Tetrapands, a New Class of Scorpion Toxins That Specifically Inhibit Store-operated Calcium Entry in Human Embryonic Kidney-293 Cells*

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Received for publication, July 28, 2003, and in revised form, October 17, 2003
Published, JBC Papers in Press, October 28, 2003, DOI 10.1074/jbc.M308234200

Venoms from 14 snakes and four scorpions were screened for inhibitory activities toward store-operated Ca\(^{2+}\) entry (SOCE) in human embryonic kidney-293 cells. An inhibitory activity was found in venom from the African scorpion Pandinus imperator. The active agent of this venom was purified by gel filtration and reverse-phase high pressure liquid chromatography methods. Sequence information on the purified fraction, by automatic Edman degradation and mass spectrometry analysis, identified the activity as being contained in two tetrapeptides, which we have named tetrapands. We demonstrate that synthesized tetrapands have inhibitory activity for SOCE in human embryonic kidney-293 cells while having no effect on either thapsigargin- or carbachol-stimulated release of Ca\(^{2+}\) stores. These toxins should be extremely useful in future studies to determine downstream events regulated by SOCE as well as to determine whether multiple pathways exist for thapsigargin-stimulated Ca\(^{2+}\) entry.

A large number of receptors utilize Ca\(^{2+}\) as a second messenger to initiate downstream physiological processes. In the case of G protein-coupled receptors, the Ca\(^{2+}\) response generally is biphasic with an initial rapid spike of Ca\(^{2+}\), due to release of internal Ca\(^{2+}\) stores, followed by a longer, more sustained response. In some cases, the sustained response takes the form of a stable Ca\(^{2+}\) plateau, while in other cases sustained Ca\(^{2+}\) oscillations are observed; both the Ca\(^{2+}\) plateau and sustained Ca\(^{2+}\) oscillations are dependent on a continued influx of external Ca\(^{2+}\). Generally this Ca\(^{2+}\) influx is mediated by non-voltage-gated channels (1–4), and a great deal of effort is being exerted to characterize these channels and the proteins responsible for forming these channels. There appear to be multiple types of non-voltage-gated Ca\(^{2+}\) channels stimulated by agonists of G protein-coupled receptors, one of which, the store-operated channels (SOCs),\(^1\) mediates what was originally referred to as capacitative Ca\(^{2+}\) entry (5) but now often called store-operated Ca\(^{2+}\) entry (SOCE). These channels are activated in response to depletion of internal Ca\(^{2+}\) stores. While there are a number of plausible theories on the mechanism by which store depletion activates these channels (6, 7), there is still no complete model for this process. Likewise some progress has been made in identifying members of the TRPC channel family as the likely protein mediators of SOCE, but there is still much controversy in the literature concerning which of the TRPC family members are store-operated and which are receptor-operated. In addition, there is some question as to whether more than one subtype of SOCE might exist (8) as well as a relative dearth of information concerning the downstream consequences of SOCE.

One limitation in the characterization of SOCs and the investigation of the downstream consequences of SOCE has been the lack of an inhibitor with high affinity and high specificity for SOCs. While a number of substances have been used to inhibit SOCE, these agents in general lack the specificity required to fully characterize these channels and to determine unequivocally which downstream physiological events are regulated by Ca\(^{2+}\) entering via SOCs. For example, SKF96365 has frequently been used to inhibit SOCE and to investigate events regulated downstream of SOCE (9). However, SKF96365 has been demonstrated to inhibit Na\(^{+}\) channels (10), K\(^{+}\) channels (11), and maitotoxin-induced Ca\(^{2+}\) entry (12, 13) and to facilitate nicotinic receptor desensitization (14). Other agents utilized as inhibitors of SOCs, such as carbamoylchitridiazole (15) and 2-aminoethoxydiphenylborate (2-APB) (16), have also been demonstrated to have inhibitory effects on other channels and transport molecules with carbamoylchitridiazole being reported as an inhibitor of voltage-gated Ca\(^{2+}\) channels (17) and 2-APB being reported to inhibit InsP\(_3\)-mediated Ca\(^{2+}\) release (18–20), K\(^{+}\) currents (21), sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (22), gap junctions (23), and Ca\(^{2+}\)-induced Ca\(^{2+}\) release from isolated mitochondria (24) and even to activate a novel calcium-permeable cation channel (25). Given all of the

1 The abbreviations used are: SOC, store-operated channel; SOCE, store-operated Ca\(^{2+}\) entry; HEK, human embryonic kidney; HPLC, high pressure liquid chromatography; TRPC, transient receptor potential channel; 2-APB, 2-aminoethoxydiphenylborate; InsP\(_3\), inositol 1,4,5-trisphosphate; AM, acetoxyethyl ester; HBSS, Hank\'s balanced salt solution; OAG, 1-ooleoyl-2-acetetyl-sn-glycerol; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; CCh, carbachol.

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One fertile source of ion channel inhibitors has been the venom of poisonous snakes, scorpions, spiders, and sea snails. Peptide toxins have been purified from complex venoms that block Na⁺ channels (26), K⁺ channels (27), voltage-gated Ca²⁺ channels (28), and receptor-operated channels such as the N-methyl-D-aspartate receptor (29). From the utilization of these peptide toxins, much has been learned about the subunit composition of these channels, their mode of activation and inactivation, and the downstream events regulated by ion movements through these channels. To date, toxins for non-voltage-gated channels such as SOCs have not been identified. Clearly the discovery of a peptide toxin specific for SOCs would allow a level of study of these channels that has not been possible with current inhibitors. In this report, we describe our search for a peptide toxin in venoms of snakes and scorpions, the purification of the toxin from the *Pandinus imperator* scorpion venom, the identification of the toxin sequence, and the synthesis and functional effect of the peptide toxins we have named tetrapandins. These toxins should be extremely useful in future studies to determine downstream events regulated by SOCE as well to determine whether multiple pathways exist for thapsigargin-stimulated Ca²⁺ entry.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fura-2 free acid, fura-2 AM, and Fluronic F-127 were purchased from Molecular Probes; thapsigargin was purchased from LC Laboratory. Hanks' balanced salt solution (HBSS), Ca²⁺-, Mg²⁺-, HCO₃⁻-free HBSS, Dulbecco's modified Eagle's medium, penicillin/streptomycin, η-glutamine, and trypsin-EDTA were purchased from Invitrogen. Chelex-100 came from Bio-Rad, and 1-oleoyl-2-acetyl-sn-glycerol (OAG), along with other chemicals, were purchased from Sigma.

**Vemon Purification Procedure**—All snake species, kept alive at the Kentucky Reptile Zoo (Slade, KY), were milked manually by having Sigma. Invitrogen. Chelex-100 came from Bio-Rad, and 1-oleoyl-2-acetyl-sn-glycerol (OAG), along with other chemicals, were purchased from Sigma.

**Venom Purification Procedure**—All snake species, kept alive at the Kentucky Reptile Zoo (Slade, KY), were milked manually by having them bite into a rubber membrane, which was stretched over a glass funnel. The venom was collected in a glass vial, and the harvest was immediately frozen after extraction and lyophilized within 1 week. The powdered venom from each species was dissolved in 1 ml of distilled water and kept at −20 °C.

Scorpions of the species *P. imperator*, kept alive in the laboratory, were milked by electric stimulation, and their venom was dissolved in twice distilled water and centrifuged at 15,000 × g for 15 min. The supernatant was freeze-dried and kept at −20 °C until used. When required, samples of the soluble venom were loaded on to a Sephadex G-50 column (medium size, from Amersham Biosciences) and eluted with 20 mM ammonium acetate buffer, pH 4.7, following earlier published results (30). The active fraction (see below) was further separated using a semipreparative C18 reverse-phase column (Vydac, Hesperia, CA) of an HPLC system using a linear gradient from solution A (0.12% trifluoroacetic acid in water) to 60% solution B (0.10% trifluoroacetic acid in acetonitrile). The active fraction was further subjected to HPLC separation using an analytical Vydac C18 reverse-phase column eluted with the same gradient. Two pure peptides were obtained from this column and were further characterized.

**Amino Acid Sequence and Mass Spectrometry Analysis**—The peptides were the HPLC system were sequenced using automatic Edman degradation in a ProSequencer apparatus (Beckman LF3000) following the protocol described by the manufacturer. The molecular weights of purified components were determined by means of a Finnigan LCQ™ quadrupole ion trap mass spectrometer (San Jose, CA). Confirmation of sequence and molecular masses were obtained using a MALDI-TOF MS apparatus ( Voyager DE-PRO, Applied Biosystems, Framingham, MA). Peptides were crystallized using the dried droplet technique and α-cyano-4-hydroxycinnamic as matrix. Postsource decay fragment ion spectra were acquired after isolation of the appropriate precursor by using timed ion selection. Fragment ions were refocused onto the detector by stepping the voltage applied to the reflectron and acquiring distinct spectral segments using an acquisition digitizer at a digitization rate of 500 MHz. Individual spectra were superimposed by using the Data Explorer Version 4.0 software (Applied Biosystems).

**Peptide Synthesis and Aggregation**—The chemical synthesis of the peptides was performed by the solid phase method of Merrifield (31) using Fmoc (N-(9-fluorenylethoxycarbonyl)-derivatized amino acids. Novosyn, TGA resins (600 mg, 0.12 mol) were used for the solid phase synthesis. Once the peptides were synthesized, they were detached from the resin, and the corresponding protecting groups of the residues were eliminated by means of reactive K (84% trifluoroacetic acid, 5% phenol, 5% H₂O, 5% thioanisole, 1% dithiothreitol) for 1 h. Deprotected peptides were precipitated with t-butyl methyl ether, washed three times with ether, redissolved in 20% acetic acid, and lyophilized as described. After Novosyn, the peptides were purified by HPLC using conditions described for purification of the native peptides (see above). Their structures were confirmed by mass spectrometry analysis. The exact amino acid sequence for each one of the peptides was confirmed by digesting them to a final concentration of 500 pmol/5 μl of 50% acetonitrile with 1% acetic acid and directly applying them into the Finnigan LCQ™ ion trap spectrometer using a Surveyor MS syringe pump delivery system. The eluate at 10 μl/min was split to allow only 5% of the sample to enter the nanospray source. The spray voltage was set to start at 1.7 kV, and the capillary temperature was set at 115 °C. For MS/MS experiments, the fragmentation source was operated with 25–35V of collision energy and 35–45% (arbitrary units) of normalized collision energy, and the scan was operated with wide band activated. All spectra were obtained in the positive ion mode. The data acquisition and the deconvolution of data were performed with the Xcalibur (Sequitur) software. The MS/MS spectra were analyzed using PeptideTools software.

The synthetic peptide was dissolved in either HBSS or Ca²⁺-, Mg²⁺-free HBSS (pH 7.4) and then filter-sterilized. The sterile peptide solutions were either used directly, or they were mixed for 2 days at room temperature on a Glas-Col rotator (Fisher) and then used for calcium imaging experiments.

**Cell Culture**—HEK-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. Cells were grown in an incubator at 37 °C with humidified 5% CO₂ and 95% air.

**Ca²⁺ Imaging**—The intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was measured in cells loaded with the fluorescent indicator fura-2. Cells were plated onto 25-mm coverslips 1 day before the experiment. On the next morning, cells were washed twice with HEPES-buffered HBSS, loaded with 30 μM fura-2 AM that was dissolved in HBSS, and then unloaded in HBSS supplemented with 1 mg/ml bovine serum albumin + 0.25% Pluronic F-127, and then unloaded in HBSS for another 30 min. The coverslips were mounted as the bottom of a chamber that was placed on the stage of a Nikon Diaphot inverted epifluorescence microscope equipped with a Fluor 10× objective. Cells in the chamber were perfused via an eight-channel syringe system. A suction pipette maintained a constant volume of solution (~0.5 μl) in the chamber.

**Imaging System**—The imaging system (dual wavelength fluorescence imaging system, Intracellular Imaging Inc., Cincinnati, OH) was used to measure [Ca²⁺]ᵢ during the experiment. Excitation light from a xenon light source was alternately passed through 340 and 380 nm narrow pass filters mounted in a Sutter filter wheel (Lambda 10-C). The 510 nm emissions were captured by a cooled CCD camera (Cohu 4915). The images were transmitted to a computer and processed with the imaging software InCyt IM²™ Version 4.8. [Ca²⁺]ᵢ, was calculated by measuring the ratio of the two emission intensities for excitation at 340 and 380 nm. Calcium standard solutions, which were prepared with fura-2 potassium salt, were used to create a graph of fluorescence ratio (F₅₁₀/F₃₄₀) as a function of Ca²⁺ concentration (nM). This graph was then used to convert fluorescence ratios in an experiment to calcium concentrations. In experiments in which Ba²⁺ influx was measured, the data are reported as the ratio (F₅₁₀/F₃₄₀) since the Ba²⁺ calibration curve for fura-2 differs from the calibration curve for Ca²⁺. During the experiment, an averaged response of ~800 cells from a single field on each coverslip was represented as one trace. For initial experiments, intracellular Ca²⁺ stores were depleted in the presence of extracellular Ca²⁺, and the effect of the toxin was monitored by measuring the amount of reduction of the Ca²⁺ plateau. In later experiments where Ba²⁺ entry was monitored as a measure of the level of SOCE, the thapsigargin-stimulated Ba²⁺ entry was obtained by subtracting the slope of the Ba²⁺ leak (before stimulation) from the slope of Ba²⁺ influx (after stimulation) for each coverslip.

Nominally Ca²⁺-, Mg²⁺-, and HCO₃⁻-free HBSS with Chelex-100 beads. After filtering out the Chelex-100 beads, MgCl₂ was added to a final concentration of 1 mM.
RESULTS

Our initial step was to screen a variety of snake and scorpion venoms in search of an inhibitory effect on SOCE in HEK-293 cells. This was done by investigating the effect of the various venoms on thapsigargin-stimulated Ca\(^{2+}\) entry. HEK-293 cells were stimulated with 1 \(\mu\)M thapsigargin (TG, 1 \(\mu\)M). Once the stable calcium plateau levels were reached, a series of different P. imperator (PI) venom dilutions were added (A). Similar fura-2 imaging experiments were performed with 10 \(\mu\)M valinomycin being applied shortly before and along with the venom (B).

Venom was collected from the African scorpion P. imperator, and a series of dilutions were made in HBSS. HEK-293 cells were perfused with HBSS, and the [Ca\(^{2+}\)] was monitored by fura-2 imaging. After the establishment of a base line, cells were stimulated with thapsigargin (TG, 1 \(\mu\)M). The data in Fig. 1A illustrate the effects of several doses of P. imperator venom on thapsigargin-stimulated Ca\(^{2+}\)-entry. HEK-293 cells were stimulated with thapsigargin (TG, 1 \(\mu\)M). Once the stable calcium plateau levels were reached, a series of different P. imperator (PI) venom dilutions were added (A). Similar fura-2 imaging experiments were performed with 10 \(\mu\)M valinomycin being applied shortly before and along with the venom (B).

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The initial step in characterization of the active agent in the P. imperator venom was to fractionate the venom on a gel filtration column. The Sephadex G-50 column separated several families of components based on molecular sizes (Fig. 2). Tubes corresponding to five subfractions (I–V) were pooled and lyophilized. They were all screened to determine their activity for reducing the thapsigargin-stimulated Ca\(^{2+}\) extrusion mechanisms rather than on Ca\(^{2+}\) entry pathways. Since Ba\(^{2+}\) is not pumped by Ca\(^{2+}\)-ATPases (32, 33), an inhibition of Ba\(^{2+}\) entry by the venom would indicate that the effect is on a Ca\(^{2+}\) entry pathway. We observed that the venom had a significant inhibitory effect on Ba\(^{2+}\) entry ( venom data not shown, but data for purified toxin shown later).

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Although SOCs are not directly regulated by membrane depolarization, the level of SOCE can be affected by a reduction of the driving force following membrane depolarization. To determine whether this was the case, we investigated whether the addition of valinomycin, which locks the membrane in a hyperpolarized state, would eliminate the effect of the P. imperator venom. The data in Fig. 1B show that the addition of valinomycin led to a slight increase in plateau Ca\(^{2+}\) levels but did not block the effect of the P. imperator venom on Ca\(^{2+}\) plateau levels. We also investigated the effect of the P. imperator venom on thapsigargin-stimulated Ba\(^{2+}\) entry to rule out the possibility that the venom is working on Ca\(^{2+}\) extrusion mechanisms rather than on Ca\(^{2+}\) entry pathways. Since Ba\(^{2+}\) is not pumped by Ca\(^{2+}\)-ATPases (32, 33), an inhibition of Ba\(^{2+}\) entry by the venom would indicate that the effect is on a Ca\(^{2+}\) entry pathway. We observed that the venom had a significant inhibitory effect on Ba\(^{2+}\) entry ( venom data not shown, but data for purified toxin shown later).

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Subfraction 7 was then submitted to automatic Edman degradation, giving the amino acid sequence Leu-Trp-Lys-Thr with a molecular mass of 546.6 atomic mass units, and peptide 2 eluting at 23.27 min had the amino acid sequence Leu-Trp-Ser-Gly with a molecular mass of 461.5 atomic mass units, and peptide 2 eluting at 23.82 min had the amino acid sequence Leu-Trp-Lys-Thr-Ser-Gly with a molecular mass of 581.6 atomic mass units. Two distinct sequences were finally obtained. Peptide 1 and peptide 2 in a tube and submitted them to constant rotation for various periods of time ranging from hours to days and then tested the potency of the mixture at various time periods. We found that after 2 days of constant rotation a substantially lower dose of peptide was required for inhibition of SOCE. However, this observation was not unique to the mixture of peptide 1 and peptide 2 as we found that placing either peptide 1 alone or peptide 2 alone under similar conditions resulted in a substantial increase in their potency. The data in Fig. 6B show a representative dose response for peptide 2 treated under such conditions. With this material, one can observe a slight inhibitory effect at a peptide dose as low as 1 nM and a maximum effect at a peptide dose of 1 μM. At this maximum dose the Ca\(^{2+}\) plateau is reduced by ~50%.

To confirm that the modified peptide is inhibiting Ca\(^{2+}\) entry and not stimulating Ba\(^{2+}\) entry, its effect on Ba\(^{2+}\) entry was measured. For this experiment, we incubated the cells in HBSS and then switched to a Ba\(^{2+}\)-containing, Ca\(^{2+}\)-free medium to obtain a brief measure of Ba\(^{2+}\) leak flux. The cells were then switched to 0 Ca\(^{2+}\) medium followed by 0 Ca\(^{2+}\) with 1 μM thapsigargin to deplete internal stores. Following the return of Ca\(^{2+}\) to basal levels, 2 mM Ba\(^{2+}\) was again added to monitor total Ba\(^{2+}\) entry. The SOCE was determined by subtracting the Ba\(^{2+}\) leak from the total Ba\(^{2+}\) entry. As shown in Fig. 7A, the peptide at a dose of 1 μM inhibited Ba\(^{2+}\) entry by ~50%. A statistical analysis of a series of experiments at a peptide toxin concentration of 1 μM is shown (Fig. 7B). The slope of Ba\(^{2+}\) entry in the control was 0.00072 ± 0.00004 s\(^{-1}\) (n = 13), and in the presence of modified peptide toxin 2 (1 μM) it was 0.00038 ± 0.00002 s\(^{-1}\) (n = 11). These values are significantly different (p = 0.0005). To determine whether the peptide toxin was having its inhibitory effect by depolarizing the cells and thereby decreasing the driving force for Ca\(^{2+}\) entry, we ran similar experiments in the presence of valinomycin, which locks the membrane potential in a hyperpolarized state. We found that the addition of valinomycin slightly increased the thapsigargin-stimulated Ba\(^{2+}\) entry, consistent with the predicted effect of membrane hyperpolarization. However, in the presence of valinomycin the modified synthetic peptide 2 still inhibited SOCE by a comparable percentage (Fig. 8). In the presence of valinomycin, Ba\(^{2+}\) entry increased to 0.00084 ± 0.00001 s\(^{-1}\) (n = 4); the addition of modified peptide toxin 2 (1 μM) in the presence of valinomycin reduced Ba\(^{2+}\) entry to 0.00046 ± 0.00006 s\(^{-1}\) (n = 4). The difference in these values is statistically significant (p = 0.03).

To determine the specificity of the peptide toxin, we investigated its effect on another type of Ca\(^{2+}\) entry in HEK-293 cells. We determined the effect of the peptide toxin on Ca\(^{2+}\) entry stimulated by OAG. While in our previous studies (34) both TRPC1 and TRPC3 appear to mediate SOCE, in later studies we observed that only TRPC3 mediates OAG-stimulated Ca\(^{2+}\) entry (35). Following addition of 1 μM modified peptide toxin 2 to cells stimulated with OAG, no effect on the stimulated Ca\(^{2+}\) entry was observed (Fig. 9). Thus, the peptide toxin appears to be highly specific for Ca\(^{2+}\) entry activated by Ca\(^{2+}\) store depletion.
As mentioned in the Introduction, 2-APB not only inhibits SOCE but also inhibits release of Ca\(^{2+}\)/H\(^{100}\) from internal stores. Thus, the question arose as to whether tetrapandins would also inhibit Ca\(^{2+}\) release from intracellular stores. To investigate this question, we monitored either thapsigargin-stimulated or CCh-stimulated Ca\(^{2+}\) release from intracellular stores. Control cells were incubated for 30 min in HBSS and washed quickly in Ca\(^{2+}\)-free HBSS, and then thapsigargin was added in a Ca\(^{2+}\)-free HBSS medium to release Ca\(^{2+}\) from intracellular stores. In parallel, cells were preincubated for 30 min in HBSS containing either 100 μM 2-APB or 1 μM tetrapandin 2 (both represent maximum doses for inhibition of Ca\(^{2+}\) entry) and washed quickly in Ca\(^{2+}\)-free HBSS, and then thapsigargin was added in a Ca\(^{2+}\)-free HBSS medium to release Ca\(^{2+}\) from intracellular stores. In
average peak height for the thapsigargin control was $143 \pm 5.8$ nM ($n = 8$) compared with $143 \pm 13.9$ nM ($n = 8$) for tetrapandin 2-treated cells or $84.3 \pm 9.9$ nM ($n = 10$, statistically different from control, $p < 0.0002$) for 2-APB-treated cells. Similarly, if CCh was utilized to release Ca$^{2+}$ from InsP$_3$-sensitive stores (Fig. 10B), tetrapandin 2 had no effect on the Ca$^{2+}$ release.
Fig. 7. Inhibition of thapsigargin-stimulated Ba\(^{2+}\) entry by modified synthetic toxin peptide. A, HEK-293 cells were incubated in HBSS to establish a baseline prior to switching into Ca\(^{2+}\)-free HBSS. A Ca\(^{2+}\)-free, 2 mM Ba\(^{2+}\) HBSS solution was added briefly to the cells to measure the Ba\(^{2+}\) leak. Following a return to Ca\(^{2+}\)-free HBSS, cells were stimulated with thapsigargin (TG, 1 \(\mu M\)), and after cytosolic Ca\(^{2+}\) returned to basal levels, 2 mM Ba\(^{2+}\) was added. SOCE was defined as the leak-subtracted, TG-stimulated Ba\(^{2+}\) influx. In half of the coverslips, the modified synthetic peptide 2 (1 \(\mu M\)) was mixed with 2 mM Ba\(^{2+}\) solution, and it was applied after thapsigargin depletion of Ca\(^{2+}\) stores (thin line). The results obtained were compared with the control (thick line) for which a comparable amount of vehicle was added. B, the Ba\(^{2+}\) entry values were plotted as percentage of control. At a dose of 1 \(\mu M\) modified peptide toxin 2, SOCE was reduced by 48 \(\pm\) 2%. *, significantly different from control (\(p < 0.0001\)).

Fig. 8. Effect of valinomycin on inhibition of SOCE by modified synthetic toxin peptide. Ba\(^{2+}\) entry experiments similar to those described in Fig. 7 were performed in the presence of 10 \(\mu M\) valinomycin. Statistical analysis of the data with the mean value of the leak-subtracted Ba\(^{2+}\) entry representing the amount of SOCE is shown. *, significantly different from control (\(p < 0.0001\)). **, significantly different from valinomycin alone (\(p < 0.03\)).

(right panel), while 2-APB had a substantial inhibitory effect (center panel). The average peak height for the CCh control was 245.6 \(\pm\) 26.7 \(\text{nM}\) (\(n = 5\)) compared with 276.8 \(\pm\) 18.8 \(\text{nM}\) (\(n = 4\)) for the CCh response in cells preincubated with tetrapandin 2 or 53.2 \(\pm\) 7.9 \(\text{nM}\) (\(n = 9\)) for cells preincubated with 2-APB.

We also wanted to compare the effect of tetrapandins to that of 2-APB on the plateau phase of the CCh-stimulated Ca\(^{2+}\)
response. Cells were treated with 100 μM CCh in HBSS and allowed to establish a stable plateau phase. In control cells, the HBSS was replaced with Ca²⁺/Mg²⁺-free HBSS to establish that the plateau phase is dependent on a continued influx of Ca²⁺ (Fig. 11A). For the 2-APB and tetrapandin conditions, these drugs were added during the plateau phase in the continued presence of HBSS. The data in Fig. 11B show that addition of 2-APB resulted in a substantial reduction of the plateau Ca²⁺ level. A similar response was observed with the addition of tetrapandin 2 (Fig. 11C). The removal of Ca²⁺ from the external medium brought the Ca²⁺ value 97 ± 1.3% (n = 5) of the way back down to basal levels. In comparison, 2-APB brought the Ca²⁺ value 76 ± 3.3% (n = 6) of the way back down to basal levels, while tetrapandin 2 brought the Ca²⁺ value 70.4 ± 5.0% (n = 9) of the way back down to basal levels.

**DISCUSSION**

In this study, we found an inhibitory effect on SOCE by venom from the African scorpion *P. imperator*. This inhibitory activity appears to be uncommon among venoms from snakes and other scorpions as we found it in only one of 14 snake venoms and in one of four scorpion venoms tested. This finding encouraged us to proceed since it indicated that we were not observing a nonspecific effect of venoms in general, such as...
membrane depolarization due to toxin action on other ion channels. This was further supported by the finding that the venom still inhibited thapsigargin-stimulated Ca\textsuperscript{2+} entry even in the presence of valinomycin, which locks the membrane potential in a hyperpolarized state.

We have outlined the steps involved in the purification of a peptide toxin from the *P. imperator* venom as well those involved in the sequencing and chemical synthesis of a peptide toxin that inhibits SOCE. Two short peptides were found to be present in the HPLC subfraction 7. Both peptides were tetrapeptides (LWSG and LWKT) and were found to share two amino acids in common.

These peptide toxins were named tetrapandins 1 and 2 (peptides 1 and 2, respectively) from the fact that they come from the scorpion of the genus *Pandinus* and have only four amino acids. This is quite unique among the venom components isolated and characterized thus far in the literature. Most scorpion venoms contain peptides that modify or block the function of ion channels such as voltage-dependent K\textsuperscript{+} and Na\textsuperscript{+} channels, although other venoms also have peptides capable of impairing the function of Cl\textsuperscript{−} channels or Ca\textsuperscript{2+}-sensitive ryanodine channels (for a review, see Ref. 36). Actually, from the venom of the same *P. imperator*, several interesting peptides were isolated previously, such as imperatoxin a and b (for a review, see Ref. 37) and scorpine, a defensin and antimalaria agent (38). The findings described here should not be taken as unusual since of the 100,000 components estimated to exist in scorpion venoms alone, only over 300 components have been studied thus far (36, 39, 40). Most of the studies reported in the literature deal with peptides from 26 to 76 amino acid residues long, but very few, if any, studies have been performed with the low molecular weight components of these venoms, which elute from the Sephadex G-50 column in fraction V.

Tetrapandins 1 and 2 have a lipophilic segment as well as a hydrophilic segment. The side chains of Leu and Trp are hydrophobic, whereas the C-terminal carboxylic groups and side chains of Lys and hydroxyl groups of Ser and Thr are hydrophilic. Since these peptides have a hydrophobic segment and knowing that biological membranes are permeable to hydrophobic substances, it is possible that these peptides might cross the cell membrane. This would provide the possibility that their effect could be at either an external or internal channel site to inhibit SOCE.

Both synthetic peptides have inhibitory action on SOCE when they are freshly prepared, although their potency is somewhat low. However, upon continued rotation for 2 days both peptides greatly increase their potency for inhibition of SOCE. After modification, the peptide toxins can initiate their effects at a dose of 1 nM, which is considerably lower than the 1 \(\mu\)M dose required for a threshold effect to be seen with freshly prepared toxin. At present, we are not sure whether the peptides are chemically modified (e.g. air-oxidized) to a new structure or whether they aggregate during the incubation period. It is tempting to speculate that Trp oxidation to \(\text{N}-\text{formylkynurenine}\) might be responsible for such an increase in biological activity since a significant amount of the peptide purified from *P. imperator* venom showed signs of Trp oxidation. On the other hand, since they are amphiphilic substances (partially hydrophilic and hydrophobic) some kind of aggregation (dimerization or micelle formation) could occur with these peptides that could be responsible for the increased activity seen after agitation at room temperature. Thus, while a number of experiments are still needed to elucidate the exact modification of the synthetic peptides required to obtain the highest potency, we have clearly obtained a preparation that should be quite useful for the further exploration of SOCs.
Tetrapandins, a New Class of Scorpion Toxins

One interesting aspect of this peptide toxin is that at a maximum dose it inhibits only 50% of either the thapsigargin-stimulated Ca\(^{2+}\) plateau phase or the thapsigargin-stimulated Ba\(^{2+}\) entry. This is in direct contrast to the effects of 2-APB, which has been shown to inhibit ~90% of the thapsigargin-stimulated Ba\(^{2+}\) entry in these cells (35). However, 2-APB also is known to inhibit release of Ca\(^{2+}\) from internal stores. Our data indicate that the tetrapandins are more specific than 2-APB in that they have no effect on release of internal Ca\(^{2+}\) stores under conditions where 2-APB dramatically reduces Ca\(^{2+}\) store release. This is true whether the store Ca\(^{2+}\) is released by thapsigargin or by InsP\(_3\) production in response to CCh stimulation (Fig. 10).

The 50% maximal reduction of thapsigargin-stimulated Ca\(^{2+}\) entry may suggest either a partial block of SOCs or full inhibition of one of multiple Ca\(^{2+}\) entry pathways activated by thapsigargin. Previous studies have suggested that there may be more than one pathway activated in response to thapsigargin (8). For example, our previous TRPC3 antisense data (34) indicate that suppression of ~60% of the TRPC3 protein reduces SOCE by only 32%. Thus, extrapolation of this data to full TRPC3 protein suppression suggests that TRPC3 may mediate at most 45–50% of the total SOCE. Thus, it is possible that the toxin may be inhibiting in a selective manner either the TRPC3-sensitive or the TRPC3-insensitive portion of the thapsigargin-stimulated Ca\(^{2+}\) entry. We are currently using small interfering RNA technology to try to achieve a more efficient reduction of TRPC3 levels in HEK-293 cells. If we can suppress TRPC3 levels by ~90%, then we will be in a better position to address the question of whether tetrapandins are suppressing TRPC3 levels by

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**Acknowledgments**—The assistance of Dr. Cesar V. F. Batista on MS analysis, biologist Cipriano Balderas in milking the scorpions, and Grzegorz Gurda in some preliminary imaging experiments is greatly acknowledged. We also thank the Kentucky Reptile Zoo (Slade, KY) and its director Jim Harrison for providing the snake venoms for our investigation.

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