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SPECIAL REPORT

Chloride channel blockers decrease intracellular pH in cultured renal epithelial LLC-PK$_1$ cells

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The effects of chloride channel blockers upon intracellular pH (pH$_i$) were examined in renal epithelial monolayers of LLC-PK$_1$ cells. A significant intracellular acidification was found with addition of 100 μM 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB), niflumic acid, flufenamate and diphenylamine-2-carboxylate (DPC) but not with 4,4'-diisothiocyanatostilbene-2-2'disulphonic acid (DIDS). The effects of these agents upon pH$_i$ was dose-dependent with apparent $K_{0.5}$ values of: 16.7±0.3 μM, 34.2±0.9 μM and 740±13 μM for niflumic acid, flufenamate and DPC respectively. The results indicate that at concentrations commonly used to block channel activity these chloride channel blockers have profound effects upon pH$_i$.

Keywords: Chloride channel blockers; LLC-PK$_1$ cells; intracellular pH

Introduction Chloride channel blockers have proved important tools in the characterization of epithelial chloride channels. On the basis of their differential sensitivity to a range of chloride channel blockers, a number of distinct chloride channels have now been identified (Greger, 1990; Chao & Mochizuki, 1992). Recently it has been shown that some epithelial chloride channels are sensitive to inhibition at acidic intracellular pH (pH$_i$) values (Guinamard *et al.*, 1995; Park & Brown, 1995) which, since many of the chloride channel blockers currently in use are weak acids, raises the possibility that part of the inhibitory actions of these agents may result from their effects upon pH$_i$ rather than from specific interactions with the channel. Here we describe the effects of the putative chloride channel blockers 5-nitro-2-(3-phenylpropylamino-benzoate (NPPB), niflumic acid, flufenamate, diphenylamine-2-carboxylate (DPC) and 4,4'-diisothiocyanatostilbene-2-2'disulphonic acid (DIDS) upon pH$_i$ in cultured renal epithelial monolayers of LLC-PK$_1$ cells.

Methods LLC-PK$_1$ cells were cultured in Medium 199 (with 1.25 g l^{-1} $NaHCO_3$ and L-glutamine) supplemented with 3% (v/v) foetal calf serum (FCS) and gentamycin (50 μg ml^{-1}). Intracellular pH (pH$_i$) was measured as previously described (Thwaites *et al.*, 1993): Epithelial monolayers were grown on filter inserts, loaded with BCECF-AM (5 μM), and pH$_i$ measured by fluorescence (excitation at 440/490 nm, emission at 520 nm) using a photon counting system (Newcastle Photometric Systems). Data are expressed as mean±s.e.mean. Statistical comparison of mean values was made by analysis of variance (ANOVA) with a Dunnet post test.

Results Figure 1a shows the effect of apical addition of a range of putative chloride channel blockers upon pH$_i$. Addition of 100 μM DPC to the apical perfusate resulted in a marked intracellular acidification from pH 7.54±0.03 to 7.28±0.03 (n=3, P<0.01); pH$_i$ rapidly returned towards control values upon removal of the DPC pulse. Similar significant intracellular acidifications were found with 100 μM challenges of niflumic acid (Δ pH$_i$ 0.46±0.02, P<0.001), NPPB (Δ pH$_i$ 0.61±0.03, P<0.001) or flufenamate (Δ pH$_i$ 0.30±0.01, P<0.001). DIDS (100 μM) was without effect upon pH$_i$. Similar effects upon pH$_i$ were observed with basolateral challenge (data not shown). At physiological pH values, these weak acids exist in both protonated (AH) and anionic (A$^-$) forms. To identify which species was responsible for the

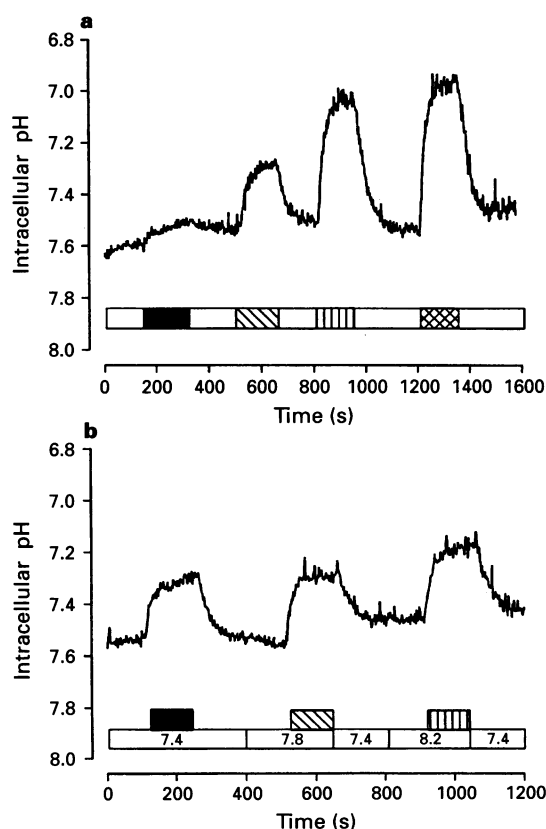

Figure 1 The effects of chloride channel blockers upon pH$_i$. (a) The effect of apical addition of (100 μM): DIDS (at solid bar), DPC (hatched bar), niflumic acid (vertically hatched bar) or NPPB (cross-hatched bar) at pH 7.4. A single trace representative of 3 separate experiments. (b) To test the relative permeabilities of the neutral (AH) and anionic (A$^-$) forms of flufenamate, cells were exposed to pulses of flufenamate at a constant [AH] of 30 nM but a range of [A$^-$] from 70 μM to 570 μM. At a perfusate pH of 7.4 (solid bar) [A$^-$] was 70 μM. At pH 7.8 (hatched bar) [A$^-$] was 200 μM. At pH 8.2 (vertically hatched bar) [A$^-$] was 570 μM. A single trace representative of 3 separate experiments.

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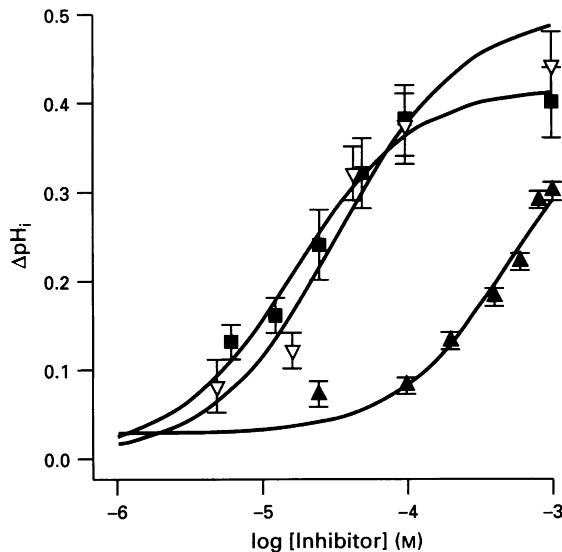

Figure 2 Intracellular acidification as a function of chloride channel blocker concentration. Dose-response curves were constructed for the ability of niflumic acid (■), flufenamate (▽) and DPC (▲) to decrease pH_i. The results are the mean ± s.e.mean of 5 determinations.

intracellular acidification, pH_i was monitored in cell monolayers exposed to apical pulses of flufenamate (p*K*a 3.9) in which the concentration of unionised flufenamate [AH] was kept constant at 30 nM, but by manipulation of external pH, the anionic form [A^-] was varied over a 6 fold range. Figure 1b shows that the acidification of pH_i in response to addition of flufenamate was solely dependent upon the magnitude of the inwardly directed (AH) gradient and was not affected by the size of the imposed (A^-) gradient suggesting that AH is the permeant species.

Figure 2 shows that the increase in pH_i in response to addition of niflumic acid, flufenamate or DPC was concentration-dependent and saturable. From the data shown in Figure 2 apparent $K_{0.5}$ values were calculated to be 16.7 ± 0.3 μM, 34.2 ± 0.9 μM and 740 ± 13 μM for niflumic acid, flufenamate and DPC respectively. At high concentrations NPPB was found to quench BCECF fluorescence.

Discussion We have presented evidence to show that the chloride channel blockers: niflumic acid, flufenamate, NPPB and DPC cause profound changes in pH_i in renal LLC-PK_1 cells. Furthermore, the order of potency of these agents (niflumic acid ≥ flufenamate > > DPC) and the calculated $K_{0.5}$ values on pH_i are of the same order as chloride channel blockade (Chao & Mochizuki, 1992). Given that many chloride channels appear to be modulated by pH_i (Duszyk *et al.*, 1995; Park & Brown, 1995), the usefulness of these drugs as specific chloride channel blockers in intact cells must now be open to question since it may prove difficult to differentiate between channel inhibition and the effects of pH_i upon channel activity.

Importantly, the effects of these agents upon pH_i cannot be explained solely by the accumulation of protons within the cells as a result of AH entry and subsequent dissociation to A^- and protons. For a 100 μM pulse of niflumic acid (p*K*a 4.76), the maximum release of protons into the cytoplasm is of the order of 48 μM l^{-1} cell water, several orders of magnitude less than the apparent buffer capacity of 12.8 mmol l^{-1} pH $unit^{-1}$ at pH 7.4. This implies that the intracellular acidification observed must result from a functional effect of these agents subsequent to AH entry, possibly at the level of miotochondria (Lukacs *et al.*, 1991) or upon cell metabolism (Fanelli *et al.*, 1995), such that balance between metabolic proton generation and proton utilisation is perturbed.

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References

CHAO, A.C. & MOCHIZUKI, H. (1992). Niflumic and flufenamic acids are potent inhibitors of chloride channels in mammalian airway. *Life Sci.*, **51**, 1453–1457.

DUSZYK, M., LIU, D., FRENCH, A.S. & MAN, S.F.P. (1995). Evidence that pH-titratable groups control the activity of a large epithelial chloride channel. *Biochem. Biophys. Res. Commun.*, 215, 355–360.

FANELLI, A., BERLIN, W.K. & GROLLMAN, E.F. (1995). Inhibition of iodide transport in rat thyroid cells using N-substituted anthranilic acid-derivatives. *Thyroid*, **5**, 223–230.

GREGER, R. (1990). Chloride channel blockers. *Methods Enzymol.*, **191**, 793–809.

GUINAMARD, R., PAULAIS, M. & TEULON, J. (1995). Inhibition of a small-conductance cAMP-dependent Cl^- channel in mouse thick ascending limb at low internal pH. *J. Physiol.*, **490**, 759–765.

LUKACS, G.L., NANDA, A., ROTSTEIN, O.D. & GRINSTEIN, S. (1991). The chloride channel blocker 5-nitro-2-(phenylpropylamino) benzoic acid (NPPB) uncouples mitochondria and increases the proton permeability of the plasma membrane in phagocytic cells. *FEBS Lett.*, **288**, 17–20.

PARK, K. & BROWN, P.D. (1995). Intracellular pH modulates the activity of chloride channels in isolated lacrimal gland acinar cells. *Am. J. Physiol.*, **268**, C647–C650.

THWAITES, D.T., BROWN, C.D.A., HIRST, B.H. & SIMMONS, N.L. (1993). Transepithelial glycylsarcosine transport in intestinal Caco-2 cells mediated by the expression of H^+ coupled carriers at both apical and basolateral membranes. *J. Biol. Chem.*, **268**, 7640–7642.

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