Upregulation of Na^+–K^+–$2Cl^-$ cotransporter activity in rat parotid acinar cells by muscarinic stimulation

Richard L. Evans and R. James Turner*

Clinical Investigations and Patient Care Branch, National Institute of Dental Research, Building 10, National Institutes of Health, Bethesda, MD 20892-1190, USA

1. The effects of fluid secretory stimuli on the Na^+–K^+–$2Cl^-$ cotransporter in rat parotid acini were investigated. Cotransporter activity was measured using NH_4^+ as a K^+ surrogate and following cotransporter-mediated NH_4^+ fluxes by monitoring intracellular pH.

2. A dramatic upregulation (15- to 20-fold) of acinar Na^+–K^+–$2Cl^-$ cotransporter activity was induced by muscarinic, α_1-adrenergic and peptidergic stimuli. A half-maximal effect of the muscarinic agonist carbachol was observed at ~0·5 μM.

3. Our results indicate that the rise in intracellular calcium concentration ($[Ca^{2+}]_i$) which accompanies these stimuli is both a necessary and a sufficient condition for this effect; but it is not a consequence of the KCl loss and concomitant isotonic shrinkage caused by increased $[Ca^{2+}]_i$ as it persists when these effects are prevented.

4. The effect of muscarinic stimulation on the cotransporter can, however, be blocked by inhibitors of phospholipase A_2 (4-bromophenacylbromide and manoalide), by a general inhibitor of arachidonic acid metabolism (5,8,11,14-eicosatetraynoic acid) and by specific inhibitors of the cytochrome P450 pathway (methoxsalen and ketoconazole).

5. These latter results argue strongly for the involvement of a product of the cytochrome P450 pathway of arachidonic acid metabolism in upregulation of the salivary Na^+–K^+–$2Cl^-$ cotransporter.

6. Owing to the complexity of the arachidonic acid cascade a wide variety of agents could potentially interfere with this upregulation of the cotransporter, and thereby result in decreased salivary fluid production. We suggest that such an effect could underlie the dry mouth (xerostomia) that occurs as an unexplained side-effect of many commonly prescribed medications.

Na^+–K^+–$2Cl^-$ cotransporters play a vital role in salt and water movements across a number of secretory and absorptive epithelia (e.g. exocrine glands, airway, kidney and gut; for reviews see O'Grady, Palfrey & Field, 1987 and Haas, 1994). Due to their experimental accessibility and rich hormonal responsiveness, salivary glands have proved to be a particularly useful system for studying the involvement and regulation of this important transporter in epithelial function. In these glands the cotransporter is located on the basolateral membrane of the acinar cells where it concentrates Cl^- in the cytoplasm above electrochemical equilibrium. The production of salivary fluid is thought to occur as follows. The application of a fluid secretory stimulus to the acinar cells leads to a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) which in turn causes the opening of Ca^{2+}-activated Cl^- and K^+ channels in the apical and basolateral membranes, respectively. These Ca^{2+}-dependent increases in K^+ and Cl^- conductance allow KCl to flow out of the cell resulting in an accumulation of Cl^- ions and their associated negative electrical charge in the acinar lumen. Na^+ then follows Cl^- paracellularly to preserve electroneutrality and the resulting osmotic gradient for NaCl causes a transepithelial movement of water from interstitium to lumen (Cook & Young, 1989; Turner, 1993). The effect of a fluid secretory stimulus on rat parotid acinar cells is quite dramatic; within the first 30 s of stimulation their intracellular chloride ion concentration falls from ~60 to ~40 mM and their volume decreases by ~20% due to KCl loss (Turner, 1993). With sustained stimulation the cells only partially recover from this initial shrinkage and salt loss (Foskett & Melvin, 1989; Melvin & Turner, 1992) indicating that solute entry, a major component of which occurs via the Na^+–K^+–$2Cl^-$ cotransporter, is the rate-limiting step in transepithelial salt and water transport.

* To whom correspondence should be addressed.

Consistent with its central role in acinar function, previous studies from our laboratory have shown that the activity of the rat parotid Na^+–K^+–$2Cl^-$ cotransporter is upregulated by a variety of physiological and potentially physiologically relevant stimuli (Paulais & Turner, 1992*a*,*b*; Ferri, Evans, Paulais, Tanimura & Turner, 1996). These include β-adrenergic stimulation, hypertonic shrinkage, as well as treatment with aluminium fluoride (an activator of G proteins) or calyculin A (a protein phosphatase inhibitor). These agents each produce an increase of cotransport activity of 3- to 6-fold, apparently via quite different mechanisms of action (Paulais & Turner, 1992*a*,*b*; Tanimura, Kurihara, Reshkin & Turner, 1995). In the present paper we characterize a much larger upregulatory effect (~20-fold) on the cotransporter associated with the application of Ca^{2+} mobilizing stimuli. In particular, this upregulation is associated with muscarinic stimulation, the main fluid secretory stimulus for the gland. This dramatic elevation in Na^+–K^+–$2Cl^-$ cotransporter activity serves the obvious physiological function of increasing acinar transepithelial Cl^- transport, and thereby fluid secretion, during stimulation. Briefly stated, our results indicate that this upregulatory effect is due mainly to an intrinsic increase in Na^+–K^+–$2Cl^-$ cotransporter activity arising from the action of a product of the cytochrome P450 pathway of arachidonic acid metabolism. Owing to the complexity of the arachidonic acid cascade and the intracellular events associated with its activation (Bonventre, 1992; Barrett & Bigby, 1995) a wide variety of agents could potentially interfere with this upregulation of the cotransporter and thereby result in decreased fluid production. We suggest that such an effect could underlie the dry mouth (xerostomia) that occurs as an unexplained side-effect of many commonly prescribed medications (Sreebny & Schwartz, 1986).

A preliminary account of part of this work has been presented to The Physiological Society (Evans & Turner, 1995).

METHODS

Materials

Collagenase P was obtained from Boehringer Mannheim Corporation. Bovine serum albumin (BSA, Type V), 4-bromophenacylbromide (4-BPB), niflumic acid, carbachol, L-phenylephrine, phorbol 12-myristate 13-acetate (PMA), prazosin hydrochloride, quinine, substance P, [D-Arg^1,D-Trp^{79},Leu^{11}]-substance P (spantide), and TEA were from Sigma Chemical Company. Calmidozolium, K252a, staurosporine, ouabain, thapsigargin and 2′,7′-bis[2-carboxyethyl]-5[6′]-carboxyfluorescein pentaacetoxymethyl ester (BCECF AM) were from Calbiochem Corporation and bumetanide was a gift from Hoffmann–LaRoche Laboratories (Nutley, NJ, USA). Ketoconazole, 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), manoalide, methoxsalen, 20-hydroxyeicosatetraenoic acid (20-HETE), (±)5,6-epoxyeicosatrienoic acid ((±)5,6-EET), (±)11,12-epoxyeicosatrienoic acid ((±)11,12-EET) and indomethacin were from Biomol (Plymouth Meeting, PA, USA). Cypermethrin, deltamethrin and Compound 5 were from L. C. Laboratories (Woburn, MA, USA), and fura-2 AM was from Molecular Probes. BCECF AM and fura-2 AM were stored at −20 °C as 2 mM stock solutions in dimethyl sulphoxide (DMSO). All other chemicals were prepared as stock solutions in distilled water, DMSO or ethanol as required, and added to cell suspensions so that the final concentrations of DMSO or ethanol did not exceed 1%. (In control experiments we have established that these concentrations of ethanol and DMSO are without effect on resting acinar Na^+–K^+–$2Cl^-$ cotransporter activity or on the upregulation of the cotransporter by muscarinic stimulation studied here.)

Solutions

Unless otherwise indicated, experiments were carried out in a physiological salt solution (PSS) which contained (mM): NaCl, 135; KCl, 5·8; $CaCl_2$, 1·8; $MgSO_4$, 0·8; NaH_2PO_4, 0·73; glucose, 11; Hepes, 20 (held at pH 7·4 with NaOH); glutamine, 2; and 0·01% BSA. In Ca^{2+}-free PSS, 0·1 mM EGTA was substituted for $CaCl_2$.

Acinar preparation

Male Wistar rats (Harlan Sprague–Dawley Inc., Indianapolis, IN, USA) were anaesthetized by inhalation of diethyl ether and killed by cardiac puncture. Dispersed acini were prepared from the parotid glands by collagenase digestion as previously described (Tanimura *et al.* 1995). The final acinar suspension was washed and resuspended in PSS containing 1% BSA (5 ml per animal) and rested for 15 min at 30 °C before loading with BCECF or fura-2 (see below). Throughout the entire preparation and subsequent experimental periods acini were kept continuously agitated (90 cycles min^{-1}) and gassed with 100% O_2 or 95% O_2–5% CO_2 as appropriate.

Measurement of intracellular pH (pH_i) and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Acini were loaded either with the pH-sensitive fluorescent indicator BCECF or the Ca^{2+}-sensitive fluorescent indicator fura-2 by incubation in the presence of 2 μM of the membrane permeant acetoxymethylester forms of each probe for 30 min at 30 °C in PSS containing 1% BSA. Following loading the acinar preparation was washed twice with PSS and maintained at 30 °C until experimental use. Intracellular BCECF fluorescence was monitored at 37 °C using a Spex ARCM-MIC spectrofluorimeter (Spex Industries, Edison, NJ, USA) fitted to a custom-built temperature-controlled cuvette housing (Micron Systems, Sykesville, MD, USA) by alternating the excitation wavelength between 490 and 440 nm at 1 s intervals and measuring emitted fluorescence at 530 nm. The fluorescent signal was then converted to intracellular pH (pH_i) as previously described (Paulais & Turner, 1992*a*). Fura-2 fluorescence was monitored in the same way at excitation wavelengths of 340 and 360 nm, measuring emitted fluorescence at 510 nm. Each fura-2 run was individually calibrated by adding 0·25% Triton X-100 in the presence of 1·8 mM Ca^{2+} (to determine fluorescence at saturating Ca^{2+} concentration), then adding 7 mM EGTA (to determine fluorescence at limitingly low Ca^{2+} concentration) and finally adding 11 mM Mn^{2+} (to quench fura-2 and thus determine the fluorescence background). Emitted fura-2 fluorescences at 340 and 360 nm excitation were corrected for background fluorescence, as well as for a component of fura-2 leakage to the extracellular medium, then $[Ca^{2+}]_i$ was calculated using the method of Grynkiewicz, Poenie & Tsien (1985).

Na^+–K^+–$2Cl^-$ cotransporter activity

As outlined in Results and discussed in detail in Paulais & Turner (1992*a*) the initial rate of pH_i recovery of rat parotid acini from an NH_4Cl-induced acute alkaline load was used as a measure of Na^+–K^+–$2Cl^-$ cotransporter activity. Briefly, the procedure and analysis were as follows. After various experimental treatments acini were subjected to a 30 mM NH_4Cl challenge, following which

they rapidly alkalinized ($t_{½}$, ~1·5 s) then recovered exponentially toward their resting pH_i (cf. Fig. 1*A* and Paulais & Turner, 1992*a*). Typically, the first 25–60 s of this recovery phase (the shorter times were used for more rapid recoveries) were fitted to an exponential equation (Paulais & Turner, 1992*a*) using the program Sigmaplot 5.0 (Jandel Scientific, Corte Madera, CA, USA) and the initial rate of pH_i recovery was determined by extrapolation back to the time of NH_4Cl addition. In general, in each experiment all pH_i recovery traces analysed in this way extrapolated back to the same pH_i at the time of NH_4Cl addition. Thus this method of analysis yields initial rates of pH_i recovery (Na^+–K^+–$2Cl^-$ cotransporter activity) determined at the same pH_i. Unless otherwise stated, in each experiment these rates were normalized to the initial rate of pH_i recovery obtained from a control run (application of 30 mM NH_4Cl to untreated acini in PSS) carried out on the same preparation.

It is also worth pointing out that, in contrast to results reported by another group (Snowdowne, Way, Thomas, Chen & Cashman, 1992), we find that the application of 30 mM NH_4Cl to rat parotid acini has no detectable effect on $[Ca^{2+}]_i$ (not shown; monitored as described above with fura-2).

Light scattering

Light scattering was used as an index of cell volume (Manganel & Turner, 1991). Scattered light (90 deg; 500 nm) from acini suspended in a fluorescence cuvette mounted in the Spex spectrofluorimeter and maintained at 37 °C was recorded at 1 s intervals.

Data analysis and presentation

Reported experimental values are means ± S.E.M. for three or more independent determinations performed under the same conditions on different acinar cell preparations. P values $< 0·05$ (Student's t test) were taken to represent statistically significant differences.

RESULTS

Evidence that muscarinic stimulation induces upregulation of Na^+–K^+–$2Cl^-$ cotransporter activity

Previous work from our laboratory has established that the rate of pH_i recovery of rat parotid acini from an NH_4Cl-induced alkaline load provides a measure of Na^+–K^+–$2Cl^-$ cotransporter activity in these cells (Paulais & Turner, 1992*a*, *b*). This is because a significant component of this recovery is due to NH_4^+ entry into the cytoplasm on the cotransporter as a substitute for K^+, resulting in the dissipation of the extracellular to intracellular NH_4^+ gradient. Using this method we have previously documented an apparent dramatic upregulation of the acinar Na^+–K^+–$2Cl^-$ cotransporter by muscarinic stimulation (Ferri *et al.* 1996). This phenomenon is illustrated in Fig. 1*A* where we compare the pH_i recovery of rat parotid acini from an NH_4Cl-induced acute alkaline load in the presence (trace *b*) and absence (trace *a*) of the muscarinic agonist carbachol

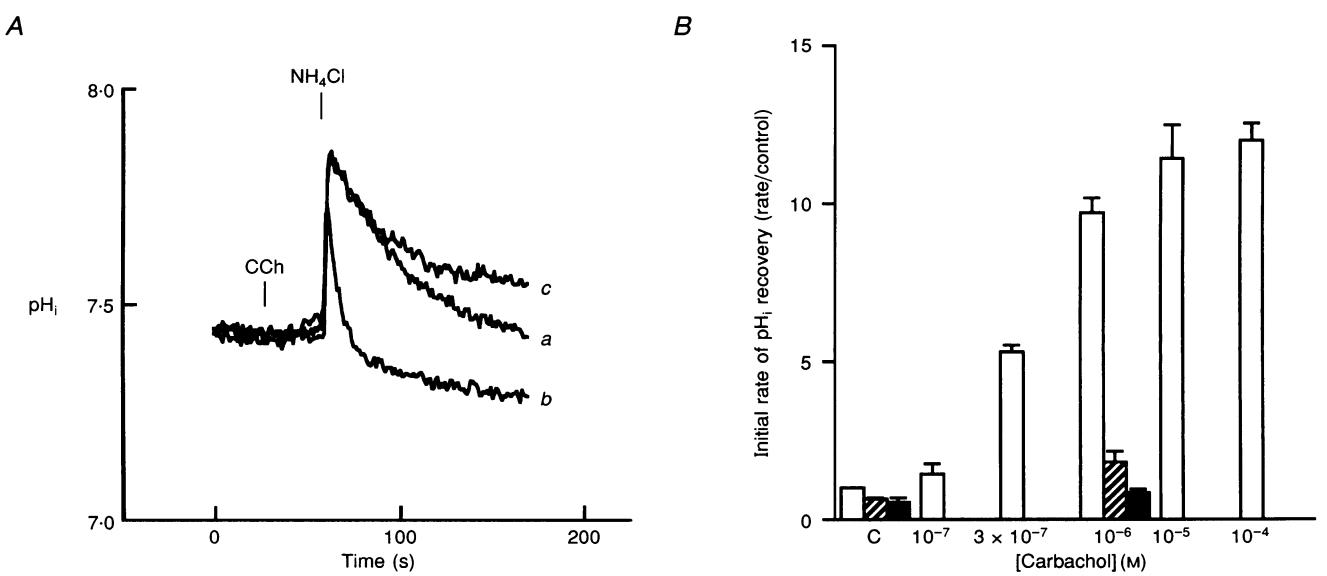

Figure 1. Effect of the muscarinic agonist carbachol (CCh) on the response of rat parotid acinar cells to an NH_4Cl-induced acute alkaline load

The results shown in *A* are representative pH_i traces (see Methods) from rat parotid acini suspended in PSS and challenged with 30 mM NH_4Cl as indicated. The traces are from otherwise untreated acini (trace *a*), acini preincubated for 30 s with 10^{-6} M carbachol before the addition of NH_4Cl (trace *b*), or acini preincubated with both 10^{-6} M carbachol and 10^{-4} M bumetanide (trace *c*; bumetanide was added 100 s before NH_4Cl). *B*, the effects of carbachol concentration and the Na^+–K^+–$2Cl^-$ cotransporter inhibitor bumetanide (10^{-5} and 10^{-4} M) have been quantified (□, no bumetanide; ▨, 10 μM bumetanide; ■, 100 μM bumetanide). Experiments were carried out as described above. In each experiment initial rates of pH_i recovery were calculated as described in Methods and normalized to the initial rate obtained in a control experiment (C; 30 mM NH_4Cl addition to untreated acini in PSS) carried out on the same preparation. The mean normalized results of 3 or more independent experiments under each condition are shown.

(10^{-6} M, added 30 s before the NH_4Cl challenge). In this figure we also demonstrate that the enhanced rate of pH_i recovery induced by carbachol is markedly inhibited by 10^{-4} M bumetanide (trace *c*), a specific inhibitor of the Na^+–K^+–$2Cl^-$ cotransporter. This effect of carbachol is also completely blocked by the muscarinic antagonist atropine (10^{-5} M, $n = 3$; not shown).

In the remainder of the paper, results of the type shown in Fig. 1*A* are quantified by determining the initial rates of pH_i recovery from the NH_4Cl-induced alkaline load as described in Methods. Figure 1*B* illustrates the dose dependence of the effect of carbachol analysed in this way. A half-maximal effect of carbachol is observed at ~0·5 μM, a value which is in good agreement with other effects of this agent on the physiological responses of rat parotid acinar cells to muscarinic stimulation (Manganel & Turner, 1991). As also shown quantitatively in this panel, the enhanced pH_i recovery induced by carbachol is completely bumetanide sensitive. Taken together with previous results from our laboratory linking the bumetanide-sensitive component of acinar pH_i recovery from an acute NH_4Cl-induced alkaline load with the Na^+–K^+–$2Cl^-$ cotransporter (Paulais & Turner, 1992*a*,*b*), these data provide strong evidence for a dramatic increase in cotransporter activity associated with muscarinic stimulation. At 10^{-6} M carbachol the bumetanide sensitive component of pH_i recovery is increased 19-fold relative to that observed in unstimulated cells. In additional experiments (not shown; $n = 3$) we have confirmed that ouabain (1 mM) has no significant effect on the enhancement of pH_i recovery induced by carbachol, indicating that this effect is not secondary to the activation of Na^+–K^+-ATPase.

Effects of other fluid secretory agonists and their antagonists

As already emphasized, the Na^+–K^+–$2Cl^-$ cotransporter plays a central role in generating the transepithelial Cl^- flux responsible for driving fluid secretion by salivary acinar cells. Accordingly, an upregulation of cotransporter activity by a fluid secretory stimulus such as carbachol is of considerable physiological significance and interest. The effects of two other agonists known to induce a fluid secretory response in salivary acinar cells are shown in Fig. 2. Enhanced pH_i recovery from an NH_4Cl-induced alkaline load analogous to that observed with carbachol is seen after stimulation with the α_1-adrenergic agonist phenylephrine (10^{-5} M) or the peptidergic agonist substance P (10^{-9} M). Figure 2 also demonstrates that the effects of these agents are blocked by their respective antagonists prazosin (10^{-5} M) and spantide (10^{-6} M). Thus similar effects on the Na^+–K^+–$2Cl^-$ cotransporter can apparently be mediated by stimulation of muscarinic, α_1-adrenergic and substance P peptidergic receptors.

The effect of carbachol is due to increased $[Ca^{2+}]_i$

The application of carbachol to salivary acinar cells results in activation of phospholipase C which in turn hydrolyses the lipid precursor phosphatidylinositol 4,5-bisphosphate to produce the intracellular messengers diacylglycerol and inositol 1,4,5-trisphosphate ($InsP_3$). Diacylglycerol is an activator of protein kinase C while $InsP_3$ releases Ca^{2+} from intracellular stores. The emptying of these stores causes a transient rise in intracellular calcium concentration as well as an increase in plasma membrane Ca^{2+} permeability. Provided physiological concentrations of Ca^{2+} are present in the extracellular solution, this latter effect results in Ca^{2+} entry which serves to maintain $[Ca^{2+}]_i$ above resting levels after dissipation of the transient $InsP_3$-induced increase. In the absence of extracellular Ca^{2+}, $[Ca^{2+}]_i$ returns to resting levels following the initial $InsP_3$-induced transient (see below). A number of observations presented below indicate that it is the carbachol-induced rise in $[Ca^{2+}]_i$, and not activation of protein kinase C, which leads to the upregulation of acinar Na^+–K^+–$2Cl^-$ cotransporter activity documented here.

In Fig. 3 we show the results of a series of experiments in which acinar suspensions were stimulated with 10^{-6} M carbachol in either Ca^{2+}-replete (+ Ca^{2+}) or Ca^{2+}-free

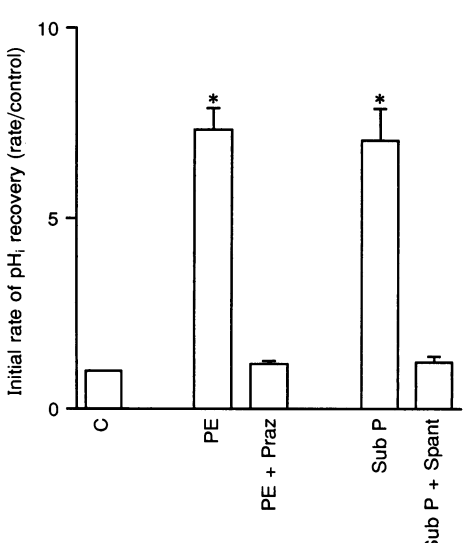

Figure 2. Effects of α_1-adrenergic and substance P peptidergic agonists and antagonists on the initial rate of acinar pH_i recovery from an NH_4Cl-induced alkaline load

The experimental procedure and data analysis were as described for Fig. 1 ($n > 3$ for all treatments). Phenylephrine (PE; 10^{-5} M) and substance P (Sub P; 10^{-9} M) were added 30 s before NH_4Cl; prazosin (Praz; 10^{-5} M) and spantide (Spant; 10^{-6} M) were added 140 s before NH_4Cl. Initial recovery rates significantly different from control (C) are indicated by *.

(– Ca^{2+}) PSS and the initial rate of pH_i recovery from an alkaline load measured 30 or 200 s later. These experiments show that in a Ca^{2+}-containing medium the carbachol-induced enhancement of pH_i recovery is greater at 30 than at 200 s after agonist stimulation, while in Ca^{2+}-free PSS the effect of carbachol is present at 30 but absent at 200 s. Under our experimental conditions in Ca^{2+}-replete PSS we find that $[Ca^{2+}]_i$ reaches its maximum value of 2·2 ± 0·3 times ($n = 3$) resting levels (57 ± 10 nM) approximately 2 s after the application of 10^{-6} M carbachol. It then falls to 1·7 ± 0·1 times resting levels at 30 s, and to a sustained plateau of 1·4 ± 0·1 times resting levels by 200 s after application of carbachol. After the application of 10^{-6} M carbachol in Ca^{2+}-free PSS, $[Ca^{2+}]_i$ peaks at 2·2 ± 0·3 times ($n = 4$) resting levels (46 ± 10 nM) then falls to 1·6 ± 0·1 times resting levels at 30 s, and has returned to resting levels by 200 s. Comparison of these $[Ca^{2+}]_i$ responses in Ca^{2+}-replete and Ca^{2+}-free media to the pattern of cotransporter upregulation illustrated in Fig. 3 indicates a correlation between increased intracellular Ca^{2+} levels and increased cotransporter activity.

The association of cotransporter upregulation with increased $[Ca^{2+}]_i$ is also supported by an additional experiment shown in Fig. 3, demonstrating that enhanced pH_i recovery from an NH_4Cl-induced alkaline load is likewise found 120 s following acinar treatment with 10^{-6} M thapsigargin. This compound is a microsomal Ca^{2+}-ATPase inhibitor that results in increased $[Ca^{2+}]_i$ without interacting with plasma membrane receptors and without activating protein kinase C. After the application of 10^{-6} M thapsigargin we find that acinar $[Ca^{2+}]_i$ slowly increases, reaching a maximum value of 2·2 ± 0·2 times ($n = 6$) resting levels approximately 120 s later. Thus, this concentration of thapsigargin results in a $[Ca^{2+}]_i$ response similar in magnitude (albeit not temporally) to that seen with 10^{-6} M carbachol (see above).

In further experiments designed to investigate a possible role of protein kinase C in the carbachol-induced enhancement of Na^+–K^+–$2Cl^-$ cotransporter activity we have shown that treatment of acini with phorbol 12-myristate 13-acetate (10^{-7} M), an activator of protein kinase C, had no detectable effect on cotransporter activity either in the presence (10^{-6} M) or absence of carbachol (data not shown; $n = 4$ for both). In addition, neither K252a (6×10^{-7} M) nor staurosporine (3×10^{-7} M), both potent inhibitors of protein kinase C, had a significant effect on the upregulatory effect of thapsigargin (Ferri *et al.* 1996). These results, together with those illustrated in Fig. 3, strongly support the hypothesis that increased $[Ca^{2+}]_i$ is both a necessary and sufficient condition for the upregulation of the cotransporter associated with muscarinic stimulation.

Evidence that Ca^{2+}-dependent upregulation of the cotransporter is not due to stimulation-induced salt loss or cell shrinkage

As discussed in the Introduction, secretagogue stimulation of rat parotid acinar cells results in a significant fall in acinar intracellular Cl^- levels ($[Cl^-]_i$) and an associated cell shrinkage, both due to KCl loss via Ca^{2+}-activated K^+ and Cl^- channels (because K^+ is the major cation in the cytoplasm there is little decrease in intracellular K^+ concentration as a result of isosmotic KCl loss). Since these effects are a consequence of increased $[Ca^{2+}]_i$, either or both of them could be responsible for the upregulation of the cotransporter activity documented above. In order to determine the mechanism underlying this upregulation it is obviously important to distinguish between the role of elevated $[Ca^{2+}]_i$ *per se* and that of the salt loss and cell shrinkage which arise from it. Our strategy to resolve this issue was to prevent salt loss using appropriate channel blockers, thus preventing both the fall in $[Cl^-]_i$ and cell shrinkage while leaving the $[Ca^{2+}]_i$ response intact. For these experiments we used thapsigargin to mimic the effect of carbachol in order to avoid possible inhibitory effects of channel blockers on the muscarinic $[Ca^{2+}]_i$ response.

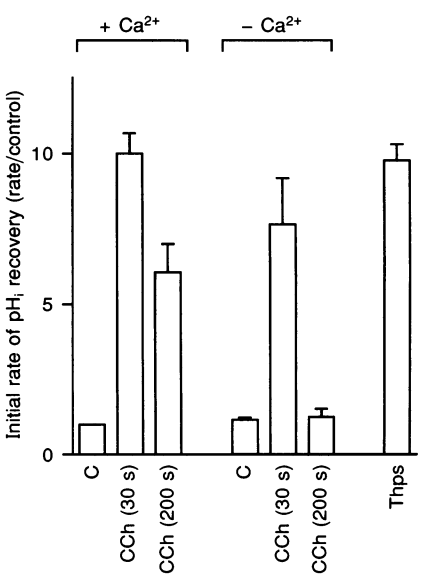

Figure 3. Effects of intracellular Ca^{2+} concentration on the initial rate of acinar pH_i recovery from an NH_4Cl-induced alkaline load

The experimental procedure and data analysis were as described for Fig. 1 ($n > 4$ for all treatments). Acinar suspensions were stimulated with 10^{-6} M CCh in either Ca^{2+}-containing (+ Ca^{2+}) or nominally Ca^{2+}-free PSS (– Ca^{2+}) and the initial rate of pH_i recovery from an NH_4Cl challenge measured 30 or 200 s later, as indicated. Also shown is the effect of acinar treatment with the microsomal Ca^{2+}-ATPase inhibitor thapsigargin (Thps; 10^{-6} M) applied 120 s before the NH_4Cl challenge.

We first verified that treatment with 10^{-6} M thapsigargin elicited a similar cell shrinkage in rat parotid acini to that seen with 10^{-6} M carbachol. (As already noted above these two agents yield similar increases in $[Ca^{2+}]_i$.) The effect of thapsigargin is illustrated in Fig. 4*A* using light scattering as an index of cell volume (see Methods). The decrease in light scattering induced by thapsigargin was $8{\cdot}2 \pm 0{\cdot}3$ ($n = 7$) *vs.* $9{\cdot}8 \pm 0{\cdot}9\%$ ($n = 4$) for 10^{-6} M carbachol (not shown). Also in Fig. 4*A* we see that treatment with the K^+ channel blockers tetraethylammonium (10 mM) or quinine (0·1 mM) results in a partial inhibition of thapsigargin-induced shrinkage while treatment with a larger concentration of quinine (0·5 mM) or the Cl^- channel blocker niflumic acid (White & Aylwin, 1990; 30 μM) results in a complete blockade. (Note that because the losses of intracellular K^+ and Cl^- via their respective channels are electrically coupled, it is only necessary to block one of these pathways in order to block KCl loss and the resulting cell shrinkage.) The experiments shown in Fig. 4*B* demonstrate that none of the above treatments has a statistically significant effect on the enhanced pH_i recovery induced by thapsigargin. Since 0·5 mM quinine and 30 μM niflumic acid completely inhibit acinar shrinkage and thus acinar KCl loss, we conclude that these effects make little contribution to the upregulation of the cotransporter associated with increased $[Ca^{2+}]_i$. In particular, we note that neither the increased chemical driving force for Cl^- entry resulting from decreased $[Cl^-]_i$, nor possible proposed modulatory effects of $[Cl^-]_i$ on the cotransporter (Breitwieser, Altamirano & Russell, 1990; Robertson & Foskett, 1994; Haas & McBrayer, 1994; Moore, George & Turner, 1995; Lytle & Forbush, 1996), can account for the upregulation by Ca^{2+} mobilizing stimuli studied here.

Evidence against the involvement of several other Ca^{2+}-dependent phenomena in the upregulation of the cotransporter induced by muscarinic stimulation

In a further series of experiments (results not shown) we found that acinar treatment with the Ca^{2+}-dependent protein phosphatase 2B (calcineurin) inhibitors, deltamethrin (10^{-5} M, $n = 4$) and cypermethrin (10^{-5} M, $n = 4$) had no effect on the upregulation of the cotransporter induced by 10^{-6} M carbachol. In addition, neither the calmodulin antagonist calmidozolium (10^{-6} M, $n = 4$) nor the Ca^{2+}–calmodulin-dependent protein kinase inhibitor Compound 5 (10^{-6} M, $n = 4$) significantly affected the increased cotransporter activity induced by 10^{-6} M thapsigargin. Thus none of these Ca^{2+}-dependent mechanisms appear to be involved in the upregulation of cotransporter activity studied here.

Inhibitors of arachidonic acid metabolism block Ca^{2+}-dependent upregulation of the cotransporter

It has recently been established that increased $[Ca^{2+}]_i$ leads to the translocation of the cytosolic form of phospholipase A_2 to the cell membrane (Clark *et al.* 1991) where it catalyses

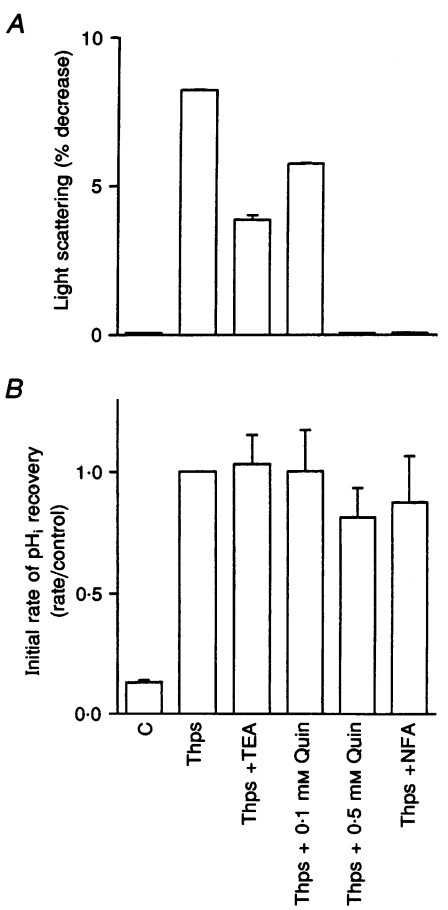

Figure 4. Upregulation of the Na^+–K^+–$2Cl^-$ cotransporter is independent of salt loss and cell shrinkage

Acini were stimulated with thapsigargin (Thps; 10^{-6} M) alone, or with thapsigargin in the presence of the K^+ and Cl^- channel blockers tetraethylammonium (TEA; 10 mM), quinine (Quin; 0·1 and 0·5 mM) or niflumic acid (NFA; 30 μM), all added 30 s before thapsigargin. Measurements of the initial rate of pH_i recovery (*B*) were carried out 120 s after the application of thapsigargin as described in Fig. 1 except that results were normalized to the initial rate of pH_i recovery observed in the presence of thapsigargin alone. None of the pH_i recovery rates observed in the presence of thapsigargin plus the various channel blockers was significantly different from that found with thapsigargin alone. Light scattering (*A*), an index of cell volume, was measured as described in Methods and expressed as the percentage decrease of the signal observed before the application of thapsigargin. All results are the means of 3 or more independent experiments carried out under the same conditions.

the release of arachidonic acid from membrane lipids and initiates the now familiar arachidonic acid cascade (Bonventre, 1992; Barrett & Bigby, 1995). Due to this direct link between $[Ca^{2+}]_i$ and arachidonic acid metabolism we explored the possible involvement of phospholipase A_2 and eicosanoids in the upregulation of the acinar Na^+–K^+–$2Cl^-$ cotransporter observed here. These experiments were carried out in the absence of extracellular Ca^{2+} to avoid any complications which might have arisen from putative effects of arachidonic acid metabolites on Ca^{2+} entry (Alvarez, Montero & Garcia-Sancho, 1992). We first examined the effects of 4-BPB (20 μM) and manoalide (10 μM), inhibitors of phospholipase A_2 itself. Acinar treatment with either of these compounds resulted in a significant inhibition of the upregulatory effect of carbachol (Fig. 5). In control experiments (not shown; $n > 3$ for all) we have established that neither these, nor any of the other compounds tested in Fig. 5, have any significant inhibitory effect on the pH_i recovery of unstimulated acini (resting Na^+–K^+–$2Cl^-$ cotransporter activity) or on the magnitude of the carbachol-induced Ca^{2+} response.

Intracellular arachidonic acid is broken down by three separate enzyme pathways to produce a plethora of metabolites that are known or presumed to be involved in regulating a variety of cellular responses (Bonventre, 1992; Barrett & Bigby, 1995). They are the cyclo-oxygenase pathway (which produces prostaglandins and thromboxanes), the lipoxygenase pathway (which produces leukotrienes) and the cytochrome P450 mono-oxygenase pathway (which produces a variety of epoxides). We next tested the effects of ETYA (40 μM), an arachidonic acid analogue which inhibits all three of these pathways of arachidonic acid metabolism. As indicated in Fig. 5, this compound also markedly blocked the effect of carbachol. Finally, we examined the effects of inhibitors of the individual pathways of arachidonate metabolism. NDGA, which inhibits the lipoxygenase pathway and indomethacin, which inhibits the cyclo-oxygenase pathway were without effect at 100 μM. But two inhibitors of the cytochrome P450 mono-oxygenase pathway, ketoconazole (100 μM) and methoxsalen (100 μM; a suicide inhibitor structurally and mechanistically different from ketoconazole), both resulted in a significant blockade of the effect of carbachol on Na^+–K^+–$2Cl^-$ cotransporter activity.

DISCUSSION

In the present study we provide strong evidence that the dramatic upregulation of rat parotid Na^+–K^+–$2Cl^-$ cotransporter activity induced by fluid secretory stimuli (Figs 1 and 2) is mediated by a product (or products) of the cytochrome P450 pathway of arachidonic acid metabolism. Briefly, our results indicate that the rise in $[Ca^{2+}]_i$ which accompanies muscarinic and other fluid secretory stimuli is both a necessary and a sufficient condition for enhancement of cotransport activity (Fig. 3). This upregulation is not a result of the KCl loss and concomitant isotonic shrinkage caused by Ca^{2+} mobilizing agents since it persists when these effects are prevented (Fig. 4). The effect of muscarinic stimulation on the cotransporter can, however, be blocked by inhibitors of phospholipase A_2 (4-BPB and manoalide), by a general inhibitor of arachidonic acid metabolism (ETYA) and by specific inhibitors of the cytochrome P450 pathway (methoxsalen and ketoconazole; Fig. 5). These latter results argue strongly for the involvement of an arachidonic acid metabolite, and specifically a product of the P450 pathway, in upregulation of the salivary Na^+–K^+–$2Cl^-$ cotransporter.

The principal role of increased $[Ca^{2+}]_i$ in this effect is presumably the Ca^{2+}-dependent translocation of the cytosolic form of phospholipase A_2 to the cell membrane (Clark *et al.* 1991) where it releases arachidonic acid from membrane lipids. Although other pathways may contribute to the intracellular pool of free arachidonate, the activity of phospholipase A_2 is thought to be the rate-limiting step for eicosanoid production in most cells (Bonventre, 1992;

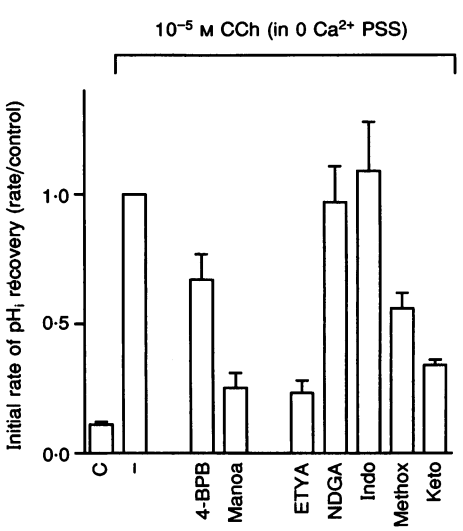

Figure 5. Effects of inhibitors of phospholipase A_2 and the arachidonic acid cascade on the upregulation of the salivary Na^+–K^+–$2Cl^-$ cotransporter induced by muscarinic stimulation

The experimental procedures were as described in Fig. 1 ($n > 3$ for all treatments) except that measurements were carried out in Ca^{2+}-free PSS. C, control; –, 10^{-5} M carbachol without inhibitors. The inhibitors used were 4-bromophenacyl bromide (4-BPB; 20 μM), manoalide (Manoa; 10 μM), 5,8,11,14-eicosatetraynoic acid (ETYA; 40 μM), nordihydroguaiaretic acid (NDGA; 100 μM), indomethacin (Indo; 100 μM), methoxsalen (Methox; 100 μM) and ketoconazole (Keto; 100 μM). All inhibitors were added 140 s before the NH_4Cl challenge except for manoalide and methoxsalen which were added 10 min before NH_4Cl.

Barrett & Bigby, 1995). In addition to this, however, increased $[Ca^{2+}]_i$ is also known to stimulate the mitochondrial generation of NAD(P)H (McCormack, Halestrap & Denton, 1990) which is required, along with molecular (atmospheric) oxygen, for the production of epoxides via the cytochrome P450 pathway (Fitzpatrick & Murphy, 1989). Hence $[Ca^{2+}]_i$ may actually play a dual role in the upregulation of the Na^+–K^+–$2Cl^-$ cotransporter studied here.

Although the importance of the cyclo-oxygenase and lipoxygenase products of arachidonic acid metabolism has been recognized for some time, it is only recently that evidence for biological effects of products of the cytochrome P450 pathway has accumulated (Fitzpatrick & Murphy, 1989; Bonventre, 1992; Barrett & Bigby, 1995). The products of this pathway are typically numerous, lipophilic and short-lived. Accordingly, in these cases it has often been difficult to identify the physiologically relevant metabolites and to determine their mode of action. In several instances P450 metabolites have been implicated in the regulation of ion transport systems such as ion channels (Alvarez *et al.* 1992; Kersting, Kersting & Spring, 1993), Na^+–K^+-ATPase (Schwartzman, Balazy, Masferrer, Abraham, McGiff & Murphy, 1987) and the Na^+–K^+–$2Cl^-$ cotransporter itself (Escalante, Erlij, Falck & McGiff, 1991, 1994). In these latter studies, Escalante *et al.* found that 20-HETE and its metabolite 20-COOH-AA, the principal products of the cytochrome P450 pathway in the rabbit thick ascending limb of the loop of Henle, *inhibited* the Na^+–K^+–$2Cl^-$ cotransporter in this tissue. In our hands neither 20-HETE (at 1 μM, the same concentration employed by Escalante *et al.* 1991, 1994) nor two other commercially available cytochrome P450 products, (±)5,6-EET and (±)11,12-EET (also tested at 1 μM), had any effect on resting Na^+–K^+–$2Cl^-$ cotransporter activity in rat parotid acini (assays were carried out using the NH_4^+-challenge method described here after 10 min of incubation with the compound in PSS at 37 °C; data not shown). This difference between our results and those reported by Escalante *et al.* (1991, 1994) may be due to the fact that different isoforms of the Na^+–K^+–$2Cl^-$ cotransporter are expressed in absorptive tissues such as the thick ascending limb and secretory tissues such as salivary glands (Kaplan, Mount & Delpire, 1996).

The cytochrome P450 product (or products) involved in the upregulation of the rat parotid Na^+–K^+–$2Cl^-$ cotransporter and the mechanism of action of this compound on the cotransporter protein remain to be determined. A recent report from our laboratory documented a small carbachol-induced increase in phosphorylation of a 175 kDa protein identified as the Na^+–K^+–$2Cl^-$ cotransporter in our rat parotid acinar preparation (Tanimura *et al.* 1995). However, this increase was not seen after treatment of acini with 10^{-6} M thapsigargin which, as shown here, induces a similar effect to that of carbachol on transport activity. Thus, these results argue against a role for phosphorylation in the muscarinic agonist-induced upregulation of the cotransporter.

In a recent publication Robertson & Foskett (1994) monitored Na^+ entry into rat parotid acinar cells using an intracellular fluorescent Na^+ indicator. They found that net Na^+ influx was negligible in resting cells but was rapidly increased by carbachol or by a thapsigargin-induced rise in $[Ca^{2+}]_i$. A portion (~30%) of this increased influx was bumetanide sensitive and, therefore, presumably due to the Na^+–K^+–$2Cl^-$ cotransporter (most of the remaining influx was via the Na^+–H^+ exchanger). However, these authors also found that when cells were stimulated in a high K^+ medium, which prevented KCl loss and cell shrinkage, this increased Na^+ influx was not observed. To explain this result they hypothesized that a fall in $[Cl^-]_i$ in addition to the carbachol-induced rise in $[Ca^{2+}]_i$ was required to activate Na^+ entry pathways. Because these authors measured *net* Na^+ influx rather than *unidirectional* Na^+–K^+–$2Cl^-$ cotransporter activity as assayed here, their results do not relate directly to ours. Nevertheless, it is clear from our data (Fig. 4) that a fall in $[Cl^-]_i$ is *not* required for the Ca^{2+}-dependent increase in cotransporter activity documented here. Thus our results suggest that something other than a modulating effect of $[Cl^-]_i$ must be responsible for the apparent decrease in net Na^+ influx via the Na^+–K^+–$2Cl^-$ cotransporter observed by these authors in high K^+ medium.

As already stressed, the Na^+–K^+–$2Cl^-$ cotransporter plays a major (and possibly dominant) role in driving the trans-epithelial Cl^- flux responsible for salivary fluid secretion. Thus the dramatic secretagogue-induced upregulation of cotransport activity documented here is clearly of central importance to salivary gland function. However, because of the complexity of the arachidonic acid cascade and the intracellular events associated with its activation, it is possible that a wide variety of chemical agents and/or cellular phenomena could potentially interfere with this upregulatory effect and thereby result in decreased saliva production. There are, in fact, a large number of commonly prescribed medications having dry mouth (xerostomia) as an unexplained side-effect (Sreebny & Schwartz, 1986). We suggest that interference with Na^+–K^+–$2Cl^-$ cotransporter regulation may account for some of these problems. In this regard we should mention that dry mouth has not been reported as a significant side effect of orally administered ketoconazole; however, patients taking this potent anti-fungal agent are typically seriously medically compromised (Como & Dismukes, 1994) and may not be aware of, or consider relevant, problems of this type.

ALVAREZ, J., MONTERO, M. & GARCIA-SANCHO, J. (1992). Cytochrome P450 may regulate plasma membrane Ca^{2+} permeability according to the filling state of the intracellular Ca^{2+} stores. *FASEB Journal* **6**, 786–792.

BARRETT, K. E. & BIGBY, T. D. (1995). New roles for eicosanoids as regulators of epithelial function and growth. *News in Physiological Sciences* **10**, 153–160.

BONVENTRE, J. V. (1992). Phospholipase A_2 and signal transduction. *Journal of the American Society of Nephrology* **3**, 128–150.

BREITWIESER, G. E., ALTAMIRANO, A. A. & RUSSELL, J. M. (1990). Osmotic stimulation of Na^+–K^+–Cl^- cotransport in squid giant axon is $[Cl^-]_i$ dependent. *American Journal of Physiology* **258**, C749–753.

CLARK, J. D., LIN, L. L., KRIZ, R. W., RAMESHA, C. S., SULTZMAN, L. A., LIN, A. Y., MILONA, N. & KNOPF, J. L. (1991). A novel arachidonic selective cytosolic PLA_2 contains a Ca^{2+}-dependent translocation domain with homology to PKC and GAP. *Cell* **65**, 1043–1051.

COMO, J. A. & DISMUKES, W. E. (1994). Oral azole drugs as systemic antifungal therapy. *New England Journal of Medicine* **330**, 263–272.

COOK, D. I. & YOUNG, J. A. (1989). Fluid and electrolyte secretion by salivary glands. In *Handbook of Physiology*, section 6, *The Gastrointestinal System*, vol. III, chap. 1, ed. FORTE, J. G., pp. 1–23. Oxford University Press, New York.

ESCALANTE, B., ERLIJ, D., FALCK, J. R. & MCGIFF, J. C. (1991). Effect of cytochrome P450 arachidonate metabolites on ion transport in rabbit kidney loop of Henlé. *Science* **251**, 799–802.

ESCALANTE, B., ERLIJ, D., FALCK, J. R. & MCGIFF, J. C. (1994). Cytochrome P450 arachidonate metabolites affect ion fluxes in rabbit medullary thick ascending limb. *American Journal of Physiology* **266**, C1775–1782.

EVANS, R. L. & TURNER, R. J. (1995). Up-regulation of the Na^+–K^+–$2Cl^+$ co-transporter by muscarinic stimulation in parotid acinar cells isolated from the rat. *Journal of Physiology* **482.P**, 7–8*P*.

FERRI, C., EVANS, R. L., PAULAIS, M., TANIMURA, A. & TURNER, R. J. (1996). Evidence that upregulation of the parotid Na^+–K^+–$2Cl^-$ cotransporter by hypertonic shrinkage is not duplicated by isotonic shrinkage. *Molecular and Cellular Biochemistry* (in the Press).

FITZPATRICK, F. A. & MURPHY, R. C. (1989). Cytochrome P450 metabolism of arachidonic acid: formation and biological actions of 'epoxygenas'-derived eicosanoids. *Pharmacological Reviews* **40**, 229–241.

FOSKETT, J. K. & MELVIN, J. E. (1989). Activation of salivary secretion: coupling of cell volume and $[Ca^{2+}]_i$ in single cells. *Science* **244**, 1582–1585.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HAAS, M. (1994). The Na^+–K^+–Cl^- cotransporters. *American Journal of Physiology* **267**, C869–885.

HAAS, M. & MCBRAYER, D. G. (1994). Na–K–Cl cotransport in nystatin-treated tracheal cells: regulation by isoproterenol, apical UTP, and $[Cl]_i$. *American Journal of Physiology* **266**, C1440–1452.

KAPLAN, M. R., MOUNT, D. B. & DELPIRE, E. (1996). Molecular mechanisms of NaCl cotransport. *Annual Review of Physiology* **58**, 649–668.

KERSTING, U., KERSTING, D. & SPRING, K. R. (1993). Ketoconazole activates Cl^- conductance and blocks Cl^- and fluid absorption by cultured cystic fibrosis (CFPAC-1) cells. *Proceedings of the National Academy of Sciences of the USA* **90**, 4047–4051.

LYTLE, C. & FORBUSH, B. (1996). Regulatory phosphorylation of the secretory Na–K–Cl cotransporter: modulation by cytoplasmic Cl. *American Journal of Physiology* **270**, C437–448.

MCCORMACK, J. G., HALESTRAP, A. P. & DENTON, R. M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiological Reviews* **70**, 391–425.

MANGANEL, M. & TURNER, R. J. (1991). Rapid secretagogue-induced activation of Na/H exchange in rat parotid acinar cells: possible interrelationship between volume regulation and stimulus secretion coupling. *Journal of Biological Chemistry* **266**, 10182–10188.

MELVIN, J. E. & TURNER, R. J. (1992). Cl^- fluxes related to fluid secretion by the rat parotid: involvement of Cl^-/HCO_3^- exchange. *American Journal of Physiology* **262**, G393–398.

MOORE, M. L., GEORGE, J. N. & TURNER, R. J. (1995). Anion dependence of bumetanide binding and ion transport by the rabbit parotid Na^+–K^+–$2Cl^-$ co-transport: evidence for an intracellular anion modifier site. *Biochemical Journal* **309**, 637–642.

O'GRADY, S. M., PALFREY, H. C. & FIELD, M. (1987). Characteristics and functions of Na–K–Cl cotransport in epithelial tissues. *American Journal of Physiology* **253**, C177–192.

PAULAIS, M. & TURNER, R. J. (1992*a*). β-Adrenergic upregulation of the Na^+–K^+–$2Cl^-$ cotransporter in rat parotid acinar cells. *Journal of Clinical Investigation* **89**, 1142–1147.

PAULAIS, M. & TURNER, R. J. (1992*b*). Activation of the Na^+–K^+–$2Cl^-$ cotransporter in rat parotid acinar cells by aluminum fluoride and phosphatase inhibitors. *Journal of Biological Chemistry* **267**, 21558–21563.

ROBERTSON, M. A. & FOSKETT, J. K. (1994). Na^+ transport pathways in secretory acinar cells: membrane cross talk mediated by $[Cl^-]_i$. *American Journal of Physiology* **267**, C146–156.

SCHWARTZMAN, M., BALAZY, M., MASFERRER, J., ABRAHAM, N., MCGIFF, J. & MURPHY, R. C. (1987). 12(*R*)-Icosatetraenoic acid: a cytochrome P450 dependent arachidonate metabolite that inhibits Na^+/K^+-ATPase in the cornea. *Proceedings of the National Academy of Sciences of the USA* **84**, 8125–8129.

SNOWDOWNE, K. W., WAY, B., THOMAS, G., CHEN, H. Y. & CASHMAN, J. R. (1992). pH_i controls cytoplasmic calcium in rat parotid cells. *Biochimica et Biophysica Acta* **1108**, 145–152.

SREEBNY, L. M. & SCHWARTZ, S. S. (1986). A reference guide to drugs and dry mouth. *Gerodontology* **5**, 75–99.

TANIMURA, A., KURIHARA, K., RESHKIN, S. J. & TURNER, R. J. (1995). Involvement of direct phosphorylation in the regulation of the rat parotid Na^+–K^+–$2Cl^-$ cotransporter. *Journal of Biological Chemistry* **270**, 25252–25258.

TURNER, R. J. (1993). Ion transport related to fluid secretion in salivary glands. In *Biology of the Salivary Glands*, ed. DOBROSIELSKI-VERGONA, K., pp. 105–127. CRC Press, Boca Raton, FL, USA.

WHITE, M. M. & AYLWIN, M. (1990). Niflumic and flufenamic acids are potent reversible blockers of Ca^{2+}-activated Cl^- channels in *Xenopus* oocytes. *Molecular Pharmacology* **37**, 720–724.

Acknowledgements

We thank Drs Jorge Arreola and James E. Melvin for suggesting the use of niflumic acid as a Cl^- channel blocker, and Drs Bruce J. Baum and Philip C. Fox for helpful discussions and encouragement during the course of this work.

Author's email address

R. J. Turner: rjturner@nih.gov

Received 23 September 1996; accepted 7 November 1996.