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AMYLASE RELEASE FROM DISSOCIATED MOUSE PANCREATIC ACINAR CELLS STIMULATED BY GLUCAGON: EFFECT OF MEMBRANE STABILIZERS

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SUMMARY

1. The effect of membrane stabilizers and cytochalasin-B on amylase secretion, basal and induced by ionophore A23187, CCK-PZ, bethanechol and glucagon, was studied in dissociated mouse pancreatic acinar cells.

2. Cytochalasin-B did not affect basal or secretagogue-stimulated amylase secretion.

3. Membrane stabilizers [thymol (10^{-7}–10^{-4} M), chlorpromazine (10^{-7}–10^{-4} M) and propranolol (10^{-7}–10^{-5} M) did not alter basal release of amylase. At higher concentrations of thymol (10^{-3} M), chlorpromazine (10^{-3} M) and propranolol (10^{-4} M), dissociated acinar cells were lysed as indicated by an increase in release of lactic dehydrogenase (LDH).

4. Ionophore A23187, CCK-PZ (maximal effective concentrations, 0·01 u. ml.$^{-1}$), bethanechol (maximal effective concentrations, 10^{-4} M) and glucagon increased amylase secretion in a dose-dependent fashion. Concentrations of CCK-PZ and bethanechol beyond optimal levels decreased amylase secretion. Concentrations of ionophore A23187 and glucagon when tested beyond 10^{-6} M and 10^{-4} M respectively increased the release of LDH. In concentrations that were non-toxic, membrane stabilizers blocked the stimulating effect of chlolecystokinin-pancreozymin and bethanechol on amylase secretion but did not alter the response to A23187 and glucagon.

5. Unlike bethanechol, glucagon neither increased the uptake of ^{45}Ca nor did it alter the release of ^{45}Ca from cells previously loaded with $^{45}CaCl_2$.

6. These data provide evidence that stimulus–secretion coupling in dissociated pancreatic acinar cells is basically similar to cells *in situ*. The effect of glucagon is consistent with the model in which hormone-dependent mobilization of Ca^{2+} from intra- or extracellular sources is bypassed leading to digestive enzyme secretion.

INTRODUCTION

A group of membrane-active amphiphilic drugs have been employed to study the amylase release process from the pancreas (Singh, Black & Webster, 1973; Beaudoin, Marois, Dunnigan & Morisset, 1974; Williams, Poulsen & Lee, 1977). At lower concen-

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trations, these drugs stabilize membranes and lead to inhibition of stimulus–secretion coupling; higher concentrations lead to membrane destabilization and lysis of cells. It has been observed that glucagon increases amylase secretion from dissociated mouse pancreatic acinar cells (Singh, 1978, 1980*b*). Recently, other investigators (see Manabe & Steer, 1979) reported a concentration-dependent stimulation of amylase secretion in response to glucagon from mouse pancreatic fragments. These findings indicate a direct stimulatory effect of glucagon which is contrary to its inhibitory (possibly indirect) effect on pancreatic secretion stimulated by secretin or secretin + CCK-PZ *in vivo* (Dyck, Rudick, Hoexter & Janowitz, 1969; Nakajima & Magee, 1970; Konturek, Tasler & Obtulowicz, 1973, 1974; Shaw & Heath, 1973; Clain, Barbezat, Waterworth & Bank, 1978).

It is not clear at present whether the mechanism of secretion in dissociated cells is similar or dissimilar to acinar cells *in situ* (see Case, 1978). It was reported by some (Williams, 1977) but not by others (Amsterdam & Jamieson, 1974*a*) that the cell dissociation procedure causes loss of organization of the apical membrane complex of microtubules and microfilaments, leading to partial loss of luminal specialization of the plasma membrane of acinar cells. In the present study, the ability of membrane stabilizers to inhibit steps in stimulus–secretion coupling was utilized to validate dissociated acinar cells as a model for secretion and to study the mechanism of glucagon-stimulated amylase secretion *in vitro*.

METHODS

Preparation of dissociated mouse pancreatic acinar cells. All studies were carried out on pancreases from male outbred Swiss mice (ARS/Sprague Dawley, Madison, WI, U.S.A.).

Glands were collected from five to eight mice which had an average weight of 25 g. After an overnight fast, the animals were decapitated and exsanguinated. The glands were trimmed free of mesentery and fat and weighed to ~ 800 mg as the starting material. The basic incubation solution used to isolate cells was Krebs Ringer HEPES medium containing 120 mM-NaCl, 6 mM-KCl, 15 mM-HEPES, 1 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 14 mM-glucose, 2 mM-L-glutamine, 0·01 % w/v soybean trypsin inhibitor (chromatographically purified) and 2 % v/v minimal Eagle's medium amino acid supplement (Eagle, 1959). The digestion medium also contained 0·1 mM-$CaCl_2$, collagenase 0·75 mg ml.$^{-1}$ (Sigma, Type I) and 1·5 mg ml.$^{-1}$ hyaluronidase (Sigma, Type I). Dissociated acinar cells were prepared by modifications of techniques developed by Amsterdam & Jamieson (1974*a, b*) as described in detail in an earlier publication (Singh, 1980*b*). Cell counts showed that more than 95 % of the cells were acinar and were intact by trypan Blue exclusion studies. Characterization of isolated cells, including intracellular ion content and response to secretagogues, is reported elsewhere (Singh, 1980*b*).

Secretory studies with CCK-PZ, bethanechol, glucagon, A23187 and surface-active drugs. The cell suspension was pre-incubated for 30 min at 37 °C with the gas phase of 100 % O_2 at 60 oscillations min^{-1}. The cells were pelleted by centrifugation for 5 min at 50 × *g* and resuspended in ten volumes of the incubation medium. Before the start of secretory studies, the cell suspension was distributed in 0·5 ml. aliquots. One 0·5 ml. aliquot was used to determine time-zero amylase and lactic dehydrogenase (LDH) by centrifugation in a Beckman Microfuge-B at ~ 10,000 rev/min for 1 min. The pellet was resuspended in 1 ml. distilled H_2O, transferred to a tared aluminium foil planchet, dried overnight at 80 °C and the dry weight determined. In some experiments aliquots of cell suspensions were treated with equal volume of 2·1 N-perchloric acid and protein content of the pellets was determined by the biuret method (Gornall, Bardawill & David, 1969). The rest of the 0·5 ml. aliquots of cell suspension were used for secretory studies. Appropriate concentrations of secretagogues (CCK-PZ, bethanechol, glucagon, A23187), cytochalasin-B, and membrane stabilizers (thymol, chlorpromazine and propranolol) were added to the cell suspension in a final incubation volume of 5 ml. ($\sim$ 2 mg dry wt. $ml.^{-1}$ or 0·6 mg protein $ml.^{-1}$) and incubated at 37 °C (60 oscillations min^{-1}) for 60 min under gas phase of 100 % O_2 (pH 7·4). At the end of the incubation, the medium and cells were separated by centrifugation in a Beckman Microfuge-B. The cells were suspended in twice-distilled water and sonicated (Sonic Dismembrator, Fisher, Model 300). The media and sonicated cells were used for amylase assay by the method described by Bernfeld (1955) using Lintner's starch as a substrate.

Because A23187 and cytochalasin-B were prepared in stock solution with dimethylsulphoxide (DMSO), studies involving ionophore A23187 and cytochalasin-B also contained 0·1 % DMSO in the control flasks. Previous studies had shown that DMSO at 0·1 % concentration has no effect on amylase secretion from rat pancreas (Singh, 1979). Lactic dehydrogenase was measured spectrophotometrically by the method described by Kornberg (1955) as used previously (Singh, 1979).

Measurement of ^{45}Ca uptake and release. These studies were done by modifications of methods described by Lucas, Schmidt, Kromas & Loffler (1978).

^{45}Ca uptake. For ^{45}Ca uptake studies, 0·5 ml. aliquots of cells were pre-incubated for 30 min with or without secretagogues to reach steady-state condition and the final incubation was carried out with or without secretagogues in a volume of 5 ml. of media containing ^{45}Ca, 0·4 $\mu Ci\ ml.^{-1}$. Aliquots of 0·2 ml. were taken out at 10 min intervals for up to 100 min and were transferred to Eppendorf tubes and centrifuged for 1 min at 10,000 × *g*. The cell pellets were washed 3 times with Krebs Ringer HEPES buffer (without $CaCl_2$ and $MgSO_4$ but containing 2 mM-EGTA so as not to disturb the cell pellet). The cell pellets were suspended by stirring in 50 μl. water to which 0·4 ml. 50 % of Hyamine in ethanol (v/v) was added and the mixture incubated at 56 °C for 120 min. Counting of the samples was done by the method used previously (Singh, 1980*a*).

^{45}Ca release. 0·5 ml. aliquots of the final suspension were pre-incubated for 30 min as in the secretory studies. The cells were distributed to vials containing 0·4 μCi $^{45}CaCl_2$ in a final volume of 5 ml. After 60 min incubation, secretagogues were added at the desired concentration without changing the medium. At 2, 5, 10 and 20 min intervals, 0·2 ml. aliquots of cell suspension were removed, spun at × 10,000 – *g* for 1 min and treated similarly to the samples in the ^{45}Ca uptake studies.

Measurement of the extracellular space. Following a 30 min pre-incubation, cell suspensions were incubated with 0·4 μCi ^3H[sucrose] in the presence or absence of glucagon or bethanechol for 15, 30, 45 and 60 min. Aliquots of 0·2 ml. were placed in Eppendorf tubes and centrifuged at $\sim$ 10,000 *g* for 1 min. ^3H radioactivity in the pelleted cells was determined as in ^{45}Ca uptake studies. Extracellular space was calculated from the specific activity of ^3H[sucrose] in the medium and expressed as $\mu l.\ mg^{-1}$ pelleted protein, following the procedure of Lucas *et al.* (1978).

^{45}Ca (specific activity 25·5 mCi mg^{-1}) was purchased from International Chemical Nuclear Pharmaceuticals Inc., Cleveland, OH, U.S.A. [^3H]sucrose (specific activity 4·79 Ci m-$mole^{-1}$) was purchased from New England Nuclear, Boston, MA, U.S.A. Glucagon was purchased from the Sigma Chemical Co., St Louis, MO, U.S.A. Cholecystokinin-pancreozymin (CCK-PZ) was purchased from the Gastrointestinal Hormone Research Unit, Karolinska Institute, Stockholm, Sweden, and ionophore A23187 was a gift from Dr R. J. Hosley of the Eli Lilly Company Inc., Indianapolis, IN, U.S.A. Chlorpromazine was kindly provided by the Smith, Kline & French Laboratories, Sunnyvale, CA, U.S.A. All other chemicals were obtained from commercial sources and were of the highest purity available.

Calculations. The activities of amylase and LDH released into the medium at the end of 60 min incubation minus the amylase and LDH activities of the supernatant at the start of the incubation plus the amount remaining in the tissue at the end of the incubation were considered as total amylase and LDH and were defined as 100 %. Secretion of amylase and release of LDH into the medium were expressed as % of total. Data on ^{45}Ca uptake and release (after correction for extracellular space) were calculated as n-mole ^{45}Ca per mg protein based on the specific activity of the loading medium and expressed as such at various times. Student's *t* test (Snedecor & Cochran, 1975) for unpaired groups was used to analyse the data presented as means ± S.E. of means.

RESULTS

Fig. 1 shows that thymol at concentrations of 10^{-7}–10^{-4} M did not significantly alter basal amylase secretion and LDH release from dissociated mouse pancreatic acinar cells; at 10^{-3} M, it increased amylase secretion (+315 %) and LDH release

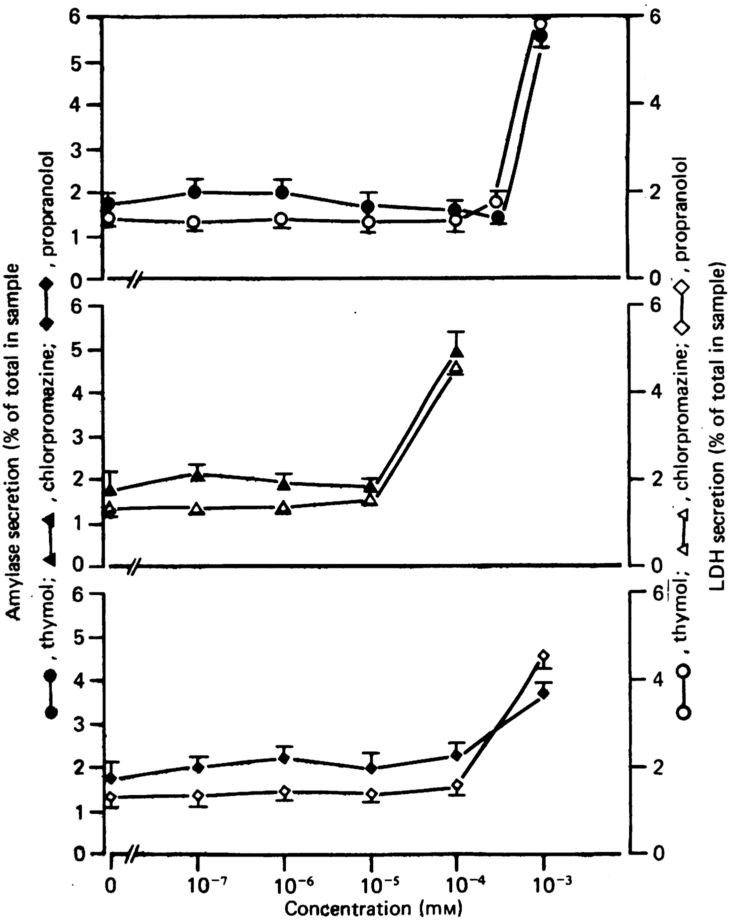

Fig. 1. Effect of membrane stabilizers on basal amylase secretion and LDH release from dissociated acinar cells of mouse pancreas. Amylase secretion is plotted as a function of the added concentration of membrane stabilizers. All values are mean ± S.E. of mean of four experiments.

(+332 %). Chlopromizine at concentrations of 10^{-7}–10^{-5} M did not significantly alter basal amylase secretion and LDH release; at 10^{-4} M, it significantly increased amylase secretion (+287 %) and LDH release (+228 %). Propranolol at concentrations of 10^{-7}–10^{-4} M did not significantly alter basal amylase secretion and LDH release; at 10^{-3} M, it increased amylase secretion (+216 %) and LDH release (+232 %).

Fig. 2 shows that cholecystokinin-pancreozymin (CCK-PZ) increased amylase secretion from dissociated mouse pancreatic acinar cells in a dose-dependent fashion, with a peak effect observed with 0·01 μ. ml.$^{-1}$ (+110 %) and decreases at 0·1 and

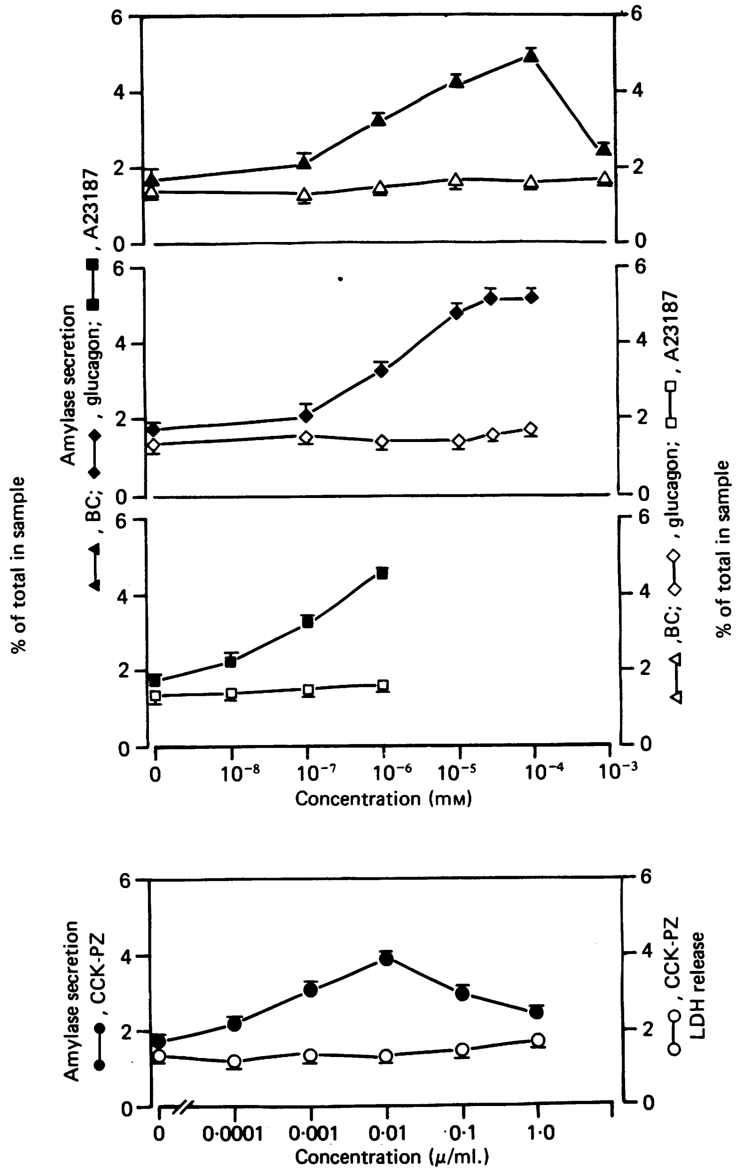

Fig. 2. Effect of secretagogues on stimulated amylase secretion and LDH release from dissociated acinar cells of mouse pancreas. Amylase secretion was stimulated with bethanechol (BC), glucagon, ionophore A23187, CCK-PZ and plotted as a function of the added concentration of secretagogues. All values are mean ± S.E. of mean of four to eight experiments.

1 u. ml.$^{-1}$. Bethanechol also increased amylase secretion in a dose-dependent fashion, with a peak effect observed at 10^{-4} M (+178 %). Glucagon increased amylase secretion in a progressive manner with +192 % increase at 10^{-4} M. Ionophore A23187 employed in concentrations (10^{-6} M) which have been shown not to cause Ca^{2+}-dependent damage to acinar cells (see Chandler & Williams, 1977) also increased amylase

TABLE 1. Effect of membrane stabilizers and cytochalasin-B on basal, CCK-PZ, bethanechol, glucagon and A23187 stimulated amylase secretion from dissociated acinar cells of mouse pancreas

	Amylase secretion (% of total in medium)				
Addition	Basal	CCK-PZ (0·01 u. ml.$^{-1}$)	Bethanechol (10^{-4} M)	Glucagon (10^{-4} M)	A23187 (10^{-6} M)
0	1·78 ± 0·19	3·75 ± 0·33[b]	4·96 ± 0·43[d]	5·21 ± 0·61[b]	4·52 ± 0·49[b]
Cytochalasin-B (5 μg ml.$^{-1}$)	1·68 ± 0·18	3·26 ± 0·31[b]	3·92 ± 0·41[c]	5·38 ± 0·99[a]	4·49 ± 0·49[b]
Thymol (10^{-4} M)	1·65 ± 0·21	1·75 ± 0·19	2·41 ± 0·31	5·37 ± 0·83[b]	4·21 ± 0·48[b]
Chlorpromazine (10^{-5} M)	1·89 ± 0·27	2·41 ± 0·26	1·97 ± 0·28	4·61 ± 0·54[b]	4·81 ± 0·51[b]
Propranolol (10^{-4} M)	1·73 ± 0·26	1·71 ± 0·18	2·55 ± 0·34	3·88 ± 0·49[a]	4·63 ± 0·41[c]

All values are mean ± S.E. of mean of four experiments. Statistical comparisons are between amylase secretion without additions or in media containing indicated concentration of cytochalasin-B or a membrane stabilizer (thymol, chlorpromazine and propranolol) to amylase secretion stimulated with indicated concentrations of the CCK-PZ, bethanechol, glucagon or A23187 in identical media. *a*, $P < 0{\cdot}05$; *b*, $P < 0{\cdot}025$; *c*, $P < 0{\cdot}01$; *d*, $P < 0{\cdot}005$.

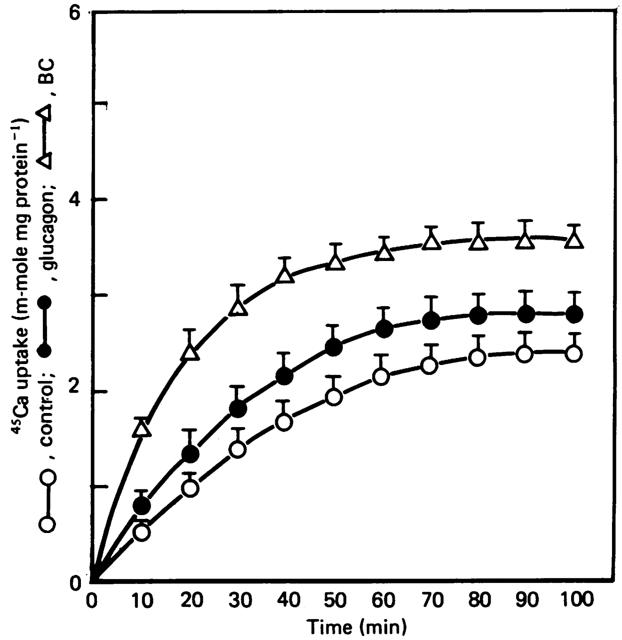

Fig. 3. ^{45}Ca uptake curves from dissociated acinar cells of mouse pancreas due to glucagon (5×10^{-5} M) or bethanechol (10^{-4} M). Each point is the mean of six experiments. For experimental details see Methods. Incubations were done in a medium without KH_2PO_4, and $MgCl_2$ was substituted for $MgSO_4$.

secretion (+154%). LDH release was not increased significantly by any of these secretagogues.

Table 1 shows the effect of membrane stabilizers and cytochalasin-B on amylase secretion stimulated by maximal doses of secretagogues. Cytochalasin-B (5 μg ml.$^{-1}$)

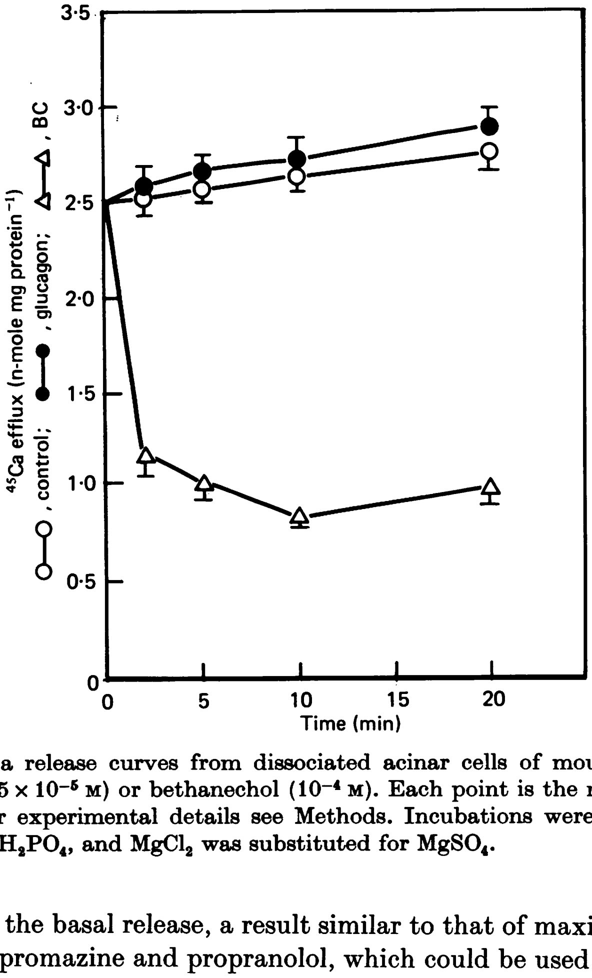

Fig. 4. ^{45}Ca release curves from dissociated acinar cells of mouse pancreas due to glucagon (5×10^{-5} M) or bethanechol (10^{-4} M). Each point is the mean of four experiments. For experimental details see Methods. Incubations were done in a medium without KH_2PO_4, and $MgCl_2$ was substituted for $MgSO_4$.

did not affect the basal release, a result similar to that of maximal concentrations of thymol, chlorpromazine and propranolol, which could be used without cell lysis (see Fig. 2). Cholecystokinin-pancreozymin and bethanechol-induced amylase secretion was inhibited by thymol, chlorpromazine and propranolol but not by cytochalasin-B. Amylase secretion induced by glucagon (10^{-5} M) or ionophore A23187 was not inhibited by any of these agents.

Since membrane stabilizers did not inhibit A23187- and glucagon-stimulated secretion it was of interest to study the effect of glucagon on ^{45}Ca uptake and release. Fig. 3 shows ^{45}Ca uptake by dissociated pancreatic acinar cells. After pre-incubation of dissociated cells with glucagon or bethanechol in the presence of 2·5 mM-$CaCl_2$, addition of $^{45}CaCl_2$ resulted in accumulation of ^{45}Ca, reaching a plateau at 60 min. Bethanechol increased the rate of uptake of $^{45}CaCl_2$ but the increase in the net rate of uptake of ^{45}Ca by glucagon was not significantly different from controls at the time points studied.

Fig. 4 shows the effect of glucagon and bethanechol on net ^{45}Ca content of dissociated pancreatic acinar cells pre-loaded with $^{45}CaCl_2$ in the presence of 2·5 mM-$CaCl_2$ for 60 min to reach steady-state conditions. Addition of bethanechol decreased the intracellular ^{45}Ca content to 34 % of control value at 10 min. Glucagon did not significantly affect ^{45}Ca efflux.

The extracellular space of the cell pellet as determined by [^3H]sucrose at any time point (see Methods) was less than 0·12 μl. mg^{-1} protein and was not affected by bethanechol or glucagon. For both control, bethanechol and glucagon studies on ^{45}Ca uptake, the error for determining intracellular space amounted to < 6 %.

DISCUSSION

Due to theoretical advantages in studying physiological phenomena in single cells (especially the ability to control the extracellular environment rapidly and predictably and a very small extracellular space so that small fluxes of ions critical for secretion can be detected), investigators have tried to use dissociated pancreatic acinar cells obtained by combined procedures of digestion of tissue by enzymes, mechanical disruption and Ca^{2+} chelation with EGTA or EDTA (Amsterdam & Jamieson, 1974*a*; Gardner, Conlon, Klaeveman, Adams & Ondetti, 1975; Williams, Cary & Moffat, 1976; Kondo & Schulz, 1976; Kempen, DePont & Bonting, 1977; Renckens, Schrijen, Swarts, DePont & Bonting, 1978; Case & Clausen (see Case, 1978); Singh, 1978, 1980*b*). However, in practice, it has been observed that dissociated cells respond poorly to physiological secretagogues (Kondo & Schulz, 1976), possibly due to damage of cell surface receptors (Case, 1978) and need about a tenfold greater concentration of agonists than intact tissue to elicit enzyme secretion (Amsterdam & Jamieson, 1974*b*; Williams *et al.* 1976). In addition, freshly prepared cells leak enzymes in the early post-preparation phase (Gardner *et al.* 1975), suggesting that plasma membrane function may have been altered by dissociation procedures. Williams (1977) reported that isolated cells, while retaining over-all polarity, lost microfilaments and microvillous structures so characteristic of apical membrane, whereas other investigators reported no such loss (Amsterdam & Jamieson, 1974*b*). Kempen *et al.* (1977) reported decreased recovery of membrane-associated enzyme activity viz. adenylate-cyclase and high K_m phosphodiesterase from isolated rat pancreatic cells. On electron microscopy dissociated cells did not show evidence of exocytosis (Williams *et al.* 1976) which has been considered as a hallmark for secretion by some (Jamieson & Palade, 1977) and contested by those who believe in a diffusion-like process accounting for protein secretion by the pancreas (Isenman & Rothman, 1979). Since previous studies would point to membrane damage during dissociation of cell, the present study was done to determine the effect of membrane-active drugs on isolated cells to validate them as a model for such studies, while maintaining a distinction between the active secretion from passive leakage of enzymes.

Local anaesthetics are known to act as membrane stabilizers due to their surface-active properties (Singh *et al.* 1973; Beaudoin *et al.* 1974; Williams & Lee, 1974). Williams *et al.* (1977) reported that this activity was not unique to local anaesthetics and could be shown with such drugs as chlorpromazine, propranolol and the simple detergent, thymol. These agents inhibited A23187 and bethanechol-induced secretion, blocked bethanechol-induced depolarization and stimulation of ^{45}Ca efflux in mouse pancreatic acinar cells. In the present study, the role of these agents in the mechanism of amylase secretion from dissociated acinar cells was studied with particular emphasis on glucagon-induced enzyme secretion. As is true of their action on pancreatic fragments, basal amylase secretion from dissociated cells was not affected by the membrane stabilizers, whereas stimulated secretion was abolished. Higher concentrations of membrane stabilizers proved toxic to acinar cells, resulting in destabilization or lysis of the cell membranes as evidenced by increased release of LDH. The lack of response of dissociated cells to cytochalasin-B in the basal or stimulated state was consistent with previous observations on pancreatic cells reported by Williams (1977). Cytochalasin-B did not block glucagon-induced amylase secretion in dissociated cells.

Since bethanechol-induced depolarization is considered to be due to opening of ionic channels for Na^+ and K^+ (Nishiyama & Petersen, 1975), the inhibitory action of membrane stabilizers on amylase secretion in the present study may be due to lack of Na^+–Ca^{2+} exchange in the pancreatic acinar cells. This observation indicates that cell surface receptors in the cells dissociated by the present technique were preserved. Surface-active drugs prevented amylase secretion stimulated by bethanechol and CCK-PZ, possibly due to blocking of the depolarization and a rise in intracellular Ca^{2+}. This is further supported by the observation that artificial introduction of Ca^{2+} into the acinar cells with A23187 increased amylase secretion even in the presence of membrane stabilizers. This finding is in contrast to the previous observations and the reason for this discrepancy between pancreatic fragments (Williams *et al.* 1977) and dissociated cells (present study) is not apparent. Glucagon increased amylase secretion from dissociated cells. ^{45}Ca uptake and release studies revealed that glucagon, unlike bethanechol, did not increase ^{45}Ca release or ^{45}Ca uptake in pancreatic acinar cells. The present studies show that the process of secretion from dissociated cells in response to physiological secretagogues is similar to acinar cells *in situ*. The results with glucagon are consistent with a model in which hormone-dependent mobilization of Ca^{2+} from intracellular or extracellular sources is bypassed, leading to digestive enzyme secretion.

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