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THE EFFECT OF HARMALINE ON UNIDIRECTIONAL POTASSIUM FLUXES AND OUABAIN BINDING IN RENAL CELL CULTURES

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Harmaline inhibits K^+ influx into primary cell cultures of ground squirrel kidneys to a greater extent than either ouabain or furosemide. A concentration of 200 μM harmaline was required to inhibit half of the total K^+ influx; this effect was also seen at low temperature (5°C), and in another species (hamster). Although kinetic analysis of K^+ influx indicates that harmaline does not compete with extracellular K^+, harmaline did reduce the binding of [^{3}H]ouabain to the cells. K^+ efflux was also reduced. Therefore, harmaline may inhibit the furosemide-sensitive Na^+/K^+ cotransport system as well as the ouabain-sensitive Na^+/K^+ pump.

Introduction

The indole alkaloid harmaline has been described by Canessa et al. [1] as an Na^+-competitive inhibitor of ($Na^+ + K^+$)-ATPase; Dunn and Hunt [2] found that harmaline inhibited Na^+ efflux from human erythrocytes almost as effectively as ouabain. Harmaline has also found use as an inhibitor of transport across epithelial tissues, such as intestine [3], frogskin [4], and renal tubules [5]. In a previous study [6], we found that harmaline was a consistent inhibitor of K^+ influx into ground squirrel kidney cells in primary culture. Furthermore, it was suggested that harmaline inhibited both Na^+-K^+ and K^+-K^+ exchanges in these cells.

The purpose of this study was to compare the effects of harmaline with those of ouabain on K^+ fluxes, and to investigate the interaction of the two drugs. The results showed that harmaline is a potent inhibitor of K^+ influx and that its potency is undiminished at low temperature and in an ouabain-insensitive species (hamster).

Materials and Methods

Culture conditions

The method of culture and flux measurement have been described previously [6]. Briefly, primary cultures were produced by disaggregating kidney cortex slices in 0.25% trypsin, and the cells were plated on 35 or 60 mm plastic plates. The animals used were 13-lined ground squirrels (*Citellus tridecemlineatus*) and golden hamsters (*Mesocricetus auratus*). Cells were incubated in Dulbecco's modified Eagle's medium with 10% calf serium for 3 days in a CO_2 incubator.

Ion fluxes

Flux measurements were carried out in Dulbecco's modified Eagle's medium without serum or in bicarbonate-buffered medium: 109 or 114 mM NaCl, 0.0 or 5.5 mM KCl, 1.74 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.97 mM $NaHPO_4$, 43 mM $NaHCO_3$, pH 7.4 in a 5% CO_2 atmosphere. Ouabain (30 μM for ground squirrel and 4 mM for

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hamster cell cultures) was added during a 10 min preincubation period to insure maximal inhibition. Influx measurement was begun by adding a small amount of ^{42}K in bicarbonate-buffered medium, and the reaction was stopped 5 min later by 3 ice-cold washes with a solution containing 300 mM sorbitol, 0.8 mM $MgSO_4$, and 1.2 mM $CaCl_2$, followed by 2.0 ml of 10% trichloroacetic acid. At 5°C, similar procedures were used except that 45 min was used as an initial temperature equilibration period, flux was measured for 15 min, and 300 μM ouabain was necessary to attain a maximal inhibition. Efflux was measured by loading cells with ^{42}K for 2–4 h, washing three times in warm bicarbonate-buffered medium, then adding nonradioactive medium for a measured time period, and stopping the reaction as described above. The isotopic contents of the plates of cells were subtracted from the content of a plate of cells stopped at zero time. Specific activity was obtained from the cpm of a sample of the medium (K^+ concentration of the medium = 5.5 mM). The trichloroacetic acid-soluble extract was removed and Cerenkov radiation was counted in a Beckman LSC-100 liquid scintillation counter. The acid-insoluble residue on the plates was extracted overnight in 1 M NaOH, and protein was measured by the procedure of Lowry et al. [7]. Cell numbers were obtained from extra plates of cells exposed to trypsin-EDTA for 40 min, and counted in a Coulter counter. After isotope decay, K^+ contents of the cells were measured with an IL flame photometer (Waltham, MA); estimates of cell K^+ content by flame photometry agreed within 10% with estimates from cells equilibrated with ^{42}K for 2–4 h.

Computation of fluxes

Since measurement of K^+ content showed that cells quickly lost net K^+ when exposed to harmaline, especially at 37°C, all influx computations were based on non-steady state treatments. It was determined that fluxes from 0.5 to 5.0 min at 37°C and from 1 to 15 min at 5°C provided the best initial rate estimates of ^{42}K influx. All ^{42}K radioactivity values were corrected for isotope decay. The following equation was used to compute initial rate K^+ influx [8]:

$$^{i}M_K = (Q \cdot dS_c/dt)/(S_m - S_c) \quad (1)$$

where Q = average quantity of K^+ in cells (μmol/plate); S_c = average specific activity of cells, and S_m = specific activity of medium (cpm/μmol); and dS_c/dt = rate of change of specific activity. Rates of K^+ efflux were calculated using the following equations:

$$^{o}M_K = (d(^{42}K)/dt) \cdot S_c^{-1} \quad (2)$$

$$d(^{42}K)/dt = A_{cell,o} - A_{cell,t} \quad (3)$$

where S_c = specific activity of cells (cpm/μmol) at time 0; $d(^{42}K)/dt$ = change in radioactivity in cells (cpm/min); $A_{cell,t}$ = activity of cells after 't' min washout. Both influx and efflux values were divided by the average cell protein content (g/plate). Final values were expressed as: μmol K^+/g per min.

Ouabain binding

In general the procedures of Baker and Willis [9] were followed. Ouabain binding experiments were carried out in bicarbonate-buffered medium without K^+, and when present, harmaline was added at the same time as the [^{3}H]ouabain. After 1 h (37°C) or 5 h (5°C), the plates of cells were washed three times in ice-cold bicarbonate-buffered medium without K^+, and 1.0 ml of 10% trichloroacetic acid was added. Ten ml of scintillation fluid (toluene/Triton X-100, 2 : 1; 5 g PPO/l; 0.03 g POPOP/l) were added and ^3H was counted in a Nuclear Chicago Mark II scintillation counter. Harmaline was well washed out by the time the samples were counted, but the external standard (^{133}Ba) channels' ratio was always monitored for quenching. The [^{3}H]ouabain was made up in K^+-free medium; concentrations were adjusted with non-radioactive ouabain carrier to the same ^3H specific activity to facilitate estimation of nonspecific ouabain binding. The cpm of a plate of cells with a 1 mM excess nonradioactive ouabain was subtracted from all the other cpm values. The difference should consist largely of high affinity, K^+-sensitive binding [9]. Because the kinetic experiments required accurate concentrations for ouabain within a narrow range, concentrations were estimated spectrophotometrically at 220 nm, as suggested by Joiner and Lauf [10]. Binding was expressed as nmol ouabain per gram cellular protein.

Materials

Ouabain octahydrate and harmaline HCl were purchased from Sigma (St. Louis, MO); [^{3}H]ouabain from New England Nuclear (10–20) mCi/ml; Boston, MA); and furosemide was donated by Hoechst-Roussel Pharmaceuticals (Somerset, NJ). All other chemicals were reagent grade.

Results

K^+ influx

Maximal inhibition of K^+ influx by 1 mM harmaline is greater than that caused by maximal ouabain inhibition in ground squirrel kidney cells at 37°C (Fig. 1, ouabain concentration 30 μM). Furthermore, maximal inhibition caused by the two inhibitors together was always greater than the maximal inhibition produced by ouabain alone, but was not greater than that caused by harmaline alone. The degree of inhibition of K^+ influx by harmaline was not altered by either low temperature or a change in the species of animals used (Table I). Inhibition by ouabain at low temperature and in hamster cells was very similar to the pattern seen in Fig. 1, although higher concentrations of ouabain were required for maximal inhibition (300 μM at 5°C and 4 mM in hamster cells at 37°C, data not shown). Since high concentrations ($>$ 1 mM at 38°C, $>$ 5 mM at 5°C) cause the cells to detach from the plates, the maximally inhibitory concentration of harmaline cannot be determined in this system. Thus, a true I_{50} (half-maximal inhibition) could not be measured, but the concentration of harmaline which inhibited 50% of the total K^+ influx was $2 \cdot 10^{-4}$ M in both species at 37°C and in ground squirrel kidney cells at 5°C (Fig. 1, Table I).

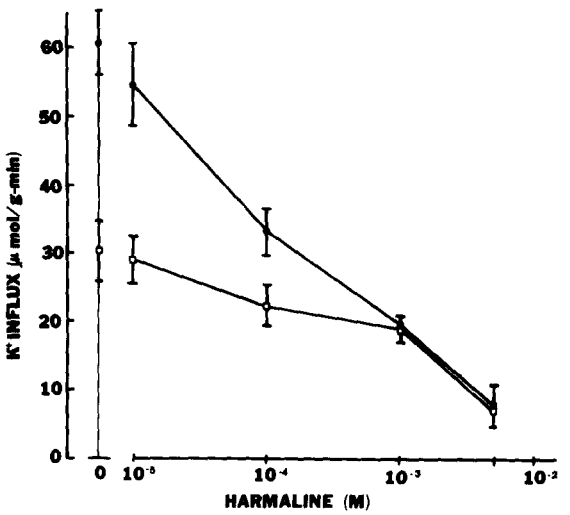

Fig. 1. Initial rate of ^{42}K influx into ground squirrel kidney cultures at 37°C in the absence (●) or presence (□) of 30 μM ouabain. Values are mean ± S.E.; N = 4–9 per point, one culture per animal.

Na^+-for-Na^+ and K^+-for-K^+ exchange fluxes have been demonstrated in several types of cell cultures, such as Ehrlich ascites tumor cells; these exchange fluxes represent a component of total

TABLE I

EFFECT OF TEMPERATURE AND SPECIES ON HARMALINE INHIBITION OF K^+ INFLUX

Data are expressed as percent inhibition of control K^+ influx in the absence of harmaline (mean ± S.E., n = 4–7). GS37, ground squirrel kidney cells at 37°C; control value 60.48 ± 4.47. GS5, ground squirrel cells at 5°C; control value 5.59 ± 0.24. H37, hamster kidney cells at 37°C; control value 35.78 ± 2.95 μmol K^+/g per min. HME, harmaline.

HME (mM)	Percent inhibition			K^+ influx (μmol/g per min)		
	GS37	H37	GS5	GS37	H37	GS5
0.00	0	0	0	60.48 ± 4.47	35.78 ± 2.95	5.59 ± 0.24
0.01	9.8 ± 9.7	8.3 ± 8.5	19.1 ± 8.2	54.58 ± 5.87	32.80 ± 3.04	4.52 ± 0.46
0.10	45.2 ± 5.5	38.9 ± 3.7	38.6 ± 6.4	33.13 ± 3.34	21.85 ± 1.33	3.43 ± 0.36
1.00	67.7 ± 1.7	74.0 ± 2.4	69.0 ± 3.6	19.55 ± 1.06	9.31 ± 0.86	1.73 ± 0.20
2.50	–	77.7 ± 2.0	–	–	7.97 ± 0.70	–
5.00	87.2 ± 4.8	–	8.21 ± 2.0	7.73 ± 2.88	–	1.00 ± 0.11

flux that is sensitive to the diuretic drug furosemide [11]. Because furosemide-sensitive self-exchange of K^+ also occurs in ground squirrel kidney cells [6], the interactions between the inhibitors furosemide, ouabain, and harmaline were examined in ground squirrel cells during measurements of K^+ influx. In an additional experiment, we essentially confirmed our earlier findings [6]: the furosemide-sensitive component represented 23% of the total influx, the ouabain-sensitive component represented 41%, and that both inhibitors together inhibited 93% of all K^+ influx. Harmaline, even at a submaximal concentration (1.0 mM), can inhibit K^+ influx to a greater extent than ouabain or furosemide alone. Furthermore, neither combination (harmaline + furosemide, or harmaline + ouabain) was more inhibitory than 1.0 mM harmaline alone. However, maximal reduction of K^+ influx by harmaline was less than that caused by ouabain and furosemide together (69%, Fig. 1).

K^+ efflux

Since harmaline is a more effective inhibitor of K^+ influx than ouabain, harmaline could possibly have an effect on passive transport of K^+. Therefore, K^+ efflux experiments were performed in the presence and absence of harmaline. In the absence of harmaline an efflux of 16.43 ± 1.22 μmol K^+/g per min was found. With the addition of 0.1, 0.25 and 1.00 mM harmaline the efflux values were 16.57 ± 1.62, 16.49 ± 1.89 and 19.53 ± 1.12 μmol K^+/g per min (±S.E., $n = 7$), respectively. Measurements of the time course of K^+ efflux showed that the logarithm of ^{42}K loss was linear with time for at least 20 min. The efflux rate constants calculated from times at which 50% of the radioactivity remained were: 2.25 h^{-1} for control cells and 3.20 h^{-1} for cells treated with 1.0 mM harmaline. The results showed that there was no significant effect of harmaline on K^+ efflux at concentrations less than 1.0 mM, where the efflux rate was 19% greater than control levels ($P < 0.05$). Measurements of K^+ content from these same experiments are shown in Fig. 2. It is evident that the net loss of cellular K^+ depended upon the concentration of harmaline present. The absolute values of the fluxes above do not correspond with the previous influx values (see Fig. 1), which were measured at different times, and with different plating densities. Therefore an experiment was performed on one culture from the same animal, and the plates for influx and efflux were handled in an identical manner, except for the time of ^{42}K addition. These results are shown in Table II, along with the net movements of cellular K^+ as estimated by flame photometry. There was a net loss of cell K^+ at higher harmaline concentrations, caused by an inhibition of K^+ influx and an increase in K^+ efflux.

Competition for K^+

The inhibition of the enzyme $(Na^+ + K^+)$-ATPase by harmaline is competitive for Na^+ [1, 12], but the interaction with $(Na^+ + K^+)$-ATPase

TABLE II

UNIDIRECTIONAL AND NET FLUXES OF POTASSIUM IN GROUND SQUIRREL KIDNEY CELLS IN THE PRESENCE OF HARMALINE

Results are the means of triplicates from one experiment, ΔK^+, flame = net difference in cell K^+ contents between plates of cells incubated with and without harmaline, as measured by flame photometry. All flux times were 10 min.

Harmaline (mM)	Flux (μmol K^+/g per min)			ΔK^+, flame (μmol/g per min)	
	Influx ^iM_K	Efflux ^oM_K	Difference ($^iM_K - {}^oM_K$)	Influx plates	Efflux plates
0.00	21.79	21.40	+0.39	-	-
0.10	19.41	21.61	−2.20	−1.84	−2.56
0.25	19.85	24.92	−5.07	−8.21	−7.00
1.00	8.43	26.05	−17.62	−16.63	−12.61

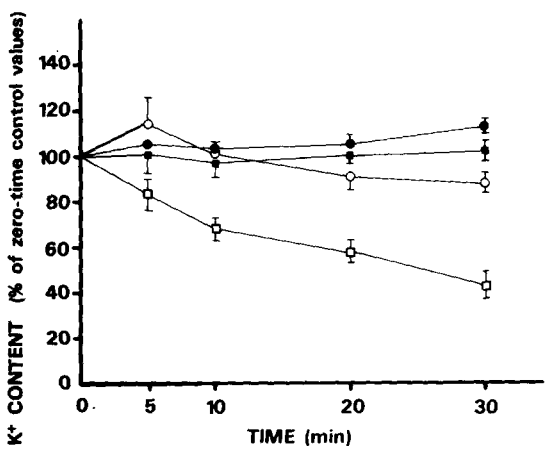

Fig. 2. Potassium content in the presence of harmaline. The content of potassium in group squirrel kidney cells at 38°C was measured by flame photometry, and expressed as mean ± S.E. ($n = 7$). Symbols: control (●), 0.10 (■), 0.25 (○), 1.00 (□) mM harmaline.

may also involve a lowering of the enzyme's affinity for K^+ [13]. Therefore, the effect of harmaline on K^+ influx at various concentrations of external K^+ was measured. The data were plotted according to the method of Lineweaver and Burk (see Ref. 14), but parabolic curves were observed. Since

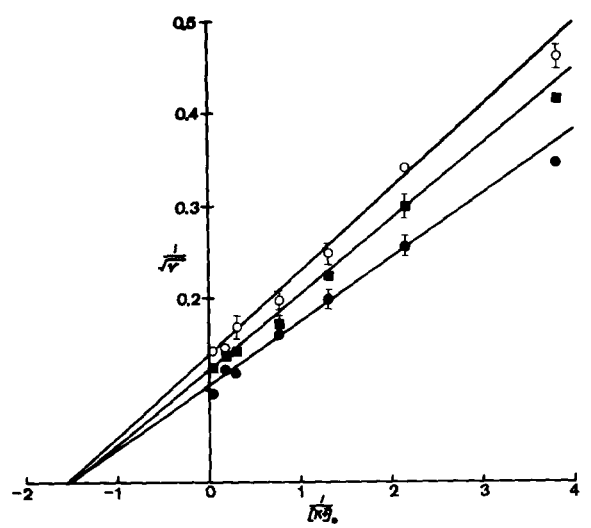

Fig. 3. Effect of harmaline on transport kinetics. Initial rate influx of ^{42}K was measured in the presence of external K^+ concentrations ranging from 0.26 to 20 mM. Data from one representative culture are presented in a modified Lineweaver-Burk form. Symbols: control (●), 0.10 (■) and 0.25 (○) mM harmaline.

the Na^+/K^+ pump probably has two equivalent sites for external K^+ [15], the reciprocal K^+ concentration was plotted against the reciprocal square root of total K^+ influx. As seen in Fig. 3, straight lines were obtained, and the value for $K_{1/2}(K^+)$ was not affected by harmaline. The maximal velocity, however, was lowered by harmaline.

Ouabain binding

The effect of harmaline on ouabain binding to ground squirrel kidney cells is shown in Fig. 4, wherein two types of binding were seen: a saturable component, and a linear component which was more evident at ouabain concentrations greater than 10^{-6} M. Apparently, harmaline interferes with only the saturable component of ouabain

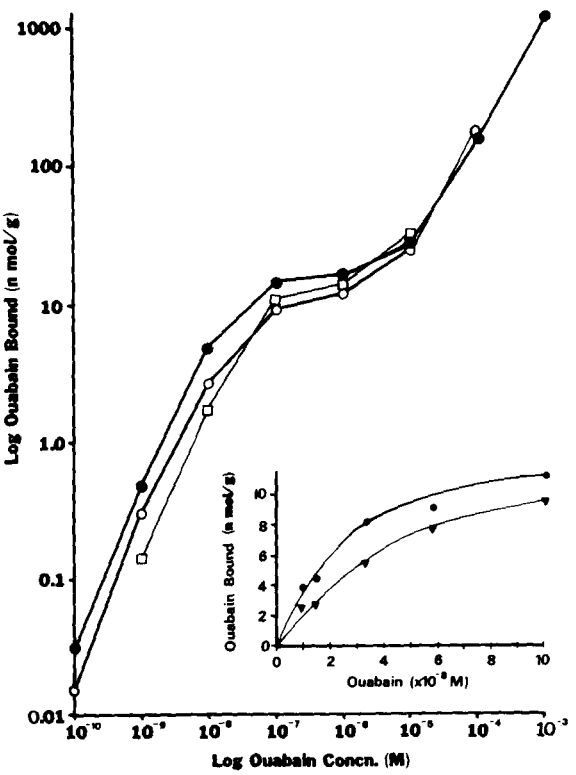

Fig. 4. Effect of harmaline on ouabain binding to ground squirrel kidney cells at 37°C. Data on the double logarithmic plot are the means from 2–8 animals; coefficients of variation ((S.D./mean) × 100) ranged from 39 to 105%. Symbols: control (●), 0.25 (○), and 1.00 (□) mM harmaline. Inset: Ouabain binding in the concentration range of 10^{-8} to 10^{-7} M ouabain. Data are the means of duplicates from one culture; ranges are approximately the size of the symbols. Symbols: control (●), 0.5 (▼) mM harmaline.

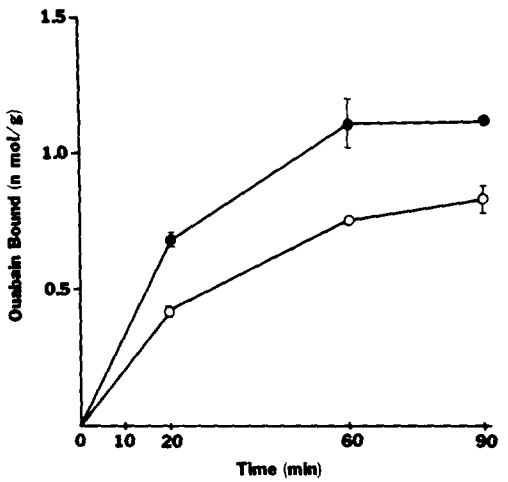

Fig. 5. Effect of harmaline on the rate of ouabain binding to ground squirrel kidney cells at 38°C. Points represent mean, with range, of duplicate samples from one culture. Ouabain concentration = $1 \cdot 10^{-8}$ M. Symbols: control (●), and 0.25 (○) mM harmaline.

binding. The data of Fig. 4 suggest that the half-maximal concentration (K_b) for [^{3}H]ouabain binding lies between 10^{-8} and 10^{-7} M, so binding experiments were performed within this range (Fig. 4, inset). Although these values at one hour may not be equilibrium binding levels, the K_b for ouabain was approx. $3 \cdot 10^{-8}$ M, which is similar to a value of $1.4 \cdot 10^{-8}$ M in HeLa cells [16].

Harmaline also reduces the rate of ouabain binding to the enzyme ($Na^+ + K^+$)-ATPase [12]; this effect was also observed in intact ground squirrel kidney cells (Fig. 5). In this experiment using 10^{-8} M ouabain, binding seems to have reached equilibrium within 90 min, yet 0.25 mM harmaline prevented 25% of the ouabain binding at that time. In similar experiments, harmaline retarded binding with ouabain concentrations of $2 \cdot 10^{-8}$ and $5 \cdot 10^{-8}$, but not with $1 \cdot 10^{-7}$ M.

Discussion

Whereas previous studies of harmaline effects on cation transport have dealt with Na^+ transport, this study focuses on inhibition of K^+ fluxes in cultured cells. The principal finding is that harmaline is a more effective inhibitor of K^+ influx than either ouabain or furosemide in these cells, and in fact the level of inhibition caused by high concentrations of harmaline approaches the combined effect of ouabain and furosemide [6]. Potassium ion influx occurs by three routes, the pump, the cotransporter and the leak. Thus, harmaline seems to inhibit the first two components. We shall discuss these two possibilities in turn.

The extent of inhibition of K^+ influx and the reduction of cell K^+ concentration indicate that at least part of the harmaline effect is in blocking the Na^+/K^+ pump. This conclusion is consistent with those of studies on the enzyme ($Na^+ + K^+$)-ATPase [1,12,13], on transepithelial ion transport [3,4], and on transmembrane Na^+ fluxes [1,2]. The interference by harmaline with binding of ouabain to intact cells (Fig. 4) and to isolated ($Na^+ + K^+$)-ATPase [12] at low but inhibitory concentrations of ouabain is also consistent with the conclusion that harmaline is interacting with the Na^+/K^+ pump. However, intracellular Na^+ and ATP levels also affect ouabain binding to human red cells [17]. Since harmaline can increase cellular Na^+ levels [2], and can complex with ATP in solution [12], the harmaline actions in reducing the rate and extent of ouabain binding may be indirect, rather than a direct competition for the ouabain binding site.

Although there is clear evidence that harmaline competes for Na^+ binding sites on the ($Na^+ + K^+$)-ATPase [1,18], other investigators have claimed that the effects of harmaline are more complex, and may involve interaction with the K^+-activation site of the isolated ($Na^+ + K^+$)-ATPase enzyme [5,12,19]. On the other hand, our kinetic analysis of the effect of harmaline on K^+ influx (Fig. 3) implies that harmaline does not directly affect K^+-activation sites.

Another action of harmaline is an inhibition of the furosemide-sensitive K^+ fluxes. In our previous paper [6], we attributed this to an inhibitory action of harmaline on K^+-K^+ and Na^+-Na^+ exchanges [11]. Although external K^+ may still exchange one-for-one with internal K^+ under certain conditions [20], it now seems more likely that the furosemide-sensitive K^+ influx in ground squirrel kidney cells is another example of the Na^+-K^+-$2Cl^-$ cotransporter which has been described for Ehrlich ascites cells and other cell types [21].

For the cotransport system there are also possibilities for complex interactions with harmaline, especially in intact cells. For example in certain systems, cotransport exhibits cyclic AMP dependence (avian erythrocytes, Ref. 22) and metabolic dependence (ascites cells, Ref. 20), through either of which harmaline might exert its effects. It is quite possible, however, that the effect of harmaline on furosemide-sensitive K^+ flux involves the interaction of harmaline with Na^+ binding sites on the cotransport molecule(s).

Potassium efflux occurs via at least two paths, basic electrochemical leak and the cotransport system. One should, therefore, expect to see an inhibition of efflux at least at low concentrations of harmaline and its absence in these studies (see Results and Table II) is puzzling. The absence of effect may be attributable to cotransport having a lower susceptibility to harmaline than the pump has. Such a difference in sensitivity in the two systems is suggested by the results in Fig. 1 showing a steeper slope of inhibition by harmaline in the ouabain-free cells than in the ouabain-treated cells. Thus, at low concentrations of harmaline, pump inhibition would raise cell Na^+ concentration, stimulating cotransport and offsetting harmaline inhibition of cotransport.

The enhancement of K^+ efflux at higher harmaline concentrations (1 mM) is probably due to a non-specific increase in membrane permeability. Harmaline has been shown to increase membrane permeability in red cells at 10^{-2} M [2] and in BHK cells at 5 mM under certain conditions (Becker, J.H., unpublished data). These two effects (Na^+ stimulation of cotransport, membrane disruption) could also account for the failure of harmaline to cause a maximal reduction of K^+ influx equal to that caused by ouabain and furosemide in combination.

As a substitute for ouabain, harmaline has the disadvantages of requiring high (millimolar) concentrations, inhibiting other ion transport systems (Na^+/K^+ cotransport), and altering membrane permeability. On the other hand, harmaline has the advantages of not being influenced by temperature or by differences in species (Table I). Moreover, the ability to inhibit several ion transport systems may make harmaline a useful pharmacological tool.

Acknowledgements

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