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Quinine inhibits multiple Na^+ and K^+ transport mechanisms in Ehrlich ascites tumor cells

Thomas C. Smith and Charles Levinson

Department of Physiology, University of Texas Health Science Center, San Antonio, TX (U.S.A.)

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The interaction of quinine with K^+ and Na^+ transport mechanisms has been investigated in Ehrlich ascites tumor cells. Quinine affects both Ca^{2+}-dependent K^+ channel and total K^+ influx. Activation of Ca^+-dependent K^+ channels by propranolol is abolished by quinine (1 mM). In addition, quinine inhibits the ouabain-sensitive component of K^+ influx with an apparent K_i of 0.32 ± 0.02 mM and the furosemide-sensitive component with a K_i of 0.24 ± 0.01 mM. Furthermore, a significant fraction (52%) of Na^+ influx is inhibited by quinine. The same component is sensitive to amiloride, suggesting that it represents Na^+/H^+ antiport. Concomitant with the inhibition of K^+ and Na^+ transport, quinine stimulates ATP hydrolysis by 57%. The results suggest that quinine exerts broad, nonspecific effects on cellular mechanisms which serve to regulate cation transport in Ehrlich cells.

Introduction

Activation of the Ca^{2+}-dependent K^+ channels has been postulated to contribute to a diverse set of cellular responses [1–3], including alterations in membrane potential [4], cell volume regulation [5,6] and endocrine pancreatic and hepatic secretory activity [7]. Definitive assessment of the physiological role of the Ca^{2+}-dependent channels has been complicated by the presence of additional, parallel active and passive pathways mediating K^+ transport. These include active uptake mediated by the Na^+/K^+'pump', passive Cl^- dependent cotransport in steady-state cells, and cotransport with Na^+ and Cl^- in cells with altered volume or perturbed ion gradients [8–10].

Frequently, it is necessary to infer the presence of Ca^{2+}-dependent K^+ channels in a particular cellular preparation only on the basis of indirect evidence. A prominent test for their presence has been the appraisal of the effects of blocking the channel on membrane potential and K^+ fluxes (see Ref. 11). In the case of the Ca^{2+}-dependent K^+ channels in human erythrocytes, the Cl^- salts of quinine and quinidine serve as effective inhibitors [12]. These agents have been widely used in other tissues to define K^+ movements through this channel (see Ref. 13). For example, in Ehrlich ascites tumor cells, quinine-sensitive membrane hyperpolarization in response to increases in intracellular Ca^{2+} has implied their existence [6], while in lymphocytes and thymocytes quinine-induced depolarization of the resting membrane has provided evidence (see Ref. 11). However, Lew and Ferreira have emphasized [13] that although quinine and quinidine inhibit K^+-channel activity in many cells, their actions are not limited to this pathway. They also function as local anesthetics, antiarrhythmic agents, and as inhibitors of Na^+/H^+ antiport in certain epithelia [13,14].

Recent studies in Ehrlich ascites tumor cells have suggested that the response to hypotonic challenge depends on activation of Ca^+-dependent K^+ channels [6]. The sensitivity of the regulatory volume decrease to quinine has been taken as strong supportive evidence for channel activation. Whether other ion-transport pathways are involved depends at least in part on the specificity on the quinine inhibition. The present studies are concerned with characterizing the effects of quinine on unidirectional Na^+ and K^+ fluxes in Ehrlich cells. The results demonstrate that quinine has a broad inhibitory action against cation-transport systems.

Materials

Radioisotopes, ^{22}Na and ^{86}Rb, were obtained from New England Nuclear. DL-Propranolol, ouabain and

Correspondence: T.C. Smith, Department of Physiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7756, U.S.A.

quinine were purchased from Sigma Chemical Co., while furosemide was the generous gift of Hoechst Pharmaceuticals. All other reagents were of the highest purity available.

Methods

Experiments were performed on Ehrlich-Lettre ascites tumor cells (hyperdiploid strain) grown in white, male mice (Ha/ICR) by weekly transplantation. The cells were harvested from tumor-bearing animals by aspiration and washed free of ascites fluid as previously described [15]. The wash and incubation medium was a physiological saline solution containing (mM): 150 NaCl/6 KCl/2 $CaCl_2$/0.2 $MgCl_2$/10 Hepes-NaOH (pH 7.4; 285–300 mosM). Cells were incubated at a density of 15–20 mg dry weight/ml suspension for at least 30 min at 21–23°C to establish a steady state.

Unidirectional K^+ uptake was measured using ^{86}Rb as a tracer [16,17]. The initial experiments were designed to screen a number of known transport inhibitors (ouabain, furosemide, quinine) and activators (propranolol) for their effects on K^+ uptake. Steady-state cell suspension was added to flasks containing the test agents and a tracer amount of isotope (0.3–0.5 μCi/ml cell suspension). Periodically during the next 6 min, aliquots (0.2 or 0.5 ml) of cell suspension were removed and centrifuged (30 s at 15000 × g) through isosmotic choline dihydrogen citrate [18] or isosmotic choline chloride solution. The supernatant was removed and the cell pellets mixed with 1 ml 1% (v/v) ice-cold perchloric acid. Additional samples of cell suspension and medium were taken for the determination of wet and dry cell weights and intracellular water [19,20] and for the analysis of ^{86}Rb, Na^+, K^+ and Cl^-. Correction for ^{86}Rb trapped in the extracellular space was made by adding cell suspension to ice-cold choline dihydrogen citrate solution containing the appropriate amount of tracer and centrifuging immediately or by a regression line relating [^{3}H]methoxyinulin space to the wet cell weight.

The initial influx of K^+, expressed as mEq/kg dry wt. per min, was determined from $(d[^{86}Rb]/dt)/SA$, where $d[^{86}Rb]/dt$ is the slope of the time-dependent uptake of ^{86}Rb, and SA is the specific activity (cpm/mmol) of the extracellular K^+ (Rb^+).

The effects of test agents (amiloride, quinine) on unidirectional Na^+ uptake were determined in media with reduced [Na^+] using isosmotic replacement of NaCl in the standard saline solution with choline chloride. Cell suspension was incubated in this medium for 30 min, washed and again resuspended in a Na^+-free environment. This procedure reduces the Na^+ content of the cell suspension to less than 1 mM [21]. Aliquots (1.0 ml) of the cell suspension were added to 1.5 ml polyethylene centrifuge tubes containing the test agents dissolved in standard saline solution (with tracer ^{22}Na; 0.4 mCi/mmol). ^{22}Na uptake in these cells is linear for at least 4 min if active Na^+ efflux is inhibited [22]. All tubes also contained ouabain (final concentration 2 mM). Incubation was continued for 3 min. Aliquots (0.2 ml) were then removed and centrifuge through isosmotic choline chloride as described above. The supernatants were removed and the cell pellets mixed with 0.5 ml 2% perchloric acid. Correction for ^{22}Na trapped in the extracellular space was estimated from a regression line relating [^{3}H]methoxyinulin space to pellet wet weight. Samples of the cells suspension were also taken for determination of cell wet and dry weights and water content as described above.

The initial influx of Na^+ is given by $(d[^{22}Na]/dt)/SA$, where $d[^{22}Na]/dt$ represents the ^{22}Na incorporation kg dry wt. per min and SA is the specific activity of the extracellular Na^+.

The cell pellets were extracted with perchloric acid in an ice bath for 60 min and subsequently centrifuged (15000 × g) for 2 min to remove acid-insoluble residue. The Na^+ and K^+ contents of the extracts and media were determined by emission flame photometry using 15 mM Li^+ as an internal standard. Aliquots of the perchloric acid extracts and media were assayed for ^{86}Rb and ^{22}Na radioactivity with either a gamma or liquid scintillation counter. In some studies, ATP content of the cell extracts was determined as previously described [23].

The membrane potential of Ehrlich ascites tumor cells was measured using glass microelectrodes filled with 300 mM potassium acetate. The apparatus and techniques have been described in detail [24–26].

Glass dishes were prepared to promote adherence of the cells to the glass surface and to facilitate impalement. Briefly, glass Petri plates (diameter 10 cm) were soaked in NaOH-saturated methanol for 24 h at 21–33°C. The plates were thoroughly rinsed with distilled water and with physiological saline prior to use. The tumor cells were prepared by adding 0.2 ml cell suspension to 10 ml of test solution, and were placed in the Petri plate. Since the majority of cells adhered to the prepared surface, the impalements were greatly facilitated. The potential difference between the cell cytoplasm and medium was recorded as previously described [24]. Our criteria for the validity of potential measurements have been discussed in detail [26]. These include consideration of the characteristics of the recordings upon insertion and withdrawal of the electrode, as well as attainment of a stable recording. Approximately 60% of our attempts at cell impalement yielded responses which are consistent with these criteria.

Corrections of the experimentally determined values of the membrane potential for the influence of junction potentials were made by applying a modified form of the Henderson equation [27]. For these calculations we

used the ion mobility ratios $U_{Na}/U_K/U_{Cl} = 0.682:1.0:1.038$. Ionic activity coefficients were taken as 0.73 for K^+ and Cl^- and 0.71 for Na^+ in the medium, while in the cytoplasm they were 0.41, 0.67 and 0.18 for K^+, Cl^- and Na^+, respectively [26]. The maximum correction was -2.1 mV.

All values are expressed as the mean ± S.E. Student's t test was used to evaluate statistical significance. $P < 0.05$ has been taken as the criterion for significance.

Results and Discussion

K^+ entry into Ehrlich cells is usually considered to occur by three, parallel transport pathways: (1) active uptake mediated by Na^+/K^+-ATPase, usually defined as the 'ouabain-inhibitable' component; (2) passive, Cl^--dependent cotransport, usually defined as the 'furosemide-sensitive' component; and (3) passive, channel-mediated diffusion [16]. Whether the diffusional component represents a 'ground permeability' which is insensitive to quinine or baseline activity of the Ca^{2+}-sensitive channel is unclear. We have approached apportioning the K^+ entry among these mechanisms by evaluating the effects of the defining inhibitors on influx (Fig. 1A). In control steady-state Ehrlich cells, the K^+ uptake is 29.5 ± 0.8 mEq/kg dry wt. in 5 min. Significant fractions of this influx are sensitive to inhibition by ouabain (31.2 ± 8.4%), furosemide (69.8 ± 8.6%) and quinine (37.0 ± 7.0%). It is noteworthy that the sum of the individual inhibitor-sensitive components (138 ± 14% control) greatly exceeds the total uptake. This suggests that either the inhibitors are not specific or indirect interactions between the pathways yield erroneously high estimates of inhibition.

One possible mode of indirect interaction is through alterations in the membrane potential (V_m). The value of V_m in Ehrlich cells is somewhat controversial, since indirect estimates using chemical probes and direct electrophysiological measurements yield quantitatively different values (see Refs. 28, 29). However, there is qualitative agreement that the ouabain-sensitive pathway and the Ca^{2+}-sensitive channel are electrogenic in Ehrlich cells [4,30]. We have determined the effect of quinine on V_m using glass microelectrodes. In steady-state cells, $V_m = -24.7 \pm 1.5$ mV (S.E.: $n = 54$). Addition of quinine (1 mM) depolarizes the membrane to $V_m = -9.4 \pm 0.6$ mV (S.E.: $n = 24$). Thus, it is possible that membrane depolarization in response to either ouabain or quinine could give secondary reduction in the parallel pathways. We also tested the interaction of quinine and propranolol, a putative activator of Ca^{2+}-dependent K^+ channels [6], on V_m. Treatment of the cells with propranolol (0.4 mM) hyperpolarizes the membrane to $V_m = -41.3 \pm 2.9$ mV (S.E.: $n = 14$). This response is completely abolished in the presence of 1 mM quinine, $V_m = -10.8 \pm 1.0$ mV (S.E.: $n = 16$).

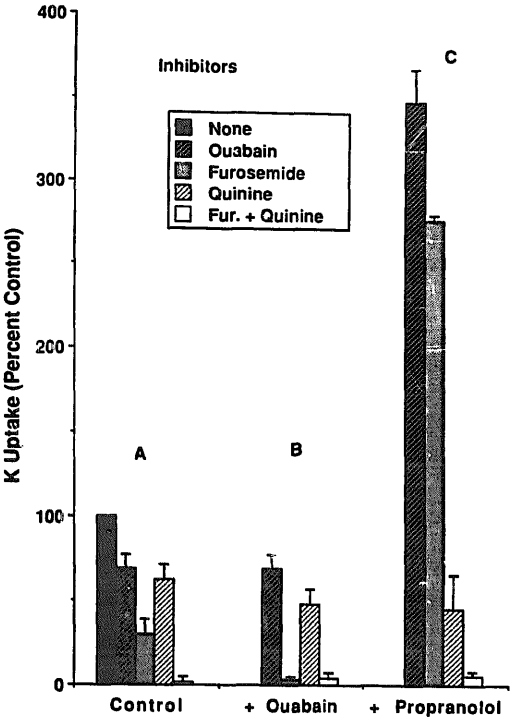

Fig. 1. K^+ uptake in Ehrlich ascites tumor cells. Ehrlich cells were pre-incubated in physiological saline to establish steady-state cellular electrolyte and water contents. Samples of the cell suspension were then transferred to flasks containing physiological saline (±) the test inhibitors and tracer amounts of ^{86}Rb (0.3–0.5 μCi/ml). K^+ uptake was estimated from the cellular accumulation of ^{86}Rb during 5 min and the results reported as percent control (in the absence of inhibitors or activators). The concentrations of the test agents were: ouabain = 2 mM, furosemide = 2 mM, quinine = 1 mM, and propranolol = 0.4 mM. Values represent the mean ± S.E. from duplicate samples in four separate experiments.

Clearly, propranolol activation of the channels occurs through a quinine-sensitive mechanism.

We have further tested interactions between the pathways by evaluating the effects of inhibitor combinations (Fig. 1B). In the presence of ouabain, furosemide gives an added inhibition (65.6 ± 9.0% control) which is not different from its effect alone. Furthermore, the inhibition achieved by the combination of ouabain and furosemide (96.8 ± 2.0%) is not different from the sum of their individual effects (101 ≡ 13% control). The nearly total inhibition of K^+ uptake by this combination is in excellent agreement with previous studies [31]. Thus, there is no apparent overlap between ouabain and furosemide, either direct or indirect. On the other hand, the inhibitory effects of quinine is reduced in the presence of ouabain, to only 20.6 ± 5.9% control. This clearly indicates that at least a portion of the 'ouabain-sensitive' flux in control cells contributes to the 'quinine-sensitive' flux component. Moreover, since the ouabain-insensitive flux is subject to nearly complete inhibition by furosemide, its inhibition by quinine suggests either a lack of specificity of furosemide and/or quinine for their respective pathways

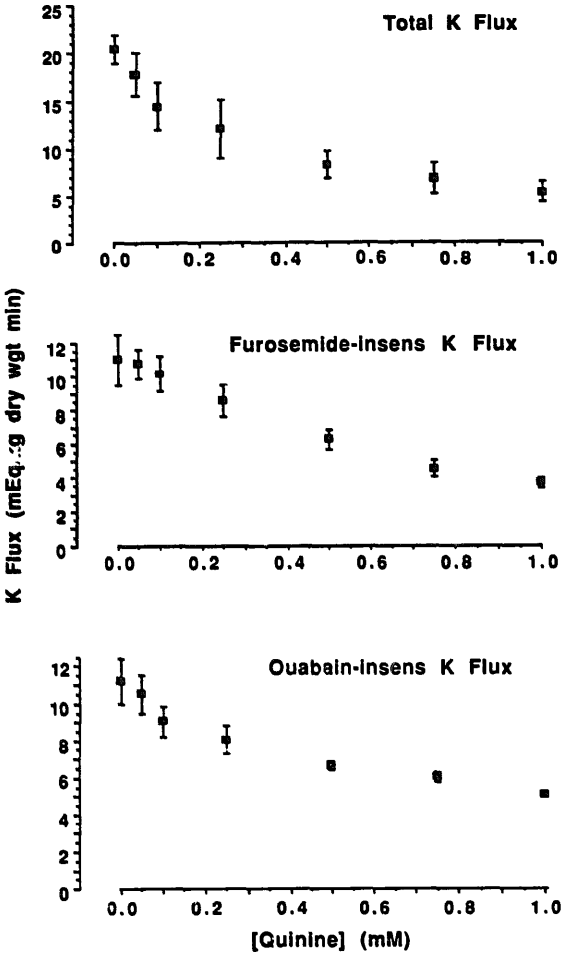

Fig. 2. The effect of quinine on K^+ influx in Ehrlich ascites tumor cells. Ehrlich cells were pre-incubated in physiological saline to establish steady-state cellular electrolyte and water contents. K^+ influx was determined from the uptake of ^{86}Rb (0.3–0.5 μCi/ml) in the presence of quinine (concentration range: 0–1.0 mM). Determination of the effects of quinine on the total flux (upper panel) was made in the absence of added inhibitors. Its effects on the furosemide-insensitive (middle panel) and ouabain-insensitive (lower panel) components of the flux were evaluated in the presence of furosemide (2 mM) and ouabain (2 mM), respectively. Values are the mean ± S.E. from duplicate samples in three experiments.

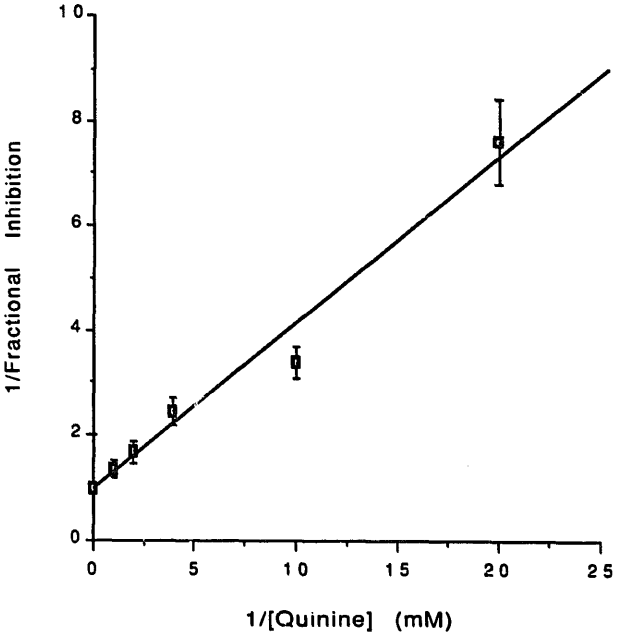

Fig. 3. Inhibition of total K^+ influx by quinine in Ehrlich ascites tumor cells. The data of Fig. 2 (upper panel) have been replotted showing the relationship between fractional inhibition of the total K^+ influx and quinine concentration. Fractionation inhibition = $(J_o - J_{inh})/J_o$ where J_o is the total K^+ influx in the absence of quinine and J_{inh} is the flux in its presence.

or an undefined secondary coupling between the fluxes.

All three inhibitors together give no additional inhibition compared to the combination of ouabain and furosemide. Thus, our data provide no evidence for activity of the quinine-sensitive K^+ influx in steady-state cells. Consequently, we tested for its presence in cells exposed to propranolol (Fig. 1C). Although propranolol stimulates K^+ uptake more than 3-fold in the presence of ouabain (to 357 ± 20% control; Fig. 1C), again its effect is completely abolished by quinine. Moreover, the magnitude of the furosemide-sensitive component is the same as found in either control or ouabain-treated cells. This strongly supports the conclusion that furosemide is without effect on Ca^{2+}-dependent channels in these cells. Taken together, these data are consistent with the view that quinine is an effective inhibitor of Ca^{2+}-dependent K^+ channels. However, its actions are not restricted to this pathway. Both the 'ouabain-sensitive' and 'furosemide-sensitive' fluxes are also altered by quinine.

We have extended these studies to evaluate the dependence of K^+ influx on quinine concentration (Fig. 2). The total K^+ influx is progressively reduced as [quinine] is increased over the range 0–1 mM. Analysis of the ouabain- and furosemide-sensitive components show that the separate pathways are similarly quinine-sensitive. The maximum extent of the inhibition can be estimated from a double-reciprocal plot of the fractional inhibition and [quinine] (Fig. 3). The intercept of the plot for total K^+ influx is approx. equal to unity. Thus, the entire influx is subject to inhibition by quinine. Separate plots for the ouabain- and furosemide-sensitive components, of course, give similar results. The [quinine] which gives 50% inhibition is 0.32 ± 0.02 mM (S.E.; $n = 3$) for the ouabain-sensitive flux and 0.24 ± 0.01 mM (S.E.; $n = 3$) for the furosemide-sensitive flux. The difference in the potencies for quinine inhibition of these two K^+-flux components is significant ($P < 0.02$). However, conclusions based on the apparent difference in sensitivity to quinine must be tempered by the recognition that alterations in V_m may affect the kinetic parameters of electrogenic transport pathways [32].

Quinine has also been shown to inhibit Na^+/H^+ antiport in renal tissue [14]. Consequently, we have

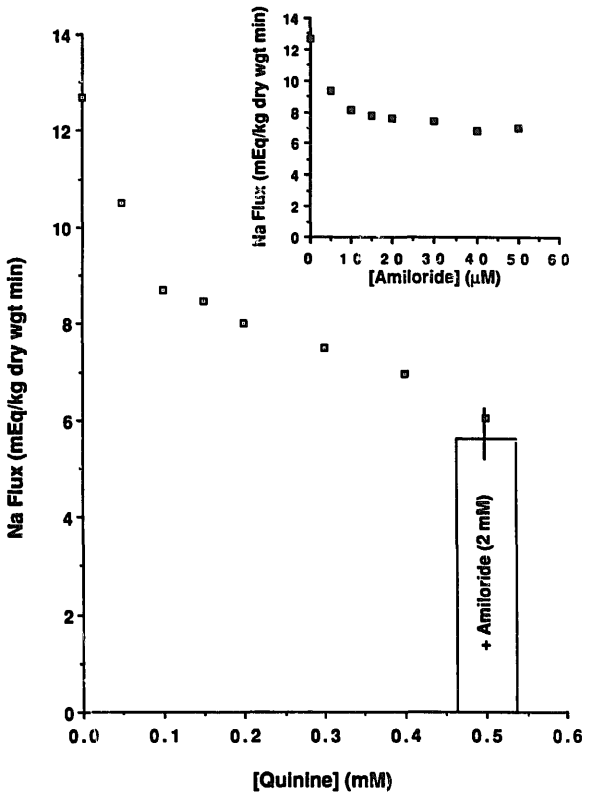

Fig. 4. The effect of quinine and amiloride on Na^+ influx in Ehrlich ascites tumor cells. Ehrlich cells were washed and incubated in choline chloride to render both the cells and the medium Na^+-free. Samples of the cell suspension were then transferred to 1.5 ml centrifuge tubes containing physiological saline (±) the test inhibitors and tracer amounts of ^{22}Na (0.4 mCi/mmol). Final [Na^+] was 40 mEq/l. Na^+ uptake was estimated from the cellular accumulation of ^{22}Na during 3 min. The bar (+amiloride) shows the Na^+ influx in the presence of quinine (0.5 mM) + amiloride (2 mM).

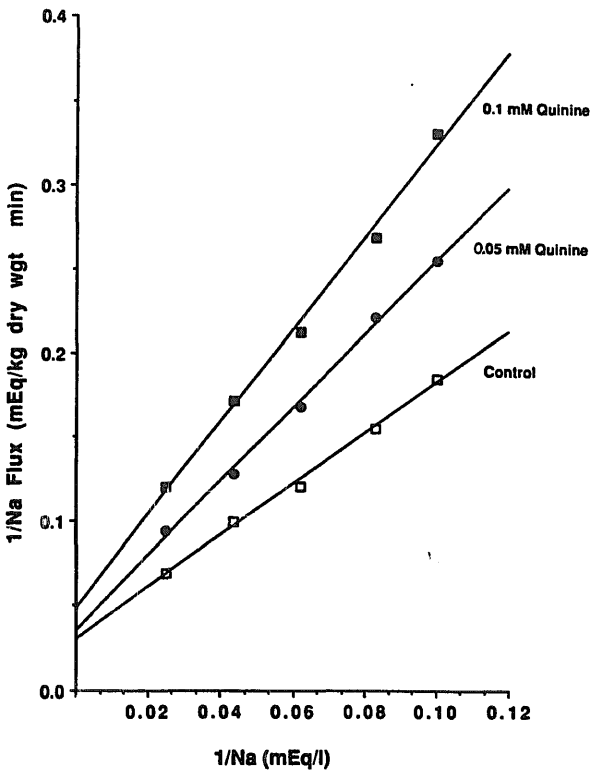

Fig. 5. Lineweaver-Burk analysis of the effect of quinine on Na^+ influx in Ehrlich ascites tumor cells. Na^+ influx was determined from uptake of tracer ^{22}Na in the absence and presence of quinine (0.05 and 0.10 mM).

tested its effect on unidirectional Na^+ influx in Ehrlich cells (Fig. 4). These experiments were performed in medium with reduced [Na^+] (40 mEq/l) to optimize the amiloride-sensitivity of the influx. Under these conditions, 52% of the Na^+ influx is inhibitable by amiloride with an apparent K_i of 4.8 μM. Quinine also inhibits the influx to a similar extent. At [quinine] = 0.5 mM, Na^+ influx is reduced by 52%. Addition of amiloride (2 mM) in the presence of quinine (0.5 mM) gives no further inhibition, supporting the view that quinine and amiloride inhibit the same Na^+ transport pathway. Quinine interaction with amiloride-sensitive Na^+ transport (Fig. 5) is without effect on the apparent Michaelis-Menten constant for the amiloride-sensitive Na^+ influx at either 0.05 or 0.1 mM quinine ($K_m = 50.5 \pm 2.8$ mM). However, quinine does significantly reduce the maximum amiloride-sensitive Na^+ influx. In control cells, $K_{max} = 32.3 \pm 3.2$ mEq/kg dry wt. per min while the addition of quinine (0.05 and 0.10 mM) reduces K_{max} to 24.4 ± 2.0 and 18.9 ± 0.9 mEq/kg dry wt. per min ($P < 0.05$ and < 0.01, respectively). These results are consistent with non-competitive inhibition by quinine with an apparent K_i of 0.14 ± 0.01 mM (S.E.; $n = 4$).

The results of these studies demonstrate that quinine inhibits a diverse set of alkali cation-transport systems. The question raised by the data is whether quinine acts by: (1) having independent effects on the separate transport systems; (2) affecting an electrogenic system, which then affects other parallel systems through altered V_m; or (3) altering a common regulatory system (e.g., ATP), which expresses generalized inhibitory effects. Unfortunately, tests of these possibilities are not definitive at present. Our results suggest that ouabain-sensitive and furosemide-sensitive K^+ fluxes and amiloride-sensitive Na^+ flux are affected by quinine with different potencies based on the significant difference ($P < 0.02$) in their apparent K_i value. In the light of the concomitant membrane depolarization, these differences do not provide definitive evidence for independent effects of quinine on separate transport systems. The differences in the K_i values may only reflect secondary electrostatic linkages to the separate pathways. However, our finding that the furosemide-sensitive K^+ flux is unaffected by propranolol (Fig. 1) makes it likely that its inhibition by quinine is direct. This conclusion is consistent with the presumed electroneutrally of the furosemide-sensitive cotransport pathway for

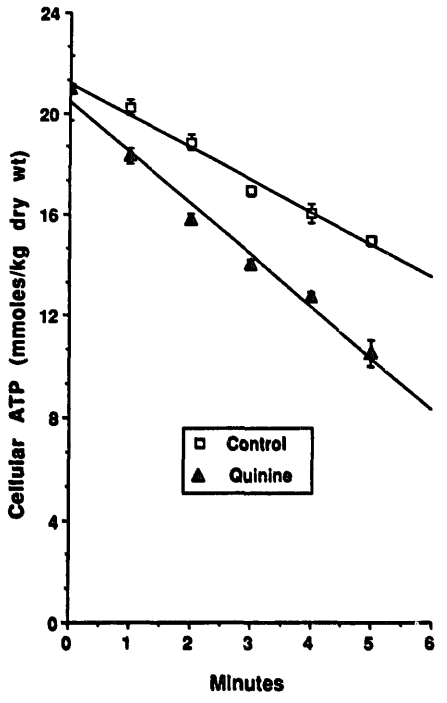

Fig. 6. Effect of quinine on cellular ATP in Ehrlich ascites tumor cells. Steady-state suspensions of Ehrlich cells in physiological saline were prepared as described in Fig. 1. Samples of the suspension were transferred to flasks containing rotenone (10 μM) and 2-deoxyglucose (5 mM) in the absence or presence of quinine (2 mM). Aliquots of the cell suspension were taken as specified times and the ATP content determined by bioluminescence. Values represent the mean from duplicate samples in three separate experiments. Error bars representing the standard errors of the mean (S.E.) are given except if they are smaller than the symbols.

K^+ [10], and points to a more direct interaction of quinine with the cotransporter.

Finally, we have considered the possibility that quinine exerts its inhibitory effects through a common regulatory mechanism. Of the pathways we have examined, only the Na^+/K^+ active transport mechanism (ouabain-sensitive K^+ influx) derives energy directly from ATP hydrolysis. However, there is abundant evidence suggesting a role for cellular ATP, perhaps in a regulatory capacity, in each of the other pathways [1,5,9,33]. Consequently, we determined the effect of quinine on the cellular content and utilization of ATP. Steady-state Ehrlich cells have an ATP content of 21.0 ± 1.4 mmol/kg dry wt. (S.E.: $n = 4$). Exposure of these metabolically competent cells to quinine (2 mM) for 10 min is without effect on ATP content (19.9 ± 0.9 mmol/kg dry et.; S.E.; $n = 4$). Since the experimental conditions of these studies mimic those used for our transport measurements, it is clear that the generalized quinine-sensitivity of cation transport is not simply secondary to altered ATP availability. It remains possible, however, that quinine alters ATP utili ation of the transport systems. We have extended the studied to evaluate ATP turnover in the presence of quinine. Ehrlich cells, when exposed to a combination of rotenone (10 μM) and 2-deoxyglucose (5 mM), are rapidly depleted of ATP (Fig. 6). The rate of disappearance provides a measure of the total cellular ATPase activity. Addition of quinine to these cells accelerates the loss by 57%. Although no evidence establishes a link between the generalized cation-transport inhibition by quinine and the attendant alteration in ATPase activity of the cells, it is interesting to speculate that quinine may exert its broad effects on transport by altering ATP interactions with the transport systems.

Taken together, the evidence from this investigation argues strongly against the use of quinine for evaluating the role of Ca^{2+}-dependent K^+ channels in Ehrlich ascites tumor cells. Quinine also leads to membrane depolarization and inhibition of the Na^+/K^+ active transport mechanism, $Na^+ + K^+ + 2Cl^-$ cotransport and Na^+/H^+ antiport. It is not clear whether these effects involve direct interaction of quinine with the transport systems or whether secondary coupling is involved. Possible linkages of the parallel systems through the changes in the membrane potential or by ATP dependence need to be further explored.

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