Isolation of a Member of the Neurotoxin/Cytotoxin Peptide Family from *Xenopus laevis* Skin Which Activates Dihydropyridine-sensitive Ca\(^{2+}\) Channels in Mammalian Epithelial Cells*

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We have used a sensitive bioassay of calcium-mediated volume changes in mammalian absorptive intestinal epithelial cells to screen extracts of the skin of the amphibian *Xenopus laevis* for the presence of factors affecting ion transport. A 66-residue peptide, purified using reversed-phase high performance liquid chromatography techniques, caused isotonic volume reduction of guinea pig jejunal villus cells in suspension. This volume reduction required extracellular Ca\(^{2+}\) and was prevented by the dihydropyridine-sensitive Ca\(^{2+}\) channel blocker nifedipine. Structural analysis demonstrated the presence of eight cysteines and a primary structure homologous to that of the neurotoxin/cytotoxin family found in the venom of certain poisonous snakes. The structure of the peptide was identical to that of xenoxin-1 purified from dorsal gland secretions of *X. laevis* (Kolbe, M., Huber A., Cordier, P., Rasmussen, U., Bouchon, B., Jaquinod, M., Blasak, R., Detot, E., and Kreil, G. (1993) *J. Biol. Chem.* 268, 16458–16464). Xenoxin-1 (10 nM) caused volume changes that required extracellular Ca\(^{2+}\) and were comparable in magnitude and direction to changes caused by BayK-8644 (100 nM), a dihydropyridine-sensitive Ca\(^{2+}\) channel agonist. The initial rate of dihydropyridine-sensitive Ca\(^{2+}\) influx was substantially increased by xenoxin-1. Staurosporine (10 nM) prevented volume changes caused by ATP (250 µM) but had no effect on volume changes caused by BayK-8644 or xenoxin-1. We conclude that xenoxin-1 directly activated dihydropyridine-sensitive Ca\(^{2+}\) channels in villus cells and that a mammalian homologue to xenoxin-1 may exist.

Frog skin, which is a multilayered epithelium, is a rich source of peptides with diverse biological functions (1–3). Originally used in tracer flux experiments (4), frog skin has recently been used as a model system for studying volume regulation (5). Frog skin peptides are similar or identical to hormones and neurotransmitters of mammalian origin. Numerous amphipathic antibacterial peptides have also been purified from amphibian skin exudates and tissue extracts (6). Since frog skin is an epithelium, it was originally speculated that some peptides could be regulators of electrolyte homeostasis (7).

The isotonic volume of mammalian intestinal epithelial cells is regulated by salt transport (8). Increasing the rate of salt efflux (i.e. K\(^+\) and Cl\(^−\) ions) results in a volume reduction of these epithelial cells (9). Previously, we have shown that members of the defensin family of peptides will differentially activate isotonic volume changes in mammalian intestinal epithelial cells (10). These 29–34-residue cationic peptides contain a consensus distribution of six cysteine residues aligned in a highly conserved fashion (11). Homogeneous populations of viable mature villus epithelial cells can be isolated in suspension (8, 12, 13), and their volume can be accurately determined using electronic cell sizing (14). We have used isotonic volume changes in this cell preparation as a bioassay to facilitate the purification of peptides that activate electrolyte efflux from extracts of amphibian skin.

In this report, we describe the isolation and characterization of a peptide from the dorsal skin of *Xenopus laevis*, which activates dihydropyridine-sensitive Ca\(^{2+}\) channels in guinea pig villus epithelial cells. This peptide shows structural similarity to the cytotoxin and neurotoxin family of peptides found previously only in the venom of poisonous snakes. While these studies were in progress, an identical peptide was characterized by other investigators as xenoxin-1 (15).

**MATERIALS AND METHODS**

Reagents—The acetoxyethylmerister of 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA) was obtained from Molecular Probes (Eugene, OR). Nifedipine was from Research Biochemicals (Natick, MA) and N-methyl-D-glucamine from Aldrich. EGTA was from Sigma and RPMI 1640 (10×) medium from Life Technologies, Inc. (Burlington, Ontario). Dicyclophosphatase was from Pfoltz and Bauer (Waterbury, CT), and \(^{45}\)CaCl\(_2\) was purchased from ICN Biomedicals (Montreal).

High Performance Liquid Chromatography (HPLC)—Chromatography was undertaken using a Waters Associates (Milford, MA) HPLC system consisting of two 510 pumps and a gradient controller. Column eluates were monitored for UV absorbance at 210 and 280 nm using LDC (Riviera Beach, FL) and Beckman (Palo Alto, CA) detectors connected in series. HPLC solvents and reagents were prepared as described previously (16). Reversed-phase HPLC was undertaken using Waters C\(_{18}\) µBondapak and C\(_{18}\) Vydac TP201 (Coe Palmer, Chicago, IL) columns, which were eluted as described previously (17).

Tissue Extraction and Peptide Purification—A female *X. laevis* was purchased from Boreal (St. Catharines, Ontario) and sacrificed by decapitation. The dorsal skin was removed and homogenized in 50 ml of

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*The abbreviations used are: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; HPLC, high performance liquid chromatography; PCK, protein kinase C.*
an acidic extraction medium consisting of 1 M hydrochloric acid containing 5% (v/v) formic acid, 1% (w/v) sodium chloride and 1% (v/v) trifluoroacetic acid (16). Following centrifugation, the supernatant was subjected to a reversed-phase extraction procedure using 10 Waters C_{18} Sep-Pak cartridges as described previously (16). The cartridge eluates containing 1% (v/v) trifluoroacetic acid were combined and lyophilized. This material was homogenized in 5 ml of 0.1% trifluoroacetic acid. Following centrifugation the supernatant was loaded on the gel permeation HPLC columns using a trace enrichment procedure as described previously (18). The column was eluted isocratically using 40% aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid (17). Biologically active fractions were pooled and subjected to two reversed-phase HPLC procedures using C_{18} μBondapak and Vydac columns each eluted with linear gradients of aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid throughout.

**Villus Enterocyte Isolation and Volume Determinations**—Villus cells were isolated from segments of adult male (200–300 g) guinea pig jejunum by mechanical vibration as described previously (8, 9). Isolated cells were resuspended at 0.8–1.5 × 10^6 cells/ml in RPMI 1640 medium (without HCO_3) containing bovine serum albumin (Cohn fraction V) at 1 mg/ml and 20 mM sodium Heps, pH 7.3 at 37 °C. Viability, assessed by trypan blue exclusion was 85%, 5 h after suspension in medium. Cell volume was measured using a Coulter Counter (ZM) with an attached Channelizer (C-256) as described previously (8, 9). Villus cell volume measurements were taken electronically over a range of tonicities corrected positively (r = 0.967) with isotonic measurements of cell water (9). Relative cell volume was determined as the ratio of cell volume after agonist addition to the volume under basal conditions in isotonic medium immediately before challenge. Cell volume measurements were made using 30,000 cells/ml in Na^+ free medium, which contained (in mM): 140 NaCl, 3 KCl, 1 CaCl_2, 1 MgCl_2, 10 β-glucose, and 10 Heps (pH 7.3, 280 mosm/kg H_2O). Na^+ -free medium was prepared by iso-osmotically replacing NaCl with the chloride salt of N-methyl-N-glucamine. Nominally Ca^2+-free Na^+ medium contained 150 μM EGTA.

**Uptake of 45Ca**—We measured the initial rate of 45Ca influx into villus cells using a modification of a procedure previously described for human lymphocytes (18). We incubated the villus cells with 10 μM acetylatedlylester of BAPTA (BAPTA-AM) for 30 min in RPMI medium, then centrifuged the cells and resuspended them in BAPTA-AM-free medium. Each villus cell preparation was divided in half and resuspended at a final concentration of 5–6 mg of protein/ml in prewarmed uptake medium (Na^+ -medium supplemented) with bovine serum albumin at 1 mg/ml in a continuously stirred cuvette. For some experiments this medium contained 0.1 μM nifedipine. Uptake was initiated with the addition of 45Ca at a concentration of 10 μCi/ml, followed in some experiments by xenon-1- (10 μM). Immediately afterward, a 500-μl cell suspension was removed and added to 500 μl of ice cold Ca^2+-free (150 μM EGTA) uptake medium, which served as a “stop” solution. This took less than 5 s and was taken to represent the extracellular Ca associated with the cell pellet. Uptake was terminated after 4 s by diluting 500 μl of cell suspension in an equal volume of ice-cold stop solution, which was then gently layered on a 100 μl layer of di-n-butylphthalate:di-n-nonylphthalate (3:2 by volume) and centrifuged in an Eppendorf microcentrifuge for 20 s. Aliquots of the supernatant were saved for counting and the cell pellet was processed as described previously (8). Prior to the addition of 45Ca, duplicate samples were taken and processed as above, but following aspiration of the supernatant and oil, 100 μl of Triton X-100 was added to the pellet. After vigorous shaking to induce lysis, protein content was determined using the Bio-Rad protein assay reagent using bovine γ-globulin as a standard. Uptake experiments were performed in the presence and absence of 0.1 μM nifedipine, then xenon-1- (10 μM) in the presence and absence of nifedipine. In preliminary experiments, we determined that 45Ca influx followed first order kinetics in the BAPTA-loaded cells for 100 s. The extracellular 45Ca associated with the cell pellet was subtracted from the 80-s values. Rates were expressed as picoforms /min/mg protein and are representative of five experiments performed in duplicate.

**Amino Acid Analysis**—Purified peptide and cleavage fragments were hydrolyzed in the gas phase using a Waters Pico-Tag workstation as described previously (20) and subjected to amino acid analysis using a Beckman 6300 Series autoanalyzer.

**Peptide Fragmentation**—Purified peptide was pyridylethylated according to a previously published method (20). The pyridylethyl cysteine derivative was purified by reversed-phase HPLC and subjected to both sequence analysis and endoprotease fragmentation. Arg-C endoprotease (Boehringer Mannheim) cleavage of the alkylated peptide was undertaken in 100 μl of 500 mM Tris (pH 8.5) at 37 °C for 5 h using a peptide to enzyme ratio of 20:1 (w/w). Arg-C protease fragments were purified by reversed-phase HPLC using a Waters C_{18} μBondapak column eluted with a linear gradient of 0–40% aqueous acetonitrile over 1 h containing 0.1% (v/v) trifluoroacetic acid throughout.

**Gas-phase Sequencing**—Alkylated peptide and cleavage fragments were subjected to automated Edman degradation using a Porton Instruments gas-phase sequenator located at the Sheldon Biotechnology Center of McGill University.

**Electrospray Ionization Mass Spectrometry**—Mass spectra of purified peptide were obtained as described previously using an API III triple-stage mass spectrometer with ion-spray interface (Sciex, Thornhill, Ontario, Canada) located at the Biotechnology Research Institute of the National Research Council of Canada in Montreal.

**Statistics**—Data are reported as means ± S.E. of 4–10 experiments performed in duplicate. Differences in means were determined using Student’s t test.

**RESULTS**

**Peptide Isolation and Characterization**—A portion equivalent to 5% of the unfractionated X. laevis skin extract was tested in the bioassay and was found to cause a volume reduction (15%) of the suspended villus epithelial cell preparation (Fig. 1). The remaining skin extract was subjected to gel permeation HPLC (Fig. 1). Material causing volume reduction was localized to fractions 16 and 17, which corresponded to a molecular weight of approximately 6000. Material within these fractions was purified further using reversed-phase HPLC as shown in Fig. 2A. Upon testing these fractions in the villus enterocyte bioassay, one region of bioactivity was found which corresponded exactly with a large peak of UV absorbance. This material was subjected to further reversed-phase HPLC procedures until homogeneity was achieved (Fig. 2B). Amino acid analysis revealed that the active compound was a peptide rich in cysteine with the following composition: Asx (4.7), Thr (7.2), Ser (2.7), Gla (6.6), Pro (0.9), Gly (5.8), Ala (3.7), 1/2 Cys (6.0), Val (1.2), Met (3.7), Ile (2.5), Leu (6.5), Tyr (0), Phe (0.9), His (0), Lys (7.5), and Arg (2.2).

The isolated peptide was deemed to be homogeneous since repeated rounds of reversed-phase HPLC failed to dissociate UV absorbing material from biological activity. Furthermore, electrospray ionization mass spectrometric analysis indicated the presence of a single compound with molecular weight of 7227.43. The purified peptide was alkylated and subjected to amino-terminal micro-sequence analysis. This yielded 43 amino acids of the 66-residue peptide. The structure of the peptide was completed by sequence analysis of fragments gen-
erated by Arg-C endoprotease cleavage. The complete amino acid sequence of the active peptide thus obtained was as follows: LKCVNLQANGIKMTQECAKEDTKCLTLRSLKKT-LKFCASGRTCTTMKIMSLPGEQITCCEGNMCNA. The predicted mass of the determined structure (7227.33) corresponds closely to that observed by mass spectrometric analysis. The structure is identical to that of xenoxin-1 purified from the exudate of the skin of *X. laevis* (15).

**Xenoxin-1 Stimulates Ca\(^{2+}\)-dependent Volume Changes**—The effect of xenoxin-1 (10 nM) on isotonic volume of villus epithelial cells is illustrated in Fig. 3A. Addition of the peptide to cells suspended in isotonic medium containing Na\(^+\) (140 mM) caused a volume reduction. The final relative volume of cells to which xenoxin-1 was added was less than untreated controls (0.95 ± 0.01 versus 0.99 ± 0.01, p < 0.001, Fig. 3A). In nominally Ca\(^{2+}\)-free medium (150 μM EGTA), the volume reduction brought about by xenoxin-1 was prevented. The final relative volume of the cells in Ca\(^{2+}\)-free medium with xenoxin-1 was greater than cells treated with xenoxin-1 in Ca\(^{2+}\)-containing medium (1.00 ± 0.01 versus 0.95 ± 0.01, p < 0.001). In Na\(^+\)-free medium, xenoxin-1 also stimulated rapid volume reduction

![Graph](image1.png)

**FIG. 2** Reversed-phase HPLC of material causing isotonic volume reduction of villus cells. A, fractions 16 and 17 from the gel filtration HPLC of the skin of *X. laevis* (Fig. 1) were pooled and loaded onto the C\(_{18}\) μBondapak reversed-phase HPLC column, which was eluted with a linear gradient of increasing acetonitrile containing 0.1% trifluoroacetic acid. B, material contained in fractions 19, 20, and 21 in panel A were found to cause isotonic volume reduction of villus cells and these fractions were pooled, loaded onto a C\(_{18}\) Vydac reversed-phase HPLC column, which was eluted with a linear gradient of increasing acetonitrile containing 0.1% trifluoroacetic acid. Material contained in fraction 32 was shown to be biologically active and subjected to structural analysis.

![Graph](image2.png)

**FIG. 3** Effect of xenoxin-1 on isotonic volume of villus cells. A, ●, xenoxin-1 (10 nM); ○, no additions; △, xenoxin-1 added in Ca\(^{2+}\)-free (150 μM EGTA) medium (n = 10). All experiments were conducted in medium containing Na\(^+\) (140 mM). B, ●, xenoxin-1 (10 nM); ○, no additions; △, xenoxin-1 added in Ca\(^{2+}\)-free medium (n = 10). All experiments were conducted in Na\(^+\)-free medium. C, nigel-dipine (0.1 μM) prevents xenoxin-1-stimulated (10 nM) volume reduction in Na\(^+\)-containing medium (●) and Na\(^+\)-free medium (○) (n = 4). Volume was measured electronically, and expressed relative to isotonic controls.
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FIG. 4. Effect of BayK-8644 on isotonic volume of villus cells. C, BayK-8644 (100 nM); ●, BayK-8644 in Ca\(^{2+}\)-free (150 μM EGTA) medium, n = 6. Volume was measured electronically, and expressed relative to isotonic controls.

(Fig. 3B). Within 2 min of xenoxin-1 addition, the cells were smaller than untreated controls (relative volume 0.90 ± 0.01 versus 0.95 ± 0.01, p < 0.001). At the conclusion of the experiment, the xenoxin-1-treated cells were substantially smaller than untreated cells (final relative volume 0.82 ± 0.01 versus 0.89 ± 0.1, p < 0.001). This volume reduction was also prevented in Ca\(^{2+}\)-free medium. The final relative volume of cells treated in Ca\(^{2+}\)-free medium with xenoxin-1 was greater than in Ca\(^{2+}\)-containing medium (0.90 ± 0.01 versus 0.82 ± 0.01, p < 0.001). We assessed the effect of dihydropyridine-sensitive Ca\(^{2+}\) channel blocker on the volume changes stimulated by xenoxin-1 in either Na\(^{+}\)-containing or Na\(^{+}\)-free medium (Fig. 3C). Niguldipine (0.1 μM), a potent inhibitor of dihydropyridine-sensitive Ca\(^{2+}\) channels (21, 22), prevented the xenoxin-1-stimulated volume reduction in Na\(^{+}\)-containing medium (final relative volume: 1.00 ± 0.01, p < 0.001). In Na\(^{+}\)-containing medium, niguldipine alone had no effect on cell volume over the course of the experiment. In Na\(^{-}\)-free medium, niguldipine also blocked the xenoxin-1-stimulated volume reduction (final relative volume: 0.92 ± 0.01 versus 0.82 ± 0.01, p < 0.001). The data suggest that xenoxin-1 activates a dihydropyridine-sensitive Ca\(^{2+}\) channel, and subsequently with the Ca\(^{2+}\) influx from the extracellular Ca\(^{2+}\) compartment, osmolyte loss is activated leading to isotonic volume reduction of these epithelial cells.

Bay K-8644 and XenoXin-1 Stimulate Ca\(^{2+}\)-dependent Volume Changes—To determine whether directly activating dihydropyridine-sensitive Ca\(^{2+}\) channels would generate volume changes, we used a well characterized (23) and selective agonist of these channels; 1-4-dihydro-2,6-dimethyl-5-nitro-4-(2-(trifluoromethyl)phenyl)-3-pyridine carboxylic acid methyl ester (BayK-8644). BayK-8644 (100 nM), added to villus cells suspended in isotonic Na\(^{+}\)-containing medium, caused a volume reduction (Fig. 4). The final relative volume of these cells was less than untreated controls (0.93 ± 0.01 versus 1.00 ± 0.01, p < 0.001, n = 6). The BayK-8644 effect was dose-dependent with an EC\(_{50}\) of 40 nM (data not shown). In Ca\(^{2+}\)-free medium (150 μM EGTA), the BayK-8644 volume reduction was prevented. The final relative volume of these cells was greater than in the presence of extracellular Ca\(^{2+}\) (0.93 ± 0.01 versus 0.93 ± 0.01, p < 0.001, n = 6).

We speculated that if xenoxin-1 were activating Ca\(^{2+}\) influx in a similar manner to BayK-8644, it should be possible to cause volume increases when conditions were fixed to make anion-permeability rate-limiting for volume changes. Such conditions occur when the cation ionophore, gramicidin (0.5 μM) is added to villus cells suspended in isotonic Na\(^{-}\)-containing medium (9, 12). As illustrated in Fig. 5A, gramicidin addition caused cell swelling. Addition of BayK-8644 (100 nM) to these cells caused them to swell to a greater volume (final relative volume: 1.20 ± 0.02 versus 1.12 ± 0.01, p < 0.05, n = 6). In Ca\(^{2+}\)-free medium (150 μM EGTA), the BayK-8644-stimulated cell swelling was attenuated (final relative volume: 1.13 ± 0.02, p < 0.05, n = 6). There was no difference in the final relative volume of cells in Ca\(^{2+}\)-free medium treated with gramicidin alone compared with gramicidin- and BayK-8644-treated cells (1.11 ± 0.01 versus 1.13 ± 0.02, NS, n = 6). After gramicidin permeabilization, addition of xenoxin-1 (10 nM) caused cells to swell to a greater volume compared with gramicidin alone (final relative volume 1.18 ± 0.02 versus 1.12 ± 0.01, p < 0.05, n = 6, Fig. 5B). The xenoxin-1-stimulated cell swelling was attenuated in Ca\(^{2+}\)-free medium (150 μM EGTA), final relative volume 1.13 ± 0.02, p < 0.05, n = 6). Addition of BayK-8644 and xenoxin-1 together in Ca\(^{2+}\)-containing medium caused a volume increase no different than that observed with either agonist alone (final relative volume: 1.21 ± 0.02, n = 6). Together, these results suggested that xenoxin-1, like BayK-8644, activated dihydropyridine-sensitive Ca\(^{2+}\) channels in the villus cells and that the observed volume changes were subsequent to the stimulation of calcium influx.

FIG. 5. BayK-8644 or xenoxin-1 stimulate volume increase when anion-permeability is rate-limiting. A, gramicidin (0.5 μM) added, followed at 2 min by BayK-8644 (100 nM) in either Ca\(^{2+}\)- (1 mM) or Na\(^{+}\)-containing medium (●) or Ca\(^{2+}\)-free (150 μM EGTA), Na\(^{+}\)-containing medium (○). Gramicidin (0.5 μM) alone, Ca\(^{2+}\)-free (150 μM EGTA), Na\(^{+}\)-containing medium, ▲, (n = 6). B, gramicidin (0.5 μM) added, followed by xenoxin (10 nM) at 2 min in either Ca\(^{2+}\)-containing (1 mM), Na\(^{+}\)-containing medium (●) or Ca\(^{2+}\)-free (150 μM EGTA), Na\(^{+}\)-containing medium (○) (n = 6). Volume measured electronically, expressed relative to untreated control cells.
**Xenoxin-1 Stimulates Dihydropyridine-sensitive \( {\text{45Ca}}^{2+} \) Influx**—To determine whether xenoxin-1-stimulated \( {\text{45Ca}}^{2+} \) influx that was sensitive to a dihydropyridine, we directly measured the initial rate of \( {\text{45Ca}}^{2+} \) influx after addition of xenoxin-1 in the absence or presence of nigelidine (Fig. 6). Nigelidine (0.1 \( \mu \)M) inhibited the initial rate of \( {\text{45Ca}}^{2+} \) influx in control cells (40 ± 9 versus 155 ± 38 pmol of \( {\text{45Ca}}^{2+} \)/min/mg of protein, \( p < 0.05, n = 5 \)). Xenoxin-1 (10 nM) substantially increased the rate of influx in comparison with control cells (848 ± 117 pmol of \( {\text{45Ca}}^{2+} \)/min/mg protein, \( p < 0.05, n = 5 \)). Nigelidine (0.1 \( \mu \)M) abolished the increase in rate stimulated by xenoxin-1 (73 ± 24 pmol of \( {\text{45Ca}}^{2+} \)/min/mg of protein, \( p < 0.01, n = 5 \)). Thus, xenoxin-1 stimulated a 6-fold increase in dihydropyridine-sensitive \( {\text{45Ca}}^{2+} \) influx into villus cells.

**Staurosporine Blocks ATP but Not Xenoxin-1-stimulated Volume Reduction**—In remaining experiments, we assessed whether protein kinase C (PKC) was required for the xenoxin-1-stimulated volume reduction. We first assumed that ATP would interact with a purinergic receptor coupled to phospholipase C hydrolysis, which would release diacylglycerol and mobilize PKC, which would in turn activate Cl\(^-\) conductance causing volume reduction. Addition of ATP (250 \( \mu \)M) to villus cells in \( \text{Na}^+ \) medium caused a volume reduction that peaked at 10 min and remained stable for an additional 20 min. The final relative volume of cells treated with ATP was less than untreated controls (0.92 ± 0.01, \( p < 0.001, n = 6 \)). The PKC inhibitor staurosporine (10 nM) prevented the ATP-stimulated volume reduction (final relative volume: 0.99 ± 0.01, \( p < 0.001, n = 6 \)). Staurosporine (10 nM) had no effect on volume reduction caused by xenoxin-1 (10 nM, 0.95 ± 0.01, \( n = 6 \)) or BayK-8644 (100 nM, 0.93 ± 0.01, \( n = 6 \)).

**DISCUSSION**

In this report, we have shown that xenoxin-1, a peptide belonging to the neurotoxin/cytotoxin family of peptides (1, 15), isolated from the skin of *X. laevis*, stimulates dihydropyridine-sensitive \( {\text{45Ca}}^{2+} \) influx and dihydropyridine-sensitive volume changes in \( \text{Na}^+ \)-absorbing epithelial cells isolated from guinea pig jejunum. We conclude that a biological activity of xenoxin-1 is its ability to activate dihydropyridine-sensitive \( {\text{Ca}}^{2+} \) channels in mammalian epithelium.

Xenoxin-1 was purified in this study because of its ability to stimulate isotonic volume changes in jejunal villus epithelial cells. A well characterized dihydropyridine-sensitive \( {\text{Ca}}^{2+} \) channel agonist, BayK-8644, caused comparable volume changes when added to these cells. In general, isotonic epithelial cell volume is maintained because the rate of salt influx is equivalent to the rate of efflux (8, 9, 14). Accordingly, volume reduction occurs because salt efflux is greater than salt influx. Previously, we have shown that both the calcium ionophore A23187 and certain members of the defensin peptide family cause an isotonic volume reduction, which was blocked by either a Cl\(^-\) channel or K\(^+\) channel blocker (10). This was consistent with osmolyte loss as being responsible for the volume reduction. However, the volume reductions stimulated by either the ionophore or defensin peptide were prevented in the absence of extracellular calcium, suggesting that \( {\text{Ca}}^{2+} \) influx was proximal to the activation of the K\(^+\) and Cl\(^-\) channels. In the current experiments, the volume reduction stimulated by xenoxin-1 or BayK-8644 were prevented in the absence of extracellular calcium. In other systems, BayK-8644 has been shown to increase \( {\text{Ca}}^{2+} \) influx by increasing the mean open time of dihydropyridine-sensitive \( {\text{Ca}}^{2+} \) channels (23). The volume reduction of villus cells caused by BayK-8644 suggests that activating dihydropyridine-sensitive \( {\text{Ca}}^{2+} \) channels in these cells results in the activation of K\(^+\) and Cl\(^-\) channels, which cause the volume reduction. Consequently, the loss of volume reduction brought about by BayK-8644 in the absence of extracellular calcium is consistent with calcium influx being required for volume changes initiated by BayK-8644. Because all the volume changes stimulated by xenoxin-1 also required calcium, we conclude that calcium influx was the stimulus for the volume altering effects of xenoxin-1.

Xenoxin-1 stimulated dihydropyridine-sensitive \( {\text{45Ca}}^{2+} \) influx into the villus cells. Our earlier studies have shown that both dihydropyridines and benzothiazapines will prevent volume regulation following hypotonic dilution of the cells (24). Binding studies of basolateral membrane vesicles derived from rabbit villus epithelial cells have characterized both phenylalkylamine- and dihydropyridine-sensitive Ca\(^{2+}\) channels in these cells (25). Furthermore, addition of the dihydropyridine agonist BayK-8644 in the current studies caused both isotonic volume reduction, and when cells were permeabilized to sodium by gramicidin, a Ca\(^{2+}\)-dependent volume increase. The latter volume change has been characterized as rate-limited by anion permeability (9, 12). Because xenoxin-1 caused Ca\(^{2+}\)-dependent volume changes that were comparable in both magnitude and direction as those caused by the specific agonist BayK-8644, and no greater effects were observed when both agonists were added together, our data strongly suggest that xenoxin-1 stimulates dihydropyridine-sensitive Ca\(^{2+}\) channels in these epithelial cells.

We considered three models to explain the volume reduction caused by xenoxin-1. They are as follows: xenoxin-1 causing direct activation of Cl\(^-\) channels, xenoxin-1 activating a receptor that is coupled to phospholipase C hydrolysis, and xenoxin-1 directly activating dihydropyridine-sensitive Ca\(^{2+}\) channels. In the first model, Cl\(^-\) channel activation would depolarize the cell thus activating voltage-sensitive K\(^+\) channels so that the net effect is K\(^+\) and Cl\(^-\) efflux resulting in volume reduction. However, mobilization of extracellular Ca\(^{2+}\) would not be required to activate this osmolyte efflux. In the second model, xenoxin-1, activating a receptor coupled to phospholipase C hydrolysis, would liberate intracellular Ca\(^{2+}\) and generate diacylglycerol, which would in turn activate protein kinase C. In villus cells, as we have previously determined (12), PKC activation of Cl\(^-\) conductance may be inhibited by staurosporine. Again, the net effect would be K\(^+\) and Cl\(^-\) efflux, which would lead to a volume reduction. In the current experiments, activation of a purinergic receptor by ATP-stimulated volume reduction, which was prevented by the PKC inhibitor staurosporine, suggesting that this pathway was operative in the volume reduction. Neither the xenoxin-1- nor the BayK-
electrolyte homeostasis. Our results suggesting that peptides isolated from frog skin might regulate water and activity, as well as anti-protease and anti-bacterial activity inhibited. Niguldipine did block the increased rate of 45Ca2+

epithelial cells that if either the constitutively active K+ channels in both villus (8, 9) and crypt (14) under isotonic conditions in both villus (8, 9) and crypt (14) epithelial cells that if either the constitutively active K+ or Cl− channels of these cells were inhibited by the appropriate blockers, the cells began to swell because of continued influx of Na+ and Cl−. Niguldipine had no effect on the isotonic volume of the cells suggesting that neither the K+ nor Cl− conductances were inhibited. Niguldipine did block the increased rate of 45Ca2+ influx stimulated by xenoxin-1, consistent with the capacity of such compounds to increase closure intervals of dihydropyridine-sensitive Ca2+ channels (31). Therefore, we conclude that xenoxin-1 activated dihydropyridine-sensitive Ca2+ channels in villus epithelial cells.

Xenoxin-1 has been shown to be negative for α-neurotoxin activity, as well as anti-protease and anti-bacterial activity (15). Frog skin is an epithelium, and it was speculated earlier that peptides isolated from frog skin might regulate water and electrolyte homeostasis (7). Our results suggesting that xenoxin-1 activates dihydropyridine-sensitive Ca2+ channels in intestinal epithelial cells allow us to speculate on a physiological role for a mammalian homologue of xenoxin-1 in the gut. In the mammalian small intestine, transepithelial Na+ and Cl− flux in the mucosal to serosal direction is exquisitely sensitive to intracellular calcium concentrations. Lowering [Ca2+] will increase this sodium flux while elevating it inhibits the flux (32). These effects are thought to be mediated through protein kinase C or Ca2+/calmodulin kinase II (32, 33). Most peptides identified from X. laevis skin or skin secretions are homologous to vertebrate hormones and neurotransmitters, providing evidence for the evolutionary relationship of the amphibian skin/ gut/brain axis. Indeed, it is now appreciated that the neurotoxin/cytotoxin peptides are members of a protein superfamily that includes the glycosyl phosphotidylinositol-anchored membrane proteins CD59, the Ly-6 family, and the urokinase receptor (34). We speculate that a mammalian homologue of xenoxin-1, if present in the gut, would participate in electrolyte homeostasis by dihydropyridine-sensitive Ca2+ channel activation. Transient increases in villus epithelial cell [Ca2+]i, would inhibit sodium and chloride influx in these cells.

In conclusion, using a sensitive bio-assay of calcium-mediated volume changes in intestinal epithelial cells, we have purified to homogeneity xenoxin-1 from the skin of X. laevis. This peptide, which is a member of the cytotoxin/neurotoxin family of peptides, activates dihydropyridine-sensitive Ca2+ channels in mammalian gut epithelial cells.

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