Involvement of Direct Phosphorylation in the Regulation of the Rat Parotid Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} Cotransporter*

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We identify a 175-kDa membrane phosphoprotein (pp175) in rat parotid acini whose properties correlate well with the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter previously characterized functionally and biochemically in this tissue. pp175 was the only phosphoprotein immuno precipitated by an anti-Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter antibody and the only membrane protein whose phosphorylation state was conspicuously altered after a brief (45-s) exposure of acini to the \beta-adrenergic agonist isoproterenol. Phosphopeptide mapping provided evidence for three phosphorylation sites on pp175, only one of which was labeled in response to isoproterenol treatment. The half-maximal effect of isoproterenol on phosphorylation of pp175 (∼20 nM) was in excellent agreement with its previously demonstrated up-regulatory effect on cotransport activity. Increased phosphorylation of pp175 was also seen following acinar treatment with a permeant cAMP analogue and with forskolin, conditions that have likewise been shown to up-regulate the cotransporter. Combined with earlier results from our laboratory, these data provide strong evidence that the up-regulation of the cotransporter by these agents is due to direct phosphorylation mediated by protein kinase A. AlF\textsubscript{4}\textsuperscript{−} treatment, which results in an up-regulation of cotransport activity comparable with that observed with isoproterenol (∼6-fold), caused a similar increase in phosphorylation of pp175. However, hypertonic shrinkage and treatment with the protein phosphatase inhibitor calyculin A, which also up-regulate the cotransporter (∼3-fold and ∼6-fold, respectively) caused no change in the phosphorylation level. Furthermore, although acinar treatment with the muscarinic agonist carbachol results in a dramatic up-regulation of cotransport activity and a concomitant phosphorylation of pp175, no phosphorylation of pp175 was seen with the Ca\textsuperscript{2+}-mobilizing agent thapsigargin, which is able to fully mimic the up-regulatory effect of carbachol on transport activity. Taken together, these results indicate that direct phosphorylation is one of the mechanisms involved in secretagogue-induced regulation of the rat parotid Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter.

Because of its experimental accessibility, relative homogeneity and rich hormonal responsiveness, the rat parotid gland is rapidly becoming one of the more popular mammalian experimental models for the study of the mechanism(s) and regulation of epithelial fluid and electrolyte secretion (1, 2). Work from a number of laboratories has established that salt and water secretion by the acinar cells, which comprise the bulk of this gland, is due to transepithelial Cl\textsuperscript{−} movement (1–3). The active step in this process is Cl\textsuperscript{−} entry across the acinar basolateral membrane, a large component of which has been shown to be due to Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransport (4, 5).

Consistent with its importance role in secretion, we have shown that the activity of the rat parotid Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter is regulated by a number of physiological and other potentially physiologically relevant stimuli. We first demonstrated a substantial (∼6-fold) up-regulation of cotransporter activity following \beta-adrenergic stimulation and provided good evidence that this was due to a phosphorylation event mediated by cyclic AMP-dependent protein kinase (6). This up-regulation is paralleled in vivo by an increase in salivary flow seen when sympathetic (adrenergic) stimulation, arising, for example, from mastication, is superimposed on parasympathetic (muscarinic) stimulation (7), the main fluid secretory stimulus for the gland. In a later publication (8) we demonstrated that the rat parotid Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter is up-regulated (again ∼6-fold) by aluminum fluoride (AlF\textsubscript{4}\textsuperscript{−}), an activator of G-proteins, and by calyculin A, a protein phosphatase inhibitor. Based on several factors, including diverse sensitivity to blockade of up-regulation by protein kinase inhibitors and the observation that AlF\textsubscript{4}\textsuperscript{−} does not induce CAMP generation in the rat parotid, we have argued that the mechanisms of action of AlF\textsubscript{4}\textsuperscript{−} and calyculin A on the cotransporter are different from that of \beta-adrenergic stimulation and from one another (8).

More recently (9) we have shown that Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransport activity in these cells is also increased by muscarinic stimulation (∼15-fold) and by hypertonic shrinkage (∼3-fold). Our data suggest that these latter effects are also unrelated to one another and unrelated to the effect of \beta-adrenergic stimulation (see “Discussion”). At this time our understanding of these up-regulatory events is still incomplete, and the physiological significance of some of these stimuli remains to be determined. However, our results clearly demonstrate that the rat parotid Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter is under tight regulatory control, in all likelihood by multiple intracellular signaling pathways, and thus that it provides a particularly rich experimental system for the study of transport regulation by hormonal and other stimuli.

In the present paper we explore these phenomena further by studying the effects of these various up-regulatory stimuli on the phosphorylation state of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransport protein itself. Although it is generally accepted that phosphorylation events play an important role in cellular signaling, relatively few studies have actually directly explored their possible

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involvement in the regulation of facilitative membrane trans-
port proteins. We show here that there is a good correlation between the transport activity and increased phosphorylation following β-adrenergic stimulation and AIF₂ treat-
ment of rat parotid acini, suggesting that the regulation of the cotransporter by these stimuli is due to direct phosphoryl-
ation. Somewhat surprisingly, however, this was not the case for the other stimuli studied, in spite of the fact that some of these agents have been shown to increase both the transport activity and the phosphorylation state of Na⁻⁻⁺-K⁺-2Cl⁻ cotrans-
porters in lower species (11, 12). These observations indicate that the Na⁻⁻⁺-K⁺-2Cl⁻ cotransporter in the rat parotid is reg-
ulated both via direct phosphorylation and via other, as yet unidentified, mechanisms.

**EXPERIMENTAL PROCEDURES**

Materials and Media—Male Wistar strain rats, weighing 250–300 g, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Carrier-free ³²P (10 μCi/ml) and [¹⁴C]bumetanide (80.8 Ci/mmol) were obtained from Amersham Corp. Collagenase, P, protease inhibitors, and dibutylryl camp were from Boehringer Mannheim. (−)-Isoproterenol, phorbol 12-myristate 13-acetate (PMA), V⁸ protease, and bovine serum albumin (albumin number A6003) were purchased from Sigma. Calyculin A and forskolin were from Calbiochem. Phosphatidylinerine (number 840032, from bovine brain; supplied in chloroform) was from Avanti Polar Lipids (Birmingham, AL). Molecular weight standards, prepared 4–20% SDS-PAGE gels, and prepoured 16% Tricine gels were obtained from Novex Electro-Technology (San Diego, CA). Protein G-Sepharose beads were from Pierce. All other chemicals were from standard com-
mercial sources and were reagent grade or the highest purity available.

The digestion medium was Earle's minimum essential medium (Biogenesis, Rockville, MD) containing 0.22 units/ml collagenase P, 2 mM glucose, and 1% bovine serum albumin. The physiological salt solution (PSS) contained 135 mM NaCl, 5.8 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄, 0.73 mM NaH₂PO₄, 11 mM glucose, 20 mM HEPES (pH 7.4 with NaOH), 2 mM glutamine, and 1% bovine serum albumin. The digestion medium and PSS were continuously gassed with 95% O₂, 5% CO₂ and 8% H₂O, respectively. The stop solution for the ³²P labeling studies was 0.3% Triton X-100 and no NaCl and kept on ice for 30 min. This sample was then centrifuged at 100,000 × g for 30 min. The supernatant and pellet from this second high speed spin are referred to as the “Triton extract” and the “Triton-insoluble fraction” respectively. As shown below (see “Results”) the Triton extract was the only fraction that contained the phosphopeptide of interest (the Na⁺⁻⁺-K⁺-2Cl⁻ cotransporter), and thus only this fraction was usually re-
tained and analyzed.

Gel Electrophoresis and Autoradiography—SDS-PAGE was performed essentially as described by Laemmli (13) using a 4% polyacry-
lamide stacking gel and a 4–20% (continuous gradient) polyacrylamide separating gel. Tricine-SDS (16%) electrophoresis was carried out ac-
cording to Schagger and von Jagow (14). Samples were equilibrated for 2 min in 25% SDS, 50 mM Tris-Cl (pH 6.8), 4% glycerol, 100 mM dithiothreitol, and 0.04% bromophenol blue and centrifuged before electrophoresis. Gels were stained with Coomas-
ise Blue, dried, and visualized by autoradiography using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY). Gels used in phosphopeptide mapping studies were washed twice with 15 min in 20% ethanol before drying to remove acetic acid. Autoradiographs were scanned using a Molecular Dynamics computing densitometer (Molecular Dynamics, Sunnyvale, CA) to quantitate ³²P labeling. Linearity of the densitomet-
ric scans was confirmed using autoradiographic ¹⁴C Micro-Scales (Am-
ersham Corp.). Samples of Triton extract electrophoresed for quantita-
tion of ³²P labeling typically contained 15–150 μg of protein (the protein concentration of the Triton extract was 0.5 mg/ml).

Production of Antiserum against Parotid Bumetanide Binding Protein—The following method was used to produce sheep antiserum di-
rected against a rabbit parotid bumetanide binding protein previously iden-
tified in our laboratory as the bumetanide moiety (and perhaps all) of the Na⁺⁻⁺-K⁺-2Cl⁻ cotransporter in this tissue (15). Deglycosylated 
bumetanide binding protein (Mᵦ = 135,000) was purified from rabbit 
parotid basolateral membranes as described previously (15). A suitable 
quantity of protein (100 μg for the first injection and 30–40 μg for subsequent injections) was diluted to 1 ml with phosphate-buffered saline and combined with 1 ml of complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. Injections were carried out at the NIH Animal Care Center (Ungulate Section) in Poolesville, Maryland. The primary injection was subcutaneous, and subsequent injections (3, 8, 19, and 36 weeks later) were intramuscular; all injec-
tions were done at multiple sites. Anti-bumetanide binding protein antibody titer in serum samples was monitored by Western blot anal-
ysis against purified bumetanide binding protein and rabbit parotid baso-
lateral membranes. In these samples the antiserum strongly la-
beled proteins of Mᵦ = 135,000 and 160,000–175,000, respectively. The range of molecular weights observed for the labeling of native mem-
branes (160–175 kDa) was related to the gel system used. With the large format Bio-Rad gels used in our previous publication (15) we 
observed an Mᵦ of 160,000 (in this same publication we showed that the parotid bumetanide binding protein had a native Mᵦ of 160,000 and a deglycosylated Mᵦ of 135,000), while with the prepevoked Novex minigels used in the present work we consistently observed a Mᵦ of ~175,000.

Immunoprecipitation of ³²P-Labeled Proteins—Protein G-Sepharose 
beads were prewashed twice with washing buffer (extraction buffer titrated to pH 8.6 with Tris and containing 0.1% SDS and 300 μM NaCl) and in low pH buffer (150 mM glycine-HCl (pH 2.3), containing 150 mM NaCl and 0.5% Triton X-100), and then in extraction buffer and then suspended in extraction buffer containing 1% ovalbumin.

A 500 μl aliquot of Triton extract was incubated overnight at 4°C with immune or non-immune sheep serum (7 μg/100 μl of extract protein). Prewashed protein G-Sepharose beads (10 μl of beads/μl of
serum) were then added. After 30 min of additional incubation, the beads were collected by centrifugation and washed six times with washing buffer. The tube was changed for the last spin. Protein retained by the washed beads was then eluted with 100 μl of electrophoresis sample buffer.

Immunoprecipitation of [3H]Bumetanide Binding Activity—Triton extracts for bumetanide binding studies were prepared by a modification of the procedure given above. This modification was based on our previous observation that the bumetanide binding activity of the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter could be preserved in detergent solutions by the addition of suitable exogenous lipids (16, 17). The procedure was as follows. A “particulate fraction” was prepared as described above from cells that had not been labeled with \textsuperscript{32}P. However, instead of extraction buffer, the particulate fraction was resuspended in Buffer K (100 mM mannitol, 10 mM HEPES, 1 mM EDTA, 195 mM potassium gluconate, and 5 mM KCl buffered to pH 7.4 with Tris). This material was centrifuged again at 100,000 × g for 1 h, and the resulting pellet was resuspended in Buffer K at a protein concentration of 2.5–3.5 mg/ml, fast frozen in aliquots, and stored above liquid nitrogen. On the day of the bumetanide binding experiment, frozen samples were thawed and diluted with Buffer K to a protein concentration of 2 mg/ml. This suspension was mixed with the same volume of 0.6% Triton X-100 in Buffer K, left on ice for 10 min, and then transferred to a glass tube in which a suitable volume of phosphatidylserine (final concentration 0.15%) had been evaporated. This mixture was sonicated to a Branson B-12 bath sonicator (−60°C immersion) and centrifuged at 100,000 × g for 1 h. The resulting supernatant, which is analogous to the “Triton extract” described above for \textsuperscript{32}P labeling studies, is referred to here as the “lipid-stabilized Triton extract.”

Immunoprecipitation of [3H]Bumetanide binding activity from the above lipid-stabilized Triton extracts was carried out using immune and nonimmune IgG preabsorbed onto protein G-Sepharose beads. This was done in order to avoid any possible interference of serum with the immunoprecipitations (see “Results”). Protein G beads were washed twice with Buffer K containing 0.3% Triton X-100 and 0.15% phosphatidylserine (sonicated to clarity as above) and then resuspended in the same buffer containing 2% BSA and incubated for 40 min with immune or nonimmune sheep serum (10 μl of beads/μl of serum; total volume ~300 μl). The beads were then washed three times in Buffer K plus 0.3% Triton X-100 and 0.15% phosphatidylserine, added to the lipid-stabilized Triton extract (70 μl of beads/100 μg of extract protein), and incubated for 2 h at 4°C. After removal of the beads by centrifugation, [3H]bumetanide binding activity remaining in the resulting supernatant was determined by the method given below.

[3H]Bumetanide Binding Assay—Equilibrium bumetanide binding was measured using a nitrocellulose filtration assay as described previously (16, 18). Briefly, a 20-μl aliquot of sample was combined with 20 μl of incubation medium consisting of either Buffer K containing 10 μCi/ml [3H]bumetanide or the same medium with all potassium replaced by Na+. After a 15-min incubation the reaction was terminated by the addition of ice-cold stop solution followed by Millipore filtration (HAWP 0.45 μm). Other procedures were as described previously (16, 18). [3H]Bumetanide binding observed in the absence of Na\textsuperscript{+} was subtracted from that observed in its presence to yield the Na\textsuperscript{+}-dependent component of binding. In previous studies (16, 18) we have demonstrated that this Na\textsuperscript{+}-dependent component of binding represents the specific binding of bumetanide to its inhibitory site on the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter.

Phosphopeptide Mapping—Triton extracts for phosphopeptide mapping studies were prepared as described above for \textsuperscript{32}P-labeling studies, except that the extraction buffer contained 1% Triton X-100 and the protein concentration of the extract was ~3 mg/ml. Following SDS-PAGE (~90 μg of extracted protein/lane) the band corresponding to the Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter (pp175, see “Results”) was identified using autoradiography, cut from the dried gel, and swollen in 1 ml of a buffer containing 50 mM NH\textsubscript{4}HCO\textsubscript{3} (pH 8.0), 1 mM dithiothreitol, and 20 μg/ml V8 protease. After 6 or 12 h of incubation at 37°C protease digestion was terminated by heating the sample to 100°C for 5 min. The liquid was set aside, and the gel slice was then sequentially incubated in 500 μl of distilled water for 2 h, 500 μl of distilled water for 1 h, 500 μl of 0.1% SDS for 1.5 h, and 500 μl of 0.1% SDS for 1 h. All of these samples were combined (total volume 3 ml) and dried in a Savant DNA Speed Vac (Savant Instruments Inc., Farmingdale, NY). This final V8 protease digest was then taken up in sample buffer and subjected to Tricine-SDS gel electrophoresis.

Data Analysis—All experiments were repeated three or more times with similar results. Data are given as means ± S.E.
diagrams of the Triton extracts (large arrow); its phosphorylation state is likewise markedly increased by isoproterenol, and its presence in the Triton extract indicates that it is an integral membrane protein.

A number of factors discussed in the remainder of the paper provide strong evidence that the 175-kDa phosphoprotein (pp175) identified above is (a major part or all of) the rat parotid Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter.

Demonstration of a Strong Correlation between Phosphorylation of pp175 and cAMP-dependent Up-regulation of the Parotid Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) Cotransporter—In our previous report (6) we demonstrated that the half-maximal effect of isoproterenol for up-regulation of the rat parotid Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter was seen at \(-20 \text{ nM}\), measured after 37.5 s of agonist incubation at 37°C. The dose response of isoproterenol for the phosphorylation of pp175, measured under essentially identical experimental conditions, is illustrated in Fig. 2A. The half-maximal effect of isoproterenol for phosphorylation is also seen at \(-20 \text{ nM}\), in excellent agreement with its effect on the up-regulation of cotransport activity.

In parotid acinar cells, cAMP is thought to be the major intracellular messenger mediating the effects of \(\beta\)-adrenoreceptor stimulation. In our earlier work (6) we also demonstrated that significant up-regulation of cotransport activity was seen following acinar treatment with permeant analogues of cAMP and with forskolin, which increases intracellular cAMP by direct activation of the catalytic subunit of adenylate cyclase. Consistent with the effects of these agents on transport, in Fig. 2B we showed that increased phosphorylation of pp175 is likewise seen when acini are treated with the permeant cAMP analogue dibutyryl cAMP and with forskolin.

The strong correlation established in Fig. 2 between the effects of isoproterenol and cAMP on phosphorylation of pp175 and their previously documented effects on Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport activity (6) supports the hypotheses that pp175 is the rat parotid Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter and that its up-regulation by isoproterenol is due to direct phosphorylation.

Immunoprecipitation of pp175 by an Anti-Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) Cotransporter Antibody—In order to further confirm that pp175 is indeed the rat parotid Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter we carried out several studies using a polyclonal antiserum raised against a rabbit bumetanide binding protein previously identified in our laboratory as the bumetanide binding moiety of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter in this tissue (see "Experimental Procedures" and Ref. 15). In Western blots of purified rat and rabbit parotid basolateral membranes this antiserum recognizes a broad band with \(M_r \sim 175,000\), which closely resembles the autoradiographic images of pp175 in Fig. 1.\(^a\) In addition, as illustrated in Fig. 3, this antiserum quantitatively immunoprecipitates the Na\(^{+}\)-dependent component of bumetanide binding (the specific binding of bumetanide to the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter) from a lipid-stabilized Triton extract of the rat parotid particulate fraction. In Fig. 3 we compare the Na\(^{+}\)-dependent component of bumetanide binding in the lipid-stabilized Triton extract with the activity of the same extract after immunoprecipitation with nonimmune serum (N) or immune serum (I) raised against the rabbit parotid bumetanide binding protein. No significant Na\(^{+}\)-dependent bumetanide binding activity remains in the supernatant after immunoprecipitation with immune serum (7.6 ± 5.3% of control; \(n = 3\)), while all binding is retained after precipitation with nonimmune serum. In addition, densitometric scans of Coomassie Blue-stained SDS-PAGE gels of the supernatants remaining after immune or nonimmune precipitation were virtually superimposable except for the region around \(M_r \sim 175 \text{ kDa}\), where a broad minor protein band was missing from the sample treated with immune serum (not shown).

When this polyclonal antiserum was used in immunoprecipitation studies with Triton extracts from \(^{32}\text{P}\)-labeled cells it specifically precipitated pp175. This result is illustrated in Fig. 4. Here we compare autoradiographs of SDS-PAGE gels of material immunoprecipitated with nonimmune (N) or immune (I) serum from extracts of \(^{32}\text{P}\)-labeled acini pretreated with (+) or without (−) 1 \(\mu\text{M}\) isoproterenol. A single phosphoprotein appearing as a diffuse band centered at \(M_r \sim 175,000\) whose phosphorylation is markedly increased by isoproterenol treatment is seen in the precipitate from immune serum. No phosphoproteins were immunoprecipitated by nonimmune serum.

\(^a\) S. J. Tessler, M. L. Moore, S. J. Reshkin, and R. J. Turner, unpublished results.
Fig. 3. Immunoprecipitation of Na\(^+\)-dependent bumetanide binding activity by antiserum against the parotid bumetanide binding protein. A lipid-stabilized Triton extract of the particulate fraction of rat parotid acini was prepared and subjected to immunoprecipitation using protein G-Sepharose beads preincubated with nonimmune sheep serum or immune serum raised against the rabbit parotid bumetanide binding protein (see “Experimental Procedures”). The Na\(^+\)-dependent component of \(^{3}H\)bumetanide binding (see “Experimental Procedures”) in the lipid-stabilized Triton extract before immunoprecipitation (Control) and in the supernatant remaining after immunoprecipitation with immune (I) or nonimmune (N) serum are shown. The results are the means ± S.E. of three independent experiments. The data from each experiment were normalized to the binding observed in the lipid-stabilized extract before immunoprecipitation (5.28 ± 0.75 pmol/mg protein, n = 3); the data were also corrected for the dilution arising from addition of protein G-Sepharose beads.

Fig. 4. Immunoprecipitation of pp175 by antiserum against the parotid bumetanide binding protein. Rat parotid acini labeled with \(^{32}\)P were incubated with (+) or without (−) 1 μM isoproterenol for 45 s at 37°C. The Triton extract of the particulate fraction was then immunoprecipitated with immune (I) or nonimmune (N) sheep serum as described under “Experimental Procedures.” The figure shows autoradiographs of SDS-PAGE gels of the initial Triton extracts on the left, the immunoprecipitates from untreated cells in the center, and the immunoprecipitates from isoproterenol-treated cells on the right, as indicated.

In addition, when the supernatants remaining after immunoprecipitation with immune serum were examined by SDS-PAGE and autoradiography, no \(^{32}\)P-labeled band at 175 kDa was detectable (not shown), indicating that all of the labeled protein at 175 kDa is recognized by the anti-Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter antibody.

Fig. 5. Phosphopeptide mapping of pp175 using V8 protease. Rat parotid acini were labeled with \(^{32}\)P, and then incubated with (+) or without (−) 1 μM isoproterenol for 45 s at 37°C as usual. Digestion of pp175 with V8 protease (for 6 or 12 h, as indicated) and Tricine-SDS electrophoresis were then carried out as described under “Experimental Procedures.” The figure shows an autoradiograph of a representative Tricine-SDS gel. The positions of the molecular weight markers are indicated on the left of the autoradiograph.

Phosphopeptide Mapping—Fig. 5 shows the result of digestion of pp175 with V8 protease. In this experiment \(^{32}\)P-labeled pp175 from acini treated with (+) or without (−) isoproterenol was incubated with V8 protease for 6 or 12 h as indicated (see “Experimental Procedures”), and the resulting digests were separated by Tricine-SDS gel electrophoresis and visualized by autoradiography. Three \(^{32}\)P-labeled bands are clearly seen in Fig. 5 at 16.6 ± 1.1, 7.5 ± 0.5, and 5.7 ± 0.1 kDa, respectively (n = 4 for these data and all those given below). Scanning densitometry revealed that only the 16.6-kDa peptide showed a significant change in phosphorylation with isoproterenol treatment (with pp175 from isoproterenol-treated acini, densities of the 16.6-, 7.5-, and 5.7-kDa bands determined after 6 h of digestion with V8 protease were 3.6 ± 0.9, 0.97 ± 0.10, and 1.21 ± 0.16 times their levels without isoproterenol treatment, respectively; after 12 h of digestion these ratios were 2.74 ± 0.48, 0.82 ± 0.09, and 0.79 ± 0.07, respectively).

On average little difference was found between the phosphorylation patterns observed after 6 and 12 h of V8 protease digestion. The density of labeling of the 7.5-kDa band did, however, decrease significantly between 6 and 12 h of protease treatment (20 ± 5% and 31 ± 9% decreases were found in digests from control and isoproterenol-treated cells, respectively), presumably indicating continued slow digestion of this peptide by V8 protease. No other significant increase or decrease in labeling with time of protease treatment was observed. In particular, paired t tests provided no evidence for a systematic shift of \(^{32}\)P from the 7.5-kDa to the 5.7-kDa peptide with time. This observation argues against the possibility that the latter peptide may be a digestion product of the former. Since all of the labeled protein at 175 kDa is recognized by our anti-Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter antibody (see above), all three of these labeled peptides are presumably associated with the transporter. Thus the results illustrated in Fig. 5 are consistent with the presence of at least three phosphorylation sites on pp175, only one of which is phosphorylated in response to isoproterenol treatment.

Effects of Other Stimuli on Phosphorylation of pp175—The effects of acinar treatment with AlF\(_4\)\(^-\), calyculin A, and hyper tonic medium on phosphorylation of pp175 are illustrated in Fig. 5. Interestingly, although treatment with the concentrations of AlF\(_4\) and calyculin A used in Fig. 6 both yield comparable up-regulations of cotransport activity to that produced by...
NaNO₃. Previous work from our laboratory has shown that the by diluting acini in PSS 1:1 with PSS in which NaCl was replaced with reduced by 1

In this paper we identify a 175-kDa membrane phosphoprotein whose properties correlate well with intracellular chloride concentration have been shown to produce significant change in the phosphorylation level of pp175.

In their study of the phosphorylation of the shark rectal gland experimental maneuvers that reduce intracellular chloride concentration have been shown to result in up-regulation of Na⁺⁻K⁺⁻2Cl⁻ cotransport activity and phosphorylation of the Na⁺⁻K⁺⁻2Cl⁻ cotransporter protein (11). However, as also illustrated in Fig. 6, switching rat parotid acini to low chloride medium has no effect on the phosphorylation level of pp175.

Recent work in our laboratory (9) has demonstrated a dramatic up-regulation of rat parotid Na⁺⁻K⁺⁻2Cl⁻ cotransport activity by muscarinic stimulation (−15-fold and −25-fold after 30 s of stimulation with 1 μM and 10 μM of the muscarinic agonist carbachol, respectively). In Fig. 7 we show that this up-regulation is paralleled by increased phosphorylation of pp175. However, in additional experiments (9) we have shown that the up-regulatory effect of carbachol on the cotransporter can be duplicated by the micromolar Ca²⁺-ATPase inhibitor thapsigargin, which raises intracellular calcium concentration to levels comparable with that seen with carbachol, but without interacting with plasma membrane receptors, and without activating protein kinase C. We illustrate in Fig. 7 that treatment of acini with 1 μM thapsigargin under conditions that yield an up-regulation of cotransport activity comparable with that produced by 1 μM carbachol (Ref. 9 and data not shown), results in no significant phosphorylation of pp175. In addition, treatment of acini with the active phorbol ester PMA to activate protein kinase C yields no phosphorylation of pp175 in the presence or absence of thapsigargin (Fig. 7).

**DISCUSSION**

In this paper we identify a 175-kDa membrane phosphoprotein (pp175) in the rat parotid whose properties correlate well with the Na⁺⁻K⁺⁻2Cl⁻ cotransporter characterized functionally in this gland (6) and with Na⁺⁻K⁺⁻2Cl⁻ cotransporters previously identified biochemically in salivary glands (15) and other tissues (11, 20–23). pp175 was the only membrane protein whose phosphorylation state was conspicuously altered after a brief (45-s) exposure to the cAMP-mobilizing secretagogue isoproterenol (Fig. 1). We have previously demonstrated that isoproterenol treatment results in a substantial up-regulation of Na⁺⁻K⁺⁻2Cl⁻ cotransport activity in the rat parotid and provided strong evidence that this was due to a phosphorylation event mediated by protein kinase A (6). Consistent with the identification of pp175 as the Na⁺⁻K⁺⁻2Cl⁻ cotransporter, the half-maximal effect of isoproterenol on phosphorylation of pp175 (−20 nm; Fig. 2A) was in excellent agreement with its half-maximal effect on cotransport activity (6). Phosphopeptide mapping provided evidence for three phosphorylation sites on pp175 (Fig. 5), only one of which was labeled in response to isoproterenol treatment.

Increased phosphorylation of pp175 was also seen following acinar treatment with a permeant cAMP analogue and with forskolin (Fig. 2B), conditions that have also been shown to up-regulate the cotransporter, presumably by the same mechanism as isoproterenol (6). In addition, pp175 was the only phosphoprotein immunoprecipitated by an antibody raised against the rabbit parotid Na⁺⁻K⁺⁻2Cl⁻ cotransporter (Fig. 4). This antibody also quantitatively immunoprecipitated sodium-dependent bumetanide binding activity from a detergent extract of the rat parotid (Fig. 3) consistent with the expected properties of an anti-cotransporter antibody. Taken together with its molecular weight, which is in the expected range (150,000–195,000) of previously identified Na⁺⁻K⁺⁻2Cl⁻ cotransporters (15, 20–23), the above results provide convincing evidence that pp175 is the Na⁺⁻K⁺⁻2Cl⁻ cotransporter of the rat parotid.

The effects of cAMP-dependent secretagogues on the phosphorylation state of Na⁺⁻K⁺⁻2Cl⁻ cotransporters recently identified in the shark rectal gland (Ref. 11; a 195-kDa phospho-protein) and the avian salt gland (Ref. 23; a 170-kDa phosphoprotein) have also been studied. Consistent with the results presented here, in both these tissues a strong correlation between apparent up-regulation of transport activity and cotransporter phosphorylation was observed (11, 23). In their study of the phosphorylation of the shark rectal gland Na⁺⁻K⁺⁻2Cl⁻ cotransporter, Lytle and Forbush (11) also showed that transport up-regulation by osmotic shrinkage is
accompanied by parallel increases in transporter phosphorylation. In addition, they showed that maneuvers that decrease intracellular chloride concentration in this tissue result in increased cotransporter phosphorylation. This latter observation is consistent with previous suggestions from this group that the decreased intracellular chloride concentration that accompanies secretion by the gland may itself play a role in the activation of the cotransporter (10, 11, 22). In the avian salt gland Torchia et al. (12, 23) have demonstrated that Ca$^{2+}$-mobilizing secretagogues also result in cotransporter phosphorylation. This is apparently due to the combined effect of increased intracellular calcium concentration and activation of protein kinase C, since it can be mimicked by the application of a Ca$^{2+}$-ionophore plus an active phorbol ester but not by either of these treatments alone (12). Treatment with the protein phosphatase inhibitor okadaic acid also resulted in cotransporter phosphorylation in the avian salt gland (12).

The above results from the shark rectal gland and avian salt gland are consistent with the hypothesis that direct phosphorylation, possibly at different sites by different stimuli, plays a central role in the regulation of Na$^{+}$-K$^{+}$-2Cl$^{-}$ cotransporter activity in these tissues. However, the situation is clearly more complex in the rat parotid. Although the up-regulation of transport activity seen with isoproterenol and AlF$_4^-$ (both ~6-fold) correlates well with the phosphorylation of pp175 induced by these agents (Figs. 2 and 6), this is not the case for treatment with the protein phosphatase inhibitor calyculin A or hypertonic shrinkage (Fig. 6). Despite the fact that both these latter treatments result in significant up-regulation of the cotransporter (~6-fold and ~3-fold, respectively; Ref. 8 and Footnote 1), neither causes significant phosphorylation of pp175. Furthermore, although acinar treatment with the muscarinic agonist carbachol results in a dose-dependent phosphorylation of pp175 (Fig. 7), no phosphorylation is produced by the Ca$^{2+}$-mobilizing agent thapsigargin, which is able to fully mimic the dramatic up-regulatory effect of carbachol on transport activity (~15-fold for treatment with either 1 mM carbachol or 1 mM thapsigargin; Ref. 9). Moreover, treatment of acini with the active phorbol ester PMA to activate protein kinase C yielded no phosphorylation of pp175 in the presence or absence of thapsigargin (Fig. 7). Taken together, these latter results indicate that the phosphorylation of pp175 seen with muscarinic stimulation is not required for up-regulation of cotransport activity in the rat parotid and, in addition, suggest that this phosphorylation is not due to protein kinase C.

Finally, two observations made here indicate that the increase in cotransporter phosphorylation associated with decreased intracellular chloride concentration in the shark rectal gland (see above) is not seen in the rat parotid: (i) increased phosphorylation of pp175 is not observed after suspension of acini in low chloride medium (Fig. 6), and (ii) increased phosphorylation of pp175 is not observed after thapsigargin treatment (Fig. 6), which is expected to lead to a secretion-induced decrease in intracellular chloride concentration similar to that observed with muscarinic stimulation (4).

As already indicated, the experimental evidence available to date suggests that the up-regulations of the parotid Na$^{+}$-K$^{+}$-2Cl$^{-}$ cotransporter by treatment with β-adrenergic agonists, muscarinic agonists, hypertonic shrinkage, AlF$_4^-$, and calyculin A, all occur via different intracellular mechanisms. Briefly stated, this conclusion is based on the observations that the effects of β-adrenergic and muscarinic stimulation are secondary to increased intracellular levels of cAMP (6) and Ca$^{2+}$ (9), respectively, while the effects of hypertonic shrinkage, AlF$_4^-$, and calyculin A are apparently independent of both of these intracellular messengers (8). The effects of these latter three stimuli on the cotransporter can, however, be distinguished by their sensitivities to inhibition by the compound K252a (Ki = 0.6 μM, ~20 μM, and ~20 μM, respectively; Ref. 8 and Footnote 1). It is nevertheless always possible that the effects of some of these stimuli may be related. For example, two stimuli may act at different steps in the same up-regulatory pathway, resulting in the apparent differences discussed above.

We also considered the possibility that agents which resulted in an up-regulation of cotransport activity without a concomitant increase in the phosphorylation of pp175 might be acting via phosphorylation of another membrane-associated protein. However, close examination of autoradiographs of Triton extracts from cells treated with thapsigargin, hypertonic shrinkage, or calyculin A did not reveal changes in the phosphorylation pattern of proteins at any molecular weight.

The results presented here support the hypothesis that the up-regulations of the rat parotid Na$^{+}$-K$^{+}$-2Cl$^{-}$ cotransporter by β-adrenergic stimulation and AlF$_4^-$ treatment are due to direct phosphorylation of the transporter itself, whereas other mechanisms are clearly involved in the up-regulatory effects of muscarinic stimulation, hypertonic shrinkage, and calyculin A treatment.

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