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K^+ Influx Components in Ascites Cells: The Effects of Agents Interacting with the $(Na^+ + K^+)$-Pump

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Summary. Several agents known to interact with the $(Na^+ + K^+)$-pump were tested for their effects on the components of steady-state K^+ flux in ascites cells. $^{86}Rb^+$ was used as a tracer for K^+, and influx was differentiated into a ouabain-inhibitable "pump" component, a Cl^--dependent and furosemide-sensitive "exchange" component, and a residual "leak" flux. All agents tested (ouabain, quercetin, oligomycin, phosphate) affected both the "pump" flux and the Cl^--linked flux. These findings suggest a linkage between the activity of the Na/K ATPase and the Cl^--dependent K^+ exchange flux. In the discussion we point out that the mechanism of this linkage could be direct; e.g., Cl^--dependent exchange may represent a mode of operation of the Na/K ATPase. However, data from this and other systems tend to suggest an indirect linkage between the Na^+ pump and a KCl symporter, perhaps via a change in the level of intracellular ATP.

Monovalent cations have been shown to cross animal cell plasma membranes by several pathways: alone, or in co- or countertransport with other ions or molecules. Such transport may or may not be coupled to the action of an ATP-driven "pump". For instance, K^+ is actively taken up and Na^+ extruded by a ouabain-sensitive $(Na^+ + K^+)$-pump[1]; a passive leak pathway is available for both ions [11]. In addition, there is evidence for ouabain-insensitive cotransport of Na^+ and K^+, especially for avian erythrocytes [17]; this type of cotransport may also account for a ouabain-insensitive Na^+ transport system, "Pump II", first described for human red blood cells [12]. Some observations point to the existence of a $(K^+ + Cl^-)$-cotransport mechanism, at least in Ehrlich cells [6, 7]. And in most animal cells investigated so far, Na^+ is cotransported with certain amino acids [4].

In several cell types Na^+ and K^+ have been shown to cross the membrane in a process denoted "exchange diffusion" [3,13,23,24]: this process involves mediated, energy-independent transport of Na^+ or K^+ across the membrane in exchange for an identical amount of the same ion species. Presumably, any of the transport systems mentioned above under certain conditions may mediate exchange diffusion of each one of the ions or molecules involved. Conversely, cation exchange diffusion may be considered an alternate mode of operation of one or more co- or countertransport systems which normally are utilized in a hetero-cotransport or -exchange manner. Thus, red blood cells under certain conditions exhibit ADP-dependent and ouabain- and oligomycin-sensitive Na^+ exchange diffusion (for references, *see* [8]); K^+ exchange diffusion in red blood cells is inhibited by ouabain, and requires the presence of inorganic phosphate (P_i) at the inner surface of the membrane [20]. Both these Na^+- and K^+-exchange diffusion mechanisms are inhibited by antiserum to the $(Na^+ + K^+)$-pump [9]. All those characteristics tend to link this specific exchange phenomenon to the $(Na^+ + K^+)$-pump. Evidence that this pump is indeed capable of exchange diffusion has been obtained from a reconstituted system containing highly-purified $(Na^+ + K^+)$-ATPase [10].

In Ehrlich cells, total K^+ influx at normal extracellular ion concentrations has been accounted for by three components: a ouabain-inhibitable pump flux, furosemide-sensitive exchange diffusion, and a residual leak flux [23]. Also in our strain of ascites cells we have demonstrated a furosemide-sensitive K^+ exchange fraction [1]. This fraction appears to share the characteristics of the $(K^+ + Cl^-)$-cotransport sys-

[1] *Abbreviations:* P_i, inorganic phosphate; $(Na^+ + K^+)$-pump, pumping activity associated with the $(Na^+ + K^+)$-activated ATPase (E.C. 3.6.1.3).

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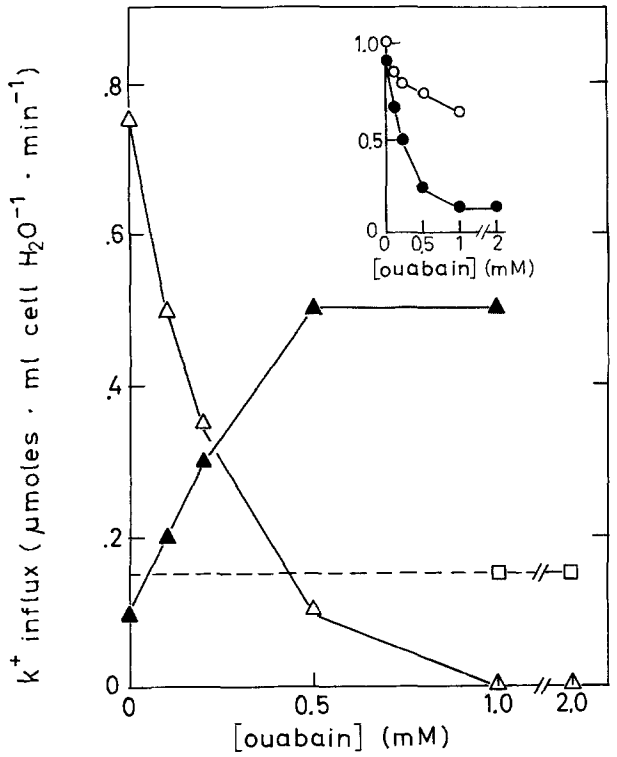

Fig. 1. K^+ influx components as a function of ouabain concentration. Ouabain was added 1 min before $^{86}Rb^+$. The experiment was performed as described in Methods. K^+ flux components were calculated from the $^{86}Rb^+$ uptake data presented in the insert as described in the text. Insert: K^+ influx in: ○—○, Cl^--saline; ●—●, NO_3^--saline. Main figure: △—△, ouabain-inhibitable flux (difference in $^{86}Rb^+$ influx in NO_3^--saline plus or minus 1 mM ouabain); ▲—▲, Cl^--dependent flux (difference in $^{86}Rb^+$ influx in Cl^--saline and NO_3^--saline); □—□, residual flux ($^{86}Rb^+$ influx in NO_3^--saline in the presence of 1 mM ouabain)

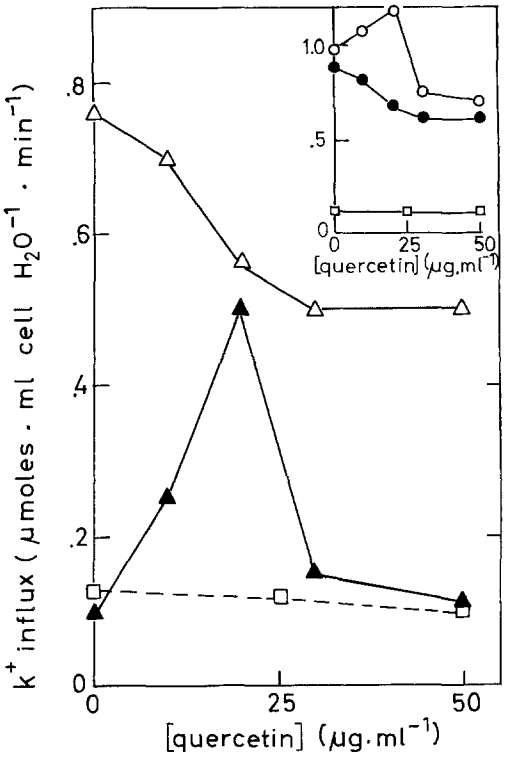

Fig. 2. K^+ influx components as a function of quercetin concentration. Quercetin was added 5 min, and ouabain (1.2 mM) 1 min, before $^{86}Rb^+$. The experiment was performed and the data evaluated as indicated in the text to Fig. 1. Insert: K^+ influx in: ○—○, Cl^--saline; ●—●, NO_3^--saline; □—□, NO_3^--saline plus, 1.2 mM ouabain. Main figure: △—△, ouabain-sensitive K^+ flux; ▲—▲, Cl^--dependent flux; □—□, residual flux

tem recently described for Ehrlich cells [6,7]: both exchange and net cotransport are inhibited by furosemide and depend upon the presence of Cl^-; in both cases Br^- is partially able, and NO_3^- is unable to substitute as a main anion [1,7]. That in our system Cl^- is actually required for K^+ exchange diffusion rather than NO_3^- being a furosemide-like inhibitor is strongly suggested by the fact that the concentration dependence of the anion effect is identical for such diverse anions as I^-, NO_3^- and SO_4^{2-} [1].

Previously we have observed [1] that the Cl^--dependent K^+ exchange diffusion in our cells was stimulated by ouabain, an inhibitor of the $(Na^+ + K^+)$-pump [19]. In this communication we present a more systematic investigation of the effects on Cl^--dependent K^+ flux of agents known to interact with the $(Na^+ + K^+)$-pump. We found that all agents tested affected both ouabain-inhibitable and Cl^--dependent K^+ fluxes in our cells. Our results indicate that interaction between the $(Na^+ + K^+)$-pump on the one hand and the $(K^+ + Cl^-)$-cotransport system on the other hand may complicate the interpretation of unidirectional flux measurements.

Materials and Methods

Ascites tumor cells (a nonspecific strain kindly donated by Dr. J. Molnar from the University of Illinois) were grown in the abdominal cavity of Swiss Webster mice. Cells were washed in either Cl^--containing saline (Cl^--saline) or NO_3^--containing saline (NO_3^--saline). The cell concentration was determined by spinning duplicate samples for 10 min in capillary tubes at 1,000 rpm. Extracellular (^{14}C-sucrose-accessible) space under these conditions was 20%; dry wt, after dehydration at 100 °C overnight, was 16%. Results are expressed per ml cell water: to this end, the final intracellular H_2O space in all cases has been taken as (100–20–16=) 64% of the original total pellet volume, without considering possible volume changes after addition of the different inhibitors.

Washed cells were suspended at a concentration of 1 $^1/_2$%, and incubated at 37 °C for 90 min to establish steady-state ion concentrations. Inhibitors and P_i were added as indicated. Uptake experiments were performed at 37 °C, and started by adding $^{86}Rb^+$

Table 1. Effects of compounds interacting with the $(Na^+ + K^+)$-pump on K^+ influx components

	K^+ influx					K^+ influx components					
Saline:	Cl^-	NO_3^-		Cl^- + furosemide		Ouabain-sensitive		Cl^--dependent		Furosemide-sensitive	
Ouabain 1.2 mM:	−	−	+	−	+	Flux	% control	flux	% control	flux	% control
Expt. 1a control (K_i at 6′/24′)	1.50 (131/130)	0.75 (131/126)	0.20 (120/107)			0.55		0.75			
+Quercetin 25 μg·ml^{-1} (K_i at 6′/24′)	1.95 (122/120)	0.60 (122/116)	0.15 (107/97)			0.45	82	1.35	180		
+Quercetin 50 μg·ml^{-1} (K_i at 6′/24′)	1.10 (114/117)	0.55 (112/108)	0.10 (98/87)			0.45	82	0.55	73		
Expt. 1b control	2.13			1.36	0.10	1.26				0.77	
+Quercetin 40 μg·ml^{-1}	1.57			1.02	0.20	0.82	65			0.55	71
Expt. 2a control (K_i at 8′/32′)	1.55 (131/126)	1.10 (119/113)	0.20			0.90		0.45			
+Oligomycin 60 μg·ml^{-1} (K_i at 8′/32′)	1.05 (124/121)	0.80 (117/117)	0.20			0.60	67	0.25	56		
Expt. 2b control (K_i at 8′/32′)	2.05 (133/133)			1.10 (129/126)	0.20 (136/114)	0.90				0.95	
+Oligomycin 50 μg·ml^{-1} (K_i at 8′/32′)	1.40 (137/130)			0.85 (138/136)	0.15 (138/126)	0.70	78			0.55	58
Expt. 3 control (K_i at 8′/32′)	1.60 (138/137)	1.15 (125/125)	0.15	1.20 (137/131)	0.18 (130/117)	1.0		0.45		0.40	
+P_i 10 mM (K_i at 8′/32′)	2.05 (140/138)	1.90 (123/122)	0.15	1.98 (139/135)	0.16 (127/107)	1.75	175	0.15	33	0.07	18

Fluxes are expressed in μmol·ml cell H_2O^{-1}·min^{-1}. Flux components were calculated as in the experiments of Figs. 1 and 2. Quercetin, oligomycin, and ouabain were added 1–2 min before $^{86}Rb^+$. Furosemide (1.2 mM) and P_i were present during the 90-min preincubation (*see* Methods). In Expt. 3 the salt concentration in the control incubations was increased with 12.5 mM NaCl or $NaNO_3$ for osmotic balance.

Between brackets: K^+ content (in μmol·ml cell H_2O^{-1}) at the indicated two time points after addition of $^{86}Rb^+$. K^+ content was determined as described in Methods; values are the average of duplicates, which did not differ by more than 5%.

(5–20 nCi/ml) at zero time: this isotope has been shown [18] to act as a tracer for K^+ in Ehrlich cells. At fixed intervals, varying between 4 and 8 min over a total of 24–40 min, uptake was terminated on a total of 4 to 6 duplicate 4-ml samples of cell suspension by centrifuging at 1,500 rpm for 1 min. Radioactivity was extracted from the precipitate with 5% trichloroactetic acid and counted on a Picker Liquimat® scintillation counter. Duplicates did not differ by more than 5%. $^{86}Rb^+$ uptake was linear in time for at least 24 min in the absence and 40 min in the presence of ouabain (*compare* [1], Fig. 1). Occasionally uptake curves bent off within this time course: those experiments were discarded. The rate of unidirectional K^+ uptake was calculated by linear regression from the slope of the initial linear part of the $^{86}Rb^+$ influx curves: the correlation coefficient r exceeded 0.97 in all cases. Typically, the ordinate intercepts of the $^{86}Rb^+$ influx curves corresponded with apparent compartments of 3–5 ml·(ml cell H_2O)$^{-1}$ in Cl^--saline, and 1–2 ml·(ml cell H_2O)$^{-1}$ in NO_3^--saline, or in Cl^--saline in the presence of furosemide; both ranges of values greatly exceeded the extracellular space estimated with ^{14}C-sucrose under identical conditions (0.32–0.35 ml·(ml cell H_2O)$^{-1}$). The nature of the rapidly-exchanging compartment(s) responsible for the difference is at present not clear.

Cellular K^+ content was determined in part of the samples by flame photometry. For the calculation it was assumed that in all cases pellets were obtained with an intracellular space of 9.6 μl and an extracellular space of 3.2 μl per ml cell suspension centrifuged (these values follow from the data on cell concentration, intra- and extracellular spaces given above).

Solutions and Chemicals

Cl^--saline contained (in mM): NaCl, 100; KCl, 5; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50, neutralized with NaOH, 25; $CaCl_2$, 1; $MgSO_4$, 1; glucose, 20; final pH, 7.4–7.6; temperature, 37 °C. In NO_3^--saline, NaCl and KCl were replaced by $NaNO_3$ and KNO_3, respectively. $^{86}Rb^+$ was obtained from New England Nuclear, ouabain from Sigma. Oligomycin (Sigma) was added in ethanolic solution, and quercetin (Nutritional Biochemical Company) in dimethylsulfoxide; identical amounts of pure solvent (final concentration not exceeding 1% vol/vol) were added to controls. Crystalline furosemide was kindly provided by Dr. H.L. Dettelbach from Hoechst.

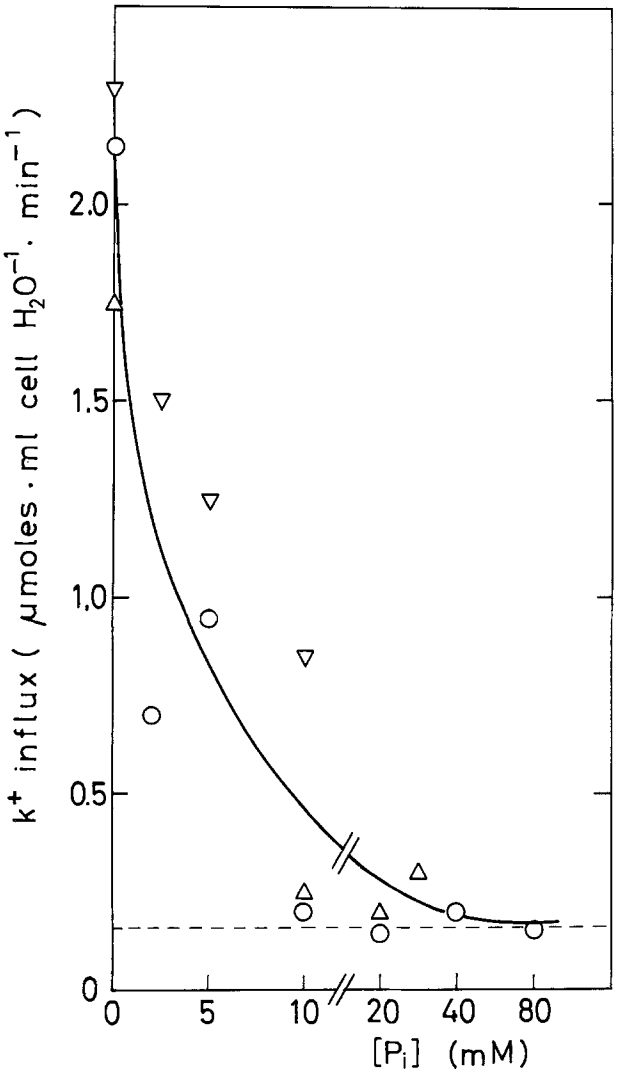

Fig. 3. Effect of P_i on K^+ influx in the presence of 1.2 mM ouabain. P_i (brought to pH 7.5 with NaOH) was substituted for part of the NaCl contained in the saline to give the indicated final P_i concentrations (to keep osmotic balance, 1.25 mM NaCl was omitted for each mM of P_i added). Cells were preincubated at 37° for 90 min in the indicated P_i concentrations. Ouabain (1.2 mM) was added 1 min before $^{86}Rb^+$. Different symbols denote different experiments. Dashed line: influx in NO_3^--saline in the presence of 1.2 mM ouabain

Results

Cardiac glycosides have long been known to interact with the $(Na^+ + K^+)$-pump [19]. We measured K^+ influx in ascites cells in Cl^--saline and NO_3^--saline as a function of ouabain concentration (Fig. 1, insert). Note that the influx in both saline systems decreased with increasing ouabain concentrations, but that the effect of ouabain was more pronounced in NO_3^--saline. From the data presented in the insert, "ouabain-sensitive", "Cl^--dependent" and "residual" contributions were derived and plotted in the main figure. First, the Cl^--dependent component was calculated as the difference in influx rate in Cl^--saline and NO_3^--saline (filled triangles). K^+ influx in NO_3^--saline can be seen to level off above 1 mM ouabain (insert). Consequently, the influx in NO_3^--saline at concentrations of ouabain exceeding 1 mM was taken as the residual component (squares); the difference in influx rate in NO_3^--saline at any concentration of ouabain with that at 1–2 mM ouabain was identified as ouabain-sensitive component (open triangles). Note that while the ouabain-sensitive activity was inhibited, Cl^--dependent activity increased, as observed before for a saturating ouabain concentration [1].

Quercetin is a bioflavonoid which has been claimed to restore cancer-related coupling defects in both mitochondrial H^+- and plasma membrane $(Na^+ + K^+)$-ATPase [22]. Figure 2 shows the K^+ influx in Cl^--saline and in NO_3^--saline, plus or minus 1.2 mM ouabain (insert), and the derived K^+ flux components, as a function of quercetin concentration (main figure). The flux components were calculated from the data in the insert as described for Fig. 1: Cl^--dependent K^+ flux (filled triangles) was again taken as the difference in influx rate in Cl^--saline and NO_3^--saline, and the influx in NO_3^--saline was differentiated into a ouabain-sensitive (open triangles) and a residual component (squares) by the action of 1.2 mM ouabain, at the indicated quercetin concentrations. From the main figure it can be seen that quercetin suppressed the ouabain-sensitive K^+ flux up to 33% at 50 $\mu g \cdot ml^{-1}$. The Cl^--dependent flux was stimulated at the lower quercetin concentrations and subsequently reverted to the basic level. Table 1, Expt. *1a*, presents another experiment involving quercetin. Although the absolute values of the K^+ flux components differ from those in Fig. 2, the data again show a clear stimulation of Cl^--dependent K^+ flux at lower quercetin concentration.

Oligomycin is thought to block the conversion of a high-energy phosphorylated pump intermediate into a low-energy form [25]. Table 1, Expt. *2a* shows that oligomycin, at a concentration of 60 $\mu g \cdot ml^{-1}$, inhibited the ouabain-sensitive K^+ flux by 33% and the Cl^--dependent flux by 44%.

As one of the reaction products of $(Na^+ + K^+)$-dependent ATPase activity, inorganic phosphate (P_i) is inherently involved in the action of the $(Na^+ + K^+)$-pump. Table 1, Expt. *3* summarizes the effect of 10 mM P_i on the respective flux components: at this concentration P_i stimulated the ouabain-sensitive fraction by 75%, and suppressed the Cl^--dependent fraction by 66%. The effect of P_i on Cl^--dependent K^+ flux was also investigated in the presence of a saturating amount of ouabain: also under those conditions P_i inhibited Cl^--dependent K^+ flux, with an apparent K_i in the order of 2–5 mM (Fig. 3).

Table 1, Expts. *1b, 2b* and *3* show that the effect of replacement of Cl^- by NO_3^-, where tested, was comparable to that of addition of furosemide to Cl^--saline. We had already shown this to be the case in the presence of a saturating amount of ouabain [1].

Table 1 also presents, where measured, values for the intracellular K^+ contents. All values were contained within one broad range, from approximately 130 mM in the absence, down to approximately 100 mM after 32 min in the presence, of 1.2 mM ouabain.

As can be seen from the experiments presented here, the absolute rates of Cl^--dependent (or furosemide-sensitive) K^+ influx were rather variable (up to a factor 5). The reason for this is not clear; however, we did notice an inverse relationship between the magnitude of the Cl^--dependent component and the blood content of the peritoneal aspirate from which the cells were isolated. In contrast, residual fluxes only varied up to a factor 2, without apparent correlation to the incubation conditions.

Discussion

In our strain of ascites cells, more than 80% of K^+ influx under normal conditions is accounted for by the sum of a ouabain-sensitive and a Cl^--dependent component ([1]; this paper). In Ehrlich cells, the ouabain-sensitive component has been characterized as a net flux, mediated by the $(Na^+ + K^+)$-pump; the furosemide-sensitive component (equivalent to the Cl^--dependent flux: [1] and this paper) as exchange diffusion; and the residual flux as passive diffusion [18,23]. In first approximation this same flux characterization also holds for our strain of ascites cells; in fact, we have previously applied it [1]. However, the following qualifications should be made:

1) We have not strictly proven that the residual influx is purely diffusional: it may still contain as yet unclassified pump or exchange components. Although this is an important point, it is not relevant for the present discussion.

2) The ouabain-sensitive flux may, on top of a net pump flux, contain a $(Na^+ + K^+)$-pump-mediated exchange fraction. Specifically, enhancement of ouabain-sensitive K^+ influx in the presence of P_i (Table 1, Expt. *3*) probably must be ascribed to an induction of $(Na^+ + K^+)$-pump-mediated exchange diffusion rather than to an increase in net K^+ transport: the latter would have been reflected in a higher steady-state K^+ content.

3) From the data on cellular K^+ content (Table 1) it may be inferred that under our conditions Cl^--dependent K^+ influx must largely have been balanced by Cl^--dependent K^+ efflux. However, this in itself does not qualify the Cl^--dependent K^+ flux as exchange diffusion in the sense originally envisioned by Ussing [24]: i.e., as a tightly coupled exchange of permeants in the absence of any net flux at all substrate levels. Presumably, as stated in the introduction, the Cl^--dependent K^+ flux is mediated by a $(K^+ + Cl^-)$-cotransport system; since this system, at least in Ehrlich cells [6,7], is capable of net KCl transport, our Cl^--dependent K^+ exchange should probably be classified "competitive" rather than "compulsory", in Stein's [21] terminology.

The main conclusion to be drawn from our data is that several compounds acting on the $(Na^+ + K^+)$-pump at the same time influenced Cl^--dependent K^+ flux. There are three possible classes of explanation for these observations: (i) Cl^--dependent K^+ exchange may represent a mode of operation of the $(Na^+ + K^+)$-pump itself, unrelated to the operation of the KCl symporter described by Geck and associates [6,7]; (ii) there could be a direct linkage between the Na/K ATPase and the KCl symporter; and (iii) the operation of the KCl symporter may be linked indirectly to the Na/K ATPase through changes in such substances as ATP, inorganic phosphate or cell sodium.

Our data do not allow us to draw a clear distinction between these mechanisms. However, the fact that ^{86}Rb uptake was linear with time in the presence of ouabain suggests that changes in intracellular sodium do not mediate the enhanced Cl-dependent exchange seen in the presence of this drug. Had this been the case the time courses should have shown a distinct upward bend. A similar effect of ouabain on K^+ exchange diffusion has been observed [2] in virally-transformed 3T3 cells. In these cells the effects of ouabain on pump- and exchange activities have been temporally separated, which indicates that at least there the interaction between $(Na^+ + K^+)$-pump and K^+ exchange system is indirect. It may be possible that inhibition of the $(Na^+ + K^+)$-pump leads to a local increase in ATP level, which in its turn, could stimulate Cl^--dependent exchange: ATP seems to be required for the $(K^+ + Cl^+)$-symport, presumably in a regulatory role [6,7]. In line with this explanation, the inhibitory effects on Cl^--dependent K^+ flux of quercetin at higher concentrations and of oligomycin (Fig. 2 and Table 1) could be due to their interference with mitochondrial ATP generation [14,15].

In contrast to our observations, ouabain had no effect on furosemide-sensitive K^+ fluxes in Ehrlich cells [6,23]. This discrepancy may be due to a difference in cell strain, or in experimental conditions, notably the presence (in our case) or absence [6,23] of glucose: this metabolite has been shown [16] to affect K^+ flux patterns in ascites cells.

Phosphate inhibited Cl^--dependent K^+ flux both in the absence (Table 1, Expt. *3*) and presence (Fig. 3) of ouabain. As suggested above, its net effect on K^+ flux in the absence of ouabain was probably rather complex: it appeared to partially convert a Cl^--dependent into a $(Na^+ + K^+)$-pump-mediated exchange flow. In view of the fact that P_i was shown to be required for K^+ exchange diffusion in a reconstituted $(Na^+ + K^+)$-ATPase system [10], its stimulating effect on $(Na^+ + K^+)$-pump-mediated exchange was probably direct; conversely, our data do not allow any conclusions as to the nature of its inhibitory effect on Cl^--dependent K^+ flux.

Whatever the mechanism of cation exchange diffusion, its occurrence must be kept in mind in studies pertaining to the energetics of cation transport. For example, K^+ pump flux is commonly taken as the ouabain-inhibitable component of unidirectional K^+ influx in Cl^--containing Ringer; this is only justified under conditions where K^+ exchange diffusion is shown to be absent, or not influenced by ouabain. Another example is the measurement of the coupling of active K^+ transport to glycolysis in the presence of quercetin [22]: the published data do not exclude the possibility that the apparent increase in coupling in the presence of quercetin may result from increased K^+ exchange diffusion rather than increased net K^+ pumping (*compare* [22], Fig. 1, with our Fig. 2).

Summarizing our experiences with ascites cells, it appears that problems arising from K^+ exchange diffusion may at least partially be avoided by substituting NO_3^- for Cl^- as the main anion. We feel this procedure to be preferable to the addition of furosemide: although we obtained the same results in either furosemide-containing Cl^--saline or NO_3^--saline, the possibility of complicating drug interactions in the presence of furosemide cannot *a priori* be excluded. The effect of replacing Cl^- by NO_3^- on K^+ flux may be quite general: NO_3^--sensitive K^+ flux components have also been demonstrated in erythrocytes [5] and in chinese hamster ovary cells [1 a].

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