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Authors
Ball, Andrew J
McCluskey, J T
Flatt, P R
et al.

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Chronic exposure to tolbutamide and glibenclamide impairs insulin secretion but not transcription of K$_{\text{ATP}}$ channel components

Andrew J. Ball*, Jane T. McCluskey, Peter R. Flatt, Neville H. McClenaghan

School of Biomedical Sciences, University of Ulster, Coleraine BT52 1SA, Northern Ireland, UK

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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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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$_{\text{ATP}}$ 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-

* Corresponding author. Present address: UCSD Cancer Center, 9500 Gilman Drive, La Jolla, CA 92039-0816, USA. Tel.: +1-858-822-4178; fax: +1-858-822-4181.
E-mail address: ajball@ucsd.edu (A.J. Ball).

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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<sub>ATP</sub> 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<sub>ATP</sub> 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<sup>®</sup> reagent and Superscript One-Step RT-PCR system were from GibcoBRL (Paisley, Strathclyde, UK), rat insulin standard was from Novo-Nordisk ( Bagsvaerd, Denmark), and [125I]-bovine insulin was from Lifescience (Wartho, 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<sup>−1</sup> t-glutamine, and supplemented with 10% (v/v) foetal calf serum, 100 IU ml<sup>−1</sup> penicillin and 0.1 g l<sup>−1</sup> streptomycin at 37°C with 5% CO<sub>2</sub> 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<sup>5</sup> 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<sub>4</sub>, 1.28 CaCl<sub>2</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 Hepes and 1 M NaHCO<sub>3</sub> (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<sup>5</sup> 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-saline solution (1.5%, v/v, HCl, 75%, v/v, ethanol, 23.5%, v/v, H<sub>2</sub>O) 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<sub>ATP</sub> 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<sup>®</sup> 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<sub>4</sub>, 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 aggaataggggaaacagcg (forward) and agtgctccccagcacaagtc (reverse), resulting in a 500 bp fragment. SUR1 primer sequences were cctccatcaccctcccat (forward) and agaaaagctcctgccgacag (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-
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, 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). ΔP < 0.05, ΔΔP < 0.001 when compared with culture in absence of tolbutamide.

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).

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, 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). ΔP < 0.05, ΔΔP < 0.001 when compared with culture in absence of glibenclamide.

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).
Table 1
Effects of chronic sulphonylurea exposure upon cellular insulin content

<table>
<thead>
<tr>
<th>Sulphonylurea concentration (µM)</th>
<th>Exposure time (h)</th>
<th>Recovery time (h)</th>
<th>Cellular insulin content (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Tolbutamide</td>
</tr>
<tr>
<td>0</td>
<td>72</td>
<td>0</td>
<td>60.1 ± 1.9</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>0</td>
<td>58.9 ± 2.2</td>
</tr>
<tr>
<td>25</td>
<td>72</td>
<td>0</td>
<td>27.3 ± 0.7***</td>
</tr>
<tr>
<td>100</td>
<td>72</td>
<td>0</td>
<td>28.3 ± 0.8***</td>
</tr>
<tr>
<td>0</td>
<td>144</td>
<td>0</td>
<td>58.7 ± 2.2</td>
</tr>
<tr>
<td>5</td>
<td>144</td>
<td>0</td>
<td>59.6 ± 3.2</td>
</tr>
<tr>
<td>25</td>
<td>144</td>
<td>0</td>
<td>58.0 ± 2.8</td>
</tr>
<tr>
<td>100</td>
<td>144</td>
<td>0</td>
<td>22.0 ± 1.3***</td>
</tr>
<tr>
<td>0</td>
<td>144</td>
<td>72</td>
<td>61.0 ± 2.7</td>
</tr>
<tr>
<td>5</td>
<td>144</td>
<td>72</td>
<td>55.3 ± 1.8</td>
</tr>
<tr>
<td>25</td>
<td>144</td>
<td>72</td>
<td>55.8 ± 3.4</td>
</tr>
<tr>
<td>100</td>
<td>144</td>
<td>72</td>
<td>53.9 ± 0.9**</td>
</tr>
<tr>
<td>0</td>
<td>144</td>
<td>144</td>
<td>57.5 ± 3.0</td>
</tr>
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<td>5</td>
<td>144</td>
<td>144</td>
<td>54.0 ± 3.4</td>
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<tr>
<td>25</td>
<td>144</td>
<td>144</td>
<td>50.7 ± 1.2**</td>
</tr>
<tr>
<td>100</td>
<td>144</td>
<td>144</td>
<td>60.0 ± 5.7***</td>
</tr>
</tbody>
</table>

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 144 h, media being changed every 24 h. Following 144 h culture in the presence of 5, 25 or 100 µM tolbutamide, culture media were replaced by standard RPMI medium for a further 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. ΔP < 0.05, ΔΔΔP < 0.01, ΔΔΔΔP < 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
and glibenclamide were reduced 72–144 h after drug-free conditions restored basal insulin secretion. How-

ever, the acute secretory responses to tolbutamide or glibenclamide increased basal insulin secretion, corre-

sponding to inhibition of insulin secretion than equimolar tolbutamide [3], and by penetrating a more potent agent than tolbutamide [3] , and by penetrating the beta-cell may have a slower wash-out from intracellular drug-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 \( \mu\)M 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 \( \mu\)M 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 \( \mu\)M) and glibenclamide (5, 25 and 100 \( \mu\)M) 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 \( \mu\)M sulphonylurea persisted but insulin content of cells cultured at 25 \( \mu\)M tolbutamide or 5 or 25 \( \mu\)M 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 \( \text{K}_{\text{ATP}} \) 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<sub>ATP</sub> 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<sub>ATP</sub> 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<sub>ATP</sub> channels is altered during chronic exposure to insulinitropic 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<sub>ATP</sub> 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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