Na\(^+\), K\(^+\), and Cl\(^-\) Transport in Resting Pancreatic Acinar Cells

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ABSTRACT To understand the role of Na\(^+\), K\(^+\), and Cl\(^-\) transporters in fluid and electrolyte secretion by pancreatic acinar cells, we studied the relationship between them in resting and stimulated cells. Measurements of [Cl\(^-\)]\(i\) in resting cells showed that in HCO\(_3^-\)-buffered medium [Cl\(^-\)]\(i\) and Cl\(^-\) fluxes are dominated by the Cl\(^-\)/HCO\(_3^-\) exchanger. In the absence of HCO\(_3^-\), [Cl\(^-\)]\(i\) is regulated by NaCl and NaK2Cl cotransport systems. Measurements of [Na\(^+\)]\(i\) showed that the Na\(^+\)-coupled Cl\(^-\) transporters contributed to the regulation of [Na\(^+\)]\(i\), but the major Na\(^+\) influx pathway in resting pancreatic acinar cells is the Na\(^+\)/H\(^+\) exchanger. 86Rb influx measurements revealed that >95% of K\(^+\) influx is mediated by the Na\(^+\) pump and the NaK2Cl cotransporter. In resting cells, the two transporters appear to be coupled through [K\(^+\)]\(i\), in that inhibition of either transporter had small effect on 86Rb uptake, but inhibition of both transporters largely prevented 86Rb uptake. Another form of coupling occurs between the Na\(^+\) influx transporters and the Na\(^+\) pump. Thus, inhibition of NaK2Cl cotransport increased Na\(^+\) influx by the Na\(^+\)/H\(^+\) exchanger to fuel the Na\(^+\) pump. Similarly, inhibition of Na\(^+\)/H\(^+\) exchange increased the activity of the NaK2Cl cotransporter. The combined measurements of [Na\(^+\)]\(i\), and 86Rb influx indicate that the Na\(^+\)/H\(^+\) exchanger contributes twice more than the NaK2Cl cotransporter and three times more than the NaCl cotransporter and a tetraethylammonium-sensitive channel to Na\(^+\) influx in resting cells. These findings were used to develop a model for the relationship between the transporters in resting pancreatic acinar cells.

INTRODUCTION

Pancreatic acinar cells secrete digestive enzymes and isotonic fluid rich in NaCl (Schulz, 1987; Case, 1989). Whereas enzyme secretion (Gardner and Jensen, 1986; Williams and Blevins, 1993) stimulus-secretion coupling (Hootman and Williams, 1987; Petersen and Findlay, 1987) and second messenger regulation (Muallem, 1992) have been studied extensively, the mechanism of fluid and electrolyte secretion by acinar cells is poorly understood.
Early studies examined the properties of fluid secretion by the perfused pancreas. In the presence of HCO$_3^-$ and cholecystokinin (CCK), where most secretion is believed to be of acinar origin, pancreatic fluid secretion was inhibited $\sim$70% by a high concentration of amiloride, reduction of serosal [Na$^+$], or high concentration of acetamide isothiocyanostilbene disulfonic acid (SITS) (Seow, Lingard, and Young, 1986; Ishikawa and Kanno, 1988, 1989). Tetraethylammonium (TEA) and Ba$^{2+}$ reduced fluid secretion by $\sim$30% (Evans, Pirani, Cook, and Young, 1986). Spectrofluorometric studies on the cellular level demonstrated the presence of Na$^+$/H$^+$ (Dufresne, Bastie, Vaysse, Creach, Holland, and Ribet, 1985; Muallem and Loessberg, 1990a) and Cl$^-$/HCO$_3^-$ (Muallem and Loessberg, 1990a) exchange mechanisms in acinar cells. Strong evidence in several species points to a Ca$^{2+}$-activated Cl$^-$ channel as mediating the Cl$^-$ exit at the apical membrane (AM) (Petersen and Findlay, 1987; Peterson, 1992). In the rat and mouse pancreatic acini, in addition to K$^+$ selective channels, the basolateral membrane (BLM) contains a Ca$^{2+}$-activated, voltage-independent, nonselective cation channel that depolarizes stimulated cells (Maruyama and Petersen, 1982; Thorn and Petersen, 1992). TEA and Ba$^{2+}$ partially inhibit fluid secretion, probably by inhibition of the nonselective channels.

The ion transport pathways mentioned above, together with a paracellular transport pathway for Na$^+$, were proposed to mediate fluid and electrolyte secretion by acinar cells (Schulz, 1987; Case, 1989; Petersen, 1992). However, unlike pancreatic duct cells, acinar cells secrete fluid and electrolyte very well in the absence of HCO$_3^-$ (Kanno and Yamamoto, 1977; Petersen and Ueda, 1977; Seow et al., 1986), where their Cl$^-$/HCO$_3^-$ exchanger is not active (Muallem and Loessberg, 1990a,b). It was suggested that in the absence of HCO$_3^-$, Cl$^-$ entry at the BLM is mediated by the NaK2Cl cotransporter, since furosemide at high concentrations inhibited CCK-stimulated fluid secretion (Seow et al., 1986; Ishikawa and Kanno, 1989). However, a more recent report by one of these groups found no effect of serosal furosemide on fluid or K$^+$ secretion to both the luminal and the serosal fluids (Ishikawa and Kanno, 1991). Further complications arise from findings with isolated mouse pancreatic acini in which cholinergic stimulation caused $^{86}$Rb and net K$^+$ efflux by the cotransporter (Singh, 1984; Petersen and Singh, 1985). Hence the existence and possible role of the NaK2Cl cotransporter in acinar cells are not clear.

In the first stage of the present studies, we attempted to determine the relationship between the Na$^+$, K$^+$, and Cl$^-$ transporters by measuring the intracellular concentrations of Na$^+$, K$^+$, and Cl$^-$, and the undirectional influx of $^{86}$Rb. The results identified the major transporters regulating [Na$^+$]$_i$, [K$^+$]$_i$, and [Cl$^-$]$_i$ in resting acinar cells and point to a coupling between the transporters through the intracellular concentrations of Na$^+$ and K$^+$.

**MATERIALS AND METHODS**

**Chemicals**

Chemicals were purchased from the following sources: tributyltin, gramicidin, monensin, ouabain, (TEA) and digitonin came from Sigma Chemical Co. (St. Louis, MO). Nigericin was from Boehringer Mannheim (Indianapolis, IN). Dimethylamiloride was from Research Biochemicals Inc. (Natic, MA). Fura 2/AM, SPQ, SBFI/AM, and Na$^+$-Green/AM were from Molecular Probes.
Solutions

The solutions used in the present experiments and their composition are listed in Table 1. In the text, when referring to a particular solution, we also indicate the major salt and the pH buffer as follows: solution A (NaCl, HEPES); solution B (Cl−-free, HEPES); solution C (Na+−free, HEPES); solution D (K+−free, HEPES); solution E (NaCl, HCO₃); solution F (Cl−-free, HCO₃); solution G (SCN−-, HEPES). All solutions containing HCO₃ were equilibrated with 5% CO₂/95% O₂ to pH 7.4 at room temperature. The osmolarity of all solutions was adjusted to 310 mosM with the major salt.

Preparation of Pancreatic Acini

Acini were prepared according to a previously published method (Zhang, Tortorici, and Muallem, 1994). The pancreas of a 100−150-g rat was removed and injected with 10 ml of a solution composed of solution A, 0.02% soybean trypsin inhibitor, and 0.1% bovine serum albumin (PSA). Fat, connective tissue, and blood vessels were removed, the fluid was drained by blotting, and the pancreas was finely minced. The minced pancreas was added to 15 ml PSA containing 2.5 mg collagenase P and incubated for 5−6 min at 37°C. The resultant acini were washed twice with PSA and filtered through a 150-μm nylon mesh. For fluorescence measurements, acini from one pancreas were suspended in 8 ml PSA and kept on ice until use. For measurement of S6Rb fluxes, the acini were suspended in 10 ml PSA and used immediately.

Dyes Loading

For [Ca²⁺], measurements, 2 ml of acinar cell suspension was incubated with 2.5 μM Fura 2/AM for 20 min at 37°C. For [Na⁺], measurements, 1.5 ml acinar cell suspension was incubated with 5 μM SBFI/AM at room temperature for 45−60 min. Alternatively, 1 ml acini suspension was incubated with 2 μM Na⁺-Green for 15 min at room temperature. At the end of each loading period, the cells were washed once with 35 ml PSA, resuspended in ~5 ml PSA, and kept on ice until plat-
ing. For each experiment, ~0.5 ml dye-loaded acini were plated on thin-glass coverslips that formed the bottom of a perfusion chamber.

Several preliminary experiments were performed to obtain suitable conditions for measurement of \([\text{Cl}^-]\). Acinar cells were loaded with SPQ or MQAE. In six-cell preparations, dye leak with SPQ was lower than with MQAE. Dye loading at 37°C resulted in reduced signal/noise. Incubation of the cells with SPQ at room temperature for 15 min and then at 0°C for 30 min or longer resulted in maximal loading and reproducible signal intensity between experiments performed with the same cell preparation. Therefore, the following conditions were used to measure \([\text{Cl}^-]\), in acinar cells. About 3 ml acinar cell suspension was centrifuged, and the acini were resuspended in 1 ml PSA containing 10 mM SPQ. After 15 min incubation at room temperature, the acini were diluted with 1 ml PSA and kept on ice for at least 30 min and then until plating on coverslips. Before each plating, 0.1 ml of SPQ-loaded acini were added to 0.4 ml solution A in the perfusion chamber.

**Fluorescence Measurements**

Dye-loaded acini were allowed to attach to the glass coverslips for ~2 min at room temperature, after which perfusion of a warm (37°C) solution A commenced to remove unattached cells. Fluorescence recording started after at least a 10-min perfusion (at a rate of 10–12 ml/min) with solution A to allow establishment of ionic gradients. The perfusion chamber was placed on the stage of an inverted microscope attached to a Delta Scan Fluorimeter, which provided a dual wavelength excitation light. Fluorescence was recorded from 2–3 cells within a single acinus composed of 10–20 cells. Fura 2 fluorescence was recorded at excitation wavelengths of 355 and 380 nm and SBFI fluorescence at 340 and 385 nm. The fluorescence of SPQ and Na⁺-Green was recorded with single excitation wavelengths of 380 and 490 nm, respectively, since these dyes are not ratio dyes. The emitted light was directed to a photomultiplier tube by an appropriate dichroic mirror, and emission cut off filter sets. Light intensity was measured at a resolution of 2/s. The fluorescence ratios of 355/380 for Fura 2 were calibrated exactly as detailed before (Zhao, Star, and Muallem, 1994). The procedure used to calibrate the fluorescence of the other dyes is described in Results. All experimental protocols were repeated at least three times with different cell preparations.

**Measurement of \(^{86}\text{Rb}^{+}\) Influx**

Acini in PSA were incubated for 20 min at 37°C. To initiate \(^{86}\text{Rb}^{+}\) uptake, the acini were diluted 1:1 into a warm PSA containing \(^{86}\text{Rb}^{+}\) (~4.10⁵ cpm/ml) and twice the final desired concentration of any agent tested. At the indicated times (usually after 4 min incubation at 37°C), duplicate 0.5 ml samples of acini were transferred to 8 ml stop solution containing 150 mM NaCl, 1 mM LaCl₃, 10 mM HEPES (pH 7.4 with NaOH), and 1 mg/ml BSA. The stop solution was in glass tubes placed in an ice-cold water bath. At the end of the experimental sampling, the acini were collected by a 20-s centrifugation at 200 g and washed twice more with an ice-cold stop solution in the same manner. The pellets were dissolved in 0.5 ml of 0.2 M NaOH by 30 min incubation at 60°C. 10–20 µl samples from representative tubes (at least six) were removed for determination of protein; the remaining volume was transferred to scintillation vials for counting. The results were calculated in terms of nmoles K⁺/mg protein and are presented as mean ± SEM for the number of experiments performed.

**RESULTS**

The first goal of the present study was to characterize the major Cl⁻, Na⁺, and K⁺ transporters in acinar cells, their relative contribution to maintaining the cytosolic activities of the relevant ions, and the relationship between them. This was achieved
by measuring the intracellular activities of Na⁺ and Cl⁻ and the properties of ⁸⁶Rb influx.

**Cl⁻ Transport**

The intracellular Cl⁻ concentration ([Cl⁻]ᵢ) was measured with the Cl⁻-sensitive dye SPQ. Fig. 1 a shows the behavior of SPQ in acinar cells and the procedure used to calibrate the fluorescence. The fluorescence was relatively stable during continuous perfusion and dye leak was ~12% per hour. Maximal anion-dependent fluorescence signal was obtained by exposing cells incubated in Cl⁻-free medium to the highly permeable anion SCN⁻. To calibrate the dye in the cells, [Cl⁻]ᵢ was clamped by bathing the cells in medium containing 145 mM K⁺ (Kᵢ⁺ = Kₒ⁺), the K⁺/H⁺ exchange ionophore nigericin, the Cl⁻/OH⁻ exchanger tributyltin, and different Cl⁻ concentrations. Under these conditions [Cl⁻]ᵢ follows [Cl⁻]ₒ. Calibration procedures similar to that in Fig. 1 a yielded a Stern-Volmer constant (Kₛ) for SPQ in the cells of 17.3 ± 0.8 M⁻¹ (n = 5), which is close to that reported before with other cell types (Chao, Dix, Sellers, and Verkman, 1989; Chao, Widdicombe, and Verkman, 1990; Foskett, 1990). Previous studies showed that the Kₛ for SPQ in solution
is affected by pH and the type of buffer used (Vasseur, Frargne, and Alvarado, 1993). We therefore tested the effect of pH on dye fluorescence. After clamping pH$_i$ at 7.2 and [Cl$^-_i$] at 0, 20, or 100 mM with the ionophores/high K$^+$ medium, the cells were perfused with the same solutions buffered to pH of 7.6. Fluorescence intensity changed by <5% and only at 0 [Cl$^-_i$] conditions (not shown). The sensitivity of SPQ and other Cl$^-$-sensitive dyes to carboxylate groups probably accounts for the minimal effect of pH$_i$ on the dye in the cells (Koncz and Dougidas, 1994).

Using a K$_q$ of 17.3 and measurement of total anion-dependent fluorescence, the [Cl$^-_i$] of resting acinar cells was found to be 65.4 ± 4.2 mM ($n = 58$).

Fig. 1 b compares the rate of Cl$^-$ efflux and influx in the presence and absence of HCO$_3^-$.

![Figure 2](image)

**Figure 2.** Cl$^-$ fluxes by the NaK2Cl cotransporter. In experiments a and b, SPQ-loaded acinar cells in solution A (NaCl) were perfused with Cl$^-$-free solution B. As indicated, solution A (a) or solution B (b) contained 0.1 mM bumetanide. For the experiments in c and d, cells in solution A (HEPES) were perfused with solution E (HCO$_3^-$) for at least 10 min before solution changes. All solutions in experiments c and d were buffered with CO$_2$/HCO$_3^-$. Where indicated, the cells were perfused with solutions E (NaCl) or F (HCO$_3^-$, Cl$^-$ free) and with or without 0.1 mM bumetanide.

The results in Fig. 3 a provide further evidence for NaK2Cl co-transport in acinar
cells by showing that removal of external K⁺ was as effective as bumetanide in inhibiting Cl⁻ influx.

The partial inhibition of Cl⁻ fluxes by bumetanide and removal of external K⁺ suggest the presence of additional Cl⁻ transport mechanisms in acinar cells. Fig. 3, b–d, shows that some of these fluxes are probably mediated by NaCl cotransport. Removal of extracellular Na⁺ almost completely inhibited Cl⁻ influx into Cl⁻-depleted cells (Fig. 3 b). In addition, the NaCl cotransport inhibitor thiazide (TZ) (Gamba, Miyanoshita, Lombardi, Lytton, Lee, Hediger, and Hebert, 1994) partially inhibited Cl⁻ efflux (Fig. 3 c) and Cl⁻ influx (Fig. 3 d) in acinar cells. Effects of TZ could be demonstrated in HEPES (Fig. 3) but not in HCO₃⁻-buffered medium (not shown), due to the dominance of the Cl⁻/HCO₃⁻ exchanger in Cl⁻ fluxes.

Na⁺ Transport

The previous section showed the transport of Cl⁻ by two Na⁺-dependent mechanisms. To evaluate their relative role in Na⁺ transport and the presence of other Na⁺ transporters, intracellular Na⁺ concentration ([Na⁺]ᵢ) was measured with the Na⁺-sensitive fluorescent dye SBFI. Fig. 4 a shows an in situ calibration of the SBFI fluorescence ratio. The membrane was permeabilized to monovalent ions with the ionophores gramicidin and monensin. Under these conditions, changes of Na⁺ resulted in rapid and stable changes in the fluorescence ratio. Assuming a 1:1 stoichiometry for the interaction of Na⁺ with SBFI (Minta and Tsien, 1989; Harootunian, Kao, Eckert and Tsien, 1989), the dye in pancreatic acinar cells showed an apparent Kd for Na⁺ of ~39 ± 7 mM (n = 4). Using this Kd, [Na⁺]ᵢ in resting pancreatic acinar cells was found to be 11.7 ± 0.5 mM (n = 36).
Fig. 4 b shows that removal of Na\(^+\) resulted in depletion of Na\(^+\)\(_i\) and that addition of Na\(^+\)\(_o\) caused [Na\(^+\)]\(_i\) to increase to normal levels without a noticeable overshoot. Na\(^+\) efflux and influx rates remained the same when this procedure was repeated up to three times. The membrane permeability to Na\(^+\) was estimated from inhibition of the Na\(^+\) pump with ouabain, which is the major Na\(^+\) efflux mechanism in resting cells. Addition of 100 \(\mu\)M ouabain caused [Na\(^+\)]\(_i\) to increase at an initial rate of \(\sim 1.56 \pm 0.11\) mM/min, which was then gradually reduced to 0.68 \(\pm 0.08\) mM/min \((n = 6)\). Similar results were obtained when the Na\(^+\) pump was inhibited by removal of external K\(^+\) (see Zhao and Muallem, 1995). The low Na\(^+\) permeability of resting cells was maintained for many minutes. In fact, incubation of resting acinar cells with ouabain for 30, 60, and 120 min caused [Na\(^+\)]\(_i\) to increase to 21 \(\pm 4\), 46 \(\pm 6\), and 102 \(\pm 7\) mM \((n = 3)\), respectively. However, removal and addition of
Na\(^+\) to the incubation medium of ouabain-treated acini resulted in relatively rapid Na\(^+\) fluxes, but again without causing an overshoot (Fig. 4 b).

The most reproducible protocol to identify mechanisms of Na\(^+\) transport in acinar cells was to measure Na\(^+\) influx into cells incubated in the absence of Na\(^+\). Fig. 5, a–c, shows that TZ and TEA had no measurable effect on Na\(^+\) influx. The concentrations of TZ and TEA used were optimal in affecting \(^{86}\)Rb uptake in resting and stimulated cells (see below). Increasing TZ up to 0.5 mM and TEA up to 20 mM had no further effect on Na\(^+\) influx or \(^{65}\)Rb uptake (not shown). On the other hand, bumetanide inhibited the rate and extent of Na\(^+\) influx by \(\sim 82\%\), and removal of bumetanide resulted in recovery of normal [Na\(^+\)]\(_i\) (Fig. 5 d). These findings were corroborated by testing the effect of external K\(^+\) and Cl\(^-\) on Na\(^+\) influx. Fig. 6 shows that removal of external K\(^+\) (Fig. 6 b, top) or external Cl\(^-\) (Fig. 6 d, bottom) reduced the rate of Na\(^+\) influx by \(\sim 37\%\). In the absence of external K\(^+\), [Na\(^+\)]\(_i\) did not stabilize at a reduced level, probably due to inhibition of the Na\(^+\) pump (Fig. 6 b). In the absence of Cl\(^-\) and the presence of Na\(^+\), [Na\(^+\)]\(_i\) stabilized below resting level and addition of Cl\(^-\) caused a further increase in [Na\(^+\)]\(_i\).

The results in Figs. 5 and 6 indicate that a small portion (30–35%) of Na\(^+\) influx into acinar cells is mediated by the NaK2Cl cotransporter, whereas most of the influx is mediated by a K\(^+\)- and Cl\(^-\)-independent mechanism. This is likely to be the Na\(^+\)/H\(^+\) exchanger. It was not possible to test directly the contribution of the exchanger to Na\(^+\) influx using SBFI since amiloride and all analogues tested entered the cells and dominated the fluorescence. Another alternative was to use the new Na\(^+\)-sensitive fluorescent dye Na\(^+\)-Green. Fig. 7 shows the behavior of this dye in pancreatic acinar cells. In \(\sim 31\%\) (29/94) of the experiments, the dye appeared to be located in the cells, probably in the cytoplasm (Fig. 7 a). Hence, removal and re-addition of Na\(^+\) resulted in relatively small and slow changes in the fluorescence.
Exposing the cells to digitonin caused an initial increase in fluorescence, probably due to an increased permeability of the plasma membrane to Na⁺ before permeabilization to Na⁺-Green. Subsequently, the cells lost ~84 ± 9% (n = 8) of their fluorescence. On the other hand, in ~69% (65/94) of the experiments, a significant amount of the dye (between 65 and 96%) was localized in a digitonin-insensitive space. This portion of the dye rapidly responded to changes in external Na⁺ before and after digitonin treatment (Fig. 7 b). In addition, the changes in fluorescence due to removal and addition of Na⁺ were not affected by any combination of Na⁺ transport inhibitors or removal of K⁺ and Cl⁻ from the medium. Therefore, we believe most of the dye was localized in the external surface of the plasma membrane. We attempted to improve dye loading with little success. The parameters tested (between 2 to 3 experiments each) were the incubation temperature (20–37°C), loading time (1–60 min), dye concentration (0.5–10 μM), number of cells (20–200 mg/ml), and structural units (single cells, small and large acinar clusters). At 37°C and at incubation times above 20 min, the dye behaved as if it was mostly extracellular. Loading freshly prepared cells at room temperature for 15 min resulted in a sufficient fluorescence signal to allow reliable measurement of [Na⁺]i changes when the dye was mostly intracellular (31% of cells). The effect of the other parameters tested was not obvious. In a preparation where the dye was intracellular or extracellular, most of the cells in this preparation showed similar dye localization.

The advantage of using Na⁺-Green is that the contribution of the Na⁺/H⁺ exchanger to Na⁺ fluxes can be directly studied using amiloride analogues. This transporter has a prominent role in Na⁺ homeostasis in resting and stimulated cells (see below) so that direct evaluation of its contribution is important. Fig. 7 d shows that complete inhibition of the Na⁺/H⁺ exchanger with 25 μM dimethylamiloride...
DMA) reduced the rate of Na⁺ influx by 67 ± 4.6% \((n = 11)\). Bumetanide together with DMA inhibited the influx by 86 ± 6.6% \((n = 5)\). Removal of the blockers resulted in recovery of resting [Na⁺]. It was not possible to reliably calibrate the Na⁺-Green signals because this dye does not have isosbestic point with respect to Na⁺, which prevented correction for dye leak, photobleaching, and extracellular binding. Nonetheless, when most of the dye was intracellular, it seemed to accurately report the contribution of the Na⁺/H⁺ exchanger to Na⁺ homeostasis.

The fluorescent probes report the net changes in the concentration of the relevant ions. To study the relationship between the transporters we had to measure their activity at steady state. To achieve that, we followed the unidirectional influx

![Diagram](image-url)
of K⁺ using ⁸⁶Rb, which did not require a prior change of the steady state level of any ion. These results are summarized in Fig. 8. Preliminary experiments showed that ⁸⁶Rb uptake was linear for at least 6 min at 37°C. The first 4 min of the uptake is illustrated in Fig. 8. Inhibition of the Na⁺ pump of resting cells with 0.5 mM ouabain reduced the rate of ⁸⁶Rb by ~14%. Similar results were obtained with 0.1 or up to 2 mM ouabain. Bumetanide inhibited the uptake by ~29%. However, the combination of ouabain and bumetanide inhibited the uptake by 97%. Hence, there was a large overlap between the ouabain-sensitive and bumetanide-sensitive ⁸⁶Rb uptake into pancreatic acinar cells.

\[ \text{Inhibition of the Na⁺/H⁺ exchanger with DMA enhanced, rather than reduced, ⁸⁶Rb uptake by 18.6 ± 3.4\% (} n = 4, P < 0.05). Furthermore, in the presence of DMA ouabain inhibited ⁸⁶Rb uptake by 33.4 ± 1.9\% and bumetanide inhibited ⁸⁶Rb uptake by 66.9 ± 2.7\%. Thus, in the presence of DMA, ouabain and, in particular, bumetanide alone were more effective in inhibiting ⁸⁶Rb uptake. A similar trend was observed with TEA and TZ. The increase in Na⁺ uptake due to TEA or TZ was between 6 and 10\% but did not reach statistical significance. Although ouabain appears more effective in the presence of either blocker, the effect was relatively small. However, both blockers significantly increased the fraction of bumet-
anide-sensitive $^{86}$Rb uptake (Fig. 8). In the presence of TEA, bumetanide-sensitive uptake (21.3 nmol/mg protein/4 min) was about twice that in the absence of TEA and accounted for 49.7 $\pm$ 3.8% ($n = 3$) of the uptake. In the presence of TZ, bumetanide inhibited the uptake by 40.4 $\pm$ 4.2% ($n = 3$).

**DISCUSSION**

In addition to digestive enzymes, pancreatic acinar cells secrete isotonic, plasma-like fluid (Schulz, 1987; Case, 1989). This requires the vectorial, transepithelial transport of Na$^+$ and Cl$^-$. Most of the Cl$^-$ transport is transcellular, whereas $\sim$70% of Na$^+$ is transported through a paracellular pathway (Kuijpers and dePont, 1987; Case, 1989). It is generally accepted that Cl$^-$ exits the AM through Ca$^{2+}$-activated Cl$^-$ channels (Petersen and Findlay, 1987; Petersen, 1992). However, the Cl$^-$ entry pathway(s) at the basolateral membrane is not known. To identify the dominant pathway of Cl$^-$ entry during stimulation of fluid secretion, it was necessary to characterize the properties of Cl$^-$ transport in acinar cells. Cl$^-$ transport is directly or indirectly coupled to the transport of Na$^+$ and K$^+$. In fact, a tight coupling between Na$^+$ and Cl$^-$ influx into stimulated acinar cells has been reported (O'Doherty and Stark, 1983). Therefore, it was also necessary to examine the role of Na$^+$ and K$^+$ in Cl$^-$ transport and the relationship between the various transporters controlling the cytosolic concentrations of Cl$^-$, Na$^+$, and K$^+$. Indeed, the overall conclusion of the present studies is that the various transporters appear to be sensitive to the concentration of intracellular K$^+$ and Na$^+$, and electrolyte secretion is regulated through modulation of [Na$^+$].

**Cl$^-$ Transport**

Measurements with SPQ showed that in resting acinar cells, [Cl$^-$]$_i$ was 65.4 mM, which is approximately fourfold above equilibrium. This value is close to the 68.9 mM reported in mouse acinar cells using Cl$^-$ selective microelectrodes (O'Doherty and Start, 1983). Several transporters were found to support the uphill transport of Cl$^-$ in acinar cells. The first is a NaK2Cl cotransport system. Previous studies reported the presence of a furosemide-sensitive K$^+$ and $^{86}$Rb efflux in mouse pancreatic acini (Singh, 1984; Petersen and Singh, 1985). Here we show that in the absence of HCO$_3^-$ this transporter mediates $\sim$70% of Cl$^-$ and 30% of Na$^+$ uptake into the cells. Thus, Cl$^-$ influx and efflux could be partially inhibited by bumetanide or removal of external K$^+$ or Na$^+$, and Na$^+$ influx was partially inhibited by bumetanide and removal of external K$^+$ or Cl$^-$. Another Cl$^-$ transporter not described before in pancreatic acini is the NaCl cotransporter. TZ is a relatively specific inhibitor of this transporter (Gamba et al., 1994). TZ reduced Cl$^-$ influx and efflux in acinar cells. Removal of K$^+o$ inhibited, but did not prevent, Cl$^-$ influx into Cl$^-$-depleted acinar cells. Only in the absence of Na$^+_o$ (and in the presence or absence of K$^+_o$) was Cl$^-$ influx largely inhibited. This also indicates that in the absence of HCO$_3^-$, the Na$^+$-dependent cotransporters mediate most of Cl$^-$ fluxes in acinar cells.

A different behavior was found in the presence of HCO$_3^-$ Under these conditions, the Cl$^-$/$\text{HCO}_3^-$ exchanger dominated the Cl$^-$ fluxes. Not only did HCO$_3^-$
more than double the rates of Cl⁻ influx and efflux, but bumetanide had minimal effect (Fig. 2), whereas TZ and TEA had no apparent effect (not shown) on Cl⁻ fluxes. Following pH₄, we have previously shown the presence of a Cl⁻/HCO₃⁻ exchanger in acinar cells that transports HCO₃ but not OH⁻ (Muallem and Loessberg, 1990a). The measurement of [Cl⁻], provides the first evidence that the Cl⁻/HCO₃⁻ exchanger dominates Cl⁻ fluxes in acinar cells. An important implication of these findings is that the mechanism of fluid and electrolyte secretion by pancreatic acini is fundamentally different from that of the various salivary acinar cells (Petersen, 1992). In salivary acinar cells, the NaK2CI cotransporter is the major Cl⁻ influx pathway in the BLM (Foskett, 1990; Robertson and Foskett, 1994) and plays a central role in electrolyte secretion by these cells (Young, Cook, van Lennep, and Roberts, 1987; Petersen, 1992). Our findings indicate that in the presence of HCO₃⁻, the cotransporter had a minimal role in transcellular Cl⁻ transport in pancreatic acinar cells and that most of the Cl⁻ entry across the BLM is mediated by the Cl⁻/HCO₃⁻ exchanger. Indeed furosemide had minimal effect (Scow et al., 1986) or no effect (Ishikawa and Kanno, 1991) on fluid and electrolyte secretion by pancreatic acinar cells. Continuous functioning of the Cl⁻/HCO₃⁻ exchanger during electrolyte secretion requires the parallel activation of the Na⁺/H⁺ exchanger to maintain stable pHᵢ. Activation of the Na⁺/H⁺ exchanger can also fuel the Na⁺ pump. Measurements of [Na⁺]ᵢ confirmed the coupling between the Na⁺/H⁺ exchanges and the Na⁺ pump.

Na⁺ Transport

Measurements with SBFI showed that in resting acinar cells, [Na⁺]ᵢ was ~11.7 mM. Previous measurements with Na⁺-selective microelectrodes reported a resting [Na⁺]ᵢ of between 8.3 and 10.5 mM (O’Doherty and Stark, 1982, 1983). The Na⁺ permeability of resting cells was low, as evident from the rate of [Na⁺]ᵢ increase after inhibition of the Na⁺ pump with ouabain or removal of K⁺o. Therefore, to identify the transporters mediating Na⁺ influx, it was necessary to deplete the cells from Na⁺. In addition to the NaK2CI and the NaCl cotransporters, a voltage-independent, nonselective cation channel (Maruyama and Petersen, 1982; Petersen, 1992) and a Na⁺/H⁺ exchange mechanism (Muallem and Loessberg, 1990a) can mediate Na⁺ influx into acinar cells. The present studies show that in the presence of Cl⁻, the NaCl cotransporter and a TEA-sensitive channel had minimal effect on Na⁺ influx into Na⁺-depleted cells. A significant part of the influx was dependent on the presence of K⁺o and Cl⁻, and inhibited by bumetanide, suggesting that the NaK2Cl cotransporter mediates some of the Na⁺ influx into Na⁺-depleted cells, even when the cells contained Cl⁻. However, most of the Na⁺ influx (65–70%) was sensitive to amiloride and therefore mediated by the Na⁺/H⁺ exchanger.

In resting cells, all the Na⁺ transporters operated at a fraction of their maximal capacity. This is evident from the low Na⁺ permeability of the cells and the small effect of the various blockers on [Na⁺]ᵢ. This is despite an inwardly directed driving force for the NaCl and NaK2Cl cotransporters. It is well established that the Na⁺/H⁺ exchanger is sensitive to pHᵢ (Aronson, Nee, and Suhm, 1982; Grinstein and Foskett, 1990), with minimal activity at physiological pHᵢ. It is likely that the other transporters are also regulated to maintain low activity in resting cells. Recently we
have shown that the NaK2Cl cotransporter is sensitive to [Na\(^+\)], (Whisenant, Khademazad, and Muallem, 1993). Similar findings were reported in ciliary epithelial cells (Dong, Delamere, and Coca-Prados, 1994). In parotid acinar cells, all major Na\(^+\) influx transporters appear to be sensitive to [Cl\(^-\)], (Robertson and Foskett, 1994). A combination of these regulatory mechanisms is likely to minimize the activity of the Na\(^+\) influx transporters in resting acinar cells.

**Coupling of Transporters**

Measurement of \(^{86}\)Rb fluxes revealed an intimate relationship between the various transporters (Fig. 8). The model in the accompanying manuscript (Zhao and Muallem, 1995) illustrates these findings. All the Na\(^+\) transporters feed the Na\(^+\) pump to fuel Na\(^+\) efflux and K\(^+\) influx. Because the transport of Na\(^+\) is coupled to other ions, the transporters also respond to changes in the concentrations of these ions. For example, the NaCl cotransporter responds to the internal concentration of Na\(^+\) and Cl\(^-\) and the NaK2Cl cotransporter to the intracellular concentrations of all ions. The first of these relationships is noted when neither bumetanide nor ouabain alone can inhibit \(^{86}\)Rb influx, whereas in the presence of both blockers, K\(^+\) influx is completely blocked. Obviously, when the NaK2Cl cotransporter is inhibited, another transporter is mediating Na\(^+\) influx into the cells to fuel the Na\(^+\) pump. This transporter appears to be the Na\(^+\)/H\(^+\) exchanger (Fig. 8). When the Na\(^+\) pump is blocked, the NaK2Cl cotransport activity is increased (compare the bumetanide sensitive uptake in the presence and absence of ouabain). Hence, it seems that the activities of the cotransporter and the Na\(^+\) pump are modified to complement each other with respect to K\(^+\) influx. In resting cells, the coupling is unlikely to be through [Na\(^+\)], since inhibition of Na\(^+\) influx by the cotransporter should reduce Na\(^+\) pump activity, and an increase in [Na\(^+\)], due to inhibition of the Na\(^+\) pump is expected to inhibit net Na\(^+\) uptake by the cotransporter (Whisenant et al., 1993). Thus, it is likely that in resting acinar cells, the Na\(^+\)/K\(^+\) pump and the NaK2Cl cotransporter are coupled through [K\(^+\)]. In this case the two transporters should be sufficiently sensitive to detect small changes in [K\(^+\)], in the presence of 140–145 mM K\(^+\). One way to achieve that would be if the transporters are localized next to each other to affect the local concentration of K\(^+\). The influence of [K\(^+\)], on NaK2Cl cotransport and the Na\(^+\) pump is not well understood, although effects of [K\(^+\)], on partial reactions mediated by the two transporters have been described (Glynn, 1985; Lauf, McManus, Haas, Forbush, Duhm, Flatman, Saier, and Russell, 1987).

A major form of coupling appears to be through the supply of Na\(^+\) to the Na\(^+\) pump. Inhibition of Na\(^+\) influx through the nonselective channel (TEA), the NaCl cotransporter (TZ) or the Na\(^+\)/H\(^+\) exchanger (DMA) increased the total but, in particular, the bumetanide-sensitive \(^{86}\)Rb uptake. Since changes in coupling stoichiometry of the Na\(^+\) pump by the blockers are not likely, the increased \(^{86}\)Rb uptake indicates that the activity of the NaK2Cl cotransporter is increased to compensate for the reduction in Na\(^+\) influx. Since Na\(^+\) influx is now coupled to K\(^+\) influx, the total and bumetanide-sensitive fluxes are increased. The overall effect is increased coupling between the activities of the Na\(^+\) pump and the NaK2Cl cotransporter. In addition, these findings show that the relative contribution of the different trans-
porters to Na⁺ influx can be evaluated from their effect on bumetanide-sensitive ⁸⁶Rb uptake. Thus, the Na⁺/H⁺ exchanger contributes twice more than the co-
transporter and three times more than the NaCl cotransporter and the TEA-sensitive 
channel to Na⁺ influx in resting cells (Fig. 8; Zhao and Muallem, 1995).

Coupling among the transporters through the concentrations of Na⁺ and K⁺ in-
dicates that most of the transporters are likely to be regulated by these ions 
through interaction with a cytosolic domain. It is also likely that agonists that stimu-
late electrolyte secretion by pancreatic acinar (and probably other) cells do so by 
modification of Na⁺ and/or K⁺. The companion article (Zhao and Muallem, 1995) 
describes how pancreatic secretagogues regulate the activity of specific transporters 
to modulate [Na⁺], and the overall process of fluid and electrolyte secretion by aci-
nar cells.

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