Apamin is often cited as one of the few substances selectively acting on small-conductance Ca\(^{2+}\)-activated potassium channels (K\(\text{Ca}_2\)). However, published pharmacological and structural data remain controversial. Here, we investigated the molecular pharmacology of apamin by two-electrode voltage-clamp in *Xenopus laevis* oocytes and patch-clamp in HEK293, COS7, and CHO cells expressing the studied ion channels, as well as in isolated rat brain neurons. The microtitre broth dilution method was used for antimicrobial activity screening. The spatial structure of apamin in aqueous solution was determined by NMR spectroscopy. We tested apamin against 42 ion channels (K\(\text{Ca}_2\), K\(\text{V}\), N\(\text{a}_V\), nAChR, ASIC, and others) and confirmed its unique selectivity to K\(\text{Ca}_2\) channels. No antimicrobial activity was detected for apamin against Gram-positive or Gram-negative bacteria. The NMR solution structure of apamin was deposited in the Protein Data Bank. The results presented here demonstrate that apamin is selective for K\(\text{Ca}_2\) channels and provides ample functional data support the use of apamin as a K\(\text{Ca}_2\)-selective pharmacological tool and as a template for drug design.

**KEYWORDS**
apamin, *Apis mellifera*, bee venom, calcium-activated potassium channel, ion channel, spatial structure

**1 Introduction**

Highly selective molecules that can interact with specific ion channel isoforms serve as invaluable molecular tools for fundamental and applied pharmacology (Hille, 2001; Wulff et al., 2019). The history of using such substances goes hand-in-hand with the discovery and investigation of their molecular targets (Hille, 2001). Indeed, selective molecular probes helped to identify and characterize a number of physiologically critical ion channels (Kuzmenkov and Vassilevski, 2018). For example, marine guanidinium and
scorpion polypeptide toxins were used in pioneering studies for the purification of voltage-gated Na⁺ channels (Naᵥ) (Hartshorne and Catterall, 1981; Barhanin et al., 1983; Hanke et al., 1984). Moreover, Naᵥ are traditionally classified based on isoform sensitivity to tetrodotoxin (Narahashi, 2008). A plethora of other ion channel-selective compounds has been extracted from different animal venoms (Herzig et al., 2020).

In the 1960s, Habermann examined the venom of the honeybee Apis mellifera and purified a minor peptide component that he named “apamin” (Habermann and Reiz, 1965; Habermann, 1972); the toxin was shown to induce muscle spasms, jerks, and convulsions when injected into mice (LD₅₀ = 4 mg kg⁻¹, i.v.). The symptoms are apparently of central origin because the toxicity increases by a factor of 1,000–10,000 and the progression of poisoning is faster in case of i.c.v. injection, but the clinical picture stays the same (Habermann, 1977, Habermann, 1984). Primary structure determination showed that apamin contains 18 amino acid residues (Haux et al., 1967; Shipolini et al., 1967), with two intramolecular disulfide bonds (Cys1–Cys11 and Cys3–Cys15) (Callewaert et al., 1968). Moreover, the C-terminal residue is amidated; this post-translational modification is common for peptide toxins from animal venoms (Kuzmenkov et al., 2015).

Studies of the spatial structure of apamin began as early as the late 1970s, but no atomic coordinates have been deposited in the Protein Data Bank. The first NMR study reported an erroneous assignment of the secondary structure elements (Bystrov et al., 1980), which was corrected in further works (Wemmer and Kallenbach, 1983; Pease and Wemmer, 1988). The most recent work (Le-Nguyen et al., 2007) presented both a solution NMR structure of apamin and an X-ray structure of its N-terminally acetylated analog, which differ in the packing of the N-terminal region, the length of the α-helix, and the χ angles of the disulfide bridges. In addition, whereas apamin is monomeric in solution, the X-ray structure is a dimer, and that dimeric structure was deposited in the Cambridge Crystallographic Data Centre (https://www.ccdc.cam.ac.uk; database identifier: NIWFEF).

Apamin played a key role in the identification and characterization of a novel subset of Ca²⁺-activated K⁺ ion channels (KᵥCa) (Banks et al., 1979; Lazdunski, 1983; Garcia et al., 1991). Since the early 1980s, this toxin has been widely utilized to differentiate KᵥCa channels into "apamin-sensitive" and "apamin-insensitive" because it selectively affected only the small-conductance KᵥCa channels (KᵥCa2.2, SK2, or SKᵥCa) and not intermediate-conductance (KᵥCa3.1) or large-conductance (KᵥCa3.1) KᵥCa channels. (Burgess et al., 1981; Romey and Lazdunski, 1984; Pennefather et al., 1985). After the cloning of SK channels from rat and human brain and their expression in Xenopus laevis oocytes, the direct activity of apamin was shown on these molecular targets (Köhler et al., 1996; Grunnet et al., 2001a). A number of pharmacological studies using X. laevis oocytes and mammalian cells indicated that among the three SK isoforms apamin was more potent on KᵥCa2.2 (SK2 or SKᵥCa2; IC₅₀ = 0.03–0.14 nM) and less potent on KᵥCa2.1 (SK1 or SKᵥCa1; IC₅₀ = 0.7–12 nM) (Köhler et al., 1996; Shah and Haylett, 2000; Strobaek et al., 2000; Grunnet et al., 2001a), whereas KᵥCa3.2 (SK3 or SKᵥCa3) channels showed an intermediate sensitivity (IC₅₀ = 0.6–4.0 nM) (Ishii et al., 1997; Grunnet et al., 2001b; Grunnet et al., 2001a).

Those investigations led to a prevailing view in the literature that apamin is a selective probe for just SK channels. Quite surprisingly, data to support this claim by showing absence of activity on other targets are missing. Moreover, some published studies actually claim various off-target activities of apamin. For example, it was assumed that apamin affects Ca²⁺ and/or Na⁺ channels in the embryonic chicken heart (Bkaily et al., 1985; Bkaily et al., 1991; Bkaily et al., 1992). On the contrary, in another study human cardiac Ca²⁺, Na⁺, or K⁺ channels were not affected, with the exception of SK channels (Yu et al., 2014). Apamin was also reported to affect inward-rectifier K⁺ channels Kᵥ3.1/3.4 and the voltage-gated K⁺ channel Kv1.3 (Jin and Lu, 1998; Voos et al., 2017); however, the activity of the toxin on those channel isoforms expressed individually is yet to be shown.

To verify the molecular pharmacology of such an important compound as apamin and to resolve the contradictory claims, we performed a large-scale electrophysiological profiling of this toxin against various molecular targets. We conclude that apamin is indeed a selective inhibitor of small-conductance KᵥCa channels.

### 2 Materials and methods

#### 2.1 Materials

For consistency, we used apamin purified from the honeybee Apis mellifera venom (product number A1289) and synthetic mellitin (M4171) purchased from Sigma-Aldrich. TRAM-34 was synthesized as described (Wulff et al., 2000). Other low-molecular-weight compounds were purchased from Sigma-Aldrich: acetylcholine chloride (ACh; A6625), capsicain (M2028), capsazepine (C191), glycine (G7126), kainic acid monohydrate (K0250), and N-methyl-D-aspartic acid (NMDA; M3262).

#### 2.2 Nomenclature of targets and ligands

Ion channel targets and their ligands are presented according to IUPHAR/BPS Guide to PHARMACOLOGY (http://www.guidetopharmacology.org) and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021). For potassium channel ligands of protein and peptide nature, readers are advised to consult the Kalium database (https://kaliumdb.org) (Kuzmenkov et al., 2016; Tabakmakher et al., 2019).
2.3 Analytical chromatography

Apamin purity was confirmed using reversed-phase (RP) HPLC on a Vydac 218TP54 C18 column (4.6 × 250 mm; Separations Group) in a linear gradient of acetonitrile concentration (0%–60% in 60 min) in the presence of 0.1% trifluoroacetic acid (TFA). 1525 Binary HPLC pump and 2489 UV/Visible detector under the control of Breeze 2 software (all from Waters) were used for chromatography.

2.4 Mass spectrometry

Molecular mass measurements were performed using MALDI on an Ultraflex III TOF-TOF instrument (Bruker). 2,5-Dihydroxybenzoic acid (Sigma-Aldrich) was used as a matrix. Measurements were carried out in the reflector mode, which enabled isotopic resolution. Calibration was performed using the ProteoMass Peptide MALDI-MS Calibration Kit (Sigma-Aldrich). Mass spectra were analyzed with the Data Analysis 4.3 and Data Analysis Viewer 4.3 software (Bruker).

2.5 Peptide concentration measurements

Apamin and melittin concentrations were determined by UV spectrophotometry. To obtain absorption spectra of those substances in the UV range, lyophilized peptides were dissolved in 0.5 ml of water (Milli-Q produced on a Merck Millipore Water Purification System). We used a UV-1800 spectrophotometer (Shimadzu) and quartz cuvettes with an optical path length of 1.0 cm; water served as a reference solution. Apamin does not contain aromatic amino acid residues; therefore, absorbance at 205 nm was used to measure the concentration (Anthis and Clore, 2013). Melittin contains tryptophan, and so its absorbance was measured at 280 nm and the calculations were performed accordingly (Beaven and Holiday, 1952).

2.6 Antimicrobial assay

Apamin was tested against Gram-positive (Enterococcus faecalis ATCC 29212, Staphylococcus aureus subsp. aureus ATCC 29213) and Gram-negative bacteria (Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853) following the previously described modification of the microtitre broth dilution method (Vassilevski et al., 2010). Those bacterial strains were also subjected to the treatment by melittin in the same concentration range to serve as a positive control.

Briefly, bacteria were cultured in a low-salt LB medium. The two-fold microtitre broth dilution assay was carried out in 96-well sterile plates in a final volume of 100 μL. Mid-exponential-phase cultures were diluted to a final concentration of 10⁵ colony-forming units/ml. Pure peptides were dissolved in 10 μL of water and added to 90 μL of the bacterial dilution. The samples, a non-treated control and a sterility control were tested in five independent experiments (n = 5). The microtitre plates were incubated for 24 h at 37°C, and growth inhibition was determined by measuring the absorbance at 620 nm. Minimum inhibitory concentration (MIC) is expressed as the lowest concentration of peptide that caused 100% bacterial growth inhibition.

2.7 NMR spectroscopy

For NMR studies 1 mg of apamin was dissolved in 320 μL of H₂O/D₂O mixture (95:5) and placed into a 5-mm Shigemi NMR tube. The sample pH was adjusted to 3.2. NMR spectra of apamin were recorded using a 600-MHz Bruker Avance III NMR spectrometer, equipped with a triple-resonance cryogenic probe, at 25°C. We recorded the following spectra: DQF-COSY, NOESY (120 ms), ROESY (200 ms), TOCSY (80 ms), 13C and 15N-HSQC at natural abundance. 3JHNHA were measured by the line shape analysis of cross-peaks in NOESY spectra, whereas 3JHAHB couplings of AMX spin systems were determined by the line shape analysis of cross-peaks in 2D TOCSY spectra.

3D structure calculation was performed using the simulated annealing/molecular dynamics protocol as implemented in the CYANA software package version 3.98 (Güntert et al., 1997). The disulfide linkages were introduced based on previously published data (Callewaert et al., 1968). 100 structures were obtained starting from random conformations and the 10 best were then selected for further analysis. Visual inspection of the calculated structures and figure drawings were performed using PyMOL (Schrödinger) and MOLMOL (Koradi et al., 1996) software.

2.8 Expression of ion channels in X. laevis oocytes

The following genes encoding ion channel subunits were expressed in Xenopus oocytes: for voltage-gated potassium channels, Kv [rKv1.1 (GenBank accession number: NM_173095), rKv1.2 (NM_012970), hKv1.3 (NM_002232), rKv1.4 (NM_012971), rKv1.5 (NM_012972), rKv1.6 (NM_023954), hKv2.1 (NM_004975), hKv3.1 (NM_004976), rKv4.3 (NM_031739), hKv7.1 (NM_000218), hKv7.2/7.3 (NM_004518/NM_004519), hKv10.1 (EAG1; NM_172362), hKv11.1 (hERG; NM_000238), Shaker-IR from Drosophila melanogaster (NM_167595; amino acids 6–46 deleted), and KQT-1 from Caenorhabditis elegans (NM_171710), inward-rectifier potassium channels, Kir [mKir3.1/3.2 or GIRK1/2 (NM_008426/
2.9 Expression of ion channels in eukaryotic cells

HEK293 cells stably expressing hKv1.1 (BK, GenBank accession number: NM_002247), hKv2.1 (SK1, NM_002248), and hKv3.1 (IK, AH009923) or COS7 cells stably expressing hKv2.3 (SK3, AJ251016) were generated and cultured as previously described (Sankaranarayanan et al., 2009). HEK293 cells stably expressing rKv2.2 (SK2, NM_019314) were a gift from Dr. Mio Zhang (Chapman University, Irvine).

CHO cells were used for transient expression of acid-sensing ion channels (ASIC). These cells were cultured in a CB-150 CO2 incubator (Binder) at 37°C in a humidified atmosphere of 5% CO2. Cells were maintained under standard culture conditions (DMEM/F12, 10% fetal bovine serum, and 50 mg l−1 gentamicin) in 35 mm2 Petri dishes. Transfection was performed using 0.5 μg of plasmids encoding rASIC1a (NM_024154), rASIC2a (NM_001039523, NM_000747, NM_000751), αβ2 (NM_002744, NM_002748), and α7 (NM_000746).

Linearized plasmids bearing the ion channel genes were transcribed using the mMESSAGE mMACHINE SP6 or T7 transcription kits (Ambion) to prepare the respective cRNA. The harvesting of stage V–VI oocytes from anesthetized female X. laevis frogs was described previously (Liman et al., 1992; Peigneur et al., 2021). Oocytes were injected with 50 nL of cRNA at a concentration of 1 ng nl−1 using a microinjector (Drummond Scientific). The oocytes were incubated at 16°C in ND96 solution containing (in mM): NaCl, 96; KCl, 2; CaCl2, 1.8; MgCl2, 2; and HEPES, 5 (pH 7.4), supplemented with 50 mg l−1 gentamicin sulfate.

2.10 Isolation of rat neurons

Wistar rats (12–18 days old, both sexes) were deeply anesthetized with isoflurane and sacrificed by cervical dislocation followed by decapitation. The brains were quickly removed and immersed in ice-cold (2°C–4°C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 124; KCl, 5; CaCl2, 1.3; MgCl2, 2; NaHCO3, 26; Na2HPO4, 1.24; and D-glucose, 10; aerated with carbogen (95% O2, 5% CO2). Transverse slices, comprising hippocampus and striatum, were cut with a 7000 SMZ-2 vibratome (Campden Instruments) and stored at room temperature (22°C–24°C) in ACSF aerated with carbogen. Neurons were isolated from the slices by vibrobiossociation (Vorobjev, 1991). The effects of apamin on ionotropic glutamate receptors (iGluR) were studied on hippocampal pyramidal neurons of the CA1 area expressing GluN2A/B NMDA receptors (Monyer et al., 1994; Foster et al., 2010) and Ca2+-impermeable GluA2-containing AMPA receptors (Wenthold et al., 1996; Seifert et al., 2000) and on giant cholinergic interneurons of the striatum expressing Ca2+-permeable GluA2-lacking AMPA receptors (Buldakova et al., 1999). The experiments on ASIC were carried out on hippocampal interneurons of the lacunosum-molecular and radiatum layers of the CA1 region, which express ASIC1a/2 heteromers (Weng et al., 2010).

## 2.11 Two-electrode voltage-clamp

Recordings were performed at room temperature (18°C–22°C) using a Geneclamp 500 amplifier (Molecular Devices) controlled by a pClamp data acquisition system (Axon Instruments). Whole-cell currents from oocytes were recorded 1–4 days after cRNA injection. Bath solution composition was ND96, or HK containing (in mM): NaCl, 2; KCl, 96; CaCl2, 1.8; MgCl2, 2; and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept at 0.7–1.5 MΩ. Elicited currents were sampled at 1 kHz and filtered at 0.5 kHz (for potassium currents) or sampled at 20 kHz and filtered at 2 kHz (for sodium currents) using a four-pole low-pass Bessel filter. Leak subtraction was performed using a –P/4 protocol.

Currents were evoked by a 100 ms (Nav) or 500 ms (Kv) depolarization to the voltage corresponding to the maximal activation of the channels in control conditions from a holding potential of –90 mV. For nAChR experiments, the oocytes were voltage-clamped at a holding potential of –70 mV and continuously superfused with ND96 via gravity-fed tubes at 0.1–0.2 ml min−1, with 5 min incubation times for the bath-applied peptides. ACh was applied via gravity-fed tubes until peak current amplitude was obtained (1–3 s), with 1–2 min washout periods between applications. The nAChR were gated by a particular time duration pulse of ACh for the respective nAChR subtype (200 μM for α1β1δ and α4β2; 100 μM for α7) at 2 ml min−1. Data were sampled at 500 Hz and filtered at 200 Hz. TRP currents were measured in ND96 at –90 mV during 400 s. Capsaicin (2 μM) was used as
an agonist and capsazepine (10 μM) as an antagonist of TRPV1. Peak current amplitude was measured prior to and following the application of the peptide. All data were obtained in at least five independent experiments (n ≥ 5).

2.12 Patch-clamp of HEK293 or COS7 cells

All experiments were conducted with an EPC-10 amplifier (HEKA) in the whole-cell configuration with a holding potential of –80 mV. Pipette resistances averaged around 2.5 MΩ. Solutions of apamin in Na⁺ Ringer were freshly prepared during the experiments from 100 μM stock solutions in Roswell Park Memorial Institute medium (RPMI). For current measurements, we used an internal pipette solution containing (in mM): K⁺ aspartate, 160; MgCl₂, 2.08; HEPES, 10; EGTA, 10; and CaCl₂, 8.55 (1 μM free Ca²⁺); pH 7.2; osmolarity 310 mOsm. Free Ca²⁺ concentrations were calculated with MaxChelator (developed by Chris Patton, Stanford University) assuming a temperature of 25°C, pH 7.2, and ionic strength of 160 mM. Na⁺ Ringer was used as an external solution containing (in mM): NaCl, 160; KCl, 4.5; CaCl₂, 2; MgCl₂, 1; and HEPES, 10; pH 7.4; osmolarity, 315 mOsm. Please note that the provided concentrations are what was weighed in to achieve an initially hyperosmolar solution that was diluted with water to 310 mOsm for the internal and 315 mOsm for the external solution as measured with a VAPRO Vapor pressure osmometer (Wescor). A slight 5 mOsm difference in osmolarity between internal and external solution improves the sealing rate.

Kc₃.2 and Kc₃.1 currents were elicited by 200-ms voltage ramps from −120 to 40 mV applied every 10 s, and the fold decrease of slope conductance at −80 mV was taken as a measure of apamin-induced channel inhibition. TRAM-34 was used to block Kc₃.1. Kc₃.1 channel activity was recorded with a step protocol, in which cells were stepped to +40 mV for 100 ms at 30-s intervals. Concentration-dependent current inhibition was fitted with the Hill equation using Prism 8 (GraphPad Software). All data were collected in at least five independent experiments (n ≥ 5).

2.13 Patch-clamp of rat neurons or CHO cells

In this case, the holding potential was set at −70 mV. Signals were filtered at 10 kHz and sampled at 20 kHz. Drugs were applied using an RSC-200 (BioLogic) perfusion system. The extracellular solution contained (in mM): NaCl, 143; KCl, 5; CaCl₂, 2.5; D-glucose, 10; and HEPES, 10; and the pH was adjusted to 7.4 with HCl. 10 mM MES was used in the extracellular solution in experiments on ASIC. The patch pipettes (2.5–3.5 MΩ) were made from borosilicate glass (WPI) using a P-97 puller (Sutter Instruments). The pipette solution contained (in mM): CsF, 100; CsCl, 40; NaCl, 5; CaCl₂, 0.5; EGTA, 5; and HEPES, 10; and the pH was adjusted to 7.2 with CsOH. All experiments were performed at room temperature (22°C–24°C). NMDA receptors were activated by 100 μM NMDA and 10 μM glycine. AMPA receptors were activated by 100 μM kainate. ASIC of rat neurons were activated by pH drops from pH 7.4 to 6.5. ASIC1α, ASIC2a, and ASIC3 homomers were expressed in CHO cells and activated by pH 6.5, 5.0, and 6.8, respectively. Lyophilized apamin was freshly dissolved in extracellular solution prior to the experiment. After recording the control response, the patched cell was superfused for 40 s with extracellular solution containing apamin, and then the response to co-application of the agonist and apamin was recorded. Offline data analyses were performed using Origin 9.1 (OriginLab) software. All data were collected from at least five experiments (n ≥ 5).

2.14 Data analysis and statistics

Data and statistical analysis comply with recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). All data points in the apamin concentration-response curves on Kc₃.2 channels are means ± standard deviation (SD) from at least five independent experiments. IC₅₀ values are reported with 95% confidence intervals (CI). The CI is based on the fit for the averaged currents. Student’s two-tailed unpaired t-test was used to assess the significance of the effect of apamin in rat neurons (current in the presence of apamin vs current in control). A value of p < 0.05 was considered statistically significant.

3 Results

3.1 Confirmation of apamin sample purity

Natural apamin isolated from bee venom was purchased from Sigma and subjected to analytical RP-HPLC. It presented a single symmetrical Gaussian peak (Figure 1A) corresponding to >95% purity. The fraction corresponding to the individual component was further inspected by MALDI mass spectrometry. The measured monoisotopic molecular mass ([M + H]+ = 2026.7 Da; Figure 1A, inset) did not differ significantly from the calculated value for apamin (2026.9 Da; Δ = 0.2 Da).

3.2 Apamin spatial structure in solution

As there is no apamin structure in the PDB, we solved it with NMR spectroscopy in solution based on the recorded J-couplings and NOE distances (Supplementary Figure S1). NMR chemical shifts, experimental restraints, and the spatial structure were deposited to the BMRB and PDB databases under the accession
codes 34641 and 7OXF, respectively. The obtained structure fits all the experimental data and is characterized by a low backbone RMSD value (0.24 Å), which confirms that it is well converged (Supplementary Table S1).

Apamin forms a short α-helix (Ala9–Gln16) and a β-turn (Asn2–Ala5; Figures 1B–D). Additionally, the amide group of Lys4 forms a hydrogen bond with the side chain carbonyl of Asn2, which is in agreement with the previously published decelerated hydrogen-deuterium exchange of Lys4 amide (Bystrov et al., 1980). Analysis of the structure reveals no electrostatic, π-cation, or stacking interactions that could stabilize the observed apamin conformation.

### 3.3 Antimicrobial activity

The antibacterial assay showed no activity of apamin against either Gram-positive or Gram-negative bacteria up to a concentration of 50 μM. Melittin, on the other hand, displayed the expected antimicrobial effect. Its MIC values on *E. faecalis, S. aureus, E. coli*, and *P. aeruginosa* were 0.5, 3, 1.5, and 6 μM, respectively.

### 3.4 Apamin pharmacology

We estimated the activity of apamin against five KCa, one Kv, 15 Kv, 10 Na, three ASIC, and one TRP, as well as three nAChR (Table 1; Figures 2, 3). The inhibitory effect was detected only for the three isoforms of KCa2 (SK2 or SKCa) channels (Figures 2A–C). We constructed concentration-response curves for the susceptible channels (Figure 2D) and confirmed that apamin displays the expected nanomolar and subnanomolar affinity to these channels. The IC50 values were 4.1 nM, 87.7 pM, and 2.3 nM, respectively for KCa2.1, KCa2.2, and KCa2.3 (Table 1). Apamin had no effect on the intermediate-conductance KCa3.1 (IK) or large-conductance KCa1.1 (BK) currents at 5 μM (Figures 2E,F, respectively).
TABLE 1 Apamin potency against tested ion channels. KCa channels were expressed in HEK293 cells (KCa2.3, in COS7 cells); Kv, NaV, TRP, and nAChR were expressed in X. laevis oocytes; homeric ASIC1a, ASIC2a, and ASIC3 were expressed in CHO cells; native NMDA and Ca2+-impermeable AMPA receptors were investigated in hippocampal CA1 pyramidal cells; native Ca2+-permeable AMPA receptors were studied in giant cholinergic interneurons of the striatum; and native ASIC1a/2 heteromers were investigated in hippocampal interneurons of the lacunosum-moleculare and radiatum layers of the CA1 region.

| Channel Type                     | IC50 with 95% Confidence Interval |
|----------------------------------|-----------------------------------|
| **Ca2+-activated K+ Channels (KCa)** |                                  |
| KCa1.1                           | 4.1 nM 95% CI 3.3–5.0 nM         |
| KCa2.1                           | N.E. [1.02 ± 0.07 (n = 5)]       |
| KCa2.2                           | 87.7 pM 95% CI 74.2–103.3 pM     |
| KCa2.3                           | 2.3 nM 95% CI 1.8–2.9 nM         |
| KCa3.1                           | N.E. [0.99 ± 0.05 (n = 5)]       |
| **Voltage-gated K+ channels (KV)** |                                  |
| KV1.1                            | N.E. [0.97 ± 0.02 (n = 5)]       |
| KV1.2                            | N.E. [0.99 ± 0.03 (n = 5)]       |
| KV1.3                            | N.E. [1.01 ± 0.04 (n = 5)]       |
| KV1.4                            | N.E. [0.99 ± 0.02 (n = 5)]       |
| KV1.5                            | N.E. [0.95 ± 0.04 (n = 5)]       |
| KV1.6                            | N.E. [1.02 ± 0.01 (n = 5)]       |
| KV1.7                            | N.E. [1.05 ± 0.03 (n = 6)]       |
| KV3.1                            | N.E. [0.99 ± 0.02 (n = 5)]       |
| KV4.3                            | N.E. [0.97 ± 0.03 (n = 5)]       |
| KV7.1                            | N.E. [0.93 ± 0.05 (n = 5)]       |
| KV7.2/7.3                        | N.E. [1.00 ± 0.01 (n = 5)]       |
| KV10.1                           | N.E. [1.02 ± 0.04 (n = 6)]       |
| KV11.1                           | N.E. [1.07 ± 0.06 (n = 5)]       |
| **Inwardly rectifying K+ channels (Kir)** |                             |
| Kir3.1/3.2                       | N.E. [1.02 ± 0.04 (n = 6)]       |
| **Voltage-gated Na+ channels (NaV)** |                              |
| NaV1.1                           | N.E. [0.93 ± 0.04 (n = 5)]       |
| NaV1.2                           | N.E. [0.97 ± 0.02 (n = 6)]       |
| NaV1.3                           | N.E. [0.98 ± 0.06 (n = 5)]       |
| NaV1.4                           | N.E. [1.06 ± 0.08 (n = 5)]       |
| NaV1.5                           | N.E. [1.00 ± 0.03 (n = 5)]       |
| NaV1.6                           | N.E. [1.09 ± 0.06 (n = 5)]       |
| NaV1.7                           | N.E. [0.93 ± 0.08 (n = 5)]       |

**Voltage-gated Na+ channels (NaV)**

| NaV1.1                           | N.E. [0.93 ± 0.04 (n = 5)]       |
| NaV1.2                           | N.E. [0.97 ± 0.02 (n = 6)]       |
| NaV1.3                           | N.E. [0.98 ± 0.06 (n = 5)]       |
| NaV1.4                           | N.E. [1.06 ± 0.08 (n = 5)]       |
| NaV1.5                           | N.E. [1.00 ± 0.03 (n = 5)]       |
| NaV1.6                           | N.E. [1.09 ± 0.06 (n = 5)]       |
| NaV1.7                           | N.E. [0.93 ± 0.08 (n = 5)]       |

**Transient receptor potential channels (TRP)**

| TRPV1                            | N.E. [1.20 ± 0.09 (n = 5)]       |

**Nicotinic acetylcholine receptors (nAChR)**

| α1β1γδ                            | N.E. [1.06 ± 0.03 (n = 6)]       |
| α4β2                              | N.E. [0.95 ± 0.07 (n = 6)]       |

**Acid-sensing ion channels (ASIC)**

| ASIC2a native                     | N.E. [0.99 ± 0.12 (n = 10)]     |
| ASIC1a                            | N.E. [0.99 ± 0.03 (n = 6)]      |
| ASIC2a                            | N.E. [1.01 ± 0.03 (n = 7)]      |

**Glutamate receptors (GluR)**

| AMPA (Ca2+ impermeable)            | N.E. [0.98 ± 0.03 (n = 7)]     |
| AMPA (Ca2+ permeable)              | N.E. [1.01 ± 0.02 (n = 6)]     |
| NMDA                               | N.E. [0.96 ± 0.07 (n = 14)]    |

N.E., no effect at 5 μM concentration. The current ratio (Iapamin/Icontrol) with an indication of SD, and of the n is displayed in the square brackets.
The effects of apamin on native iGluR (Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-impermeable AMPA receptors as well as NMDA receptors) and ASIC were studied in isolated neurons of rat brain. Extracellular application of apamin alone did not produce noticeable effects on the holding currents. We compared the whole-cell currents through the receptors in control and in the presence of apamin. At a concentration of 5 µM, it produced no effects on the three types of iGluR and ASIC. In the case of NMDA receptors we noticed that the current in the presence of apamin tended to be slightly lower than in control (in some cells up to \approx 15\%), although this difference was not statistically significant (\text{I}_{\text{apamin}}/\text{I}_{\text{control}} = 0.96 \pm 0.07, p > 0.05, n = 14; Supplementary Figure S2).

4 Discussion

4.1 Apamin’s 3D structure

Apamin was investigated several times, by different groups, using both crystallography and NMR spectroscopy. To our surprise, no atomic coordinates were deposited in the PDB, limiting the utility of those studies to further progress. We, therefore, performed a solution NMR study, solved our own structure (Figures 1C,D), and deposited it in the PDB (accession code: 7OXF).

At least four papers reported the spatial structure of apamin. The first study used NMR and was published by Bystrov and coworkers in 1980 (Bystrov et al., 1980). However, the reported structure appears erroneous due to the wrong positioning of the secondary structure elements (for instance, the \(\alpha\)-helix was proposed to be formed in the region 6–13, which is incorrect). Two later NMR studies were published in 1983 and 1988 (Wemmer and Kallenbach, 1983; Pease and Wemmer, 1988) and reported a conformation that seems almost correct, with the \(\alpha\)-helix in the region 9–17, and a \(\beta\)-turn formed by residues 2–5. Finally, the most recent work by Aumelas and coworkers (Le-Nguyen et al., 2007) reported an NMR structure of apamin and an X-ray structure of its analog. The NMR structure from that study is in perfect agreement with the apamin conformation observed in our work. The X-ray structure, on the other hand, presents a dimer and differs in the conformation of the N-terminus, the length of the \(\alpha\)-helical segment (9–18 in the case of X-ray and 9–16 in NMR), and the dihedral angles of the disulfide bonds. These differences are apparently due to crystal packing constraints.
4.2 Apamin shows no antimicrobial effects

Honeybee *Apis mellifera* venom is well known to display antibacterial and antifungal effects, which are mostly due to its major component melittin, one of the best-studied cytolytic peptides (Habermann, 1972; Raghuraman and Chattopadhyay, 2007). Expectations of similar activity in apamin are reasonable because animals usually produce several similar toxins or even “libraries” of toxins, which is the case for both neurotoxic and cytolytic components (Vassilevski et al., 2009). However, apamin testing against Gram-positive and Gram-negative bacteria revealed no effect up to a very high concentration of 50 μM. At the same time, melittin shows prominent antimicrobial effects at low micromolar concentrations, and since its content in bee venom is ≈20 times higher, the chances that apamin shares a similar mode of action are very low.

**FIGURE 3**
Electrophysiological profiling of apamin. Shown are representative traces of currents through the corresponding ion channels in control (gray) and after application of 5 μM toxin (blue). In the case of nAChR, TRPV1, and GIRK1/2, blue bars indicate apamin application. In nAChR, arrows indicate agonist (ACh) application. In TRPV1, the open bar shows agonist (capsaicin, CAP) application, and gray bar, antagonist (capsazepine, CZP) application. In GIRK1/2, presentation of different bath solutions is shown as line segments. In ASIC traces, application of the activating pH is shown with gray bars. And in GluR traces, gray bars depict the application of agonists. Ca_{impr}, Ca^{2+}-impermeable; Ca_{pr}, Ca^{2+}-permeable.
4.3 Apamin’s revisited pharmacology

Small-conductance Ca\(^{2+}\)-activated K\(^+\) channels (K\(_{Ca}\)2, SK, or SK\(_{Ca}\)) are a group of three α-subunit isoforms (K\(_{Ca}\)2.1–2.3) that can form mature channels of either homo- or heterotetrameric structure (Köhler et al., 1996; Strassmaier et al., 2005). Similarly to many other K\(^+\) channels, each SK α-subunit contains six transmembrane segments (S1–S6, with S5 and S6 contributing to the pore domain). Unlike K\(_{V}\) channels or large-conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK or K\(_{Ca}\)1.1), the gating of SK channels is insensitive to transmembrane voltage. K\(_{Ca}\)2 channels have no Ca\(^{2+}\) binding sites in their α-subunit, but they form a stable complex with calmodulin that acts as their Ca\(^{2+}\) sensor (Xia et al., 1998). All three isoforms of K\(_{Ca}\)2 are widely expressed in the central nervous system (Stocker and Pedarzani, 2000; Chen et al., 2004) and found in sensory neurons and the heart (Köhler et al., 1996; Xu et al., 2003; Tuteja et al., 2005; Skisbye et al., 2014). K\(_{Ca}\)2.2 is also important in the liver (Feranchak et al., 1996; Xu et al., 2003; Tamarina et al., 2003; Chen et al., 2004). According to the Mouse Brain Atlas (http://www.mousebrain.org) (Zeilis et al., 2018), K\(_{Ca}\)2.1 is highly expressed in neurons of the cerebral cortex, midbrain red nucleus, hindbrain, and sensory neurons; K\(_{Ca}\)2.2 in the cerebral cortex, thalamus, hippocampus, spinal cord, midbrain, hindbrain, erector muscle, and sensory neurons, as well as glial cells; and K\(_{Ca}\)2.3 in the diencephalon, nuclei of cranial nerves, medulla, thalamus, hypothalamus, ventral midbrain, hindbrain, cerebellum, enteric and erector muscle neurons, as well as enteric fibroblasts.

The number of known polypeptide or peptide toxins acting on K\(_{Ca}\)2 with high (nanomolar) affinity is limited. According to the Kalium database (https://kaliumdb.org) (Kuzmenkov et al., 2016; Tabakmacher et al., 2019), six of these substances were identified in scorpion venom (AmP05, maurotoxin, Pi-1, scyllatoxin, taminap, and Ts9). Scyllatoxin (α-KTx 5.1) from Leiurus quinquestriatus hebraeus (Shakkottai et al., 2001) and taminap (α-KTx 5.4) from Mesobuthus tamulus (Pedarzani et al., 2002), in particular, show high potency against K\(_{Ca}\)2.2 with IC\(_{50}\) values in the subnanomolar range. Interestingly, apamin shares a similar sequence motif RXCQ with several scorpion toxins that inhibit K\(_{Ca}\) channels: X = R in apamin and AmP05, M in scyllatoxin, and P in Pi-1. In all these toxins the RXCQ motif is found in an α-helix. Replacement of “X” in the RXCQ motif of scyllatoxin with a positively charged residue (e.g., lysine or 2,4-diaminobutyric acid) resulted in derivatives with enhanced selectivity for K\(_{Ca}\)2.2 over K\(_{Ca}\)2.3 (Sabatier et al., 1993, 1994; Shakkottai et al., 2001). The importance and generality of this motif will be clarified in the future.

Structure-activity studies showed that one of the two adjacent arginine residues (Arg13 and Arg14) and Gln17 of apamin are key determinants of its activity (Vincent et al., 1975; Sandberg, 1979; Labbé-Jullié et al., 1991). Mutagenesis suggested that one of these Arg residues interacts with Asp341 (S5–P region) of K\(_{Ca}\)2.2 channel pore region, whereas Gln17 interacts with Asn368 (P–S6; numbering according to rK\(_{Ca}\)2.2, UniProt accession number: P70604) (Ishii et al., 1997). Two additional positions in the pore region were subsequently proposed to be involved in apamin sensitivity [His337 or His485 of K\(_{Ca}\)2.2 and K\(_{Ca}\)2.3 (Q9UG16), respectively, and Asn345 of K\(_{Ca}\)2.2; all in S5–P] (Lamy et al., 2010). Those studies advocated in favor of apamin acting like a pore blocker. On the other hand, some investigations reported that the S3–S4 extracellular loop may be an essential molecular determinant of apamin sensitivity suggesting an allosteric mode of action and not direct pore blockage. Thus, a point mutation (Thr216Ser) significantly influences K\(_{Ca}\)2.1 (Q92952) sensitivity towards apamin (Nolting et al., 2007). In addition, the three-amino-acid motif in the S3–S4 loop of K\(_{Ca}\)2 channels (216TYA218 in hK\(_{Ca}\)2.1, 244SYA246 in hK\(_{Ca}\)2.2, and 393SYT395 in hK\(_{Ca}\)2.3) was implicated in forming the binding interface for apamin (Weatherall et al., 2011). In the absence of a solved 3D structure of a K\(_{Ca}\)2 channel, it is difficult to predict the details of molecular interactions with apamin. One tempting possibility is that the long S3–S4 loop protrudes to the pore domain and together they form a common binding site for apamin.

It is apamin from the honeybee venom (Habermann and Reiz, 1965; Habermann, 1972) that is the most prominent peptide ligand of K\(_{Ca}\)2. Indeed, since the early 1980s apamin has been used as the main pharmacological agent to distinguish K\(_{Ca}\)2 channels from other K\(^+\) channels (Burgess et al., 1981; Roney and Lazdunski, 1984; Pennefather et al., 1985). Subsequent studies on cloned K\(_{Ca}\)2 confirmed that these three channel isoforms are the molecular targets of apamin (Köhler et al., 1996; Shah and Haylett, 2000; Strøbaek et al., 2000; Grunnet et al., 2001a). However, later studies also claimed some off-target activities of apamin. One series of publications reported inhibition of Ca\(^{2+}\) and Na\(^{+}\) channels in embryonic heart tissues (Bkaily et al., 1985, 1991, 1992). And another study pointed to K\(_{V}\)1.3 as a target (Voos et al., 2017). Our extensive electrophysiological measurements disagree with or directly disprove these claims. Apamin does not present any activity on neither of the expressed Na\(^{+}\) channels nor K\(_{V}\)1.3.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author. NMR chemical shifts, experimental restraints, and the spatial structure of apamin were deposited to the BMRB (accession code 34641) and PDB databases (7OXF).

Ethics statement

This study strictly complied with the International Guiding Principles for Biomedical Research Involving Animals and the
guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 18. III. 1986). Animal studies are reported in compliance with the ARRIVE 2.0 guidelines (Percie du Sert et al., 2020). The use of the frogs was in accordance with the license number LA1210239 to Toxicology and Pharmacology, KU Leuven, and was approved by the Ethical Committee for animal experiments of KU Leuven (P186/2019). Wistar rats were bred and reared at a local animal facility of the Sechenov Institute of Evolutionary Physiology and Biochemistry. Animals were housed in ventilated plastic cages and had ad libitum access to food and water. 12/12 h light/dark cycle, temperature and humidity control in the rooms were secured. All experiments with rats were approved by the Local Bioethics Committee of the Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences (Protocol no. 4/2021, 29 April 2021). Maximum efforts were made to minimize animal suffering and the number of animals used in the experiments.

Author contributions

AK and AV designed research; AK, SP, JN, ELP-J, KM, MN, HW, JT, and AV analysed data; AK, SP, ELP-J, KM, and MN performed research; AK performed the purification; KM established the NMR structure; SP, ELP-J, JN, and MN performed the pharmacological profiling of apamin; HW and JT supervised the pharmacology; AA supervised the NMR spectroscopy; AK, SP, JN, KM, MN, HW, and AV wrote the paper.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.977440/full#supplementary-material

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