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Effects of Nystatin on Membrane Conductance and Internal Ion Activities in *Aplysia* Neurons

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Summary. Two methods were used to study effects of the antibiotic, nystatin, on giant neurons of *Aplysia.* In the first method the effects of various concentrations of nystatin on the current-voltage relationship were evaluated at a fixed time after exposure to the antibiotic using a two-microelectrode voltage clamp. Nystatin increased membrane conductance in a dose-dependent manner. The dose-response relation was very steep, with little or no effect below 15 mg/liter and an effect too large to measure at concentrations greater than 30 mg/liter. Upon return to antibiotic-free solution, membrane conductance returned to pre-treatment levels within 30 minutes. The second type of experiment involved use of ion-specific microelectrodes to measure changes of intracellular univalent ion activities which attended the nystatin-induced permeability. Nystatin-induced permeability changes mainly involved univalent cations, but Cl permeability was also increased. Nystatin may therefore be used to selectively rearrange the internal ionic milieu to study the effect of such a change on membrane transport or electrical properties.

The polyene antibiotic, nystatin, is known to induce a large increase in the permeability of lipid bilayer membranes (Kinsky, 1970; Finkelstein & Holz, 1973). Single ionic channels induced at low concentrations of this antibiotic have recently been observed (Ermishkin, Kasumov & Potzeluyev, 1976). The permeability increase is restricted to univalent ions and to uncharged substances whose Stokes-Einstein radii do not exceed 4 Å (Holz & Finkelstein, 1970). Similar effects of nystatin on the red blood cell membrane have also been demonstrated (Cass & Dalmark, 1973). Furthermore, by applying the nystatin in an appropriate solution, these latter workers showed that they could drastically change the intracellular concentrations of sodium, potassium and chloride, yet after removal of nystatin the membrane permeability and active potassium flux appeared normal.

This result suggested that nystatin might prove to be a useful tool for altering internal ionic concentrations for the study of ionic dependencies of various neuronal properties. We have therefore studied the effects of nystatin on membrane conductance and intracellular ion concentrations in single neurons of *Aplysia.* We were particularly interested in the magnitude of the permeability changes and their reversibility.

In the present paper we report that the effect of nystatin to increase membrane conductance and permeability is rapid, large, and readily reversible. By applying the antibiotic in the appropriate solution, it was possible to effect large changes in the intracellular concentrations of potassium and chloride. The time course of the recovery of the intracellular potassium and chloride concentrations was dependent on the cation used to displace potassium. When sodium was used, both potassium and chloride returned to pretreatment levels with a half-time of about 75 min, whereas if lithium replaced potassium the half-time was about 240 min.

A preliminary account of some of these results has been presented to the Biophysical Society (Russell, Eaton & Brodwick, 1976).

Materials and Methods

Biological Preparation

Aplysia californica were obtained from Pacific Biomarine Supply Co., Venice, California and maintained in a seawater aquarium at 14 °C. After the abdominal or pleural ganglion was removed, it was treated in one of two ways. In some cases it was immediately pinned to a clear Sylgard (Dow-Corning) resin in the bottom of an acrylic plastic chamber and the connective tissue capsule above the giant neuron was removed by cutting with a small razor-blade knife. In other cases, the ganglion was placed in an artificial seawater solution (ASW) which contained 5 mg/ml trypsin (Sigma Chemical Co.). The ganglion was incubated in this solution at 33 °C for 30–40 min in order to soften the connective tissue capsule. The ganglion was then quickly transferred to cold (5 °C) ASW, washed several times and then pinned out in the Sylgard resin of the plastic chamber. The capsule was now easily removed using forceps and small iridectomy scissors. The effects of nystatin were the same regardless of the preparatory procedure. However, two general differences were noted: (1) the input resistance of the cells was slightly less after trypsin treatment, and (2) spontaneous post-synaptic potentials were essentially abolished by trypsin pretreatment.

Electrical Recording

Membrane potential (V_m) was measured differentially with conventional glass microelectrodes having tip diameters of less than 1 μm and resistances of 3–10 MΩ when filled with 3 M KCl. The microelectrodes were connected to an electrometer with negative

capacitance compensation, and the output was displayed on both an oscilloscope and a penwriter from which voltage could be read with an accuracy of 0.5 mV.

In the voltage-clamp experiments, a second, current-passing, electrode with a resistance of 2–5 MΩ was introduced into the cell. Only currents at long times compared with the action potential currents were observed. For these currents the potential variation was less than 5%. No attempt was made to clamp the action potential currents or to maintain potential control at depolarizations more than 15–20 mV from the resting potential. The clamping circuit was similar to that used by Brown, Hagiwara, Koike and Meech (1970).

The fabrication, testing, and use of Cl^-- and K^+-sensitive microelectrodes have been previously described (Brown, Walker & Sutton, 1970; Walker, 1970; Russell and Brown, 1972*a*,*b*). The ion-sensitive electrodes were made daily, and each was calibrated in artificial seawater and in a series of KCl solutions varying in activity from 605 to 7 mM, prior to and after the experiment. Selectivities for competing ions were determined from equimolar substitution for K^+ or Cl^- at constant ionic strength. In the case of the K^+-sensitive electrode, the selectivity for K^+ over Na^+ was 100–125:1 and over Li^+ it was about 500:1. The Cl^--sensitive electrode was at least 100 times more sensitive to Cl^- than to SO_4^{2-}. Since the selectivities over competing ions were so great, no corrections were made in the reported K^+ or Cl^- activity data.

The impedance of the ion-sensitive electrodes was 10^{10} to 10^{11} Ω. Consequently, the electrode potentials were measured with a varactor bridge which had an input impedance of 10^{14} to 10^{15} Ω. The output of the varactor was read directly from a digital voltmeter with an accuracy of 1.0 mV.

After calibration the ion-sensitive electrodes were positioned over the giant neuron, and the voltage reading in the external saline (V_0) was noted. The electrode was then advanced into the cell. If the electrode was inside the cell, current pulses passed across the membrane resulted in equal voltage response of the ion-sensitive electrode and the membrane potential-sensing electrode. Intracellular ion activity (a_x^i) was then calculated as follows:

$$a_x^i = (a_x^0)\left[\exp\frac{nFz}{RT}(V_i - V_0 - V_m)\right]$$

where a_x^i, V_0 and V_m are defined above; a_x^0 is the extracellular ion activity, V_i is the electrode potential inside the cell, z the valence of the ion, and n a slope factor to correct for nonideal electrode slopes.

In many of the experiments involving measurement of intracellular ion activities, the current-passing electrode contained 0.3 M K_2SO_4, since the presence of two 3 M KCl-filled micropipettes often caused a noticeable increase in a_{Cl}^i.

Solutions

Detergent-free nystatin was obtained from Sigma Chemical Co., St. Louis, Missouri. It was dissolved in methanol to form a stock solution of 5 mg/ml. Aliquots of this stock solution were added to the various saline solutions to produce the final nystatin concentrations mentioned in the text. For the nystatin experiments, the final concentration of methanol never exceeded 0.8% by volume. In separate experiments, we showed that such concentrations of methanol alone were without significant effects on membrane conductance or intracellular potassium levels.

In these experiments nystatin was applied to neurons bathed in the saline solutions whose composition is given in Table 1. Several considerations went into the design of

Table 1. Solutions (mM)

	KCl	NaCl	LiCl	$MgCl_2$	$CaCl_2$	$MgSO_4$	Sucrose	Bes	Tris	HEPES
Artificial Seawater (ASW)	10	494	—	20	10	30	—	—	10	
Loading solutions:										
NaCl loading solution	—	300	—	—	—	100	394	5.0	0.3	
LiCl loading solution	—	—	300	—	—	100	394	5.0	0.3	
$MgCl_2$ loading solution	—	—	—	150	—	—	515	5.0	0.3	
$CaCl_2$ loading solution	—	—	—	—	150	—	515	5.0	0.3	
Selectivity solutions:										
Univalent-free	—	—	—	—	10	350	600	—	—	10
Chloride	—	—	—	350	10	—	100	—	—	10
Alkali metals ($350X^+$)[a]	—	—	—	—	10	—	Varies[b] with ion	—	—	10
pH 7.4										

[a] $X^+ = Li^+$, Na^+, K^+, Rb^+ or Cs^+ as the sulfate salt.
[b] Total osmolality of all solutions adjusted between 1000–1100 mOsm.

these particular solutions, namely, the choice of ions we wanted to move, and the need to maintain intracellular osmolality and pH. Relying on the data of Holz and Finkelstein (1970) and Cass and Dalmark (1973), we assumed that only univalent ions would permeate and that large, uncharged molecules such as sucrose would not. Therefore, the osmolality of all the solutions was maintained at 1050 mOsm/kg by addition of an appropriate amount of sucrose. The osmolality was checked using a Wescor Model 5100 vapor pressure osmometer. No measurable change in cellular volume could be detected when nystatin was applied in these solutions. The intracellular pH of snail neurons is reported to be 7.5 (Thomas, 1974) and of squid axons, 7.3 (Boron & DeWeer, 1976; therefore, the pH of the nystatin-containing salines was set at 7.4. $MgSO_4$ was included in all solutions because *Aplysia* neurons do not tolerate divalent cation-free solutions. However, the presence of sulfate in the media means that the levels of free alkali-metal cations will be somewhat reduced as a result of the formation of the complex anion, XSO_4^-. The extent to which this complex is formed varies only slightly among the various univalent cations; therefore this complexation was not a problem for determining conductance ratios, but was important for determining absolute permeabilities. For the reaction:

$$X^+ + SO_4^{2-} \rightleftharpoons XSO_4^-$$

the association constants (in units of moles^{-1}) are: Li, 5.2; Na, 8.1; K, 10; Rb, 8.4 and Cs, 8.4 (Sillen & Martell, 1971).

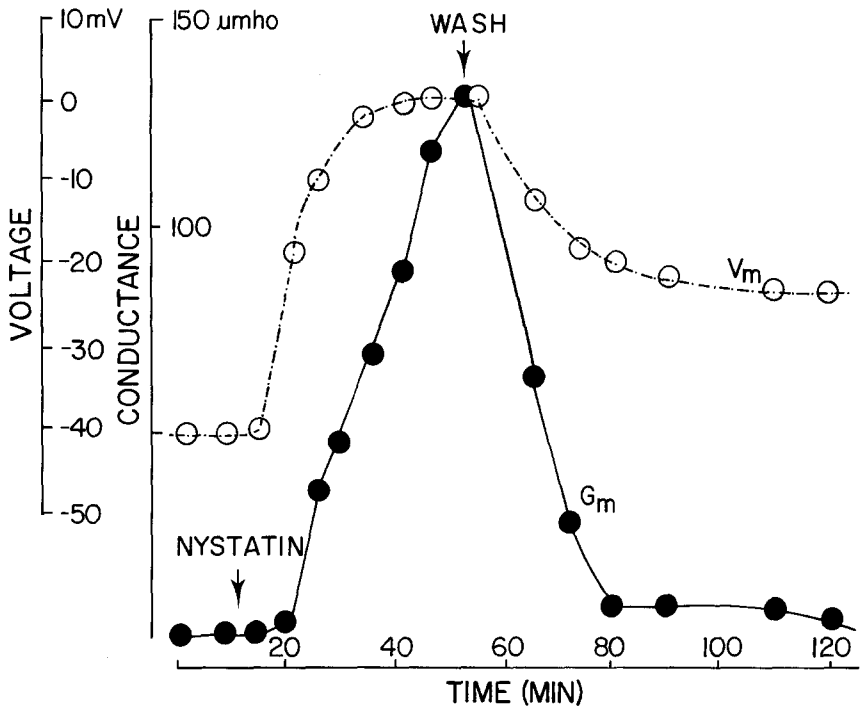

Fig. 1. Effect of nystatin on membrane potential and conductance. The open circles are membrane potential while the filled circles are membrane conductance. At the arrow labeled "nystatin" 22 mg/liter of nystatin in seawater (ASW) was applied. At the arrow labeled "wash" the cell was washed with nystatin-free ASW

Results

Effect of Nystatin on Membrane Conductance and Potential

The concentrations of nystatin which when added to both sides of a thin lipid bilayer give large conductance increases were without effect on *Aplysia* neuron conductance, whereas concentrations in excess of 15 mg/liter were effective in producing conductance increases. This is similar to the situation for red blood cells, epithelia and the one-sided addition to lipid bilayers (Marty & Finkelstein, 1975). Fig. 1 illustrates the effects of applying 20 mg/liter nystatin in ASW on membrane potential (V_m) and conductance.

The onset of the effect was extremely rapid, the time to initial effect corresponding to the wash time of the chamber. The resting membrane potential became significantly more positive, finally reaching a steady-state level at a value just slightly more positive than zero. The conductances, on the other hand, continued to increase for the duration of

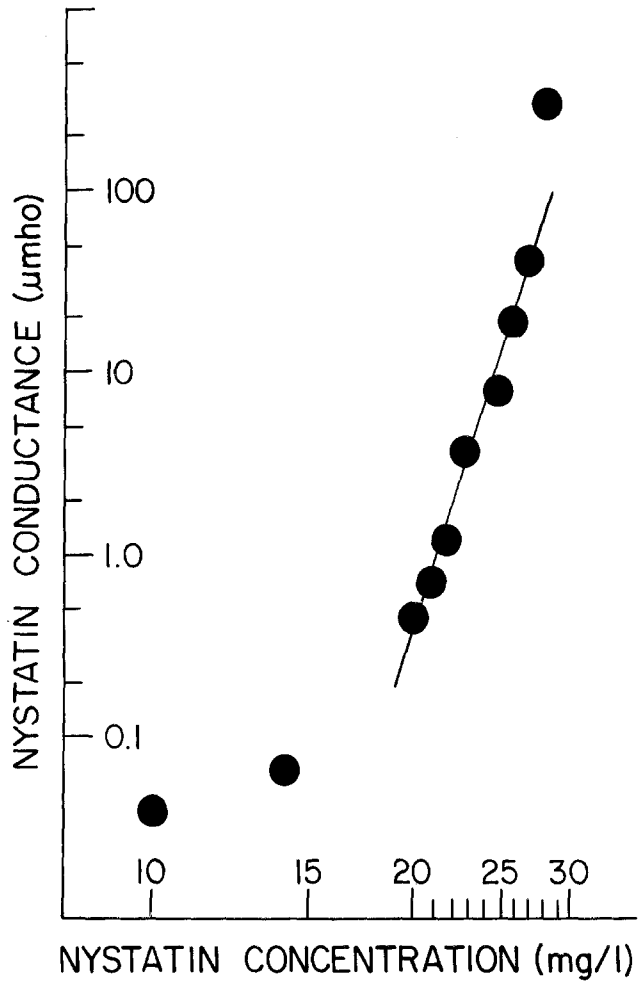

Fig. 2. Dose-response relationship for nystatin applied in ASW. The slope of the line is 3

the exposure to nystatin. In the particular case illustrated, the cell was washed with nystatin-free ASW after the membrane potential had reached a steady state. Under these conditions recovery of the membrane conductance was excellent, even though the membrane potential did not recover completely in the same period of time. However, after an additional 2–3 hr, membrane potential usually recovered completely. The lag in recovery of the membrane potential presumably reflects the time required for the cell to re-establish normal intracellular ionic concentrations.

As mentioned above, we were never able to demonstrate a stable conductance level after nystatin application in ASW. The conductance steadily increased, and if one waited long enough the cell would swell

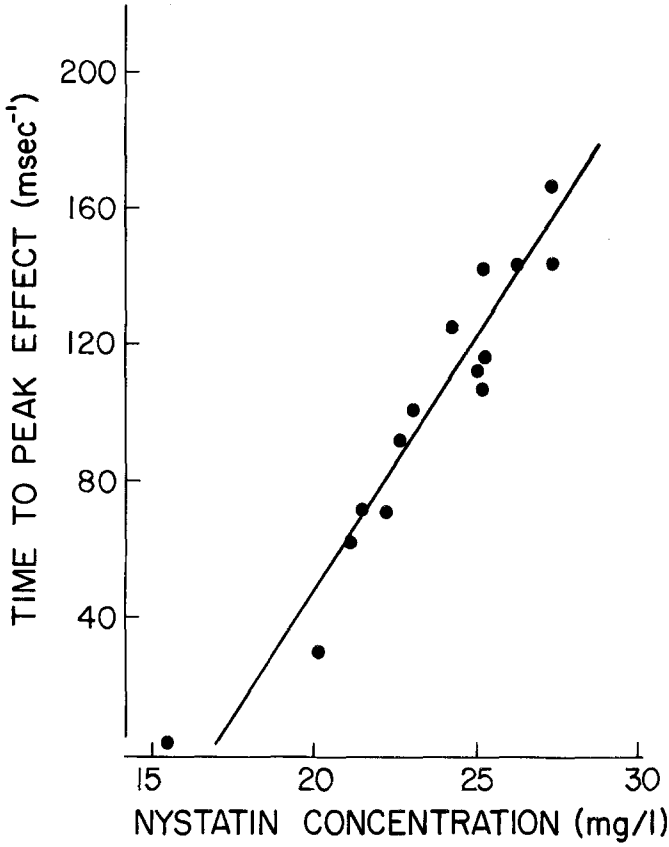

Fig. 3. Time to peak effect on membrane potential *vs.* nystatin concentration. The reciprocal of the time to reach the steady-state potential (near 0 mV) is plotted against concentrations of nystatin. The line is the least-squares regression line

and burst. For this reason, a determination of a dose-response relation at steady state was not possible. Therefore, two methods were used. The first was to wait a prescribed period of time (15 min) after the application of nystatin and measure the membrane conductance at that time. The second method was to measure the time until the membrane potential reached a steady state near zero at each nystatin concentration. The rationale of the latter method was that the voltage plateau represented a standard state of similar ion selectivity and membrane conductance, presumably reflecting equal membrane concentrations of nystatin. The dose-response curve from a single cell using the first method is shown in Fig. 2. The dose-response relation is extremely steep. The solid straight line has a slope of 3. The results of the second method obtained from the same cell are depicted in Fig. 3, showing an approximately linear relationship between nystatin concentration and time to standard state.

Selectivity of Nystatin-Induced Conductance

The virtual absence of membrane potential in seawater after nystatin treatment raises the question of ionic selectivity of the induced conductance. In red blood cells (Cass & Dalmark, 1973) and in bilayer membranes treated with nystatin from one side (Marty & Finkelstein, 1975), the induced conductance pathway is cation selective, while in the case of symmetrical application of nystatin to both sides of the bilayer membrane the conductance is anion selective. The question of selectivity is then relevant from two standpoints; the selectivity properties will not only have a bearing on the sidedness of the action of nystatin on the neuronal membrane, but will also tell us which internal ion concentrations we can expect to easily modify.

For determination of selectivity, the conductance of the membrane while bathed in a nystatin-containing $MgSO_4$ solution (univalent-free solution, Table 1) was used as a reference. The reports of previous workers (Cass & Dalmark, 1973) and our own observations (*see below*) show that divalent ions are essentially impermeable through the nystatin-induced pathway. The exposure time to nystatin during these experiments was limited to less than 10 min for any single determination. Nystatin was not re-applied for another test until membrane conductance had returned to at least 50% of its pretreatment value. Measurements with ion-selective electrodes indicate that at these nystatin concentrations and for such short exposure times, no measurable change in intracellular K^+ or Cl^- levels occur.

When all of the $MgSO_4$ was replaced with $MgCl_2$ (700 mM Cl^-), the membrane potential shifted to more negative values by 23 ± 1 mV ($n=4$); however, the membrane conductance increased by only a factor of 1.14 ± 0.05 when measured at similarly negative values (-80 mV). The latter result suggests that the Cl^- is only slightly more permeable than SO_4^{2-}. On the other hand, when 150 mM $MgSO_4$ was replaced with K_2SO_4 the membrane potential became more positive by 32 ± 4 mV ($n=8$). This change in potential represents not only that due to the nystatin-induced potassium conductance increase, but also the natural depolarization one would expect from a nerve cell membrane with elevated external potassium. In order to obtain an estimate of how much of the conductance increase was due to the action of nystatin the following formula was used:

$$N_K = \frac{g_{K_2SO_4}(\mathrm{NYS}) - g_{K_2SO_4}}{g_{MgSO_4}(\mathrm{NYS}) - g_{MgSO_4}} \tag{1}$$

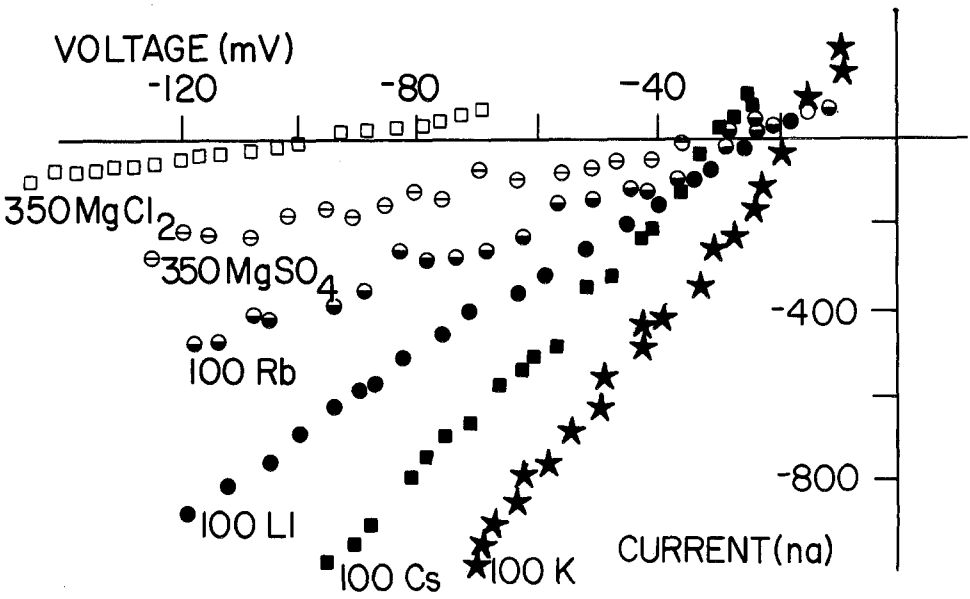

Fig. 4. Current-voltage (I–V) relationships in nystatin. The I–V relationships for 100 mM of the sulfate salt of the alkali metals was obtained after application of 25 mg/liter nystatin. The I–V relationship for a $MgSO_4$ solution and $MgCl_2$ solution with nystatin are also shown for reference

where N_K is the ratio of the nystatin-induced conductance in K_2SO_4 solution to the nystatin-induced conductance in $MgSO_4$ solution, $g_{K_2SO_4}$(NYS) is the conductance in 150 mM K_2SO_4 + 200 mM $MgSO_4$ solution with 25 mg/liter nystatin and g_{MgSO_4}(NYS) is the conductance with 350 mM $MgSO_4$ solution and the same concentration of nystatin. $g_{K_2SO_4}$ and g_{MgSO_4} are the same parameters without added nystatin.

The nystatin-induced conductance in the K^+-containing solution was 4.70 ± 0.35 ($n=8$) times larger than the nystatin-induced conductance in the K^+-free solution. Thus, application of 300 mM K^+ produced a fourfold greater conductance increase than application of 700 mM Cl^- after nystatin treatment. The disparity is even greater when one considers that of the 300 mM potassium added to the solution as K_2SO_4, only 180 mM was present as the free ion (*see* Materials and Methods). Thus, the nystatin-treated neuronal membrane is cation-selective.

In order to examine the selectivity among alkali metal cations, we sequentially replaced 100 mM $MgSO_4$ with the sulfate salts of Li, Na, K, Rb or Cs. We used Eq. (1) to calculate the relative conductance of the

Table 2. Membrane conductance induced by 25 mg/liter nystatin in monovalent cation-containing solutions relative to that in $MgSO_4$-containing solutions

	Li	Na	K	Rb	Cs
	2.5 ± 0.4	3.6 ± 0.6	3.1 ± 0.5	1.7 ± 0.2	3.1 ± 0.7
n	13	12	19	15	6

membrane when it was bathed in solutions containing each of these cations. Fig. 4 shows the current-voltage relationships for a single cell bathed with nystatin and the alkali cation solutions mentioned above. Table 2 summarizes the results from all cells tested. The relative conductances obtained from four of the five cations were statistically indistinguishable. Only Rb substitution yielded a relative conductance significantly less than the other alkali metals.

Temperature Effect on Nystatin-Induced Conductance

In artificial bilayer membranes, nystatin-induced conductance decreases about 10^4 times for a 10 °C temperature rise (Cass, Finkelstein & Krespi, 1970). However, in red blood cells, Cass and Dalmark (1973) have reported that ion permeability increases by only a factor of 2.2 when the temperature is lowered by 10 °C.

We have examined the effects of cooling on nystatin-induced conductance in *Aplysia* neurons in two ways. One was to examine the effect of cooling on the rate of conductance increase. As seen in the first part of Fig. 5 the maximum rate of conductance increase at 22 °C was about 2.3 times greater than that at 10 °C. The second testing method was to cool a neuron that was being treated with nystatin. In the latter part of Fig. 5 it can be seen that such cooling resulted in a fall of membrane conductance. Thus, the results of both methods indicate that, for the *Aplysia* neurons, the Q_{10} for nystatin-induced conductance is positive, rather than negative. The reasons for this qualitative discrepancy are unknown. The effects of the temperature change on the untreated membrane conductance are quite small compared to the conductance of nystatin-treated membrane (*see* Fig. 5) and thus seem unlikely to be responsible for the conductance decrease upon cooling.

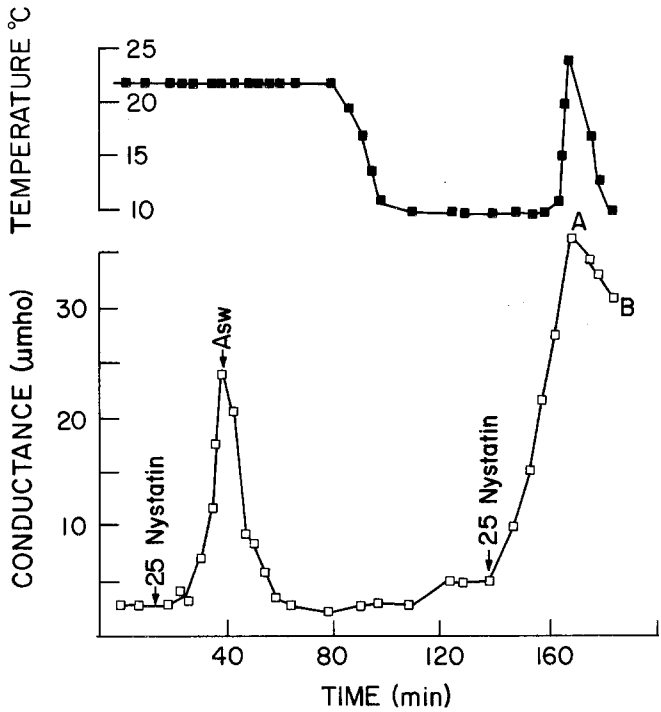

Fig. 5. Effect of temperature on nystatin-induced conductance. The rate of conductance change after application of 25 mg/liter (at arrow) nystatin is shown at two different temperatures. Between *A* and *B* the temperature is reduced, inducing a significant decrease in nystatin-induced conductance

Effects of Nystatin on Intracellular K^+ and Cl^- Activities

When nystatin was added to the ASW in concentrations of less than 35–40 mg/liter only small, slow changes in intracellular ionic activities were noted as measured with ion-selective electrodes. However, nystatin concentrations of 35–40 mg/liter always resulted in rapid changes in intracellular ionic activity. So rapid, in fact, that they often led to irreversible damage due to cell swelling and bursting. Therefore, in order to obtain measurable changes which were reversible, the external bathing solutions used contained only the salt of interest, plus, in the case of the univalent salts, some $MgSO_4$. The total diffusible ion concentration was less than half that in ASW, hence significant cellular swelling was not a problem. The compositions of these solutions are noted in Table 1 as loading solutions.

The general protocol was to apply the nystatin in the appropriate loading solution for 10–15 min. By this time, the membrane input re-

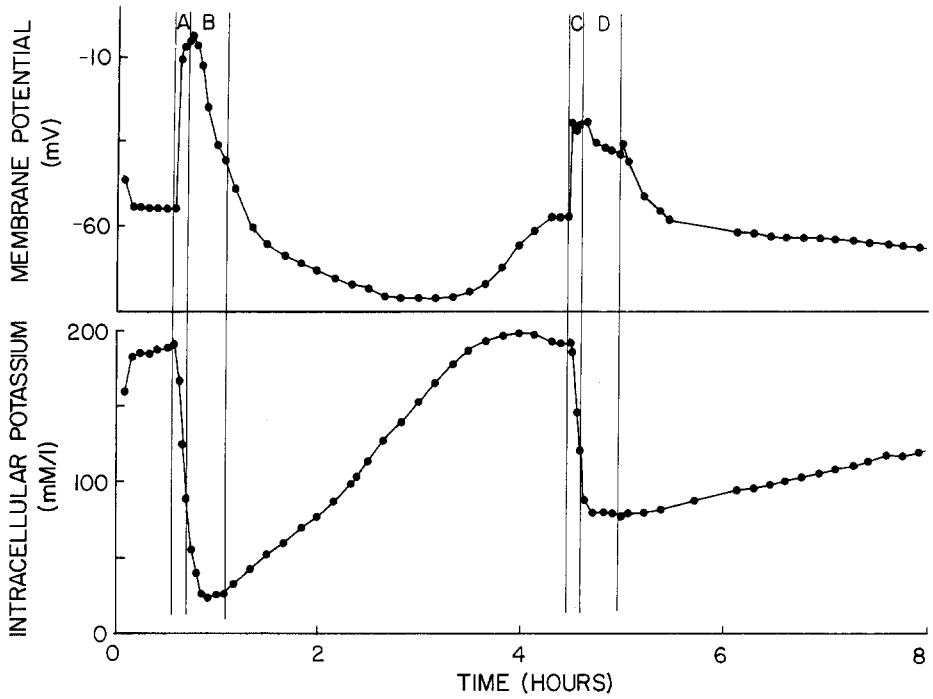

Fig. 6. Effect on intracellular potassium ion activity of NaCl and LiCl loading solution in the presence of nystatin. During segment *A* NaCl loading solution plus 40 mg/liter nystatin was applied to the neuron. Segment *B* represents the wash-out period, during which the cell was superfused with nystatin-free NaCl loading solution. After recovery, the neuron was treated with LiCl loading solution plus 40 mg/liter nystatin (segment *C*) followed by a nystatin wash-out period in LiCl loading solution (segment *D*). Temp. = 19 °C

sistance, as measured by passing hyperpolarizing current pulses from a 0.5 M K_2SO_4-filled microelectrode was too low to measure. Then the nystatin was removed by flushing with a nystatin-free loading solution for about 20–30 min. By that time the input resistance had returned to 25–50 % of control values. Thus, the cell was in contact with the loading solution throughout the period of significantly reduced membrane resistance. The recovery of the neuron was then monitored while it was being bathed with ASW.

When the univalent cation (Na^+, Li^+) loading solutions were applied with 40 mg/liter of nystatin, a rapid depolarization was observed which was greater for the Na^+ than for the Li^+ solution (e.g. *see* Fig. 6). This depolarization coupled with the fact that the external solution was nominally K^+-free resulted in a large net outward driving force on K^+. Under these conditions it was possible to lower a_K^i very quickly to quite low levels (Fig. 6). Recovery of a_K^i occurred readily after treatment with

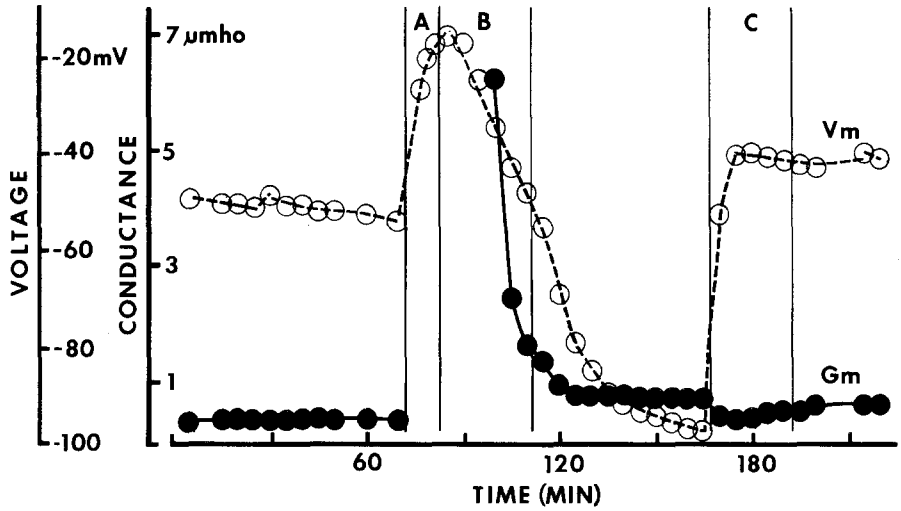

Fig. 7. Effect of ouabain on V_m and input conductance in a Na^+-loaded neuron. Input conductance was calculated as the reciprocal of resistance and measured using a 0.5 M K_2SO_4-filled microelectrode which passed a 1-sec pulse of 2.5 nA of hyperpolarizing current every minute. This resistance was not corrected for nonlinearity of the current-voltage relationship but merely measures the slope resistance at the observed membrane potential. The membrane conductance could not be measured during the nystatin treatment and the early part of nystatin wash-out because of current-passing limitations. The neuron was bathed in ASW until segment *A* when NaCl loading solution plus 40 mg/liter nystatin was applied. During segment *B* the nystatin was removed by washing with nystatin-free NaCl loading solution. Then the neuron was washed with ASW until a clear hyperpolarization was achieved. During segment *C*, ouabain (2×10^{-4} M) was applied. Temp. = 20 °C

the Na^+-loading solution. Recovery from Na^+ loading was accompanied by considerable hyperpolarization which rapidly decreased as a^i_K reached pretreatment levels. This hyperpolarization was probably the result of an increased electrogenic pumping activated by the high $[Na]_i$ since it could readily be inhibited by ouabain (Fig. 7). Li^+ solutions resulted in a much slower recovery unaccompanied by membrane potential hyperpolarization. Li^+ seemingly was less effective in activating [Na+K]-ATPase transport system.

The effects of nystatin applied in these univalent cation-loading solutions on a^i_{Cl} can be seen in Fig. 8. Because of the membrane depolarization, the driving force on Cl^- was inward even though the $[Cl]_0$ of the univalent cation loading solutions was substantially less than that found in ASW. Thus, a 30–40 mM increase in a^i_{Cl} resulted from such treatment. As was the case for K^+, the effects following Na^+ loading solution were reversible within $1^1/_2$–2 hours and recovery was character-

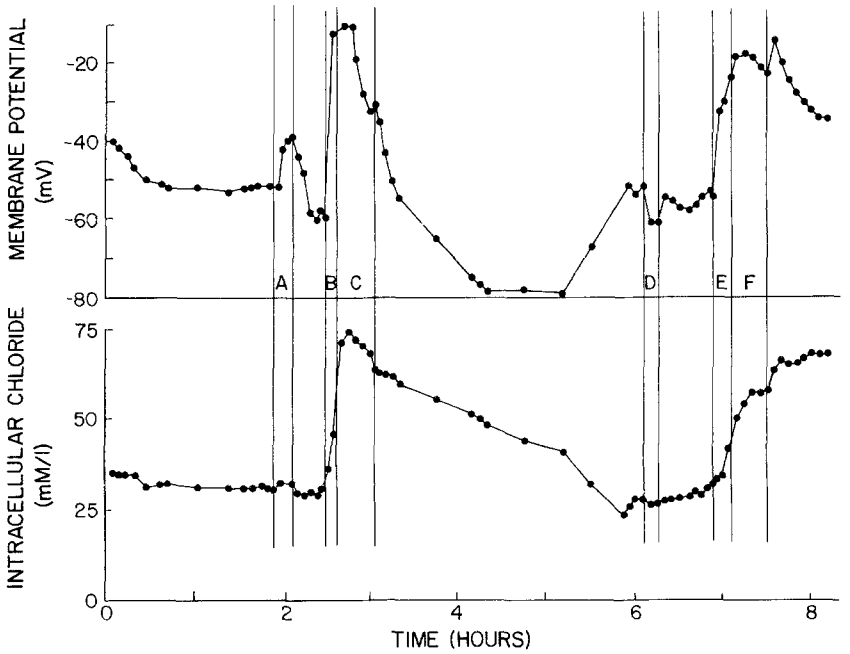

Fig. 8. Effect on intracellular chloride ion activity of NaCl and LiCl loading solutions with and without nystatin. The neuron was superfused with ASW until segment *A* when NaCl loading solution without nystatin was applied. After a short return to ASW the NaCl loading solution plus 40 mg/liter nystatin was applied during segment *B*. The cell conductance was then allowed to recover in NaCl loading solution during segment *C*. The same protocol was followed for LiCl loading solution. Segment *D* = LiCl loading solution alone; Segment *E* = LiCl loading solution + 40 mg/liter nystatin; Segment *F* = LiCl loading solution. Temp. = 19 °C

ized by membrane hyperpolarization. However, following Li^+-loading complete return of a^i_{Cl} to pretreatment levels was very slow. Whether this reflects the lack of membrane hyperpolarization or some direct link between the movements of Cl^-, K^+ and Na^+ is not known.

Application of 40 mg/liter nystatin in the divalent cation (Ca^{2+}, Mg^{2+}) solutions caused V_m to hyperpolarize (*see* Fig. 9). Thus, in this case the electrochemical driving forces on both K^+ and Cl^- were outward. However, neurons treated with the divalent loading solutions lost substantially less K^+ than those treated with nystatin in the Na^+- or Li^+-loading solutions. The reasons for this we believe are as follows. Since macroscopic electroneutrality had to be preserved, the only way a net loss of K^+ could occur was either for a cation to enter or an anion to leave the cell simultaneously with the K^+. Thus, the amount and rate of K^+ movement was ultimately linked to the compositions of the loading

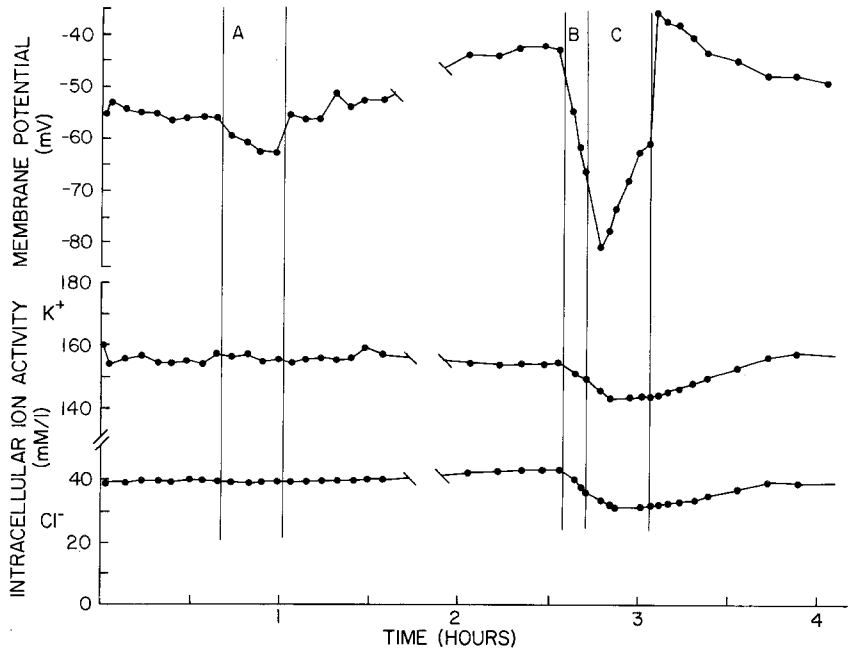

Fig. 9. Effect of 40 mg/liter nystatin applied in $CaCl_2$ loading solution on internal potassium and chloride ion activities measured simultaneously in the same neuron. During segment *A*, $CaCl_2$ loading solution without nystatin was applied. During segment *B*, the neuron was superfused with $CaCl_2$ loading solution plus 40 mg/liter nystatin. Segment *C* represents the period of wash with nystatin-free $CaCl_2$ loading solution. At 96 min all electrodes were removed from the neuron. They were replaced in the cell at 120 min. Temp. = 20 °C

solution and the cytoplasm. In the case of Na^+ or Li^+ loading it appears that these univalent cations were able to move easily through the nystatin-induced pathways to exchange for K^+. The greatly reduced loss of K^+ when Ca^{2+} or Mg^{2+} were the only external cations suggests that neither Ca^{2+} nor Mg^{2+} were able to readily move through the nystatin channel and hence net K^+ loss could occur only by simultaneous loss of Cl^-. In support of this concept is the fact that in three neurons where a^i_K and a^i_{Cl} were measured simultaneously during nystatin treatment, a^i_K fell by 14.3 ± 4.7 mM and a^i_{Cl} fell by an almost identical amount, 12.3 ± 4.4 mM (e.g. *see* Fig. 9). The slight difference may reflect experimental measuring error, a very small divalent cation influx or an unknown internal anion efflux.

A further demonstration of the dependence of net K^+ movements on the ionic milieus can be seen from the results of calculations of the apparent nystatin-induced permeabilities based on the net fluxes of K^+

Table 3. Apparent nystatin-induced potassium and chloride permeabilities in *Aplysia* giant neurons

	Loading solution:			
	NaCl	LiCl	$CaCl_2$	$MgCl_2$
P_K (10^{-8} cm/sec)	207.1 (D)[a] 204.3 (I)	196.2 (D) 202.1 (I)	34.3 (B) 36.1 (F) 103.9 (G)	39.4 (E) 35.4 (H)
P_{Cl} (10^{-8} cm/sec)	33.0 (C) 31.7 (I)	30.7 (C) 28.3 (I)	21.8 (B) 23.1 (F) 31.3 (G)	26.4 (A) 27.3 (E) 15.4 (J)
V_m (mV)	−16 (C) −15 (D) −16 (I)	−23 (C) −36 (D) −28 (I)	−80 (B) −66 (F) −66 (G)	−46 (A) −56 (E) −54 (H) −54 (J)

[a] Denotes the particular experimental neuron from which the data were obtained.

and Cl^-. Net fluxes were calculated according to the following formula:

$$M_x = \frac{(a_x^i)_1 - (a_x^i)_2}{t} \cdot V/A \tag{2}$$

where M_x was the net flux in $M/cm^2 \cdot sec$; $(a_x^i)_1$ and $(a_x^i)_2$ were the directly measured intracellular ion activities at the beginning and end of the flux period; t = time in seconds; V/A = volume to surface area ratio for the giant neurons. Assuming that membrane infoldings give a surface area 13-fold greater than for a smooth sphere (Russell & Brown, 1972*a*) one obtains an average V/A of 6.5×10^{-7} cm. Then the permeabilities were calculated according to the constant field equation as follows:

$$P_x = M_x \cdot \frac{RT}{V_m F} \cdot \frac{1 - \exp(z V_m F/RT)}{a_x^0 - a_x^i \exp(z V_m F/RT)} \tag{3}$$

where R, T and F have their usual meanings; V_m = membrane potential; a_x^i and a_x^0, the directly measured internal and external ion activities and P_x, the nystatin-induced permeability of ion x (either K or Cl^-). Since the loading solutions (Table 1) contain no K^+, a_K^0 will equal zero when P_K is calculated.

The results of such calculations for experiments carried out on 10 neurons can be seen in Table 3. The apparent nystatin-induced P_{Cl} was the same regardless of the cation present in the loading solution or the

direction of the V_m change. However, P_K was much greater when a univalent, presumably permeable, external cation was present than when a divalent, presumably impermeable, external cation was present. The single apparent exception to this was in the experiment labelled G where the apparent P_K was about three times higher than that derived from other divalent cation-treated cells. However, in this case a^i_{Cl} was considerably higher than in the other cells so that much more Cl^- left the cell during the nystatin treatment, hence a greater K^+ loss with the Cl^- over a comparable period of time would be expected compared to the other cells. Since the driving force on K^+ was the same in this cell as the others, the greater K^+ loss was reflected as a greater apparent P_K.

Discussion

The concentration of nystatin required to induce a significant increase of membrane conductance in the *Aplysia* neuronal membrane was much greater than that required for artificial bilayer membranes when nystatin was applied to both sides (Cass, Finkelstein & Krespi, 1970). The requirement for higher nystatin concentration has already been noted for its one-sided addition to artifical bilayers (Marty & Finkelstein, 1975), and red blood cells (Cass & Dalmark, 1973). In view of its potential use as a tool to modify intracellular ion composition, it is particularly useful to know that the conductance increase can be abolished by 10–30 min of washing with nystatin-free media. The relatively slower recovery of membrane potential is probably associated with changes in intracellular ion concentrations, most importantly, the decrease of the intracellular potassium concentration.

Qualitatively, it is clear that the conductance induced by nystatin is cation selective; however, quantitative estimates of the ratio of cation to anion selectivity are difficult to make. The relative anion conductance of the membrane seems to be quite small. But this may be confused by the native conductances of the membrane, since inward Cl^- current occurs in the same region of the current-voltage relationship as the large, natural K^+ outward current. Attempts to block this K^+ outward current with tetraethylammonium ion or tetracaine were not successful enough to reveal an underlying Cl^- conductance. The large shift in membrane potential when $MgSO_4$ is replaced by $MgCl_2$ may seem to argue for a larger chloride conductance than that measured near the zero current point of the current-voltage relationship. However, this discrepancy may

be explained by the fact that E_{Cl} shifts about +160 mV when $MgSO_4$ is replaced by $MgCl_2$. The 14% increase in g_{Cl} together with the large positive increment of driving force can adequately account for the effect on V_m. At higher concentrations of nystatin, a Cl^- permeability approximately three times larger than that reported (Eaton, Russell & Brown, 1975) in the normal cell can be demonstrated by the net flux measurements. At any rate, even though the relative anion permeability is low, it is large enough to permit significant net movements of chloride across the neuronal membrane.

Among the univalent cations there appears to be no large selectivity differences, although the selectivity for rubidium may be somewhat lower than for the other alkali metal cations. The lack of selectivity might be expected if the nystatin conductance pathway acts as an aqueous pore to cations (*see* Ermishkin, Kasumov & Potzeluyev, 1976). In that case selectivity differences would result only from differences in free solution mobility and might be too small to measure in this preparation.

It may be useful to compare the results of applying nystatin in the NaCl loading solution with those obtained in LiCl loading solution. Application of nystatin in either solution resulted in a large net loss of intracellular potassium and a somewhat smaller increase of intracellular chloride. However, the pattern of recovery was quite dependent upon whether the neuron had been loaded with sodium or lithium. When internal potassium had been exchanged for sodium, the return of intracellular potassium towards control values was three to four times faster than when lithium had replaced the intracellular potassium. Furthermore, recovery in sodium-loaded neurons was characterized by a long-lasting hyperpolarization, not seen when lithium was the replacement cation. This hyperpolarization must have been due to an electrogenic pump since the V_m at this time was more negative than the calculated equilibrium potential for potassium, chloride or sodium and it could be entirely blocked by ouabain. V_m exceeded E_K until the internal potassium activity reached a steady value, whereupon V_m depolarized. After loading with lithium, we did not note an after-hyperpolarization even though E_{Li} would be very negative under the conditions of this experiment. Thus, $V_m = E_K$ throughout the recovery from lithium loading. These results suggest that lithium at best only poorly supports potassium uptake and that it cannot stimulate electrogenic pumping. This agrees with results showing that Li^+ could not be actively transported by the Na—K pump (Maizels, 1968) and that ouabain had no effect on Li^+ efflux into Li^+-free media (Haas, Schooler & Tosteson, 1975).

The recovery of intracellular chloride also depended on the cation replacing intracellular potassium. Recovery following loading with lithium was very slow, in some cases, too slow to measure, even though a significant driving force ($V_m - E_{Cl}$) existed. The return of intracellular chloride levels following sodium loading was much faster. This might be due to the increased driving force resulting from the aforementioned membrane hyperpolarization although voltage clamp experiments show that V_m has very little effect on net movements of Cl^- (Russell, *unpublished observations*). Thus, the different rates of net Cl efflux may reflect a relation between net Cl efflux and the activity of the Na—K pump. In support of such a relation, it is known that ouabain can partially inhibit net Cl efflux following a latency of about an hour (Russell & Brown, 1972*a*).

A technique for loading neurons with various univalent ions has been demonstrated. By utilizing high concentrations of nystatin one can effect large changes in internal ionic composition in only a few minutes. The consequences of such changes on electrophysiological and active transport processes can thus be studied since the effects of nystatin on conductance are reversible in about 30 min.

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