Diffusion of nystatin in plasma membrane is inhibited by a glass-membrane seal

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ABSTRACT In perforated patch recording, the pore former nystatin is incorporated into a cell-attached patch, to increase its conductance. The possibility of lateral diffusion of nystatin through the membrane and under the glass-membrane seal was examined by reversing the nystatin gradient. Namely, a cell-attached patch on a cell was examined while placing nystatin into the bath. The reversal potential and current-voltage relationship of single Ca^{2+} activated K^+ channels in the patch were readily changed by varying the K^+ concentration in the bath, showing that nystatin was active in the cell membrane outside of the patch. However, the patch itself did not become leaky. The absence of a conductance induced in the patch by the nystatin in the rest of the plasma membrane of the cell suggests that the lateral diffusion of nystatin is inhibited by the glass-membrane seal.

INTRODUCTION

Perforated patch recording was developed as a method to record macroscopic currents from a cell without the loss or dilution of the cytoplasm (Horn and Marty, 1988; Korn et al., 1991; Horn and Korn, 1991). The method involves the incorporation of the pore former nystatin into the membrane of a cell-attached patch. The conductance induced in the patch provides electrical access to the interior of the cell without the exchange of either multivalent ions or substances larger than 0.8 nm. Nystatin does not cross the bilayer and enter the cell (Korn et al., 1991), but could be capable of diffusing laterally within the bilayer and under the seal between the glass pipette and the membrane. The lack of an appreciable leakage conductance in the cell in perforated patch experiments is evidence that nystatin does not cross the seal (Korn et al., 1991). However, as nystatin traverses the seal, its dilution and desorption into the bath may explain the absence of such leakage, especially when considering the steep dependence of the nystatin conductance on nystatin concentration (Lewis et al., 1977; Kleinberg and Finkelstein, 1984). Therefore, in spite of the absence of a significant leakage, nystatin may pollute the plasma membrane under study, where it could have functional effects, either subtle or obvious.

The effects of dilution are minimized if the size of the cell is small in a perforated patch experiment. In the extreme case, a perforated vesicle (Levitan and Kramer, 1990), the absence of leak is harder to explain if the nystatin can diffuse freely in the plane of the membrane (Korn et al., 1991). The experiments here attempt to test whether the glass-membrane seal is capable of inhibiting the lateral diffusion of nystatin. The effects of diffusion were enhanced by reversing the nystatin gradient of a perforated patch experiment. Thus, the conductance of a cell-attached patch was examined while incorporating nystatin into the extra-patch membrane by adding it to the solution bathing the cell. The small area of the patch maximizes the rate of the rise of nystatin concentration, if it is able to diffuse under the seal. Unlike the situation in perforated vesicle recording, the nystatin in the present experiments was added rapidly and at a high concentration to the bath, where it incorporates rapidly into the plasma membrane of the cell (Horn and Marty, 1988). The presence of a nystatin conductance in the extra-patch membrane was verified by measuring the current-voltage relationship for the amplitude of single K^+ channel currents in the patch while changing $[K^+]$ in the bath.

METHODS

Electrophysiology

Cell-attached patches were obtained on tissue cultured GH_3 cells using standard patch clamp methods (Hamill et al., 1981; Korn and Horn, 1989). These cells are approximately spherical with a diameter ~20 μm. Sylgard (Dow Corning Corp., Midland, MI) coated borosilicate patch pipettes were used. The pipette resistance in the bath solution was ~1.0 MΩ, and the apparent seal resistances usually were >10 GΩ. The bath solution was a 50/50 mixture of HiNa and HiK. HiNa was (in mM): 100 Na-methylsulfate, 50 NaCl, 12 $CaCl_2$, 1 $MgCl_2$, and 10 HEPES, pH 7.3. HiK solution was identical except that K^+ was substituted for Na^+. Corrections were made for junction potentials. The data were acquired and analyzed using pCLAMP (Axon Instruments, Foster City, CA). The temperature was 22°C.

Both ramps and constant voltages were presented to the patches. The conductance of patches, when channels were closed, was measured by voltage ramps from the holding potential of +50 mV to −100

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mV. The current voltage relationship for single open K^+ channels was determined from amplitudes measured both during ramps (Yellen, 1982) and during constant voltages. The positive holding potential for ramps was designed to increase the probability that a K^+ channel would be open at the ramp's onset.

Nystatin (Sigma Chemical Company, St. Louis, MO) was added to the desired solutions at a supersaturated concentration of 167 μg/ml from a dimethyl sulfoxide stock solution of 50 mg/ml, as previously described (Korn et al., 1991). To insure the highest possible activity of the nystatin-containing solutions, all records were obtained within 1 h of addition of the nystatin. Previous experiments with perforated patch recording showed little deterioration of nystatin potency over this period (Korn et al., 1991). The potency of the nystatin was tested directly in several experiments by standard whole cell recording after doing the above series of experiments on several patches. In all cases, the introduction of nystatin into the bath caused a rapid, large increase in conductance in the cell under observation, as in earlier experiments on lachrimal gland cells (Horn and Marty, 1988). Solutions were presented to a cell by a ~50-μm glass pipette containing four gravity-fed outlet tubes. Solenoid valves were used to switch among the solutions. The time for complete solution exchange was <3 s, and the flow was continuous for the duration of the experiment.

Theory

Calculation of the diffusion of nystatin into the patch from the plasma membrane of the cell involved the following assumptions. (*a*) Bath application leads to a rapid incorporation (on the order of seconds) of nystatin into the plasma membrane of the cell (Horn and Marty, 1988). (*b*) Nystatin that has entered the cell-attached patch by lateral diffusion may desorb from the patch membrane into the pipette solution with a time constant of 10 to 20 min (Cass et al., 1970; Russell et al., 1977; Tillotson and Horn, 1978). The slow rate of desorption may be related to the hydrophobicity of nystatin. The rate of desorption is much smaller than the expected rate of diffusion into the patch (see below), and was thus ignored for the calculations. (*c*) Nystatin reversibly enters, and desorbs from, the plasma membrane of the cell, but does not cross the membrane into the cytoplasm (see references and discussion in Korn et al., 1991). (*d*) The typical size of a patch of membrane is ~5 μm^2 (Sakmann and Neher, 1983), equivalent to a disk with a radius of 1.26 μm. (*e*) The conductance of nystatin varies roughly as the fifth power of its concentration (Lewis et al., 1977; Kleinberg and Finkelstein, 1984). (*f*) The lateral mobility of nystatin in the membrane was assumed to be similar to that of a phospholipid or small lipophilic peptides (see discussion, below).

The nystatin concentration in the plasma membrane outside the patch was not known. Therefore, all calculations of concentration were normalized to a value of 1.0 at the rim of the patch, the region where the membrane contacted the glass pipette. The diffusion of nystatin into the patch was assumed to be radial, and followed Eq. 5.22 in Crank (1975); namely,

$$[\text{nystatin}] = 1 - (2/a)\ \text{n=1}\infty \exp(-D\alpha\text{n}2t) J_0(r\alpha_n)/[\alpha_n J_1(a\alpha_n)], \quad (1)$$

where a is the radius of the patch, D is the diffusion coefficient of nystatin (assumed to be 10^{-8} cm^2/s), r is the radial distance within the patch, and t is time after presentation of a nystatin concentration of 1.0 at the rim of the patch. $J_i(x)$ is the Bessel function of the first kind of order i, and the α_ns are the roots of

$$J_0(a\alpha_n) = 0.$$

The fractional quantity of nystatin which enters the patch in time t is given by Eq. (5.23) of Crank (1975),

$$\text{quantity} = 1 - \sum_{n=1}^{\infty} 4 \exp(-D\alpha_n^2 t)/[(a\alpha_n)^2]. \quad (2)$$

Eqs. 1 and 2 were solved by use of truncated series (10 terms). The Bessel functions were determined numerically (Press et al., 1986).

RESULTS AND DISCUSSION

Fig. 1 shows the current response to a 1.2-s voltage ramp from +50 to −100 mV in a cell-attached patch, before and 7 min after addition of 167 μg/ml nystatin to a HiK bath solution. The chord conductance (from −50 to +50 mV) was 62 pS both before (trace marked "control") and after adding nystatin to the bath. Similar results were obtained in six patches in which the duration of exposure to nystatin ranged from 3 to 10 min. The patch conductance before addition of nystatin was 82.2 ± 14.8 pS (mean ± standard deviation). The increase of slope conductance in these patches, after exposures of ≥3 min to nystatin to the bath, was 24.2 ± 30.2 pS (range: 21 pS decrease to a 69 pS increase). It should be noted that a slight increase in conductance is expected, due to the nystatin-induced condutance in series with the patch.

The presence of nystatin in the extra-patch membrane was verified by changing the $[K^+]$ in patches containing active Ca^{2+} activated K^+ channels, while maintaining a high concentration of nystatin in the bath. Fig. 2 shows the ramp currents from a patch exposed to 50/50 (Fig. 2*A*) and HiK (Fig. 2*B*) salines in the presence of

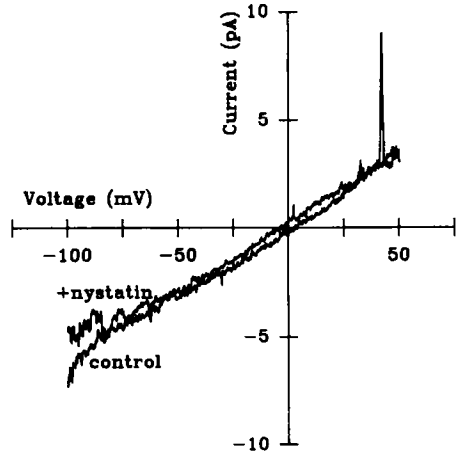

FIGURE 1 Effect of nystatin on patch current induced by 1.2 s voltage ramps from a holding potential of +50 mV. The bath contained HiK before (control) and 7 min after addition of nystatin. The spike in one of the records was caused by a brief opening of a Ca^{2+} activated K^+ channel in the patch.

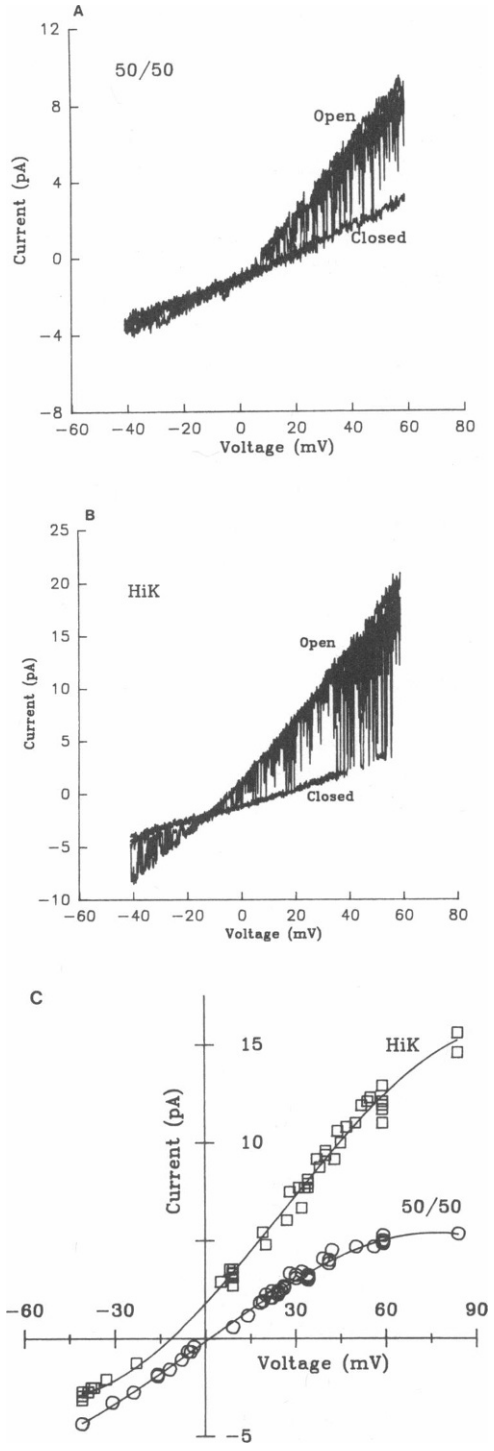

FIGURE 2 Effect of bath [K^+] on single K^+ channel currents in the presence of nystatin. *A* shows three superimposed records of currents in response to 460-ms voltage ramps from +60 to −40 mV, repeated over a period of 6 min in the continuous presence of nystatin. The bath contained 50/50 saline, and the open channel current reversed near 0 mV. *B* shows, in the same patch, the effect of replacing 50/50 with HiK, in the continuous presence of nystatin. Two traces are superimposed. *C* shows the current-voltage relationship for single K^+ channels, obtained from both ramps and holding potentials. Data from two patches are combined.

nystatin. Fig. 2 *C* shows the amplitude of single channel currents as a function of voltage for the two bath solutions. The observed shift in reversal potential (12 mV) is close to that expected under the assumption that these channels are perfectly selective for K^+ (16 mV).

The time course of the shift in the single channel current-voltage relationship was followed in several cases. After changing the [K^+] in the bath, the K^+ currents typically reached a steady-state value over a period of 20–40 s (data not shown), presumably due to the time required for the exchange of K^+ between the bath and the cytoplasm (for comparison, see Russell et al., 1977). Accordingly, the outward current through the K^+ channels was increased by an increase of [K^+] in the bath.

How fast should nystatin diffuse into a patch? Fig. 3 *A* shows the expected increase in relative concentration of nystatin in a patch whose rim (at the region of the seal) is suddenly exposed to a step increase in [nystatin]. The five curves show the time course of [nystatin] increase at five radial distances into the patch, from the center in equal increments towards the edge. The relative increase in [nystatin] is more than 95% complete after 1 s at all locations. Fig. 3 *B* shows the total accumulation of nystatin within the patch and the expected conductance of the membrane, assuming a 5^{th} power concentration dependence. Again, the increase is largely complete within 1 s. The expected conductance of a patch loaded with nystatin is large and would be difficult to miss above the background. Specifically, the series resistance observed in perforated patch experiments is 5–20 MΩ, equivalent to a patch conductance of 50–200 nS, at least three orders of magnitude greater than the conductance found in the experiments in this paper (Fig. 1). All data suggest, therefore, that nystatin is inhibited from diffusing past the pipette-membrane seal.

The assumption that nystatin diffusion is radial is a simplification, because the membrane in a typical cell-attached patch lies along a region of glass before forming the dome across the mouth of the pipette (Sokabe and Sachs, 1990). It is possible, therefore, that the diffusion of nystatin is rate limited by diffusion along the glass wall rather than across the rim of the pipette. This geometrical consideration has no qualitative effect on the conclusions of this paper. However, the diffusion path is both longer and more complicated than in the simple model used here.

Perhaps the most tenuous assumption in the above analysis is that the diffusion coefficient of nystatin is as large as 10^{-8} cm^2/s. However, values of $< 5 \times 10^{-12}$ cm^2/s

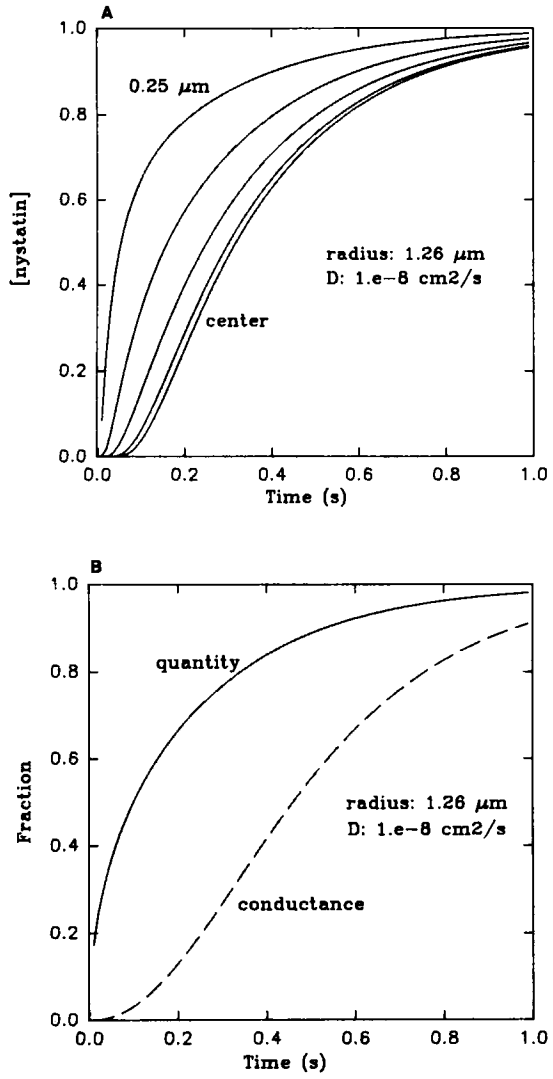

FIGURE 3 Theoretical diffusion rate of nystatin into the patch from the extra-patch membrane. *A* shows the normalized [nystatin] within the patch, at five concentric locations, in response to a concentration jump at the rim of a pipette. *B* shows the total quantity of nystatin diffusing into the patch along with the expected relative conductance.

must be assumed before the expected accumulation of nystatin in the patch approaches the rate of desorption into the pipette (calculations not shown). These values are lower than found even for large, relatively immobile membrane proteins, such as lectin receptors, surface antigens, and acetylcholine receptors (Cherry, 1979). Therefore, the most parsimonious conclusion is that the seal blocks nystatin movement. How this occurs is not clear. Perhaps a hydrophilic portion of the molecule extends into the bath and is restricted in the seal region. This could explain the inability of nystatin to diffuse freely past a tight junction in epithelial cells (Lewis et al., 1977). Another possibility is that the lipids in the seal region are somehow frozen and inhibit the movement of any substance embedded in the bilayer. This possibility can be tested by the methods used in this paper, using other hydrophobic compounds whose presence could be detected in a cell-attached patch.

The above results provide some assurance that nystatin is not mucking up the cell in perforated patch recordings. They also suggest that other substances, either attached to or imbedded within the plasma membrane, may not be able to travel freely across a glass-membrane seal. For example, the block of N-type Ca^{2+} channels by α-adrenergic agonists depends on the activation of GTP binding proteins (Lipscombe et al., 1989), but the effects on single channels are only observed when the agonist is placed inside the pipette during cell-attached recordings. Activation of receptors outside of the patch has no effect on the channels in the patch, suggesting that activated *G* proteins cannot diffuse across the seal. These observations suggest some caution in the interpretation of experiments involving cell-attached patches.

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