The Virally Encoded Fungal Toxin KP4 Specifically Blocks L-Type Voltage-Gated Calcium Channels

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ABSTRACT

KP4 is a virally encoded fungal toxin secreted by the P4 killer strain of Ustilago maydis. Previous studies demonstrated that this toxin inhibits growth of the target fungal cells by blocking calcium uptake rather than forming channels, as had been suggested previously. Unexpectedly, this toxin was also shown to inhibit voltage-gated calcium channel activity in mammalian cells. We used whole-cell patch-clamp techniques to further characterize this activity against mammalian cells. KP4 is shown to specifically block L-type calcium channels with weak voltage dependence to the block. Because KP4 activity is abrogated by calcium, KP4 probably binds competitively with calcium to the channel exterior. Finally, it is shown that chemical reagents that modify lysine residues reduce KP4 activity in both patch-clamp experiments on mammalian cells and in fungal killing assays. Because the only lysine residue is K42, this residue seems to be crucial for both mammalian and fungal channel activity. Our results defining the type of mammalian channel affected by this fungal toxin further support our contention that KP4 inhibits fungal growth by blocking transmembrane calcium flux through fungal calcium channels, and imply a high degree of structural homology between these fungal and mammalian calcium channels.

Interstrain inhibition in Ustilago maydis was discovered during heterokaryon experiments (Puhalla, 1968). The inhibitory factors (killer toxins) were shown to be secreted proteins encoded by double-stranded RNA mycoviruses (Hankin and Puhalla, 1971). Killer toxins have been identified in eight genera of yeast (Young, 1987b), but the killer toxins of U. maydis are the only ones known in a filamentous fungus.

KP4 is a single polypeptide of 105 amino acids produced by the UMV4 virus that infects the P4 strain of U. maydis (Park et al., 1994). It is the only U. maydis toxin not processed by Kex2p, and there is no sequence similarity to other toxins (Ganesa et al., 1991; Park et al., 1994). Although most of the yeast toxins are acidic (Bussey, 1972) and the KP6 and KP1 toxins have neutral pI values (Levine et al., 1979), KP4 is extremely basic, with a pI >9.0 (Ganesa et al., 1989). KP4 is an α/β sandwich protein with a relatively compact structure (Gut et al., 1995). From a tenuous structural similarity to the scorpion toxin AuHII from Androctonus australius, it was suggested (Gu et al., 1995) and then subsequently shown (Gage et al., 2001) that KP4 inhibits calcium uptake in fungal cells and that KP4 effects on fungal cells are reversible. To further support this structural similarity, Lys58 in AaHII and the analogous lysine in KP4, Lys42, were both shown to be crucial for activity (Gage et al., 2001).

Calcium is a ubiquitous signaling molecule that plays an important role in the life cycle of both mammalian and fungal cells. In mammalian cells, calcium is involved in processes such as gene expression (Bean, 1989), neuronal migration, and neurotransmitter release (Catterall, 1998). In fungi, calcium is involved in, but not limited to, bud formation (Davis, 1995), hyphal elongation (Jackson and Heath, 1993), and cAMP regulation (Iida et al., 1990a). In both mammalian and fungal systems, cytosolic calcium levels are normally maintained between 100 and 200 nM, whereas extracellular calcium concentrations are normally between 0.1 and 10 mM (Hlacamhi and Eilam, 1989; Iida et al., 1990b; Clapham, 1995). This calcium gradient is maintained through a series of Ca²⁺ channels, antiporters, and pumps (Tsig and Tsim, 1990; Cunningham and Fink, 1994).

In mammals, there are two primary classes of calcium channels: low-voltage-activated and high-voltage-activated (HVA) channels. The primary low-voltage-activated channel is the T-type channel, which is found in a wide range of cell types (Catterall, 1998). HVA channels include L-, N-, P-, Q-, and R-type channels (Catterall, 1998). L-type channels are the primary HVA channel type in muscle and cardiac cells, whereas N-, P-, Q-, and R-type calcium channels are located primarily in neuronal cells (Catterall, 1995). L-type channels

ABBREVIATIONS: HVA, high-voltage-activated; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.
also play a role in hormone release in endocrine cells (Milani et al., 1990) and in gene expression in neurons (Bean, 1989). N-, P-, and Q-type channels play roles in neurotransmitter release (Catterall, 1998).

Relative to the mammalian systems, very little information is known about calcium channels in fungi. Two genes that have been identified as possible calcium channels in Saccharomycodes cerevisiae are MIDI and CCHI. Both the CCH1 and MIDI gene products have been shown to be involved in calcium import in S. cerevisiae (Iida et al., 1994; Fisher et al., 1997; Locke et al., 2000). The sequence of CCH1 is similar to the α1 subunit of animal voltage-gated calcium channels. MIDI does not have any sequence similarity to known ion channels, but has been reported to be a stretch-activated cation channel (Kanzaki et al., 1999). It has been suggested that the CCH1 and MIDI gene products interact to regulate calcium import (Fisher et al., 1997; Locke et al., 2000).

Animal toxins have played a key role in the characterization of mammalian voltage-gated calcium channels. L-type calcium channels were first identified using dihydropyridine compounds and a large number of organic compounds are known to modulate the function of L-type channels. In contrast, N- and P-type calcium channels were first identified using, respectively, ω-conotoxin GVI A from the cone snail Conus geographus and ω-agatoxin IVA from the spider Agelesenopsis aperta [reviewed by Olivera (1994)]. N-type calcium channels were the first dihydropyridine-insensitive channels identified (Fox et al., 1987). Since the discovery of ω-conotoxin GVIA, many other peptide toxins that target calcium channels have been identified in the venoms of mollusks, arthropods, and snakes. The differential specificity of these toxins has played an important role in increasing understanding of the function and pharmacology of calcium channels.

Here we continue these studies and demonstrate that KP4 specifically blocks L-type voltage-gated calcium channels. This result clearly eliminates the possibility that KP4 affects mammalian calcium channels in a nonspecific manner. We further show that KP4 acts in a weakly voltage-dependent fashion. Finally, chemical modification studies demonstrate a tight correlation between KP4 activity against fungal and mammalian cells. The most likely modification site of these reagents is K42 and therefore also supports our contention of the structural homology between KP4 and AaIII.

**Materials and Methods**

**KP4 Purification**

KP4 was purified as reported previously (Gu et al., 1994, 1995). In brief, the toxin was isolated from the supernatant of the KP4 toxin expressing strains of U. maydis (or S. cerevisiae) grown in complete U. maydis media (2.5% bacto-peptone, 1% dextrose, 0.15% ammonium nitrate, 0.1% yeast extract) for 7 to 10 days. Cells were removed by centrifugation at 10,000g for 30 min. The supernatant was stirred overnight with CM Sephadex-25 beads (Amersham Biosciences, Piscataway, NJ) that were equilibrated with 25 mM sodium acetate, pH 5.5. The toxin was eluted with 1 M NaCl using a Pharmacia GradiFrac system. The eluant was concentrated using a Minitan II Ultrafiltration System (Millipore, Bedford, MA) with 1-kDa cutoff membranes and dialyzed against a 10 mM malonic acid, pH 6.0 buffer. KP4 was then purified using a high-resolution cation-exchange chromatography (Mono-S; Amersham Biosciences) matrix attached to a fast-performance liquid chromatography system in the same buffer and using NaCl for elution. The toxin was further purified with size exclusion chromatography using an Amersham Biosciences Superdex-75 gel filtration column. Toxin activity was tested throughout the purification using the killing-zone activity assay described below and purity was assessed using Homogenous 20 SDS gels on an Amersham Biosciences Phastgel system. Only a single band representing KP4 was observable when silver staining was used to observe the protein bands.

**Killing-Zone Activity Assay**

KP4-sensitive P2 cells were grown overnight in complete U. maydis media. P2 cells (~1 ml/100 ml) were added to warm complete U. maydis media containing 2% bacto-agar and poured into 100- x 20-mm culture dishes. Once the agar solidified, wells were cut into the agar and 10 µl of the test solutions were added to each well. The plates were then incubated at 30°C for ~36 h. KP4 activity presents a clear zone around the point of application.

**Cell Culture**

PC12 cells. wtPDGF-R expressing PC12 cells (Vaillancourt et al., 1995) were cultured on rattail collagen-coated plastic tissue culture dishes. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) with 12.5% heat inactivated horse serum derived from platelet-poor plasma (to prevent differentiation; Sigma-Aldrich, St. Louis, MO) and 2.5% fetal bovine serum (FBS; Atlas Biological, Fort Collins, CO) at 37°C with 5% CO2. Cell medium was exchanged every other day and cells were passaged weekly. Cells were plated in 35-mm rattail collagen-coated dishes for electrophysiology. Cells were differentiated by addition of fresh 100 ng/ml nerve growth factor to the cell medium every other day for 7 days.

GH3 Cells. GH3 cells were cultured on plastic tissue culture dishes. Cells were grown in DMEM/Ham’s F-12 medium (Invitrogen) with 10% FBS (Atlas Biological, Fort Collins, CO) at 37°C with 5% CO2. Cell medium was exchanged every other day and cells were passaged weekly. Cells were plated in 35-mm tissue culture dishes for electrophysiology.

**Expression**

Wild-type Ca2.1 (de Weille et al., 1991), Ca1.2 (Snutch et al., 1991), and Ca2.3 (Soong et al., 1993) channel subunits were expressed with CaV1.2 (Pragnell et al., 1991) and CaV1.3 (Ellis et al., 1988) channel subunits and enhanced green fluorescent protein (CLONTECH, Palo Alto, CA) in tsA-201 cells. Cells were transfected using the reagent Geneporter2 (Gene Therapy Systems, San Diego, CA) and an equimolar ratio of the three channel subunit cDNAs along with 0.8 µg of enhanced green fluorescent protein for a total of 4 µg of DNA. Transfected cells were detected by fluorescence at 510 nm with excitation at 480 nm.

**Electrophysiology**

A standard whole-cell bath solution was used for the PC12 and GH3 experiments: 10 mM BaCl2, 135 mM tetraethylam-
monium-Cl, 4 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 0.001 mg tetrodotoxin, pH 7.2. For experiments in which CaCl₂ was substituted for BaCl₂, CaCl₂ concentration was 5 mM and tetraethylammonium-Cl was 150 mM. In experiments using tsA-201 cells, a bath solution consisting of 150 mM Tris, 4 mM MgCl₂, 10 mM BaCl₂, pH 7.3 was used. In all experiments, the patch pipette solution consisted of 150 mM CsCl, 2 mM MgATP, 0.5 mM GTP, 2 mM BAPTA, and 10 mM HEPES, pH 7.2. Data acquisition and analysis were performed using Pulse and PulseFit software (InstruTECH Corporation, Port Washington, NY). All current recordings except the 1-Hz train were leak subtracted using a standard P/N procedure (summed amplitude of the peak pulse current/number of pulses) and filtered at 5 kHz before being saved directly to disk. A holding potential of −90 mV was used for all experiments except where indicated. Currents were recorded using either an Axopatch 200B (Axon Instruments, Union City, CA) or a Patch Clamp L/M EPC-7 amplifier (List Medical, Darmstadt, Germany). Application of drug and KP4 was by pressure application from a blunt-tipped, fire polished micropipet positioned about 5 to 10 μm from the cell. Purified KP4 was lyophilized and resuspended in the bath solution to the desired concentration. Nimodipine was prepared fresh daily from a 5 mM stock in ethanol. Concentrations of drug and KP4 denote the final concentrations with bath solution. Application of bath solution alone or bath plus KP4/drug vehicle had no effect on calcium current amplitudes or kinetics.

### Table 1

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Charge Carrier</th>
<th>V&lt;sub&gt;test&lt;/sub&gt;</th>
<th>Block</th>
<th>n</th>
<th>Block – 5 μM nimodipine in bath</th>
<th>n</th>
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<tbody>
<tr>
<td>PC12, undifferentiated</td>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>10</td>
<td>21 ± 5</td>
<td>17</td>
<td>0.4 ± 0.4</td>
<td>9</td>
</tr>
<tr>
<td>PC12, NGF-differentiated</td>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>10</td>
<td>16 ± 2</td>
<td>15</td>
<td>0.3 ± 0.3</td>
<td>6</td>
</tr>
<tr>
<td>GH₃</td>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
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<td>53 ± 7</td>
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<tr>
<td>GH₃</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0</td>
<td>0.8 ± 5</td>
<td>12</td>
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### Results

**Specificity of KP4 for L-Type Calcium Channels.** Previous electrophysiology experiments had demonstrated that KP4 modulated voltage-gated calcium channel currents but had no effect on either voltage-gated sodium or potassium channels (Gu et al., 1995). Application of identical concentrations of KP4 to different cell lines expressing varying calcium channel subtype populations produced different levels of modulation.

**Chemical Modification of KP4**

A 10 μM KP4 solution of KP4 in 5 mM sodium phosphate, pH 8.0, was used for chemical modification studies. To this solution, acetic anhydride was added to reach a final concentration of 10 mM (1:1000 molar ratio). The reaction was incubated at 25°C for 4 h and then dialyzed against 10 mM sodium phosphate, pH 7.0 at 4°C, overnight. The sample was then treated with 0.5 M hydroxylamine, pH 7.0, for 5 h at 25°C to reverse modification of tyrosine residues. The sample was then dialyzed against water and lyophilized. The extent of modification was determined by the fluorometric method described previously (Stocks et al., 1986). Although it is possible that the chemical modification might denature the protein, such denaturation was not made evident by precipitation. Also, the protein has been shown to be remarkably stable in that it resists thermal denaturation, inactivation by organic solvents, and proteolytic cleavage. Therefore, it is highly unlikely that these chemical reagents denature KP4.

![Fig. 1](image-url) Effect of KP4 on differentiated PC12 cells. A, data traces showing the effects of 14 μM KP4 on the whole-cell calcium current of differentiated PC12 cells. B, raw data traces showing the effects of 14 μM KP4 on the whole cell calcium current of differentiated PC12 with 5 μM nimodipine in the bath solution. KP4 effects are blocked by the presence of nimodipine. Similar effects are seen for undifferentiated PC12 cells.
whole-cell current block. This indicates that KP4 might have different affinities for the different types of calcium channels producing more or less block depending on the proportion of channel subtypes in a given cell line. If KP4 were nonspecific, the level of modulation should be dependent on KP4 concentration and independent of channel type.

The magnitude of KP4 effects correlated with the known expression levels of L-type calcium channels in the various cell lines tested, indicating that KP4 might specifically bind L-type calcium channels. To test this hypothesis, the effects of 14 μM KP4 on whole-cell calcium currents in PC12 cells were measured in the presence of 5 μM nimodipine, a dihydropyridine calcium channel blocker. Nimodipine (5 μM) was also placed in the micropipet applicator with the 14 μM KP4 to prevent dilution of the local nimodipine concentration. If KP4 specifically blocks L-type calcium channels, then KP4 should have no measurable effect in the presence of nimodipine. Table 1 summarizes the results of this experiment (representative raw data traces for differentiated PC12 cell experiments are shown in Fig. 1). KP4 (14 μM) has no apparent effect on cells treated with 5 μM nimodipine. This suggests that KP4 may be specific for L-type calcium channels.

To further demonstrate that KP4 is specific for L-type calcium channels, we looked at KP4 specificity versus heterologously expressed individual calcium channel subtypes. Three different calcium channel subtypes were tested: Ca, 1.2, an L-type calcium channel; Ca, 2.1, a P/Q-type calcium channel; and Ca, 2.3, an R-type calcium channel. In the case of expressed Ca, 1.2 channels (Fig. 2A), 1 μM KP4 blocks 52% of the current. The IC_{50} of KP4 for expressed calcium channels was determined to be 1.24 μM (Fig. 2D). However, concentrations of up to 100 μM KP4 have no effect on either Ca, 2.1 or Ca, 2.3 (Fig. 2, B and C). KP4 clearly is specific for L-type channels of the Ca, 1 family.

Mechanism of KP4 Block of Ca, 1.2. Phenylalkylamine and benzothiazepine compounds often act in a voltage-dependent manner (Hering et al., 1997; Motoike et al., 1999). Voltage-dependent block has several characteristics: shifts in the steady-state inactivation curve, changes in the \( \tau_{\text{inactivation}} \), and use-dependent block. Drugs that block in a voltage-dependent manner do so with different efficacy depending on the holding potential.

By ascertaining whether KP4 affected calcium currents in a voltage-dependent manner, the pharmacological effects of

![Fig. 2. Effect of KP4 on heterologously expressed calcium channels. Effects of 1 μM KP4 on the whole cell calcium current from heterologously expressed Ca, 1.2 channels (Figure A). KP4 blocks 54% of the current (n = 8). Effects of 100 μM KP4 on the whole cell calcium current from heterologously expressed Ca, 2.1 (Figure B; n = 7) and Ca, 2.3 (Figure C; n = 8) channels, respectively. KP4 clearly does not affect the current in either Ca, 2.1 and Ca, 2.3 channels. D, the dose-response curve of KP4 on Ca, 1.2 channels. Each point represents the results from three to nine experiments. The IC_{50} for KP4 is 1.24 μM.](image-url)
KP4 could be compared with these drugs. In these first studies, the effects of KP4 on steady state inactivation of \( \alpha_{1C} \) calcium channels were measured. As shown in Fig. 3A, 1 \( \mu \)M KP4 induces a negative shift in the steady state inactivation with a shift in the half-inactivation voltage \( (V_{1/2}) \) from \(-19.8 \pm 2.1 \) mV to \(-28.5 \pm 3.8 \) mV \( (n = 5) \). For comparison, verapamil and diltiazem induce negative shifts of 30 mV \( (Cai et al., 1997) \), which is much larger than the \(-9 \) mV shift caused by KP4.

In the next set of experiments, it was ascertained whether KP4 blocked calcium channels in a use-dependent manner. Use-dependent block is an important feature of therapeutically useful calcium channel antagonists. Phenylalkylamines, such as verapamil and D600, show an accumulation of block on the order of 50 to 80% during repetitive depolarization \( (Johnson et al., 1996) \). A 1-Hz train of 20 100-ms depolarizations to \(-10 \) mV was applied in the presence of 1 \( \mu \)M KP4 (Fig. 3B). KP4 induces a 10% accumulation of block under these conditions. This is smaller than the accumulation of block observed for verapamil and D600.

Finally, if KP4 acted in a voltage-dependent manner, changes in the holding potential should affect the activity of KP4. As Fig. 3C shows, a 30 mV shift in holding potential from \(-90 \) mV to \(-60 \) mV causes an increase in block of from 53 \( \pm 5 \) to 62 \( \pm 2 \)% or a change of \(-9 \)%. In contrast, the block caused by verapamil increases \(-50 \)% when the holding potential is shifted from \(-100 \) to \(-60 \) mV \( (Cai et al., 1997) \). From these experiments, KP4 seems to act in a weakly volt-

![Fig. 3. Effects of KP4 on the biophysical properties of Ca\(_{\text{a,1,2}}\) channels.](image)

For these experiments, tsA 201 cells were transfected with Ca\(_{\text{a,1,2}}\), Ca\(_{\beta_{1a}}\), and Ca\(_{\alpha_2\delta}\) channel subunit cDNA. Barium currents were evoked by 100-ms depolarizations to various voltages \( (-50 \text{ to } +50 \) mV) from a holding potential of \(-60 \) mV in the presence or absence of 1 \( \mu \)M KP4. A, steady-state inactivation curve from a representative cell before and after acute treatment with 1 \( \mu \)M KP4. Absolute currents have been normalized to the maximum current. Treatment with 1 \( \mu \)M KP4 causes a shift in the \( (V_{1/2}) \) of \(-9 \) mV toward more negative potentials. The error on the control cells was \( \pm 2 \) mV and \( \pm 4 \) mV for the KP4 treated cells. B, use-dependent block with 1 \( \mu \)M KP4. Accumulation of block by 1 \( \mu \)M KP4 was measured with a 1 Hz train. An accumulation of block of \(-10 \)% is seen. C, effects of a shift in holding potential on block of calcium current by 1 \( \mu \)M KP4. KP4 (1 \( \mu \)M) blocks 53.87 \( \pm 4.71 \)% of the absolute current at a holding potential of \(-90 \) mV. This increases to 62.26 \( \pm 2.11 \)% when the holding potential is shifted to \(-60 \) mV. These results indicate that KP4 acts in a weakly voltage-dependent manner. D, KP4 does not modify the activation of Ca\(_{\text{a,1,2}}\) channels. For this figure, the channel conductance \( (g_{\text{ch}}) \) was calculated using the equation: \( g_{\text{ch}} = \frac{I_{\text{Ba}}}{(E - E_{\text{rev}})} \), where \( I_{\text{Ba}} \) is the peak barium current evoked by the test potential \( E \), and \( E_{\text{rev}} \) is the estimated Ca\(_{\text{a,1,2}}\) channel reversal potential. The conductance-voltage relationships are plotted as relative conductance \( \left( g_{\text{ch}} / g_{\text{max,control}} \right) \) versus test potentials. The data were fit to the equation: \( \frac{g_{\text{ch}} / g_{\text{max,control}}}{1 + \exp \left[-(V - V_{g0.5})/k\right]} \), where \( V \) is the test potential used to evoke barium current, \( V_{g0.5} \) is the potential where channel conductance is half-maximal \( (0.77 \pm 0.50 \) mV in the absence \( \bullet \) and \( 1.83 \pm 1.67 \) mV in the presence \( \circ \) of 1 \( \mu \)M KP4), and \( k \) is the slope factor \( (6.1 \) in the absence and 7.2 in the presence of 1 \( \mu \)M KP4). Results shown are mean values \( \pm \) S.E. \( (n = 6) \). Because both curves are essentially identical, KP4 does not seem to modify the voltage dependence of activation.
age dependent manner. Figure 3D shows that KP4 does not alter the voltage dependence of channel activation. This is in contrast to hanatoxin modulation of K^+ channels (Swartz and MacKinnon, 1997) and α-(Calahan, 1975) and β-(Jonas et al., 1986) scorpion toxin modulation of sodium channels.

**Calcium Effects on Current Modulation of KP4.** In *U. maydis*, the activity of KP4 is abrogated by increasing extracellular calcium concentrations (Gage et al., 2001). As little as 10 mM CaCl_2 reduces the killing activity of KP4 and 100 mM CaCl_2 completely abrogates its effects. If the fungal and mammalian channels are functionally homologous, then the effects of KP4 on mammalian calcium channels should also be abrogated by extracellular calcium. To test for this, KP4 activity was measured in a bath solution, where the normal concentration of 10 mM barium chloride was replaced with 5 mM calcium chloride. Table 1 shows the modulation of the peak current in GH3 cells treated with 14 μM KP4 in both a Ba^{2+} and Ca^{2+} bath. When Ba^{2+} is replaced by Ca^{2+} in the bath solution, KP4 activity is abolished (Table 1, raw data traces shown in Fig. 4). This is consistent with our finding in *U. maydis* that KP4 effects are abrogated by exogenous calcium and further demonstrates that the effects of KP4 on mammalian and fungal calcium channels are analogous.

**Chemical Modification of Lysine 42.** K42 has been shown to play an important role in KP4 activity (Gage et al., 2001). In the case of the scorpion toxin AaHII, chemical modification of lysine residues abrogated its channel blocking activity (Sampieri and Habersetzer-Rochat, 1978). To further test the structural/functional homology between AaHII and KP4 and to again demonstrate that the effects of KP4 on fungal calcium channels is analogous to its effects on mammalian channels, similar chemical modification studies were performed on KP4. Acetic anhydride modifies primary amines, the hydroxyl group of tyrosine, the thiol group of cystine, and the amino group of histidine. Under these reaction conditions, histidine and cysteine spontaneously deacetylate and the tyrosine can be deacetylated with hydroxylamine. This leaves only acetylated primary amines. The degree of modification can be determined fluorometrically (Fig. 5A) from the ratio of the slopes of the modified and the unmodified KP4. Eighty-five percent of the primary amines (1.7 modification sites per KP4) were modified in Modified KP4 sample 1, which was used for fungal killing assays. 65% of the primary amines (1.3 modification sites per KP4) were modified in Modified KP4 sample 2, which was used for electrophysiology.

Acetylation clearly abrogates KP4 activity against both fungal and mammalian cells. The effects of acetylation on KP4 activity were examined using the fungal killing assay (Fig. 5B). Modified toxin is ~85% less active than the unmodified KP4, consistent with the degree of modification. At a concentration of 36 μM, acetylated KP4 has very weak activity, whereas there is no apparent activity at 3.6 μM. To determine the effects of chemical modification on KP4’s inhibition of mammalian calcium channels, GH3 calcium currents were measured. Modified KP4 (14 μM) blocked 17 ± 4% of the calcium current (Fig. 5C). This is 33% of the block found for wild-type KP4. Because 65% of the primary amines are modified, the decrease in block correlates well with the degree of modification. These results clearly demonstrate that KP4 activity against fungal cells correlates well with inhibition of mammalian calcium channels. Furthermore, because it is more than likely that the sole lysine, K42, is being modified by the acetic anhydride, K42 seems to be crucial for toxin activity. All of these results together strongly suggest a structural/functional homology between KP4 and the scorpion toxin AaHII and between fungal and mammalian L-type calcium channels.

**Discussion**

The results presented here demonstrate that the virally encoded fungal toxin KP4 inhibits mammalian L-type voltage-gated calcium channels. Although it might seem unusual...
that the activity of a calcium channel blocker crosses animal and fungal kingdoms, it is not without precedence because venoms from mollusks, arthropods, and snakes contain peptides that inhibit mammalian voltage-gated calcium channels. What is unusual here, however, is that KP4 is the first toxin shown to act across such phylogenetically divergent organisms yet at a specific molecular target.

As shown here, KP4 specifically targets L-type calcium channels. Both undifferentiated and differentiated PC12 cells express multiple calcium channel types, L, N, and P/Q, and both have modest L type current components (Usowicz et al., 1990; Lievano et al., 1994). KP4 blocks 20% of the total whole-cell calcium current in undifferentiated PC12 cells, and this is consistent with the contribution of the L-type current to the total in these cells. The percentage contribution of L current to total calcium current is reduced in differentiated cells because they have a relatively larger proportion of N type current. Consistent with this reduced L current percentage KP4 was observed to block less of the total current in differentiated cells. When PC12 cells are treated with the L-type channel blocker, nimodipine, the cells become insensitive to KP4. This is strong evidence that KP4 targets L-type calcium channels. When KP4 activity is tested against heterologously expressed calcium channels, KP4 is active against Ca_{1.2} but not against Ca_{2.1} and Ca_{2.3} channels. Therefore, we conclude that KP4 is specific for L-type calcium channels. What is particularly interesting is that, of all of the calcium channels found in mammalian cells, these results suggest that it is the L-type calcium channels that most closely resemble fungal channels being targeted by KP4.

From the studies on the pharmacological effects of KP4 on mammalian cells, it is also apparent that KP4 blocks Ca_{1.2} in a manner distinct from other calcium channel blocking agents. Unlike the small-molecule calcium channel inhibitors, verapamil, and D600, KP4 induces only a small shift in the voltage-dependence of inactivation (\( V_{1/2} \)) but not a significant change in \( \gamma \) _inactivation_. An increase in the frequency of stimulation from 0.05 to 1 Hz results in only a slight increase in block of Ca_{1.2} by KP4. Finally, KP4 demonstrates a very small but significant preference for block of Ca_{1.2} channels held at more depolarized membrane potentials. Therefore, KP4 seems to act in a weakly voltage-dependent manner. The characteristics of Ca_{1.2} channel block by KP4 are thus dif-

Fig. 5. Effects on the activity of KP4 from chemical modification. A, determination of the degree of chemical modification of KP4 samples used for fungal killing assays and electrophysiology. The degree of modification is determined by the ratio of the slopes. Modified KP4 sample 1, used for fungal killing assays, was 85% modified. Modified KP4 sample 2, used for electrophysiology, was 65% modified. B, fungal killing assay demonstrating the effects of modification on activity. These results show that KP4 is ~85% less active than native KP4, consistent with the degree of modification. C, current-voltage curve of a representative GH_3 cell treated with 14 \( \mu \)M modified KP4. D, raw data traces showing the effects of 14 \( \mu \)M KP4 on the whole cell calcium current of GH_3 cells. Modified KP4 blocks 17.46 ± 4.11% of the raw current (n = 6). Modified KP4 blocks whole-cell calcium current ~33% as well as native KP4, consistent with the degree of modification.
ferent from those of phenylalkylamine and benzothiazepine drugs but similar to those reported for block of Ca.1,2 by the spider toxin ω-agatoxin IIIA (Cohen et al., 1992). These observations, along with the abrogation of KP4 block by high extracellular Ca\(^{2+}\) concentrations, suggest that KP4 may bind to the extracellular side of the Ca.1,2 pore region, much like charybdotoxin block of K\(^+\) channels (MacKinnon and Miller, 1989) or tetrodotoxin block of Na\(^+\) channels (Terlau et al., 1991).


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