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# Regulation by tolbutamide and diazoxide of the electrical activity in mouse pancreatic $\beta$-cells recorded *in vivo*

[1]Ana Gomis & [2]Miguel Valdeolmillos

Instituto de Neurociencias-Departamento de Fisiología, Universidad Miguel Hernandez, Facultad de Medicina, Campus de San Juan, 03550 Alicante, Spain

**1** The glucose-dependence of $\beta$-cell electrical activity and the effects of tolbutamide and diazoxide were studied in anaesthetized mice.

**2** In untreated animals there was a direct relationship between glycaemia and the burst pattern of electrical activity. Animals with high glucose concentration showed continuous electrical activity. The application of insulin led to a steady decrease in blood glucose concentration and a transition from continuous to oscillatory activity at $7.7 \pm 0.1$ mM glucose (mean $\pm$ s.d.) and a subsequent transition from oscillatory to silent at $4.7 \pm 0.6$ mM glucose.

**3** At physiological blood glucose concentrations the electrical activity was oscillatory. The injection of tolbutamide (1800 mg $kg^{-1}$) transformed this oscillatory pattern into one of continuous electrical activity. The increased electrical activity was associated with a decrease in blood glucose concentration from $7.1 \pm 0.9$ (control) to $5.5 \pm 1.0$ mM (10 min after tolbutamide injection). The effects of tolbutamide are consistent with a direct blocking effect on the $K_{ATP}$ channel that leads to membrane depolarization.

**4** The injection of diazoxide (6000 mg $kg^{-1}$) hyperpolarized the cells and transformed the oscillatory pattern into a silent one. This is consistent with a direct stimulant effect by diazoxide on the $K_{ATP}$ channel. The use of tolbutamide or diazoxide correspondingly led to the lengthening or shortening of the active phase of electrical activity, respectively. This indicates that *in vivo*, such activity can be modulated by the relative degree of activation or inhibition of the $K_{ATP}$ channel.

**5** These results indicate that under physiological conditions, tolbutamide and diazoxide have direct and opposite effects on the electrical activity of pancreatic $\beta$-cells, most likely through their action on $K_{ATP}$ channels. This is consistent with previous work carried out on *in vitro* models and explains the drugs hypo- and hyperglycaemic effects.

**Keywords:** Electrical activity; $\beta$-cell; tolbutamide; diazoxide; glucose; endocrine pancreas; insulin; sulphonylureas

## Introduction

Sulphonylureas have been used for over forty years in the treatment of non-insulin-dependent diabetes mellitus. The mechanism by which tolbutamide promotes an increase in insulin secretion has been the subject of intense research and it is now accepted to be mediated by its action on adenosine 5′-triphosphate (ATP)-dependent potassium channels ($K_{ATP}$) (for reviews see Henquin, 1992; Ashcroft & Ashcroft 1992; Edwards & Weston 1993; Panten *et al*., 1996).

Sulphonylureas bind to a receptor which is present in pancreatic $\beta$-cells and block the $K_{ATP}$ channel (Sturgess *et al*., 1985; Trube *et al*., 1986), leading to membrane depolarization. At subthreshold glucose concentrations, this initiates the characteristic burst pattern of $\beta$-cell electrical activity, whereas at intermediate glucose levels, sulphonylureas lead to the lengthening of the active phases (Henquin & Meisner, 1982; Cook & Ikeuchi 1989). In both cases, the stimulated electrical activity results in the opening of voltage-dependent calcium channels, producing a parallel increase in intracellular calcium concentration (Santos *et al*., 1991; Gilon & Henquin 1992; Nadal *et al*., 1994) and the stimulation of insulin release (Gilon *et al*., 1993; Bergsten *et al*., 1994). In addition to the effects of sulphonylures mediated by changes in ionic permeability, there is recent evidence indicating that sulphonylureas may also stimulate insulin release acting at a site distal to their effect on the electrical activity (Flatt *et al*., 1994; Eliasson *et al*., 1996), although there are conflicting data (Garcia-Barrado *et al*., 1996).

Besides their clinical importance, sulphonylureas have been a fundamental tool for the isolation and molecular characterization of its target, the sulphonylurea receptor (SUR) (Aguilar & Bryan *et al*., 1995) and the associated potassium permeation subunits, which are members of a new subfamily of the $K_{ir}$ (potassium inward rectifier) channel gene family (Inagaki *et al*., 1995; Sakura *et al*., 1995).

The sulphonamide diazoxide is used clinically in the treatment of familiar hyperinsulinism, insulinoma and in hypoglycaemia due to overtreatment with sulphonylureas (Ferner & Neil, 1988; Baker *et al*., 1991). The mechanism of action is the opposite to that of tolbutamide. Diazoxide opens $K_{ATP}$ channels and hyperpolarizes pancreatic $\beta$-cells (Trube *et al*., 1986; Dunne *et al*., 1987; Kozlowski *et al*., 1989). This leads to a decrease in intracellular calcium concentration and the inhibition of insulin secretion.

Recent work has characterized the electrical activity of islets of Langerhans *in vivo*, showing that $\beta$-cells display glucose-dependent oscillatory activity (Sánchez-Andrés *et al*., 1995; Valdeolmillos *et al*., 1996; Gomis *et al*., 1996). Such studies have also shown that glucose-dependent electrical activity is maximally sensitive to changes in blood glucose concentration within the normal range of glycaemia, about 5 to 7 mM. The maximal sensitivity obtained in parallel experiments *in vitro* is 12 to 15 mM. Such a shift in the dose-response curve *in vivo* with respect to the *in vitro* situation, suggests that there are factors other than glucose, affecting glucose-sensitivity *in vivo* which are not present *in vitro*.

The recording of electrical activity *in vivo* makes it possible to test directly the effects of drugs in the intact animal, where

[1] Present address: Neurobiology Division, MRC Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH.
[2] Author for correspondence.

all the factors regulating electrical activity are present. The aim of the present investigation was to study the effect of tolbutamide and diazoxide on the electrical activity of mouse pancreatic β-cells at different blood glucose concentrations.

## Methods

The methods used for *in vivo* recording have been described elsewhere (Sánchez-Andrés *et al*., 1995). Briefly, albino mice (8–10 weeks old, 25–35 g weight) bred in our animal house were anaesthetized by intraperitoneal injection of 90 mg $kg^{-1}$ Nembutal. The degree of anaesthesia was checked periodically during the experiment by exploration of cutaneous reflexes. The experiments were carried out according to institutional animal care guidelines. During the experiment the animal was laid on its back on a heated bed maintained at 37°C. The animal was laparotomized and the duodenal part of the pancreas dissected free from adherence. The vena cava and the abdominal aorta were cannulated at their most caudal parts for injection of the drugs and blood sampling, respectively. For electrical recording, the pancreas was gently exteriorized on top of a Sylgard (Dow Corning, U.S.A.) platform. The platform was attached to a micromanipulator. The pancreas was isolated from respiratory and peristaltic movements by fixing it to the Sylgard with dissection pins.

Pancreatic β-cells were impaled with high resistance glass microelectrodes (80–120 MΩ) filled with a solution of potassium citrate 3 M + potassium chloride 100 mM, and the electrical activity recorded with an Axoclamp 2A (Axon Instruments, U.S.A.) Unfiltered records were acquired at a frequency of 300 Hz and stored on a microcomputer with Axotape for analysis off-line. Samples of blood (25 μl) were analysed for glucose concentration by the glucose oxidase method, with a Beckman glucose-analyser-2. The animals used in this study had free access to food and water. In control experiments, glycaemia was measured at 15 min intervals in animals anaesthetized and handled in the same way as experimental animals except that the electrical activity was not recorded, and was shown to be relatively constant and within the normal range.

Tolbutamide and diazoxide (Sigma) were dissolved in sterile saline solution and injected at the indicated concentration as a single bolus. In control experiments, the injection of the same volume of saline solution had no effect on the electrical activity. For drug concentration calculations, a 2 ml blood circulating volume was assumed.

## Results

Figure 1 shows the effect of tolbutamide on the electrical activity of a β-cell recorded *in vivo*. The electrical activity consisted of alternating hyperpolarized and depolarized phases. During the plateau depolarized phase there were superimposed calcium action potentials that were not individually resolved in the figure due to the time scale (see Figure 1 in Sánchez-Andrés *et al*., 1995). The blood glucose concentration measured before the injection of tolbutamide was 8.4 mM and the depolarized phases had a duration of 14 s. At the time indicated by the arrow, tolbutamide (20 μl of a 5 mM solution) was injected into the vena cava as a single bolus, leading to an immediate increase in the electrical activity consisting of a train of action potentials lasting for approximately two minutes, after which the cell re-established its previous oscillatory pattern. The first oscillation after the train of action potentials was longer (35 s) than the control ones. After a few minutes, the duration of the depolarized phase was similar to its duration before the injection of tolbutamide. However, note that at this time, the blood glucose concentration was 6.2 mM, considerably lower than before the injection of tolbutamide. In five similar experiments, the blood glucose concentration decreased from a control value of $7.1 \pm 0.9$ mM (mean ± s.d.) to $5.5 \pm 1.0$ mM ten minutes after the injection of tolbutamide.

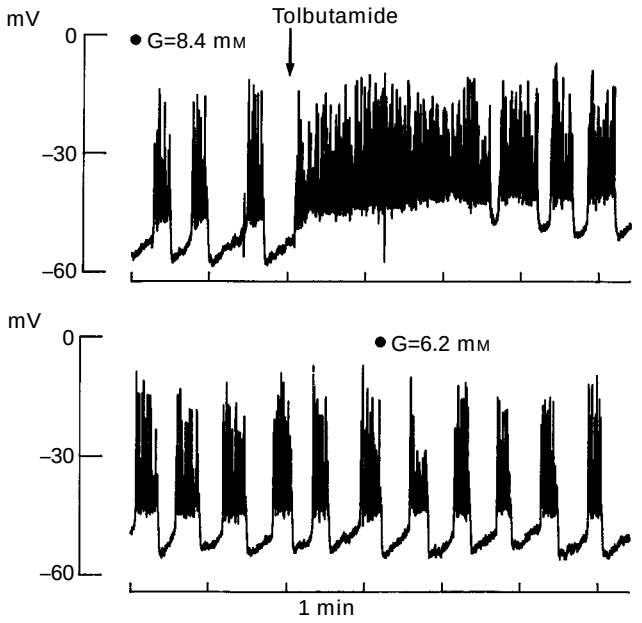


**Figure 1** Effect of low doses of tolbutamide on the electrical activity recorded *in vivo*. The arrow indicates the time at which 20 μl of a solution of tolbutamide 5 mM was injected as a single bolus. Essentially the same results were obtained in other 5 animals. Unless stated otherwise, in all figures, the lower panel is a continuation of the trace shown in the upper panel. The letter G denotes the time at which a blood sample was taken and the glycaemia measured.

The effect of tolbutamide was dose-dependent, and a larger dose of the sulphonylurea transformed the oscillatory electrical pattern into a continuous phase of active electrical activity. Figure 2 shows the effect on electrical activity of a larger dose of tolbutamide (50 μl of a 10 mM solution) injected as a single bolus. As in the previous figure, tolbutamide led to the appearance of continuous electrical activity. The frequency of spikes after the injection was higher during the first minutes of drug action and gradually decreased with time. However, even 20 min after the injection, the cell was permanently active with no signs of silent phases appearing (not shown).

The effect of tolbutamide *in vitro* is dependent of the cells metabolic status. Hence, it was of interest to test whether *in vivo* tolbutamide was able to elicit electrical activity at very low glucose concentrations, when the membrane potential of the β-cell is permanently hyperpolarized. At normal blood glucose concentrations electrical activity is oscillatory. Therefore, in order to decrease the blood glucose concentration it was necessary to treat the animals with insulin.

Figure 3 shows the effect of insulin on the electrical activity in an animal with initially high blood glucose levels. In this example, the blood glucose concentration before the start of the experiment was 11 mM, which was associated with a permanently active electrical pattern. The application of insulin, gradually decreased the blood glucose level to a point at which the electrical pattern was transformed into an oscillatory one. In 4 experiments, this transition took place

at a blood glucose concentration of $7.7 \pm 0.14$ mM (mean $\pm$ s.d., $n = 4$). As the blood glucose concentration was steadily decreasing, the frequency of bursts of electrical activity decreased in parallel, to a point where the cells remained hyperpolarized. The blood glucose concentration at which the cells changed pattern from oscillatory to permanently hyperpolarized was $4.7 \pm 0.6$ mM (mean $\pm$ s.d. $n = 18$ experiments).

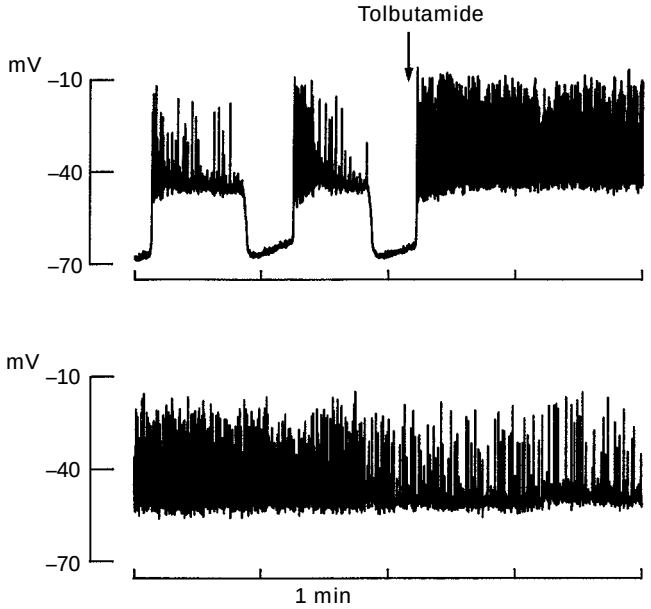


**Figure 2** Effect of higher doses of tolbutamide on the electrical activity. At the time indicated by the arrow, 50 μl of a solution of tolbutamide 10 mM were injected. In this experiment, the mean frequency of spikes during the active phases before the injection of tolbutamide was 1.3 spikes $s^{-1}$. During the first two minutes after tolbutamide, the frequency increased to 5.5 spikes $s^{-1}$ and gradually decreased over 4 min to 1.2 spikes $s^{-1}$ (last minute shown in the figure). This recording is representative of the results obtained in 3 experiments in 3 different animals.

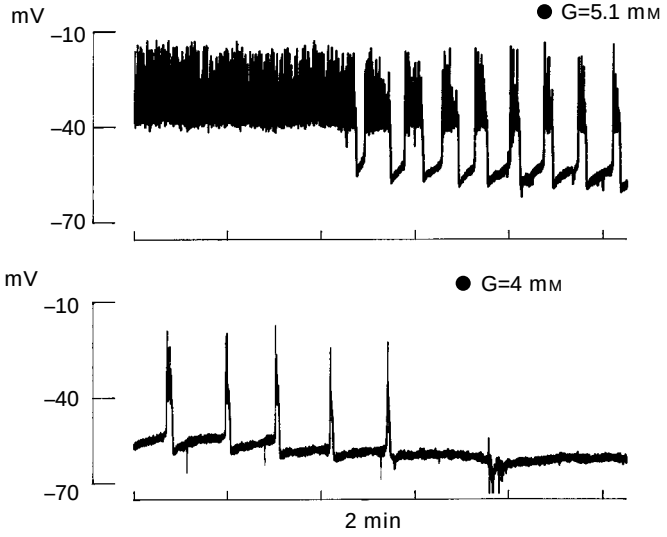


**Figure 3** Effect of insulin treatment on the electrical activity. At the beginning of the experiment, blood glucose concentration was 11.0 mM, and was associated with continuous electrical activity. Seven minutes before the record shown, 0.42 i.u. of insulin were injected through the vena cava as a single bolus. The samples of blood glucose were obtained where indicted by the dots. This experiment is representative of the effects obtained from 28 different animals.

Figure 4 shows the effect of tolbutamide on the electrical activity in an animal previously treated with insulin. The beginning of the record shows the cell when it was hyperpolarized. Tolbutamide (50 μl of a 10 mM solution) was injected at the time indicated by the arrow, eliciting an early transitory phase of continuous electrical activity of approximately 7 min duration. The cell then began to oscillate with a regular pattern that remained largely unchanged for the rest of the recording time. In this experiment the cell was still oscillating 11 min after the injection of tolbutamide (not shown). In other experiments, tolbutamide was only able to produce a transitory phase of continuous electrical activity (see figure legend).

In the next series of experiments, we tested the effects of diazoxide on the electrical activity in animals with different glucose concentrations. Figure 5 shows the effect of diazoxide on the oscillatory patern in an animal with normal glucose concentration. Diazoxide (50 μl of a 16 mM solution), injected through the vena cava during the active phase, caused an immediate hyperpolarization of the cell that subsequently remained silent for approximately one minute. After this time, the cell restarted oscillations which initially had a longer silent phase that gradually recovered to the initial ratio between active and silent phase (see figure legend).

In those situations where the initial glucose concentration was high and the electrical pattern was of continuous electrical activity, the injection of diazoxide at low doses (30 μl of a 16 mM solution) transformed the continuous activity pattern into an oscillatory one, as shown in Figure 6. In this example, the oscillatory electrical activity elicited by diazoxide remained

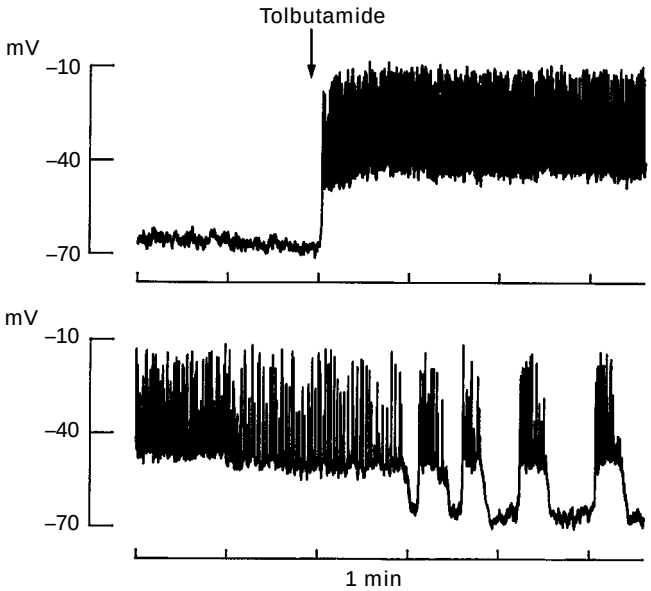


**Figure 4** Effect of tolbutamide on the electrical activity in animals with low glucose concentration. The animal was treated with insulin (0.42 i.u.) 10 min before the start of the recording. The first two minutes of the record show the cell in a continuous silent phase. At the time indicated, 50 μl of tolbutamide 10 mM were injected. In 3 out of 5 similar experiments the cells showed an initial phase of continuous electrical activity followed by the appearance of oscillations with gradually shorter active phases. After a variable time in the range of several minutes, the cells again hyperpolarized (not shown). In two other experiments, tolbutamide was only able to elicit a transitory phase of continuous electrical activity with no oscillations (not shown).

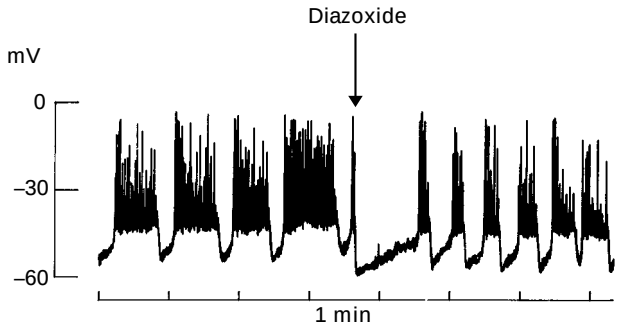


**Figure 5** Effect of diazoxide on the oscillatory electrical activity. At the time indicated by the arrow 50 μl of diazoxide 16 mM were injected as a single bolus. In this experiment, the duration of the active phase ranged from 25 to 38 s and the silent phase from 10 to 14 before the injection of diazoxide, making an active/silent ratio of 2.6. This ratio was only 0.65 in the first oscillation after diazoxide and increased to 2.3 in the last oscillation shown in the figure. The figure is representative of 7 experiments in 7 different animals.

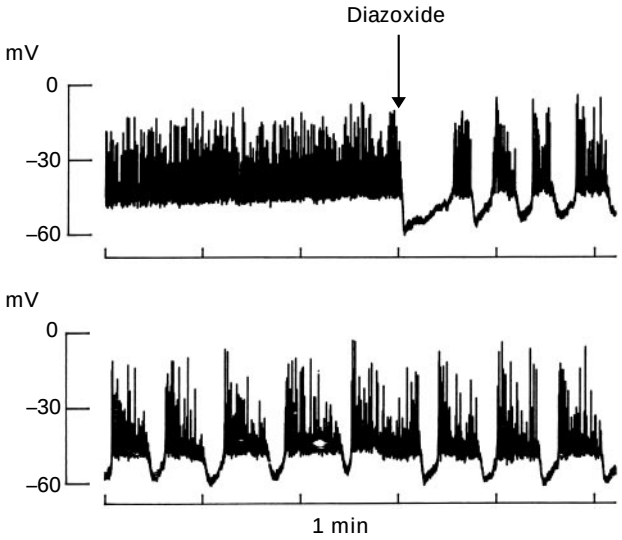


**Figure 6** Effects of diazoxide on the continuous electrical activity pattern. At the time indicated by the arrow 30 μl of a solution of diazoxide 16 mM were injected. The figure is representative of 3 experiments in 3 different animals.

for the rest of the record, lasting for about 30 min after diazoxide injection (not shown).

Figure 7 shows that higher doses of diazoxide (30 μl of a 64 mM solution) transformed a permanently active phase into a permanently silent phase without an intermediate oscillatory pattern. This permanent silent phase lasted for long periods of time (up to 20 min in one experiment if no other manoeuvres were performed). It was not the consequence of cell inexcitability due to cell damage resulting from high doses as, when an animal treated with a high concentration of diazoxide was subsequently challenged with tolbutamide, as shown in the lower panel of the figure, or with glucose (not shown) it was able to respond, at least transiently, with a burst of electrical activity.

## Discussion

The results presented in this paper show that pancreatic β-cells recorded in the intact animal respond to tolbutamide and

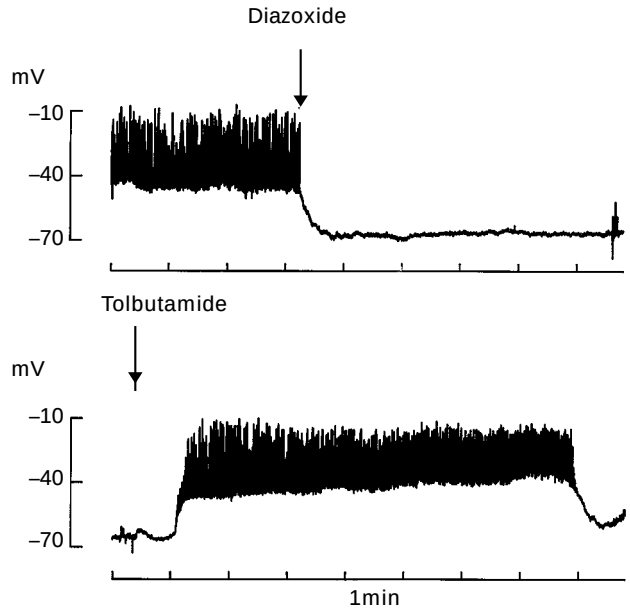


**Figure 7** Effects of maximal doses of diazoxide on a continuous electrical activity pattern. After the injection of diazoxide (30 μl of a 64 mM solution) the cell remained hyperpolarized until it was challenged with tolbutamide (20 μl of a 5 mM solution). The figure is representative of 3 experiments in 3 different animals.

diazoxide with the activation and blockade, respectively, of electrical activity. These effects are consistent with those previously shown *in vitro*, reinforcing the proposed mode of action of these drugs.

The link between electrical activity and insulin secretion is well documented, showing a tight correlation between the active phase of electrical activity (Atwater *et al.*, 1980), intracellular calcium oscillations (Santos *et al.*, 1991; Valdeolmillos *et al.*, 1993) and pulsatile insulin release (Rosario *et al.*, 1986; Gilon *et al.*, 1993). The electrical activity recorded *in vivo* follows in many respects the pattern found *in vitro*, which consisted of alternating depolarized and hyperpolarized phases the duration of which was glucose-dependent. However, an important difference is that the glucose-dependence of the electrical activity *in vivo*, unlike that of *in vitro* studies, was highly sensitive to small changes in glycaemia within the physiological range (5 to 7 mM). The results presented here support a model in which changes in electrical activity are due to the activation and inhibition of $K_{ATP}$ channels, because they can be reproduced by small doses of tolbutamide and diazoxide.

The pharmacological blockade by tolbutamide of a number of $K_{ATP}$ channels produces a change in the relationship between blood glucose concentration and the electrical activity of β-cells. This change is exemplified by the experiment shown in Figure 1, where very different glucose concentrations (8.4 and 6.2 mM) were associated with a very similar pattern of electrical activity.

The opposite effects were observed when diazoxide was applied to an animal with high blood glucose and continuous electrical activity. In this condition, diazoxide induced first the appearance of an oscillatory pattern and then hyperpolarization of the cell. In either case, the activation or inhibition of the β-cells takes place irrespective of the blood glucose levels, indicating that *in vivo* the action of such drugs is largely independent of the metabolic status of the cells.

The application of tolbutamide or diazoxide produced a biphasic effect on the electrical activity, being maximal during

the first few minutes and then continuing to act over an extended period of time. The same biphasic effect has been observed when glucose is injected (Sánchez-Andrés *et al*., 1995), and may reflect the time taken for drugs to redistribute and reach a steady plasma concentration. Alternatively, the two phases may reflect intrinsic properties in the response of islets of Langerhans, since these two phases have also been observed in experiments with isolated islets of Langerhans, when glucose is increased or tolbutamide added.

Insulin treatment of animals with initially high blood glucose concentrations, allowed us to determine precisely the blood glucose concentration (7.7 mM) at which the electrical activity was transformed from continuous to oscillatory. Likewise, the blood glucose concentration at which the electrical activity changes from an oscillatory to a permanently silent one was 4.7 mM. Such values match well with the sigmoid relationship between blood glucose concentration and the degree of electrical activity recorded *in vivo* (Sánchez-Andrés *et al*., 1995).

As a whole, the changes in the electrical pattern observed with tolbutamide and diazoxide suggest that the duration of the active and silent phases is largely dictated by the activity of $K_{ATP}$ channels. Furthermore, it seems that the activation or inhibition of only a proportion of them can drastically modify these patterns.

The results presented here are relevant to the therapeutic use of sulphonylureas, given that the sensitivity of rodent β-cells to ATP and tolbutamide seems to be similar to that of human cells (Ashcroft *et al*., 1989; Misler *et al*., 1989). Furthermore, the dosages used here (25–135 μg injected in a circulating blood volume of 2 ml) are within the pharmacological range currently used in the treatment of diabetes (above 27 $\mu g/ml^{-1}$ of plasma; Melander, 1987).

The sulphonylurea receptors are distributed in a variety of tissues such as central nervous system, heart and endothelium. In this context, the *in vivo* recording of electrical activity may be an important tool in determining the selectivity of new antidiabetic agents for pancreatic β-cells. Furthermore, electrical recording *in vivo* is a precise means of following the time course of drug action.

In conclusion, our results indicate that the therapeutic effects of tolbutamide and diazoxide are the consequence of their inhibitory and stimulant effects on $K_{ATP}$ channels. Both drugs modify the relationship between blood glucose concentration and the degree of electrical activity. The *in vivo* recording of electrical activity in pancreatic β-cells may help to define the mechanisms of action of new drugs aimed at interacting specifically with $K_{ATP}$ channels.

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