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Agonist-Induced Activation of Na^+/H^+ Exchange in Rat Parotid Acinar Cells

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Summary. The present studies were designed to test our previous suggestion that Na^+/H^+ exchange was activated by muscarinic stimulation of rat parotid acinar cells. Consistent with this hypothesis, we demonstrate here that intact rat parotid acini stimulated with the muscarinic agonist carbachol in HCO_3^--free medium show an enhanced recovery from an acute acid load as compared to similarly challenged untreated preparations. Amiloride-sensitive ^{22}Na uptake, due to Na^+/H^+ exchange, was also studied in plasma membrane vesicles prepared from rat parotid acini pretreated with carbachol. This uptake was stimulated twofold relative to that observed in vesicles from control (untreated) acini. This stimulation was time dependent, requiring ~15 min of acinar incubation with carbachol to reach completion, and was blocked by the presence of the muscarinic antagonist atropine (2×10^{-5} M) in the pretreatment medium. The effect of carbachol was dose dependent with $K_{0.5} \sim 3 \times 10^{-6}$ M. Stimulation of the exchanger was also seen in vesicles prepared from acini pretreated with the α-adrenergic agonist epinephrine, but not with the β-adrenergic agonist isoproterenol, or with substance P. Kinetic analysis indicated that the stimulation induced by carbachol was due to an alkaline shift in the pH responsiveness of the exchanger in addition to an increased *apparent* transport capacity. Taken together with previous results from this and other laboratories, these results strongly suggest that the Na^+/H^+ exchanger and its regulation are intimately involved in the fluid-secretory response of the rat parotid.

Key Words exocrine gland · pH regulation · Na^+/H^+ exchange · fluid secretion · muscarinic agonist

Introduction

Salivary glands have been used extensively as model systems for the study of exocrine fluid and electrolyte secretion [1, 4, 9, 11–13, 16–19, 21, 28]. Considerable evidence now indicates that a mechanism first proposed by Silva et al. [23] to explain salt secretion by the shark rectal gland applies, at least to a good first approximation, to these and many other secretory epithelia [20]. In this model, Cl^- is driven into the secretory cell against its electrochemical gradient via a basolateral $Na^+/K^+/Cl^-$ cotransporter utilizing the sodium chemical gradient generated by Na^+/K^+ ATPase. Stimulation by a secretagogue results in the opening of basolateral K^+ channels and apical Cl^- channels, allowing the cell to lose KCl and generating a net transepithelial Cl^- flux from interstitium to lumen. Na^+ then follows Cl^- into the lumen via the tight junctions to preserve electroneutrality and water follows the NaCl osmotic gradient.

Although the qualitative features of the above model have been confirmed in salivary glands, recent reports indicate that additional ion transport pathways also participate in the fluid-secretory response [4, 9, 10, 13, 18, 21, 24]. Several studies have provided evidence that Na^+/H^+ exchange plays a significant role both in fluid secretion and in intracellular pH regulation during muscarinic stimulation of salivary acinar cells [4, 9, 10, 13, 18, 21]. More specifically, it has been suggested that parallel basolateral Na^+/H^+ and Cl^-/HCO_3^- exchangers are responsible for driving a portion of the acinar Cl^- secretion and that the Na^+/H^+ exchanger is also involved in a bicarbonate-dependent component of fluid secretion [4, 13, 18, 21, 24]. With regard to the latter, Melvin et al. [13] have recently presented data which indicate that parotid acinar cells undergo a significant HCO_3^- loss in response to muscarinic stimulation, and that the Na^+/H^+ exchanger plays an essential role in buffering the cytoplasm against this effect.

In addition to the above more direct indications of the involvement of Na^+/H^+ exchange in salivary fluid secretion, it has also been observed that the intracellular pH of both perfused rat submandibular glands [21] and isolated rat parotid acini [13] rises following muscarinic stimulation. In analogy with observations from a number of other cell types, it has been suggested that this effect may be due to activation of the exchanger [13, 21]. Increased Na^+/H^+ exchange activity has been observed in response to a variety of stimuli in nonsalivary tissues

(e.g., hormones, growth factors, fertilization, osmotic shrinkage) and this effect is thought to play an important role in the associated cellular response [2, 6, 15].

In the present study, we demonstrate directly that Na^+/H^+ exchange activity is enhanced in basolateral membrane vesicles prepared from rat parotid acini stimulated with the muscarinic agonist carbachol. The time course of this phenomenon is in good agreement with the time course of the rise of intracellular pH observed previously in our laboratory in intact acini following carbachol stimulation [13]. These and other data provide strong evidence for the activation of the Na^+/H^+ exchanger in rat parotid acini during the fluid-secretory response.

Materials and Methods

Materials

Hyaluronidase (type 1-S) was from Sigma (St. Louis, MO), collagenase (Type CLSPA) was from Cooper Biochemical (Malvern, PA), ^{22}Na was purchased from New England Nuclear (Boston, MA) and amiloride was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). BCECF/AM[1] (the acetoxymethyl ester of BCECF) was obtained from Calbiochem (La Jolla, CA). All other chemicals were from standard commercial sources and were reagent grade or the highest purity available.

Acinar Preparation

Dispersed parotid acini were prepared from male Wistar strain rats (Harlan-Sprague-Dawley, Indianapolis, IN) by collagenase/hyaluronidase digestion according to a previously published procedure from our laboratory [12]. Final parotid acinar preparations to be used for BCECF studies were washed and resuspended (5 ml/animal) in a physiological salt solution (PSS) containing: 110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.33 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 20 mM NaHEPES, 2 mM glutamine and 0.01% bovine serum albumin. Final parotid acinar preparations to be used for vesicle transport studies were washed and resuspended (30 ml/animal) in Earle's Minimal Essential Medium containing 0.01% bovine serum albumin. All acini were left for at least 15 min at 37°C before further treatment. Throughout the acinar preparation procedure and the subsequent experimental period, the acini were continuously gassed with 95% O_2/5% CO_2 (HCO_3^--containing media) or 100% O_2 (HCO_3^--free media). Except during fluorescence measurements, the preparation was kept agitating in a Dubnoff shaker (100 cycles/min).

[1] *Abbreviations:* HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MES, (2[N-morpholino] ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; BCECF, 2',7'-*bis*-(2-carboxyethyl)-5(6)-carboxyfluorescein.

Intracellular pH Measurements Using BCECF

Isolated acini were loaded with the fluorescent pH indicator BCECF by incubation with BCECF/AM (final concentrations 2 μM BCECF/AM and 0.1% DMSO in PSS) for 30 min at 25°C. After loading, the acini were washed three times with PSS to remove extracellular dye, then resuspended in PSS (~5 ml/animal) and left in the Dubnoff shaker at 25°C until use.

Immediately before beginning an experiment, a 1 ml aliquot of BCECF-loaded acini was washed and resuspended in HCO_3^--free PSS (PSS with $NaHCO_3$ replaced by NaCl) at 37°C, then transferred to a cuvette in the thermostatically controlled (37°C) cuvette holder of an SLM 8000C spectrofluorimeter equipped with a magnetic stirrer and a gas line. BCECF fluorescence was monitored in "ratio mode" by alternating the excitation wavelength between 490 and 440 nm at 2-sec intervals and measuring emitted fluorescence at 530 nm as previously described [13].

Membrane Vesicle Preparation

Experiments typically involved treatment of intact acini with various agents (*see* figure captions for details) followed by cell disruption, isolation of a membrane vesicle fraction, and ^{22}Na flux measurements. Treatments were carried out on 10-ml aliquots of acini (*see above*) maintained at 37°C with continuous agitation in the Dubnoff shaker.

The membrane vesicle fraction was prepared from dispersed rat parotid acini using the following abbreviated form a basolateral membrane vesicle preparation procedure previously developed in our laboratory [10]. After the experimental treatment of the acini, the extracellular medium was removed by centrifugation (400 $g \times 30$ sec) and the acini were resuspended in 10 ml of homogenization buffer (10 mM HEPES buffered with Tris to pH 7.4 and containing 10% sucrose, 1 mM EDTA and 0.1 mM PMSF) at 4°C (all subsequent steps were likewise carried out at 4°C). The acini were then homogenized with one 10-sec burst in a Polytron (Brinkman Instruments, Westbury, NY) set at power level 5. This homogenate was centrifuged at 2500 $\times$ g for 5 min in a Beckman J2-21 centrifuge fitted with a JA-20 rotor and the pellet was discarded. The supernatant was centrifuged at 22,000 $\times$ g for 20 min. The resulting pellet (the "membrane vesicle fraction") was suspended in a few milliliters of an appropriate buffer (unless otherwise noted, 50 mM HEPES buffered with Tris to pH 7.0, containing 100 mM mannitol and 1 mM EDTA), passed once through a 25-gauge needle and once through a 30-gauge needle, then diluted to 50 ml/g starting parotid tissue and centrifuged at 48,000 $\times$ g for 20 min. This pellet was suspended in the same buffer at a protein concentration of 1.5 mg/ml, incubated at 23°C for 30 min, and stored on ice for $\geq$1 hr before use.

Enzyme Assays

The vesicle preparation procedure was monitored for purity by assaying for the activities of enzymes characteristic of basolateral membranes (K^+-stimulated *p*-nitrophenyl phosphatase), luminal membranes (dipeptidyl peptidase IV) and mitochondria (succinic dehydrogenase) carried out as previously described [25]. Protein was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine gamma globulin as the standard.

Transport Studies

The uptake of ^{22}Na into freshly prepared vesicles was measured at 23°C using a rapid filtration technique. Briefly, a 10-μl aliquot of vesicles was placed in a 12 × 75 test tube and at time zero a 100-μl aliquot of incubation medium containing ^{22}Na and other constituents as required was added. The incubation medium used in all transport studies was 50 mM HEPES buffered with Tris to pH 8.0, containing 100 mM mannitol and 1.1 mM ^{22}Na gluconate (30 μCi/ml) with or without 1.1 mM amiloride. After 15 sec, the reaction was terminated by the addition of 1.5 ml of ice-cold stop solution (*see below*). After addition of the stop solution, the vesicles were collected on a Millipore filter (HAWP 0.45 μm) which was then washed with a further 6.0 ml of stop solution, placed in a scintillation vial with 1 ml of water and 10 ml of Filtron X (National Diagnostics) and counted for radioactivity. Each experimental point was carried out in triplicate or quadruplicate.

The stop solution was 10 mM HEPES buffered with Tris to pH 7.4 and containing 100 mM mannitol, 150 mM K acetate and 1 mM amiloride. The entire stopping and washing procedure took less than 30 sec, during which the vesicles were in contact with the stop solution for less than 15 sec. From control experiments in which the time the vesicles were left in the stop solution was prolonged, we have established that no significant loss of ^{22}Na occurs during the washing and stopping procedure [10].

Data Analysis and Presentation

In all experiments, ^{22}Na uptake was measured in the presence and absence of 1 mM amiloride (*see above*) and the amiloride-sensitive component of uptake for each vesicle preparation was calculated by subtraction. Amiloride-sensitive uptakes have been normalized to those obtained from vesicles prepared simultaneously from control (untreated) acini. Absolute uptakes in vesicles from control acini varied somewhat from preparation to preparation (*see* figure captions); however, the effects of the various treatments studied on normalized uptakes were quite consistent. The results illustrated in the figures and tables are the means ± SEM for (normalized) amiloride-sensitive uptakes measured in the indicated number of experiments carried out under identical conditions. The amiloride-insensitive component of ^{22}Na uptake was not affected by any of the experimental treatments employed (*data not shown*). P values < 0.05 as determined from the Student's t test were taken to represent statistically significant differences.

Results

Evidence for Activation of the Na^+/H^+ Exchanger in Intact Acini

As mentioned earlier, we have previously provided convincing evidence that the Na^+/H^+ exchanger plays a central role in regulating the intracellular pH of rat parotid acinar cells following muscarinic stimulation [13]. In that report, we also suggested that the exchanger may be activated in stimulated cells. Evidence supporting this hypothesis is provided in Fig. 1. This figure illustrates the recovery of intracellular pH by BCECF-loaded rat parotid acini in HCO_3^--free medium following an acute acid load induced by exposure of the cells to sodium propionate. We have previously demonstrated that this recovery is markedly retarded in the presence of amiloride and, thus, is apparently due to the Na^+/H^+ exchanger [13]. Figure 1 illustrates that acini preincubated with the muscarinic agonist carbachol (10^{-5} M) show an enhanced recovery from a propionic acid load relative to untreated controls. This observation is consistent with enhanced Na^+/H^+ exchange activity resulting in more effective intracellular buffering in these cells. The α-adrenergic agonist epinephrine (10^{-5} M), which like carbachol elicits a copious fluid-secretory response [1, 28], also renders the cells more resistant to intracellular acidification (Fig. 1). These effects are documented more quantitatively in Table 1. The results in Table 1 also show that the effect of carbachol is blocked by the muscarinic antagonist atropine (2×10^{-5} M).

It also should be pointed out that, in contrast to our earlier observation of an increase in acinar intracellular pH (≈0.1 pH unit, $T_{1/2} \approx 5$ min) following muscarinic stimulation in complete PSS [13], we observed no significant increase in intracellular pH following carbachol or epinephrine stimulation in the HCO_3^--free, high chloride medium employed in the experiments shown in Fig. 1 (intracellular pH =

Table 1. Recovery of rat parotid acinar cells from an acute acid load after treatment with various agents

Treatment	Response[a]	n
Control	55.3 ± 4.6	11
Atropine (2×10^{-5} M)	48.3 ± 7.7	4
Carbachol (10^{-5} M)	28.5 ± 5.1*	8
Atropine + Carbachol	44.5 ± 5.6	4
Epinephrine (10^{-5} M)	34.3 ± 5.4*	5

[a] BCECF fluorescence traces corresponding to the recovery of rat parotid acini from sodium propionate-induced acid loads were obtained as described in the caption of Fig. 1. Acini were pretreated with the agents indicated for 15 min prior to acid loading. For each BCECF trace, the average fluorescence (in ratio units—*see* Materials and Methods) over the 100-sec interval before the addition of sodium propionate was calculated. At each 2-sec interval following the addition of sodium propionate (the trace consists of fluorescence ratios measured every 2 sec), the difference between this value and the measured fluorescence ratio was then determined. The quantity listed under "Response" is the sum of these differences over the 380-sec interval following the addition of propionate. This quantity is a measure of the area enclosed by the BCECF trace as the acini acidify and recover in response to acid loading and, thus, is a measure of the ability of the acini to respond to an acid load. The calculated "Responses" from the number of BCECF traces indicated have been averaged to produce the table. The asterisks indicate responses that are significantly different from the "Control."

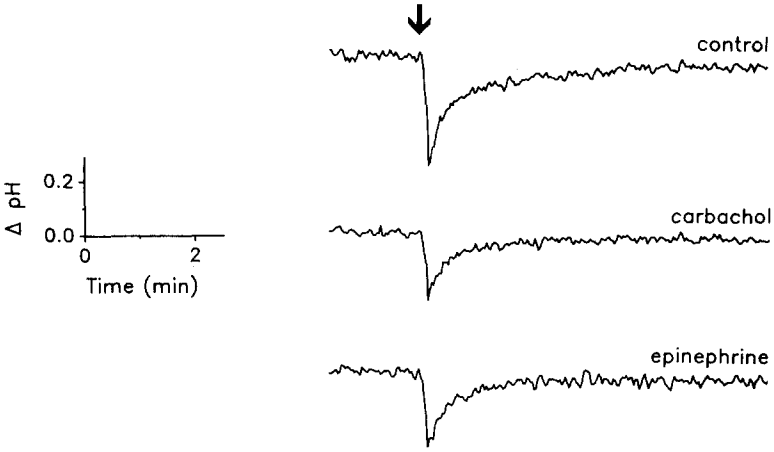

Fig. 1. The recovery of treated and untreated rat parotid acini from an acute acid load. At the time indicated by the arrow BCECF-loaded acini in HCO_3^--free PSS were exposed to an acute acid load by adding 30 μl of a 3.03 M sodium propionate stock solution to 3 ml of acinar suspension. The traces represent BCECF fluorescence (*see* Materials and Methods) from untreated (control, n = 11) acini and from acini pretreated with carbachol (10^{-5} M, n = 8) or epinephrine (10^{-5} M, n = 5) for 15 min before exposure to propionate. The results shown are the averaged BCECF traces from the number of experiments indicated

7.33 ± 0.05 immediately before sodium propionate addition for all conditions in Fig. 1 and Table 1). This difference may be related to the dramatic increase in intracellular sodium concentration which is known to accompany muscarinic stimulation [7, 22, 28]. The majority of this sodium presumably enters the acinar cell with chloride via the Na/K/Cl cotransporter [25]. Thus, intracellular sodium levels may be higher in the high chloride medium used here than in PSS, reducing the driving force for Na^+/H^+ exchange and rendering the exchanger unable to drive the intracellular pH above resting levels.

Evidence for Activation of the Na^+/H^+ Exchanger in Isolated Membranes

In order to confirm that the enhanced buffering capacity of carbachol-stimulated acini is due to enhanced Na^+/H^+ exchange, we have examined the activity of this transporter in membranes isolated from treated and untreated cells. We have previously documented the existence of a potent, amiloride-sensitive, electroneutral Na^+/H^+ exchanger in a vesicle preparation from the rat parotid [10]. This Na^+/H^+ exchange activity was present in the membrane vesicle fraction employed here (*see* Materials and Methods) and copurified with the basolateral membrane marker, K^+-stimulated *p*-nitrophenyl phosphatase, when this fraction was run on a Percoll density gradient [10]. All data presented here represent the amiloride-sensitive component of ^{22}Na uptake (*see* Materials and Methods) measured in the presence of a pH gradient (unless otherwise noted pH_{in} = 7.0, pH_{out} = 8.0). We have been unable to detect any significant component of conductive amiloride-sensitive sodium transport in either the membrane vesicle preparation employed here or in the more purified basolateral membrane fraction obtained from it by Percoll density centrifugation (*data not shown, see* ref. 15 for experimental methods). Thus, we attribute the amiloride-sensitive component of ^{22}Na uptake studied here to electroneutral Na^+/H^+ exchange.

In Fig. 2, we compare the amiloride-sensitive component of ^{22}Na uptake into vesicles prepared from control (untreated) acini, and from acini exposed to 10^{-5} M carbachol for 15 min before vesicle preparation. Uptake into vesicles from carbachol-treated acini is more than twice that of control values ($P < 0.002$). Moreover, as also shown in Fig. 2, this effect of carbachol is completely blocked by treatment of acini with the muscarinic antagonist atropine. The increase in amiloride-sensitive sodium uptake induced by carbachol cannot be accounted for by differences in purity between vesicles from treated and untreated acini since enzymatic markers for basolateral, luminal and mitochondrial membranes are not significantly different for these two preparations (Table 2). It is also worth re-emphasizing at this point that, in the experiment illustrated in Fig. 2 and in subsequent results reported here, only the intact acini were subjected to the various experimental treatments; the vesicle preparation procedure was identical for all acini irrespective of pretreatment.

Figure 3 shows the amiloride-sensitive component of ^{22}Na uptake into vesicles prepared from rat parotid acini pretreated with carbachol (10^{-5} M), isoproterenol (10^{-5} M), carbachol plus isoproterenol (both 10^{-5} M), epinephrine (10^{-5} M) or substance P (10^{-7} M). Epinephrine, like carbachol stimulates amiloride-sensitive sodium uptake ($P < 0.002$). The β-adrenergic agonist isoproterenol, which mainly induces protein secretion [28], is without effect in both the presence and absence of carbachol. Sub-

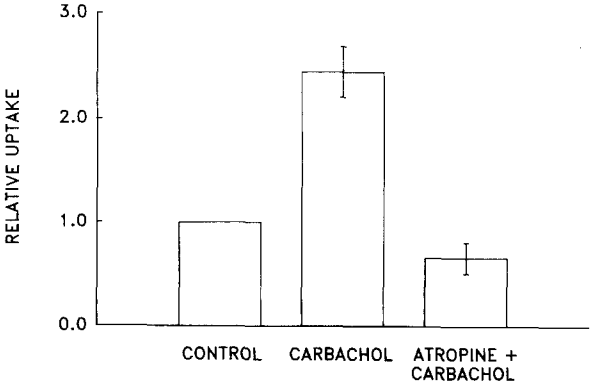

Fig. 2. Amiloride-sensitive uptake of ^{22}Na by vesicles prepared from control and carbachol-treated rat parotid acini. Vesicles were prepared from control (untreated) acini (average uptake = 0.86 ± 0.10 nmol/min/mg protein, n = 3), from acini pretreated with 10^{-5} M carbachol for 15 min before vesicle preparation (n = 3) and from acini pretreated with 10^{-5} M carbachol plus 2×10^{-5} M atropine for 15 min (n = 3)

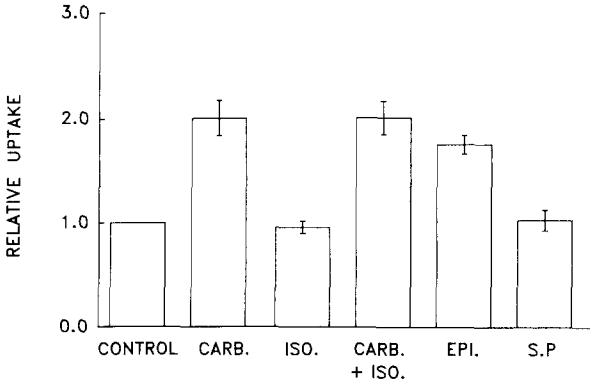

Fig. 3. Amiloride-sensitive uptake of ^{22}Na by vesicles prepared from acini pretreated with various agonists. Vesicles were prepared from control acini (average uptake = 0.46 ± 0.03 nmol/min/mg protein, n = 9) or from acini pretreated for 15 min with carbachol (10^{-5} M, n = 7), isoproterenol (10^{-5} M, n = 4), carbachol plus isoproterenol (both at 10^{-5} M, n = 3), epinephrine (10^{-5} M, n = 6) or substance P (10^{-7} M, n = 4)

Table 2. Enrichment of marker enzymes in vesicles prepared from control and carbachol-treated rat parotid acini

Enzyme	Enrichment[a]			
	Control	n	Carbachol treated	n
K^+-stimulated p-nitrophenyl phosphatase	2.30 ± 0.09	10	2.65 ± 0.19	10
Dipeptidyl peptidase IV	2.04 ± 0.18	9	2.32 ± 0.16	9
Succinic dehydrogenase	2.01 ± 0.25	9	2.09 ± 0.26	9

[a] Vesicles were prepared from control (untreated) acini and from acini pretreated for 15 min with 10^{-5} M carbachol before vesicle preparation. Enzymatic activities (*see* Materials and Methods) were measured in vesicles and the corresponding acinar homogenates. Relative enrichments (vesicles/homogenate) for the number of preparations indicated have been averaged to produce the table. The enzyme enrichments of vesicles from treated acini are not significantly different from those of controls.

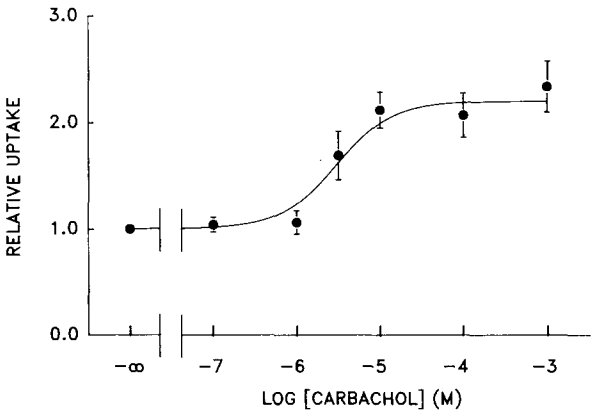

Fig. 4. Amiloride-sensitive uptake of ^{22}Na by vesicles prepared from acini pretreated with various concentrations of carbachol. Vesicles were prepared from control acini (average uptake = 0.55 ± 0.04 nmol/min/mg protein, n = 6) and from acini pretreated for 15 min with various concentrations of carbachol as indicated

stance P is also without effect on sodium uptake (*see* Discussion).

The dependence of the stimulation of amiloride-sensitive sodium uptake on carbachol concentration during acinar pretreatment is illustrated in Fig. 4. The effect of carbachol is half-maximal at approximately 3 μM, a value in good agreement with the concentration of this agonist which yields a half-maximal chloride-secretory response in intact rat parotid acini [12].

The time course of the stimulatory effect of carbachol is investigated in Table 3. Here acini were preincubated with 10 μM carbachol for 2, 7, 15 and 30 min before vesicle preparation. No significant stimulation of amiloride-sensitive sodium uptake is detectable after 2 min of incubation, a partial stimulation may be present at 7 min, and the effect of carbachol is complete at 15 min.

Mechanism of Activation of Na^+/H^+ Exchange

Figure 5*a* shows amiloride-sensitive sodium uptake into vesicles prepared from control and carbachol-treated rat parotid acini measured as a function of

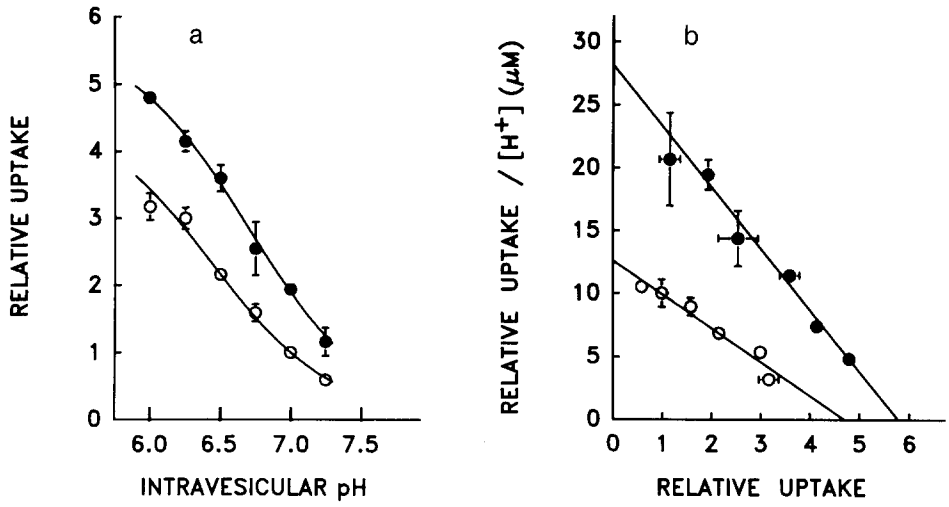

Fig. 5. Intravesicular pH dependence of amiloride-sensitive ^{22}Na uptake by vesicles prepared from control (○) and carbachol-treated (15 min at 10^{-5} M) (●) acini. Vesicles were prepared in a buffer containing 25 mM HEPES, 25 mM MES, 100 mM mannitol and 1 mM EDTA buffered with Tris to the pH indicated. ^{22}Na uptake was measured at extravesicular pH 8.0 ($n \geq 3$ for all points). Uptakes from each experiment were normalized to the flux observed at intravesicular pH 7.0 (average uptake = 0.90 ± 0.09 nmol/min/mg protein, n = 7) and averaged to produce the figure. The lines drawn through the data points are least squares fit to the Eadie Hofstee plots in (*b*). These fits are given by V_{max} = 4.70 ± 0.30 (relative units), $K_{0.5}$ = 0.372 ± 0.033 μM (control, r = 0.985), ○) and V_{max} = 5.78 ± 0.06, $K_{0.5}$ = 0.205 ± 0.011 μM (treated, r = 0.994, ●)

Table 3. Amiloride-sensitive uptake of ^{22}Na by vesicles prepared from acini treated with carbachol for various times[a]

Time of pretreatment in carbachol min	Relative uptake	n
0 (control)	1.00	5
2	1.01 ± 0.09	3
7	1.28 ± 0.23	3
15	2.07 ± 0.08	4
30	1.86 ± 0.28	2

[a] Vesicles were prepared from control (untreated) acini (average uptake = 0.59 ± 0.08 nmol/min/mg) or from acini pretreated with 10^{-5} M carbachol for the times indicated.

intravesicular pH (pH_{out} = 8.0 for all points). This plot demonstrates clearly that this component of sodium uptake is strongly pH gradient dependent, confirming that it is due to Na^+/H^+ exchange. Uptake into vesicles prepared from treated acini is higher than into controls at all intravesicular pHs tested ($P < 0.005$). Figure 5*b* is an Eadie Hofstee plot of the same data. The data fall on reasonably good straight lines in this plot (*see* figure caption) indicating that they obey the Michaelis-Menten equation over this range of experimental conditions. Owing to recent reports of non-Michaelis-Menten-type behavior of Na^+/H^+ exchange in experiments of this type in other tissues [3, 5, 8, 14, 26, 27], we have also carried out nonlinear least squares fits of these data to the Hill equation. However, the resulting Hill coefficients were not significantly different from unity for vesicles from either control or stimulated acini, indicating, as suggested by Fig. 5*b*, that the data do indeed obey the Michaelis-Menten equation rather well.

The least squares fits to Fig. 5*b* (*see* figure caption) indicate that both the maximum velocity of transport (V_{max}) and the half-saturation constant for intravesicular $[H^+]$ ($K_{0.5}$) are significantly changed ($P < 0.0003$) in vesicles prepared from carbachol-treated acini. The intravesicular pH at which amiloride-sensitive sodium uptake is half-maximal under the experimental conditions of Fig. 5 can be calculated from the least squares parameter $K_{0.5}$ to be 6.43 ± 0.04 for control vesicles and 6.69 ± 0.02 for vesicles from carbachol-treated acini. Thus, carbachol treatment causes an alkaline shift of 0.26 ± 0.04 units in the dependence of the exchanger on intravesicular pH.

Discussion

The present studies were designed to test our previous suggestion that Na^+/H^+ exchange was activated by muscarinic stimulation of rat parotid acinar cells [13]. We demonstrate here that intact rat parotid acini stimulated with the muscarinic agonist carbachol show an enhanced recovery from an

acute acid load as compared to similarly challenged untreated preparations (Fig. 1 and Table 1). Since this recovery is known to be amiloride-sensitive and, thus, presumably due to the Na^+/H^+ exchanger [13], these observations provide strong evidence for increased exchanger activity in stimulated cells. The α-adrenergic agonist epinephrine, which produces a fluid-secretory response similar to that of carbachol in the rat parotid [1, 28], also enhances pH recovery (Fig. 1 and Table 1).

These observations were explored further by preparing basolateral membrane vesicles from stimulated and unstimulated acini and assaying for sodium transport activity. Our results show that amiloride-sensitive sodium uptake via the Na^+/H^+ exchanger is enhanced in membrane vesicles prepared from rat parotid acini stimulated with either carbachol or epinephrine (Figs. 2 and 3). The effect of carbachol on the exchanger is dose dependent (Fig. 4) and completely blocked by the muscarinic antagonist atropine (Fig. 2). The concentration at which carbachol produces half-maximal stimulation of the exchanger ($\sim 3\ \mu M$) is in good agreement with carbachol concentrations associated with a half-maximal fluid-secretory response [12]. No stimulation of the exchanger is observed in vesicles prepared from acini treated with the β-adrenergic agonist isoproterenol or with substance P (Fig. 3). The fact that substance P is without effect, even though this compound induces fluid secretion, may be related to the desensitization of its receptor over the relatively long incubation times required to stimulate the exchanger (Table 3).

Kinetic analysis of the dependence of the exchanger on intravesicular pH indicated that both its *apparent* transport capacity and its half-saturation constant for $[H^+]_{in}$ are modified in vesicles from carbachol-treated acini (Fig. 5). Both of these changes are such that transport is increased at all intravesicular pHs. Thus, at a fixed extracellular-to-intracellular sodium chemical gradient, one would expect the exchanger in carbachol-stimulated acini to be able to drive the intracellular pH to higher levels than observed in resting cells. Recent results from our laboratory have, in fact, shown that the intracellular pH of rat parotid acinar cells incubated in a physiological salt solution increases gradually following carbachol stimulation, reaching values ~ 0.1 pH units above resting levels after 10 min [13]. The time scale of this response corresponds well with the time course of the activation of the exchanger observed here (Table 3). Taken together, our results indicate that the carbachol-induced alkalinization observed in intact acini is due to activation of the Na^+/H^+ exchanger and are strongly suggestive of a significant role of this transporter and its regulation in the fluid-secretory response of the rat parotid.

In contrast to several recent reports on other tissues [3, 5, 8, 14, 26, 27], we do not observe a non-Michaelis-Menten-type dependence of Na^+/H^+ exchange activity on $[H^+]_{in}$ (Fig. 5). This behavior is commonly associated with a sigmoidal dependence of sodium flux on $[H^+]_{in}$ yielding Hill coefficients greater than one [3]. This is thought to be due to the presence of an intracellular allosteric proton (or hydroxyl) modifier site [2, 3, 6, 15]. Although Hill coefficients of ~ 2–3 (indicative of a sharp decrease in exchanger activity with increasing pH_{in}) have been observed [5, 26, 27], this is not the case for all cell types. Hill coefficients ≈ 1.2 have been observed in renal brush-border membrane vesicles [8] and in a cultured renal cell line [14]. In fact, the Hill coefficient in the latter was reduced to 1.0 by parathyroid hormone treatment [14]. Despite the fact that our data clearly rule out a dramatic sigmoidal dependence of Na^+/H^+ exchange activity on $[H^+]_{in}$ in our preparation, we cannot exclude the possibility that this type of behavior may be present under other experimental or physiological conditions.

Although the increase in V_{max} of Na^+/H^+ exchange activity seen in Fig. 5 could be the result of the activation of previously dormant transporters, this cannot explain the decrease in the $K_{0.5}$ for intravesicular $[H^+]$. Both effects could, however, be the result of a modification of existing transporters. An increase in the translocation rate constant for the loaded carrier could, for example, simultaneously increase V_{max}, by increasing the cycling rate of the transporter, and decrease $K_{0.5}$, by increasing the availability of the transporter on the intravesicular surface. The changes in transport activity we observe with carbachol treatment are presumably due to a covalent modification of the Na^+/H^+ exchanger since they are preserved through an extensive series of washing and dilution steps (*see* Materials and Methods).

A complete understanding of the role of the Na^+/H^+ exchanger in the secretory physiology of the rat parotid acinar cell will obviously require further experimentation; however, several observations and suggestions can already be made. First, as previously stated, earlier results from our laboratory indicate that rat parotid acinar cells secrete significant amounts of HCO_3^- in response to muscarinic stimulation and that the Na^+/H^+ exchanger plays a major role in buffering the cytoplasm against this effect [13]. We have suggested that this HCO_3^- loss drives a HCO_3^--dependent component of fluid secretion in parallel with Cl^--dependent secretion. Stimulation of the exchanger would aid in buffering the acid load resulting from HCO_3^- loss. An associ-

ated increase in intracellular pH, would raise intracellular HCO_3^- (by 25% per 0.1 pH unit) and consequently increase HCO_3^--dependent fluid secretion. Increased intracellular HCO_3^- could also increase Cl^- entry via the basolateral Cl^-/HCO_3^- exchanger and thereby stimulate chloride-dependent fluid secretion as well. The relevance of these phenomena to the fluid-secretory response in the parotid are currently under investigation in our laboratory.

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