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Importance of Cellular Calcium Stores in Glucose-stimulated Insulin Release

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INTRODUCTION

There is now overwhelming evidence that stimulus-secretion coupling in the pancreatic β-cell is mediated by an increase of the concentration of ionised Ca^{++} in the cytosol (3,9,19). As direct measurements of changes in the concentration of cytosolic Ca^{++} in the β-cell have not yet been possible, changes in this Ca^{++} concentration have been inferred from measurements of $^{45}Ca^{++}$ fluxes in islets. Glucose has been shown to increase net retention of $^{45}Ca^{++}$ by islets which have not attained isotopic equilibrium (3,9,17). When islets were allowed to equilibrate with $^{45}Ca^{++}$ during 24h at a low glucose concentration it was found that glucose rapidly increased the total content of 45 calcium. Under these conditions this can be equated with an increase in total calcium content of the islets (11). Two actions of glucose have been demonstrated which both could contribute to raise cytosol Ca^{++} and, in turn, total islet calcium content. First, glucose stimulates the initial uptake of $^{45}Ca^{++}$, reflecting Ca^{++} influx (17). This influx is probably occurring via voltage-sensitive Ca^{++} channels in the plasma membrane which are gated upon depolarisation of the membrane by glucose (9,19). Secondly, glucose inhibits $^{45}Ca^{++}$ efflux from islets preloaded to isotopic equilibrium and perifused in the absence of extracellular Ca^{++} (2,5). This inhibition of net Ca^{++} efflux may be due to an interference with Na/Ca counter-transport at the plasma membrane (4, 14). An inhibition of Ca^{++} sequestration or direct mobilisation of the ion from calcium storing organelles in the β-cell has been proposed as a third mechanism by which glucose may raise cytosol Ca^{++} (6,14,18,19). It has so far not been possible to

demonstrate directly this effect on glucose.

The activation of several enzyme systems by Ca^{++} has been shown to be mediatey by the calcium regulator protein, calmodulin. In islets, calcium-calmodulin has been shown to activate adenylate cyclase (13,16), Ca^{++}-ATPase (10) and protein phosphorylation (1). Calmodulin appears to be present in islets (15) and trifluoperazine, an inhibitor of calcium-calmodulin action (8) was found to inhibit glucose-stimulated insulin release (1,7,15). In the present study the involvement of calmodulin in glucose-induced changes in islet cell Ca^{++} handling and insulin release were investigated by the use of trifluoperazine. The effects of this drug were compared with those of verapamil, a blocker of voltage-sensitive Ca^{++} channels. The results indicate that calmodulin may be involved in the process by which glucose acts on intracellular calcium stores to raise cytosol Ca^{++}.

METHODS

Collagenase isolated islets from male Wistar rats were maintained in tissue culture for 46h as described elsewhere. In experiments involving the measurement of $^{45}Ca^{++}$ efflux, the islets were equilibrated with the isotope during the entire culture period (5,14).

Dynamic insulin release and $^{45}Ca^{++}$ efflux were measured in a perifusion apparatus by placing 40 islets per chamber. The perifusate consisted of Krebs-Ringer bicarbonate buffer containing 1mM Ca^{++}, 0.5% bovine serum albumin, and 2.8mM glucose.

$^{45}Ca^{++}$ uptake and insulin release were measured by incubating the islets on top of an oil layer in microfuge tubes, as described in detail elsewhere (17,18). The buffer consisted of a modified Krebs-Ringer bicarbonate buffer (KRB-Hepes) containing 10mM Hepes, 5mM $NaHCO_3$, 0.5% bovine serum albumin, and 2.8mM glucose. The buffer was supplemented with 0.8μCi of $^{45}Ca^{++}$ at a final concentration of 1mM and 1.4μCi [6,6^{3}H]sucrose at a concentration of 4μM serving as an extracellular space marker. 10 islets were incubated for 5min and then centrifuged through the oil layer. Insulin release was measured in the supernatant and $^{45}Ca^{++}$ uptake in the islet pellet. In this incubation system basal and glucose-stimulated $^{45}Ca^{++}$ uptake is linear with time over the 5min of incubation (17). Insulin release was measured

by radioimmunoassay using rat insulin as standard (17).

RESULTS AND DISCUSSION

1. Effects of verapamil

In a first series of experiments the importance of Ca^{++} uptake for the stimulation of insulin release by glucose, was examined using verapamil. As can be seen in Fig. 1, 16.7mM glucose-

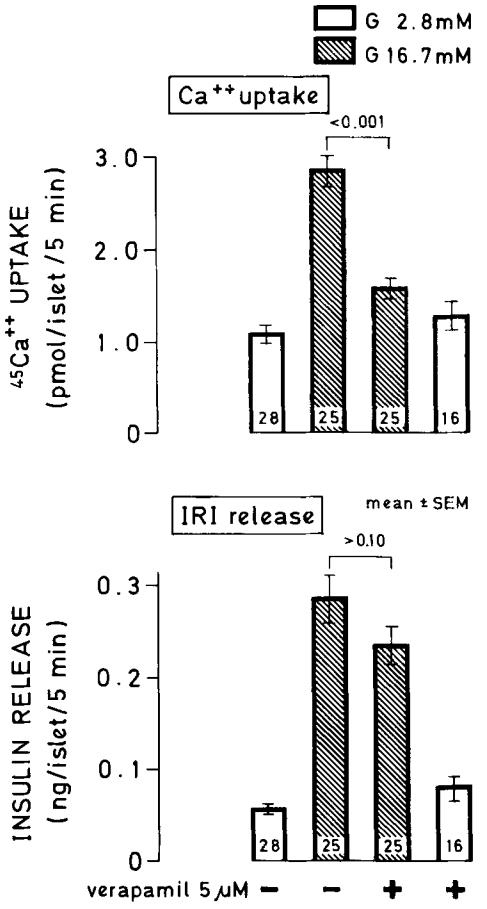


Fig. 1

Effect of verapamil (5µM) on basal and glucose-stimulated $^{45}Ca^{++}$ uptake and insulin release measured over 5 min

stimulated Ca^{++} uptake by 166%. In the presence of 5µM verapamil, the stimulant action of glucose on Ca^{++} uptake was inhibited by 83%. There was no longer a significant increase in Ca^{++} uptake

when comparing the uptake in the presence of verapamil at 2.8mM glucose with that at 16.7mM glucose. In the same experiments glucose stimulated insulin release by 400%. In marked contrast to Ca^{++} uptake verapamil failed to significantly decrease glucose-stimulated insulin release.

In the experiments shown in Fig. 2 the effects of verapamil

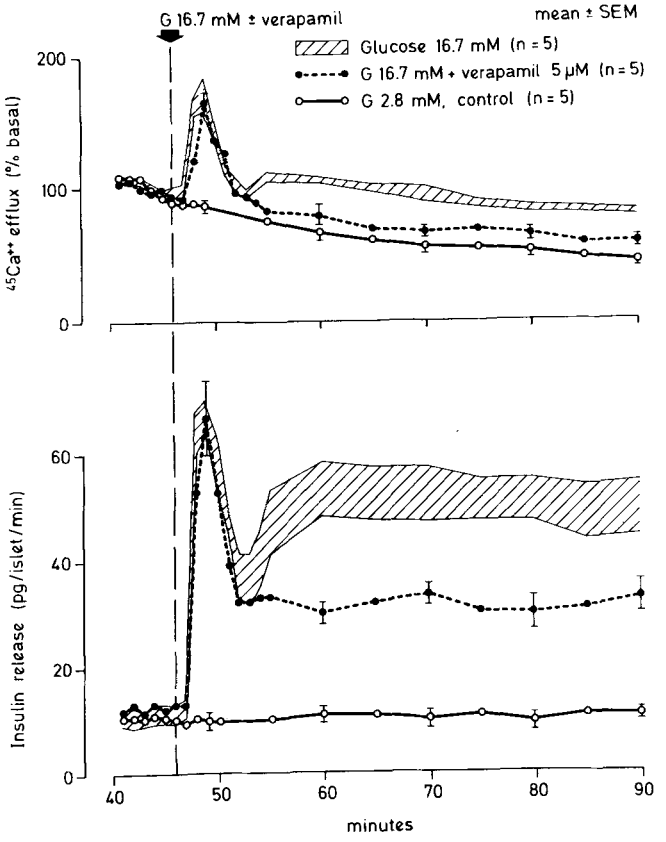


Fig. 2

Effect of verapamil on glucose-stimulated $^{45}Ca^{++}$ efflux and insulin release. 5µM verapamil was added at the same time (46min) as the glucose concentration was increased from 2.8mM to 16.7mM. $^{45}Ca^{++}$ efflux expressed as a percentage of the mean efflux rate at 41-46min.

on the dynamics of insulin release and $^{45}Ca^{++}$ efflux from pre-loaded islets were tested. An increase of the glucose concentration in the perifusate from 2.8 to 16.7mM caused a biphasic stimulation of insulin release. The peak of the first phase was

reached 3min after the shift and the nadir between the two phases after another 3min. The addition of 5μM verapamil, together with 16.7mM glucose failed to inhibit the first phase of glucose-stimulated insulin release. However, in the presence of verapamil the rate of insulin release did not increase above the rate reached at the nadir. Thus, verapamil reduced the second phase of insulin release to a simple plateau, the second phase being inhibited by approximately 50%. As the insulin release shown in Fig. 1 was measured over 5min, it corresponds to the first phase release of the dynamic system. The failure of verapamil to inhibit the first phase of insulin release, despite the inhibition of Ca^{++} uptake during this period, strongly suggests that the stimulation of Ca^{++} uptake by glucose does not contribute to the generation of the first phase of insulin release. Nonetheless, an increase of cytosol Ca^{++} is likely to be generating the first phase of insulin release. This is suggested from the measurements of $^{45}Ca^{++}$ efflux also shown in Fig. 2. 16.7mM glucose caused a biphasic increaseof the $^{45}Ca^{++}$ efflux from the islets. The pattern of $^{45}Ca^{++}$ efflux paralleled that of glucose-stimulated insulin release. The efflux was most marked during the first phase. The gradual decrease during the second phase probably reflects a decrease of the specific radioactivity in the islets. Verapamil only caused slight changes in Ca^{++} efflux during the first phase period while the efflux was inhibited by more than 60% during the second phase. As it has been demonstrated previously that the $^{45}Ca^{++}$ is not released together with the insulin during exocytosis, the pattern of $^{45}Ca^{++}$ efflux may reflect changes in the concentration of Ca^{++} in the cytosol of the islet cells (5,17,19). The lack of effect of verapamil on first phase insulin release was not due to a delayed onset of action of the drug. This is evident from the marked inhibition of Ca^{++} uptake in Fig. 1 and from the results shown in Fig. 3.
In these experiments the addition of verapamil together with the glucose stimulus, or 5min prior to the additionof glucose, was compared. 5min pre-perifusion with verapamil also failed to inhibit first phase of insulin release. Others have reported that verapamil inhibits both phases of insulin release. However, in those studies the pancreatic preparations were exposed to

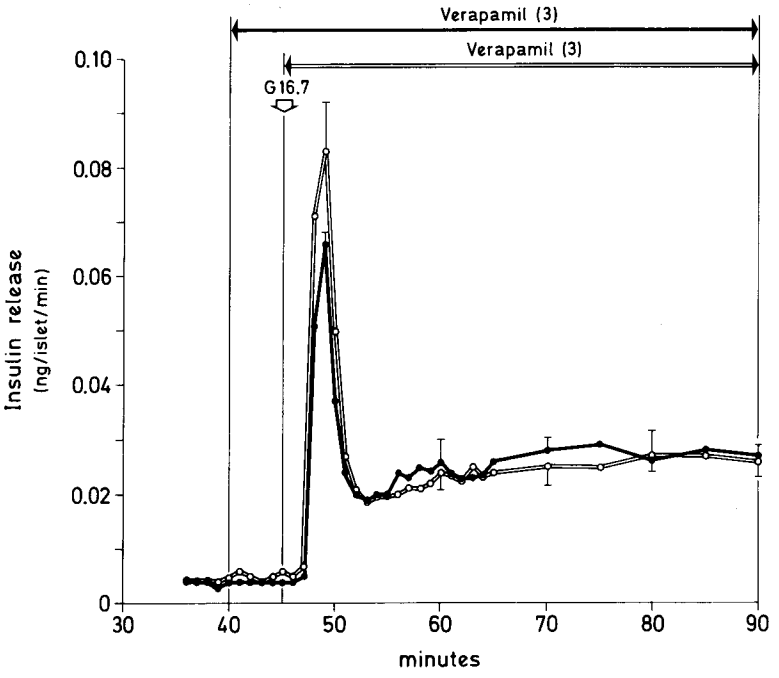


Fig. 3

Effect of verapamil (5μM) on insulin release stimulated by 16.7mM glucose. In paired experiments verapamil was added either together with glucose or 5min prior to glucose

verapamil for lengthy periods of time prior to the addition of glucose (9). The inhibition of first-phase insulin release observed under those conditions could therefore be due to a depletion of a critical, labile calcium pool in the ß-cells or reflect the uptake of verapamil by the cells. The former explanation is the most attractive one and will be further considered below.

The results obtained with verapamil suggest that glucose utilises cellular calcium stores for the generation of the first phase of insulin release, and part of the second phase, while the remainder of the second phase depends on the stimulation of Ca^{++} uptake from the extracellular fluid.

2. Effects of trifluoperazine

The inhibitor of calcium-calmodulin action, trifluoperazine, has been shown to inhibit glucose-stimulated insulin release (1, 7,15). The drug has also been reported to inhibit protein

phosphorylation in intact cells induced by depolarising concentrations of K^+ (12) and calmodulin-induced phosphorylation in islet cell homogenates (1). The effects of trifluoperazine on Ca^{++} uptake, insulin release and $^{45}Ca^{++}$ efflux have been investigated.

The effects of trifluoperazine on Ca^{++} uptake and insulin release are shown in Table 1. 100μM trifluoperazine did not alter Ca^{++} uptake or insulin release in the presence of 2.8mM glucose. 16.7mM glucose increased Ca^{++} uptake by 200%. Glucose-stimulated Ca^{++} uptake was inhibited by 34% when trifluoperazine was added together with the glucose stimulus. When the islets were exposed to trifluoperazine 5min prior to the addition of 16.7mM glucose, a further inhibition of Ca^{++} uptake was observed (54% inhibition). Under these conditions glucose-induced insulin release was inhibited by 51%.

In the following series of experiments the effects of trifluoperazine on the dynamics of insulin release and $^{45}Ca^{++}$ efflux were examined. As shown in Fig. 4, the addition of 100μM trifluoperazine at the time of glucose stimulation did not significantly inhibit the first phase of glucose-stimulated insulin release. The second phase of insulin release was inhibited by 56%. There was a striking similarity between the pattern of inhibition of insulin release induced by trifluoperazine and that caused by verapamil (Fig. 2). In the presence of trifluoperazine the rate of insulin release remained constant from the nadir position to the end of the perifusion. The first peak of $^{45}Ca^{++}$ efflux, which was maximal 3min after the addition of high glucose, was significantly inhibited by trifluoperazine. The inhibition of $^{45}Ca^{++}$ was more marked during the second phase.

To examine whether the lack of inhibition of first phase insulin release was due to a delay in the onset of action of trifluoperazine, the islets were exposed to the drug 5min prior to the addition of 16.7mM glucose. The results of these experiments are shown in Fig. 5. Under these conditions the first phase of glucose-stimulated insulin release was almost completely abolished by trifluoperazine. When comparing the incremental integrated insulin release between min 47 and 51 to that of the control experiments shown in Fig. 4, the release was inhibited by 86%.

TABLE 1

Effect of trifluoperazine on basal and glucose-stimulated Ca^{++} uptake and insulin release

Preincubation (5min)	Incubation (5min)	$^{45}Ca^{++}$ uptake (pmol/islet/5min)	Insulin release (pg/islet/5min)	n
G 2.8mM	G 2.8mM	0.55 ± 0.08	49 ± 10	13
G 2.8mM + TFP	G 2.8mM + TFP	0.46 ± 0.06	35 ± 10	11
G 2.8mM	G 16.7mM	1.64 ± 0.13	246 ± 19	14
G 2.8mM	G 16.7mM + TFP	1.27 ± 0.09*	-	14
G 2.8mM + TFP	G 16.7mM + TFP	1.06 ± 0.08**	132 ± 17**	13

Batches of 10 islets were preincubated and incubated in the absence or presence of 100μM trifluoperazine (TFP). Radioactive Ca^{++} only present during the 5min incubation.

* $p < 0.05$ and ** $p < 0.001$ versus G 16.7mM alone.

G, glucose

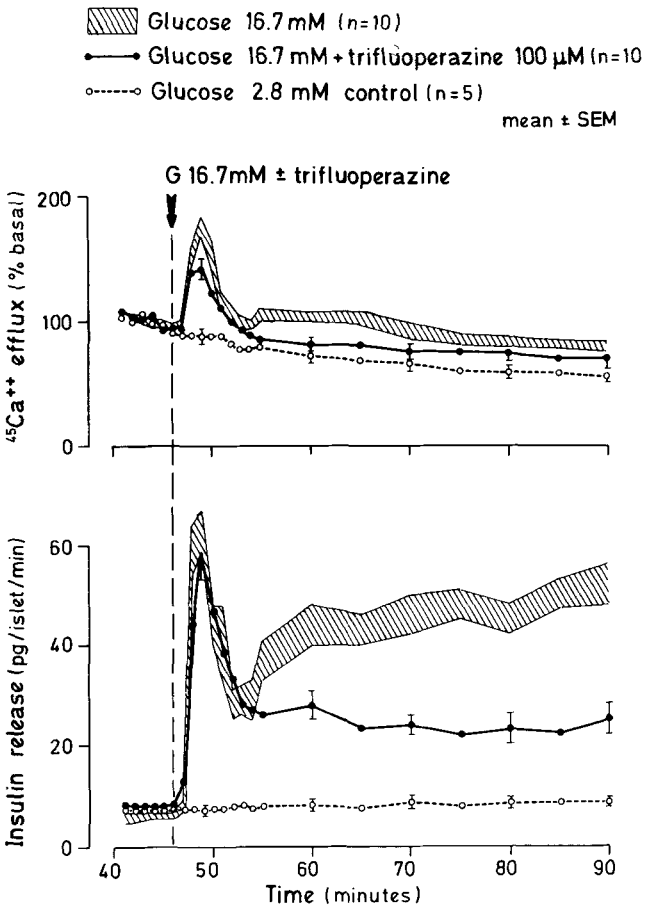


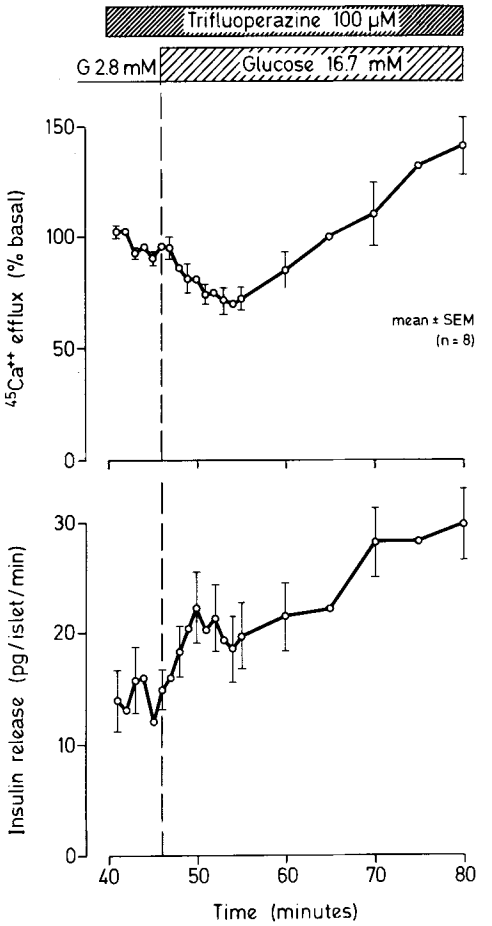
Fig. 4

Effect of trifluoperazine (100µM) on glucose-stimulated $^{45}Ca^{++}$ efflux and insulin release. Trifluoperazine was added to the perifusate at the time of glucose stimulation.

The incremental, integrated insulin release was 161±12pg/islet, n = 10, and 29±3, n = 8, for controls and trifluoperazine respectively. The rate of insulin release increased slightly during second phase, yielding an inhibition of the incremental insulin release by 68%. The insulin release was 1455±87 pg/islet, n = 10, and 463±30, n = 8, for controls and trifluoperazine respectively. These values should be compared with the insulin release during second phase when trifluoperazine was added together with the glucose-stimulus (Fig. 4). Under these conditions the incremental insulin release was 646±76 pg/islet, n = 10. The increased length of exposure of the islets to trifluoperazine had thus dramatic effects on the first phase of insulin release, but only a slightly further inhibitory effect on the second phase. The pattern of $^{45}Ca^{++}$ efflux was also completely altered under these conditions. In the presence of trifluoperazine there was a complete inhibition of the stimulation of $^{45}Ca^{++}$ efflux during the first phase.

Fig. 5

Effect of trifluoperazine on glucose-stimulated $^{45}Ca^{++}$ efflux and insulin release when added 5min prior to glucose-stimulation.



Instead, $^{45}Ca^{++}$ efflux gradually declined to reach a nadir at 55min. Thereafter the efflux of $^{45}Ca^{++}$-gradually increased to reach rates slightly higher than those reached with glucose alone at the end of the perifusion (compare Figs. 4 and 5).

The effect of trifluoperazine mainly on the first phase of insulin release and $^{45}Ca^{++}$ efflux thus depends on the time of addition of this drug. This may, at least in part, be due to a delayed onset of action. The earliest detectable effect is the inhibition of $^{45}Ca^{++}$ efflux at 3min (Fig. 4). Exposure of the islets for 5min appears to be sufficient to cause optimal inhibition of insulin release by trifluoperazine. However, while the first phase of insulin release was almost abolished under these conditions, the second phase was only partially inhibited. This pattern of inhibition by trifluoperazine contrasts markedly to that observed with verapamil. In the case of verapamil, the

first phase of glucose-induced insulin release was not inhibited even when the drug was added prior to the stimulus (Fig. 3). Glucose-stimulated Ca^{++} uptake was inhibited by both verapamil and trifluoperazine, albeit more markedly in the presence of verapamil. These observations suggest that the inhibition of first phase insulin release by trifluoperazine is not due to the inhibition of Ca^{++} uptake.

In addition to the inhibitory action on Ca^{++} uptake, trifluoperazine may interfere with islet cell Ca^{++} handling by a different mechanism. Although the process underlying the stimulation of $^{45}Ca^{++}$ efflux by glucose is not well understood, the efflux may, at least in part, reflect an action of glucose on intracellular calcium stores. The strong inhibition of $^{45}Ca^{++}$ efflux by trifluoperazine (Fig. 5) suggests that the drug may act by interfering with this intracellular action of glucose. From several previous studies, including the use of verapamil (18,20) or islets with increased intracellular calcium stores following incubation in high concentrations of inorganic phosphate (6,20), it was concluded that glucose generates the first phase of insulin release and part of the second phase by an action on cellular calcium stores. Interference with this action of glucose could therefore be expected to result in the pattern of inhibition of insulin release seen with trifluoperazine (Fig. 5).

Trifluoperazine was used to assess the involvement of calmodulin in stimulus-secretion coupling in the β-cell. Three lines of evidence support the specificity of the action of the drug. First, while insulin release in response to glucose or glyceraldehyde is inhibited by the drug, that due to raised cyclic AMP levels is not (7). Secondly, two processes probably less dependent on the concentration of cytosol Ca^{++} than the release mechanism, are not affected by trifluoperazine. These are glucose metabolism by the islets (1,15) and glucose-stimulated insulin biosynthesis (1). Thirdly, a good correlation between the potency of inhibition of glucose-stimulated insulin release and the inhibition of brain phosphodiesterase activity, has been reported for several phenothiazines, including trifluoperazine (1). The definition of the sites of action of trifluoperazine may help to clarify the sites of involvement of calmodulin in the glucose-induced insulin

release. As insulin release stimulated by cyclic AMP was not affected by trifluoperazine, it is unlikely that calmodulin is involved in the process of exocytosis per se (7,19). Ca^{++} uptake stimulated by either glucose (Table 1) or depolarising concentrations of K^+ (C.B. Wollheim, unpublished observations) was inhibited by trifluoperazine. Calmodulin appears thus to be involved in the activation of Ca^{++} channels in the plasma membrane. A second site of involvement of calmodulin seems to be the mechanism by which glucose acts on cellular calcium stores to raise cytosol Ca^{++} (19). Further studies are required to delineate the enzyme systems involved in these processes.

3. Effect of ouabain

By using verapamil it was shown that the stimulation of Ca^{++} uptake is not involved in the generation of first-phase insulin release induced by glucose (18) but is involved in that induced by K^+ (20) or acetylcholine (21). If glucose acts on intracellular calcium stores to generate the first phase of insulin release, it should be possible to stimulate release in the absence of extracellular Ca^{++}. However, even when Ca^{++} was removed from the perifusate only at the time of glucose addition, there was an immediate, dramatic inhibition of insulin release. As can be seen in Fig. 6, this manoeuvre caused an inhibition of both phases of insulin release. The inhibition was 75% and 88% for first and second phase, respectively (14). This rapid inhibition of insulin release by the removal of extracellular Ca^{++} has been thought to be due to a rapid loss of calcium from a critical, labile pool in the β-cells (3,14). It may be this, hypothetical, calcium pool upon which glucose acts to mobilise calcium for the first phase insulin release.

Like other cell types the β-cell plasma membrane appears to have an Na/Ca counter-transport mechanism. In this process the maintenance of a high Na^+ gradient across the plasma membrane favours the extrusion of Ca^{++} against its electrochemical gradient (4,14). Dissipation of the Na^+ gradient results in a decrease of Ca^{++} efflux. Ouabain, which inhibits the Na-pump, and increases intracellular Na, causes an initial inhibition of Ca^{++} efflux followed by a delayed stimulation of the efflux. It was found that ouabain immediately inhibited Ca^{++} efflux, while glucose only

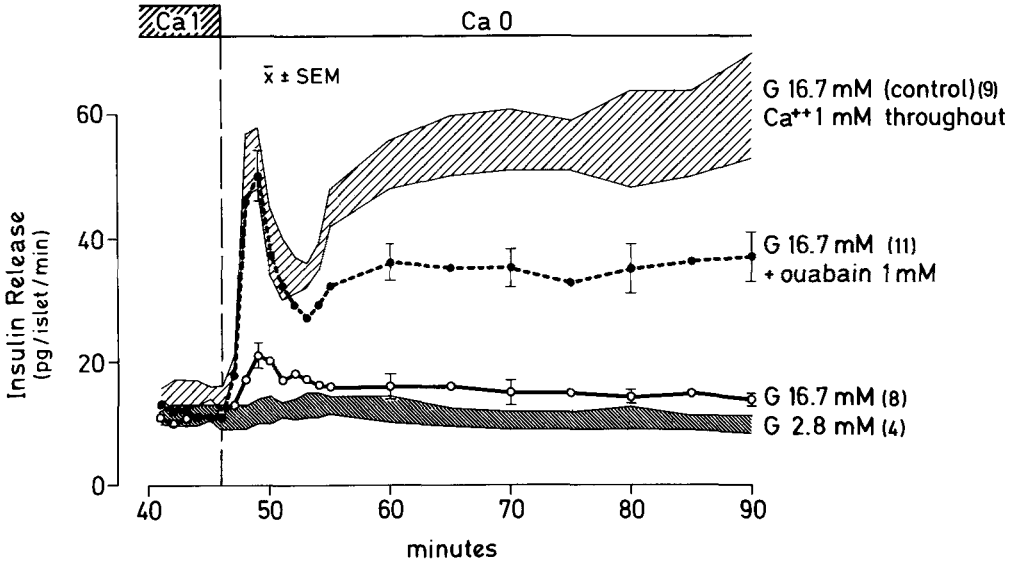


Fig. 6

Effect of Ca^{++} removal and ouabain on glucose-stimulated insulin release. 1mM ouabain was added at the time of Ca^{++} removal together with 16.7mM glucose. For the medium without Ca^{++}, $CaCl_2$ was omitted from the buffer in the 0 Ca^{++} conditions

inhibited the efflux after a 1min lag (14). The immediate inhibition of Ca^{++} efflux could prevent a loss of cellular calcium and, consequently, allow glucose to use cellular calcium to initiate insulin release, even in the absence of extracellular Ca^{++}. Indeed, the addition of ouabain together with 16.7mM glucose at the time of Ca^{++} removal resulted in a complete restoration of the first phase of insulin release (Fig. 6). The second phase was restored to approximately 60%. This release pattern resembles that observed with verapamil (Fig. 2), or, under certain conditions, with trifluoperazine (Fig. 4). A common feature of all these experimental situations is that the Ca^{++} uptake from the extracellular fluid is inhibited. Although the initial inhibition of Ca^{++} efflux by ouabain was similar to that of glucose, the addition of ouabain in the presence of 2.8mM glucose and in the absence of Ca^{++} only caused 10% of the insulin release during first phase, and only 30% during second phase of the release seen with ouabain, and 16.7mM glucose in Fig. 6 (14). Thus, inhibition of Ca^{++} efflux

alone is not sufficient to raise cytosol Ca^{++} to levels high enough to induce insulin release. In contrast, glucose is able to elicit first phase insulin release in the presence of ouabain, even when 0.1mM EGTA was added to the medium (14). Trifluoperazine completely inhibited the stimulation of insulin release under these conditions (C.B. Wollheim, unpublished observations). These observations favour the conclusion that glucose increases cytosol Ca^{++} by acting on cellular calcium stores.

4. Summary and conclusions

The addition of trifluoperazine together with high glucose resulted in a decrease of glucose-stimulated Ca^{++} uptake. Although Ca^{++} uptake was less inhibited than in the presence of verapamil, both drugs caused a similar pattern of inhibition of insulin release. The first phase of glucose-stimulated insulin release was not affected, while second phase was inhibited by approximately 50%. Addition of trifluoperazine prior to the addition of glucose revealed an additional effect of the drug. Under these conditions the first peak of glucose-stimulated $^{45}Ca^{++}$ efflux was completely absent. This was associated with a complete inhibition of the first phase, and a partial inhibition of the second phase of insulin release. Trifluoperazine appears to interfere on the one hand with the opening of voltage-sensitive Ca^{++} channels in the plasma membrane, and on the other hand, with the mechanism by which glucose acts on cellular calcium stores. In the absence of extracellular Ca^{++}, glucose-stimulated insulin release was almost completely abolished. Inhibition of the Na^{+}-pump by ouabain enabled glucose to elicit a normal first phase and a half-normal second phase even in the absence of extracellular Ca^{++}. Ouabain, which like glucose decreases the efflux of Ca^{++}, cannot mimick the effect of glucose on the early phase of insulin release.

It is concluded from these results that glucose acts on cellular calcium stores to raise cytosol Ca^{++} for the generation of the first phase, and part of the second phase, of insulin release. The stimulation of Ca^{++} uptake by glucose contributes to the full development of the second phase. The action of glucose on intracellular calcium stores involves calmodulin, since it is sensitive to trifluoperazine.

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