

## Beta-adrenergic upregulation of the Na(+)-K(+)-2Cl- cotransporter in rat parotid acinar cells.

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**Research Article**

We used the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6')-carboxyfluorescein to monitor the recovery of the intracellular pH (pHi) of rat parotid acini from an NH<sub>4</sub><sup>+</sup>-induced alkaline load. This recovery was markedly inhibited by the loop diuretic bumetanide and by Cl<sup>-</sup> removal, indicating that it is largely due to NH<sub>4</sub><sup>+</sup> entry via the basolateral Na(+)-K(+)-2Cl<sup>-</sup> cotransporter. The rate of recovery of pHi was enhanced threefold by pretreatment (37.5 s) with isoproterenol (K<sub>1/2</sub> = 21.5 nM) or norepinephrine (in the presence of phentolamine), and blocked by the beta 1-specific antagonist atenolol, indicating an upregulation of cotransport activity by beta 1-adrenergic stimulation. The effect of isoproterenol was prevented by protein kinase inhibitors and mimicked by cAMP analogues, and by maneuvers known to increase cytosolic cAMP levels in these cells, consistent with the involvement of protein kinase A. Physiologically, such an upregulation of the acinar Na(+)-K(+)-2Cl<sup>-</sup> cotransporter would lead to an increase in acinar chloride uptake across the basolateral membrane, and consequently, an increase in overall chloride and fluid secretion. Prevention of this upregulation by beta-blockers and possibly by other commonly used clinical agents may account for the dry mouth and dry eyes experienced by some patients taking these medications.

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# $\beta$ -Adrenergic Upregulation of the $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ Cotransporter in Rat Parotid Acinar Cells

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## Abstract

We used the pH-sensitive fluorescent dye 2',7'-bis(2-carboxyethyl)-5(6')-carboxyfluorescein to monitor the recovery of the intracellular pH ( $\text{pH}_i$ ) of rat parotid acini from an  $\text{NH}_4^+$ -induced alkaline load. This recovery was markedly inhibited by the loop diuretic bumetanide and by  $\text{Cl}^-$  removal, indicating that it is largely due to  $\text{NH}_4^+$  entry via the basolateral  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter. The rate of recovery of  $\text{pH}_i$  was enhanced threefold by pretreatment (37.5 s) with isoproterenol ( $K_{1/2} = 21.5$  nM) or norepinephrine (in the presence of phentolamine), and blocked by the  $\beta_1$ -specific antagonist atenolol, indicating an upregulation of cotransport activity by  $\beta_1$ -adrenergic stimulation. The effect of isoproterenol was prevented by protein kinase inhibitors and mimicked by cAMP analogues, and by maneuvers known to increase cytosolic cAMP levels in these cells, consistent with the involvement of protein kinase A. Physiologically, such an upregulation of the acinar  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter would lead to an increase in acinar chloride uptake across the basolateral membrane, and consequently, an increase in overall chloride and fluid secretion. Prevention of this upregulation by  $\beta$ -blockers and possibly by other commonly used clinical agents may account for the dry mouth and dry eyes experienced by some patients taking these medications. (*J. Clin. Invest.* 1992, 89:1142–1147.) Key words: exocrine gland • fluid secretion • loop diuretic • chloride secretion • xerostomia

## Introduction

$\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporters are found in a wide variety of cell types (1, 2). This transport system is thought to play a central role in salt and water movements in many absorptive and secretory epithelia. It is also involved in volume regulatory phenomena in both epithelial and nonepithelial cells. As is characteristic of other transporters that are extensively utilized in nature,  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransport activity is regulated in a very tissue-specific manner (1, 2). Thus, in some cell types, increases in cyclic nucleotide levels, intracellular calcium concentration, or protein kinase C activity stimulate the cotransporter, whereas in others, these same agents may have exactly the opposite effect, or possibly no effect at all. At present, the significance of these tissue specific differences remains largely un-

explored; however, it is clear that these observations raise interesting questions related to the mechanisms involved in the regulation of this important transporter at both the whole cell and individual protein level.

Studies from a number of laboratories, including our own, indicate that a  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter is responsible for driving the bulk of the acinar chloride secretion associated with fluid production in salivary glands (3–7). This process is thought to conform to the following scheme (8, 9). The  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter concentrates  $\text{Cl}^-$  above electrochemical equilibrium in the acinar cytoplasm, using the extracellular to intracellular sodium gradient generated by  $\text{Na}^+/\text{K}^+$  ATPase. Stimulation by secretagogues raises intracellular calcium levels, causing the opening of basolateral  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and (putative) apical  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels. This allows  $\text{K}^+$  and  $\text{Cl}^-$  to flow out of the cell down their respective electrochemical gradients,  $\text{Cl}^-$  into the lumen, and  $\text{K}^+$  into the interstitium.  $\text{Na}^+$  then follows  $\text{Cl}^-$  paracellularly to preserve electroneutrality, and water follows  $\text{NaCl}$  into the lumen osmotically.

In some tissues, the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter is quiescent in resting cells; however, this is not the case in salivary glands (10). Thus, the above model does not necessarily require upregulation of cotransporter activity by secretagogues (although such an upregulation would enhance transepithelial  $\text{Cl}^-$  movement, and thus fluid secretion). Nevertheless, several observations indicated to us that the cotransporter might be regulated by certain agonists. When  $^{36}\text{Cl}^-$ -loaded rat parotid or submandibular acini are stimulated with maximal doses of muscarinic ( $\text{Ca}^{2+}$  mobilizing) agonists they undergo an initial rapid ( $t_{1/2} < 10$  s) and dramatic (30–50%) loss of intracellular  $\text{Cl}^-$  (via the apical  $\text{Cl}^-$  channel), followed by a partial recovery to intracellular  $\text{Cl}^-$  levels significantly below resting values (4, 7, 11). In  $\text{HCO}_3^-$ -free medium, this recovery is essentially completely blocked by the loop diuretics bumetanide and furosemide, indicating that it is due to  $\text{Cl}^-$  entry via the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter (4, 7). This recovery coincides with the transition between the initial and the sustained phases of fluid secretion, and presumably reflects the adjustment of the cells to a new steady state characteristic of a continuous secretory response. When the same experiments were carried out with the combined  $\alpha$ -adrenergic ( $\text{Ca}^{2+}$  mobilizing) and  $\beta$ -adrenergic (cAMP generating) agonist epinephrine, a similar pattern of  $^{36}\text{Cl}^-$  loss and recovery was seen. However, in contrast to the sustained decrease in  $\text{Cl}^-$  content observed with muscarinic agonists, in this case, acini were observed to recover to near resting intracellular  $\text{Cl}^-$  levels (11, 12). Interestingly, this enhanced recovery of  $\text{Cl}^-$  content was blocked by the  $\beta$ -adrenergic antagonist propranolol (11). In additional experiments it was found that stimulation of both rat submandibular (13) and rat parotid (J. E. Melvin and R. J. Turner, unpublished observations) acini with the  $\beta$ -adrenergic agonist isoproterenol caused a gradual in-

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crease in  $^{36}\text{Cl}^-$  content that was prevented by furosemide (13). These results are suggestive of an upregulation of the acinar  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporter by  $\beta$ -adrenergic stimulation.

The purpose of the present study was to directly assess the effect of  $\beta$ -adrenergic stimulation on the  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporter in rat parotid acini. In order to do so, we employed a novel method for monitoring cotransporter activity. Briefly stated, we have taken advantage of the fact that  $\text{NH}_4^+$  can substitute for  $\text{K}^+$  on the cotransporter (14–16). This made it possible for us to follow cotransporter activity via the changes in intracellular pH associated with cotransporter-mediated dissipation of  $\text{NH}_4^+$  gradients. We demonstrate here that the acinar  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporter is indeed upregulated by  $\beta$ -adrenergic stimulation, apparently via a cyclic AMP/protein kinase A-dependent pathway. Prevention of this upregulation by  $\beta$ -blockers, and possibly by other commonly used clinical agents, may account for the dry mouth (xerostomia) and dry eyes experienced by some patients taking these medications (17, 18).

## Methods

**Materials.** Collagenase (type CLSPA) was obtained from Worthington Biomedical Corporation (Newark, NJ). (–)Isoproterenol, DL-propranolol, epinephrine, norepinephrine, atenolol, carbachol, phentolamine, ouabain, and IBMX<sup>1</sup> (3-isobutyl-1-methylxanthine) were from Sigma Chemical Co. (St. Louis, MO). 8-4 CPT cAMP (8-[4 chlorophenylthio]-adenosine 3',5'-cyclic monophosphate) and 8 Br cAMP (8-bromo adenosine 3',5'-cyclic monophosphate) were from Boehringer Mannheim Biochemicals (Indianapolis, IN) and bumetanide was a gift from Hoffmann-LaRoche Laboratories (Nutley, NJ). Forskolin, 1,9-dideoxyforskolin, K252a, H9, staurosporine, and BCECF/AM (2',7'-bis[2-carboxyethyl]-5[6']-carboxyfluorescein pentaacetoxymethyl ester) were from Calbiochem Corp. (San Diego, CA). BCECF/AM was stored at  $-20^\circ\text{C}$  as a 2 mM stock solution in dimethyl sulfoxide.

**Solutions.** Experiments were typically carried out in a physiological salt solution (PSS) containing 135 mM NaCl, 5.8 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , 0.73 mM  $\text{NaH}_2\text{PO}_4$ , 11 mM glucose, 20 mM HEPES, 2 mM glutamine, and 0.01% BSA. In  $\text{Cl}^-$ -free PSS, gluconate was used to replace chloride, and the ionic calcium activity of the medium was matched to that of PSS by adding additional calcium salts (19). In  $\text{Ca}^{2+}$ -free PSS, 0.1 mM EGTA was substituted for  $\text{CaCl}_2$ . All solutions were titrated to pH 7.4 with NaOH.

**Acinar preparation.** Dispersed parotid acini were prepared from the parotid glands of two male, 250–350 g Wistar strain rats (Harlan Sprague-Dawley Inc., Indianapolis, IN) by collagenase digestion, as previously described (20), except that hyaluronidase was omitted from the digestion medium. Final parotid acinar preparations were washed and resuspended (5 ml/animal) in PSS, then rested for 10 min at  $25^\circ\text{C}$  before loading with BCECF (see below). Throughout this and the subsequent experimental period, the acini were kept continuously agitating (85 cycles/min in a shaking water bath) and gassed with 100%  $\text{O}_2$ .

**Intracellular pH measurements.** Isolated acini were loaded with the pH-sensitive fluorescent dye BCECF by incubation with  $2\ \mu\text{M}$  of the membrane permeant species BCECF/AM for 30 min at  $25^\circ\text{C}$ . Intracellular BCECF fluorescence was monitored at  $37^\circ\text{C}$  in “ratio mode,” using a spectrofluorimeter (model 8000C; SLM Instrs. Inc., Urbana, IL) (20). The fluorescent signal was calibrated to yield intracellular pH ( $\text{pH}_i$ ) values, as previously described (20).

1. Abbreviations used in this paper: BCECF, (2',7'-bis[2-carboxyethyl]-5[6']-carboxyfluorescein; BCECF/AM, BCECF pentaacetoxymethyl ester; 8Br cAMP, 8-bromo cAMP; 8-4 CPT cAMP, (8-[4 chlorophenylthio]-cAMP); IBMX, (3-isobutyl-1-methyl-xanthine);  $\text{pH}_i$ -intracellular pH; PSS, physiological salt solution.

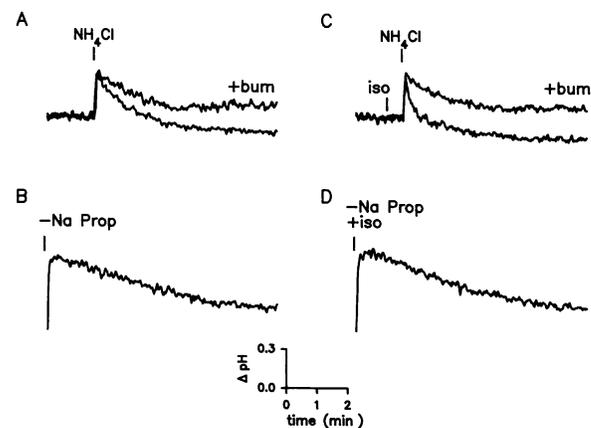
**Data analysis and presentation.** Fluorescence traces were converted to numerical ASCII files, using software supplied with the 8000C spectrofluorimeter (SLM Instrs.), then transferred into the program SigmaPlot 4.0 (Jandel Sci., Corte Madera, CA) for conversion to  $\text{pH}_i$  values and analysis (see Results).

Each experimental value given in the figures or text is the mean  $\pm$  SEM for three or more independent determinations carried out under identical conditions on different acinar preparations. *P* values  $< 0.05$  (Student's *t* test) were taken to represent statistically significant differences.

## Results

**Effect of bumetanide, isoproterenol, and other treatments on the recovery of rat parotid acinar cells from an  $\text{NH}_4^+$ -induced acute alkaline load.** The behavior of the  $\text{pH}_i$  of rat parotid acinar cells following the addition of 30 mM  $\text{NH}_4\text{Cl}$  to the extracellular medium is illustrated in the lower trace of Fig. 1 A. In this ‘control’ run (acini in PSS) the cells first rapidly alkalinize ( $t_{1/2} < 3$  s,  $\Delta\text{pH}_i = 0.32 \pm 0.01$ ;  $n = 47$ ) then recover to a final  $\text{pH}_i$  ( $7.36 \pm 0.01$ ;  $n = 47$ ) which is significantly below resting levels ( $7.56 \pm 0.01$ ;  $n = 47$ ). This initial intracellular alkalinization is a common result of  $\text{NH}_4^+$  addition to cells, and is caused by the rapid equilibration of the membrane permeant species  $\text{NH}_3$  and its intracellular combination with  $\text{H}^+$  to form  $\text{NH}_4^+$ . From this  $\Delta\text{pH}_i$  in response to a 30 mM  $\text{NH}_4\text{Cl}$  challenge we calculate that the intrinsic buffering power (21),  $\beta_i$ , of these cells is  $44.9 \pm 1.4$  mmol/liter per pH U.

Since most cells recover from an acute alkaline load via  $\text{HCO}_3^-$ -dependent mechanisms, and since our experiments were carried out in  $\text{HCO}_3^-$ -free medium (see Methods), one might expect a large part of the  $\text{pH}_i$  recovery observed after  $\text{NH}_4^+$ -induced alkalinization in Fig. 1 A to be due to dissipation



**Figure 1.** The effects of bumetanide, isoproterenol and  $\text{NH}_4^+$  on the response of rat parotid acini to an acute alkaline load. The results shown in A and C are representative BCECF traces from rat parotid acini suspended in PSS and challenged with 30 mM  $\text{NH}_4\text{Cl}$ , at the times indicated (dilution 1%). In these experiments, acini were studied in the presence (upper trace) and absence (lower trace) of 0.1 mM bumetanide (*bum*; added 75 s before  $\text{NH}_4^+$ ). In C,  $3 \times 10^{-8}$  M isoproterenol (*iso*) was added 37.5 s before  $\text{NH}_4\text{Cl}$ . The results shown in B and D are representative BCECF traces from acini which were preequilibrated (10 min) in PSS containing 30 mM sodium propionate, then centrifuged and diluted at time zero into propionate-free PSS containing no additions (B) or  $3 \times 10^{-8}$  M isoproterenol (D). An acute alkaline load is generated in these cells by the rapid diffusion of the permeant species propionic acid out of the cytoplasm.

of the extracellular to intracellular  $\text{NH}_4^+$  gradient. In other words, the rate of  $\text{pH}_i$  recovery should be, at least in part, a measure of acinar membrane  $\text{NH}_4^+$  permeability.

It has been shown that  $\text{NH}_4^+$  can substitute for  $\text{K}^+$  on the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter in several tissues (14–16), including salivary glands (R. J. Turner and J. N. George, unpublished observations). We demonstrate in the upper trace of Fig. 1 *A* (+bum) that the  $\text{pH}_i$  recovery of rat parotid acinar cells is markedly slowed by 0.1 mM bumetanide, a well known inhibitor of this transporter (quantitation of these results is discussed below). In additional experiments (not shown) we have verified that SITS (1 mM), an inhibitor of  $\text{Cl}^-/\text{OH}^-$  exchange, has no significant effect on this recovery. Since the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter is thought to be highly active in salivary tissue, these results suggest that a significant component of  $\text{pH}_i$  recovery following  $\text{NH}_4^+$ -induced alkalization of these cells occurs via  $\text{NH}_4^+$  entry on this transport system. Further evidence for this hypothesis is provided in Fig. 1 *B*. Here we have alkalized the acinar cells by propionate removal, rather than  $\text{NH}_4^+$  addition (see Fig. 1, *legend*). The magnitude of the resulting initial alkalization using this procedure ( $\Delta\text{pH}_i = 0.37 \pm 0.03$  above resting  $\text{pH}_i$ ;  $n = 3$ ) is similar to that seen in Fig. 1 *A*, but the rate of  $\text{pH}_i$  recovery more closely resembles that seen in bumetanide-treated acini. This result suggests that the bumetanide-inhibitable component of  $\text{pH}_i$  recovery seen in Fig. 1 *A* is also  $\text{NH}_4^+$ -dependent, consistent with the involvement of a bumetanide-sensitive  $\text{NH}_4^+$  transport pathway.

The effect of preincubating (37.5 s) the acinar suspension with the  $\beta$ -adrenergic agonist isoproterenol ( $3 \times 10^{-8}$  M) before  $\text{NH}_4^+$  addition is illustrated in Fig. 1 *C*. Isoproterenol treatment dramatically accelerates the rate of  $\text{pH}_i$  recovery with little effect on the magnitude of the initial alkalization ( $\Delta\text{pH}_i = 0.29 \pm 0.01$ ;  $n = 15$ ) or the value of the final  $\text{pH}_i$  ( $7.34 \pm 0.01$ ;  $n = 15$ ). The acceleration of the rate of  $\text{pH}_i$  recovery produced by isoproterenol is blocked by propranolol ( $10^{-5}$  M; not shown) and dramatically inhibited by bumetanide (Fig. 1 *C*). No effect of isoproterenol on  $\text{pH}_i$  recovery is seen in cells alkalized by propionate removal (Fig. 1 *D*).

The time courses of  $\text{pH}_i$  recovery illustrated in Fig. 1 conform well to a single exponential decay (not shown). Accordingly, we have used least-squares curve-fitting techniques to determine initial  $\text{pH}_i$  recovery rates, and thus quantitate our results. More specifically, we have fit our individual recovery traces to the equation

$$\text{pH}_t = (\text{pH}_{\text{initial}} - \text{pH}_{\text{final}})e^{-kt} + \text{pH}_{\text{final}} \quad (1)$$

where  $\text{pH}_t$  is the value of  $\text{pH}_i$  at time  $t$ ,  $\text{pH}_{\text{initial}}$  is the initial (alkaline)  $\text{pH}_i$  induced by the addition of  $\text{NH}_4^+$  (at  $t = 0$ ),  $\text{pH}_{\text{final}}$  is the final resting  $\text{pH}_i$  after  $\text{NH}_4^+$  addition, and  $k$  is the recovery rate constant. In the least squares analyses  $\text{pH}_{\text{initial}}$ ,  $\text{pH}_{\text{final}}$  and  $k$  were all treated as free parameters. The initial  $\text{pH}_i$  recovery rate is the slope of the  $\text{pH}_i$  vs. time curve ( $d\text{pH}_i/dt$ ) at  $t = 0$ , which from equation (1) is given by  $k(\text{pH}_{\text{initial}} - \text{pH}_{\text{final}})$ . Under control conditions (30 mM  $\text{NH}_4\text{Cl}$  added to untreated acini in PSS) we find this initial recovery rate to be  $0.31 \pm 0.07$  pH U/min ( $n = 47$ ). This value can be converted to an initial  $\text{NH}_4^+$  flux rate of  $13.9 \pm 3.1$  mmol/min, using the value of  $\beta_i$  derived above.

Fig. 2 shows the combined results of a number of experiments performed as outlined in Fig. 1 and analyzed as just described. In each experiment, the initial rates of  $\text{pH}_i$  recovery

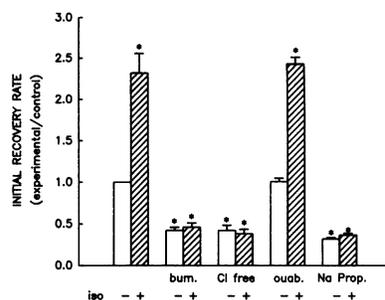


Figure 2. Effects of various treatments on the isoproterenol-induced enhancement of  $\text{pH}_i$  recovery from an acute alkaline load. The experimental procedures were as described for Fig. 1. Initial rates of  $\text{pH}_i$  recovery were calculated as described in the text. Rates have

been normalized to the initial rate obtained from a control run (30 mM  $\text{NH}_4\text{Cl}$  addition to untreated acini in PSS) carried out on the same preparation. Averaged normalized initial recovery rates from three or more independent experiments are shown in the presence (hatched bars) and absence (open bars) of isoproterenol ( $3 \times 10^{-8}$  M), in the presence of bumetanide (bum, 0.1 mM), in  $\text{Cl}^-$ -free PSS (Cl free; all  $\text{Cl}^-$  replaced with gluconate and challenged with 15 mM  $[\text{NH}_4]_2\text{SO}_4$ ), in the presence of 1 mM ouabain (ouab.) and in the absence of  $\text{NH}_4^+$  (Na prop.; alkalization by propionate removal as described in Fig. 1). In these experiments, ouabain was added 75 s before  $\text{NH}_4\text{Cl}$  (cf. Fig. 1) to minimize any dissipation of transmembrane ion gradients. \*Initial recovery rates which are significantly different from control.

were normalized to the initial rate obtained from a control run (30 mM  $\text{NH}_4\text{Cl}$  addition to untreated acini in PSS) carried out on the same preparation. These results show clearly that the  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load is strongly retarded by bumetanide, and that the enhancement of this recovery produced by isoproterenol is completely bumetanide inhibitable. These effects of bumetanide are duplicated by the removal of medium chloride (Fig. 2), a maneuver which would also result in suppression of  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransport activity (6). Furthermore, when the acinar cells are alkalized by propionate removal rather than  $\text{NH}_4^+$  addition, as in Fig. 1, *B* and *D*,  $\text{pH}_i$  recovery is significantly slower than that observed in the presence of  $\text{NH}_4^+$ , and no effect of isoproterenol is seen (Fig. 2). Taken together, the above results provide strong evidence that the bumetanide inhibitable,  $\text{NH}_4^+$ -dependent component of  $\text{pH}_i$  recovery illustrated in Fig. 1 is due to  $\text{NH}_4^+$  entry via the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter, and that the stimulation of this recovery induced by isoproterenol is due to an upregulation of cotransporter activity. Fig. 2 also shows that 1 mM ouabain has no effect on the stimulation of  $\text{pH}_i$  recovery induced by isoproterenol, indicating that this phenomenon is not due to upregulation of  $\text{Na}^+/\text{K}^+$  ATPase and a concomitant change in driving forces for the cotransporter, or to  $\text{NH}_4^+$  entry on the  $\text{Na}^+/\text{K}^+$  ATPase itself.

The effect of isoproterenol concentration on the initial rate of  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load is examined in Fig. 3. A half-maximal effect is observed at  $2.15 \times 10^{-8}$  M (see Fig. 3 legend). This value is close to, but somewhat lower than, typical  $K_{1/2}$  values for isoproterenol-induced protein (amylase) secretion in this tissue ( $\approx 10^{-7}$  M) (22–24).

*Effects of intracellular cyclic AMP levels and protein kinase inhibitors.* In parotid acinar cells, cAMP is thought to be the major intracellular messenger mediating the events associated with  $\beta$ -adrenoreceptor activation (25). In Fig. 4 *A* we illustrate that the permeant cAMP analogues 8 Br cAMP and 8-4 CPT cAMP mimic the effect of isoproterenol on  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load. Simulation of recovery is also

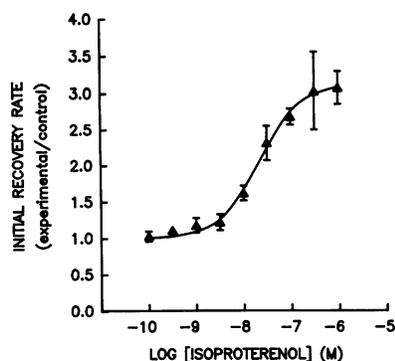


Figure 3. Effect of isoproterenol concentration on the initial rate of  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load. The experimental procedure was as described in Fig. 1 C, using the concentrations of isoproterenol indicated ( $n \geq 3$  for all conditions). The line drawn through the points is a nonlinear least squares fit to the

data given by  $K_{1/2} = 21.5 \pm 1.0$  nM, and a maximal initial  $\text{pH}_i$  recovery rate of  $3.13 \pm 0.02$  times the control rate.

seen with forskolin ( $10^{-5}$  M), which increases intracellular cAMP levels by direct activation of the catalytic subunit of adenylate cyclase (24). The inactive forskolin analogue 1,9-dideoxyforskolin was without effect. The phosphodiesterase inhibitor IBMX ( $10^{-4}$  M) also mimics the effect of isoproterenol, and has a marked synergistic effect when applied in combination with a submaximal dose of isoproterenol ( $3 \times 10^{-9}$  M).

In Fig. 4 B we demonstrate that the stimulation of  $\text{pH}_i$  recovery induced by isoproterenol is blocked by the protein kinase inhibitors H9, staurosporin, and K252a. No significant effect of these compounds was observed on the recovery of untreated acini (data not shown). These results together with those shown in Fig. 4 A provide strong evidence that the upregulation of the  $\text{Na}^+ \text{-K}^+ \text{-2Cl}^-$  cotransporter studied here is due to a phosphorylation event mediated by protein kinase A.

**Effects of calcium.** Several reports have suggested that changes in intracellular calcium concentrations may play some role in the response of rat parotid acinar cells to  $\beta$ -adrenergic agonists (26–28). Accordingly, we carried out experiments to explore the role of calcium, if any, in the effects studied here. In these experiments (Table I) we found that the isoproterenol-de-

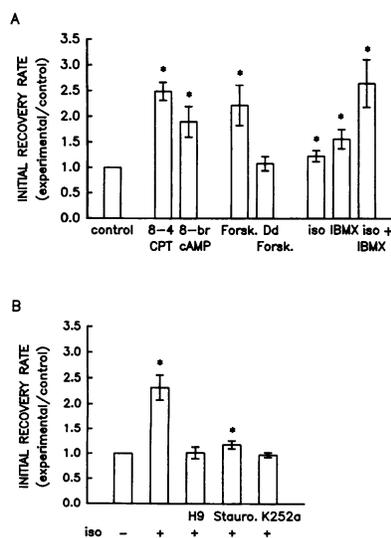


Figure 4. Effects of intracellular cAMP levels (A) and protein kinase inhibitors (B) on the initial rate of  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load. The experimental procedure was as described for Fig. 1 C ( $n \geq 3$  for all conditions). 8–4 CPT cAMP ( $8\text{-}4$  CPT;  $2.5 \times 10^{-3}$  M), 8-Br cAMP ( $2.5 \times 10^{-3}$  M), forskolin (Forsk.;  $10^{-5}$  M), dideoxyforskolin (Dd Forsk.;  $10^{-5}$  M), and isoproterenol (iso;  $3 \times 10^{-9}$  M in A and  $3 \times 10^{-8}$  M in B) were added

37.5 s before  $\text{NH}_4\text{Cl}$ , IBMX ( $10^{-4}$  M) was added 75 s before  $\text{NH}_4\text{Cl}$ , and H9 (100  $\mu\text{M}$ ), staurosporine (stauro.; 1  $\mu\text{M}$ ), and K252a (20  $\mu\text{M}$ ) were added 20 min before  $\text{NH}_4\text{Cl}$ . \*Initial recovery rates which are significantly different from control.

pendent stimulation of  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load was not significantly affected by either the removal of extracellular calcium, or the depletion of intracellular  $\text{IP}_3$ -sensitive calcium stores (accomplished by preincubation of acini with the  $\text{Ca}^{2+}$  mobilizing agonist carbachol in  $\text{Ca}^{2+}$ -free medium—see Table legend). These results argue strongly against a role for intracellular calcium concentration in the upregulation of the  $\text{Na}^+ \text{-K}^+ \text{-2Cl}^-$  cotransporter studied here.

Table I. Effect of Calcium on the Isoproterenol-induced Enhancement of  $\text{pH}_i$  Recovery from an  $\text{NH}_4^+$ -induced Alkaline Load\*

Experimental condition	Initial recovery rate (experimental/control)		n
	-iso	+iso	
	<i>10 nM</i>		
PSS	1.0	$1.63 \pm 0.10$	16
EGTA	$0.95 \pm 0.10$	$1.59 \pm 0.27$	3
Carbachol pretreated	$1.08 \pm 0.02$	$1.75 \pm 0.11$	3

\* Experiments were carried out as described in Fig. 1, A and C. 'PSS' denotes acini in PSS, 'EGTA' denotes acini in  $\text{Ca}^{2+}$ -free PSS (all calcium salts replaced with 0.1 mM EGTA) and 'Carbachol pretreated' denotes acini incubated in  $\text{Ca}^{2+}$ -free PSS containing  $10^{-5}$  M carbachol for 10 min, then centrifuged and resuspended in  $\text{Ca}^{2+}$ -free PSS for the experiment. None of the recovery rates observed in the presence of isoproterenol (+iso) are significantly different from each other.

The effect of isoproterenol is mediated by the  $\beta_1$  receptor. Isoproterenol is a combined  $\beta_1$  and  $\beta_2$  adrenergic agonist. We show in Fig. 5, however, that the effect of isoproterenol is mimicked by both epinephrine (a combined  $\beta_1$  and  $\beta_2$  agonist) and norepinephrine (a  $\beta_1$  agonist), with approximately equal potency (experiments with epinephrine and norepinephrine were carried out in the presence of the  $\alpha$ -adrenergic antagonist phenolamine to limit their effects to  $\beta$ -adrenergic receptors). We also demonstrate that the effect of isoproterenol is blocked by the  $\beta$ -adrenergic antagonist atenolol at a concentration ( $3 \times 10^{-7}$  M) where it is highly specific for the  $\beta_1$  receptor subtype (29). Thus, the effect of isoproterenol on the  $\text{Na}^+ \text{-K}^+ \text{-2Cl}^-$  cotransporter appears to be mediated by  $\beta_1$  adrenoceptors.

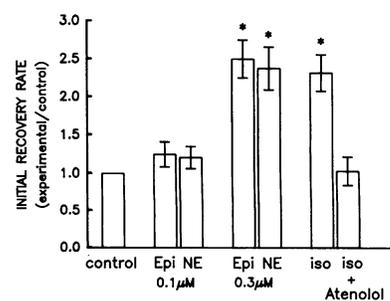


Figure 5. Effects of  $\beta$ -adrenergic agonists and antagonists on the initial rate of  $\text{pH}_i$  recovery from an  $\text{NH}_4^+$ -induced alkaline load. The experimental procedure was as described for Fig. 1 C ( $n \geq 3$  for all conditions). Epinephrine (Epi; 0.1  $\mu\text{M}$  and 0.3  $\mu\text{M}$ ), norepinephrine

(NE; 0.1  $\mu\text{M}$  and 0.3  $\mu\text{M}$ ), and isoproterenol (iso; 30 nM) were added 37.5 s before  $\text{NH}_4\text{Cl}$ , and atenolol (0.3  $\mu\text{M}$ ) was added 75 s before  $\text{NH}_4\text{Cl}$ . The effects of epinephrine and norepinephrine were assayed in the presence of  $10^{-5}$  M phenolamine. \*Initial recovery rates which are significantly different from control.

## Discussion

We demonstrate here that the recovery of rat parotid acini from an  $\text{NH}_4^+$ -induced acute alkaline load is dramatically enhanced by pretreatment with the  $\beta$ -adrenergic agonist isoproterenol (Fig. 1). This enhancement of  $\text{pH}_i$  recovery is completely inhibited by the loop diuretic bumetanide, and by chloride removal, and is absolutely dependent on the presence of  $\text{NH}_4^+$  (Figs. 1 and 2). Taken together with the fact that  $\text{NH}_4^+$  has been shown to share the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter in several cell types (14–16), including salivary glands, these results provide strong evidence that this enhancement of  $\text{pH}_i$  recovery is due to increased  $\text{NH}_4^+$  entry into the acinar cytoplasm via the cotransporter, and thus indicate that isoproterenol treatment results in an upregulation of cotransporter activity.

The effect of isoproterenol is mimicked by permeant cAMP analogues and forskolin, and is potentiated by the phosphodiesterase inhibitor IBMX (Fig. 4 A), indicating that it is associated with the increased intracellular cAMP levels known to occur following  $\beta$ -adrenergic stimulation of this tissue (24, 25, 30). The dose of isoproterenol yielding a half maximal effect on the cotransporter ( $2.15 \times 10^{-8}$  M; Fig. 3) is in reasonable agreement with the half maximal effect of this agent on amylase secretion, a phenomenon which is also cAMP-mediated in the parotid (25, 31). Furthermore, since atenolol (a specific  $\beta_1$  antagonist) blocks the effect of isoproterenol, and since both epinephrine (a combined  $\beta_1$  and  $\beta_2$  adrenergic agonist) and norepinephrine (a relatively specific  $\beta_1$  agonist) mimic the effect of isoproterenol at comparable doses (Fig. 5), it appears that this upregulation, like amylase release (23, 30, 31), is mediated via the  $\beta_1$  adrenoreceptor subtype (32). The fact that the effect of isoproterenol is blocked by the protein kinase inhibitors H9, staurosporine and K252a (Fig. 4 B) provides strong evidence that this effect is due to a phosphorylation event presumably mediated by protein kinase A.

As already mentioned, there is evidence for both up- and downregulation of  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransport activity in a variety of cell types (1, 2). In spite of the intense interest in this transporter, however, little has been established concerning the molecular mechanisms involved in these events. In addition, relatively few reports have distinguished between upregulation of cotransport activity which arises simply from changes in intracellular ion concentrations, and thus in thermodynamic driving forces for cotransport, and upregulation which arises from true changes in intrinsic transporter properties, resulting, for example, from direct modifications (e.g., phosphorylation) of the cotransporter or an associated regulatory protein.

A number of observations indicate that the effect of isoproterenol on the parotid  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter arises from a true modification of cotransport activity, rather than a secondary effect due to changes in ionic driving forces. In this regard, it is important to bear in mind that, in contrast to a number of other secretory tissues, fluid secretion in salivary glands arises primarily from  $\text{Ca}^{2+}$  mobilizing agonists, rather than cAMP generating agents such as isoproterenol (9). Thus, whereas muscarinic and  $\alpha$ -adrenergic stimulation of salivary acini ( $\text{Ca}^{2+}$  mobilizing agents) result in a significant decrease in intracellular chloride content, which is associated with the fluid secretory response (4, 7, 11), stimulation with isoproterenol ( $10^{-5}$  M) results in no initial change in intracellular chloride, followed by a slow increase in chloride content (20–30%) which is first detectable after  $\sim 2$  min of stimulation (13) (J. E. Melvin and

R. J. Turner, unpublished observations). As already mentioned, this latter effect has been shown to be blocked by furosemide in rat submandibular acini (13), and thus is almost certainly secondary to activation of the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter. A small component of calcium mobilization from  $\text{IP}_3$ -sensitive stores has, however, been observed in rat parotid acini in response to relatively large doses ( $\geq 5 \mu\text{M}$ ) of isoproterenol (26–28). This phenomenon may be responsible for the small component of fluid secretion associated with  $\beta$ -adrenergic stimulation of intact perfused salivary glands by similar concentrations of this agonist (33). Although these effects occur at concentrations of isoproterenol several orders of magnitude greater than those relevant to the phenomenon studied here, we have nevertheless verified that the effect of isoproterenol we observe is still seen after depletion of  $\text{IP}_3$ -sensitive  $\text{Ca}^{2+}$  stores (Table I). We have also verified that the activation of cotransporter activity is not secondary to an increased driving force for sodium generated by  $\text{Na}^+\text{/K}^+$  ATPase (Fig. 2). All of these results argue strongly in favor of a true change in intrinsic cotransport properties induced by  $\beta$ -adrenergic stimulation.

Further studies will be required to characterize the molecular events involved in the upregulation of the cotransporter. In this regard, Pewett et al. (34) have recently demonstrated that an  $M_r \sim 150,000$  protein thought to represent part or all of the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter in the avian erythrocyte is a phosphoprotein. Although no correlation between cotransport activity and phosphorylation of this protein has yet been established, this result supports the conjecture that phosphorylation and dephosphorylation of the cotransporter itself may be involved in its regulation.

In vivo salivary glands typically receive simultaneous muscarinic,  $\alpha$ -adrenergic, and  $\beta$ -adrenergic stimulation. Thus one would expect upregulation of the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter to accompany the fluid secretory response. Physiologically, upregulation of the parotid  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter would lead to an increase in acinar chloride uptake across the basolateral membrane, and consequently, an increase in overall chloride and fluid secretion by the acinar cell (see Introduction). Larsson and Olgart (35) have, in fact, recently demonstrated that agents that increase intracellular cAMP levels in the rat parotid do indeed markedly enhance carbachol-induced salivary secretion (100–250%) consistent with this scheme. Whether similar effects occur in human salivary tissues remains to be determined. However, it is often reported that many commonly used drugs result in complaints (17, 18) of dry mouth (xerostomia) and/or dry eyes, the underlying causes of which, in many cases, are unknown. Among these drugs are a number of  $\beta$ -blockers (17, 18) that could produce these side effects by preventing the upregulation of the  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter characterized here. Indeed, any drug that interfered with the  $\beta$ -receptor/cAMP/protein kinase A cascade might produce such symptoms. From the perspective of symptomatic treatment of these exocrine disorders (e.g., Sjögren's syndrome; reference 36), the results presented here suggest that a therapy combining both  $\text{Ca}^{2+}$ -mobilizing and cAMP-generating stimuli would be most effective.

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