Puroindolines Form Ion Channels in Biological Membranes

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ABSTRACT Wheat seeds contain different lipid binding proteins that are low molecular mass, basic and cystine-rich proteins. Among them, the recently characterized puroindolines have been shown to inhibit the growth of fungi in vitro and to enhance the fungal resistance of plants. Experimental data, using lipid vesicles, suggest that this antimicrobial activity is related to interactions with cellular membranes, but the underlying mechanisms are still unknown. This paper shows that extracellular application of puroindolines on voltage-clamped *Xenopus laevis* oocytes induced membrane permeabilization. Electrophysiological experiments, on oocytes and artificial planar lipid bilayers, suggest the formation, modulated by voltage, of cation channels with the following selectivity: $Cs^+ > K^+ > Na^+ > Li^+ >$ choline = TEA. Furthermore, this channel activity was prevented by addition of Ca^{2+} ions in the medium. Puroindolines were also able to decrease the long-term oocyte viability in a voltage-dependent manner. Taken together, these results indicate that channel formation is one of the mechanisms by which puroindolines exert their antimicrobial activity. Modulation of channel formation by voltage, Ca^{2+}, and lipids could introduce some selectivity in the action of puroindolines on natural membranes.

INTRODUCTION

Wheat seeds contain a number of low molecular mass, basic cystine-rich proteins that are able to interact with lipids (Douliez et al., 2000). The first protein of this type, isolated by Balls in 1940 (Balls and Hale, 1940), has a molecular mass of ~5 kDa and contains eight cysteines connected by disulfide bridges. It is a member of the thionin family, proteins that are ubiquitous in the plant kingdom (Florack and Stiekema, 1994). More recently, other lipid binding proteins, called nsLTPs, have been characterized in wheat. They were identified by their ability to enhance, in vitro, the intermembrane transfer of phospholipids (Kader, 1996) and, like thionins, are found ubiquitously in plants. Today at least two families, nsLTP1 and nsLTP2, have been identified, differing by their molecular masses, 9 and 7 kDa, respectively. They contain four disulfide bonds and are able to bind lipids without apparent specificity.

Another group of proteins was isolated from seeds using phase partition with a nonionic detergent, a method generally used to extract transmembrane proteins (Blochet et al., 1993). There are, at least, two major isoforms, called PIN-a and PIN-b, as they contain a unique tryptophan-rich domain and are isolated from wheat endosperm (indole ring of tryptophan and *puros*, for wheat in Greek). They have molecular masses around 13 kDa and contain five disulfide bridges. PIN-a and PIN-b display 60% similarity and slight sequence homology with nsLTPs except in the tryptophan-rich region (Gautier et al., 1994). Circular dichroism and Fourier transform infrared spectroscopy studies have revealed that both puroindolines and nsLTPs are α-helical proteins (Le Bihan et al., 1996). In puroindolines, but not nsLTPs, an additional disulfide bond encloses the nonhomologous tryptophan-rich domain. All these data suggest that puroindolines and nsLTPs are structurally related. Unlike thionins and nsLTPs, indolines are restricted to the *Triticeae* and *Aveneae* tribes (Gautier et al., 2000; Tanchak et al., 1998). In wheat, they are considered as a genetic marker of endosperm texture (Giroux and Morris, 1998). In contrast with nsLTPs, puroindolines interact with lipids by forming an interface, since binding occurs only above the critical micellar concentration (Husband et al., 1995; Le Guerneve et al., 1998). Therefore, their lipid binding properties more closely resemble those of thionins than those of nsLTPs (Douliez et al., 2000).

By analogy with thionins (Bohlmann, 1994; Florack and Stiekema, 1994), it was suggested that puroindolines could play a role in the mechanisms of plant defense against their microbial pathogens (Blochet et al., 1993; Douliez et al., 2000). In this regard, puroindolines, as thionins, have been shown to display antifungal activities in vitro (Dubreil et al., 1998), and transgenic rice, constitutively expressing wheat puroindolines, appears more resistant to fungi (Krishnamurthy et al., 2001). Recently, PIN-a and α1-purothionin have also been found to induce nodal swelling in myelinated axons (Mattei et al., 1998). Thus, puroindoline toxicity could be related to membrane permeabilizing properties, and recent experiments do suggest that this is indeed the case for

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Abbreviations used: nsLTP, nonspecific lipid transfer protein; PIN-a, puroindoline a; PIN-b, puroindoline b; BAPTA, 1,2-bis(2-Aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; TEA, tetraethylammonium; octyl-POE, octyl-polyoxyethylene.

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β-purothionin, which appears to be able to form ion channels in lipid bilayers at low concentrations (Hughes et al., 2000).

In this paper, using *Xenopus laevis* oocytes, we demonstrate that puroindolines are able to permeabilize biological membranes in a voltage-dependent manner. Reconstitution experiments in planar lipid bilayers demonstrate that this permeabilization can be attributed to the formation of selective ion channels. These results also provide interesting clues to understand the difference in potencies between thionins and indolines.

EXPERIMENTAL METHODS

Wheat protein purification

PIN-a and PIN-b were purified from *Triticum aestivum* seeds as previously described (Dubreil et al., 1997). In ion exchange chromatography, purothionins (α and β) eluted, with 0.7 M NaCl, just after PIN-b. Detergent-free proteins were freeze-dried and stored in darkness at room temperature.

Reconstitution in planar lipid bilayers

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) grade 1 from egg yolk and phosphatidylserine (PS) grade 1 from bovine spinal cord were purchased from Lipid Products (Surrey, UK). In conductance experiments, virtually solvent-free planar lipid bilayers were formed by the Montal and Mueller technique (Montal and Mueller, 1972). The membrane was formed over a 100–150 μm hole in a Teflon film (10 μm thick), pretreated with a mixture of 1:40 (v/v) hexadecane/hexane, separating two half glass cells. Lipid monolayers were spread on the top of an electrolyte solution (1 M KCl, 10 mM Tris, pH 7.4) in both compartments. Bilayer formation was achieved by lowering and raising the electrolyte level in one or both sides and monitoring by capacitance responses. The lipid solution at 1 mg/ml was either a mixture of PC/PE 7:3 (w/w) or PC/PE/PS 7:3:1 (w/w). Voltage was applied through an Ag/AgCl electrode to the *cis*-side. From a stock solution in H_2O (400 μM), appropriate puroindoline concentrations were prepared in H_2O or 1% Octyl-POE (Bachem, Switzerland). Currents were amplified and potentials were applied simultaneously by a patch-clamp amplifier (RK 300, Bio-Logic, Claix, France). Single channel currents were monitored on an oscilloscope (TDS 3012, Tektronix,) and stored on a CD recorder (DRA 200, Bio-Logic, Claix, France) for offline analysis. For further analysis, CD data were treated by Windac32 (http://www.shareit.com) and Biotools (Bio-Logic, Claix, France) software. Single-channel conductances were estimated by dividing the single-channel current by the holding potential. All experiments were performed at room temperature. Data were filtered at 1 kHz before digitization at 11.4 kHz for analysis.

Electrophysiological recordings on *Xenopus laevis* oocyte

Ovaries were surgically removed from *Xenopus laevis* females (Elevage de Lavalette, Montpellier, France), and oocytes were isolated after enzymatic dissociation (collagenase, type IA, Sigma, La Verpillere, France) and extensive washing, as already described (Chaloin et al., 1998). In experiments using oocytes expressing the *Shaker* K^+ channel, oocytes were injected with 50 nl of mRNA (at 0.4 ng/nl) synthesized from linearized plasmids containing the *Shaker* K^+ channel cDNAs using T7 polymerase and an in vitro transcription kit (mMessage mMachine, Ambion). After 24–48 h, oocytes were placed individually in a 50-μl recording chamber. Macroscopic whole-cell currents were recorded under two electrode voltage-clamp using the GeneClamp 500 amplifier (Axon Instruments, Union City, CA) as in Charnet et al. (1994) connected to the bath with the virtual-ground bath-clamp headstage and 3 M KCl agar-bridges. Voltage and current electrodes were filled with 3 M KCl and had a resistance of 1–2 MΩ. Junction potentials (typically less than 3–5 mV) were canceled. In some experiments, contaminating endogenous Ca^{2+}-activated Cl^- currents were suppressed by injecting BAPTA (in mM: BAPTA 100, Hepes 10, CsOH 10; pH 7.2) into oocytes by means of a third microelectrode and a pneumatic injector. The final intra-oocyte BAPTA concentration was estimated to 2–5 mM. Voltage-command, sampling, acquisition, and analysis were done using a Digidata 1200 and the pClamp program (version 6.01, Axon Instruments). All experiments were performed at room temperature (20–25°C).

The recording solution had the following composition (in mM): NaCl, 100; Hepes, 5; $MgCl_2$, 2; pH 7.0 with NaOH (Na100 solution). For ionic selectivity measurements, NaCl was replaced by an equimolar concentration of either KCl, TEA-Cl, LiCl, CsCl, or choline-Cl, and pH was adjusted using KOH, TEAOH, LiOH, CsOH, or NaOH, respectively (K100, TEA100, Li100, Cs100, or choline100 solutions). For chloride permeability, NaCl was replaced with equimolar Na-acetate, and $MgCl_2$ was removed from both solutions. The tested protein was applied directly to the bath at the final concentration (usually 20–50 μM) after proper dilution of a stock solution (500 μM in water) into the desired solution. Washing-out of the protein was done by a gravity-driven perfusion of the chamber with the same solution without protein. Membrane conductance was measured during voltage-ramps (−80 to +20 mV, 450 ms), applied from the holding potential of −80, −100, or −120 mV every 3 s, by least squares fitting of the inward current. Reversal potentials were measured as the zero-current potential after digital subtraction of traces recorded before protein application, for each ionic condition.

Reversal potentials (Erev(X)) were measured in Na100, K100, TEA100, Li100, Cs100, or choline100 solutions, and ionic permeability ratios of puroidoline-induced channels (P_X/P_{Na}) were deduced for each cation from the shift in reversal potentials Erev(X) − Erev(Na) using the Goldman-Hodgkin-Katz equation (Hille, 1992):

$$\mathrm{Erev(X)} - \mathrm{Erev(Na)} = RT/F \ln(P_X[X]_o/P_{Na}[Na]_o)$$

$$P_X/P_{Na} = [Na]_o/[X]_o \, e^{[(\mathrm{Erev(X)}-\mathrm{Erev(Na)})F/RT]},$$

where $[X]_o$ is the extracellular concentration of ion X; and R, T, and F have their usual meaning (RT/F ~25.3 mV at 20°C).

Single-channel recordings were performed on cell-attached patches at room temperature (22 – 24°C) using a depolarizing extracellular solution (100 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 10 mM EGTA, pH adjusted to 7.4 with KOH). In this solution, the oocyte resting membrane potential was close to 0 mV. PIN-a (50 μM) was included in the pipette solution (Na100). Pipettes, made from KG33 borosilicate glasses (Garner Glass, Claremont, CA), treated with Sylgard™ and fire polished, had a resistance of 10–15 MΩ when filled with Na100. Data were recorded with a Geneclamp500 amplifier (Axon Instruments, 100 G-CV5 headstage), using Fetchex 6.02 (Axon Instruments) in event-driven mode and digitized by a Labmaster interface. Single-channel conductances were determined at a pipette-voltage of +150 mV by fitting Gaussians to amplitude histograms (pStat, Axon Instruments).

For cell toxicity studies, *Xenopus* oocytes, expressing or not the *Shaker* K^+ channel, were placed into a petri dish in Na100 solution under a binocular microscope equipped with a Hamamatsu CCD digital camera. At time 0, PIN-a was added (final concentration 50 μM) and images were stored every 5 min. Cell volume was estimated by measuring the oocyte diameter.

Results are presented as mean ± standard deviation and Student's t-test has been used to determine the significance of the difference between two means.

RESULTS

Puroindolines induce changes in oocyte membrane conductance

The purified PIN-a preparation was homogenous, as determined by MALDI-TOF mass spectrometry and 15% SDS-PAGE electrophoresis using Coomassie blue staining (Fig. 1

A left and *right*, respectively). Molecular masses of 12776/12944 and ~16 kDa determined by these two methods, respectively, were in agreement with a previous report describing the existence of a heterogeneity at the C-terminus of the protein and overestimation of the molecular masses in SDS gel (Blochet et al., 1993). A first attempt to test the effect of puroindolines on cellular membranes was made by direct application of the proteins to voltage-clamped *Xenopus* oocytes. This system, intensively used for heterologous expression, can also allow, in voltage-clamp conditions, the analysis of changes in membrane permeability induced by pore-forming molecules. Isolated oocytes were held at a membrane potential of −100 mV, and voltage ramps (from −80 to +20 mV in 450 ms) were applied every 3 s in Na100 recording solution. Addition of PIN-a (50 μM) to the bath solution induced, after 5–10 s, a gradual increase of the oocyte membrane conductance, as revealed by the increase of the membrane currents recorded during the ramp protocol (Fig. 1 *B*).

In these conditions, the increase in the membrane conductance induced by PIN-a did not reach a steady state, but developed continuously to finally exceed the range of the voltage-clamp amplifier. In control condition, without PIN-a added in the bath, stable recordings were routinely obtained over a period >20 min, with no change in membrane conductance. Washing the recording chamber, for 10–15 min, with PIN-a-free solution did not allow any recovery of the effects but, nevertheless, stopped the increase in membrane conductance leading to the establishment of a steady-state level of high membrane permeability (not shown, but see Na100 washout in Fig. 4 *C*). Current-voltage curves of the PIN-a induced conductance could be constructed by digital subtraction of currents recorded during the voltage ramp protocol before application of PIN-a. After application of the protein, currents were found to be slightly inward rectifying and had a reversal potential (Erev) of about −10 mV (Fig. 1 *C*, $n = 14$, see Table 1). This value did not change over time and during persistent application of PIN-a, when current was increasing. This suggests that the current was more likely due to formation of ion channels, with well-defined biophysical properties independent of the time of application of PIN-a, rather than to a global destabilization of the membrane. We therefore hypothesized that PIN-a could form ion channels in oocyte membranes. Similar results were found using PIN-b, in the same conditions, instead of PIN-a (not shown). It should be noted, however, that formation of ion channels by puroindolines appears at protein concentrations 20-fold higher than those required for purothionins (20–50 μM versus 1 μM, not shown, but see Hughes et al., 2000).

Puroindolines form ion channels into planar lipid bilayers

To provide direct and strong evidence for the pore-forming activity of PIN-a, rather than other mechanisms such as membrane destabilization or regulation of the activity of endogenous channels, we decided to characterize the single-channel activity of PIN-a in artificial lipid bilayers. Single-channel experiments were carried out in 1 M KCl at room temperature with PC/PE (7:3, v/v) membranes.

As soon as puroindoline was first added (final concentration 0.2 nM) to the conductance measurement cell (*cis* chamber), a typical single-channel activity began to develop rapidly (Fig. 2 *A*). As already observed on macroscopic recordings in *Xenopus* oocytes, the membrane rapidly became leaky, preventing long-term recordings and precise analysis of single-channel events. Current amplitudes analyzed at different applied voltages however, revealed the existence of two main conductance levels of ~50–100 and ~150–200 pS. Fig. 2 *A* shows a typical example of this type of recording. Here, the lowest level corresponds to a channel permanently open, with additional channel activity, of 60, 177, and 285 pS, superimposed. Occasionally, larger values of ~600 and ~800 pS could be temporarily observed, although they were often masked by the deterioration of the recording conditions. It should be noted that, in these conditions, it has been already shown (and checked again in our conditions, data not shown) that Triton X114, used for extraction of PIN-a, was not able to induce any channel activity, thus suggesting strongly that channel formation was indeed due to PIN-a. At higher PIN-a concentrations (>20 nM), channel formation and subsequent membrane destabilization were too fast to allow any clear measurement of channel activity. As puroindolines are positively charged proteins, we tried to evaluate the role of negatively charged lipids on the incorporation of the protein into the membrane. We thus added phosphatidylserine into the membrane bilayers with the following stoichiometry PC/PE/PS 7:3:1 v/v. For a similar range of protein concentrations (0.05–0.2 nM), the same well-defined single-channel conductances of ~100 pS, ~300 pS, and ~400 pS were observed (Fig. 2 *B*). As already described with the PC/PE membrane, the insertion of the protein into bilayers increased with time, and larger channels could be observed after one hour. In this case nevertheless, incorporation was much slower and occurred without deterioration of the recording quality. Several experiments ($n = 4$) demonstrated additional reproducible conductance values of 600, 1400, 2400, 3200, and 6400 pS in 1 M KCl.

These results confirm that PIN-a is able to form ion channels in artificial bilayers. The progressive time- and concentration-dependent increase in the number and amplitude of conductance levels is reminiscent of the gradual increase of the membrane permeability observed, at the whole-cell level, in *Xenopus* oocytes, and thus suggests the formation of oligomers.

Single-channel events could also be recorded in cell-attached patches from *Xenopus* oocytes. External application of PIN-a (included in the recording pipette) induced the apparition of brief openings of different conductance levels

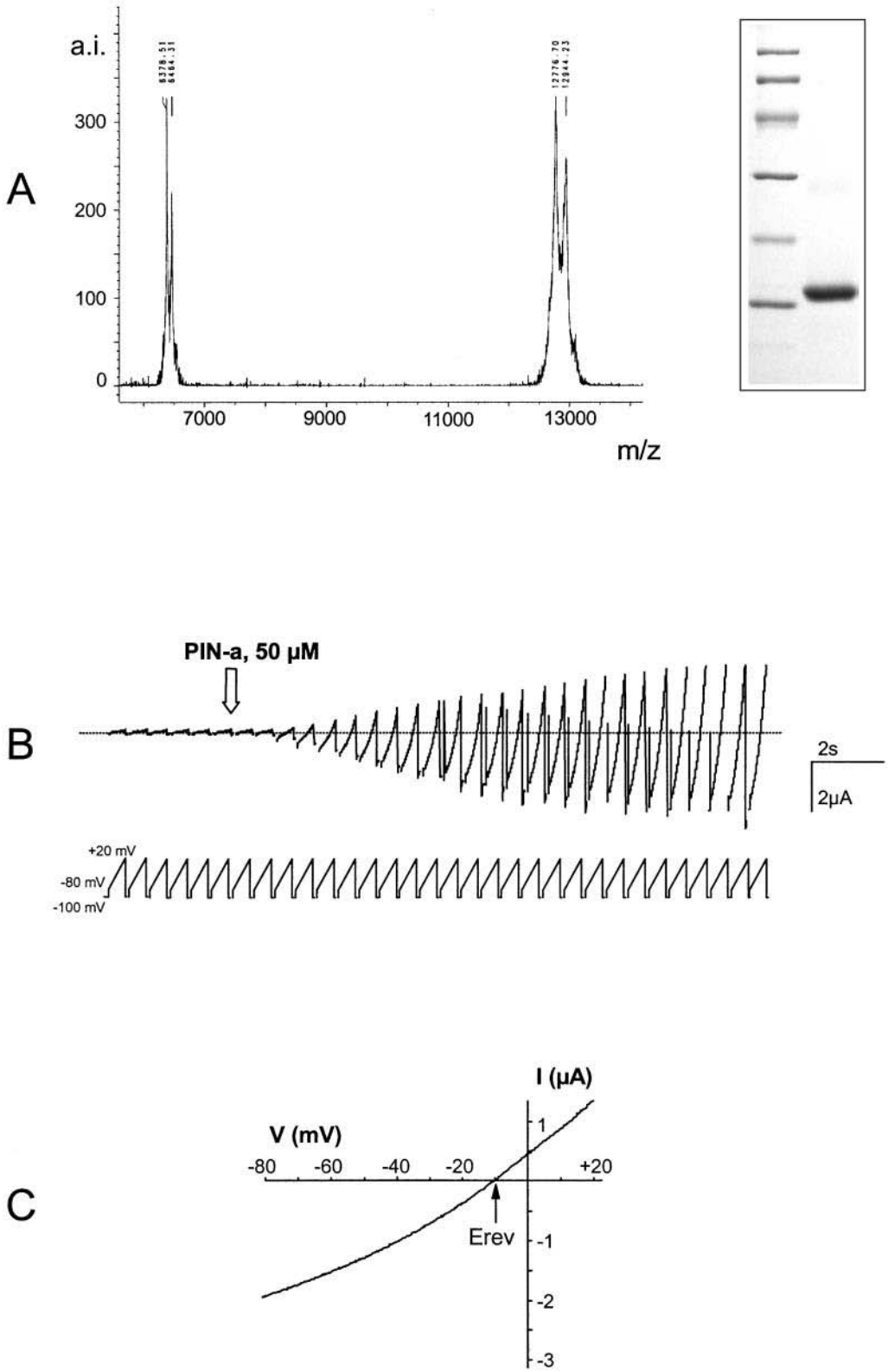

FIGURE 1 Puroindolines permeabilize the oocyte membrane. (*A*, *left*) Mass spectrometry profile of PIN-a, displaying the two peaks at 12776 and 12944 characteristic of the C-terminus heterogeneity of PIN-a. The higher molecular weight corresponds to PIN-a with an Ile-Gly extension (Blochet et al., 1993). (*A*, *right*) In 15% SDS-PAGE, PIN-a was resolved as a single band of approximate molecular weight of 16 kDa (*right, right lane*). Molecular weight markers from top to bottom: 97, 66, 45, 30, 20.1, and 14.4 kDa (*left lane*). (*B*) Oocyte membrane currents were recorded during voltage ramps from −80 to +20 mV (one ramp/3 s, protocol and traces are displayed after concatenation for graphic purposes) at a holding potential of −100 mV. Addition of PIN-a (50 μM) directly to the recording chamber (*arrow*) induced a marked increase in membrane conductance that developed after 5–10 s. (*C*) Macroscopic current-voltage curve measured during application of PIN-a using a voltage-ramp from −80 to +20 mV. The current is shown after digital subtraction of leak current recorded before puroindoline application. Erev is the reversal potential (−10 mV).

from 10 to 50 pS in the Na100 solution (see Fig. 2 *C*). Occasionally, the baseline level permanently switched to a higher conducting state, suggesting that channel opening may become permanent (Fig. 2 *C*). As already observed at the whole-cell level, and in artificial membrane bilayers, deterioration of the patches occurred within minutes.

TABLE 1 Ionic selectivity of PIN-a-induced channels

	Cs	K	Na	Li	Choline	TEA
Erev (mV)	0.5 ± 2.4	−1.9 ± 1.3	−9.5 ± 1.5	−12.3 ± 0.38	−16.9 ± 1.2	−18.1 ± 2.5
	$n = 3$	$n = 4$	$n = 14$	$n = 3$	$n = 3$	$n = 3$
P_x/P_{Na}	1.49	1.34	**1**	0.90	0.74	0.71

Erev: measured reversal potential; P_x/P_{Na}: ionic permeability ratios of cation X relative to sodium permeability; and n: number of independent experiments (see Experimental procedures for details).

Selectivity of puroindoline-induced ion channels

Since long recordings were difficult to obtain at the single channel level, we returned to macroscopic recordings in *Xenopus* oocytes to characterize more precisely the ionic selectivity of these channels. Voltage-ramps, like those described in Fig. 1, were applied under different ionic conditions, where the main extracellular cation Na^+ was replaced by equimolar concentrations of various monovalent cations, differing in their hydration size (Cs^+, K^+, Li^+, choline, TEA). Fig. 3 *A* shows the macroscopic current-voltage curves recorded after PIN-a application (50 μM) in some of the distinct recording solutions. In each case, background currents were suppressed by digital subtraction of the current trace recorded before application of PIN-a.

As seen in Fig. 3 *A* and summarized in Table 1, current reversal potentials varied from 0 mV (Cs) to −18 mV (TEA) suggesting selectivity of the channel for monovalent cations. Similar experiments replacing Cl^- anions by acetate, in the presence of 100 mM Na^+, and no $MgCl_2$ added, did not evidence any change in the reversal potential (Erev = −13.2 ± 3.3 mV for NaCl and −13.2 ± 1.5 mV for Na-acetate), demonstrating the absence of anion permeation. Calculation of the shift in reversal potential recorded using Na^+ and the different ionic conditions (Erev(X) − Erev(Na)) allowed to deduce, for each ion, the permeability relative to Na^+ (P_x/P_{Na}, see experimental procedures and Table 1). The cationic selectivity order was found to be the following: $Cs^+ > K^+ > Na^+ > Li^+ >$ choline = TEA, similar to the Eisenman selectivity sequences I or II, where selectivity is driven by the de-hydration energy of the cation (Hille, 1992).

Voltage dependence of puroindoline-induced ion channels

Modification of oocyte membrane potential between ramps had drastic consequences on the capability of PIN-a to induce channel formation. At −60 mV or −80 mV, 50 μM PIN-a had almost no effect on membrane permeability (Fig. 3 *B*), whereas the same application to oocytes held at −100 mV clearly increased membrane currents within 5 to 10 s, currents which then slowly developed during several minutes. The amplitude and the kinetics of these effects were even more pronounced when the oocyte was held at −120 mV (Fig. 3 *B*), suggesting that membrane insertion, channel formation, and/or channel opening were voltage-modulated. When these changes in membrane conductances were plotted over time after PIN-a addition (Fig. 3 *C*, *left*), the increase in macroscopic membrane conductance was clearly faster for hyperpolarized voltages. We then evaluated the speed of the change in membrane conductance by measuring, for each voltage, the early slope of these changes just after PIN-a addition (dashed line in Fig. 3 *C*, *left*). As seen on the right part of Fig. 3 *C*, these changes in membrane conductance were strongly voltage-dependent, with an e-fold change for 14 mV. Although such calculations can be viewed as a crude estimation of the real voltage-dependence of these effects, they nevertheless underlined the strong potentiating effect of negative voltages.

Effects of calcium on ion channels induced by PIN-a

Because the antimicrobial activities of the puroindolines has been shown to be altered by addition of Ca^{2+} in the medium (Dubreil et al., 1998), we decided to analyze in more detail the effect of this cation on the ability of puroindolines to form ion channels. We found that the presence of 5 mM Ca^{2+} in the recording solution inhibited the effects of bath application of PIN-a or PIN-b (not shown, but see Fig. 5 *A*). This inhibition was seen at all membrane potentials tested (−100 mV and −120 mV, not shown) suggesting that membrane insertion of puroindoline, channel formation, and/or channel opening were blocked. When puroindoline was applied in the absence of Ca^{2+}, the changes in membrane permeability were rapid and hardly reversible upon washing with a PIN-a free solution (Na100, see Experimental Methods). However, partial reversibility, after puroindoline application, could be obtained only when the oocyte was bathed with Na100 + 5 mM Ca^{2+} (Fig. 4 *A*). Several experimental data indicate that this effect was not due to a block of the channel by Ca^{2+} ions. First, immediately after perfusion of Ca^{2+}, a transient increase in current amplitude was noticed before the current decay took place (indicated by an asterisk in the time course of changes in conductance, Fig. 4 *A*, *right*).

This transient increase was interpreted as permeation of Ca^{2+} through the channels formed by puroindolines and subsequent activation of the endogenous Ca^{2+}-activated Cl^- current since it disappeared upon injection of BAPTA into

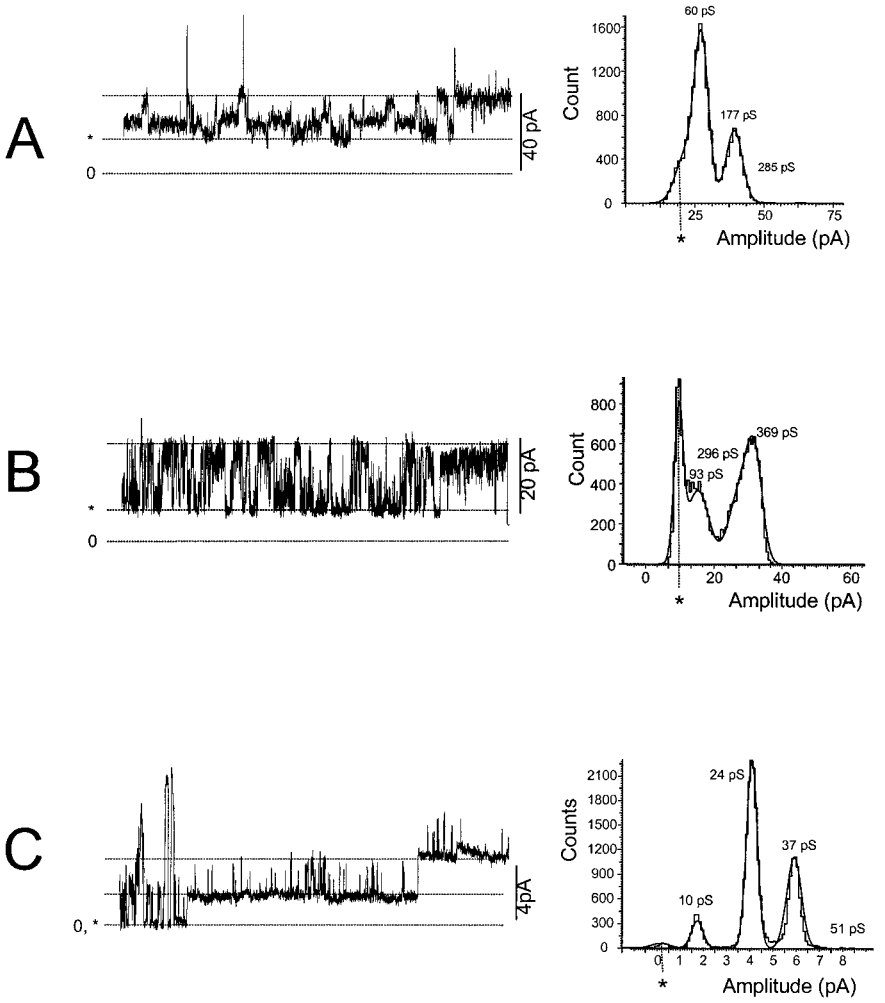

FIGURE 2 Single channel current induced by PIN-a. (*A*) Application of PIN-a, diluted in H_2O, to the *cis* chamber of the bilayer apparatus induced the apparition of single amplitude events (*left panel*), reflecting the formation of ion channels. The bilayer membrane was composed of PC/PE (7:3 w/w), and the applied voltage was −100 mV. The all-point amplitude histogram (*right panel*), calculated from the trace on the left, revealed the existence of different conducting levels (see text). The total recording time was 7 s. The asterisk refers to the current baseline (first component in the multi-gaussian fit) used to calculate the conductance, and the 0 refers to the absolute 0 current. (*B*) Recording conditions identical to *A*, except that the membrane composition includes phosphatidylserine (PC/PE/PS 7:3:1 w/w) and the applied voltage was −60 mV. Recording time 6.5 s (PIN-a diluted in 1% OPOE). In each case, conductances are estimated using the applied membrane potential. (*C*) Single-channel events were also recorded when PIN-a (50 μM in Na100) was added to the pipette solution of a cell-attached patch recorded from *Xenopus* oocytes. The extracellular solution was 100 mM KCl, 10 EGTA, 5 Hepes, to cancel the oocyte membrane potential, and the pipette voltage was +150 mV. The amplitude histograms denoted the existence of multiple conducting levels. The asterisk refers to the current baseline.

the oocyte (not shown). Second, the analysis of the changes in current reversal-potential during application of PIN-a and washout in the presence of Ca^{2+} (Fig. 4 *B*, analysis of the recording made during the experiments displayed in Fig. 4 *A*) showed a shift from −60 mV to −7 mV as soon as the first channels were formed and then did not vary significantly during the increase in current amplitude (compare the evolution of conductance in Fig. 4 *A* with that of reversal potential in Fig. 4 *B*). During washout with Ca^{2+}, however, the reversal potential shifted to −15 mV, an intermediate value between reversal potentials of PIN-a-induced currents and Ca^{2+}-activated chloride currents (−25 mV, in Dascal, 1987). Interestingly, when Ba^{2+} was used instead of Ca^{2+}, little reversibility could be obtained (Fig. 4 *C*), indicating that the inhibition of the channel formation and/or opening was Ca^{2+}-selective.

Since Ca^{2+} did not inhibit the puroindoline-induced membrane conductance directly by occluding the pore of the channel (as suggested by the activation of the Ca^{2+}-activated Cl^- current, see above), we suspected inhibition of either protein translocation in the membrane or channel formation/opening after translocation. To answer this question, we applied PIN-a on oocytes bathed with a Na100 + 5 mM Ca^{2+} solution (Fig. 5 *A*).

As expected in such conditions, no change in membrane permeability was observed. The oocyte was then extensively washed using the Na100 + 5 mM Ca^{2+} and PIN-a free solution for 2 to 3 min without any change in membrane current. The bathing solution was then switched to Na100 without added Ca^{2+}, and surprisingly, the usual increase in conductance was recorded after a latency of 20–30 s. This result suggests that Ca^{2+} did not prevent protein translocation into the membrane but rather blocked channel aggregation or openings. Indeed, this change in conductance was partially reversible upon perfusion with a Ca^{2+} containing solution (see Fig. 5 *A* for current traces and Fig. 5 *B* for the kinetics of the effects). The dual role of Ca^{2+}, as inhibitor of channel translocation/opening and charge carrier, prevented a more detailed analysis of Ca^{2+} permeation through the PIN-a channels.

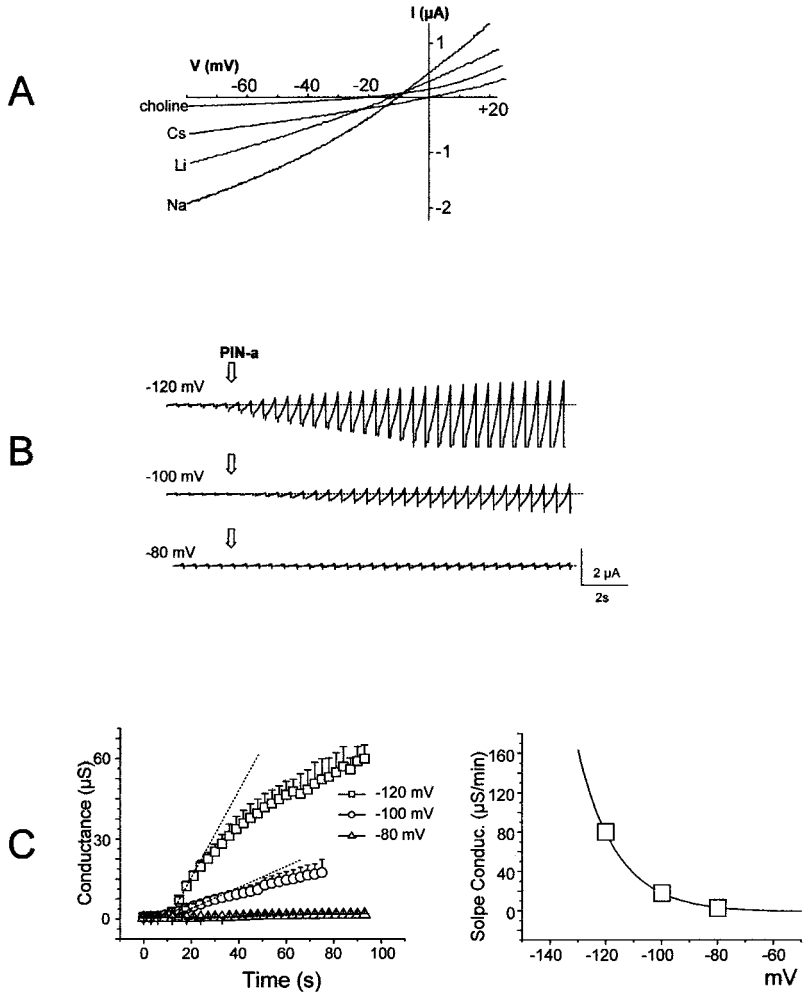

FIGURE 3 Ionic selectivity of the channels and voltage-dependence. (*A*) Macroscopic current-voltage curves recorded during application of PIN-a (50 μM) in different recording solutions (Na100, Li100, Cs100, and choline100). Current recordings and voltage protocols are identical to Fig. 1 *A*. Note the shift in the reversal potential obtained in the different conditions. (*B*) Voltage modulated effect of PIN-a. The typical voltage ramps (as described in Fig. 1) were applied from holding potentials of either −80, −100, or −120 mV, and PIN-a was added to the bath at the time indicated by an arrow. Kinetics and amplitude of oocyte membrane conductance changes were then recorded. Hyperpolarized holding potentials clearly potentiated the PIN-a effect. (*C*) Graphical representation of the kinetics of PIN-a induced changes in oocyte membrane conductance at different holding potentials. Dashed lines represent the slopes of the conductance changes at early PIN-a effects and at −80, −100, and −120 mV. (*D*) The voltage-dependence of the changes in conductance was estimated by plotting the slope of conductance changes (in μS/min) at different potentials against the membrane potential. The exponential decay predicts an e-fold change in conductance for 14 mV.

Oocyte cytotoxicity of puroindoline

We finally tested the cytotoxicity of puroindoline directly onto isolated *Xenopus* oocytes. Oocytes were incubated in a petri dish containing Na100 solution and 50 μM PIN-a and placed under a binocular stereomicroscope equipped with a CCD camera. One picture of the oocytes was taken every 5 min for at least 1 h. Incubation of normal oocytes in this solution (Fig. 6 *A*), did not result in any major morphological changes of the oocyte, i.e., pigmentation appeared normal and cell volume was constant (less than 1% variation in the oocyte diameter).

These data suggested a lack of puroindoline toxicity in contradiction with the permeabilizing properties of the protein. However, as mentioned before, the effects of PIN-a were modulated by voltage (Fig. 3, *B* and *C*), and the oocyte resting potential was rather low in these conditions (−30/−40 mV). We thus repeated this experiment on artificially hyperpolarized oocytes. This was done by expressing the *Shaker* K^+ channel via mRNA injection. Twenty-four hours after mRNA injection, oocytes expressed K^+ currents of more than 20 μA, and their resting potentials were hyperpolarized to potentials more negative than −60 mV. When placed in a petri dish containing the Na100 solution and 50 μM PIN-a, these oocytes rapidly displayed (in 10–35 min) rings of depigmentation, indicating membrane perturbations (see arrows on picture taken 45 min after puroindoline application in Fig. 6 *B*). Concomitantly, an increase in the oocyte diameter of 10% to 15% was recorded. The toxic effect of puroindolines therefore was observed only with hyperpolarized oocytes suggesting that, in vivo, puroindoline-induced cell modifications were controlled by voltage. All these modifications were, however, less dramatic than the effects of purothionins which, in the same conditions, induced oocyte death in 30 min (Fig. 6 *C*) with appearance of the first rings of depigmentation in less than 10 min using a 10-fold lower protein concentration.

DISCUSSION

PIN-a and PIN-b belong, with purothionins and nsLTPs, to a group of seed lipid binding proteins that share common characteristics. They are basic, cysteine-rich, amphiphilic proteins, and some of them have antimicrobial activities

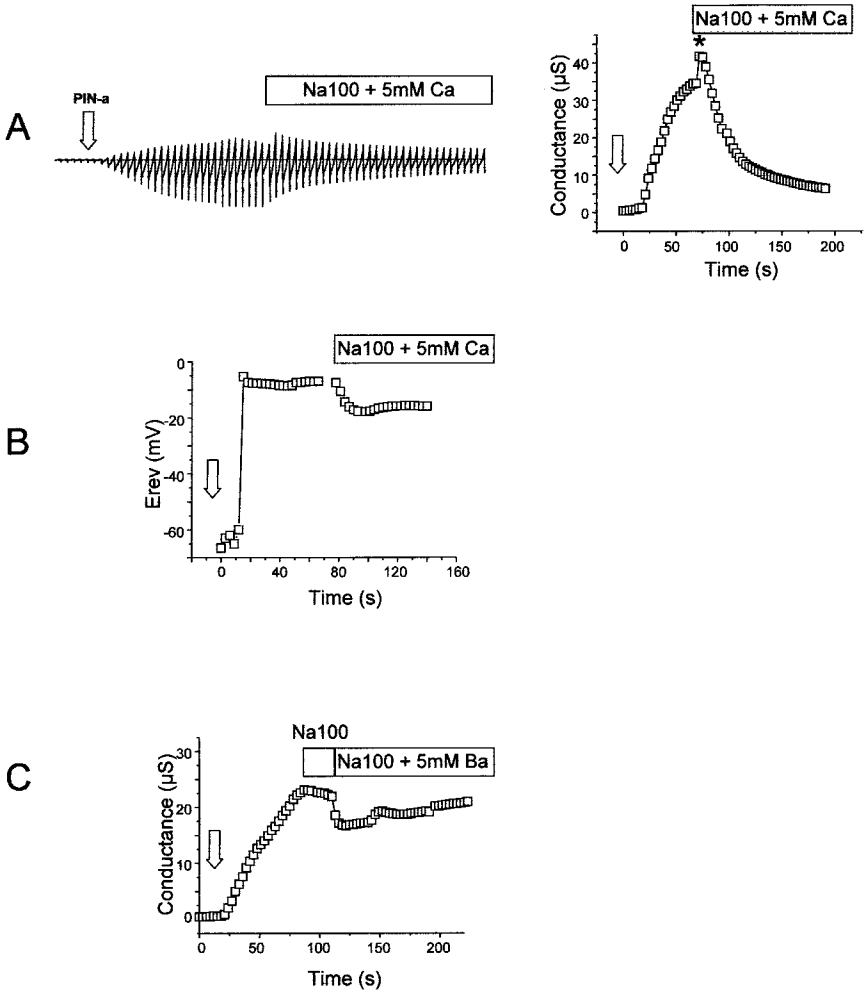

FIGURE 4 Antagonistic action of calcium on channel formation. (*A, left*) Current traces recorded after bath application of PIN-a and subsequent washout of the protein by perfusion of Na100 + 5 mM $CaCl_2$ solutions (marked by the *open box, left*). Voltage ramps were applied using the same protocol as in Fig. 1. (*A*, *right*) Graphical representation of the kinetics of the conductance changes shown in panel *A*. Note that the reversibility of the effect of PIN-a upon perfusion of the Na100 + 5 mM $CaCl_2$ solution is preceded by a transient increase in conductance (noted by an asterisk). (*B*) Kinetics of the changes in the current reversal potential recorded during the experiment depicted in *A*. Note that perfusion of Ca^{2+} induced a modification in the reversal potential. (*C*) Kinetics of the conductance changes recorded in the same conditions as in *A* and *B*, but using first Na100 and then Na100 + 5 mM Ba^{2+}. Reversibility in this case was not obtained. Arrows: Time of addition of PIN-a.

(Douliez et al., 2000). Antifungal properties of puroindolines have already been described in vitro, but the precise mechanisms of their toxicity are not known (Dubreil et al., 1998). Alteration of membrane permeability by these proteins has been reported (Mattei et al., 1998), and channel formation was suspected on the basis of similarities with purothionin, which has been shown to form ion channels in biological membranes (Hughes et al., 2000). However, although puroindoline-induced nodal swelling in axons has been attributed to the formation of pores (Mattei et al., 1998), direct experimental evidence of such pores was lacking.

In this study, we clearly demonstrate that 1), PIN-a is able to form ion channels in artificial and biological membranes; 2), these channels display some selectivity toward monovalent cations; 3), voltage and Ca^{2+} ions modulate channel formation and/or opening; and 4), PIN-a displays some voltage-dependent cytotoxic activity toward *Xenopus* oocytes.

We show here that the concentrations of PIN-a necessary to permeabilize the oocyte membrane (50 μM at -100 mV) are close to those reported for the nodal swelling of myelinated axons or for the inhibition of fungal growth (10–100 μM; Dubreil et al., 1998; Mattei et al., 1998). This concentration is, however, variable, depending on the membrane voltage and possibly the lipid composition of the membrane. Similar results have been found for β-purothionin, another cytotoxic protein able to form ion channels, but at lower concentrations (1 μM, Hughes et al., 2000). This suggests that the two related proteins could share the same basic mechanism for their biological activity, but with different potencies. The stronger hydrophobic character of thionins compared to puroindolines (Kyte and Doolittle hydropathic index, PIN-a -0.489, β–purothionin -0.396) may support a better partition coefficient for lipids leading to better insertion into the membrane. Another important factor may be the conformation of the protein itself in solution, or the stability of the open channel conformation.

The behavior of PIN-a in artificial bilayers, typified by a strong incorporation in the membrane at negative voltages gradually leading to membrane breakdown, is also reminiscent of the effect of β–purothionin (Hughes et al., 2000). In *Xenopus* oocytes, the same individual events were found,

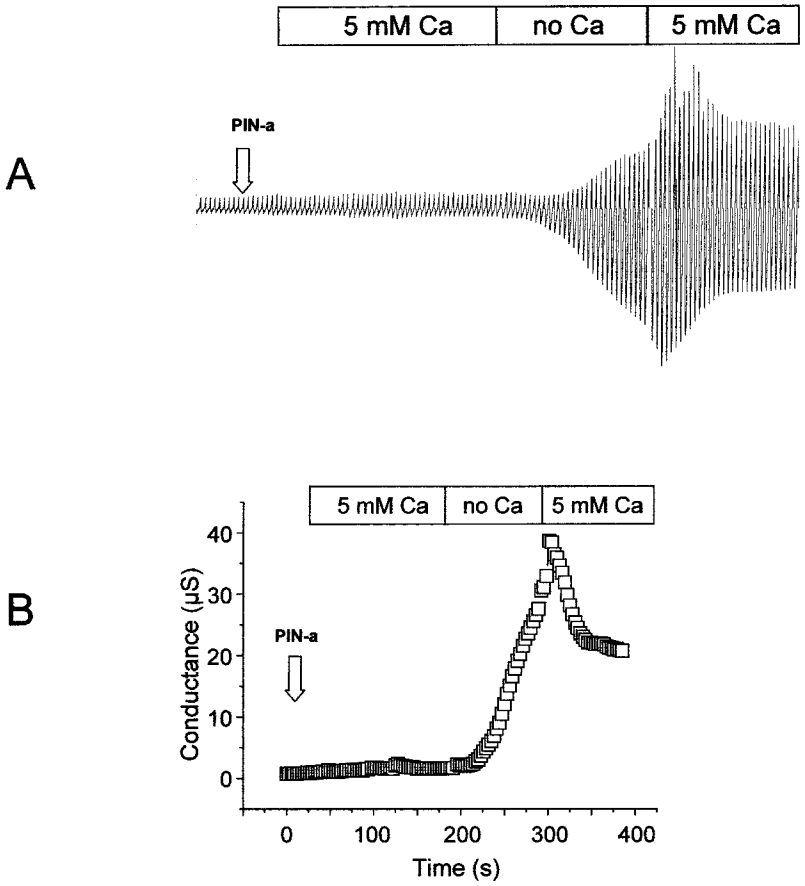

FIGURE 5 Puroindoline effect is masked by calcium addition. (*A*) Voltage ramps were applied to an oocyte bathed with a Na100 + 5 mM Ca^{2+} solution. Addition of PIN-a (50 μM, *arrow*) in these conditions did not induce any increase in membrane conductance. After extensive washing in Na100 + 5 mM Ca^{2+}, perfusion of an Na100 Ca^{2+}-free solution induced an increase in membrane conductance without any addition of puroindoline. (*B*) Graphical representation of the changes in membrane conductance recorded during this experiment. The increase in membrane conductance recorded after perfusion of the Ca^{2+}-free solution without any addition of puroindoline suggested that Ca^{2+} inhibited channel activity but not membrane insertion of the protein. Similar results were found on three other oocytes.

although conductances were diminished by a factor of ~10. We did not attempt to understand the origin of this difference, but the different ionic activity used in lipid bilayers (1 M KCl) and cell-attached patches (100 mM NaCl) is undoubtedly one of the major causes of this difference acting directly, and possibly indirectly, via modifications in the hydration state of the molecule (Biswas et al., 2001). These results, however, strongly support our hypothesis that ion channel formation is at the basis of the increase in membrane permeability induced by PIN-a at the macroscopic level.

We also found that the macroscopic reversal potential of the current did not vary during the development of the PIN-a effect. This suggests that the increase of membrane conductance occurred mainly through addition of new channels with fixed biophysical properties rather than formation of supramolecular assemblies. This hypothesis agrees with the lower single-channel conductance values found in PC/PE and PC/PE/PS bilayers (~100 pS in 1 M KCl) and with the cooperative adsorption of puroindolines (Biswas et al., 2001). Similar values have been obtained for bundles of alamethicins (Hanke and Boheim, 1980), and suggest that the oligomer responsible for the unitary channel may be built around 4 α-helices of PIN-a. In this scheme, higher conductance levels could occur via the association and cooperative opening of a variable number of such units, giving rise to a family of channels with different subconductance states. Such cooperativity, together with the high affinity of the protein for lipids, may explain the instability of the membrane patches observed during our recordings. These channels are strictly selective for cations, without any clear evidence for noticeable chloride permeation. The recorded selectivity series for monovalent cations follows the Eisenman selectivity sequence I or II, indicating that energy of dehydration is the limiting factor for ion permeation, and that direct electrostatic interactions between permeant ions and channel walls are weak. Interestingly, the development of a Ca^{2+}-induced Cl^- current at the early phase of Ca^{2+} perfusion suggests that PIN-a channels could also be permeable to divalent cations. This permeability, however, is probably low, since the negative shift observed in the reversal potential upon perfusion of Ca^{2+} most likely reflected the activation of the Ca^{2+} activated Cl^- (reversal potential of -25 mV) rather than the participation of the Ca^{2+} influx through PIN-a channel (the equilibrium potential for Ca^{2+} is $+119$ mV in 5 mM external Ca^{2+} and assuming an internal Ca^{2+} concentration of 0.4 μM, see Dascal, 1987).

Clearly, formation of ion channels by puroindolines is strongly potentiated by negative voltages. Hyperpolarized membrane potentials may bring the energy necessary for

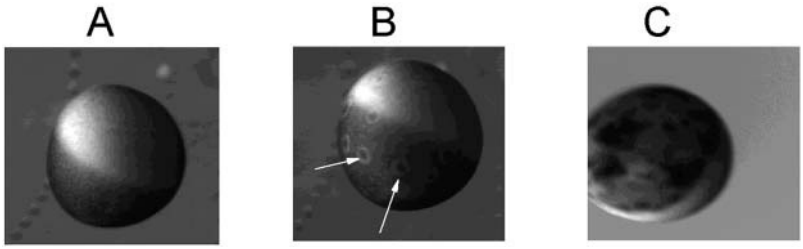

FIGURE 6 Cellular toxicity of puroindoline is voltage-modulated. (*A*) Noninjected oocytes (resting potential around −35 mV) bathed in Na100, 1 h after addition of PIN-a (50 μM). (*B*) *Shaker* K^+ channel-expressing oocytes (resting potential <-60 mV), 45 min after addition of PIN-a in Na100. Arrows indicate rings of depigmentation. (*C*) *Shaker* K+ channel-expressing oocytes 30 min after addition of purothionin in Na100 solution.

the translocation of the positively charged PIN-a and the subsequent formation of ion channels as described for src (Murray et al., 1998). Such an effect could have important physiological implications, targeting toxicity toward cell types with hyperpolarized membrane potentials. For example, the membrane potential of *Neurospora crassa* is around −175 mV, a potential compatible with channel formation (Thevissen et al., 1996). The fact that PIN-a induced membrane disruption only on hyperpolarized oocytes (expressing K^+ channels) confirms the likelihood of this mechanism.

A second important point is the strong inhibition of channel formation by extracellular Ca^{2+} (5 mM). Our preliminary data indicate that this inhibition was not voltage-dependent and could not be released at positive potentials (data not shown), thus discarding mechanisms such as open-channel block. The reversibility of the Ca^{2+}-induced inhibition upon washout of Ca^{2+} seems to indicate that Ca^{2+} did not inhibit protein adsorption and/or translocation, but rather influenced channel gating. Whether puroindolines can bind Ca^{2+} ions directly or indirectly (via other proteins) is presently unknown, but such direct binding has been suspected for purothionins. However, in the latter case, the effect of Ca^{2+} was not reversible and Ca^{2+} chelation has even been suggested (Hughes et al., 2000). Therefore, the mechanism of Ca^{2+} inhibition or affinity could be different for these two types of proteins. The Ca^{2+} dependence of channel formation in these two cases has to be related to previous data showing that calcium could modulate antimicrobial activities and toxicity of thionins and puroindolines (Carrasco et al., 1981; Dubreil et al., 1998; Molina et al., 1993; Vernon and Rogers, 1992). Thus, Ca^{2+}-dependent inhibition, coupled to the voltage-dependent formation and/or activity of the channel, could play an important role in the physiological activity of puroindolines in seeds.

Finally, we observed that the oocyte membrane disruption was accompanied by a moderate swelling of the oocyte (10–15%). These effects were seen only when the oocytes were hyperpolarized by overexpression of the *Shaker* K^+ channels. These effects probably reflect water influx into the oocytes, consecutive to ionic homeostasis imbalance as previously shown in neurons (Mattei et al., 1998). We also found that purothionins can induce channel formation at lower concentrations than puroindolines, in agreement with their different potency to induce channel formation in voltage-clamped oocytes. Indeed, these different behaviors could be one of the causes responsible for the greater cellular toxicity of purothionins (Broekaert et al., 1997; Hughes et al., 2000).

Our study constitutes a first attempt to explain the cellular toxicity of puroindolines. We show that their antifungal properties could be related to their ability to form ion channels in membranes, resulting in modifications of the transmembrane ionic gradients necessary to maintain cellular homeostasis. Thus, our results demonstrate that the membrane permeabilizing activity of puroindolines and purothionins occurs through similar mechanisms and suggest that puroindolines should participate, with the other antifungal active proteins isolated in wheat seeds (Broekaert et al., 1997), in the defense against pathogens. Among them, enzymes such as chitinases, by hydrolyzing the fungi walls and providing an easiest access to the plasma membrane, may potentiate the in vivo effects of PIN-a, as shown for other antimicrobial proteins (Jach et al., 1995). These synergistic mechanisms have been reviewed recently (Punja, 2001). The specific expression of indolines in seeds of *Triticeae* and *Aveneae* tribes (Douliez et al., 2000) suggests that these cereals may possess specific responses against microbial attack.

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