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AN ALTERED RESPONSE OF VIRALLY TRANSFORMED 3T3 CELLS TO OUABAIN

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Summary

The effect of ouabain on K^+ transport was examined in 3T3 and virally transformed 3T3 cells. A 10 min exposure to ouabain (10^{-3} M) produced approximately 40% inhibition of the unidirectional K^+ influx in all cell lines. In 3T3 cells the response was not significantly altered by up to 70 min exposure to the drug. In contrast, the continued exposure of transformed cells to ouabain produced a time-dependent increase in the K^+ influx. This increased influx was shown to be accompanied by an increase in the K^+ efflux. The results suggest that, in transformed cells, ouabain produces both an inhibition of Na^+-K^+ exchange and a stimulation of K^+-K^+ exchange. The latter was shown to be more readily reversible than the former.

Introduction

The physical and biochemical properties of the plasma membrane of normal and virally transformed cells are different. Transformed cells show greater agglutinability with concanavalin A [1], and contain altered proteins [2], glycoproteins and glycolipids [3,4]. Transformed cells often show increased rates of membrane transport [5,6]. Recently, several reports have appeared in which K^+ transport in normal and transformed cells was compared [7—12]. In such studies it is desirable to separate the total K^+ influx into its active and passive components. For this purpose, ouabain, an inhibitor of ($Na^+ + K^+$)-ATPase [13], is usually used. During the course of experiments designed to investigate the effects of cell population density on K^+ transport in 3T3 and virally transformed 3T3 cells [14] we observed a qualitative difference in the response of the transformed cells to ouabain.

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Materials and Methods

Cell culture procedures. Swiss 3T3 and SV3T3 cells were originally obtained from Flow Laboratories Ltd. Py3T3 cells were kindly supplied by Dr. E. Steinhardt, Institute of Virology, University of Glasgow. Each cell line was restarted from a frozen supply at approximately 10-week intervals. Cells were grown in Dulbecco-Vogt modified Eagle's medium [15] with 10% calf serum at 37°C in 5% CO_2. Stock cultures were grown in Roux bottles and subcultered every three to four days. For experimental purposes, $5 \cdot 10^5$ cells were seeded in plastic culture dishes (Nunc, 9 cm diameter) and used two days later during exponential growth.

Measurement of K^+ uptake. Cells were incubated at 37°C for periods of time in $^{86}Rb^+$-labelled Krebs solution [16] and then washed in ice-cold Krebs solution to remove extracellular activity. The washed cells were detached from the dish by incubation at 37°C with 2 ml of a 0.025% solution of trypsin in Krebs solution minus calcium and magnesium. 10 ml Krebs solution were added to the dish and a suspension of single cells was formed by aspiration. A 1 ml sample of the cell suspension was used to determine the cell number and mean cell volume (Coulter Counter) as described previously [17]. The $^{86}Rb^+$ content of the remaining suspension was measured by detection of Cerenkov radiation in a liquid scintillation counter [18].

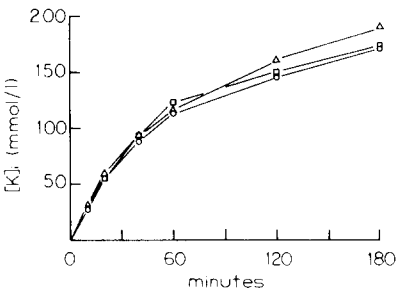
Measurement of K^+ washout. Cells were incubated at 37°C for 3 h in $^{86}Rb^+$-labelled Krebs solution and then washed in ice-cold Krebs solution to remove extracellular activity. 10 ml Krebs solution were added to the dish which was then incubated at 37°C. After 5 min the collecting solution was quickly poured into a scintillation vial for $^{86}Rb^+$ counting and a further 10 ml of inactive Krebs solution were added to the dish. This procedure continued for a total of 30 min. The cells were then trypsinised and the cell number, mean cell volume, and remaining cellular $^{86}Rb^+$ activity determined.

Results

$^{86}Rb^+$ was used as a K^+ tracer in this work. Preliminary experiments using $^{42}K^+$, and reports from other laboratories [7,19], justified the substitution of $^{86}Rb^+$ for the short half-life $^{42}K^+$ isotope.

The time-course of total K^+ uptake in 3T3, Py3T3 and SV3T3 cells is shown in Fig. 1. The results indicate that K^+ uptake was linear within 10 min in all cell lines and 10 min was, therefore, chosen as a suitable incubation period for measurement of the initial rate of K^+ uptake (influx). In this experiment the 3T3 cells were at a density of $1.5 \cdot 10^4$ cells/cm^2 and the transformed cells at $2.8 \cdot 10^4$ cells/cm^2. Under these conditions there was no difference in the K^+ influx between the cell lines.

It is well known that ouabain causes an inhibition of Na^+-K^+ exchange across cell membranes [13]. In 3T3 cells a ouabain concentration of 10^{-3} M was required to produce a 40% reduction of the K^+ influx. The response was not significantly altered by up to 60 min pretreatment with the drug (Fig. 2). This result demonstrates, in agreement with others [20,21], that cultured mouse cells retain the low sensitivity of the species to ouabain. When the experiment was



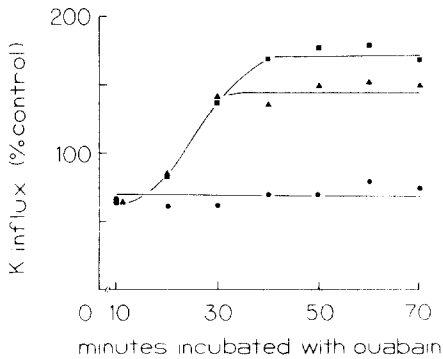


Fig. 1. The time-course of K^+ uptake by 3T3 (○———○), Py3T3 (□———□) and SV3T3 (△———△) cells. The K^+ concentration of the Krebs solution was 5.4 mmol/l and the $^{86}Rb^+$ activity was approximately 0.2 μCi/ml.

Fig. 2. The effect of ouabain on the K^+ influx in 3T3 (●———●), Py3T3 (■———■) and SV3T3 (▲———▲) cells. The growth medium was removed and the cells were incubated at 37°C in 10 ml Krebs solution with or without ouabain (10^{-3} M) for 0—60 min. This solution was then replaced by 10 ml $^{86}Rb^+$-labelled Krebs solution, with or without ouabain, and the K^+ influx measured over 10 min. Each point represents the value for the K^+ influx in the presence of ouabain expressed as a percent of the control values measured in the absence of ouabain. A representative experiment of four separate experiments performed is presented.

repeated using Py3T3 and SV3T3 cells it was found that the initial inhibition (40%) of the K^+ influx was followed by an increase in the K^+ influx (Fig. 2). The chloride influx was not increased after a 60 min incubation with 10^{-3} M ouabain. This result suggests that the effect on the K^+ influx was not due to membrane damage.

The effect of ouabain on the efflux of K^+ from 3T3 and transformed 3T3 cells was also investigated. The effect of various bathing solutions on the rate of loss of $^{86}Rb^+$ from preloaded Py3T3 and 3T3 cells is shown in Table I. Extracellu-

TABLE I

THE EFFECT OF OUABAIN ON THE RATE OF LOSS OF K^+ FROM 3T3 AND PY3T3 CELLS

The washout of $^{86}Rb^+$ from preloaded cells was followed into each of the following solutions: (1) control Krebs solution, (2) Krebs solution + 10^{-3} M ouabain, (3) K^+-free Krebs solution, (4) K^+-free Krebs solution + 10^{-3} M ouabain. The amount of $^{86}Rb^+$ lost during six successive 5 min periods is expressed as a fraction of the amount of $^{86}Rb^+$ present at the beginning of each 5 min period. The fractional loss of $^{86}Rb^+$ from 3T3 cells was not significantly affected by any of the conditions tested ($P > 0.10$) and the mean values (±S.E.) are shown. The fractional loss of $^{86}Rb^+$ from Py3T3 cells into solutions 1, 3 and 4 was not significantly different ($P > 0.10$) and the mean values (±S.E.) are shown. The fractional loss of $^{86}Rb^+$ from Py3T3 cells was significantly increased ($P < 0.01$) by the presence of ouabain in the collecting solution.

Cell line	Collecting solution	Fraction $^{86}Rb^+$ lost					
		Collecting interval (min)					
		0—5	5—10	10—15	15—20	20—25	25—30
3T3	(1), (2), (3), (4)	0.116 (±0.010)	0.138 (±0.008)	0.128 (±0.005)	0.130 (±0.012)	0.124 (±0.004)	0.116 (±0.006)
Py3T3	(1),(3),(4)	0.133 (±0.015)	0.180 (±0.009)	0.172 (±0.006)	0.160 (±0.006)	0.134 (±0.015)	0.122 (±0.002)
	(2)	0.148	0.232	0.266	0.295	0.326	0.335

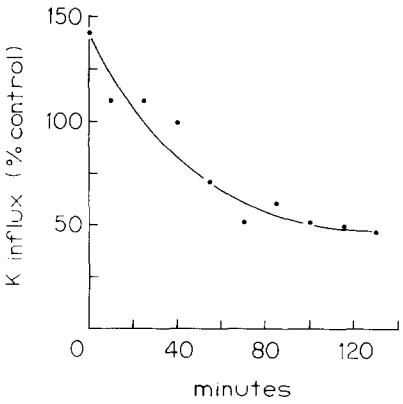


Fig. 3. Reversibility of the response of virally transformed cells to ouabain. SV3T3 cells were incubated with 10 ml Krebs solution with or without ouabain (10^{-3} M) for 60 min. All dishes then received 10 ml Krebs solution minus ouabain for periods ranging from 0 to 120 min. The K^+ influx was measured by incubation with $^{86}Rb^+$-labelled Krebs solution for 10 min. Each point represents the values for the K^+ influx after ouabain treatment expressed as a percent of the value obtained for the control. The value at 0 min was measured with ouabain present in the $^{86}Rb^+$-labelled Krebs solution.

lar ouabain increased the rate of $^{86}Rb^+$ loss from the transformed cells but not from the untransformed cells (Table I). Similar results were obtained using $^{42}K^+$ (data not shown). The increased K^+ loss from the transformed cells was abolished by the removal of K^+ from the bathing solution (Table I). This result indicates that the effect of ouabain on the K^+ fluxes of transformed cells is due to the establishment of K^+-K^+ exchange. Such exchange was not present in untreated cells since the efflux of K^+ was not affected by the removal of extracellular K^+ (Table I).

It appears then that the K^+ influx of ouabain-treated transformed cells is initially reduced by the inhibition of Na^+-K^+ exchange and then rises after the onset of K^+-K^+ exchange. We wished to test whether the inhibition of Na^+-K^+ exchange continued after the establishment of K^+-K^+ exchange. To this end, Py3T3 cells were treated with 10^{-3} M ouabain and the intracellular Na^+ and K^+ concentrations were measured by flame photometry [16]. Over a 60 min period the $[Na^+]_i$ rose exponentially from 20 to 70 mmol/l intracellular water and the $[K^+]_i$ fell exponentially from 200 to 130 mmol/l intracellular water. Therefore, in the transformed cells, ouabain causes a simultaneous reduction of Na^+-K^+ exchange and stimulation of K^+-K^+ exchange. In a similar experiment using 3T3 cells $[Na^+]_i$ rose from 14 to 72 mmol/l of intracellular water and $[K^+]_i$ fell from 168 to 102 mmol/l intracellular water.

The reversibility of these effects has been examined. SV3T3 cells were treated with ouabain for 60 min and then transferred to a control Krebs solution. The K^+ influx was measured at 15-min intervals up to 120 min after the removal of ouabain. The K^+ influx decreased during the first 60 min and then remained constant at a value 40% below that of the untreated controls (Fig. 3). Thus, the ouabain-induced K^+-K^+ exchange is more readily reversible than the inhibition of Na^+-K^+ exchange.

Discussion

Ouabain has been shown to bind to cells in two ways: (1) a saturable, specific binding to Na^+ pump sites which is sensitive to $[K^+]_o$ and (2) a non-specific uptake which is not affected by $[K^+]_o$ and does not saturate [22]. The slow onset and rapid reversal on the K^+-K^+ exchange suggests that this effect is not caused by ouabain attachment to the specific binding sites. We propose that the K^+-K^+ exchange arises from a non-specific interaction of ouabain with the transformed cell's membrane. In an attempt to obtain further evidence on this question we tried to measure the characteristics of [^{3}H]ouabain binding to 3T3 and Py3T3 cells. It is difficult to obtain accurate measurements of specific ouabain binding for cells which are insensitive to the drug [22]. We were unable to separate the two components of binding because of the high level of non-specific uptake at 10^{-3} M ouabain. We were also unable to detect differences in the time-course of total ouabain binding to normal and transformed cells.

Thus the nature of the interaction with ouabain responsible for the stimulation of K^+-K^+ exchange in tranformed cells remains unknown. It is possible that a change in the properties of membrane lipids after viral transformation [4] is involved. This interpretation is supported by a preliminary observation that treatment of SV3T3 cells with ouabain for 60 min at 18°C did not induce K^+-K^+ exchange. Under these conditions the K^+ influx was reduced 38% by ouabain, demonstrating that inhibition of Na^+-K^+ exchange was maintained at the reduced temperature.

Spaggiare et al. [7] reported a value of 50% for the inhibition of the unidirectional K^+ influx (measured over 10 min) in SV3T3 cells. However, they did not measure the K^+ influx in cells treated with the drug for longer periods of time. Kimelberg and Mayhew [8,9] have measured the K^+ "uptake" in 3T3 and SV3T3 cells exposed to ouabain for 60 min. However, in their experiments $^{86}Rb^+$ was present during the whole period of exposure to ouabain, whereas in the experiments reported here $^{86}Rb^+$ was present only during the final 10 min of incubation. The problems associated with the use of long uptake times have already been noted [7]. Because of the substantial isotope backflux these uptake data are not comparable to unidirectional influx measurements. It is, therefore, impossible to determine whether the SV3T3 cells of Kimelberg and Mayhew display the anomalous response to ouabain seen in our cells. If so, their conclusion that the transformed cells have a larger ouabain-sensitive K^+ uptake than normal cells may not be justified. In our experiments, the values for the ouabain-sensitive K^+ influx were similar when normal and transformed cells were compared at low cell densities.

Previous results on this question have been variable. The ouabain-sensitive K^+ influx has been reported to be increased [10], unaltered [12,14] or decreased [7,11] after virus-transformation. Similar variability has been observed for the effect of virus-transformation on (Na^+ + K^+)-ATPase activity [8,9,23—27]. Tupper [28] has recently evaluated the effect of subcultivation on the ouabain-sensitive K^+ influx in 3T3 and SV3T3 cells. The influx changed little over 50 passages of the transformed cell. In contrast, over a similar number of passages, the influx in the 3T3 cells increased 5-fold. Thus, the

ouabain-sensitive K^+ influx in 3T3 cells may be less, greater or equivalent to that in the transformed cells. It is proable that this finding accounts for much of the variation in previous studies.

At the moment we cannot say whether stimulation of K^+-K^+ exchange by ouabain is a characteristic feature of virally transformed cells. The experiments need to be repeated using other cell lines, preferably more sensitive to ouabain so that lower concentrations of the drug may be used. Negative selection on a cloned population of transformed 3T3 cells permits the recovery of revertants whose growth [29] and transport [30] characteristics are similar to those of normal cells. It will be interesting to examine the response of such cells to ouabain.

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