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INDIRECT EVIDENCE FOR THE PRESENCE OF NON-SPECIFIC ANION CHANNELS IN RABBIT MANDIBULAR SALIVARY GLAND ACINAR CELLS

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SUMMARY

1. Intracellular pH (pH_i) was measured using the fluorescent pH-sensitive dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein in acini isolated from the rabbit mandibular salivary gland.

2. Stimulation of the acinar cells with acetylcholine (ACh) evoked an intracellular acidosis, the size of which was dependent on the HCO_3^- concentration in the bathing medium. A half-maximal acidosis was observed at approximately 10 mM-HCO_3^-. ACh also evoked an acidosis in HCO_3^--free solutions containing acetate; a half-maximal acidosis was observed at about 10 mM-acetate.

3. Propionate, lactate and butyrate were also able to support the ACh-evoked acidosis to varying extents. In contrast, formate, pyruvate and salicylate did not support the ACh-induced acidosis to any great extent.

4. Acetazolamide greatly reduced the size of the acidosis in HCO_3^--buffered medium, but had no effect in acetate-buffered medium, suggesting that the inhibitory effect of acetazolamide was due to a specific inhibition of carbonic anhydrase activity.

5. The Cl^- channel blockers diphenylamine-2-carboxylic acid (DPC, 1 mM) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (0·5 mM) abolished the ACh-evoked acidosis in both HCO_3^-- and acetate-buffered media.

6. The data are consistent with the presence in the acinar cell of relatively non-specific anion channels sensitive to DPC and its derivatives. Such channels, activated on stimulation with ACh, would allow HCO_3^- and other weak acid ions to leave the cell, leading to the observed acidosis. The existence of such channels, located in the apical membrane, could explain why HCO_3^- or acetate can sustain fluid secretion in the intact perfused rabbit mandibular gland in the absence of Cl^-.

INTRODUCTION

Primary fluid and electrolyte secretion in exocrine glands and other secretory epithelia is currently thought to be driven by active accumulation of Cl^- in the cell through the action of a Na^+–K^+–$2Cl^-$ co-transporter located on the basolateral membrane (see Silva, Stoff, Field, Fine, Forrest & Epstein, 1977; Greger, Schalatter & Gögelein, 1986). Cl^- then leaves the cell down an electrochemical gradient through

Cl^- channels located in the apical membrane. These channels are activated when secretion is stimulated by neurotransmitters such as acetylcholine (ACh; Marty, Tan & Trautmann, 1984; Findlay & Petersen, 1985). A shortcoming of this model, at least with regard to the salivary gland, is that it cannot explain why HCO_3^- is able to support fluid secretion in the intact perfused rabbit mandibular gland when accumulation of Cl^- into the cell is prevented (Case, Hunter, Novak & Young, 1984; Case, Howorth & Lau, 1986).

We have recently shown that cholinergic stimulation of acini isolated from the rabbit mandibular salivary gland evokes a transient intracellular acidosis (Lau, Elliott & Brown, 1989; Steward, Seo & Case, 1989). The most likely explanation for the acidosis appears to be that stimulation with ACh activates channels permeable to HCO_3^- in the acinar cell membrane. These channels could be primarily selective to HCO_3^-, analogous to those proposed for the bull-frog choroid plexus (Saito & Wright, 1984). Alternatively, HCO_3^- might be able to permeate the Cl^- channels thought to be present in the apical membrane of acinar cells in the mandibular gland (Lau & Case, 1988) and other exocrine glands (Marty *et al.* 1984; Findlay & Petersen, 1985; Marty, 1987). Evidence obtained in other tissues suggests that some classes of Cl^- channels may actually be permeable to HCO_3^- and other small non-halide anions (Bormann, Hamill & Sakmann, 1987; Kaila & Voipio, 1987; Reinhardt, Bridges, Rummel & Lindemann, 1987). If the Cl^- channels in the mandibular gland acinar cell have similar properties, they should mediate an efflux of HCO_3^- down its electrochemical gradient when they are activated. This could explain the striking observations that, in the perfused rabbit mandibular gland, either HCO_3^- (Case *et al.* 1984) or acetate (Novak & Young, 1989) can sustain fluid secretion in the complete absence of Cl^-.

In the present study we have further characterized the events which underlie the ACh-induced intracellular acidosis, in particular the specificity of the mechanisms responsible for the movements of HCO_3^- and other weak acid anions. Taken together, the results suggest that acinar cells may contain relatively non-specific anion channels which are activated on stimulation with acetylcholine.

METHODS

Rabbit mandibular salivary gland acini were isolated by collagenase digestion as previously described (Lau *et al.* 1989). Briefly, male half-lop rabbits were killed with an overdose of sodium pentobarbitone and both mandibular salivary glands were removed. They were trimmed free of fat, finely minced and incubated at 37 °C in a Ca^{2+}-free, HCO_3^--free isolation buffer (see Table 1) containing 0·1 mg ml^{-1} collagenase, 1 mg ml^{-1} hyaluronidase and 1% (w/v) bovine serum albumin (BSA), which was continuously shaken and top-gassed with 100% O_2. Following one hour's incubation the tissue pieces were washed three times in a 1% BSA isolation buffer containing 2 mM-$CaCl_2$, dissociated with a plastic pipette tip, and filtered through nylon mesh. The acini in the resulting filtrate were then purified by centrifugation through 4% BSA isolation medium (twice) and 1% BSA isolation medium (twice) before being finally resuspended in standard HCO_3^--buffered medium (Table 1). The viability of acini prepared by this method has been discussed in a previous paper (Lau *et al.* 1989).

Measurement of intracellular pH. The acini were loaded with 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubating them with 0·5 μM of the acetoxymethyl ester of BCECF for 20 min at 37 °C. Fluorescence measurements were made at 37 °C in a stirred, thermostatted cuvette in a Perkin-Elmer LS-3 spectrofluorimeter. BCECF fluorescence (excitation and emission wavelengths of 490 and 526 nm respectively) was related to pH_i either (in a few experiments) by

using nigericin and KCl to equilibrate extra- and intracellular pH or (in the majority of experiments) by lysing the cells with 0·2% (v/v) Triton X-100 (Lau *et al.* 1989). Solution pH was measured using a Corning 130 pH meter accurate to 0·01 pH units and a Schott miniature combination pH electrode. Calibration of the electrode at the experimental temperature of 37 °C was carried out at the beginning of each experiment using pH 4·0 and 7·0 standard buffers (Sigma). As in our previous study (Lau *et al.* 1989), a correction of 0·15 pH units was applied to the pH_i values obtained by the Triton method in order to correct for the slight shift in emission wavelengths between extracellular and intracellular dye (Rink, Tsien & Pozzan, 1982; Paradiso, Negulescu & Machen, 1986). The intracellular dye concentration was estimated to be 0·1–1 μM. Leakage of BCECF out of the cells occurred at a rate of approximately 5% of intracellular dye per 10 min. This gives rise to an apparent alkalinization of at the most 0·02 pH units per 10 min during the experiments, as discussed by Lau *et al.* (1989). In view of the small size of this error, none of the pH_i values in the paper have been corrected for the effects of leakage.

Measurement of intracellular Ca^{2+} activity. The changes in intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) evoked by ACh were used to assess the viability and responsiveness of the acinar cells when incubated in the different bathing solutions. $[Ca^{2+}]_i$ was measured using Fura-2, which was loaded into the acini by incubating them for 20 min at 37 °C in a solution containing 5 μM-Fura-2-acetoxymethyl ester. Fluorescence was measured as for BCECF, but with excitation at 340 nm and emission at 500 nm. Fluorescence (F) was related to $[Ca^{2+}]_i$ by lysing the cells with Triton X-100 to give F_{max} (the fluorescence generated with the dye saturated with Ca^{2+}), and then completely quenching the Ca^{2+} signal by adding 6 mM-EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid, buffered with 18 mM-Tris base) to obtain F_{min}. $[Ca^{2+}]_i$ was calculated using the following equation, assuming a K_D of 224 nM (Grynkiewicz, Poenie & Tsien, 1985).

$$[Ca^{2+}]_i = K_D \frac{(F - F_{min})}{(F_{max} - F)}.$$

No correction was made for the effects of dye leakage.

Solutions. All solutions contained (in mM unless otherwise stated): Na^+, 118; K^+, 6; Mg^{2+}, 1·2; Ca^{2+}, 2; SO_4^{2-}, 1·2; HPO_4^-, 1·2; glucose, 15; amino acids (Eagle's medium supplement), 1% (v/v); glutamine, 0·3 mg ml^{-1}; bovine serum albumin (Fraction V, Sigma), 0·1% (w/v). The solutions contained variable amounts of Cl^-, HCO_3^-, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) and weak acid anions, as given in Table 1. Table 1 also gives the compositions of the various gas mixtures with which the solutions were equilibrated (note that the ratio of CO_2 to HCO_3^- was kept constant so that all solutions had the same pH of 7·4). HCO_3^- concentrations in the solutions used to study the HCO_3^- dependence of the ACh-induced acidosis (see Fig. 1) were measured (as total CO_2 in mM following conversion of HCO_3^- to CO_2 with HCl) using a Corning 965 carbon dioxide analyser. Once BSA was added to the solutions they could only be top-gassed, so that in the nominal 5, 10, 25 and 75 mM-HCO_3^- buffers the measured HCO_3^- concentrations were: 2·5±0·2, 5·6±0·08, 22·5±0·5 and 65·7±0·5 mM (mean±S.E.M., $n = 4$) respectively. The pH of all solutions was 7·4. The pH values of all experimental solutions were checked immediately before the solutions were used.

Acetylcholine (Sigma) was prepared freshly each day as a 1 mM stock solution in distilled water. BCECF-AM was obtained from Calbiochem (La Jolla, CA, USA), Fura-2-AM from Molecular Probes Inc. (Junction City, OR, USA), collagenase (Type CLPSA) from Worthington Biochemical (Freehold, NJ, USA), and hyaluronidase (Type 1-S) and acetazolamide from Sigma. Diphenylamine-2-carboxylic acid (DPC), 5-nitro-2(3-phenylpropylamino)-benzoic acid (NPPB) and 3',5-dichlorodiphenylamine-2-carboxylic acid (compound 131) were gifts from Professor R. Greger, Physiologisches Institut, Albert Ludwigs Universität, Freiburg, FRG. *N*-Ethylisopropyl-amiloride (EIPA) was a gift from Dr T. Friedrich, Max-Planck-Institut für Biophysik, Frankfurt, FRG. DPC, NPPB, compound 131 and EIPA were all stored frozen as stock solutions in dimethylsulphoxide. All other chemicals were analytical grade reagents obtained from Sigma (Poole, UK) except for the Eagle's essential amino acid supplement (×50 concentrate), which was obtained from Gibco Laboratories.

Statistics. All results are given as mean±standard error of the mean (S.E.M.). All tests for differences between two means were made by two-tailed t tests.

RESULTS

The HCO_3^- dependence of the acetylcholine-induced acidosis

Stimulation of acini with ACh led to a transient intracellular acidosis, in agreement with our previous work (Lau *et al.* 1989). Acetylcholine (1 μM) was used to stimulate the acini in all experiments in the present study. This dose of ACh evoked an acidosis of $0{\cdot}10 \pm 0{\cdot}01$ pH units (mean $\pm$ S.E.M., $n = 16$) in acini bathed in medium containing 25 mM-HCO_3^-.

TABLE 1. Composition of solutions

Solution	Concentration (mM)					
	Na^+	Cl^-	HCO_3^-	HEPES	Weak acid anion	Gas mixture
HCO_3^--free isolation buffer	123	113	–	25	–	100, O_2
Standard 25 mM-HCO_3^- buffer	119	97	25	12·5	–	95/5, O_2/CO_2
5 mM-HCO_3^- solution	119	117	5	12·5	–	99/1, O_2/CO_2
10 mM-HCO_3^- solution	119	112	10	12·5	–	98/2, O_2/CO_2
75 mM-HCO_3^- solution	119	47	75	12·5	–	85/15, O_2/CO_2
Weak acid solution	119	89	–	25	25	100, O_2
Sodium acetate solution	118	9	–	25	105	100, O_2

Anion and buffer composition of solutions. The columns give the concentrations (in mM) of the major anionic and buffer species in the various solutions, and the gas mixtures with which the solutions were equilibrated (%). To prepare solutions with acetate concentrations ranging from 5 to 100 mM the sodium acetate solution was mixed with the HCO_3^--free isolation buffer in the appropriate proportions. Details of the components common to all solutions (cations, glucose, BSA, etc.) are given in the text.

Figure 1 shows the effect of varying external HCO_3^- concentration at a constant external pH of 7·4 on acinar cell pH_i. Figure 1*A* shows experimental traces recorded for acini from a single preparation bathed in the 10, 25 and 75 mM-HCO_3^--buffered media. Changes in the concentration of the extracellular HCO_3^- did not have any significant effect on resting pH_i (resting pH_i in solutions containing 25 mM-HCO_3^- was $7{\cdot}12 \pm 0{\cdot}02$, see Table 2; this is identical to that reported previously, see Lau *et al.* 1989). However, as the concentration of HCO_3^- in the bathing medium was increased, the size of the acidosis evoked by ACh increased. The peak change in pH_i is plotted as a function of the actual measured external HCO_3^- concentration in Fig. 1*B*. A half-maximal effect of HCO_3^- was observed at approximately 10 mM.

The effects of replacing HCO_3^- with weak acids on the ACh-induced acidosis

Some weak acid anions, including HCO_3^- and acetate, are transported by the perfused rabbit mandibular gland (Case, Conigrave, Favaloro, Novak, Thompson &

Young, 1982). We therefore examined the possibility that other weak acid anions might mimic the behaviour of HCO_3^- in supporting the ACh-evoked transient acidosis.

Weak acids enter the cell by passive diffusion in the undissociated form, and then dissociate to give an intracellular acidosis (see Roos & Boron, 1981). The size of the

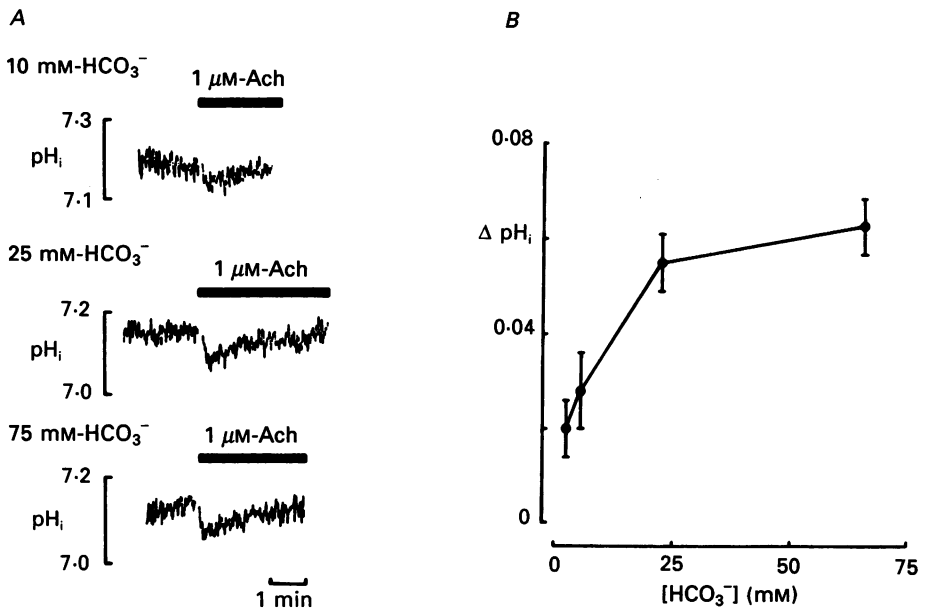

Fig. 1. Dependence of the ACh-induced intracellular acidosis on medium HCO_3^- concentration. Acini were pre-incubated in the (nominally) 5, 10, 25 and 75 mM-HCO_3^- buffers (see Table 1 and Methods) for 5–10 min before the addition of 1 μM-ACh. *A*, fluorescence traces obtained for acini taken from a single-cell preparation in the (nominal) 10, 25 and 75 mM-HCO_3^- buffers. 1 μM-ACh was added as indicated. *B*, averaged values of the size of the acidosis evoked by 1 μM-ACh (mean ± S.E.M., $n = 4$) plotted as a function of the experimentally determined extracellular HCO_3^- concentration (see Methods for details).

acidosis produced by addition of the weak acid, and the rate at which it developed, varied. For instance, Fig. 2 shows that propionate appeared to partition into the cells more rapidly than acetate. This can probably be attributed to their relative lipid permeabilities (Jackson, Williamson, Dombrowski & Garner, 1978). Figure 2 also shows that the acidosis was followed by a recovery of pH_i towards the control value. This recovery of pH_i was blocked by 100 μM-ethylisopropyl-amiloride (EIPA; Fig. 2, bottom trace). Addition of EIPA to the cuvette caused a small acidosis (0·07 + 0·01 pH units, $n = 4$), consistent with the presence of the Na^+–H^+ exchange in the acinar cells. The inhibition of the recovery of pH_i following the acetate-induced acidosis by EIPA suggests that Na^+–H^+ exchange is involved in this recovery. The same finding has been reported for many other cells following acid loading by a variety of means (see Frelin, Vigne, Ladoux & Lazdunski, 1988, for a recent review).

Table 2 gives the values of resting pH_i measured at least 5 min after the resuspension (including two washes) of the acini in solutions containing a range of weak acid anions (all present at a concentration of 25 mM). The measured pH_i

showed some variability, with pH_i of acini equilibrated in the solutions containing butyrate, lactate and salicylate being more acid than pH_i of acini in control (HCO_3^--buffered) medium. The extent to which the weak acids supported the ACh-induced acidosis also varied, as summarized in Table 2. The values in this table are expressed

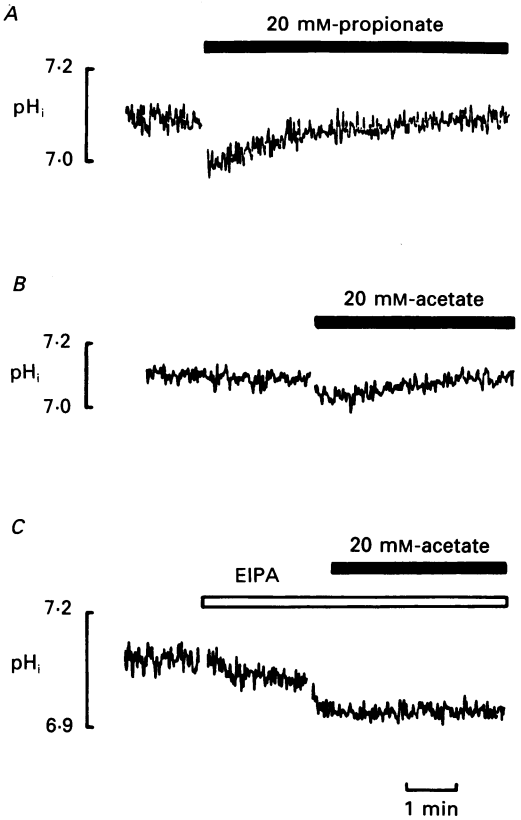

Fig. 2. The effects of weak acid anions on pH_i in mandibular acinar cells. The weak acids propionate (*A*) and acetate (*B* and *C*) were added to the cuvette at a final concentration of 20 mM from concentrated stock solutions (2 M-weak acid–sodium salt, 12·5 mM-HEPES–NaOH, pH 7·4). Acini initially equilibrated in the 25 mM-HCO_3^--buffered medium in all three traces. The traces are all representative of at least four experiments. Similar experiments were carried out with all the weak acids listed in Table 2.

in terms of the change in the intracellular H^+ activity (ΔH_i^+), rather than in terms of the change in pH_i, in order to take account of the differences in resting pH_i in the solutions containing the various weak acids. Propionate, acetate (see Fig. 3) and butyrate were all capable of supporting an acidosis similar in size to that obtained in HCO_3^--buffered solutions. Lactate suported the acidosis to a lesser extent, but with salicylate, formate and pyruvate the acidosis was much smaller than that measured in HCO_3^--buffered solutions ($P < 0{\cdot}001$). In the case of pyruvate it did not significantly differ from zero ($P > 0{\cdot}1$).

The responsiveness to stimulation of acini bathed in the various different weak acid-containing media was assessed by measuring $[Ca^{2+}]_i$ in the acini. Stimulation of salivary acinar cells causes a biphasic increase in $[Ca^{2+}]_i$, consisting of an initial peak

followed by a decrease to a plateau level elevated with respect to control (Merritt & Rink, 1987; Gray, 1988). The $[Ca^{2+}]_i$ response of rabbit mandibular acinar cells to cholinergic stimulation also follows this pattern (Brown, Dho, Elliott, Hamill-Keays, Lau & Sawhney, 1988). We examined the ACh-evoked changes in $[Ca^{2+}]_i$ in acini

TABLE 2. Summary of weak acid results

Weak acid anion	Resting pH_i (pH units)	ΔH_i^+ ($\times 10^{-9}$ M)	n
HCO_3^-	7·12 ± 0·02	18·6 ± 1·1	16
Propionate	7·06 ± 0·01	17·9 ± 1·4	4
Butyrate	7·00 ± 0·02*	16·8 ± 1·7	4
Acetate	7·13 ± 0·03	14·9 ± 1·8	6
Lactate	7·04 ± 0·01*	9·7 ± 2·3*	4
Salicylate	6·97 ± 0·04*	5·4 ± 1·1**	4
Formate	7·15 ± 0·02	4·5 ± 0·7**	3
Pyruvate	7·04 ± 0·02	3·2 ± 1·2**	4
Chloride	7·23 ± 0·02	4·3 ± 0·8	14

The effects of substituting weak acid anions for bicarbonate. The columns show the resting pH_i and the size of the transient intracellular acidosis evoked by 1 μM-ACh. The acini were equilibrated in the weak acid-containing medium for 5 min prior to the start of the measurements. All values given are mean ± S.E.M. The number of experiments is given in the last column of the table. Asterisks indicate significant differences from the responses observed in acini in HCO_3^--buffered medium (given in the first line of the Table): * = $P < 0·05$; ** = $P < 0·01$. Tests for differences between group means were made by unpaired t tests. The rsult for Cl^- solutions are adapted from Lau *et al.* (1989). 10 μM-ACh was used to elicit an acidosis in these experiments; it should be noted, however, that 1 and 10 μM-ACh gave an acidosis almost identical in size (see Fig. 3 of Lau *et al.* 1989).

bathed in media containing all of the weak acids studied (except for salicylate which dramatically quenched Fura-2 fluorescence). Table 3 shows results obtained in HCO_3^--containing medium and in media containing acetate and pyruvate, weak acids which respectively did and did not support the ACh-evoked intracellular acidosis. In each case, ACh evoked the characteristic increase of $[Ca^{2+}]_i$ to a peak followed by a decrease to a plateau level. The values for $[Ca^{2+}]_i$ obtained at all three stages of the response were similar for control acini (HCO_3^--containing medium) and for acini bathed in acetate- or pyruvate-containing media. Quantitatively similar results were obtained in two or more experiments using each of the other weak acids studied. This strongly suggests that the variation in the ACh-evoked acidosis in cells bathed in the weak acid-containing media was not due to differences in responsiveness to stimulation.

The acetate dependence of the ACh-induced acidosis

As with the dependence of the ACh-evoked acidosis in HCO_3^--buffered media on the medium HCO_3^- concentration (Fig. 1), the acidosis evoked by ACh in acetate-containing media was dependent on the concentration of acetate present. Cells were resuspended in solutions (all with a pH of 7·4) which contained acetate at concentrations ranging from 5 to 100 mM, 5–10 min before the addition of 1 μM-ACh. Resting pH_i was not significantly different from that measured with 25 mM-acetate in these experiments (7·13 ± 0·03, $n = 6$; see Table 2). However, Fig. 4 shows that

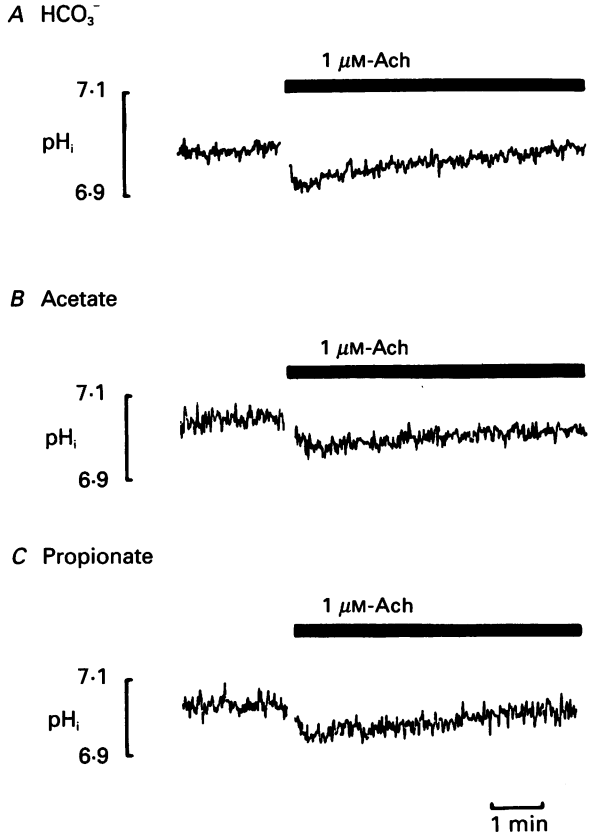

Fig. 3. Anions of weak acids support the ACh-induced acidosis. Acini were resuspended 5–10 min before the start of the records into media containing the following weak acids: *A*, 25 mM-HCO_3^-; *B*, 25 mM-acetate; *C*, 25 mM-propionate. 1 μM-ACh was added to the cuvette as indicated.

TABLE 3. Effect of acetylcholine on $[Ca^{2+}]_i$ (nM)

	Resting	Peak	Plateau	*n*
HCO_3^-	96±15	240±38	156±32	6
Acetate	77±15	233±39	147±30	4
Pyruvate	71±8	369±81	181±36	4

The effect of stimulation on $[Ca^{2+}]_i$ in acini bathed in solutions containing HCO_3^-, acetate or pyruvate. $[Ca^{2+}]_i$ was measured using Fura-2 as described in Methods. The cells were stimulated with 1 μM-ACh. The columns give values of $[Ca^{2+}]_i$ before stimulation, at the peak of the stimulation-evoked increase in $[Ca^{2+}]_i$ and at the (subsequent) plateau level. All values are quoted as mean±S.E.M. in nM. The number of experiments is given in the final column of the table. Note that no correction was made for the possible effects of dye leakage, so that the values of $[Ca^{2+}]_i$ should be regarded as comparative between the different experimental groups rather than absolute (see Methods).

the size of the ACh-induced acidosis increased with increasing medium acetate concentration, with a half-maximal acidosis occurring at a concentration of 10 mM.

The effects of acetazolamide on the ACh-induced intracellular acidosis

Acetazolamide (at a concentration of 0·1 or 1 mM) reduces the size of the ACh-induced acidosis in HCO_3^--buffered medium (Lau *et al.* 1989). Figure 5 shows the

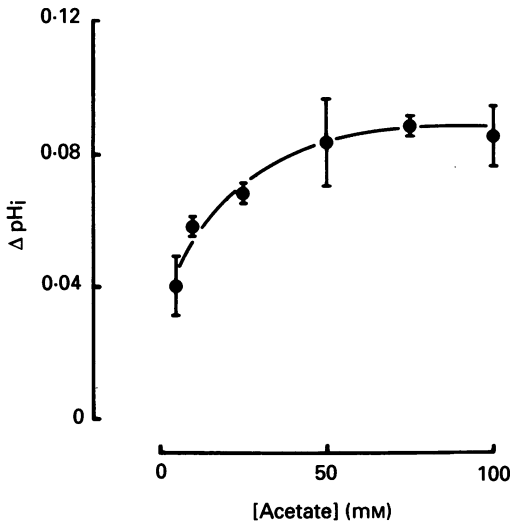

Fig. 4. Dependence of the ACh-induced acidosis on medium acetate concentration. Acini were resuspended in medium containing concentrations of acetate from 5 to 100 mM: these were obtained by mixing the HCO_3^--free isolation medium with the 105 mM-sodium acetate medium (see Table 1) in the appropriate proportions. 1 μM-ACh was added 5–10 min after resuspension of the acini in the acetate-containing medium. All the data were collected from four preparations of acini, and the results are given as means ± S.E.M. (n = 4). The curve was fitted by eye. The format of the figure is similar to Fig. 1*B*.

effects of 1 mM-acetazolamide on cells bathed in HCO_3^- or acetate-buffered media were compared. Pre-treatment of acini bathed in HCO_3^--containing medium with 1 mM-acetazolamide markedly reduced the ACh-evoked acidosis (Fig. 5*A*), in agreement with our previous report (Lau *et al.* 1989). In contrast, when the cells were bathed in a HCO_3^--free medium containing acetate the acidosis was unaffected by acetazolamide (Fig. 5*B*). Averaged results from a number of experiments are given in Table 4 (note that the changes in pH_i are expressed as ΔH_i^+ in order to take account for the differences in resting pH_i).

The effect of chloride channel blockers on the ACh-induced acidosis

Following stimulation by ACh, HCO_3^- and acetate may leave the acinar cells via ion channels (Lau *et al.* 1989). If these putative anion channels are similar to Cl^- channels present in mandibular acinar cells (Marty *et al.* 1984; Findlay & Petersen, 1985) and in other epithelia (reviewed by Gögelein, 1988), then compounds which can block Cl^- channels might be expected to reduce or abolish the ACh-evoked acidosis. Diphenylamine-2-carboxylate (DPC), compounds derived from it, e.g. NPPB and

compound 131, are effective Cl^- channel blockers in some tissues, particularly renal epithelia (Wangemann, Wittner, DiSteffano, Englert, Lang, Schlatter & Greger, 1986). The efflux of $^{36}Cl^-$ from rat parotid gland acinar cells evoked by stimulation is inhibited by 1 mM-DPC (Melvin, Kawaguchi, Baum & Turner, 1987), suggesting that the Cl^- channels in salivary glands are susceptible to this blocker.

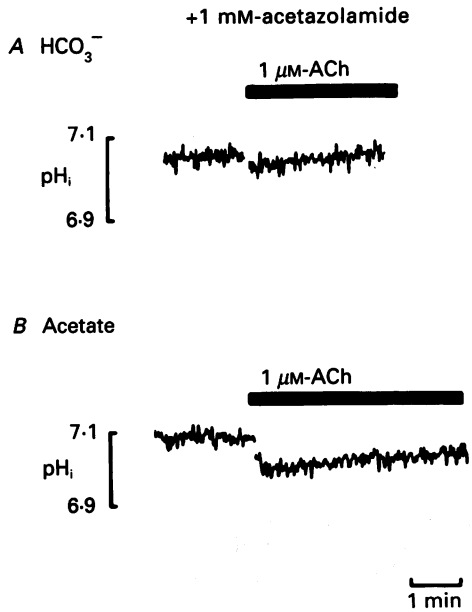

Fig. 5. Acetazolamide (1 mM) inhibits the ACh-induced acidosis in acini bathed in medium containing HCO_3^-, but not in medium containing acetate. Acini were resuspended in medium containing either 25 mM-HCO_3^- or 25 mM acetate buffers and incubated in the presence of 1 mM-acetazolamide for 10 min before the addition of 1 mM-ACh as indicated. To allow comparisons with controls to be made, the acini used in these experiments were from the same cell preparation as those used for Fig. 3*A* and *B*.

Acini equilibrated in medium containing either 25 mM-HCO_3^- or 25 mM-acetate were pre-incubated with DPC for 10 min. The size of the acidosis evoked by ACh in HCO_3^--buffered media was reduced or almost completely abolished by 0·5 mM-DPC (Fig. 6*B*) or 1 mM-DPC (Fig. 6*C*), respectively. Exposure to 1 mM-DPC for shorter periods than 10 min failed to inhibit the ACh-evoked acidosis fully. DPC (0·1 mM) had no effect on the acidosis ($n = 4$; not shown). Averaged results on the effect of 1 mM-DPC are given in Table 4, which also shows that the acidosis evoked by ACh in acetate-containing medium was greatly reduced by 1 mM-DPC. The effects of the more potent Cl^- channel blocker NPPB (Wangemann *et al.* 1986) were also studied in both HCO_3^-- and acetate-containing media. NPPB quenched BCECF fluorescence, attenuating the sensitivity to pH (by approximately 20%). This has the effect of reducing the accuracy of the calibration. It was clear, however, that 0·5 mM-NPPB almost completely abolished the ACh-evoked acidosis in the presence of either HCO_3^- or acetate (Table 4). NPPB had no significant effect on the acidosis at concentrations of less than 0·2 mM ($P > 0·1$; $n = 4$). In four experiments, compound 131 (0·5 mM)

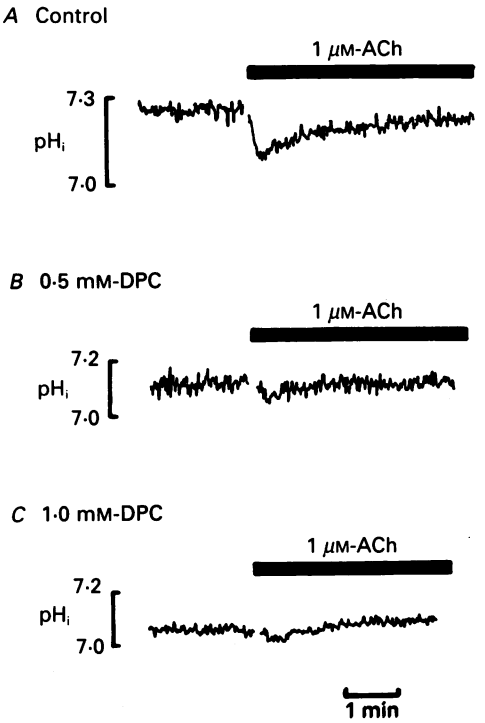

Fig. 6. DPC reduces the ACh-induced acidosis. Acini resuspended in HCO_3^--containing medium were pre-incubated for 10 min with: *A*, 1% (v/v) dimethylsulphoxide (the vehicle for DPC); *B*, 0·5 mM-DPC; or *C*, 1 mM-DPC. 1 μM-ACh was added as indicated. The traces shown are for a single preparation of acinar cells.

TABLE 4. Inhibitors of the ACh-induced acidosis: ΔH_i^+ ($\times 10^{-9}$ M)

	HCO_3^-		Acetate	
	Control	+Inhibitor	Control	+Inhibitor
Acetazolamide (1 mM)	19·0±3·8 (5)	4·0±1·2* (5)	16·2±2·6 (5)	17·7±4·5 (5)
DPC (1 mM)	13·8±1·9 (8)	4·7±1·9* (8)	19·0±1·7 (4)	4·0±1·5** (4)
NPPB (0·5 mM)	15·0±0·8 (4)	2·4±2·4* (4)	20·5±4·3 (4)	1·4±1·4* (4)

Acetazolamide and the Cl^- channel blockers were added to the acini 10 min before the addition of the ACh. All values are presented as means±S.E.M. with the number of experiments in parentheses. Tests for differences between group means were made by unpaired *t* tests (* = $P <$ 0·05 and ** $P < 0·01$).

completely blocked the HCO_3^--supported acidosis (data not shown). It also caused a 50% inhibition of the acidosis at concentrations of 0·1–0·2 mM ($n = 4$), but had no effect at 0·01 mM.

The Cl^--channel blockers caused an apparent intracellular acidification in resting cells; for instance, pH_i after 10 min incubation with 1 mM-DPC was 6·95±0·03 ($n = 8$) compared with a control value of 7·18±0·03 ($n = 8$). It is unclear why the drugs

should cause an acidosis, and in view of the quenching of BCECF fluorescence by the drugs we are unsure whether the acidosis is a real effect. We have previously reported the somewhat similar observation that millimolar concentrations of amiloride, which also quench BCECF fluorescence, cause apparently anomalous values of resting pH_i (Lau *et al.* 1989). Calibrating BCECF fluorescence with the dye inside (nigericin/KCl) rather than outside the cell (Triton) did not make any difference to the apparent acidification caused by the Cl^- channel blockers (data not shown). One observation which led us to suspect that the acidification might be an artifact was that the shift in resting pH_i was apparent after very short (1 min) periods of exposure of the acini to the drug. This was in contrast to the effect of the drugs in blocking the ACh-evoked acidosis, which was only observed after 10 min incubation of the cells with the drug.

DISCUSSION

Stimulation of salivary gland acinar cells with acetylcholine causes a transient intracellular acidosis, probably as a result of the efflux of HCO_3^- ions from the cell (Lau *et al.* 1989). In the present paper we have shown that some other weak acid anions (acetate, butyrate, propionate and lactate) also support this transient acidosis in the absence of HCO_3^-. The size of the acidosis evoked by ACh is dependent on the concentration of the weak acid anion in the bathing medium, and this concentration dependence is similar for HCO_3^- and acetate (half-maximal acidosis at approximately 10 mM). It thus seems likely that it is also the loss of these weak acid anions from the cell which gives rise to the acidosis. We propose that the acinar cell can transport a variety of small anions, and that these anions may share common transport mechanisms with HCO_3^-.

All the weak acids examined entered the cell in the un-ionized form, as judged by the development of an intracellular acidosis when they were added to the bathing medium. However, they differed widely in their ability to support the ACh-induced intracellular acidosis. For instance, salicylate, which crossed the membrane most readily of all the weak acids tested, was one of the least effective in supporting the ACh-induced acidosis. It is clear that the ease with which the weak acids permeate the cell in the un-ionized form is not the critical property for determining their ability to support transport of acid equivalents. Nor can these differences between the weak acids be explained by effects on the responsiveness of the cells to stimulation, since none of the weak acid anions appeared to compromise the ability of ACh to evoke a rise in intracellular free Ca^{2+} (although it remains to be determined whether they affect metabolism or intracellular buffering capacity). The explanation of why some weak acid anions (acetate, propionate, butyrate and lactate) support the ACh-evoked intracellular acidosis, while others (pyruvate, salicylate and formate) do not, must lie elsewhere, most likely in the selectivity of an anion transport mechanism (see below).

Table 2 shows that ACh actually appeared to evoke a very small change in pH_i in the presence of the ineffective weak acid anions (salicylate, formate and pyruvate). It should perhaps be noted, however, that the small ΔH_i^+ observed with salicylate, formate and pyruvate is similar in size to that observed with Cl^- (Table 2). Transport of Cl^- at least should not cause an acidosis, and the

small ΔH_i^+ evoked by ACh in media containing Cl^- alone, salicylate, formate or pyruvate may therefore reflect acid production by cellular metabolism, and/or movements of small amounts of HCO_3^- produced as a by-product of cellular metabolism.

The carbonic anhydrase inhibitor acetazolamide (1 mM) clearly inhibited the transport of acid equivalents in cells bathed in medium containing HCO_3^- but not in medium containing acetate. As with any drug used at a concentration as high as 1 mM, the possibility of non-specific effects must be borne in mind. However, we have previously shown (Lau *et al.* 1989) that acetazolamide was nearly as effective at a lower concentration (0·1 mM) at which it is relatively specific for carbonic anhydrase (Maren, 1977). The lack of effect of 1 mM-acetazolamide on the ACh-evoked acidosis in acetate-containing medium also tends to rule out non-specific effects of the drug. The effect of acetazolamide on the ACh-evoked acidosis tends to suggest that HCO_3^- is mainly accumulated in the cell via hydration of CO_2 catalysed by carbonic anhydrase.

Of the weak acid anions tested in the present study, only acetate has been examined in any detail with respect to its transport by the isolated perfused rabbit mandibular gland. Acetate enhanced the rate of fluid secretion, and appeared in the saliva at a concentration of 10–20 mM, when it was substituted for HCO_3^- in the perfusate (Case *et al.* 1982), and was recently shown to support secretion in the complete absence of Cl^- (Novak & Young, 1989). The evidence in the present study that acinar cells can transport acetate suggests that the acini are the site of acetate secretion by the whole gland.

Acetate and other small carboxylic acid anions are known to mimic the effects of HCO_3^- in other epithelia, for instance in supporting fluid secretion in the pancreas (Swanson & Solomon, 1975; Case, Hotz, Hutson, Scratcherd & Wynne, 1979) and in enhancing the absorption of Na^+ and Cl^- in the gall bladder (Petersen, Wood, Schulze & Heintze, 1981). It is unlikely, however, that exactly the same mechanisms are involved in these tissues as in the salivary acinar cell; Cl^-–HCO_3^- exchange has been implicated in the effects of carboxylic acids in both pancreas and gall-bladder (Petersen *et al.* 1981; Novak & Greger, 1988), while it is clearly not involved in mediating HCO_3^- efflux in the salivary acinar cell (Lau *et al.* 1989).

Possible routes for anion efflux

We have previously shown that the ACh-induced intracellular acidosis results from the efflux of HCO_3^- ions from the acinar cell on stimulation (Lau *et al.* 1989) and that this efflux is not mediated by either Cl^-–HCO_3^- exchange (Lau *et al.* 1989) or by Na^+-dependent mechanisms, for instance Na^+–HCO_3^- co-transport (Brown, Donohue, Elliott & Lau, 1989). By a process of elimination this leads us to hypothesize that ACh activates ion channels which are permeable to HCO_3^-. HCO_3^- should then leave the cell down its electrochemical gradient (see below). The fact that the acidosis can still be evoked when HCO_3^- is replaced by acetate, propionate, butyrate or lactate would tend to suggest that the putative anion channels are also permeable to these anions.

There is no electrophysiological evidence available that bears directly on the question of a HCO_3^- conductance in the rabbit mandibular gland. The response of the acinar cells to stimulation is a transient depolarization, presumably due largely

to conductive Cl^- efflux (Lau & Case, 1988), but equally consistent with conductive HCO_3^- efflux. This Cl^- efflux is thought to be from cell to lumen through apically located Cl^- channels, with much of the driving force coming from the membrane potential (Lau & Case, 1988).

It has recently been clearly demonstrated (by patch-clamp methods) that channels in nerve cells which were originally found to be permeable to Cl^- are in fact also permeable to a range of small (halide and non-halide) anions (Bormann *et al.* 1987). HCO_3^- efflux through receptor-gated Cl^- channels has also been reported in crayfish muscle fibres (Kaila & Viopio, 1987). Channels permeable to Cl^- are known to be present in acinar cells of the exocrine glands (reviewed by Marty, 1987; Petersen & Gallagher, 1988) including the rabbit mandibular gland (Lau & Case, 1988). The permeability of these acinar cell Cl^- channels to the anions used in the present study has not been investigated, and it is quite possible that these channels are in fact relatively non-specific anion channels. In other epithelia, anion channels with a low selectivity for Cl^- over HCO_3^- (1:0·4) have been described in rat colonic cells (Reinhardt *et al.* 1987), while the existence of a conductance primarily selective for HCO_3^- over Cl^- in the choroid plexus has been proposed by Saito & Wright (1984).

Mechanism of action of DPC and its derivatives

DPC and its analogues block Cl^- channels in some epithelia (see Gögelein, 1988, for a recent review). The inhibition of the ACh-induced intracellular acidosis by these drugs in the present study is thus consistent with their blocking conductive HCO_3^- efflux through anion channels. The inhibition of the acidosis by DPC and NPPB in either HCO_3^-- or acetate-containing medium suggests that the drugs blocked the efflux of both these anions.

A potential problem with the use of the Cl^- channel blockers in the present work is that quite high concentrations (0·1–1 mM) of all three blockers were required to block anion efflux. These concentrations are greatly in excess of those found to be effective in blocking Cl^- conductive pathways in the loop of Henle (Wangemann *et al.* 1986), although they are similar to the concentrations required to block Cl^- conductances in airway epithelia (Welsh, 1986*a*, *b*). Nevertheless, the concentrations clearly are sufficiently high to raise the question of whether the drugs were having non-specific effects. Two actions of DPC and its derivatives other than blockade of Cl^- channels have been reported. Firstly, DPC may block anion exchange (Reuss, Costantin & Bazile, 1987). This cannot, however, explain the action of DPC (or its derivatives) in the present experiments, since Cl^-–HCO_3^- exchange plays no part in the ACh-evoked intracellular acidosis (Lau *et al.* 1989). Secondly, Cl^- channel blockers have been shown to block Ca^{2+}-activated non-specific cation channels in pancreatic acinar cells (Gögelein & Pfannmüller, 1988). However, salivary acinar cells do not contain this channel type, but rather the more common Ca^{2+}-activated K^+-selective channel (Maruyama, Gallagher & Petersen, 1983). DPC (1 mM) has been shown to block ACh-evoked $^{36}Cl^-$ efflux from rat parotid salivery gland acini (Melvin *et al.* 1987). Since this stimulated Cl^- efflux almost certainly results from the opening of Cl^- channels (Lau & Case, 1988), the known effects of DPC in salivary gland acinar cells are consistent with its action being to block Cl^- channels.

At least two possible explanations can be proposed for the need to use high

concentrations of the Cl^- channel blockers to inhibit anion efflux. First, it may reflect differences in the ability of these inhibitors to block different classes of Cl^-/anion channel (see the previous paragraph, and also Gögelein, 1988). Second, it may reflect restricted access of the drugs to anion channels on the apical membrane, which presumably faces a collapsed duct lumen. Such restricted access might explain why the effects of the Cl^- channel blockers took up to 10 min to develop (see Results). Some further support for the restricted-access hypothesis comes from the fact that DPC and its derivatives do not affect the Cl^- conductance present in *Necturus* enterocytes in experiments on the intact tissue (Giraldez, Sepúlveda & Sheppard, 1988) but are able to block Cl^- channels in isolated membrane patches (F. V. Sepúlveda, personal communication).

In summary, rabbit mandibular salivary gland acinar cells contain an anion transport mechanism which mediates the efflux of HCO_3^- and some monocarboxylate anions. This anion transport mechanism is activated on stimulation with acetylcholine. Since HCO_3^- efflux is not mediated by anion exchange (Lau *et al.* 1989), and since Na^+ removal has no effect on the efflux of either HCO_3^- (Brown *et al.* 1989) or acetate (P. D. Brown, M. Donohue, A. C. Elliott & K. R. Lau, unpublished observations), we propose that this mechanism may be a non-specific anion channel. Since mandibular acinar cells are thought to possess Cl^- channels activated on stimulation with ACh (Lau & Case, 1988), and in view of the evidence that 'Cl^- channels' in other tissues are permeable to other small anions, including HCO_3^- and acetate (Bormann *et al.* 1987), we propose that the 'non-specific anion channels' may be identical to the Cl^- channels which are a key element of current models of exocrine secretion. A channel of this kind would provide a route for transcellular transport of HCO_3^- and acetate in the acinar cell and could thus explain the ability of HCO_3^- and acetate to support fluid secretion in the isolated perfused rabbit mandibular gland.

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