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Chronic exposure to tolbutamide and glibenclamide impairs insulin secretion but not transcription of K_{ATP} channel components

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Abstract

Clonal insulin-secreting BRIN-BD11 cells were used to examine effects of chronic 72–144 h exposure to the sulphonylureas tolbutamide and glibenclamide on insulin release, cellular insulin content, and mRNA levels of the Kir6.2 and SUR1 subunits of the beta-cell K_{ATP} channel.

Chronic exposure for 72–144 h to 5–100 μM tolbutamide and glibenclamide resulted in a time- and concentration-dependent irreversible decline in sulphonylurea-induced insulin secretion. In contrast, the decline in cellular insulin content induced by chronic exposure to high concentrations of sulphonylureas was readily reversible. Chronic exposure to tolbutamide or glibenclamide had no effect upon transcription of the Kir6.2 or SUR1 subunits of the pancreatic beta-cell K_{ATP} channel.

Whilst further studies are required to understand the precise nature of the chronic interactions of sulphonylurea with the insulin exocytotic mechanism, these observations may partially explain the well-known progressive failure of sulphonylurea therapy in type 2 diabetes.

Keywords: Sulphonylureas; Clonal pancreatic beta-cells; Insulin release; K_{ATP} channels

1. Introduction

Insulinotropic sulphonylureas such as tolbutamide and glibenclamide have found widespread application in drug therapy of type 2 diabetes mellitus [1,2]. Following acute or short-term administration, they exert a hypoglycaemic action, mainly due to a direct stimulation of insulin secretion [3]. This stimulatory action is mediated via the pancreatic beta-cell K_{ATP} channel [4,5], which functions as a heterooctameric protein of four Kir6.2 and four SUR1 subunits [6,7]. Prevailing opinion holds that Kir6.2 acts as the pore of the K_{ATP} channel complex, while SUR1 endows Kir6.2 with sensitivity to sulphonylureas, as well as diazoxide and MgADP [4,5]. Binding of sulphonylurea to SUR1 leads to K_{ATP} channel closure, evoking a sequence of events including membrane depolarisation and elevation of cytoplasmic Ca^{2+} due to increased Ca^{2+} influx through voltage-dependent calcium channels, ultimately leading to exocytosis of insulin-containing secretory granules [5,6].

Although sulphonylureas are known to exert acute stimulatory effects upon insulin secretion [3], several studies have suggested that chronic treatment with these drugs leads to a decline in their insulinotropic activity [8–11], an observation which has been attributed to a direct desensitisation of the pancreatic beta-cell to the actions of these drugs [12,13]. This desensitisation effect has been suggested to be due to a decline in beta-cell K_{ATP} channel activity [14,15]. Such observations may be of clinical significance given the tendency of sulphonylurea therapy for type 2 diabetes to progressively fail [16].

In vitro studies of the chronic effects of sulphonylureas on insulin secretion have been limited by the decline in insulin production by pancreatic islets during extended periods in tissue culture [17]. This difficulty has been largely surmounted by the development of insulin-secreting cell lines which are stable over time in tissue culture [18,19]. One such cell line is BRIN-BD11, developed by electrofusion of New England Deaconess Hospital (NEDH) rat pancreatic beta-cells with RINm5F cells [20], a cell line originally derived from an NEDH rat insulinoma [21]. The BRIN-BD11 cell line has been shown to possess key com-

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ponents of the insulin secretory mechanism [18,19,22–24], and has been utilised to examine the effects of prolonged administration of a range of insulinotropic drugs, including sulphonylureas, on insulin secretion [24–27]. These studies have indicated that prolonged (18 h) exposure of insulin-secreting cells to sulphonylurea induces a specific and readily reversible desensitisation of sulphonylurea action without causing an intrinsic defect in the K_{ATP} channel [25–27]. This study aims to examine the effects of longer periods of exposure of BRIN-BD11 cells to these drugs on insulin secretion and cellular insulin content. Additionally, molecular biology techniques have been used to examine the effects of chronic sulphonylurea treatment on mRNA transcript levels of components of the beta-cell K_{ATP} channel, namely Kir6.2 and SUR1.

2. Methods

2.1. Chemicals

Reagents of analytical grade and deionised water (Purite, Oxon, UK) were used. RPMI-1640 tissue culture medium, foetal bovine serum, antibiotics, TRIZOL® reagent and *Superscript One-Step* RT-PCR system were from GibcoBRL (Paisley, Strathclyde, UK), rat insulin standard was from Novo-Nordisk (Bagsvaerd, Denmark), and [^{125}I]-bovine insulin was from Lifescreen (Watford, UK). All other chemicals were from Sigma and BDH Chemicals Ltd. (both of Poole, Dorset, UK).

2.2. 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 mM glucose and 0.3 g l^{-1} L-glutamine, and supplemented with 10% (v/v) foetal calf serum, 100 IU ml^{-1} penicillin and 0.1 g l^{-1} 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 containing 1 mM EDTA, and seeded at 1.5×10^5 cells per well into 24-multiwell plates. Monolayers of cells were then cultured for 18 h at 37 °C. 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 1 M $NaHCO_3$ (pH 7.4) supplemented with 0.1% bovine serum albumin and 1.1 mM glucose [20]. 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 legends to figures. After 20 min incubation at 37 °C, aliquots of test buffer were removed and stored at −20 °C for insulin radioimmunoassay [28].

2.3. Determination of cellular insulin content

After harvesting, BRIN-BD11 cells were resuspended in tissue culture medium, seeded at a density of 2.5×10^5 cells per 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.

2.4. RT-PCR analysis of Kir6.2 and SUR1 expression

Amplification of the Kir6.2 and SUR1 sub-units of BRIN-BD11 cell K_{ATP} channels was carried out using the *Superscript One-Step* RT-PCR system (GibcoBRL, UK). Using this method both cDNA synthesis and PCR are performed in a single tube. BRIN-BD11 cell mRNA was isolated using TRIZOL® reagent (GibcoBRL, UK). One hundred nanograms of RNA template was added to a sterile Eppendorf tube along with reaction mixture containing 2.4 mM of each dNTP and 2.4 mM $MgSO_4$, enzyme mixture consisting of superscript II reverse transcriptase and Taq polymerase and 200 pmol of each sense and antisense primer. Kir6.2 primer sequences were aggtaattgggcaaaagcag (forward) and agtgtcccccagacaaagtg (reverse), resulting in a 500 bp fragment. SUR1 primer sequences were ctcttcatcaccttccccat (forward) and agaaaggtcctttgcagcag (reverse), resulting in a 1000 bp fragment.

RNA was first denatured at 45 °C for 30 min and 94 °C for 2 min. cDNA was amplified over 2 h with 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Ten microliters of the final amplification mixture was separated in a 2% agarose gel containing ethidium bromide.

2.5. 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 $P < 0.05$.

3. Results

3.1. Effects of chronic tolbutamide exposure on sulphonylurea-stimulated insulin secretion

Both tolbutamide (1.9-fold increase; $P < 0.001$) and glibenclamide (2.9-fold increase; $P < 0.001$) stimulated insulin release from BRIN-BD11 cells cultured under standard conditions (Fig. 1). Culture with 25–100 μM tolbu-

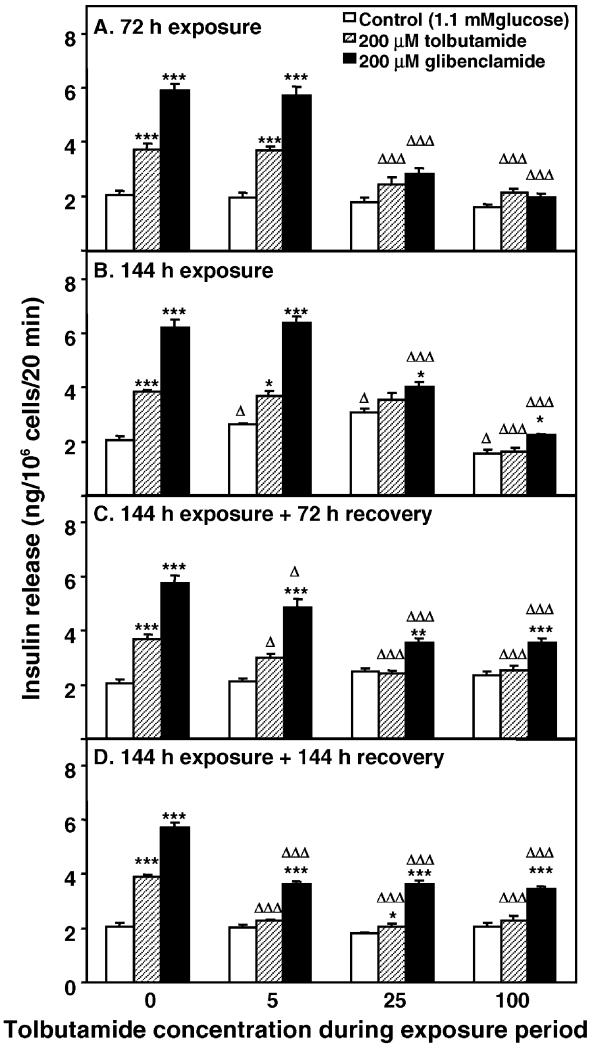

Fig. 1. BRIN-BD11 cells were cultured for 72 or 144 h in either standard RPMI-1640 tissue culture medium, or RPMI supplemented with 5, 25 or 100 μM tolbutamide, media being changed every 24 h. Following 144 h culture with tolbutamide, culture media were replaced by standard RPMI medium for a further 72 or 144 h, media being changed every 24 h. Following 40 min preincubation, effects of 200 μM tolbutamide or glibenclamide were tested during a 20 min acute exposure period in the presence of 1.1 mM glucose. Values are mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control (1.1 mM glucose). $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.001$ when compared with culture in absence of tolbutamide.

tamide for 72–144 h significantly reduced acute secretory responsiveness to tolbutamide and glibenclamide (Fig. 1A and B).

The stimulatory effect of tolbutamide on insulin release was abolished following 144 h exposure to 25 or 100 μM tolbutamide, whilst glibenclamide retained an insulinotropic action (1.3- and 1.5-fold increase; each $P < 0.05$) (Fig. 1B). When these secretory data are expressed as a percentage of cellular insulin content (as reported in Table 1), the situation is exactly mirrored, with tolbutamide lacking a secretagogue effect and glibenclamide evoking an insulinotropic response (1.3- and 1.5-fold increase; $P < 0.01$ and $P < 0.001$).

Exposure to standard culture conditions for 72–144 h (recovery time) following 144 h exposure to tolbutamide resulted in decreased tolbutamide-stimulated insulin secretion (Fig. 1C and D). Tolbutamide was without acute insulinotropic effect in cells which had been previously exposed to 25 or 100 μM of the drug, whilst glibenclamide retained an insulinotropic action (1.4- and 1.5-fold increase, respectively; both $P < 0.001$) (Fig. 1C and D). Cells which had previously been cultured in the presence of 100 μM tolbutamide for 144 h exhibited no difference in secretory responsiveness between 72 and 144 h recovery periods (Fig. 1D).

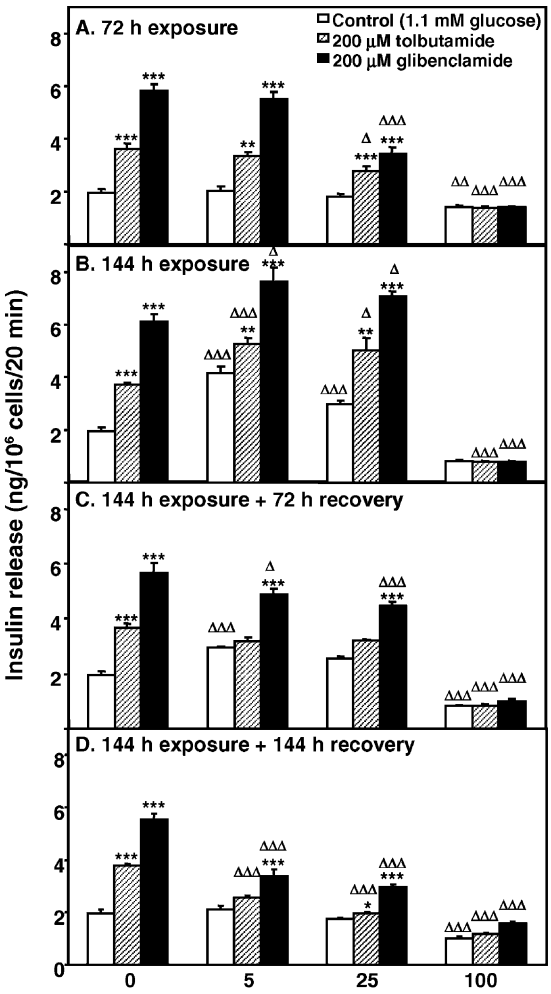

Fig. 2. BRIN-BD11 cells were cultured for 72 or 144 h in either standard RPMI-1640 tissue culture medium, or RPMI supplemented with 5, 25 or 100 μM glibenclamide, media being changed every 24 h. Following 144 h culture with glibenclamide, culture media were replaced by standard RPMI medium for a further 72 or 144 h, media being changed every 24 h. Following 40 min preincubation, effects of 200 μM tolbutamide or glibenclamide were tested during a 20 min acute exposure period in the presence of 1.1 mM glucose. Values are mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when compared with control (1.1 mM glucose). $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ when compared with culture in absence of glibenclamide.

Table 1
Effects of chronic sulphonylurea exposure upon cellular insulin content

Sulphonylurea concentration (μM)	Exposure time (h)	Recovery time (h)	Cellular insulin content (ng/10^6 cells)	
			Tolbutamide	Glibenclamide
0	72	0	60.1 ± 1.9	60.1 ± 1.9
5	72	0	58.9 ± 2.2	37.0 ± 2.2***
25	72	0	27.3 ± 0.7***	29.5 ± 0.5***
100	72	0	28.3 ± 0.8***	16.1 ± 0.5***
0	144	0	58.7 ± 2.2	58.7 ± 2.2
5	144	0	59.6 ± 3.2	56.7 ± 2.8
25	144	0	58.0 ± 2.8	52.4 ± 2.7
100	144	0	22.0 ± 1.3***	19.0 ± 0.7***
0	144	72	61.0 ± 2.7	61.0 ± 2.7
5	144	72	55.3 ± 1.8	55.3 ± 4.1
25	144	72	55.8 ± 3.4	62.9 ± 3.6$^{\Delta}$
100	144	72	53.9 ± 0.9$^{*,\Delta\Delta\Delta}$	23.2 ± 1.1$^{***,\Delta\Delta}$
0	144	144	57.5 ± 3.0	57.5 ± 3.0
5	144	144	54.0 ± 3.4	56.7 ± 4.4
25	144	144	50.7 ± 1.2$^{\Delta}$	53.9 ± 2.2
100	144	144	60.0 ± 5.7$^{\Delta\Delta\Delta}$	27.7 ± 1.0$^{***,\Delta\Delta\Delta}$

BRIN-BD11 cells were cultured for 72 or 144 h in either standard RPMI-1640 tissue culture medium, or RPMI supplemented with 5, 25 or 100 μM tolbutamide or glibenclamide, media being changed every 24 h. Following 144 h culture with tolbutamide, culture media were replaced by standard RPMI medium for a further 72 or 144 h, media being changed every 24 h. Cellular insulin content values are mean ± S.E.M. ($n = 6$). $^{*}P < 0.05$, $^{***}P < 0.001$ when compared with culture in absence of sulphonylurea. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ when compared with 0 h recovery.

3.2. Effects of chronic glibenclamide exposure on sulphonylurea-stimulated insulin secretion

Secretory responsiveness of BRIN-BD11 cells to 200 μM tolbutamide or glibenclamide was unaffected by 72 h exposure to 5 μM glibenclamide, whereas after 144 h at 5 or 25 μM glibenclamide there was enhanced stimulation of insulin release (Fig. 2A and B). However, secretory responsiveness was progressively attenuated by increasing concentrations and duration of exposure to glibenclamide. Indeed, culture for 72–144 h with 100 μM glibenclamide abolished the insulinotropic actions of both tolbutamide and glibenclamide (Fig. 2A and B). This abolition is also noted when secretory output data is expressed as a percentage of cellular insulin content (as reported in Table 1).

Culture for 72–144 h exposure to standard culture conditions after exposure to glibenclamide reduced basal and sulphonylurea-induced insulin release (Fig. 2C and D). This effect was evident following culture with 5 μM glibenclamide and increased in severity with increasing glibenclamide concentration and duration of the subsequent culture period.

3.3. Effects of chronic exposure to sulphonylureas on glucose-induced insulin release

72–144 h exposure to 100 μM tolbutamide or 100 μM glibenclamide resulted in a time-dependent maximal 30 or 43% ($P < 0.001$ or $P < 0.001$, respectively) decrease in 16.7 mM glucose-induced insulin release. A 72 h 'recovery' period partially restored the secretory response to 16.7 mM glucose (1.4-fold increase; $P < 0.01$) after 144 h prior exposure to 100 μM tolbutamide. The effects of 100 μM glibenclamide were less readily reversed. There was no recovery by 72 h, but glucose-stimulated insulin release was increased 1.4-fold increase ($P < 0.01$) after 144 h recovery.

3.4. Effects of chronic exposure to sulphonylureas on cellular insulin content

Exposure of BRIN-BD11 cells to 25 or 100 μM tolbutamide during 72–144 h culture decreased cellular insulin content (Table 1). This effect was reversed by 72–144 h recovery under standard culture conditions. Glibenclamide at 5, 25 or 100 μM similarly decreased cellular insulin content during 72–144 h cultures. The effect was more pronounced than equimolar tolbutamide, but reversible by 72–144 h subsequent culture following all but the highest (100 μM) glibenclamide concentration (Table 1).

3.5. Effects of chronic exposure to sulphonylureas on expression of Kir6.2 and SUR1

RNA samples from BRIN-BD11 cells under each culture condition tested were analysed by RT-PCR for K_{ATP} channel subunits Kir6.2 and SUR1. These studies revealed the presence of both Kir6.2 and SUR1 transcripts in BRIN-BD11 cells (Fig. 3). Kir6.2 and SUR1 mRNA remained detectable following 144 h culture in the presence of 5, 25 or 100 μM tolbutamide or glibenclamide (Fig. 3A and C). Following 144 h exposure to these sulphonylureas, cells were cultured for a further 144 h under standard conditions. No differences

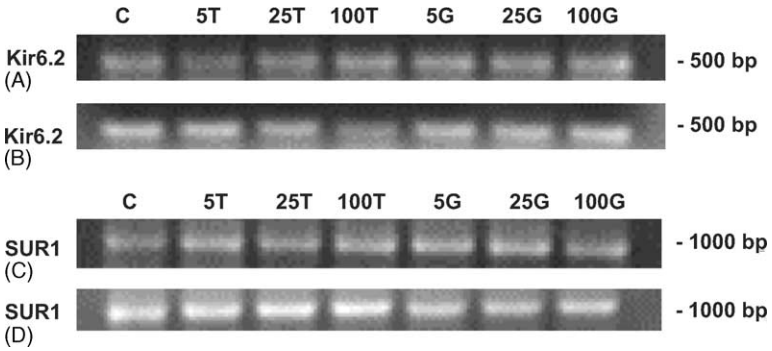

Fig. 3. RT-PCR analysis of Kir6.2 (A, B) and SUR1 (C, D) mRNA in BRIN-BD11 cells following chronic sulphonylurea exposure. Cells were cultured for a period of 144 h in the presence of tolbutamide or glibenclamide (A, C), following which media were removed and cells cultured for a further 144 h under standard (sulphonylurea-free) culture conditions (B, D). 144 h exposure to C—control (standard culture conditions); 5T—5 μM tolbutamide; 25T—25 μM tolbutamide; 100T—100 μM tolbutamide; 5G—5 μM glibenclamide; 25G—25 μM glibenclamide and 100G—100 μM glibenclamide.

in Kir6.2 or SUR1 transcription levels were noted following these conditions (Fig. 3B and D).

4. Discussion

Previous studies [25–27] have demonstrated a reversible inhibitory effect of 18 h sulphonylurea exposure on insulin secretion from BRIN-BD11 cells. Consistent with these observations, 72 h exposure to 100 μM tolbutamide was found to inhibit sulphonylurea-induced insulin release. Both basal and sulphonylurea-induced insulin secretion were inhibited following 144 h exposure but subsequent culture under drug-free conditions restored basal insulin secretion. However, the acute secretory responses to tolbutamide or glibenclamide of cells previously cultured for 144 h with 100 μM tolbutamide were less readily reversed. This indicates that chronic exposure to tolbutamide caused lasting damage to component(s) of the insulin secretory mechanism required for sulphonylurea-induced insulin release.

Culture with 100 μM glibenclamide caused a greater inhibition of insulin secretion than equimolar tolbutamide. Despite a partial restoration of insulin secretion following 144 h subsequent exposure to standard culture following previous exposure to 100 μM glibenclamide, basal and sulphonylurea-induced insulin secretion remained markedly impaired. This is not surprising, given that glibenclamide is a more potent agent than tolbutamide [3], and by penetrating the beta-cell may have a slower wash-out from intracellular binding sites.

Chronic exposure to lower concentrations of tolbutamide and glibenclamide had more complex effects upon insulin release. 144 h exposure to 5 or 25 μM tolbutamide or glibenclamide increased basal insulin secretion, corresponding with previous observations using MIN-6 cells [15]. This was associated with increased acute secretory effects of tolbutamide and glibenclamide from cells cultured with glibenclamide. Acute effects of both tolbutamide and glibenclamide were reduced 72–144 h after drug-free culture following previous 144 h exposure to 5 or 25 μM tolbutamide. The apparent inhibition of insulin release seems to be triggered during the period of sulphonylurea exposure, as cells cultured in absence of sulphonylurea responded normally. Collectively, these data indicate that the impairment of sulphonylurea-induced insulin secretion following long-term sulphonylurea exposure is at best partially reversible. Consistent with this view, 72–144 h recovery after sulphonylurea exposure was associated with significant but not absolute restoration of acute glucose-induced insulin release. The extent of reversibility was less following glibenclamide, possibly reflecting its intracellular accumulation by the beta-cells.

Unlike nutrient secretagogues, sulphonylureas do not stimulate insulin biosynthesis, but rather only stimulate release of preformed insulin, leading to beta-cell degranulation [1,29,30]. Accordingly, chronic 72 h exposure of BRIN-BD11 cells to tolbutamide (25 and 100 μM) and glibenclamide (5, 25 and 100 μM) decreased cellular insulin content. The more marked depletion by glibenclamide was expected, given that it is a more potent insulin secretagogue than tolbutamide [3]. The effects of 100 μM sulphonylurea persisted but insulin content of cells cultured at 25 μM tolbutamide or 5 or 25 μM glibenclamide were normalised after recovery for 144 h. Interestingly, restoration of cellular insulin content corresponded with unexpected increases in sulphonylurea-stimulated insulin release, implying that these events may be linked. However, a clear dissociation between the chronic effects of sulphonylureas on insulin secretion and cellular insulin content existed under other experimental conditions. This is particularly interesting in light of the fact that sulphonylurea receptors have been reported to be localised upon insulin secretory granules [31], but further studies must be undertaken to determine if this is due to an effect of sulphonylureas on the exocytotic mechanism.

As sulphonylureas regulate insulin secretion by interacting with K_{ATP} channels [4,5], it was clearly valuable to examine the effects of chronic drug exposure on the

transcription of components of these channels. RT-PCR analysis revealed expression of both Kir6.2 and SUR1 mRNA in BRIN-BD11 cells, consistent with previous observations [22]. Kir6.2 represents the ATP-sensitive pore of the beta-cell K_{ATP} channel, whereas the SUR1 subunit confers sensitivity to sulphonylureas [4,5]. No differences in transcript levels of either subunit was noted under any culture conditions tested. Thus, transcription of these K_{ATP} channel components escapes the detrimental effects of chronic sulphonylurea exposure, even at concentrations which markedly reduce insulin secretion and cellular insulin content. However, it remains a possibility that the functional integrity of K_{ATP} channels is altered during chronic exposure to insulinotropic drugs.

In conclusion, this study has demonstrated that chronic exposure to tolbutamide and glibenclamide has detrimental effects on insulin secretion and cellular insulin content without affecting transcription of K_{ATP} channel components. Some actions are irreversible, possibly contributing to the tendency of sulphonylurea therapy of type 2 diabetes to progressively fail. This lends further weight to an earlier call [13] to consider intermittent, rather than continuous sulphonylurea therapy, in type 2 diabetes.

Acknowledgements

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