Effects of Muscarinic, Alpha-Adrenergic, and Substance P Agonists and Ionomycin on Ion Transport Mechanisms in the Rat Parotid Acinar Cell

The Dependence of Ion Transport on Intracellular Calcium

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ABSTRACT The relationship between receptor-mediated increases in the intracellular free calcium concentration ($[Ca]_i$) and the stimulation of ion fluxes involved in fluid secretion was examined in the rat parotid acinar cell. Agonist-induced increases in $[Ca]_i$ caused the rapid net loss of up to 50–60% of the total content of intracellular chloride (Cl_i) and potassium (K_i), which is consistent with the activation of calcium-sensitive chloride and potassium channels. These ion movements were accompanied by a 25% reduction in the intracellular volume. The relative magnitudes of the losses of K_i and the net potassium fluxes promoted by carbachol (a muscarinic agonist), phenylephrine (an alpha-adrenergic agonist), and substance P were very similar to their characteristic effects on elevating $[Ca]_i$. Carbachol stimulated the loss of K_i through multiple efflux pathways, including the large-conductance Ca-activated K channel. Carbachol and substance P increased the levels of intracellular sodium (Na_i) to more than 2.5 times the normal level by stimulating the net uptake of sodium through multiple pathways; Na-K-2Cl cotransport accounted for > 50% of the influx, and ~ 20% was via Na-H exchange, which led to a net alkalinization of the cells. Ionomycin stimulated similar fluxes through these two pathways, but also promoted sodium influx through an additional pathway which was nearly equivalent in magnitude to the combined uptake through the other two pathways. The carbachol-induced increase in Na_i and decrease in K_i stimulated the activity of the sodium pump, measured by the ouabain-sensitive rate of oxygen consumption, to nearly maximal levels. In the absence of extracellular

calcium or in cells loaded with the calcium chelator BAPTA (bis[o-aminophe-noxy]ethane-N,N,N',N'-tetraacetic acid) the magnitudes of agonist- or ionomycin-stimulated ion fluxes were greatly reduced. The parotid cells displayed a marked desensitization to substance P; within 10 min the elevation of $[Ca]_i$ and alterations in K_i, Na_i, and cell volume spontaneously returned to near baseline levels. In addition to quantitating the activation of various ion flux pathways in the rat parotid acinar cell, these results demonstrate that the activation of ion transport systems responsible for fluid secretion in this tissue is closely linked to the elevation of $[Ca]_i$.

INTRODUCTION

Neurotransmitters stimulate the secretion of fluid by exocrine glands via alteration of membrane permeability. The data obtained from isotopic flux studies and, more recently, from patch-clamp recordings suggest that fluid secretion in the parotid cell involves the activation of various ion fluxes across the apical and basolateral membranes. For many years it was known that salivary glands lose K to the surrounding medium (Burgen, 1956). Muscarinic and alpha-adrenergic agonists were observed to stimulate the efflux of K or ^{86}Rb (Martinez et al., 1976; Putney, 1976) and chloride (Nauntofte and Poulsen, 1986; Melvin et al., 1987) from the parotid. Using patch-clamp techniques to measure single channel K currents in salivary acinar cells, Maruyama et al. (1983a) demonstrated that the probability of the channel opening was increased by elevating Ca at the intracellular side of the membrane, and electrophysiological studies also have demonstrated agonist-induced activation of K channels and chloride channels in other secretory cells (Maruyama et al., 1983b; Marty et al., 1984, Findlay and Petersen, 1985).

Various studies have suggested that Na-K-2Cl cotransport was involved in saliva formation. The replacement of extracellular chloride with less permeable anions and the addition of furosemide (to block Na-K-2Cl cotransport) to Cl-containing media was noted to reduce the volume of saliva secreted by isolated rat submandibular glands (Martinez and Cassity, 1985) and to reduce sodium uptake into rat parotid acini (Poulsen and Kristensen, 1982). Furosemide- and bumetanide-sensitive ^{36}Cl and ^{86}Rb uptakes were reported in rat parotid acini (Kawaguchi et al., 1986; Nauntofte and Poulsen, 1986), and K- and Cl-dependent and furosemide-sensitive uptakes of ^{22}Na were observed using basolateral vesicles isolated from rabbit parotid glands (Turner et al., 1986).

In addition, there was indirect evidence that Na-H exchange also contributes to sodium entry in submandibular salivary cells (Case et al., 1984; Young et al., 1987). Direct evidence of Na-H exchange has been reported in pancreatic acinar cells (Hellmessen et al., 1985) and in basolateral membrane vesicles prepared from rat parotid gland (Manganel and Turner, 1988). The intracellular pH of parotid acinar cells was recently reported to be increased by beta-adrenergic agonists (Jonsson et al., 1987); however, these agonists are not believed to play a major role in stimulating fluid secretion in parotid cells.

Thus, according to the model that has been derived from the studies of various secretory epithelia, including shark rectal gland (Silva et al., 1977), fluid secretion by the parotid acinar cell is believed to be dependent on the concerted activity of mul-

tiple ion transport systems. The results presented here, as well as those reported previously, support the general model in which agonists stimulate saliva formation by elevating $[Ca]_i$, which in turn activates basolateral K and luminal Cl channels, resulting in the net loss of K_i and Cl_i from the cell. This is followed by the uptake of Na (and the reuptake of K and Cl) via the Na-K-2Cl cotransport system. The resulting increase in Na_i activates the Na,K-ATPase in the basolateral membrane, which leads to the extrusion of the elevated Na_i and helps reaccumulate the lost K. Accordingly, the primary acinar secretion is believed to result from the net transcellular transport of Cl into the lumen, and the paracellular transport of Na through the tight junctions, accompanied by para- or transcellular water flux.

In addition to serving as a model for fluid and electrolyte secretion, the parotid cell has served as a model system to study the stimulation of phosphatidylinositol (PI) turnover by receptor-mediated agonists, which promote the production of diacylglycerol and inositol (1,4,5)-trisphosphate (IP_3) by the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) via activation of phospholipase C (Aub and Putney, 1985; Downes and Stone, 1986). The link between receptor activation and the stimulation of fluid and electrolyte secretion is believed to be the elevation of the intracellular free calcium concentration ($[Ca]_i$) mediated by IP_3-induced mobilization of intracellular Ca stores in the endoplasmic reticulum and Ca influx across the plasma membrane (Aub and Putney, 1985; Putney, 1986; Merritt and Rink, 1987a). These receptors have been shown to be coupled to phospholipase C by a regulatory GTP-dependent protein (Taylor et al., 1986), and the effects of various phospholipase C-linked agonists on elevating $[Ca]_i$ in rat parotid cells has been the focus of attention in a number of laboratories (Takemura, 1985; Nauntofte and Dissing, 1986; Merritt and Rink, 1987a, b), including ours (McMillian et al., 1987, 1988).

In view of these reports, our goal in the present studies was to examine the relationship between the elevation of $[Ca]_i$ and the activation of ion fluxes involved in fluid secretion by the parotid gland. We measured the effects of phospholipase C-linked agonists on Na, K, and Cl fluxes, pH_i, oxygen consumption, intracellular volume, and $[Ca]_i$ in dispersed rat parotid acinar cells. The receptor-mediated alterations in ion fluxes were compared with those induced by raising $[Ca]_i$ with the Ca ionophore ionomycin, and the effects of Ca-mobilizing stimuli were also examined in the absence of extracellular Ca and when the intracellular Ca was buffered with BAPTA (bis[o-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid). In all of the studies, the quantitative relationship between the rise in $[Ca]_i$ and the activation of the ion transport systems involved in fluid secretion demonstrated that activation was closely linked to alterations in the $[Ca]_i$.

Preliminary reports of these studies were published in abstract form in the *Journal of General Physiology*, 1986, 88:55a and in *Kidney International*, 1988, 33:173.

MATERIAL AND METHODS

Cell Preparation

The protocol used to prepare isolated parotid cells from the rat parotid gland is a modification of the method of Kanagasuntheram and Randle (1976) and has been described previously (McMillian et al., 1987). In brief, parotid glands were removed from 200–300-g male

rats and carefully dissected to remove lymph nodes, fat, and other nonparotid tissue. The glands were then minced, briefly exposed to trypsin, washed, and exposed to collagenase for up to 1 h. At the end of this process the cells were subjected to several centrifugations to produce a final suspension that was a mixture of single cells and small clusters of multiple cells. As judged by trypan blue exclusion, the preparation was highly viable (< 5% staining). Prior to use, the cells were maintained on ice in a HEPES/Ringer solution of the following composition, in millimolar: NaCl, 120; KCl, 5; MgCl$_2$, 2.2; CaCl$_2$, 1; HEPES, 20; betahydroxybutyrate, 5; glucose, 10; and bovine serum albumin (BSA), 0.1%; pH 7.4. As noted below, in some experiments a portion of the original cell suspension was washed and resuspended in various solutions of different composition.

Intracellular Free Ca

Fura 2-acetoxymethylester (AM) (Molecular Probes, Eugene, OR) was used to measure [Ca]$_i$. An aliquot of the cell preparation was pelleted and resuspended in fresh HEPES/Ringer solution containing 2% BSA, 0.5 μM fura 2-AM, 0.1% Pluronic F127 detergent (Molecular Probes), and 10 μM probenecid. The cell suspension was gently rotated using an Adams Nutator (Clay Adams, Parsippany, NJ) at room temperature for ~ 60 min. At the end of the loading period, the cells were washed two times and resuspended in oxygenated, BSA-free HEPES/Ringer containing 10 μM probenecid and stored on ice. [Ca]$_i$ was monitored at 32°C using a fluorescence spectrophotometer (LS5; Perkin-Elmer Corp., Norwalk, CT) at an excitation/emission wavelength pair of 340/510 (5/10-nm slit widths). The contribution of extracellular fura 2 to the fluorescent signal was evaluated at the start of each measurement by adding 100 μM MnCl$_2$ to quench the entire extracellular fura 2 signal, followed by 200 μM diethylenetriaminepentaacetic acid (Eastman Kodak Co., Rochester, NY) to chelate the added Mn^{2+} as previously described (McMillian et al., 1987). F_{max} and F_{min} values were obtained using ionomycin (0.5–1 μM) and 2 mM MnCl$_2$, respectively, at the end of each measurement. The contribution of cell autofluorescence was negligible, as was the fluorescence of any of the agents added to the cells.

Intracellular pH

pH$_i$ was measured using the pH-sensitive dye (2',7')-bis(carboxyethyl)-5,6) carboxyfluorescein (BCECF) (Grinstein et al., 1984). Cells were loaded with 2.5 μM BCECF-AM (Molecular Probes) in a similar manner to that described above for fura 2-AM. After loading at room temperature for ~60 min the cells were washed twice and resuspended in the appropriate solution at 37°C. pH$_i$ was monitored at 37°C using a fluorescence spectrophotometer (LS5; Perkin-Elmer) using the excitation/emission pair of 506/530 nm (5/10-nm slit widths). The pH$_i$ was calculated using the nigericin technique (Thomas, 1979).

Rate of Oxygen Consumption (QO$_2$)

The rate of respiration of the parotid cell suspension was monitored using a Clark-type oxygen electrode (model 5331; Yellow Springs Instrument Co.; Yellow Springs, OH) and an oxymeter (model 53 oxygen monitor; Yellow Springs Instrument Co.) to monitor the disappearance of oxygen from a closed, thermostated chamber. The oxygen tension was calculated by measuring the amount of room air oxygen that was dissolved in 150 mM NaCl at 37°C. A sample of the suspension was collected from the chamber at the end of each measurement so that the QO$_2$ could be normalized to the protein content of the cells. In general, an aliquot of the cell suspension was washed twice, resuspended in the appropriate solution, and incubated at 37°C for 20 min before monitoring the QO$_2$.

^{22}Na Studies

Portions of the original cell preparation were washed twice and resuspended in the appropriate solution. In general, the cells were added to a water-jacketed system (5302 Macro Bath Assembly; Yellow Springs Instrument Co.) and were maintained at 37°C in a single stirred chamber for ~ 10 min after which ^{22}Na (Amersham Corp., Arlington Heights, IL; Dupont, Wilmington, DE) was added to the cells (1.2–2.6 μCi/ml cell suspension). The intracellular pool of Na equilibrated with ^{22}Na within ~ 10 min, after which time the ^{22}Na content of the cells remained constant for up to 60 min. Stimulating agents were added at least 15 min after the addition of ^{22}Na. To evaluate Na uptake, 200-μl aliquots of the cell suspension were collected at different times and added to 1.5-ml microfuge tubes containing 800 μl of solution above 400 μl of an oil mixture (dinonylphthalate:silicone oil, 1:1). The upper aqueous "wash" solution was identical in composition to the solution in which the cells were suspended. Immediately upon collection, each tube was spun at 14,000 g for ~ 10 s in an Eppendorf centrifuge (model 5414; Brinkmann Instruments Co., Westbury, NY) to pellet the cells. To normalize ^{22}Na uptake, the protein content was determined in pellets from two 200-μl samples of the cell suspension collected in similar fashion before adding the isotope. The protein content of the suspensions generally ranged between 0.5–0.75 mg/ml. At the end of the experiment, the aqueous layer and part of the oil layer were drawn off, the tubes were inverted, and the tips containing the cell pellet were cut off. The ^{22}Na activity of each pellet was measured in a gamma counter (5500; Beckman Instruments, Inc., Palo Alto, CA) using a fully open window setting. Background counts were subtracted from all values.

In many experiments, after the addition of ^{22}Na, the cell suspension was split into separate portions, each of which was stirred and maintained at 37°C and exposed to separate stimuli or inhibitors (see Results). After the stimuli were added to the cell suspension (time zero), samples were rapidly collected. The time assigned to each sample was that which was noted at the initiation of the centrifugation, which generally was 2–3 s after removal from the cell suspension. To measure the unidirectional Na influx rate, 2.5 mM ouabain was added to the cell suspension ~ 10–20 s before the stimulus. In these experiments, the initial uptake rate was calculated from the linear portion of the increase in ^{22}Na, which generally included at least three or four values, including the basal ^{22}Na content in cells before stimulation. The basal value was calculated from the average ^{22}Na content of at least three samples collected before the addition of the stimulating agent. When maximally effective concentrations of carbachol or ionomycin were added to cells suspended in normal medium, the ^{22}Na content of the cell reached a maximum value within 2 min, and the initial linear period of the uptake occurred within the first minute after these agents were added to the cell suspension.

The intracellular ^{22}Na content of the cells was calculated by subtracting the contribution of the extracellular ^{22}Na from the total ^{22}Na content using the extracellular space determination (see below) and the specific activity of the isotope in the extracellular medium. Where noted, the [Na]_i was calculated using the intracellular ^{22}Na content and the intracellular volume.

^{36}Cl Efflux Studies

The cells were equilibrated with ^{36}Cl (~ 0.6 μCi/ml) for at least 20 min at 37°C, and 200-μl samples were collected at appropriate times. The samples were collected rapidly by centrifugation through oil and a wash solution in a manner identical to that used for the ^{22}Na experiments. The tips of the microfuge tubes containing the cell pellets were cut off, placed in glass scintillation vials containing 8 ml of Liquiscint (National Diagnostics, Manville, NJ), and extensively vortexed. Aliquots of the extracellular medium were also collected to determine the specific activity. The ^{36}Cl activity was measured in a scintillation counter (LS 1801; Beckman). Before the addition of any stimuli, at least three samples were collected to measure the

control value of the Cl content. Intracellular Cl content was calculated similarly to that for Na.

K Fluxes

The net release and uptake of K by the parotid cells in suspension was monitored using a K-sensitive extracellular electrode (Microelectrodes, Inc., Londonderry, NH). Cells were incubated for 15–20 min at 37°C, and then placed in a well-stirred thermostated chamber. An increase or decrease in the extracellular K concentration was interpreted as a net cellular K efflux or influx, respectively. To measure the total intracellular K content, digitonin (25 μM) was added to lyse the cells and release the intracellular K. Where appropriate, the results were normalized to the protein content of the suspension.

Volume Determinations

Cells were equilibrated for at least 15 min at 37°C with a combination of $[^3H]H_2O$ (50 μCi/ml) and $[^{14}C]$sucrose (1 μCi/ml). Aliquots of the cell suspension were collected and spun through 400 μl of the oil mixture described above. An aliquot of the extracellular medium above the oil was removed for radioactive analysis. The remaining volume was drawn off, and the tube was rinsed at least three times with distilled water to remove all traces of radioactivity above the oil. The oil layer was removed, 1.2 ml of 7% perchloric acid was added to the tissue pellet for at least 24 h to extract the isotopes, and the solution was then removed for radioactive analysis. The pellets were solubilized and the protein contents were determined (see below). After extraction, < 0.3% of the radioactivity remained associated with the pellet. The $[^3H]H_2O$ and $[^{14}C]$sucrose volume of the pellet was determined by standard dual isotope analysis, and the values were normalized to the protein content. The intracellular volume was calculated as the difference between the total water ($[^3H]H_2O$) space and the extracellular ($[^{14}C]$sucrose) space.

In some experiments the alteration of the intracellular volume was measured at various times after the addition of a stimulus (time zero). In these experiments, the results from each experiment were compiled into four collection periods in addition to the control (unstimulated) period: 0–0.25, 0.25–3, 3–7, and 7–12 min after the addition of the stimulus. During each collection period, one to five samples were taken, and the results from the entire series of experiments are presented as the mean of the individual experiments. The control values were calculated from samples collected in triplicate or quadruplicate before the addition of the stimuli.

Chelation of Intracellular Free Ca Using BAPTA

The effects of intracellular Ca on various measurements were evaluated by loading the cells with BAPTA, a Ca chelator (Tsien, 1980) to buffer the intracellular Ca. In these experiments the cells were exposed to 25 μM BAPTA-AM, the cell permeable form of the chelator, for at least 20 min before any of the measurements.

Solutions

The composition of the normal solution in which the cells were resuspended was as follows, in millimolar: NaCl, 116.4; KCl, 5.4; $MgSO_4$, 0.8; NaH_2PO_4, 1; Na HEPES, 25; $CaCl_2$, 1.8; Na butyrate, 1; glucose, 5.6, pH 7.4. In Cl-substitution experiments, Na isethionate, KNO_3, and $Ca(NO_3)_2$ were substituted for NaCl, KCl, and $CaCl_2$, respectively. For Ca-free experiments, $CaCl_2$ was replaced with equimolar NaCl. All solutions were pH 7.35–7.4 and 300 ± 5 milliosmolar.

Materials

Male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY) were used for all experiments. ^{36}Cl, [^{14}C]sucrose, and [^{3}H]H$_2$O were purchased from Dupont (Wilmington, DE). Other chemicals and their sources include: nystatin (mycostatin) (Calbiochem-Behring Corp., San Diego, CA), dinonylphthalate (ICN Biochemicals, Inc., Plainview, NY), silicone oil (Aldrich Chemical Co., Milwaukee, WI), and TEA (tetraethylammonium Cl) (Sigma Chemical Co., St. Louis, MO). Dimethylamiloride (DMA) was synthesized as described previously (Cragoe et al., 1967). All other chemicals were reagent grade or better.

Protein Determination

Tissue pellets were dissolved in 0.2 M NaOH/0.1% sodium dodecyl sulfate and protein content was determined by the procedure of Lowry et al. (1951) using BSA as the protein standard.

Statistics

Values are given as the mean ± SE. In all cases, the number of determinations of any measurement represents the results from that number of parotid preparations.

RESULTS

Effect of Ca-mobilizing Agents on [Ca]ᵢ

The basal level of [Ca]ᵢ (fura 2 fluorescence) of rat parotid cells in suspension, ~ 200 nM, was elevated by phospholipase C-linked agonists. As shown in Fig. 1 *A*, the elevation of [Ca]ᵢ in cells exposed to the muscarinic agonist carbachol rapidly reached a maximum value, and then declined to a somewhat lower value, which, in the absence of a muscarinic antagonist (e.g., atropine), was maintained for up to 60 min (McMillian et al., 1987). The addition of atropine returned the [Ca]ᵢ to basal levels. In contrast, the elevated [Ca]ᵢ of cells exposed to substance P was not maintained at high levels; it spontaneously returned close to the unstimulated level within 10 min, as previously noted by us (McMillian et al., 1987) and others (Merritt and Rink, 1987*b*). The maximum elevations of [Ca]ᵢ by carbachol and substance P exceeded the baseline level of [Ca]ᵢ by about two- and threefold, respectively (Fig. 2). Phenylephrine, an alpha-adrenergic agonist, had only a slight effect on [Ca]ᵢ, raising it by ~ 35% (Fig. 2). The extent to which agonist-treated cells maintained elevated levels of [Ca]ᵢ appeared to be closely related to the relative stimulation of the production of (1,4,5)IP$_3$, which was similar initially for carbachol and substance P, but which was not maintained with substance P (McMillian et al., 1987; Merritt and Rink, 1987*b*; Sugiya et al., 1987, 1988). The characteristic changes in [Ca]ᵢ produced by carbachol and substance P were used (see below) to investigate how ion fluxes varied in response to these two different patterns of Ca mobilization and maintenance.

The contribution of [Ca]ᵢ to ion movement also was evaluated by diminishing the rate of elevation of [Ca]ᵢ by loading the cells with BAPTA, a Ca chelator (Tsien, 1980). Under the conditions of these experiments (i.e., exposing the cells to 25 μM BAPTA-AM), the resting [Ca]ᵢ was not significantly affected. However, loading the cells with BAPTA substantially blunted the increases in [Ca]ᵢ promoted by the agonists. Instead of a rapid large increase in [Ca]ᵢ (Fig. 1 *A*), there was a small initial
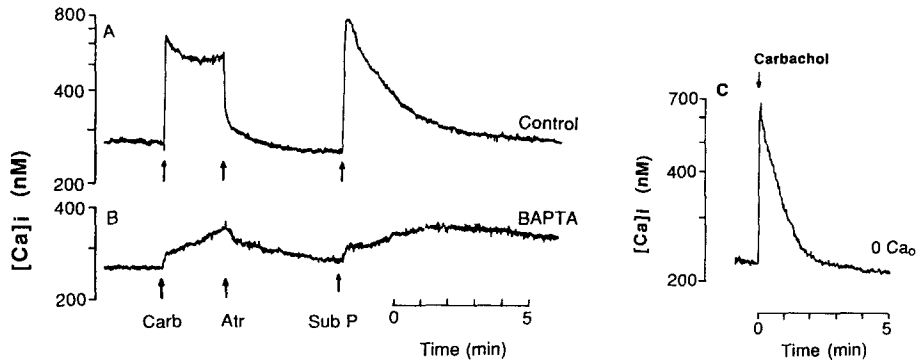
FIGURE 1. The effects of carbachol and substance P on $[Ca]_i$ under normal conditions and when Ca mobilization was altered. $[Ca]_i$ was measured using fura 2. Fig. 1, A and B. Shown is a typical comparison of the effects of carbachol $(2 \times 10^{-5} M)$ and substance P $(1 \times 10^{-8} M)$ on normal (control) rat parotid cells (A) and on cells exposed to 25 μM BAPTA-AM (B). The cells in both traces were taken from the same batch of cells loaded with fura 2, but those in Fig. 1 B were subsequently exposed to 25 μM BAPTA-AM (see Methods). The results shown on these traces are typical of five similar experiments. The muscarinic antagonist atropine $(1 \times 10^{-6} M)$ was added after carbachol. Fig. 1 C. Carbachol $(2 \times 10^{-5} M)$ was added to rat parotid acinar cells suspended in Ca-free medium. This trace is typical of seven experiments, and demonstrates the transient increase seen in the absence of extracellular Ca. See text for further details.

increase in $[Ca]_i$ followed by a slow rise after the addition of carbachol or substance (Fig. 1 B). This treatment did not alter the initial production of IP_3 (not shown). BAPTA treatment also slowed the rate of elevation of $[Ca]_i$ in cells exposed to iono-mycin (not shown), and thus altered both IP_3-mediated and ionophore-mediated increases in $[Ca]_i$.

Another way in which the relationship of $[Ca]_i$ to ion fluxes was examined was to perform experiments in the absence of extracellular Ca. In these experiments, cells
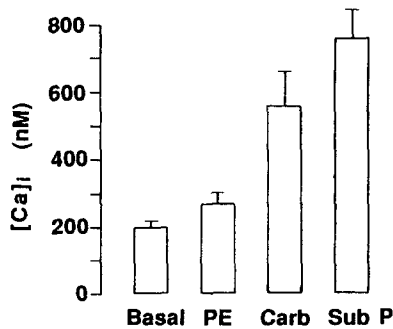

FIGURE 2. The effect of alpha-adrenergic, muscarinic, and substance P agonists on the free $[Ca]_i$ in rat parotid acinar cells in suspension. $[Ca]_i$ was measured using fura 2 (see Methods). Shown are the peak $[Ca]_i$ values reached after the addition of maximally effective concentrations of agonists, which were added sequentially to the same suspension in the order presented on the figure. The antagonists atropine $(1 \times 10^{-6} M)$ and phentolamine $(1 \times 10^{-5} M)$ were added after carbachol $(2 \times 10^{-5} M)$ and phenylephrine $(1 \times 10^{-5} M)$, respectively, to return the elevated $[Ca]_i$ to normal levels. The substance P concentration was $1 \times 10^{-8} M$. Basal values represent the $[Ca]_i$ before the addition of agonists $(n = 7)$.

were either suspended in Ca-free medium or normal medium to which 5 mM EGTA was added. Under these conditions, the basal [Ca]$_i$ (240 ± 39, n = 9) was not significantly different from normal (267 ± 43, n = 18). The maximal carbachol-induced elevation of [Ca]$_i$ (703 ± 163, n = 9) was also similar to that found in the presence of external Ca (675 ± 118, n = 18); but the increase in [Ca]$_i$ was very transitory (Fig. 1 C). These findings are in agreement with recent results reported by Merritt and Rink (1987a) using a similar cell preparation. It has been suggested that the initial elevation of [Ca]$_i$ is due primarily to the release of Ca from intracellular stores via (1,4,5)IP$_3$, and that the subsequent maintenance of elevated [Ca]$_i$ requires Ca influx across the plasma membrane. However, the relative contribution of the intra- and extracellular Ca pools to the elevation of [Ca]$_i$ may be more complicated than this, since recent kinetic experiments have demonstrated that Ca influx may contribute to the elevation of [Ca]$_i$ within 100 ms of exposure to carbachol (Merritt and Rink, 1987a).

Intracellular Volume and Extracellular Space

To accurately measure changes in both the content and concentration of intracellular ions, it was necessary to measure the intracellular and extracellular space of samples collected from the cell suspension after centrifugation through oil (see Methods). For control (nonstimulated) cells, the total water space was 5.56 ± 0.23 μl/mg protein (n = 7 preparations). This represented the entire volume occupied by the intracellular and extracellular space in the pellet. The extracellular space measured using [^{14}C]sucrose was 2.00 ± 0.17 (7) μl/mg protein. The difference between these values, 3.56 ± 0.14 (7) μl/mg protein, represented the intracellular volume. The values for the cellular water content and the fraction (36.0%) of trapped extracellular volume in the pellet were similar to those reported by others (Landis and Putney, 1979; Nauntofte and Poulsen, 1986) using a similar cell preparation. Alterations of intracellular volume in response to different agonists are discussed below.

Alteration of K$_i$ Content by Agonists

Since one of the initial ionic events in response to agonists is the activation of Ca-sensitive K channels (Maruyama et al., 1983b; Marty et al., 1984), the effects of the three phospholipase C-linked agonists in stimulating the net efflux of K from the parotid cell were measured. Secretagogues were added at concentrations that produced maximal increases in [Ca]$_i$. The effects of agonists and ionomycin on K release are shown in Fig. 3 in parallel with their relative effects on [Ca]$_i$. Carbachol initially stimulated a net K efflux, which lasted for ~ 1 min. Net reuptake of K followed, restoring about one-half of the K$_i$ that was initially lost. The addition of atropine returned the K$_i$ content to the prestimulated level. Substance P had a similar effect on the efflux of K, except that the net loss of K$_i$ was somewhat less. Notably, the pattern of K movement mimicked the transient alteration of [Ca]$_i$ by substance P; the K$_i$ content spontaneously returned nearly completely to the prestimulated level within 5–10 min. Phenylephrine acted much like carbachol, but was less effective, and the addition of the alpha-adrenergic antagonist phentolamine restored K$_i$. Ionomycin produced the largest loss of K$_i$ (see values in Fig. 3 legend). Thus, the effects of the three agonists on the initial loss of K$_i$, as well as the extent of recovery

were very similar to the relative magnitude and temporal pattern of their effects on $[Ca]_i$. Muscarinic and alpha-adrenergic agonist-induced losses of K_i and the desensitization of the effect of substance P on K and ^{86}Rb effluxes have been noted in previous studies using rat parotid and rat submaxillary gland slices (Martinez et al., 1976; Putney, 1976; Gallacher, 1983; Friedman et al., 1985).

The similarities between the net alterations in K_i and the elevation of $[Ca]_i$ by the different agonists (Fig. 3), as well as the effects of ionomycin on K efflux, suggested
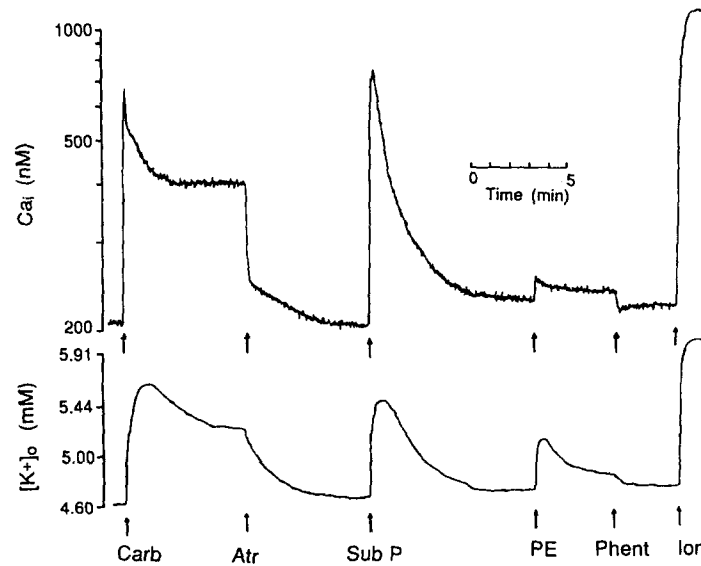

FIGURE 3. The effects of several Ca-mobilizing agents on $[Ca]_i$ and net K release and reuptake in rat parotid acinar cells in suspension. K was monitored using an extracellular K-sensitive electrode, and $[Ca]_i$ was determined using fura 2. The $[Ca]_i$ and K results shown here were obtained at different times using separate parotid cell preparations. A net K efflux or influx is indicated by an increase or decrease, respectively, in the extracellular K concentration. Note that the alterations in K display a close relationship to the kinetics and magnitude of the changes in $[Ca]_i$ produced by the different stimuli. The mean alterations in $[Ca]_i$ by these agonists are shown in Fig. 1. The net loss of K was also calculated as a percentage of the total cellular K released upon exposure of the cells to digitonin at the end of the experiment (see Methods), as follows: carbachol (2×10^{-5} M), $61.9 \pm 3.5\%$ ($n = 6$); substance P (1×10^{-8} M), $52.9 \pm 7.0\%$ (5); phenylephrine (1×10^{-4} M), $28.8 \pm 5.4\%$ (3); ionomycin (1×10^{-6} M), $84.8 \pm 5.3\%$ (3). The total digitonin-releasable K content was 501 ± 44 (6) nmol/mg protein. Atropine (1×10^{-6} M) and phentolamine (1×10^{-5} M) were added where indicated on the figure.

that the agonist-initiated decreases in K_i occurred via efflux through Ca-activated channels. Additional studies were performed to identify the K efflux pathway(s). In these studies, the effects of various inhibitors were examined on both the fractional loss of cellular K as well as the rate of K efflux from the cells (Table I). A substantial portion of the carbachol-induced loss of K was blocked by TEA, which blocks the BK (big potassium) or large-conductance Ca-activated K channel (Suzuki et al.,

1985) and which blocked the large conductance Ca-activated K current in our previous patch-clamp studies of these cells (McMillian et al., 1988). 15 mM TEA was more effective than 2 mM TEA, and both concentrations reduced the fractional loss of K as well as the rate of K efflux. Ba (5 mM) blocked 30–40% of the carbachol-stimulated K efflux. The removal of the extracellular cellular Ca substantially reduced the carbachol-stimulated K efflux. The inhibition was even greater when the rate of Ca elevation was blunted using BAPTA. Under these conditions, the rate of K efflux was reduced > 90%, which suggested that nearly all of the efflux was dependent on the elevation of $[Ca]_i$. These results suggest that as much as 60% of the K efflux occurred through the TEA-sensitive BK channel, and that there is more than one pathway of K efflux. Additional studies (see below) suggested that a portion of the K efflux was due to the activation of a nonselective cation channel.

TABLE I

Effects of Various Conditions on the Release of K from Parotid Acinar Cells Stimulated by Carbachol

	Fractional K release	K release rate
	% inhibition	
TEA (15 mM)	55.1 ± 4.1 (10)	58.7 ± 5.0 (9)
TEA (2 mM)	36.7 ± 4.6 (5)	29.9 ± 9.3 (6)
Barium	31.3 ± 7.3 (5)	40.1 ± 11.1 (5)
0 Caₒ	51.0 ± 2.5 (4)	33.2 ± 9.4 (4)
BAPTA	63.4 ± 10.0 (3)	93.2 ± 3.1 (3)

Carbachol (20 μM) was added to rat parotid acinar cells suspended in solution A, and K release was measured as shown in Fig. 3. When present, TEA and $BaCl_2$ (5 mM) were added 2–3 min before the addition of carbachol. BAPTA-treated cells were exposed to BAPTA-AM (25 μM) for at least 20 min before carbachol. In 0 Caₒ experiments, cells were suspended in nominally Ca-free solution A, and 500 μM EGTA was added ~2 min before carbachol. The effects of carbachol added to cells in the presence of inhibitors were normalized to the results obtained using cells from the same preparation in the absence of any inhibitors. The fractional release of K was the amount of K released by carbachol relative to that of digitonin (see Methods). Carbachol released 61.8 ± 2.2% (15) of the cytosolic K of control cells at a release rate of 3496 ± 287 (16) nmol · mg·min. The number of different preparations is shown in parentheses.

Alteration of Cl_i Content

The addition of Ca-mobilizing stimuli also produced a rapid reduction in the Cl_i content (Fig. 4). The normal level of Cl_i was 183.5 ± 11.7 (n = 6) nmol/mg protein, corresponding to a Cl_i concentration of 51.5 mM. Within the first time period examined (~ 10 s after the addition of stimulus), the Cl_i content was reduced 50.2 ± 2.7% (n = 6), 51.0 ± 8.7% (3), 21.4 (2), and 47.4 ± 3.6% (6) by carbachol (2 × 10⁻⁵ M), substance P (1 × 10⁻⁸ M), phenylephrine (1 × 10⁻⁴ M) and ionomycin (1 × 10⁻⁶ M), respectively. The reduction in Cl_i by carbachol was similar to that reported by others using a suspension of parotid acini (Nauntofte and Poulsen, 1986; Melvin et al., 1987). Unlike the receptor-mediated agonists, ionomycin produced a biphasic effect; after an initial decrease, a marked increase occurred within 30 s of exposure to ionomycin, and the Cl_i content reached levels which were ~ 20% greater than the initial levels (Fig. 4).

In cells loaded with BAPTA to buffer the rise in $[Ca]_i$, Cl efflux stimulated by carbachol and ionomycin was greatly slowed. While the net loss of Cl_i was normally maximal within 10 s after the addition of either stimulus, at this time in BAPTA-loaded cells the Cl_i content was reduced by only ~ 5% of its initial level (Fig. 4). The pronounced lag in the stimulated Cl efflux in BAPTA-treated cells was similar to the lag in $[Ca]_i$ elevation (Fig. 1 B). These findings are consistent with the movement of Cl via a Ca-activated Cl channel in the rat parotid acinar cell.

Alterations of Intracellular Volume

As might be expected from the rapid large losses of K and Cl that were initiated by carbachol and substance P, there were also rapid alterations of intracellular volume upon the exposure of parotid acinar cells to these agonists. Carbachol caused about a 25% decrease in intracellular volume (Fig. 5). The decrease was maximal within
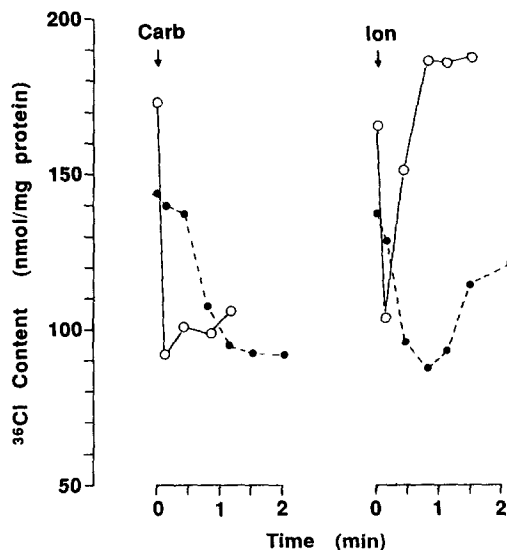

FIGURE 4. The effects of carbachol $(2 \times 10^{-5} M)$ and ionomycin $(1 \times 10^{-6} M)$ on the Cl content of rat parotid acinar cells equilibrated with ^{36}Cl. Shown are the results of a typical experiment for control cells (O) and for cells loaded with BAPTA (●). The Cl content of unstimulated cells (time zero) was determined from triplicate samples, and the subsequent points represent single determinations. Stimuli were added at time zero, and samples were collected as described in Results. The Cl_i content was slightly reduced in BAPTA-loaded cells (82.0 ± 1.6% (3) of control values). This may have been due to a Donnan-type effect of trapped intracellular BAPTA acting as an impermeant anion.

10 s after the addition of carbachol and was maintained for at least 10 min, although a slight (5%) return toward normal levels occurred. A similar fractional decrease in parotid cell volume by carbachol was reported by Nauntoufte and Poulsen (1986). Substance P promoted a similar decrease in intracellular volume; but unlike carbachol, this effect was not maintained and the volume returned nearly to normal within 10 min (Fig. 5). Thus, the alterations of intracellular volume reflect the characteristic differences in the alterations of $[Ca]_i$ elicited by substance P and carbachol.

Although ionomycin also promoted net K (Fig. 3) and Cl (Fig. 4) efflux in parotid cells, its effect on cell volume was somewhat different from the receptor-mediated agonists. Ionomycin produced a smaller volume decrease (~ 16%) than did carbachol or substance P, which was followed by swelling to a volume ~ 25% larger than

the normal cell volume (Fig. 5). Presumably, the rapid influx of Cl (Fig. 4) and Na (see below) promoted by ionomycin was more than sufficient to replace the initial losses of K and Cl, but further experiments will be necessary to establish the exact mechanism of ionomycin-induced swelling.

Alterations of Na_i by Agonists

The effect of secretagogues on Na_i was measured by equilibrating the cells with ^{22}Na and then exposing them to Ca-mobilizing stimuli. Before stimulation, the Na_i content was 66.1 ± 3.8 ($n = 30$) nmol/mg protein. Using the intracellular volume determination, this represents an intracellular Na concentration of 18.6 mM. The effects of carbachol and substance P on Na_i are shown in Fig. 6. Within ~ 1 min after the addition of either agonist to the cell suspension, the ^{22}Na content rose to > 2.5 times that of the unstimulated cells, and then declined. Since the cell volume decreased (Fig. 5) while the Na increased, the Na_i was elevated to a concentration
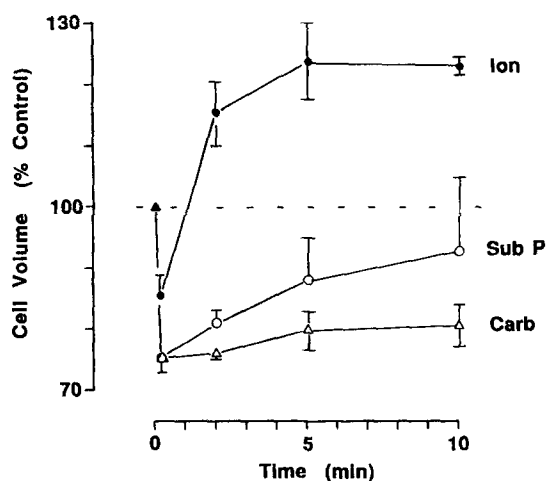

FIGURE 5. The effect of carbachol (△) (2×10^{-5} M), substance P (O) (1×10^{-8} M), and ionomycin (●) (1×10^{-6} M) on the intracellular volume of rat parotid acinar cells in suspension. Intracellular volume determinations were measured using [^{3}H]H_2O and [^{14}C]sucrose (see Methods), and are normalized to the volume measured in the absence of stimuli (▲), represented by the broken line, which was 3.56 ± 0.14 μl/ mg protein (see Results). For carbachol and substance P, $n =$ 4 preparations; for ionomycin, $n = 3$ preparations.

> 47 mM. Similar elevations of the Na concentration by carbachol were reported by Poulsen and Kristensen (1982). In cells exposed to substance P, Na_i returned to the prestimulated level within 10 min. However, even after 10 min the carbachol-treated cells maintained an elevated level of Na_i that was about twice that of the unstimulated level (Fig. 6). The characteristic alterations of Na_i promoted by substance P and carbachol were quite similar to the characteristic effects of these agonists on [Ca]_i (Fig. 1 A), although they lagged somewhat behind. The reduction in Na_i may involve activation of the Na pump or inactivation of Na entry (see below).

Initial Rate of Na Influx

In the absence of stimuli, the Na concentration within the parotid cell is constant because the influx of Na is equal to the efflux. Na efflux is primarily due to the activity of the Na,K-ATPase (the Na pump) in the basolateral membrane. In the

results presented in Fig. 6, the agonists produced an increase in Na_i because the entry of Na into the cell was initially more rapid than the efflux of Na. The increase in Na_i, as well as the decrease in K_i (Fig. 3) (see Discussion), rapidly activates the Na pump (see below), promoting Na efflux. Therefore, to measure unidirectional initial rates of Na influx, the increase in ^{22}Na content promoted by Ca-mobilizing agents was measured in the presence of ouabain to block Na efflux. The agonist-stimulated rates of ^{22}Na accumulation were ~ 85% larger in the presence of ouabain than in its absence (Fig. 7), although the ^{22}Na content increased only very slowly if ouabain was added alone (Fig. 8). The rates of the carbachol-stimulated and the substance P-stimulated Na influx were very similar and were more than 30 times faster than that observed with ouabain alone (Fig. 8). Phenylephrine stimulated the influx of Na much less than either carbachol or substance P, but at a rate that was still well above
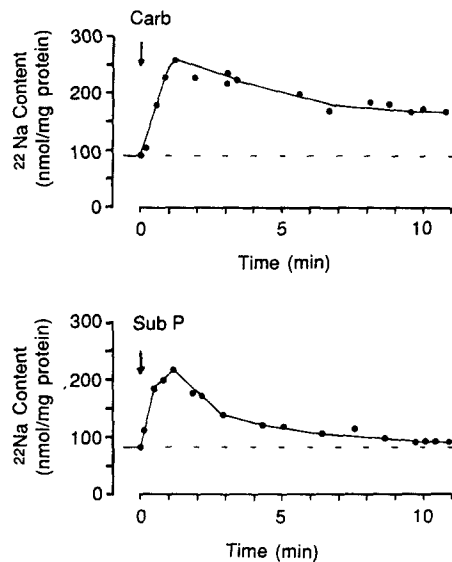

FIGURE 6. The effects of carbachol $(2 \times 10^{-5}$ M$)$ and substance P $(1 \times 10^{-8}$ M$)$ on the Na content of rat parotid acinar cells in suspension. The cells were equilibrated with ^{22}Na before the addition of agonist. The Na content of unstimulated cells (time zero) was determined from at least three samples collected before the addition of agonist and is represented by the broken line. Each point after the addition of agonist represents the Na content measured in a single sample. The results from a typical experiment are shown. The maximum value was reached 1.4 ± 0.2 (8) and 1.4 ± 0.1 (6) minutes after the addition of carbachol and substance P, respectively. In these experiments, the Na content of cells exposed to carbachol rose from 77.0 ± 5.5 (8) nmol/mg protein (time zero) to a maximum of 203.3 ± 10.7 (8), and then declined to 140.8 ± 21.0 (4) 10 min after the addition of agonist. Cells exposed to substance P rose from 71.8 ± 6.4 (6) nmol/mg protein (time zero) to a maximum of 180.6 ± 9.6 (6), and then declined to 74.2 ± 7.6 (4) 10 min after the addition of agonist.

the basal (ouabain alone) rate. The relative stimulatory effects of carbachol, substance P, and phenylephrine on ^{22}Na uptake (Fig. 8) were similar to their relative effects on $[Ca]_i$ (Fig. 2), and the Ca ionophore ionomycin, which increases $[Ca]_i$ more than any of these three agonists, stimulated ^{22}Na uptake to a rate almost twice that of carbachol or substance P (Fig. 8).

The degree to which these agents stimulated Na uptake did not appear to result from the sensitivity of separate populations of cells. The effect of the simultaneous addition of carbachol and substance P was not additive, and increased the rate of uptake only 6.1% (n = 2) above the larger of the two individual rates of uptake. Similarly, the rate of ^{22}Na uptake promoted by the combination of ionomycin and
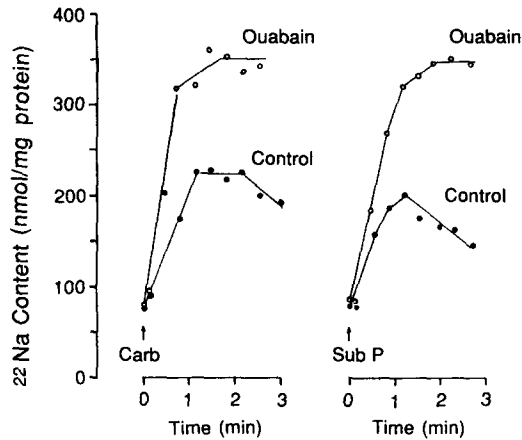
FIGURE 7. The effect of ouabain on the accumulation of Na by rat parotid acinar cells exposed to carbachol $(2 \times 10^{-5} M)$ and substance P $(1 \times 10^{-8} M)$. Ouabain $(2.5 mM)$ was added 20 s before each agonist. The Na content of unstimulated cells (time zero) was determined from at least three samples. Each point after the addition of agonist represents a single sample. The initial rate of Na accumulation after the addition of carbachol was 88 ± 23% (*n* = 4 preparations) larger in the presence of ouabain, and the rate of accumulation after the addition of substance P was 99 ± 19% (*n* = 4 preparations) larger in the presence of ouabain.

carbachol was only 5.0% (*n* = 2) greater than that observed in the presence of iono-mycin alone. These findings are further evidence that maximal concentrations of these stimuli were used in these studies.

Effect of Agonists on Oxygen Consumption

The rate of oxygen consumption (QO_2) was measured to estimate the degree of agonist-stimulated Na pump activity of the parotid cell. Because mitochondrial oxidative phosphorylation supplies the majority of the ATP required for active transport in many epithelial tissues, there is a tight coupling between the activity of the Na pump and cellular oxygen consumption, and the ouabain-sensitive QO_2 is a quantitative measurement of the Na pump activity (Mandel and Balaban, 1981). The QO_2 was greatly stimulated (Fig. 9) by the addition of the cationophore nystatin, which increases intracellular Na and decreases intracellular K (Soltoff and Mandel,
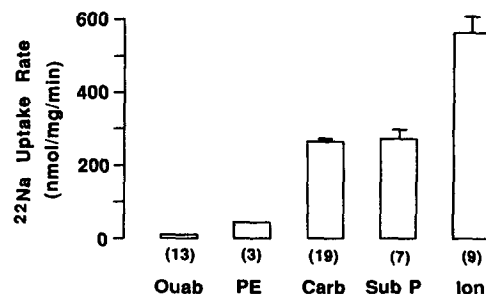

FIGURE 8. The effects of phen-ylephrine $(1 \times 10^{-4} M)$, carbachol $(2 \times 10^{-5} M)$, substance P $(1 \times 10^{-8} M)$, and ionomycin $(1 \times 10^{-6} M)$ on the initial rate of unidirectional Na uptake into rat parotid acinar cells in suspension in the presence of ouabain. The cells were equilibrated with ^{22}Na before the addition of the stimuli, and ouabain $(2.5 mM)$ was added 20 s before the stimuli (see Methods). Also shown is the rate of Na accumulation in the presence of ouabain alone. The number of cell preparations is shown at the bottom of each bar.

1984). The nystatin-induced stimulation of respiration was blocked by ouabain. Thus, the nystatin-stimulated ouabain-sensitive QO_2 (Fig. 10) is a measurement of the Na pump activity when Na entry into the cell was not rate-limiting and when the intracellular Na/K ratio exerts a maximal stimulation of the Na pump (Soltoff and Mandel, 1984).

There was an increase in the QO_2 of the parotid cell suspension in response to the addition of phospholipase C-mediated agonists. The stimulation of respiration was prevented by the prior addition of either ouabain, which reduced the basal QO_2 to a rate slightly below that of the unstimulated state, or of the appropriate antagonist (atropine for carbachol, phentolamine for phenylephrine), which by itself did not alter the basal QO_2 (not shown). The agonist-stimulated QO_2 returned to the unstimulated rate ($\pm$ 10%) after the addition of the appropriate antagonist (Fig. 9).
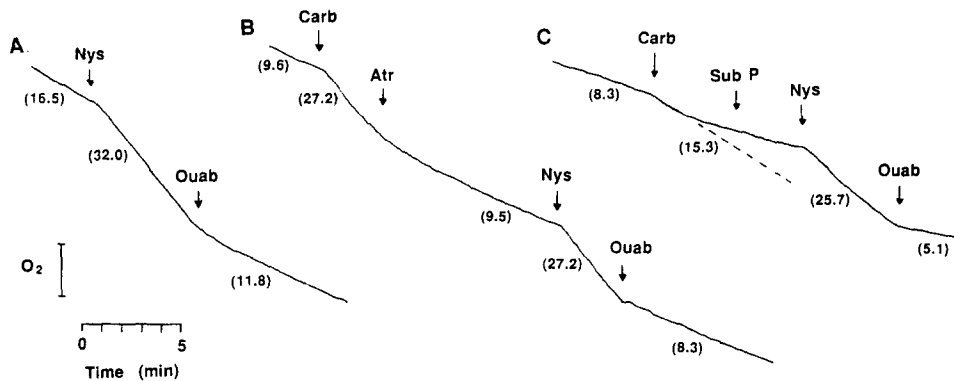

FIGURE 9. The effect of various stimuli on the oxygen consumption of rat parotid acinar cells in suspension. Shown is a reduction in the O_2 present in a closed system. In A and B, the cells were suspended in normal media. In C, cells were suspended in Ca-free media that contained 500 μM EGTA. The following concentrations of agents were added: nystatin, 0.4 mM; ouabain, 3 mM; carbachol, 2×10^{-5} M; atropine, 1×10^{-6} M; substance P, 1×10^{-8} M. The numbers in parentheses are the rate of oxygen consumption (in nanomoles O_2 per milligram of protein per minute), and were calculated from the linear portion of disappearance of O_2. In C, the calculation was based on the initial portion of the transitory stimulation, represented by the dotted line, after the addition of carbachol. These traces are representative of those obtained in at least four separate preparations.

When ouabain was added to the suspension after the addition of agonist, the QO_2 was reduced to a rate below the basal level. Not surprisingly, the relative effectiveness of the agonists in stimulating the ouabain-sensitive QO_2 (Fig. 10) was similar to that for both ^{22}Na uptake (Fig. 8) and $[Ca]_i$ (Fig. 2). The relative stimulation of the ouabain-sensitive QO_2 suggests the degree to which the agonists increase the intracellular Na concentration or the Na/K ratio within the parotid cells (see Discussion). Carbachol stimulated the ouabain-sensitive QO_2 almost as much as did nystatin (Fig. 10), which suggests that the Na pump activity was stimulated to nearly maximal rates. Assuming a mitochondrial P/O_2 ratio of 6:1 and a Na pump stoichiometry of 3 Na^+:1 ATP, the theoretical Na^+/O_2 ratio is 18/1. This value was used to calculate the pump-mediated Na efflux based on the ouabain-sensitive QO_2, and is indicated

on the right-hand ordinate of Fig. 10. In the presence of maximal concentrations of carbachol, the calculated Na pump activity was ~ 325 nmol $Na^+ \cdot$ mg protein$^{-1} \cdot$ min^{-1}.

Contribution of Na-K-2Cl Cotransport

Additional experiments were carried out to identify the Na entry pathway(s) in the parotid cell. Various reports have suggested that a portion of Na entry across the basolateral membrane of the parotid cell (see Introduction) and in secretory cells (Petersen and Maruyama, 1984) and many other cells (O'Grady et al., 1987) occurs through the Na-K-2Cl cotransport system. Therefore, the effects of furosemide (which blocks Na-K-2Cl cotransport in a number of systems, O'Grady et al., 1987) and the removal of extracellular Cl on the carbachol-stimulated ^{22}Na uptake and QO$_2$ were examined. Since the effects promoted by carbachol were large and long-lasting, carbachol was taken as representative of phospholipase C-mediated agonists.
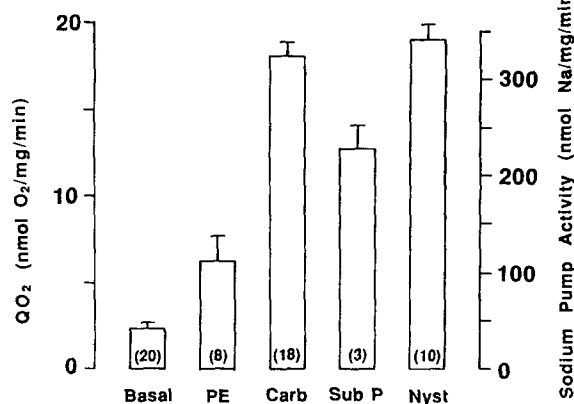

FIGURE 10. The effect of various stimuli on the ouabain-sensitive rate of oxygen consumption (QO$_2$) in rat parotid acinar cells. Shown is the unstimulated (basal) rate, as well as the maximum QO$_2$ in response to phenylephrine $(1 \times 10^{-4}$ M), carbachol $(2 \times 10^{-5}$ M), substance P $(1 \times 10^{-8}$ M), and nystatin (0.4 mM). The corresponding Na pump activity was calculated from the ouabain-sensitive QO$_2$ by assuming a Na/O$_2$ ratio of 18:1 (see text), and is presented on the right-hand ordinate. The number of preparations is shown at the bottom of each bar.

The carbachol-stimulated ^{22}Na uptake rate was reduced by ~ 55% in the cells exposed to 1 mM furosemide (Fig. 11, Table II). Bumetanide (100–200 μM), a diuretic which also blocks Na-K-2Cl cotransport (O'Grady et al., 1987), also blocked the carbachol-stimulated ^{22}Na uptake to a similar degree (not shown). In the absence of extracellular Cl, the carbachol-stimulated rate was reduced by 67%, and the addition of furosemide to cells suspended in Cl-free solution reduced the stimulation by an additional 11% (Fig. 11).

Similar effects were observed when the rates of oxygen consumption were measured (Fig. 11). The presence of 1 mM furosemide, the removal of extracellular Cl, and the addition of 1 mM furosemide in the absence of Cl reduced the carbachol-stimulated ouabain-sensitive QO$_2$ by 49, 57, and 66%, respectively. The addition of bumetanide also had an inhibitory effect comparable to furosemide on the carbachol-stimulated ouabain-sensitive QO$_2$ (not shown). High concentrations of furose-

mide have been reported to directly or indirectly (e.g., by metabolically compromising the mitochondria) alter the Na,K-ATPase activity of some cells (Manuel and Weiner, 1976). However, this was not the case for parotid cells, since the stimulation of the ouabain-sensitive QO_2 by nystatin was not significantly affected by furosemide or Cl-free medium (Fig. 11).

The stimulation of [22]Na uptake by ionomycin was also reduced by 1 mM furosemide (Fig. 11, Table II). The relative inhibition of ionomycin by furosemide (22%) was less than that of carbachol (54%), but more importantly, the absolute magnitudes of the furosemide-sensitive portion of [22]Na influx (~ 140 nmol·mg pro-
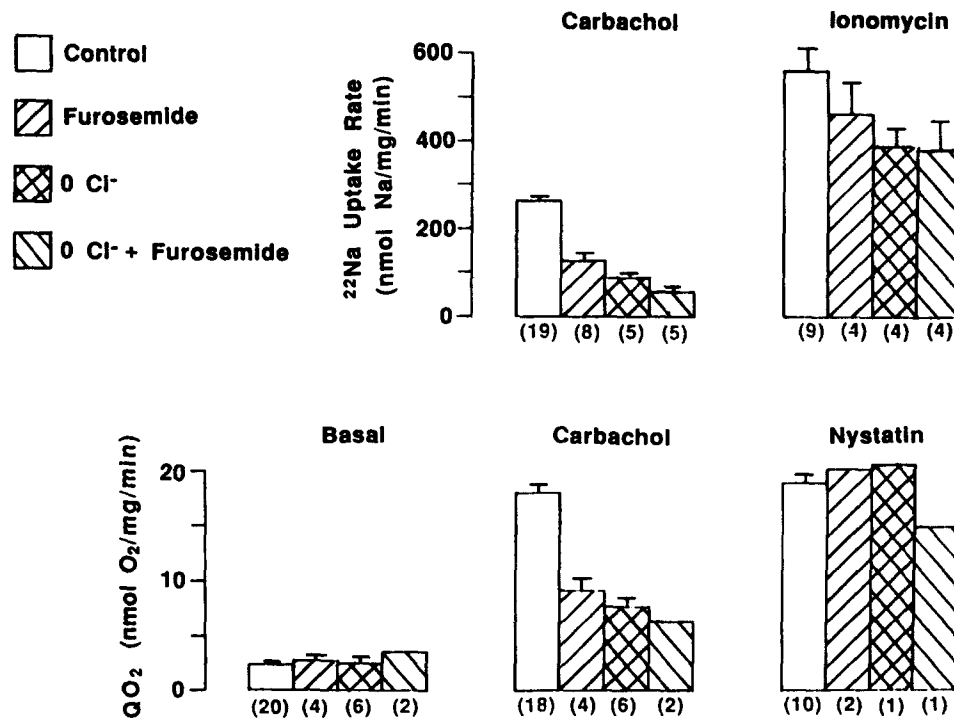

FIGURE 11. The effects of furosemide (1 mM) and/or the absence of extracellular Cl on the stimulation of [22]Na uptake and the ouabain-sensitive QO_2 by carbachol $(2 \times 10^{-5}$ M) and ionomycin $(1 \times 10^{-6}$ M) or nystatin (0.4 mM). Furosemide was present for at least 10 min before the addition of stimuli. The basal QO_2 values were calculated from the QO_2 in the absence of stimuli. The number of preparations are shown at the bottom of each bar.

tein^{-1}·min^{-1}) were very similar for carbachol and ionomycin (Table II). The removal of extracellular Cl (31% inhibition) was more effective than furosemide in reducing the ionomycin-stimulated [22]Na uptake rate, as was the combined effect of furosemide and Cl-free medium (33% inhibition) (Fig. 11). In all of these conditions, the absolute values of the reductions in the rate of [22]Na influx were nearly identical for carbachol and ionomycin, suggesting that they promoted an equivalent influx of Na via Na-K-2Cl cotransport. However, even when furosemide was added in the absence of extracellular Cl, ionomycin still stimulated [22]Na uptake at a substantial

rate, more than 350 nmol Na·mg protein^{-1}·min^{-1}, indicating that a substantial amount of the ionomycin-induced increase in Na uptake occurred through a mechanism other than Na-K-2Cl cotransport (see Discussion).

These results suggest that the majority of Na entry into the parotid cell stimulated by carbachol occurred via the Na-K-2Cl cotransport system. The relative contribution of this system to Na entry under basal (unstimulated) conditions was not directly measured in this study. Although samples were sometimes collected immediately upon the addition of ^{22}Na to the cell suspension before the addition of agonists, the normal Na concentration of the parotid cell was low, $\sim$ 19 mM (see above), and an accurate measurement of the unstimulated rate of ^{22}Na entry could not be reliably obtained under the conditions of these experiments. The basal ouabain-sensitive QO_2 (Fig. 11) or the rate of ^{22}Na uptake in the presence of ouabain (Fig.

TABLE II

Effect of Furosemide and/or DMA on the Stimulated Rate of ^{22}Na Uptake into Rat Parotid Acinar Cells

| | Furosemide | | | DMA | | | II. Control | Δ | | |
	I. Control	Exptal.	Δ	Control	Exptal.	Δ		Furo.	DMA	Furo. + DMA
				nmol Na/mg protein per min						
Carb.	265.0	123.0	142.0	264.0	216.3	47.7	257.8	110.2	40.9	145.5
	± 14.1	± 14.0	± 13.2	± 26.1	± 26.3	± 8.1	± 25.4	± 16.3	± 18.2	± 18.5
	(10)	(10)	(10)	(8)	(8)	(8)	(4)	(4)	(4)	(4)
Ion.	590.1	463.1	127.0	503.5	458.0	45.5				
	± 68.1	± 70.9	± 12.9	± 27.0	± 47.0	± 21.2				
	(4)	(4)	(4)	(3)	(3)	(3)				

The uptakes were determined in paired (± inhibitor) experiments. The cells were first equilibrated with ^{22}Na, and then split into two (or more) portions, one (or more) of which was exposed to the inhibitor(s). About 20 s before the addition of the stimulating agent, ouabain (2.5 mM) was added to inhibit the Na pump so that the unidirectional influx could be measured. In the presence of ouabain alone, the rate was 8 ± 1 (13) nmol Na/mg protein per min. Concentrations: carbachol (Carb.) 2 × 10^{-5} M; ionomycin (Ion.) 1 × 10^{-6} M; furosemide (Furo.) 1 × 10^{-3} M; DMA, 5 × 10^{-6} M (Exptal., experimental.) Although the absolute rates of Na uptake were very different for carbachol and ionomycin, the furosemide-sensitive or the DMA-sensitive rates were very similar for both stimuli, indicating that they activated Na-K-Cl cotransport and Na-H exchange to a similar extent. In series II the effects of furosemide and DMA were measured separately and in combination. The effects of the two inhibitors were additive, indicating that they each blocked a single uptake pathway.

8), which are measurements of the Na pump activity under unstimulated conditions, were small and were not measureably reduced by furosemide or chloride removal in these experiments. However, the ^{22}Na content of unstimulated cells was reduced by furosemide by a small but significant amount (Table III). Additionally, we observed that the Na content of cells suspended in Cl-free medium (± furosemide) was lowered by $\sim$ 40% (Table III). Furosemide has been reported to reduce the rate of ^{36}Cl uptake into unstimulated rat parotid acini by $\sim$ 63% (Nauntofte and Poulsen, 1986). Therefore, the Na-K-2Cl cotransport system appears to be the major pathway by which Na enters the parotid cell both in the unstimulated state and when the cells are stimulated by carbachol and other phospholipase C-mediated agonists.

Contribution of Na-H Exchange to Na Entry

Neurotransmitters, hormones, and growth factors alkalinize a variety of cells by activating the Na-H exchange system (Grinstein and Rothstein, 1986). To determine whether this system was activated in parotid cells, pH_i measurements were made. In the absence of any stimuli, the pH_i was 7.20 ± 0.04 (n = 7). The addition of carbachol or substance P to the parotid cell suspension elevated the pH_i (Fig. 12). The alkalinization was always preceded by a slight decrease in pH_i, which occurred within the first 10–15 s after the addition of agonist. In the absence of external Na or in the presence of 5 μM DMA, a potent amiloride analogue that has been demonstrated to inhibit Na-H exchange in other cells (Besterman et al., 1985), the agonists did not produce an alkalinization of the cells. Under these conditions, in which the Na-H exchanger was inhibited, carbachol or substance P produced a larger acidification than usual (Fig. 12). Similar acidification responses have been observed in other cells and were attributed to an increase in $[Ca]_i$, and may involve Ca-H exchange (Ives and Daniel, 1987). After carbachol or substance P elevated the pH_i

TABLE III

Effect of Furosemide (1 mM) and/or the Removal of Extracellular Cl on the Na Content of Unstimulated Parotid Cells

		Na content (nmol/mg protein)			
A.	Control	Furosemide	Δ		
	77.5 ± 5.2	71.8 ± 5.8	5.7 ± 1.8		
B.	Control	0 Cl	Δ	0 Cl + Furosemide	Δ
	66.3 ± 7.9	40.2 ± 6.3	26.1 ± 2.1	39.9 ± 5.6	26.4 ± 3.8

The Na content was determined as described in Methods. The values were corrected for trapped extracellular Na using the [^{14}C]sucrose space (see text). The data are presented in paired fashion. Section A shows the results obtained by equilibrating a suspension of parotid cells with ^{22}Na and separating it into two portions, one of which was exposed to furosemide (n – 13 preparations). In section B, a portion of each cell preparation was suspended in normal Cl-containing medium (control) or Cl-free medium plus or minus furosemide (n – 7 preparations).

of cells suspended in normal medium, the addition of DMA returned the elevated pH_i toward normal levels (Fig. 12). Monensin, an ionophore that exchanges Na^+ and H^+, also increased the pH_i of parotid cells and was not blocked by DMA (Fig. 12). These findings suggest that a Na-H exchange system is present in rat parotid acinar cells and can be activated by muscarinic agonists and substance P. The presence of a Na-H exchanger in these cells was also recently reported by Manganel and Turner (1988), who found that ^{22}Na uptake into basolateral membrane vesicles prepared from rat parotid glands was stimulated by an outwardly directed pH gradient and was greatly reduced by 1 mM amiloride.

The relative contribution of Na-H exchange to the agonist-induced uptake of Na was evaluated by examining the effect of DMA on the ^{22}Na uptake rate (Table II). DMA (5 μM) reduced the effect of carbachol by 18%. The DMA-sensitive component of the ionomycin-stimulated ^{22}Na uptake rate was quantitatively similar (Table

II), although it made up a small fraction (9%) of the total ionomycin-stimulated uptake. Together, the furosemide-sensitive and DMA-sensitive components accounted for 72% of the carbachol-stimulated ^{22}Na uptake and 29% of the ionomycin-stimulated ^{22}Na uptake. In several experiments furosemide and DMA were added both singly and in combination, and the effects of the inhibitors were found to be additive (Table II). This indicated that the two inhibitors were indeed acting on separate Na entry pathways. In agreement with these findings, 1 mM furosemide did not significantly reduce pH gradient-stimulated ^{22}Na uptake into rat parotid basolateral membrane vesicles (Manganel and Turner, 1988).

Inhibition of Na Uptake by TEA

The elevation in Na$_i$ appeared to occur subsequent to the elevation of [Ca]$_i$ and the losses of K$_i$ and Cl$_i$. To examine whether these increases in Na$_i$ required a prior loss
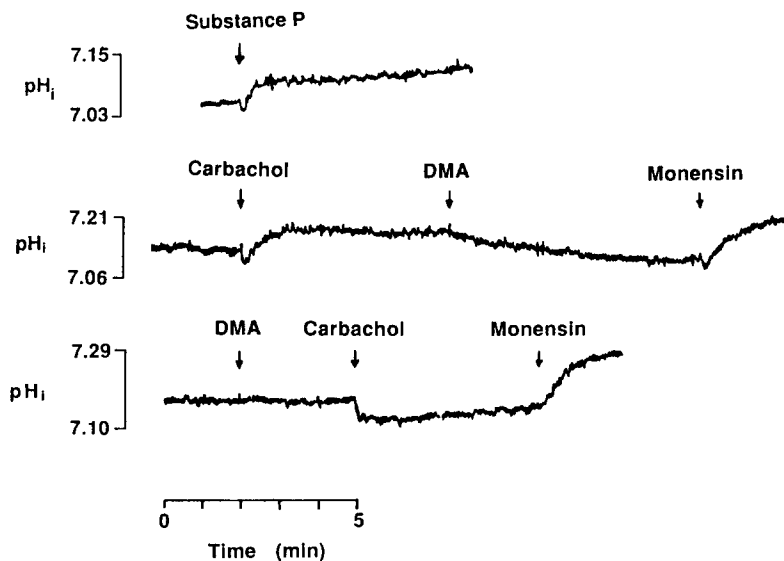

FIGURE 12. The effects of substance P $(1 \times 10^{-8}$ M), carbachol $(1 \times 10^{-4}$ M), and monensin (6 μM) on the pH$_i$ of rat parotid acinar cells in suspension. Where indicated, 5 μM DMA was added. pH$_i$ was determined using the pH-sensitive dye BCECF as described in the Methods. The pH$_i$ in unstimulated cells was 7.199 $\pm$ 0.040 (7). The addition of carbachol and substance P produced an initial decrease in pH$_i$ by 0.023 $\pm$ 0.005 (7) and 0.023 $\pm$ 0.007 (3) units, respectively, followed by an elevation in pH$_i$ to 0.041 $\pm$ 0.004 (7) and 0.065 (2) units, respectively, above the unstimulated value.

of K$_i$, Na uptake studies were performed in the presence of 15 mM TEA, which blocked the majority of carbachol-stimulated K efflux (Table I). The initial rate of carbachol-stimulated Na uptake was reduced by 60.3 $\pm$ 13.8% (3), suggesting that a substantial portion of the elevation of Na$_i$ depends on the prior rapid loss of K$_i$. The activation of Na entry may be part of a volume regulatory response in parotid cells, as it is in various other kinds of cells (see Discussion).

Effect of Ca on ^{22}Na *Uptake and* QO_2

Removal of extracellular Ca. The removal of extracellular Ca inhibited the carbachol-stimulated ^{22}Na unidirectional uptake rate (ouabain present) by ~70%, and the ionomycin-stimulated uptake rate was reduced by 76% relative to the rate measured in the presence of Ca (Fig. 13). Moreover, even though ouabain was present, the ^{22}Na content increased to only about one-half the usual maximum reached in Ca-containing medium. For example, although carbachol increased Na_i from a basal
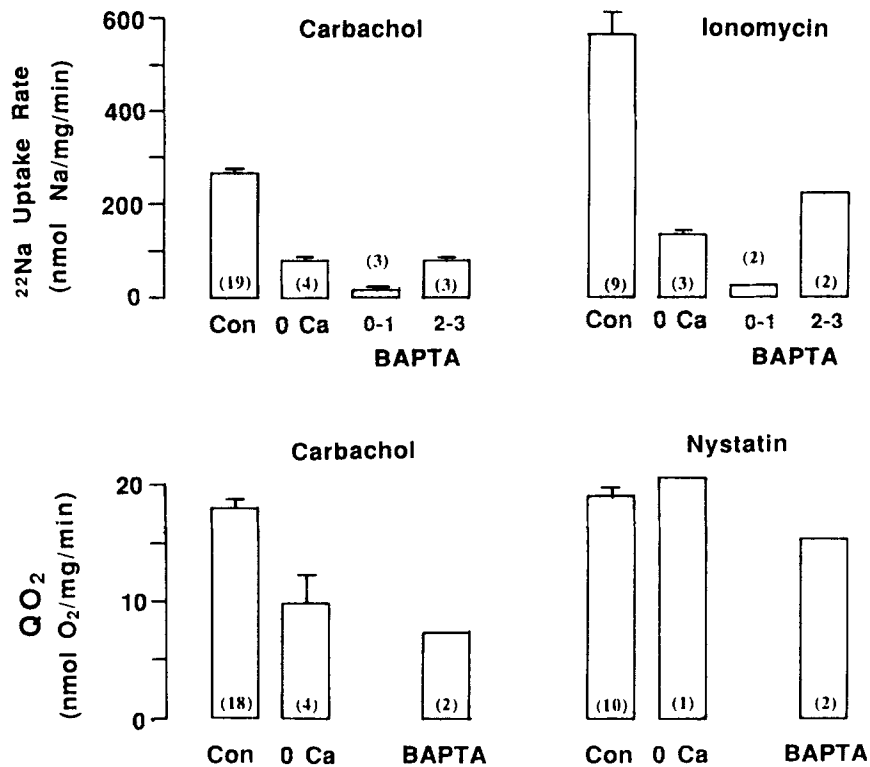

FIGURE 13. The effects of carbachol $(2 \times 10^{-5}$ M) and ionomycin $(1 \times 10^{-6}$ M) on the rate of ^{22}Na uptake and the ouabain-sensitive QO_2 of rat parotid cells in suspension. Cells were suspended in normal medium (control) or in Ca-free medium containing 500 μM EGTA (0 Ca), or in normal medium treated with 25 μM BAPTA-AM (BAPTA). For the BAPTA-treated cells, the ^{22}Na uptake rate was calculated over the first minute (0–1) as well as a second interval between ~2–3 min (2–3) after the addition of the stimuli. The number of preparations are shown at the bottom of each bar.

value of 67.2 ± 6.8 (3) to 329.2 ± 16.4 (3) nmol/mg protein in cells in normal medium, in Ca-free medium Na_i was elevated from a basal level of 60.5 ± 14.5 (4) to only 195.9 ± 8.2 (3) nmol/mg protein. The ionomycin-stimulated accumulation of ^{22}Na also did not continue beyond a level that was much lower than usual (not shown). These results suggest that in the absence of extracellular Ca, carbachol (Fig. 1 C) and ionomycin can release sufficient intracellular Ca to partially stimulate Na

entry into the cell, but that this entry occurs at a reduced rate and cannot be maintained for a long period of time.

The effects of agonists on the QO$_2$ of cells suspended in Ca-free medium also clearly indicate the transitory nature of the stimulation under these conditions (Fig. 9 C). After the addition of carbachol, the rate of oxygen consumption increased only briefly before returning to the unstimulated level. The addition of phenylephrine or substance P also caused a transitory stimulation of the QO$_2$ under these conditions (not shown). Similar observations of the short-lived effects of carbachol on oxygen consumption of guinea pig parotid acini suspended in Ca-free solution were reported by Hootman and Williams (1985). After the initial transient increase in QO$_2$ produced in rat parotid cells by carbachol returned to the basal rate, the addition of substance P did not cause a further stimulation (Fig. 9 C), even in the presence of an antagonist (atropine) to carbachol (not shown). This suggests that the Ca released by the initial stimulation was not replenished in the absence of extracellular Ca. Consistent with the reduction in the rate of ^{22}Na uptake and K efflux in Ca-free medium, the removal of extracellular Ca also reduced the carbachol-stimulated ouabain-sensitive QO$_2$ to ~ 50% of that found in normal medium (Fig. 13). In the absence of extracellular Ca the nystatin-stimulated ouabain-sensitive QO$_2$ was not significantly affected (Fig. 13), indicating that Na pump activity was not limited by oxidative phosphorylation under these conditions.

Unlike the reductions in ^{22}Na uptake and QO$_2$ of cells in the absence of external Ca (Fig. 13), the stimulation of inositol phosphate production by muscarinic agonists was similar to that found in cells suspended in normal media over the first several minutes (Aub and Putney, 1985; McMillian, unpublished data). Thus, the transitory nature of the carbachol-stimulated increases in Na entry in the absence of extracellular Ca was not due to a diminished production of IP$_3$, but was due to the inability of the cell to keep [Ca]$_i$ at levels sufficient to maintain the normal degree of activation of such Ca-dependent processes as the opening of K channels.

Buffering of [Ca]$_i$. Additional studies of the effects of [Ca]$_i$ on Na entry and Na pump stimulation were performed by loading the parotid cells with BAPTA to blunt the elevations in [Ca]$_i$. BAPTA-loaded cells exposed to carbachol and ionomycin displayed characteristic time lags in ^{22}Na accumulation, and the rates of ^{22}Na uptake were greatly reduced (Fig. 13). During the first minute of carbachol exposure, when the maximum ^{22}Na content normally was reached (Fig. 7), the carbachol-stimulated ^{22}Na uptake rate (measured in the presence of ouabain) was reduced by 93% in BAPTA-loaded cells (Fig. 13). Between the second and third minute after the addition of carbachol to BAPTA-loaded cells, the rate of ^{22}Na entry into the cells increased to ~ 80 nmol·mg·protein^{-1}·min^{-1} (Fig. 13), which was still only ~30% of the normal Na uptake rate measured during the first minute. There also was a pronounced delay in the onset of the stimulation of oxygen consumption by carbachol (not shown), which was reduced by 60% compared with cells not exposed to BAPTA (Fig. 13). The ionomycin-stimulated ^{22}Na uptake rate measured during the first minute was ~ 95% less than the normal ionomycin-stimulated rate (Fig. 13), a similar proportional reduction to that observed for carbachol. Between the second and third minute, the uptake of ^{22}Na increased to a rate that was ~ 40% of the normal initial ionomycin-stimulated rate.

The nystatin-stimulated ouabain-sensitive QO_2 of BAPTA-loaded cells was only slightly different from that of normal cells (Fig. 13), indicating that the delays in the carbachol- or ionomycin-stimulated increases in QO_2 were due to the decrease in Na entry and accumulation rather than to a direct effect of BAPTA-loading on either the Na,K-ATPase or on the ability of mitochondrial metabolism to supply ATP to the Na pump. BAPTA-loaded cells also had similar ATP contents to control cells (not shown).

DISCUSSION

The goal of these experiments was to quantitate the alterations of various ion fluxes stimulated by Ca-mobilizing secretagogues in the rat parotid acinar cell, and to study the relationship of $[Ca]_i$ to the activation of the ion transport systems. The experiments presented here demonstrate the coupling between increases in $[Ca]_i$ and the activation of multiple ion transport systems. The elevation of $[Ca]_i$ produced rapid reductions in K_i and Cl_i, accompanied by decreases in intracellular volume, and were followed by increases in Na_i. In response to carbachol, elevations of $[Ca]_i$ and alterations of intracellular electrolytes were relatively sustained, while responses to substance P desensitized and returned toward prestimulated levels. The majority of the carbachol-stimulated uptake of Na was through Na-K-2Cl cotransport and Na-H exchange. Ionomycin and carbachol were equally effective in stimulating Na uptake through both pathways, suggesting that the activation of these Na uptake systems was initiated by a rise in $[Ca]_i$.

As has been well documented for parotid cells (Aub and Putney, 1985; Irvine et al., 1985; McMillian et al., 1987) and for many other cells (for review, see Michell, 1986), the receptor-mediated agonists used in this study stimulate the hydrolysis of PIP_2 and generate diacylglycerol and inositol polyphosphates, including $(1,4,5)IP_3$. In other studies we observed that the activation of muscarinic, alpha-adrenergic, and substance P receptors increased the production of IP_3, and that the relative effects of these agonists on this increase were similar to their effects on elevating $[Ca]_i$ (McMillian et al., 1987, 1988). Merritt and Rink (1987a) observed a dose-dependent lag in the elevation of $[Ca]_i$ within the first second after the addition of carbachol to rat parotid acinar cells, and suggested that the lag was probably related to the time required for the accumulation of an effective level of IP_3 to be generated. However, due to kinetic and technical constraints, evidence that generation of IP_3 precedes the increase in $[Ca]_i$ has not yet been obtained in intact cells.

Activation of K and Cl Channels by Elevating [Ca]_i

The mechanism by which the elevation of $[Ca]_i$ produces a stimulation of fluid and electrolyte secretion involves both Ca-activated K channels in the basolateral membrane and chloride channels in the apical membrane (Marty et al., 1984; Petersen and Maruyama, 1984). Patch-clamp studies have provided direct evidence of Ca-activated K channels in secretory epithelia. In the experiments reported here, the coupling between the elevation of $[Ca]_i$ and the opening of K channels was demonstrated by monitoring the magnitude and temporal course of alterations of K_i pro-

duced by different receptor-mediated agonists and the Ca ionophore ionomycin. Notably, the effects of the various stimuli on altering the net flux of K were very closely related to their characteristic effects on [Ca]$_i$ (Fig. 3), and the alterations in [Ca]$_i$ preceded the net alterations of K$_i$ (and Cl$_i$ and Na$_i$). The relative effects of these stimuli on the loss of K$_i$ (ionomycin > carbachol > substance P > phenylephrine) were similar to those on [Ca]$_i$ (ionomycin > substance P > carbachol > phenylephrine). The different order of carbachol and substance P in the two comparisons probably has to do with the transitory nature of the elevation of [Ca]$_i$ by substance P. That is, the relatively short time that the substance P-induced elevation of [Ca]$_i$ was maintained limited the efflux of K$_i$ through the K channels.

Previously, in patch-clamp experiments we observed that carbachol or the Ca ionophore A23187 activated a TEA-sensitive current (the BK channel) and a current attributed to a nonselective cation channel that was activated at lower elevations of [Ca]$_i$ than those required for the BK channel (McMillian et al., 1988). Nonselective cation channels have been reported in a number of systems (Partridge and Swandulla, 1988), and may be activated by relatively low elevations of [Ca]$_i$ (Maruyama and Petersen, 1984). The effect of TEA on K efflux in the present study (Table I) indicated that as much as 60% of the efflux was through the BK channel. The remaining portion, or a fraction of it, may be through the nonselective cation channel. A portion of the Na uptake that was not blocked by furosemide or DMA (Table II) may occur through this channel. The fact that a measurable portion of the normal K efflux (Table I) occurred in the presence of TEA or when the normal rapid elevation of [Ca]$_i$ was slowed by BAPTA suggests that the activation of this channel (or one with a greater sensitivity to [Ca]$_i$ than the BK channel) may account for a substantial fraction of the agonist-stimulated K efflux. Multiple Ca-sensitive K channels have been observed in some cells (Hoshi and Aldrich, 1988), and may also contribute to the efflux of K promoted by carbachol in parotid cells.

The stimulation of Cl$_i$ efflux by the receptor-mediated agonists was also related to their relative effects on elevating [Ca]$_i$ (i.e., phenylephrine was the least effective in both). However, unlike the results obtained for the loss of K$_i$, ionomycin was no more effective than carbachol or substance P in promoting a net reduction of Cl$_i$. This appeared to be due to the rapid net uptake of Cl that occurred shortly after the addition of ionomycin to the cell suspension (Fig. 4), which limited the extent of the initial net efflux. This rapid uptake of Cl differed from the relatively slower uptake observed after the first minute of exposure to carbachol (see Fig. 4), which is due to Na-K-2Cl cotransport. In studies by other investigators, a substantial net increase in cellular Cl was not observed within the first minute after the exposure of parotid acinar cells to carbachol (Nauntofte and Poulsen, 1986; Melvin et al., 1987), probably because Cl efflux was larger than or equivalent to Cl influx at these times. Based on the [22]Na uptake studies shown in Table I or Fig. 11, Cl uptake via Na-K-2Cl cotransport would be expected to be identical for carbachol and ionomycin. Additional experiments will be required to further characterize the mechanism of the rapid ionomycin-stimulated Cl uptake.

The dependence of ion fluxes on the [Ca]$_i$ was further highlighted in the studies in which the cells were loaded with BAPTA to slow the rate of elevation of [Ca]$_i$. A major effect of BAPTA treatment was to delay the net losses of Cl$_i$ (Fig. 4) and K$_i$

(Table I) that were promoted by receptor activation or by the Ca ionophore ionomy-cin, which suggests that the effluxes of these ions were directly dependent on the increase in $[Ca]_i$. The rapid ionomycin-promoted Cl uptake was also delayed in BAPTA-treated cells (Fig. 4).

Iwatsuki et al. (1985) have reported that a Cl-sensitive current was activated by acetylcholine and the Ca ionophore A23187 in patch-clamp studies using rat and mouse parotid acinar cells, and similar observations were made by others using rat lacrimal gland cells (Marty et al., 1984; Findlay and Petersen, 1985). The results presented here suggest that carbachol and ionomycin activated a rapid net efflux of Cl through a Ca-sensitive Cl channel in rat parotid acinar cells, although alternative explanations are possible. For example, the Cl efflux (or a portion of it) may be promoted by hyperpolarization of the membrane potential due to the loss of K through Ca-activated K channels.

Relationship between Na Uptake and the Elevation of $[Ca]_i$

The relative effects of the receptor-mediated agonists on stimulating Na uptake (Fig. 8) and on maintaining elevated levels of Na_i (Fig. 6) were very similar to their relative effects on $[Ca]_i$ (Figs. 2 and 3). Ionomycin produced the largest increase in Na uptake, demonstrating that the elevation of $[Ca]_i$ in the absence of receptor-mediated changes in phosphatidylinositol breakdown was sufficient to alter Na_i. Further support for the involvement of $[Ca]_i$ in the activation of Na influx was indicated by the effects of extracellular Ca removal or treatment with the Ca chelator BAPTA. Both the carbachol- and ionomycin-stimulated [22]Na uptake rates were markedly reduced in Ca-free medium (Fig. 13), as was the increase in the total Na content (see Results). Moreover, there were substantial delays in the stimulation of Na uptake into BAPTA-treated cells (Fig. 13), similar to the delays in the rise of $[Ca]_i$ (Fig. 1 B). The agonist-induced increases in pH_i in BAPTA-loaded cells also were delayed (not shown), suggesting that Na entry via Na-H exchange also was dependent on the elevation of $[Ca]_i$. Similar findings have been reported in several other systems, including human fibroblasts, in which the activation of Na-H exchange by growth factors appears to require a rise in $[Ca]_i$ (Owen and Villereal, 1982).

It should be noted, however, that the increases in Na uptake were only indirectly dependent on $[Ca]_i$, since they appeared to require prior (Ca-dependent) activation of the efflux pathways and the net loss of K_i and Cl_i. The Na_i content was only slightly elevated 10 s after the addition of agonist (Fig. 6), at which time the entire decrease in Cl_i had already occurred (Fig. 4). In addition, the reduction by TEA of the efflux of K_i severely inhibited the stimulation of [22]Na uptake by carbachol (see Results). The maximal stimulation of Na uptake occurred only when K channels and (probably) Cl channels were first activated, leading to reduction of the cellular K and Cl contents.

Ca-mobilizing Agonists Activated Na-K-2Cl Cotransport and Na-H Exchange

The majority of the carbachol-stimulated [22]Na influx occurred via a pathway that was furosemide- or Cl-sensitive (Fig. 11), suggesting the activation of the Na-K-2Cl cotransport system. A second pathway of Na entry, that of Na-H exchange, also was

activated by carbachol and substance P, and contributed to Na uptake by a much smaller fraction, ~ 18% (Table II). For carbachol-stimulated cells, the combined rates of Na uptake through the furosemide-sensitive and DMA-sensitive pathways did not completely account for the total rate of Na uptake (Table II). This partly appeared to be due to the incomplete inhibition of Na-K-2Cl cotransport in these cells by 1 mM furosemide. This was supported by our findings that the removal of extracellular Cl or the combined effects of furosemide and Cl removal reduced carbachol-stimulated ^{22}Na uptake by a larger amount than did the addition of furosemide to cells suspended in Cl-containing medium (Fig. 11). Turner et al. (1986) reported that the K- and Cl-dependent uptake of ^{22}Na into rabbit parotid basolateral membrane vesicles was inhibited by only ~ 80% by 1 mM furosemide. Thus, in rat parotid acinar cells the activation of Na-K-2Cl and Na-H exchange account for > 70% of the Na uptake that is stimulated by carbachol. As mentioned above, a portion of the remaining uptake may be due to Na influx through the nonselective cation channel. Not surprisingly, multiple pathways of Na entry also have been reported in other exocrine acinar cells. Based on studies of the effects of transport inhibitors on the rate of fluid secretion in rat submandibular glands, the relative contribution of Na-K-2Cl cotransport and Na-H exchange to Na entry was calculated as 8:5 (Young et al., 1987). Measurements made using an intracellular Na electrode suggested that Na-H exchange, Na-K-Cl cotransport, and organic substrate-coupled Na cotransport all appeared to contribute to the elevation of Naᵢ in acetylcholine-stimulated mouse lacrimal acinar cells (Saito et al., 1987).

It should be noted that the present series of experiments were performed in nominally HCO_3-free media. The main anion normally present in the solutions used in these experiments was Cl (see Methods). A $Cl-HCO_3$ antiport system, which normally exchanges intracellular HCO_3 for extracellular Cl, has been postulated to exist on the basolateral membrane of exocrine acinar cells and to contribute to Cl entry and fluid secretion (Case et al., 1984; Young et al., 1987). In addition, a component of ^{22}Na uptake was attributable to $Na-HCO_3$ cotransport in monkey kidney epithelial cells (BSC-1) (Jentsch et al., 1985). Thus, the inclusion of HCO_3 in the suspending medium might alter the relative contribution of Na-K-2Cl cotransport to the total unidirectional uptake of Na. However, in studies performed using perfused rabbit mandibular gland, which shares many aspects of fluid secretion with the parotid gland, the replacement of perfusate HCO_3 with Cl, isethionate, or HEPES did not alter salivary secretion or composition (Case et al., 1984; Martinez, 1987). Thus, the net processes involved in fluid and electrolyte secretion should not be compromised in the absence of HCO_3.

Specificity of Dimethylamiloride

Additional comments must be made concerning the use of DMA to identify the component of Na uptake that occurs via Na-H exchange. It has been reported that concentrations of amiloride analogues that inhibit Na-H exchange can also block the activation of protein kinase C (Besterman et al., 1985) and oxidative phosphorylation (Soltoff et al., 1985). Therefore, it was important to rule out any nonspecific effects of DMA. In fact, in initial experiments we observed that 50 μM hexamethyleneamiloride, another amiloride analogue, completely blocked carbachol-stimu-

lated ^{22}Na uptake (not shown). However, this concentration also reduced the nystatin-stimulated QO_2 and reduced the cellular ATP content, which indicated that hexamethyleneamiloride indirectly blocked the Na,K-ATPase by metabolically compromising the mitochondria. In addition, hexamethylamiloride also reduced the increase in the production of inositol phosphates by carbachol, suggesting that phospholipase C activation was inhibited. However, the concentration of DMA (5 μM) used in this study was sufficient to block the elevation of pH_i promoted by carbachol (Fig. 12) without reducing the nystatin-stimulated QO_2, the cellular ATP content, and the carbachol-stimulated inositol polyphosphate production (not shown). Consequently, the effects of DMA on ^{22}Na uptake appear to be due specifically to the inhibition of Na-H exchange.

Cellular Volume Decrease and the Activation of Na-K-2Cl Cotransport

The initial decrease in the intracellular volume of the parotid acinar cells appears to be due to the rapid loss of K and Cl without an immediate compensating influx of osmotic equivalents. In some cells, for example lymphocytes (Grinstein et al., 1984) and Ehrlich ascites tumor cells (Hoffman et al., 1984), K and Cl efflux pathways are activated as a regulatory mechanism to decrease the volume of cells swollen in hypotonic media. Although the specific mechanisms by which Na-K-2Cl cotransport is activated in the parotid cell is not known, in various cells Na-K-2Cl cotransport is stimulated by alterations in cell volume, as well as by hormones and neurotransmitter agonists (for review, see O'Grady et al., 1987). The activation of Na-K-2Cl cotransport by changes in volume may be due to changes in the concentration of an impermeant cytoplasmic solute or to physical stresses placed on components of the plasma membrane (Grinstein et al., 1984). In the rat parotid acinar cell, the stimulation of Na-K-2Cl cotransport may be related to the cell shrinkage that occurs in response to the rapid loss of K_i (Fig. 3) and Cl_i (Fig. 4). Although ionomycin increased the intracellular volume of parotid cells (Fig. 5) while stimulating Na uptake via the Na-K-2Cl pathway to a similar extent as carbachol (Fig. 11), the swelling was preceded by the loss of K_i (Fig. 3) and Cl_i (Fig. 4), and by a decrease in cell volume (Fig. 5). This is consistent with a requirement for reduction of cell volume to activate Na-K-2Cl cotransport.

The role of the extracellular K concentration in activating Na-K-2Cl cotransport must also be considered. The loss of K into the lateral intracellular spaces in vivo may substantially increase the extracellular K concentration above the plasma concentration. In fact, in a study in which the extracellular K concentration of cat and dog submaxillary glands was monitored using an extracellular K electrode, electrical stimulation of the parasympathetic nerve caused the extracellular K concentration to increase from ~ 2 mM to > 15 mM (Poulsen and Bledsoe, 1978). In the experiments described in this paper, activation of Ca-sensitive K channels produced an increase in the extracellular K concentration, the extent of which depended on the relative mass of parotid cells in the suspension. In the measurements of net K flux (Fig. 3), for which a relatively large number of cells (2–5 mg/ml) were required, there generally was an increase of ~ 1 mM K (from ~ 5 to ~ 6 mM) in the extracellular [K]. Fewer cells (0.5–0.75 mg/ml) were used in the ^{22}Na uptake experiments designed to measure the uptake of Na through Na-K-2Cl cotransport (Fig. 11), and

a much lower elevation (≤ 0.3 mM) of extracellular K would have resulted. Therefore, large increases in the extracellular K concentration alone do not appear to be sufficient to account for the activation of Na-K-2Cl cotransport in the experiments reported here, but the combined loss of intracellular K and the elevation of extracellular K do create a more favorable gradient for K entry. The K dependence of Na uptake into basolateral membrane vesicles prepared from rabbit parotid glands exhibited a $K_{0.5}$ for K of ~ 30 mM (Turner et al., 1986). Although vesicle studies identify the presence of this transport system, they do not indicate how it is activated in intact cells. Additional studies are necessary to more accurately determine the role of cellular shrinking in the stimulation of Na-K-2Cl cotransport in the rat parotid acinar cell.

Ionomycin Activates Na Influx by an Additional Pathway(s)

The Ca ionophore ionomycin caused the largest increase in [Ca]$_i$ and also produced the largest stimulation of Na uptake (Fig. 8). In a previous report, ionomycin was observed to initiate an increase in ^{22}Na accumulation in a manner nearly identical to carbachol (Poggioli et al., 1982). Although we observed that the furosemide-sensitive and DMA-sensitive components of the ionomycin-stimulated ^{22}Na uptake were nearly identical to those of carbachol (Table II), the total ionomycin-stimulated unidirectional uptake of Na was larger than the combined influx through two defined pathways. The results suggest that increases in [Ca]$_i$ to levels equivalent to those produced by carbachol were sufficient to maximally activate Na-K-2Cl cotransport, but that the larger ionomycin-induced increases in [Ca]$_i$ activated an additional pathway(s) of Na entry. Although this pathway was not dependent on extracellular Cl (Fig. 11), it was sensitive to the removal of extracellular Ca and the chelation of intracellular Ca (Fig. 13). This component of Na influx was not coupled to amino acids or to glucose, since the former were not present in the medium and phloretin or the removal of glucose did not substantially reduce the stimulated unidirectional Na influx (not shown). As with carbachol, one possible route of Na entry is through the activation of the Ca-sensitive nonselective cation channel. Although the activation of such a channel in rat lacrimal gland required higher levels of [Ca]$_i$ than that required for the activation of the BK channel (Marty et al., 1984), this channel appeared to be activated at relatively low elevations of [Ca]$_i$ in rat parotid acinar cells (McMillian et al., 1988), and it seems unlikely that Na influx through this channel can account for the additional component of Na influx. Na-Ca exchange may also contribute to this component of Ca-sensitive Na entry, but this remains to be examined directly.

An alternative possibility to be considered is that ionomycin may itself function as a monovalent cationophore and thereby directly mediate the entry of Na into the parotid cell. In rat liver mitochondria, ionomycin and A23187 were reported to directly mediate monovalent ion fluxes (Kauffman et al., 1980), although a subsequent report by another group found that the stimulation of monovalent cation fluxes in mitochondria was secondary to an effect of the A23187 on Mg (Dordick et al., 1980). Since the rate of ionomycin-stimulated Na entry into parotid cells was greatly reduced in the absence of extracellular Ca or in cells loaded with BAPTA (Fig. 13), the additional component of Na influx is not likely to be due to the effect

of ionomycin as a Na ionophore but rather to its effects on elevating $[Ca]_i$ and to the activation of an additional pathway for Na uptake.

Stimulation of the Na Pump Activity by Alterations in Na_i and K_i

The results of the oxygen consumption studies shown in Figs. 9 and 10 indicate that the agonists used in this study stimulated the Na pump of the parotid acinar cell in a manner quantitatively related to their effect on $[Ca]_i$. In the absence of any added stimuli, the Na pump in intact parotid cells is unsaturated with respect to the Na_i concentration. The agonists stimulated an influx of Na (Fig. 8) and an increase in Na_i (Fig. 6). Although a rise in Na_i will increase the activity of the Na pump, it can also be altered by changes in the intracellular K concentration (Soltoff and Mandel, 1984). This is due to the competition between Na and K at the intracellular site to which Na normally binds to the Na pump (Na,K-ATPase). In fact, the monovalent cationophore nystatin, which increases Na_i and decreases K_i (Soltoff and Mandel, 1984), maximally stimulated the parotid Na pump (Fig. 10). At submaximal concentrations of Na, a given concentration of Na will be more effective in stimulating the Na pump if the K concentration is low rather than high. From the results presented in this paper, it appears that a large proportion of agonist-induced reduction of intracellular K via activation of K channels (Fig. 3) occurs before there is a large increase in Na_i. Therefore, both the decreases in K_i as well as increases in Na_i, which increases the Na:K ratio within the cell, appear to play physiological roles in activating the Na pump activity in the parotid acinar cell. It is likely that other reports (e.g., Hootman et al., 1987) of the stimulatory effects of $[Ca]_i$ on the Na pump are explainable by the effects of $[Ca]_i$ on alterations of the relative ratio of Na:K.

Stoichiometry of Na Transport and Oxygen Consumption

A theoretical maximum of 18 mol of Na will be transported out of the cell by the Na pump for each mol of O_2 consumed (see Results), and the $K:O_2$ ratio will be 12:1 for K uptake by the Na pump. Using the Na ratio, the Na pump activity calculated from the carbachol-stimulated ouabain-sensitive QO_2 was ~ 325 nmol Na · $mg^{-1} \cdot min^{-1}$, a value similar to the carbachol-stimulated rate of ^{22}Na uptake (265 nmol Na· $mg^{-1} \cdot min^{-1}$) into the parotid cell. The similarity of these numbers indicates that the maximum rates of Na influx and efflux are closely matched. Therefore, it is not surprising that a ratio of about 15:1, close to the theoretical maximum, is achieved using the ^{22}Na uptake rate (265) and the ouabain-sensitive QO_2 (18.0) measured in carbachol-stimulated cells. Interestingly, the furosemide-sensitive (142:8.9) and chloride-sensitive (178:10.3) portions of these measurements gave a similar ratio (16.0 and 17.3, respectively). In a recent study in which ouabain-sensitive K (^{42}K) entry and oxygen consumption were measured using unstimulated parotid acinar cells, a $K:O_2$ ratio of 33:1 was observed (Poulsen and Nauntofte, 1987). Compared to the theoretical ratio of 12:1, the large experimentally-derived ratio suggests that K entry occurs by other pathways (e.g., Na-K-2Cl cotransport) in addition to uptake by the Na pump. Similar experiments in the presence of furosemide should result in a ratio that is closer to 12:1.

Substance P Exhibits Desensitization

Substance P-initiated alterations of [Ca]$_i$, K$_i$, Na$_i$, and intracellular volume decayed with time, representing desensitization of the cell to this agonist. Substance P has been reported to produce transient effects on salivary secretion (Martinez and Martinez, 1981) and the release of K from the parotid gland (Friedman et al., 1985). In a previous study using our preparation of parotid cells, we observed that the stimulation by substance P of IP$_3$ accumulation was in fact greater than that provided by carbachol during the first 20 s of exposure, but that the stimulation of IP$_3$ accumulation by substance P did not continue beyond the first minute (McMillian et al., 1987). Sugiya et al. (1987, 1988) and Merritt and Rink (1987b) have also made similar observations. This appears to account for the transitory elevation of [Ca]$_i$ and the subsequent transitory stimulation of the Ca-activated processes involved in the alterations of Na$_i$, K$_i$, QO$_2$, and intracellular volume. Presumably, the maintenance of extended responses to these agonists depends, at least in part, on their relative ability to maintain the production of (1,4,5)IP$_3$ (and perhaps other inositol polyphosphate compounds, see Morris et al., 1987) and thus maintain an elevated level of [Ca]$_i$. However, while homologous desensitization of the substance P responses appears to occur at the receptor level (McMillian et al., 1987; Sugiya et al., 1987, 1988), the precise mechanism remains unknown. In contrast, the carbachol-induced increase in [Ca]$_i$ and changes in intracellular electrolytes and volume were better maintained (Figs. 1, 3, 5, and 6). Therefore, although both agonists promote quantitatively similar initial effects, the effects of the muscarinic agonist are long lasting, while the effects of substance P are transient.

Conclusions

In the present studies, the involvement of [Ca]$_i$ in activating ion fluxes was demonstrated by several findings, including parallel patterns of alterations of [Ca]$_i$ and ion fluxes, and the similar effects of receptor-mediated agonists and the Ca ionophore ionomycin. In addition, ion fluxes were reduced under conditions that limited the time course and extent of [Ca]$_i$ elevation. In all cases, the elevation of [Ca]$_i$ was noted to play a critical role in the initial processes involved in fluid and electrolyte secretion in the rat parotid acinar cell. Since activation of the ion fluxes could be fully mimicked by ionomycin, it appeared that the only absolute requirement was the increase in [Ca]$_i$, and that the production of (1,4,5)IP$_3$ or other biochemical intermediates is not mandatory for the initial ion transport events.

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