Muscarinic Agonists Induce Phosphorylation-independent Activation of the NHE-1 Isoform of the Na⁺/H⁺ Antiporter in Salivary Acinar Cells*

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Cholinergic agonists stimulate isotonic fluid secretion in the parotid gland. This process is driven by the apical exit of Cl⁻, which enters the cells partly via Cl⁻/HCO₃⁻ exchange across the basolateral membrane. Acidification of the cytosol by the extrusion of HCO₃⁻ is prevented by the concomitant activation of the Na⁺/H⁺ exchanger (NHE), which is directly activated by cholinergic stimulation. Multiple isoforms of the NHE have been described in mammalian cells, but the particular isoform(s) present in salivary glands and their mechanism of activation have not been defined. Reverse transcriptase-polymerase chain reaction with isoform-specific primers was used to establish that NHE-1 and NHE-2, but not NHE-3 or NHE-4, are expressed in parotid glands. The presence of NHE-1 was confirmed by immunoblotting and immunofluorescence, which additionally demonstrated that this isoform is abundant in the basolateral membrane of acinar cells. The predominant role of NHE-1 in carbachol-induced Na⁺/H⁺ exchange was established pharmacologically using HOE694, an inhibitor with differential potency toward the individual isoforms. Because muscarinic agonists induce stimulation of protein kinases in acinar cells, we assessed the role of phosphorylation in the activation of the antiporter. Immunoprecipitation experiments revealed that, although NHE-1 was phosphorylated in the resting state, no further phosphorylation occurred upon treatment with carbachol. Similar phosphopeptide patterns were observed in control and carbachol-treated samples. Together, these findings indicate that NHE-1, the predominant isoform of the antiporter in the basolateral membrane of acinar cells, is activated during muscarinic stimulation by a phosphorylation-independent event. Other processes, such as association of Ca²⁺/calmodulin complexes to the cytosolic domain of the antiporter, may be responsible for the activation of Na⁺/H⁺ exchange.

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1 The abbreviations used are: DMA, dimethylamiloride; BCECF, 2,7'-bis(carboxyethyl)-5(6)-carboxyfluorescein; [Ca²⁺]i, cytosolic free calcium concentration; HOE694, 3-(methylsulfonyl)-4-piperidino-benzo-yl-guanidine methanesulfonate; NHE, Na⁺/H⁺ exchanger; pH, intra-cellular pH; PBS, phosphate-buffered saline; SBFI, sodium-binding benzofuran isophthalate; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.
Muscarinic Stimulation of Na⁺/H⁺ Exchange

EXPERIMENTAL PROCEDURES

Materials—DNA was a generous gift from Dr. T. Kleyman (Department of Medicine, University of Pennsylvania). 3-(Methylsulfonyl-4-piperidino-benzoyl)guanidinesulfonate (HOE694) was kindly provided by Dr. A. Durckheimer, Hoechst AG, Frankfurt, Germany. The acetoxyethyl esters of sodium-binding benzofuroxan (SBFI) and of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) were obtained from Molecular Probes Inc. (Eugene, OR). Nigericin was provided by Dr. A. Durckheimer, Hoechst AG, Frankfurt, Germany. HOE694 was a generous gift from Dr. T. Kleyman (Department of Medicine, University of Pennsylvania). NEM-9 (bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)) was obtained from Molecular Probes Inc. (Eugene, OR). Nigericin was from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other chemicals were obtained from Sigma or other standard commercial sources.

Solutions—Unless otherwise indicated, experiments were conducted in nominally HCO₃⁻-free medium (solution A) consisting of (in mM) 135 NaCl, 144.6 CaCl₂, 5.4 KCl, 0.73 PO₄3⁻, 0.8 SO₄2⁻, 0.5 MgCl₂, 1.8 CaCl₂, 20 HEPES, 2 glucose, and 10 glucose, pH 7.4, at 37 °C. Where specified, 25 mM HCO₃⁻ replaced Cl⁻ and the solution was gassed with 95% O₂, 5% CO₂ (solution B). Acid loading was accomplished by pre-pulsing the cells for the indicated time in medium where 40 mM NH₄Cl replaced Na⁺ (solution C). In the Na⁺-free medium (solution D) all Na⁺ was iso-osmotically replaced with N-methyl-D-glucamine (NMDG).

Preparation of Acinar Cells—Parotid acinar cells from male Wistar rats were isolated by sequential treatment of the glands with trypsin (3) and 0.5% NBD-bis (carboxyethyl)-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester for 5 min at 37°C or with 7 mM NEM-9 (substitution for KCl) in solution A, where indicated. During all experiments, cells were viewed continuous with solution A on the stage of an inverted microscope equipped with an olive (di)cellule column (Pharmlab). Parotid mRNA was then reverse-transcribed and the complementary DNA amplified by the polymerase chain reaction, using the GeneAmp RNA PCR kit (Perkin-Elmer) and a Perkin-Elmer DNA thermal cycler Model 480. After completion of the PCR reaction (35 cycles), a 10-μl sample of the PCR tube was analyzed by electrophoresis on a 0.8% agarose gel pre-stained with 0.5 μg/ml ethidium bromide and the gel was photographed under UV illumination. Four isoformspecific sets of primers, which were hybridized to unique regions of the rat NHE-1, NHE-2, NHE-3, and NHE-4 primers were as follows: NHE-1, 5′ primer: CCT ACG TGG AGG CCA CAAC, 3′ primer: CAG CCA AGG GCT CTA CC, size of the PCR product: 429 base pairs (bp); NHE-2, 5′ primer: GCT GTCC TCT GCA GG GT, 3′ primer: CTT TGA GCA GAC AGT CG, size of PCR product: 880 bp; NHE-3, 5′ primer: CTT CTG CCT CTA GCT GC, 3′ primer: CAA GGA CAG CAT CTC GG, size of PCR product: 574 bp; NHE-4, 5′ primer: CTG ACG TCT GTG GTC, 3′ primer: C GAG GAA ATG CAG C G, size of PCR product: 381 bp. All four sets of primers yielded the expected PCR products when pCMV plasmids containing the full-length clone of the corresponding isoform were used as a template, but did not yield discernible products when any of the other isoforms was used as a template.

Immunoblotting and Immunoprecipitation—The preparation and purification of anti-NHE-1 antibodies and the method used for immunoblotting of membranes have been described in detail elsewhere (21). For immunoprecipitation, acinar cells were labeled for 2 h at 37 °C in nominally phosphate-free medium containing (50)μMphosphohydroxiphosphate (500 μCi/ml). Cells were then treated with or without carbachol in medium A containing 5% CO₂ at 37 °C. The reaction was stopped by sedimentation, followed by resuspension in immunoprecipitation buffer. The samples were extracted for 30 min at 4 °C and sedimented for 30 min at 100,000 × g at 0 °C. Immunoprecipitation proceeded as described previously (21).

Peptide Mapping—Samples were immunoprecipitated as above and eluted from the beads by boiling for 5 min in 125 mM Tris-HCl, pH 6.8, 0.5% SDS, 10% glycerol, and 0.001% bromphenol blue. After cooling to room temperature, 25 μg/ml chymotrypsin was added to the eluate. Digestion was stopped at the indicated times by addition of mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively, and boiling for 5 min. The peptides were resolved by SDS-PAGE using 15% acrylamide, the gels were dried and autoradiograms obtained using Kodak X-Omat AR film.

Immunofluorescence—Cells attached to coverslips or frozen tissue sections were rinsed twice with PBS and then fixed by incubation with 3% paraformaldehyde for 10 min. Fixation was terminated by rinsing and incubating with 100 mM glycine in PBS, pH 7.4, for 15 min. Cells were then permeabilized by incubation with a solution of 0.1% Triton-X and 0.1% (w/v) bovine serum albumin in PBS (TA-PBS solution) for 15 min, followed by three washes with the same solution. Blocking was then performed by incubation for 20 min in TA-PBS containing 5% goat serum. The coverslips were then washed three more times with TA-PBS. All the preceding steps were at room temperature. The cells were then incubated overnight with a 1:100 dilution of anti-NHE-1 antibodies in TA-PBS at 4 °C. Where indicated, the primary antibody was omitted to control for specificity of staining. After three more washes in TA-PBS, the samples were incubated with a 1:200 dilution of fluorescently labeled donkey anti-rabbit antibody in TA-PBS for 50 min at room temperature. The cells were then washed three times with PBS and mounted in 50% glycerol containing 1% n-propyl gallate.

Other Methods—Protein was determined using the Pierce BCA Reagent. All experiments were performed at least three times. Representative radiograms or confocal images are illustrated. Quantitative data were compared by ANOVA and differences were considered significant when the p value was <0.05.
transient acidification was superseded by a secondary alkalinization. Exposure of cells to 10 mM methyl-D-glucammonium). In agreement with earlier observations (4, 5), we found that muscarinic stimulation of parotid acinar cells using quantitative imaging of the fluorescence of intracellular BCECF. In these experiments, the amount of Na\(^{+}\) that enters the cell through the antiporter is virtually quiescent in resting (unstimulated) cells.

**Effect of Carbachol on Na\(^{+}\)/H\(^{+}\) Exchange**

**pH Dependence**—The preceding results suggest that treatment with carbachol converts the antiporter from a quiescent to an active mode. Further insight into this transition was gained by analyzing the properties of Na\(^{+}\)/H\(^{+}\) exchange in resting and stimulated cells. Because the antiporter is not detectable in untreated cells at normal pH, its activity was unmasked by acid-loading the cytosol, using an NH\(_4\)Cl pre-pulse. A representative experiment is shown in Fig. 2A. Acinar cells were pulsed with the weak base, which was then removed while simultaneously replacing Na\(^{+}\) with N-methyl-D-glucammonium\(^{+}\) (solution D). Under these conditions, the cells underwent rapid acidification and failed to recover within the period studied, due to the absence of Na\(^{+}\). Upon readdition of Na\(^{+}\), however, a rapid alkalinization ensued. In otherwise untreated cells, pH recovered to near the original basal level. By contrast, if the cells were treated with carbachol prior to Na\(^{+}\) readdition (open circles in Fig. 2A), the recovery surpassed the resting pH level, resulting in a net cytosolic alkalinization, reminiscent of that recorded in Fig. 1. Calculation of the rates of Na\(^{+}\)-induced H\(^{+}\) (equivalent) extrusion in cells with or without muscarinic stimulation are summarized in Fig. 2B. Two features are noteworthy: first, that following acid loading the rates of recovery were...
are very large (upwards of 100 mM/min), comparing very favorably with other cell types where absolute antiport rates have been reported (e.g. Ref. 24). This likely reflects the specialized function of these secretory cells. Second, it is apparent that the pH sensitivity of the antiporter is increased following exposure to carbachol. Although the rates of both control and carbachol-treated cells were similar at more acidic pH, exchange is clearly noticeable in the stimulated cells at pH 7.45. Similar shifts in the activation threshold or "setpoint" of the antiport have been reported in other systems (see Ref. 25 and NHE-4 primers did not yield discernible products in repeated trials (Fig. 3, lanes 8 and 11). No discernible products were detected when a specific primer set was used with any of the non-corresponding isoforms as template (not shown). When cDNA obtained by reverse transcription of rat parotid mRNA was used as a template, the NHE-1 primers yielded a product of ~500 bp (Fig. 3, lane 2), while a smaller yield of the expected product (~700 bp) was also observed for NHE-2. The NHE-3 and NHE-4 primers did not yield discernible products in repeated trials (e.g. Fig. 3, lanes 8 and 11). Omission of reverse transcriptase prevented appearance of the 500- and 700-bp products, ruling out contamination with genomic DNA. Thus, the predominant isoforms expressed in parotid glands are NHE-1 and NHE-2, with no detectable NHE-3 and NHE-4.

The presence of NHE-1 was further documented immunochromically. Acinar cell membranes were probed with an antibody raised against the C-terminal 157 amino acids of NHE-1. The specificity of the antibody was first ascertained comparing Chinese hamster ovary cells transfected with NHE-1 with their untransfected, antiport-deficient counterparts (Fig. 4). The anti-

FIG. 3. Parotid glands express NHE-1 and NHE-2 transcripts. mRNA was extracted from partially purified parotid acinar cells and used as a template for RT-PCR with isoform-specific primers. M, molecular weight markers. P, template was linearized pCMV plasmid containing the full sequence of rat NHE-1 (lane 1), NHE-2 (lane 4), NHE-3 (lane 7), or NHE-4 (lane 10), hybridized with the corresponding primers. AC, template was parotid acinar mRNA, which was reverse transcribed and hybridized with primers specific for NHE-1 (lane 2), NHE-2 (lane 5), NHE-3 (lane 8), or NHE-4 (lane 11). AC, controls using similar template and primers as AC, but reverse transcriptase was omitted. Representative of three separate experiments.

FIG. 4. NHE-1 protein is expressed in parotid glands. Microsomal fractions were separated by gel electrophoresis and immunoblotted with a polyclonal antibody specific for the C-terminal domain of the human NHE-1 isoform. AC mem, acinar cell membranes; AC mem*, acinar membranes after extraction of extrinsic proteins in alkaline carbonate solution; NHE-1*, membranes from antiport-deficient CHO cells stably transfected with the NHE-1 isoform; NHE-1, untransfected antiport-deficient CHO cells. Representative of three separate experiments.

 ISOFORMS OF NHE IN ACINAR CELLS — To better understand the mechanism underlying NHE activation by muscarinic agonists, it was important to establish which isoform(s) of the antiport operate in acinar cells. To this end, we extracted mRNA from parotid glands and assessed the expression of the four well known isoforms of the antiporter (NHE-1 to 4) by RT-PCR (Fig. 3). Isoform-specific primers which hybridized to unique regions of the rat NHE-1, NHE-2, NHE-3, and NHE-4 were used. All four sets of primers yielded the expected PCR products when linearized pCMV plasmids containing the full-length cDNA clone of the corresponding isoform were used as template (Fig. 3, lanes 1, 4, 7, and 10). No discernible products were detected when a specific primer set was used with any of the non-corresponding isoforms as template (not shown). When cDNA obtained by reverse transcription of rat parotid mRNA was used as a template, the NHE-1 primers yielded a product of ~500 bp (Fig. 3, lane 2), while a smaller yield of the expected product (~700 bp) was also observed for NHE-2. The NHE-3 and NHE-4 primers did not yield discernible products in repeated trials (e.g. Fig. 3, lanes 8 and 11). Omission of reverse transcriptase prevented appearance of the 500- and 700-bp products, ruling out contamination with genomic DNA. Thus, the predominant isoforms expressed in parotid glands are NHE-1 and NHE-2, with no detectable NHE-3 and NHE-4. The presence of NHE-1 was further documented immunochromically. Acinar cell membranes were probed with an antibody raised against the C-terminal 157 amino acids of NHE-1. The specificity of the antibody was first ascertained comparing Chinese hamster ovary cells transfected with NHE-1 with their untransfected, antiport-deficient counterparts (Fig. 4). The an-

FIG. 2. pH dependence of the antipor in carbachol-activated cells. A, effect of carbachol (10 μM) on the rate of pH recovery from an acid load. Cells suspended in Na+-rich medium (solution A) were pulsed with 40 mM NH₄Cl (solution C) and then perfused with Na+-free medium (solution D) with (open circles) or without (solid circles) carbachol. pH recovery was next induced by reintroducing extracellular Na+. Traces are data from single cells representative of at least cells 20 cells from six different animals. B, pH dependence of the Na+-induced net H⁻ (equivalent) flux (JₚNa). This was calculated as the sum of the Na+-dependent rate of pHᵢ recovery and the rate of acid loading observed when Na⁺ was removed, multiplied by the buffering power, which was determined independently throughout the pH range of interest as described under “Experimental Procedures.” No spontaneous acid loading was detected in control cells when Na⁺ was removed (n = 25). Straight lines were fitted by least squares using Cricket Graph 3.1.2. and data were analyzed using Statworks. Control cells: closed circles (fitted by the equation y = 1072–146x; r = 0.76, p < 0.001, n = 36). Carbachol-stimulated cells: open circles (fitted by y = 2125–283x; r = 0.88, p < 0.001, n = 28).

ISOFORMS OF NHE IN ACINAR CELLS — To better understand the mechanism underlying NHE activation by muscarinic agonists,
tibody recognized a major band of 110–115 kDa, the expected size of mature NHE-1, in the transfectants but not in the deficient precursor cells. A smaller and sharper band also present in the transfectants but missing in the controls is in all likelihood the incompletely (core) glycosylated form of NHE-1, a biosynthetic precursor. A third polypeptide, present in both samples, is likely nonspecific. As shown in the leftmost lanes of Fig. 4, one major and one minor polypeptide were also recognized by the antibody in acinar cell membranes. Both polypeptides remained associated with the membranes following alkaline extraction of extrinsic components, suggesting that they are transmembrane proteins. The predominant immunoreactive band of acinar cells likely represents the mature form of NHE-1, which is known to be heterogeneously glycosylated (12), accounting for its diffuse mobility on SDS-PAGE. The smaller, sharper band may be the core-glycosylated biosynthetic precursor.

Parotid glands are composed of acini and ducts. Because these were not separated for preparation of RNA or for membrane isolation, it cannot be definitively stated that NHE-1 is present in the acinar cells. To verify this point, the distribution of NHE-1 in parotid slices was assessed immunochemically, using the polyclonal antibody described above. Representative confocal fluorescence images are shown in Fig. 5. The low power image of panel A demonstrates that NHE-1 is present in both ductal and acinar cells. In both instances, the staining is observed predominantly on the basolateral membrane, which can be more clearly discerned in panels B and C. Plasmalemmal immunoreactivity was also evident in isolated acinar cells (Fig. 5D). Such staining likely reflects the presence of NHE-1 on the basolateral membrane, inasmuch as this occupies by far the largest fraction of surface area in acinar cells (1). Whether NHE-1 is present also in the apical membranes cannot be defined unambiguously, but in several instances staining appeared to be minimal in the region of the membrane facing the lumen of the acinus (e.g. arrow in Fig. 5B), suggesting that the apical membranes are largely devoid of NHE-1. Discontinuities in the staining of the luminal membrane are also apparent in ductal cells, suggesting preferential distribution of NHE-1 on the basolateral side.

The preceding results indicate that NHE-1 is present in acinar cells, but do not clarify the contribution of this isoform to the Na\(^{+}/H\(^{+}\) exchange activity across the basolateral mem-

brane. The fraction of the exchange mediated by NHE-1 was assessed pharmacologically in isolated acinar cells using HOE694. This compound inhibits NHE-1, NHE-2, and NHE-3 at widely differing concentrations (26), thus providing a means of discerning between the isoforms. As shown in Fig. 6A, the antiport activity of acinar cells, measured as the Na\(^{+}\)-dependent recovery of pH, from an acid load, could be effectively inhibited by low doses of HOE694. The concentration required for half-maximal inhibition was ~0.06 \(\mu\)M (Fig. 6B), similar to that reported to inhibit NHE-1 (26), and much lower than that needed to inhibit either NHE-2 or NHE-3 (\(K_{0.5}\) of 5 and 650 \(\mu\)M, respectively). Together, the biochemical and functional findings indicate that NHE-1 is the primary Na\(^{+}/H\(^{+}\) antiporter of rat acinar cells. Of note, low doses of HOE694 inhibited the pH recovery effectively both before and after treatment with carbachol. In carbachol-stimulated cells the recovery was inhibited by >95% by 3 \(\mu\)M HOE694, from 4.6 ± 0.53 to 0.2 ± 0.02 pH/min (n = 10; measured at pH 6.8). These data imply that NHE-1 is the isoform mediating muscarinic activation of the antiport.

**Mechanism of Activation of Na\(^{+}/H\(^{+}\) Exchange**

Having established that NHE-1 is the isoform activated by muscarinic agonists in acinar cells, we proceeded to explore the mechanism(s) underlying this form of regulation. Phosphorylation of serine residues within the cytoplasmic (C-terminal) domain has been postulated to mediate receptor-mediated activation of NHE-1 in cultured cells and in platelets (see Refs. 12 and 25, for reviews). Because muscarinic stimulation triggers protein kinase activity in parotid cells, we compared the phosphorylation of NHE-1 before and after challenge with carbchol (Fig. 7A). Freshly isolated cells were labeled with \(^{32}\)P\text{-}orthophosphate for 2 h and, after washing, they were incubated for 2 min with or without 10 \(\mu\)M carbachol at 37°C. Finally, the cells were solubilized and NHE-1 was immunoprecipitated and analyzed by SDS-PAGE and radiography. Immunoblotting with anti-NHE-1 antibody was used to ensure that comparable amounts of the protein were precipitated from control and treated cells.\(^2\) One of four similar experiments is
affected the total $^{32}$P content only moderately and could go undetected.

Phosphorylation sites may exist in the resting state. In this case phosphorylation of a different site. Alternatively, multiple phosphorylation events are likely. In other words, the inactivation had not been elucidated.

The above experiments indicate that net phosphorylation of NHE-1 does not change when cells are acutely stimulated by muscarinic agonists. While the overall phosphate content of NHE-1 appears to remain constant, it is conceivable that phosphorylation of one site occurs and is accompanied by dephosphorylation of a different site. Alternatively, multiple phosphorylation sites may exist in the resting state. In this case phosphorylation or dephosphorylation of a single residue may affect the total $^{32}$P content only moderately and could go undetected when the total radioactivity is compared. To test these possibilities, we carried out phosphopeptide mapping of radio-labeled immunoprecipitates from untreated and stimulated cells. The precipitates were eluted from the beads and denatured with SDS, then hydrolyzed using chymotrypsin. The protease yielded 4 phosphopeptides of molecular mass between 4 and 6 kDa. This pattern was essentially identical whether the reaction had reached completion. Importantly, the phosphopeptide composition and relative intensity of the bands were identical in control and carbachol-treated samples (Fig. 8). These findings indicate that dephosphorylation of one site with concomitant phosphorylation of another is unlikely. In addition, no evidence was found for preferential dephosphorylation of any one of the phosphopeptides resolved by chymotryptic cleavage of NHE-1.

**DISCUSSION**

Primary fluid secretion by rat salivary glands is driven osmotically by transepithelial salt gradients. As in most secretory epithelia, these gradients are generated by Na$^+$-coupled anion uptake across the basolateral membrane. In salivary glands, such secondary active uptake of anions is mediated not only by Na$^+$-K$^+$-2Cldimeteranion transport, but also by Cl$^-$-HCO$_3$ exchange coupled to Na$^+$/H$^+$ exchange via the intracellular pH. By alkalizing the cytosol, NHE is important also in driving HCO$_3$ secretion in guarding the cells against metabolic acidosis. Despite recognition of the important role of the antiporter in fluid secretion in salivary glands, the identity of the isofrom activated by muscarinic stimulation and the molecular details of this activation had not been elucidated.

The present studies provide evidence that NHE-1 is the predominant isofrom in the parotid gland and that it is preferen-
entially localized to the basolateral membrane of acinar cells. This site is in agreement with the reported location of NHE-1 in other epithelia and is consistent with a role for the antiporter in promoting secondary active, pH$_1$-coupled Cl$^-$ entry into the cell. That this isof orm is the major contributor to Na$^+$ uptake in carbachol-stimulated cells was shown using HOE694. At low micromolar or submicromolar concentrations, this benzoylguanidine derivative greatly inhibits NHE-1, while leaving the other isoforms unaffected. In our studies, low doses of HOE694 markedly inhibited Na$^+$-induced H$^+$ extrusion, implicating NHE-1 in the process.

Transcripts encoding NHE-2 have been detected in kidney medulla and cortex, jejunum, ileum, duodenum, stomach, and adrenal gland (11, 14, 27, 28), although the subcellular distribution of this isoform remains controversial. NHE-2 was also detected in the parotid, using RT-PCR. Its location and regulation were not pursued here partly because of unavailability of effective antibodies, but mainly because it seems to contribute little to the muscarinic response of the acinar cells, as judged by the effects of HOE694. NHE-2 may be located on the apical surface of the ductal cells, which failed to stain for NHE-1.

Activation of parotid cells by carbachol resulted in an apparent alkaline shift of the pH dependence of NHE, suggesting increased affinity for intracellular H$^+$. A similar mode of activation was reported earlier in submandibular glands and in a variety of systems where NHE-1 mediates transport. In contrast, there is evidence that activation of NHE-2 entails primarily an increase in maximal velocity, at constant affinity (11). Albeit indirect, this evidence further supports the notion that NHE-1 is the isof orm activated in acinar basolateral membranes.

Despite earlier controversy, it is now generally accepted that NHE-1 can be activated by elevation of intracellular [Ca$^{2+}$]. In the parotid acinar cells, increased [Ca$^{2+}$], was sufficient to mimic the stimulation effect by muscarinic agonists: treatment with thapsigargin induced a Na$^+$-dependent cytosolic alkalinization (not illustrated). Interestingly, the apical isoforms of epithelia (NHE-2 and/or NHE-3) have been suggested to become inhibited when [Ca$^{2+}$] increases (29, 30). This would further argue for a predominant role of NHE-1 in parotid cells.

NHE-1 has been shown to be constitutively phosphorylated in other cells and additional phosphate groups are acquired upon stimulation by growth factors, phorbol esters, or okadaic acid (31, 32). It has been previously postulated that phosphorylation was mediated at least part of the biological responses of NHE-1. It was conceivable that the muscarinic activation, and that elicited by thapsigargin, were similarly mediated by activation of Ca$^{2+}$-dependent kinases and/or inhibition of phosphatases. However, no change in phosphorylation of NHE-1 was detectable when the cells were stimulated. This observation is not without precedent, since the osmotic activation of the antiporter had been demonstrated earlier to occur in the absence of phosphorylation (21) and ionomycin-induced elevation of [Ca$^{2+}$] similarly activated the exchanger without altering the phosphorylation state of NHE-1 (33, 34).

The simplest hypothesis available to explain our observations is that muscarinic stimulation of the antiporter results, at least in part, from the elevation of [Ca$^{2+}$], and formation of Ca$^{2+}$-calmodulin complexes that bind and activate the antiporter directly. Wakabayashi and colleagues (33, 34) reported the existence of two calmodulin-binding domains in NHE-1, which upon binding Ca$^{2+}$-calmodulin are believed to induce a conformational change in the protein that displaces an autoinhibitory domain, thereby stimulating the exchanger. Indeed, deletion of the putative autoinhibitory segment resulted in constitutively activated exchangers. We attempted to demonstrate the applicability of this model to acinar cells. At the concentrations required to block calmodulin, compound W7 completely inhibited the alkalinization of carbachol-stimulated cells. However, recovery of unstimulated cells from an acid load was also impaired. This may reflect a constitutive effect of calmodulin on NHE-1 of unstimulated acinar cells, and/or a direct (nonspecific?) effect of W7 on the antiporter, which complicates the assessment of the role of calmodulin. Another calmodulin inhibitor, calmidazolium, induced a variable acidification of the cells, particularly after stimulation with carbachol. The confounding nature of this acidification again precluded evaluation of the role of calmodulin in muscarinic stimulation. Other approaches to evaluate the role of calmodulin are currently being considered.

In summary, carbachol stimulation of salivary acinar cells results in marked activation of NHE-1, which resides predominantly, if not exclusively, in the basolateral membrane. Such activation is independent of phosphorylation and can be at least partially mimicked by simply raising the concentration of [Ca$^{2+}$], to levels similar to those observed upon muscarinic stimulation. Although direct proof is as yet unavailable, we speculate that interaction of the antiporter with Ca$^{2+}$-calmodulin is largely responsible for the stimulation.

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FIG. 8. Phosphopeptide composition of NHE-1 isolated from control and carbachol-treated cells. Untreated (Con) and carbachol-stimulated (Car) cells were extracted and immunoprecipitated with NHE-1 antibodies. The radiolabeled immunoprecipitates were subjected to proteolytic degradation by chymotrypsin, as described under “Experimental Procedures.” The resulting phosphopeptides were resolved by SDS-PAGE using 15% acrylamide. The position of major phosphopeptides is indicated by arrows. The location of molecular mass markers is also noted. Representative of four similar experiments.
Muscarinic Stimulation of Na\(^+\)/H\(^+\) Exchange

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