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CALCIUM ION UPTAKE IN ISOLATED PANCREAS CELLS INDUCED BY SECRETAGOGUES

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SUMMARY

1. Secretagogues of pancreatic enzyme secretion: pancreozymin, carbamylcholine, gastrin I, the octapeptide of pancreozymin, caerulein and the Ca^{2+} ionophore A 23187 stimulate ^{45}Ca uptake into isolated rat pancreatic cells, whereas adrenaline, isoproterenol, secretin, dibutyrylic cyclic adenosine 3′,5′-monophosphate and dibutyrylic cyclic guanosine 3′,5′-monophosphate have no effect on ^{45}Ca uptake.

2. A graphical analysis of the Ca^{2+} uptake curves reveals at least two phases: a fast phase, probably due to binding of Ca^{2+} to the membrane and a slow phase representing Ca^{2+} transport into cells. Both phases are stimulated by pancreozymin and carbamylcholine.

3. The ^{45}Ca-exchangeable pool size is increased by both carbamylcholine and pancreozymin, whereas a significant increase of total content of cell calcium was too small to be detected.

4. Atropine blocks the stimulatory effect of carbamylcholine completely but not that of pancreozymin. The Ca^{2+} antagonist D600 blocks the stimulatory effects of both carbamylcholine and pancreozymin only partially.

5. The data suggest that secretagogues of pancreatic enzyme secretion act by increasing the rate of Ca^{2+} transfer into the cell most probably through an increase of the cell membrane permeability for Ca^{2+}.

INTRODUCTION

It is well established that Ca^{2+} is necessary for enzyme secretion from the exocrine pancreas. Omission of Ca^{2+} from the extracellular fluid causes a reduction of the pancreozymin and acetylcholine-induced enzyme secretion [1–3]. On the other hand, amylase release is stimulated by conditions which lead to an increased Ca^{2+} concentration in the cell, such as rapid elevation of the extracellular Ca^{2+} concentration [4] or increased Ca^{2+} influx produced by the divalent cation ionophore A 23187 [5, 6]. Although the importance of extracellular Ca^{2+} in the mechanism of enzyme secretion has been accepted, it still remains undecided whether in the course of hormone-stimulated enzyme secretion, intracellular Ca^{2+} is increased by an increased influx of Ca^{2+} from the extracellular medium, or if Ca^{2+} is released from intracellular

stores. The current view that extracellular Ca^{2+} is not a major source for triggering enzyme secretion stems mainly from the following lines of evidence: 1) Omission of Ca^{2+} from the bath medium of pancreas preparations in vitro did not result in an immediate inhibition of the hormone-induced enzyme secretion but in a delayed one [3]. 2) Ca^{2+} efflux from ^{45}Ca-preloaded isolated pancreas of baby rats and from pancreatic slices was stimulated by pancreatic secretagogues whereas an increased Ca^{2+} uptake could not be detected under the influence of pancreozymin or acetylcholine [7, 8]. 3) No electrophysiological evidence was found for an acetylcholine-evoked increase in Ca^{2+} conductance of the acinar cell membrane [9]. Recent observations in our laboratory using the isolated perfused cat pancreas suggest that the effect of acetylcholine is mediated by an increased influx of extracellular Ca^{2+} into the cell [30]. However, it was observed that the effect of pancreozymin can occur independently of extracellular Ca^{2+}. The effect of pancreozymin might be mediated exclusively by cyclic nucleotides which probably also act by releasing Ca^{2+} from intracellular stores, such as mitochondria; this was demonstrated in other tissues (kidney, liver and heart) [10]. The purpose of the present study is to assess the role of extracellular Ca^{2+} in the mechanism of pancreozymin and acetylcholine action, by measuring the Ca^{2+} uptake directly into isolated pancreatic cells of the rat. In contrast to the current view that secretagogues of pancreatic enzyme secretion do not induce Ca^{2+} uptake from extracellular medium [7, 8], our data show that both pancreozymin and carbamylcholine, as well as other stimulants of enzyme secretion, increase the Ca^{2+} influx into the cells.

MATERIALS AND METHODS

1. *Materials*

Reagents were obtained from the following sources: crude collagenase, 152 units/mg, from Worthington Biochemical Corp., Freehold, N.Y., U.S.A.; salt-free sheep-test hyaluronidase, 1000 units/mg, from Boehringer, Mannheim, Germany; chromatographically purified soybean trypsin inhibitor from Serva, Heidelberg, Germany; essentially fatty acid-free bovine plasma albumin from Sigma, St. Louis, Mo., U.S.A.; choline chloride, carbamylcholine · HCl from Merck, Darmstadt, Germany; pancreozymin from the GIH Research Unit, Karolinska Institute, Stockholm, Sweden; gastrin I from Imperial Chemical Industries Ltd., Cheshire, England; caerulein from Farmatalia, Milano, Italy; L-adrenalin and DL-isoproterenol · HCl from Serva, Heidelberg, Germany; dibutyrylic cyclic adenosine-monophosphate and dibutyrylic cyclic guanosine monophosphate from 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. Miquel A. Ondetti (The Squibb Institute for Medical Research, Princeton, N.J., U.S.A.). Bovine plasma albumin for the incubation medium was dialyzed against 0.9 % NaCl solution before use. The animals used were young rats of either sex weighing 150–210 g, and they were normally fed before experiments.

2. *Cell isolation*

The cells were isolated according to the method of Amsterdam and Jamieson [11] with a small modification: 1.5–2 g of pancreas were obtained from 8–10 rats

which were killed by a blow to the neck. For all steps in the procedure, we used a Krebs-Ringer-bicarbonate solution equilibrated with 95 % O_2 and 5 % CO_2 containing 15 mM glucose and 0.1 mg of soybean trypsin inhibitor per ml.

a) First digestion: the pancreas was trimmed free of fat and mesentery, and 10 ml of the enzyme mixture (0.75 mg of crude collagenase/ml and 1.5 mg hyaluronidase/ml in Krebs-Ringer-bicarbonate solution containing 0.1 mM Ca^{2+} and 1.2 mM Mg^{2+}) was inoculated into the interstitium of the tissue by means of a syringe with a fine needle. The distended gland and excess enzyme solution were transferred to a 100 ml Erlenmeyer flask, equilibrated with 95 % O_2 and 5 % CO_2 and incubated at 37 °C for 15 min with agitation at 130 oscillations/min.

b) Removal and replacement of divalent cations: the supernatant was decanted and the tissue was then incubated twice for 5 min at 37 °C in 20 ml of Krebs-Ringer-bicarbonate solution without Ca^{2+} and Mg^{2+} and containing 2 mM EDTA. Divalent cations were subsequently added back by briefly washing the tissue twice with 20 ml of Krebs-Ringer-bicarbonate solution containing 0.1 mM Ca^{2+} and 1.2 mM Mg^{2+}.

c) Second digestion: Fresh collagenase and hyaluronidase (10 ml with 0.1 mM Ca^{2+} and 1.25 mM Mg^{2+}) at concentrations of 1.25 mg/ml and 2.0 mg/ml respectively, were added and the cells were incubated for 40–45 min at 37 °C.

d) Final dissociation and harvesting of the cells: After step c, cells were liberated by sequential passage two times through Eppendorf 1 ml pipettes with tips cut to diameters of 3 mm and 1.5 mm respectively. The isolated cells were filtered three times through one layer of gauze, and layered in centrifuge tubes over 10 ml columns of Krebs-Ringer-bicarbonate solution with 1.25 mM Ca^{2+} and 1.2 mM Mg^{2+} containing 4 % bovine plasma albumin. The tubes were centrigfued at $50 \times g$ for 5 min, thus yielding pellets of packed cells. After two further washes in 10 ml of the above solution and two filtrations between the washes, the pooled cells were suspended in 2–3 ml of Krebs-Ringer-bicarbonate solution with 1.25 mM Ca^{2+}, 1.2 mM Mg^{2+}, 10 mM glucose, 1 mg/ml of bovine plasma albumin and 0.1 mg/ml of trypsin inhibitor.

3. *Ca^{2+} uptake experiments*

0.5 ml of the cell suspension (20–30 mg protein) 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^{2+}, 1.2 mM Mg^{2+}, 10 mM glucose, 1 mg/ml of 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 as certain optimal oxygenation and agitated at 80 oscillations/min. The cells were preincubated for 30 min in Krebs-Ringer-bicarbonate containing 1.25 mM $CaCl_2$ to assure steady-state conditions. In one set of experiments hormones were added to the preincubation medium and in another set of experiments, hormones were added after the preincubation period at the beginning of the incubation. After 30 min of preincubation, 5 μCi of ^{45}Ca were added to the medium and the cells were incubated for 1–2 h. At different periods, an aliquot (0.5 ml) of the cell suspension was removed and squirted into a 25 ml conical glass centrifuge tube containing 20 ml of ice-cold isotonic choline chloride solution. Choline chloride was preferred to NaCl as Na^+ could have some effect on Ca^{2+} efflux as has been described [36]. Each tube was immediately centrifuged for 45 s at $1000 \times g$, and the centrifuge was stopped with an electric brake. The supernatant solution was rapidly decanted and the tubes were inverted for further

drainage. The whole procedure, from the time of sampling to the separation of cell pellets from the choline chloride solution lasted approximately 2 min. After the tubes were thoroughly drained, 1 ml of twice-distilled water was added and the cells were homogenized with a high intensity ultrasonic probe. Aliquots of the homogenate were analyzed for cell protein by the method of Lowry et al. [12] and for Ca^{2+} by means of an Eppendorf flame photometer. The homogenate was dissolved in 10 ml Bray's solution and the radiation of ^{45}Ca was counted in a Tri-Carb Packard scintillation spectrometer model 3380.

4. *Counting of cells*

The isolated cells were counted in a Neubauer chamber for blood cell counting.

5. *Measurement of the volumes of cell pellets after incubation of the cells in the presence and absence of hormones*

Cells were incubated in a Krebs-Ringer-bicarbonate solution for 60 min with and without hormones, then centrifuged at $50 \times g$ for 5 min and the volume of cell pellet was measured with calibrated pipettes.

6. *Measurement of cell water content*

Cells were put into isotonic choline-chloride buffer containing [^{14}C]inulin and centrifuged at $50 \times g$ for 5 min. The cell pellets were weighed and kept for 2 days at 50 °C in a desiccator containing silica gel until the weight was constant. The dried cells were sonicated in 1 ml of twice-distilled water, Bray's solution was added and the radioactivity of [^{14}C]inulin was counted in a Packard scintillation spectrometer to determine the volume of trapped extracellular fluid. The intracellular water content was calculated from the difference of the trapped extracellular volume and the total water content.

7. *Tests of viability and function of isolated cells*

a) *Exclusion of trypan blue.* Cells were examined microscopically to estimate the amount of deformed and destroyed cells as well as released subcellular particles: nuclei, mitochondria and zymogen granules (Fig. 1). The percentage of cells excluding trypan blue (1 % in Krebs-Ringer-bicarbonate buffer) was also determined.

b) *Amylase release.* Cells were incubated at 37 °C in Krebs-Ringer-bicarbonate containing 1.25 mM Ca^{2+}, 1.2 mM Mg^{2+}, 10 mM glucose, 1 mg/ml bovine plasma albumin and 0.1 mg/ml trypsin inhibitor. Aliquots of the cell suspension were removed at different periods of incubation and centrifuged at $100 \times g$ for 2 min. The supernatant was again centrifuged at $500 \times g$ for 1 min to remove cell debris and then submitted to the amylase assay using the method of Street and Close [13]. Cell pellets obtained after the first centrifugation were submitted to protein determination according to the method of Lowry et al. [12]. Amylase activity was expressed in International Units per mg protein.

Assessment of the effect of a possible pinocytosis on Ca^{2+} uptake into the cells

Cells were incubated with and without hormones in the test medium containing [^{3}H]inulin (about 100 000 cpm/ml corresponding to 0.6 μM/l). After 30 min of pre-

incubation and at 10 min intervals during the following hour, aliquots of cells were removed and centrifuged in cold choline chloride buffer.

The radioactivity of [^{3}H]inulin trapped in the cell pellets was counted. The volume of incubation medium trapped in the pellet and the concentration of Ca^{2+} in this volume were calculated. Thus the amount of extracellular Ca^{2+} plus Ca^{2+} taken up by a possible pinocytosis was estimated.

9. *Calculations*

A mathematical model of the three-compartment closed system and the calculations of the different kinetic parameters have been published by Borle [14]. We applied this analytical procedure to the Ca^{2+} uptake experiments involving preincubation of the cells with test substances. Three compartments are considered to be in parallel, represented schematically as follows:

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

cell membrane medium cell

S_i: amount of Ca^{2+} 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 for calculation of the fluxes from the change of Ca^{2+} uptake into membrane (index 1) and cell (index 3) as a function of time:

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

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.

Ca^{2+} uptake curves were drawn by hand and the parameters of the different compartments were determined by reading the change of Ca^{2+} concentration in the cells from the Ca^{2+} uptake curve for 1 min intervals and replotting the values thus obtained on semilogarithmic paper.

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

RESULTS

Morphology and function of isolated cells

Light microscopy of the preparation shows isolated cells which retained their in situ polarity as judged by the regional concentration of zymogen granules in one sector of the cytoplasm that is opposite to that occupied by the nucleus (Fig. 1).

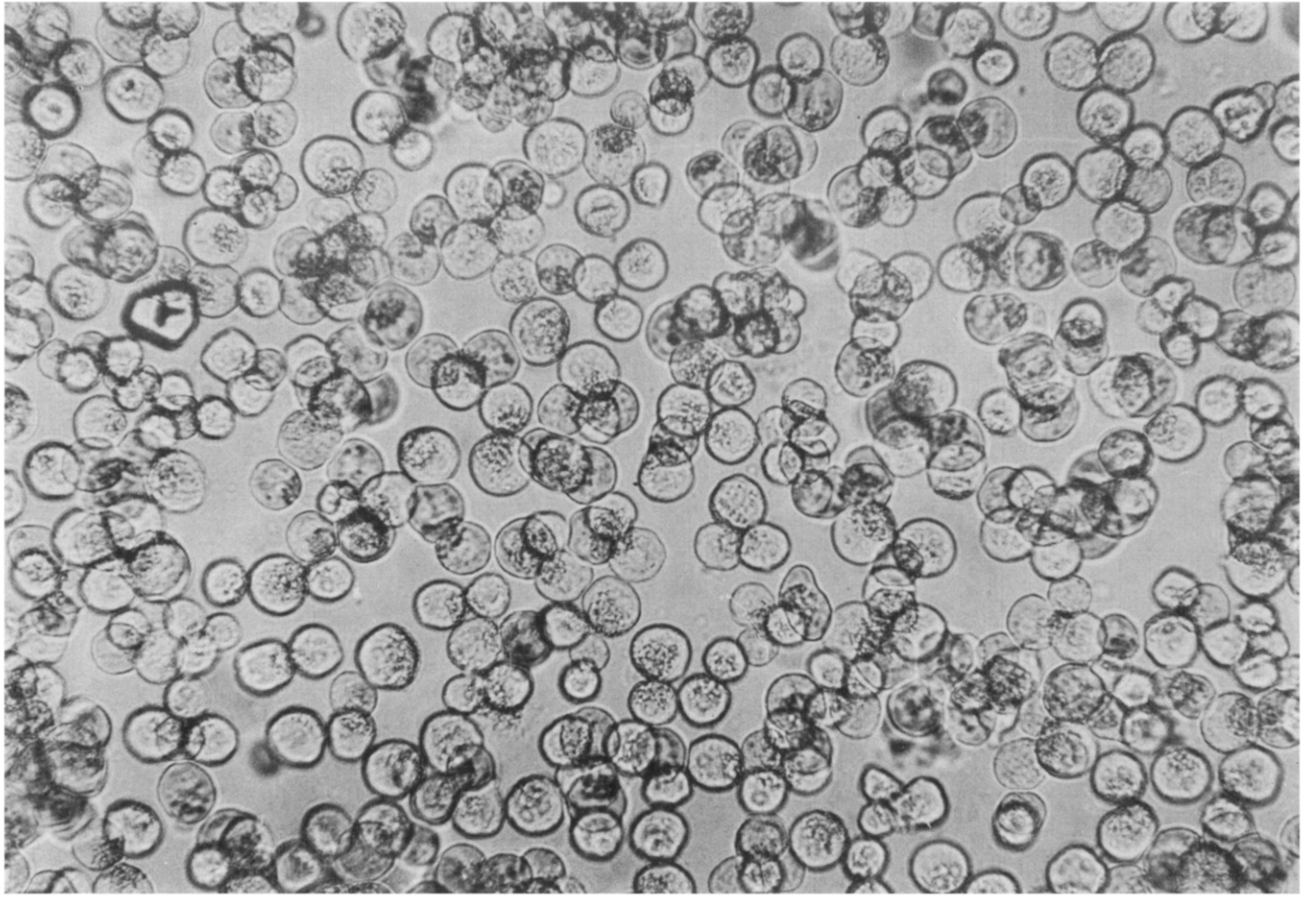

Fig. 1. Light-microscope photograph of living pancreatic exocrine cells. Magnification $\times 390$.

TABLE I

AMYLASE RELEASE STIMULATED BY HORMONES

Values represent mean ±S.E. (6 determinations out of 3 experiments) in I.U./mg protein.

	Incubation time	
	15 min	30 min
Control	0.938±0.086 (100 %)	1.4125±0.0573 (100 %)
Pancreozymin ($0.86 \cdot 10^{-7}$ M)	1.487±0.105 (+ 59 %, P < 0.005)	1.9733±0.289 (+ 40 %, P < 0.1)
Carbamylcholine (10^{-5} M)	1.525±0.105 (+ 62 %, P < 0.005)	2.288±0.098 (+ 63 %, P < 0.001)

Cell viability as evaluated by the exclusion of trypan blue dye was greater than 95 %. Amylase release, stimulated maximally by 15 and 30 min preincubation with pancreozymin and carbamylcholine, was increased by about 60 % over the control values (Table I).

Analysis of Ca^{2+} uptake

The effect of pancreozymin and of carbamylcholine on Ca^{2+} uptake into cells is shown in Fig. 2. The curves represent exponential functions, which can be demonstrated in a semilogarithmic plot versus time. Fig. 3 shows the replot of the pancreozymin stimulation curve. It reveals at least 2 components of the Ca^{2+} uptake: a fast phase, represented by the steep slope, most probably due to Ca^{2+} interaction with the

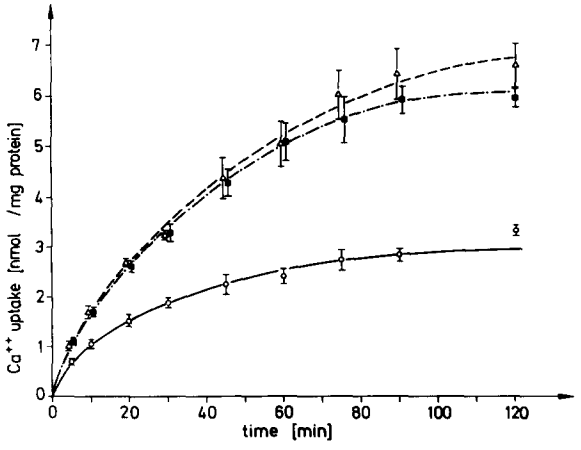

Fig. 2. Calcium uptake by pancreatic cells from 5 to 120 min. Each point represents the mean ±SEM of 6 determinations (3 separate experiments). Lines are hand-drawn curves for the best fit of the experimental points; △, in the presence of pancreozymin ($0.86 \cdot 10^{-7}$ M, 30 min preincubation); ■, in the presence of carbamylcholine (10^{-5} M, 30 min preincubation); ○, control.

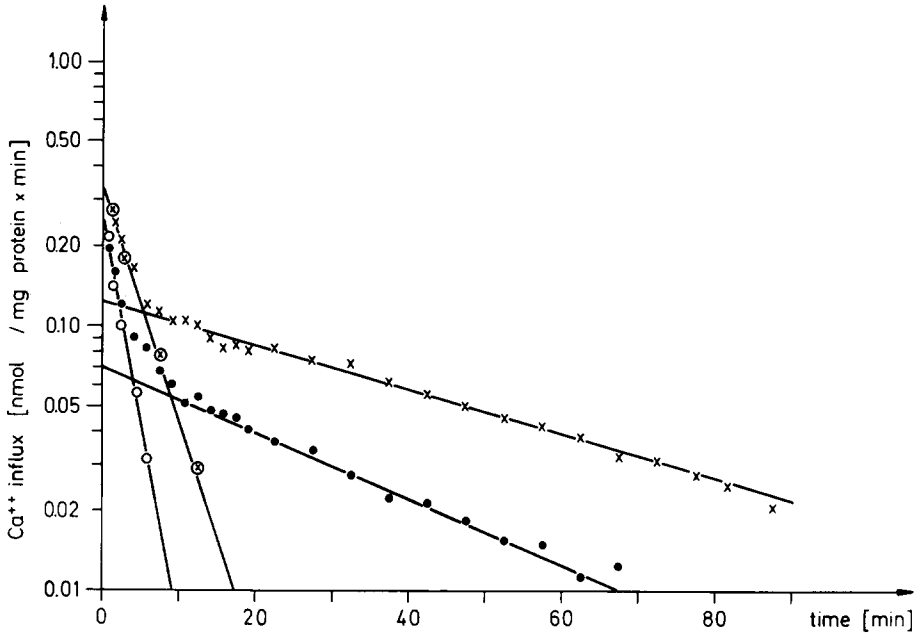

Fig. 3. Semilogarithmic plot against time of $(dR_{1+3})/(dt/E)$ derived from the hand drawn curve of Fig. 2.

cell membrane, and a slow phase which might represent Ca^{2+} uptake into the cell. From these straight lines one can assess the half times and the flow rates (intercepts on the ordinate). The rate constants and the compartment sizes can be calculated according to the equation given in Methods. The results represented in Table II show that preincubation with pancreozymin increased the flow rate of the fast phase by 31 % and that of the slow phase by 77 %. The compartment size was increased by 126 % for the fast phase and by 167 % for the slow phase. Similar effects were obtained with carbamylcholine (Table II). The half times were increased in all cases by the hormones, whereas the rate constants were decreased. This points to the possibility that the hormones increase the time necessary to reach the steady-state conditions for the Ca^{2+} exchange.

The effects of hormones on Ca^{2+} uptake were also studied on cells not preincubated with hormones (see Table II, values in parentheses). The effects on flow rate, compartment size, half times and rate constants were comparable to those obtained when the cells were preincubated with the hormones. If Ca^{45} had been exchanged completely with the cell-Ca^{2+}, the relative ^{45}Ca specific activity (cell specific activity/medium specific activity) should be equal to 1. However, our data showed that the relative specific activity of ^{45}Ca under steady state conditions was 0.049 for the control values, 0.10 for pancreozymin and 0.11 for carbamylcholine-stimulated uptake respectively, indicating that only 4.9 % of the total calcium of the cells had been exchanged under control conditions, whereas 10 % and 11 % were exchanged when incubated with hormones. Total cell Ca^{2+} was about 61 nmol/mg protein as determined by flame photometry (Table V). As compared with controls, an increase in the amount of

TABLE II

ANALYSIS OF Ca^{2+} INFLUX INTO ISOLATED PANCREATIC CELLS PREINCUBATED WITH PANCREOZYMIN AND CARBAMYLCHOLINE

Values in parentheses represent those of non-preincubated experiments.

Parameters	Control	Pancreo-zymin	Ratio pancreo-zymin/ control	Control	Carbamyl-choline	Ratio carbamyl-choline control
Slow phase:						
$\phi \left(\frac{\text{nmol}}{\text{mg prot.} \cdot \text{min}}\right)$	0.070 (0.071)	0.124 (0.098)	1.77 (1.38)	0.070 (0.073)	0.112 (0.095)	1.60 (1.31)
$t_{\frac{1}{2}}$ (min)	24.2 (26.5)	36.6 (40.0)	1.52 (1.51)	24.2 (28.8)	48.5 (36.4)	2.00 (1.25)
K (min^{-1})	0.0288 (0.0260)	0.0199 (0.0170)	0.68 (0.66)	0.0288 (0.0240)	0.0143 (0.0190)	0.50 (0.79)
$S \left(\frac{\text{nmol}}{\text{mg prot.}}\right)$	2.43 (2.71)	6.49 (5.66)	2.67 (2.09)	2.43 (3.02)	7.85 (5.00)	3.23 (1.66)
Fast phase:						
$\phi \left(\frac{\text{nmol}}{\text{mg prot.} \cdot \text{min}}\right)$	0.260 (0.450)	0.340 (0.455)	1.31 (1.01)	0.260 (0.250)	0.330 (0.380)	1.27 (1.52)
$t_{\frac{1}{2}}$ (min)	2.0 (1.1)	3.4 (1.5)	1.70 (1.25)	2.0 (2.2)	2.7 (1.7)	1.35 (0.77)
K (min^{-1})	0.356 (0.660)	0.207 (0.462)	0.58 (0.70)	0.356 (0.315)	0.253 (0.408)	0.71 (1.29)
$S \left(\frac{\text{nmol}}{\text{mg prot.}}\right)$	0.73 (0.68)	1.65 (0.99)	2.26 (1.44)	0.73 (0.791)	1.31 (0.931)	1.80 (1.17)

Ca^{2+} per mg protein of cells preincubated with hormones could not be detected. Probably the method of chemical determination is not sensitive enough to detect differences of 5 % at most, which was the calculated difference in Ca^{2+} uptake between cells incubated with hormones and controls. Based on these data (4.9 % of 61 nmol of Ca^{2+}), the compartment size $S_1 + S_3$ of exchangeable Ca^{2+} should be 2.99 nmol/mg protein, which compares well with the value of 3.16 nmol ($S_1 + S_3 = 2.43 + 0.73 = 3.16$ nmol/mg protein) obtained by compartment analysis ($S = \phi/k$, Table V). In the presence of hormones the compartment sizes obtained by tracer analysis were 7.14 and 9.16 nmol/mg protein for pancreozymin and carbamylcholine, respectively, whereas the calculated values (10 % from 61 nmol/mg protein) were 6.1 and 6.7 nmol/mg protein for pancreozymin and carbamylcholine, respectively. The values for the compartment sizes obtained by either tracer analysis or by calculation from relative specific activities are in reasonable agreement. Both approaches show hormone-induced increase of the compartment size by approximately 100–200 %. If the increase of the exchangeable Ca^{2+} compartment size under the influence of hormones would be due to an increase in cell volume, the latter would have had to increase by up to 2–3fold to that of control values. As shown in Table VI, the presence of hormones did

TABLE III

EFFECT OF HORMONES (OTHER THAN PANCREOZYMIN AND CARBAMYLCHOLINE), DIBUTYRYLIC CYCLIC NUCLEOTIDES AND THE Ca^{2+} IONOPHORE A 23187 ON Ca^{2+} UPTAKE INTO ISOLATED CELLS

Stimulation				No effect		
Substance	Concentration	Ratio slow phase/ control	Number of determinations	Substance	Concentration	Number of determinations
				Atropine	10^{-5}–10^{-4} M	4
				Adrenaline	10^{-5}–10^{-4} M	2
Gastrin I (human)	$2.27 \cdot 10^{-7}$–$2.27 \cdot 10^{-6}$ M	2.07–2.57	3	Isoproterenol	10^{-5}–10^{-4} M	2
Caerulein	0.1–0.5 μg/ml	1.49–2.12	2	Secretin	$0.33 \cdot 10^{-7}$–$16.5 \cdot 10^{-7}$ M	3
Octapeptide of pancreozymin	$5 \cdot 10^{-6}$ M	1.97	1	dbcAMP**	10^{-4}–10^{-3} M	3
Ca^{2+} ionophore (A 23187)*	5–10 μg/ml	1.50–1.80	2	dbcGMP	10^{-4}–10^{-3} M	2

* Cells were preincubated in Ca^{2+} free medium with the Ca^{2+} ionophore for 20 min. Then Ca^{2+} was added at the final concentration of 1.25 mM.

** dbc, dibutyryl cyclic.

TABLE IV

EFFECT OF D600 AND ATROPINE ON CARBAMYLCHOLINE AND PANCREOZYMIN STIMULATION OF Ca^{2+} UPTAKE INTO CELLS

Values represent mean ± S.E., (00) = number of determinations out of 2–4 experiments. Effect of hormone and hormone + inhibitor was studied in the same experiment.

Substance	Concentration	Ratio test substance/control (values after 60 min incubation)		Substance	Concentration	Ratio test substance/control (values after 60 min incubation)	
Carbamylcholine	10^{-5}–10^{-4} M	1.86 ± 0.17	(4)	Carbamyl-choline	10^{-5}–10^{-4} M	1.72 ± 0.28	(4)
Carbamylcholine + D600	10^{-5}–10^{-4} M 10^{-4}–$2 \cdot 10^{-4}$ M	1.49 ± 0.07 0.1 > P > 0.05	(4)	Carbamyl-choline + atropine	10^{-5}–10^{-4} M 10^{-5}–10^{-4} M	0.95 ± 0.04 0.05 > P > 0.02	(4)
Pancreozymin	0.86–$4.3 \cdot 10^{-7}$ M	1.99 ± 0.02	(4)	Pancreozymin	0.86–$8.6 \cdot 10^{-7}$ M	1.74 ± 0.13	(4)
Pancreozymin + D600	0.86–$4.3 \cdot 10^{-7}$ M 10^{-4}–$2 \cdot 10^{-4}$ M	1.57 ± 0.13 0.02 > P > 0.01	(4)	Pancreozymin + atropine	0.86–$8.6 \cdot 10^{-7}$ M 10^{-5}–10^{-4} M	1.63 ± 0.1 P > 0.5	(4)
D600	10^{-4}–$2 \cdot 10^{-4}$ M	1.09 ± 0.09	(3)	Atropine	10^{-5}–10^{-4} M	0.98 ± 0.2	(4)

not affect the cell volume. Also, estimation of the diameter of cells which had been incubated with hormones did not show any difference from controls when measured by means of a calibrated scale in a Leitz microscope.

When the cellular calcium concentration is expressed on the basis of the water content of the cell, it was found to be 9.45 mmol/kg cell water as calculated by data obtained by chemical analysis and 0.482, 1.090, 1.400 mmol/kg cell water for control, pancreozymin and carbamylcholine as calculated on the basis of tracer analysis. In order to compare the calcium fluxes obtained in pancreas cells with those reported in the literature for other cell systems, we estimated the surface area of the cell. As shown in Table V the mean diameter was found to be 17.9 μm$\pm$0.52 μm S.E. (25 determinations). Assuming the cells in suspension to be spheres, the surface area of one cell is 1010 μm^2 or 25.8 cm^2/mg cell protein. Calcium fluxes per surface area were calculated for the slow and fast phases. The exchange rate between compartment S_1 and medium is 0.168 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ for control, 0.218 and 0.212 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ for pancreozymin and carbamylcholine respectively.

Effect of other hormones, cyclic nucleotides and the Ca^{2+} ionophore A 23187 on Ca^{2+} uptake

As shown in Table III, other hormones which stimulate enzyme secretion in the pancreas such as gastrin, caerulein, the octapeptide of pancreozymin and the compound A 23187 also stimulate the Ca^{2+} uptake into isolated pancreas cells. Hormones which do not stimulate enzyme secretion had no influence on Ca^{2+} uptake either. Cyclic AMP and cyclic GMP or their dibutyryl derivatives, which both had a small effect on amylase release in several observations [18–23], did not increase Ca^{2+} uptake into cells; this suggests that the cyclic nucleotides do not affect the cell membrane, but that their effect may be on a later step in the stimulus-secretion coupling.

Effect of D600 on the Ca^{2+} uptake

The so-called Ca^{2+} antagonist D600, which was shown to inhibit acetylcholine-induced enzyme secretion in the isolated perfused pancreas [4] also inhibited the pancreozymin- and carbamylcholine-stimulated Ca^{2+} uptake. As compared with controls the inhibition produced by 10^{-4} M D600 was only about 40 %. Atropine is known to inhibit the acetylcholine but not the pancreozymin effect on enzyme secretion in the pancreas in vivo [24, 25] and in vitro [26, 27]. It also inhibited the Ca^{2+} uptake stimulated by carbamylcholine, but failed to inhibit the pancreozymin effect (Table IV).

Assessment of the effect of a possible pinocytosis on Ca^{2+} uptake into the cells

The volume of trapped incubation medium, as calculated from the amount of trapped [^{3}H]inulin in the pellet was less than 0.11 μl/mg protein. This volume contains 0.14 nmol of Ca^{2+}. During incubation periods of 30 to 90 min the amount of trapped extracellular medium did not increase. Moreover we could not detect any effect of pancreozymin ($0.86 \cdot 10^{-7}$ M and $0.43 \cdot 10^{-6}$ M) and carbamylcholine (10^{-5} M and 10^{-4} M) on the amount of trapped extracellular medium. This suggests that the effect of a possible pinocytosis following enzyme release was negligible on hormone-induced Ca^{2+} uptake into cells.

TABLE V

ANALYSIS OF Ca^{2+} FLUXES INTO CELL COMPARTMENTS

Values represent mean ± S.E. (00) – number of determinations

	Chemical analysis		Kinetic analysis
a) Comparison between the calcium compartment sizes of cells obtained by chemical analysis and by tracer kinetics			
Cell Ca (nmol/mg protein)			
control	61.0±8.0	(5)	3.16
pancreozymin	61.3±7.0	(6)	7.14
carbamylcholine	61.5±6.0	(6)	9.16
Cell Ca (nmol/kg cell water)			
control	9.40		0.482
pancreozymin	9.47		1.090
carbamylcholine	9.49		1.400
b) Cell parameters for calculation of Ca^{2+} fluxes			
cells/mg protein	$2.55 \pm 0.178 \cdot 10^6$	(6)	
cell diameter μm	17.9±0.52	(25)	
cell surface, μm^2/cell	1010		
cell volume cm^3/cell	$3 \cdot 10^{-9}$		
cell surface cm^2/mg protein	25.8		
water content, mg/mg protein	6.54		
c) Ca^{2+} fluxes as expressed per mg protein and per surface area			
Fast phase			
Ca^{2+} flux ϕ_{21} (nmol/mg prot. · min)			
control	0.260		
pancreozymin	0.340		
carbamylcholine	0.330		
Ca^{2+} flux ϕ_{21} (pmol $cm^{-2} \cdot s^{-1}$)			
control	0.168		
pancreozymin	0.218		
carbamylcholine	0.212		
Slow phase			
Ca^{2+} flux ϕ_{23} (nmol/mg prot. min)			
control	0.070		
pancreozymin	0.124		
carbamylcholine	0.112		
Ca^{2+} flux ϕ_{23} (pmol $cm^{-2} \cdot s^{-1}$)			
control	0.0452		
pancreozymin	0.0800		
carbamylcholine	0.0723		

TABLE VI

MEASUREMENT OF THE VOLUMES OF CELL PELLETS

The cells were incubated for 1 h with pancreozymin or carbamylcholine and without hormones. Values represent mean ± S.E. (5 determinations out of 3 experiments).

Control	5.58±0.67 μl/mg protein
Pancreozymin ($0.86 \cdot 10^{-7}$–$4.3 \cdot 10^{-7}$ M)	5.40±0.53 μl/mg protein
Carbamylcholine (10^{-5}–10^{-4} M)	5.48±0.60 μl/mg protein

DISCUSSION

The present study was undertaken in order to determine whether the secretagogues of pancreatic enzyme secretion stimulate Ca^{2+} uptake from extracellular sources or whether they act by releasing Ca^{2+} from such intracellular stores as mitochondria, microsomes or from the inner site of the cell membrane. Experiments which had been performed on the isolated pancreas and on pancreatic slices did not show increased ^{45}Ca influx following stimulation [7, 8]. Electrophysiological measurements failed to show any increase of the Ca^{2+} conductance of acinar membranes under the influence of acetylcholine or pancreozymin [9]. Thus, the current opinion of investigators working on the role of Ca^{2+} in the mechanism of pancreatic enzyme secretion is that extracellular Ca^{2+} is of no importance in hormone action but that Ca^{2+} is released from intracellular stores. From experiments on the isolated perfused cat's pancreas [4] we have concluded that there are at least two channels through which extracellular Ca^{2+} can move, an event which is related to pancreatic enzyme secretion: the channel mediating Ca^{2+} influx into the cell after rapid elevation of Ca^{2+} in the outside medium is blocked neither by atropine nor by the "Ca^{2+} antagonist" D600. The other channel, which is opened by acetylcholine, can be blocked by D600 as well as by atropine. As the effect of pancreozymin could not be blocked by these inhibitors, we had no direct evidence for a pancreozymin-influenced Ca^{2+} channel, and we concluded that extracellular Ca^{2+} is not as important for the action of pancreozymin as it is for the action of acetylcholine. In order to measure Ca^{2+} uptake into pancreatic cells directly, we decided to use isolated cells. This method probably allows the detection of smaller changes of Ca^{2+} uptake under the influence of test substances than when slice techniques are used, as a considerable extracellular space has to be taken into account in preparations such as slices, and small changes of Ca^{2+} uptake into the cells could be masked. This might be the reason why previous investigators were not able to detect an increase of Ca^{2+} influx under the influence of secretagogues [7, 8].

Our data show that, in contrast to the current view in this field, secretagogues do stimulate Ca^{2+} uptake into pancreatic cells. The data presented in Table II and III indicate that there are at least two calcium compartments as demonstrated by kinetic analysis of Ca^{2+} influx into pancreatic cells. One compartment has a rapidly exchangeable Ca^{2+} pool whereas the other one showed a slow component of Ca^{2+} influx. The slow-exchanging component has a rate of exchange which is compatible with an intracellular compartment and presumably represents uptake into the cytoplasma and into subcellular components such as mitochondria, endoplasmatic reticulum and zymogen granules. When the exchange rate of the slow phase is expressed as flux per surface area of the cell, a comparison of the values obtained in this study with those from the literature can be made. The exchange rate of the slow phase (0.0452 pmol $\cdot$ $cm^{-1} \cdot s^{-1}$ for control and 0.0800 and 0.0723 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ for pancreozymin and carbamylcholine respectively) is in agreement with the transmembrane calcium influx of 0.076 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ in squid axon [15], of 0.094 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ in sartorius muscle [16] and of 0.016–0.048 pmol $\cdot$ $cm^{-2} \cdot s^{-1}$ in heart muscle [17]. The similarity between our results and those obtained in nerve and muscle supports our assumption that the slow component of exchange in pancreas cells does represent calcium exchange between an intracellular pool and the medium, whereas the fast

phase of exchange, which is of a higher magnitude than those reported for transmembrane calcium fluxes in nerve and muscle [15–17], more probably represents a calcium compartment of the plasma membrane and interaction of Ca^{2+} with the surface of the cell membrane or the quick influx into the intracellular submembranous spaces.

Pancreozymin and carbamylcholine stimulated the Ca^{2+} influx of both the slow and the fast phases (Table II) when the cells were preincubated with the hormones. When the hormones were not added to the preincubation medium but added together with ^{45}Ca to the medium at 0 time, the qualitative effects of the hormones were comparable (values in parentheses in Table II). It is difficult to decide whether the data obtained for the fast phase could be delimited precisely enough from the slow phase to conclude anything of physiological relevance. At least one can say that both stimulants increased the influx of Ca^{2+} of the slow phase as well as its pool size, which would be compatible with the interpretation that they act by increasing the Ca^{2+} transfer from the outside into the inside of the cell by a change of the membrane permeability for Ca^{2+}.

It would be desirable to know the total Ca^{2+} content of the outer phase of the membrane as well as of the inside of the cell to see whether our data obtained by kinetic analysis correspond to those values. Such determinations would require methods to either strip the cell from the cell coat by treatment with trypsin and EGTA (ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid) as has been performed by Borle [28] or by replacing extracellular Ca^{2+} by La^{2+} which displaces Ca^{2+} from its binding sites but does not move into the cell itself [29]. Such experiments are being performed in our laboratory. As only 5 % of total cell calcium was exchangeable with ^{45}Ca, the question arises whether such an almost unexchangeable Ca^{2+} pool could release sequestered Ca^{2+} by hormone stimulation and increase the cellular Ca^{2+} concentration, so that more Ca^{2+} would be available for exchange with ^{45}Ca, resulting in an increased ^{45}Ca influx. Such a mechanism would require a coupled exchange of Ca^{2+} so that for an increased Ca^{2+} efflux an increased ^{45}Ca influx could occur. If such a coupled exchange mechanism was present in the acinar cell membrane, the extracellular Ca^{2+} concentration should determine the rate of Ca^{2+} efflux from ^{45}Ca-preloaded cells. In recent unpublished observations, we did not find any decrease of hormone-stimulated ^{45}Ca efflux from pancreatic cells when Ca^{2+} was reduced or omitted in the bath medium which contained 10^{-5} M EGTA. Considering this finding together with our observations that the Ca^{2+} influx and the pool size were increased by pancreozymin and acetylcholine, the most likely interpretation is that Ca^{2+} influx into the cell is increased by an increase of the cell membrane permeability for Ca^{2+}. However, as the kinetic analysis treats the total intracellular calcium as a homogenous compartment, it could also be possible that the rate of accumulation of ^{45}Ca is determined by an uptake or exchange step at the level of an intracellular membrane and not at the plasma membrane. If the hormone does not penetrate the cell but interacts with a receptor on the cell membrane, this intracellular rate-limiting step would have to be mediated by an intracellular messenger. This second messenger could be neither cyclic AMP nor cyclic GMP as neither of their dibutyryl derivatives had an effect on Ca^{2+} uptake (Table III), although both exert an effect on enzyme release in the intact organ [4, 30], and the involvement of cyclic nucleotides as second messengers in the action of pancreozymin is well supported experimentally [19, 23, 33–35].

All other compounds which have been shown to stimulate amylase release from the pancreas, also stimulate Ca^{2+} uptake into the cells; whereas substances which have no effect on enzyme secretion also fail to stimulate Ca^{2+} uptake (Table III). Since the effect of the Ca^{2+} ionophore A 23187 was in the same range as that of pancreozymin, its octapeptide and carbamylcholine, one can suggest that the hormones act like a Ca^{2+} ionophore by opening channels for Ca^{2+} in the cell membrane rather than in an intracellular membrane.

From our data one can consider 3 different Ca^{2+} pathways through the cell membrane, of which one is mediated by pancreozymin, one by carbamylcholine and one is independent of both. As these pathways are inhibited by the same inhibitors which also inhibit their specific effects on enzyme secretion (Table IV), one can conclude that the effect of the hormones on enzyme secretion involves increased uptake of Ca^{2+} into the cell. Cell Ca^{2+} might then act synergistically with cyclic nucleotides at a later step of the stimulus-secretion coupling, e.g. at the level of fusion of zymogen granules with the luminal acinar membrane. It has been suggested that Ca^{2+} and cyclic nucleotides are necessary in the latter process [31, 32].

From the present data we cannot say anything about the mechanism of the Ca^{2+} uptake, i.e. whether it is proceeding through channels or through carriers. In the latter case it would be interesting to know whether the hormones stimulate the calcium transport by increasing the rate of transfer of the calcium carrier complex across the membrane, by increasing the carrier concentration or by raising its binding affinity for calcium. Further work is necessary to answer those questions.

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