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Calcium Mobilizing Hormones Activate the Plasma Membrane Ca^{2+} Pump of Pancreatic Acinar Cells

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Summary. ^{45}Ca fluxes and free-cytosolic Ca^{2+}($[Ca^{2+}]_i$) measurements were used to study the effect of Ca^{2+}-mobilizing hormones on plasma membrane Ca^{2+} permeability and the plasma membrane Ca^{2+} pump of pancreatic acinar cells. We showed before (Pandol, S.J., et al., 1987. *J. Biol. Chem.* **262:**16963–16968) that hormone stimulation of pancreatic acinar cells activated a plasma membrane Ca^{2+} entry pathway, which remains activated for as long as the intracellular stores are not loaded with Ca^{2+}. In the present study, we show that activation of this pathway increases the plasma membrane Ca^{2+} permeability by approximately sevenfold. Despite that, the cells reduce $[Ca^{2+}]_i$ back to near resting levels. To compensate for the increased plasma membrane Ca^{2+} permeability, a plasma membrane Ca^{2+} efflux mechanism is also activated by the hormones. This mechanism is likely to be the plasma membrane Ca^{2+} pump. Activation of the plasma membrane Ca^{2+} pump by the hormones is time dependent and 1.5–2 min of cell stimulation are required for maximal Ca^{2+} pump activation. From the effect of protein kinase inhibitors on hormone-mediated activation of the pump and the effect of the phorbol ester 12-0-tetradecanoyl phorbol, 13-acetate (TPA) on plasma membrane Ca^{2+} efflux, it is suggested that stimulation of protein kinase C is required for the hormone-dependent activation of the plasma membrane Ca^{2+} pump.

Key Words hormones · plasma membrane · Ca^{2+} permeability · Ca^{2+} pump

Introduction

Stimulation of pancreatic acinar and other cells with Ca^{2+}-mobilizing hormones results in Ca^{2+} release from an intracellular pool into the cytosol and an increase in $[Ca^{2+}]_i$ [24, 33, 35, 53, 55, 56]. At least part of this Ca^{2+} is then extruded from the cytosol to the extracellular medium. Upon termination of cell stimulation, the internal pool is reloaded with Ca^{2+} to prepare the cells for a second cycle of stimulation [24, 27, 35]. The reloading process is absolutely dependent on the presence of medium Ca^{2+} and involves movement of Ca^{2+} from the medium into the cytosol and then incorporation into the pool.

Recently we demonstrated that Ca^{2+} reloading of the internal pool requires the activity of a La^{3+}-sensitive Ca^{2+} entry pathway, which is located in the plasma membrane of pancreatic acinar cells [35]. Extracellular free-Ca^{2+} measurements in the perfused liver [44] and Ca^{2+}-mediated $^{86}R6$ fluxes and $[Ca^{2+}]_i$ measurements in parotid glands [2, 24] suggest that a similar Ca^{2+} entry pathway is required for Ca^{2+} reloading of the internal pool of other cells.

A hormone-mediated increase in plasma membrane Ca^{2+} permeability of pancreatic acinar cells was previously reported [10, 18, 19, 28, 29, 35, 50]. Ca^{2+} influx through this pathway, however, appears to have only a small effect on $[Ca^{2+}]_i$ since the cells are able to reduce $[Ca^{2+}]_i$ to near resting levels within 3–5 min after stimulation [5, 25, 33, 35, 36]. The following possibilities may explain the rather small rise in $[Ca^{2+}]_i$: (i) the increase in plasma membrane Ca^{2+} permeability due to activation of the La^{3+}-sensitive Ca^{2+} entry pathway is rather small, (ii) alternatively, activation of plasma membrane Ca^{2+} efflux mechanism(s) during cell stimulation may compensate for the increased plasma membrane Ca^{2+} permeability. The present studies address these possibilities.

We show here that hormone stimulation increases the plasma membrane Ca^{2+} permeability of pancreatic acinar cells by about sevenfold. To compensate for the increased Ca^{2+} permeability, the plasma membrane Ca^{2+} pump in these cells is activated. Activation of the plasma membrane Ca^{2+} pump is probably a protein kinase C-mediated event.

Materials and Methods

Materials

HEPES and bovine serum albumin (fraction V) were from Boehringer Mannheim. Soybean trypsin inhibitor, EGTA, carbachol, and atropine were from Sigma. Purified collagenase (type CLSPA) was from Cooper Biomedical. Ionomycin was from Calbiochem. $^{45}CaCl_2$ (4–50 Ci/g Ca) was from New England Nuclear. Fura-2/AM was from Molecular Probes (Junction City, OR). H7 (1-(5-isoquinolinesulfonyl)-2 methylpiperazine dihydrochloride) and HA 1004 (N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride) were from Seikagaku America (St. Petersburg, FL).

The incubation solution (solution A) contained 20 mM HEPES (at pH 7.4), 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM sodium pyruvate, 10 mM ascorbate, 0.1% (wt/vol) bovine serum albumin, and 0.01% (wt/vol) soybean trypsin inhibitor.

Methods

Preparation of Pancreatic Acini

Dispersed pancreatic acini were prepared from rats (75–150 g) by the procedure previously described [4, 26]. In brief, the pancreas was removed and injected with 10 ml of solution A also containing 10 mM $NaHCO_3$. Excess 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 (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. Then the digestion media was removed, replaced with 10 ml of fresh digestion media and the tissue was digested for another 10 min. The acini were then washed twice with solution A by alternate 5-sec. centrifugation at 150 × *g* and resuspension. The acini were suspended in 10 ml solution A, passed through a nylon mesh and washed once more with solution A. The acini were suspended in 5–50 ml solution A and kept at room temperature until used.

Measurement of Free Cytosolic Ca^{2+}

Acini from one pancreas were suspended in 5 ml solution A and were incubated with 2 μM Fura-2/AM for 20 min at 37°C. The cells were then washed twice with 35 ml solution A and resuspended in 2–3 ml solution A. Then 50–100 μl of cell suspension were transferred to 1.9 ml prewarmed media and fluorescence measurements were performed while the cells were continually stirred and maintained at 37°C. Fluorescence was measured with a Perkin–Elmer spectrofluorometer model 650–40 with excitation and emission wavelengths set at 340 and 500 nm, respectively. $[Ca^{2+}]_i$ was calculated as described before using a K_d of 220 nM for the Fura-2 · Ca^{2+} dissociation constant [14]. 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 1 hr incubation at room temperature), samples of cells sufficient for 2–4 experiments were washed prior to $[Ca^{2+}]_i$ measurements.

^{45}Ca Fluxes

Acini from the pancreas of one rat were suspended in 20–30 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 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 1 M NaOH for 10 min and ^{45}Ca was counted using standard liquid scintillation counting.

Results

Effect of Agonists on Plasma Membrane Ca^{2+} Permeability

The plasma membrane permeability for Ca^{2+} of rat pancreatic acinar cells was estimated from measurements of the rate of ^{45}Ca uptake. Figure 1 shows the properties of ^{45}Ca uptake into control, carbachol-stimulated, and carbachol-stimulated, atropine-inhibited (cycled) acini. Extrapolation of $^{45}Ca^{2+}$ uptake to zero time shows that under all conditions there was similar, small rapid ^{45}Ca incorporation. This rapid ^{45}Ca incorporation is not into the hormone-sensitive pool since it could not be mobilized by hormones (*see below*). It probably represents ^{45}Ca trapped in the extracellular space. Subsequent ^{45}Ca uptake into control acini was linear for at least 10 min. ^{45}Ca uptake into carbachol-stimulated and cycled acini was faster during the first 3 min and reached higher levels compared to control acini.

We showed previously [28, 29, 35 and *see below*] that this pattern of ^{45}Ca uptake reflects (i) the slow labeling of the hormone mobilizable intracellular Ca^{2+} pool with ^{45}Ca in control acini, (ii) the reuptake of Ca^{2+} and the labeling of the pool to isotopic equilibrium in cycled acini, and (iii) the partial reloading of the pool with Ca^{2+} and its labeling with ^{45}Ca in stimulated acini. Thus, at the end of reloading, Ca^{2+} content of the internal pool of cycled acini is similar to that in control acini and Ca^{2+} content can be determined from the specific radioactivity of ^{45}Ca in cycled acini. On the other hand, in the continuous presence of agonist, the pool is only partially loaded with Ca^{2+}. Ca^{2+} levels in the pool of stimulated acini can be determined from ^{45}Ca content after 5–10 min of labeling. Since $[Ca^{2+}]_i$ is constant when ^{45}Ca uptake into stimulated acini is initiated (*see* Fig. 4), ^{45}Ca uptake into stimulated acini reflects Ca^{2+}/Ca^{2+} exchange between medium, cytosol and the intracellular pool. ^{45}Ca uptake into cycled acini reflects Ca^{2+}/Ca^{2+} exchange and net Ca^{2+} accumulation by the pool. Hence, to derive the rates of ^{45}Ca uptake under the different condi-

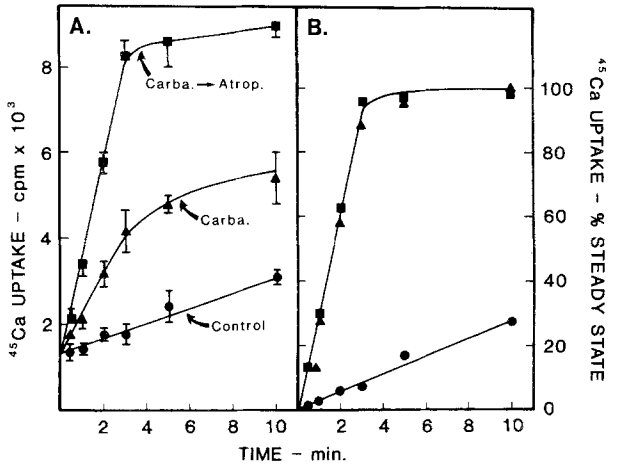

Fig. 1. Effect of carbachol stimulation on plasma membrane $^{45}Ca^{2+}$ influx. Pancreatic acinar cells suspended in solution A containing 2 mN $CaCl_2$ were preincubated with (▲, ■) or without (●) 0.2 mM carbachol for 5 min at 37°C. At $t = 0$ of the illustrated experiments, ^{45}Ca (about 2.10^5 cpm/ml) (●, ▲) and 20 μM atropine (■) were added. At the indicated times, 0.5 ml samples were removed to 10 ml of cold stop solution containing 1 mM $LaCl_3$. The cells were washed and ^{45}Ca content of the cells was analyzed as described in Materials and Methods. (*A*) The results were expressed as cpm in cells. (*B*) To calculate ^{45}Ca uptake as a percentage of steady state, ^{45}Ca content of cycled (■) acini after 10 min incubation at 37°C was taken as the equilibrium level for control and cycled acini. The ^{45}Ca content of carbachol-stimulated acini (▲) after 10 min incubation at 37°C was taken as the equilibrium level for carbachol-stimulated acini. ^{45}Ca level that was determined by extrapolating ^{45}Ca uptake to $t = 0$ was subtracted from ^{45}Ca uptake under all conditions. The figure shows the mean ± SD of 5 experiments

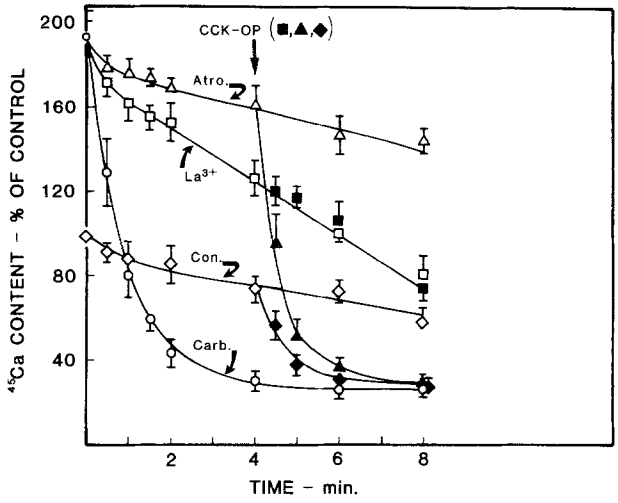

Fig. 2. Effect of carbachol stimulation on plasma membrane ^{45}Ca efflux. Pancreatic acini in solution A containing 2 mM $CaCl_2$ were preincubated with (○, △, □) or without (◇) 0.2 mM carbachol for 5 min at 37°C and then with ^{45}Ca for an additional 10 min as described in Fig. 1 legend. Samples were removed to determine the initial ^{45}Ca content of control (◇) and carbachol-stimulated acini (○). Then the acini were collected by 10-sec centrifugation at 150 *g* and medium containing ^{45}Ca was removed. The acini were suspended in solution A containing 2 mM of unlabeled $CaCl_2$ (◇) and 0.2 mM carbachol (○), 0.2 mM carbachol and 20 μM atropine (△), 0.2 mM carbachol and 50 μM La^{3+} (□). After 4 min of incubation at 37°C, 10^{-8} M CCK-OP were added to acini incubated with carbachol and La^{3+} (■), carbachol and atropine (▲) or control acini (◆). At the indicated times, samples were removed to determine ^{45}Ca content of the cells as described in Materials and Methods. The figure shows the mean ± SD of 3 experiments

tions, it is necessary to express ^{45}Ca uptake as a percentage of the steady-state levels of Ca^{2+} in the pool under the different conditions and to subtract the initial nonspecific ^{45}Ca incorporation. Figure 1*B* shows the results of such expression of the data in Fig. 1*A*. It can be seen that the rates of ^{45}Ca uptake into both carbachol-stimulated and cycled acini is similar and is about 7.14 ± 0.3 times faster than into control acini.

Another protocol that demonstrates the increased plasma membrane Ca^{2+} permeability of stimulated cells is given in Fig. 2. In these experiments, we measured the rate of ^{45}Ca efflux from resting and stimulated cells. Control or carbachol-stimulated acini were labeled with ^{45}Ca for 10 min, as described in Fig. 1. The acini were then collected by centrifugation, medium containing ^{45}Ca was removed and the acini were suspended in medium containing unlabeled Ca^{2+}. It can be seen that ^{45}Ca efflux from control acini was slow compared to ^{45}Ca efflux from carbachol-stimulated acini. In order to demonstrate that most of the ^{45}Ca is stimulated cells was present in the hormone-sensitive pool, the effect of atropine and subsequent stimulation with CCK-OP on ^{45}Ca efflux was tested. Addition of atropine to the carbachol-stimulated acini caused rapid (less than 30 sec) trapping of ^{45}Ca within the cells. That ^{45}Ca was first incorporated and then rapidly trapped in the hormone-sensitive pool is concluded from the ability of CCK-OP to release the ^{45}Ca from the cells (Fig. 2, filled triangles). These results also indicate that during the first 3 min after addition of atropine to stimulated cells, the rate of Ca^{2+} uptake into the pool (Fig. 1, filled squares) is approximately 12-fold faster than the rate of Ca^{2+} efflux from the pool (Fig. 2, open triangles).

The rapid ^{45}Ca efflux from stimulated cells can result from Ca^{2+}/Ca^{2+} exchange across the plasma membrane and then across the internal pool membrane, from net Ca^{2+} efflux from the cell, or both. To distinguish between these possibilities, we tested the effect of La^{3+} on the rate of ^{45}Ca efflux. Figure 2 shows that 50 μM La^{3+} inhibited ^{45}Ca efflux from carbachol-stimulated cells. Addition of CCK-OP had no effect on the rate of ^{45}Ca efflux in the presence of La^{3+}. Thus, La^{3+} inhibits both hor-

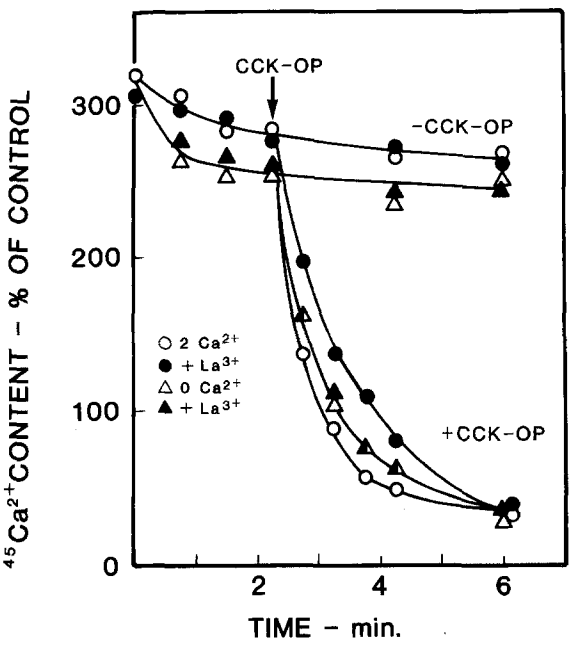

Fig. 3. Effect of La^{3+} and medium Ca^{2+} on net Ca^{2+} efflux. The hormone-sensitive pool of pancreatic acini was labeled with ^{45}Ca by cycling as described in Fig. 1 (carbachol and atropine treatment). Samples were taken to determine initial ^{45}Ca content. The acini were collected by centrifugation and medium containing ^{45}Ca was removed. The acini were suspended in fresh solution A containing 2 mM of unlabeled $CaCl_2$ (○) also containing 50 μM $LaCl_3$ (●) or 2 ml of Ca^2-free solution A (△) also containing 50 μM $LaCl_3$ (▲). After 2 min incubation at 37°C, a portion of acini from all incubation conditions were transferred to tubes containing CCK-OP to give a final concentration of 10^{-8} M. At the indicated times, samples were removed to determine ^{45}Ca content of cells. For clarity the error bars are not shown. This experiment is 1 out of 3 with similar observations.

mone-mediated ^{45}Ca uptake (35) and ^{45}Ca efflux from the cells. To demonstrate that 50 μM La^{3+} had no effect on net, pump-mediated Ca^{2+} efflux, we measured the effect of La^{3+} on CCK-OP-induced ^{45}Ca release. Figure 3 shows that when acini labeled with ^{45}Ca by cycling were incubated in medium containing unlabeled Ca^{2+} or Ca^{2+}-free medium, the rate of ^{45}Ca efflux was slow and similar to that of control acini (Fig. 2). Addition of CCK-OP induced an increase in $[Ca^{2+}]_i$ (*see below*) and rapid release of ^{45}Ca from the cells. Upon addition of CCK-OP, Ca^{2+} efflux occurs when $[Ca^{2+}]_i$ was being reduced (the transient response in Fig. 4) and Ca^{2+} was pumped out of the cells. Measurement of Ca^{2+} content of the cells under these conditions show that there is a net loss of Ca^{2+} from the cells [10, 13, 18, 22, 28, 29].

La^{3+} at 50 μM had no effect of ^{45}Ca efflux from cells suspended in Ca^{2+}-free medium, but slightly decreased the rate of ^{45}Ca efflux from cells incubated in Ca^{2+}-containing medium. Since the CCK-OP-induced ^{45}Ca efflux into Ca^{2+}-free medium represents only the net Ca^{2+} efflux from high $[Ca^{2+}]_i$, the results in Fig. 3 indicate that La^{3+} at 50 μM does not inhibit active Ca^{2+} efflux from the cells. Ca^{2+} efflux into Ca^{2+}-containing medium is likely to be due to net Ca^{2+} efflux and Ca^{2+}/Ca^{2+} exchange. This might explain the small inhibition of Ca^{2+} efflux by La^{3+} under these conditions. Thus, the results in Figs. 2 and 3 suggest that Ca^{2+}/Ca^{2+} exchange can be blocked with 50 μM La^{3+}, while net Ca^{2+} efflux is La^{3+}-insensitive and La^{3+} inhibition can be used to distinguish between the two modes of Ca^{2+} fluxes.

The results presented in Figs. 1–3 show that stimulation of the cells with Ca^{2+}-mobilizing hormones increased the plasma membrane Ca^{2+} permeability by about sevenfold and that this increased permeability is maintained for as long as the cells were stimulated. The concentration of free-cytosolic Ca^{2+} before and 5 min after carbachol stimulation was then measured. Figure 4 shows that resting $[Ca^{2+}]_i$ was 124.7 ± 3.4 nM (n = 89). Stimulation of the cells with carbachol transiently increased $[Ca^{2+}]_i$ to 1044.9 ± 35.4 nM. After 5 min of stimulation, the cells reduced Ca^{2+} to 149.1 ± 5.3 nM. That $[Ca^{2+}]_i$ in stimulated acini was maintained at somewhat higher than resting levels is shown by the ability of atropine to induce a small reduction in $[Ca^{2+}]_i$ back to resting levels. A statistically significant increase in $[Ca^{2+}]_i$ above resting levels could be measured only at medium Ca^{2+} concentrations above 1.5 mM. At lower medium Ca^{2+} concentrations (0.5 mM and below), the stimulated acini reduced $[Ca^{2+}]_i$ to below the prestimulated levels [28, 29, 35]. When the cells were incubated in the presence of atropine and medium Ca^{2+} for 5 min, complete reloading of the internal pool occurred [35] and the ability of CCK-OP to maximally increase $[Ca^{2+}]_i$ was restored.

Effect of Agonists on Active Ca^{2+} Efflux

Despite the sevenfold increase in plasma membrane Ca^{2+} permeability in the continuous presence of agonist, the cells were able to reduce and maintain $[Ca^{2+}]_i$ at near resting levels. The steady-state levels of $[Ca^{2+}]_i$ is determined only by the rates of Ca^{2+} entry and efflux across the plasma membrane. To compensate for the increased plasma membrane Ca^{2+} permeability, it is therefore likely that the rate of active Ca^{2+} efflux is increased during the continuous presence of agonist. To directly test this prediction, we measured the effect of the Ca^{2+} ionophore, ionomycin, on the $[Ca^{2+}]_i$ levels of resting and stimulated cells.

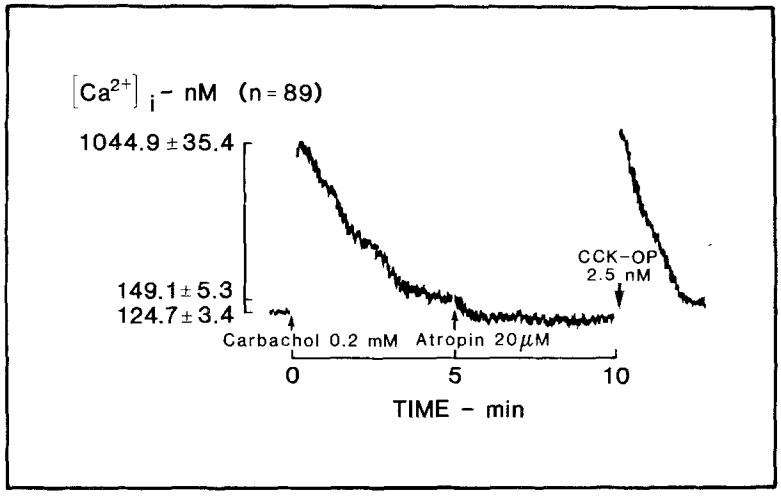

Fig. 4. Effect of agonists and antagonist on $[Ca^{2+}]_i$. Pancreatic acinar cells were loaded with Fura-2 and $[Ca^{2+}]_i$ was determined as described in Materials and Methods. The acini were added to 2 ml of solution A containing 2 mM $CaCl_2$. Where indicated the acini were treated with carbachol, atropine and CCK-OP. To calculate the mean ± SD of $[Ca^{2+}]_i$ at different times, the measured changes in $[Ca^{2+}]_i$ of acini stimulated with 0.1–1 mM carbachol were used (n = 89) since similar responses were obtained with these concentrations of carbachol

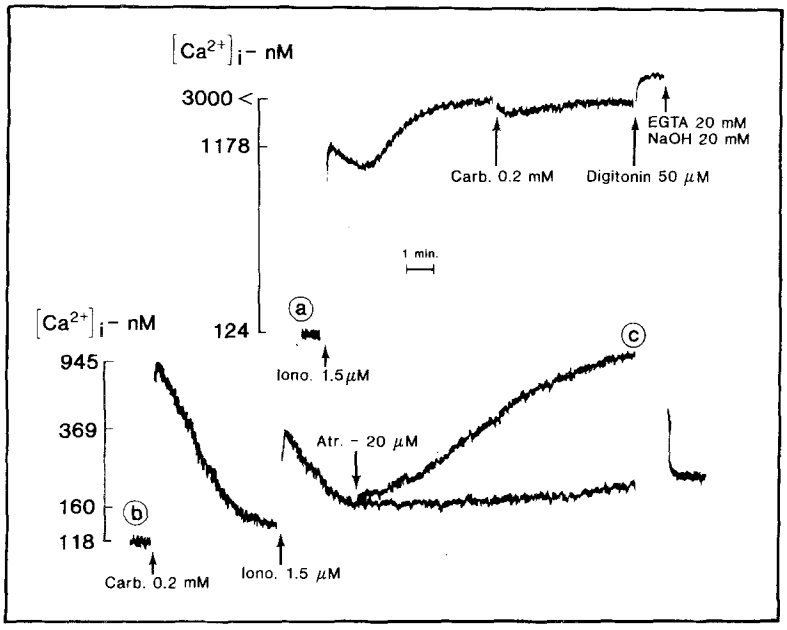

Fig. 5. Effect of ionomycin on $[Ca^{2+}]_i$ of resting and stimulated cells. Fura-2-loaded acini were suspended in albumin-free solution A containing 2 mM $CaCl_2$. Acini were then exposed to 1.5 μM ionomycin, 0.2 mM carbachol, 50 μM digitonin, 20 mM EGTA, and 20 mM NaOH where indicated (*a*). Acini were also stimulated with 0.2 mM carbachol prior to addition of 1.5 μM ionomycin (*b*). Acini treated with carbachol and ionomycin were also exposed to 20 μM atropine where indicated (*c*). The experiments shown in *a* and *b* represent 1 of 31 others and the experiment shown in *c* represents 1 of 8 others

Figure 5*a* shows that addition of 1.5 μM ionomycin to resting cells increased $[Ca^{2+}]_i$ from 124 to approximately 1178 nM. After an initial reduction, $[Ca^{2+}]_i$ slowly increased and stabilized at about 3 μM. Addition of 0.2 mM carbachol at that point resulted in only a small reduction in $[Ca^{2+}]_i$. When the cells were first stimulated with 0.2 mM carbachol, a typical transient increase in $[Ca^{2+}]_i$ was observed. Addition of 1.5 μM ionomycin to acini prestimulated with carbachol for 5 min induced an initial increase in $[Ca^{2+}]_i$ to 369 nM, but then the acini reduced and maintained $[Ca^{2+}]_i$ at 160 nM, (186 ± 14, n = 31) (Fig. 5*b*). Addition of 20 μM atropine to carbachol- and ionomycin-treated cells reversed the ability of the carbachol-stimulated cells to overcome the increased plasma membrane Ca^{2+} permeabilty induced by ionomycin (Fig. 5*c*). After addition of atropine, $[Ca^{2+}]_i$ slowly increased and stabilized at only about 1 μM. Thus, a similar increase in Ca^{2+} permeability as induced by addition of ionomycin increased $[Ca^{2+}]_i$ to about 3 μM in resting cells, but only to 160 nM in stimulated cells. Since ionomycin completely permeabilize the intracellular pool membrane to Ca^{2+}, and in the presence of Ca^{2+} ionophore the intracellular pools do not accumulate Ca^{2+} [28, 29, 39, 47], this experiment suggests that plasma membrane located Ca^{2+} efflux mechanism(s) in the stimulated cells were activated and allowed the cells to better resist the large increase in plasma membrane Ca^{2+} permeability.

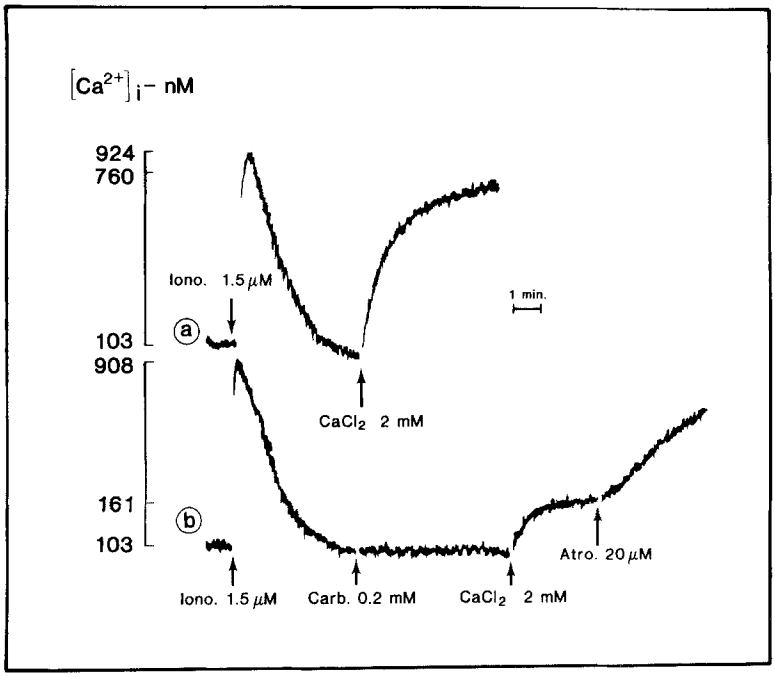

Fig. 6. Effect of ionomycin and added $CaCl_2$ on $[Ca^{2+}]_i$. Fura-2-loaded acini were suspended in albumin-free and Ca^{2+}-free solution A(no EGTA added). The acini were then exposed to 1.5 μM ionomycin and then 2 mM $CaCl_2$ (*a*) or 0.2 mM carbachol, 2 mM $CaCl_2$ and 20 μM atropine (*b*) were added at the indicated times. The experiment shown is 1 out of at least 21 others with similar observations

Figure 5*a* shows that addition of hormone to ionophore-pretreated cells was ineffective in reducing $[Ca^{2+}]_i$. In addition, when antagonist was added to hormone- and ionomycin-treated cells, $[Ca^{2+}]_i$ increased only to 1 rather than 3 μM (Fig. 5*b*,*c*). This suggested that very high $[Ca^{2+}]_i$ concentrations interfere with the ability of the hormone to activate Ca^{2+} efflux. Figure 6 provides evidence to support this possibility. When cells suspended in Ca^{2+}-free medium were exposed to 1.5 μM ionomycin, $[Ca^{2+}]_i$ increased to about 924 nM but then $[Ca^{2+}]_i$ was reduced to resting levels within 2.5 min (Fig. 6*a*). Hence, the initial and rapid ionomycin-induced increase in $[Ca^{2+}]_i$ represented Ca^{2+} release from intracellular stores. Addition of 2 mM $CaCl_2$ to the medium resulted in Ca^{2+} influx into the cells, which was slower than the rate of Ca^{2+} release from intracellular stores by ionomycin. Under the experimental conditions of Fig. 6*a*, the ionomycin-induced Ca^{2+} influx increased $[Ca^{2+}]_i$ only to about 760 nM (744 ± 38 nM, n = 29). Figure 6*b* shows that when ionophore-treated cells in Ca^{2+}-free medium were exposed to carbachol for 5 min they were able to maintain $[Ca^{2+}]_i$ at about 161 nM (163 ± 23, n = 21) subsequent to the addition of 2 mM $CaCl_2$. In addition, atropine reversed the effect of carbachol and induced an increase in $[Ca^{2+}]_i$.

The protocol of Fig. 6 was used to study the dependence of activation of Ca^{2+} efflux on carbachol concentration and the incubation time. Figure 7 shows that when acini in Ca^{2+}-free medium were exposed to 1.5 μM ionomycin and then to 20 or 200 μM carbachol for 5 min, addition of 2 mM $CaCl_2$ increased $[Ca^{2+}]_i$ to 171 ± 8 nM (n = 6). Addition of 2mM $CaCl_2$ to cells incubated with 2.5 μM or 0.5 μM carbachol increased $[Ca^{2+}]_i$ to 297 ± 15 nM and 513 ± 20 nM (n = 3), respectively. Thus, the ability of the cells to resist the effect of ionophore was a function of carbachol concentration in a range similar to that found for carbachol-mediated increase in $[Ca^{2+}]_i$ [28, 33, 36]. Figure 8 shows the time course of hormone-mediated activation of Ca^{2+} efflux. Cells in Ca^{2+}-free medium were exposed to 1.5 μM ionomycin, and after 2 min 2.5 nM CCK-OP were added. At different times (0–3 min) after addition of CCK-OP, 2 mM $CaCl_2$ were added to the medium. When $CaCl_2$ and CCK-OP were added together, $[Ca^{2+}]_i$ increased to levels similar to those measured in unstimulated cells (Fig. 8*a*,*b*). Addition of $CaCl_2$ to cells stimulated with CCK-OP for increasing periods of time was followed by a lower increase in $[Ca^{2+}]_i$ (Fig. 8*c*–*g*). Incubation of the cells with CCK-OP for at least 1.5 min (n = 3) was required for maximal activation of Ca^{2+} efflux. Similar results were obtained in 2 experiments with 0.2 mM carbachol as the stimulant.

Figure 3 shows that hormone-mediated net Ca^{2+} efflux from the cells was independent of medium Ca^{2+} concentration. Figure 9 shows the effect of removal of medium Na^+ on the ability of stimulated cells to resist the Ca^{2+} permeability induced by Ca^{2+} ionophore. Cells were suspended in Ca^{2+}-free, tetramethylammonium (TMA) Cl-containing medium. Atropine was added to prevent Ca^{2+} release from intracellular stores by binding of TMA^+ to the muscarinic-cholinergic receptor [26]. Addi-

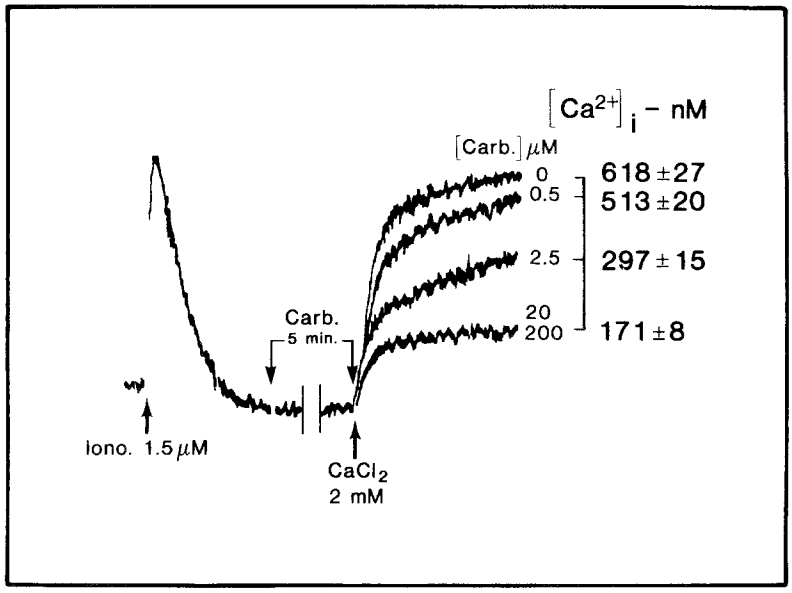

Fig. 7. Effect of different concentrations of carbachol on ionomycin and Ca^{2+}-induced increases in $[Ca^{2+}]_i$. Fura-2-loaded acini in albumin- and Ca^{2+}-free solution A were exposed to 1.5 μM ionomycin. The acini were then incubated for 5 min with 0, 0.5, 2.5, 20 or 200 μM carbachol. At the end of the incubations, 2 mM $CaCl_2$ were added to the preceding conditions and Fura-2 fluorescence was recorded. The illustrated figure shows the effect of ionomycin and 200 μM carbachol on $[Ca^{2+}]_i$ and the results of 5 superimposed experiments with the different carbachol concentrations. The given $[Ca^{2+}]_i$ are the mean ± SD of 3 determinations

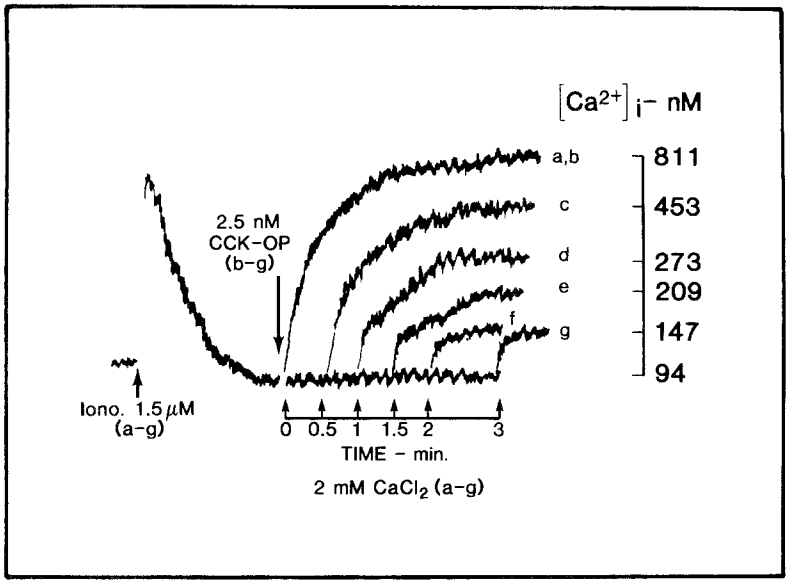

Fig. 8. The time dependency of CCK-OP-mediated activation of Ca^{2+} efflux. Acini loaded with Fura-2 and suspended in albumin-free, Ca^{2+}-free solution A were exposed to 1.5 μM ionomycin. When $[Ca^{2+}]_i$ was reduced to near resting levels, 2 mM $CaCl_2$ alone (*a*) or 2 mM $CaCl_2$ and 2.5 nM CCK-OP (*b*) were added. Acini treated with ionomycin were stimulated with CCK-OP for 0.5 (*c*) 1 (*d*) 1.5 (*e*) 2 (*f*) and 3 (*g*) min prior to addition of 2 mM $CaCl_2$. The effect of ionomycin and 3 min of incubation with CCK-OP on $[Ca^{2+}]_i$ before addition of $CaCl_2$ is shown. The experiment shown represents 1 out of 3 experiments with similar results

tion of 2 mM $CaCl_2$ to cells treated with ionomycin for 5 min increased Ca^{2+} to 958 nM. When ionophore-treated cells were stimulated with CCK-OP for 5 min, addition of 2 mM $CaCl_2$ increased $[Ca^{2+}]_i$ to only 209 nM. This experiment indicates that the hormones probably stimulated the plasma membrane Ca^{2+} pump and not the Na^+/Ca^{2+} exchanger.

Mode of Activation of the Plasma Membrane Ca^{2+} Pump

To study the mode by which the hormones might activate the Ca^{2+} pump in pancreatic acinar cells, the effect of the protein kinase inhibitors H7 and HA1004 [1, 15, 51, 57] on the effect of CCK-OP was measured. Figure 10 shows that incubation of cells with 50 μM H7 for 0–4 min had no effect on the ability of CCK-OP to trigger Ca^{2+} release from internal stores. Addition of 2 μM ionomycin and then 2 mM $CaCl_2$ to CCK-OP stimulated cells increased $[Ca^{2+}]_i$ to 232 nM, while similar additions to control cells increased $[Ca^{2+}]_i$ to approximately 1613 nM. When the cells were preincubated with H7 prior to stimulation with CCK-OP, the cells partially lost their ability to resist the increased permeability induced by ionomycin. The effect of H7 was time dependent so that 3–4 min of incubation with H7 were required to obtain the reversal of the CCK-OP effect. Treatments with higher concentrations of H7

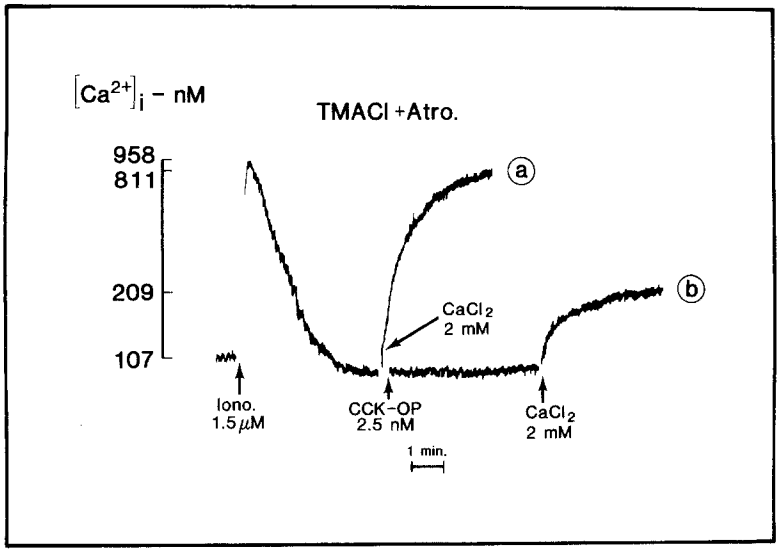

Fig. 9. Effect of cellular and medium Na^+ on Ca^{2+} efflux. Fura-2-loaded acini were washed twice with solution containing (mM): TMACl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 0.5, glucose 10, pyruvic acid 10, atropine 0.02, and HEPES/Tris 20 (at pH 7.4). After 20 min incubation at room temperature, the acini were washed and incubated in Ca^{2+}-free solution prewarmed to 37°C. Where indicated, 1.5 μM ionomycin and then 2 mM $CaCl_2$ (*a*) or 1.5 μM ionomycin, 2.5 nM CCK-OP and 2 mM $CaCl_2$ (*b*) were added to the medium. $[Ca^{2+}]_i$ was determined as described in Materials and Methods. The experiment shown represents 1 out of 2 others

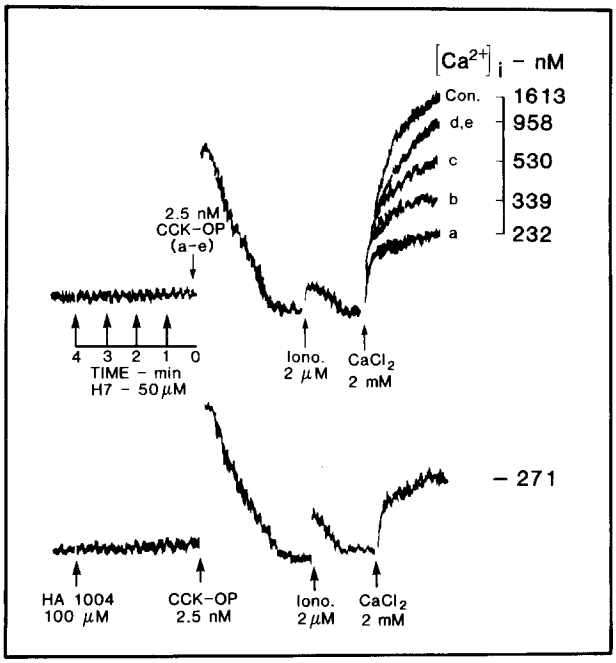

Fig. 10. Effect of H7 and HA 1004 on CCK-OP-mediated activation of the plasma membrane Ca^{2+} pump. Acini loaded with Fura-2 were suspended in albumin-free, Ca^{2+}-free solution A. The acini were incubated with 50 μM H7 for 7 min (no CCK-OP stimulation) and then exposed to 2 μM ionomycin and 2 mM $CaCl_2$ where indicated (control in upper tracings). Acini were also preincubated with 50 μM H7 for 0 (*a*), 1 (*b*), 2 (*c*), 3 (*d*), and 4 (*e*) min prior to stimulation with 2.5 nM CCK-OP. The stimulated acini were then exposed to 2 μM ionomycin and 2 mM $CaCl_2$ where indicated. The effect of 4 min of incubation with H7, CCK-OP and ionomycin on $[Ca^{2+}]_i$ prior to $CaCl_2$ addition is shown. Acini were preincubated with 100 μM HA 1004 for 4 min prior to stimulation with CCK-OP and addition of ionomycin and $CaCl_2$ (bottom tracing). The experiments shown in the upper tracings are 1 out of 3 similar experiments and the experiment in the bottom tracing is 1 out of 5 similar observations

and for longer periods of time did not further increase the effect of H7 (*not shown*). Thus, pretreatment with H7 only partially reversed the effect of CCK-OP. Figure 10 (lower tracing) shows the specificity of the effect of H7. Thus, preincubation of the cells with 100 μM of HA 1004 had no effect on the ability of CCK-OP-stimulated cells to resist the increased Ca^{2+} permeability induced by ionomycin.

Figure 11 shows the effect of H7 when added prior to or after cell stimulation on Ca^{2+} pump activation by CCK-OP. When the cells were incubated with H7 for 4 min prior to stimulation with CCK-OP, addition of ionomycin and 2 mM $CaCl_2$ increased $[Ca^{2+}]_i$ to about 723 nM. However, when the cells were first stimulated with CCK-OP and then exposed to 50 μM H7 for 4 min, addition of ionomycin and $CaCl_2$ increased $[Ca^{2+}]_i$ to only 258 nM. Longer incubation with H7 (up to 12 min), similarly could not reverse the effect of CCK-OP prestimulation (*not shown*).

H7 is an inhibitor of protein kinase C-, cAMP-, and cGMP-dependent protein kinases [15]. HA 1004 is a potent inhibitor of cAMP- and cGMP-dependent protein kinases but a poor inhibitor of protein kinase C [1]. Therefore, the results in Figs. 10 and 11 suggest that stimulation of the Ca^{2+} pump by hormones is mediated by protein kinase C. To further test this possibility, we measured the effects of protein kinase C stimulation on the ability of the cells to resist the effect of ionomycin. Figure 12 shows that incubation of the cells for 5 min with 4α phorbol had no effect on the level of $[Ca^{2+}]_i$ induced by addition of ionomycin and $CaCl_2$. On the other hand, when the cells were incubated with 0.2 μM TPA for 5 min, addition of ionomycin and $CaCl_2$ increased $[Ca^{2+}]_i$ to only 423 nM. Finally, H7 but

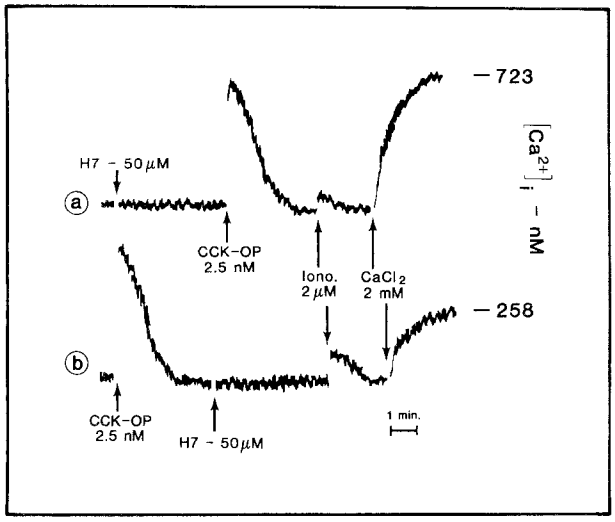

Fig. 11. Effect of sequential addition of H7 and CCK-OP on CCK-OP-mediated stimulation of the Ca^{2+} pump. Acini suspended in albumin-free, Ca^{2+}-free solution A were incubated for 4 min at 37°C with 50 μM H7 prior to (*a*) or after (*b*) stimulation of the cells with 2.5 nM CCK-OP. Then, where indicated, 2 μM ionomycin and 2 mM $CaCl_2$ were added to the medium. The experiment shown in (*a*) is 1 out of 12 others and the experiment shown in (*b*) is 1 out of 3 others with similar observations

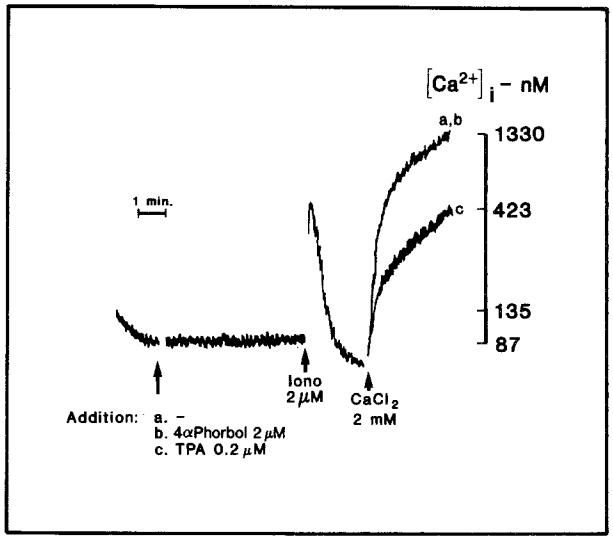

Fig. 12. Effect of phorbol esters on ionomycin induced-increase in $[Ca^{2+}]_i$. Fura-2-loaded acini were suspended in albumin-free, Ca^{2+}-free solution A. Then acini were incubated for 5 min at 37°C with no further additions (*a*-control) with 2 μM 4 phorbol (*b*), or 0.2 μM TPA (*c*). Then the acini were exposed to 2 μM ionomycin and 2 mM $CaCl_2$ as indicated. The experiment with 4α phorbol is 1 out of 3 and with TPA is 1 out of 16 similar experiments

not HA1004 completely blocked the effects of TPA (*not shown*).

Discussion

A plasma membrane Ca^{2+} entry pathway is activated when cells are stimulated with Ca^{2+}-mobilizing hormones [12, 20, 23, 24, 33, 35, 38, 43, 48, 53, 55, 56]. This La^{3+}-sensitive Ca^{2+} entry pathway remains activated as long as the internal Ca^{2+} pool is not completely reloaded with Ca^{2+} [2, 24, 35, 44]. In the present studies, ^{45}Ca influx and efflux measurements were used to quantitate the hormone-mediated increase in plasma membrane Ca^{2+} permeability of rat pancreatic acinar cells. The increased Ca^{2+} permeability can be demonstrated under Ca^{2+}/Ca^{2+} exchange (carbachol stimulation, Figs. 1 and 2) and net Ca^{2+} uptake (cycled, Fig. 1) conditions. The increased Ca^{2+} permeability was similar under the two measurement conditions, indicating that the rate of pool reloading with Ca^{2+} is determined by the rate of Ca^{2+} influx across the plasma membrane. Both $^{45}Ca^{2+}$ influx [35] and ^{45}Ca efflux (Fig. 2), but not net Ca^{2+} pump-mediated Ca^{2+} efflux (Fig. 3) could be completely blocked by 50 μM La^{3+}. Thus the La^{3+}-sensitive ^{45}Ca fluxes represent Ca^{2+} movements through a plasma membrane Ca^{2+} entry pathway. The ^{45}Ca flux measurements indicate that hormonal stimulation increases the plasma membrane permeability of pancreatic acinar cells by approximately sevenfold. Similar increases in plasma membrane Ca^{2+} permeability were reported when hepatocytes were stimulated with Ca^{2+}-mobilizing hormones [23, 43].

Termination of cholinergic stimulation with atropine resulted in reloading of the hormone-sensitive pool with Ca^{2+}. Comparing the rates of Ca^{2+} influx into (Fig. 1, filled squares) and efflux (Fig. 2, open triangles) out of the pool during reloading reveals that the rate of Ca^{2+} influx is approximately 12-fold faster than the rate of Ca^{2+} efflux. Hence, after addition of atropine, net Ca^{2+} influx into the pool continues for 3 min while Ca^{2+} efflux from the pool is reduced to control levels within less than 30 sec. These findings have implications in terms of the localization of the plasma membrane Ca^{2+} entry pathway through which Ca^{2+} reloading occurs. Reloading of internal stores with Ca^{2+} at the termination of cell stimulation takes place at or below resting levels of $[Ca^{2+}]_i$ ([24, 27, 28, 35] and Fig. 4). To explain these observations, a model describing the activities of the different Ca^{2+}-transporting pathways in the plasma and internal pool membrane of the resting and stimulated cells has been proposed [24, 41, 42]. An important feature of this model is that the plasma membrane Ca^{2+} entry pathway is located in specific regions where anatomic association between the plasma and the pool membrane exists. This organization provides direct access of

the pool interior to extracellular Ca^{2+}. When the cells are stimulated, Ca^{2+} is released into the cytosol and the internal pool is depleted of Ca^{2+}. Depletion of the pool from Ca^{2+} results in activation of the reloading pathway and allows Ca^{2+} reloading by Ca^{2+} influx from the medium directly into the pool without Ca^{2+} first entering the cytosol and then pumped into the pool [24, 41, 42]. Such a mechanism requires that under the condition of the experiment in Fig. 2, the ^{45}Ca should not be rapidly trapped in the pool after addition of atropine. Rather, the direct connection between the intracellular Ca^{2+} pool and the medium should have resulted in a rapid ^{45}Ca efflux due to Ca^{2+}/Ca^{2+} exchange between medium and pool Ca^{2+}. Thus, the dissociation between the rates of Ca^{2+} influx and efflux from the pool during the reloading period argue against a direct connection between the pool interior and the mechanism. It is likely that for reloading, Ca^{2+} enters across the plasma membrane into the cytosol and is only then incorporated into the hormone-sensitive pool by the pool Ca^{2+} pump.

Despite the maintained sevenfold increased plasma membrane Ca^{2+} permeability, the stimulated cells were able to reduce and maintain $[Ca^{2+}]_i$ at near resting levels within 3–5 min of stimulation. $[Ca^{2+}]_i$ at steady state is determined by the pump-leak turnover rates across the plasma membrane. Since $[Ca^{2+}]_i$ of resting and 5-min stimulated cells were similar and cell stimulation increased the leak rate by a factor of about 7, the overall pumping rate must have been increased by a similar factor in stimulated cells. The stimulation of Ca^{2+} efflux by the hormones is reflected in the effect of ionomycin on the $[Ca^{2+}]_i$ levels of resting and stimulated cells. Identical increase in plasma membrane Ca^{2+} permeability induced by ionomycin increased $[Ca^{2+}]_i$ to about 800 nM in resting cells and to only about 160 nM in stimulated cells. Hence, due to the increased rate of Ca^{2+} efflux, the stimulated cells were able to reduce and maintain $[Ca^{2+}]_i$ at lower levels compared to resting cells. A similar approach of measuring the effect of Ca^{2+} ionophore on $[Ca^{2+}]_i$ was used to show that stimulation of neutrophils [45] and platelets [39, 47] with Ca^{2+}-mobilizing agonists results in stimulation of plasma membrane Ca^{2+} efflux mechanisms. In addition, stimulation of other cells with Ca^{2+}-mobilizing hormones increases the plasma membrane Ca^{2+} permeability [12, 20, 23, 38, 43, 48] and stimulated cells reduce $[Ca^{2+}]_i$ to near resting levels after about 5 min of stimulation [27, 40, 49]. Therefore, it is likely that activation of the plasma membrane Ca^{2+} pump by Ca^{2+}-mobilizing hormones also occurs in other cell types.

Two active Ca^{2+} efflux mechanisms have been described in pancreatic acinar [16] and other cell types [7]—a Na^+/Ca^{2+} exchange mechanism and a plasma membrane Ca^{2+} pump. It is likely that stimulation of pancreatic acinar cells increased the activity of the plasma membrane Ca^{2+} pump and not that of the Na^+/Ca^{2+} exchanger. This is concluded from the findings that in the presence of La^{3+}, net Ca^{2+} efflux from the stimulated cells was similar in the presence and absence of medium Ca^{2+} (Fig. 3). Further, removal of cellular and medium Na^+ did not prevent the CCK-OP-mediated stimulation of Ca^{2+} efflux and the ability of the cells to resist the ionomycin-dependent increase in Ca^{2+} permeability (Fig. 9).

Stimulation of the plasma membrane Ca^{2+} pump did not appear to require the initial, hormone-mediated increase in $[Ca^{2+}]_i$. When intracellular stores were depleted from Ca^{2+} by the treatment of the cells with ionomycin in Ca^{2+}-free medium, $[Ca^{2+}]_i$ was reduced to below resting levels and addition of the hormones had no further effect on $[Ca^{2+}]_i$. However, after 5 min of exposure to the hormones under low $[Ca^{2+}]_i$ conditions, the plasma membrane Ca^{2+} pump was activated as is reflected by the ability of the cells to maintain relatively low $[Ca^{2+}]_i$ in the presence of medium Ca^{2+} and ionomycin. On the other hand, in the presence of very high $[Ca^{2+}]_i$, pump activity and pump stimulation were inhibited. Addition of ionophore to cells suspended in Ca^{2+}-containing medium increased $[Ca^{2+}]_i$ to about 3 μM (Fig. 5) while addition of the same concentration of $CaCl_2$ to cells treated with ionomycin increased $[Ca^{2+}]_i$ to about 800 nM (Fig. 6). Once $[Ca^{2+}]_i$ was increased to about 3 μM, subsequent stimulation of the cells with carbachol was not followed by reduction of $[Ca^{2+}]_i$ to near resting levels. These observations indicate that activation of the plasma membrane Ca^{2+} pump does not require an increase in $[Ca^{2+}]_i$ to above resting levels and very high $[Ca^{2+}]_i$ inhibits the Ca^{2+} pump of both resting and stimulated cells.

Plasma membrane Ca^{2+} pump activation upon stimulation and inactivation upon termination of cell stimulation are relatively slow processes. Incubation of the cells with the hormones for 1.5–2 min was required for maximal pump stimulation. Shorter incubation periods resulted in partial pump activation.

When carbachol stimulation of pancreatic acinar cells was terminated with atropine, about 7–10 min of incubation were required before $[Ca^{2+}]_i$ was increased to the levels expected from the effect of ionomycin on $[Ca^{2+}]_i$ in unstimulated cells. A possible reason for the slow transition from activated to inactivated Ca^{2+} pump might be the continued increase in plasma membrane Ca^{2+} permeability. Despite the removal of agonist, the plasma membrane

Ca^{2+} entry pathway remained activated until complete reloading of the internal pool with Ca^{2+} occurred [2, 24, 35, 44]. For reloading, net Ca^{2+} uptake into the cytosol and then into the pool occurs. The increased plasma membrane Ca^{2+} pumping activity can prevent large increases in $[Ca^{2+}]_i$ during the reloading period.

Activation of plasma membrane Ca^{2+} pumps by protein kinase-mediated processes were previously reported. Phosphorylation of heart sarcolemmal [8] and red blood cell [30] plasma membrane Ca^{2+} pump by a cAMP-dependent protein kinase activated this pump. Stimulation of smooth muscle cells with 8-Bromo-cGMP resulted in activation of the plasma membrane Ca^{2+} pump [17]. Stimulation of protein kinase C with phorbol esters increased the rate of Ca^{2+} efflux from neutrophils [45], GH_3 cells [11] and platelets [39, 47], and the rate of ATP-dependent Ca^{2+} uptake into plasma membrane vesicles of neutrophils [21]. Finally, protein kinase C-mediated phosphorylation of the purified plasma membrane Ca^{2+} pump of the red blood cell resulted in activation of $(Ca^{2+} + Mg^{2+})$ ATPase activity and Ca^{2+} uptake into inside out vesicles [52]. Stimulation of pancreatic acinar cells with Ca^{2+}-mobilizing hormones was shown to increase the turnover of phosphatidylinositols [9, 37, 54]. The increased phosphatidylinositol turnover is followed by an increased level of diacylglycerol [34], which activates protein kinase C [31]. We, therefore, tested the effect of direct stimulation of protein kinase C on Ca^{2+} pump activity in pancreatic acinar cells.

It appears that in these cells, the hormone-induced activation of the plasma membrane Ca^{2+} pump is mediated by activation of protein kinase C. This is concluded from the following. Preincubation of the cells with the protein kinase inhibitor H7 reduced the ability of stimulated cells to resist the increased Ca^{2+} permeability. The protein kinase inhibitor HA 1004 was without any effect. H7 at 50 μM inhibits all protein kinases including protein kinase C, while HA 1004 at 100 μM inhibits all protein kinases, but with little inhibition of protein kinase C [1, 15]. In addition, preincubation of the acini with the phorbol ester TPA had a similar effect to that found with the hormones, while the nonactive phorbol ester, 4α phorbol, was without any effect. The effect of TPA could be inhibited with H7 but not with HA 1004. When taken together, these findings suggest that stimulation of protein kinase C was involved in the hormone-mediated activation of the plasma membrane Ca^{2+} pump.

Pretreatment with a TPA concentration sufficient to maximally stimulate enzyme secretion and activate protein kinase C [6, 32] was not as effective as the hormones in activating the plasma membrane Ca^{2+} pump. In addition, preincubation of the cells with H7 only partially prevented the hormone-mediated activation of the Ca^{2+} pump. The partial effects of the drugs used can result from their incomplete activation or inhibition of protein kinase C. However, it is also possible that hormone-mediated activation of the plasma membrane Ca^{2+} pump requires the activation of protein kinase C, but activation of protein kinase C alone is not sufficient to induce the overall effect of the hormones on the plasma membrane Ca^{2+} pump.

The results provided here for pancreatic acinar cells and that reported for GH_3 cells [11], neutrophils [45] and platelets [39, 47] suggest that Ca^{2+}-mobilizing hormones activate the plasma membrane Ca^{2+} pump. This is in contrast with previous reports showing that Ca^{2+}-mobilizing hormones had no effect on the rate of ATP-dependent Ca^{2+} uptake into basolateral membrane vesicles isolated from pancreatic acinar cells [3, 46]. This may be due to the preparation used to study Ca^{2+} pump activation, i.e., intact cells *vs.* isolated basolateral membrane vesicles. Ca^{2+} pump activation appear to be the result of a protein kinase C-mediated phosphorylation process in the different cell types tested. It is, therefore, possible that Ca^{2+} pump activation was lost during the isolation of basolateral membrane vesicles. Further studies, possibly isolation of vesicles in the presence of phosphatase inhibitors, will be required to demonstrate the effect of Ca^{2+}-mobilizing hormones on ATP-dependent Ca^{2+} uptake into basolateral membrane vesicles.

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