to Helen,

Stéphane and Mia

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RESUME

L'influence des catécholamines sur le pancréas insulaire n'est pas un fait récent. Toutefois, personne n'est encore parvenu à élucider le mode d'action intime de l'épinephrine sur la cellule-β. Ce travail se veut une amorce à une meilleure compréhension des mécanismes régissant l'interaction entre ce catécholamine et la cellule-β.

Au cours de travaux préliminaires, des rats reçurent une injection d'antisérum anti-insulinique et d'albumine humaine marquée à l'iode^{131}. Le débit sécrétoire d'insuline endogène fut déduit de la réduction progressive du pool d'anticorps circulants nonneutralisés. Le débit sécrétoire fut nettement moindre chez les rats obligés à nager que chez les animaux témoins. Cet effet inhibiteur de l'exercice musculaire fut levé par injection préalable de phentolamine. Ces données suggérèrent que l'effet inhibiteur de l'effort sur la sécrétion d'insuline est secondaire à une libération de catécholamines.

Utilisant des fragments de pancréas, il nous fut possible de confirmer l'inhibition du débit sécrétoire induite par l'épinephrine. En effet, nous avons montré que la phentolamine (et non le propranolol) pouvait contrer les effets inhibiteurs de l'épinephrine sur la sécrétion insulinique lorsque stimulée par le glucose, ce qui nous permit d'affirmer qu'une telle inhibition est probablement induite par

une stimulation des récepteurs alpha de la cellule-β. Il nous fut également possible de montrer que cette inhibition occasionnée par l'épinephrine ne correspondait pas nécessairement à un appauvrissement intracellulaire en AMP-cyclique; en effet, des conditions expérimentales provoquant une accumulation intracellulaire de ce nucléotide furent sans effet sur l'inhibition provoquée par l'épinephrine.

Par la suite, nous avons montré que le glucose ne constituait pas le seul agent insulinotrope contre lequel l'épinephrine exercait son effet inhibiteur. En effet, nous avons remarqué que tous les agents réputés insulinotropes (acides aminés, sulfonylurées, fortes concentrations de K^+, AMP-cyclique, théophylline) subissaient cette inhibition.

Au cours de l'évaluation du rôle des cations sur les processus sécrétoires, nous avons remarqué une amplification de l'effet stimulant du glucose sur la sécrétion insulinique tant par la ouabaine que par de fortes concentrations de K^+ (10 à 34 mEq/l). Par contre, un effet contraire se produisit lorsque le Na^+ fut partiellement remplacé par le Li^+, le Tris, ou par de fortes quantités de K^+ (87 à 115 mEq/l). La substitution du sucrose au NaCl abaissa significativement cette forte sécrétion observée lors d'un enrichissement en K^+ (24 mEq/l). Ces données suggérèrent que les cations alkalins pourraient influencer les processus sécrétoires en modifiant le jeu des cations alkalino-terreux sur

la cellule-β. L'absence de réponse au Ba^{++} lors de l'échange du Na^{+} contre du Li^{+}, du Tris, ou de fortes quantités de K^{+} favorise une telle interprétation. L'absence de Ca^{++} dans le milieu d'incubation offre un argument supplémentaire en faveur de cette interprétation; en effet, dans de telles conditions, les taux sécrétoires élevés obtenus par substitution du Na^{+} par la choline (ou par des concentrations moyennes de K^{+}) se sont affaissés très significativement.

L'effet inhibiteur de l'acidose extracellulaire sur l'effet stimulant du glucose pourrait également être attribuable à une pénétration réduite du Ca^{++} dans la cellule.

L'effet stimulant du glucose sur le débit sécrétoire fut levé tant en l'absence de Ca^{++} qu'en présence de fortes quantités de Mg^{++}. Ces observations suggérèrent que le Mg^{++} d'une part et que le Ca^{++} ou le Ba^{++} d'autre part pourraient agir comme antagonistes sur les processus sécrétoires. L'inhibition engendrée par l'épinephrine sur les effets stimulants du glucose ne fut en aucun temps modifiée ni par le Ba^{++} ni par le Ca^{++}. Nous avons conclu que l'influx de Ca^{++} dans la cellule-β pouvait stimuler la décharge insulinique et que l'épinephrine pouvait supprimer cette décharge en empêchant cet influx calcique.

Afin de mieux comprendre les relations pouvant exister entre le Ca^{++}, le glucose, et l'épinephrine, nous avons mis au point une technique permettant la mesure des efflux de Ca^{++} de la cellule-β. Ayant préalablement préincubé les i-

lôts de Langerhans en présence de calcium^{45} , nous avons remarqué un parallélisme entre le débit d'insuline provoqué par le glucose et celui de calcium^{45} , probablement associé aux granules sécrétoires. Lors de la suppression du débit insulinique (absence ou presque de calcium extracellulaire, présence d'eau lourde) le glucose imprimait une réduction immédiate de l'efflux de calcium^{45} . Ces effets du glucose étaient tous réversibles. Ces données nous laissèrent croire que le glucose aurait pu accroître la teneur intracellulaire en calcium en inhibant son transfert vers le milieu extracellulaire. Il nous apparut également que l'effet inhibiteur de l'épinephrine ne correspondait pas exactement à l'inhibition observée par un appauvrissement en glucose; il semblerait plutôt que l'épinephrine augmente l'affinité protoplasmique de la cellule-β pour le calcium.

Une série d'évènements semble être à l'origine de la sécrétion insulinique provoquée par le glucose dans la cellule-β; nous pourrions, chronologiquement, les établir comme suit:

1) métabolisme glucidique conduisant à l'élaboration d'un signal métabolique;
2) captation de calcium par la cellule-β en réponse à ce signal;
3) interaction entre le calcium et un réseau microtubulaire-microfilamenteux responsable des mouvements émiocytotiques des granules d'insuline.

On attribue au db-cAMP et à la théophylline la capacité de

potentialiser la réponse insulinique provoquée par le glucose. S'appuyant sur les concepts ci-haut mentionnés, nous avons tenté lors des experiences subséquentes de situer l'action première de l'AMP-cyclique. Trois hypothèses furent considérées:

1) une facilitation du métabolisme glucidique;
2) une modification de la distribution intracellulaire du calcium;
3) une interaction avec le réseau microtubulaire-microfilamenteux.

La première de ces trois hypothèses nous apparut peu vraisemblable vu l'absence d'effets du db-cAMP et de la théophylline sur la captation glucidique induite par le glucose. La dernière de ces hypothèses fut également rejetée, premièrement en raison de l'indifférence de la théophylline face aux effets délétères de la colchicine sur le réseau microtubulaire-microfilamenteux, et, deuxièmement, vu que les effets insulinotropes du glucose et de la théophylline étaient indifféremment affectés par la vincristine et la colchicine. Les données suivantes nous ont suggéré un effet de l'AMP-cyclique sur la distribution intracellulaire du calcium: contrairement au glucose qui demeura incapable d'exercer ses effets insulinotropes en milieu pauvre en calcium, le db-cAMP et la théophylline se révélèrent capables de restorer l'effet insulinotrope du glucose. De plus, sur ilôts perfusés, la théophylline occasionna une augmentation très sensible de l'efflux de calcium^{45} quelle que soit la concentration en glucose du milieu. Nous avons donc conclu

que l'effet insulinotrope de l'AMP-cyclique pourrait être occasionné par une redistribution du calcium intracellulaire, laquelle favoriserait un échange plus facile entre le calcium intra- et extracellulaire.

Finalement, au moyen d'une technique impliquant la ligature de canaux pancréatiques, nous avons montré un effet glucagonotrope de l'épinephrine. Cet effet pu être observé à basse (0.3 mg/ml) et à haute (3.0 mg/ml) teneurs en glucose, en présence de concentrations très faibles d'épinephrine (100 nM).

ABSTRACT

It has been known for many years that, under certain circumstances, catecholamines induce changes in the rate of insulin release from the beta-cells; but nobody has yet characterized the intimate effects of epinephrine upon the pancreas. This work was undertaken in an attempt to gain further understanding upon the mode of action of epinephrine-induced inhibition of insulin release.

In a preliminary series of experiments, rats were injected with guinea-pig anti-insulin serum and ^{131}I-labelled human albumin. The amount of endogenously secreted insulin was estimated from the progressive reduction in the pool of circulating unneutralized antibodies. In animals compelled to swim, insulin secretion occured at a much lower rate than in control rats. Prior injection of phentolamine abolished the exercise-induced inhibition of insulin release. These findings supported the concept that such an inhibition is due to endogenously released catecholamines.

Using in vitro approaches, the adrenergic nature of the insulin inhibition was confirmed. Indeed, it was found that phentolamine (and not propranolol) prevented the blocking action of epinephrine upon glucose-induced insulin secretion, suggesting an alpha receptor-mediated inhibition. It was equally shown that the epinephrine-induced inhibition does

not necessarily correspond to a decrease in the intracellular level of cAMP since suitable exogenous cAMP additions, or conditions known to favor its intracellular accumulation, were unable to prevent an epinephrine-induced inhibition of insulin release.

Experiments were then designed to demonstrate that epinephrine did not exert a preferential inhibitory effect over glucose-stimulated insulin release. Indeed, it was found that all the insulinotropic* agents tested (amino acids, sulfonylureas, high K^+ concentrations, cAMP, theophylline) were equally affected by the presence of epinephrine.

When evaluating the role of cations upon the insulin secretory process, it was found that glucose-induced insulin secretion was enhanced at high K^+ concentrations (10 to 34 mEq/l), and in the presence of ouabain. It was reduced when Na^+ was partially replaced by either Li^+, Tris, or larger amounts of K^+ (87 to 115 mEq/l). The elevated rate of secretion observed at high K^+ concentration (24 mEq/l) was markedly reduced by replacement of NaCl by sucrose. These data suggested that Na^+ entry in the beta-cell favors insulin release. However, the stimulant action of glucose was increased when Na^+ was replaced by choline and persisted in a Na^+- free medium, indicating that the influx of Na^+ *per se* was not necessary for secretion to occur. It was suggested that alkali cations could influence insulin secretion through an altered handling of alkaline earth cations by the beta-cell. In favor of this hypothesis, it was shown that

* Agent inducing insulin release

the beta-cell became unresponsive to Ba^{++} when Na^{+} was replaced by Li^{+}, Tris, or large amounts of K^{+} ; whereas, the elevated rates of secretion, observed when Na^{+} had been replaced by choline or by moderate amounts of K^{+}, were markedly reduced by the omission of Ca^{++} from the incubation medium. The inhibitory effect of extracellular acidosis upon glucose-induced secretion could also have been attributed, at least in part, to a reduced entry of Ca^{++} in the beta-cell. Omission of Ca^{++} , or high Mg^{++} concentrations, inhibited the stimulant action of glucose upon insulin secretion. Addition of Ba^{++} restored a normal rate of secretion both in the absence of Ca^{++} and at high Mg^{++} concentrations. These data did suggest that Mg^{++} on the one hand, and Ca^{++} or Ba^{++} on the other, might act as antagonists upon the secretory process. Inhibition of glucose-induced insulin secretion by epinephrine was not modified by Ba^{++} nor Ca^{++}. It was hypothesized that Ca^{++} influx in the beta-cell might trigger the release of insulin, and that epinephrine might suppress insulin secretion by blocking such an influx.

An attempt was then made to clarify the inter-relations existing between Ca^{++}, glucose, and epinephrine. Having devised a technique allowing for the measurement of calcium ion effluxes from the beta-cell, isolated islets preincubated in the presence of ^{45}calcium were perifused and the effluent radioactivity continuously measured. It was found that glucose-induced insulin release was associated with a

concomittant release of ^{45}calcium, possibly enclosed within the secretory granules. When insulin release was abolished, namely at low extracellular calcium concentration, or in the presence of heavy water, glucose provoked an immediate reduction in ^{45}calcium. The effect of glucose was reversible. These data suggested that glucose might have increased the intracellular concentration of calcium by inhibiting the outward transport of calcium across the membrane of the beta-cell. It also appeared that the effect of epinephrine did not exactly correspond to the effect of glucose suppression. Indeed, it rather seemed that epinephrine could have increased the sequestering ability of the beta-cell for calcium.

Glucose-induced insulin release is thought to result from the following sequence of events in the beta-cell: Glucose metabolism leading to the production of a metabolic signal, Net calcium uptake by the beta-cell in response to the signal, and interaction between calcium and a microtubular-microfilamentous system leading to emiocytosis of the secretory granules. Dibutyryl-cAMP and theophylline are known to potentiate glucose-induced insulin release. Based on the above-mentioned concepts, it was considered in the following series of experiments that the primary site of action of cAMP in the beta-cell could correspond to either a facilitation of glucose metabolism, a modification of calcium distribution, or an interaction with the microtubular-microfilamentous system. The first of these hypotheses ap-

peared unlikely because db-cAMP and theophylline, in sharp contrast with other agents known to affect glucose metabolism in the beta-cell, did not modify glucose-induced calcium uptake by isolated islets. The last hypothesis also appeared unlikely since theophylline did not interfere with the deleterious effect of colchicine on the microtubular-microfilamentous system, and since vincristine or colchicine did not differentially affect the respective insulinotropic action of glucose and theophylline. An effect of cAMP upon calcium distribution in the beta-cell was suggested by the following findings. Whereas glucose was unable to promote insulin release in the absence of extracellular calcium, the addition of db-cAMP or theophylline to the calcium-depleted media restored the insulinotropic action of glucose. Moreover, theophylline caused a dramatic increase in ^{45}calcium efflux from perifused islets, whether in the presence or absence of glucose. It is concluded that the insulinotropic action of cAMP could be due to a glucose-independent translocation of calcium within the beta-cell, from an organelle-bound pool to a cytoplasmic pool of ionized calcium readily available for transport across the cell membrane.

Finally, using a duct-ligation technique, we were able to demonstrate a glucagonotropic effect of epinephrine detectable at both low (0.3 mg/ml) and high (3.0 mg/ml) glucose levels, and observable in the presence of very low concentrations of this catecholamine (100 nM).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1.1 Introduction

2.1 Introduction

Anderson and Long (1948) were the first to report an epinephrine-induced inhibition of the insulin release from perfused rat pancreas. From their observations, it was generally accepted that the inhibitory effect resulted from the vasoconstrictive property of epinephrine. Many years later, Coore and Randle (1964) reported a direct inhibitory action of epinephrine upon glucose-induced insulin secretion upon incubation of pieces of rabbit pancreas. The inhibitory effect of epinephrine on insulin secretion has since been extensively confirmed, using both in vivo and in vitro approaches, in a great variety of species, including man (Karam et al., 1966; Porte et al., 1966a), monkey (Kris et al., 1966; Miller et al., 1969), dog (Campbell et al., 1966; Kosaka et al., 1964; Seltzer and Crout, 1966), pig (Hertelendy et al., 1966), sheep (Hertelendy et al., 1969), rabbit (Coore and Randle, 1964; Wong et al., 1967), and rat (Malaisse et al., 1967a; Kansal et al., 1967).

2.2 Problem

These observations raised questions about the physiological significance of an epinephrine-induced inhibition of insulin secretion and of its mode of action upon the beta-cell. Various

experimental and clinical observations already have suggested a relation between adrenergic mechanisms and the regulation of insulin secretion. Suffice to mention a) the observed terminal fibers of the sympathetic and parasympathetic nervous system reaching into the islets of Langerhans and being in close proximity with alpha- and beta-cells (Sergeyeva, 1940; Creutzfeldt, 1949; Coupland, 1958; Heinzen, 1960; Esterhuizen, 1968; Watari, 1968); b) that when compared to other organs, except for the adrenals, relatively high amounts of extractable catecholamines are found in pancreatic tissue (Falck and Hellman, 1963, 1964; Cegrell et al., 1964; Loubatières et al., 1964); c) the well-documented frequent association of pheochromocytoma and glucose intolerance (Molinatti et al., 1964; Wilber et al., 1966; Colwell, 1969; Porte, 1969; Vance et al., 1969).

Because the level of blood and urinary catecholamines is increased by stress and physical exercise (Gray and Beetham, 1957; Vendsalu, 1960; Goldfien et al., 1961; Klepping et al., 1964; Neal et al., 1968; Weil-Malherbe et al., 1968; DeSchaepdryver et al., 1969; Kotchen et al., 1971; Myles et al., 1971), these experimental conditions have been utilized to investigate insulin secretion. Most reports indicate that the level of circulating insulin is either unchanged or decreased in normal subjects or animals exposed to stressful situations or obliged to perform a physical work (Cochran et al., 1966; Rasio et al., 1966; Schalch, 1967; Mason et al., 1968; Nikkila et al., 1968; Federspil et al., 1969; Schwarz et al., 1969). But yet the involvement of an adrenergic factor in this phenomenon remains to

be assessed.

Concerning the mode of action of epinephrine in the beta-cell, it has been suggested that the inhibition in insulin release results from the activation of alpha-adrenergic receptors by epinephrine (Kansal et al., 1967; Malaisse et al., 1967c; Porte, 1967a). In favor of this concept, it was shown that epinephrine, a potent activator of these receptors (Ahlquist, 1948) is a potent inhibitor while norepinephrine and isoproterenol, which are much weaker activators of alpha receptors, are also much weaker inhibitors of insulin secretion (Porte et al., 1966b; Malaisse et al., 1967a), and that the inhibition caused by epinephrine is abolished by alpha-adrenergic blocking agents while it persists in the presence of beta-receptor antagonists. The latter observations first established in vitro (Malaisse et al., 1966, 1967a) have since been confirmed in vivo (Porte, 1967a; Hertelendy et al., 1969; Turtle et al., 1967a). But little is known concerning the process by which activation of alpha-receptors influences the insulin secretory rate.

At least three hypotheses have been proposed to explain the relation between alpha-adrenergic receptors and insulin release. A first one, from Turtle and Kipnis (1967b), suggests that the inhibitory effect of epinephrine upon glucose-induced insulin secretion might be due to a decreased cellular concentration in cyclic adenosine monophosphate (cAMP). A second hypothesis, from Hellman and his coworkers (1969a), postulates that the inhibitory effect of epinephrine is occasioned by changes oc-

curing in the glycolytic enzyme sequence "phosphoglyceraldehyde dehydrogenase - phosphoglycerate kinase", leading to a block in the beta-cell secretory activity. A third and last hypothesis, from Milner and Hales (1969, 1970), assumes that epinephrine interferes with the cationic fluxes across the beta-cell membrane, fluxes which are thought to be essential for secretion to proceed.

2.3 Purpose of the work

The aim of the present contribution is to characterize the inhibitory effect of epinephrine in terms of both its physiological significance and its possible mode of action. In a first study (chapter 1.2) we have re-examined the influence of exercise upon insulin secretion in vivo by investigating its adrenergic nature, combining the performance of a physical task with the administration of an alpha-adrenergic blocking agent. The alpha-adrenergic nature of epinephrine-induced inhibition of insulin release was then confirmed by in vitro studies (chapter 1.3). Further studies (chapter 1.4) extended the inhibitory action of epinephrine to a variety of insulinotropic agents. Knowing that the absence of extracellular ionized calcium also exerts a universal blocking effect comparable to the one exerted by epinephrine, the interactions of epinephrine and cations upon insulin secretory processes were investigated (chapter 1.5). These studies lead us to postulate that calcium ions might be of primary importance for the beta-cell to secrete. In the meantime, Malaisse-Lagae and Malaisse

(1971a) reported a series of experiments where the release of insulin appeared to be coupled with a necessary accumulation of calcium into the beta-cell, confirming the key-role of that alkaline earth cation upon the secretory processes. Since those data referred only to the net movements of the cation across the beta-cell, an attempt was made to dissociate inward from outward calcium movements across the beta-cell membrane. To achieve this, a technique was devised allowing for the measurements of calcium effluxes from adequately treated islets of Langerhans (chapter 1.6). The measurement of those calcium effluxes made possible a better characterization of the glucose and of the epinephrine actions upon the beta-cell calcium metabolism.

Having investigated and characterized the alpha-adrenergic nature of the epinephrine-induced inhibition upon the release of insulin, the next chapter (1.7) was devoted to an analysis of the beta-cellular changes induced by the stimulation of beta-adrenergic receptors. Since it is well established that a stimulation of the latter induces an increase in the intracellular concentration of cyclic adenosine monophosphate, we shall propose a site of action for cAMP and its probable impact upon the beta-cell calcium metabolism. Finally, an incursion on the "second" pancreatic hormone was made where epinephrine effect upon glucagon secretion was investigated (chapter 1.8). It is showed that in addition to its inhibitory influence upon the release of insulin, epinephrine also stimulates glucagon release from the pancreatic alpha-cells.

The physiological significance of such a glucagonotropic effect of epinephrine is emphasized.

1.2 Effect of phentolamine administration upon insulin secretion during physical exercise

2.1 Introduction

The influence of muscular exercise upon insulin secretion has been investigated on several occasions. In healthy subjects, the level of circulating insulin is either unchanged or decreased during exercise (Rasio et al., 1966; Cochran et al., 1966; Schalch, 1967; Nikkila et al., 1968; Schwarz et al., 1969; Sutton et al., 1969; Giffin, 1970). Insulin release inhibition in these subjects might be difficult to detect since the level of circulating insulin under basal conditions is usually at the lower limit of sensitivity of most methods of plasma-insulin assay (Wright, 1967). Moreover, exercise in human subjects might be associated with a decreased metabolic clearance of insulin, and this could mask inhibition of insulin release if judged solely by the level of the circulating hormone (Conard et al., 1969). A decreased concentration of circulating insulin has also been documented in rats compelled to swim (Federspil et al., 1969), monkeys exposed to a psychological stress (Mason et al., 1968), and man submitted to a surgical stress (Majid et al., 1971). Also the amount of insulin secreted during stress and muscular exercise has been measured in rats injected with guinea-pig anti-insulin serum (GPAIS); when these animals were forced to swim or were sub-

mitted to repeated electrical shocks, the amount of secreted insulin was dramatically reduced, despite the concomittant hyperglycemia due to the induction of insulin deficiency (Wright et al., 1968). From all these observations the inhibition of insulin secretion was ascribed to stress- or exercise-induced endogenous release of catecholamines.

The rising level of circulating and urinary catecholamines or catecholamine derivatives observed during muscular exercise or during stress (Gray and Beetham, 1957; Vendsalu, 1960; Goldfien et al., 1961; Klepping et al., 1964; Neal et al., 1968; Weil-Malherbe et al., 1968; DeSchaepdryver et al., 1969; Kotchen et al., 1971; Myles et al., 1971), coupled with the in vitro observations of an effective inhibitory effect of epinephrine upon the beta-cell secretory activity (Malaisse et al., 1967a; Porte, 1967a), provide good indications for such an in vivo blocking action of epinephrine upon insulin release. However, it remains to be demonstrated that the reduction in insulin release during physical exercise indeed is due to an adrenergic mechanism.

To achieve such a demonstration, the following experiment was devised, in which use will be made of phentolamine, an alpha-adrenergic antagonist (Meier et al., 1949; Freis et al., 1951; Gould et al., 1971) which would antagonize the effect of catecholamines upon the beta-cell secretory activity.

2.2 Materials and methods

3.1 Experimental procedure

Female albino rats (Rega, Heverlee, Belgium) were fed on

a standard diet (Aliments Protector, Brussels, Belgium) and had free access to food and water up to the time of use. They were alloted to three groups with comparable mean body weight (157 ± 3 g for group A; 158 ± 2 g for group B; and 157 ± 2 g for group C). At zero time, all animals were injected intravenously (tail vein) with a same volume (0.6 ml) of guinea-pig anti-insulin serum (GPAIS, lot 513; prepared and kindly donated by Dr. P.H. Wright, Indiana University, Indianapolis, Ind.) and a trace amount of ^{131}I-labeled human albumin (approximately 12 µC ; CEA-CEN, Sorin Italy). The amount of GPAIS injected was able to bind approximately 2.1 units of bovine insulin. Blood (0.4 to 0.6 ml) was drawn from the severed end of the tail immediately before, and 2 and 50 minutes after this injection. For at least 30 minutes before and 50 minutes after the single intravenous injection, all the rats were kept in warm cages with free access to water, and none were handled except at times of bleeding or injection.

After the bleeding at the 50th minute, the rats were submitted to one of the following treatments. Group A : these rats were replaced in the warm cages and were not handled until they were killed at 70 minute. Group B : these rats were placed in a tank of deep (18 inches) tepid (31 - 35°C) water, and forced to swim for a period of 20 minutes. After swimming for 20 minutes, none of these animals showed any sign of exhaustion. Group C : these rats were injected intraperitoneally at the 45th minute with phentolamine (0.5 mg ; Regitin, CIBA*),

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* see footnote next page.

and subsequently submitted to the same treatment as for group B. The impression was gained that the rats injected with phentolamine were less able to perform exercise. Thus, out of 12 animals in group C, one rat died after 12 minutes of swimming; 2 other rats had to be removed from the tank after 7 to 13 minutes of exercise, because of obvious signs of distress.

A last sample of blood was obtained from the severed neck when each animal was killed by decapitation at the 70th minute after the injection of GPAIS.

3.2 Analytical methods

All samples of blood were collected in heparinized tubes. The volume and hematocrit of each sample were measured and the plasma immediately separated. The plasma sugar concentration was determined by an automated microtechnique based on that described by Hoffman (1937). An aliquot (0.1 ml) of each sample of plasma was examined for its content in ^{131}I-labeled albumin, the volume of distribution of albumin being taken as a measurement of the volume of distribution of the anti-insulin antibodies (Wright et al., 1966b). Thereafter, the same aliquot was assayed for unneutralized anti-insulin antibodies, using the method of Wright et al., (1966a, 1967). The amount of ^{125}I-labeled bovine insulin used for this assay was selected so that the interference of ^{131}I did not exceed 0.5 per cent

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The commercial preparation of phentolamine also contained glucose, so that the rats in group C were injected intraperitoneally with 17.5 mg of glucose at the 45th min. This might explain, in part at least, why the plasma sugar concentration noticed at the 70th min was slightly higher in group C than in group B.

of the actual radioactivity of ^{125}I. From the volume of distribution of the labeled albumin and simultaneously measured concentration of unneutralized antibodies in the plasma, the total amount of unneutralized antibodies in each rat was determined at each time of bleeding. Knowing the total amount of antibodies injected and taking into account losses encountered at each bleeding, the rate of neutralization of the injected antibodies between successive bleedings was calculated and is equated here to the rate of endogenous insulin secretion for that period. This method has been previously described in greater detail (Wright et al., 1966b).

2.3 Results

3.1 Initial period

As shown in Table 2-I, the administration of guinea-pig anti-insulin serum caused a rise in the plasma sugar concentration from 125 ± 2 to 277 ± 7 mg / 100 ml. During the same period (0 to 50 minutes), the hematocrit fell and the volume of distribution of labeled albumin rose. Between the 2nd and the 50th minute, insulin secretion amounted to 252 ± 27 mU. All the values recorded during the initial period were of the same order of magnitude as those found during the same period by Wright and his coworkers (1966b, 1968).

3.2 Experimental period

In the control rats of group A, the plasma sugar concentration continued to rise between the 50th and 70th minute (

Table 2-I. Effect of exercise and phentolamine upon insulin secretion

PERIOD	TIME (min)	RATS Group	(N)	PLASMA SUGAR (mg /100 ml)	HEMATOCRIT (per cent)	ALBUMIN VOLUME (ml)	INSULIN SECRETED (mUnits)
INITIAL	0	A+B+C	(21)	125 ± 2	43.4 ± 0.2		
	2	A+B+C	(21)	131 ± 3	38.7 ± 0.3	6.56 ± 0.15	
	50	A+B+C	(21)	277 ± 7	35.9 ± 0.6	8.33 ± 0.24	252 ± 27
EXPERIMENTAL	70	A	(6)	347 ± 9	31.4 ± 1.2	8.56 ± 0.67	260 ± 24
		B	(7)	259 ± 28	38.7 ± 1.0	7.68 ± 0.43	79 ± 17
		C	(8)	308 ± 27	34.3 ± 0.9	8.97 ± 0.32	223 ± 31

Mean values (± SEM) for plasma sugar concentration, hematocrit, and volume of distribution of labeled albumin are stated at four times of bleeding, before (0 min) and after (2, 50, and 70 min) injection of anti-insulin serum. Mean amounts of endogenously secreted insulin are stated for the whole series of animals (N = 21) during the initial period (2 to 50 min); and for the smaller groups (N = 6 to 8) during the experimental period (50 to 70 min), when rats remained untreated (group A) or were compelled to swim (groups B and C). In group C, phentolamine (5 mg) was injected intraperitoneally at the 45th min.

paired difference : + 51 ± 9 mg / 100 ml). In the swimming rats of group B, no further change in plasma sugar concentration occured during the experimental period (paired difference : - 11 ± 16 mg / 100 ml); so that their mean plasma sugar level at the 70th minute was significantly ($P < 0.02$) lower than that of the control animals. In most of the rats of group C, the plasma sugar concentration rose during the experimental period; however, their mean plasma sugar value at the 70th minute was not significantly different ($P > 0.2$) from that of either the control (group A) or swimming (group B) rats.

Differences were also found between the 3 groups of rats in the behavior of the hematocrit and the distribution of albumin during the experimental period. Swimming prevented the further fall in hematocrit found in control rats, and caused a minor decrease in the albumin volume. Prior injection of phentolamine apparently minimized these hemodynamic effects of exercise.

The rate of insulin secretion was much lower in the swimming than in the control rats ($P < 0.001$). Administration of phentolamine restored a normal secretory response. Thus, the mean rate of insulin secretion in group C was not significantly different ($P > 0.3$) from that of group A, and much higher ($P < 0.005$) than that of group B. Even when a restricted number of animals from group B (N=5) and C (N=6) were selected in order to match their mean plasma sugar concentration at the 70th minute (279 ± 25 vs 282 ± 28 mg / 100 ml),

the rate of insulin secretion during the experimental period remained significantly lower ($P < 0.02$) in the rats of group B (66 ± 15 mU) than in these of group C (199 ± 37 mU).

2.4 Discussion

The present findings confirm that exercise inhibits insulin secretion, even when the beta-cell is exposed to a sustained hyperglycemia (Wright et al., 1968). The role of endogenous catecholamines in this process was examined by combining the performance of exercise with the administration of an alpha-adrenergic blocking agent. In human subjects who perform exercise during an oral glucose tolerance test, the administration of phentolamine does not prevent the insulin level from decreasing during the imposed physical exercise (Reinheimer et al., 1968). However, the significance of these findings is obscured by the fact that the observed insulin decrease occured concomittantly with a marked fall in the blood sugar concentration. In the rats injected with GPAIS, phentolamine abolished the inhibition of insulin secretion otherwise induced by exercise.

It is unlikely that the present changes in insulin secretion induced by exercise and phentolamine are due to concomittant variations in the plasma sugar concentration. First, in the range of glucose concentration observed between the 50th and the 70th minute (259 to 347 mg/100 ml), insulin secretion in vitro occurs at a nearly maximal rate (Malaisse et al., 1967b). Second, in rats injected with GPAIS and compelled to swim,

insulin secretion is markedly depressed even when the plasma sugar concentration remains at the same level as that found in control animals (Wright et al., 1968). Third, in the present experiments, a significant difference in the amount of secreted insulin by groups B and C was also recorded when animals from these groups were selected on the basis of a comparable degree of hyperglycemia.

The most likely explanation for the present findings is that phentolamine abolished the exercise-induced inhibition of insulin release by some direct effect upon the beta-cell. This interpretation is consistent with the hypothesis that exercise-induced inhibition of insulin secretion involves the activation of the beta-cell's alpha-adrenergic receptors by endogenously released catecholamines (Ashmore, 1970; Robertson and Porte, 1971).

1.3 The alpha-adrenergic nature of epinephrine-induced inhibition of insulin secretion

2.1 Introduction

In 1905, Langley introduced the concept of some hypothetical structures or systems located in, on or near effector cells, to explain the action of curare on skeletal muscle. Almost half a century later, Ahlquist (1948) suggested the existence of two distinct adrenergic responsive sites which he named respectively alpha and beta adrenoceptive receptors. The same concept was extended to the beta-cells when it was found that alpha-adrenergic blocking agents abolished the inhibitory effect of epinephrine upon the insulin release, whereas beta-adrenergic blocking agents failed to do so. These observations, first established in vitro (Malaisse et al., 1966, 1967a), have since received in vivo confirmation in man (Porte, 1967a), sheep (Hertelendy et al., 1969), and rat (Turtle et al., 1967a).

By means of in vitro approach to the pancreas, the next series of experiments want to achieve the followings:

1) to confirm the epinephrine-induced inhibition of insulin release;
2) to ascertain the alpha-adrenergic nature of the receptors involved;
3) to evaluate the effect of an exogenous load of cyclic-AMP upon the beta-cell secretory activity;

4) to verify the hypothesis presented by Turtle and Kipnis previously introduced in the first chapter.

2.2 Materials and methods

3.1 Rats

Female albino rats (150 to 200 g; Rega, Heverlee, Belgium) were fed on a standard diet (Aliments Protector, Brussels, Belgium) and had free access to food and water up to the time of use.

3.2 Experimental procedure

Immediately after decapitation of the rat, the pancreas was carefully dissected out and placed in a Petri dish containing cold buffer (0°C). The glandular portion was dissected free of obvious lymph nodes and adipose tissue, and then cut up into small pieces, each weighing between 7 and 10 mg. In all experiments, four such pieces were gently blotted and placed in small Erlenmeyer flasks (10 ml) containing the incubation medium (2.0 ml); pieces for each flask were chosen at random. The flasks were placed in an incubation water bath (Dubnoff type), gassed (95% O_2:5% CO_2) for the first five minutes, and incubated at 36°C for a period of 60 or 90 minutes. After incubation, the pieces of pancreatic tissue were quickly removed from the flasks and weighed, and the antibody content of all media were simultaneously examined.

3.3 Incubation media

The pieces of pancreatic tissue were incubated in a bicarbonate-buffered medium (Na^+, 139; K^+, 5; Ca^{++}, 2; Mg^{++}, 2; Cl^-, 124; HCO_3^-, 24 mEq. per liter) containing added bovine serum albumin (0.5 per cent, w/v; Bovine albumin, Fraction V, Sigma Chemical Co., St.Louis. Mo.), and an excess of guinea-pig anti-insulin serum (GPAIS, lot 404). To this, as required, was added glucose ($C_6H_{12}O_6 \cdot H_2O$; E. Merck A.G., Darmstadt, Germany), epinephrine (Adrénaline gauche, Rhone-Poulenc, Paris, France), theophylline (Theophylline-7-acetic acid; Fluka A.G., Buchs, Switzerland), dibutyryl-cAMP (N^6-2'0-dibutyryl-adenosin-3',5'-monophosphate, cyclisch; Boehringer, Mannheim, Germany), ATP (Adenosine-5'-triphosphate; Sigma Chemical Co.), phentolamine (Regitin; CIBA Pharmaceutical Co., Summit, N.J.), propanolol (Inderal; Ayerst Laboratories Inc., New York, N.Y.), or phenoxybenzamine (Dibenzyline; Smith, Kline & French Laboratories, Philadelphia, Penna). The medium was equilibrated against a mixture of oxygen (95 per cent) and carbon dioxide (5 per cent). When glucose was absent or present in high concentrations, iso-osmolarity was maintained by an appropriate increase or reduction of the sodium chloride concentration of the incubation medium.

3.4 Immunoassay of incubation medium

To equal volumes (1.0 ml) of incubation medium containing partially neutralized guinea-pig anti-insulin serum (GPAIS) was added a constant volume (0.5 ml) of an albuminated (1% bovine serum albumin), neutral (pH 7.0) phosphate buffer

(Sorenson, 0.1 M), containing a fixed amount of labeled (^{125}I-insulin; CEA-CEN, Sorin, Italy) and unlabeled (Pork-Beef Insulin, NOVO Co., Copenhagen, Denmark) insulins.

The resulting solution was incubated at room temperature (20 to 25°C) for thirty minutes. Then a continuously stirred suspension (1.0 ml; 10% w/v) of finely divided cellulose (MN 30; Macherey, Nagel and Co., Duren, Germany), prepared in phosphate buffer (0.1 M, pH 7.0) was added and well mixed. A further thirty minutes later, the cellulose was deposited by centrifugation for 10 minutes in a clinical centrifuge. Equal aliquots (0.5 ml) of the resulting clear supernatant solution were removed from each tube and examined for their radioactive content.

2.3 Results

3.1 Effects of epinephrine and its alpha-adrenergic mediation

The in vivo insulin inhibition previously observed in rats compelled to swim was attributed to an endogenous release of catecholamines, since it was prevented by administration of phentolamine (Brisson et al., 1971a).

The first series of experiments, summarized in Figure 3.1, indicate that epinephrine is capable of blocking the in vitro release of insulin. Moreover, phenoxybenzamine and not propranolol was able to antagonize that epinephrine-induced inhibition of insulin release. It is also shown that phenoxy-

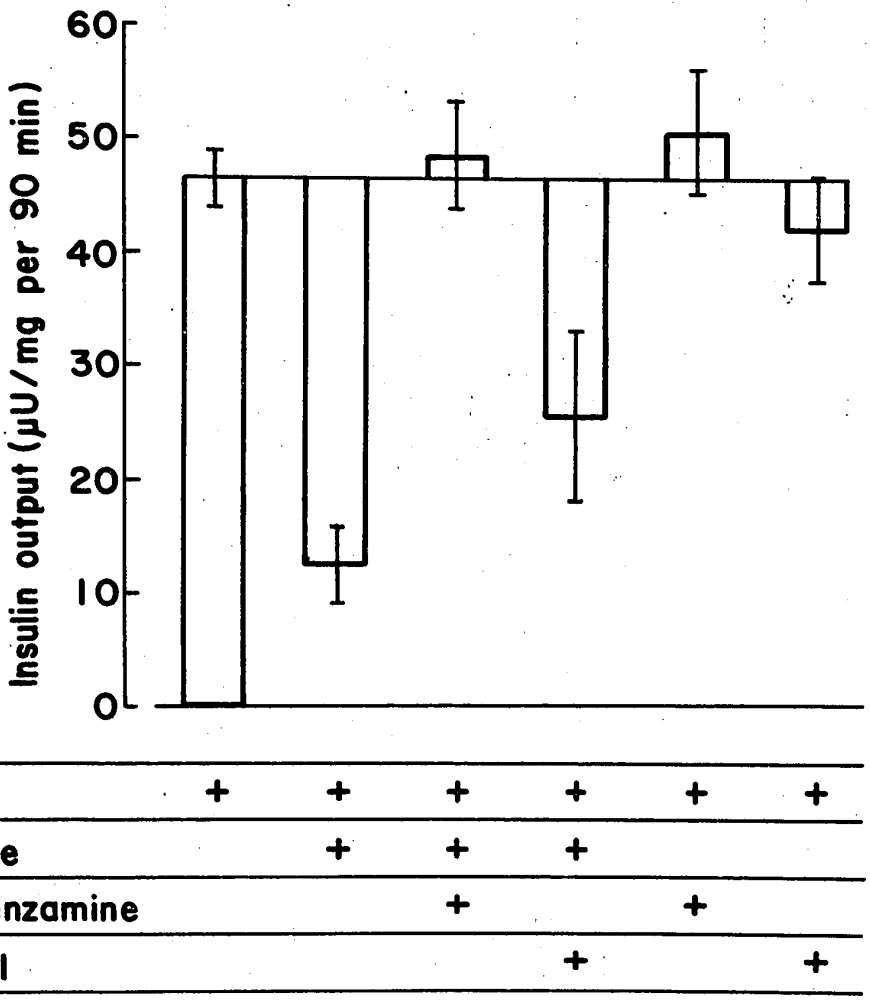

Figure 3-1. Effects of glucose and adrenergic agents upon insulin secretion in vitro. The control output induced by glucose (1.5 mg/ml) is indicated by the column on the left. The other columns represent the variations in output as compared to the control output induced by the adrenergic agents. Mean values ± SEM.

benzamine and propanolol do not exert any effect of their own upon the release of insulin.

3.2 Combined effects of epinephrine, db-cAMP and theophylline

The inhibitory effect of epinephrine has been attributed to a decrease in the intracellular level of cAMP (Turtle et al., 1967b). In order to test this hypothesis, the combined effects of epinephrine and db-cAMP upon insulin secretion were investigated.

The data reported in Table 3-I indicate that db-cAMP, even at a very high concentration (1.0 mM) failed to affect the basal rate of insulin secretion measured in the absence of glucose. In the presence of glucose, however, db-cAMP significantly increased the secretion rate, the enhancing effect of db-cAMP being most marked at the highest glucose concentrations. In control experiments, ATP failed to affect insulin secretion whatever the glucose concentration; the possible insulinotropic effect of other nucleotides (Levine et al., 1970; Watkins et al., 1971) was not investigated. The observed action of db-cAMP probably corresponded to its maximal effect, since it was not decreased by lowering the concentration of the nucleotide.

In view of these findings, the combined effect of epinephrine and db-cAMP was investigated in the presence of glucose. As shown in Table 3-II, epinephrine markedly reduced glucose-induced secretion, whether in the absence or presence of db-cAMP. The enhancing action of db-cAMP upon glucose-

Table 3-I. Effects of glucose, db-cAMP, and ATP upon insulin secretion*

Glucose concentration of media (mg/100ml)	Control insulin secretion µU/mg per 90min	Change in insulin secretion (µU/mg per 90 min) induced by drugs		
		db-cAMP		ATP
		1.0 mM	0.5 mM	1.0 mM
---	18.4 ± 2.0 (48)¶	- 2.2 ± 4.7 (16)		+ 1.4 ± 2.6 (32)
100	21.9 ± 1.8 (25)	+ 10.2 ± 3.2† (16)		+ 1.6 ± 2.2 (9)
300	104.6 ± 3.0 (98)	+ 43.2 ± 6.8T (32)	+ 48.9 ± 11.9T (32)	+ 5.8 ± 9.0 (34)
500	133.5 ± 4.8 (82)	+ 44.7 ± 9.0T (32)		- 5.2 ± 7.8 (50)

* The mean rate of secretion (± SEM) by tissue incubated in medium containing glucose alone (control output) is quoted together with the mean changes in secretion rate induced by db-cAMP and ATP and their statistical significance.
¶ The number of observations.
† $P < 0.01$
T $P < 0.001$

Table 3-II. Effects of epinephrine, db-cAMP, theophylline, and phentolamine upon glucose-induced insulin secretion*

Duration of incubation (min)	Epinephrine (μM)	Other drugs (mM)	Insulin output† (μU/mg per 60 or 90 min)
Control 60	----		90.5 ± 12.8 (26)
60	10.0		18.5 ± 7.8¶ (26)
60	----	db-cAMP (0.5)	153.0 ± 13.7Ψ (26)
60	10.0	db-cAMP (0.5)	45.2 ± 7.0Ψ (26)
Control 90	----		157.9 ± 13.0 (22)
90	10.0		35.6 ± 6.3¶ (22)
90	10.0	phentolamine (1.0)	150.4 ± 14.5 (18)
90	----	theophylline (1.4)	293.9 ± 19.1¶ (22)
90	10.0	theophylline (1.4)	49.3 ± 9.2¶ (22)
90	10.0	theophylline (1.4) & phentolamine (1.0)	250.9 ± 38.7Ψ (9)

* Experimental conditions are shown in the left part of the table.

† Mean values (± SEM) for insulin secretion rate induced by glucose (300 mg per 100 ml) are shown together with the number of observations (in parentheses) and the significance of difference between control (first line) and experimental values.

¶ $P < 0.001$

Ψ $P < 0.01$

induced secretion was significantly ($P < 0.05$) less marked in the presence than in the absence of epinephrine (+ 26.7 ± 6.2 vs + 62.5 ± 17.3 µU per milligram per 60 minutes).

Comparable results were obtained when theophylline was used in order to raise the intracellular concentration of cAMP (Table 3-II). In these experiments, epinephrine abolished the potentiating effect of theophylline. Thus, the residual enhancing action of theophylline in the presence of epinephrine (+ 13.7 ± 10.8 µU per mg per 90 min) did not reach statistical significance.

In contrast with the failure of db-cAMP or theophylline to overcome the epinephrine-induced inhibition of insulin secretion, phentolamine did restore rates of secretion comparable to those found in the absence of epinephrine (Table 3-II).

2.4 Discussion

3.1 Inhibition of insulin secretion through activation of alpha-adrenergic receptors

In a preceding chapter (1.2) it was described that physical exercise induces a fall in the level of circulating insulin. It was assumed that the decrease in insulin secretion corresponded to an adrenergic inhibition exerted on the beta-cell by endogenously released catecholamines since it was overcome by phentolamine. The present findings bring in vitro confirmation of the adrenergic nature of the inhibition previously observed in vivo.

Malaisse and his coworkers (1966, 1967a) and Porte (1967a) had already observed that alpha-adrenergic blocking agents (but not beta-adrenergic blocking agents) could prevent the epinephrine-induced inhibition of insulin secretion provoked by glucose. That first series of experiments confirm the potent inhibitory effect exerted by catecholamines upon the beta-cell secretory activity and its mediation through activation of alpha-adrenergic receptors.

3.2 Combined effects of epinephrine and db-cAMP

Turtle and Kipnis (1967b) have ascribed the inhibition of insulin secretion by epinephrine to a decrease in the cellular content of cAMP. The present data indicate that, even in the presence of db-cAMP, epinephrine exerted a marked inhibitory effect upon insulin secretion. Comparable results were observed when theophylline was used instead of db-cAMP in order to raise the intracellular level of cAMP. The concentrations of db-cAMP and of theophylline used in these experiments were sufficient to potentiate markedly the stimulant action of glucose (Table 3-II). These results indicated that flooding the beta-cell with exogenous db-cAMP did not prevent the inhibitory effect of epinephrine. Incidentally, db-cAMP also failed to cause sustained insulin secretion in the absence of glucose (Table 3-I). This finding, which indicates that intracellular accumulation of cAMP (or db-cAMP) is not sufficient *per se* to initiate insulin release, confirms previous observations on the role for

the adenylcyclase system in insulin secretion (Malaisse et al., 1967c). Indeed, none of the agents which are thought to increase the cellular level of cAMP (isoproterenol, glucagon, enteroglucagon, ACTH, TSH, theophylline, caffeine, and cAMP itself) are able to cause sustained insulin release in the absence of a metabolic substrate; whereas, all of them enhance the stimulant action of glucose, mannose, and certain amino acids (Malaisse et al., 1967c, 1968a, 1969, 1970a).

1.4 The ubiquitous inhibitory effect of epinephrine

2.1 Introduction

For many years glucose has generally been considered as the main stimulant for insulin secretion. Evidence is now being gained that there exists a variety of other agents having insulinotropic properties.

A few years ago, a phenomenon of hereditary sensitivity to ℓ-leucine was reported (Cochrane et al., 1956); that hypersensitivity to leucine was reflected by a severe hypoglycemia after ingestion of this amino acid or a leucine-rich meal. More recently, further observations have confirmed and emphasized the role of amino acids in the regulation of insulin secretion (Cochrane, 1960; Yalow and Berson, 1960; Floyd et al., 1966a, 1966b; Malaisse et al., 1968a; Hertelendy et al., 1968; Lambert et al., 1969; Milner, 1969a, 1969b).

Apart from glucose and amino acids, a class of chemical compounds derived from sulfanilic acid and exhibiting potent insulinotropic effects are the hypoglycemic sulfonylureas (see Müller-1969- for a review). Their widespread clinical use has promoted extensive research in an attempt to elucidate their mode of action but still, very little is known about their exact effects upon the beta-cell intimate metabolism (Loubatières, 1969; Brisson and Malaisse, 1971b).

Finally, the analogy reported by Douglas (1968) between

the biophysical events of excitation-contraction coupling in skeletal muscle and the analogous events of stimulation-secretion coupling occuring in endocrine glands, has prompted many workers to investigate the involvement of ions in the insulin secretory processes. Our study will here be restricted to potassium ions, alteration of which in the incubation medium was reported to affect the release of insulin (Malaisse et al., 1971a).

It thus became interesting to verify if epinephrine exerts preferentially its inhibitory effect upon glucose-stimulated insulin release, or if it also alters the effects of the above-mentionned insulinotropic agents.

In the preceeding chapter the concept of an adenylcyclase system was introduced when discussing Turtle and Kipnis hypothesis. Malaisse and his coworkers (1967c) have presented a possible physiological regulation of the insulin secretory activity through that intracellular pathway. Such modulations would be brought mainly by glucagon, ACTH, or TSH. Since those contribute to an increase in the intracellular level of cAMP, dibutyryl-cAMP and theophylline, achieving the same intracellular cAMP accumulation, will be included in that analysis.

Since all the following information was gathered from incubation of pancreatic tissue and of isolated islets of Langerhans, and since the method for incubation of pancreatic fragments has already been presented in detail, the method for isolation of islets of Langerhans will here be described.

2.2 Materials and methods

3.1 Rats

Female albino rats (150 to 200 g; Rega, Heverlee, Belgium) were fed on a standard diet (Aliments Protector, Brussels, Belgium) and had free access to food and water up to the time of use.

3.2 Experimental procedure

When pancreatic fragments were used, the procedure described in the preceeding chapter was followed. When islets of Langerhans were required, the method described by Lacy and Kostianovsky (1967) was applied with minor modifications. Briefly, rats were killed by decapitation and, through a cannula inserted in the bile duct, previously clamped at its distal end, 8.0 to 10.0 ml of Hanks solution (1949) were forced to upstream the pancreatic ducts since the pancreas drains directly into the common bile duct (Keen et al., 1965) provoking an interstitial edema. The pancreas is then dissected out, placed in a Petri dish containing Hanks solution where it is freed from obvious lymph nodes and adipose tissue. Cut into small pieces with scissors, it is transferred into a test tube to which an appropriate amount of collagenase (Worthington Biochemical Corporation, Freehold, N.J.) is incorporated. The suspension is immediately and vigourously shaken in a water bath (37°C) for 6 to 8 minutes. After that digestion period, the islets are washed by successive sedimentations using a clinical centrifuge. The exocrine tissue-free sediment is then ready for the transfer of islets into the

incubation medium using a binocular dissecting microscope.

3.3 Incubation media

The islets of Langerhans were incubated in groups of eight islets each in small flasks (2.0 ml) containing equal volumes (1.0 ml) of a bicarbonate-buffered medium (Na^+, 139; K^+, 5; Ca^{++}, 2; Mg^{++}, 2; Cl^-, 124; HCO_3^-, 24 mEq. per liter). The incubation medium also contained bovine serum albumin (500 mg%, w/v; Bovine albumin, Fraction V, Sigma Chem.Co.) and, as required, leucine (L-leucine; E. Merck A.G., Darmstadt, Germany), BS-4231 (Glisoxepide ; 4-{ 4- [β-(5-methyl - isoxazole - 3 - carboxamido) -ethyl] - benzolsulfonyl }- 1, 1-hexamethylene - semicarbazide; Bayer-Schering, Germany), db-cAMP, theophylline, and epinephrine. When the potassium ion concentration was increased, an appropriate amount of sodium ions was removed to maintain a physiological osmotic pressure in the incubation medium.

3.4 Immunoassay of incubation medium

To equal volumes (0.5 ml) of incubation medium, guinea-pig anti-insulin serum was added. The solution is then shaken and incubated at 37°C for a thirty minute equilibration period. The antibody-insulin solution is then cooled down to room temperature and a constant volume (0.5 ml) of an albuminated (1% bovine serum albumin), neutral (ph 7.0) phosphate buffer (Sorenson, 0.1 M), containing a fixed amount of labeled and unlabeled insulin was incorporated.

The resulting solution was incubated at room temperature

(20 to 25°C) for thirty minutes. Then, a continuously stirred suspension (1.0 ml; 10% w/v) of finely divided cellulose (MN 30; Macherey, Nagel & Co., Duren, Germ.) prepared in phosphate buffer (0.1 M, pH 7.0) was added and well mixed. A further thirty minutes later, the cellulose was sedimented by centrifugation for 10 minutes in a clinical centrifuge. Equal aliquots (0.5 ml) of the resulting clear supernatant solution were removed from each tube and examined for their radioactive content.

2.3 Results

The data reported here summarize our studies of epinephrine-induced inhibition of insulin release when stimulated by different insulinotropic agents.

It is shown that:

a) in the absence of glucose, epinephrine abolishes the stimulant effect of leucine upon the insulin release by isolated islets of Langerhans (Fig. 4-1);

b) at an intermediate (1.0 mg/ml) and at a high (3.0 mg/ml) glucose concentration, epinephrine suppresses the enhancing effect of a potent sulfonylurea, BS-4231, when studied on fragments of pancreatic tissue (Fig. 4-2; Fig. 4-3);

c) when, on fragments of pancreas, insulin release was stimulated by increased concentrations of potassium ions, epinephrine markedly reduced the insulin output in both the absence (Fig. 4-4) and the presence (Fig. 4-5) of glucose.

d) in the presence of a high glucose level, the enhancing effect of dibutyryl-cAMP when observed on fragments of pancreas (Fig. 4-6) or on isolated islets of Langerhans (Fig. 4-7), and the potentiating action of theophylline measured with pancreatic fragments (Fig. 4-8), are abolished by the addition of epinephrine into the incubation medium.

2.4 Discussion

We have in the preceding chapters presented the role of the blood glucose level upon the insular function and the antagonizing effect of catecholamines upon its secretory activity. The just reported experiments were performed to investigate whether epinephrine was exerting a preferential inhibition upon glucose-induced insulin release or if that inhibition could be extended to a variety of agents known to promote the insulin secretion rate.

3.1 Epinephrine-induced inhibition of leucine-induced insulin release

The amino acid-induced increase in the blood insulin level has been repeatedly described (Grumbach and Kaplan, 1960; Mabry et al., 1960; Yalow and Berson, 1960; Marks and Klein, 1961; Weisenfeld and Goldner, 1961; Samols and Marks, 1963; Floyd et al., 1964; Fajans et al., 1967; Hellman et al., 1971). The blood amino acid level has been postulated to play an important role in the regulation of insulin secretion (Cochrane, 1960; Floyd et al., 1966b). It now appears that the physio-

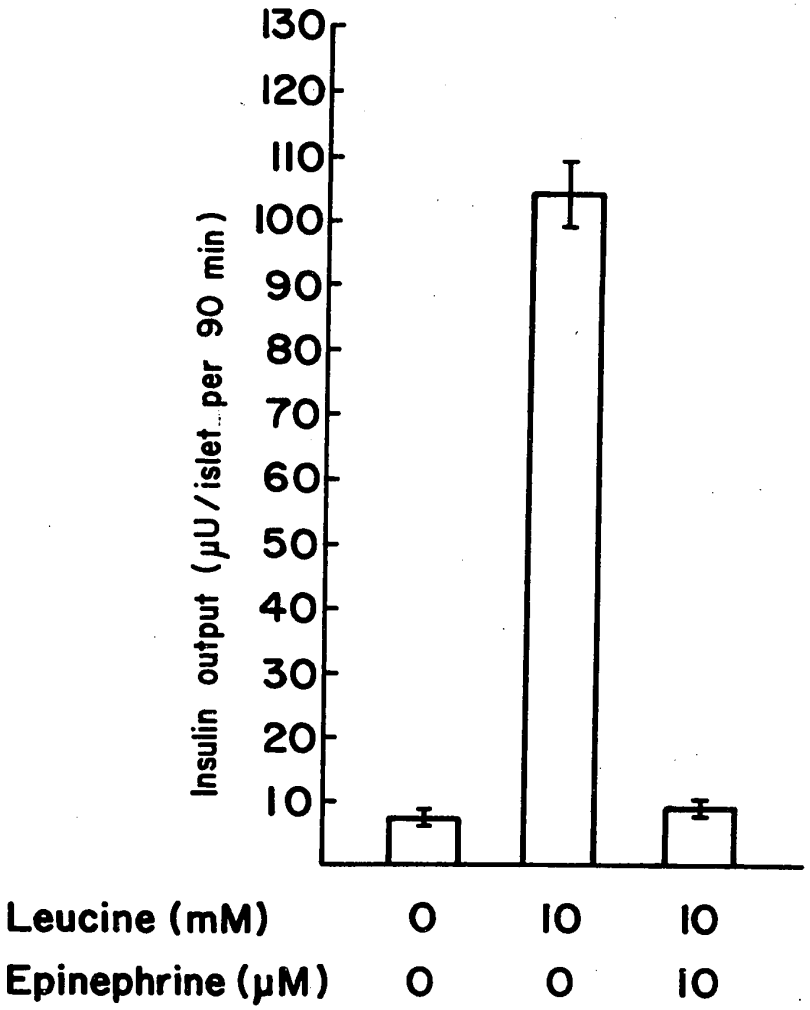

Figure 4-1. Effects of epinephrine upon leucine-induced insulin secretion by isolated islets of Langerhans. Each column represents the mean (± SEM) for 10 individual observations.

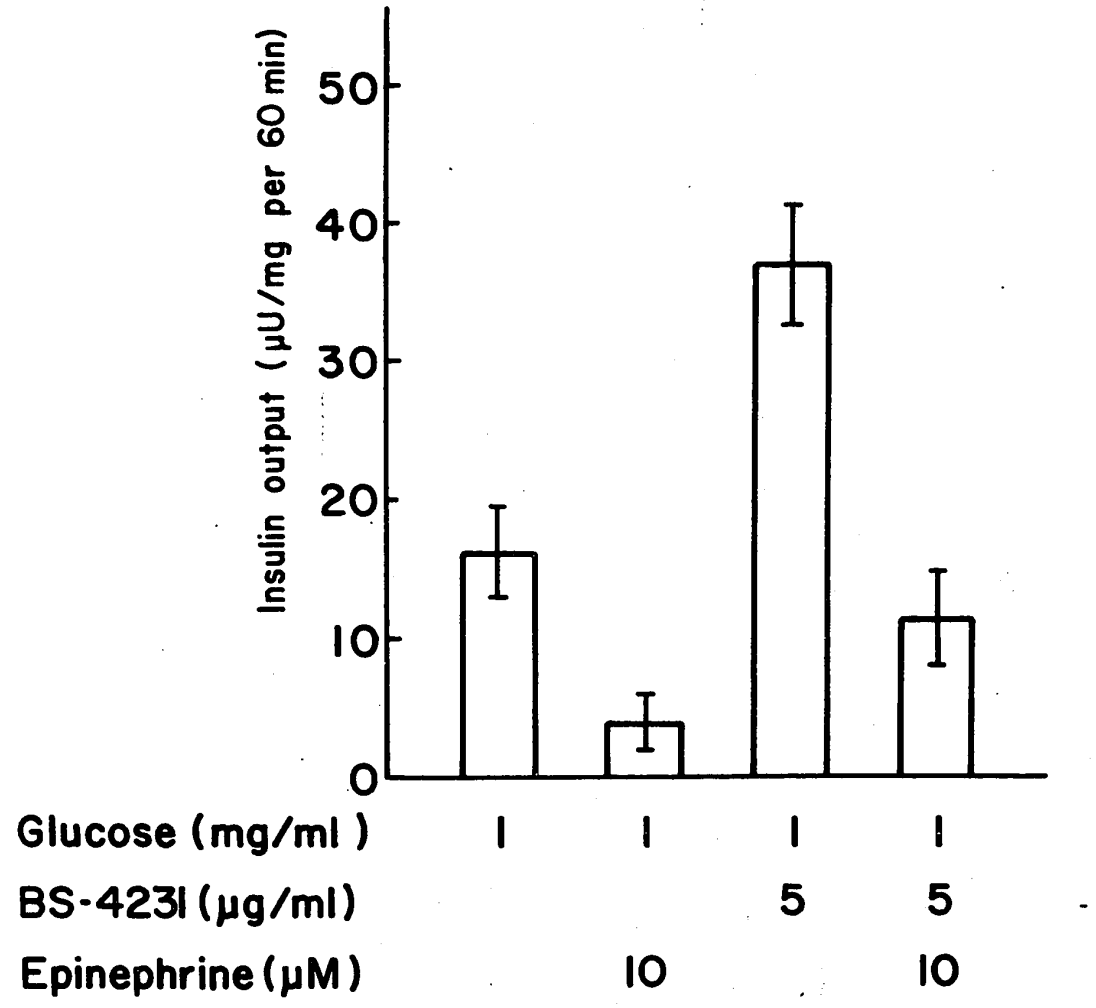

Figure 4-2. Effects of epinephrine upon insulin secretion induced by a moderate glucose concentration in the absence or the presence of BS-4231. Each column represents the mean value (± SEM) for 13 individual observations.

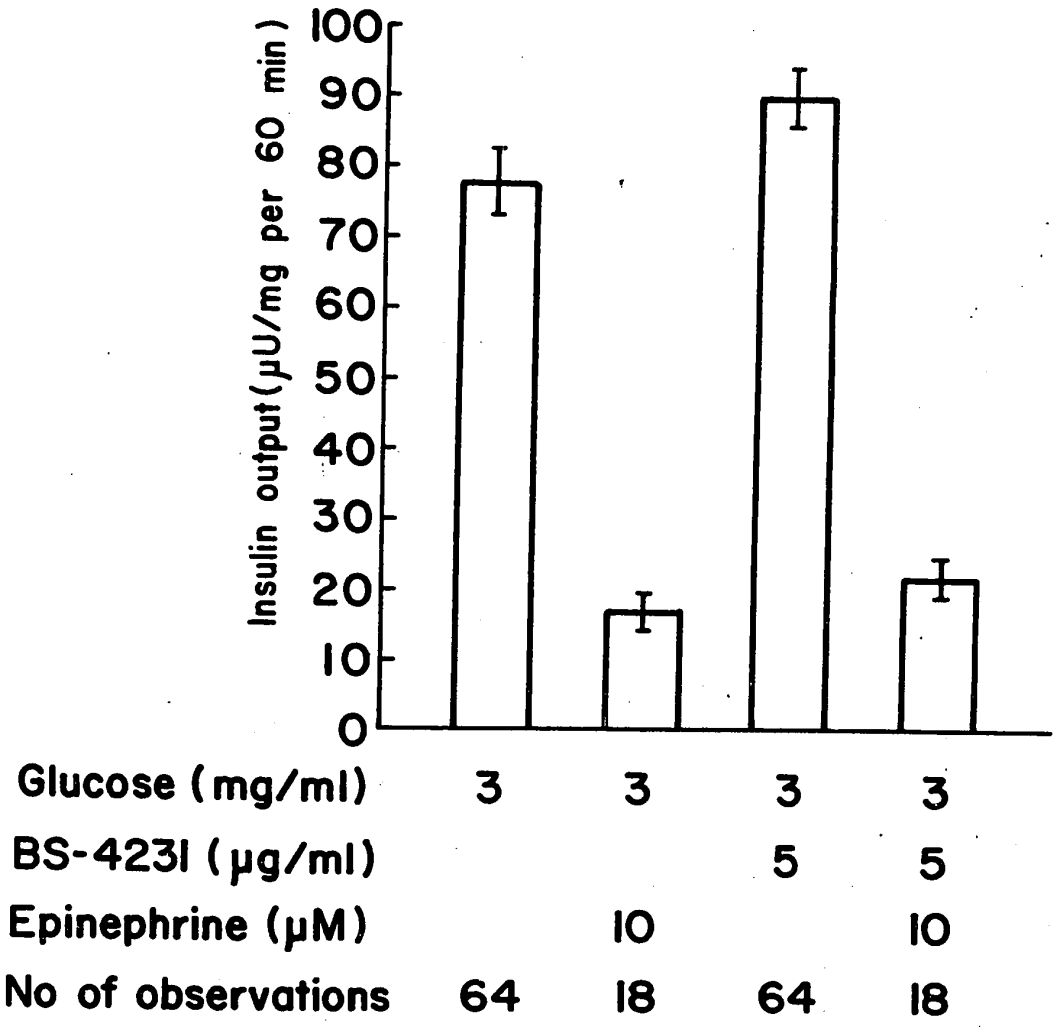

Figure 4-3. Effects of epinephrine upon insulin secretion induced by a high glucose concentration in the presence or the absence of BS-4231. Each column represents the mean value (± SEM) for the stated number of observations.

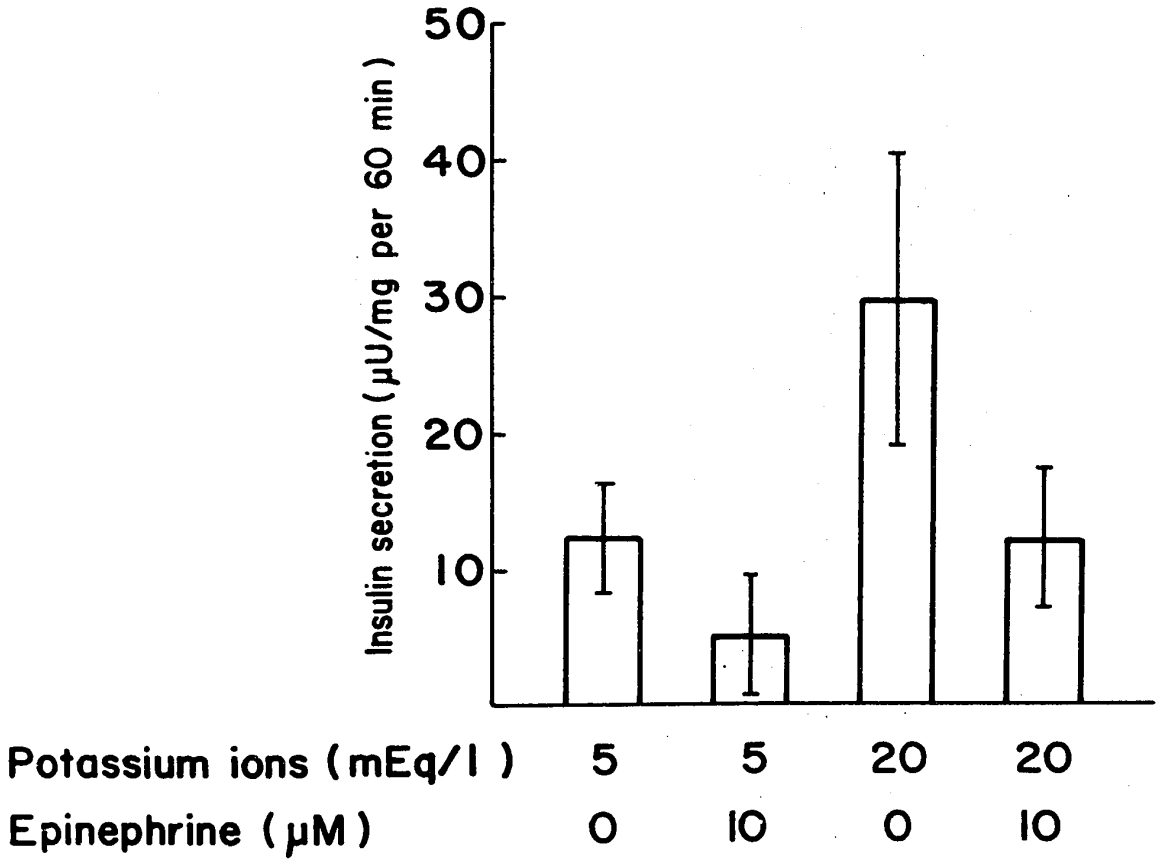

Figure 4-4. Effects of epinephrine upon insulin secretion when the potassium ion concentration is increased from 5 to 20 mEq. per liter in the absence of glucose. Each column represents the mean value (± SEM) for 13 observations.

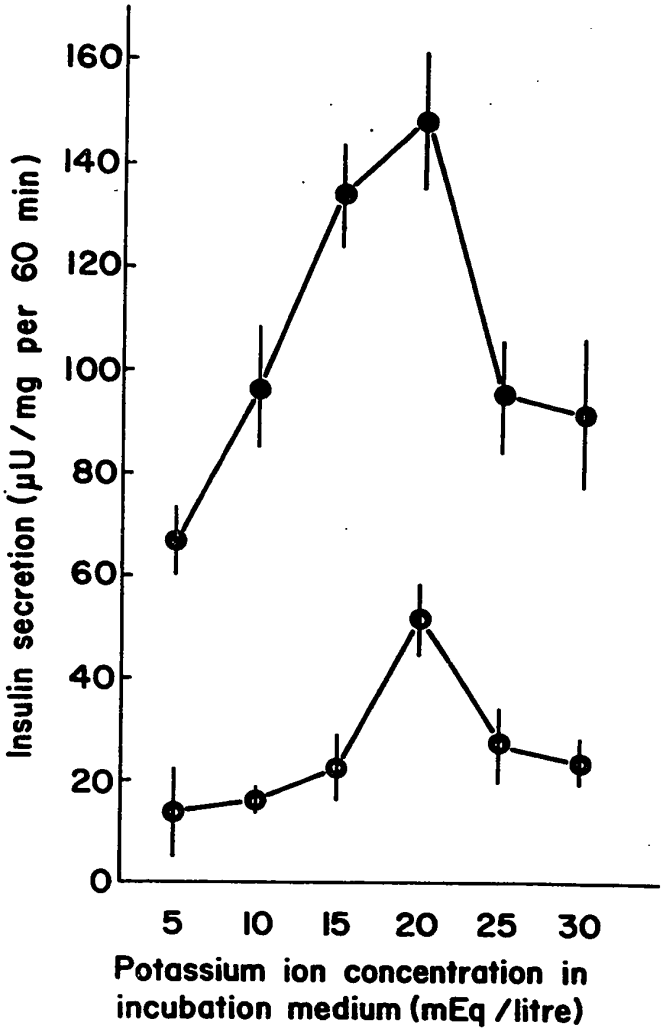

Figure 4-5. Insulin secretion in media of increasing potassium ion concentration with (open circles) and without (closed circles) epinephrine (10 μM). Each point represents the mean value (± SEM) for 13 observations. All experiments were performed in presence of glucose (3.0 mg/ml).

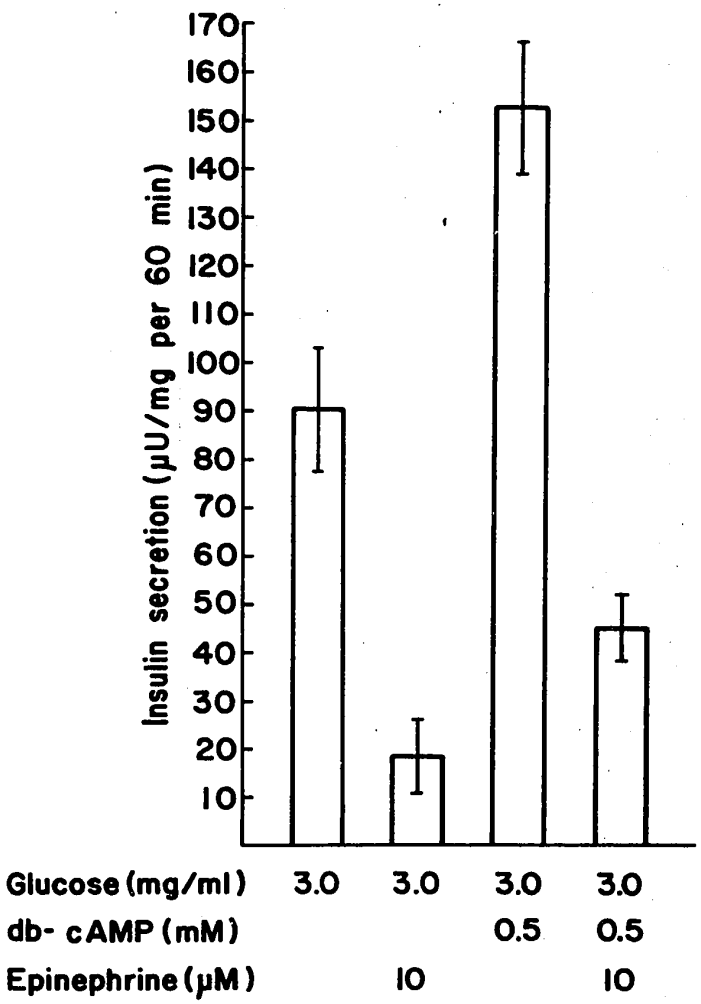

Figure 4-6. Effects of epinephrine upon glucose- and db-cAMP- induced insulin secretion. Each column represents the mean value (± SEM) for 26 observations.

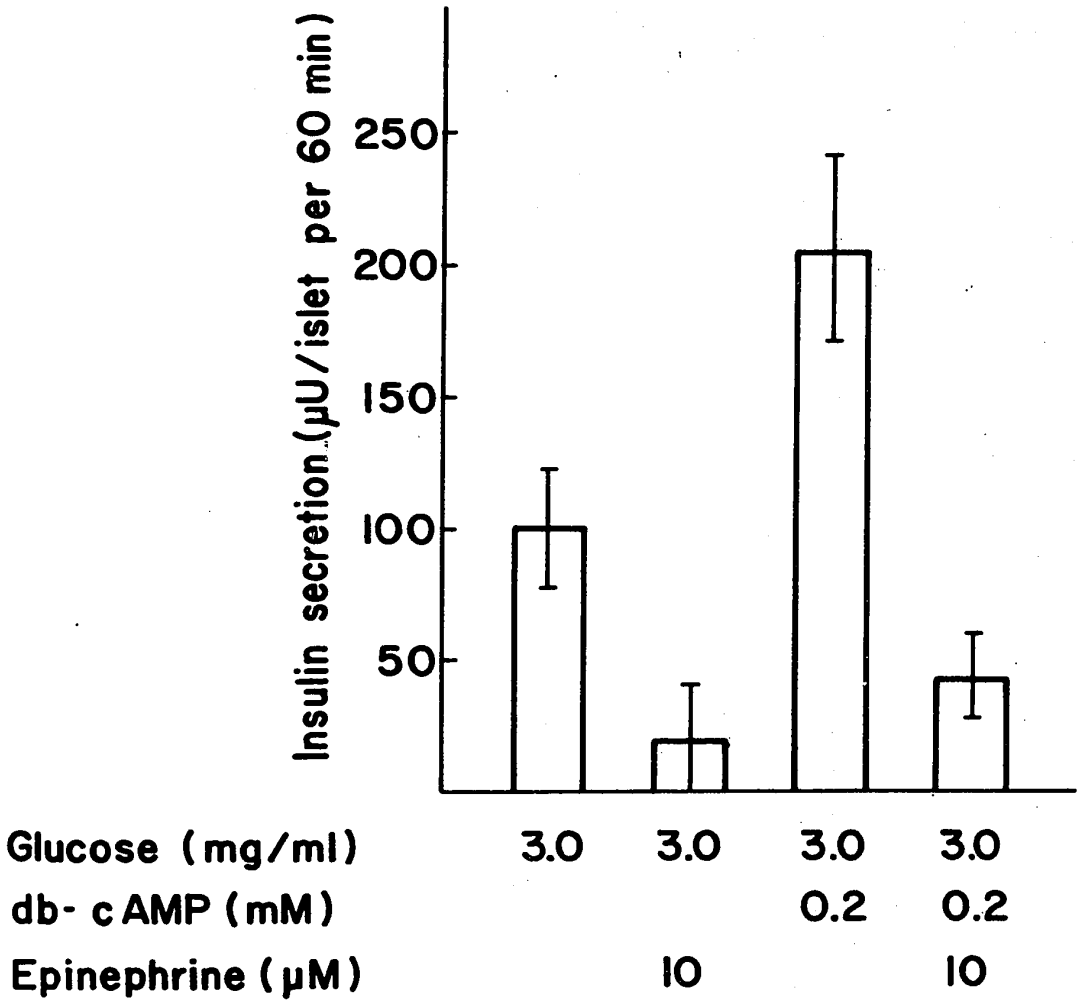

Figure 4-7. Effects of epinephrine upon glucose- and db-cAMP- induced insulin secretion by islets of Langerhans. Each value represents the mean (± SEM) for 9 or 18 individual observations.

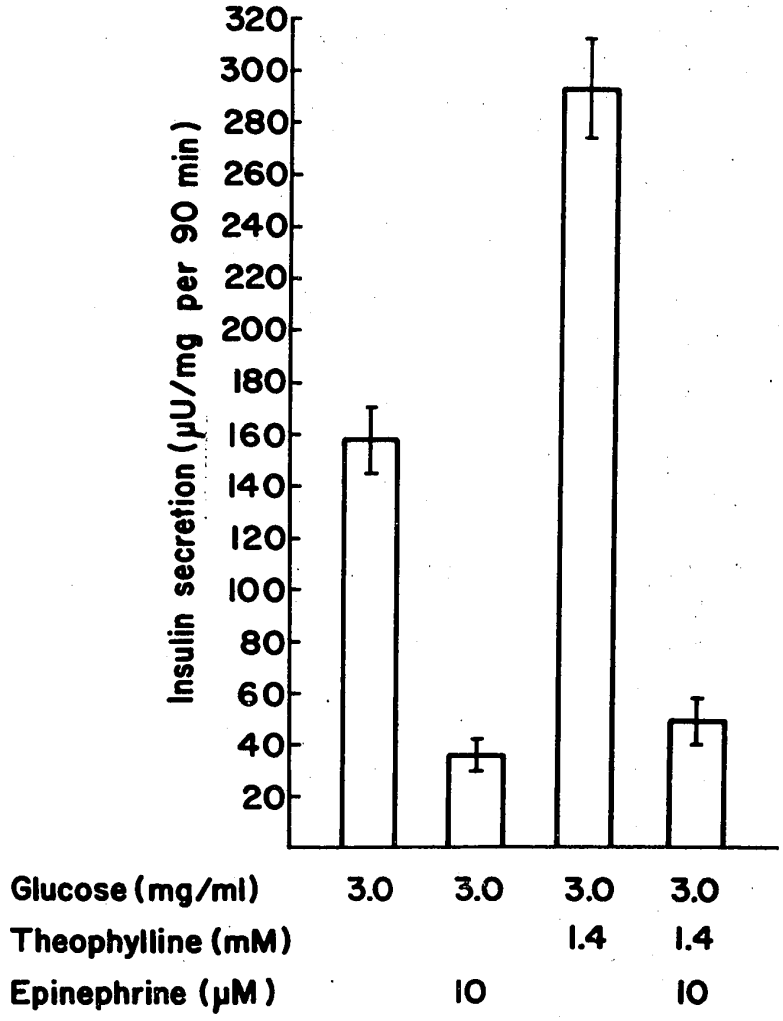

Figure 4-8. Effects of epinephrine upon glucose- and theophylline-induced insulin secretion. Each column represents the mean value (± SEM) for 22 observations.

logical implications of amino acid induction of insulin release are of greater importance in premature infant (Grasso et al., 1968) and in lower animals (Cahill, 1965). Even in obese subjects, where the plasmatic level of certain amino acids is increased, it has been postulated that hyperaminoacidemia could provide the feedback signal to the beta-cell through which insulin resistance in obesity is accompanied by an appropriately augmented secretory response (Felig et al., 1969).

When, in vitro, epinephrine action is investigated upon leucine-stimulated insulin release from islets of Langerhans, a complete blockade of the release is observed (Fig. 4-1), suggesting that any stressful situations encountered by the organism and provoking an increased streaming of catecholamines will probably suppress the effect of circulating amino acids upon the insular secretory activity.

3.2 Epinephrine-induced inhibition of sulfonylurea-induced insulin release

A few years ago, it was shown that sulfonylureas act within the cell as uncoupling agents of the oxidative phosphorylation (Pentillä, 1966; DeBeer et al., 1967). Such effect would upset the cellular "phosphate potential" and increase glycogenolysis and glycolysis in the beta-cell (Hellman,1970).

We have recently demonstrated that the action of a new potent sulfonylurea, BS-4231, was compatible with that hypothesis, and that the different responses of glucose and of BS-4231 to mannoheptulose and diazoxide inhibitions did not

rule out a common catabolic pathway for both insulinotropic agents (Brisson et al., 1971a).

Figures 4-2 and 4-3 show that epinephrine affects in a similar manner the insulinotropic effect of BS-4231 and of glucose whether the latter is present in high (Fig. 4-3) or moderate (Fig. 4-2) concentrations.

3.3 Epinephrine-induced inhibition of enhanced insulin release by potassium additions

The role of potassium in physiological processes has been known for many years (Fenn, 1940). More recently, the number of hormones shown to be released by high potassium concentrations in the extracellular fluid has steadily increased: vasopressin (Douglas and Poisner, 1964), thyrotrophin and adrenocorticotrophic hormone (Vale and Guillemin, 1967), luteinizing hormone (Samli and Geschwind, 1968), follicle-stimulating hormone (Jutisz and Paloma de la Llosa, 1970), and growth hormone (MacLeod and Fontham, 1970). Elevated potassium concentrations have also been shown to stimulate the release of insulin from both perfused (Grodsky and Bennett, 1966) and cultured (Lambert et al., 1969) rat pancreas, and from rabbit (Hales and Milner, 1968a) and rat(Malaisse et al., 1971a) pancreas in vitro. Excess potassium ions also were found to stimulate amylase secretion from both exocrine pancreas (Argent et al., 1971) and parotid gland (Bdolah et al., 1964).

The marked depletion of total body potassium known to occur in severe diabetes (Telfer, 1966) where secretion of

insulin is impaired, or in temporary diabetic state produced by the thiazide diuretics (Rapoport and Hurd, 1964) could be attributed to a direct effect of potassium on pancreatic insulin release. The recent observation of impaired insulin release after glucose in subjects with hypokalemia in man and the improvement of the insulin release mechanism after potassium therapy (Conn, 1965) is consistent with our observations that potassium ion can also act independently of glucose to promote insulin release. In all cases, whether glucose is present (Fig. 4-5) or not (Fig. 4-4), the addition of epinephrine to the media markedly reduced the enhancing effects of the added alkali metal ions upon the release of insulin.

3.4 Epinephrine-induced inhibition of db-cAMP- and of theophylline-induced insulin release

The inhibitory effect of epinephrine has been attributed to a decrease in the intracellular level of cAMP (Turtle et al., 1967b). It is here shown that even when the incubation medium is supplemented with db-cAMP (Fig. 4-6 and Fig. 4-7), or when the accumulation of endogenous cAMP is favored by a blocking of the phosphodiesterase enzyme activity by theophylline (Fig. 4-8); epinephrine abolishes the stimulant action upon the release of insulin whether pieces of pancreas or isolated islets of Langerhans are utilized.

2.5 Conclusion

The present data clearly demonstrate that epinephrine abo-

lishes the stimulatory action of all insulinotropic agents. Because the primary site of action of these various insulinotropic agents is thought to be vastly different from each other, the ubiquitous inhibitory effect of epinephrine suggests that this agent acts upon a late step in the sequence of events leading to insulin release. It is assumed that this late event is common to all insulinotropic factors. For instance, it could correspond to an alteration of the cationic fluxes across the beta-cell membrane which are thought to trigger the emiocytosis of the secretory granules. This hypothesis is considered in greater details in the following chapter.

1.5 Epinephrine and cations upon insulin secretion

2.1 Introduction

From a study on incubated pieces of rabbit pancreas, Milner and Hales (1967a) and Hales and Milner (1968a) have concluded that Na^+ is a prerequisite for the stimulation of insulin secretion, and that depolarization and/or influx of Na^+ across the beta-cell membrane might lead to insulin secretion. Thereafter, the same authors suggested that entry of Na^+ in the beta-cell might be associated with an increase in the intracellular concentration of Ca^{++} which in turn might cause insulin release (Hales et al., 1968b; Milner et al., 1970).

In the light of these observations, this chapter will examine the relative importance of the cations involved in insulin release and evaluate their respective influence on the secretory apparatus.

2.2 Materials and methods

The methods used to measure insulin secretion in vitro from either pieces of pancreatic tissue or isolated islets of Langerhans have been described in previous chapters. Rates of secretion induced by glucose (2.0 or 3.0 mg/ml, except if otherwise stated) under a variety of experimental conditions were compared to those found within the same experiment in a medium of "normal" ionic composition (i.e., Na^+, 139; K^+,5; Ca^{++}, 2; Mg^{++}, 2; Cl^-, 124; and HCO_3^-, 24 mEq. per liter; pH 7.4).

In those experiments designed to study the effects of Na^+ upon insulin secretion, NaCl was replaced by equimolar amounts of KCl, LiCl, or choline chloride. For complete replacement of Na^+ by choline, both NaCl and $NaHCO_3$ were replaced by equimolar amounts of the corresponding choline salt. For partial replacement of Na^+ by tris-(hydroxymethyl) aminomethane (Tris), NaCl was replaced by an equimolar amount of Tris dissolved in water (Trisaminol; Roger Bellon, Neuilly, France). The pH of the latter solution was adjusted at 7.4 by addition of HCl (ca. 0.85 mole of HCl per mole of Tris), so that the final pH, osmolarity, and Cl^- content of the incubation medium were little or not affected under this experimental condition.

In another series of experiments performed with isolated islets of Langerhans (8 islets / 1.0 ml), the replacement of $NaHCO_3$ (24 mM) by an equimolar amount of $KHCO_3$, and that of NaCl (115 mM) and KCl (5 mM) by an equimolar amount of sucrose (240 mM) enabled to prepare a Na^+- free medium (Table 5-I). For purpose of comparison, the control medium contained the same high concentration of K^+ (24 mEq. per liter; obtained by partial replacement of NaCl by KCl). In these experiments, isolated islets were used instead of pieces of pancreatic tissue, for the latter underwent obvious alterations and released proteolytic factors interfering with the insulin assay, when incubated in the sodium-free medium. When the incubation medium contained high concentrations of Ca^{++}, the mixture of labeled and unlabeled insulins and the cellulose suspension used during the assay procedure were prepared in a borate buffer instead of the usual phosphate buffer, in order to avoid

precipitation.

Also added to the media, as required, were acetazolamide (Diamox; Lederle, Brussels, Belgium), ouabain (Ouabaïne Arnaud; Nativelle, France), and epinephrine.

2.3 Results

3.1 Effects of sodium

When sodium was replaced by K^+, a biphasic pattern was observed (Fig. 5-1). At decreasing Na^+ concentrations from 139 to 124 mEq. per liter and increasing K^+ concentrations from 5 to 20 mEq. per liter, a progressive enhancement of glucose-induced secretion occured, with a peak rate averaging twice the control value. At lower Na^+ and higher K^+ concentrations, a progressive reduction of glucose-induced secretion was observed. The lowest rate of secretion (at Na^+ 24 and K^+ 120 mEq. per liter) averaged 25 per cent of the control value.

When Na^+ was replaced by Li^+, only the progressive reduction in the rate of insulin secretion was noticed (Fig. 5-1). A significant inhibition of glucose-induced secretion occured when as little as 15 mEq. per liter of Na^+ were replaced by Li^+. Replacement of Na^+ by Tris also caused a progressive inhibition of the stimulant action of glucose (Fig. 5-1). The lowest rate of secretion observed on replacement of Na^+ by either Li^+ or Tris were comparable to those found at high K^+ concentration.

By contrast, when Na^+ was replaced by choline, a progressive increase in the rate of secretion occured (Fig. 5-1).

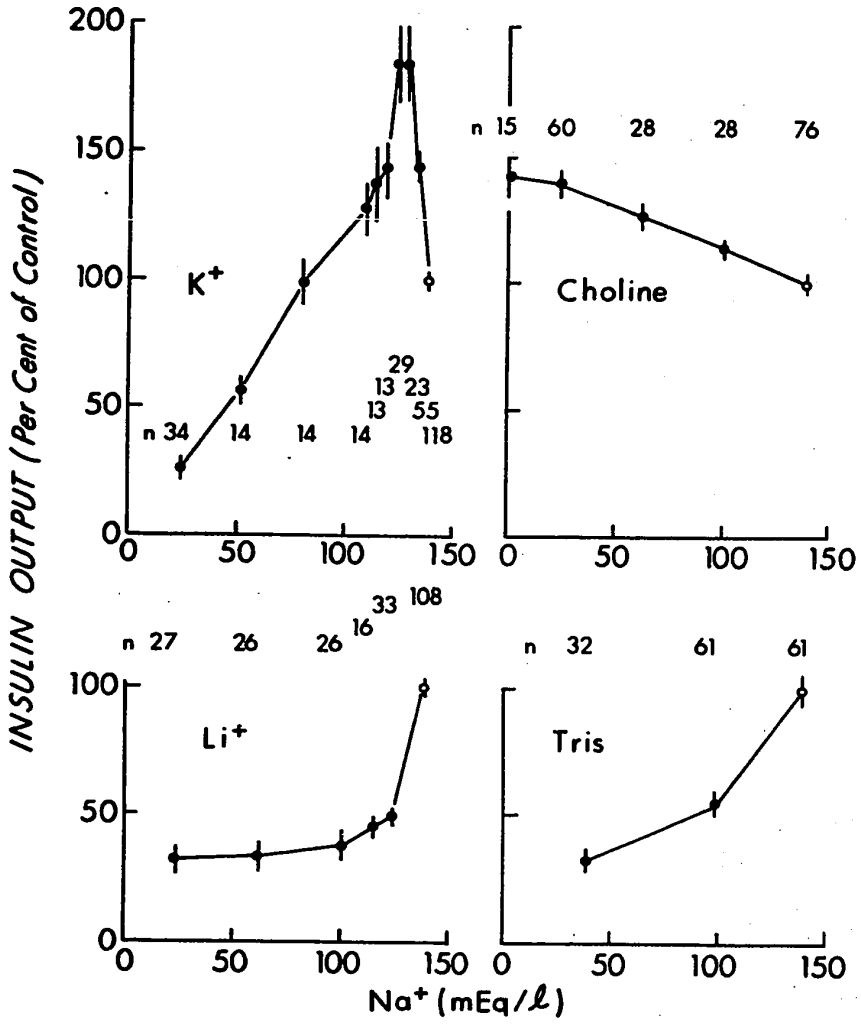

Figure 5-1. Mean values (± SEM) for insulin secretion rate induced by glucose (300 mg/100 ml; 90 min incubation) in pieces of pancreas are shown as percent of the control value (open circle) found in the same experiment, in a medium of normal composition (Na^+ 139, and K^+ 5 mEq. per liter; no Li^+, Tris, or choline). The number of individual observations (n) is indicated in each case.

Even when Na^+ was completely replaced by choline, the rate of insulin secretion induced by glucose remained significantly higher than that observed in the control medium.

In media of "normal" ionic composition (i.e., Na^+ 139 and K^+ 5 mEq. per liter), the mean rate of insulin secretion by isolated islets of Langerhans increases from 30 ± 8 to 289 ± 6 µU/ islet per 90 min, as the glucose concentration of the incubation medium is raised from 0.3 to 3.0 mg/ml (Malaisse et al., 1968b). When Na^+ was partially replaced by K^+ (i.e., Na^+ 120, and K^+ 24 mEq. per liter), a much higher rate of glucose-induced secretion was recorded (478 ± 11 µU / islet per 90 min; Table 5-I). At the same high K^+ concentration (24 mEq. per liter), the omission of Na^+ caused a dramatic decrease in the secretion rate (Table 5-I). However, such low insulin output (77 ± 6 µU / islet per 90 min) still represented more than twice the basal rate of insulin release observed at identical ionic concentrations, in the absence of glucose (32 ± 4 µU / islet per 90 min).

3.2 Effect of ouabain

The effect of ouabain on insulin secretion was studied because this glycoside had been shown to inhibit specifically a sodium pump in a wide variety of cells (Schatzmann, 1953; Skou, 1965). The effect of ouabain (20 to 50 µM) upon insulin secretion was tested over a wide range of glucose concentrations (0 to 3.0 mg/ml; Table 5-II). Ouabain had no effect except at glucose concentrations larger than 100 mg/

Table 5-I.

Insulin secretion in a Na^+-free medium

Na^+ (mEq./l)	K^+ (mEq./l)	Cl^- (mEq./l)	Sucrose (mM)	Glucose (mM)	Insulin output (μU/islet/90min)
120	24	124	-	16.7	478 ± 11 (18)
-	24	4	240.0	16.7	77 ± 6 (18)
-	24	4	256.7	-	32 ± 4 (18)

The mean insulin output (± SEM) by isolated islets of Langerhans (8 islets / 1.0 ml of incubation medium) is quoted together with the number of individual observations (in parenthesis). Also shown are the Na^+, K^+, Cl^-, sucrose and glucose concentrations of the incubation media. All media were "normal" in other respects (i.e., Ca^{++}, 2; Mg^{++}, 2; HCO_3^-, 24 mEq. per liter; pH 7.4; 310.7 mOsmole per liter).

Table 5-II.

Effect of ouabain upon insulin secretion

Glucose (mg/100 ml)	Control output (μU/mg per 60 min)	Effect of ouabain (+ μU/mg/60 min) 20 μM	50 μM
-	6.5 ± 1.4 (22)	+ 1.1 ± 2.5 (9)	+ 2.1 ± 4.5 (13)
100	6.9 ± 3.8 (13)		+ 10.5 ± 5.3 (13)
150	29.2 ± 3.2 (34)	+ 5.1 ± 2.5 (18)	+ 16.0 ± 4.1* (16)
300	47.9 ± 3.7 (34)	+ 4.2 ± 7.2 (18)	+ 12.1 ± 8.5 (16)

The mean secretion rate (± SEM) by pieces of pancreatic tissue incubated in media containing glucose alone (control output) is quoted together with the mean changes in secretion rate induced by ouabain and their statistical significance (* = $P < 0.01$). The number of observations is shown in parenthesis.

100 ml.

3.3 Effects of alkaline earth cations

Omission of Ca^{++} from the incubation medium markedly reduced the rate of insulin release evoked by glucose. As little as 0.5 mEq. per liter of Ca^{++} was sufficient to ensure a normal rate of insulin secretion. At high Ca^{++} concentrations, the rate of insulin release was the same as that observed in the control medium (Fig. 5-2A).

Omission of Mg^{++} did not significantly modified the stimulant action of glucose upon insulin secretion. A progressive inhibition of glucose-induced insulin secretion was observed when the Mg^{++} concentration was raised up to 20 mEq. per liter (Fig. 5-2B); these experiments were carried out in the presence of a normal Ca^{++} concentration. When a subnormal Ca^{++} concentration was used, the inhibition of insulin secretion induced by raising the Mg^{++} concentration from 2.0 to 12.0 mEq. per liter was twice that observed in the presence of a normal Ca^{++} concentration (48 per cent inhibition at Ca^{++} = 0.4 mEq. per liter vs 23 per cent inhibition at Ca^{++} = 2.0 mEq. per liter). These findings suggest that Ca^{++} and Mg^{++} might act competitively upon insulin secretion.

However, as shown in Fig. 5-2C, the inhibition of glucose-induced insulin secretion observed at high Mg^{++} concentration was not overcome in the presence of increasing Ca^{++} concentrations. Since both Mg^{++} and Ca^{++} salts were added to an otherwise normally composed medium, these experiments were

Insulin secretion vs alkaline earth cations

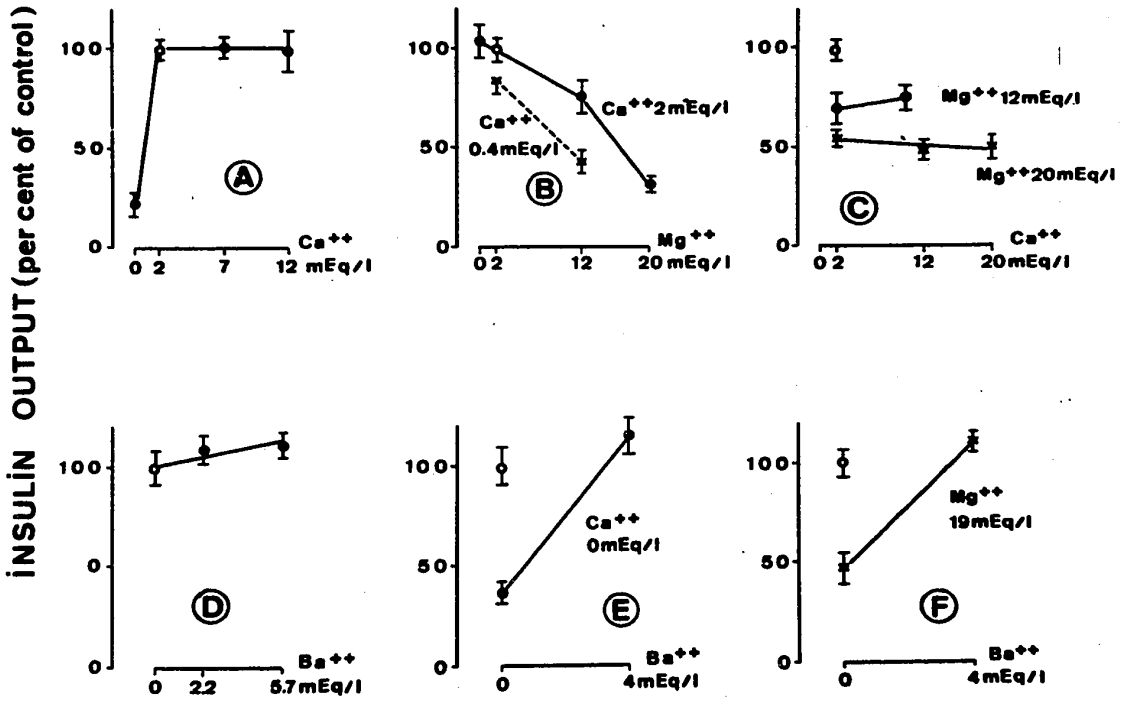

Figure 5-2. Mean values (± SEM) for insulin secretion rate induced by glucose (300 mg/100 ml; 90 min incubation) at various Ca^{++}, Mg^{++}, and Ba^{++} levels (closed circles and crosses) are shown as percent of the control value (open circle) found in the same experiment, in a medium of normal composition (Ca^{++} 2, and Mg^{++} 2 mEq. per liter; no Ba^{++}). Each value represents the mean of 14 or more individual observations.

carried out in hyperosmolar medium.

The normal medium contains no Ba^{++}. Addition of Ba^{++} did not significantly modify the stimulant action of glucose (Fig. 5-2D). The reduced rates of insulin secretion observed either in the absence of Ca^{++}, or in the presence of excess Mg^{++} were both restored to normal when Ba^{++} was also added to the incubation medium (Fig. 5-2E; Fig. 5-2F).

3.4 Combined effects of sodium and alkaline earth cations

The data reported in Table 5-III deal with the possible interaction of Na^{+} and alkaline earth cations. They indicate, firstly, that the addition of Ba^{++} (4 to 8 mEq. per liter) to the incubation medium failed to significantly enhance the low rates of secretion observed when Na^{+} was partially replaced by either K^{+}, Li^{+}, or Tris (Table 5-III, expt 1 to 4).

Secondly, when Na^{+} was partially replaced by either Li^{+} or Tris, an increase in the Mg^{++} content of the medium up to 8 mEq. per liter failed to significantly affect the rate of insulin secretion (Table 5-III, expt 5 and 6); whereas, in the same experiments, the same Mg^{++} concentration (8 mEq. per liter) slightly but significantly reduced (-25.6 ± 10.6 μU / mg per 90 min; d.f. = 50; $P < 0.02$) the rate of insulin secretion induced by glucose in media containing Na^{+} in a normal concentration (i.e., 139 mEq. per liter).

Thirdly, a reduction in the Ca^{++} content of the medium from 2.0 to 0.5 mEq. per liter, a condition which in the present system is known not to modify insulin secretion at a

Expt. N°	Control output (µU/mg/90 min)	Na^+ replacement ion	(mEq/l)	Ca^{++}	Mg^{++} (mEq/l)	Ba^{++}	Insulin output (µU/mg/90 min)
1.	92.3 ± 8.9 (16)	K^+	115	2	2	0	26.1 ± 3.8 (16)
		K^+	115	2	2	6	32.3 ± 3.5 (16)
2.	117.6 ± 8.9 (13)	Li^+	77	2	2	0	29.5 ± 7.1 (13)
		Li^+	77	2	2	4	36.5 ± 3.5 (13)
		Li^+	77	2	2	8	32.5 ± 4.8 (13)
3.	99.9 ± 7.9 (16)	Li^+	23	2	2	0	44.9 ± 4.2 (16)
		Li^+	23	2	2	6	48.5 ± 4.6 (16)
4.	107.5 ± 13.4 (16)	Tris	40	2	2	0	39.6 ± 14.7 (16)
		Tris	40	2	2	8	54.9 ± 8.1 (16)
5.	106.5 ± 9.9 (13)	Li^+	15	2	2	0	45.0 ± 6.6 (13)
		Li^+	15	2	8	0	47.3 ± 8.5 (13)
6.	131.1 ± 11.2 (13)	Tris	40	2	2	0	74.1 ± 5.5 (13)
		Tris	40	2	8	0	64.0 ± 7.4 (13)
7.	95.2 ± 8.5 (13)	Li^+	38	2	2	0	15.3 ± 5.1 (13)

7.	95.2 ± 8.5 (13)	Li^+	30	2	2	0	15.3 ± 3.1 (13)
8.	89.2 ± 8.3 (13)	Li^+	15	2	2	0	49.8 ± 7.1 (13)
		Li^+	15	0.5	2	0	49.2 ± 6.7 (13)
9.	120.9 ± 10.9 (13)	Li^+	15	2	2	0	44.7 ± 7.9 (13)
		Li^+	15	0	2	0	20.9 ± 7.1 (13)
10.	93.6 ± 12.8 (18)	Choline	115	2	2	0	168.0 ± 22.9 (18)
		Choline	115	0	2	0	42.7 ± 5.9 (16)
11.	97.2 ± 14.6 (16)	K^+	14	2	2	0	141.8 ± 13.8 (16)
		K^+	14	0	2	0	30.9 ± 5.7 (16)

Table 5-III. Combined effects of alkali and alkaline earth cations

From left to right, the table indicates respectively the reference number of the experiment; the control rate of insulin secretion (mean (± SEM) induced by glucose (300 mg/100 ml) in media of normal composition (Na^+ 139, K^+ 5, Ca^{++} 2, Mg^{++} 2 mEq/l; no Ba^{++}, Li^+, Tris or choline); the ion used for Na^+ replacement and the amount of Na^+ replaced; the Ca^{++}, Mg^{++}, and Ba^{++} concentrations of the incubation media; and the corresponding rate of insulin secretion (mean ± SEM) induced by glucose (300 mg/100 ml). For each experiment, the number of observations is shown in parenthesis.

normal Na^+ concentration (Fig. 5-2A), also failed to reduce secretion when Na^+ was partially replaced by Li^+ (Table 5-III, expts 7 and 8). Only by omitting Ca^{++} from the medium could such secretion rate be further decreased (Table 5-III, expt. 9). Omission of Ca^{++} also markedly reduced the high rate of secretion observed in media in which Na^+ was partially replaced by choline or K^+ (Table 5-III, expts 10 and 11).

3.5 Effect of pH

The stimulant effect of glucose upon insulin secretion was reduced at low pH and increased at high pH (Table 5-IV, expt. 1). These changes in insulin output are thought to result from changes in pH rather than from the corresponding changes in the bicarbonate concentration of the media. Thus, at a pH of 7.4, glucose (1.5 mg/ml) induced the same rate of insulin secretion in a 18 mM phosphate buffer (38.2 ± 3.9 μU / mg per 90 min, n=18) as in the usual bicarbonate-buffered medium (35.9 ± 3.7 μU / mg per 90 min, n=18). Moreover, the rate of insulin secretion (103.8 ± 6.1 μU / mg per 90 min, n=34) induced by glucose (3.0 mg / ml) was not significantly affected (87.3 ± 6.6 μU / mg per 90 min, n=34) in the presence of a very high concentration (20 mM) of acetazolamide, suggesting that the beta-cell, like other endocrine glands (Maren, 1967) is devoid of carbonic anhydrase.

The addition of Ba^{++} (4 to 8 mEq. per liter) to the incubation medium did not affect the low rate of insulin secretion induced by glucose in acidic media (Table 5-IV, expt. 2). By

Table 5-IV.

Effect of pH upon insulin secretion

Expt N°	pH	Ca^{++} mEq/l	Mg^{++} mEq/l	Ba^{++} mEq/l	Insulin output (µU/mg/90 min)
1	6.3	2	2	-	24.4 ± 2.9 (14)
	6.9	2	2	-	74.8 ± 3.5 (14)
	7.4	2	2	-	95.4 ± 5.4 (14)
	8.0	2	2	-	103.1 ± 6.9 (14)
2	6.1	2	2	-	29.9 ± 3.4 (32)
	6.1	2	2	4	29.4 ± 4.3 (16)
	6.1	2	2	8	27.1 ± 5.4 (16)
3	7.9	2	2	-	96.6 ± 6.1 (32)
	7.9	-	2	-	50.5 ± 4.9 (32)

Mean insulin secretion rates (± SEM) by incubated pieces of pancreatic tissue are quoted together with the number of observations (in parenthesis). Experimental conditions are shown in the left part of the table. All media contained glucose in a concentration of 200 mg/100 ml.

Table 5-V.

Effects of epinephrine, Ca^{++}, and Ba^{++} upon glucose-induced insulin secretion.

Epinephrine (μM)		Ca^{++} (mEq/l)	Ba^{++} (mEq/l)	Insulin output (μU/mg per 90 min)	
Control	---	2	-	106.5 ± 6.1	(28)
	2.0	2	-	44.7 ± 5.1¶	(28)
	2.0	10	-	55.6 ± 3.5¶	(28)
Control	---	2	-	90.1 ± 6.7	(18)
	12.5	2	-	28.0 ± 2.8¶	(18)
	12.5	2	4	26.6 ± 4.6¶	(18)

Mean values (± SEM) for insulin secretion rate induced by glucose (300 mg per 100 ml) are shown together with the number of observations (in parentheses) and the significance of differences between control (first line) and experimental values (¶ = $P < 0.001$).

contrast, the omission of Ca^{++} caused a significant inhibition ($P < 0.001$) of the much higher rate of secretion induced by glucose in alkalotic media (Table 5-IV, expt. 3).

3.6 Combined effects of epinephrine and alkaline earth cations

The data in Table 5-V indicate that neither a high Ca^{++} concentration, nor the addition of Ba^{++} were able to modify the inhibitory effect of epinephrine upon glucose-induced secretion.

2.4 Discussion

3.1 Sodium influx and insulin secretion

The existence of a Na^+ pump in the beta-cell is suggested by its membrane potential (Malaisse, unpublished observations; Dean et al., 1968). It has been postulated that depolarization of the beta-cell and Na^+ influx might lead to insulin secretion (Milner et al., 1967a; Hales et al., 1968b). Our present data also point to a role for Na^+ in the regulation of insulin release.

Firstly, glucose-induced secretion was increased at high K^+ concentrations (10 to 34 mEq per liter; Fig. 5-1). A stimulant effect of high K^+ concentrations upon insulin secretion has also been observed in the perfused rat pancreas (Grodsky et al., 1966), and in incubated pieces of rabbit pancreas (Hales et al., 1968b) or the foetal rat (Lambert et al., 1969). As suggested by Baker (1968) K^+ could increase the Na^+ efflux from the ouabain-insensitive, calcium-dependent sodium pump and so favor influx of calcium. More likely, it seems that the increased secretion induced by high K^+ could here correspond to

cell depolarization and concomittant entry of Na^+ in the beta-cell. Indeed, the abnormally high rate of secretion observed under this experimental condition dramatically fell when Na^+ was omitted from the incubation medium (Table 5-I). Hales et al., (1968a) also reported that pieces of pancreas which had been incubated in a Na^+-free medium for three successive periods and from which all extracellular Na^+ had presumably been removed could no longer be stimulated by K^+. More support can also be obtained from Douglas et al. (1967) who reported that excess K^+ in the extracellular medium does in fact depolarize the chromaffin cells.

Secondly, when Na^+ was partially replaced by either K^+, Li^+ or Tris, the stimulant effect of glucose was decreased. Variable amounts of Na^+ had to be replaced to provoke inhibition of secretion (Fig. 5-1). When Na^+ was replaced by K^+, more than half of the usual amount of Na^+ had to be substituted. Smaller amounts of Tris were required. This may seem surprising since Tris has been reported to promote insulin secretion (Zaharko, 1969). However, recent observations suggest that such stimulant effect of Tris might result from its alkalinizing property (Broeckaert, 1970). When Na^+ was replaced by Li^+, a substitution of 15 mEq was sufficient to cause inhibition of glucose-induced secretion. Hales et al. (1968b) also found that the inhibition of insulin secretion due to Na^+ replacement by Li^+ was irreversible, probably because Li^+ is slowly pumped out of the cell (Maizels, 1954; Keynes et al., 1959).

Lastly, ouabain increased insulin output (Table5-II). Stimulation of insulin secretion by ouabain has been previous-

ly observed both in vivo (Triner et al., 1968), and in vitro (Milner et al., 1967a; Lambert et al., 1969). The concentration of ouabain found to be effective in the present system is comparable to that used by Milner and Hales in their experiments with rabbit pancreatic tissue (1967a). In view of the low concentration used, the stimulant action of ouabain is likely to be due to its effect on Na^+ and K^+ active transport, rather than to any effect of the sugar moiety of the glycoside. Since the beta-cell is thought to be freely permeable to glucose (Malaisse et al., 1968c), the effect of ouabain is also unlikely to result from an increase in the membrane transport of glucose, as it occurs in cardiac or skeletal muscle (Bihler, 1968). That ouabain indeed inhibits the beta-cell sodium pump is demonstrated by the data of Howell and Taylor (1968) who found that ouabain inhibits $^{42}K^+$ uptake by isolated islets. To test the postulate that ouabain stimulates insulin secretion by inhibiting a sodium pump, Hales and Milner (1968a) omitted potassium from incubation media. They knew that active pumping of sodium across the red cell membrane is inhibited by the absence of extracellular K^+ (Dunham et al., 1961; Whittam, 1962). They found that insulin secretion was stimulated by incubation in a potassium-free medium but the stimulation was not as great as that caused by ouabain. They assumed it was due to partial pump inhibition caused by the presence of a low concentration of K^+ still remaining in the incubation media. Also, the failure of omission of potassium to augment the stimulation caused by ouabain suggested to them that both stimuli acted through a common mechanism. The optimal action of ouabain upon insulin

secretion in the present system was observed at a glucose concentration of 1.5 mg/ml, thus at a level where small increments in glucose concentration cause marked increases in insulin output (Malaisse et al., 1967b).

Malaisse and his colleagues(1967a, 1971d) have shown that insulin secretion was stimulated from rat pancreas incubated in vitro by cholinergic drugs, and that this stimulation was blocked by atropine. Hales and Milner (1968a) demonstrated that the possibility for some changes in insulin secretion to be mediated by ionic fluxes in the autonomic nervous system appeared to be excluded by the fact that K^+ and ouabain remained effective stimuli in the presence of atropine.

All these findings suggest that the Na^+ flux across the membrane of the beta-cell might be a rate-limiting factor of the stimulant action of glucose upon insulin secretion. There seems thus little doubt that entry of Na^+ in the beta-cell favors the release of insulin, as first suggested by Hales and Milner.

3.2 Possible dissociation of Na^+ influx and insulin secretion

Our data indicate that Na^+ *per se* is not necessarily involved in the secretory process. Thus, insulin secretion in response to glucose occured at a higher than normal rate when Na^+ was either partially or completely replaced by choline. This observation implies either that choline is an effective substitute for Na^+, or that a dissociation might occur, under

certain experimental conditions, between the entry of Na^+ in the beta-cell and the stimulation of insulin secretion.

Indeed, many reports have suggested a dissociation between sodium influx and cellular response. In different systems, it appeared that calcium deprivation favored internal Na^+ accumulation (Frankenhaeuser and Hodgkin, 1957; Paton et al., 1971) but yet the secretory activity was in every case decreased, suggesting that the internal sodium accumulation per se was not responsible for the release.

Such dissociation was also noticed by Douglas and his collegues when investigating the chromaffin cell. They concluded that depolarization in response to acetylcholine seemed to be due mainly to inward sodium current but that sodium did not appear as the sole factor involved for acetylcholine retained some depolarizing action when all sodium was removed and replaced by sucrose (Douglas, 1968). That residual effect seemed to be largely due to inward movement of calcium since it increased with increasing extracellular concentration of Ca^{++} (Douglas et al., 1967). Those findings were harmonizing with earlier evidence showing that acetylcholine increased ^{45}calcium uptake in the adrenal medulla (Douglas et al., 1962).

Such dissociation is also suggested by the effects of pH upon insulin output (Table 5-IV). Thus, extracellular acidosis inhibited glucose-induced secretion, although this condition is known to favor Na^+ influx in the cell, at least in other tissues (Adler et al., 1965). Admittedly, the evidence

is weak for acidosis could also affect the secretory process by inhibiting a number of intracellular reactions, e.g., glycolysis (Harris et al., 1969).

More convincingly, glucose was able to stimulate insulin secretion in the complete absence of Na^+ (Table 5-I). In the Na^+ - free medium, the rate of insulin secretion induced by glucose was more than twice that found in the absence of glucose, and it represented approximately one fourth of that found under normal ionic conditions.

These findings suggest that, although Na^+ influx is usually an adequate stimulus for insulin release, it might neither be sufficient nor necessary for secretion to occur.

3.3 Combined effects of alkali and alkaline earth cations

A number of observations indicate that alterations in the flux of sodium ions across the cell membrane affect the transmembrane flux and intracellular distribution of Ca^{++} (Birks, 1963; Palmer et al., 1967; Simpson, 1968; Birks and Cohen, 1968; Glitsch et al., 1970). Moreover, the existence of a calcium-dependent sodium pump has been recently recognized (Baker, 1968). One should, therefore, consider whether the inhibition of insulin secretion observed at low Na^+ concentrations might not be mediated through changes in the handling of alkaline earth cations by the beta-cell, as recently suggested by Hales and Milner (1968b) and supported by Glitsch et al., (1970) and De Meis (1971). The data summarized in Table 5-III indicate that the beta-cell is not more and may even be less sensitive to Ba^{++} or Mg^{++} at low than at normal Na^+

concentrations. These apparently negative findings do not rule out our working hypothesis. Indeed, it remains possible that the ineffectiveness of Ba^{++} and of Mg^{++} upon the reduced rate of secretion observed at low Na^{+} concentrations corresponds in fact to an inhibition in the influx of alkaline earth cations in the beta-cell, as thought to occur in other secreting cells (Douglas, 1968). The effect of low Na^{+} concentrations upon the beta-cell secretory activity thus appears to be comparable to that induced by epinephrine and reported in a previous chapter.

Inversely, the abnormally high rate of insulin secretion observed on replacement of Na^{+} by either K^{+} or choline could correspond to an increased influx of Ca^{++} in the beta-cell. Two evidences can be found in favor of this hypothesis. Firstly, when Na^{+} was partially replaced by either K^{+} or choline, although the omission of Ca^{++} caused a marked reduction of the stimulant action of glucose, the rate of insulin secretion remained higher than the basal value (Table 5-III, expts 10 and 11). Replacement of Na^{+} by either K^{+} or choline might thus have facilitated the influx of some extracellular Ca^{++} still present in the pieces of pancreas, since these were neither preincubated in Ca^{++}- free media, nor exposed to any chelating agent. In adrenal and fat tissue ACTH activity is dependent on the presence of calcium in the medium, but there seems to be enough calcium bound to membrane sites in subcellular particles from these tissues to make addition of calcium to the incubation mixtures unnecessary (Taunton et al., 1969; Birnbaumer et al., 1969). In such systems tissue cal-

cium must be carefully depleted with a chelating agent to demonstrate the calcium requirement for ACTH action (Birnbaumer and Rodbell, 1969; Bar and Hechter, 1969; Lefkowitz et al., 1970). Secondly, epinephrine, which inhibits the Ca^{++} uptake by the beta-cell (Malaisse-Lagae et al., 1971a), abolishes the enhancing action of K^{+} in high concentrations upon glucose-induced secretion (Fig.4-5) and even in glucose-free medium (Fig. 4-4).

3.4 Effects of pH

The influence of pH upon insulin secretion could also be due, in part at least, to changes in the flux of Ca^{++} across the cell membrane. Thus, in other biological systems, parallel influx of Ca^{++} and efflux of H^{+} have been reported (Harris et al., 1969; Epstein, 1968).. Such process could account for the high rate of secretion induced by glucose in alkalotic media, and its inhibition at low Ca^{++} concentration (Table 5-IV). Under the latter experimental condition, although the omission of Ca^{++} caused a marked reduction of the stimulant action of glucose, the rate of insulin secretion remained higher than the basal value. Again, the high pH might have facilitated the entry of residual extracellular Ca^{++} still present in the pieces of pancreas. Inversely, inhibition of alkaline earth cations influx at low pH would account for both the low rate of insulin secretion and the uneffectiveness of Ba^{++} observed in acidic media.

3.5 Effects of alkaline earth cations

The results obtained with alkaline earth cations (Fig. 5-2) are in good agreement with those reported by Grodsky et al. (1966), Curry et al. (1968a, 1968b), Bennett et al. (1969), and by Milner and Hales (1967b). They indicate that the presence of Ca^{++} is required in order for glucose to stimulate insulin secretion. Ba^{++} can be substituted for Ca^{++}. Mg^{++} exerts an effect opposite to that of Ca^{++}. Its presence is apparently not required for the process of secretion to occur, and high Mg^{++} concentrations inhibit the stimulant action of glucose. This inhibitory effect of Mg^{++} was enhanced at a subnormal Ca^{++} concentration, suggesting that Mg^{++} and Ca^{++} might compete for a common receptor. A parallel phenomenon was observed with perfused adrenal glands. Indeed, Mg^{++} depressed ACTH-evoked secretion in the presence of normal calcium (Jaanus et al., 1970). In favor of this concept, Malaisse et al. (1970b) have recently demonstrated that glucose-induced Ca^{++} uptake by isolated islets of Langerhans is inhibited at high Mg^{++} concentrations. The inhibitory effect of Mg^{++} was abolished in the presence of Ba^{++}, but not at high Ca^{++} concentrations. Thus, in media of comparable osmolarity (338 to 342 mOsm. per liter), as little as 4.0 mEq. per liter of Ba^{++} restored a normal secretion in the presence of 19 mEq. per liter of Mg^{++}; whereas, 10 mEq. per liter of Ca^{++} failed to do so in the presence of 12 mEq. per liter of Mg^{++}. Therefore, the failure of Ca^{++} to overcome the inhibitory effect of Mg^{++} might be due to a lesser affinity of Ca^{++} than

Ba^{++} for the postulated receptor. Taken as a whole, the present data indicate that Ca^{++} and Ba^{++} on the one hand, and Mg^{++} on the other act as antagonists upon the process of insulin secretion. A similar alkaline earth cation interrelationship has been noticed when studying the release of norepinephrine from nerve endings (Keen and Bogdanski, 1970). An equivalent antagonist effect of magnesium upon secretion was also previously noticed by Douglas and Rubin (1963, 1964).

3.6 Combined effects of epinephrine and alkaline earth cations

The inhibitory effect of epinephrine was not overcome at high Ca^{++} or Ba^{++} concentrations (Table 5-V). That could indicate an interference by epinephrine with the handling of alkaline earth cations by the beta-cell. We have shown previously that epinephrine inhibitory effect upon insulin secretion was mediated through an activation of alpha-adrenergic receptor system. The present experimental data are compatible with the hypothesis that Ca^{++} plays an important role in the stimulus-secretion coupling of glucose-induced insulin secretion (Malaisse et al., 1970a, 1970b), the influence of alkali cations and pH upon the secretory process being eventually mediated, in part at least, through an altered handling of Ca^{++} by the beta-cell. Whether such an altered Ca^{++} handling by epinephrine represents the primary events in the activation of alpha-adrenergic receptors by this agent remains to be investigated.

1.6 Effects of glucose and of epinephrine upon ^{45}calcium efflux from isolated islets of Langerhans

2.1 Introduction

It has been estimated that the Ca^{++} concentration in the sarcoplasm of relaxed muscles is about 0.5 µM, and that the Ca^{++} concentration required to set off a contraction is estimated to be about 5.0 µM. So, to couple excitations from motor neurons with muscle contractions, the muscle cell is equipped with a sleevelike system of vesicles specialized in Ca^{++} trapping, the sarcoplasmic reticulum. Upon excitation, Ca^{++} is released from these reticular formations and accumulates in the sarcoplasm. When the proper calcium concentration is reached contraction occurs (Lehninger, 1971).

In a sequence of events that appear to be more and more analogous to the excitation-contraction coupling, glucose would induce insulin to be released from the beta-cell. Indeed, Malaisse-Lagae and her colleagues (1971a, 1971b, 1971c) very recently reported that the 'exciting' glucose induces an accumulation of calcium in isolated islets of Langerhans. From these studies it now appears that, as for the muscle cell, the beta-cell must built up in its cytoplasm a certain calcium concentration in order to provoke emiocytotic movements of insulin.

Nobody has ever assigned to the beta-cell's endoplasmic reticulum a function similar to the one assumed by its sarcoplasmic equivalent. Indeed, it seems that the muscle reticular calcium-handling properties are, for the beta-cell, restricted to the cell membrane itself.

We here present evidences that the rate of calcium efflux from the beta-cell cytoplasm could be of primary importance in favoring the intracellular accumulation of calcium necessary for the insulin exocytotic movements. We shall also refute an epinephrine-induced inhibition through the sole mediation of transmembrane alterations of calcium fluxes.

2.2 Materials and methods

3.1 Isolated islets

All experiments were performed on isolated islets of Langerhans removed from fed albino rats (150 to 200 g; Rega, Heverlee, Belgium).

3.2 Perifusion media*

All perifusions were carried out at 37°C in a bicarbonate-buffered medium containing bovine serum albumin (0.5 %), and equilibrated against a mixture of oxygen (95 %) and car-

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* In Table 6-I are listed the composition of the various perifusates and the corresponding symbols used in Fig. 6-1 to Fig. 6-15.

Table 6-I

Explanations for symbols used in Fig. 6-1 to Fig. 6-15.

G3	:	medium containing glucose at a concentration of 3.0 mg / ml.
αG	:	medium containing no glucose
NCa	:	medium containing calcium at a "normal" concentration (2.0 mEq. per liter)
noCa	:	medium into which no calcium is incorporated
αCa	:	medium into which no calcium is incorporated and enriched with EGTA (2.0 mM)
Ba	:	medium containing barium (4.0 mEq. per liter)
D_2O	:	medium in which all H_2O is replaced by D_2O
EPI	:	medium containing epinephrine (10 μM).

bon dioxide (5 %). This medium had the following ionic composition: Na^+, 139; K^+, 5; Ca^{++}, 2; Mg^{++}, 2; Cl^-, 124; and HCO_3^-, 24 mEq. per liter. Series of experiments were performed where calcium ions from the incubation media were simply omitted or replaced by barium chloride (4.0 mEq. per liter). Upon assay of such media the total calcium content averaged nevertheless 0.22 ± 0.02 mEq. per liter; hence, experiments were also carried out where the calcium-depleted media were enriched with EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid; 2.0 mM; Sigma Chemical Co.). Also added to the media, as required, were glucose, epinephrine, ^{45}calcium (50 µC/ml; $^{45}CaCl_2$; The Radiochemical Center, Amersham, England). In one series of experiments the incubation media were prepared in deuterium oxide (D_2O ; E.G. Merck, Darmstadt, Germany).

3.3 Perifusion of islets

4.1 The perifusion apparatus

The islets were perifused in an apparatus illustrated in Figure 6-1. The control (A) and experimental (B) media used for perifusion were maintained at 37°C in a water bath (C), and continuously mixed with an incoming mixture (D) of oxygen (95 %) and carbon dioxide (5 %). The reservoirs (A & B) were connected to a small perifusion chamber (E ; volume = 250 µl; kindly donated by Dr. I.A. Mirsky, University of Pittsburg, Pittsburg, Penna.) through a Y-shaped valve (F) allowing rapid switching from one medium to the other. The

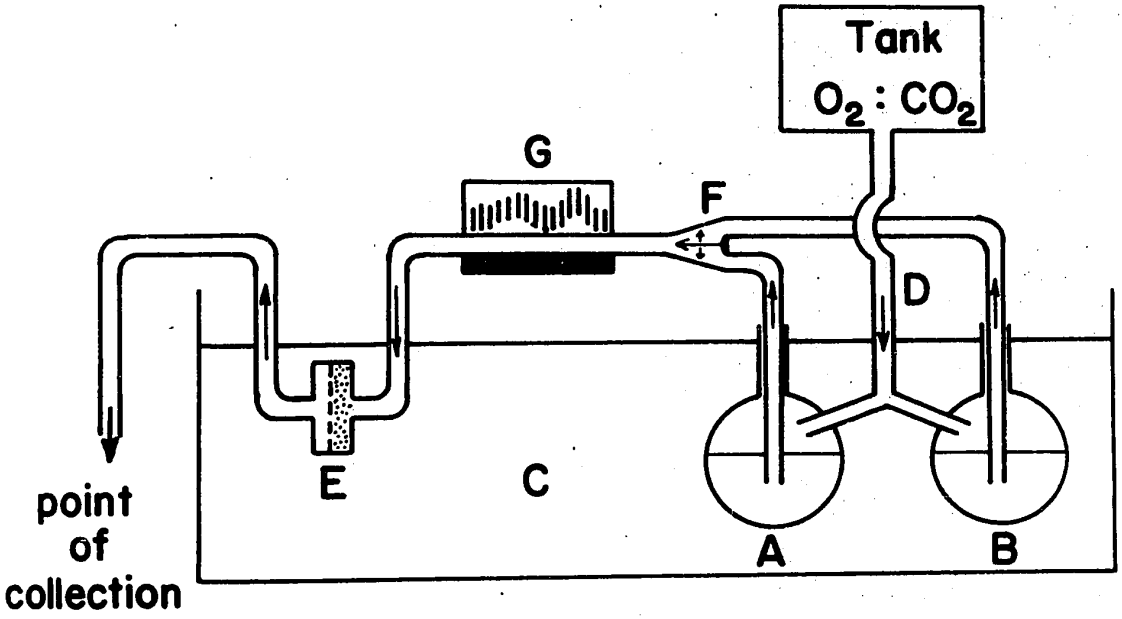

Fig. 6-1. The perifusion apparatus, where the control (A) and experimental (B) media used for perifusion were maintained at 37 °C in a water bath (C), and continuously mixed with an incoming mixture (D) of oxygen (95 %) and carbon dioxide (5 %). The reservoirs (A & B) were connected to a small perifusion chamber (E) through a Y-shaped valve (F) allowing rapid switching from one medium to the other. The perifusate was delivered at a constant rate by means of a finger pump (G).

perifusate was delivered at a constant rate (0.83 to 0.88 ml/min) by means of a finger pump (G ; Sigmamotor pump, model T-8SH, Sigmamotor Inc., Middleport, N.Y.).

4.2 Insulin measurements

For measurement of insulin release, a group of 200 isolated islets were placed in the chamber and perifused at 37 °C for two hours. The control and experimental media contained glucose, respectively at a concentration of 0.3 and 3.0 mg/ml. The effluent was continuously collected over periods of 1 to 4 min each, its insulin content being assayed from 0.5 ml aliquot by the method of Wright et al. (1967) previously described. The results are expressed in Fig. 6-2 as μU of secreted insulin / islet per minute.

4.3 ^{45}calcium measurements

For measurement of ^{45}calcium efflux, groups of 200 islets each were preincubated for 60 minutes at 37 °C in media (1.0 ml) containing glucose (3.0 mg/ml) and ^{45}calcium (50 μC/ml ; $^{45}CaCl_2$). After preincubation, the islets were submitted to repeated washes (X5) in order to remove extracellular ^{45}calcium, transferred to the perifusion chamber, and perifused at 37 °C for 60 to 70 minutes. During the first 45 minutes, the perifusate came from the "control" reservoir. At the 45th min, the perifusate was derived from the "experimental" reservoir which contained either the same medium as that used during the first period (control experiments)

or a modified medium whose effects are to be tested. As required, those media were glucose-rich or glucose-free, and contained normal concentrations (2.0 mEq. per liter) or no calcium chloride. The effluent was continuously collected in counting vials over successive periods of one minute each. To each vial, 10 ml of scintillation fluid (Insta-Gel; Packard Instrument Co. Inc., Downers Grove, Ill.) were added before determining their radioactive content (Nuclear Chicago "Mark II" liquid scintillation counter). In each experiment, ^{45}calcium efflux (cpm/min) was expressed in per cent of the value found at the 45th min of perifusion.

2.3 Results

3.1 Control experiments

4.1 Insulin secretion

Figure 6-2 illustrates the secretory response of the perifused islets to glucose. When the glucose concentration of the perifusate was raised from 0.3 to 3.0 mg/ml, a rapid increase in insulin output was noticed. This early response was followed by a progressive build-up of the secretory rate. After exposure for 30 to 40 minutes to the high glucose concentration, insulin output reached a plateau corresponding to a mean secretory rate of 2 µU / islet per min. This value is of the same order of magnitude as that observed on prolonged incubation of isolated islets (Brisson et al., 1972). A rapid and marked decrease in insulin output occured when the glucose concentration of the perifusate was lowered. This secretory pattern was taken as an indication of the functional

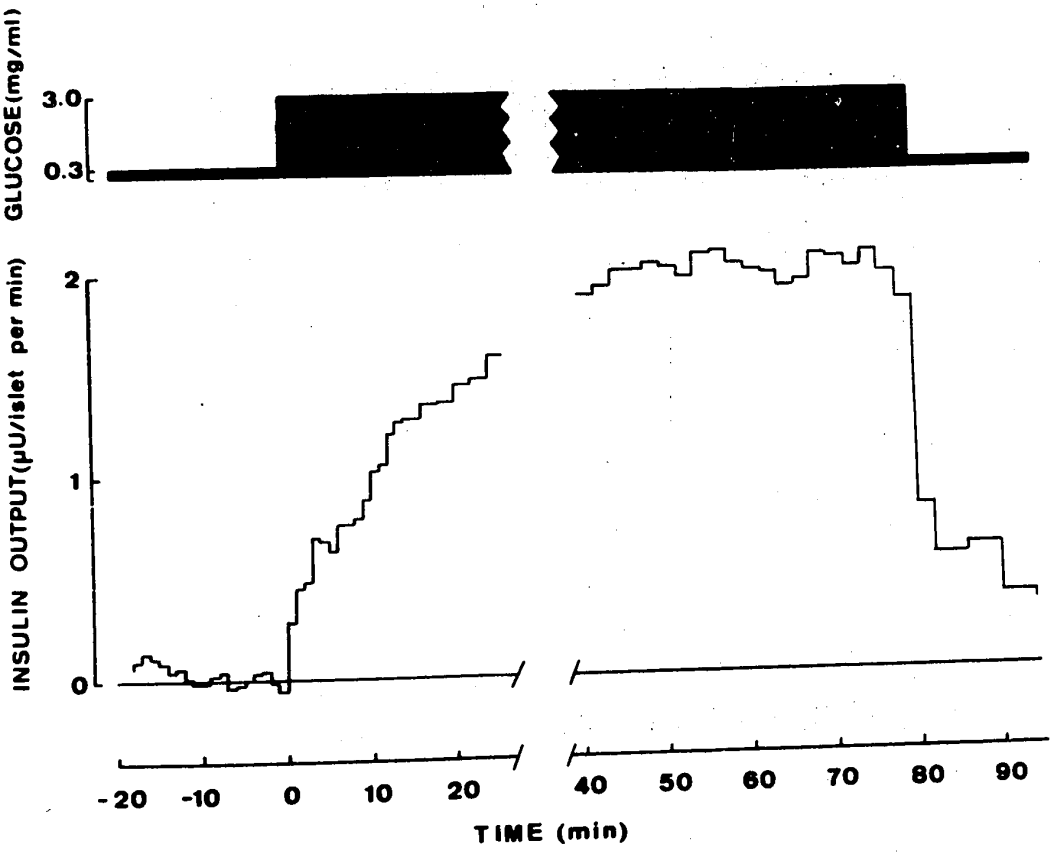

Figure 6-2. Insulin output by perifused islets. Mean values for respectively 6 (left part) and 4 (right part) experiments. In each experiment, a group of 200 islets was placed in the perifusion chamber at -25 min. The glucose concentration of the perifusate is shown in the upper part of the figure.

integrity of the perifused islets.

4.2 Calcium efflux in glucose-free media

5.1 In the presence of a normal calcium concentration

When the islets, preincubated in the presence of ^{45}calcium, were perifused with a solution freed from glucose (Fig. 6-3), the efflux of ^{45}calcium rapidly decreased during the first half-hour of perifusion (data not shown). Thereafter, the decrease in ^{45}calcium efflux occured at a slower rate, the mean value progressively decreasing between the 31st and the 70th minute from 119 ± 3 to 74 ± 1 per cent of the reference value observed at the 45th min.

5.2 In a calcium-free, EGTA-treated medium

Figure 6-4 illustrates the ^{45}calcium efflux when prelabeled islets were perifused in a glucose-free medium from which no calcium chloride had been added and to which EGTA (2.0 mM) had been supplemented. Taking the same reference point (45th min) one observed a moderate change in the rate of ^{45}calcium efflux, being 148 ± 12 at the 31st min and 98 ± 4 at the 70th min.

3.2 The effect of glucose

At normal calcium concentration and when glucose (3.0 mg/ml) was added to the perifusate, a biphasic change in ^{45}calcium efflux was observed (Fig. 6-5). The efflux first fell. In individual experiments, the lowest value was recor-

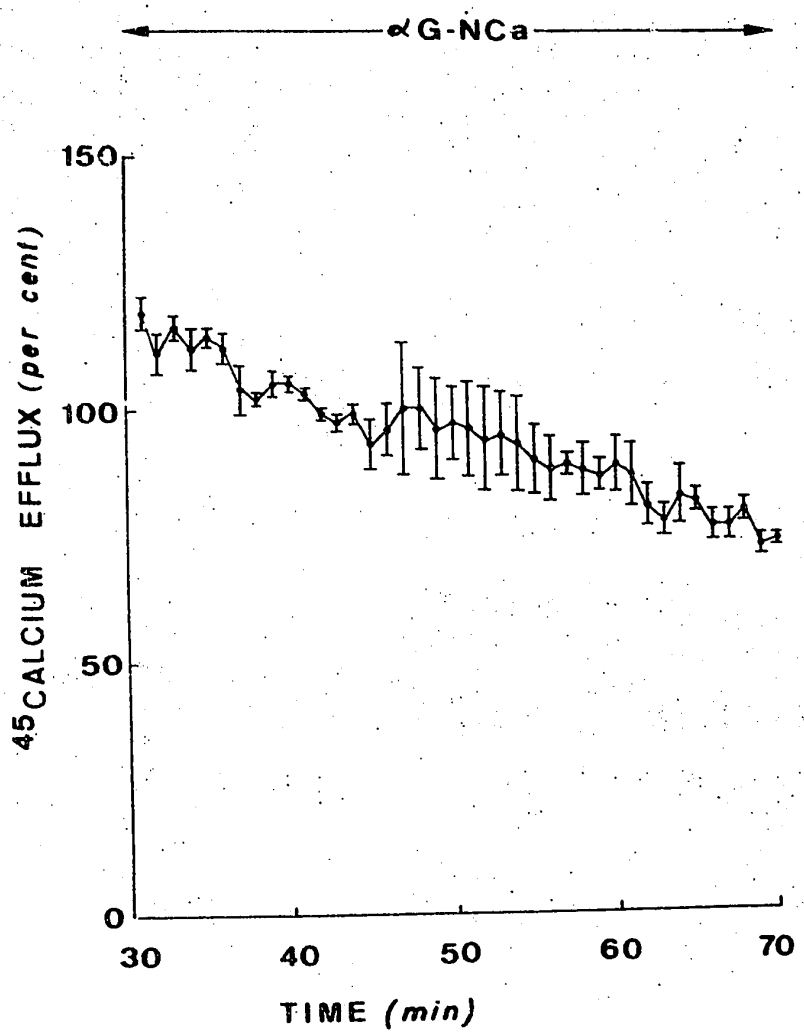

Fig. 6-3. Efflux of ^{45}calcium in the absence of glucose (αG) and at a normal Ca^{++} concentration (NCa). Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

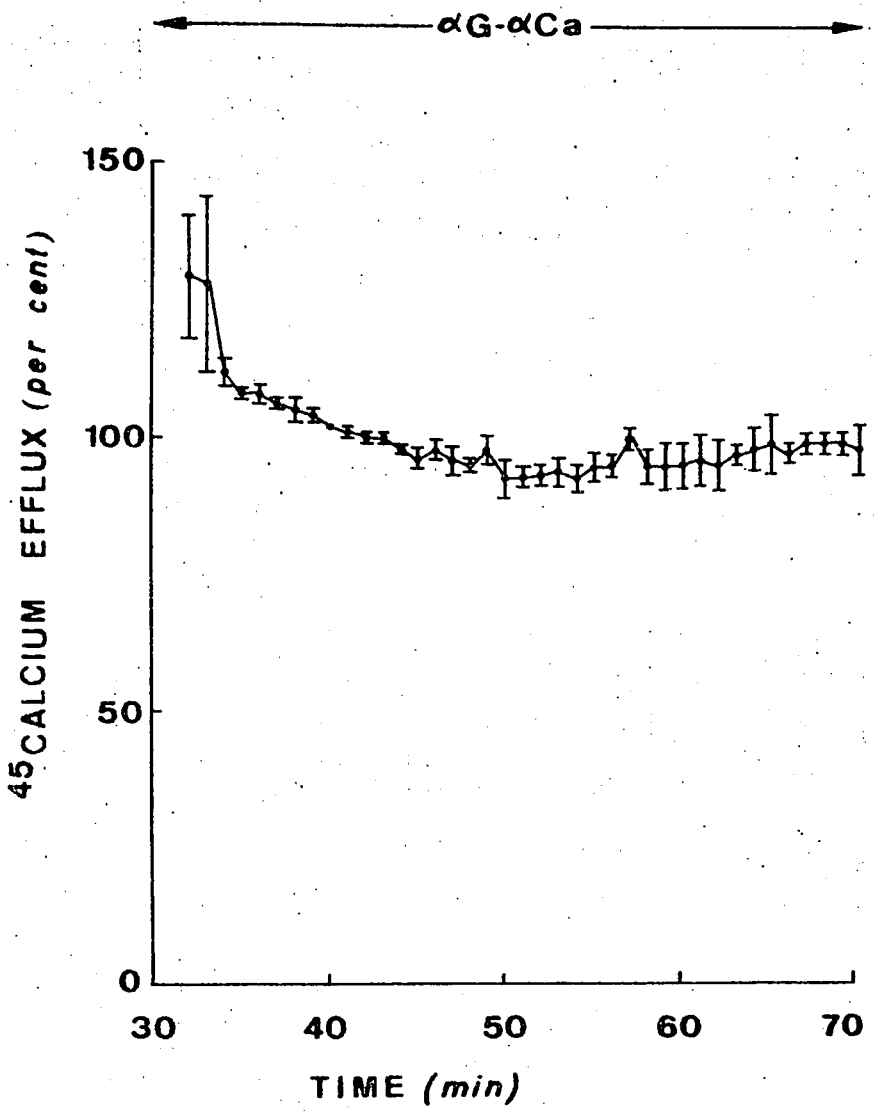

Fig. 6-4. Efflux of ^{45}calcium in the absence of glucose (αG) and in Ca^{++}-free medium (αCa). Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

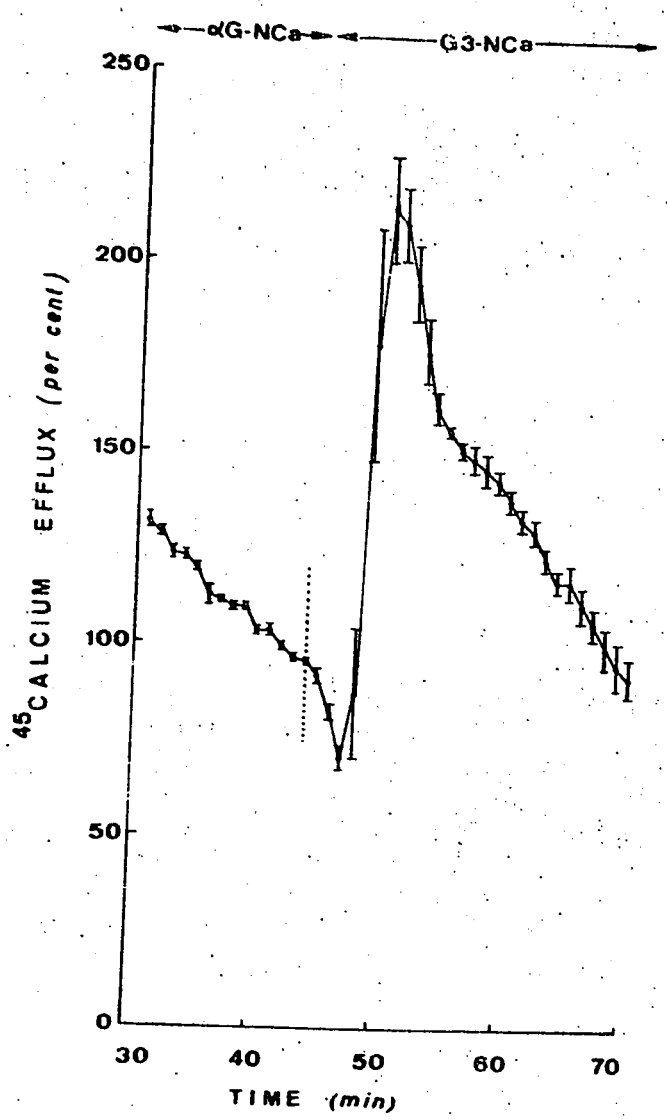

Fig. 6-5. Effect of a glucose enrichment of the perifusate (from no glucose - αG - to glucose 300 mg/100 ml - G3) on ^{45}calcium efflux in a medium of normal Ca^{++} concentration. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

ded at the 3rd or 4th min after the switch and averaged 68 ± 4 %, as distinct from 91 ± 5 % in the control experiments ($P < 0.02$; Fig. 6-3). This initial fall was followed by a dramatic increase. In individual experiments, the highest value was observed either 5 or 6 min after the switch and averaged 231 ± 9 %.

In view of these results, it was considered that the initial fall might correspond to an inhibitory effect of glucose on calcium efflux from the beta-cell. By inhibiting the outward transport of calcium across the cell membrane, glucose might increase the level of calcium in the cytosol up to a critical value which in turn might trigger insulin release. The secondary rise in the amount of ^{45}calcium leaving the islets might correspond to a release of ^{45}calcium associated with the release of insulin. Further experiments were designed to test these hypotheses.

3.3 The effect of glucose in the absence of insulin release

Glucose does not stimulate insulin release in the absence of extracellular calcium (see Chapter 1.5 & 1.7). As shown in Fig. 6-6, when glucose was added to a perifusate containing no calcium, the immediate fall in ^{45}calcium efflux was noticed, but no secondary rise occured. Four minutes after the addition of glucose, the efflux of ^{45}calcium averaged 67 ± 4 %, as distinct from 95 ± 1 % in the control experiments ($P < 0.001$; Fig. 6-4). This finding is compatible with the

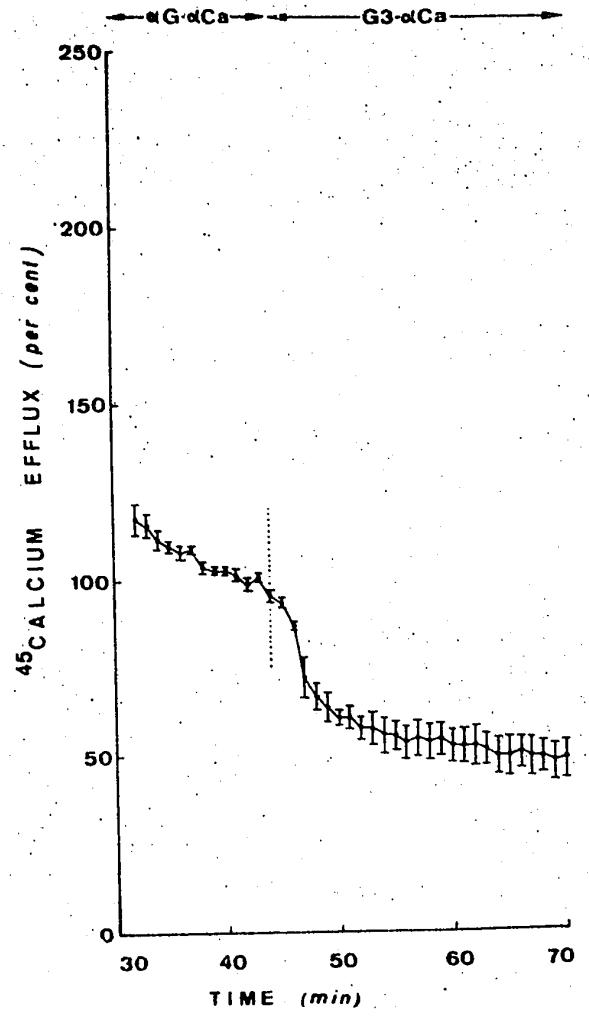

Fig. 6-6. Effect of a glucose enrichment of the perifusate (from no glucose - αG - to glucose 300 mg/100 ml - G3) on ^{45}calcium efflux in a Ca^{++} free medium. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

hypotheses that (i) glucose inhibits the outward transport of calcium across the cell membrane; and (ii) the secondary elevation in ^{45}calcium release observed at normal calcium concentration is concomittant to insulin release. However, it is also plausible that different pools of calcium are mobilized by glucose respectively in the presence and absence of extracellular calcium. In order to rule out the latter possibility, we decided to investigate the effect of glucose on ^{45}calcium efflux at normal calcium concentration but in the absence of insulin release.

3.4 The influence of D_2O

Most agents known to suppress glucose-induced insulin release apparently do so by reducing the net uptake of calcium by the beta-cell (Malaisse-Lagae and Malaisse, 1971a). Therefore, they cannot be used as inhibitors of insulin release in experiments designed to study the unaltered effect of glucose on calcium movement in isolated islets.

To our knowledge, the only agents which are able to inhibit glucose-induced insulin release without affecting the net uptake of calcium by the beta-cell are microtubules-stabilizers and mitotic spindle-inhibitors (Malaisse et al., 1971b). For instance, D_2O is known to inhibit the insulinotropic action of glucose, leucine and sulfonylureas (Malaisse et al., 1971b, 1971d; Malaisse-Lagae et al., 1971b), the degree of inhibition expressed in per cent being almost identical to the relative concentration of D_2O also expressed in

per cent. However, D_2O fails to affect the stimulant action of either glucose or sulfonylureas on ^{45}calcium accumulation in the beta-cell (Malaisse et al., 1971b, 1971d). These results have been taken as an indication that D_2O, a microtubules-stabilizer, renders the microtubular system unresponsive to the triggering action of calcium.

Heavy water thus represents a unique opportunity to study the unaltered effect of glucose on ^{45}calcium efflux at physiological calcium concentration and in the absence of insulin release. As shown in Fig. 6-7, in the presence of D_2O and at normal calcium concentration, glucose provoked a rapid and marked fall in ^{45}calcium efflux. Within 6 minutes, the efflux averaged 66 ± 7 % as distinct from 97 ± 7 % in the control experiments (P = 0.025 ; Fig. 6-3). Almost identical results were obtained when the effect of glucose was tested in the presence of D_2O and absence of calcium (Malaisse, personal communication). These findings indicate that (i) the major effect of glucose is to inhibit ^{45}calcium efflux; (ii) a comparable inhibition is observed in the presence and absence of extracellular calcium; and (iii) no secondary peak of released ^{45}calcium occurs when insulin secretion is abolished.

3.5 The influence of barium

Barium is able to restore the insulinotropic action of glucose in calcium-depleted media (see Chapter 1.5). Therefore, we decided to investigate the effect of glucose on

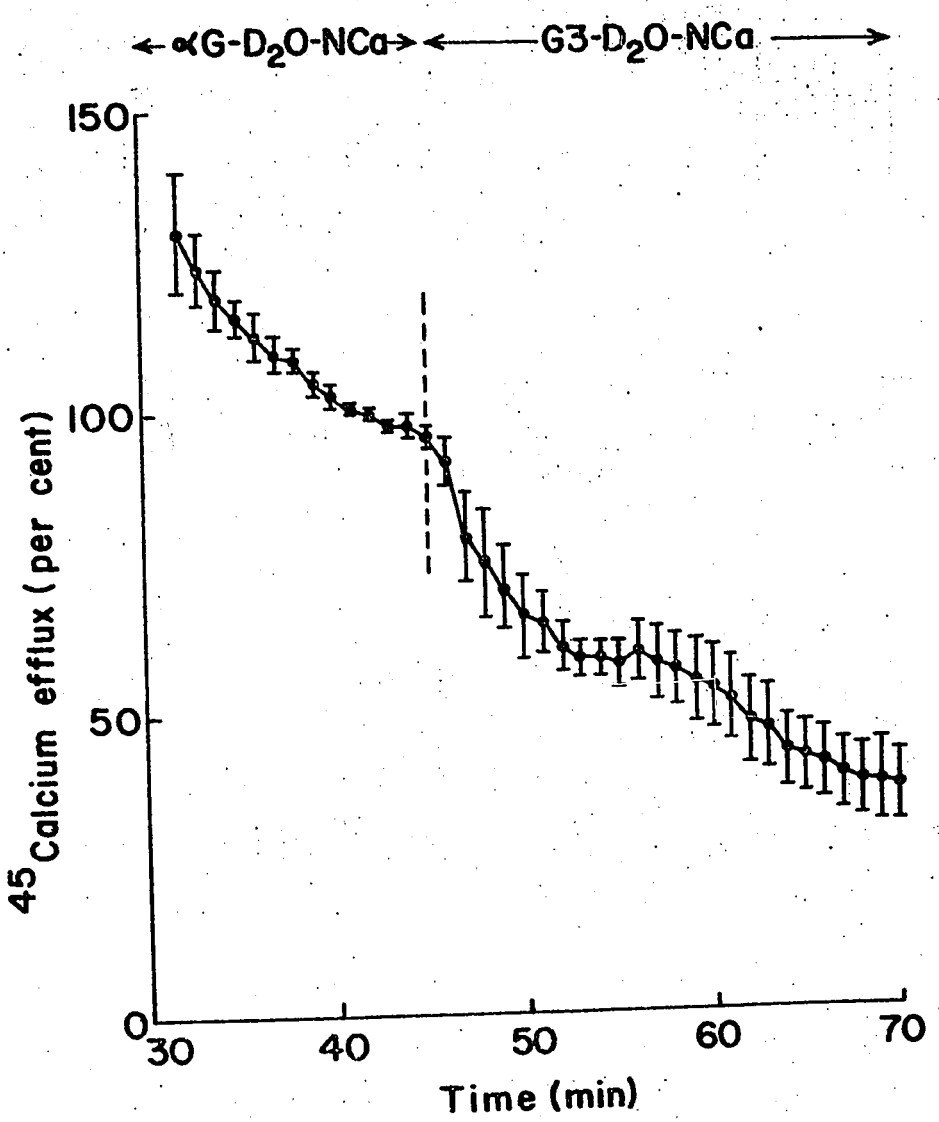

Fig. 6-7. Effect of a glucose enrichment of the perifusate (from no glucose - αG - to glucose 300 mg/100 ml - G3) on ^{45}calcium efflux in a normocalcique medium where H_2O had been replaced by D_2O. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

^{45}calcium efflux in the presence of barium and absence of calcium. In a preliminary series of experiments, it was found that the addition of barium to calcium-depleted media provoked a marked increase in ^{45}calcium efflux (Fig. 6-8). Such an increase might be due in part to a release of ^{45}calcium associated with insulin release, since barium stimulates insulin secretion even at low glucose concentration (Hales and Milner, 1968b). It is also likely that the increase in ^{45}calcium efflux is due in part to a displacement of ^{45}calcium by barium from some cellular site(s).

When the islets had been exposed for 44 min to such a medium containing barium and no calcium, the addition of glucose provoked a significant fall in ^{45}calcium efflux. For instance, at the 70th min, ^{45}calcium efflux averaged 39 ± 3 % as distinct from 74 ± 1 % in the control experiments carried out at normal calcium concentration ($P < 0.001$; Fig. 6-3). When the data obtained in the presence of barium (Fig. 6-9) were compared to those obtained either in the absence of calcium (Fig. 6-6) or in the presence of D_2O (Fig. 6-7), it became apparent that, in the presence of barium, the glucose-induced decrease in ^{45}calcium efflux was partially and transiently masked by a minor secondary elevation. This comparison is illustrated in Table 6-II. At the 50th min of perifusion (i.e. at a time corresponding to the peak value in Fig. 6-5), the mean level of ^{45}calcium was significantly higher ($P < 0.05$ or less) in the presence of barium (Fig. 6-9) than in the absence of calcium (Fig. 6-6) or presence

Table 6-II

Effect of glucose on ^{45}calcium efflux

Minute	G3-αCa	G3-NCa-D_2O	G3-noCa-Ba
46	87 ± 1	92 ± 4	87 ± 2
48	67 ± 4	75 ± 8	90 ± 3
50	61 ± 2	66 ± 7	79 ± 2
70	49 ± 5	37 ± 6	39 ± 3

Mean values (± SEM) for ^{45}calcium efflux at various time (min) are expressed in per cent (see Materials and Methods) and refer to 4 individual experiments.

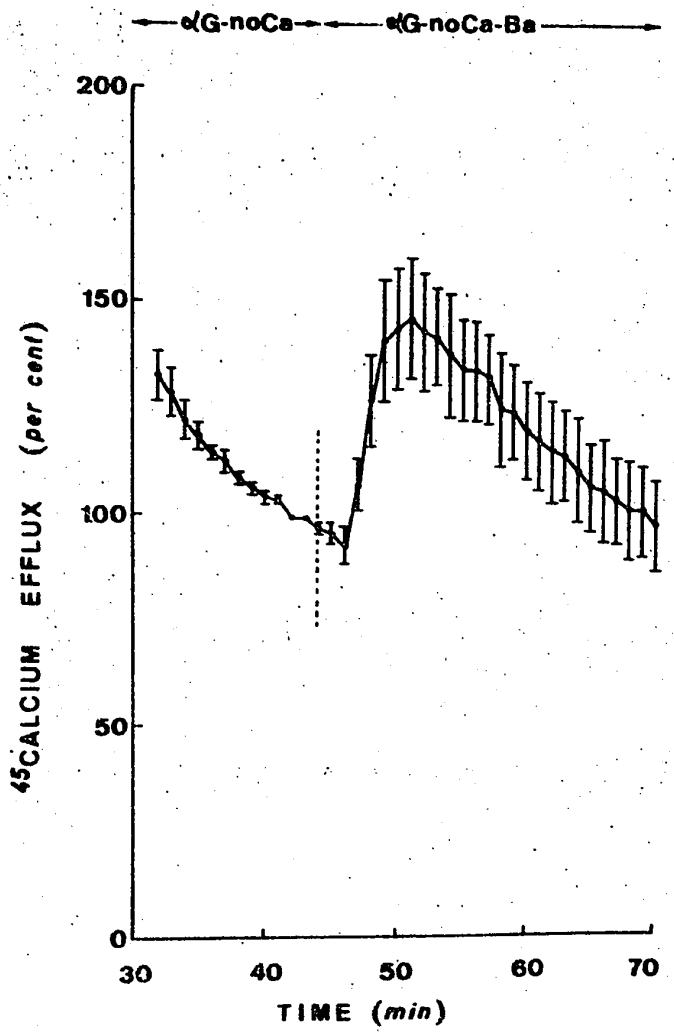

Fig. 6-8. Effect of Barium (Ba) on ^{45}calcium efflux in a glucose-free, calcium-depleted medium. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

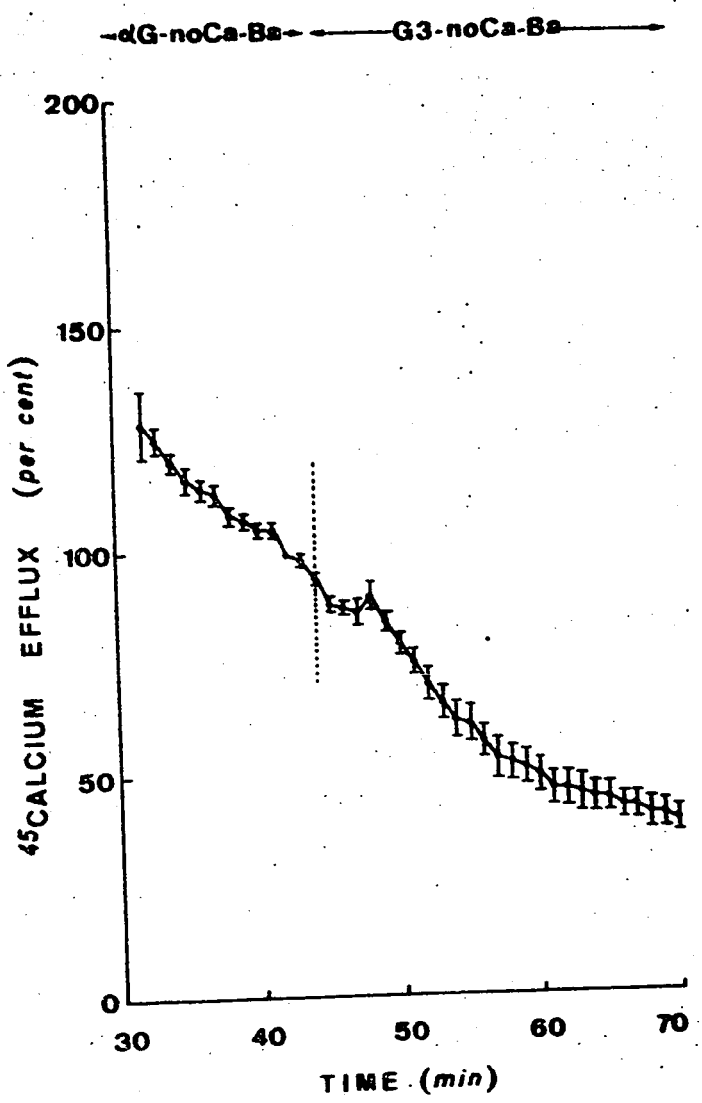

Fig. 6-9. Effect of a glucose enrichment (from glucose 0 - αG - to glucose 300 mg/100 ml - G3) of the perifusate on ^{45}calcium efflux in the presence of barium (Ba). Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

of D_2O (Fig. 6-7). In contrast, no significant difference in calcium efflux could be detected, under these various experimental conditions, either 2 min after the addition of glucose (46th min) or at a much later time (70th min).

These data once again indicate that glucose significantly reduces calcium efflux. They also suggest (i) that the calcium which is released simultaneously with insulin corresponds to a pool of calcium which can be partially displaced by barium; and (ii) that barium, by restoring insulin release, tends also to partially restore the secondary peak of ^{45}calcium release.

3.6 The effect of glucose suppression

Fig. 6-10 and Fig. 6-11 illustrate the smooth curves obtained in control experiments performed at high glucose concentration (3.0 mg/ml) respectively in the presence and absence of calcium. In the presence of calcium, a sharp decrease in ^{45}calcium release was observed a few minutes after the removal of glucose (Fig. 6-12). In view of the above-mentioned hypothesis (see Results, section 3.2), this fall could be due to a suppression of both insulin release (see Chapter 1.7; Brisson et al., 1972) and associated ^{45}calcium release.

In the absence of calcium, namely under a condition known to suppress glucose-induced insulin secretion, the omission of glucose provoked an immediate and sustained increase in ^{45}calcium efflux. The difference between experimental (Fig.

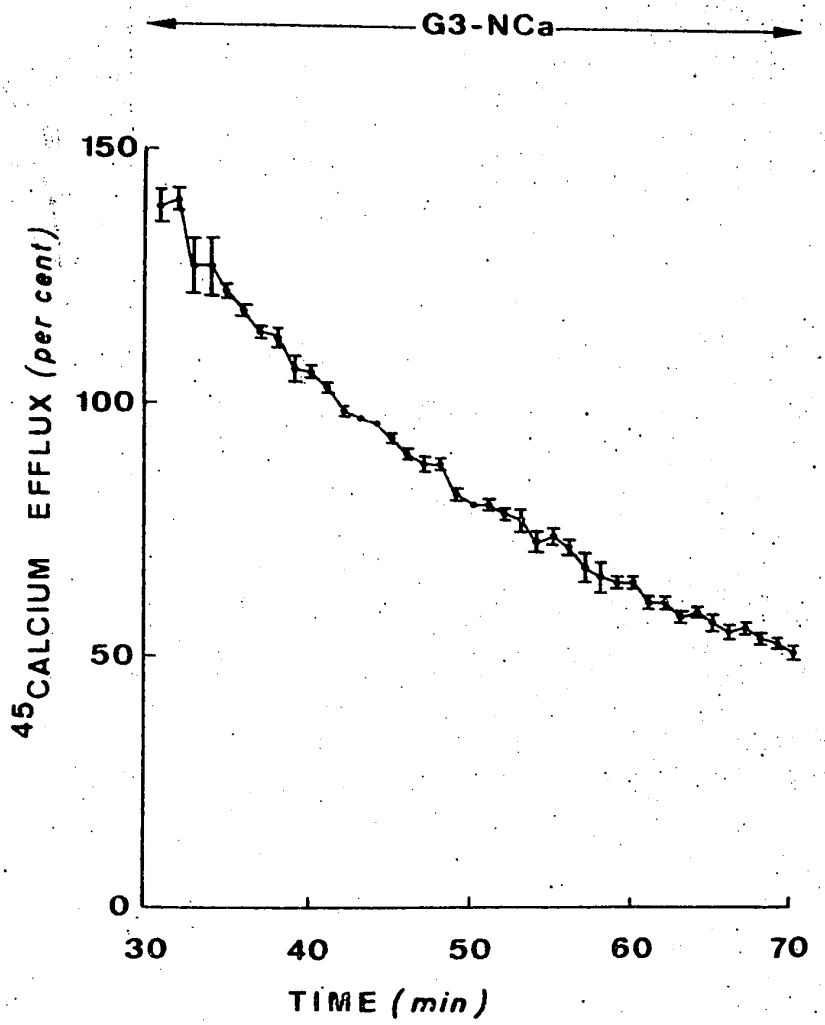

Fig. 6-10. Efflux of ^{45}calcium in the presence of glucose 300 mg/100 ml (G3) at a normal calcium concentration. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

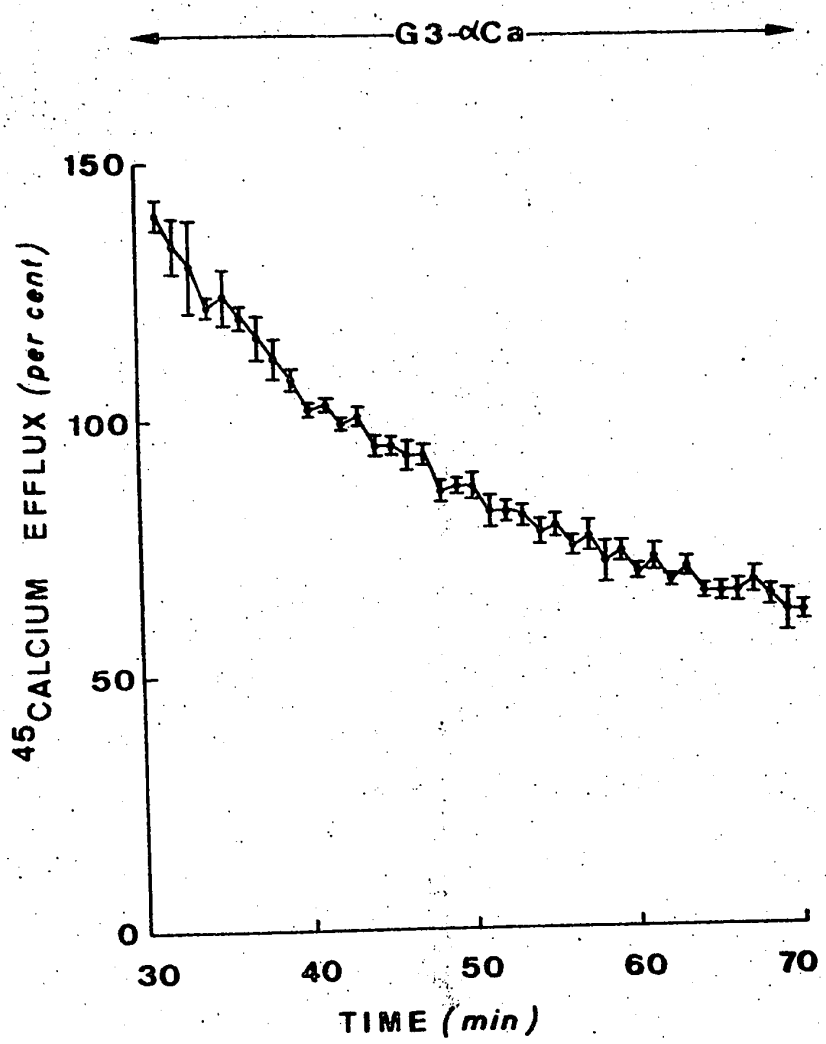

Fig. 6-11. Efflux of ^{45}calcium in the presence of glucose 300 mg/100 ml (G3) in a Ca^{++}-free medium. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

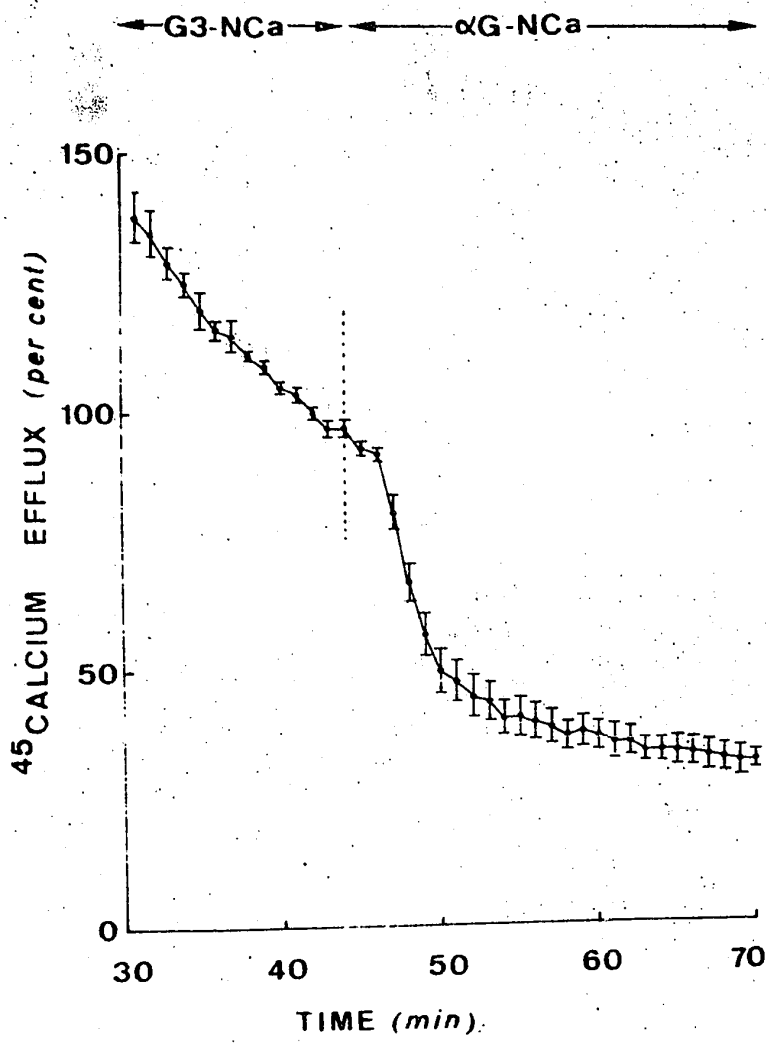

Fig. 6-12. Effect of glucose deprivation (from glucose 300 mg/100 ml - G3 to glucose 0 - α G) on ^{45}calcium efflux in a medium of normal calcium concentration. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

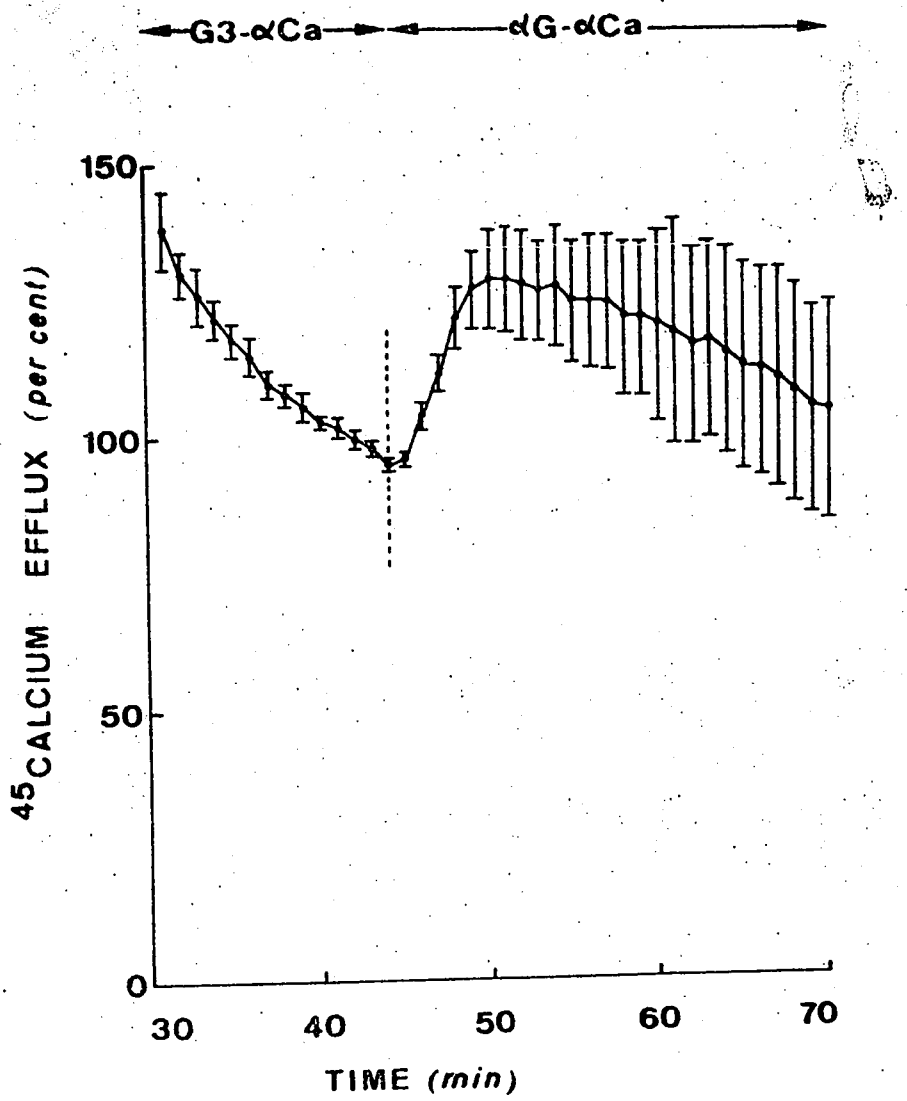

Fig. 6-13. Effect of a glucose deprivation (from glucose 300 mg/100 ml - G3 to glucose 0 - α G) on ^{45}calcium efflux in a Ca^{++}-free medium. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

6-13) and control values (Fig. 6-11) was already significant at the end of the 47th min, i.e. within 2 minutes after the switch. This finding suggest that, when glucose is removed from the perifusion medium, there is an immediate increase in the outward transport of calcium across the cell membrane. In other words, the inhibitory effect of glucose on calcium efflux from the beta-cell is rapidly reversible.

As suggested by Hellman and his colleagues (1969a) epinephrine inhibits insulin release by suppressing the utilization of glucose in the beta-cell. Since such an effect could correspond to a glucose deprivation of the islet cells, it was decided to examine whether or not the calcium efflux from the beta-cell when perifused with epinephrine would parallel the efflux previously observed upon glucose deprivation.

3.7 The influence of epinephrine

When a glucose-rich (3.0 mg/ml) otherwise normal perifusion solution was suddenly enriched with epinephrine (10 µM ; Fig. 6-14), the islet impregnation induced a ^{45}calcium efflux rate similar to the one observed upon glucose withdrawal from a similarly normal perifusate (Fig. 6-12). Indeed, a marked and rapid fall in the ^{45}calcium efflux was induced which was not significantly different than the one observed upon glucose suppression (48 ± 4 % vs 55 ± 4 % five minutes after the switch). On the other hand, when repeated in calcium-depleted perifusates, epinephrine (10 µM ;

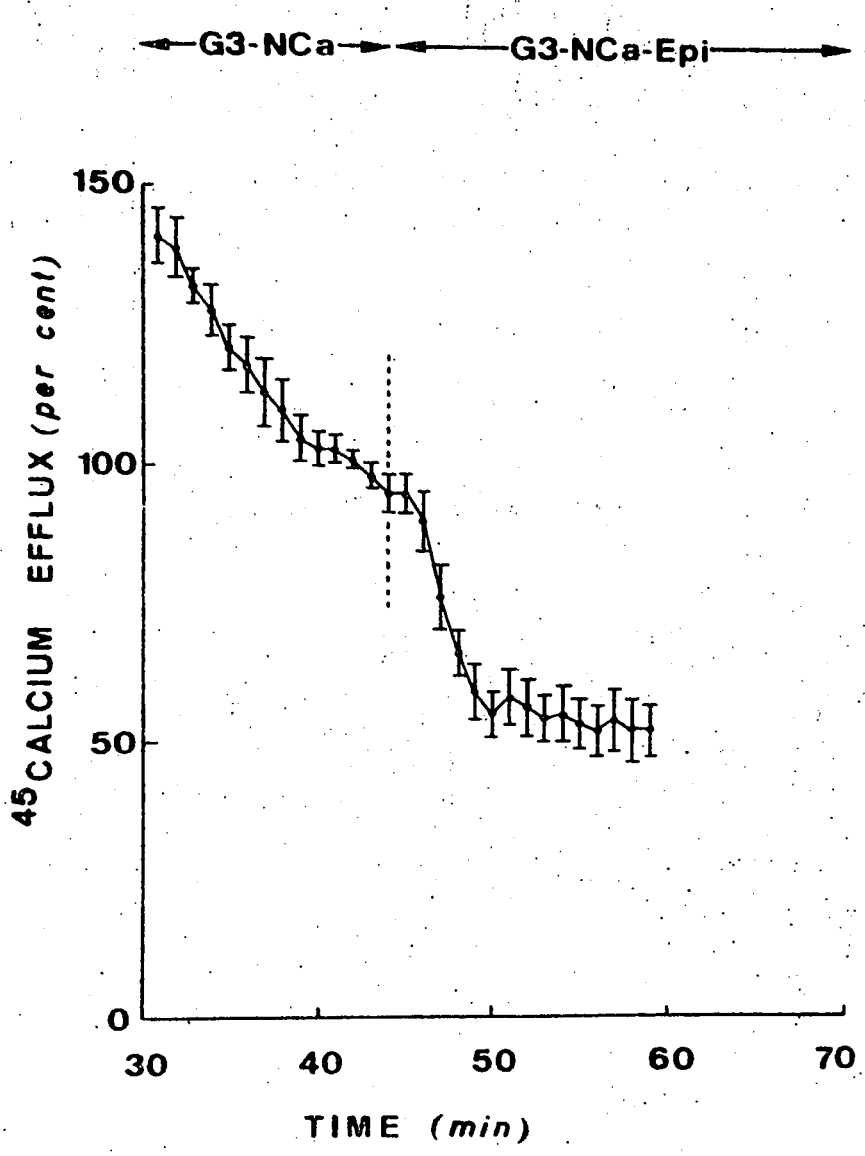

Fig. 6-14. Effect of epinephrine (Epi) on ^{45}calcium efflux in the presence of glucose 300 mg/100 ml (G3). Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

Fig. 6-15) did not induce ^{45}calcium efflux changes parallel to the ones observed upon glucose withdrawal under similar ionic conditions (Fig. 6-13). Instead of a marked increase followed by a sustained ^{45}calcium efflux rate, epinephrine was capable to provoke only a transient increase which was resumed rapidly to assume a rate similar to the ones observed with control experiments (Fig. 6-11).

2.4 Discussion

The aim of the present study was to investigate the effect of glucose and epinephrine on ^{45}calcium efflux from the beta-cell. The interpretation of these results relied on a number of assumptions. Firstly, it was assumed that the modifications in ^{45}calcium efflux were representative of a pure beta-cell population. Secondly, it was postulated that the ^{45}calcium which appeared in the perifusate corresponded to either calcium transported across the intact membrane of the beta-cell from the cytosol into the interstitial fluid, or calcium released together with insulin at the time and site of emiocytosis. And thirdly, it was assumed that the outward transport of ^{45}calcium was indicative of the movement of unlabelled calcium.

The first of these assumptions appeared to be reasonable since islets of Langerhans are composed predominantly of beta-cells.

The second assumption was based mainly on the biphasic pattern of ^{45}calcium release evoked by glucose at normal cal-

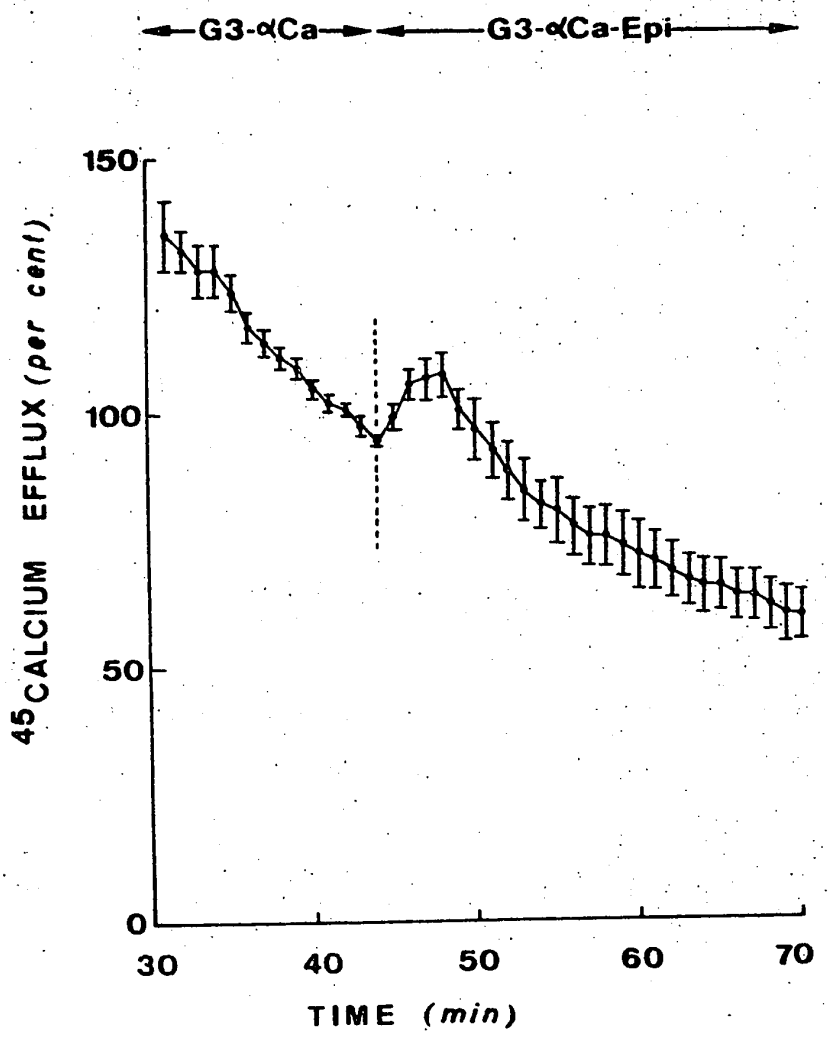

Fig. 6-15. Effect of epinephrine (Epi) on ^{45}calcium efflux in the presence of glucose 300 mg/100 ml (G3) in a Ca^{++}-free medium. Mean values (±SEM) are expressed in per cent of the value found at the 45th minute of perifusion and refer to 4 individual experiments.

cium concentration (Fig. 6-5). A secondary peak of ^{45}calcium release was only seen under conditions known to preserve the insulinotropic action of glucose, namely at normal calcium concentration or in the presence of barium. This secondary peak was quite obvious at normal calcium concentration (Fig. 6-5) and much more modest when calcium had been replaced by barium as the main alkaline earth cation (Fig. 6-9). No secondary peak was observed under conditions known to abolish the insulinotropic action of glucose, i.e. in the absence of calcium (Fig. 6-6) or in the presence of D_2O (Fig. 6-7) which suppresses insulin secretion by over-stabilizing the microtubular system of the beta-cell (Malaisse et al., 1971b; Malaisse-Lagae et al., 1971b, 1971d).

Recently, Malaisse (personal communication) has recovered, from islets incubated with ^{45}calcium, significant amounts of ^{45}calcium associated with the subcellular fraction said to contain most of the secretory granules. The presence of calcium in secretory granules has been recognized in other secretory cells (Borowitz et al., 1965; Schumann, 1966; Wallach and Schramm, 1971). Ultrastructural studies also suggest the presence of calcium in the insulin secretory granules of the beta-cell (Orci, unpublished observations). It seemed therefore reasonable to assume that the secondary peak corresponded to a release of ^{45}calcium associated with or concomittant to the release of insulin. For instance, it might have corresponded to ^{45}calcium enclosed within the membranous sacs of secretory granules. Other possibilities could

have been considered. Whatever its nature, this particular pool of ^{45}calcium could apparently be partially displaced by barium.

When no secretion of insulin could occur, the ^{45}calcium which appeared in the perifusate was thought to represent cations transported across the cell membrane from the cytosol into the interstitial fluid. Glucose apparently inhibited this outward transport of calcium (Fig. 6-6; Fig.6-7). The inhibitory effect of glucose was rapidly reversible (Fig. 6-12).

It is important to notice that the pattern of glucose-induced inhibition of ^{45}calcium efflux was almost superimposable at normal calcium concentration (Fig.6-7) and in calcium-depleted media (Fig. 6-6). This finding suggested that the glucose-induced change in ^{45}calcium efflux was not dependent on the presence of extracellular calcium since it was still observed after a prolonged exposure (45 min) of the islets to media enriched with EGTA (Fig. 6-6). It is likely, therefore, that the glucose-induced change in ^{45}calcium efflux indeed corresponded to an inhibition of the outward transport of calcium (third assumption) rather than to a sudden decrease in the specific activity of the calcium actually transported from the cytosol into the interstitial fluid. These data, however, did not rule out the possibility for glucose to also increase the influx of calcium in the beta-cell (Sehlin et al., 1972).

As suggested by Fig. 6-15, epinephrine mode of inhibition

differed from the one induced by glucose depletions. It seemed that epinephrine could affect the sequestering calcium ability of the beta-cell protoplasm. Indeed, it appeared that epinephrine would deplete the intracellular calcium pool by stabilizing or translocating the calcium ions in a manner that would be the opposite of the effects of cAMP and which are discussed in the next chapter. Evidences for such a dissociation between glucose and epinephrine had previously been obtained when we observed, in absence of substrate, a marked epinephrine-induced inhibition upon potassium-stimulated insulin release (see chapter 1.4, Fig. 4-4).

1.7 A proposed role for the adenyl cyclase-phosphodiesterase system in the beta-cell

2.1 Introduction

It was recently postulated that glucose, when metabolized in the beta-cell, stimulates calcium uptake; and that calcium in turn triggers the release of insulin by activating a microtubular-microfilamentous system thought to control the migration of secretory granules and their expulsion from the cell in the process of emiocytosis (Malaisse and Malaisse-Lagae, 1970c; Lacy, 1970). Thus, glucose-induced insulin release is dependent on at least 3 factors, namely the integrity of glucose metabolism in the beta-cell, the presence of a sufficient amount of extracellular calcium, and the functional integrity of the microtubular system. Other insulinotropic agents, such as amino acids and sulfonylureas, also stimulate calcium uptake by the beta-cell and also require an intact microtubular system in order to promote insulin release (Malaisse-Lagae et al., 1971b; Malaisse et al., 1971d).

The initial aim of the present study was to extend these observations to another class of stimulatory agents, which are thought to exert their insulinotropic action by increasing the level of adenosine-3',5'-cyclic monophosphate (cAMP) in the beta-cell. Within the framework of the above-mentioned model, it was considered that the primary site of action of

cAMP could correspond to either a stimulation of glucose metabolism, a modification of calcium transport and distribution in the beta-cell, or an effect on the microtubular system. These three hypotheses are here explored.

2.2 Materials and methods

3.1 Pancreatic tissue and isolated islets

All experiments were performed with either small pieces of pancreatic tissue (approximately 8 to 10 mg each) or isolated islets of Langerhans removed from fed female albino rats (150 - 200 g; Rega, Heverlee, Belgium). The method for both procedures was described in previous chapters.

3.2 Incubation and perifusion media

All incubations and perifusions were carried out at 37°C in a bicarbonate-buffered medium containing albumin (0.5 per cent, w/v; Fraction V, Sigma Chemical Co., St.Louis, Mo.), and equilibrated against a mixture of O_2 (95 %) and CO_2 (5 %). This medium has the following ionic composition: Na^+, 139; K^+, 5; Ca^{++}, 2; Mg^{++}, 2; Cl^-, 124; and HCO_3^-, 24 mEq. per liter. A series of experiments was performed with media into which no $CaCl_2$ had been incorporated. Also added to the medium, as required, were glucose, galactose, leucine and pyruvate (E. Merck A.G., Darmstadt, Germany); theophylline; db-cAMP (N^6-2'O-dibutyryl-adenosin-3',5'-monophosphate, cyclisch; Boehringer, Mannheim, Germany); vincristine sulfate (Oncovin; Eli Lilly, Indianapolis, Ind.); colchicine (Sigma Chemical Co., St.Louis, Mo.); diazoxide (Schering, Berlin,

Germany); epinephrine (Adrénaline gauche; Rhône-Poulenc, Paris, France); d-mannoheptulose (Schering, Berlin, Germany); 2,4-dinitrophenol (E.Merck A.G., Darmstadt, Germany); and tetracaine hydrochloride (Winthrop, New York, N.Y.).

3.3 Insulin secretion

Insulin release by pancreatic tissue was measured on incubation of small pieces of pancreas, in groups of 4 pieces each, in media (2.0 ml) containing guinea-pig anti-insulin serum (kindly donated by Dr. P.H. Wright, Indiana University, Indianapolis). The rate of insulin release was deduced from the partial neutralization of the antibodies, and calculated as μU secreted insulin per mg of tissue per 90 min incubation (Malaisse et al., 1967b).

Isolated islets were incubated in groups of 8 islets each in media (1.0 ml) containing no anti-insulin serum. The insulin content of these media was assayed by a method described in a previous chapter and detailed elsewhere (Wright et al., 1967), rates of secretion being expressed as μU secreted insulin per islet per 90 min incubation.

3.4 Calcium uptake

The method used for measurement of ^{45}calcium uptake by isolated islets has been described in detail elsewhere (Malaisse-Lagae et al., 1971a). Briefly, in each experiment, 2 groups of 150 to 200 islets each were incubated for 90 min in media (1.0 ml) containing $^{45}CaCl_2$ (12.5 μC/ml; The Radiochemical Center, London, England). After incubation, the

islets were submitted to repeated washes in order to remove extracellular ^{45}calcium, and transferred in groups of 5 islets each in counting vials for measurement of their radioactive content by liquid scintillation. Calcium uptake was expressed either as pg/islet per 90 min, or in per cent of the control value found in paired group of islets.

3.5 Perifusion of islets

As previously explained (chapter 1.6) the control and experimental media used for perifusion were kept at 37°C and continuously mixed with O_2 (95 %) and CO_2 (5 %). The reservoirs were connected to a small perifusion chamber (volume: 250 µl) through a Y-shaped valve. The perifusate was delivered at a constant rate (0.83 to 0.88 ml/min) by mean of a finger pump (Fig. 6-1 of the previous chapter).

For measurement of ^{45}calcium efflux, groups of 200 islets each were preincubated for 60 min at 37°C in media (1.0 ml) containing glucose (16.7 mM) and ^{45}calcium (50 µC/ml). After preincubation, the islets were submitted to repeated washes in order to remove extracellular ^{45}calcium, transferred to the perifusion chamber, and perifused at 37°C for 80 min. During the first 45 min, the perifusate was derived from a first reservoir and contained no theophylline. At the 45th minute, the perifusate was derived from a second reservoir which contained either the same medium as that used during the first period (control experiments) or a medium enriched with theophylline (3.2 mM). In these experiments, all media used for perifusion were glucose-free. The effluent was continu-

ously collected in counting vials, over successive periods of 1 min each. To each vial, 10 ml of scintillation fluid (Insta-Gel; Packard Instrument Company, Downers Grove, Ill.) were added. In each experiment, ^{45}calcium efflux (cpm/min) was expressed in per cent of the value found at the 45th min of perifusion.

2.3 Results

3.1 Effects of theophylline and db-cAMP on insulin secretion and calcium uptake by isolated islets

Table 7-I illustrates the effect of glucose, theophylline and db-cAMP on insulin secretion by isolated islets. Insulin release increased as the glucose concentration of the incubation medium was raised from 5.6 to 16.7 mM. Theophylline and db-cAMP failed to affect insulin secretion in the absence of glucose or at low glucose levels, but markedly enhanced glucose-induced insulin release at high glucose concentrations. Table 7-II illustrates the effect of the same agents on calcium uptake by the isolated islets. Glucose stimulated calcium uptake. Both theophylline and db-cAMP inhibited calcium uptake in the absence of glucose; slightly enhanced calcium uptake at a low glucose concentration (5.6 mM); and failed to significantly affect calcium uptake at higher glucose levels. These data, illustrated in Fig. 7-1, clearly indicate that the insulinotropic action of theophylline and db-cAMP observed at high glucose levels cannot be accounted for by an additional increment in the net accumulation of calcium induced by glucose.

Table 7-I

Effect of glucose, theophylline and db-cAMP upon insulin secretion by isolated islets

Glucose (mM)	Control output (i) (μU/islet per 90 min)		Drug-induced change (+ or - μU/islet per 90 min) (ii) Theophylline (1.4 mM)		db-cAMP (0.2 to 0.4 mM)	
Nil	10.8 ± 3.0	(38)	- 1.7 ± 1.4	(8)	+ 3.0 ± 1.4	(10)
1.7	13.1 ± 2.8	(32)	+ 3.2 ± 2.8	(16)		
2.8	13.7 ± 2.2	(26)	- 1.8 ± 2.4	(16)		
4.2	15.5 ± 1.7	(16)	+ 22.5 ± 2.9*	(14)		
5.6	29.7 ± 1.8	(42)	+ 32.8 ± 4.9*	(12)	+ 18.5 ± 5.0*	(16)
8.3	91.1 ± 7.5	(17)	+ 146.5 ± 7.4*	(8)	+120.8 ± 10.5*	(8)
16.7	236.3 ± 6.6	(93)	+ 253.5 ± 14.2*	(13)	+212.4 ± 35.7*	(18)

(i) Mean values (± SEM) for glucose-induced insulin release; number of observations in parentheses.

(ii) Effects of theophylline or db-cAMP are shown as mean difference (± SEM) from control values obtained in equal numbers and within the same experiment(s); also shown are the number of observations (in parentheses) and the statistical significance (* = P < 0.001) of the observed changes

Table 7-II.

Effect of glucose, theophylline and db-cAMP upon ^{45}calcium uptake by isolated islets.

Glucose (mM)	Control uptake (i) (pg/islet per 90 min)	Experimental value (per cent of control) (ii) Theophylline (1.4 mM)	db-cAMP (1.0 mM)
Nil	66 ± 5 (39)	73.5 ± 4.6* (26)	72.5 ± 4.9* (13)
5.6	93 ± 6 (51)	145.5 ± 9.9* (26)	124.8 ± 8.8¶ (25)
8.3	173 ± 8 (58)	107.5 ± 4.3 (26)	103.6 ± 4.4 (22)
16.7	249 ± 12 (62)	96.3 ± 2.8 (32)	103.9 ± 4.4 (26)

(i) Mean absolute values (± SEM) for glucose-induced ^{45}calcium uptake; number of observations in parentheses.

(ii) Experimental values obtained in the simultaneous presence of glucose and either theophylline or db-cAMP are quoted as per cent of their paired control value; also shown are the number of individual observations (in parentheses) and the statistical significance (* = $P < 0.001$; ¶ = $P < 0.01$) of the observed changes.

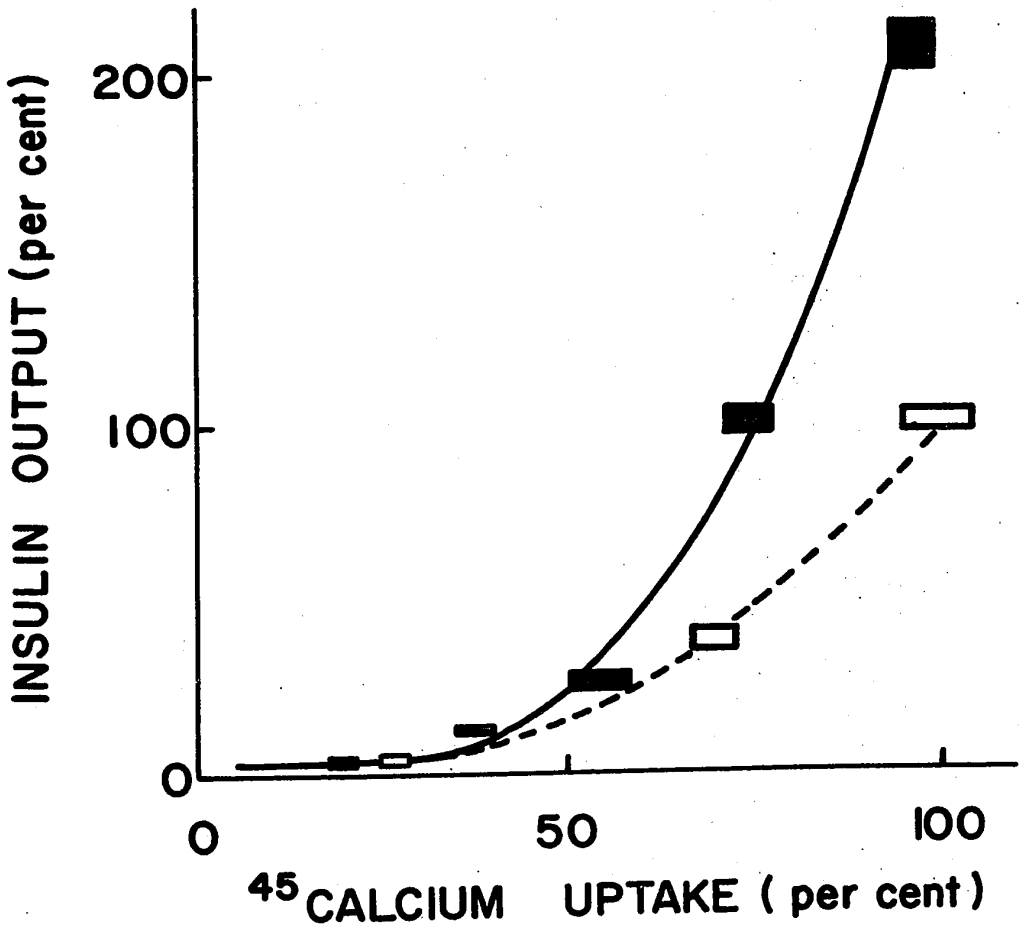

Fig. 7-1. Relationship between ^{45}calcium uptake and insulin output by isolated islets.
Each rectangle represents the mean (± SEM) for ^{45}calcium uptake and insulin output, both parameters being expressed in per cent of the value found at high glucose concentration (16.7 mM). Open rectangles refer to increasing glucose concentrations (nil, 5.6, 8.3 and 16.7 mM) in the absence of theophylline; closed rectangles refer to the same glucose concentrations in the presence of theophylline (1.4 mM).

3.2 Induction of insulin secretion in calcium-depleted media

When no calcium was incorporated into the incubation medium, both glucose and leucine failed to stimulate insulin release from isolated islets of Langerhans (Table 7-III, lines 2 and 3). Theophylline, db-cAMP, or the combination of these two agents also failed to significantly affect insulin release in the calcium- depleted media (Table 7-III, lines 4 to 6). However, in the simultaneous presence of glucose and db-cAMP, a marked stimulation of insulin release was noticed (Table 7-III, line 7). The secretion rate was even higher with the combination of glucose and theophylline (Table 7-III, line 8). The rate of insulin release observed under the latter experimental condition (85 ± 4 µU / islet per 90 min) remained, however, much lower than that induced by glucose alone at a normal calcium level (236 ± 7 µU/islet per 90 min; see Table 7-I). The insulinotropic action of the combination of glucose and theophylline was abolished by mannoheptulose, dinitrophenol, epinephrine, diazoxide, or tetracaine (Table 7-III, lines 9 to 13). Other metabolic substrates than glucose were also tested. A significant enhancement of insulin release was induced by theophylline in the presence of leucine (Table 7-III, line 15 *vs* 3); whereas, neither galactose nor pyruvate in association with theophylline were able to significantly enhance the low rate of insulin release observed in the presence of theophylline alone (Table 7-III, lines 14 and 16 *vs* 5).

Table 7-III.

Insulin secretion by islets of Langerhans incubated in calcium-depleted media

Line N°	Substrate (mM)	Potentiator (mM)	Inhibitor (mM)	Insulin output(i) (µU/islet/90 min)	P (ii)
1				9.8 ± 1.1 (103)	
2	Glucose (16.7)			11.9 ± 1.2 (82)	vs 1 : N.S.
3	Leucine (20.0)			8.5 ± 3.0 (9)	vs 1 : N.S.
4		db-cAMP (1.0)		4.3 ± 1.3 (10)	vs 1 : N.S.
5		theophylline (1.4)		12.5 ± 1.2 (22)	vs 1 : N.S.
6		db-cAMP (1.0) and theophylline (1.4)		8.1 ± 1.2 (10)	vs 1 : N.S.
7	Glucose (16.7)	db-cAMP (1.0)		27.6 ± 2.0 (34)	vs 2 : <0.001
8	Glucose (16.7)	theophylline (1.4)		85.4 ± 3.5 (40)	vs 2 : <0.001
9	Glucose (16.7)	theophylline (1.4)	mannoheptulose (12.0)	6.3 ± 1.2 (10)	vs 8 : <0.001
10	Glucose (16.7)	theophylline (1.4)	dinitrophenol (0.4)	8.6 ± 1.8 (10)	vs 8 : <0.001
11	Glucose (16.7)				

	[illegible]	theophylline (1.4)		85.4 ± 3.5 (40)	vs 2 : <0.001
9	Glucose (16.7)	theophylline (1.4)	mannoheptulose (12.0)	6.3 ± 1.2 (10)	vs 8 : <0.001
10	Glucose (16.7)	theophylline (1.4)	dinitrophenol (0.4)	8.6 ± 1.8 (10)	vs 8 : <0.001
11	Glucose (16.7)	theophylline (1.4)	epinephrine (0.01)	7.3 ± 1.1 (10)	vs 8 : <0.001
12	Glucose (16.7)	theophylline (1.4)	diazoxide (0.43)	2.3 ± 1.4 (10)	vs 8 : <0.001
13	Glucose (16.7)	theophylline (1.4)	tetracaine (0.5)	17.3 ± 1.9 (10)	vs 8 : <0.001
14	Galactose (16.7)	theophylline (1.4)		14.9 ± 2.7 (10)	vs 5 : N.S.
15	Leucine (20.0)	theophylline (1.4)		28.0 ± 2.5 (10)	vs 3 : <0.001
16	Pyruvate (20.0)	theophylline (1.4)		17.7 ± 3.3 (10)	vs 5 : N.S.

(i) All experiments were performed with media into which no calcium had been incorporated; mean values (± SEM) for insulin output are shown together with the number of observations (in parentheses).

(ii) The statistical significance of difference (N.S. = not significant) always refer to a group comparison performed on equal numbers of data obtained within the same experiment(s).

The present experiments thus confirm that glucose and leucine are unable to stimulate insulin release in the absence of sufficient extracellular calcium. They also suggest that db-cAMP or theophylline modify the internal milieu of the beta-cell in such a way that the normal supply of extracellular calcium is no longer necessary for glucose or leucine to stimulate insulin secretion.

3.3 Calcium efflux from perifused islets

In view of the preceding findings, a possible effect of theophylline on the intracellular distribution of calcium was considered. In order to test this hypothesis, we decided to examine the influence of theophylline upon ^{45}calcium efflux from perifused islets.

When the islets preincubated in the presence of ^{45}calcium were perifused with a glucose-free solution, the efflux of ^{45}calcium rapidly decreased during the first half-hour of perifusion (data not shown). Thereafter, the decrease in ^{45}calcium efflux occured at a slower rate, the mean value progressively decreasing between the 32nd and 81st min from 119 ± 3 to 70 ± 3 per cent of the reference value observed at the 45th min (Fig. 7-2). The addition of theophylline to the perifusate provoked a marked and sustained increase in ^{45}calcium efflux. The rise in ^{45}calcium efflux was already detectable within the first minute of exposure to theophylline. At its maximal value, ^{45}calcium efflux in the presence of theophylline averaged twice the control value found in the absence of methylxanthine ($P < 0.03$).

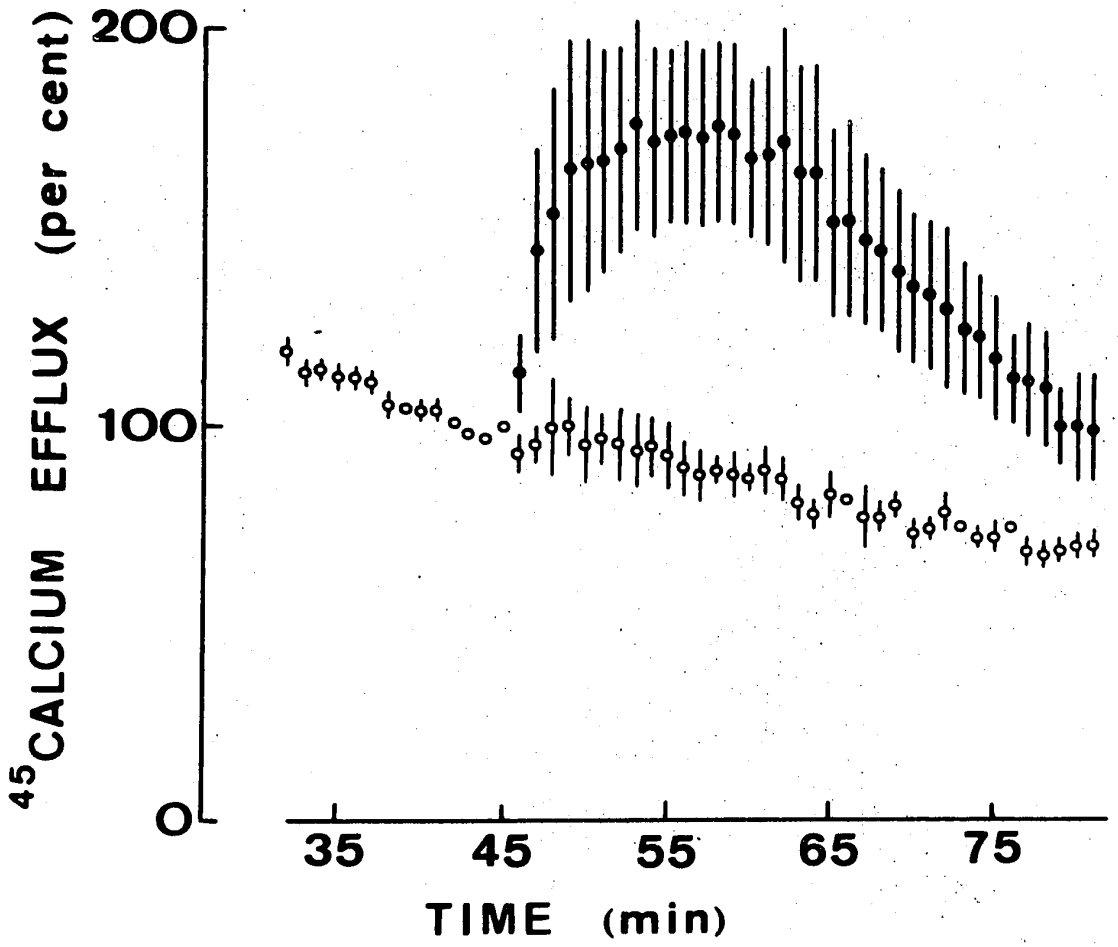

Figure 7-2. ^{45}calcium efflux from perifused islets. In each experiment 200 islets were preincubated in the presence of ^{45}calcium, and placed in the perifusion chamber at time zero. The perifusate was a glucose-free bicarbonate - buffered medium. Open circles refer to data obtained in the absence of theophylline, and closed circles to data obtained in the presence of theophylline (3.2 mM). In each experiment, ^{45}calcium efflux (cpm/min) was expressed in per cent of the value found at the 45th min. Mean values (± SEM) refer to 7 experiments up to the 45th min; and, respectively, to 4 (closed circles) and 3 (open circles) experiments thereafter.

3.4 Effects of theophylline, vincristine and colchicine upon insulin release

In the experiments summarized in Table 7-IV, pieces of pancreatic tissue were preincubated for 90 min in media containing glucose (5.6 mM) and, as required, vincristine, colchicine and theophylline. After this preincubation, the pieces were incubated for 90 min in a second medium containing a higher amount of glucose (16.7 mM) with or without theophylline. Insulin secretion was measured only during the final incubation period.

When the pieces of pancreatic tissue were preincubated in the presence of either vincristine or colchicine and, thereafter, transferred to a new medium containing no inhibitor, their secretory response to glucose was significantly impaired (Table 7-IV, lines 3 and 5 *vs* line 1). The addition of theophylline to the final incubation medium significantly enhanced glucose-induced insulin secretion, whether preincubation had been carried out in the absence or presence of a mitotic-spindle inhibitor Table 7-IV, lines 2 *vs* 1, 4 *vs* 3, and 6 *vs* 5). However, after preincubation with either vincristine or colchicine, the rate of insulin secretion induced by the combination of glucose and theophylline was significantly lower than that observed with the same combination after preincubation in the absence of any inhibitor (Table 7-IV, lines 4 and 6 *vs* line 2). Prior exposure to vincristine inhibited to a comparable degree the secretory responses to glucose alone (47.4 per cent inhibition) and to the combination of glucose and theophylline (42.5 per cent inhibition).

Table 7-IV.

Effect of theophylline and mitotic-spindle inhibitors upon insulin secretion by incubated pieces of pancreatic tissue

Line N°	Preincubation (i) (Glucose: 5.6 mM) Added drug (mM)	Incubation (i) (Glucose: 16.7mM) Added drug (mM)	Insulin output(ii)	P
1	Nil	Nil	100.0 ± 10.0 (44)	
2	Nil	Theophylline (1.4)	167.8 ± 16.9 (26)	vs 1 : <0.01
3	Vincristine (0.03)	Nil	52.6 ± 10.2 (13)	vs 1 : <0.01
4	Vincristine (0.03)	Theophylline (1.4)	96.6 ± 15.0 (13)	vs 2 : <0.01; vs 3 : <0.05
5	Colchicine (1.0)	Nil	60.4 ± 12.7 (28)	vs 1 : <0.05
6	Colchicine (1.0)	Theophylline (1.4)	110.9 ± 12.9 (13)	vs 2 : <0.01; vs 5 : <0.02
7	Colchicine (1.0) & theophylline (1.4)	Nil	50.5 ± 11.1 (28)	vs 1 : <0.01; vs 5 : N.S.

(i) Both the preincubation and incubation were carried out for 90 min at 37°C.
(ii) Mean values (± SEM) for insulin output during incubation are expressed in per cent of the mean control rate of secretion observed within the same experiment; also shown are the number of observations (in parentheses) and the statistical significance (P) of differences between mean values (N.S. = not significant).

Prior exposure to colchicine also reduced to a same extent the secretory responses to glucose alone (39.6 per cent inhibition) and to the combined stimuli (34.0 per cent inhibition). After preincubation in the simultaneous presence of colchicine and theophylline, the secretory rate induced by glucose was not significantly different from that observed after exposure to colchicine alone (Table 7-IV, lines 5 & 7).

These data suggest that the alteration of the microtubular system induced by colchicine or vincristine does not differentially affect the respective insulinotropic action of glucose and theophylline; and that theophylline itself does not interfere with the deleterious effect of colchicine on the microtubular system.

2.4 Discussion

It is now firmly established that the intracellular accumulation of cAMP stimulates insulin release from the pancreatic beta-cell. Samols, Marri and Marks (1965) were the first to demonstrate that glucagon, an activator of adenyl cyclase, promotes insulin secretion. Turtle and Kipnis (1967b) observed that glucagon indeed increases the level of cAMP in isolated islets. Numerous reports (Turner and McIntyre, 1966; Vecchio et al., 1966; Grodsky et al., 1967; Lambert et al., 1967; Porte, 1967b; Sussman and Vaughan, 1967; Turtle et al., 1967a; Malaisse and Malaisse-Lagae, 1970a) have also documented the insulinotropic action of various agents thought either to activate adenyl cyclase (isoproterenol, glucagon, enteroglucagon, ACTH, TSH) or to inhibit phosphodiesterase

(theophylline, caffeine); cAMP and db-cAMP were also shown to cause insulin release (Sussman and Vaughan, 1967; Malaisse et al., 1967c, 1970d).

The present study aims at locating the primary site of action of cAMP in the beta-cell. Three hypotheses are considered, namely a stimulation of glucose metabolism, an effect of cAMP upon calcium metabolism, and an interaction between the nucleotide and the microtubular protein.

3.1 Cyclic AMP as a modulator of glucose metabolism

Samols and his colleagues (1966) were also the first to suggest that cAMP exerts its insulinotropic action by facilitating glucose metabolism in the beta-cell. This concept appeared to be substantiated when it was shown that all agents thought to increase the cellular level of cAMP, and even cAMP itself or db-cAMP, are only able to cause sustained hypersecretion of insulin in vitro if the beta-cell is provided with a sufficient supply of either extracellular or intracellular glucose (Malaisse et al., 1967c, 1970a, 1970d). Moreover, whenever the metabolism of glucose in the beta-cell is inhibited by such agents as mannoheptulose and 2-deoxyglucose, the enhancing effect of glucagon or theophylline upon glucose-induced insulin release is also suppressed (Malaisse et al., 1967c, 1970a).

As soon as these data were published, it was observed that glucagon and theophylline do not only potentiate glucose-induced insulin release, but also enhance the insulinotropic action of certain amino acids, such as leucine and glycine (Malaisse et al., 1968a. 1970a). Since the latter results were

recorded in the absence of glucose, they did not support the concept of a specific effect of cAMP on glucose metabolism (Malaisse et al., 1970a).

The measurement of calcium uptake by isolated islets offered a new opportunity to test the possible effect of cAMP on glucose metabolism. Indeed, it was shown elsewhere that calcium uptake is regulated by the amount of glucose available to and metabolized in the beta-cell (Malaisse-Lagae et al., 1971a). Thus, glucose stimulates the accumulation of calcium by inhibiting calcium efflux from the beta-cell (chapter 1.6; Malaisse and Brisson, 1971c); and inhibitors of glucose metabolism, such as mannoheptulose and 2-deoxyglucose, inhibit the stimulant action of glucose upon calcium uptake (Malaisse-Lagae et al., 1971a). Therefore, if cAMP were to enhance glucose-induced insulin secretion by accelerating glucose metabolism in the beta-cell, it should theoretically also enhance glucose-induced calcium uptake.

In the present experiments, a modest but significant potentiation of glucose-induced calcium uptake by either theophylline or db-cAMP was only observed at a 5.6 mM glucose concentration which is close to the threshold value for the stimulant action of glucose upon both calcium uptake (Malaisse-Lagae et al., 1971a) and insulin release (Turtle et al., 1967a). It is, therefore, possible that, at this particular glucose level, cAMP indeed accelerate some rate-limiting step of glucose metabolism, as suggested elsewhere (Malaisse et al., 1967c). However, at higher glucose concentrations, no significant effect of either theophylline or db-cAMP upon

glucose-induced calcium uptake could be detected (Table 7-II), suggesting that the marked insulinotropic action of these agents at high glucose levels is not due to a facilitation of glucose metabolism in the beta-cell.

The available biochemical data also do not suggest that cAMP has any major effect on glucose metabolism in the beta-cell. Thus, Hellerström and Gunnarsson (1970) have shown that, whereas glucose (16.7 mM) increases by about 50 per cent the endogenous respiration of isolated islets, both glucagon and db-cAMP slightly depress the oxygen uptake of the islets incubated in the presence of glucose (11.1 mM). Ashcroft and Randle (1969) and Randle et al., (1970) reported that, in the presence of glucose (7.4 mM), glucagon produces a slight fall in the rate of glucose oxidation by mouse islets and does not change their glucose-6-phosphate concentration. Finally, according to Hellman and Idahl (1969b), glucose increases the ATP content of isolated islets, whereas the level of ATP remains unaltered when the beta-cell is stimulated by db-cAMP.

3.2 Cyclic AMP as a modulator of calcium metabolism

Rasmussen and Tenenhouse (1968) have suggested that the effects of cAMP in a variety of systems are due mainly to a modification of calcium metabolism at the cellular level. Thus, the insulinotropic action of cAMP could be due to an altered handling of calcium by the beta-cell. If insulin secretion is controlled by the cellular concentration of calcium as suggested elsewhere (Malaisse-Lagae et al., 1971a), cAMP could enhance insulin release by provoking a translocation of calcium within the beta-cell, from a pool stored in

some organelles to a cytoplasmic pool.

Two arguments can be found in favor of the latter hypothesis. Firstly, theophylline causes a rapid and sustained increase in ^{45}calcium efflux from prelabelled islets. This finding illustrated in Fig. 7-2 is reminiscent of the rapid increase in ^{45}calcium efflux induced by glucagon or cAMP in the perfused liver (Friedmann and Park, 1968). It suggests a sudden enrichment of a pool of ionized calcium readily available for transport across the cell membrane. Secondly, db-cAMP and theophylline are able to compensate, to a certain extent, for a lack of extracellular calcium. Thus, in calcium-depleted media, the amount of extracellular calcium available to the beta-cell is obviously insufficient to allow glucose or leucine to exert their normal insulinotropic action; whereas, in the presence of db-cAMP or theophylline, the insulinotropic effect of these metabolic substrates is partially restored (Table 7-III).

The concept of a cAMP-induced redistribution of calcium would be consistent with the effects of db-cAMP and methylxanthines in adipose tissue where these agents are also thought to cause a shift of calcium from a bound to a free pool (Efendic et al., 1970). The recent demonstration of adenyl cyclase activity in cardiac sarcoplasmic reticulum (Entman et al., 1969) suggests that the well-known calcium-translocating effect of caffeine in muscle might also be mediated through cAMP accumulation. Dr. Malaisse-Lagae and her coworkers (1971d) have recently underlined the analogy between the processes of stimulus-secretion coupling in the pancreatic beta-cell and excitation-contraction coupling in muscle.

The present data apparently extend this analogy to calcium-translocating effect of cAMP which results respectively in enhanced insulin release and muscular contracture (Rasmussen and Tenenhouse, 1968).

3.3 Glucose-independency of cAMP-induced calcium translocation

The increase in ^{45}calcium efflux induced by theophylline is apparently independent of glucose, since it was observed in a glucose-free medium. Such effect of theophylline contrasts with that of glucose which was recently shown to inhibit ^{45}calcium efflux from the isolated islets (Malaisse and Brisson, 1971c). Because glucose and cAMP apparently influence calcium metabolism in the beta-cell through different mechanisms, it is unlikely that glucose exerts its own insulinotropic effect solely by provoking an intracellular accumulation of cAMP, as suggested by some authors (Cerasi and Luft, 1970). Other arguments against such idea are the unability of db-cAMP *per se* to mimic the sustained stimulant action of glucose on insulin release (Malaisse et al., 1970d), the maintenance of a significant insulinotropic action of glucose despite full activation of phosphodiesterase by imidazole (Malaisse et al.,1968d), and the failure of glucose to affect the cellular level of cAMP in isolated islets (Kipnis, 1970; Montague and Cook, 1971).

3.4 Substrate-dependency of cAMP-induced insulin release

If cAMP translocates calcium within the beta-cell and increases the concentration of this cation in the cytosol,

and furthermore if the level of calcium in the cytosol regulates insulin secretion, it is apparently surprising that the nucleotide is unable to cause sustained insulin release in the absence of glucose (Malaisse et al., 1967c, 1970d). This glucose-dependency does probably not correspond to an energy requirement of the beta-cell. Thus, pyruvate, which is known to increase ATP level in the beta-cell (Georg et al., 1970), cannot replace glucose as a substrate for the insulinotropic action of cAMP (Table 7-III). Moreover, leucine, which is a suitable substrate for such action, has apparently not to be metabolized in order to stimulate insulin release (Knopf et al., 1963).

It could be speculated that the cAMP-induced translocation of calcium is only able to promote sustained secretion of insulin when the beta-cell is simultaneously exposed to a suitable insulinotropic substrate (e.g. glucose or leucine) which itself favors calcium accumulation in the cytosol by inhibiting calcium efflux. That glucose indeed acts in such a manner has been recently demonstrated (Malaisse and Brisson, 1971c).

This hypothesis would account for all available experimental data. Firstly, it would explain why the accumulation of cAMP does not cause insulin secretion in the absence of either an extracellular or intracellular source of insulinotropic substrate (Malaisse et al., 1967c, 1970a). Actually, in the absence of glucose, the cAMP-induced translocation of calcium and subsequent efflux of calcium from the beta-cell should reduce the total load of cellular calcium. That this is indeed the case is suggested by the significant lowering

of calcium uptake induced by either theophylline or db-cAMP in the absence of glucose (Table 7-II). Secondly, the proposed model would explain why the enhancing action of cAMP upon insulin secretion is more marked at high than at low glucose levels (Malaisse et al., 1967c). Indeed, the insulinic response evoked by the translocation of a fixed amount of calcium will depend on the actual balance between calcium influx and efflux i.e., on the ability of the beta-cell to retain intracellularly part or all of the translocated calcium. In support of this concept, we have observed (Brisson and Malaisse, unpublished results) that the increase in ^{45} calcium efflux from isolated islets induced by theophylline is much less pronounced in the presence than in the absence of glucose. Thirdly, the model implies that any agent which suppresses the insulinotropic action of glucose also abolishes the enhancing action of glucagon or theophylline. That this is indeed the case has already been documented (Malaisse et al., 1967c, 1970a), and is here confirmed (Table 7-III) with mannoheptulose, dinitrophenol, diazoxide, epinephrine, and tetracaine.

Teleologically, the proposed model offers certain advantages. For instance, the substrate dependency of cAMP-induced insulin release is likely to protect the organism against excessive insulin secretion which could otherwise result from the release of pancreatic glucagon in glucopenic situations (Ohneda et al., 1969).

3.5 Cyclic AMP as a modulator of tubulin metabolism

Lacy and his colleagues (1968, 1970) have suggested that the microtubular-microfilamentous system of the beta-cell

plays a role in the insulin-secretory process. Further investigations have confirmed that the integrity of the microtubular system is required in order for glucose and other agents to exert their stimulant action upon insulin release (Malaisse-Lagae et al., 1971d; Malaisse et al., 1971b). Because the microtubular protein might serve as a substrate for a cAMP-dependent protein kinase (Goodman et al., 1970), the insulinotropic effect of the nucleotide could conceivably be due to such interaction between cAMP and the tubulin molecule. Moreover, it has also been reported that cAMP interferes with the binding of colchicine to the microtubular protein (Gillespie and Levine, 1970). However, the data summarized in Table 7-IV indicate that theophylline does not interfere with the deleterious effect of colchicine and that the impairment of the microtubular system induced by mitotic-spindle inhibitors does not preferentially affect the respective insulinotropic actions of either glucose or theophylline. These data do not suggest, although they do not entirely rule out, a primary effect of cAMP on the microtubular system. At least, the present findings are compatible with the concept that the participation of the microtubular-microfilamentous system represents an obligatory and late event in the secretory sequence whatever agent is used to promote insulin release (Malaisse et al., 1971b).

3.6 Conclusions

The present experimental data were taken as an indication that cAMP provokes a redistribution of calcium in the beta-cell. This effect of cAMP is apparently independent of the

presence of glucose. It might thus represent a fundamental action of cAMP likely to occur in other systems. As such, it could account for the ubiquitous stimulatory effect of cAMP upon calcium-dependent secretory processes (Sutherland and Robison, 1969).

1.8 Effect of epinephrine upon alpha-cell secretory activity

2.1 Introduction

It is now well established that glucagon induces hyperglycemia in animals and man through its action on liver glycogenolysis (Sutherland, 1950) and gluconeogenesis (Exton et al., 1966). Epinephrine has similar metabolic effects on the liver (Sutherland, 1950; Exton et al., 1966) and it is often thought that both hormones play a physiological role as glycogenolytic agents in blood glucose homeostasis. However, several authors have reported that when epinephrine was directly infused in the portal vein its effects on blood glucose and hepatic glycogenolysis were much less pronounced than when the hormone was administered into the systemic circulation (Baudouin et al., 1936; Sherlock, 1949; Ezdinli et al., 1966). Moreover, Sokal and his coworkers demonstrated both in vitro and in vivo that, in contrast to glucagon, doses of epinephrine within the physiologic range had only small and transient effects on liver glycogen and phosphorylase activity (Sokal et al., 1964; Weintraub et al., 1969). According to these observations, they concluded that glucagon is the only agent promoting glycogenolysis in the liver under physiologic conditions, and suggested that the effect of moderate doses of epinephrine might be indirect, possibly mediated through a stimulation of glucagon secretion.

In order to test this hypothesis, we decided to examine the effect of epinephrine upon glucagon secretion in vitro.

Variable results were obtained when glucagon secretion was measured from either pieces of intact pancreatic tissue or isolated islets obtained by collagenase digestion of the rat pancreas. These erratic results could be due respectively to the release of proteolytic factors from the acinar tissue in case of the pancreatic fragments, and to damage of the peripherally-located alpha-cells in case of the isolated islets. In view of these technical difficulties, we turned to the pancreatic duct ligation technique so as to induce atrophy of the exocrine tissue without impairing endocrine islet function (Mialhe and Meyer, 1961).

2.2 Materials and methods

Pancreatic tissue was removed from fed female albino rats (125 to 150 g; Rega, Heverlee, Belgium) in which the pancreatic duct had been ligated 3 to 4 weeks previously. Small pieces (4 to 10 mg) were incubated in 2 ml bicarbonate-buffered medium containing bovine albumin (5mg/ml), Trasylol (833 KIU / ml; Bayer Co., Germany), and equilibrated against a mixture of oxygen (95 %) and carbon dioxide (5 %). Glucose (0.3 to 3.0 mg/ml) and epinephrine (0.1 to 10.0 µM) were added as required. The glucagon content of the media was determined by immunoassay (Leclercq-Meyer et al., 1970) and the rate of glucagon release (IRG) expressed as pg / mg tissue / 90 min incubation. The insulin content of the media was measured by the method of Wright et al., (1967) previously out-

lined, and the rate of insulin release (IRI) expressed as μU / mg / 90 min of incubation.

2.3 Results

The results (Table 8-I) show that mean rate of IRG secretion decreased as the glucose concentration of the incubation media was raised from 0.3 to 3.0 mg / ml, a significant difference ($P < 0.02$) being observed when tested by paired comparison. Concomitantly, glucose induced the classical increase in IRI output, attesting the functional integrity of our preparation. Epinephrine abolished the stimulant action of glucose on IRI output, and significantly enhanced IRG release. The glucagonotropic effects of epinephrine were observed both at low and high glucose levels and could still be detected in the presence of a very low concentration of this catecholamine (0.1 μM).

2.4 Discussion

Our experiments clearly demonstrate that epinephrine and glucose have opposite effects on glucagon and insulin secretions in vitro. The increase in IRG secretion observed at a low glucose level confirms previous data from both in vitro (Vance et al., 1968; Chesney et al., 1969) and in vivo (Ohneda et al., 1969; Buchanan et al., 1969; Aguilar-Parada et al., 1969) experiments, though the relative changes in glucagon output at various glucose concentations are often of minor amplitude and sometimes of doubtful significance (Edwards et al., 1969).

Table 8-I.

In vitro release of glucagon (IRG) and insulin (IRI) from duct-ligated rat pancreas

glucose	control output		epinephrine	increment	
	IRG	IRI		IRG	IRI
mg / ml	pg / mg / 90 min	µU / mg / 90 min	µM	pg / mg / 90 min	µU / mg / 90 min
0.3	565 ± 115 (17)	153 ± 15 (17)	10.0	+ 287 ± 82* (17)	+ 28 ± 15 (17) NS
1.5	498 ± 83 (20)	236 ± 22 (20)	0.1	+ 413 ± 99¶ (20)	- 35 ± 19 (20) NS
			1.0	+ 236 ± 88§ (20)	-103 ± 19¶ (20)
			10.0	+ 390 ±106* (20)	-113 ± 22¶ (20)
3.0	339 ± 72 (17)	959 ± 83 (17)	10.0	+ 181 ± 60* (17)	-787 ± 83¶ (17)

Mean values (± SEM) are shown together with the number of observations (in parentheses). Significant differences are indicated § when P < 0.02, * when P < 0.01, and ¶ when P < 0.001.

A stimulant effect of epinephrine on pancreatic glucagon release would be in agreement with the hypothesis of Sokal who suggested that, in vivo, catecholamines induce hepatic glycogenolysis through a stimulation of glucagon secretion. Such a mechanism could take place in states of glucopenia, during muscular exercise, or in any stressful situations where a rise in circulating catecholamines has been documented (Gray and Beetham, 1957; Vendsalu, 1960; Goldfien et al., 1961; Klepping et al., 1964; Neal et al., 1968; Weil-Malherbe et al., 1968; DeSchaepdryver et al., 1969; Kotchen et al., 1971; Myles et al., 1971). It is noteworthy that such situations are accompanied by a fall in plasma IRI concentrations which we have ascribed in previous chapters to an inhibitory effect of endogenously released catecholamines on insulin secretion (Cochran et al., 1966; Schalch, 1967; Wright et al., 1968; Federspil et al., 1969; Pruett, 197o). It is well known that glucagon and insulin have opposite effects upon both liver glycogenolysis and adipose tissue lipolysis. Therefore, the simultaneous stimulation of glucagon and inhibition of insulin release by endogenously released catecholamines will ensure maximal mobilization of glucose from the liver and free fatty acids from adipocytes. The hormonal regulation would then be optimally oriented for the supply of energetic substrates to tissues such as the brain and the exercising muscles.

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