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Ca^{++} Fluxes in Isolated Cells of Rat Pancreas. Effect of Secretagogues and Different Ca^{++} Concentrations

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Summary. Secretagogues of pancreatic enzyme secretion, the hormones pancreozymin, carbamylcholine, gastrin I, the octapeptide of pancreozymin, and caerulein as well as the Ca^{++}-ionophore A 23187 stimulate ^{45}Ca efflux from isolated pancreatic cells. The non-secretagogic hormones adrenaline, isoproterenol, secretin, as well as dibutyryl cyclic adenosine 3′,5′-monophosphate and dibutyryl cyclic guanosine 3′,5′-monophosphate have no effect on ^{45}Ca efflux. Atropine blocks the stimulatory effect of carbamylcholine on ^{45}Ca efflux completely, but not that of pancreozymin. A graphical analysis of the Ca^{++} efflux curves reveals at least three phases: a first phase, probably derived from Ca^{++} bound to the plasma membrane; a second phase, possibly representing Ca^{++} efflux from cytosol of the cells; and a third phase, probably from mitochondria or other cellular particles. The Ca^{++} efflux of all phases is stimulated by pancreozymin and carbamylcholine. Ca^{++} efflux is not significantly effected by the presence or absence of Ca^{++} in the incubation medium. Metabolic inhibitors of ATP production, Antimycin A and dinitrophenol, which inhibit Ca^{++} uptake into mitochondria, stimulate Ca^{++} efflux from the isolated cells remarkably, but inhibit the slow phase of Ca^{++} influx, indicating the role of mitochondria as an intracellular Ca^{++} compartment. Measurements of the $^{45}Ca^{++}$ influx at different Ca^{++} concentrations in the medium reveal saturation type kinetics, which are compatible with a carrier or channel model. The hormones mentioned above stimulate the rate of Ca^{++} translocation.

The data suggest that secretagogues of pancreatic enzyme secretion act by increasing the rate of Ca^{++} transport most likely at the level of the cell membrane and that Ca^{++} exchange diffusion does not contribute to the $^{45}Ca^{++}$ fluxes.

In a previous paper [9] we have shown that secretagogues of pancreatic enzyme secretion stimulate $^{45}Ca^{++}$ influx into isolated pancreas cells. The results were compatible with the interpretation that in stimulation of pancreatic enzyme secretion an increase in the Ca^{++} permeability of the cell membrane is involved. If this assumption is correct, the secretagogues should increase ^{45}Ca efflux of preloaded cells as well as ^{45}Ca influx. Data of $^{45}Ca^{++}$ efflux should, therefore, compare with those of our previous $^{45}Ca^{++}$ influx study [9] concerning rate constants and compartment

* With the technical assistance of C. Hornung.

size. However, previous Ca^{++}-influx measurements did not distinguish between the modes of ^{45}Ca uptake – simple diffusion, interaction with membrane components, or even tracer exchange. By studying the effect of different extracellular calcium concentrations on the kinetic parameters of $^{45}Ca^{++}$ influx and efflux, we hoped to characterize the mechanism of the Ca^{++} transport. Since other authors gave a restricted interpretation of a secretagogue-induced increased Ca^{++} efflux from cells by a liberation of Ca^{++} from mitochondria [6] and disregarded the possibility of increased Ca^{++} permeability of the outer cell membrane, we mimicked the effect of Ca^{++} liberation from the mitochondria by applying Antimycin A and dinitrophenol. This procedure, as expected, increased the ^{45}Ca efflux from the cells but diminished the ^{45}Ca influx. Our results indicate a carrier- or channel-mediated translocation of Ca^{++} through the outer cell membrane which is stimulated by pancreozymin and acetylcholine and saturated at higher Ca^{++} concentration in the medium.

Materials and Methods

Materials

Reagents were obtained from the following sources: crude collagenase (152 U/mg, Worthington Biochemical Corp., Freehold, N. Y., USA, or 230 U/mg, Sigma, St. Louis, Mo., USA); salt-free sheep-testes hyaluronidase (1,000 U/mg, Boehringer, Mannheim, Germany); chromatographically purified soybean trypsin inhibitor (Serva, Heidelberg, Germany); essentially fatty acid-free bovine-plasma-albumin (Sigma, St. Louis, Mo., USA); 2,5-dinitrophenol, choline chloride and carbamylcholine × HCl (Merck, Darmstadt, Germany); pancreozymin (GIH Research Unit, Karolinska Institute, Stockholm, Sweden); gastrin I (Imperial Chemical Industries Ltd., Cheshire, England); caerulein (Farmaceutici Italia, Milano, Italy); Antimycin A, L-adrenalin and DL-isoproterenol × HCl (Serva, Heidelberg, Germany); dibutyryl cyclic adenosine-monophosphate and dibutyryl cyclic guanosine monophosphate (Boehringer, Mannheim, Germany). $^{45}CaCl_2$ (1 mCi/ml distilled water) was purchased from Amersham Buchler GmbH, Braunschweig, Germany. The octapeptide of pancreozymin was a gift from Dr. Miguel A. Ondetti (The Squibb Institute for Medical Research, Princeton, N. J., USA). Synthetic secretin was received from Professor E. Wünsch (Max-Planck-Institut für Biochemie, Martinsried bei München), and the compound A 23187 was a gift from The Lilly Research Laboratories, Indianapolis, Indiana, USA). Bovine plasma albumin for the incubation medium was dialysed against 0.9 % NaCl solution before use. Unfasted young rats of either sex were used weighing 150 to 210 gm.

Cell Isolation

The cells were isolated according to the method of Amsterdam and Jamieson [1] with a small modification as described in a previous publication [9]. The procedure involves (a) first digestion with collagenase and hyaluronidase for 15 min; (b) removal of divalent cations

with 2 mM EDTA; (c) second digestion with collagenase and hyaluronidase for 40 to 45 min; and (d) final dissociation and harvesting of the cells by sequential passage of the cells through pipettes and centrifugation of the cells through a column of 4% bovine plasma albumin.

Ca^{++} Efflux Experiments

Preloading of ^{45}Ca. 0.5 ml of the cell suspension (20 to 30 mg protein which corresponds to $5.1–7.7 \times 10^7$ cells) was put into a 50 ml Erlenmeyer flask which contained 10 ml of incubation media composed of Krebs-Ringer-bicarbonate solution with 1.25 mM Ca^{++}, 1.2 mM Mg^{++}, 10 mM glucose, 1 mg/ml bovine plasma albumin, and 0.1 mg/ml of trypsin inhibitor. All through the procedure, the incubation media were equilibrated with 95% O_2 and 5% CO_2 at 37 °C to ensure optimal oxygenation and agitated at 80 oscillations/min. The cells were preincubated for 30 min in the medium with or without hormones to assure steady-state conditions. After 30 min of preincubation, 5 μCi of ^{45}Ca were added to the medium and the cells were further incubated for 90 min. Hormones were already present during these 120 min of preincubation. Longer preincubation periods were not possible since the cells collapsed after having been kept for 3–4 hr *in vitro*.

Efflux Experiment. After incubation the cell suspensions were divided into equal amounts and transferred to 20 ml conical glass centrifuge tubes and centrifuged at $1,000 \times g$ for 15 sec at room temperature. The cell pellets were washed twice with efflux media (Krebs-Ringer bicarbonate with or without calcium and with or without test substances), equilibrated with 95% O_2 and 5% CO_2 at 37 °C, and finally suspended in 2 ml efflux media, incubated at 37 °C with 95% O_2 and 5% CO_2, and agitated at 80 oscillations/min to prevent cells from being sedimented.

The tubes were centrifuged at $1,000 \times g$ for 15 sec at room temperature, and the supernatant was poured into a vial for counting the radioactivity. Then the pellet was resuspended in 2 ml of fresh efflux medium, and the whole procedure was repeated at different time courses of incubation ranging from 1 to 120 min. After the efflux experiments the cell pellets were suspended in 2 ml of distilled water, sonicated, aliquots were taken out for protein determination [11], and the rest was submitted for radioactivity count with 10 ml of Bray's solution in a tri-carb Packard scintillation spectrometer model 330. The total radioactivity of the supernatant of each collection plus the final pellet homogenate was expressed as 100% radioactivity present in the cell at the beginning of the efflux experiment.

Ca^{++} Influx Experiment

Preincubation and incubation was the same as described above for Ca^{++} efflux experiments. Cells were preincubated at different Ca^{++} concentrations for 30 min with or without test substances and at 0 time ^{45}Ca was added to the incubation medium. At different periods, an aliquot (0.5 ml) of the cell suspension was removed and transferred into a 25 ml conical glass centrifuge tube containing 20 ml of ice-cold isotonic choline chloride solution, and each tube was immediately centrifuged for 45 sec at $1,000 \times g$; the supernatant solution was rapidly decanted, and the tubes were inverted for further drainage. The cell pellets were suspended in 1 ml of distilled water and sonicated. An aliquot of the sonicated homogenate was removed for protein determination and the rest submitted for radioactivity count, as was already described in a preceding paper [9].

Calculations

A mathematical model of the three-compartment closed system and the calculations of the different kinetic parameters have been published by Borle [3]; our application of this

analytical procedure to the Ca^{++} uptake experiments involving preincubation of the cells with test substances was described in a preceding report [9]. In the case of efflux, the same method of calculation as in influx was applied. Three compartments are considered to be parallel, represented schematically as follows:

$$S_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftarrows}} S_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftarrows}} S_3$$

cell membrane medium cell

S_i = amount of Ca^{++} in compartment i.

Assuming the specific activity of ^{45}Ca in the medium and all fluxes to be constant at any time in the steady state condition, the following equation can be used to calculate the fluxes from the change of Ca^{++} uptake into membrane (index 1) and cell (index 3):

$$\frac{dR\,1+3}{dt/E} = -\phi_{12} \cdot e^{-k_{12}t} - \phi_{32} e^{-k_{32}t}$$

k_{ij} = rate constants.
E = specific activity of tracer in equilibrium (i.e., at infinite time).
ϕ_{ij} = flux of calcium from compartment i to compartment j.
R_i = amount of tracer in compartment i.

$^{45}Ca^{++}$ efflux curves were drawn by hand. From these curves the Δc of Ca^{++} was read at 1 min intervals. These Ca^{++} efflux values were plotted on semilogarithmic paper and the efflux parameters determined.

The graphical analysis reveals at least three compartments: a first phase (Phase 1), a second phase (Phase 2) and a third phase (Phase 3). The parameters of the compartments (flux = ϕ (nmol/mg protein × min) and half-time = $t_{1/2}$ (min)) were read from the semi-log plot and the apparent rate constant k (min^{-1}) was calculated from the half-time: $t_{1/2} = \frac{\ln 2}{k}$. The compartment size S_i was calculated from the formula $S_i = \frac{\phi_i}{k_i}$.

Results

Analysis of Ca^{++} efflux

The effect of pancreozymin and carbamylcholine on Ca^{++} efflux from the cells is shown in Fig. 1. Both substances augment the efflux of ^{45}Ca from preloaded cells as compared to the control. The curves represent exponential functions, which can be replotted on a semilogarithmic scale (Fig. 2). This reveals at least 3 components of Ca^{++} efflux obtainable by graphical analysis. The steeper slope characterizes phase 1, and two slower phases (2 and 3) are represented by the shallower slopes. From the straight lines, flow rates (intercept on the ordinate) and rate constants are derived, allowing calculation of compartment sizes. The results presented in Table 1 show the values obtained from efflux experiments after preloading the cells with ^{45}Ca in the presence of hormones. The values of phase 1

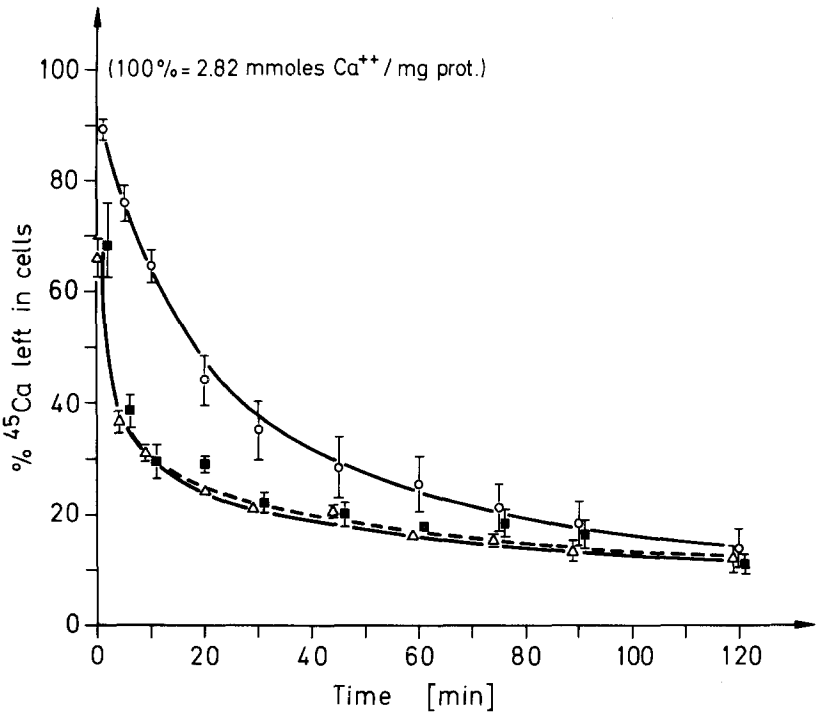


Fig. 1. Effect of pancreozymin and carbamylcholine on ^{45}Ca efflux from isolated pancreatic cells. Cells were preincubated without hormones for 30 min and then ^{45}Ca (10 μCi/10 ml) was added to preload the cells for another 90 min before starting the efflux experiment. o——o, indicates ^{45}Ca efflux in the absence of hormones, ■– – –■ in the presence of pancreozymin (0.86×10^{-7} M), and △——△ in the presence of carbamylcholine (10^{-5} M), respectively. Each point represents the mean ± SEM of 5 to 6 experiments

are not accurate because some ^{45}Ca of phase 1 efflux is lost during washing (which took about 3 min) of the ^{45}Ca-preloaded cell. Therefore, the values of phase 1 are omitted from the calculations of the flux parameters in Table 1. This table shows that the flux rates of phase 2 increase after stimulation with pancreozymin and carbamylcholine, while those of phase 3 decrease. Since the fluxes of phase 3 are small compared to phase 2, only the latter ones have been taken for calculating the ratios. Thus, the flux rate of phase 2 increases after pancreozymin by 1.78 times compared to the control; the corresponding value for carbamylcholine is 1.58.

It became apparent that preincubation with hormones increases the compartment size. The total compartment size is given by the total exchangeable ^{45}Ca, as measured directly by the cumulative efflux over 120 min (Table 2), which averages 4.38 when preincubated with pancreozymin and 4.15 when preincubated with carbamylcholine. Thus, the

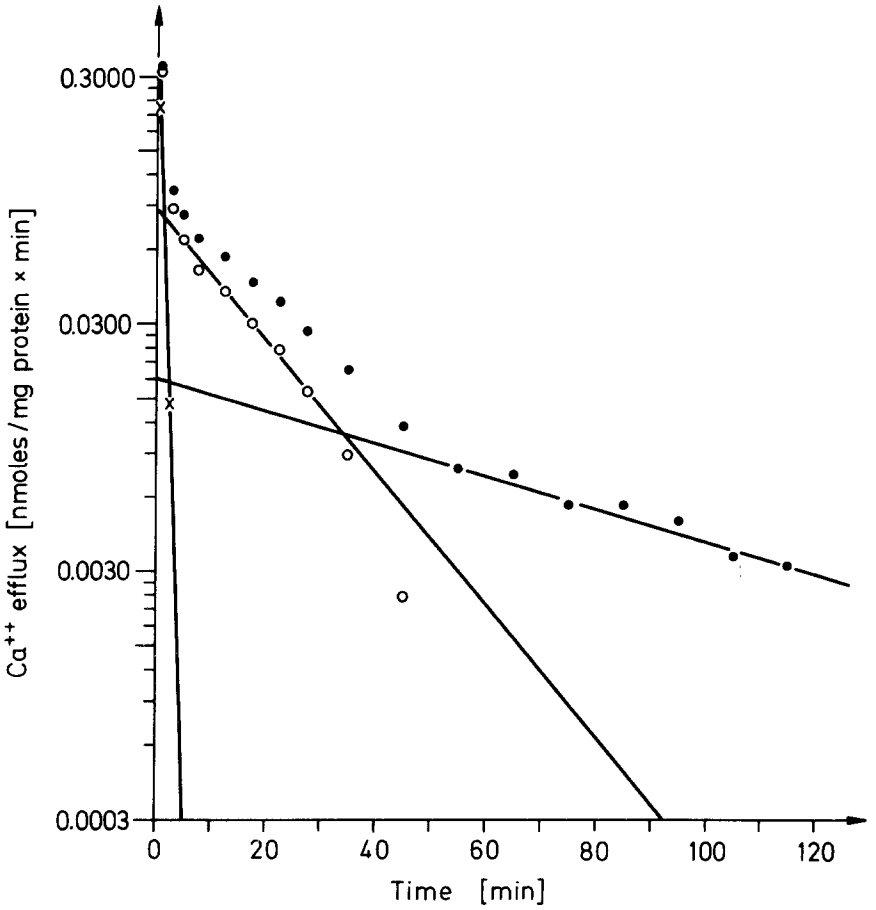


Fig. 2. The points (●) of the upper curve have been obtained by reading the change of % ^{45}Ca left in the cells for 5 min intervals from the control curve in Fig. 1 and replotting the values thus obtained on semilogarithmic paper. The straight line through the —●— points between 55 and 115 min represents points at equilibrium time (third phase of the efflux curve). The second phase (○) was obtained by subtracting values on the line of the third phase from the points of the upper curve at each time. The first phase (×) was obtained by subtracting values from the second phase at each time

exchangeable cell calcium compartment size increases by about 80% if the cells are preincubated with the hormone for 120 min.

In order to compare the parameters of calcium efflux with the previously published parameters of calcium influx into isolated pancreatic cells, the control values, the pancreozymin values, and the carbamylcholine values of Table 1 are listed again with the phase 2 values of influx (Table 3). The efflux rates in phase 2 are comparable with the influx rates of the slow phase. However, compartment sizes in efflux of phase 2 are much smaller

Table 1. Analysis of Ca^{++} efflux from isolated pancreatic cells[a]

arameters	Phases	Control	With pancreozymin	With carbamylcholine	With Ca^{++}-ionophore	Ratio (pancreozymin/control)	Ratio (carbamylcholine/control)
$\left(\frac{\text{nmoles}}{\text{mg protein} \times \text{min}}\right)$	phase 2	0.081	0.144	0.128	0.121	1.78	1.58
	phase 3	0.017	0.021	0.018			
$_{/2}$ (min)	phase 2	11.3	5.0	8.6	4.5		
	phase 3	45.5	66.5	58.5			
(min^{-1})	phase 2	0.061	0.138	0.080	0.154		
	phase 3	0.015	0.010	0.012			
$\left(\frac{\text{nmoles}}{\text{mg protein}}\right)$	phase 2	1.331	1.043	1.600	0.790	0.78	1.20
	phase 3	1.125	2.043	1.507			

[a] Pancreozymin, Carbamylcholine and the Ca^{++}-ionophore A23187 were added in the preincubation period. Each value was obtained from the graphical analysis of 4 to 6 experiments as shown in Fig. 1. The values with the Ca^{++}-ionophore A23187 are taken from one representative experiment.

Table 2. Total exchangeable calcium as measured directly by the cumulative efflux over 120 min[a]

Control	With pancreozymin	With carbamylcholine
2.72	4.33	4.15

[a] Values (nmoles/mg protein) are the mean of 4 to 6 experiments.

Table 3. Comparison between the half times, the calcium fluxes and the compartment sizes of Phases 2 and 3 as determined by influx and efflux experiments[a]

Parameter	Hormone	Influx phase 2 (slow phase)	Efflux		Total of phases 2 and 3 (efflux)
			phase 2	phase 3	
Flux rate	control	0.070	0.081	0.017	–
$\phi \left(\frac{\text{nmoles}}{\text{mg protein} \times \text{min}}\right)$	pancreozymin	0.124	0.144	0.021	–
	carbamylcholine	0.112	0.128	0.018	–
Compartment size	control	2.43	1.33	1.13	2.46
$S \left(\frac{\text{nmoles}}{\text{mg protein}}\right)$	pancreozymin	6.49	1.04	2.04	3.08
	carbamylcholine	7.85	1.60	1.51	3.11
half time	control	24.2	11.3	45.5	–
$t_{1/2}$ (min)	pancreozymin	36.6	5.0	66.5	–
	carbamylcholine	48.5	8.6	58.5	–

[a] The values for influx were cited from a preceding report [9].

than those obtained in the influx slow phase. The total compartment size in efflux of both phase 2 plus phase 3, however, is comparable with that of the slow phase of influx in the case of control (without hormone), but only half the influx values after pancreozymin and carbamylcholine stimulation. The reason is that washing of the cells before starting the efflux experiment took more time than in influx experiments; therefore, some ^{45}Ca is lost, and the total exchangeable calcium is smaller in efflux than in influx experiments.

Effect of Other Hormones, Dibutyryl Cyclic Nucleotides, the Ca^{++}-Ionophore (A23187), and Metabolic Inhibitors on Ca^{++} Fluxes

As shown in Table 4, those substances which stimulated Ca^{++} influx in the preceding report [9] showed stimulation of a Ca^{++} efflux, i.e., gastrin I, caerulein, the octapeptide of pancreozymin and the Ca^{++}-ionophore A23187. Dibutyryl cyclic AMP, which has an effect on enzyme release in the perfused pancreas [14], has no effect on either Ca^{++} efflux or on influx, the latter being shown in our previous study [9].

The detailed data on the effect of the Ca^{++} ionophore A23187 on the Ca^{++} efflux can be seen from Tables 1 and 4. The presence of the Ca^{++} ionophore changes each parameter of phase 2 (flux rate, half time, rate constant and compartment size) in the same direction as does pancreozymin, carbamylcholine and the octapeptide of pancreozymin.

The metabolic inhibitors Antimycin A and dinitrophenol strongly stimulate Ca^{++} efflux (Table 4). These inhibitors were tested also in Ca^{++} uptake experiments. As shown in Fig. 3, Antimycin A, which is an inhibitor of oxidative phosphorylation, inhibits the slow phase of Ca^{++} uptake in the presence and absence of carbamylcholine (same results were obtained with pancreozymin), but does not affect the fast phase of Ca^{++} uptake. A similar pattern of inhibition of Ca^{++} uptake was observed in the presence of dinitrophenol.

Effect of Atropine on the Ca^{++} Efflux

Atropine, which inhibited completely the effect of carbamylcholine on Ca^{++} uptake [9], also completely inhibits the carbamylcholine effect on Ca^{++} efflux, whereas it has no effect on pancreozymin-stimulated Ca^{++} efflux (Table 5).

Table 4. Effect of hormones (other than pancreozymin and carbamylcholine), dibutyryl nucleotides, Ca^{++} ionophore, 2,6-dinitrophenol and Antimycin A on Ca^{++} efflux out of isolated cells[a]

Substance (concentration)	Ratio $\frac{\text{Phase 2 efflux}}{\text{Control efflux}}$	
	$+Ca^{++}$	$-Ca^{++}$
effective		
(1) gastrin I (10^{-6} M)	$\frac{0.0806}{0.0538}$ (1.5)	$\frac{0.0876}{0.0533}$ (1.64)
(2) caerulein (0.5 µg/ml)	$\frac{0.1212}{0.0707}$ (1.71)	$\frac{0.1293}{0.0808}$ (1.60)
(3) octapeptide of pancreozymin (5×10^{-6} M)	$\frac{0.128}{0.0538}$ (2.38)	$\frac{0.112}{0.0533}$ (2.10)
(4) Ca^{++} ionophore (5 µg/ml) (A23187)	$\frac{0.121}{0.0538}$ (2.25)	$\frac{0.1080}{0.0533}$ (2.03)
(5) 2′,6′ dinitrophenol (0.5 mM)	$\frac{0.1640}{0.0566}$ (2.90)	$\frac{0.1747}{0.0560}$ (3.12)
(6) Antimycin A (10^{-5} M)	$\frac{0.1687}{0.0566}$ (2.98)	$\frac{0.183}{0.056}$ (3.27)
not effective		
(7) adrenaline (10^{-4} M)	$\frac{0.0448}{0.0410}$ (1.09)	$\frac{0.0470}{0.0504}$ (0.93)
(8) isoproterenol (10^{-4} M)	$\frac{0.0394}{0.0410}$ (0.96)	$\frac{0.0544}{0.0504}$ (1.08)
(9) secretin (16.5×10^{-7} M)	$\frac{0.0768}{0.0707}$ (1.09)	$\frac{0.0848}{0.0808}$ (1.05)
(10) dibutyryl cyclic AMP (1 mM)	$\frac{0.0578}{0.0530}$ (1.09)	$\frac{0.0566}{0.0578}$ (0.98)
(11) dibutyryl cyclic GMP (1 mM)	$\frac{0.0591}{0.0530}$ (1.11)	$\frac{0.0615}{0.0578}$ (1.06)

[a] Unit of values = nmoles/mg protein × min. $+Ca^{++}$ or $-Ca^{++}$ indicates the medium Ca^{++} concentration 1.25 mM and 0 mM. Values of (1), (3) and (4), (2) and (9), (5) and (6), (7) and (8), (10) and (11) were obtained in the same experiment, respectively.

Effect of Ca^{++} Concentration on Efflux

Omission of Ca^{++} from the medium has no effect on Ca^{++} efflux. As Tables 4 and 5 show, no significant difference is obtained between Ca^{++} effluxes (phase 2) in Ca^{++}-containing or Ca^{++}-free media. The mean values (nmoles/mg protein × min) of all experiments taken together are

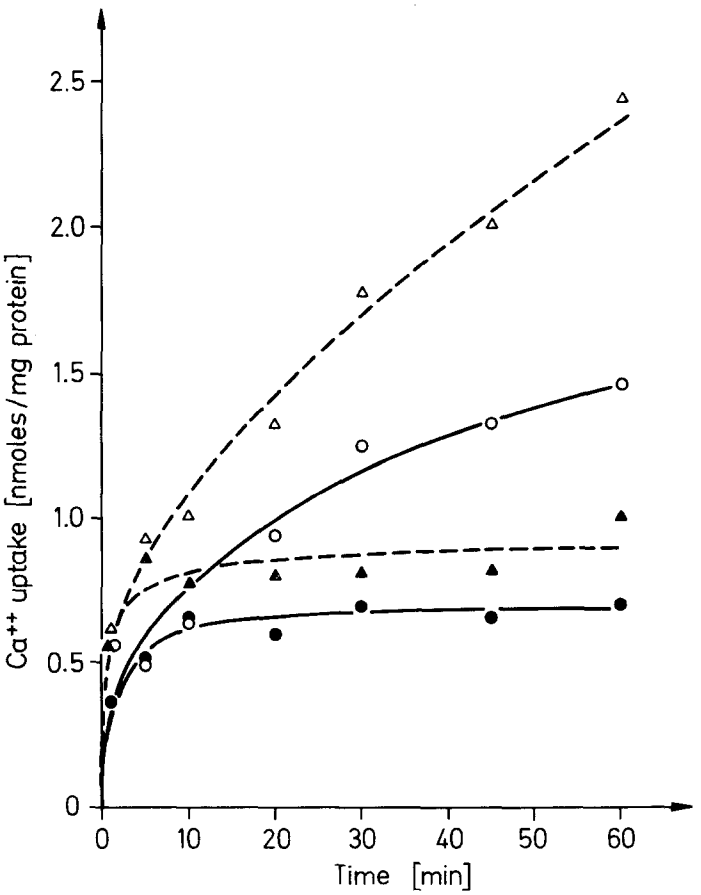


Fig. 3. Effect of Antimycin A on Ca^{++} uptake in the presence and absence of carbamylcholine. The figure shows a representative experiment out of 3. The cells were preincubated for 30 min with 1.25 mM unlabelled Ca^{++} in the presence and absence of carbamylcholine. At the end of preincubation period (time = 0) ^{45}Ca (5 μCi/10 ml) and Antimycin A were added to the media. ○ control (without hormones); ● with Antimycin; (2×10^{-6} M); △ with carbamylcholine (10^{-5} M); ▲ with carbamylcholine (10^{-5} M) + Antimycin A (2×10^{-6} M)

0.056 ± 0.0046 and 0.057 ± 0.005 ($p > 0.8$) in the controls with or without Ca^{++} in the media, and 0.129 ± 0.009 and 0.129 ± 0.010 ($p > 0.8$) for hormone stimulation. In Ca-free media, the cellular ^{45}Ca activity left in the cells after 10 min of efflux was not significantly different regardless of EGTA (0.5 mM) addition to the efflux media. In the presence of hormones (pancreozymin and carbamylcholine) cellular ^{45}Ca activity was 34.2% with and 34.5% without EGTA of ^{45}Ca left in cells ($p > 0.8$, paired observations of 3 experiments); in the absence of hormones, this was 68.8% with and 70.7% without EGTA ($p > 0.5$, paired observations of 3 experiments).

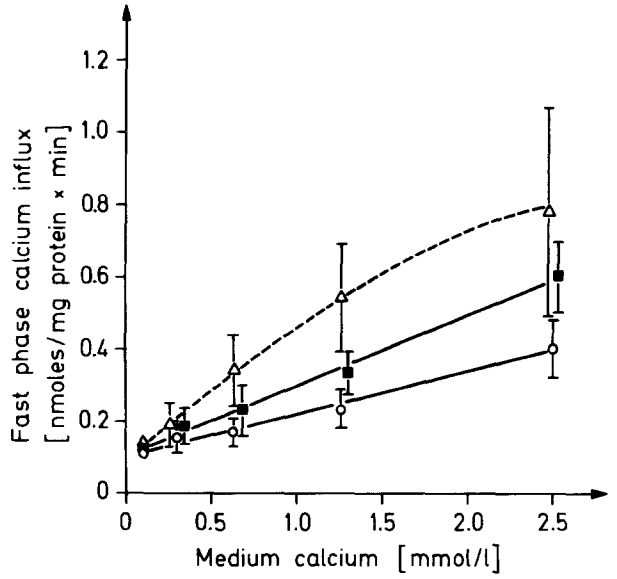
Table 5. Atropine effect on pancreozymin and carbamylcholine stimulation of Ca^{++} efflux[a]

(Single experiments)	Ratio $\frac{\text{Phase 2 efflux}}{\text{control efflux}}$	
	$+Ca^{++}$	$-Ca^{++}$
(1) control	$\frac{0.0657}{0.0657}$ (1.0)	$\frac{0.0424}{0.0424}$ (1.0)
control + atropine (10^{-5} M)	$\frac{0.0615}{0.0657}$ (0.94)	$\frac{0.0445}{0.0424}$ (1.05)
carbamylcholine (10^{-5} M)	$\frac{0.1272}{0.0657}$ (1.94)	$\frac{0.1100}{0.0424}$ (2.59)
carbamylcholine (10^{-5} M) + atropine (10^{-5} M)	$\frac{0.0572}{0.0657}$ (0.87)	$\frac{0.0445}{0.0424}$ (1.05)
(2) control	$\frac{0.0537}{0.0537}$ (1.0)	$\frac{0.0600}{0.0600}$ (1.0)
control + atropine (10^{-5} M)	$\frac{0.0453}{0.0537}$ (0.84)	$\frac{0.0540}{0.060}$ (0.9)
pancreozymin (0.86×10^{-7} M)	$\frac{0.1200}{0.0537}$ (2.24)	$\frac{0.1240}{0.0600}$ (2.07)
pancreozymin (0.86×10^{-7} M) + atropine (10^{-5} M)	$\frac{0.1280}{0.0537}$ (2.38)	$\frac{0.1330}{0.0600}$ (2.22)

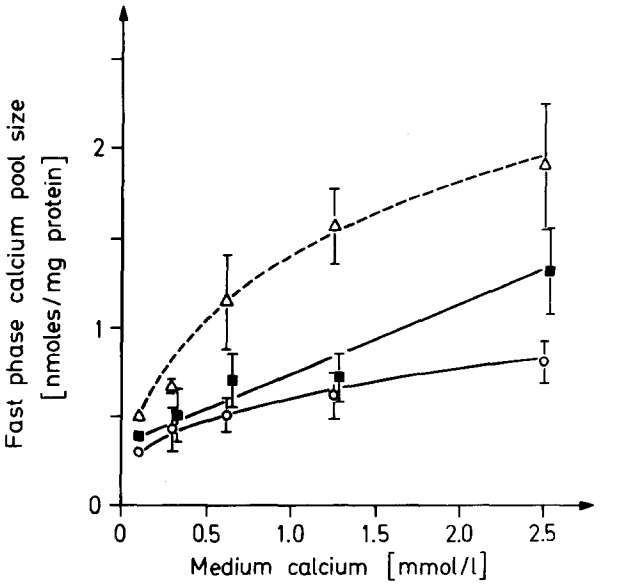
[a] Unit of values = nmoles/mg protein × min. $+Ca^{++}$ and $-Ca^{++}$ indicate the medium Ca^{+} concentration 1.25 mM and 0 mM.

Effect of Different Ca^{++} Concentration on Influx at a Steady State

As shown in Fig. 4*A*, the fast phase calcium influxes show linearity in the controls and during pancreozymin stimulation, yet tend to saturate during carbamylcholine stimulation when the external Ca^{++} concentration was elevated from 0.1 mM to 2.5 mM. The fast phase calcium pool sizes (Fig. 4*B*) show a similar pattern to the fast phase calcium influxes. Calcium influxes of the slow phase (Fig. 4*C*) show in all three cases a tendency to saturate. Lineweaver-Burk plots of the curves of Fig. 4*C* give values of K_m and V_{max} as listed in Table 6. For the slow phase of ^{45}Ca influx both pancreozymin and carbamylcholine increase K_m values by about 82% and 190% and V_{max} by about 115% and 197%, respectively. The slow phase calcium pool sizes show a tendency to saturate in all three cases, as with the slow phase of calcium influx (Fig. 4*C*).

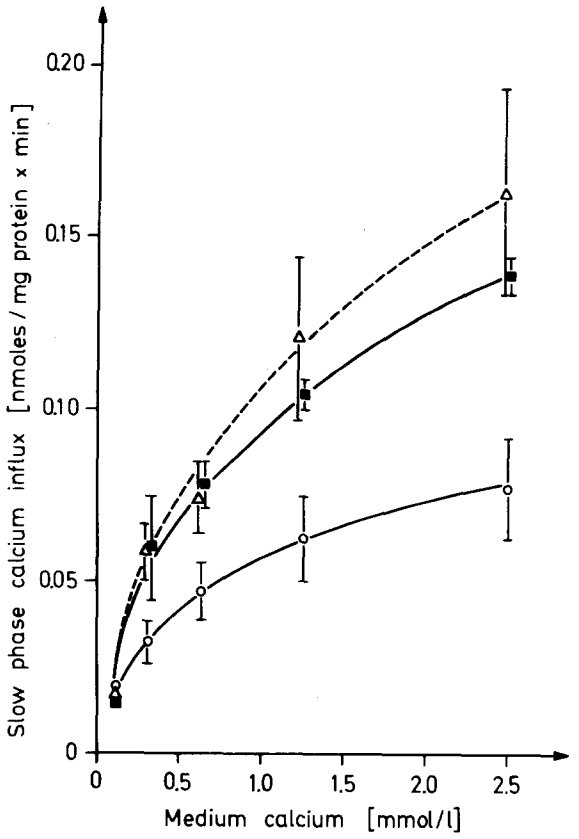


(*A*)

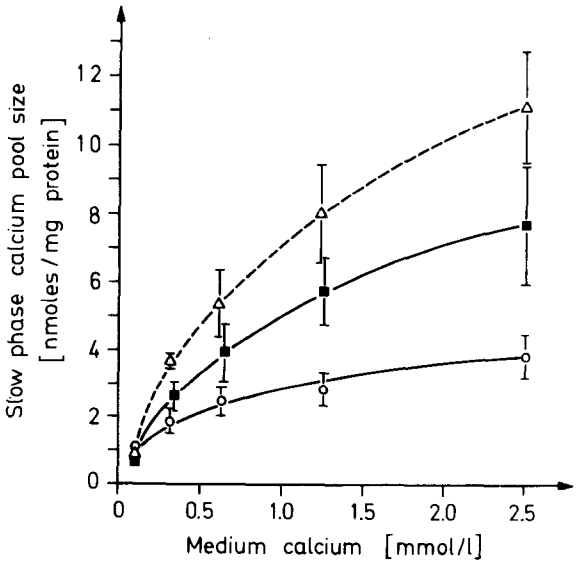


(*B*)

Fig. 4. Effect of different medium Ca^{++} concentrations on Ca^{++} influx. (*A*) Fast phase (phase 1) calcium influx versus medium calcium concentration. (*B*) Fast phase (phase 1) calcium pool size *vs.* medium calcium concentration. (*C*) Slow phase (phase 2) calcium influx *vs.* medium calcium concentration. (*D*) Slow phase (phase 2) calcium pool size *vs.* medium calcium concentration. ○ control; ■ in the presence of pancreozymin (0.86×10^{-7} M); △ in the presence of carbamylcholine (10^{-5} M). The points at 0.1 mM Ca^{++} are single experiments for hormones and the mean of two experiments for control. All other values represent the mean ± SEM out of 3 to 5 experiments


Slow phase calcium influx [nmoles / mg protein x min]
0.20
0.15
0.10
0.05
0
0
0.5
1.0
1.5
2.0
2.5
Medium calcium [mmol/l]

(*C*)


Slow phase calcium pool size
[nmoles/mg protein]
12
10
8
6
4
2
0
0
0.5
1.0
1.5
2.0
2.5
Medium calcium [mmol/l]

(*D*)

Table 6. K_m and V_{max} obtained by the analysis of Lineweaver-Burk plots replotted from Fig. 4 *C*

	Control	Pancreozymin	Carbamylcholine
V_{max} (nmoles/mg protein × min)	91	196	270
K_m (mM Ca^{++})	0.55	1.00	1.59

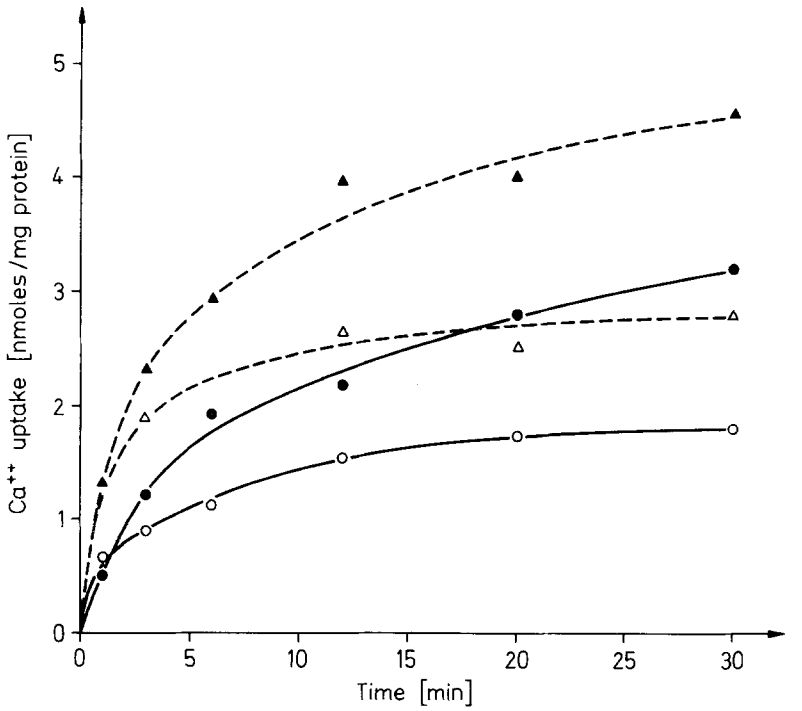


Fig. 5. Effect of sudden addition of Ca^{++} to the incubation medium on Ca^{++} uptake. Closed symbols (● ▲) refer to ^{45}Ca uptake into cells 40 min preincubated in a Ca^{++}-free medium, ^{45}Ca (1.25 mM) and pancreozymin (0.86×10^{-7} M) were added at the beginning of the uptake period (time = 0): ▲– – –▲ with and ●——● without addition of pancreozymin. Open symbols (○ △) = ^{45}Ca uptake into cells which had been preincubated with 1.25 mM Ca^{++} in the media for 40 min. ^{45}Ca (5 μCi/10 ml) and pancreozymin were added at time 0 to start the uptake experiment: △– – –△ with, ○——○ without pancreozymin

Effect of Sudden Addition of Ca^{++} on Influx

So far we have described ^{45}Ca influx in a steady state condition. However, under nonsteady state conditions, when isolated pancreatic cells were preincubated in Krebs-Ringer bicarbonate without Ca^{++},

addition of $^{45}Ca^{++}$ (1.25 mM) and pancreozymin causes an enormous stimulation of ^{45}Ca uptake as compared with the steady state conditions (Fig. 5). As this experiment was not done under steady state conditions, calculation of these data are not valid and, therefore, have not been made.

Discussion

The present study was undertaken in order to confirm the role of extracellular Ca^{++} in pancreatic enzyme secretion induced by secretagogues. Furthermore, kinetic parameters of Ca^{++} transport were derived to characterize the mechanism of the transport process.

Ca^{++} Efflux as Compared with Ca^{++} Influx

In a previous study we had shown that secretagogues of pancreatic enzyme secretion, such as pancreozymin, caerulein, gastrin, the octapeptide of pancreozymin, acetylcholine, and a Ca^{++} ionophore, stimulate Ca^{++} uptake into isolated cells most likely by increasing the cell membrane permeability for Ca^{++}. The evidence for this interpretation was an increased rate of ^{45}Ca influx into the cells under steady state conditions and an increased ^{45}Ca pool size after pancreozymin and acetylcholine-stimulation. The present study shows that, similar to the studies of Ca^{++} influx into isolated cells, the curves of Ca^{++} efflux represent exponential functions with more than one component. Thus, a plot of the pancreozymin stimulated ^{45}Ca efflux curve reveals at least three phases: a first phase, a slower second phase and an even slower third phase, which we did not detect in the Ca^{++} influx experiments. As shown in Table 3 the ^{45}Ca influx and efflux rates of the slow phase and phase 2, respectively, are very similar, which suggests that for both unidirectional flux rates the same compartment might be involved.

In the preceding paper we had presented some lines of evidence that the slow component represents the calcium flux between an intracellular compartment and the extracellular fluid, whereas the fast phase is more probably due to the binding of Ca^{++} to the surface of the cell membrane. For the slow phase compartments cytoplasm, mitochondria, endoplasmatic reticulum, and zymogen granules could be considered.

Since the present technique of isotope "desaturation" is not fast enough to measure the fast component of efflux with an acceptable

precision, only the fluxes of the slower phases (phases 2 and 3) were considered in this paper and compared to the slow phase of Ca^{++} influx of the previous paper [9]. As Table 3 shows, the Ca^{++} flux rates of the slow phase of influx and phase 2 of efflux are comparable. However, the half times of the slow phase of Ca^{++} influx match rather those for phase 3 of efflux than those of phase 2. Also, the compartment sizes as calculated for the slow phase of influx and phase 2 of efflux experiments are not equal. However, if one compares the total compartment size of phase 2 plus phase 3 of efflux, the value, at least in the controls, compares better with that of the slow phase of influx. This suggests that the slow phase obtained by influx experiments comprises both the second and the third phase in efflux experiments. The reason for these discrepancies might be due to different washing procedures as explained in the *Result* section, but also because the assumption of two or more compartments in parallel might not be quite correct. A discrimination between a parallel and a series model can only be made concerning the fast and the slow phase. A distinction between phase 2 and phase 3 unfortunately cannot be made since the Ca^{++} concentration and the specific activity of compartment 2 is not known. Actually it should be noted that considering different phases of the flux curves is an over-simplification since overlapping of more than 2 or 3 phases probably occurs. After two hr of either ^{45}Ca influx or 1.5 hr of preloading the cells with ^{45}Ca, a first equilibration is approached of those compartments (membrane and cytoplasma) which are influenced by the secretagogue hormones. Probably a time of incubation with ^{45}Ca longer than 1.5–2 hr would reveal more and slower compartments than those described here, which are not in tracer equilibrium with ^{45}Ca of the extracellular medium within the present period of incubation. These very slow phases, however, could have some influence on the phases evaluated in these studies which then could not be considered as totally in tracer equilibrium. The fact that after 120 min of ^{45}Ca efflux about 15% of the original ^{45}Ca is still left in the cells (Fig. 1) indicates that there are other compartments with more tightly bound Ca^{++} which exchange at slower rates than those which reached equilibrium after 2 hr, the time used in our experiments. For such a slow exchangeable compartment, zymogen granules would come into question. As Ceccarelli *et al.* [7] have shown, zymogen granules are loaded relatively quickly with ^{45}Ca but keep a steady isotope concentration for more than 4–5 hr. Thus, efflux from this compartment should not be detectable in our efflux experiments, and could be only in phase 3 ($t_{1/2}$ 65 min). In this phase flow rates are small and increase only moderately after stimulation with pancreozymin and carbamylcholine.

Thus, although an exact comparison of all kinetic data obtained by influx and efflux studies cannot be made under steady state conditions, the values for the slow phase of Ca^{++} influx and phase 2 of Ca^{++} efflux compare fairly well, suggesting that this compartment is the same under control conditions as well as under hormone stimulation (Table 3).

Ca^{++} Fluxes in Relation to the Medium Ca^{++} Concentration

Another purpose of this study was to identify a possible mechanism of the Ca^{++} transport process. The rate of ^{45}Ca efflux was not dependent on the presence of Ca^{++} in the media. Neither omission of Ca^{++} from the extracellular medium nor addition of EGTA in the absence of Ca^{++} caused a significant change of the rate of $^{45}Ca^{++}$ fluxes in the presence or absence of hormones or other test substances. This would suggest that a coupled Ca^{++}/Ca^{++} exchange as has been observed in squid axon [2] does not contribute to either Ca^{++} influx or Ca^{++} efflux in our system. Similar observations have been made by Heisler *et al.* [8] using fragments of rat pancreas and by Case and Clausen using the isolated pancreas of baby rats [6].

On the other hand, increasing medium concentration increased calcium influx markedly. The effect of the extracellular calcium concentration of the slow phase of influx and pool size show saturation kinetics, whereas saturation was not so evident in the fast phase of both calcium influx and pool size, except during carbamylcholine stimulation (Fig. 4*A* and *B*). This is consistent with the interpretation that the slow phase represents a carrier or channel for limited transport of calcium through the outer plasma membrane. The calculation of V_{max} and K_m from a Lineweaver-Burk plot of calcium influx of the slow phase *vs.* Ca^{++} concentration of the medium demonstrated that both values were increased by pancreozymin and acetylcholine (Table 6).

These data parallel the effect of parathyroid hormone on the parameters of Ca^{++} influx into kidney cells [4]. As pointed out in this paper, the simultaneous increase of V_{max} and K_m could be explained by an increase in the rate constant k_2 in the Briggs-Haldane derivation, k_2 representing translocation steps of Ca X through the membrane (where Ca is the extracellular calcium and X the postulated carrier in the membrane).

The implication which can be derived is that pancreozymin and acetylcholine stimulate calcium transport by increasing the rate of transfer across the membrane and not by increasing the carrier concentration or raising its binding affinity for calcium.

Ca^{++} Fluxes through the Outer Cell Membrane as Related to the Intracellular Ca^{++} Stores

A striking parallel to the interpretation that hormones act by stimulating a Ca^{++} translocating process is the finding that an artificial carrier of high selectivity for calcium exhibits the same type of kinetics in Ca^{++} influx experiments [9], as well as in Ca^{++} efflux, as that of hormone-stimulated transport (Tables 1 and 4). The question, however, whether the hormone stimulates the membrane translocation of Ca^{++} directly or by the mediation of a second intracellular substance, remains to be answered. Thus, the involvement of cyclic nucleotides in the mechanism of enzyme secretion has been considered [8, 10, 12, 14], but their role as a second messenger has not been established unequivocally. Since Borle [5] had reported that cyclic AMP stimulates Ca^{++} efflux from mitochondria, the possibility of a similar action of cyclic nucleotides has been considered in the present study. One should expect an increase in Ca^{++} efflux from cells if the Ca^{++} concentration in the cytoplasm would be raised by release of Ca^{++} from mitochondria. As Table 4 shows, cyclic nucleotides have no action on either Ca^{++} influx nor efflux in contrast to the action of all hormones which induce enzyme secretion, but it should be considered that *db c* AMP is not equivalent to a rise in intracellular cAMP. On the other hand, mitochondrial inhibitors, Antimycin A and dinitrophenol, which are known to inhibit Ca^{++} uptake into mitochondria [13], inhibited Ca^{++} uptake into cells but stimulated Ca^{++} efflux strongly (Fig. 3, Table 3). This observation suggests that under these conditions the system might not be in a steady state; it is rather likely that the steady state efflux is perturbed by drastic changes of the intracellular free Ca^{++} concentration. Perturbing steady state conditions by altering the extracellular Ca^{++} concentration by sudden addition of Ca^{++} also increased ^{45}Ca flux into the cells much more effectively than under equilibrium conditions. In the latter situation the hormone effect, too, was more pronounced and showed a steadily increasing rate of ^{45}Ca uptake into the cells during the time of observation.

The implication which we can derive from this study and from the foregoing paper is that secretagogues of enzyme secretion stimulate Ca^{++} transport into pancreatic cells directly, neither cyclic AMP nor a Ca^{++}/Ca^{++} exchange mechanism being involved. The most likely interpretation is that hormone increased Ca^{++} efflux as well as Ca^{++} influx are due to an increase of cell membrane permeability for Ca^{++} as one of the main driving forces for unidirectional fluxes under steady state conditions.

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