Ouabain potentiates the activation of ERK1/2 by carbachol in parotid gland epithelial cells; inhibition of ERK1/2 reduces Na\(^+\)-K\(^+\)-ATPase activity

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Plourde, Deana, and Stephen P. Solttof. Ouabain potentiates the activation of ERK1/2 by carbachol in parotid gland epithelial cells; inhibition of ERK1/2 reduces Na\(^+\)-K\(^+\)-ATPase activity. Am J Physiol Cell Physiol 290: C702–C710, 2006. First published October 19, 2005; doi:10.1152/ajpcell.00213.2005.—The Na\(^+\)-K\(^+\)-ATPase and the ERK1/2 pathway appear to be linked in some fashion in a variety of cells. The Na\(^+\)-K\(^+\)-ATPase inhibitor ouabain can promote ERK1/2 activation. This activation involves Src, intracellular Ca\(^2+\) concentration ([Ca\(^2+\)]) elevation, reactive oxygen species (ROS) generation, and EGF receptor (EGFR) transactivation. In contrast, ERK1/2 can mediate changes in Na\(^+\)-K\(^+\)-ATPase activity and/or expression. Thus signaling between ERK1/2 and Na\(^+\)-K\(^+\)-ATPase can occur from either direction. Whether such bidirectionality can occur within the same cell has not been reported. In the present study, we have demonstrated that while ouabain (1 mM) produces only a small (<50%) increase in ERK1/2 phosphorylation in freshly isolated rat salivary (parotid acinar) epithelial cells, it potentiates the phosphorylation of ERK1/2 by submaximal concentrations of carbachol, a muscarinic receptor ligand that initiates fluid secretion. Although ERK1/2 is only modestly phosphorylated when cells are exposed to 1 mM ouabain or 10^(-6) M carbachol, the combination of these agents promotes ERK1/2 phosphorylation to near-maximal levels achieved by a log order carbachol concentration. These effects of ouabain are distinct from Na\(^+\)-K\(^+\)-ATPase inhibition by lowering extracellular K\(^+\), which promotes a rapid and large increase in ERK1/2 phosphorylation. ERK1/2 potentiation by ouabain (EC50 ~100 μM) involves PKC, Src, and alterations in [Ca\(^2+\)], but not ROS generation or EGFR transactivation. In addition, inhibition of ERK1/2 reduces Na\(^+\)-K\(^+\)-ATPase activity (measured as stimulation of QO2 by carbachol and the cationophore nystatin). These results suggest that ERK1/2 and Na\(^+\)-K\(^+\)-ATPase may signal to each other in each direction under defined conditions in a single cell type.

protein kinase C; intracellular Ca\(^2+\) concentration; muscarinic receptor; α1-subunit; potassium removal

Na\(^+\)-K\(^+\)-ATPase (Na\(^+\) pump) is an energy-transducing ion transporter that plays a critical role in maintaining the Na\(^+\) and K\(^+\) ion gradients across the plasma membrane in most mammalian cells. It mediates the electrogenic exchange of three intracellular Na\(^+\) ions for two extracellular K\(^+\) ions, a reaction that consumes one molecule of ATP. Its role in physiological events and the elucidation of the biochemical and molecular nature of this enzyme have been documented extensively during the past 50 years (4). Ouabain, a cardiac glycoside, binds to the α-subunit of the Na\(^+\)-K\(^+\)-ATPase (28) and inhibits its activity. Recently, ouabain was found to act not merely as an inhibitory ligand of the Na\(^+\)-K\(^+\)-ATPase but also as an initiator of cellular signals transduced by Na\(^+\)-K\(^+\)-ATPase in vascular smooth muscle cells (11), cardiac myocytes (15), renal epithelial cells (6), and other cells and cell lines (for review, see Ref. 35). In some cells, ouabain can initiate cell signaling events at concentrations that do not produce substantial inhibition of Na\(^+\)-K\(^+\)-ATPase activity or increases in intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) (6, 15). Ouabain increases cell growth and proliferation in a variety of cells by initiating a signaling scheme involving a cascade of multiple protein-protein interactions and signaling events. The activation of MAPK ERK1/2 is central to this cascade, and ouabain activates ERK1/2 at levels 3–10 times (or more) greater than basal levels. The effects of ouabain on ERK1/2 involve ROS production (15), PKC (24), Src activation (10, 11), and trans-activation of the EGFR receptor (EGFR) (10). In this paradigm, Na\(^+\)-K\(^+\)-ATPase plays a signal-transducing role similar to that played by G protein-coupled and growth factor receptors in response to ligand binding. Ouabain also triggers changes in Ca\(^2+\) signaling in renal cells (1, 23).

In addition to signals transmitted from Na\(^+\)-K\(^+\)-ATPase to ERK1/2, signals can be transmitted in the opposite direction. Na\(^+\)-K\(^+\)-ATPase activity and transmembrane Na\(^+\)-K\(^+\)-ATPase current in renal cells was blocked by inhibition of ERK1/2 (22). Inhibition of ERK1/2 blocked the insulin-promoted stimulation of Na\(^+\)-K\(^+\)-ATPase and the phosphorylation of the α-subunits of Na\(^+\)-K\(^+\)-ATPase in skeletal muscle and renal cells (2, 37). ERK1/2 also regulates the expression of the α- and β-subunits of Na\(^+\)-K\(^+\)-ATPase (9, 27).

Ouabain blocks net fluid and electrolyte secretion by epithelial cells, including secretion initiated by muscarinic receptor ligands in parotid and other salivary gland epithelial cells. The muscarinic receptor is a G protein-coupled receptor linked to PLC. Muscarinic stimulatory ligands sequentially increase the production of inositol 1,4,5-trisphosphate (InsP\(_3\)), increase the intracellular Ca\(^2+\) concentration ([Ca\(^2+\)]\(_i\)), and open Ca\(^2+\)-sensitive ion channels and other ion transport proteins that initiate fluid secretion in the parotid gland. The resulting ionic alterations, including large increases in [Na\(^+\)]\(_i\) and decreases in intracellular K\(^+\) concentration ([K\(^+\)]\(_i\)), produce an increase in Na\(^+\)-K\(^+\)-ATPase activity (5, 25). By inhibiting Na\(^+\)-K\(^+\)-ATPase, ouabain blocks receptor-mediated increases in fluid secretion and saliva formation in salivary cells (20). Muscarinic receptor-initiated changes in ion movements are accompanied by changes in the phosphorylation and activity status of signaling proteins, including an increase in the activation of ERK1/2 (29).

In initial studies, in which we used freshly isolated parotid acinar cells, we investigated whether ouabain had a stimulatory
effect on ERK1/2 activation in a manner similar to that found in many other cell systems. Although ouabain produced modest changes (generally less than a doubling in ERK1/2 phosphorylation), we found that ouabain greatly amplified the activation of ERK1/2 by submaximal concentrations of the muscarinic receptor ligand carbachol. In the present study, we examined this phenomenon in more detail and also examined changes in ERK1/2 phosphorylation by lowering extracellular K+ to block Na+-K+-ATPase. Because acute inhibition of ERK1/2 can affect the Na+-K+-ATPase activity of ex vivo cells and cells in culture (2, 22), we also examined whether ERK1/2 can affect the Na+K+-ATPase activity in freshly isolated rat parotid cells. Our studies suggest that within the same epithelial cell type, ouabain and low extracellular K+ promote distinct signals to initiate ERK1/2 activation and that ERK1/2 inhibition has a negative effect on Na+-K+-ATPase activity.

EXPERIMENTAL PROCEDURES

**Chemicals.** Carbamyl choline (carbachol) and N-acetyl-D,L-cysteine were purchased from Sigma (St. Louis, MO). Ouabain was purchased from Sigma-Aldrich and Calbiochem (San Diego, CA). 1,4-Diaminobenzenesulfonyl fluoride hydrochloride was obtained from Sigma-Aldrich and Calbiochem (San Diego, CA). 1,4-Diaminobenzene (carbonyl) and 1,4-diamino-1,2-bis(2-aminophenylthio)butadiene (U0126) were purchased from Boehringer Mannheim. 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (AG-1478) was obtained from Aldrich. 4-Amino-5-(4-chlorophenyl)-7-(4-t-butyl)pyrazolo[3,4-d]pyrimidine (PD) were purchased from Calbiochem. BAPTA-AM and fura-2 AM were purchased from Molecular Probes (Eugene, OR). Anti-phosphotyrosine antibody was a generous gift from Dr. Thomas M. Roberts (Dept. of Pathology, Dana Farber Cancer Institute, Boston, MA). PAb ERK2 (SC-154), MAb ERK2 (SC-1647), and MAb anti-α1-Na+-K+-ATPase (SC-21712) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-α1-Na+-K+-ATPase PAb (no. 05-369) was purchased from Upstate Biotechnology. PAb phospho-p44/42 MAPK (Thr202/Tyr204) and phospho-p90Rsk were purchased from Cell Signaling Technology (Beverly, MA). Secondary antibodies used with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) were IRDye 800-conjugated anti-rabbit IgG (no. 611-632-122; Rockland Immunocologicals, Gilbertsville, PA) and Alexa Fluor 680 anti-mouse IgG (A-21058; Invitrogen, Carlsbad, CA). Igepal was purchased from ICN Biomedicals (Irvine, CA). All other chemicals were of reagent grade or better.

**Cell preparation and solutions.** Animal care and the protocols followed for animals in all experiments were reviewed and approved by the Harvard Medical Area Standing Committee on Animals. Parotid acinar cells were prepared from male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, or Taconic, Germantown, NY; 150–250 g) according to previously established techniques (32). In brief, rat parotid glands were removed and treated with trypsin and collagenase to obtain a suspension of single cells and small groups of cells. Cells were suspended at ~0.25–1 mg of protein/ml in solution A, which was composed of (in mM) 116.4 NaCl, 5.4 KCl, 1 NaHPO4, 25 Na++-HEPES, 1.8 CaCl2, 0.8 MgCl2, 5 sodium butyrate, and 5.6 glucose, pH 7.4. K+-free solution A was made by replacing KCl with equimolar NaCl. Cells were maintained on ice before use.

All cell treatments were performed at 37°C in a water-jacketed chamber using a magnetic flea to stir the suspended cells. Aliquots of cells (usually 1.5 ml) were equilibrated for 2–3 min before treatment with various agents or vehicles (water or 0.1–0.2% DMSO). In general, cells were exposed to the Src family inhibitor PP2 (10 μM for 15 min), the PKC inhibitor GF-109203X (10 μM for 15 min), and the Ca2+ chelator BAPTA (25 μM for 20 min), or vehicle, followed by treatment with ouabain (1 mM) or vehicle (DMSO) for 1 min and stimuli for 2 additional min as indicated. In some experiments, cells were treated with various concentrations of ouabain before being treated with carbachol. Basal conditions represent cells treated with DMSO for 3 min. Cells exposed solely to ouabain were treated for 3 min. When experiments were conducted using K+-free solution, cells were preequilibrated at 37°C in normal solution A for 15 min, spun down, and resuspended in warmed (37°C) K+-free solution A for up to 10 min as indicated.

**Western blot analysis.** At the end of the treatment period, parotid cells were collected by rapid sedimentation in a microcentrifuge (model no. 5414; Brinkmann Instruments, Westbury, NY). The supernatant was removed, and cells were lysed by 400 μl of ice-cold lysis buffer [137 mM NaCl, 10 mM Tris base, pH 7.5, 1 mM EGTA, 1 mM EDTA, 10% glycerol (vol/vol), and 1% Igepal (vol/vol)] containing the following reagents: 1 mM vanadate, 4.5 mM sodium pyrophosphate, 47.6 mM NaF, 9.26 mM β-glycerophosphate, 0.5 mM DTT, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 2 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride. The lysates were vortexed extensively and then sedimented at 10,000 g for 15 min at 4°C. The cleared supernatants were transferred to fresh microfuge tubes and diluted with 5× Laemmli sample buffer [312.5 mM Tris·HCl (pH 6.8), 25% glycerol, 5% SDS, 1.8 M β-mercaptoethanol, and bromophenol blue (trace)], boiled for 5 min, and stored at −20°C before electrophoresis. Samples were separated using SDS-PAGE with a 10% separating gel and a 3% stacking gel as described previously (3). Proteins were transferred onto nitrocellulose membranes. Immunoblots were probed overnight with various antibodies according to the supplier’s specifications. Proteins were visualized using ECL reagents and X-ray film; alternatively, in other experiments, proteins were visualized and quantified using direct infrared fluorescence with the Odyssey Infrared Imaging System.

**Quantification of ERK1/2 activation.** In all experiments, changes in the phosphorylation status of ERK1/2 were evaluated as an indication of changes in the activation of ERK. Unless indicated otherwise, changes in ERK1/2 activation and/or phosphorylation for various conditions were normalized to ERK1/2 phosphorylation conditions under basal (nonstimulated) conditions in the absence of ouabain or other inhibitors. For each sample, phosphorylated ERK1/2 was normalized to total ERK2 to account for gel loading and/or transfer variations. Quantifications of ERK1/2/2 were performed in one of two ways. In some experiments, bands of relevant proteins on X-ray film (obtained using ECL techniques) were quantified using the National Institutes of Health’s ImageJ software. Care was taken to use exposure times that were within the linear capability of the film. For these experiments, blots were probed for phosphoproteins, stripped, and reprobed for total proteins. In other experiments in which we used the Odyssey Infrared Imaging System, blots were probed simultaneously for phosphoproteins and total protein levels using PAbS and mouse MAbS, respectively, and fluorescent anti-rabbit and anti-mouse antibodies were used for visualization and subsequent quantification.

**Measurement of [Ca2+]i in parotid cells.** Alterations in [Ca2+]i, were analyzed in parotid cells in suspension by measuring changes in the fluorescence of the Ca2+ indicator dye fura-2, similarly to the method described in previous studies (32). In brief, cells suspended in 2 μM fura-2 AM (in solution A plus 2% BSA) were incubated at 37°C for ~60 min. Cell were washed, suspended in solution A, and maintained on ice. For experiments conducted in Ca2+-free media, aliquots of fura-2-loaded cells were incubated at 37°C in solution A for 5 min, rapidly washed twice in warmed (37°C) Ca2+-free solution A, resuspended in 2 ml of this solution, and added to the cuvette in a chamber maintained at 37°C. After 3 min, 100 μM EGTA was added to the solution, and 1 min later, the experiment was commenced. For experiments conducted in Ca2+-containing solution A, aliquots of cells were incubated at 37°C in solution A for 5 min, rapidly washed twice in solution A, resuspended in 2 ml of this solution, and added to the cuvette in a chamber maintained at 37°C. Fura-2 fluorescence was
monitored at a 340/380-nm excitation ratio and 510-nm emission using a QuantaMaster fluorescence spectrophotometer (Photon Technology International, Birmingham, NJ). Generally, duplicates or triplicates of each condition were examined in each experiment, and the results were averaged. Changes in [Ca\(^{2+}\)]_i were evaluated on the basis of changes in the 340/380-nm excitation ratio. Statistical analysis of the results was performed using data compiled from all independent experiments (n = 3).

**Oxygen consumption.** QO\(_2\) measurements were similar to those reported previously (29). Porotid acinar cells suspended in solution A were prewarmed for 15 min at 37°C before being placed into a stirred 400-\(\mu\)l chamber maintained at 37°C. A Clark-type \(O_2\) electrode (model 125/05; Instech Laboratories, Plymouth Meeting, PA) was used to measure the disappearance of \(O_2\) from the closed chamber. Output was recorded using a flatbed chart recorder (model BD112; Kipp & Zonen, Bohemia, NY). At the end of each measurement, a sample of the cell suspension was collected for protein analysis (18) so that each QO\(_2\) measurement could be normalized to the protein content of the cells. The QO\(_2\) value was calculated as the linear QO\(_{2\text{max}}\) upon the addition of an agent to the chamber. The changes in QO\(_2\) (\(\Delta QO_2\)) values for porotid cells were the differences between the sustained QO\(_2\) under basal conditions and the linear QO\(_{2\text{max}}\) upon addition of different concentrations of carbachol.

For experiments conducted using U0126, cells suspended in solution A were treated with 10 \(\mu\)M drug or 0.1% DMSO for 20 min at 37°C and then placed into the chamber containing the \(O_2\) electrode. Basal QO\(_2\) was monitored, and carbachol (10\(^{-5}\) M) or nystatin (80 \(\mu\)g/ml) was added. As described above, the \(\Delta QO_2\) values were calculated as the difference between the sustained basal QO\(_2\) and linear QO\(_{2\text{max}}\) upon the addition of carbachol and nystatin. In each of three experiments, five to nine individual samples were analyzed for each condition. In each experiment, the values from U0126-treated cells were normalized to the values from control cells.

**ATP measurement.** ATP was measured in porotid acinar cells as described previously (29). Cells suspended in solution A were treated for 20 min with vehicle or U0126 (10 \(\mu\)M) at 37°C. ATP was extracted from the cells and analyzed spectrophotometrically. This procedure was used in three independent experiments.

Na\(^{+}\)-K\(^+\)-ATPase was measured based on homogenates of porotid acinar cells. In brief, freshly isolated porotid acinar cells were washed once in a phosphate-free buffer and suspended in a homogenization buffer (in mM: 150 sucrose, 10 HEPES, pH 7.4, and 4 EGTA). The cells were homogenized using a glass Dounce homogenizer (20 up-and-down strokes), and the homogenate was frozen at −80°C. Aliquots of the homogenate were added to a Na\(^{+}\)-K\(^+\)-ATPase assay mixture (pH 7.4) consisting of (in mM) 140 NaCl, 20 KCl, 30 histidine, 0.2 EGTA, 3 MgCl\(_2\), and different concentrations of ouabain. The samples were preincubated at 37°C for 5 min, and the reaction was started with the addition of 3 mM ATP. The reaction was stopped after 15 min by the addition of ice-cold TCA (3.2% final concentration). ATP hydrolysis (phosphate liberation) was measured using a modified Fiske-Subbarrow assay (8). Total Na\(^{+}\)-K\(^+\)-ATPase activity was defined as the ATPase activity that was sensitive to 3 mM ouabain, which was 48.6 ± 2.5% (n = 3) of the total ATPase activity. Each assay (n = 3) was conducted using a homogenate from a different preparation of acinar cells.

**Data analysis.** Values were calculated as means ± SE of the number (n) of independent experiments (each n representing a different cell preparation). Differences between control or basal and experimental samples for accumulated data were evaluated using a two-tailed Student’s t-test. All experiments including Western blot analysis were performed at least three times. Within each experiment to be analyzed using Western blot analysis or the Odyssey Infrared Imaging System, multiple (i.e., duplicate or triplicate) cell samples were collected for each condition and subjected to SDS-PAGE, and the average of the values obtained within each individual experiment was treated as n = 1.

**RESULTS**

Ouabain potentiates the phosphorylation of ERK1/2 by carbachol. In preliminary experiments, ouabain did not produce substantial increases in ERK1/2 activation in rat parotid acinar cells exposed to ouabain (10\(^{-6}\)-10\(^{-3}\) M) for up to 15 min (data not shown). Exposure of cells to 1 mM ouabain, a concentration sufficient to block the majority of the relatively ouabain-insensitive \(\alpha_1\)-subunit of the rodent Na\(^{+}\)-K\(^+\)-ATPase, produced less than a doubling of basal ERK1/2 phosphorylation (Fig. 1, A and B, and data not shown). However, when 1 mM ouabain was added 1 min before a concentration of carbachol (10\(^{-6}\) M) that produced only a small increase (50–100%) above basal levels in ERK1/2 activation when it was added by itself for 2 min, the combined presence of ouabain and carbachol activated ERK1/2 to a level similar to that produced by 10\(^{-5}\) M carbachol (Fig. 1, A and B). Ouabain did not increase the activation of ERK1/2 by 10\(^{-3}\) M carbachol (Fig. 1, A and B), which is a maximal concentration for ERK1/2 activation (Fig. 1C). Ouabain (1 mM) also potentiated ERK1/2 activation using 3 \(\times\) 10\(^{-7}\) M carbachol (Fig. 1B).

In these experiments, ouabain (1 mM) was added 1 min before carbachol. However, the cells did not require pretreatment with ouabain to respond in an enhanced manner. Ouabain was nearly equally effective in increasing the ERK1/2 signal when it was added simultaneously with 10\(^{-6}\) M carbachol (Fig. 1B). When paired comparisons within the same experiment were analyzed, ouabain added simultaneously with carbachol increased ERK1/2 activation to 80.1 ± 7.4% (n = 4) of the increase produced when cells were pretreated for 1 min with ouabain.

Ouabain had a concentration-dependent effect in amplifying the activation of ERK1/2 by 10\(^{-6}\) M carbachol (Fig. 2, A and B). The EC\(_{50}\) value of ouabain in promoting this effect was ~100 \(\mu\)M or higher (Fig. 2C). This finding is similar to the inhibitory potency of ouabain reported to block the Na\(^{+}\)-K\(^+\)-ATPase activity of rodent cells containing primarily the \(\alpha_1\)-isoform of Na\(^{+}\)-K\(^+\)-ATPase (4, 6). Consistent with these data, we detected the \(\alpha_1\)-subunit in porotid acinar cells using Western blot analysis with \(\alpha_1\)-specific antibodies (data not shown). We also evaluated the potency of the effect of ouabain on the Na\(^{+}\)-K\(^+\)-ATPase enzymatic activity in porotid acinar cells using a biochemical assay to measure the ouabain-sensitive rate of ATP hydrolysis. The IC\(_{50}\) of ouabain was ~100 \(\mu\)M or higher (Fig. 2D), which was similar to its potency in amplifying the activation of ERK1/2 by 10\(^{-6}\) M carbachol.

**Signaling mechanisms involved in the action of ouabain.** To gain more insight into the mechanism of the ouabain-promoted muscarinic signaling, we examined the potential involvement of several signaling proteins and signaling events that might contribute to ERK1/2 activation. Of interest is that the action of ouabain on the activation of ERK1/2 by 10\(^{-6}\) M carbachol was not blocked by 300 nM AG-1478 (data not shown), an inhibitor of EGFR kinase activity that blocks the EGFR-initiated activation of ERK1/2 in parotid cells (see below). The amplifying effects of ouabain also were not blocked by treatment of cells with 15 mM N-acetyl-cysteine, a chelator of ROS that reduces ERK1/2 activation by pervanadate (data not shown). Thus, unlike the effects of ouabain on ERK1/2 in other cells, the amplification of carbachol-initiated ERK1/2 activation in parotid cells was not dependent on EGFR kinase activity and
ROS generation. However, similar to the stimulatory effects of ouabain on ERK1/2 activation in other cell systems (11, 35), the amplification effect of ouabain on carbachol-initiated ERK1/2 activation was blocked by PP2, an inhibitor of Src kinase family members. PP2 blocked ERK1/2 activation in a concentration-dependent fashion at concentrations between 1 and 10 μM (Fig. 3A). PP2 (10 μM) reduced ERK1/2 activation by ~80% in cells exposed to 1 mM ouabain plus 10−6 M carbachol (Table 1). PP2 also reduced the activation of ERK1/2 by 10−5 M carbachol and PMA (Fig. 3B and Table 1). Thus Src contributes to the carbachol-initiated activation ERK1/2 in parotid cells in both the presence and absence of ouabain.

The effect of ouabain on ERK1/2 activation by 10−6 M carbachol was also blocked by the PKC inhibitor GF-109203X. This inhibitor, which is an effective inhibitor of members of the cPKC and nPKC subfamilies of PKC, acted in a concentration-dependent fashion (Fig. 3C) and was effective at 10 μM. GF-109203X also reduced the activation of ERK1/2 by 10−5 M carbachol and PMA (Table 1). Thus PKC is involved in ERK1/2 activation by carbachol in the presence or absence of ouabain. When cells were loaded with the Ca2+ chelator BAPTA, the potentiation of carbachol-dependent ERK1/2 activation by ouabain was reduced by ~90%, and the activation of ERK1/2 by 10−5 M carbachol and PMA also were substantially reduced (Fig. 3D and Table 1). These data indicate that elevation of [Ca2+], is part of the activation of ERK1/2 by carbachol in the presence or absence of ouabain. Of interest regarding these particular findings, increases in ERK1/2 activation produced by 10−6 M carbachol plus ouabain, as well as those produced by 10−5 M carbachol, were not blocked (data not shown) by the PKC inhibitor Gö6976 at concentrations (~1 μM) that are relatively selective for members of the cPKC subfamily, which are activated by increases in [Ca2+]i. A high concentration (10 μM) of Gö6976 did reduce ERK1/2 activation, but this concentration is considered to be too high to be specific for blocking cPKC proteins.

Removal of extracellular K+ stimulates ERK1/2 and p90 Rsk phosphorylation. The concentration dependence of ouabain on carbachol-initiated ERK1/2 activation (Fig. 2) suggests that its potentiating effects are due to inhibition of Na+–K+–ATPase. Because Na+–K+–ATPase activity is also inhibited by the removal of extracellular K+, we also examined the effects of low extracellular K+ on ERK1/2 activation (see experimental procedures) and compared this with that of carbachol. ERK1/2 phosphorylation in cells suspended in K+-free solution increased within 2 min and reached a peak at 5 min that was sustained. The readdition of 5 mM K+ at 5 min resulted in a return of ERK1/2 phosphorylation to basal levels within 5 min (Fig. 4A). In contrast, the activation by carbachol (10−5 M) peaked at 2 min and was not sustained at this level at later times. Both carbachol and K+-free conditions also increased the phosphorylation of p90Rsk (Fig. 4B), an effector of ERK1/2. Moreover, the activation of ERK1/2 by K+-free conditions, like those of carbachol, were detected in cells exposed to the EGFR inhibitor AG-1478 at a concentration (300 nM) that effectively blocked the activation of ERK1/2 and p90Rsk by EGF (Fig. 4B).

Because ouabain and low K+ both increased [Na+]i, as did carbachol, we also examined the effects of nystatin, a monovalent cationophore that promotes rapid Na+ entry into and K+
loss from cells. These ionic changes also increase Na\(^{+}\)-K\(^{-}\)-ATPase activity in nystatin-treated parotid acinar cells (32). ERK1/2 was not activated in cells treated with nystatin (80 \(\mu\)g/ml) for 2, 5, or 10 min (data not shown).

**Ouabain does not enhance Ca\(^{2+}\) signaling by muscarinic receptors.** Because exposure of the parotid cells to ouabain amplified the actions of a submaximal concentration of carbachol on ERK1/2, we wondered whether there would be an increase in the activation of M3 receptor itself. Also, because the activation of ERK1/2 by carbachol depended on an increase in [Ca\(^{2+}\)], we wondered whether ouabain would enhance the ability of 10\(^{-6}\) M carbachol to increase the release of intracellular Ca\(^{2+}\). We examined this possibility by measuring the intracellular release of Ca\(^{2+}\) in carbachol-treated cells exposed to Ca\(^{2+}\)-free solution. Added by itself, ouabain (1 mM) did not produce a detectable increase in [Ca\(^{2+}\)], during the first minute of exposure (Fig. 5) or within the first 5 min of exposure (data not shown). In control cells (i.e., those treated with vehicle) and in cells exposed to 1 mM ouabain for 1 min before carbachol treatment, 10\(^{-6}\) M carbachol caused the release of similar amounts of Ca\(^{2+}\) from intracellular storage sites, and the amounts of Ca\(^{2+}\) released were not as large as those promoted by 10\(^{-5}\) M carbachol. The peak increases in [Ca\(^{2+}\)], produced by 10\(^{-6}\) M carbachol in the absence or presence of 1 mM ouabain were 46.5 \(\pm\) 0.6% \((n = 3)\) and 45.3 \(\pm\) 1.73% \((n = 3)\), respectively, of the increase produced by 10\(^{-5}\) M carbachol. Within 2.5 min, [Ca\(^{2+}\)], returned to basal levels after exposure to all types of stimulation. In experiments conducted using cells suspended in Ca\(^{2+}\)-containing solution A, 10\(^{-6}\) M carbachol produced similar changes in [Ca\(^{2+}\)] in the presence and absence of ouabain (data not shown). Both the initial peak elevation and the sustained elevation in [Ca\(^{2+}\)], were similar in cells with or without 1 mM ouabain present, and these responses also were much less than those produced by 10\(^{-5}\) M carbachol.

**ERK inhibition blocks Na\(^{+}\)-K\(^{-}\)-ATPase activity in parotid acinar cells.** Because several studies indicated that ERK1/2 could play a positive role in Na\(^{+}\)-K\(^{-}\)-ATPase activity, we examined the effect of ERK inhibition on the QO\(_{2}\) of parotid acinar cells as an indicator of ex vivo Na\(^{+}\)-K\(^{-}\)-ATPase activity. Because of the tight coupling between mitochondrial oxidative phosphorylation (i.e., ATP production) and Na\(^{+}\)-K\(^{-}\)-ATPase activity (i.e., ATP consumption), changes in QO\(_{2}\) can be used as a quantitative estimate of Na\(^{+}\)-K\(^{-}\)-ATPase activity in parotid cells ex vivo (32) and in a variety of epithelia (19). Of interest is that the EC\(_{50}\) value (\(\sim\)1–3 \(\mu\)M) for carbachol stimulation of QO\(_{2}\) above basal levels was similar to its concentration-dependent effects on ERK1/2 (Fig. 6). To investigate the potential contribution of ERK1/2 to Na\(^{+}\)-K\(^{-}\)-ATPase

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**Fig. 2.** Concentration dependence of ouabain on ERK1/2 activation by 10\(^{-6}\) M carbachol and on inhibition of Na\(^{+}\)-K\(^{-}\)-ATPase activity. A–C: parotid acinar cells were treated for 1 min with different concentrations of ouabain before carbachol (2 min). A: representative Western blot experiment. B: quantification of ERK1/2 activation relative to basal level. Ouabain produced a concentration-dependent increase in ERK1/2 activation. C: increases in ERK1/2 activation above basal levels were normalized to the increase produced by the combination of ouabain (1 mM) + carbachol (10\(^{-6}\) M), which was defined as 100. Numbers in parentheses in B and C are the number of independent experiments conducted. D: Na\(^{+}\)-K\(^{-}\)-ATPase activity in homogenates of parotid acinar cells was measured in the presence of different concentrations of ouabain (\(n = 3\) experiments).
on various measurements. The results suggest that ERK1/2 has a positive effect on Na\(^{+}\)-K\(^{+}\)-ATPase activity. Because a number of chemical inhibitors that block kinases also can affect oxidative metabolism (29, 30), which supports the majority of Na\(^{+}\)-K\(^{+}\)-ATPase energy needs in parotid cells, we sought to determine whether U0126 affects ATP levels in parotid acinar cells. However, cells treated with U0126 (10 \(\mu\)M, 20 min) had 99.2 \pm 2.5\% (3) of the ATP levels of control cells, so cell metabolism did not appear to be compromised by U0126.

**DISCUSSION**

The results of the present study suggest functional connections between Na\(^{+}\)-K\(^{+}\)-ATPase and ERK1/2 in parotid acinar epithelial cells and bidirectional signaling between these proteins. This conclusion is based on the following observations: 1) ERK1/2 was activated by inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase by removal of extracellular K\(^{+}\), 2) activation of ERK1/2 by the muscarinic ligand carbachol was potentiated by the Na\(^{+}\)-K\(^{+}\)-ATPase ligand and inhibitor ouabain, and 3) inhibition of ERK1/2 by U0126 reduced the activation of Na\(^{+}\)-K\(^{+}\)-ATPase by carbachol and nystatin. These data suggest that inhibition and/or inhibitors of Na\(^{+}\)-K\(^{+}\)-ATPase can exert a positive effect on ERK1/2 and that ERK1/2 plays a positive role in modulating Na\(^{+}\)-K\(^{+}\)-ATPase activity. The activation of ERK1/2 by low extracellular K\(^{+}\), high concentrations of carbachol, and ouabain plus lower concentrations of carbachol were not secondary to EGFR activation. The effect of ouabain on muscarinic signaling is a new paradigm for the action of ouabain on cell signaling and is different from its effect on other cells with regard to the production of substantial increases in ERK1/2 activation.

The similarities between the ouabain concentration dependence of the potentiation of muscarinic ERK1/2 signaling (Fig. 2C) and the inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase (Fig. 2D) seemed to activity, we examined the effect of the MEK inhibitor U0126 on parotid QO2 under conditions (10 \(\mu\)M U0126, 20-min exposure) that completely blocked ERK1/2 activation by carbachol. We evaluated the effect of U0126 on the increases in the QO2 of cells exposed to 10\(^{-5}\) M carbachol and the cationophore nystatin (80 \(\mu\)g/ml), which activates Na\(^{+}\)-K\(^{+}\)-ATPase by promoting rapid Na\(^{+}\) entry and K\(^{+}\) efflux (32). U0126 produced modest but significant decreases in basal QO2 and the carbachol-stimulated increase in QO2 (Fig. 7). The effect of U0126 on the nystatin-stimulated increase in QO2 was not (but was close to being) statistically significant using a two-tailed t-test, but it was significant (\(P < 0.04\)) when we analyzed the results using a one-tailed t-test. Throughout the data analyses presented in this study, we used a two-tailed t-test because we could not predict a priori the expected effects of the inhibitors

**Table 1. Effects of various inhibitors on ERK1/2 activation in parotid acinar cells**

<table>
<thead>
<tr>
<th>Chemical Inhibitor</th>
<th>PP2</th>
<th>BAPTA</th>
<th>GF-109203X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>61.0 (+10.0) (4)*</td>
<td>103.7 (+11.0) (3)</td>
<td>82.0 (+8.0) (4)</td>
</tr>
<tr>
<td>Ouabain, 1 (\mu)M</td>
<td>34.9 (+8.1) (3)*</td>
<td>69.6 (+6.9) (4)*</td>
<td>59.6 (+7.2) (4)*</td>
</tr>
<tr>
<td>Carbachol, 10(^{-5}) M</td>
<td>42.5 (+5.5) (3)*</td>
<td>20.7 (+6.6) (4)*</td>
<td>13.6 (+5.2) (4)*</td>
</tr>
<tr>
<td>Ouabain + Carbachol</td>
<td>50.2 (+9.3) (3)*</td>
<td>10.8 (+3.9) (3)</td>
<td>25.7 (+4.8) (4)*</td>
</tr>
<tr>
<td>PMA, 100 (\mu)M</td>
<td>18.9 (+12.2) (3)*</td>
<td>52.2 (2)</td>
<td>0 (4)</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE; numbers in parentheses refer to the no. of independent experiments conducted. Cells were treated for 15–20 min with 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2, 10 \(\mu\)M), BAPTA (20 \(\mu\)M), or 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide (GF-109203X, 10 \(\mu\)M). Cells were treated with DMSO (0.1%). Subsequently, cells were treated as described in Fig. 3. For ouabain and 10\(^{-6}\) carbachol, which cause small increases in ERK1/2 activation above basal level, and for the basal condition, the effects of these inhibitory agents on ERK1/2 were calculated as follows: [ERK1/2 activation (inhibitor)]/[ERK1/2 activation (−inhibitor)] \(\times\) 100. For the other stimuli, which produce much larger increases in ERK1/2 activation, the effects of the inhibitors were calculated for the increases in ERK1/2 activation (increased activation, activation above basal). For these values, the basal ERK1/2 (1) was subtracted from each stimulated value (\(\pm\) inhibitor) and the following calculation was used: [Δ activation (± inhibitor)]/[Δ activation (−inhibitor)] \(\times\) 100. *\(P < 0.05\); †\(P < 0.01\).
indicate that this signaling effect of ouabain was due to the inhibition of Na\(^{+}\)-K\(^{+}\)-ATPase. Ouabain itself did not greatly activate ERK1/2; however, a large increase in ERK1/2 phosphorylation was promoted by inhibiting Na\(^{+}\)-K\(^{+}\)-ATPase by exposing the cells to a low extracellular K\(^{+}\)-containing solution. Some cells were exposed to K\(^{+}\)-free conditions for 5 min, followed by exposure to 5 mM K\(^{+}\) for an additional 5 min (n = 4–5 cell preparations). B: cells were pretreated with vehicle (DMSO) or 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG-1478; 300 nM) for 15 min, followed by exposure to carbachol (10 \(^{-5}\) M) for 2 min, K\(^{+}\)-free solution for 5 or 10 min, and EGF (100 ng/ml) for 2 min. Lysates were immunoblotted for phospho-ERK1/2, ERK2, and phospho-p90Rsk. Each lane represents a different sample collected from the same cell preparation. Blots are representative of 3 experiments.

Fig. 5. Effect of ouabain on the elevation of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) by 10 \(^{-6}\) M carbachol. [Ca\(^{2+}\)]\(_i\) was monitored in fura-2-loaded cells suspended in Ca\(^{2+}\)-free solution. Cells were treated sequentially with EGFTA (100 μM) immediately before start of recording, ouabain (1 mM), or DMSO, as well as two different concentrations of carbachol. Traces show averages of multiple trials conducted with one cell preparation. Ouabain did not increase the intracellular release of intracellular Ca\(^{2+}\) produced by 10 \(^{-6}\) M carbachol, and the release by 10 \(^{-5}\) M carbachol ± ouabain was much less than that produced by 10 \(^{-5}\) M carbachol. Ordinate axis is the 340/380-nm fura-2 fluorescence excitation ratio. See text for details.

Fig. 6. Concentration dependence of carbachol on ERK1/2 activation and Na\(^{+}\)-K\(^{+}\)-ATPase activation. The ERK1/2 data are reproduced from Fig. 1C. The increases in the Na\(^{+}\)-K\(^{+}\)-ATPase activities were calculated as QO\(_2\) above basal levels promoted by 10 \(^{-7}\) M carbachol was defined as QO\(_2\)\(_{max}\) (100), and other values shown are relative to this value. Basal QO\(_2\) was 11.6 ± 0.4 amol O\(_2\)·mg\(^{-1}\)·min\(^{-1}\) (n = 3), and carbachol (10 \(^{-3}\) M) ∆QO\(_2\) = 9.6 ± 1.63 amol O\(_2\)·mg\(^{-1}\)·min\(^{-1}\) (n = 3) above basal level. For ERK, the activation produced by 10 \(^{-7}\) M carbachol (n = 6) was 7.2 ± 0.8 times that of basal level.
Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibition having a causal role in the modest ERK1/2 activation by ouabain, the potentiating action of ouabain when it is present with carbachol, or the activation of ERK1/2 by low extracellular K\textsuperscript{+}.

ERK1/2 can directly and indirectly regulate Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and/or transmembrane Na\textsuperscript{+} transport in a variety of epithelial cells (2, 7, 22). The α-subunit can be phosphorylated by ERK in vitro on serine and threonine residues (2, 13). Some ERK-dependent effects were acute and were due to various mechanisms, including an increase in the cell surface expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase α-subunits (2); other effects of ERK1/2 were manifested on a longer time scale and in some cases involved alterations in the total levels of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase subunits. The reduction (11.4 ± 1.0%, n = 3; P < 0.01) of the carbachol-initiated increases in QO\textsubscript{2} (an indicator of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity) in U0126-treated cells does not indicate whether ERK could have had a direct or indirect (via an upstream ion channel or transporter) on Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. However, the reduction (10.8 ± 3.8%, n = 4; P = 0.06) of nystatin-stimulated QO\textsubscript{2} and/or Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity in the U0126-treated cells suggests that ERK may have a direct effect, because nystatin exerts its stimulatory effect by removing the substrate (Na\textsuperscript{+}) limitation to Na\textsuperscript{+}-K\textsuperscript{+}-ATPase that exists under basal conditions, which promotes an increase in oxidative phosphorylation to support enhanced parotid Na\textsuperscript{+}-K\textsuperscript{+}-ATPase activity (31, 32). Although the measurement of cell ATP levels suggested otherwise, one caveat regarding these conclusions is that these results are dependent on U0126 not affecting a component of oxidative metabolism that is unrelated to the energy-dependent activation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase.

We were unable to distinguish the upstream activators of ERK1/2 by 10^{-5} M carbachol from those upstream signaling proteins that contributed to ERK1/2 activation by ouabain plus submaximal carbachol concentrations. Notably, it did not take long for the amplification effect to be set into motion, because the cells did not have to be pretreated with ouabain (Fig. 1B). Moreover, the potentiation effect of ouabain did not involve several important signaling events that are critical for the activation of ERK1/2 by ouabain alone in other cells (35). Thus EGFR and ROS generation did not play any role in our present findings; however, Src and PKC did contribute to our findings, as they did also when ouabain activated ERK1/2 in a variety of cells. Because the Ca\textsuperscript{2+} chelator BAPTA blocked the potentiating effects of ouabain on carbachol-promoted ERK1/2 activation, we checked for effects of ouabain on overall changes in carbachol-promoted [Ca\textsuperscript{2+}], release and [Ca\textsuperscript{2+}], homeostasis; however, ouabain itself did not cause a measurable release of Ca\textsuperscript{2+} or affect Ca\textsuperscript{2+} signaling by muscarinic receptor activation (Fig. 5). Thus ouabain did not appear to promote an increase in the muscarinic receptor activation of PLC and appeared to act downstream from the muscarinic receptor. In renal cells, ouabain produced Ca\textsuperscript{2+} oscillations by activating the InsP\textsubscript{3} receptor (InsP\textsubscript{3}R) in an InsP\textsubscript{3}-independent manner, and this activation appeared to involve the association of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase with the InsP\textsubscript{3}R (23). We were precluded from detecting these kinds of changes in our cuvette-based [Ca\textsuperscript{2+}] experiments, but our data suggest that ouabain did not enhance the carbachol-initiated production of InsP\textsubscript{3}.

In many systems in which ouabain activates ERK1/2, it appears to do so by assembling a complex of signaling proteins in caveolin microdomains (6, 16, 17, 33). Within minutes, the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase α-subunit (to which ouabain binds), Src, EGFR, and ERK1/2 colocalized in caveolin-ensheathed vesicles. Thus one possible explanation for the present results may be that ouabain affects the colocalization and/or interactions of the relevant signaling proteins that participate in ERK1/2 activation, and its amplification effect on lower concentrations of carbachol may not involve a different roster of signaling molecules from those that participate when a larger concentration of carbachol is used in the absence of ouabain. Another possible explanation might be that ouabain does act at the level of the muscarinic receptor. Mutational studies of the M3 muscarinic receptor (the main form present in parotid acinar cells; see Ref. 34) have indicated that its coupling to PLC can be distinct from its activation of ERK1/2 because of the different dependencies of PLC and ERK1/2 phosphorylation on receptor dimerization (26).

A variety of biological effects (e.g., differentiation, proliferation, protein expression) of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase inhibitor ouabain are dependent on ERK1/2 activation (6, 35). In salivary gland cells and cell lines, ERK1/2 affects a variety of responses, including the expression of ion and water channels (12, 36) and developmental events (21). In other cells, ERK also affects the expression and activity of several ion transport proteins, including Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (2, 13, 14, 22, 37). Thus ERK1/2 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase each can affect each other's activation. Our findings suggest that this phenomenon can occur in a defined manner within parotid epithelial cells.
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GRANTS

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REFERENCES


