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# STUDIES ON THE ACTION OF NYSTATIN ON CULTURED RAT MYOCARDIAL CELLS AND CELL MEMBRANES, ISOLATED RAT HEARTS, AND INTACT RATS*

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**Abstract**—The action of nystatin, a polyene antibiotic, was studied in rat myocardial cells, isolated rat hearts, and intact rats. Myocardial cells responded to 10 and 25 μg nystatin/ml with arrhythmias that could be minimized by elevated concentrations of $K^+$ and $Mg^{2+}$ or reversed by washing the cells. Similarly, the isolated heart responded to 100 μg nystatin/ml with arrhythmias that could be tempered by addition of elevated concentrations of $K^+$ and $Mg^{2+}$. The i.v. injection of the drug caused heart failure in intact animals at the 4-mg/kg dose level. At the subcellular level, nystatin made the myocardial cell membranes more rigid, as measured by electron spin resonance spectrometry. These findings indicate a parallel between physiocochemical changes caused by nystatin in the myocardial cell membrane and the biological changes caused by this drug in myocardial cells, isolated heart, and heart of the intact animal.

The biological action of the polyene antibiotic nystatin [1] is association with its integration into cholesterol-containing cell membranes [2, 3]. This clinically useful antifungal drug has been reported to cause a variety of biochemical and biophysical events in different cells. For example, it can cause reversible efflux of cations ($K^+$, $NH_4^+$ and $Mg^{2+}$) from yeast cells at a concentration of 15 μg/ml [4] and, at higher concentrations, efflux of dyes from plant, fungal and animal cells [5]; it facilitates the entry of certain drug molecules, such as actinomycin, tetracycline and rubiflavin, into yeast cells (but not into mouse fibroblast or Chinese hamster ovary cells) [6]; and it stimulates mouse macrophages [7].

Because of the variety of biochemical and biological effects of nystatin, its simple biophysical mode of action, and our interest in the comparison of *in vivo* and *in vitro* systems, the antibiotic was tested in a rat myocardial cell culture system, the isolated rat heart, and the intact rat. We performed experiments to investigate whether (a) nystatin would cause beat rhythm changes without affecting the overall viability of these cells; (b) any change in beat rhythm would be associated concurrently with membrane alterations; and (c) the action of nystatin in cell culture mimics that in the isolated heart system and possibly the whole animal.

## MATERIALS AND METHODS

*Myocardial cells.* Primary cultures of rat heart muscle cells were established from fifteen to twenty heart ventricles of 5-day-old Osborne–Mendel rats by the successive digestion of 1–2 mm tissue fragments in 0.075% trypsin, according to the method of Bollon *et al.* [8]. The cells were suspended in complete CMRL-1066 medium with L-glutamine (GIBCO, Grand Island, NY) containing: horse serum, 10%; fetal bovine serum (FBS), 5%; insulin (Sigma Chemical Co., St. Louis, MO), 50 μl/ml; gentamycin (Schering Corp., Kenilworth, NJ), 50 μg/ml; and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfuric acid (Hepes), 10 mM, at pH 7.4. After 120 min of incubation at 37° in a humidified atmosphere of 5% $CO_2$–95% air, the myocardial cells were separated from endothelial cells as described by Wenzel *et al.* [9]. The myocardial cells were pooled and counted, and 0.25 to 0.3 $10^6$ cells/ml contained in 3–4 ml of complete medium were plated in Falcon plastic petri dishes (60 × 15 mm). Beating activity was observed 1 day after isolation, and cultures were monitored daily at room temperature at 200× magnification with a phase-contrast microscope only after cells became adjusted to the light (1 min). After 2 days of incubation, the complete medium was replaced with 3 ml of fresh CMRL-1066 medium containing 10 mM Hepes, 10% FBS and 50 μg gentamycin/ml (pH 7.4). In experiment A, one-half of the medium was replaced by CMRL-1066 plus 5% FBS on day 4, and in Experiment B, all the medium was replaced with CMRL-1066 plus 5% FBS on day 6.

Nystatin (U.S.P. Standard; microbiological potency, 5800 units/mg) was dissolved in dimethyl sulfoxide (DMSO) and diluted with 0.01 M phosphate-buffered saline (PBS, pH 7.2) to obtain a nystatin suspension containing 10% DMSO. The beating myocardial cells, after 7 days in culture, were treated with nystatin at 10 or 25 μg/ml; the final concentration of DMSO in treated plates did not exceed 0.33% and the control plates contained

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0.33% DMSO. Beats/min were recorded for approximately 15–20 cells or cell aggregates/dish in control cultures but only 2–7 cells or cell aggregates/dish in nystatin-treated cultures because the antibiotic caused the immediate cessation of beating in many areas. The percentage of areas beating was determined by examination at 200× magnification of five to ten randomly selected areas per dish. The number of myocardial cells as individuals or aggregates in the area was recorded as well as the number of individual cells or aggregates with beating activity, expressed as a percentage, and averaged. If the entire area beat as a syncytium, the area was recorded as 100%. Within 15 min of the final observation, cells were prepared for membrane fluidity measurements by electron spin resonance (ESR) spectrometry.

In separate experiments, the effects of elevated concentrations of $K^+$ and $Mg^{2+}$ were investigated by adding KCl and $MgSO_4$ to cultures 1 hr after the addition of nystatin at 25 μg/ml. The concentration of $K^+$ was increased to 0.3 M from the original 0.06 M present in the medium, and the $Mg^{2+}$ concentration was increased to 0.4 M from the original 0.08 M.

*Electron spin resonance spectrometry.* For spin labeling, the myocardial cells were treated with 0.25% trypsin contained in 0.01 M PBS, pH 7.2, for 5–7 min, and a 0.1-ml aliquot of cells was taken for cell count as determined by the trypan blue dye exclusion test. The remaining cells were centrifuged at 2000 rpm for 3 min and washed twice with PBS at 20°. The cells were then centrifuged and suspended in 0.1 ml PBS, and the suspension was transferred to a glass centrifuge tube containing 3 μg 5-doxyl stearate (Syva Co., Palo Alto, CA) deposited from ether solution. After a contact time of 30 sec at 8–10°, the cells were sealed into a micropet capillary (Clay Adams Co., Parsippany, NJ) with Critoseal (Syva Co.).

The ESR spectra of the myocardial cells were recorded with a Varian E-9 Century series spectrometer (Varian Associates, Inc., Palo Alto, CA) operated at 9.5 kHz and 100 kHz field modulation. The temperature of the probe was maintained at 20° by a Varian variable temperature accessory.

Order parameters ($s$) were calculated from the spectra by the following expression:

$$s = (A_{\parallel} - A)/[A_{zz} - 1/2\,(A_{xx} + A_{yy})]$$

where $A_{\parallel} = 1/2\,A_{max}$ and $A = 1/2\,A_{min} + 0.8$ G. $A_{max}$ and $A_{min}$ are the maximum and minimum hyperfine splittings measured from the spectrum. $A_{xx}$, $A_{yy}$ and $A_{zz}$ are 6.36, 5.8 and 33.6 G, respectively, for 5-doxyl stearic acid; these values were obtained from the available single crystal ESR data. The order parameter provides a measure of the mean angular deviation of the hydrocarbon chain from its time-averaged orientation in the lipid bilayer. A less precise expression of the fluidity of the membranes, the $2T_{\parallel}$, was also used to express results, and is the distance, in G, between the maximum and minimum hyperfine splitting values.

ESR spectra obtained from membranes of intact cells with the above spin-labeled fatty acid showed contributions mostly from the spin label of restricted motion with negligible contributions from the freely moving spin label. The spin label concentrations were kept low enough to avoid spin–spin interaction. Accuracy of the ESR measurement was ± 0.2 G.

*Isolated rat heart.* Male Sprague–Dawley rats weighing 400–600 g were obtained from the Charles River Breeding Laboratories, Wilmington, MA, and were randomly assigned to control or experimental groups. The animals were anesthetized with sodium pentobarbital (13 mg/kg, i.p.), and a Langendorff preparation [10] was set up with 52 cm of water perfusion pressure. The heart was rapidly removed into a dish of Krebs–Henseleit solution. The heart was freed by dissection, and the aorta was attached to a cannula for continuous perfusion with Krebs–Henseleit solution (pH 7.2) at 36° and was oxygenated continuously with a mixture of 95% $O_2$ and 5% $CO_2$. The coronary flow was measured by collecting the perfusate into a graduated cylinder. Intrinsic heart rates were recorded by means of a pair of electrodes attached to the surface of the heart and connected to a Sanborn instrument for electrocardiographic (ECG) recording. The heart preparation was allowed to stabilize for 15–20 min, and the heart rate and coronary flow were then measured. Nystatin, suspended in 5% DMSO–Krebs–Henseleit solution, was injected into the aortic cannula at doses of 100–400 μg in a volume not exceeding 0.2 ml. After each drug exposure, the heart was washed with Krebs–Henseleit solution until the ECG baseline equilibrated. A heart preparation was used for repeated testing until its ECG showed the same pattern as it had before the first addition of the drug.

In studies to determine the effect of elevated concentrations of $K^+$ and $Mg^{2+}$, a Krebs–Henseleit solution was prepared to contain 10-fold concentrations of KCl (3.5 g/l) and $MgCl_2$ (2.9 g/l). The injection of this solution (3–6 ml) was started 15 sec before nystatin and was continued until the end of the nystatin administration (about 30 sec); the volume used was about the same as the coronary flow measured before the experiment. In control experiments, nystatin administration was omitted.

*Intact rats.* Male Sprague–Dawley rats, 4- to 5-months-old, weighing 500–700 g and specified as "heavy" animals, were obtained from the Charles River Breeding Laboratories. The unrestrained rats were placed in individual cages, electrodes were implanted s.c., and ECGs were recorded at 5-min intervals with a Beckman polygraph (Beckman Instruments, Fullerton, CA) at various speeds (10, 25, 50 mm/sec). The nystatin–DMSO solution (10% DMSO) was prepared fresh before each experiment and was administered at doses of 1–10 mg/kg into the tail vein. DMSO was injected into the tail vein at the high dose volume for control purposes. Experiments were carried out three times and the ECGs of the surviving animals were followed for 24 hr after injection.

## RESULTS AND DISCUSSION

*Myocardial cell cultures.* Myocardial cells have been observed to beat for more than 2–3 weeks *in vitro*. The beating rhythm of the cells can be observed with the microscope [11] or by an electronic monitoring system [12]. The inherent ability of these cells

Table 1. Effect of nystatin on myocardial cells in culture with time expressed as beating rate, percentage of areas beating, and membrane fluidity*

| | Experiment A | | | | | | Experiment B | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | | Nystatin (10 μg/ml) | | Nystatin (25 μg/ml) | | Control | | Nystatin (10 μg/ml) | | Nystatin (25 μg/ml) | |
| Measurement | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| Beats/min at post-treatment time (min) | | | | | | | | | | | | |
| 0 | 26 ± 6 (94) | 46 ± 10 (100) | 24 ± 8 (100) | 45 ± 7 (100) | 37 ± 12 (100) | 41 ± 10 (100) | 26 ± 8 (100) | 27 ± 6 (100) | 22 ± 6 (100) | 19 ± 1 (100) | 17 ± 5 (100) | 22 ± 4 (100) |
| 1–12 | | | | | 126 ± 6 (8) | 124 ± 12† (10) | | | | | 122 ± 24 (9) | 110 ± 22† (19) |
| 15–45 | 38 ± 7 (88) | 45 ± 8 (83) | 97 ± 1 (11) | 0 | | | | | 105 ± 11† (27) | 122 ± 4 (47) | | |
| 50–100 | 42 ± 11 (88) | | 85 ± 8 (20) | 0 | 132 ± 33† (18) | 0 | 45 ± 8 (83) | 32 ± 8 (88) | | | | |
| 101–200 | | 51 ± 11 (100) | | | 122 ± 45 (12) | | | | 110 ± 16 (11) | | 124 ± 9 (28) | |
| 201–311 | 38 ± 8 (82) | 41 ± 10 (84) | 0 | 59 ± 4 (20) | | 123 ± 15 (29) | 18 ± 13 (47) | 30 ± 9 (97) | | 106 ± 30† (12) | | 106 ± 26 (38) |
| No. viable cells recovered ($\times 10^6$) | 1.12 | 0.59 | 0.93 | 1.05 | 0.85 | 1.18 | 1.24 | 1.37 | 1.25 | 1.35 | 2.56 | 1.27 |
| Membrane fluidity, $2T_{\|\|}(G)$ | | 53.5‡ | | 52.2‡ | | 52.7‡ | 53.5 | 53.5 | 51.5 | | | 52.0 |
| Order parameter ($s$) | | 0.632 | | 0.627 | | 0.612 | 0.632 | | 0.620 | | | 0.612 |

* Myocardial cells were initially plated at $0.9 \times 10^6$ cells/dish in Experiment A and $0.74 \times 10^6$ cells/dish in Experiment B. The numbers 1–6 refer to tube or culture number for each experiment. Beats/min were recorded for 15–20 control cells/dish and for 2–7 treated cells/dish; values are given as means ± S.D. Percentages of areas beating are given in parentheses.

† Arrhythmia.

‡ Cell pellet combined with that from duplicate dish.

to beat is influenced by the $Ca^{2+}$ transport of the cellular membrane, which can be separated from other factors influencing the overall viability of the cells [13].

The results of two representative experiments with duplicate myocardial cell preparations per experimental condition are shown in Table 1. Nearly all the cells displayed beating activity before treatment, as reflected by 100% of areas beating. Within 1 min after exposure to nystatin at 25 μg/ml, many microscopic areas showed no beating cells and the remaining beating cells had accelerated rates at three to seven times greater than those cells before treatment. A strong arrhythmia was also observed as the cells beat rapidly, stopped and again beat rapidly. This remarkable change in beating rate is reflected by the often high standard deviations associated with these observations. The accelerated beating rate of some cells and the reduced areas of beating continued throughout the 4- to 5-hr observation period without a loss of cell number.

In contrast, the control cell cultures did not change appreciably during the observations when measured as average beats/min or as percentage of areas beating. The effects of 10 μg nystatin/ml appeared to be similar to those caused by the 25-μg/ml dose in that accelerated beating rates were produced in some cells and few or no beats were observed in some areas; however, the arrhythmia was not as pronounced at this dose level.

To test the possibility that nystatin-caused arrhythmia is due to excessive ion efflux through the cellular membrane, elevated concentrations of $K^+$ and $Mg^{2+}$ were added to the cell culture. Sutton *et al.* [4] previously demonstrated that a low concentration of a polyenic antibiotic, like nystatin, results in excessive efflux of $K^+$ and $Mg^{2+}$ from yeast cells and that the effect of this efflux could be counteracted by adding these ions to the medium in elevated concentrations. Therefore, myocardial cells were treated with elevated concentrations of the ions at the time when the arrhythmia was clearly visible. All cells treated with nystatin alone stopped beating 24 hr after treatment while cells treated with nystatin and elevated concentrations of $K^+$ and $Mg^{2+}$ were beating slowly, in a somewhat arrhythmic fashion, for an additional 24–30 hr. However, most myocardial cells treated in either of the above ways (nystatin treatment with or without elevated concentrations of $K^+$ and $Mg^{2+}$) recovered the regular beat rhythm (40–60 beat/min) in 24 hr when they were washed and the medium was changed 2 days after drug treatment. This finding indicates that nystatin can be washed out from cellular membranes, and that the cell membranes regain their original status in 24 hr.

It was postulated that integration of nystatin into the cellular membrane caused perturbations that, in turn, caused the ion efflux and possibly other changes [6, 14]. Therefore, nystatin-treated and untreated cells were harvested by trypsinization and spin labeled for ESR measurements; ESR spectrometry was used because it is an excellent tool in assessing cell membrane fluidity [15, 16]. ESR spectra are shown in Fig. 1, and the calculated fluidity parameters $2T_\parallel$ and $s$ (order parameter) are given in Table 1.

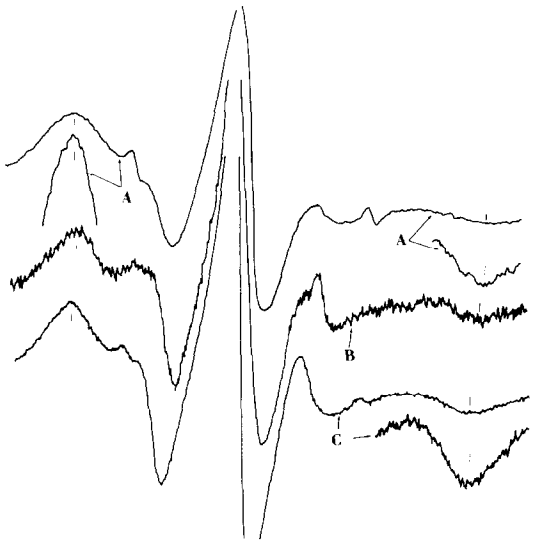


Fig. 1. ESR spectra of control and nystatin-treated myocardial cells. Key: A, untreated cells; B, nystatin 25 μg/ml; and C, nystatin 10 μg/ml.

Data derived from the ESR measurements indicated that myocardial cells treated with nystatin for 4–5 hr had cellular membranes with higher fluidity ($2T_\parallel$ = 51.5 to 52.7 G) than those of the control cells ($2T_\parallel$ = 53.5 G). The magnitude of the observed changes in the $2T_\parallel$ values (and order parameter values) is significant. ESR changes of a similar magnitude were found by Esser and Russell [17] in their macrophage activation studies and by Trudell *et al.* [18] in their studies of the effect of anesthetic agents on phospholipid vesicles. Butterfield *et al.* [19] also found a comparable magnitude of ESR spectral changes when comparing normal and myotonic erythrocyte membranes. These authors found in their studies, as we did in ours, that the observed ESR spectral changes are reproducible and consistent if experiments are carried out with appropriate controls. Because nystatin was shown to be removable from cell membranes by washing [4, 14], the ESR-indicated membrane fluidity changes must be associated with changes, indicative of permanent residual membrane changes, that remain after the 4 hr of nystatin treatment. This conclusion is supported by our finding that nystatin treatment after spin labeling and just before ESR measurement appeared to cause an opposite membrane fluidity alteration, i.e. the myocardial cell membranes became more rigid. In this latter experiment, untreated myocardial cells were trypsinized, washed and spin labeled so that there was no free spin label in the aqueous phase. The cells were then treated with 10 μg nystatin in suspension in 10 μl PBS; the subsequent ESR measurement indicated decreased membrane fluidity. Figure 2 shows the ESR spectra of the untreated cells and the nystatin-treated cells; the $2T_\parallel$ values were 53.3 and 55.0 G for the untreated and treated cells respectively. When these nystatin-treated cells were washed with PBS and again spin labeled, they regained their

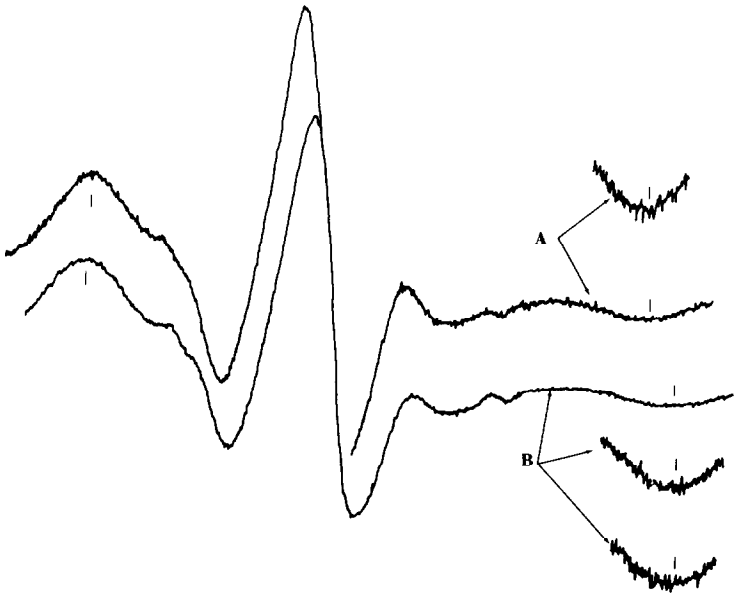


Fig. 2. ESR spectra of myocardial cells treated with nystatin after spin labeling. Key: A, untreated cells; and B, nystatin 10 μg/ml.

approximate original fluidity of $2T_{\parallel} = 53.2$ G (curve not shown).

ESR measurements of cells treated with nystatin and elevated concentrations of $K^+$ and $Mg^{2+}$ indicated that cells treated in this manner had the same membrane fluidity ($2T_{\parallel} = 52.1$ G) as those that were treated with nystatin (25 μg/ml) alone ($2T_{\parallel} = 52.0$ G) The relevance of measuring fluidity of cell membranes under conditions in which the spin probe is added to the cells before nystatin shows that the inherent properties of the membrane change toward decreased fluidity and not increased fluidity as shown with culture condition. Therefore, the change in functionality (arrhythmia and cessation of beat rate) parallels an increase of membrane fluidity and represents interaction between membrane alteration and biological function of the cell. We assume that membrane potential changes play a role in this interaction and that is the subject of future studies.

*Isolated rat heart.* Experiments were performed to investigate the possibility that the action of nystatin on the isolated rat heart preparation was analogous to its action on myocardial cells in culture. Heart rates were recorded first with the maximum dose of solvent and then with different nystatin doses. For the initial dose, 100 μg nystatin was selected because it approximated the dose used in cell cultures (3 ml medium/dish) and the volume of a rat heart was estimated to be about 3–4 ml. This 100-μg dose injected into the aortic cannula resulted in visible arrhythmias; however, ECG baseline and perfusate volume returned to the initial values of the untreated heart within 4–5 min (Fig. 3). The same overall results were obtained with doses of 200 and 300 μg, except that the arrhythmias lasted longer. A 400-μg dose caused severe arrhythmias followed by permanent cessation of the beat in all rat hearts tested. It appears that the extent of the arrhythmias and the

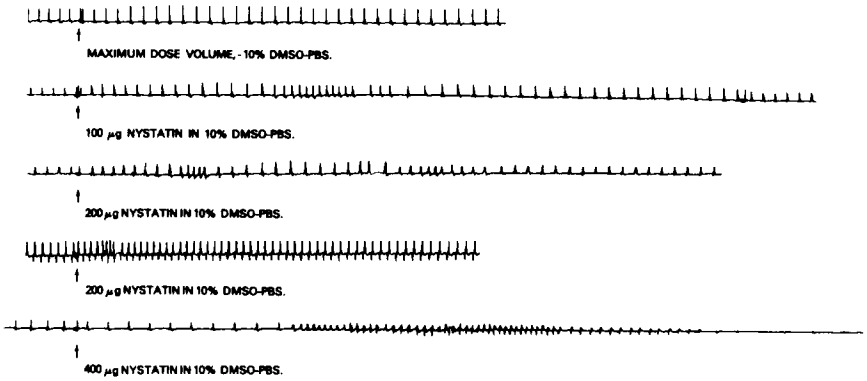


Fig. 3. ECGs obtained from isolated rat hearts treated with nystatin.

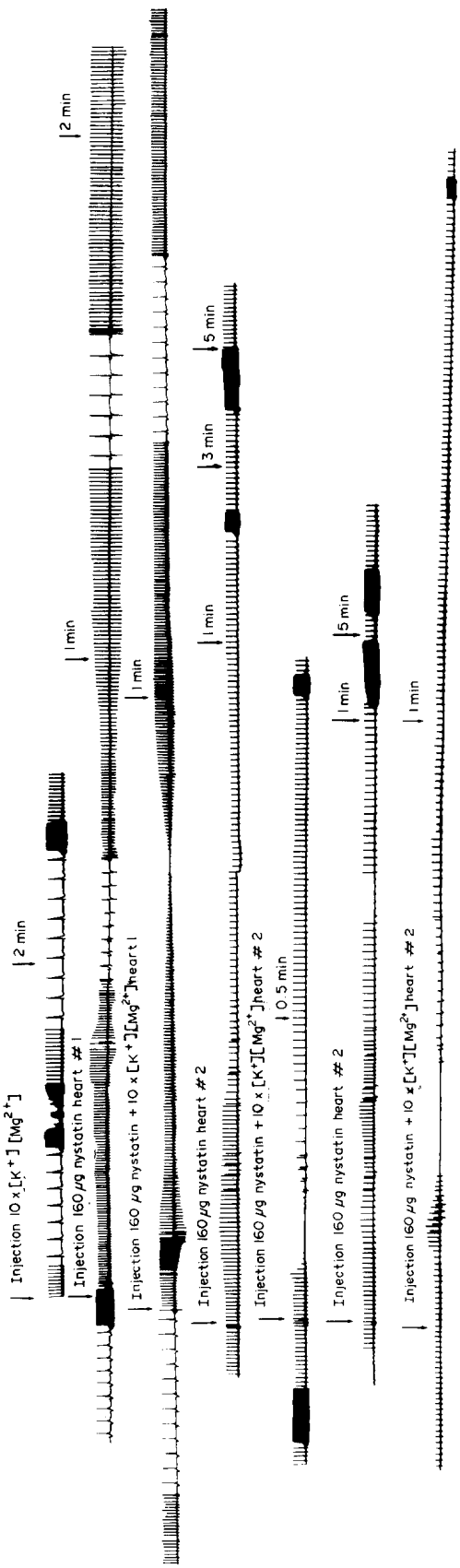


Fig. 4. ECGs obtained from isolated rat hearts treated with nystatin and elevated concentrations of $K^+$ and $Mg^{2+}$.

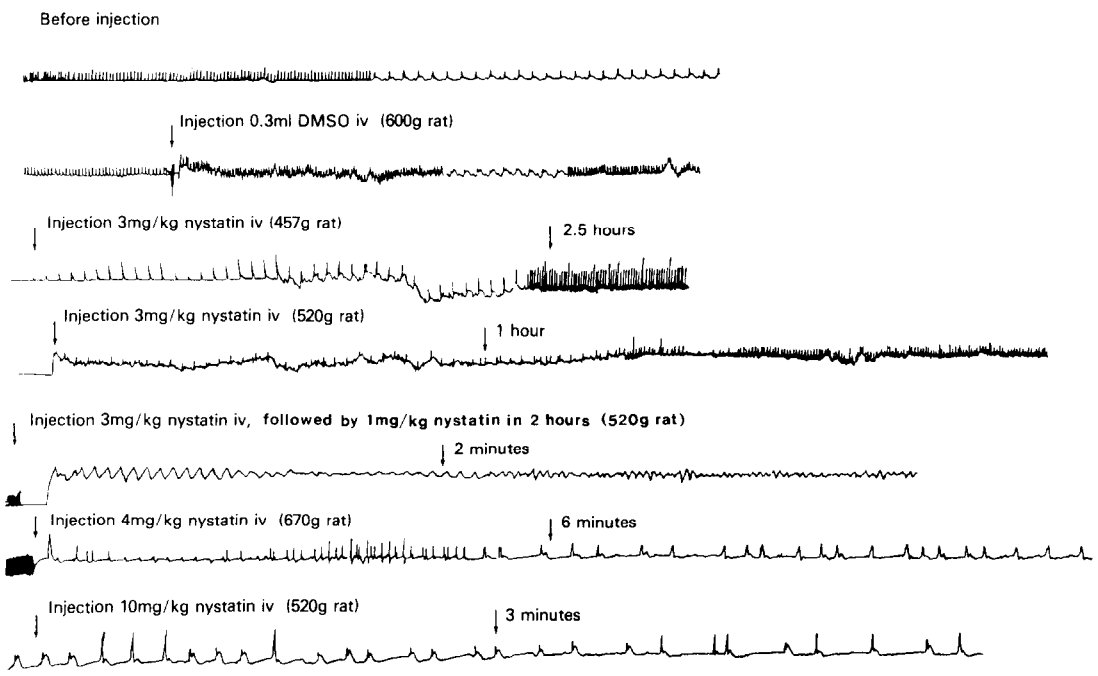


Fig. 5. ECGs obtained from intact rats treated i.v. with nystatin. ECG chart speed was adjusted during experiments according to the duration of the experiment to show ECG patterns at various times. At doses of 4 mg/kg and greater, some pulse activity was present after 3 min; however, the animals stopped breathing, indicating death of the animal in 3–5 min.

recovery periods are dose dependent between doses of 100 and 300 $\mu$g nystatin. However, slight variations in response were detected among individual heart preparations.

The effects of elevated concentrations of $K^+$ and $Mg^{2+}$ on the behaviour of nystatin-treated heart preparations were tested for comparison with those obtained with the cultured myocardial cells. A clear difference in the recovery time from the arrhythmias was observed when nystatin (160 $\mu$g) alone and nystatin and elevated concentrations of $K^+$ and $Mg^{2+}$ were administered (Fig. 4); recovery of the original ECG baseline was two to three times more rapid with the elevated concentration of ions. Krebs–Henseleit solution containing the high $K^+$ and $Mg^{2+}$ concentrations given alone caused a very short period of arrhythmia (15–20 sec). The combination of nystatin and Krebs–Henseleit solution with elevated concentrations of $K^+$ and $Mg^{2+}$ produced arrhythmias with a slightly different pattern than those obtained with either nystatin or the solution alone; however, the recovery time was invariably shorter with the combination. We concluded from these experiments that nystatin induces arrhythmias and heart rhythm changes in the isolated heart preparation similar to those observed in cultured myocardial cells. As in cell culture, nystatin seemed to disturb the ion balance of myocardial cells of the isolated heart. It should be pointed out, however, that nystatin and the additional ions were administered simultaneously to the isolated heart because of the constant washing-out effect of the perfusate.

*Intact rats.* The experiments with intact rats were performed to assess the cardiac effect of nystatin *in vivo*. For this purpose, the i.v. route was used and cardiac function was recorded by the Beckman polygraph. The baselines were recorded before and after the administration of DMSO, and nystatin doses of 1, 2, 3, 4 or 10 mg/kg were then injected. The 4- and 10-mg/kg doses were cardiotoxic, as indicated by severe arrhythmias and fibrillation in the ECG within 2–3 min and death of the animals within 3–5 min (Fig. 5). Doses of 1, 2 and 3 mg/kg caused no visual change in the ECG patterns. However, a cumulative dose of 4 mg/kg, e.g. a 3-mg/kg dose followed by a 1-mg/kg dose in 2 hr, caused the same symptoms as a single 4-mg/kg dose. We concluded from these experiments that nystatin has a direct cardiac effect in rats when administered i.v. Other routes of administration and elevating the ion concentration of the blood were not considered in these experiments for pharmacological reasons.

We concluded from the above experiments that nystatin reversibility perturbs the membrane of isolated myocardial cells and causes arrhythmia in these cells in culture and in isolated rat heart. Nystatin given i.v. to rats causes death due to heart failure. However, it should be noted that nystatin is generally used for topical treatment and not systemically. In addition, in animal studies in which macrophage activation [7] was achieved with 0.05 mg nystatin/kg, the applied dose was 50–200 times less than that used in these *in vivo* studies.

Nevertheless, it appears from this study that a parallel exists for the effect of nystatin on cultured myocardial cells, myocardial cell membranes, isolated hearts, and whole animal systems. It might be speculated that if physicochemical changes are

observed in a cell membrane upon treatment with a drug, parallel biological changes could be expected for these cells and perhaps for the corresponding organ *in vitro* and *in vivo*. Such a parallel may be brought about by drugs other than nystatin, provided the effect of the drug on myocardial cell membranes is based on the same biophysical principles and its pharmacodynamic properties are similar to those of nystatin.

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