore interesting for us to test tamapin on cloned SK channels stably expressed in Chinese hamster ovary and HEK293 cell lines.

Because tamapin displaced apamin in binding assays (Fig. 2), we first tested tamapin on SK2 channels, the ones most sensitive to apamin in binding assays and physiological recordings. SK2 channels were stably expressed in HEK293 cells. The recordings were performed in the whole-cell configuration, and the channels were activated by 1 μM free calcium present in the intracellular solution, which diffused into the cell within <1 min after reaching the recording configuration. The free calcium concentration of 1 μM guaranteed that the channels were maximally activated. SK2 currents were then elicited by voltage ramps from −100 to +40 mV (n = 45) (Fig. 3A) or by voltage pulses from −100 to +40 mV in 20-mV steps (n = 3) (Fig. 3B). In both cases, tamapin (500 pm) produced a strong reduction in the SK2 current. The current reduction was fast and largely reversible (Fig. 3C). To establish the sensitivity of SK2 channels to tamapin, we performed a concentration-response curve, which yielded an IC₅₀ of 24 pm and a Hill coefficient of 1.0 (Fig. 3D). The purified toxin was stored in a solution containing 30% acetonitrile. Therefore, as a control, we applied the maximal concentration of acetonitrile (0.15%) to which the cells were exposed during tamapin application and did not observe any significant effect on the SK currents (n = 9; three controls for each SK channel tested) (data not shown).
channels stably expressed in Chinese hamster ovary and HEK293 cells, respectively. As illustrated in Fig. 4 (A and B), tamapin blocked both SK1- and SK3-mediated currents in a reversible manner. The IC_{50} values of tamapin for SK1 and SK3 channels were estimated to be 42 and 1.7 nM, respectively. Finally, tamapin was tested on IK1 channels stably expressed in HEK293 cells. The current elicited in the presence of 1/2M intracellular Ca^{2+} was not affected by tamapin at concentrations up to 50 nM (protocol illustrated in the left panel). The SK2-mediated current in the whole-cell configuration was elicited in the presence of 1 mM Ca^{2+} in the patch pipette and was strongly suppressed by the application of 500 pM tamapin (right panel). Similar results were obtained in three cells. C, shown is the time course of the effect of tamapin (500 pM) on the SK2-mediated current measured at +40 mV. Voltage ramps were repeated every 5 s. At this concentration, the effect of tamapin was largely reversible. D, shown is the concentration-response curve for tamapin on SK2 currents. The current has been measured from voltage ramps, as the one illustrated in A, at −60 mV. Data points have been fitted with the Hill equation, giving an IC_{50} value of 24.3 pM and a Hill coefficient of 1.0.

K^{+} ions influence the binding of ^{125}I-apamin to its acceptor. Low concentrations of K^{+} (10 μM to 5 mM) increase apamin binding by a factor of −1.8, whereas higher concentrations decrease apamin binding (35, 36). A similar effect of K^{+} has been reported for ^{125}I-scyllatoxin (10). The measurements reported above were performed at an elevated external [K^{+}] (144 mM). To test whether the suppression of SK channel-mediated current is also affected by the external [K^{+}], we performed experiments using external solutions with a reduced [K^{+}] (20 mM). Under these conditions, the IC_{50} values of tamapin for SK2 were estimated to be 38.5 pM at +70 mV and 35.4 pM at +10 mV (Fig. 4C), values in the same range as those measured in high [K^{+}].

To test whether the block of SK2-mediated currents by tamapin was voltage-dependent, the IC_{50} value for tamapin inhibition was estimated at seven different voltages (−70 to +60 mV) in high symmetrical [K^{+}] (Fig. 4D) and at three different voltages in 20 mM external [K^{+}] (asymmetrical conditions) (data not shown). In both cases, the values obtained did not significantly differ at different voltages, suggesting that the block is not voltage-dependent.

We can therefore conclude that tamapin inhibits SK1, SK2, and SK3 currents, but not IK1 currents. Tamapin inhibits
Tamapin Suppresses $I_{\text{AHP}}$ in Hippocampal Pyramidal Neurons—Hippocampal pyramidal neurons present a medium-duration afterhyperpolarizing current ($I_{\text{AHP}}$) that is sensitive to apamin and scyllatoxin and that contributes to the early phase of spike frequency adaptation (4). This current is most likely mediated by the activation of SK channels composed of SK2 or SK1/SK2 subunits (4, 37). Conversely, a slower Ca²⁺-activated K⁺ current ($sI_{\text{AHP}}$) mediates the slow AHP following trains of action potentials and the slow spike frequency adaptation in these neurons, but it is not blocked by classical SK channel blockers (4, 38).

Given the effects of tamapin on recombinant SK channels, we next investigated its effect on the native SK channel-mediated $I_{\text{AHP}}$ and on the other calcium-dependent $sI_{\text{AHP}}$ current in hippocampal neurons. We performed whole-cell patch-clamp recordings from CA1 pyramidal neurons in hippocampal slices and tested the effects of tamapin on $I_{\text{AHP}}$ and $sI_{\text{AHP}}$. Tamapin at 10 nM fully suppressed $I_{\text{AHP}}$, but did not affect the amplitude or time course of $sI_{\text{AHP}}$ ($n = 5$) (Fig. 5, A and D). In the presence of 8-(4-chlorophenylthio)-cAMP, a stable cAMP analog, $I_{\text{AHP}}$ can be measured in isolation (4). Also under these conditions, 10 nM tamapin blocked $I_{\text{AHP}}$ completely ($n = 4$) (Fig. 5B). The suppression of $I_{\text{AHP}}$ unmasked an inward current (Fig. 5B), observed also upon application of apamin and scyllatoxin (4), not further investigated in this study. The time course of the tamapin effect was rather fast (3–5 min to induce full inhibition) (Fig. 5C), considering the diffusion problems linked to the application of peptide toxins to brain slices. The effect of the toxin was irreversible (Fig. 5C), similar to what was observed with apamin in the same preparation (4). Given the high affinity and selectivity of tamapin for recombinant SK2 channels, we also tested lower concentrations of the toxin on the SK channel-mediated $I_{\text{AHP}}$ in CA1 neurons in an attempt to obtain information on the relative contribution of SK2 subunits compared with SK1 and SK3 subunits to the formation of the channels mediating $I_{\text{AHP}}$. At 2 nM, a concentration well below the IC₅₀ for SK1 channels and in the range of the IC₅₀ for SK3 channels, tamapin blocked up to 80% of the $I_{\text{AHP}}$ in CA1 neurons ($n = 3$) (Fig. 5D). At 500 pM, tamapin suppressed ~50% of the $I_{\text{AHP}}$ ($n = 4$) (Fig. 5D). These results are in agreement with previous findings obtained with apamin (4) and support the notion that SK2 is predominantly involved in mediating $I_{\text{AHP}}$ in CA1 pyramidal neurons (see also Ref. 4). Also for the experiments on hippocampal CA1 neurons, as a control, we applied the maximal concentration of acetonitrile (0.15%) to which the slices had been exposed during tamapin application and did not observe any significant effect on $I_{\text{AHP}}$ or $sI_{\text{AHP}}$ (Fig. 5C).

To test the functional consequences following the application of tamapin, we performed current-clamp recordings to measure the AHP and to evaluate changes in the firing pattern of CA1 pyramidal neurons. Tamapin (10 nM) reduced the medium AHP (Fig. 6A). Additionally, it produced a slight increase in the firing frequency of these neurons and affected the early phase of adaptation ($n = 4$) (Fig. 6A). The changes in the firing properties of CA1 neurons are similar to those elicited by apamin (4) and are compatible with a blockade of the SK channel-mediated $I_{\text{AHP}}$ by tamapin.

**DISCUSSION**

Protein toxins that bind to ion channels have been invaluable tools for studying the structural basis of channel function and for investigating the physiological roles of particular channel subtypes (39–43). In this study, we have characterized a new scorpion toxin, tamapin, that acts selectively on SK channels. Tamapin competitively inhibited the binding of [125I]-apamin, suggesting that despite completely different sequences, the two toxins share, at least in part, the same binding sites on rat brain synaptosomes. Tamapin differentiates among SK channel subtypes because it presented different affinities for SK1, SK2, and SK3 channels and did not block SK4/K1 channels. Additionally, it selectively blocked the native SK channels underlying $I_{\text{AHP}}$, but not the Ca²⁺-activated K⁺ channels mediating $sI_{\text{AHP}}$ in hippocampal pyramidal neurons.

Screening for new toxins targeting Ca²⁺-activated K⁺ channels is of crucial importance because specific toxins are needed both in pharmacology and in biochemistry to understand the functional role played by specific channel subtypes and to investigate their subunit composition as well as their regional and cellular distribution. This is particularly true for SK channels, as three of the subunits that have been cloned so far (SK1, SK2, and SK3) are widely expressed in the central nervous system. Their distribution is partly overlapping, with SK1 and SK2 subunits being coexpressed in most neurons and SK3 subunits presenting a rather distinct pattern (37). The function

**Fig. 4.** Tamapin blocks SK1- and SK3-mediated (but not IK1-mediated) currents, and its effect is not voltage-dependent. A, 400-msec-long voltage ramps (protocol shown on top of the current traces) were applied to Chinese hamster ovary cells stably expressing SK1 channels in the presence of 1 μM intracellular Ca²⁺. The SK1-mediated current was partly inhibited by 50 nM tamapin. The estimated IC₅₀ for the block of SK1 channels by tamapin was 42 nM. The effect of tamapin on SK1 currents was fully reversible (Wash out). Similar results were obtained in three cells. B, the same protocol as described for A was applied to SK3 channels expressed in HEK293 cells. The SK3-mediated current was partly suppressed by 1 nM tamapin in a reversible manner (Wash out). The estimated IC₅₀ for the block of SK3 channels by tamapin was 1.7 nM. Similar results were obtained in five cells. C, the same protocol as described for A was applied to IK1 channels expressed in HEK293 cells. The IK1-mediated current was not affected by 50 nM tamapin. Similar results were obtained in three cells. D, the IC₅₀ values for the block of SK2-mediated currents by tamapin were measured at different voltages ranging from −60 to +10 mV. The IC₅₀ of tamapin for SK2 channels did not change significantly with the voltage, suggesting that the action of the toxin is not voltage-dependent. A regression line was fitted to the points. For each point, $n = 3–8$. SK2 channels at much lower concentrations compared with SK1 (1750-fold lower) and SK3 (71-fold lower). The inhibition by tamapin seems to be neither voltage- nor [K⁺]-dependent.
of molecularly identified SK channels could be analyzed only in neurons expressing predominantly one SK channel subunit so far (see, for example, Refs. 5–7). Whether native SK channels are generated by the assembly of different SK subunits and whether SK channels with different subunit compositions play distinct functional roles when coexpressed in the same neuronal subtypes are questions that could not be addressed as yet because the pharmacological tools available, such as peptide toxins (apamin and scyllatoxin) and organic compounds (cucurbit, quaternary salts of bicuculline, dequalinium, UCL 1684, and UCL 1848), block all three channel subtypes in expression systems (5, 29–31, 44–47). Tamapin presents a higher affinity for SK2 channels compared with SK1 and SK3 channels. To our knowledge, with an IC50 of 24 nM, tamapin is the most potent toxin blocker characterized so far for SK2 channels. This makes it a potentially useful tool to dissect the function of different SK channel subtypes even when expressed in the same cell type, as is the case, for example, for subicular neurons, some thalamic neurons, and facial nucleus neurons, which express SK1, SK2, and SK3 subunits (37). Indeed, the enhanced selectivity of tamapin has allowed us to gain further insight into the molecular composition of the channels underlying IAHP in CA1 pyramidal neurons, which express high levels of SK1 and SK2 subunits and low levels of SK3 transcripts (4). The strong inhibition of IAHP by low concentrations of tamapin (0.5 nM) (Fig. 5D), which do not affect SK1 channels, supports the notion that homomultimeric SK2 channels mediate IAHP in CA1 neurons. In contrast to four other scorpion toxins (maurotoxin, Pi1, PO1, and T. serrulatus/H9260 toxin) that have been reported to be very potent in 125I-apamin displacement assays (12–14, 16, 18, 34), but have little or no blocking activity on SK2 or SK3 channels (33), tamapin showed both apamin-displacing and SK channel-blocking activities. Many SK channel toxins from scorpion venoms are amidated at their C termini, and this post-translational modification often profoundly affects the pharmacological properties of the toxin. For example, both C-terminal amidated and non-amidated forms of scyllatoxin are found in the venom of L. quin-
C), the small increase in overall positive charge appears to have little effect on the potency of the toxin. Substitution of the glutamate residues that shape the toxins' backbones by forming three thiol-disulfide bridges in scyllatoxin (49, 50) and a proline in tamapin (Glu-25 and Pro-30). Structure-function studies have addressed the function of these amino acids in scyllatoxin (49, 50). Thus, chemical modifications or point mutations of the lysine residues at positions 25 and 30 or of the glutamate at position 27 lead to a substantial loss of contractile potency of scyllatoxin when applied to *Toxostoma cinctum*, without affecting significantly its ability to displace apamin binding (49, 50). On the other hand, tamapin displays full biological activity when tested on recombinant and native brain SK channels, despite the presence of oppositely charged amino acids at the corresponding positions. Furthermore, tamapin displays an affinity comparable with scyllatoxin and PO5 in competing with 125I-apamin for binding to synaptosomes (24, 48, 50). The differences in binding activity and inhibition of SK channel-mediated currents of scyllatoxin and PO5 on one hand compared with tamapin on the other suggest that, although all three toxins belong to scorpion toxin subfamily 5, the interaction of tamapin with SK channels involves regions different from those of its closest homologs. Future structure-function studies will help us understand the determinants of tamapin binding to SK channels and might lead to mutant toxins with a further improved selectivity for SK channel subtypes.

At the N-terminal region, the first amino acids of tamapin are identical to those of scyllatoxin, but different from those of PO5 (Table I). These two residues have been shown to contribute to the higher potency of scyllatoxin for SK2 and SK3 channels compared with PO5 (33), and it is therefore conceivable that they might also be important determinants of the even higher potency displayed by tamapin.

Another noticeable difference between tamapin and previously characterized SK channel toxins is seen in the RXCQ motif, which is conserved in PO5, scyllatoxin, and apamin (33, 48, 49) and which is changed into RRCE in tamapin (Table I). In particular, the methionine present at position 7 in scyllatoxin has been proposed to be important for the enhanced potency of this toxin compared with PO5, which presents an arginine residue at the same position (33). Tamapin resembles PO5 in presenting arginines at both positions 6 and 7. However, it differs significantly from the other subfamily 5 scorpion toxins in having a negatively charged glutamate residue, which replaces the glutamine in the RXCQ motif. The classical RXCQ motif is therefore not fully conserved in tamapin, and further studies will be necessary to understand the molecular basis for the high sensitivity to SK channels and, in particular, the high selectivity for SK2 of this toxin. A first indication is provided by the work of Shakkottai et al. (33), who showed that placing small, positively charged amino acids at position 7 of scyllatoxin enhances the selectivity of the mutant toxin for SK2 versus SK3 channels. Indeed, the scyllatoxin derivative [7-di-aminobutanoic acid]leiurotoxin I was shown to block SK2 homomeric channels with nanomolar potency and to exhibit a 650-fold selectivity over other related SK channels (33).

In conclusion, tamapin represents a novel, promising pharmacological tool, as it blocks SK2 channels ~1750- and 70-fold more potently than SK1 and SK3 channels, respectively, making it the most potent and selective SK2 channel natural toxin characterized so far. In the future, tamapin might be

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**Fig. 6. Tamapin affects the medium AHP and the firing pattern of CA1 pyramidal neurons.** A, tamapin (10 nM) reduced the medium AHP (mAHP) elicited by a short burst of action potentials (3 action potentials, truncated in the left and middle panels). In the right panel, the medium AHPs before (Control) and after (Tamapin) tamapin application are shown superimposed. Similar results were obtained in four cells. B, tamapin (10 nM) produced an increase in the early firing frequency and in the overall number of action potentials elicited by a prolonged depolarizing current injection of constant strength (800 ms), without affecting slow spike frequency adaptation. Membrane potential was −58 mV. Similar results were obtained in four cells. Scale bars = 5 mV and 250 ms in A and 25 mV and 300 ms in B.

*questriatus*, and the amidated form is 4-fold more potent (10). Although native PO5, with a C-terminal carboxylate, is a potent blocker of apamin-binding sites (Kᵢ = 20 pM), a synthetic PO5 derivative with a C-terminal amidated residue binds reversibly (48). Our data suggest that tamapin is amidated at the C terminus; and although we have not found a native C-terminal carboxylate form of tamapin in the venom of *Mesocestoides tammulus*, it will be of interest to examine the pharmacological properties of a synthetic tamapin derivative with a free carboxyl terminus.

In addition to tamapin, *Mesocestoides tammulus* venom contains a closely related isoform, tamapin-2, differing only in a single residue at the carboxyl terminus (His-31 instead of Tyr-31). Although this substitution makes tamapin-2 more basic (as evidenced by its later elution on an S-Sepharose ion-exchange column; see Fig. 1C), the small increase in overall positive charge appears to have little effect on the potency of the toxin in the apamin binding assay (Fig. 2). It will be interesting to see whether there are any differences in SK channel-blocking activity between the two isoforms.

We assigned tamapin to the short-chain scorpion toxin subfamily 5 (22) because it shares 77% amino acid sequence homology with scyllatoxin and 74% with PO5 (Table I). Members of subfamily 5 are characterized by the presence of six cysteine residues that shape the toxins' backbones by forming three disulfide bridges (10, 48). All six cysteine residues are conserved in tamapin, which therefore most likely presents a structure similar to scyllatoxin and PO5. Compared with the other subfamily 5 members, tamapin presents the largest sequence divergence in its C-terminal region (Table I). In the C-terminal region of tamapin, a glutamate residue, highly conserved in scyllatoxin and PO5 (Glu-27), is substituted by a lysine (Lys-27). Furthermore, two lysine residues conserved in scyllatoxin and PO5 (Lys-25 and Lys-30) are replaced by a glutamate and a proline in tamapin (Glu-25 and Pro-30). Structure-activity studies have addressed the function of these amino acids in scyllatoxin (49, 50). Thus, chemical modifications or point mutations of the lysine residues at positions 25 and 30 or of the glutamate at position 27 lead to a substantial loss of contractile potency of scyllatoxin when applied to *Toxostoma cinctum*, without affecting significantly its ability to displace apamin binding (49, 50). On the other hand, tamapin displays full biological activity when tested on recombinant and native brain SK channels, despite the presence of oppositely charged amino acids at the corresponding positions. Furthermore, tamapin displays an affinity comparable with scyllatoxin and PO5 in competing with 125I-apamin for binding to synaptosomes (24, 48, 50). The differences in binding activity and inhibition of SK channel-mediated currents of scyllatoxin and PO5 on one hand compared with tamapin on the other suggest that, although all three toxins belong to scorpion toxin subfamily 5, the interaction of tamapin with SK channels involves regions different from those of its closest homologs. Future structure-function studies will help us understand the determinants of tamapin binding to SK channels and might lead to mutant toxins with a further improved selectivity for SK channel subtypes.

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useful to 1) define the physiological role that different SK channels play in native tissue, 2) purify SK channels from native tissues and determine their subunit composition, and 3) develop the pharmacology of SK channels in view of their possible involvement in cognitive functions and diseases such as epilepsy (51–54).

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Tamapin, a Venom Peptide from the Indian Red Scorpion (Mesobuthus tamulus) That Targets Small Conductance Ca\(^{2+}\)-activated K\(^{+}\) Channels and Afterhyperpolarization Currents in Central Neurons

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