The Role of Calcium in Glucagon Release

Studies with Verapamil

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SUMMARY

The role of calcium transport into the pancreatic A_2-cell in release of glucagon was studied in the perfused in vitro rat pancreas exposed to the organic calcium–antagonist verapamil (10 and 20 μM). As judged by the inhibitory effect of verapamil, a sufficient influx of calcium was required for glucagon release to be stimulated by either arginine (10 mM) or a lowering of the glucose concentration from 16.6 to 3.3 mM. However, such was not the case for glucose to inhibit the release of glucagon or when the A_2-cell was established in a stimulated state during prolonged exposure to a low, 3.3 mM, glucose concentration. These findings suggest that the role of inwardly directed transport of calcium in the secretory process of the A_2-cell is of a complex nature, being dependent on the type of stimulus employed (arginine or glucose) and, in the case of glucose, on the static or dynamic state of the cell. The intimate mechanisms by which calcium exerts such complex effects on the secretory process in the A_2-cell remain to be elucidated. DIABETES *27*:**996-1004, October, 1978.**

Prior investigations suggest that the interrelationships between calcium and the secretory process of the A_2-cell are complex. Thus, several studies have shown that raised calcium concentrations or calcium ionophores were able to stimulate the release of glucagon.[1-6] In addition, the calcium dependency of the release of glucagon induced by arginine as well as by a mixture of "fumarate + glutamate + pyruvate" has been documented.[7-9] In contrast, however, several workers have reported on the enhancing effect of calcium deprivation upon glucagon secretion.[6,10-16]

The present work was initially undertaken in order to decide whether the increase in glucagon release seen when calcium is removed from the extracellular medium was related to diminished calcium transport into the cell. For this purpose, we infused, in the presence of fixed glucose concentrations (3.3 and 16.6 mM), the organic compound verapamil, which is thought to inhibit the transport of calcium into many tissues.[17-24] The influence of verapamil was, eventually, also studied in relation to arginine (10 mM) and to dynamic variations in the concentration of glucose (from 3.3 to 16.6 mM or the reverse). Parts of this work have been presented in abstract forms.[9,25,26]

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MATERIALS AND METHODS

Fed female albino rats were used in this study (mean body weight ± S.E.M.: 228 ± 2 gm.; n = 96). The perfusion technique has been described previously.[14-16] The pancreases were dissected under pentobarbital anesthesia (45 mg. per kilogram, intraperitoneally) according to the procedure of Loubatières et al.,[27] all adjacent organs, including the duodenum, being excluded in order to avoid contamination by enteric glucagon-like immunoreactivity. The pancreases were perfused in situ, without recycling, through the celiac and mesenteric arteries via a cannula inserted into the aorta. The animals were given intravenous heparin sodium (125 U.) immediately before cannulation.

The basic medium had the following composition: NaCl, 118.5 mM; KCl, 4.7 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; and $CaCl_2$, 2 mM. The medium was supplemented with dextran (40 gm. per liter, T 70, Pharmacia, Uppsala, Sweden) and bovine albumin (5 gm. per liter, fraction V, Sigma, St. Louis). The perfusate was equilibrated

against a mixture of oxygen and carbon dioxide (95:5), with a resulting pH of 7.4, and it entered the pancreas at a temperature of 37.5° C. The flow rate was set at around 2 ml. per minute, with a resulting pressure of 33.3 ± 0.5 mm. Hg (mean ± S.E.M., n = 96). Samples of the effluent were collected at one-minute intervals in chilled tubes containing 2,000 K.I.U. Trasylol and were frozen at −25° C. until time of assay.

The secretagogues were dissolved in saline and administered through a sidearm syringe working at a flow rate of 0.075 ml. per minute (Braun infusion pump, Melsungen, West Germany). L (+)-arginine was of analytic grade (Merck, Darmstadt, West Germany). Verapamil (Isoptine) was kindly donated by Dr. R. Lopinot (Coles-Knoll, S. A., Diegem, Belgium).

Glucagon and insulin were estimated on 0.2-ml. samples at the times shown in the figures using a combined radioimmunoassay for these hormones.[28] The hormone standards were purified beef-pork glucagon (lot 258-234-B-161-1, a gift from Dr. M. A. Root, Eli Lilly and Co., Indianapolis) and rat insulin (lot R 170, mean biologic activity: 21.4 I.U. per milligram, a gift from Dr. J. Schlichtkrull, Novo, Bagsvaerd, Denmark). The Trasylol used in both the assay and the collection tubes was kindly donated by Dr. G. Wald (Bayer, Brussels, Belgium). Glucose was titrated in the pancreatic effluent with a ferricyanide method using a first generation Technicon analyzer.

The rates of glucagon and insulin secretion were expressed in nanograms per minute. Total glucagon and insulin release was estimated by calculating the areas under the curves. Statistical analyses were conducted using Student's nonpaired *t*-test.[29]

RESULTS

The Effects of Verapamil (10 μM) upon Glucagon and Insulin Release in the Presence of a Fixed Glucose Concentration (3.3 and 16.6 mM)

In the presence of a low glucose concentration (3.3 mM), the infusion of verapamil induced a slight elevation in the rates of glucagon release, although this increase was not significant when compared with the experiments performed in the absence of verapamil (figure 1). These slightly elevated glucagon-secretory rates were not modified when the infusion of verapamil was arrested. A few points differed statistically from the control experiments, but these seemed merely related to the smaller standard error of the mean values obtained during the late period of the perfusion.

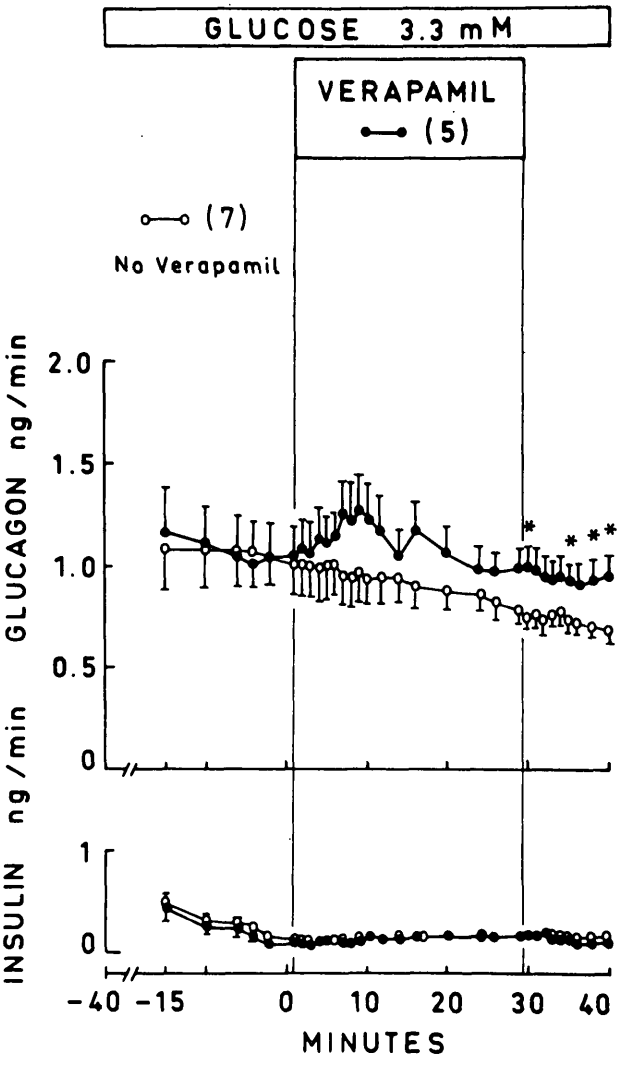

FIG. 1. The effect of verapamil (10 μM) upon glucagon and insulin release in the presence of glucose. Control experiments were performed throughout at 3.3 mM glucose in the absence of verapamil. Mean-secretory rates (± S.E.M.) are indicated together with the number of experiments performed in each group (n).

The effect of verapamil duplicated only partially that evoked by calcium deprivation and restoration in the presence of a low glucose concentration.[38] The low, basal rate of insulin release remained unaffected by the infusion of verapamil.

In the presence of a high glucose concentration (16.6 mM), the infusion of verapamil virtually failed to influence the rate of glucagon release (figure 2). Again, the results obtained with verapamil were far from duplicating those of calcium deprivation and restoration.[38] The infusion of verapamil provoked close to a 50 per cent inhibition of insulin release, the inhibitory effect being slowly reversed after with-

drawal of the drug.

Essentially similar results were obtained for both glucagon and insulin release in the presence of 3.3 as well as 16.6 mM glucose and with a higher (20 μM) concentration of verapamil (results not shown). Accordingly, the latter dose was used in all the following experiments in order to assure appropriate effectiveness of the drug.

The Effects of Verapamil (20μM) upon Arginine-induced Glucagon and Insulin Release in the Presence of a Low Glucose Concentration (3.3 mM)

In the first series of experiments, a verapamil infusion was maintained throughout the perfusions, while a 10 mM arginine stimulus was applied after a 40-minute equilibration period (figure 3, closed circles). During the equilibration period, the output of glucagon recorded in the presence of verapamil was comparable to that seen in the control experiments performed in the absence of the drug (minutes −15 to +1: 1.03 ± 0.20 ng. per minute, n = 5, versus 1.09 ± 0.16 ng. per minute, n = 6, open circles, N.S.), in agreement with the observation that verapamil by itself exerts only a limited influence upon glucagon release at the low, 3.3 mM, level of glucose (see figure 1). In the presence of verapamil, the normally biphasic and dramatic response of the A_2-cell to arginine (minutes +1 to +30, open circles: 2.95 ± 0.49 ng. per minute, n = 5) was modified strikingly. Thus, the early, secretory phase was markedly blunted, whereas the late phase was abolished. As a matter of fact, glucagon release was inhibited to below prestimulatory levels during the late phase of the ar-

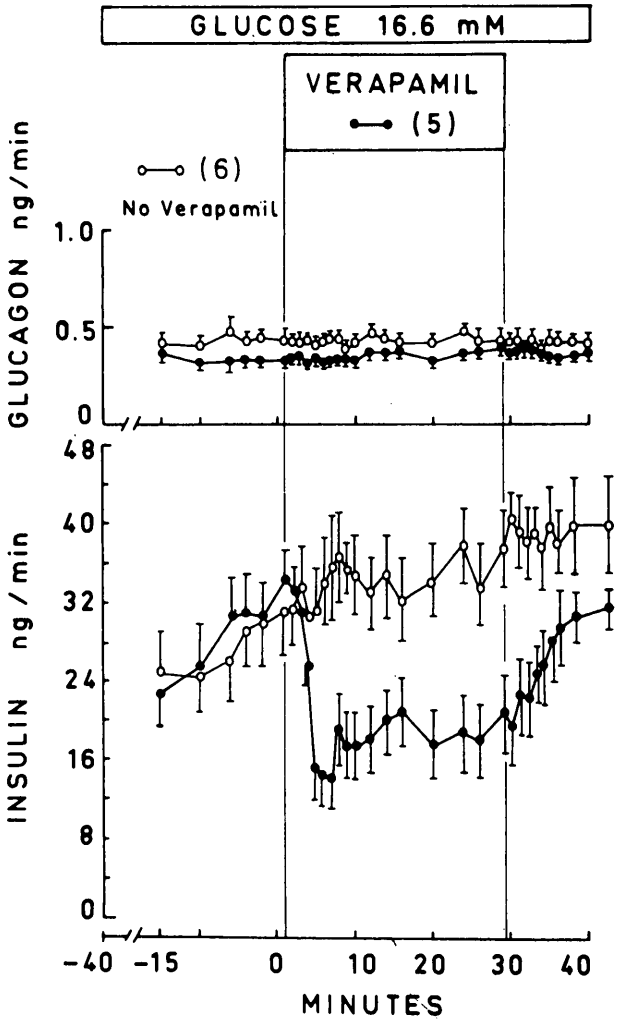

FIG. 2. The effect of verapamil (10 μM) upon glucagon and insulin release in the presence of glucose. Control experiments were performed with 16.6 mM glucose in the absence of verapamil. Presentation as in figure 1.

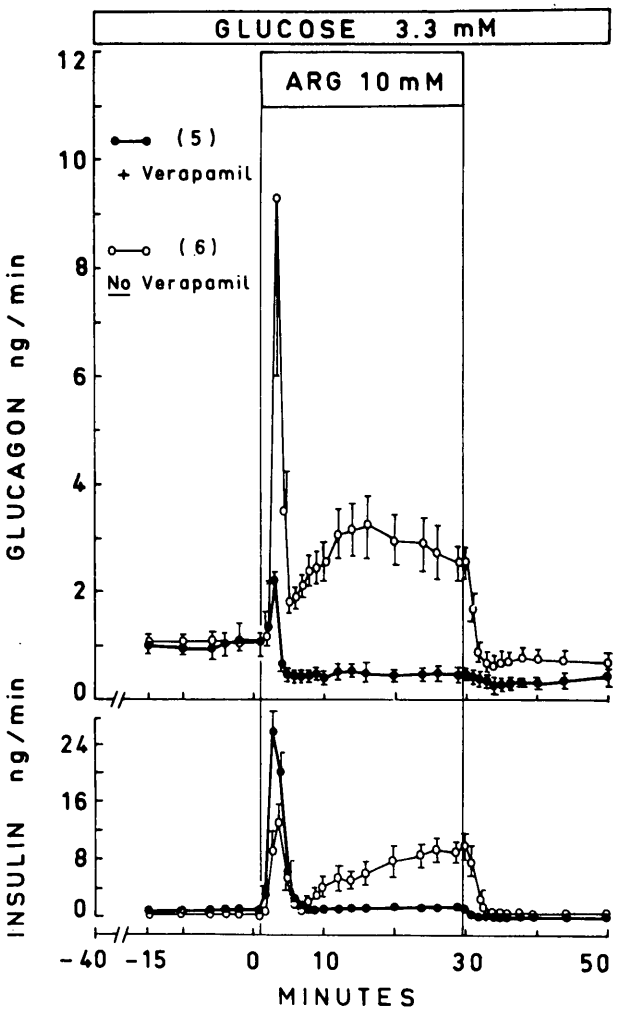

FIG. 3. The effect of arginine (ARG) upon glucagon and insulin release in the presence of glucose and in the absence or presence of verapamil (20 μM) infused throughout the experiments. Presentation as in figure 1.

ginine infusion, the secretory rates now being comparable to those usually seen in the presence of a high glucose concentration (16.6 mM) and absence of arginine (minutes +10 to +30: 0.47 ± 0.10 ng. per minute, n = 5, figure 3, closed circles, versus 0.44 ± 0.06 ng. per minute, n = 6, results from experiments performed at 16.6 mM throughout, figure 2; see also figures 7 and 8). The inhibition of glucagon release persisted after the arrest of the arginine infusion. In the presence of verapamil, the late phase of the insulin response to arginine was abolished, but, in contrast, the first phase was unexpectedly amplified (figure 3, closed circles, lower panel).

In the second series of experiments, the sequence of introduction of verapamil and arginine was reversed in order to find out whether verapamil could influence the release of glucagon once it had been initiated by the amino acid. In the pancreas exposed to 10 mM arginine throughout, the infusion of verapamil first provoked a short-lived secretory peak followed by an immediate inhibition of glucagon release (figure 4, closed circles). Again, the combination of arginine and verapamil lowered the glucagon-secretory rates to values comparable to those seen in the sole presence of a high, 16.6 mM, glucose concentration (minutes +10 to +30: 0.51 ± 0.14 ng. per minute, n = 5). This inhibition persisted for 20 minutes after the arrest of the verapamil infusion. Verapamil markedly inhibited arginine-induced insulin release, the effect of the drug also persisting in the beta cell (figure 4, closed circles, lower panel).

The Effects of Verapamil (20 μM) upon Arginine-induced Glucagon and Insulin Release in the Presence of a High Glucose Concentration (16.6 mM)

In the experiments in which an infusion of verapamil was maintained throughout the perfusion (figure 5, closed circles), the secretory rates of glucagon release recorded during the equilibration period did not differ from those seen in the experiments performed in the absence of the drug (minutes −15 to +1: 0.32 ± 0.06 ng. per minute, n = 6, versus 0.27 ± 0.05 ng. per minute, n = 6, open circles, N.S.), confirming the absence of effect of verapamil at the high, 16.6 mM, glucose concentration (figure 2). In the presence of verapamil, the late phase of arginine-induced glucagon release was suppressed (minutes +10 to + 30: 0.32 ± 0.01 ng. per minute, n = 6, versus 0.59 ± 0.06, n = 6, open circles, $P < 0.001$). In contrast, the early secretory phase was not reduced and occurred even one minute sooner than in the control experiments. In the case of insulin release, ver-

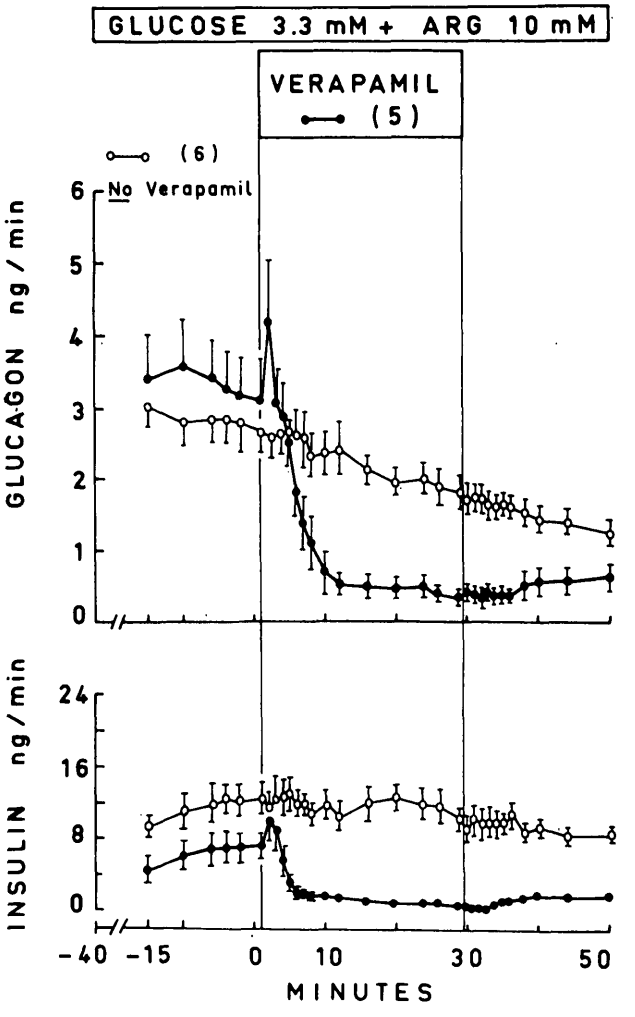

FIG. 4. The effect of verapamil (20 μM) upon arginine-induced glucagon and insulin release in the presence of glucose. The infusion of arginine (ARG) was maintained throughout the experiments. The control experiments were performed throughout in the presence of glucose and arginine and in the absence of verapamil. Presentation as in figure 1.

apamil caused a 65 per cent inhibition during the equilibration period (figure 5, closed circles, lower panel). Thereafter, the behavior of the beta cell in response to the arginine stimulus was comparable to that of the A_2-cell in that the early phase was preserved and the late phase abolished. It might be of interest to note that, in the presence of verapamil as well as in the control experiments, a transient inhibition of insulin release occurred after the arrest of the arginine infusion.

In the experiments in which the reverse sequence was adopted for the administration of arginine and verapamil, we confirmed that the calcium antagonist was rapidly able to suppress the late phase of

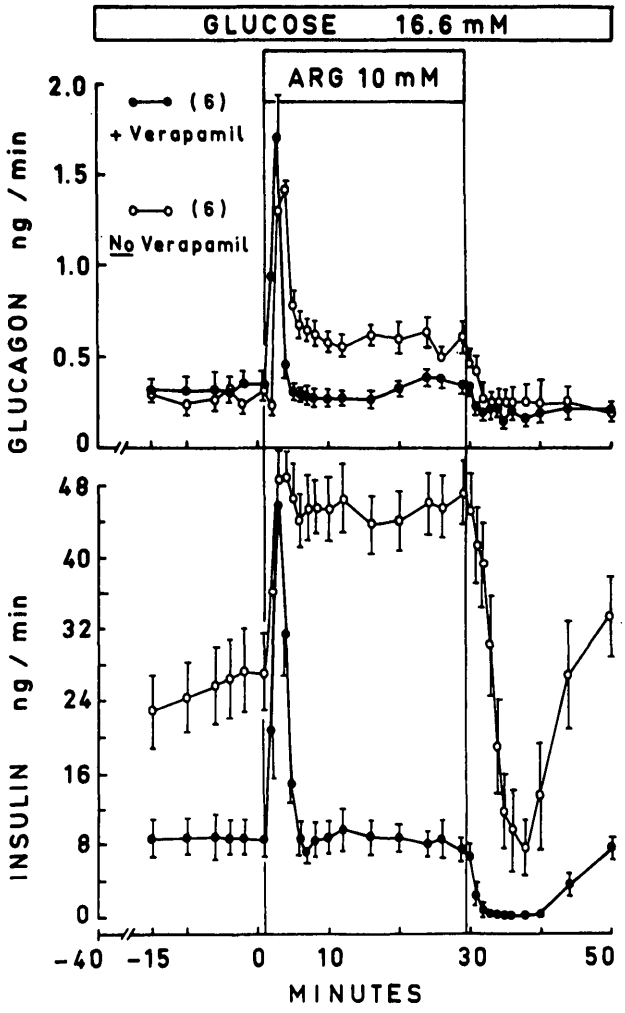

FIG. 5. The effect of arginine (ARG) upon glucagon and insulin release in the presence of glucose and in the absence or presence of verapamil (20 μM) infused throughout the experiments. Presentation as in figure 1.

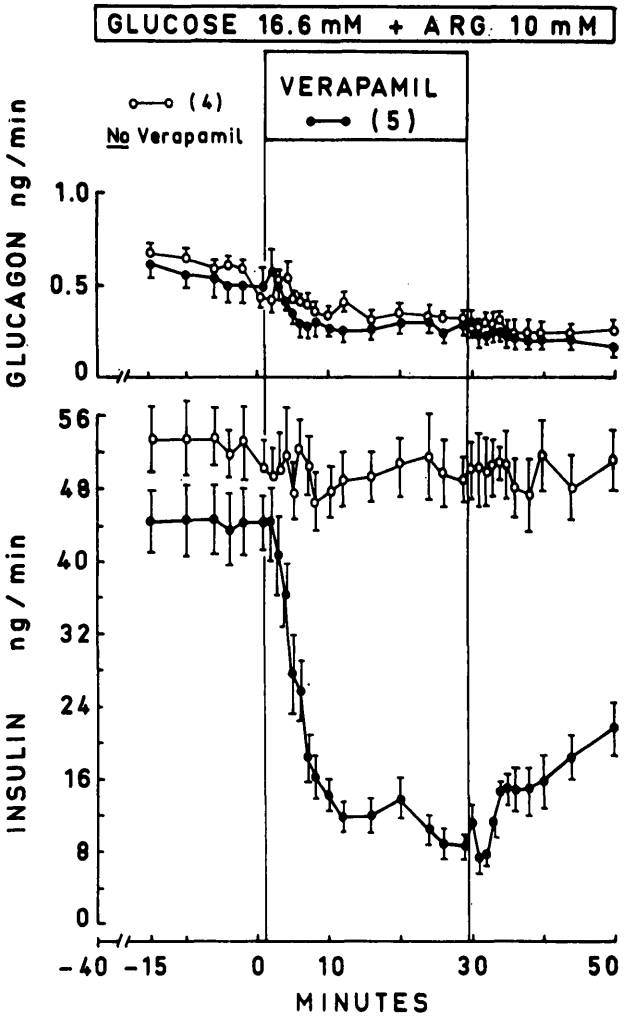

FIG. 6. The effect of verapamil (20 μM) upon arginine-induced glucagon and insulin release in the presence of glucose. The infusion of arginine (ARG) was maintained throughout the experiments. The control experiments were performed throughout in the presence of 16.6 mM glucose and 10 mM arginine and in the absence of verapamil. Presentation as in figure 1.

arginine-induced glucagon and insulin release (figure 6, closed circles).

The Effects of Verapamil (20 μM) upon Glucagon and Insulin Release in Relation to Dynamic Variations in the Concentration of Glucose

The last series of experiments was undertaken in an attempt to further characterize the interrelationships between glucose and calcium transport into the A_2-cell. We were led to such a study in view of the apparent ineffectiveness of verapamil upon glucagon release in the presence of glucose, as opposed to its potent inhibitory action in the presence of arginine. The experiments again demonstrated that verapamil by itself does not influence the release of glucagon, whether in the presence of a low, 3.3 mM, or a high, 16.6 mM, glucose concentration (figures 7 and 8, respectively, middle panel, equilibration periods). Moreover, raising the glucose concentration from 3.3 to 16.6 mM inhibited the rate of glucagon release in the presence of verapamil (figure 7, middle panel, closed circles) as well as in its absence (figure 7, middle panel, open circles). In contrast, the release of glucagon provoked by a decrease in the glucose concentration from 16.6 to 3.3 mM was clearly blunted in the presence of verapamil (figure 8, middle panel, closed circles). Verapamil consistently provoked close to a 75 per cent inhibition of insulin release (figures 7 and 8, lower panels, closed circles).

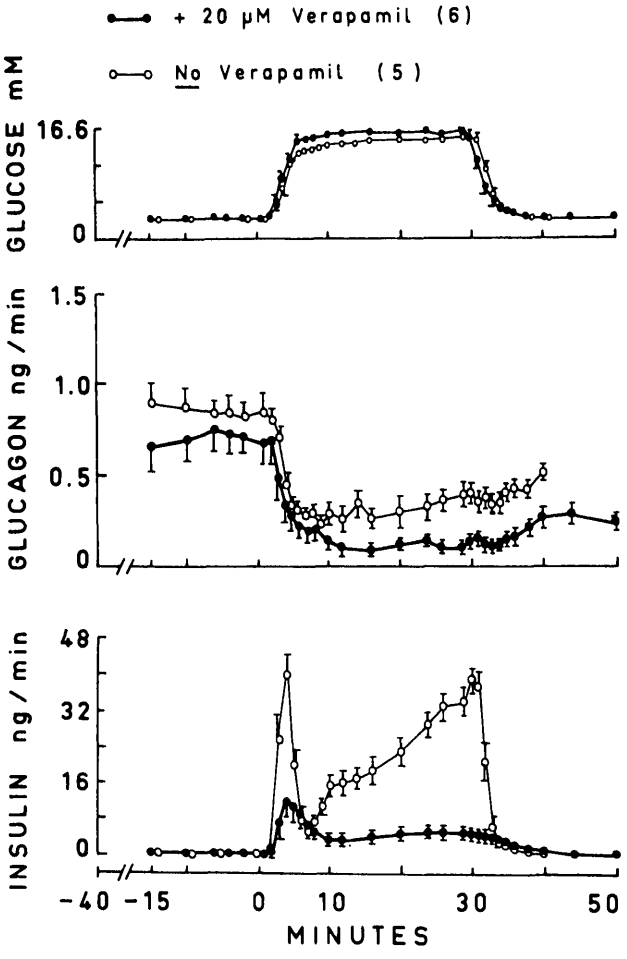

FIG. 7. The effect of raising the glucose concentration from 3.3 to 16.6 mM upon glucagon and insulin release in the absence or presence of verapamil infused throughout the experiments. Presentation as in figure 1.

DISCUSSION

The first question to be asked concerning the present experiments is, of course, was verapamil acting as a specific calcium-antagonist in the endocrine pancreas? A positive answer to this question is suggested by the results obtained with the drug in the case of glucose-induced insulin release. Indeed, verapamil consistently inhibited both the early and late phases of glucose-induced insulin release (figures 2, 7, and 8). There was evidence that the inhibitory effect of verapamil upon the late phase of glucose-induced insulin release was more pronounced when the drug was introduced before glucose (figure 7) than when it was infused after prolonged exposure of the beta cell to glucose (figure 2). Our experiments also disclose a delayed reversibility of the verapamil effect after withdrawal of the drug (figure 2). All these results are in agreement with previous findings,[30-33] which have convincingly documented the view that the major effect of verapamil in the insulin-producing cell, like in other tissues,[17-24] is to reduce the inward transport of calcium across the plasma membrane.[31,32] It seems reasonable, therefore, to assume that verapamil also acted predominantly as an organic calcium-antagonist in the glucagon-producing cell. However, one should keep in mind that verapamil, and especially the racemic form used in our study, has been reported to have other sites of action. For instance, the drug was said to interfere with the intracellular mode of action of calcium,[17,18] to inhibit K^+ uptake and efflux in cardiac Purkinje fibers,[34] to inhibit the fast Na channel,[35] and to act as a local anesthetic.[36] None of these effects has yet been described in the beta or alpha cell. The remainder of the discussion will be based, therefore, on the assumption that verapamil was indeed acting essentially as a calcium antagonist in the perfused rat pancreas.

The participation of inwardly directed transport of calcium in arginine-induced insulin release. To our knowledge, there are as yet no data available relative to the influence of verapamil upon arginine-induced insulin release. Our results demonstrate that the late phase of such a secretory process was consistently suppressed in the presence of the drug, whether at low (figures 3 and 4) or high glucose concentration (figures 5 and 6). Therefore, the integrity of inward calcium transport appears to be an absolute requirement for the late phase of arginine-induced release. The picture is different, however, as far as the first phase of arginine-induced insulin release is concerned. Indeed, this early phase was either maintained (figure 5) or amplified by verapamil (figure 3). This contrasts with the observation that verapamil consistently inhibits the first phase of the insulin secretory response to glucose, gliclazide, or Ba^{2+}.[30-37]

The participation of inwardly directed transport of calcium in glucagon release. The results obtained with verapamil in the case of glucagon release indicate that the role of calcium entry into the A_2-cell differs in relation to the type of stimulus and experimental condition under study. Several facts are outstanding.

First, the integrity of inward transport of calcium seems to be essential for the late phase of arginine-induced glucagon release, whether at low or high glucose concentration (figures 3 to 6). The effect of verapamil to inhibit arginine-induced glucagon release is consonant with previous observations indicating that

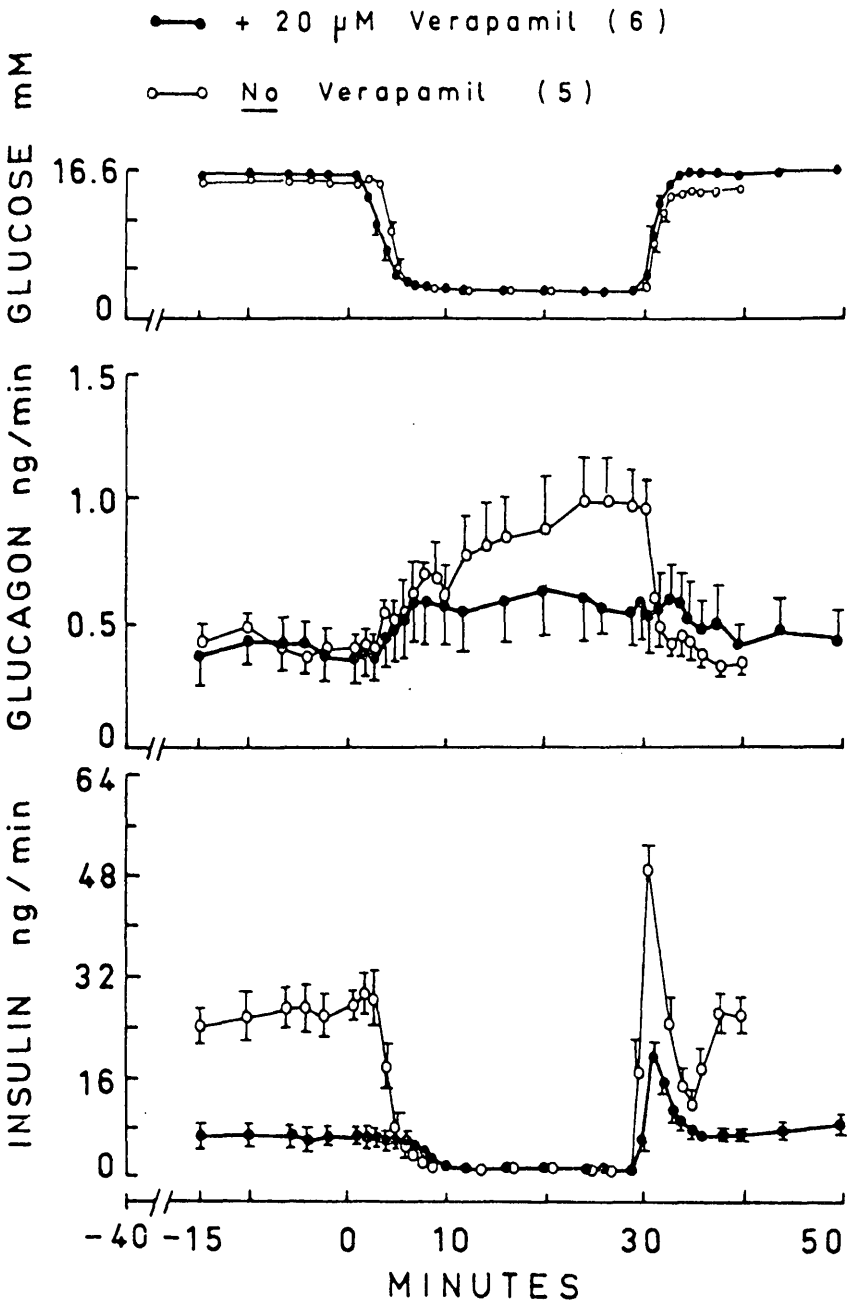

FIGURE 8

The effect of decreasing the glucose concentration from 16.6 to 3.3 mM upon glucagon and insulin release in the absence or presence of verapamil infused throughout the experiments. Presentation as in figure 1.

extracellular calcium is needed for arginine to stimulate glucagon output.[7,8] It should be noted, however, that the first phase of arginine-induced glucagon release was less dependent on inward calcium transport, since this first phase was preserved in the presence of verapamil (figure 5). This lesser requirement for calcium resembles that seen in the case of the first phase of arginine-induced insulin release. A closer examination of the experimental data suggests that verapamil did not solely abolish but even reversed the response of the A_2-cell to arginine. Indeed, during constant exposure to 3.3 mM glucose and 20 μM verapamil, the administration of arginine eventually resulted in an inhibition rather than a stimulation of glucagon release (figure 3).

There is one other experimental situation in which an apparent requirement for calcium exists—namely, when glucagon output increases in response to a sudden lowering of the glucose concentration (figure 8). Such a response of the A_2-cell is significantly reduced in the presence of verapamil. Likewise, it was previously reported that a lowering of the glucose concen-

tration fails to stimulate glucagon release at a low, extracellular calcium concentration.[16] Therefore, the enhancement of glucagon release seen in response to decreasing glucose concentrations may represent, like that evoked by arginine, a classic calcium-dependent process. Other experiments in this study, however, suggest that inward calcium transport is not invariably required for the control of the secretory process in the A2-cell. For instance, verapamil does not inhibit—and may even increase slightly and transiently—glucagon release once activated by a prolonged exposure to a low glucose concentration (figure 1), suggesting that the A2-cell no longer relies on a normal calcium influx. Moreover, glucose is perfectly able to inhibit the release of glucagon in the presence of verapamil. Likewise, under steady-state conditions, verapamil fails to exert any effect in the presence of a high concentration of glucose (figures 2, 5, and 8—equilibration periods). Let us recall that, in contrast, glucose was found to be unable to inhibit the release of glucagon at low extracellular calcium concentrations.[3,5,16] Altogether these observations suggest it is the presence of extracellular calcium per se rather than its provision to the intracellular calcium pool that is crucial for glucose to inhibit glucagon release. In conclusion, assuming that verapamil acts specifically as a calcium antagonist, a reduction in the rate of calcium entry into the A2-cell affects the process of glucagon secretion seemingly in a complex manner, which does not always reproduce the situation found at a low, extracellular, calcium concentration. It seems reasonable to postulate, therefore, that calcium has several distinct sites of action in the glucagon-secretory sequence.

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