Tamapin: a novel SK channel toxin

Tamapin: a venom peptide from the Indian red scorpion (*Mesobuthus tamulus*) which targets SK channels and AHP currents in central neurons

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Summary

The biophysical properties of calcium-activated potassium channels of the SK type are well suited to underlie afterhyperpolarizations (AHP) 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-mediated currents in pyramidal neurons of the hippocampus as well as in cell lines expressing distinct SK channel subunits. This toxin distinguishes between the SK channels underlying the apamin-sensitive $I_{\text{AHP}}$ and the calcium-activated potassium channels mediating $sI_{\text{AHP}}$ in hippocampal neurons. When compared to related scorpion toxins, tamapin displays a unique, remarkable selectivity for SK2 versus SK1 (~1750-fold) and SK3 channels (~70-fold), 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.
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Introduction

Potassium channels show an exceptional functional diversity, being implicated in neural and behavioural 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 over sixty 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 Ca²⁺-activated K⁺ channels of small conductance (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 to reveal 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 useful adjuncts to the classical bee venom toxin. These include scyllatoxin, isolated from the scorpion *Leiurus quinquestriatus* (8-10), and P05 from *Androctonus mauretanicus* (11). Both scorpions are found in desert regions of North Africa and the Eastern Mediterranean. Other more recently identified scorpion toxins which compete for apamin binding sites with high affinity include Tityus κ toxin from the venom of the Central American scorpion *Tityus serulatus* (12-14), and BmP05 from the Asian scorpion *Buthus martensii Karsch*, widely distributed throughout China (15). Finally, toxins with greatly reduced binding affinities include P01 and BmP01 (15,16). Two African scorpion toxins, maurotoxin from *Scorpio maurus* and Pi1 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 Kv 1 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
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a rich source for highly specific potassium channel blockers such as iberiotoxin (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 the 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 on recombinant SK2 channels to date.
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**Materials and Methods**

**Toxins and chemicals**

Native apamin was purified from *Apis mellifera* bee venom and monoiodo-$^{125}$I-apamin (~2200 Ci mmol$^{-1}$) was prepared as described previously (23). Low-pressure chromatography media was obtained from Amersham Pharmacia Biotech (Amersham, UK). Protease inhibitors and bovine serum albumin (BSA fraction V protease free) were purchased from Sigma (Gillingham, UK). All other chemical were reagent grade and obtained from Merck (Poole, UK).

**Purification of tamapin**

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

The sample was applied in two batches to a Sephadex G50 column (86 x 2.6 cm diam.) equilibrated with 50 mM ammonium formate, pH 3.5. The column was eluted (40 ml/h) with the equilibration buffer and elution was monitored at 280 nm. Fifteen-minute 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 50 mM sodium acetate buffer, pH 4.8 (buffer A), and applied to a cation exchange column (Pharmacia SP 16/10 Hi-load) equilibrated with buffer A. After washing the column was eluted (500 ml) with a linear gradient of 0-100% buffer B (buffer A containing 1 M NaCl) at 1 ml/min. Four-minute fractions were collected, monitored at 280 nm and fractions with apamin-like activity were pooled. Active
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fractions (eluting at 350-450mM NaCl) were re-chromatographed on the same ion exchange column, using a shallower gradient (500 ml 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 G10 column (94 x 2.6 cm diam.) 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 on to a 300 Å pore size C₈ reversed phase column (5µm, 250 x 3.2 mm diam.) 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) over 10 min, and subsequently with linear gradient of 75-100 % buffer D over 60 min. Fractions eluting (0.5 ml/min) from the column were assayed for apamin-like activity. In the second step, active fractions were applied to the same column equilibrated with 5% acetonitrile, 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 re-running pooled active fractions from the second reversed-phase column on a 5 µm Jupiter C₁₈ reversed-phase column (150 mm x 1mm diam.) equilibrated in 0.1% (v/v) trifluoroacetic acid. Bound material was eluted (flow rate 50 µl/min) with a linear gradient (20-40%) of acetonitrile in 0.1% trifluoroacetic acid. Tamapin and tamapin-2 were judged to be 97-98% pure under these conditions.

Electrospray mass spectrometry (LC-MS)

Purified tamapin and tamapin-2 were analyzed by electrospray-ionization mass spectrometry, using a triple quadrupole instrument equipped with an ionspray interface (Perkin Elmer API 300 LC-MS/MS). Samples (20 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
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the orifice voltage at 30 V. Nitrogen was used as a curtain gas and compressed air was used as the nebulizer gas.

Amino acid sequencing

Toxin samples were sequenced by automated Edman degradation, using a Perkin Elmer Applied Biosystems 494 pulsed liquid phase sequencer (Procise). Phenylthiohydantoin amino acids were identified using an on-line reversed phase PTH-C18 column in an Applied Biosystems 785A PTH amino acid 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 (approx. 10-30 nmol) were resuspended in 100 µl of denaturant buffer (6 M guanidine hydrochloride, 1 mM EDTA, 0.13 M Tris HCl, pH 8.0). After the addition of β-mercaptoethanol (2 µl) the solution was incubated under nitrogen (2 h, 37ºC). 4-Vinylpyridine (20 µl) was subsequently added and the mixture 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, 25 mM Tris, pH 8.4, containing 0.1% BSA. Aliquots of membranes (100 µg protein) were incubated (1 h on ice) in the presence of 10 pM ¹²⁵I apamin and either crude Mesobuthus tamulus venom, individual chromatographic fractions or purified toxins. The binding reaction was quenched by the addition of ice-cold incubation medium and
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rapid (<20 s) filtration through Whatman GF/B FP-100 filters pre-soaked (1 h, 4°C) in 0.5% (v/v) polyethyleneimine. The computer program EBDA (Biosoft, Cambridge, UK) was used to calculate the IC_{50} value (toxin concentration which inhibits 50% of binding of ¹²⁵I-apamin) from the raw data. IC_{50} values were then used to calculate the true equilibrium dissociation constant of the inhibitory ligand (K_{i}) using the Cheng and Prusoff equation, IC_{50} = K_{i} (1 + L*/K_{d}), where L* is the free concentration of ¹²⁵I-apamin at half displacement and K_{d} is the dissociation constant for ¹²⁵I-apamin. The experiments were designed to produce IC_{50} values in the range of 2 to 4 fold higher than the K_{i}. 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 medium (DMEM/F12) supplemented with 2 mM L-glutamine, 1 ml penicillin/streptomycin and 10% foetal calf serum. Cells were grown in a humidified atmosphere (5% CO₂, 95% air) at 37°C. For transfection, cells were grown to 50-60% confluency and 6 µg rSK2 or rSK3 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 trypsinated 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 rSK2 or rSK3 was confirmed by immunofluorescence and by patch-clamp measurements.

The SK1-CHO-FlpIn cell line was generated by using the Flp-In system (Invitrogen, Merelbeke, Belgium). Briefly, hSK1 was cloned into the pcDNA5/FRT vector. For transfection, 0.9 µg SK1-pcDNA5/FRT was co-transfected with 10 µg pOG44 into the CHO-FlpIn cells using Lipofectamine™ together with the Plus™ reagent (Invitrogen, Merelbeke, Belgium). Transfected cells were selected in medium containing 0.1 mg/ml HygromycinB. Expression of
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hSK1 was confirmed by patch clamp recordings, where the half maximal blocking concentrations of apamin and d-tubocurarine were applied.

The cell line stably expressing hSK4/hIK1 channels was a kind gift of W. J. Joiner and L. K. Kaczmarek (26).

Electrophysiology on transfected cells

Measurements were performed in the whole-cell configuration of the patch-clamp technique. HEK293 cells or CHO-Flp-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 the EPC-9 amplifier (HEKA electronic, Lambrecht, Germany), and data were acquired with the Pulse/Pulsefit software (HEKA electronic, Lambrecht, Germany) and analyzed with Igor (WaveMetrics, Lake Oswego, USA). Pipettes were pulled from borosilicate glass (Kimble Glass Inc., Vineland, USA) with a vertical patch electrode puller (List medical, Darmstadt, Germany). Pipettes had a resistance of 2-3 MΩ when filled with intracellular solution (see below). After gigaseal formation, the capacitative transients were automatically compensated.

MgCl₂ and CaCl₂ concentration were adjusted by using EqCal (Biosoft, Cambridge, UK) to obtain free calcium concentrations of 1 μM, 10 μM or 1mM. SK and IK channels were activated by whole-cell dialysis with an intracellular solution containing (in mM): 130 KCl, 10 Hepes, 10 EGTA, variable MgCl₂ and CaCl₂ (see above), adjusted to pH 7.2 with KOH. Recordings were performed in symmetric potassium or potassium-free extracellular solutions, containing (in mM): 144 KCl, 10 Hepes, 1 MgCl₂, 2 CaCl₂, adjusted to pH 7.4 with KOH (symmetric); 144 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 seconds. Alternatively, 100 or 200 ms-long voltage steps from -100 to +40 mV with
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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 on hippocampal slices

Wistar rats (19-30 days old) were anaesthetized with halotane, and after decapitation
transverse hippocampal slices (300 µm thick) were prepared by using a vibroslicer (VT1000S,
Leica). During recording, the slices were superfused with standard artificial cerebro-spinal fluid
(ACSF) containing (mM): 125 NaCl, 25 NaHCO3, 1.25 KCl, 1.25 KH2PO4, 2.5 CaCl2, 1.5 MgCl2
and 16 glucose, saturated with 95% O2 and 5% CO2, at room temperature (21-24°C). In voltage-
clamp experiments tetrodotoxin (TTX; 0.5 µM) and tetraethylammonium (TEA; 1 mM) were
added to the ACSF. In the experiments where low concentrations (0.5 and 2 nM) of tamapin
were tested, bovine serum albumin (25 µg/ml) was added to the ACSF to minimize possible non-
specific binding of the toxin to the perfusion system. As such, albumin had no effect on I_AHP
(data not shown). Whole-cell giga-seal recordings were obtained from CA1 pyramidal cells in
the slice, using the "blind" method (27). The patch pipette solution contained (mM): 135
potassium gluconate, 10 KCl, 10 HEPES, 2 Na2-ATP, 1 MgCl2, and 0.4 Na3-GTP (pipette
resistance: 3-6 MΩ); 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (8CPT-
cAMP; 50 µM) was included to measure the apamin-sensitive AHP current in isolation (see also
4). All neurons included in this study had a resting membrane potential below -55 mV (-62.1±0.7
mV; n=27). Using an EPC-9 amplifier (HEKA electronic, Lambrecht, Germany), the cells were
voltage-clamped at -50 mV, and depolarizing steps (100 ms long) of sufficient amplitude
(typically +60 or +70 mV) to elicit a robust, unclamped Ca2+ action current, were applied once
every 30 s. The access resistance (range 10-25 MΩ) and the amplitude and time course of the
Ca2+ current during the step showed only minimal variations during the recordings included in
this study. Only cells with a stable resting potential throughout the current-clamp protocols (±0.5
mV) were included in the analysis. Records were filtered at 0.25-1 kHz and digitized at 1-4 kHz.
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All data were acquired, stored and analyzed on a Power Macintosh 7100/66 using the Pulse+Pulsefit Software (HEKA electronic, Lambrecht, Germany) and Igor Pro (WaveMetrics, Lake Oswego, USA). Values are reported as mean ± SEM. Student t-test was used for statistical comparisons between groups (α = 0.05).
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**Results**

**Purification and characterization of tamapin**

Crude *Mesobuthus tamulus* venom inhibited the binding of monoiodo-\(^{125}\)I-apamin 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 (B\(_0\)). The sigmoidal shape of the binding curve in this semi-logarithmic plot suggests that there is only one molecule (or one class of molecules with similar affinity) that interacts with apamin-sensitive SK channels.

In order to purify the apamin binding activity, crude venom was initially fractionated by gel exclusion chromatography on Sephadex G50. Several unresolved peaks absorbing at 280 nm were observed (Fig. 1B), the majority with molecular masses of approximately 10kDa or less. Fractions active in the \(^{125}\)I-apamin inhibitory binding assay (fractions 27-37) were pooled and chromatographed on an S-Sepharose ion exchange column, eluting fractions with a salt gradient (0.05-1M NaCl, data not shown). Re-chromatography of the most active fractions on the same column, using a shallow salt gradient (0.35-0.55 M NaCl), yielded two peaks of inhibitory apamin-binding activity (peaks I and II, Fig. 1C). Tamapin (peak I) was finally purified to homogeneity by reversed phase chromatography on a C\(_8\) column (Fig. 1D). Tamapin was judged to be >98% pure by chromatography on a reversed phase C\(_{18}\) column, using a shallow solvent gradient (20-40% acetonitrile in 0.1% trifluoracetic acid (Fig. 1D, inset)). Homogeneity was confirmed by mass spectrometry and SDS-polyacrylamide gel electrophoresis (data not shown). A unique N-terminal residue (alanine) was detected on 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 1). The molecular mass of tamapin, as determined by electrospray-ionization mass spectrometry (3457.9±0.2, Table 2), was one mass unit less than that calculated from the amino acid sequence (3459.1, Table 2). Digestion of tamapin with either
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carboxypeptidase A or carboxypeptidase Y failed to detect any aminoacids 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 9). Tamapin shares 77% amino acid sequence similarity with scyllatoxin and 74% with P05 (Table 1), 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, P01, and Tsκ, is substantially lower (<30%).

A second active fraction (peak II, Fig. 1C) was purified to homogeneity by reverse 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) was approximately one mass unit less than that calculated from the amino acid sequence (3433.1, Table 2), suggesting that tamapin-2 might also be amidated.

Both tamapin and tamapin-2 inhibited the binding of monoiodo-125I-apamin to rat brain synaptic plasma membranes with equivalent high affinity (Kᵢ values 12 pM 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, nor 125I-charybdotoxin to rat brain membranes, nor charybdotoxin-sensitive ⁸⁶Rb⁺ fluxes in C6 glioma cells (data not shown).

Tamapin blocks cloned SK channels

Three SK channels have been cloned, SK1, SK2 and SK3, (26,28), and 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 it is not sensitive to apamin (26,32). Given their similar kinetic properties and Ca^{2+}-dependence, the functional role of SK channels with different subunit compositions in different tissues and brain regions is hard to dissect. To 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, did not display any activity as blockers in spite of 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 CHO and HEK293 cell lines.

Since tamapin displaced apamin in binding assays (Fig. 2), we first tested tamapin on SK2 channels, the most sensitive ones 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 less than a minute 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). In order to establish the sensitivity of SK2 channels to tamapin, we performed a concentration-response curve, which yielded an IC_{50} 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; 3 controls for each SK channel tested; not shown).

Tamapin was subsequently also tested on SK1 and SK3 channels stably expressed in CHO and HEK293 cells, respectively. As illustrated in Fig. 4A and B, tamapin blocked both
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SK1- and SK3-mediated currents in a reversible manner. The IC$_{50}$ of tamapin for SK1 and SK3 channels were estimated to be 42 nM 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 µM intracellular Ca$^{2+}$ was not affected by tamapin at concentrations up to 50 nM ($n = 3$; Fig. 4C), but was strongly suppressed by the application of 100 nM charybdotoxin (data not shown).

K$^+$ ions influence the binding of $^{125}$I-apamin to its receptor. Low concentrations of K$^+$ (10 µM to 5 mM) increase apamin binding by a factor of about 1.8, while 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 in elevated external [K$^+$] (144 mM). To test whether the suppression of SK-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$_{50}$ of tamapin for SK2 was 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$^+$.

In order 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 (+10 to –60 mV) in high, symmetrical [K$^+$] (Fig. 4D), and at three different voltages in 20 mM external [K$^+$] (asymmetrical conditions; not shown). In both cases, the obtained values 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, but not IK1 currents. Tamapin inhibits SK2 channels at much lower concentrations when compared to SK1 (1750-fold lower) or SK3 (71-fold lower). The inhibition by tamapin seems to be neither voltage- nor [K$^+$]-dependent.

Tamapin suppresses $I_{AHP}$ in hippocampal pyramidal neurons

Hippocampal pyramidal neurons present a medium duration afterhyperpolarizing current, $I_{AHP}$, that is sensitive to apamin and scyllatoxin and contributes to the early phase of spike
frequency adaptation (4). This current is most likely mediated by the activation of SK channels composed by SK2 or SK1/SK2 subunits (4,37). Conversely, a slower Ca$^{2+}$-activated K$^+$ current, sIAHP, mediates the slow afterhyperpolarization (sAHP) 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-mediated I$_{AHP}$ and on the other calcium-dependent sI$_{AHP}$ 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$_{AHP}$ and sI$_{AHP}$. Tamapin at 10 nM fully suppressed I$_{AHP}$, but did not affect the amplitude or time course of sI$_{AHP}$ (Fig. 5A and D; n = 5). In the presence of 8CPT-cAMP, a stable cAMP analogue, I$_{AHP}$ can be measured in isolation (4). Also under these conditions 10 nM tamapin blocked I$_{AHP}$ completely (Fig. 5B; n = 4). The suppression of I$_{AHP}$ 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 (Fig. 5C; 3-5 minutes to induce full inhibition), considering the diffusion problems linked to the application of peptide toxins to brain slices. The effect of the toxin was irreversible (Fig. 5C), similarly 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-mediated I$_{AHP}$ in CA1 neurons in the attempt to obtain information on the relative contribution of SK2 compared to SK1 and SK3 subunits to the formation of the channels mediating I$_{AHP}$. At 2 nM, a concentration well below the IC$_{50}$ for SK1 channels and in the range of the IC$_{50}$ for SK3 channels, tamapin blocked up to 80% of I$_{AHP}$ in CA1 neurons (n = 3; Fig. 5D). At 500 pM, tamapin suppressed approximately 50% of I$_{AHP}$ (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$_{AHP}$ in CA1 pyramidal neurons (see also 4). Also for the experiments on hippocampal CA1 neurons we performed
controls applying 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 $I_{AHP}$ or $sI_{AHP}$ (Fig. 5C).

In order to test the functional consequences following the application of tamapin, we performed current-clamp recordings to measure the AHP and evaluate changes in the firing pattern of CA1 pyramidal neurons. Tamapin (10 nM) reduced the medium AHP elicited by a short burst of action potentials in CA1 neurons (Fig. 6A; $n = 4$). Additionally, it produced a slight increase in the firing frequency of these neurons and affected the early phase of adaptation (Fig. 6B; $n = 4$). 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-mediated $I_{AHP}$ 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 $^{125}$I-apamin, suggesting that in spite of 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, since it presents different affinities for SK1, SK2 and SK3 channels, and it does not block SK4/IK1 channels. Additionally, it selectively blocks the native SK channels underlying $I_{AHP}$, but not the Ca$^{2+}$-activated K$^+$ channels mediating s$I_{AHP}$ in hippocampal pyramidal neurons.

Screening for new toxins targeting Ca$^{2+}$-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 (SK1-3) are widely expressed in the central nervous system. Their distribution is partly overlapping, with SK1 and SK2 subunits being co-expressed in most neurons, and SK3 presenting a rather distinct pattern (37). The function of molecularly identified SK channels could only be analyzed in neurons expressing predominantly one SK channel subunit so far (see for example 5,6,7). Whether native SK channels are generated by the assembly of different SK subunits, or whether SK channels with different subunit compositions play distinct functional roles when co-expressed 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) or organic compounds (curare, 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
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when compared to SK1 or SK3 channels. To our knowledge, with an IC$_{50}$ of 24 pM, 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 a further insight on the molecular composition of the channels underlying $I_{\text{AHP}}$ in CA1 pyramidal neurons, which express high levels of SK1 and SK2 subunits and low levels of SK3 transcripts (4). The strong inhibition of $I_{\text{AHP}}$ by low concentrations of tamapin (0.5-2 nM; Fig. 5D), which are not affecting SK1 channels, supports the notion that homomultimeric SK2 channels mediate $I_{\text{AHP}}$ in CA1 neurons.

In contrast to four other scorpion toxins (maurotoxin, Pi1, P01, and Tsk), which have been reported to be very potent in $^{125}$I-apamin displacement assays (12,13,14,16,18,34), 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 Leiurus quinquestriatus, and the amidated form is four-fold more potent (10). Although native P05, with a C-terminal carboxylate, is a potent blocker of apamin binding sites ($K_i = 20$ pM), a synthetic P05 derivative with a C-terminal amidated residue binds irreversibly (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 Mesobuthus tamulus, it will be of interest to examine the pharmacological properties of a synthetic tamapin derivative with a free carboxyl terminus.

In addition to tamapin, Mesobuthus tamulus venom contains a closely related isoform, tamapin-2, differing only in a single residue at the carboxyl terminus (His-31 instead of Tyr-31).
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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 P05 (Table 1). Members of the subfamily 5 are characterized by the presence of six cysteine residues that shape the toxins backbone 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 P05. Compared to the other subfamily 5 members, tamapin presents the largest sequence divergence in its C-terminal region (Table 1). In the C-terminal region of tamapin a glutamate residue, highly conserved in scyllatoxin and P05 (E27), is substituted by a lysine (K27). Furthermore, two lysine residues conserved in scyllatoxin and P05 (K25 and K30) are replaced by a glutamate and a proline in tamapin (E25 and P30). 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 in positions 25 and 30 or of the glutamate residue in position 27 led to a substantial loss of contractile potency of scyllatoxin when applied to taenia coli, without affecting significantly its ability to displace apamin binding (49,50). Tamapin on the other hand displayed full biological activity when tested on recombinant and native brain SK channels, in spite of the presence of oppositely charged amino acids at the corresponding positions. Furthermore, tamapin was two to ten-fold more effective than scyllatoxin and P05, respectively, in competing with ¹²⁵I-apamin for binding to synaptosomes (48,50). The differences in binding activity and inhibition of SK-mediated currents of scyllatoxin and P05 on one hand compared to tamapin on the other suggest that, although all three toxins belong to the scorpion toxin subfamily 5, the interaction of tamapin with SK channels involves different regions from those
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of its closest homologues. Future structure-function studies will help to 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 P05 (Table 1). These two residues have been shown to contribute to the higher potency of scyllatoxin on SK2 and SK3 channels when compared to P05 (33), and it is therefore conceivable that they might be also 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, conserved in P05, scyllatoxin and apamin (33,48,49), and which is changed into RRCE in tamapin (Table 1). In particular, the methionine present in position 7 in scyllatoxin has been proposed to be important for the enhanced potency of this toxin compared to P05, which presents an arginine residue in the same position (33). Tamapin resembles P05 in presenting arginines both at position 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 towards SK channels, and in particular the high selectivity for SK2 of this toxin. A first indication is provided by the work of Shakkottai and collaborators (33), where placing small, positively charged amino acids in position 7 of scyllatoxin has been shown to enhance the selectivity of the mutated toxin for SK2 versus SK3 channels. Indeed, the scyllatoxin derivative Lei-Dab7 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-fold 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
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future, tamapin might be 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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References

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**Figure Legends**

**Figure 1.** Purification of tamapin from *Mesobuthus tamulus* venom.

(A) $^{125}$I-apamin binding to rat brain synaptosomes was inhibited in a concentration-dependent manner by crude *Mesobuthus tamulus* venom. Toxin binding (B) is expressed as fraction of total binding in the absence of venom ($B_0$). (B) G50 Sephadex size exclusion chromatography of venom after acid extraction. Grey box indicates the fractions which were pooled based on the competition assay performed with $^{125}$I-apamin. (C) 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 $^{125}$I-apamin. The highest activities were found in fractions 26-28 (peak I) and 39-41 (peak II), corresponding to tamapin and tamapin-2, respectively. ● indicate absorbance at $A_{280}$; ○ indicate inhibitory binding activity. (D) C$_8$ reversed phase HPLC of peak I. Biological activity (indicated as Tamapin) was identified by the $^{125}$I-apamin competition assay as before. (INSET) C$_{18}$ reverse phase HPLC of peak marked as Tamapin in D. Ordinate scale (left), absorbance at 220 nm; ordinate (right), % acetonitrile in elution buffer; abscissa scale, time (min).

**Figure 2.** Tamapin binding to rat brain synaptosomes.

Competition assay of purified tamapin and tamapin-2 for $^{125}$I-apamin binding sites on rat brain synaptosomes. For further details, see Methods. $^{125}$I-apamin binding (B) was expressed as percent of total binding in the absence of any competing ligand ($B_0$). The assay was performed in duplicate and replicate samples typically varied between 3% and 5%.

**Figure 3.** Tamapin inhibits the current mediated by SK2 channels expressed in HEK293 cells.

(A) HEK293 cells expressing SK2 channels were measured in the whole-cell configuration of the patch clamp technique. 1 µM Ca$^{2+}$ was included in the pipette solution to activate the
channels. Voltage ramps from –100 to +40 mV (duration: 400 ms) were applied and elicited a current under control conditions. In the presence of 500 pM tamapin, the SK2-mediated current was largely suppressed. The measurements were performed in high symmetrical K⁺, resulting in a reversal potential for the SK2-mediated current of 0 mV. In the same cell, when K⁺ was eliminated from the extracellular solution, only a very small leak current could be observed. Similar results were obtained in 7 cells. The ramp protocol is illustrated schematically on top of the current traces. (B) SK2 currents were elicited by voltage pulses from –100 to +40 mV in 20 mV steps, lasting 100 ms (protocol illustrated in the left panel). The SK2-mediated current (middle panel) was elicited in the whole-cell configuration, in the presence of 1 µM Ca²⁺ in the patch pipette, and was strongly suppressed by the application of 500 pM tamapin (right panel). Similar results were obtained in 3 cells. (C) Time course of the effect of tamapin (500 pM) on the SK2-mediated current measured at +40 mV. Voltage ramps were repeated every 5 sec. At this concentration, the effect of tamapin was largely reversible. (D) 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 IC₅₀ value of 24.3 pM and a Hill coefficient of 1.0. For each point n = 3-8; error bars are SEM.

Figure 4. Tamapin blocks also SK1- and SK3-, but not IK1-mediated currents, and its effect is not voltage-dependent.

(A) 400-ms-long voltage ramps (protocol shown on top of the current traces) were applied to CHO 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 3 cells. (B) The same protocol as in 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
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block of SK3 channels by tamapin was 1.7 nM. Similar results were obtained in 5 cells. (C) The same protocol as in 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 3 cells. (D) The IC_{50} values for the block of SK2-mediated currents by tamapin were measured at different voltages ranging from –60 to +10 mV. The IC_{50} 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.

Figure 5. Tamapin suppresses the apamin-sensitive I_{AHP}, but not sI_{AHP}, in hippocampal CA1 neurons.

(A) Under control conditions (left panel), a 100-ms depolarizing pulse to +10 mV in the presence of tetrodotoxin (0.5 µM) and tetraethylammonium (1 mM) elicited two distinct Ca^{2+}-activated K⁺ currents, I_{AHP} and sI_{AHP}, in CA1 pyramidal neurons. Due to its faster time course, I_{AHP} is better displayed in the inset with an expanded time scale. Application of 10 nM tamapin led to the specific suppression of I_{AHP}, while sI_{AHP} was not significantly affected (right panel). The inhibition of I_{AHP} is better illustrated in the inset on an expanded time scale. Similar results were obtained in 5 cells. (B) I_{AHP} was measured in isolation (left panel), after suppression of sI_{AHP} by intracellular application of 8CPT-cAMP (50 µM). Under these conditions, I_{AHP} was fully suppressed by tamapin (10 nM), unmasking an inward current (right panel). Similar results were obtained in 5 cells. (C) Time course of the effect of tamapin on I_{AHP} amplitude. The hatched bar corresponds to the application of 0.15% acetonitrile. Acetonitrile had no significant effect on I_{AHP} amplitude, whereas tamapin (10 nM) fully suppressed the current within 4 min. The effect of tamapin applied to brain slices was not or only very partially reversible. Similar results were obtained in 5 cells. (D) Bar diagram summarizing the effect of tamapin at different concentrations (10 nM; 2 nM; and 0.5 nM) on the amplitude of sI_{AHP} and I_{AHP}. Error bars are SEM.
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**Figure 6.** Tamapin affects the mAHP and the firing pattern of CA1 pyramidal neurons.

**(A)** Tamapin (10 nM) reduced the mAHP elicited by a short burst of action potentials (3, truncated in the left and middle panels). In the right panel, the mAHP before (Control) and after tamapin application (Tamapin) are shown superimposed. Similar results were obtained in 4 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: -58 mV. Similar results were obtained in 4 cells.

Scale bars: in **A**: 5 mV, 250 ms; in **B**: 25 mV, 300 ms
Primary sequence of tamapin and alignment with two other SK channel toxins from scorpion venoms, scyllatoxin and PO5

The amino acids that are not conserved in the three toxin sequences are boxed in black. The RXCQ motif, conserved in PO5 (Swissprot: P31719) and scyllatoxin (Swissprot: P16341), is boxed in grey and is changed into RRCE in tamapin.
**Table 2**

Molecular masses of tamapin and tamapin-2 as determined by electrospray ionisation mass spectrometry and amino acid sequencing

<table>
<thead>
<tr>
<th></th>
<th>Mass spectrometry (±SD)</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamapin</td>
<td>3457.9 ± 0.2</td>
<td>3459.1</td>
</tr>
<tr>
<td>Tamapin-2</td>
<td>3431.4 ± 0.2</td>
<td>3433.1</td>
</tr>
</tbody>
</table>
Figure 1

**A**

Absorbance (A280)

**B**

Absorbance (A280)

**C**

Absorbance (A280)

**D**

Absorbance (A280)
Figure 2
Figure 3
Figure 4
Figure 5

A. Control vs Tamapin

B. Control vs Tamapin

C. Tamapin effect on IAHP amplitude over time

D. % Current left after Tamapin treatment

- Tamapin 10 nM: n=5
- Tamapin 10 nM: n=5
- Tamapin 2 nM: n=3
- Tamapin 0.5 nM: n=4

IAHP and sIAHP currents are indicated in the figures.
Figure 6

A
Control  Tamapin
mAHP  mAHP
Tamapin  Control

B
Control  Tamapin
Tamapin: a venom peptide from the Indian red scorpion (Mesobuthus tamulus) which targets SK channels and AHP currents in central neurons


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