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Role of Na^+/Ca^{2+} Exchange and the Plasma Membrane Ca^{2+} Pump in Hormone-Mediated Ca^{2+} Efflux from Pancreatic Acini

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Summary. The relative contributions of the Na^+/Ca^{2+} exchange and the plasma membrane Ca^{2+} pump to active Ca^{2+} efflux from stimulated rat pancreatic acini were studied. Na^+ gradients across the plasma membrane were manipulated by loading the cells with Na^+ or suspending the cells in Na^+-free media. The rates of Ca^{2+} efflux were estimated from measurements of $[Ca^{2+}]_i$ using the Ca^{2+}-sensitive fluorescent dye Fura 2 and ^{45}Ca efflux. During the first 3 min of cell stimulation, the pattern of Ca^{2+} efflux is described by a single exponential function under control, Na^+-loaded, and Na^+-depleted conditions. Manipulation of Na^+ gradients across the plasma membrane had minimal effects on resting $[Ca^{2+}]_i$, the rate constant of Ca^{2+} efflux, and $[Ca^{2+}]_i$ levels attained by the cells after 5 min of stimulation. Changing Na^+ gradients had no effect on the hormone-induced increase in $[Ca^{2+}]_i$. The results indicate that Ca^{2+} efflux from stimulated pancreatic acinar cells is mediated by the plasma membrane Ca^{2+} pump. The effects of several cations, which were used to substitute for Na^+, on cellular activity were also studied. Choline$^+$ and tetramethylammonium$^+$ (TMA^+) released Ca^{2+} from intracellular stores of pancreatic acinar, gastric parietal and peptic cells. These cations also stimulated enzyme and acid secretion from the cells. All effects of these cations were blocked by atropine. Measurements of cholecystokinin-octapeptide (CCK-OP)-stimulated amylase release from pancreatic acini, suspended in Na^+, TMA^+, choline$^+$, or N-methyl-D-glucamine$^+$ (NMG^+) media containing atropine, were used to evaluate the effect of the cations on cellular function. NMG^+, choline$^+$, and TMA^+ inhibited amylase release by 55, 40 and 14%, respectively. NMG^+ also increased the Ca^{2+} permeability of the plasma membrane. Thus, to study Na^+ dependency of cellular function, TMA^+ is the preferred cation to substitute for Na^+. The stimulatory effect of TMA^+ can be blocked by atropine.

Key Words cholinergic receptors · choline · tetramethylammonium · hormones · Ca^{2+} efflux · Na^+/Ca^{2+} exchange · Ca^{2+} pump

Introduction

Stimulation of cells, including pancreatic acinar (Pandol et al., 1985) and gastric glandular (Muallem et al., 1986) cells by agonists that release Ca^{2+} from internal stores, results in a transient increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). The transient nature of the response is due to Ca^{2+} release from intracellular stores, probably the endoplasmic reticulum (Streb et al., 1983, 1984) followed by Ca^{2+} extrusion from the cells (Matthews, Petersen & Williams, 1973; Gardner et al., 1975; Dormer et al., 1981). Two mechanisms capable of active Ca^{2+} extrusion from cells have been described—a Na^+/Ca^{2+} exchange mechanism and a plasma membrane Ca^{2+} pump (Blaustein & Nelson, 1982; Schatzmann, 1982).

The kinetic properties of these mechanisms have been studied and described in detail using isolated membranes and vesicles (Pitts, 1979; Schatzmann, 1982; Reeves & Hale, 1984). The contribution of these mechanisms to active Ca^{2+} efflux from intact cells was best evaluated in excitable cells and renal tubules (DiPolo & Beauge, 1983; Lorenzen, Lee & Windhager, 1984). These studies suggest that at resting levels of $[Ca^{2+}]_i$, Ca^{2+} efflux from the cells occurs mainly via the plasma membrane Ca^{2+} pump. The Na^+/Ca^{2+} exchange, which is a low Ca^{2+} affinity, high capacity mechanism in excitable cells, dominates Ca^{2+} efflux at high $[Ca^{2+}]_i$ (Blaustein & Nelson, 1982; DiPolo & Beauge, 1983). In renal proximal tubules, a linear relationship was obtained when $[Ca^{2+}]_i$ was plotted against triple the electrochemical potential gradient for Na^+ across the basolateral membrane (Lorenzen et al., 1985), indicating that in these cells, Na^+ gradients affect Ca^{2+} fluxes across the plasma membrane.

Information regarding the contribution of the Na^+/Ca^{2+} exchanger to Ca^{2+} efflux from resting or stimulated nonexcitable cells is limited (Borle, 1981). We therefore studied the relative contribution of the Na^+/Ca^{2+} exchanger and the plasma

membrane Ca^{2+} pump to Ca^{2+} efflux from resting and stimulated pancreatic acinar cells. At present there are no selective blockers of Na^+/Ca^{2+} exchange or the plasma membrane Ca^{2+} pump for use in intact cells. Manipulation of the Na^+ gradient across the plasma membrane is the method of choice for determining the relative contribution of the two mechanisms to active Ca^{2+} efflux from cells (DiPolo, 1979; Lorenz et al., 1984; Muallem & Sachs, 1985). To achieve that, cells are either loaded with Na^+ or suspended in Na^+-free medium. The most common cations used to substitute for Na^+ in studying Na^+-dependent transport mechanisms are choline$^+$, tetramethylammonium$^+$ (TMA^+) and N-methyl-D-glucamine$^+$ (NMG^+).

In this report we demonstrate that choline$^+$ and TMA^+ can activate cells by binding to the muscarinic-cholinergic receptors. We also evaluated the effect of these cations on cellular function. Of the cations used to substitute for Na^+, TMA^+ was least inhibitory on cell function. Thus, TMA^+ was used to estimate the contribution of the Na^+/Ca^{2+} exchange to Ca^{2+} efflux from stimulated cells. The results indicate that most, if not all, Ca^{2+} efflux from the cells is mediated by the plasma membrane Ca^{2+} pump.

Materials and Methods

Solutions

Stock solutions of 1 M tetramethylammonium$^+$ (TMA^+) Cl^-, Choline$^+$ Cl^-, N-methyl-D-glucamine$^+$ (NMG^+) Cl^- and tetraethylammonium$^+$ (TEA^+) Cl^- were prepared by titrating the OH^- form of the salts with HCl to pH 7.0. The concentration of Cl^- was measured by chloridometer and the concentrations were adjusted to 1 M. The stock solutions were used to prepare the following solutions: Solution A (mM): 140 NaCl; 5 KCl; 1.0 $MgCl_2$; 1.0 $CaCl_2$; 10 HEPES/Tris (pH 7.4); 10 glucose; 10 pyruvic acid and 0.1% bovine serum albumin (BSA). Solution B: The same composition as solution A, except that equimolar TMACl replaced the NaCl. Solution C: The same composition as solution A except that equimolar cholineCl replaced the NaCl. Solution D: The same composition as solution A except that equimolar NMGCl replaced the NaCl. Solution E: The same as solution A except that KCl was replaced with 5mM NaCl and 1 mM ouabain was included in the solution.

Preparation of Pancreatic Acini

Dispersed pancreatic acini were prepared from rats (75 to 150 g) by modification of a procedure previously described (Bruzzone et al., 1985). The pancreas was removed and injected with 10 ml of solution A containing 10 mM $NaHCO_3$ and 0.01% (wt/vol) soybean trypsin inhibitor. The fluid was drained by blotting the pancreas on filter paper. Then the pancreas was mixed with 0.5 ml of the above solution containing 1 mg/15 ml purified collagenase (type CLSPA, 660 U/mg, Cooper Biomedical) (digestion medium) and minced with scissors. The minced tissue was transferred to a 25-ml flask and 4.5 ml of digestion media was added. The flask was gassed with 100% O_2, capped and the tissue was digested for 5 min at 37°C in a shaking water bath at 160 oscillations/min. Next, the digestion medium was removed and replaced with 10 ml of fresh digestion medium. The tissue was digested for another 10 min. The dispersed acini were then washed twice with solution A containing 0.01% trypsin inhibitor by alternate 5-sec centrifugations at 150 × g and resuspensions. The acini were suspended in 10 ml of solution A, passed through a nylon mesh and washed once more with solution A. Prior to use, the acini were suspended in 5 to 50 ml of solution A and kept at room temperature.

Preparation of Gastric Glands

Rabbit gastric glands were prepared as previously described (Berglindh & Obrink, 1976). Glands from one stomach were suspended in 50 ml of solution A.

Measurement of Free Cytosolic Ca^{2+}

Acini from one pancreas were suspended in 5 ml of solution A, or 5 ml of gland suspension were incubted with 2 μM Fura 2/AM (Molecular Probes, Junction City, Ore.) for 20 min at 37°C. The cells were then washed twice with 35 ml of solution A and resuspended in 2 to 3 ml of solution A. Fifty to 100 μl of cell suspension were added to 1.9 ml prewarmed media and fluorescence measurements were made while the cells were continually stirred and maintained at 37°C. Fluorescence was measured with a Perkin-Elmer spectrofluorimeter Model 640-50, with excitation and emission set at 340 and 500 nm, respectively. $[Ca^{2+}]_i$ was calculated as previously described (Grynkiewicz et al., 1985), using a K_d of 220 nM for the Fura 2 · Ca^{2+} dissociation constant. Since dye leak was less than 1% during a typical experiment, the recordings were not corrected. To eliminate the contribution of dye leak (about 5% after one hour of incubation at room temperature) samples of cells sufficient for two to four experiments were washed prior to $[Ca^{2+}]_i$ measurements.

^{45}Ca Fluxes

Acini from the pancreas of one rat were suspended in 20 to 30 ml media and incubated with $^{45}CaCl_2$ (about 2 × 10^5 cpm/ml) at 37°C under continuous shaking. At the indicated times 0.5-ml samples were transferred to 10 ml of ice-cold, Ca^{2+}-free solution A containing 1 mM $LaCl_3$. The acini were then collected by a 30-sec centrifugation at 150 × g and washed twice more with the same solution. The acini were then dissolved by heating at 60°C in 1 ml of 1 M NaOH for 10 min, and ^{45}Ca was counted using standard liquid scintillation counting.

Other Assays

Amylase release was measured as previously described (Peikin et al., 1978). Acid and pepsin secretion were measured by the aminopyrine distribution and hemoglobin digestion techniques, respectively (Rink & Fritsch, 1974; Berglindh, Helander & Obrink, 1976).

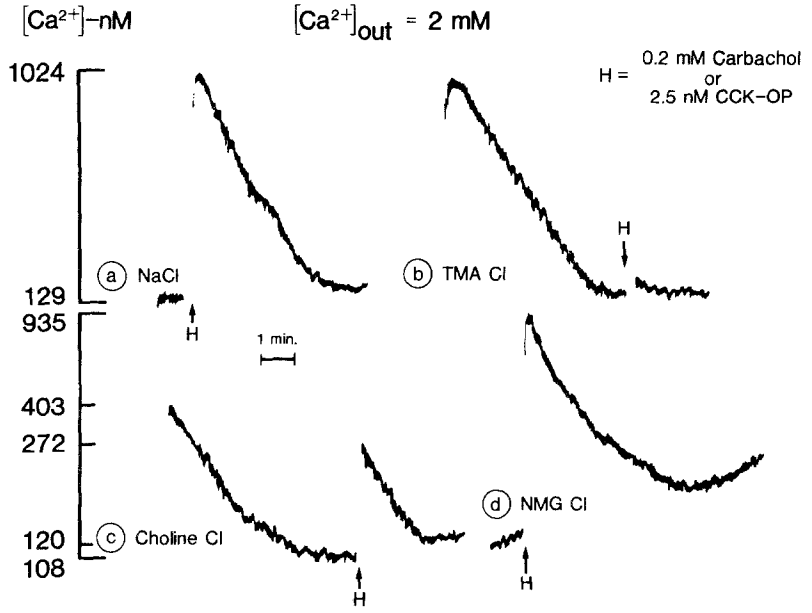

Fig. 1. Hormone and cation-dependent increase in $[Ca^{2+}]_i$. Fura 2-loaded pancreatic acini were added to solution A-NaCl (*a*), solution B-TMACl (*b*), solution C-cholineCl (*c*) or solution D-NMGCl (*d*) all of which contained 2 mM $CaCl_2$. "H" indicates when 0.2 mM carbachol or 2.5 nM CCK-OP was added to the medium. In the experiments shown the cells were stimulated with carbachol. Identical results were obtained with CCK-OP. The Figure is representative of 12 similar experiments using TMA^+ and seven experiments using $choline^+$ or NMG^+

Results

Selection of Na^+-Substituting Cation

Since there are no selective inhibitors of the Na^+/Ca^+ exchanger and the plasma membrane Ca^{2+} pump suitable for use in intact cells, the contribution of these two mechanisms to active Ca^{2+} extrusion from cells is estimated from measurements of the effect of medium and cytosolic Na^+ on Ca^{2+} efflux. Tetramethylammonium$^+$ (TMA^+), choline$^+$ and N-methyl-D-glucamine$^+$ (NMG^+) are commonly used monovalent cations in place of Na^+. The effect of these cations on free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) of rat pancreatic acinar cells is shown in Fig. 1. Addition of acini to medium containing 140 mM TMACl was followed by a transient increase in $[Ca^{2+}]_i$ to approximately 1024 nM. This value was similar to that obtained by stimulation of cells suspended in NaCl medium with a maximal dose of carbachol or CCK-OP (Fig. 1*a,b*). Further, cells suspended in TMACl medium failed to respond to stimulation by either carbachol or CCK-OP. The ability of TMA to increase $[Ca^{2+}]_i$ was dose dependent with an EC_{50} of 17 ± 0.5 mM (n = 6). When the acini were added to medium containing 140 mM cholineCl, $[Ca^{2+}]_i$ increased to about 403 nM (Fig. 1*c*). Subsequent stimulation of the acini with either carbachol or CCK-OP induced a small increase in $[Ca^{2+}]_i$. The choline$^+$-mediated increase in $[Ca^{2+}]_i$ was also dose dependent from 50 and 140 mM cholineCl. The hormone-induced signals were higher than those observed with 140 mM cholineCl (*not shown*). Addition of the acini to NMGCl-containing medium was not followed by an increase in $[Ca^{2+}]_i$. Stimulation of the cells with hormones induced an increase in $[Ca^{2+}]_i$ similar to that observed with cells suspended in NaCl-containing medium.

The effect of medium Ca^{2+} concentration on TMACl and cholineCl-mediated increase in $[Ca^{2+}]_i$ is shown in Fig. 2. Reducing medium $Ca^{2+}]_i$ to 0.1 μM did not prevent the increse in $[Ca^{2+}]_i$ induced by TMA^+ or choline Cl^+. This observation indicates that these ions released Ca^{2+} from intracellular stores of pancreatic acinar cells.

Since choline$^+$ and TMA^+ have structural similarities to acetylcholine, we next tested the effect of atropine on the TMA^+ and choline$^+$-mediated increase in $[Ca^{2+}]_i$. Figure 3 shows that 20 μM atropine completely inhibited the effect of these cations on $[Ca^{2+}]_i$. Further, the cells were now fully responsive to stimulation by CCK-OP. These results indicate that both TMA^+ and choline$^+$ stimulated the cells by binding to the cholinergic-muscarinic receptors. The CCK-OP-mediated peak increase in $[Ca^{2+}]_i$ as measured in NaCl or NMG^+ media (Fig. 1), or TMA^+ or choline$^+$ media (Fig. 3) was independent of the cation used. CCK-OP increased $[Ca^{2+}]_i$ to 1047 ± 94 in NaCl medium, 1030 ± 119 in TMACl medium, 978 ± 87 in cholineCl medium, and 934 ± 98 nM (n = 6) in NMGCl medium.

The effect of the cations on enzyme secretion was also examined. Figure 4(*A*) shows that hormone-stimulated amylase secretion is biphasic. As previously reported (Pandol et al., 1985; Bruzzone, Pozzan & Wollheim, 1986), the first rapid phase of

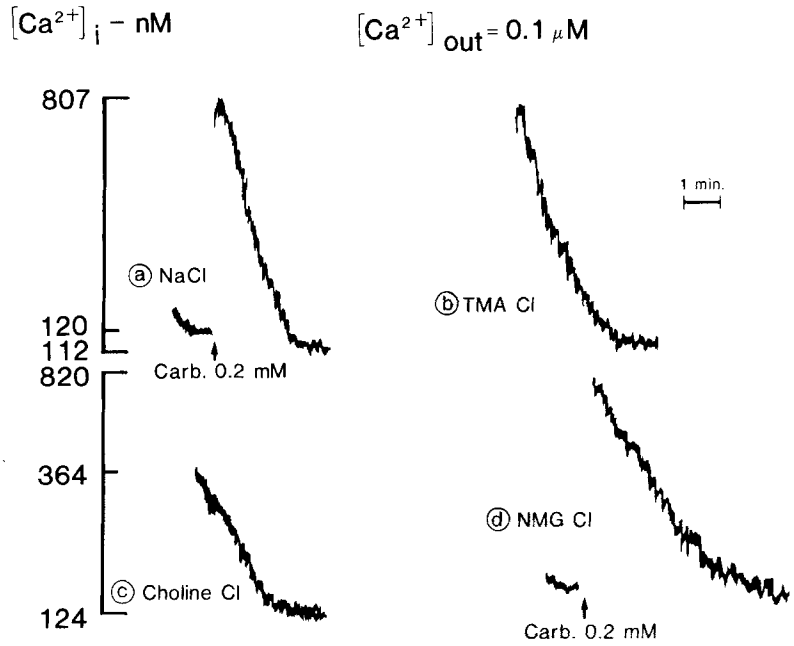

Fig. 2. Effect of medium Ca^{2+} concentration on cation-stimulated increse in $[Ca^{2+}]_i$. Experimental conditions and solutions were identical to those in Fig. 1, except that $CaCl_2$ was omitted and 0.1 mM EGTA was added to all media. Where indicated 0.2 mM carbachol was added. The experiment shown is representative of five others with similar results

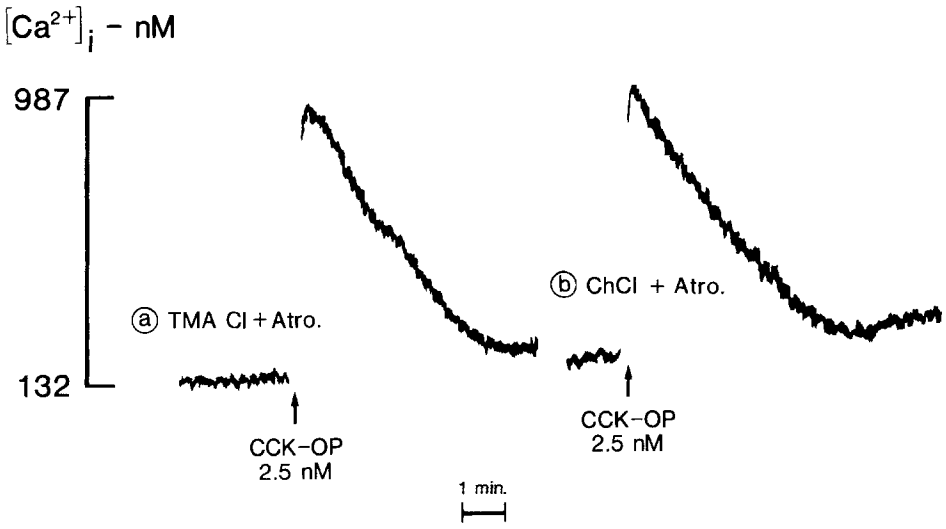

Fig. 3. Effect of atropine on TMA^+ and $choline^+$-mediated increase in $[Ca^{2+}]_i$. Pancreatic acinar cells loaded with Fura 2 were added to solution B-TMA^+ (*a*) or solutions C-$Choline^+$ (*b*) also containing 20 μM atropine. Where indicated the cells were stimulated with 2.5 nM CCK-OP

secretion is mediated by the increase in $[Ca^{2+}]_i$ while the second phase is probably mediated in part by protein kinase C activation. Suspending the cells in TMACl media also induced a biphasic rate of amylase release (Fig. 4*B*). Atropine completely blocked the TMA^+-stimulated secretion. $Choline^+$ induced only partial stimulation of amylase release which could be blocked by atropine (Fig. 4*C*), while NMG^+ had no effect on amylase release.

The effect of the different cations on cellular activity was assessed by measuring the ability of CCK-OP to stimulate amylase release. Figure 5 shows that CCK-OP did not increase the rate of amylase release beyond that induced by TMA^+ alone (*compare* Figs. 4 and 5). Amylase release in the presence of CCK-OP and $choline^+$ was higher than that measured in the presence of $choline^+$ alone. Figure 5 also shows that maximal rates of amylase release could not be obtained in either TMA^+, $choline^+$, or NMG^+ media. CCK-OP-stimulated amylase release was inhibited by 55, 40 and 14% in NMG^+, $choline^+$, and TMA^+ media, respectively. Due to the inhibitory effect of $choline^+$ and NMG^+ on cell function, TMA^+ was used as the

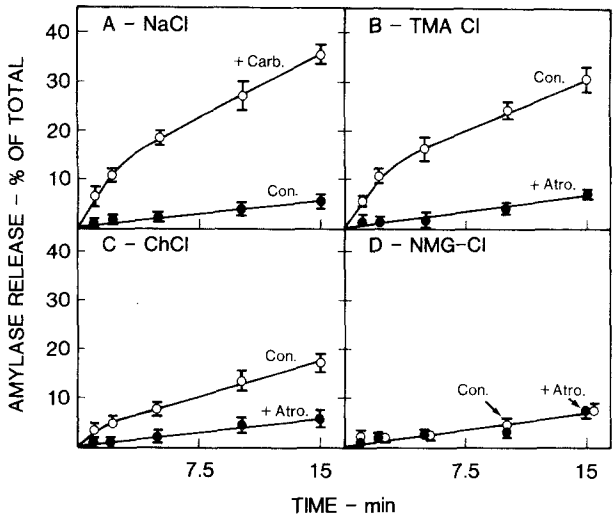

Fig. 4. Carbachol and cation-stimulated amylase release. Pancreatic acinar cells suspended in solution A were collected by a 10-sec centrifugation at 150 × *g* and the medium was removed. Experimental incubations were initiated by suspending the cells with the following solutions: (*A*) NaCl with or without 0.2 mM carbachol; (*B*) TMA^+ with or without 20 μM atropine; (*C*) $Choline^+$ with or without atropine, and (*D*) NMG^+ with or without atropine. At the indicated times 0.5-ml samples were taken, centrifuged for 10 sec in an Eppendorf centrifuge and amylase content of the supernatant was measured as described under Materials and Methods. The Figure shows the mean ± SD of five separate experiments

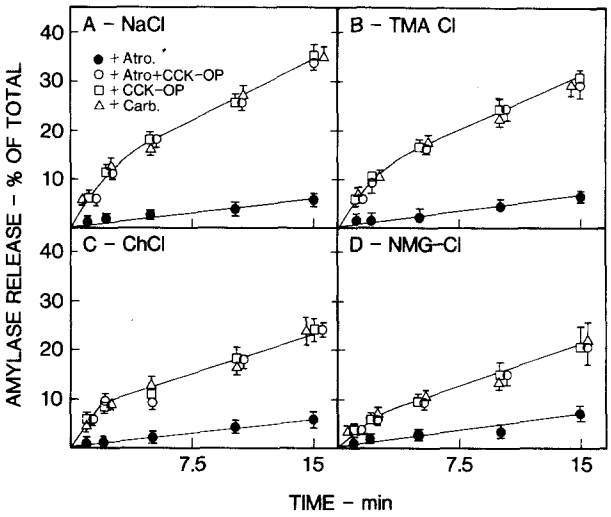

Fig. 5. CCK-OP-stimulated amylase release from cells suspended in different media. Experimental procedure was as described in Fig. 4 legend. Acini were suspended in 5 ml media of the indicted composition containing 20 μM atropine (filled circles), 20 μM atropine and 2.5 nM CCK-OP (open circles), 2.5 nM CCK-OP (open squares) or 0.2 mM carbachol (open triangles). At the indicated times, samples were taken to measure amylase release

substituting cation for Na^+, when the stimulatory effect of TMA^+ was blocked by atropine.

The effects of these cations are not confined to pancreatic acinar cells. Figure 6 shows the effect of TMA^+ on $[Ca^{2+}]_i$ of gastric glands. Suspending the glands in TMA^+ media increased $[Ca^{2+}]_i$ to the same levels as carbachol stimulation of glands suspended in NaCl media. Carbachol addition to glands suspended in TMA^+ media had no further effect on $[Ca^{2+}]_i$ (Fig. 6*b*). Similarly, CCK-OP and human gastrin (HG) (1-17) had no effect on $[Ca^{2+}]_i$ when added to glands incubated in TMA^+ media (*not shown*). The effect of TMA^+ could be blocked by atropine (Fig. 6*c*). When the glands were suspended in TMA^+ media containing atropine, they respond to CCK-OP and HG (1-17) by a typical transient increase in $[Ca^{2+}]_i$.

Since the gastric glands contain both parietal and peptic cells that can respond to cholinergic stimulation, we next tested the effect of TMA^+ on acid and pepsin secretion (Fig. 7). As expected, TMA^+ stimulated both acid and pepsin secretion similar to that of carbachol stimulation (Fig. 7*A–D*). Carbachol had no additional effect when added together with TMA^+, and the stimulatory effect of TMA^+ was blocked by atropine (Fig. 7*B,D*).

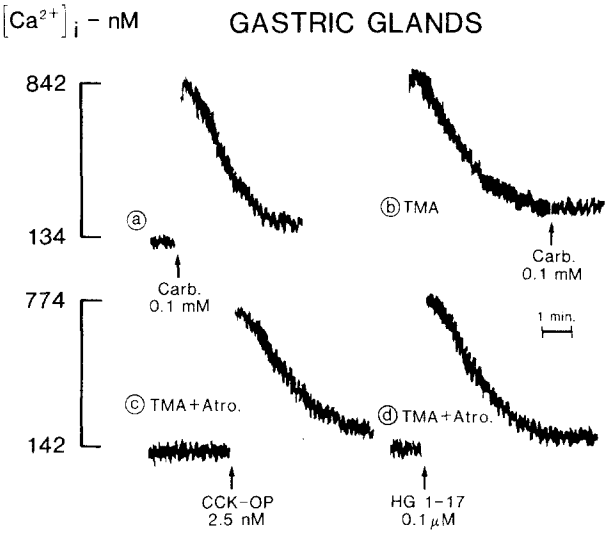

Fig. 6. TMA^+-mediated increase in $[Ca^{2+}]_i$ in gastric glands. Gastric glands loaded with Fura 2 were added to solution A-NaCl (*a*), solution B-TMACl (*b*, *c*, *d*) which also contained 20 μM atropine (*c*, *d*). Where indicated the cells were stimulated with 0.1 mM carbachol (*a*, *b*), 2.5 nM CCK-OP (*c*) or 0.1 μM human gastrin (1-17) (*d*). The Figure presents one of three similar experiments

Na^+/Ca^{2+} Exchange and the Plasma Membrane Ca^{2+} Pump

To estimate the contribution of the Na^+/Ca^{2+} exchanger to Ca^{2+} efflux, Na^+ gradients across the

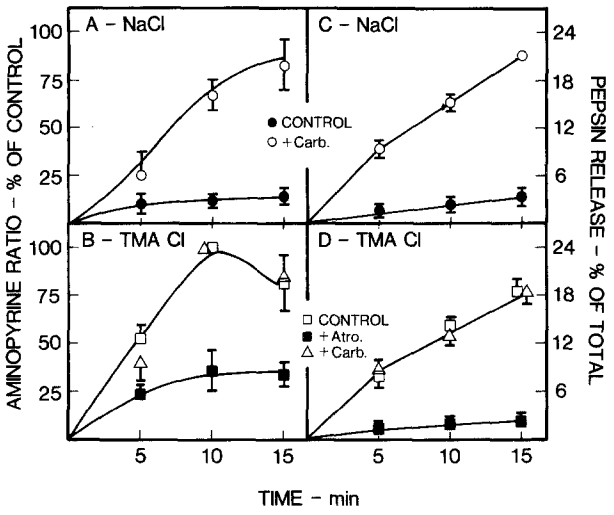

Fig. 7. TMA^+-stimulated acid and pepsin secretion from gastric glands. Gastric glands suspended in solution A were collected by 5-sec centrifugation at 150 × *g* and the medium was removed. The experimental incubations were initiated by suspending the glands with 5-ml solutions containing ^{14}C-aminopyrine. The composition of solutions was as follows: (*A*) and (*C*) solution A with (open circles) or without (filled circles) 0.1 mM carbachol. (*B*) and (*D*) solution B-TMACl (open squares) also containing 20 μM atropine (filled squares) or 0.1 mM carbachol (open triangles). At the indicated times 1-ml portions were taken and centrifuged for 10 sec in an Eppendorf centrifuge. The pellets and a sample of each supernatant were used to measure aminopyrine content and calculate aminopyrine distribution between pellet and supernatant. Aminopyrine ratio was between 42 and 78. In each experiment, aminopyrine ratio of glands suspended in TMA^+ medium and stimulated with carbachol was taken as 100% control. Another sample of the supernatant was used to measure pepsin content. The Figure shows the mean ± SD of three experiments

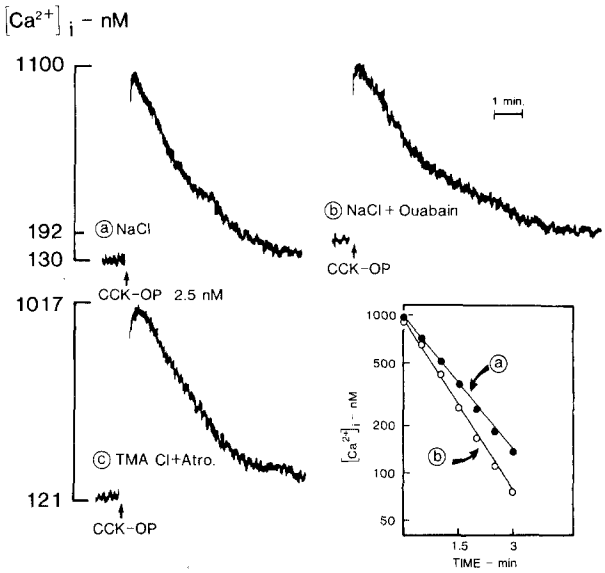

Fig. 8. Effect of Na^+ gradients on hormone-mediated changes in $[Ca^{2+}]_i$. (*a*) Fura 2-loaded cells were added to solution A and stimulated with CCK-OP. (*b*) Fura 2-loaded pancreatic acinar cells were washed once and resuspended in K^+-free solution A containing 1 mM ouabain. The cells were then incubated for 30 min at 37°C under continuous shaking, washed once more to remove extracellular dye, and then added to K^+-free solution A containing 1 mM ouabain. Where indicated, the cells were stimulated with 2.5 nM CCK-OP. (*c*) Cells were washed once with solution B (TMA^+) containing 20 μM atropine, and then resuspended in the same solution. Where indicated, the cells were stimulated with CCK-OP. Experiments (*a*) and (*b*) were used to calculate $[Ca^{2+}]_i$ every 0.5 min following the peak increase in $[Ca^{2+}]_i$. To obtain the amount of Ca^{2+} extruded by the cells, $[Ca^{2+}]_i$ after 5 min of stimulation was subtracted from the values of $[Ca^{2+}]_i$ calculated at different time points. The resulting $[Ca^{2+}]_i$ values were plotted against the incubation time (inset). The calculated rate constants at the different conditions are given in the text

plasma membrane were modified prior to hormonal stimulation (Fig. 8). Acini were loaded with Na^+ by incubation in a K^+-free medium containing 1 mM ouabain for 30 min at 37°C. This treatment increased resting $[Ca^{2+}]_i$ from 136 ± 17 to 206 ± 34 nM (n = 5). Stimulation of these cells with CCK-OP increased $[Ca^{2+}]_i$ to levels similar to that of control, untreated cells (Fig. 8*a*,*b*). In the absence of a Na^+ gradient across the plasma membrane (Fig. 8*b*), or in Na^+-free conditions (Fig. 8*c*), the cells were able to reduce $[Ca^{2+}]_i$. The reduction in $[Ca^{2+}]_i$ during the first 3 min after stimulation can be described by a single exponential function (Fig. 8 inset). The rate constants for the reduction in $[Ca^{2+}]_i$ were 0.832 ± 0.039, 0.621 ± 0.027, and 0.770 ± 0.031 min^{-1} (n = 5) for the conditions in Fig. 8(*a*), (*b*) and (*c*), respectively. Hence, manipulation of the Na^+ gradient across the plasma membrane had only a minimal effect on the ability of the cells to remove Ca^{2+} from the cytosol.

The changes in $[Ca^{2+}]_i$ reflect net Ca^{2+} efflux from the cells which is a function of unidirectional Ca^{2+} influx and efflux during hormonal stimulation. Manipulation of the Na^+ gradient may have affected Ca^{2+} influx into the stimulated cells, which would lead to an underestimation of the contribution of the Na^+/Ca^{2+} exchanger to Ca^{2+} efflux. It was, therefore, necessary to measure the rate of the unidirectional Ca^{2+} efflux from the cells. To achieve that, we examined the properties of hormone-mediated ^{45}Ca fluxes.

Figure 9 shows ^{45}Ca fluxes in rat pancreatic acini suspended in medium containing 2.0 mM $CaCl_2$. When the acini were prestimulated with carbachol for 7.5 min and subsequently exposed to atropine and ^{45}Ca (cycled acini), they incorporated approximately 3.5 times more ^{45}Ca compared to control, unstimulated acini during the first 5 min of

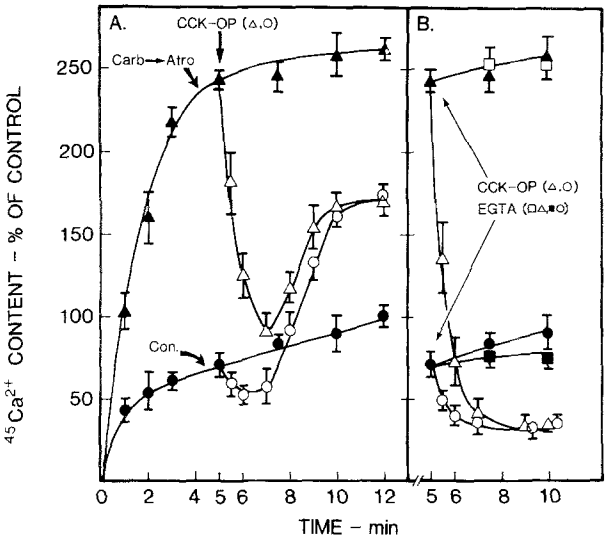

Fig. 9. Hormone-mediated ^{45}Ca fluxes in rat pancreatic acinar cells. Cells suspended in solution A containing 2 mM $CaCl_2$ were incubated with (filled triangles) or without (filled circles – control) 0.2 mM carbachol for 7.5 min. At time zero, ^{45}Ca (filled circles) or ^{45}Ca and 20 μM atropine (filled triangles – "cycled") were added. At the indicated times, samples were taken to determine ^{45}Ca content of the cells. (*A*) After 5-min incubation with ^{45}Ca, a portion of the control (open circles) or cycled (open triangles) acini was transferred to a tube containing CCK-OP to give a final CCK-OP concentration of 0.1 μM. ^{45}Ca content of the acini was determined. (*B*) After 5 min of incubation with ^{45}Ca, portions of control acini were transferred to tubes containing EGTA to give a final EGTA concentration of 1.95 mM (filled squares) or EGTA and CCK-OP (open circles). Cycled acini were transferred to tubes containing EGTA (open squares) or EGTA and CCK-OP (open triangles). ^{45}Ca content of the acini was determined where indicated by transferring 0.5 ml of cell suspension to 10 ml of stop media and washing the cells as described under Materials and Methods. ^{45}Ca content of control cells after 12-min incubation at 37°C with ^{45}Ca was taken as 100%. The Figure shows the mean ± SD of three separate experiments

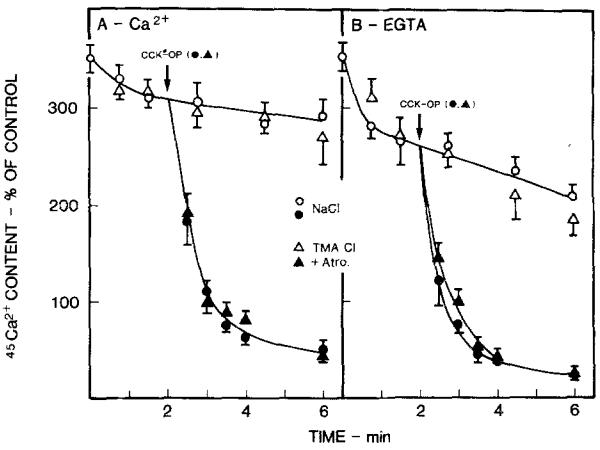

Fig. 10. CCK-OP-mediated $^{45}Ca^{2+}$ effluxes in Na^+ and TMA^+ media. The hormone-sensitive Ca^{2+} pool was labeled with ^{45}Ca by cycling the cells with carbachol, atropine, and ^{45}Ca as described in Fig. 9. After 5 min of incubation with 2 mM ^{45}Ca and 20 μM atropine, samples of cells were taken to determine initial ^{45}Ca content. The cells were divided into two portions. (*A*) The cells from one portion were collected by a 10-sec centrifugation at 150 × *g* and medium containing ^{45}Ca was removed. The cells were resuspended in solution A (open circles) or solution B (TMA) containing 20 μM atropine (open triangles), both containing 2 mM of unlabeled $CaCl_2$. After 2 min of incubation at 37°C, samples of cells in solution A (filled circles) or solution B (filled triangles) were transferred to tubes containing CCK-OP to give a final CCK-OP concentration of 0.1 μM. (*B*) Experimental protocol and symbols are as in (*A*) except that the cells were resuspended in Ca^{2+}-free solutions containing 0.1 mM EGTA. At the indicated times, samples were removed to determine ^{45}Ca content of cells as described under Materials and Methods. ^{45}Ca content of control noncycled cells after 5-min incubation with ^{45}Ca at 37°C was taken as 100% control. The Figure shows the mean ± SD of three experiments

incubation. As we previously described (Pandol et al., 1987), this phenomenon reflects the slow labeling of the hormone-sensitive intracellular Ca^{2+} pool with ^{45}Ca in control acini and the reloading of the pool with Ca^{2+} and its labeling to isotopic equilibrium in prestimulated cells. Stimulation of the cells with CCK-OP, without removal of medium ^{45}Ca, was followed by Ca^{2+} release from control and cycled acini during the first 2 min. Subsequently, the acini reincorporated Ca^{2+}, so that after 7 min of incubation, the level of ^{45}Ca was 1.85 times higher than in control acini (Fig. 9*A*). When medium ^{45}Ca was removed with EGTA during the stimulation with CCK-OP, the cells rapidly lost Ca^{2+}, and reuptake of Ca^{2+} was not observed (Fig. 9*B*). Addition of EGTA alone, which reduced medium Ca^{2+} concentration to about 50 μM, did not rapidly deplete the hormone-sensitive pool to Ca^{2+}. Therefore, hormone-mediated ^{45}Ca efflux into low Ca^{2+}medium reflects only active Ca^{2+} extrusion from the cells.

Figure 10 shows the rate of Ca^{2+} efflux from the cells suspended in Na^+ or TMA^+ media. A similar rate of CCK-OP-induced $^{45}Ca^{2+}$ efflux was observed when the cells were suspended in Na^+ or TMA^+ media containing 2 mM of unlabeled Ca^{2+} (Fig. 10*A*). In Ca^{2+}-free medium, the rate of CCK-OP-induced ^{45}Ca efflux was slightly slower in TMA^+ medium when compared to ^{45}Ca efflux into Na^+ medium (Fig. 10*B*). Using the results in Figs. 10*B*, 2*a*, and 2*b*, we determined that the rate constants for $^{45}Ca^{2+}$ efflux and for the reduction in $[Ca^{2+}]_i$ when measured in Ca^{2+}-free, Na^+-containing medium, are similar (1.873 ± 0.082 min^{-1} (n = 3)). In TMA^+ medium the rate constant was reduced by approximately 16% when calculated from ^{45}Ca efflux or $[Ca^{2+}]_i$ measurements.

Discussion

Studies of Na^+ gradient-driven transport mechanisms by cells or vesicles include the substitution of Na^+ by other cations. In most studies, it is generally assumed that these cations by themselves have no effect on cell function. The difference in cell function observed in media containing Na^+ or any of the substituted cations was attributed to the contribution of Na^+ to the measured cellular activity. In this manuscript we show that TMA^+ and $choline^+$ can activate pancreatic acinar and gastric glandular cells by binding to the cholinergic receptors of these cells. This is concluded from the following: 1) the cations induced Ca^{2+} release from intracellular stores and stimulated enzyme release and acid secretion; 2) the effects of the cations could be completely blocked by the selective cholinergic receptor antagonist, atropine; 3) once the cells were partially or completely stimulated by $choline^+$ or TMA^+, they lost their responsiveness to carbachol and CCK-OP; 4) after inhibition of the effect of these cations with atropine, a maximal CCK-OP-induced increase in $[Ca^{2+}]_i$ was observed. When taken together, these results indicate that both TMA^+ and $choline^+$ stimulated the cells by binding to cholinergic receptors.

We also tested the effect of tetraethylammonium (TEA^+) on $[Ca^{2+}]_i$ in pancreatic acinar cells. TEA^+ is commonly used as a blocker of the Ca^{2+}-activated K^+ channel in cells (Petersen, 1987). At concentrations between 2 and 10 mM, it has a negligible to small effect on $[Ca^{2+}]_i$, while 100 mM TEA^+ increased $[Ca^{2+}]_i$ similar to that observed with 140 mM $choline^+$. The effect of TEA^+ could be blocked by atropine (*not shown*). Therefore, to block the K^+ channels, TEA^+ should be used at a concentration no greater than 2 mM or it should be used together with atropine.

The cations studied were able to bind to the cholinergic receptors and activate the three cell types tested. Gastric glands have mainly two cell types, parietal and peptice cells, both of which respond to cholinergic stimulation (Muallem et al., 1986). The cells used in the present studies have an MII type cholinergic-muscarinic receptor. This type of receptor was described in different cells of the gastrointestinal tract (Hammer, 1980) and excitable tissues (Hammer & Giachetti, 1982; Vickroy et al., 1984). It is likely that all cells with MII type receptors can be stimulated with TMA^+ and $choline^+$. Another type of cholinergic receptor is the MI type which is present in excitable cells (Hammer & Giachetti, 1982; Vickroy et al., 1984). It remains to be found whether MI receptors can also bind TMA^+, $choline^+$, and TEA^+.

Stimulation of pancreatic acini with TMA^+ or $choline^+$ also increased amylase release. As expected from intracellular Ca^{2+} mobilizing agents (Pandol et al., 1985; Muallem et al., 1986), amylase release was biphasic. A first fast phase of release requires the increase in $[Ca^{2+}]_i$, and a second slower phase occurs at near resting levels of $[Ca^{2+}]_i$. An additional finding in the present study was that amylase secretion was impaired by the different cations used. Although in all media tested, CCK-OP induced a similar increase in $[Ca^{2+}]_i$, amylase release was inhibited by 55, 40 and 14% when the acini were suspended in NMG^+, $choline^+$, or TMA^+ media, respectively. Further, when cells suspended in NMG^+ media were stimulated with CCK-OP, they were not able to reduce $[Ca^{2+}]_i$ to levels observed in NaCl or TMA^+ media. Also, $[Ca^{2+}]_i$ did not stabilize after 5 min of stimulation (Fig. 1). These observations suggest that the plasma membrane Ca^{2+} permeability is increased in NMG^+ medium. Thus, $choline^+$ and NMG^+ cannot be used as a substitute of Na^+, when the Na^+ dependency of different cellular functions is to be studied. Since only a minimal effect of TMA^+ on cell function was found, this ion can be used to substitute for Na^+, providing its stimulatory effect is blocked by atropine.

Binding of the cations to cholinergic receptors induced Ca^{2+} release from intracellular stores. Therefore, it is most likely that these cations stimulated phospholipase-C, the hydrolysis of phosphatidylinositol-4,5-bisphosphate and the generation of 1,4,5-IP_3 in the cytosol (Berridge & Irvine, 1984). The agonist-induced initial increase in $[Ca^{2+}]_i$ was independent of Na^+ and K^+ content of the cytosol and the potential across the plasma membrane. Similar CCK-OP-induced increases in $[Ca^{2+}]_i$ were observed in control cells, cells depleted of Na^+ (Fig. 3) or cells loaded with Na^+ and partially depleted of K^+ (Fig. 8). Thus, it appears that the biochemical reactions leading to Ca^{2+} release from intracellular stores function similarly in Na^+- or K^+-rich cytosol. These findings are in line with previous observations demonstrating that cholinergic stimulation of phosphatidylinositol breakdown in parotid-gland fragments is independent of the ionic environment (Lynne & Michell, 1976). We (Muallem et al., 1985) and others (Joseph & Williamson, 1986) demonstrated that the IP_3-triggered Ca^{2+} release from the endoplasmic reticulum (ER) is a conductive process which requires the presence of permeable ions in the medium. The presence of a K^+-conductive pathway in the ER membrane could be demonstrated (Muallem et al., 1985). In view of the results presented here, it is likely that this pathway conducts Na^+ and K^+ thereby allowing Ca^{2+} release from the ER.

A Na^+/Ca^{2+} exchange mechanism has been

demonstrated in a variety of cells (Borle, 1981), including pancreatic acinar cells (Schulz, 1980). Measurements of Ca_i^{2+} dependency of the exchanger and a stoichiometry of 3 Na^+/1 Ca^{2+} (Pitts, 1979; DiPolo & Beauge, 1983; Reeves & Hale, 1984) suggests that the exchanger can actively extrude Ca^{2+} from the cells when $[Ca^{2+}]_i$ is high. Assuming an intracellular Na^+ concentration of approximately 15 mM and a membrane potential of approximately 60 mV (Petersen, 1987), the thermodynamic driving force for Na^+/Ca^{2+} exchange can be calculated (Eisner & Lederer, 1985). Thus, the effect of the Na^+/Ca^{2+} exchanger on $[Ca^{2+}]_i$ under different conditions can be predicted and compared to the experimental findings. Under resting $[Ca^{2+}]_i$ of 124 nM, the driving force is such that the exchanger should exchange Na_i^+ for Ca_{out}^{2+} and increase $[Ca^{2+}]_i$. Under stimulated conditions, when $[Ca^{2+}]_i$ is approximately 1 μM, the exchanger should be able to exchange Ca_i^{2+} for Na_{out}^+ and reduce $[Ca^{2+}]_i$.

The contribution of the exchanger to Ca^{2+} efflux from the cells was estimated by eliminating the Na^+ gradients and reducing the membrane potential across the plasma membrane. Incubation of the cells in K^+-free medium containing 1 mM ouabain increased $[Ca^{2+}]_i$ from 136 ± 17 to only 206 ± 34 nM. In comparison to our findings, incubation of proximal tubules, a nonexcitable tissue with potent Na^+/Ca^{2+} exchange activity, in K^+-free medium increased $[Ca^{2+}]_i$ from 82 to 237 nM (Lorenzen et al., 1984). Incubation of the tubules with ouabain increased $[Ca^{2+}]_i$ to 835 nM (Windhager et al., 1987). The combination of both treatments which loaded the cells with Na^+ and depolarized the cells, had only a modest effect on $[Ca^{2+}]_i$ in pancreatic acinar cells. These data indicate that other Ca^{2+} efflux mechanisms determine $[Ca^{2+}]_i$ in resting pancreatic acinar cells. Such is likely to be the plasma membrane Ca^{2+} pump.

Depletion or reloading the cells with Na^+ had only a small (14 to 25%) inhibitory effect on the rate constant of Ca^{2+} efflux from stimulated cells. This was the case whether the rate of Ca^{2+} efflux was estimated from $[Ca^{2+}]_i$ or ^{45}Ca efflux measurements. Williams (1980) also reported that removal of medium Na^+ had no effect on hormone-stimulated ^{45}Ca efflux from mouse pancreatic acini. We further demonstrate here that in the absence of Na^+ gradients or medium Na^+, the cells were able to reduce $[Ca^{2+}]_i$ to near resting levels. These observations indicate that also in stimulated cells, Na^+/Ca^{2+} exchange does not contribute to Ca^{2+} efflux. Recently, it was reported that activation of the Na^+/H^+ exchanger by agonists (which increase intracellular Na^+ and reduce intracellular H^+ concentrations) was followed by an increase in $[Ca^{2+}]_i$ (Grinstein & Goetz, 1985; Siffert & Akkerman, 1987). In addition, removal of extracellular Na^+ eliminated the sustained phase of the increase in $[Ca^{2+}]_i$ induced by acetylcholine stimulation of salivary acinar cells (Morris, Fuller & Gallacher, 1987). However, the increase in $[Ca^{2+}]_i$ following the stimulation of the Na^+/H^+ exchanger was secondary to cytosolic alkalinization, rather than the increase in intracellular Na^+ concentration (Grinstein & Goetz, 1985; Siffert & Akkerman, 1987).

The reduced rate of Ca^{2+} efflux under Na^+-free conditions or in cells treated with ouabain can result from partial inhibition of the plasma membrane Ca^{2+} pump rather than inhibition of Na^+/Ca^{2+} exchange. This may be inferred from the finding that Ca^{2+} efflux during the first 3 min of stimulation could be described by a single exponential function under all conditions tested. Hence, it appears that Ca^{2+} efflux is mediated by a single mechanism. Further, the reduced rate of Ca^{2+} efflux in Na^+-free medium was independent of medium Ca^{2+} concentration. Similar inhibition was found with inwardly directed Ca^{2+} gradients of 0.1 and 5 × 10^4. Therefore, our results indicate that under stimulated conditions, net Ca^{2+} efflux from pancreatic acinar cells is mediated by the plasma membrane Ca^{2+} pump and the contribution of the Na^+/Ca^{2+} exchanger is negligible under resting or stimulated conditions.

In summary, the results presented here indicate that $[Ca^{2+}]_i$ and active Ca^{2+} efflux from resting and stimulated pancreatic acinar cells are determined largely by the activity of the plasma membrane Ca^{2+} pump. A Na^+/Ca^{2+} exchange mechanism does not appear to contribution significantly to Ca^{2+} efflux across the plasma membrane of these cells.

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