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Alterations of insulin secretion following long-term manipulation of ATP-sensitive potassium channels by diazoxide and nateglinide

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Abstract

Previous studies have shown that prolonged exposure to drugs, which act via blocking K_{ATP} channels, can desensitize the insulinotropic effects of drugs and nutrients acting via K_{ATP} channels. In this study, effects of prolonged exposure to diazoxide, a K_{ATP} channel opener, on beta cell function were examined using clonal BRIN-BD11 cells. The findings were compared to the long-term effects of K_{ATP} channel blockers nateglinide and tolbutamide. Following 18 h exposure to 200 μM diazoxide, the amounts of insulin secreted in response to glucose, amino acids and insulinotropic drugs were increased. Secretory responsiveness to a variety of agents acting via K_{ATP} channels was retained following prolonged diazoxide exposure. In contrast, 18 h exposure to 100 μM nateglinide significantly attenuated the insulin secretory responses to tolbutamide, nateglinide and BTS 67 582. Glucose- and L-alanine-stimulated insulin release were unaffected by prolonged nateglinide exposure, however responsiveness to L-leucine and L-arginine was diminished. Prolonged exposure to nateglinide had no effect on forskolin- and PMA-stimulated insulin release, and the overall pattern of desensitization was similar to that induced by 100 μM tolbutamide. We conclude that in contrast to chronic long-term K_{ATP} channel blockade, long-term diazoxide treatment is not harmful to K_{ATP} channel mediated insulin secretion and may have beneficial protective effects on beta cell function.

Keywords: Clonal pancreatic β-cells; Insulin release; K_{ATP} channels; Diazoxide; Nateglinide; Tolbutamide

ATP-sensitive potassium channels are key regulators of insulin secretion by the pancreatic beta cell. Physiologically, these channels are closed as a result of changes in the intracellular ATP/ADP ratio following metabolism of glucose and other nutrients, such as certain amino acids. This elicits membrane depolarisation and elevation of cytoplasmic Ca^{2+} due to increased Ca^{2+} influx through voltage-dependent calcium channels. This, in turn, triggers a complex sequence of intracellular events resulting in insulin secretion [1]. K_{ATP} channels may also be closed pharmacologically. Several classes of insulinotropic drugs used in treatment of type 2 diabetes mellitus act via binding to sites on the K_{ATP} channel, causing channel closure, calcium influx and subsequent insulin secretion [2]. Examples of these drugs are sulphonylureas, such as tolbutamide and glibenclamide, and new drugs, such as the meglitinide analogue nateglinide [3], and the guanidine derivative BTS 67 582 [4].

Another class of drugs, which regulate K_{ATP} channel activity, are the K_{ATP} channel openers, such as diazoxide and pinacidil. By opening K_{ATP} channels they hyperpolarize the beta cell, inhibiting Ca^{2+} entry and thus, glucose-stimulated insulin secretion [5]. Although less widely used clinically than K_{ATP} channel blockers, K_{ATP} channel openers are used to treat excessive insulin secretion in cases of insulinoma and persistent hyperinsulinism and hypoglycaemia of infancy (PHHI) [6,7]. These drugs, particularly diazoxide, have also found use in studies designed to elucidate the complex mechanisms regulating insulin release.

Previous studies from our laboratory and others have shown that prolonged exposure to drugs which close K_{ATP} channels results in reduced responsiveness of these drugs to subsequent acute challenge by drugs acting at the same binding site, a phenomenon known as desensitization. This

Abbreviations: K_{ATP} channels, adenosine triphosphate-sensitive potassium channels; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate

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observation has been most extensively characterized with regard to the sulphonylureas [8–11], though other studies have extended the findings to more novel insulinotropic drugs such as imidazolines [12,13], nateglinide [14] and BTS 67 582 [4].

Relatively little is known about the effects of long-term exposure to K_{ATP} channel openers upon beta cell function and insulin secretion. A recent study using human islets [15] has shown that prolonged exposure to 11.1 mM glucose and diazoxide results in changes to cellular insulin content and glucose-induced insulin secretion, and other studies have suggested that diazoxide treatment may improve beta cell function in models of diabetes and in human subjects [16–18]. The BRIN-BD11 cell line has been extensively characterized as a model of normal insulin secretion [12,14,19], and has been utilized previously to examine the effects of prolonged administration of a range of insulinotropic drugs on insulin secretion and cellular insulin content [4,10,11,13]. This study uses the BRIN-BD11 cell line [19] to examine the effects of prolonged exposure to diazoxide upon cellular insulin content, insulin secretion, and responsiveness to a range of insulin secretagogues representing several distinct insulin secretory pathways. The long-term effects of diazoxide are contrasted with those of recently developed and well-established antidiabetic K_{ATP} channel blocking agents, namely nateglinide and tolbutamide.

1. Materials and methods

1.1. Chemicals

Reagents of analytical grade and deionised water (Purite) were used. RPMI-1640 tissue culture medium, foetal bovine serum and antibiotics were from invitrogen, rat insulin standard was from Novo-Nordisk, and [^{125}I]-bovine insulin was from Lifescreen. BTS 67 582 was obtained from Knoll Pharmaceuticals and nateglinide was a gift from Novartis Pharmaceuticals Corporation. All other chemicals were from Sigma and BDH Chemicals Ltd.

1.2. Cell culture and measurement of insulin release

Clonal pancreatic BRIN-BD11 cells (passage numbers 20–30) were used for this study. BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11.1 mmol/l glucose and 0.3 g/l L-glutamine, and supplemented with 10% (v/v) foetal calf serum, 100 IU/ml penicillin and 0.1 g/l streptomycin at 37 °C with 5% CO_2 and 95% air. Tissue culture media were removed and replaced with fresh media every 24 h. Cells were washed with Hanks' balanced saline solution (HBSS) prior to detachment from tissue culture flasks with the aid of 0.025% trypsin (v/v) containing 1 mM EDTA and seeded at 1.5×10^5 cells/well into 24-multiwell plates. Monolayers of cells were then cultured for 18 h at 37 °C. This time period was selected on the basis of previous culture experiments using sulphonylureas [11,13]. Culture medium was then replaced with 1 ml of a Krebs ringer bicarbonate (KRB) buffer, consisting of (in mM) 115 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.28 $CaCl_2$, 1.2 KH_2PO_4, 25 Hepes and 8.4% (w/v) $NaHCO_3$ (pH 7.4) supplemented with 0.1% (w/v) bovine serum albumin and 1.1 mmol/l glucose. After 40 min preincubation at 37 °C, the buffer was replaced with 1 ml of KRB test buffer containing glucose and test agents as detailed in the table and legends to figures. After 20 min incubation at 37 °C, aliquots of test buffer were removed and stored at −20 °C for insulin radioimmunoassay [20].

1.3. Determination of cellular insulin content

After harvesting, BRIN-BD11 cells were resuspended in tissue culture medium, seeded at a density of 1.5×10^5 cells/well, and allowed to attach overnight, forming monolayers in 24-well multiplates. The culture medium was then completely removed and 500 μl of acid–ethanol solution (1.5% (v/v) HCl, 75% (v/v) ethanol, 23.5% (v/v) H_2O) was added. The cells were disrupted with the aid of a Pasteur pipette and incubated overnight at 4 °C prior to centrifugation (900 rpm) and storage at −20 °C for subsequent determination of cellular insulin content by radioimmunoassay [20].

1.4. Statistical analysis

Results are presented as mean ± standard error of the mean (S.E.M.) for a given number of observations (n). Groups of data were compared by two-way ANOVA in conjunction with Bonferroni's modified t-statistics. Differences were considered significant if $P < 0.05$.

2. Results

2.1. Effects of long-term drug exposure upon cellular insulin content

Following 18 h standard culture conditions (RPMI-1640 medium; 11.1 mM glucose), BRIN-BD11 cells had mean cellular insulin content of 64.3 ± 3.1 ng/10^6 cells (Table 1). Prolonged exposure to 200 μM diazoxide for 18 h resulted in a significantly increased (1.2-fold, $P < 0.05$) cellular insulin content. Cells which had been previously exposed to 100 μM tolbutamide or nateglinide exhibited in no significant changes in cellular insulin content (Table 1).

2.2. Responsiveness to insulinotropic drugs following long-term drug exposure

As shown in Fig. 1A, tolbutamide, nateglinide and BTS 67 582 (each at 200 μM) elicited respective 1.9-, 2.0- and

Table 1
Effects of long-term drug exposure upon cellular insulin content

Drug	Exposure (h)	Cellular insulin content ($ng/10^6$ cells)
None (standard culture)	18	64.3 ± 3.1
Diazoxide (200 μM)	18	$78.2 \pm 2.4^{*}$
Nateglinide (100 μM)	18	66.7 ± 4.2
Tolbutamide (100 μM)	18	60.9 ± 2.7

Effects of 18 h culture with diazoxide, nateglinide or tolbutamide on insulin content of BRIN-BD11 cells. BRIN-BD11 cells were cultured for 18 h in either standard tissue culture medium, or in tissue culture medium supplemented with 200 μM diazoxide, 100 μM nateglinide or 100 μM tolbutamide. Insulin content values are mean ± S.E.M. ($n = 6$).
$^{*}P < 0.05$ compared with 18 h standard culture.

2.2-fold ($P < 0.001$) insulin secretory responses after culture in standard RPMI-1640 media. Eighteen-hour culture with 200 μM diazoxide significantly increased basal insulin secretion (2.2-fold; $P < 0.001$; Fig. 1B). Following diazoxide exposure, significant insulinotropic effects of tolbutamide, nateglinide and BTS 67 582 were retained (1.4-, 1.5- and 1.3-fold increase respectively; Fig. 1B). In contrast, 18 h culture with 100 μM nateglinide significantly reduced the acute secretory responses to nateglinide and BTS 67 582, whilst that of tolbutamide was abolished (all $P < 0.001$). Following 18 h tolbutamide exposure, responses to tolbutamide and nateglinide were significantly reduced ($P < 0.001$) and the insulinotropic action of BTS 67 582 was abolished (Fig. 1D). Neither tolbutamide nor nateglinide during 18 h exposure had any effect on basal insulin secretion (Fig. 1C and D).

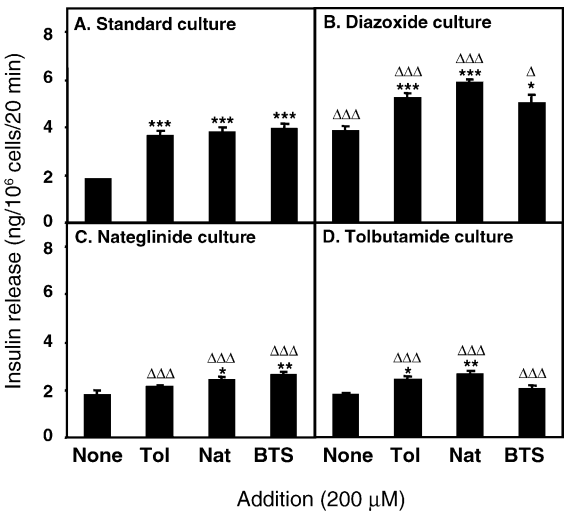

Fig. 1. Effects of 18 h culture with tolbutamide, nateglinide or diazoxide on secretory response to insulinotropic drugs. BRIN-BD11 cells were cultured for 18 h in either standard tissue culture medium (A), or in tissue culture medium supplemented with 200 μM diazoxide (B), 100 μM nateglinide (C) or 100 μM tolbutamide (D). Following 40 min preincubation with buffer containing 1.1 mM glucose, effects of 200 μM tolbutamide, nateglinide or BTS 67 582 were tested during a 20 min incubation period. Control (none) was 1.1 mM glucose. Values are mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with respect to effect in the absence of acute drug addition. $^{\triangle}P < 0.05$, $^{\triangle\triangle\triangle}P < 0.001$ compared with respect to effect following 18 h standard culture.

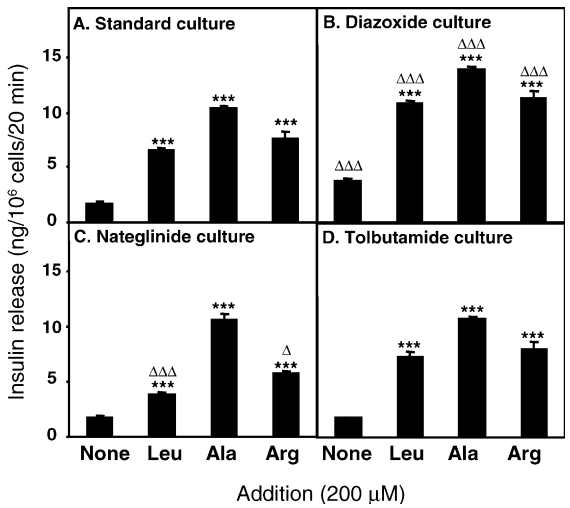

Fig. 2. Effects of 18 h culture with tolbutamide, nateglinide or diazoxide on secretory response to insulinotropic amino acids. BRIN-BD11 cells were cultured for 18 h in either standard tissue culture medium (A), or in tissue culture medium supplemented with 200 μM diazoxide (B), 100 μM nateglinide (C) or 100 μM tolbutamide (D). Following 40 min preincubation with buffer containing 1.1 mM glucose, effects of 20 mM L-leucine, L-alanine or L-arginine were tested during a 20 min incubation period. Control (none) was 1.1 mM glucose. Values are mean ± S.E.M. ($n = 6$). $^{***}P < 0.001$ compared with respect to effect in absence of acute drug addition. $^{\triangle}P < 0.05$, $^{\triangle\triangle\triangle}P < 0.001$ compared with respect to effect following 18 h standard culture.

2.3. Responsiveness to insulinotropic amino acids following long-term drug exposure

Following 18 h standard culture, L-leucine (3.7-fold increase), L-alanine (5.8-fold increase) and L-arginine (4.3-fold increase) all significantly stimulated insulin release (each $P < 0.001$, Fig. 2A). Following 18 h exposure to 200 μM diazoxide culture, there was a significant ($P < 0.001$) increase in total secretory output with each amino acid tested (Fig. 2B) when compared with standard culture conditions. Insulinotropic effects of L-leucine (2.8-fold increase), L-alanine (3.6-fold increase) and L-arginine (3.0-fold increase) remained intact (all $P < 0.001$, Fig. 2B). Following 18 h nateglinide culture, each amino acid retained a significant ($P < 0.001$) insulinotropic effect, however the secretory output in response to L-leucine (41% decrease; $P < 0.001$) and L-arginine (24% decrease; $P < 0.05$) was reduced (Fig. 2C). Insulinotropic effects of all agents were unaltered following 18 h exposure to 100 μM tolbutamide (Fig. 2D).

2.4. Responsiveness to glucose, forskolin and PMA following long-term drug exposure

Following 18 h standard culture, insulin secretion was stimulated by 16.7 mM glucose (1.4-fold increase; $P < 0.01$), 25 μM forskolin (6.4-fold increase; $P < 0.001$) or 10 nM PMA (6.9-fold increase; $P < 0.001$) (Fig. 3A). Eighteen-hour exposure to 200 μM diazoxide significantly increased the amount of insulin secreted following acute

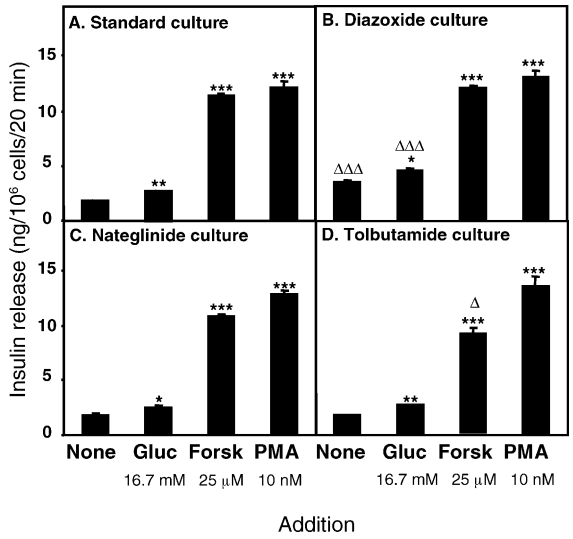

Fig. 3. Effects of 18 h culture with tolbutamide, nateglinide or diazoxide on secretory response to glucose, forskolin or PMA. BRIN-BD11 cells were cultured for 18 h in either standard tissue culture medium (A), or in tissue culture medium supplemented with 200 μM diazoxide (B), 100 μM nateglinide (C) or 100 μM tolbutamide (D). Following 40 min preincubation with buffer containing 1.1 mM glucose, effects of 16.7 mM glucose, 25 μM forskolin or 10 nM PMA were tested during a 20 min incubation period. Control (none) was 1.1 mM glucose. Values are mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with respect to effect in the absence of acute drug addition. $^{\triangle}P < 0.05$, $^{\triangle\triangle\triangle}P < 0.001$ compared with respect to effect following 18 h standard culture.

challenge by 1.1 or 16.7 mM glucose (2.1- and 1.8-fold, respectively; both $P < 0.001$; Fig. 3B). Stimulation of insulin release by 16.7 mM glucose (1.2-fold increase; $P < 0.05$) was retained following culture with diazoxide (Fig. 3B). In an additional experiment, the insulinotropic effects of 16.7 mM glucose in cells previously cultured with diazoxide were abolished when diazoxide was retained during acute incubation (data not shown in figures). Forskolin and PMA retained significant ($P < 0.001$) insulinotropic effects following 18 h diazoxide exposure, however, unlike all other conditions tested following diazoxide culture, no overall increase in amount of secreted insulin was detected in response to these agents (Fig. 3B). Culture with nateglinide had no positive or negative effect upon insulin release in response to any of the agents tested (Fig. 3C). Eighteen-hour exposure to 100 μM tolbutamide resulted in a decrease in secretory responsiveness to forskolin (16%, $P < 0.05$), while PMA- and glucose-induced insulin secretion were unaffected (Fig. 3D).

3. Discussion

This study has utilized the clonal insulin-secreting BRIN-BD11 cell line to examine the effects of prolonged exposure to diazoxide and nateglinide on insulin secretion and cellular insulin content, and compared them to the established effects of prolonged tolbutamide exposure. Each of these drugs is known to act primarily via the K_{ATP} channel complex [5,21,22], thus particular emphasis was directed to establishing the long-term effects of these agents on responses to physiological and pharmacological regulators of K_{ATP} channel activity and membrane depolarization.

Consistent with previous observations, prolonged exposure to 100 μM tolbutamide attenuated the subsequent secretory responsiveness to tolbutamide and BTS 67 582 [4,10,13]. In addition, the insulinotropic effects of the more recently developed drug, nateglinide, were markedly reduced following culture with tolbutamide. Similarly, prolonged exposure to nateglinide reduced the acute secretory responses to tolbutamide, BTS 67 582 and nateglinide itself. This effect of nateglinide is consistent with the observation that the nateglinide binding site on SUR1 shares at least one point of interaction with the binding site for tolbutamide [3]. Tolbutamide and BTS 67 582 have been reported to share signalling pathways [4] and further evidence for common mechanisms concerns the ability of prolonged exposure to BTS 67 582 to reduce the insulinotropic responses to nateglinide and tolbutamide [4].

Further experimentation revealed differences in responses to nutrient secretagogues in cells cultured with tolbutamide or nateglinide. Responsiveness to glucose and L-alanine were unaffected by long-term exposure to these drugs, whereas the effects of L-leucine and L-arginine were diminished following prolonged exposure to nateglinide but not tolbutamide. This suggests that nateglinide may alter signalling pathways shared with L-leucine and L-arginine. However, nateglinide desensitization did not alter the actions of forskolin or PMA indicating that protein kinase-mediated insulin secretion and late steps in stimulus-secretion coupling were intact.

In contrast to nateglinide and tolbutamide, diazoxide activates K_{ATP} channels, repolarising the beta cell plasma membrane, decreasing calcium influx and inhibiting glucose-induced insulin secretion [5]. Several studies have shown that diazoxide may be capable of playing a restorative role in beta cell function. When treated with diazoxide, rats subjected to a 90% pancreatectomy showed increased islet insulin content and in vitro insulin release [16]. Similarly, human islets cultured with high glucose exhibited improved cellular insulin content and glucose-induced insulin secretion and following diazoxide treatment [15,17].

Consistent with these observations, culture of BRIN-BD11 cells for 18 h with 200 μM diazoxide increased cellular insulin content. This is likely to be attributable to cellular insulin accumulation due to inhibition of insulin secretion. Thus, diazoxide has been shown not to decrease glucose metabolism in insulin-secreting cells [23], thereby allowing insulin biosynthesis to continue during diazoxide exposure [24]. It is also apparent that any possible increase in crinophagy of secretory granules [25] was more than offset by these other actions of diazoxide.

Following diazoxide culture, basal insulin secretion and total amount of insulin secreted following acute stimulation of BRIN-BD11 cells with a range of nutrients and

insulinotropic drugs were markedly and consistently increased. These data support the concept that cellular insulin stores play a role in determining the magnitude of insulin secretion [15]. The exceptions to these observations came in the form of unchanged responses to forskolin and PMA following prolonged diazoxide exposure. A possible explanation for these findings is that the increase in insulin secretion from cells previously exposed to diazoxide is a result of steps upstream of those mediated by PKA and PKC in the stimulus-secretion coupling pathway.

Following prolonged diazoxide exposure, nateglinide, tolbutamide and BTS 67 582 all retained insulinotropic responses in BRIN-BD11 cells. Thus, long-term exposure to this K_{ATP} channel opener has no apparent detrimental effect on subsequent responsiveness to drugs which close K_{ATP} channels. This may reflect the observation that diazoxide does not competitively displace sulphonylurea at the K_{ATP} channel, suggesting that diazoxide may act at a slightly different site [26]. Glucose, L-leucine, L-alanine and L-arginine also retained their insulinotropic effects following culture with diazoxide, indicating that metabolic handling and K_{ATP} channels were functionally intact. This was further confirmed by the ability of diazoxide to acutely inhibit glucose-stimulated insulin release from cells cultured with diazoxide. Activators of PKA and PKC, forskolin and PMA, also retained characteristically potent insulinotropic effects following diazoxide culture, suggesting that late stages of the insulin secretory pathway were similarly intact. Collectively, these data suggest that the functional integrity of insulin-secreting cells with regard to key secretory pathways is unaffected by prolonged exposure to diazoxide.

In summary, the data presented in this study sheds new light on the effects of prolonged exposure to nateglinide and diazoxide. Nateglinide appears to behave similarly to tolbutamide in long-term culture by down-regulating insulin-stimulatory actions of drugs which block K_{ATP} channels. In contrast, nutrient- and drug-stimulated insulin secretion mediated by K_{ATP} channels remains fully intact following prolonged diazoxide exposure. The effects of diazoxide on cellular insulin content and insulin secretion support the idea of a protective effect of diazoxide on insulin secretion.

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