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CHARACTERIZATION OF INSULIN SECRETION AFTER CONTINUOUS EXPOSURE OF NORMAL ISOLATED PERFUSED RAT PANCREAS FOR MORE THAN SIX HOURS TO GLUCOSE, THEOPHYLLINE AND TOLBUTAMIDE

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ABSTRACT

A perfusion system was conducted for more than 6 hours in response to glucose (16.7mM), theophylline (5 mM) and tolbutamide (100 μg/ml) to give an insight about the β-cell secretory capacity. Continuous exposure of the isolated pancreas to glucose (16.7 mM) caused a biphasic response. After 3 h of continuous glucose perfusion a significant sustained reduction in the insulin secretion rates to about 22% of the maximal response untill 6 h was observed.Then, after 15 min with 3 mM glucose perfusion, re-infusion of glucose 16.7 mM produced an increase in insulin secretion .Theophylline infusion for 6 h did not significanly affect the insulin secretion induced by re-infusion of theophylline after 15 min with low glucose (3 mM) concentration. The results also indicated that even after exposure of the pancreas to glucose 16.7 mM for 6 h, theophylline was able to restore the stimulatory effect after desensitization. Continuous exposure of the pancreas for long time (6 h) to tolbutamide significanly inhibit its ability to secrete insulin after 15 min of basal perfusion medium which demonstrated a desensitization phenomenon. The results revealed that, β-cells become desensitized, not exhausted, not only by glucose but also with tolbutamide. It seems also that cAMP system cann't be neglected in this model since, theophylline induced insulin secretion alone and even after long infusion with high glucose. The desensitization of the β-cell to tolbutamide may be due to sustained depolarization caused by continuous exposure to tolbutamide . The present study confirms that the elevation of plasma glucose concentration represents a pathogenic factor which causes a deleterious effect on islet β-cell function. This effect is reversed on cessation of glucose.

INTRODUCTION

It has been postulated from studies in animals and in human diabetic subjects that impaired β-cell function is a major factor in maturity onset diabetes [1-3]. This defect may develop as a result of chronic hyperglycemia or of persistent overstimulation which may leading to exhausion or depletion of the endocrine cells[4] . Normally, glucose plays a crucial role in the control of insulin secretion both by directly stimulating release[5] and by modulating the beta-cell response to most other secretagogues[6,7]. This relationship is altered in non-insulin dependent diadetes mellitus as previouly reported[8].

In addition, it has been found that [9], infusion of concentrated glucose in healthy rats for 48 -96 h resulted in the development of hyperglycemia with impaired glucose influence on insulin release and the defects in β-cells are being more pronounced as the level of hyperglycemia rose[10,11] . The abnormalities of insulin secretion characterized by marked hypersecretion of insulin at 2.5 mM glucose and failure to increase insulin secretion when perfusion is changed to 16.7 mM glucose due to desensitization [10,12,13]. On the contrary, it has been indicated by Ammon et al. [14] that, 48h contiuous glucose infusion in rats, in vivo, sensitizes the pancreatic islets toward the lower non-stimulatory glucose concentration. These abnormalities were reversible with cessation of the glucose infusion for more than 6 h before study[15] and in perfused pancreases of genetically obese diabetic rats after 4 week of hyperglycemia[16].

Moreover, Giroix et al.[17] reported that, in the diabetic pancreas the alteration of the β-cell responsiveness might be variable according to the nature of the stimulus. In addition, it has been suggested that, prolonged exposure of normal β-cells to elevated plasma glucose concentration may be an important pathogenic factor in the selective loss of the β-cell response to glucose [9].

The present study was undertaken to explore the effect of long-term stimulation in vitro on the secretory responsiveness of isolated perfused pancreas to characterize the secretion of insulin for more than 6 hours with glucose (a natural physiological stimulant), tolbutamide (a voltage-dependent Ca^{2+} channel and phosphoinositide stimulant) and theophylline (a cAMP stimulant). This trial was taken to further elucidate whether this selective insensitivity applied to intact glucose or also to other non-nutrient insulin secretagogues .

MATERIALS AND METHODS

Animals:

Adult male and female rats from local source weighing 250 - 300 g were used. The animals had free access to tap water and fed with bread and milk *ad libitum.*

Chemicals:

Chemicals used were obtained from the following sources: D-glucose, Sigma Chemicals, Egypt; Tolbutamide, Hoechst, Frankfurt, Germany; Theophylline sodium, Sigma chemicals, Egypt; Insulin RIA kit from the radioassay system Labolatories, diagnostic products corporation, Los Angeles, CA, USA, Bovine serum albumin, Behringwerke, Marburg, Germany, Pentobarbitone sodium, BDH, England, Heparin sodium, EL-Nile Co., Egypt. All other chemicals used were of analytical grade.

Isolation of the pancreas preparation:

The animals were anaesthetized with pentobarbital sodium (30 mg/ kg, I.P). The pancreas along with the stomach, the adjacent part of duodenum and the spleen were isolated surgically from adjacent organs. Cannulae of the perfusion system were attached to the coeliac axis and portal vein.

Perfusion medium:

The perfusion medium was Krebs-Ringer-Bicarbonate (KRB) buffer solusion supplemented with 0.5% bovine albumin and 54 mg/ml glucose (3mM). The concentrations of the salts in the medium (mM) were as follows; NaCl, 118.4 ; KCl, 4.8 ; $CaCl_2$, 2.5; $MgSO_4$,1.2; KH_2PO_4,1.2 and $NaHCO_3$, 25. The medium was maintaned at 37 C and gassed with a mixture of 95% O_2 and 5% CO_2 which adjust the pH of the solution to 7.35-7.4. This medium is always referred to as basal perfusion medium. the effects of the test substances on insulin secretion were investigated by adding these substances to the previously mentioned medium. The perfusion medium was introduced into the coeliac artery of the prepared pancreas by an open circuit non recycling perfusion system.

Experimental protocol:

The pancreas was first allowed to equilibrate for 15 min. with the basal medium containing 3 mM glucose. After this initial equilibration period, the perfusion medium was switched off (at zero time) by turning the three way valve to the second perfusion medium containing the test substances. The perfusion medium was introduced into the coeliac artery at a flow rate of 1ml/min and the effluent was collected from the portal vein at different time intervals (as indicated in the figures), into perchilled tubes, then frozen and stored at -20 °C till the assay of the insulin content using radioimmunoassay (RIA) according to Morgan and Lazarow[18]. Results are shown as values at time intervals (abscissa) and as the area under the curve for the phases of insulin secretion.

Series 1: Experiments in this series were designed to examine the effect of high glucose concentration (16.7 mM) on insulin secretion. After an initial equilibration period with basal medium, the perfusion medium was switched off to the second perfusion medium containing glucose (16.7 mM) for 6 h. Then the perfusion medium was switched off again to the basal medium for 15 min. Finally, glucose (16.7 mM) was introduced for 30 min by changing the medium reservoir.

Series 2- Experiments in this series were designed to study the effect of theophylline (5 mM) on insulin secretion for 6 h. The experiments were carried out as previously described under series 1 except that theophylline was introduced for 20 min.

Series 3: Experiments in this series were performed to examine the effect of infusion of glucose (16.7 mM) for 6 h on theophylline- induced insulin secretion. In this series,after an initial equilibration period with basal medium, the perfusion medium was switched off to the second perfusion medium containing glucose (16.7 mM) for 6 h. Then the perfusion medium was switched off again to the basal medium for 15 min. Finally, theophylline (5 mM) was introduced for 20 min by changing the medium reservoir.

Series 4: Experiments in this series were aimed to investigate the effect of tolbutamide (100µg/ml) infused for 6 h on insulin secretion . The experiments were carried out as previously described under series 1 except that tolbutamide was introduced for 20 min.

Statistical analysis :

Results are expressed as mean ±SEM. Tests of signifigance were carried out using Student's "t" test for paired or unpaired data. Differences between means were considered significant if probability $P < 0.05$.

RESULTS

1- Effect of Glucose 16.7 Mm:

The effect of glucose (16.7 mM) on insulin secretion was carried out first for 360 min then for 30 min (from min 375 - 405) after its cessation for 15 min (duration of glucose 3 mM infusion from 360 - 375 min which represent the basal perfusion medium) . The introduction of glucose 16.7 mM at time zero, an initial release was observed during the first 10 min reflecting first-phase of insulin secretion. Second-phase of insulin secretion increased progressively over about 3 h. Subsequently, secretion spontaneously declined about 22% of the maximal response.This response was significant and continued with the continous perfusion of glucose starting from min 240 untill 360min .

Changing the glucose concentration from 16.7 mM to glucose 3mM for 15 min, produced a decrease in insulin release. Re-infusion of glucose 16.7 mM induced a rise in insulin secretion .The increase in insulin secretion was observed till the end of perfusion period 405 min (Fig. 1).

2- Effect of Theophylline 5mM:

The effect of theophylline 5 mM on the insulin secretion was carried out first for 360 min, then for 20 min after its cessation for 15 min of 3mM glucose to test whether or not the desensitization is selective to glucose as well as to examine the cAMP system.

Addition of theophylline 5 mM to the perfusion medium at time zero for 360 min induced a swift elevation in insulin release followed by transient decrease but not significant after 2min of the perfusion time. The insulin secretion was elevated again after 60 min and this elevation was continued all over the period of the infusion time 360 min . Introduction of glucose 3 mM induced a decrease in insulin release for 15 min (from min 360-375). Re-infusion of theophylline at time

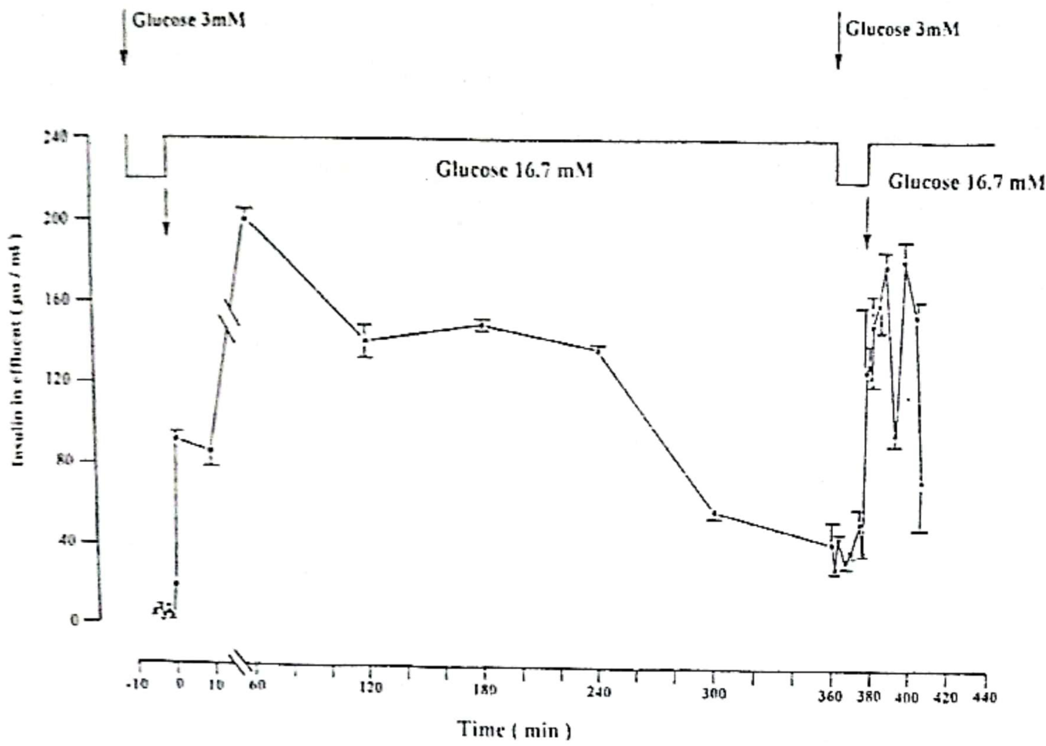

Fig.(1): Effect of long term infusion of glucose 16.7 mM on the insulin secretion from isolated perfused rat pancreas.
Values are expressed as mean ± SEM, n = (4)

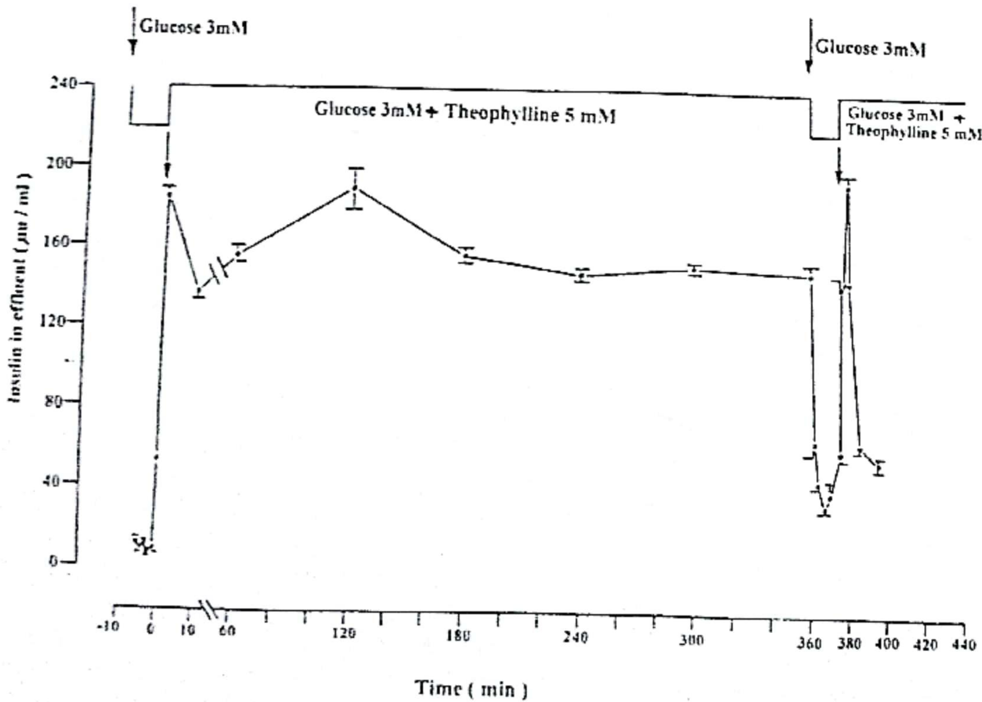

Fig.(2): Effect of long term infusion of theophylline 5 mM on the insulin secretion from isolated perfused rat pancreas.
Values are expressed as mean ± SEM, n = (4)

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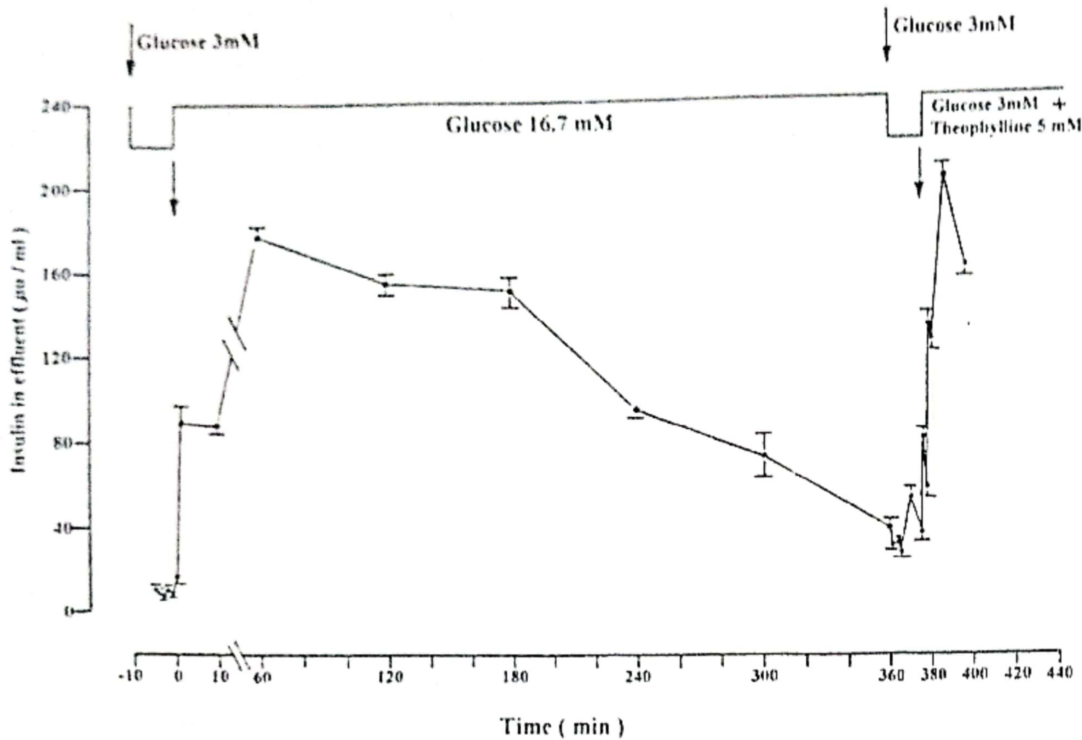

Fig.(3): Effect of long term infusion of glucose 16.7 mM on the theophylline induced 5mM-insulin secretion from isolated perfused rat pancreas.
Values are expressed as mean ± SEM, n = (4)

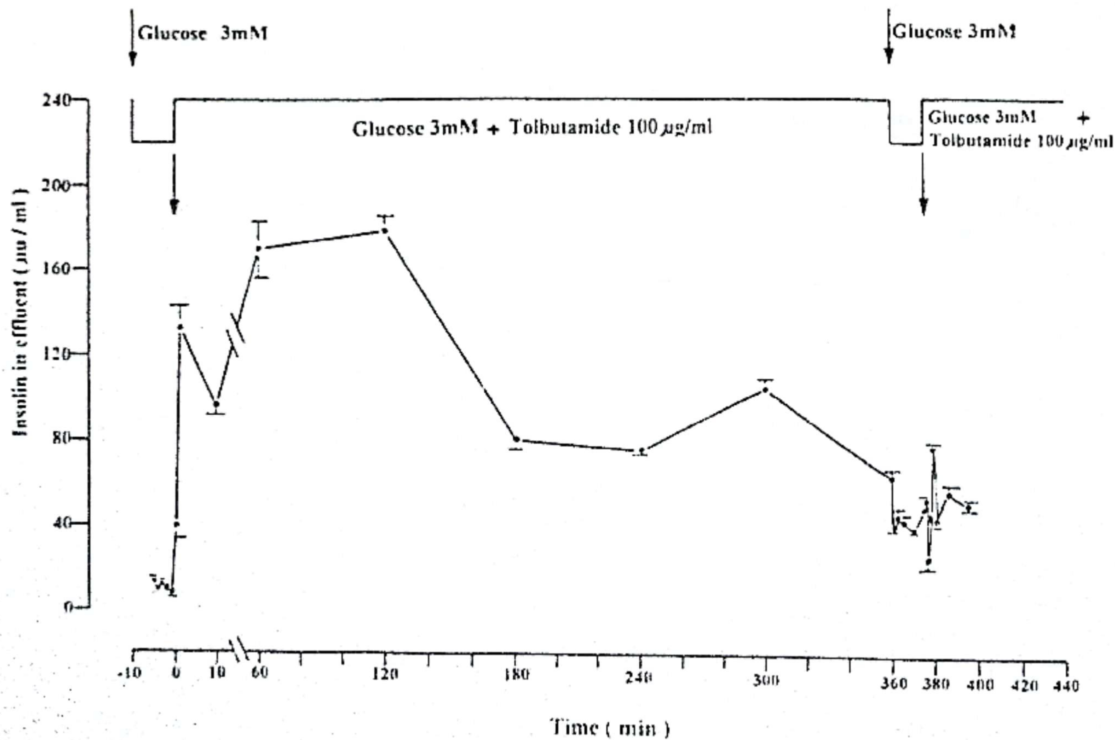

Fig.(4): Effect of long term infusion of tolbutamide 100 ug/ml on the insulin secretion from isolated perfused rat pancreas.
Values are expressed as mean ± SEM, n = (4)

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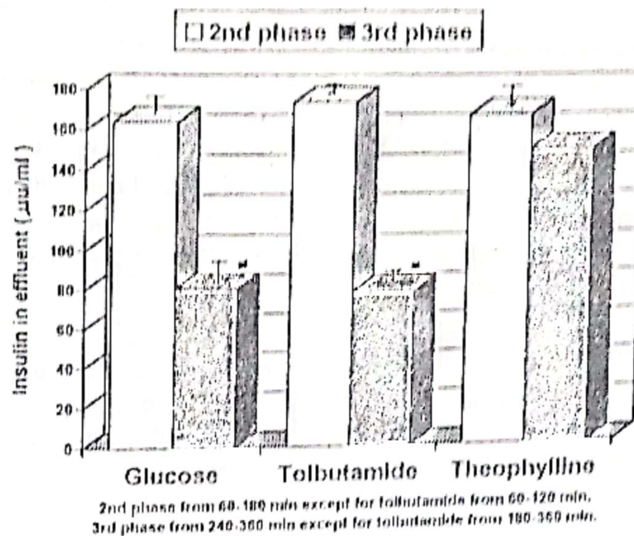

Fig. (5): Relationship between time of perfusion and amount of insulin secreted from isolated pancreas stimulated by glucose (16.7 mM), theophylline (5mM) and tolbutamide (100 µg/ml) during 6 hours of perfusion.

* Significantly different from second phase value at $P < 0.05$.

375 caused a similar elevation in insulin secretion which decline after 10 min of the perfusion time but still over the basal secretion level (Fig. 2).

3- Effect of Long Term Infusion of Glucose 16.7 mM (6 Hours) on Theophylline 5 mM- Induced-Insulin Secretion :

Figure (3) showed that the introduction of glucose 16.7 mM into the perfusion medium for 360 min produced a similar results as shown previous in experiment 1. A biphasic insulin response was observed which declined at time 240 but still over the basal secretion .

The addition of theophylline 5 mM at time 375 for 20 min after glucose 3mM infusion for 15 min (from min 360-375), induced a swift increase in insulin secretion and this increase was observed untill the end of the experiment at time 395.

4- Effect of Tolbutamide 100 µg/ml :

Figure (4) demonstrated that the addition of tolbutamide 100 µg/ml at time zero to the perfusion medium for 360 min induced two phases of insulin secretion the first phase started from 0 - 10 min .The second phase of insulin secretion was declined after 2h to about 35.9% of the maximal response . The introduction of tolbutamide for 20 min after its cessation for 15 min during the perfusion of glucose 3 mM, a significant inhibition of insulin secretion was observed untill the end of the perfusion period at 395min.

The relationship between time of perfusion and the amount of insulin secreted from isolated pancreas stimulated by glucose (16.7 mM), theophylline (5mM) and tolbutamide (100 µg/ml) during 6h of perfusion representing the second phase and the third phase was shown in Fig. (5).

DISCUSSION

The results of the present study demonstrated that continuous exposure of the isolated pancreas to glucose (16.7 mM) caused a biphasic response then after 3 h of continuous glucose stimulation the insulin secretion rates were sustained reduced to about 22% of the maximal response at 360 min. These observations are in harmony with those of Bolaffi et al [3] and Grodsky [19], who demonstrated similar pattern using perifusion, and perfusion system. Similar observations have been independently described[4]. This reduced secretion was relatively constant for 24-48 h of sustained stimulation. In this sequence of events, previous in vitro studies of the kinetics of insulin secretion in dynamic perifusion demonstrated the impaired glucose influence on insulin secretion on normal rats model and characterized by a rapid first phase and a slowly ascending second phase [20].

The second phase of insulin secretion may represent a secretagogue-induced signal desensitization of the β-cell, rather than exhausion of a β-cell compartment of stored insulin[3]. This decrease in maximum rate of release in high glucose, has been called desensitization to glucose in numerous studies [3, 21-24]

It is unlikely that the insensitivity to glucose is due to a block in the β-cell glucose metabolism prior to the triose phosphate level[17], since the insulin secretion induced by high glucose concentration (16.7 mM) in the present study was increased. These results are in harmony with that of Voyles et al.[24], who suggested that the abnormal response to high glucose (desensitization) was reversed by perfusion of the pancreas with glucose-free buffer for a 45 min as well as for 37 min [12].

Also, these data support the hypothesis that high levels of plasma glucose produce deleterious effects on islet β-cell function, whereas the absence of glucose permits rapid reversal action[13]. Furthermore, the previous studies[25,26] have correlated in vitro-induced desensitization with failure of high - glucose challenge to activate hydrolysis of islet phosphoinositol - containing lipids. So it is assumed that this response may be due to a defect in the glucose receptor in the β-cell in agreement with Timmers et al.[13]. Moreover, previous studies postulated that glucose activates several different intracellular pathways in islets[19, 27, 28].

Since it has been shown that alteration in insulin response to glucose was associated with defective glucose-induced cAMP production in the β-cells [29,30], therefore, insulin secretion in the presence of theophylline was examined in the present study. The results of the present study indicated that theophylline infused for 6 h did not affect the insulin secretion induced by re-infusion of theophylline after a period of 15 min with low glucose (3 mM) concentration. Added to that, the results indicated that even after exposure of the pancreas to the glucose 16.7 mM for 6 h, the insulin release by infusion of theophylline had not changed and showed similar response to the first phase of insulin secretion.

The present findings indicate that the generation of the cAMP in response to theophylline in the β-cells after long exposure (for 6 h) of the pancreas to the theophylline as well as to glucose 16.7mM is normal. This suggestion is supported by the findings of Frankel et al.[31] who mentioned that β-cell response to theophylline was found to be normal in the diabetic Chinese hamasters, rodents with spontanous diabetes. Moreover, the responses obtained with the β-agonist isoproternol also indicated that the ability of the adenylcyclase to generate cAMP in the β-cells of the diabetics was normal, if not increased[17].

In the same contest, studies of Nagamatsu et al.[22] on the isolated islets preincubated for 24 h with 11 mM glucose have showed a desensitization with normal proinsulin synthesis and showed that the conversion rate of proinsulin to insulin is a glucose-regulated process requiring synthesis of a pool of either converting enzyme(s) or other regulating protein before initiation of proinsulin synthesis. Nevertheless, the results of the present study were consistent with the existence of another β-cell defect that could contribute to the abnormality of the insulin response to glucose[17].

Tolbutamide is widely used in the treatment of noninsulin-dependent (type II) diabetic patients. However, After chronic therapy, their mechanism of action was not clear, since many studies have shown that in treated patients plasma insulin concentrations return toward pretreatment levels after the initial elevation [32]. Sulfonylureas stimulate insulin release by binding to a plasma membrane receptor on the β-cell surface [33].

An ATP-sensitive K^+ channel or a closely associated protein has been suggested to be sulfonylurea receptor (34,35). After sulfonylurea stimulation, K^+ efflux through this ATP-sensitive K^+ channel decreases, then causing cell depolarization and the subsequent influx of Ca^{2+} through the voltage-sensitive Ca^{2+} channels. The increase in intracellular calcium then triggers the exocytosis of the insulin granules (33,36).

Concerning the results related to the response to tolbutamide, demonstrated that continuous in vitro exposure of the pancreas for long time (6 h) to the tolbutamide impaired its ability to secrete insulin in response to itself after a period of 15 min of 3mM glucose perfusion (basal perfusion medium) which demonstrated a desensitization phenomenon. These observations are in consistent with that of Rabuazzo,et al.[32] who used rat pancreatic islets after 24-h exposure to various tolbutamide concentrations.In addition, insulin response to tolbutamide was also absent in the study of Bonner-Weir et al.[37] who used an experimental NIDDM induced in neonatal streptozotocin injection. On the contrary , Giroix et al.[17] found that insulinotropic actions of tolbutamide was normal and increased from pancreas pre exposed to 22 mM glucose, the discrepency between these modles concerning the response to tolbutamide was not clear and probably related to the severity of diabetes.

The results of the present study revealed that the desensitization of β-cell to tolbutamide, since the response to tolbutamide was almostly diminished.This response (desensitization) is may be due to the chronic membrane depolarization caused by continuous exposure to the tolbutamide which can lead the β-cell to a refractory state in which it responds less effectively to a further stimulation as reported by Rabuazzo et al.[32]. Moreover, chronic sulfonylurea therapy may lead to a selective desensitization of pancreatic β-cells to sulfonylureas[38,39] and in some patients, which may contribute to the secondary failure to these drugs [40]. In addition, tolbutamide has been shown to increase phosphoinositide hydrolysis in rat islets[41,26], an effect that appears to play an important role in the β-cell secretory response through plasma membrane diacylglycerol increase and its interaction with protein kinase-c. This mechanism has been specially related to the second phase of insulin secretion[42] and has been found to be impaired after 2 h of continuous tolbutamide exposure [26]. Moreover , the same authors suggested that, the phosphoinositide hydrolysis and insulin secretion in response to glucose stimulation are impaired in isolated rat islets by prolonged exposure to tolbutamide. Since the presence of more than one binding site for sulfonyureas has been suggested[43] and both K^+ channel activity and Ca^{2+} influx were normal in islets preexposed to tolbutamide[32]. Therefore, depending on the previous findings, these results may be also clearly interpreted based on that, tolbutamide acts on one binding site which may be impaired due to the

long duration of exposure to tolbutamide. Moreover, the results of the present study are also compatible with the view that, the basis for desensitization of stimulated secretion is complex, probably involve multiple sites within the secretory mechanisms, particularly the phosphoinositol pathway[44].

From the above mentioned data it can be suggested that, the present study confirms the desensitization of the pancreas to high glucose . A selective β-cell insensitivity to glucose has not demonstrated in this experimental model , since there is insensitivity to other secretagogues such as tolbutamide during the contiuous exposure to those compounds. It seems also that cAMP system cann't be neglected since, theophylline was able to induce insulin secretion alone and even after long infusion with high glucose. The desensitization of the β--cell to tolbutamide may be due to sustained depolarization caused by continuous exposure to tolbutamide and concequently it responds less effectively to a further stimulation.

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تقييم عملية افراز الانسولين من البنكرياس المفصول من الجرذان البالغة بعد فترة تعرض مستمره لمدة أكثر من ٦ ساعات المستحدث بالجلوكوز والثيوفللين والتولبيوتاميد

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فى هذا البحث تم دراسة عملية افراز الانسولين من البنكرياس المفصول من الجرذان البالغة باستخدام نظام التنقيط المستمر لمدة أكثر من ٦ ساعات بواسطة الجلوكوز ٧و١٦ ملي جزىء و التولبيوتاميد ١٠٠ ميكروجرام /ملليتر و الثيوفللين ٥ ملي جزىء لتوضيح كفائة خلايا البيتا في افراز الانسولين ، أظهرت النتائج أن استمرار تعرض البنكرياس المفصول من الجرذان البالغة للجلوكوز ٧و١٦ ملي جزىء يتسبب في افراز الانسولين علي مرحلتين وبعد ٣ ساعات ينخفض هذا الافراز معنويا حتي يصل الى ٢٢٪ من الافراز الأقصى حتى ٦ ساعات. كذلك بعد ١٥ دقيقة من وجود الجلوكوز ٣ ملي جزىء فان اعادة التعرض للجلوكوز ٧و١٦ ملي جزىء تحدث زيادة فى افراز الانسولين. كذلك أوضحت النتائج أن التعرض الثيوفللين لمدة ٦ ساعات لا يحدث تغيير معنوي فى قدرته على افراز الانسولين عند دخوله مرة أخرى فى المحلول. أظهرت النتائج أيضا أنه حتى بعد تعرض البنكرياس للجلوكوز ٧و١٦ ملي جزىء لمدة ٦ ساعات فان الثيوفللين قادرا على استعادة افراز الانسولين بعد فترة اضعاف الحساسية.أوضحت النتائج أيضا أن تعرض البنكرياس المفصول لمدة طويلة (٦ ساعات) للتولبيتاميد يحدث تثبيطا لمقدرته على افراز الانسولين. ويستخلص من الدراسة أن خلايا البيتا تصبح ضعيفة الحساسية وليست منهكة ليس فقط للجلوكوز ولكن أيضا للتولبيتاميد. كذلك أوضحت النتائج أن دور الاديونوزين أحادى الفوسفات الحلقي لم يتأثر فى هذا النموذج حيث أن الثيوفللين أحدث افراز للأنسولين حتى بعد تعرض البنكرياس للجلوكوز فى التركيز العالى. اضعاف الحساسية للتولبيتاميد ناتج عن حالة ازالة الاستقطاب الذى يحدثه التولبيتاميد عند تعرض البنكرياس له لمدة طويلة .كذلك تدعم هذه الدراسة أن ارتفاع تركيز الجلوكوز يؤدى الى حالة مرضية تؤدي الى خلل فى وظيفة خلايا البيتا ، وهذا التأثير يزول بزوال الجلوكوز العالى.