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Differential control of insulin secretion and somatostatin-receptor recruitment in isolated pancreatic islets

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Somatostatin receptors appear to be localized to secretory granules in pancreatic islet homogenates. Recruitment of these receptors to the islet-cell surfaces may mark the contact event between secretory granules and plasma membranes before release of insulin by fission. Isethionate, an impermeant anionic replacement for chloride, blocks the release step but does not affect receptor recruitment. By contrast, low concentrations of phenothiazine drugs, such as trifluoperazine and promethazine, inhibit both receptor recruitment and secretion. Scatchard analysis of phenothiazine effects on somatostatin receptors reveals that these drugs reduce the number of receptors but do not affect the affinity of the receptor for somatostatin. These data indicate that membrane contact and fission steps during exocytosis can be biochemically separated.

Hormone secretion by exocytosis involves initial contact between the secretory vesicle and the plasma membrane, and subsequent fission or breakage of the combined membranes to release vesicle contents (Palade, 1975; Pollard *et al.*, 1979, 1981). In the case of insulin secretion from pancreatic β-cells, we found that the major proportion of somatostatin-receptor activity was in the secretory-vesicle fraction of islet homogenates, possibly associated with the β-granules (Leitner *et al.*, 1980). We further suggested that the secretion vesicle may provide the vehicle for translocating the receptor for somatostatin from the Golgi cisternae to the plasma membrane (Mehler *et al.*, 1980; Sussman *et al.*, 1982). This has led us to the proposal that the initial contact event between secretory vesicles and plasma membranes might be investigated by monitoring the recruitment of somatostatin receptors to the cell surface after stimulation by glucose.

As a part of this investigation, we have attempted to control receptor recruitment and insulin differentially by using isethionate, an impermeant anion, as well as certain phenothiazine drugs, including TFP and PMTHZ. Since isethionate may block only the fission step in exocytosis (Pollard *et al.*, 1981), we anticipated that somatostatin-receptor recruitment might be unaffected by this drug. By contrast, the phenothiazines block a variety of Ca^{2+}-binding proteins (Pollard *et al.*, 1981) implicated in regulating the initial contact event, leading us to expect that both somatostatin-receptor recruitment and hormone release would be inhibited.

In the present paper, we report that isethionate indeed blocks glucose-induced insulin release but not somatostatin-receptor recruitment. In addition, low concentrations of either TFP or PMTHZ block both receptor recruitment and hormone secretion under the same conditions. We thus conclude that the membrane contact and fission steps of insulin secretion appear to be biochemically separable processes, accessible to analysis by using specific drugs directed against the two steps.

Abbreviations used: TFP, trifluoperazine; PMTHZ, promethazine.

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Materials and methods

Pancreatic islets

Pancreatic islets were prepared by the method of Lacy & Kostianovsky (1967) as modified by Mehler

et al. (1980) either from male Sprague–Dawley rats weighing 250–350g or from the desert sand rat *Psammomys obesus*. The desert sand rats were obtained from an inbred colony located at the Hadassah Hospital in Jerusalem. After isolation, the islets were kept in a Krebs–Ringer bicarbonate buffer containing 30mg of glucose/dl and 0.5mg of bovine serum albumin/ml. The buffer had been gassed with O_2/CO_2 (19:1) for 45min before use and the pH was 7.4.

Insulin secretion

Ten islets were placed in glass tubes (12mm × 75mm) containing 200µl of buffer, as above at 2°C. To initiate secretion, 800µl of buffer containing 300mg of glucose/dl was added and the suspension was incubated at 37°C for 30min. The reaction was terminated by reducing the temperature to 2°C for 5min and then centrifuging at 800**g** for 5min at 4°C. Insulin was assayed in an 800µl portion of the supernatant solution by a modification of the double-antibody radioimmunoassay technique (Morgan & Lazarow, 1963; Mehler *et al.*, 1980). The remaining islet pellet was washed before assay of available somatostatin receptors with 11vol. of 50mM-Tris, pH8.0, containing 0.5mg of bovine serum albumin/ml. The somatostatin receptor assay was performed as described previously (Mehler *et al.*, 1980; Sussman *et al.*, 1982). All results were corrected for non-specific binding (binding in the presence of 10µg of unlabelled somatostatin/ml), which was 0.4±0.05% of the total available radioactivity.

In the experiments employing TFP and PMTHZ, the agents are present in the initial 200µl of media to yield concentrations of 0, 1, 10, 50 or 100µM. The samples were pre-incubated at 37°C for 15min. After this pre-incubation, an 800µl portion of appropriately buffered medium was added to maintain the desired glucose and drug concentrations. The samples were then incubated for an additional 15min at 37°C. Samples for insulin assay and somatostatin binding were prepared as described above.

In those studies employing sodium isethionate (Sigma), NaCl was replaced in the Krebs–Ringer bicarbonate buffer medium on an equimolar basis to yield the desired final concentrations of the agent.

Chemicals

TFP was donated by the Smith, Kline and French Laboratories, Philadelphia, PA, U.S.A. PMTHZ was obtained from Wyeth Laboratories, Philadelphia, PA, U.S.A. Sodium isethionate was obtained from Sigma Chemical Company, St. Louis, MO, U.S.A. All other chemicals were of reagent grade.

Results

Receptor recruitment and insulin secretion in isethionate medium

Replacement of chloride in the incubation medium by the impermeant anion isethionate inhibits secretion of adrenaline (Pollard *et al.*, 1979, 1981), lysosomal enzymes (Weissman *et al.*, 1978), parathyrin (Brown *et al.*, 1978) and insulin (Somers *et al.*, 1980; Orci & Malaisse, 1980). In the present study, we also found that increasing isethionate levels progressively and completely blocked insulin secretion from isolated pancreatic islets. Indeed, 50% inhibition occurred where isethionate and chloride were in an approximately 1:1 molar ratio (see Fig. 1) ($P<0.001$ versus initial insulin release at 120mM NaCl).

However, as also shown in Fig. 1, glucose-induced somatostatin-receptor recruitment ($P<0.01$ for 30 versus 300mg/dl) was virtually unaffected by progressive replacement of NaCl by sodium isethionate. This marked the first instance in which somatostatin-receptor recruitment had been dissociated from insulin secretion in isolated islets. We tested the

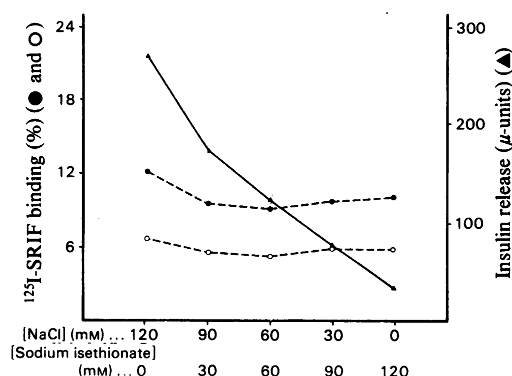

Fig. 1. *Influence of replacement of extracellular chloride by isethionate on glucose-induced insulin secretion and somatostatin-receptor recruitment*

Desert-sand-rat islets were incubated at 37°C in 200µl of Krebs–Ringer bicarbonate buffer containing 30mg (O) or 300mg of glucose/dl (●). After 30min islets were centrifuged to the bottom of the tube at 1000**g** for 10min and insulin was analysed in 100µl aliquots of the static incubation solution (▲). Somatostatin receptors were analysed by incubation of the islet tissue with ^{125}I-somatostatin, as described previously (Leitner *et al.*, 1980; Mehler *et al.*, 1980; Sussman *et al.*, 1982), for 16h at 4°C. Isethionate had no influence on either the insulin radioimmunoassay or detection of somatostatin receptors. Abbreviations used: SRIF, somatostatin; NaIs, sodium isethionate. Results represent the mean values of 10 experiments.

generality of this result with islets from Sprague–Dawley rats, and essentially identical results were obtained (results not shown).

The isethionate effect on secretion has been previously interpreted in terms of interference with the osmotic process of fission of secretory granules complexed to the plasma membrane (Pollard *et al.*, 1979, 1981). Seen from this viewpoint, the present data might indicate that the initial membrane contact event can be observed in the absence of the ultimate fission process.

Phenothiazine drug effects on somatostatin-receptor recruitment and insulin secretion

Certain phenothiazine drugs block insulin secretion (Gagliardino *et al.*, 1980; Valverde *et al.*, 1981; Janjic *et al.*, 1981) and inhibit the action of specific Ca^{2+}-binding proteins such as synexin (Pollard *et al.*, 1981) and calmodulin (Levin & Weiss, 1976). Synexin is sensitive to low concentrations of both TFP and PMTHZ, whereas calmodulin is more sensitive to TFP than to PMTHZ by a factor of at least 100 (Pazoles *et al.*, 1980).

In our present study, we therefore investigated the relative abilities of both TFP and PMTHZ to affect both insulin secretion and somatostatin-receptor recruitment. Fig. 2(*a*) shows that TFP blocked somatostatin-receptor recruitment ($P < 0.01$) and insulin release ($P < 0.01$) coincidently, with an ID_{50} of 1μM. PMTHZ (Fig. 2*b*) was also found to inhibit both receptor recruitment ($P < 0.01$) and insulin release ($P < 0.01$) coincidentally, but at a slightly lower ID_{50} than that of TFP. The two drugs were statistically indistinguishable, however. In either case, loss of receptor recruitment and insulin secretion were highly correlated ($r = 0.93$, as shown in Fig. 3).

To be certain about the generality of the effect, we again performed these experiments on pancreatic islets from Sprague–Dawley rats. Similar results were obtained, but the absolute potencies of TFP and PMTHZ were somewhat reduced. Thus the ID_{50} for TFP was 14μM, whereas that for PMTHZ was 5μM.

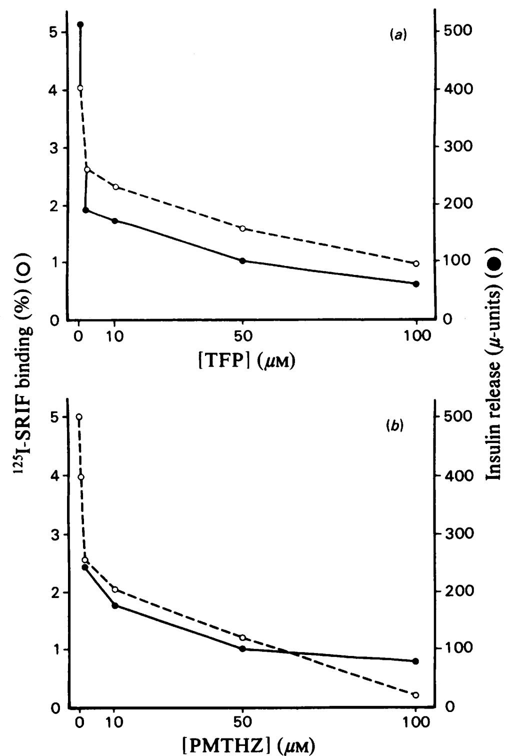

Fig. 2. *Inhibition of insulin secretion and somatostatin-receptor recruitment by TFP (a) and PMTHZ (b)*
Assay conditions were as summarized in the legend to Fig. 1, except that 10mM stock solutions of phenothiazines were freshly prepared in water and then diluted in Krebs–Ringer bicarbonate buffer before use. Phenothiazine at these concentrations had no influence on either the insulin radioimmunoassay or the somatostatin receptor assay. Abbreviation used: SRIF, somatostatin. Results for both insulin release and somatostatin-receptor recruitment represent the change in the difference between results obtained for buffer containing 300mg and 30mg of glucose/dl. The mean values of 10 experiments are presented.

Influence of phenothiazines on the binding characteristics of somatostatin receptors

To confirm that phenothiazines were inhibiting receptor recruitment and not altering receptor affinity, we measured the number and affinity of islet receptors using Scatchard analysis of competitive binding data. As shown in Fig. 4(*a*), incubation of islets with either 30 or 300mg of glucose/dl resulted in changes in the number of somatostatin receptors (R_0), with no change in the affinity of the receptors. Similarly, incubation of islets in 300mg of glucose/dl in the presence of either 10μM-TFP or -PMTHZ resulted only in a reduction in the number of available receptors, without changes in receptor affinity (Fig. 4*b*). However, at much higher dose levels, the phenothiazines were found to displace somatostatin from the islet surface-membrane receptor, with changes becoming detectable at 50μM (TFP) and at 75μM (PMTHZ). These levels were substantially above the concentration ranges affecting receptor recruitment and insulin secretion in islets of both Sprague–Dawley rats and desert sand rats.

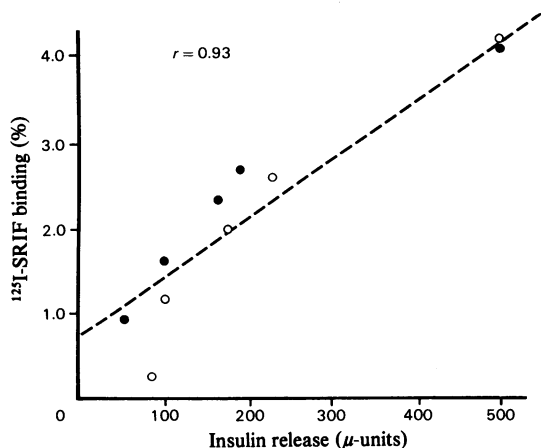

Fig. 3. *Correlation between inhibition of somatostatin-receptor recruitment and insulin secretion in different concentrations of either TFP* (○) *or PMTHZ* (●)
Results for both insulin release and somatostatin-receptor recruitment represent the change in the difference between results obtained for buffer containing 300mg and 30mg of glucose/dl. Abbreviation used: SRIF, somatostatin.

Discussion

Insulin is secreted from isolated islets of Langerhans when the islets are immersed in a medium containing a high-glucose concentration. Concurrently, somatostatin receptors become recruited to the islet surface, where they can be detected with radiolabelled somatostatin (Mehler *et al.*, 1980). We have previously shown that new somatostatin receptors probably originate from the secretory-granule membranes, which must make contact with the plasma membrane during exocytotic release of hormone (Mehler *et al.*, 1980; Sussman *et al.*, 1982). The present studies provide further support for this hypothesis, since we can now distinguish the somatostatin receptor recruitment event from that of insulin secretion itself. We find that insulin secretion but not somatostatin-receptor recruitment is blocked by replacement of chloride in the incubation medium by the impermeant anion isethionate. By contrast, phenothiazine drugs, such as TFP and PMTHZ, block both receptor recruitment and hormone secretion.

The mechanism of isethionate blockade of secretion is not fully known, but the evidence at hand

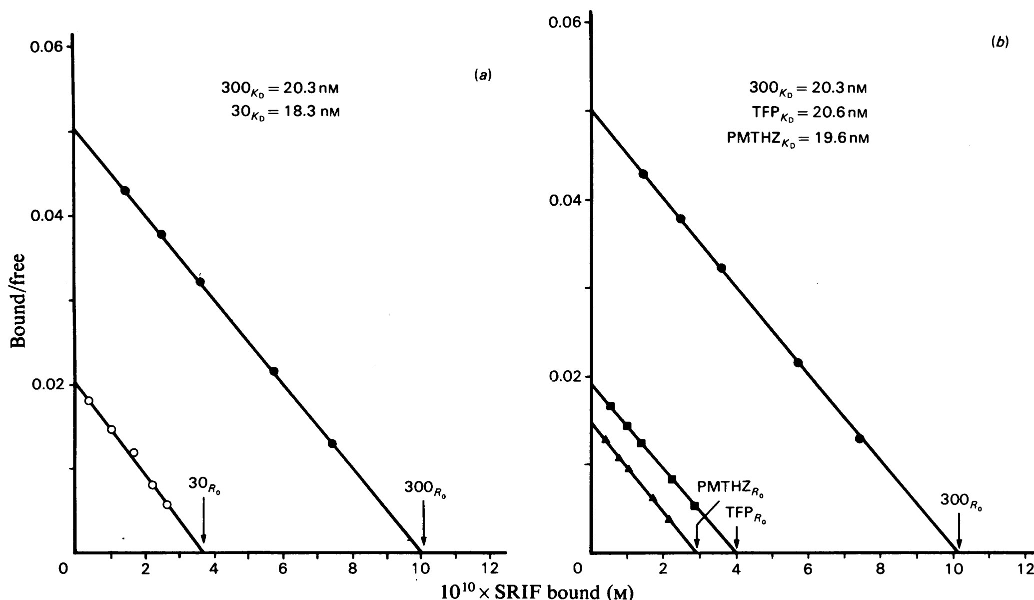

Fig. 4. *Scatchard analysis of effect of glucose concentration, TFP or PMTHZ on number and affinity of somatostatin receptors in stimulated islets*
(*a*) The symbols 30_{R_0} and 300_{R_0} on the horizontal axis indicate the concentrations of total binding sites in islets incubated in either 30 or 300mg of glucose/dl respectively. The slopes of the two curves are indicated by the symbols 30_{K_D} and 300_{K_D}, and are essentially identical. (*b*) The receptor concentration is shown for islets incubated in buffer containing 300mg of glucose/dl (i.e. 300_{R_0}). PMTHZ and TFP are shown to decrease the number of somatostatin receptors recruited (indicated by $PMTHZ_{R_0}$ and TFP_{R_0}) to the islet surface by incubation in 300mg of glucose/dl. By contrast the affinity constants of the receptors were identical in 300mg of glucose/dl (300_{K_D}), TFP (TFP_{K_D}) or PMTHZ ($PMTHZ_{K_D}$).

indicates that it may act primarily at the fission step of exocytosis. Isethionate blocks not only insulin secretion, as reported by Somers *et al.* (1980) and Orci & Malaisse (1980), but also exocytosis form other systems, including chromaffin cells (Pollard *et al.*, 1979, 1981), parathyroid cells (Brown *et al.*, 1978) and macrophages (Weissman *et al.*, 1978). At a subcellular level, isethionate blocks chemiosmotic lysis of chromaffin granules by competitively inhibiting Cl^- influx driven by an ATP-dependent electrogenic influx of protons (Pazoles *et al.*, 1980). This chemiosmotic lysis process in granules has been found to parallel the secretion of adrenaline from chromaffin cells in many ways. This has led to the proposal of an osmotic theory of secretion involving extracellular Cl^- permeation of the complex formed between granules and plasma membranes before fission (Pollard *et al.*, 1979, 1981). Of necessity, the relationship of isethionate action in islets to other systems remains indirect. But if the isethionate action on islets is indeed focused primarily on the fission step, then however it works, one would not expect the preliminary contact event to be modified. As anticipated, somatostatin-receptor recruitment is unaffected in the presence of isethionate, even though glucose-induced insulin secretion is blocked.

Phenothiazine blockade of both somatostatin-receptor recruitment and insulin secretion was not particularly surprising. TFP is known to block insulin secretion, and some have presumed it to work by action on calmodulin, supposing that calmodulin in some way mediated Ca^{2+} action and membrane contact before secretion (Gagliardino *et al.*, 1980; Valverde *et al.*, 1981; Janjic, 1981). Watkins & Cooperstein (1983) have recently reported that calmodulin promotes the binding of inside-out ^{125}I-labelled plasma membranes to secretion granules. Furthermore these workers have demonstrated a dose-dependent effect of trifluoperazine in inhibiting this calmodulin-induced binding.

However, the potency of PMTHZ, compared with that of TFP, is inconsistent with calmodulin as a target for the phenothiazines, and even excludes a trivial, local anaesthetic action as a mechanism of action of these drugs. For both inhibition of calmodulin (Levin & Weiss, 1976) and local anaesthetic effects (Poste & Reeve, 1972), the K_i values for TFP and PMTHZ are approx. 1–10 and 350μM respectively. By contrast, synexin is sensitive to both drugs in the 1–10μM range.

Synexin is widely distributed in tissues (Creutz *et al.*, 1982) and it specifically induces Ca^{2+}-dependent contact between chromaffin granules (Creutz *et al.*, 1978). Synexin binds specifically to secretory granules and plasma membranes (Pollard *et al.*, 1981; Scott *et al.*, 1983), and also promotes fusion between aggregated granules (Creutz, 1981). We are thus led to conclude that the target of phenothiazine drugs in islets may also include synexin or a synexin-like factor.

However, whatever the site of action of these drugs, it remains clear that there is a very close correlation between somatostatin-receptor recruitment and hormone secretion over a wide range of drug concentrations, as indicated in the correlation plot in Fig. 3. These data indicate that glucose-induced insulin secretion indeed appears to coincide with recruitment of somatostatin receptors derived from secretory-granule membranes upon contact between granules and plasma membranes just before fission. Blockade of fission, as with the isethionate-drug condition, does not interfere with contact. Yet blockade of contact, as in the phenothiazine-drug condition, precludes fission and hormone release.

The location of somatostatin receptors on the external granule surface indicates that the receptor site must pass across a hydrophilic bilayer in some manner to be detected from outside the cell. This is consistent with the observation that the time course for receptor recruitment is somewhat slower than for insulin secretion (Draznin *et al.*, 1982). Such a kinetic character may have physiological significance, since occupation of the receptor by somatostatin may be a mechanism for turning off insulin secretion later.

Examples of location of plasma-membrane receptors on the cytoplasmic surfaces of secretory organelles are now becoming more common (Tanabe *et al.*, 1979). There is now additional evidence suggesting the presence of other hormone receptors in association with secretion granules. Investigating prolactin cells and somatotrophic cells from the rat anterior pituitary, Rosenzweig & Kanwar (1982) have reported significant localization of dopamine (3,4-dihydroxyphenethylamine) to subcellular organelles involved in hormone secretion, including the Golgi cisternae and immature and mature secretory granules. Similarly, Hazum *et al.* (1982) observed the localization of two labelled analogues of gonadotropin releasing hormone (gonadoliberin) to the cell surface membrane and also to secretory granules and smooth membranes in close vicinity to the Golgi complex. These reports are providing morphological support for the concept of certain hormone receptors being associated with secretion granules. If this is indeed the case, then this methodological approach for studying certain intracellular events of exocytosis (i.e. granule fusion to the surface membrane and granule fission with hormone discharge) may be applicable in other tissues.

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