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# AN EFFECT OF PIRETANIDE UPON THE INTRACELLULAR CATION CONTENTS OF CELLS SUBJECTED TO PARTIAL CHRONIC (Na–K) PUMP BLOCKADE BY OUABAIN

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**Abstract**—Cultured cells have been used to study the contribution made by the ouabain-insensitive but diuretic (piretanide)-sensitive K transport system (so-called cotransport) to the maintenance of intracellular $Na^+$ and $K^+$ contents in normal cells and in cells whose Na-pump sites have been subjected to chronic partial inhibition. In cells which have normally directed gradients of $Na^+$ and $K^+$, chronic incubation in piretanide ($10^{-4}$ M) for up to 24 hr has no significant effect on the internal ion contents of HeLa (human carcinoma), MDCK (dog kidney epithelium) or $BC_3H1$ (mouse smooth muscle) cell lines. This observation is consistent with the notion that when the intracellular ion contents are in a normal steady state the net driving force acting upon the diuretic-sensitive K transport (Na + K + Cl cotransport system) is zero or very close to zero. When cells are subjected to chronic partial inhibition of the sodium pump as a consequence of growth in sublethal concentrations of ouabain ($10^{-9}$–$3 \times 10^{-7}$ M), the number of functional Na-pump sites decreases, the intracellular $Na^+$ content increases and the intracellular $K^+$ decreases in a dose dependent manner. Under these conditions, inclusion of piretanide ($10^{-4}$ M) causes a significant retardation of $Na^+$ gain and $K^+$ loss from the cells. This response is of high molar affinity ($EC_{50} = 3$–$4 \times 10^{-6}$ M) and can be obtained with the other loop diuretics, furosemide and bumetanide. The data presented are consistent with the idea that in cells subjected to chronic partial inhibition of the Na-pump, there is a piretanide-sensitive exchange of intracellular $K^+$ for extracellular $Na^+$. Such an effect of the cotransport system would not be predicted on the basis of a tightly coupled electroneutral cotransport of $Na^+$, $K^+$ and $Cl^-$ with a stoichiometry of 1Na:1K:2Cl. The data are discussed in relation to the possible role of putative circulating endogenous inhibitors of the Na-pump.

Although the main effect of the loop diuretics (e.g. piretanide) is on natriuresis, there is now increasing interest in their other extra-renal actions [1]. This arises largely from the demonstration in a diverse number of cell-types of loop diuretic inhibition of a substantial proportion of the trans-membrane passive (ouabain-insensitive) exchange of $Na^+$, $K^+$ and $Cl^-$ [2–6]. For avian erythrocytes, the operational behaviour of the loop diuretic-sensitive ion transport has been defined as an electroneutral cotransport of $Na^+$, $K^+$ and $Cl^+$ with a stoichiometry of 1Na:1K to 2Cl ions [6]. This information comes primarily from experiments where flux is driven by large chemical gradients of $Na^+$ and $K^+$, in the presence of total Na-pump blockade [6]. The overall direction of net diuretic sensitive transport is dependent upon the sum of the chemical activity gradient for $Na^+$, $K^+$ and $Cl^-$ [6]. If the net sum of chemical gradients is zero, then the cotransport system will mediate no net fluxes, allowing apparent exchange fluxes [6]. In normally functioning cells, with opposing $K^+$ and $Na^+$ gradients it is not clear what contribution the cotransport system makes to net cation balance. In human red cells it has been suggested that diuretic-inhibitable Na + K cotransport mediates a small net outward flux, thus lowering cell $K^+$ content and lowering cell volume [7]. The precise role played by the cotransport system may differ in different cell types since in epithelial cells [8] and smooth muscle cells [9], diuretic-sensitive cotransport has been implicated in the accumulation of intracellular $Cl^-$ above electrochemical equilibrium values [8, 9].

What is even less clear is the contribution made by the diuretic-sensitive cotransport system to cellular cation balance in conditions where Na-pump activity is impaired. Such a condition is of particular interest because of the increasing evidence that essential hypertension may be associated with high concentrations of a circulating inhibitor of the Na-pump [10, 11]. When there is significant modification of Na-pump function and a subsequent disequilibrium in transmembrane cation flux, it seems highly probable that diuretic-sensitive cotransport will be able to markedly contribute to the maintenence of cellular cation balance.

We have previously demonstrated both the existence of ouabain-insensitive but diuretic-sensitive K fluxes, entirely consistent with cotransport, in several cultured cell types [5], and the utility of these model systems for investigating the mechanism of diuretic action [12]. Here, we report the effects of the loop diuretic, piretanide, upon cation balance in cultured cells with fully functional Na-pump sites and in cells subjected to partial, chronic Na-pump blockade. Such an experimental model mimics the action of putative humoral inhibitors of the Na-pump [10, 11].

## MATERIALS AND METHODS

### 1. *Cultured cells*

HeLa, MDCK and $BC_3H1$ cells were maintained in serial culture at 37° as described in Aiton *et al.* [5] and Aiton and Simmons [12]. For experimental purposes monolayer cultures of cells were grown on 60 mm "Sterilin" plastic petri dishes for 3–4 days. Chronic applications of ouabain and of piretanide (with appropriate controls minus the drugs) were made by replacing the growth media (10% serum supplement) with one containing 0.1% serum supplement (either new born calf serum, HeLa; donor horse serum, MDCK; or foetal calf serum, $BC_3H1$) generally 24 hr before experimental determinations of intracellular ion contents, cell numbers and [$^3$H]ouabain binding. The lowered serum concentrations in the chronic incubation were necessary to obviate significant binding of the loop diuretics.

### 2. *Experimental measurements*

The experimental techniques used in this study have been described in detail elsewhere [5, 12, 13] and are summarized below.

2.1. *Estimation of intracellular ion contents.* Following chronic incubations, cell monolayers were rinsed with ice cold isotonic sorbitol or choline Cl solutions to remove extracellular ions. The internal ion contents were then extracted during a 2 hr incubation in 5 $cm^3$ double-distilled water at 20°. The $Na^+$ and $K^+$ content of the extract was then determined by flame photometry. Cell ion content (or concentration) was expressed relative to the cell number (or mean cell volume) which was determined on separate, parallel culture plates (see below).

2.2. [$^3$H]*Ouabain binding.* The number of functional (free) Na-pump sites in cells chronically grown in the presence and absence of piretanide was determined in HeLa cells. Validation of this technique is described in Aiton *et al.* [13]. Na-pump site density was determined as the difference in binding measured from a $2 \times 10^{-7}$ M [$^3$H]ouabain (ouabain specific activity 33 Ci/mmol, total activity used 0.4 $\mu$Ci/$cm^3$) solution containing either K-free or 15 mM-K Krebs solution with a 15 min incubation at 37° [13] after washing off the growth medium with the appropriate Krebs solution. At the end of the incubation period unbound $^3$H activity was removed by washing in ice-cold Krebs solution (4×, approx. 20 sec). Na-pump site density is expressed relative to cell number determined on the same plates used for [$^3$H]ouabain binding.

2.3. K ($^{86}$Rb) *fluxes.* $^{86}$Rb was used as an isotopic tracer for $K^+$ [5, 12, 13]. $^{86}$Rb influx (specific activity 8 mCi/mg Rb) into cell monolayers was measured during a 5 min incubation in Krebs solution containing 5.4 mM K with 0.2 $\mu$Ci/$cm^3$ $^{86}$Rb. At the end of the flux period monolayers were rinsed four times with ice-cold Krebs solution to remove extracellular isotope. Cells monolayers were then trypsinized to form a single cell suspension and aliquots were taken for determination of $^{86}$Rb activity in a liquid scintillation spectrometer (Packard model 3320) by the Cerenkov method.

2.4. *Cell number and cell volume.* Cell monolayers were trypsinized to form a single cell suspension [13]. A 1 $cm^3$ aliquot was then used to determine the cell density and mean cell volume using a Coulter Counter (model ZF) fitted with a Channelyser (model C1000).

2.5. *Solutions.* The standard Krebs solution used for [$^3$H]ouabain determinations and flux measurements contained (mM) NaCl 137, $CaCl_2$ 2.8, $MgSO_4$ 1.2, $NaH_2PO_4$ 0.6, Tris 14, HCl 12, glucose 10 (pH 7.4 at 37°), KCl was added where appropriate. Dialysed serum was used to supplement this Krebs solution (0.1%). For chronic incubations stock solutions of ouabain ($10^{-3}$ M in distilled water) loop diuretics (piretanide, furosemide and bumetanide all at 10 mM in 1 mM Tris base) and the stilbene 4-acetamide-4-isothiocyano-2,2′-stilbene disulphonic acid (SITS) (1 mM solution in 1 mM Tris base) were added directly to the culture medium to give the desired final concentrations.

### 3. *Materials*

Wherever possible Analar salts were used. Tissue culture supplies were obtained from Gibco-Biocult Ltd (Paisley, U.K.). Ouabain was obtained from Sigma (Poole, U.K.). SITS was from B.D.H. Chemicals (Poole, U.K.). Furosemide and piretanide were given by Dr. C. Osborne of Hoechst Pharmaceuticals (Hounslow, U.K.), bumetanide was given from Leo Laboratories (Princes Risborough, U.K.).

### 4. *Statistical methods*

Variation in results is routinely expressed as the standard deviation of the mean. Tests for significance of difference between mean values were made by Student's *t* test (unpaired means solution).

Table 1. Effect of piretanide (0.1 mM) upon the intracellular $Na^+$ and $K^+$ concentrations of cultured cells. Cell monolayers were incubated for 2.5 hr has described in Materials and Methods

| Experiment | Cell type HeLa | | MDCK | | $BC_3H1$ | |
|---|---|---|---|---|---|---|
| | $Na^+$ | $K^+$ | $Na^+$ | $K^+$ | $Na^+$ | $K^+$ |
| Control | 7 ± 2 | 166 ± 8 | 28 ± 2 | 148 ± 3 | 8 ± 1 | 152 ± 7 |
| + $10^{-4}$ M piretanide | 6 ± 1* | 165 ± 1* | 28 ± 1* | 137 ± 9* | 8 ± 2* | 154 ± 8* |

All data are expressed as mmol $litre^{-1}$ cell water; mean cell volumes were determined from electronic cell sizing.

* Not significantly different from control data.

Data is the mean ± S.D. from at least three determinations.

## RESULTS

### 1. *Effect of piretanide upon intracellular cation contents*

Table 1 shows that a 2.5 hr incubation in 0.1 mM piretanide, a concentration known to maximally inhibit the cotransport system [5, 14 and unpublished observations], in HeLa, MDCK and $BC_3H1$ cells, is without significant effect upon the intracellular concentrations of either $Na^+$ or $K^+$. Table 2 shows data from individual experiments in which MDCK cells were incubated for either 2.5 or 24 hr (data expressed as ion content per $10^6$ cells). Again no consistent significant effect of piretanide is observed even when possible changes in cell volume are taken into consideration. Similar data were obtained for HeLa (Table 3) and $BC_3H1$ cell cultures (data not shown). The absence of a piretanide effect suggests that the net transmembrane chemical activity gradient for $Na^+$, $K^+$ and $Cl^-$ in these cells is zero, or close to zero. In this situation where the cotransport system is known to mediate a substantial proportion of the $K^+$ exchange fluxes [6, 7], the contribution to net fluxes is small. Clearly, the precision of the data would preclude detection of very small effects (1–5%) of piretanide upon ion contents being detected.

### 2. *Effect of chronic ouabain treatment upon functional Na-pump density and intracellular ion contents*

After chronic growth in low concentrations of ouabain, HeLa cells have fewer functional pump sites than untreated cells [13]. In confirmation, Fig. 1 shows that the number of functional pump sites may be titrated out with increasing concentrations of ouabain in chronic incubations; 50 nM ouabain is sufficient to reduce the number of functional pump sites to 5% of their control value. Co-incubation of ouabain and piretanide has no significant effect upon the number of functional pump sites. This observation excludes the possibility that piretanide has a direct action on Na-pump site density and hence on ion contents (see below). Figure 2 shows that there is a reciprocal decrease in intracellular $K^+$ and increase in intracellular $Na^+$ as the concentration of ouabain in the growth medium is increased in the range 20–100 nM. It has previously been demonstrated [13] that HeLa cells are able to maintain normal cation balance with only a moderately increased level of intracellular $Na^+$ up to 10 nM ouabain, as a consequence of the stimulation of Na-pump turnover rate of remaining functional Na-pump sites. At higher ouabain concentrations, Na-pump turnover will be maximal as the intracellular $Na^+$ increases to saturate the internal $Na^+$ binding site of the pump. Table 3 demonstrates this behaviour of Na-pump fluxes measured in HeLa cells chronically exposed to 30 nM ouabain. Thus, whereas Na-pump site density is reduced to under 10% of control values (see Fig. 1), the Na-pump flux is reduced to only 17% of control values. Another important feature of the data shown in Table 3 is the magnitude of the $K^+$ unidirectional influxes sensitive to piretanide in chronically ouabain treated cells. Although there is a reduction of 35% in these fluxes (Table 3), there is no comparable change in the Na-pump mediated fluxes. Under conditions of chronic ouabain exposure the piretanide-sensitive (cotransport) fluxes now comprise the greatest proportion of transmembrane K influx in cells grown chronically in ouabain. With moderate change in intracellular cation content (up to 20 nM chronic ouabain) no significant effect of piretanide could be detected (Fig. 2) on intracellular cations (similar to the data obtained with control cells). With chronic ouabain treatment in the range of 30–100 nM, a significant effect of piretanide upon the maintenance of intracellular cation content is evident both for $K^+$ and, to a lesser extent, for $Na^+$ (Fig. 2, Table 3). This effect of piretanide indicates that piretanide-sensitive pathways can make a substantial contribution to net cation transport in non steady-state conditions. Similar results have been observed in experiments where Na-pump function has been impaired in low $K^+$ media [15]. That there is a retardation of $Na^+$ gain concurrent with retardation of $K^+$ loss is significant in that piretanide is apparently inhibiting an exchange of $K^+$ for $Na^+$. This result would not be expected from the normal operational definition of cotransport, where highly coupled fluxes operate in a single transmembrane direction [4–6].

Figure 3 shows that after chronic growth in ouabain, the action of piretanide on the intracellular ion contents is of high molar affinity. Half-maximal inhibition of retardation of $K^+$ loss (and $Na^+$ gain) by piretanide is observed at 3–4 $\mu$M. This is comparable to the half maximal inhibition of $K^+$ exchange fluxes and of KCl loss in acute experiments in HeLa cells (unpublished data). The latter are known to be operational measures of cotransport in this cell line [5]. The stoichiometry between piretanide-sensitive retardation of $K^+$ loss and $Na^+$ gain in this experiment is close to 1:1; this result

Table 2. Effect of piretanide (0.1 mM) upon the intracellular $Na^+$ and $K^+$ contents of MDCK cells incubated for both 2.5 and 24 hr. Data are expressed as the mean ± S.D., the number of replicates for each experiment was at least three

| Incubation period | Piretanide $10^{-4}$ M | Individual experiments Ion content nmol/$10^6$ cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $Na^+$ | $K^+$ | $Na^+$ | $K^+$ | $Na^+$ | $K^+$ | $Na^+$ | $K^+$ |
| 2.5 | − | 19 ± 3 | 249 ± 11 | 53 ± 5 | 238 ± 12 | 48 ± 1 | 447 ± 7 | 43 ± 3 | 225 ± 5 |
| | + | 18 ± 1* | 254 ± 10* | 58 ± 2* | 249 ± 8* | 53 ± 4* | 454 ± 18* | 44 ± 1* | 208 ± 13* |
| 24 | − | 50 ± 10 | 493 ± 23 | 31 ± 3 | 342 ± 8 | 59 ± 7 | 370 ± 11 | 79 ± 2 | 365 ± 11 |
| | + | 50 ± 7* | 468 ± 5* | 38 ± 3† | 387 ± 6‡ | 69 ± 7* | 350 ± 7* | 80 ± 4* | 377 ± 3* |

Significantly different from control values: *not significantly different; †$P < 0.10$, $>0.05$; ‡$P < 0.05$.

*Table 3. Effect of chronic growth in ouabain and piretanide on the unidirectional $K^+$ ($^{86}Rb$) influx into HeLa cells. $K^+$ ion fluxes were determined in Krebs containing 5.4 mM $K^+$, after chronic incubations and washing. The effect of piretanide is rapidly reversed by this procedure (piretanide rapidly dissociates). Chronic ouabain treatment, in contrast, is essentially irreversible [13, 22]*

| Growth condition | Na nmoles/$10^6$ cells | $K_i$ | $^{86}Rb$ influx (mmol litre cell water$^{-1}$.min$^{-1}$) 1 Total | 2 + Piretanide $10^{-4}$ M | 3 + Ouabain $10^{-3}$ M | 4 +Ouabain $10^{-4}$ M + Piretanide $10^{-3}$ M | 5 Piretanide-sensitive flux [1–2] | [3–4] | 6 Ouabain-sensitive flux [1–3] | [2–4] |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | 31 ± 10 | 508 ± 30 | 3.10 ± 0.10 | 2.20 ± 0.20 | 1.30 ± 0.10 | 0.45 ± 0.01 | 0.90 ± 0.20 | 0.85 ± 0.01 | 1.80 ± 0.15 | 1.75 ± 0.20 |
| Piretanide ($1 \times 10^{-4}$ M) | 26 ± 9[a] | 535 ± 45[a] | 3.30 ± 0.30[a] | 2.50 ± 0.20 | 1.40 ± 0.02 | 0.75 ± 0.01[d] | 0.80 ± 0.30[a] $P < 0.1 > 0.05$ | 0.65 ± 0.20[b] | 1.90 ± 0.3[a] | 1.75 ± 0.2[a] |
| Ouabain ($3 \times 10^{-8}$ M) | 263 ± 28[d] | 146 ± 4[d] | 1.30 ± 0.01[d] | 0.71 ± 0.04 | 1.00 ± 0.06 | 0.37 ± 0.05[a] | 0.59 ± 0.40 | 0.61 ± 0.07[b] | 0.30 ± 0.06 | 0.34 ± 0.07 |
| Ouabain ($3 \times 10^{-8}$ M) + piretanide | 183 ± 8[d,**] ($1 \times 10^{-4}$ M) | 306 ± 18[d,**] | 1.23 ± 0.08[d,*] | 0.74 ± 0.07 | 1.00 ± 0.02 | 0.51 ± 0.03[d,**] | 0.49 ± 0.01[b,*] | 0.49 ± 0.03[c] | 0.23 ± 0.08[d,*] | 0.23 ± 0.07[d,*] |

*Significantly different from control values (growth): [a] not significant; [b] $P < 0.05$; [c] $P < 0.010$; [d] $P < 0.001$.*
*Significantly different from ouabain treatment (growth): * not significant; ** $P < 0.01$; *** $P < 0.001$.*

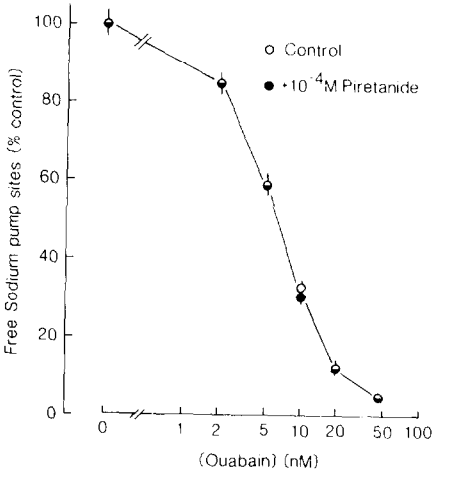


Fig. 1. Change in the number of free Na-pump sites of HeLa cells grown for 24 hr in various concentrations of ouabain, in the presence (●) or absence (○) of $1 \times 10^{-4}$ M piretanide. Culture conditions and media composition are as given in Materials and Methods. The number of free Na-pump sites are expressed as a percentage of control values after growth in the absence of ouabain ($0.65 \pm 0.04$ (SD) $\times 10^6$ sites/cell; N = 8). Data are the mean ± S.D. of at least four separate determinations.

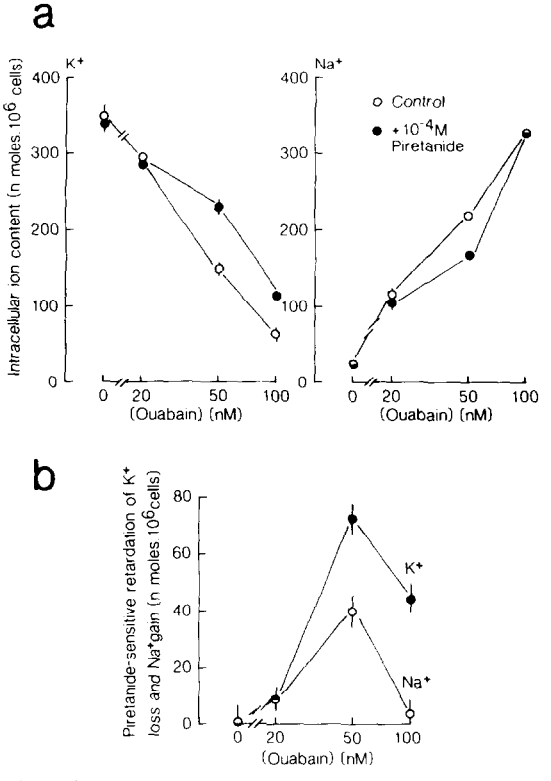


Fig. 2. (a) Dissipation of the cellular $Na^+$ and $K^+$ gradients of HeLa cells grown for 24 hr in the presence of 20–100 nM ouabain in the presence (●) and absence (○) of 0.1 mM piretanide. Each datum is the mean ± S.D. of at least four separate determinations. (b) Piretanide-sensitive retardation in the intracellular $Na^+$ (○) gain and $K^+$ (●) loss in cells grown in the presence of 20–100 nM ouabain. Data from Fig. 2(a).

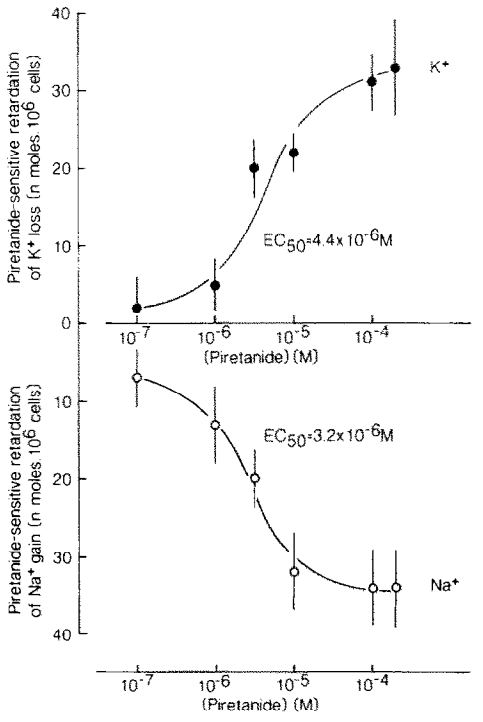


Fig. 3. Dose–response curve for the effect of piretanide upon the retardation of $Na^+$ gain (○) and loss of $K^+$ (●) in HeLa cells grown for 24 hr in the presence of 30 nM ouabain. Each datum is the mean and S.D. of at least four separate paired determinations.

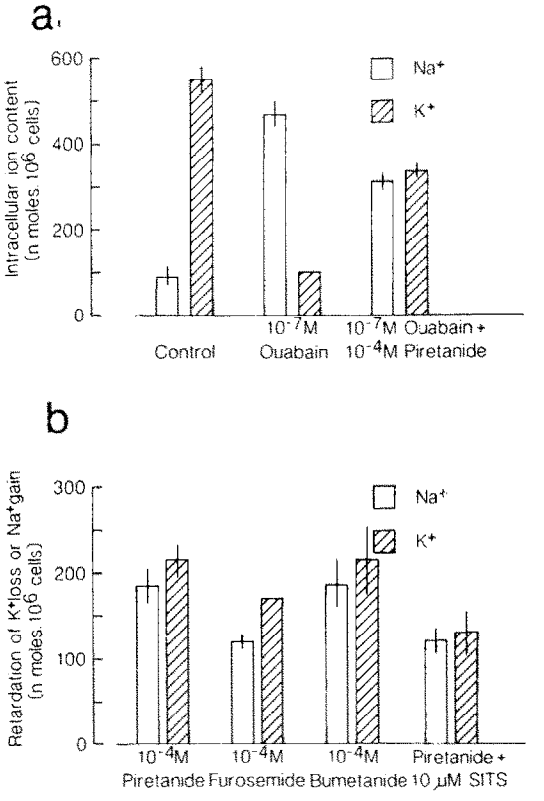


Fig. 4. (a) Effect of piretanide (0.05 mM) upon the dissipation of cellular $Na^+$ (open columns) and $K^+$ (hatched columns) of MDCK cells grown for 24 hr in the presence of 100 nM ouabain. Each column is the mean and S.D. of at least four separate determinations. (b) Diuretic-sensitive retardation of $Na^+$ gain (open columns) and $K^+$ loss (hatched columns) in MDCK cells under identical conditions to Fig. 4(a). The diuretic concentration in each case was 0.05 mM. Each column is the mean and S.D. of at least four separate determinations.

is most likely to reflect the apparent variation in stoichiometry which occurs at different levels of intracellular cation content (see Fig. 2).

Qualitatively identical effects of piretanide upon cation contents of MDCK cells chronically exposed to ouabain are observed, (Table 4, Fig. 4), though slightly higher ouabain concentrations are needed to initiate the response, reflecting a lower affinity of the dog cells to ouabain compared with HeLa [16]. Invariably an effect of piretanide upon retardation of $K^+$ loss is observed; an apparent piretanide-sensitive exchange of $K^+$ for $Na^+$ is seen in three out of the six experiments performed in MDCK cells (Table 4). The effect of piretanide is again of high molar affinity, half maximal inhibition of $K^+$ retardation and $Na^+$ gain being observed at 3 μM (data not shown). Figure 4 also demonstrates that piretanide-like activity is also observed with saturating concentrations of the other loop diuretics, furosemide

Table 4. Effect of $5 \times 10^{-5}$ M piretanide upon intracellular ion contents in MDCK cells subjected to chronic ouabain (100 nM) treatment. All data are expressed as the mean ± S.D. In each experiment there were at least four replicates

| Piretanide ($5 \times 10^{-5}$ M) | $K^+$ (nequiv/$10^6$ cells) − | + | piretanide dependent change | $Na^+$ (nequiv/$10^6$ cells) − | + | piretanide dependent change |
|---|---|---|---|---|---|---|
| Experiment | | | | | | |
| 1 | 164 ± 4 | 188 ± 8 | 23 ± 12† | 244 ± 23 | 241 ± 18 | −6 ± 35* |
| 2 | 22 ± 5 | 90 ± 14 | 70 ± 19‡ | 367 ± 10 | 371 ± 11 | 4 ± 12* |
| 3 | 157 ± 3 | 204 ± 6 | 47 ± 9‡ | 225 ± 11 | 180 ± 4 | 45 ± 14‡ |
| 4 | 26 ± 9 | 85 ± 13 | 59 ± 21‡ | 298 ± 15 | 223 ± 20 | 74 ± 26† |
| 5 | 106 ± 4 | 175 ± 4 | 69 ± 1‡ | 325 ± 11 | 328 ± 11 | 3 ± 38‡ |
| 6 | 105 ± 8 | 337 ± 11 | 233 ± 19‡ | 471 ± 28 | 302 ± 17 | 168 ± 44‡ |

Significance of the piretanide-dependent change in intracellular $Na^+$ and $K^+$ from zero change: *not significant; †significantly different, $P < 0.01$; ‡significantly different, $P < 0.001$.

and bumetanide. The effect of piretanide is unlikely to result from a general reduction in membrane $Cl^-$ permeability, thus limiting cation flux, since in the presence of the stilbene, SITS [17], a substantial piretanide effect is still observed. In the absence of piretanide SITS has no additional effect when compared to data in the presence of ouabain alone (data not shown). Taken together the data reported here are entirely consistent with the effect of piretanide being the result of inhibition of cotransport activity [4–6], rather than inhibition of other membrane transport processes [19].

## DISCUSSION

Operational definitions of the diuretic (piretanide)-sensitive cotransport system have usually been based on measurements of net ion fluxes into and out of cells whose Na-pumps have been completely inhibited by ouabain. In these conditions, large chemical gradients are established for one or more of the ions involved ($Na^+$, $K^+$ or $Cl^-$; [4, 6]). Results from these experiments indicate that cotransport is tightly coupled with a stoichiometry of 1Na:1K:2Cl [6]. The system is electroneutral and driven by the sum of the chemical gradients for $Na^+$, $K^+$ and $Cl^-$ [6]. The cotransport fluxes are bidirectional and the available evidence is consistent with the absence of *trans* effects [6]. Thus, in the presence of equal and opposite net chemical gradients, cotransport will mediate apparent exchange ion fluxes [6]. The direction of cotransport in normal cells with inverse gradients of $Na^+$ and $K^+$ has recently been the subject of interest. In human red cells evidence has been provided for outwardly directed flux (K + Na loss) in *in vivo* conditions, which may contribute to plasma $K^+$ homeostasis [7]. The system is, however, operating close to a net chemical gradient of zero and other workers [24] failed to find an effect of furosemide on cellular Na homeostasis, even though a furosemide-sensitive Na influx and efflux was demonstrated. In the present paper we present analagous data for three types of cultured cell, carcinoma (HeLa), epithelial (MDCK), and smooth muscle cells ($BC_3H1$). In each case, piretanide has no significant effect upon intracellular ion content of normal cells, providing evidence that cotransport in these cell types is operating in exchange mode. Small net ion movements may, however, not be detected with our current measurements.

There has been much recent interest in the cotransport system because of a possible link between a defect in the transporter and the aetiology of essential hypertension [19]. This field remains somewhat controversial [19]. Our current investigations were designed to model an alternative hypothesis based on the notion that the cotransport system is functioning normally but that there are endogenous circulating humoral inhibitors of the Na-pump. Evidence for such an endogeneous Na-pump inhibitor is gaining acceptance [10, 11, 21], as is the notion that this inhibitor may also be associated with the normal regulation of salt balance in non-hypertensives [10, 11, 20, 21].

With chronic applications of ouabain, regulatory phenomena are observed which are associated with the remaining (functional) pump sites. Increased $Na^+$ within the cell cytosol increases the turnover rate of the existing pump sites, and also increases the rate of *de novo* synthesis of new Na-pump sites (see Results and also [13, 22]). Both these responses tend to ameliorate the effects of chronic pump blockade [13, 22]. The blood concentration and physiological specificity of any putative Na-pump inhibitor needs to be carefully assessed due to the universal distribution of Na-pumps in all tissues. Several reports have demonstrated that significant (>50%) inhibition of Na-pump flux is observed even with non-extracted plasma samples [10]. It is conceivable that the relative rapidity of Na-pump turnover phenomena could provide a basis for physiological selectivity of a humoral substance. Such data is not available for tissues such as *in vivo* smooth muscle cells.

Our current data emphasise that the piretanide-sensitive cotransport system may be able to mediate substantial net changes in intracellular ion concentrations when cells are subjected to partial chronic Na-pump blockade. The relative magnitude of these effects varies in separate experiments, most likely reflecting variation in the cellular density of the cotransporter. In cells containing high levels of cotransporter, such as the thick ascending limbs of the loop of Henle [25], it is likely that loop diuretics will protect against the dissipation of ion gradients if active transport is compromised, as may occur in anoxia. We provide evidence that the activity of the cotransport system is responsible for an increase in intracellular $Na^+$ concentration and a decrease in intracellular $K^+$ in these conditions, since inhibition by piretanide maintains cations towards their normal physiological values. This apparent exchange is separate from the normally recognised operational modes of the cotransport system (see above). The possibility that ion transport mechanisms other than cotransport obscure the usual stoichiometries cannot be entirely excluded, though this cannot be due to the residual operation of the Na-pump since this would enhance $K^+$ uptake and $Na^+$ loss with an inwardly directed cotransport flux. The direction of cotransport flux we observe is a piretanide-sensitive outward $K^+$ transport.

Do the data provided here provide any support for possible extra-renal actions of loop-diuretics? The present data are not affected by substantial protein binding as would occur *in vivo*. Substantial cotransport inhibition will occur with free piretanide concentrations of 10 $\mu$M. In the $BC_3H1$ smooth muscle cell-line, a piretanide-sensitive cotransport is evident. Data from other cell-lines which are more convenient experimental models provide no evidence for any large qualitative differences between cell types. A substantial ouabain-insensitive $K^+$ influx component has been demonstrated in an *in vitro* experimental preparation of smooth muscle cells [10] suggesting that there may be a large component of diuretic-sensitive cotransport activity [5]. Although we were unable to detect significant piretanide-sensitive effects on cellular cations below 20 nM ouabain in the chronic experiments, we cannot exclude the possibility that piretanide modifies the

relatively small changes in internal $Na^+$ which occurs at these ouabain concentrations. Blaustein [23] has shown that significant change in the concentration of intracellular calcium can occur via Na–Ca exchange with only a few mmoles change in intracellular $Na^+$. Significant modification of $Ca^{2+}$-dependent cell function is thus possible by this mechanism. Clearly more work is needed to extend our present findings in cultured cells to smooth muscle cells *in vivo*.

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