J. Membrane Biol. 33, 185–195 (1977)

Effects of Membrane Stabilizers on Pancreatic Amylase Release

John A. Williams, Jørgen Hedemark Poulsen, and Mark Lee

Department of Physiology, University of California, San Francisco, California 94143

Received 13 September 1976

Summary. Compounds with membrane stabilizing activity were studied as to their ability to affect pancreatic amylase release and the steps in the stimulus-secretion coupling process. Chlorpromazine, propranolol, and thymol were all found to inhibit bethanechol-stimulated amylase release and at slightly higher concentrations to induce release regardless of the presence of the secretagogue. This biphasic effect was similar to that found previously for the local anesthetic tetracaine. Release by high concentrations of propranolol and tetracaine was accompanied by ultrastructural evidence of cell damage. Membrane stabilizers at concentrations which inhibited amylase release were shown to block bethanechol-induced depolarization and stimulation of $^{45}Ca^{++}$ efflux although the drugs alone partially depolarized pancreatic cells. Release of amylase induced by Ca^{++} introduced by the ionophore A23187 was also abolished. These findings indicate that membrane stabilizers independently inhibit the steps leading to a rise in intracellular Ca^{++} and the subsequent Ca^{++}-activated amylase release.

Activation of pancreatic enzyme release by cholinergic agonists and gastrointestinal hormones is believed to involve interaction of the secretagogue with a membrane receptor (Galardy & Jamieson, 1975), depolarization and release of intracellular Ca^{++} (Matthews, Petersen & Williams, 1973), with the rise in cytoplasmic Ca^{++} promoting release of zymogen granule contents by exocytosis (Palade, 1975). A similar mechanism applies to other secretory tissues although in many cases Ca^{++} enters from the outside through membrane channels opened by the secretagogue (Douglas, 1968). Local anesthetics block secretion by a number of glands including the exocrine pancreas (Beaudoin, Marois, Dunnigen & Morisset, 1974; Williams & Lee, 1974), endocrine pancreas (Bressler & Brendel, 1971; Ammon, Orci & Steinke, 1973) and the adrenal medulla (Douglas & Kanno, 1967; Jaanus, Miele & Rubin, 1967). In the case of the adrenal this has been shown to be accompanied by blockage of ACh-activated Ca^{++} channels (Douglas & Kanno, 1967). By contrast, in the pancreatic acinar cell, tetracaine was shown to block one of the

later steps in stimulus-secretion coupling, namely the release of amylase induced by Ca^{++} (Williams & Lee, 1974).

We now present data showing that inhibition of pancreatic amylase release is not specific to tetracaine but is reproduced by other drugs having in common the ability to act as membrane stabilizers (Seeman, 1972). Furthermore, these drugs inhibit at multiple sites blocking steps in stimulus secretion-coupling involving both the basal and apical membrane. Release of amylase by high concentration of membrane stabilizers is shown to occur as a result of cell damage.

Materials and Methods

All studies were carried out using pancreases from male White Swiss mice (18–22 g) which had been fasted overnight prior to use. Measurement of amylase release was carried out as previously reported (Williams & Chandler, 1975). Briefly, each pancreas was divided into 4–5 fragments weighing 15–25 mg which were incubated separately in 3 ml of Krebs-Henseleit bicarbonate (KHB) buffer at 37 °C. In all cases a 30-min preincubation period to allow drug penetration and equilibration was followed by a 30-min incubation. In studies using the Ca^{++} ionophore A23187, preincubation was in medium without added divalent cations to allow incorporation of ionophore into cellular membranes without affecting amylase release (Chandler & Williams, 1977). A23187 was dissolved in ethanol and a similar amount of ethanol was added to control flasks so that all contained 0.33% ethanol. Amylase released into the medium was assayed according to the method of Rinderknecht, Wilding and Haverback (1967) using amylose azure blue as the substrate. Amylase is reported in International units, based on the reported activity of the standard α-amylase (Sigma Type VI).

Membrane potentials of pancreatic acinar cells were measured from superfused mouse pancreas with glass microelectrodes filled with 5 M potassium acetate and having resistances of 80–150 MΩ (Poulsen & Williams, 1977). Impalements were judged satisfactory if an abrupt negative deflection was observed which was maintained for at least 30 sec. The value obtained immediately before withdrawal of the microelectrode was taken to represent the membrane potential. Serial microelectrode impalements were made in all experiments and usually about 10 cells were impaled during each phase of each individual experiment. The depolarizing effect of any drug on the membrane potential was calculated by subtracting the numerical value of the mean membrane potential in the presence of the drug (5–30 min after application) from the numerical value of the mean value of the membrane potential in the absence of the drug in each experiment, and these numbers were then averaged.

$^{45}Ca^{++}$ efflux was measured by use of perifused pancreatic fragments as in previous studies (Matthews *et al.*, 1973; Williams & Chandler, 1975).

Ultrastructure was examined in mouse pancreatic fragments which had been incubated 1 hr in KHB or KHB plus membrane stabilizer. Fixation was in 1.5% glutaraldehyde, 1% Paraformaldehyde in 0.08 M sodium cacodylate buffer (pH 7.4) for 90 min at room temperature followed by postfixation in 2% OsO_4. Tissue was embedded in British araldite and thin sections were doubly stained with lead citrate and uranyl acetate and viewed in a JEM-100B electron-microscope at 60 kV. In all cases control and drug-treated fragments were incubated and processed simultaneously. All results mentioned are the result of examination of 3–5 pancreases.

Materials used were obtained from the following sources: carbamyl-β-methycholine (bethanechol), Koch-Light Ltd; amylose azure, Calbiochem; d,l-propranolol and thymol, Sigma Co.; tetracaine, Nordsk Pharmaceut. Chlorpromazine was a gift from Smith, Kline & French and the Ca^{++} ionophore A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co.

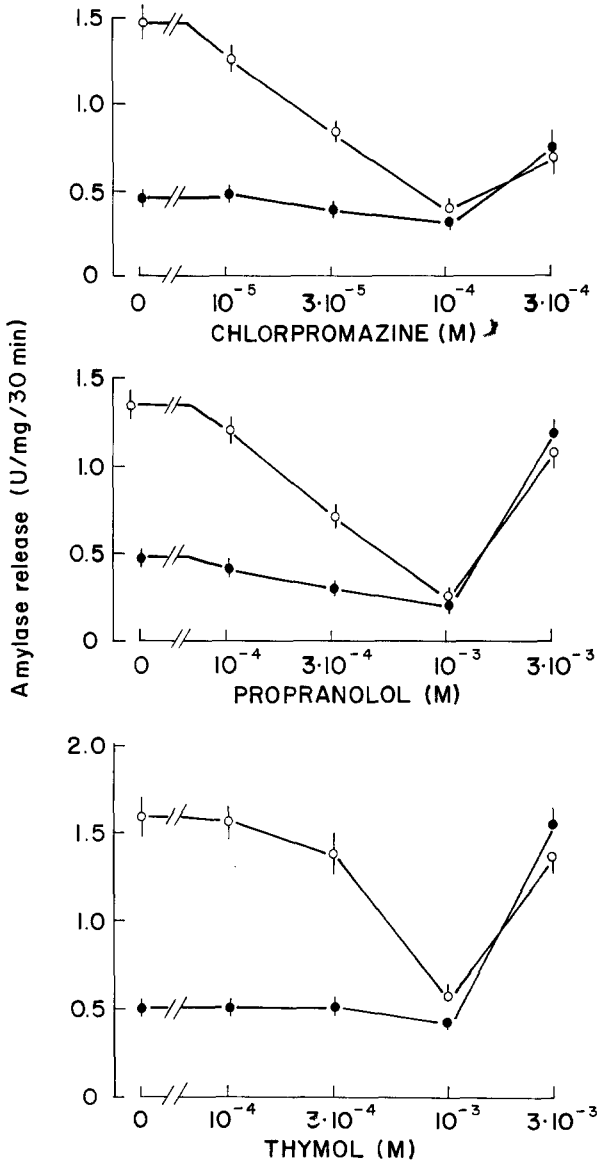

Fig. 1. Effect of different compounds with known activity as membrane stabilizers on *in vitro* pancreatic amylase release. Amylase release either unstimulated (●—●) or stimulated with bethanechol, 3×10^{-5} M (○—○) is plotted as a function of the added concentration of the membrane stabilizer. All values are the mean ±SE for 5–10 pancreases

Results

Fig. 1 shows the effects, using mouse pancreatic fragments, of three different membrane stabilizers, chlorpromazine, propranolol and thymol, on basal amylase release and that stimulated by bethanechol (a nonmetabolizable cholinergic agonist). The results with all three different agents are similar to each other and also similar to our earlier results with tetracaine (Williams & Lee, 1974). As the drug concentration is increased, the bethanechol-stimulated increment in amylase release is progressively inhibited with a much smaller decrease in basal amylase release. At concentrations of chlorpromazine (10^{-4} M), propranolol (10^{-3} M) and thymol (10^{-3} M) bethanechol stimulation of amylase release is essentially abolished. Since it is not clear how much of basal amylase release is physiological and how much is due to nonspecific damage, it cannot be determined whether basal and stimulated amylase release are affected differentially. At higher drug concentrations amylase release increases greatly but this release is similar in the presence or absence of bethanechol and, as will be shown later, is accompanied by cellular damage.

As shown in Table 1 concentrations of chlorpromazine and propranolol which inhibited bethanechol-stimulated amylase release also abolished release stimulated by the calcium ionophore A23187. As the ionophore is thought to function by increasing intracellular Ca^{++}, an effect which is insensitive to tetracaine (Williams & Lee, 1974), inhibition of ionophore-stimulated amylase release by the membrane stabilizers studied can be interpreted as due to interference with the latter steps in stimulus

Table 1. Effect of membrane stabilizers on basal and A23187-stimulated amylase release

Additions	Amylase release (U/mg/30 min)	
	Basal	A23187-stimulated
None	0.37 ± 0.03 (12)	1.07 ± 0.07 (12)
Chlorpromazine (10^{-4} M)	0.27 ± 0.02 (9)	0.33 ± 0.02 (9)
Propranolol (10^{-3} M)	0.13 ± 0.02 (9)	0.16 ± 0.01 (9)

All samples were preincubated 30 min in OCa^{++} KHB containing the membrane stabilizer and A23187 (6.7 μM) as specified. Pancreatic fragments were then transferred to incubation flasks containing similar medium plus 2.5 mM Ca^{++}. All values are the mean ± SE of the number of samples shown in parentheses.

Table 2. Effect of membrane stabilizers on resting membrane potentials and depolarization by bethanechol

Series	E_m[a]	$\Delta E_{m(\text{test})}$[b]	$\Delta E_{m(\text{bethanechol})}$[c]	n[d]
Control	-46.2 ± 1.1	–	12.8 ± 1.4	7
$[K^+]=23.5$ mM	-43.6 ± 1.5	8.1 ± 0.6	6.4 ± 0.3	3
Propranolol (10^{-3} M)	-45.2 ± 1.3	8.4 ± 1.5	0.3 ± 1.2	5
Chlorpromazine (10^{-4} M)	-44.7 ± 1.3	8.6 ± 1.1	-0.3 ± 1.2	5

[a] Resting membrane potentials recorded during superfusion with standard KHB.
[b] Depolarization evoked by substance specified in left column.
[c] Additional depolarization by bethanechol (3×10^{-5} M).
[d] Number of experiments.

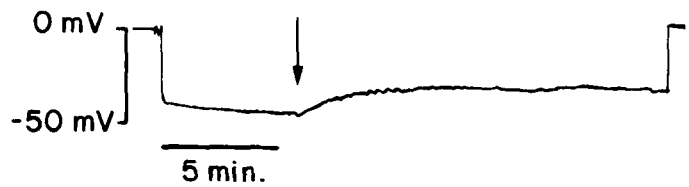

Fig. 2. Effect of propranolol (1 mM) on the membrane potential of a mouse pancreatic cell. The microelectrode was inserted at the left and the superfusate switched to medium containing propranolol at the arrow

secretion coupling. Similar findings have been made for the adrenal medulla where catecholamine release stimulated by readdition of Ca^{++} after perfusion with Ca^{++}-free medium is blocked by local anesthetics (Jaanus *et al.*, 1967).

Two of the earlier steps in pancreatic stimulus secretion coupling are depolarization and release of slowly exchanging $^{45}Ca^{++}$. The effects of propranolol and chlorpromazine on pancreatic membrane potentials were studied and results are shown in Table 2. Both agents at concentrations which maximally inhibited secretion depolarized the acinar cell by 8 mV. An example of this depolarization recorded from a single cell is shown in Fig. 2. Both agents also abolished the bethanechol-induced depolarization (Table 2). That interference with the action of bethanechol was not solely due to the drug-induced depolarization is shown by the fact that in the presence of a comparable depolarization induced by elevating the concentration of K^+ in the medium, bethanechol was still able to induce depolarization.

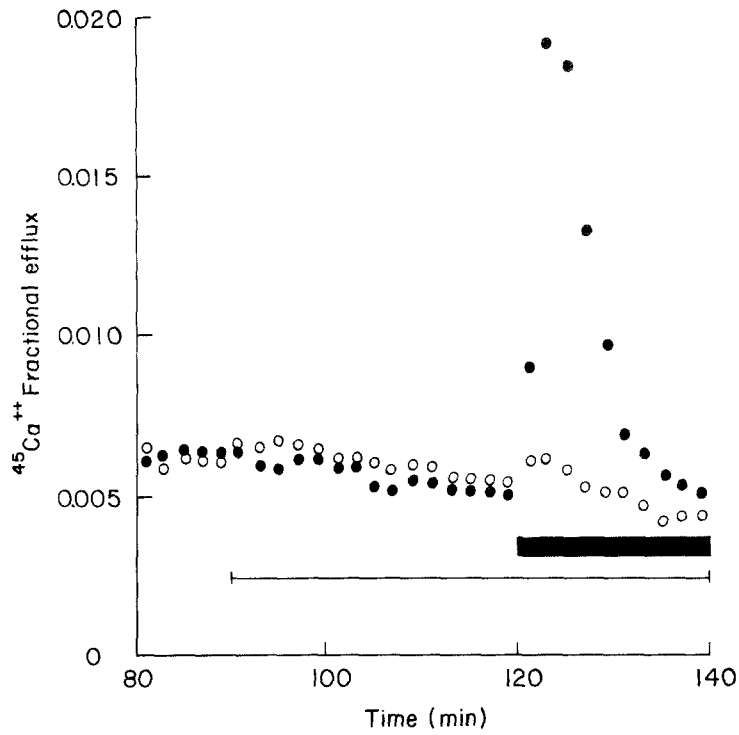

Fig. 3. Effect of chlorpromazine and bethanechol on the fractional efflux of $^{45}Ca^{++}$ from mouse pancreatic fragments. The Figure shows a representative example with a single pancreas which was loaded 1 hr with $^{45}Ca^{++}$, split into two portions, and superfused in parallel with KHB. Chlorpromazine (10^{-4} M) was added from 90–140 min to the half indicated by the open circles. Bethanechol was added as indicated by the solid black bar to both halves

Fig. 3 shows that bethanechol-stimulated release of slowly exchanging $^{45}Ca^{++}$ was blocked by prior exposure to chlorpromazine (10^{-4} M). Pooled data from similar experiments are shown in Table 3. Chlorpromazine, tetracaine, propranolol and thymol all blocked the stimulation of $^{45}Ca^{++}$ efflux by bethanechol but had little effect themselves on $^{45}Ca^{++}$ efflux.

The structural consequences of the concentration of propranolol or tetracaine which maximally inhibit bethanechol-stimulated amylase release and the higher concentrations which by themselves increase amylase release were examined. As shown in Fig. 4, the inhibitory concentration of propranolol (10^{-3} M) had no gross effect on cell ultrastructure. The complement of zymogen granules and their relation to acinar lumina is normal. At higher magnification microtubules and microfilaments were

Table 3. Effect of membrane stabilizers on basal and bethanechol-stimulated $^{45}Ca^{++}$ efflux from superfused mouse pancreas

Additions	^{45}Ca Fractional efflux (min^{-1})	
	Basal	Bethanechol-stimulated (3×10^{-5} M)
None	0.0056 ± 0.0004 (13)	0.0198 ± 0.0010 (13)
Chlorpromazine (10^{-4} M)	0.0057 ± 0.0003 (6)	0.0072 ± 0.0006 (6)
Tetracaine (6×10^{-4} M)	0.0059 ± 0.0006 (6)	0.0065 ± 0.0006 (6)
Propranolol (10^{-3} M)	0.0067 ± 0.0005 (5)	0.0071 ± 0.0007 (5)
Thymol (10^{-3} M)	0.0068 ± 0.0004 (5)	0.0069 ± 0.0007 (5)

All experiments were carried out in a manner similar to that shown in Fig. 3 except that each pancreas was divided into thirds and superfused in parallel. Membrane stabilizers were added at 90 min and bethanechol at 120 min. Basal values were measured just prior to the addition of bethanechol while bethanechol-stimulated is the maximal value. All values are the mean $\pm$SE for the number of experiments shown in parentheses.

present and mitochondria appeared normal. Two minor modifications were that much of the endoplasmic reticulum had become vesicular and there was an increased number of swollen golgi vesicles. When the concentration of propranolol was increased to that which increases amylase release (3 mM) the cells were grossly damaged (Fig. 4, lower). Most of the secretory granules have disappeared and all cell organelles appear swollen and damaged. Exactly parallel findings were observed when tissue was treated with tetracaine at an inhibitory concentration (0.6 mM) and a higher concentration (3.0 mM) which by itself caused amylase release.

Discussion

The focus of this study has been to better define the interactions with the pancreatic acinar cell of agents with membrane stabilizing (local anesthetic) activity. This is of value since local anesthetics inhibit secretion by many other cells as well as pancreatic acinar cells and therefore have been used as pharmacological tools to study the secretory process.

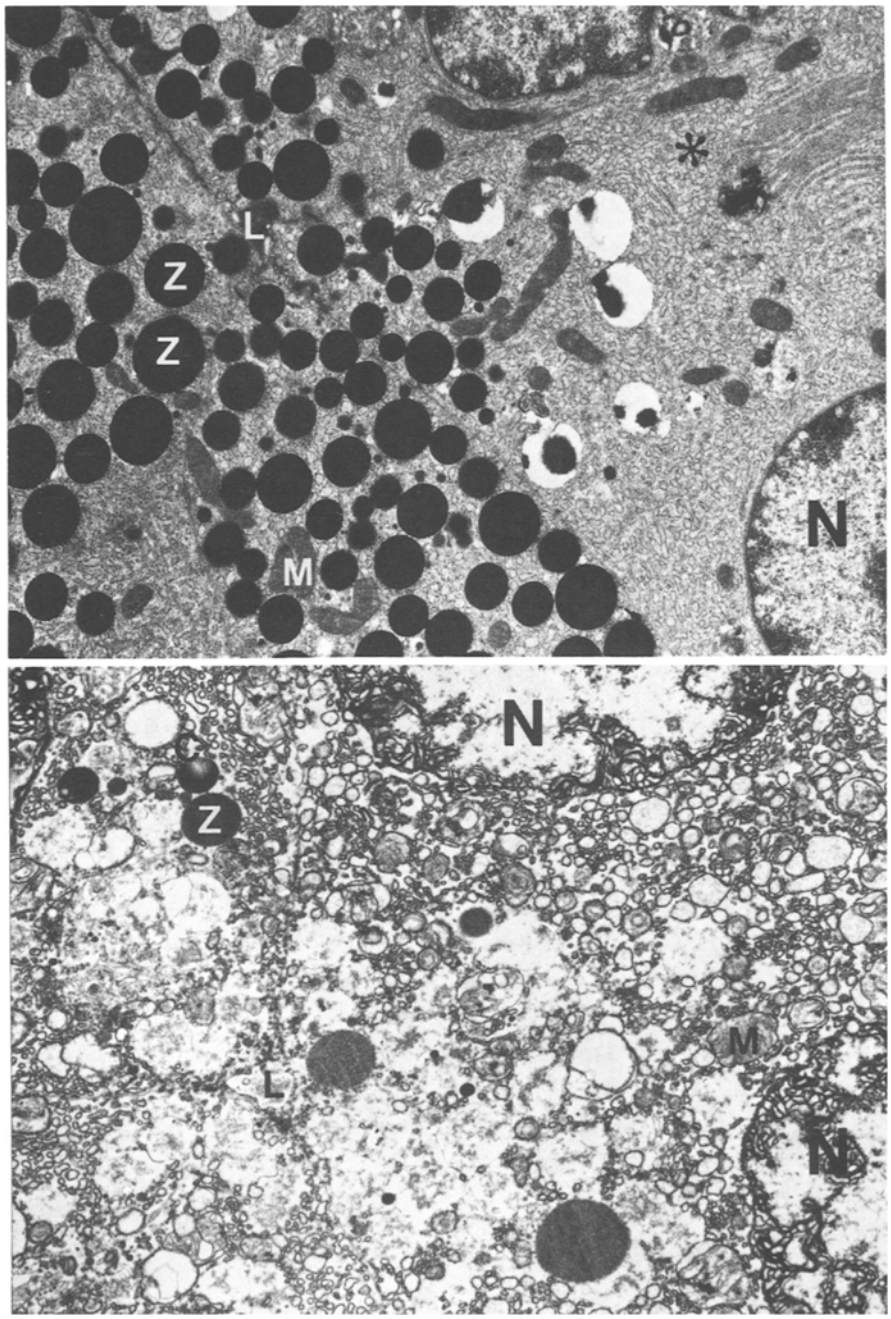

Fig. 4. Effect of propranolol on the ultrastructure of mouse pancreatic fragments incubated 1 hr in KHB containing: upper, 1 mM propranolol or lower, 3 mM propranolol. *L*=acinar lumen, *N*=nucleus, *Z*=zymogen granule, *M*=mitochondria, *=vesicular endoplasmic reticulum. Magnification for both figures ×6000

As is frequently the case, this study points out many of the limitations of using drugs as tools to study cellular processes.

It has previously been shown that a local anesthetic, tetracaine, would inhibit pancreatic amylase release stimulated by bethanechol and at slightly higher concentrations increase amylase release both in the presence and absence of the stimulator (Williams & Lee, 1974). The present study shows that this activity is not unique but can be reproduced by chlorpromazine, propranolol and the simple detergent, thymol. All of these agents are amphipathic and in the concentrations affecting amylase release possess surface-active activity. The amylase release *vs.* drug concentration curves (Fig. 1) in fact show a striking similarity to the curves for inhibition and potentiation of hypotonic hemolysis of erythrocytes (Seeman & Weinstein, 1966) and release of contents from isolated lysosomes or chromaffin granules (Seeman, 1972). In these simpler systems it is thought that the lower concentrations of the agent act to stabilize the membrane while high concentrations destabilize or lyse the membrane. We have confirmed by ultrastructural evaluation that release of pancreatic amylase by higher concentrations of membrane stabilizers does involve cell damage and lysis (Fig. 4).

In the pancreatic acinar cell stabilizing concentrations of propranolol and tetracaine did not produce gross alterations of cellular fine structure although there was a tendency for the endoplasmic reticulum to become vesiculated. The concentrations of the agents which inhibited bethanechol-stimulated release were found to inhibit three steps in pancreatic acinar cell stimulus secretion coupling: bethanechol-induced depolarization, Ca^{++} release and Ca^{++}-induced amylase release. These steps are felt to involve both the baso-lateral cell membrane upon which presumed secretagogue receptors lie and across which electrical potentials are measured and the apical or luminal cell membrane across which Ca^{++}-activated amylase release takes place. Inhibition of all these processes thus indicates an action of the membrane stabilizer on both of these membranes. This conclusion is similar to that in an earlier study of thyroid secretion in which chlorpromazine inhibited both TSH-stimulated cyclic AMP generation (a presumed basal membrane function) and the ability of cyclic AMP to increase colloid endocytosis across the apical cell membrane (Williams & Wolff, 1971). The ultrastructural studies reported here showing effects of lytic concentrations of membrane stabilizers are also consistent with a general action on all cellular membranes.

The effects of membrane stabilizers on membrane potentials have been studied in several tissues previously. All of the agents studied here

will block action potentials in myelinated nerve fibers (Seeman, 1972). Both chlorpromazine and tetracaine at stabilizing concentrations slightly depolarized frog skeletal muscle fiber (Hellenbrecht, 1971). Tetracaine (1 mM) has been previously shown to depolarize the pancreatic acinar cell (Matthews & Petersen, 1973) while 0.33 mM had little effect on resting potential of gerbil chromaffin cells (Douglas & Kanno, 1967). Thus the 8 mV depolarization of pancreas cells in response to propranolol and chlorpromazine is in agreement with previous work. Of more importance is the ability of both propranolol and chlorpromazine to block the bethanechol-induced depolarization. Similar findings have been reported for tetracaine (Matthews & Petersen, 1973). As the bethanechol-induced depolarization is believed due to opening of ionic channels for Na^+ and K^+ (Nishiyama & Petersen, 1975), the inhibitory action of membrane stabilizers is analogous to inhibition of action potentials in nerve cells.

The conclusions to be drawn from the present work are that any amphipathic compound with membrane stabilizing activity will probably inhibit pancreatic protein secretion and this must be considered a possibility whenever secretion of any type is inhibited by a foreign compound. Furthermore, this inhibition is nonspecific in that it affects a number of cell functions. Lastly, it should be re-emphasized that enzyme or hormone release from an in vitro tissue is not always secretion since release can result from tissue damage as well as activation of physiological processes.

This work was supported by NIH research grant GM-19998, the Cystic Fibrosis Foundation, the Danish Medical Research Council and the NOVO Foundation.

References

Ammon, H.P.T., Orci, L., Steinke, J. 1973. Effect of chlorpromazine (CPZ) on insulin release *in vivo* and *in vitro* in the rat. *J. Pharmacol. Exp. Ther.* **197**:423

Beaudoin, A.R., Marois, C., Dunnigan, J., Morisset, J. 1974. Biochemical reactions involved in pancreatic enzyme secretion. I. Activation of the adenylate cyclase complex. *Can. J. Physiol. Pharmacol.* **52**:174

Bressler, R., Brendel, K. 1971. Effect of local anesthetics on insulin secretion by pancreas pieces. *Diabetes* **20**:721

Chandler, D.E., Williams, J.A. 1977. Intracellular uptake and α-amylase and lactate dehydrogenase releasing actions of the divalent cation ionophore A23187 in dissociated pancreatic acinar cells. *J. Membrane Biol.* **32**:201

Douglas, W.W. 1968. Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. *Br. J. Pharmacol. Chemother.* **34**:451