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Bursting electrical activity in pancreatic β-cells: evidence that the channel underlying the burst is sensitive to Ca^{2+} influx through L-type Ca^{2+} channels

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Abstract. In glucose-stimulated pancreatic β-cells, the membrane potential alternates between a hyperpolarized silent phase and a depolarized phase with Ca^{2+} action potentials. The molecular and ionic mechanisms underlying these bursts of electrical activity remain unknown. We have observed that 10.2–12.8 mM Ca^{2+}, 1 μM Bay K 8644 and 2 mM tetraethylammonium (TEA) trigger bursts of electrical activity and oscillations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence of 100 μM tolbutamide. The $[Ca^{2+}]_i$ was monitored from single islets of Langerhans using fura-2 microfluorescence techniques. Both the high-Ca^{2+}- and Bay-K-8644-evoked $[Ca^{2+}]_i$ oscillations overshot the $[Ca^{2+}]_i$ recorded in tolbutamide. Nifedipine (10–20 μM) caused an immediate membrane hyperpolarization, which was followed by a slow depolarization to a level close to the burst active phase potential. The latter depolarization was accompanied by suppression of spiking activity. Exposure to high Ca^{2+} in the presence of nifedipine caused a steady depolarization of approximately 8 mV. Ionomycin (10 μM) caused membrane hyperpolarization in the presence of 7.7 mM Ca^{2+}, which was not abolished by nifedipine. Charybdotoxin (CTX, 40–80 nM), TEA (2 mM) and quinine (200 μM) did not suppress the high-Ca^{2+}-evoked bursts. It is concluded that: (1) the channel underlying the burst is sensitive to $[Ca^{2+}]_i$ rises mediated by Ca^{2+} influx through L-type Ca^{2+} channels, (2) both the ATP-dependent K^+ channel and the CTX- and TEA-sensitive Ca^{2+}-dependent K^+ channel are highly unlikely to provide the pacemaker current underlying the burst. We propose that the burst is mediated by a distinct Ca^{2+}-dependent K^+ channel and/or by $[Ca^{2+}]_i$-dependent slow processes of inactivation of Ca^{2+} currents.

Key words: Pancreatic β-cell – Islet of Langerhans – Bursting electrical activity – Intracellular Ca^{2+} concentration – External calcium – Calcium oscillations

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Introduction

Raising glucose concentration above 7 mM depolarizes the pancreatic β-cell membrane and evokes electrical activity [2, 6, 17, 26]. The pattern of glucose-induced electrical activity changes with the sugar concentration: within the range 7–17 mM the electrical activity occurs in the form of bursts of spikes consisting of alternating depolarized (active) and hyperpolarized (silent) phases, whereas at higher concentrations the firing pattern becomes essentially continuous [24]. Ca^{2+}-dependent action potentials are fired along the depolarized phases of the bursts, resulting in Ca^{2+} entry and in cyclic oscillations of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) [34, 39]. This oscillatory $[Ca^{2+}]_i$ activity may underlie the pulsatile insulin release that has been measured from single mouse islets of Langerhans [33].

Bursting electrical activity is commonly regarded as the major process by which the β-cell imports Ca^{2+} from the extracellular medium. However, the molecular and ionic mechanisms underlying the burst remain unknown. According to a model originally proposed by Atwater and colleagues [3, 5] and further developed mathematically by other groups [12, 29], the β-cell bursting behaviour has been attributed to a feed-back loop involving a large-conductance Ca^{2+}-dependent K^+ channel [maxi K(Ca) channel] and $[Ca^{2+}]_i$. However, more recently this model has been challenged by the apparent lack of activity of the maxi K(Ca) channel within the range of membrane potential where bursting activity occurs [21, 37]. A Ca^{2+}-dependent K^+ current distinct from that carried by the maxi K(Ca) channel has been recently implicated in the control of β-cell membrane potential [1], but its possible involvement in regular glucose-induced β-cell bursting remains unknown.

The capacity to generate the pacemaker current necessary to hyperpolarize/depolarize the β-cell membrane during the bursts has also been attributed to the sulphonylurea-sensitive, ATP-dependent K^+ channel [K(ATP) channel] [16] and to voltage- and $[Ca^{2+}]_i$-dependent inactivation of the voltage-sensitive Ca^{2+} current [11, 19,

36]. However, we have recently found that high external Ca^{2+} levels (10−13 mM) trigger bursts of electrical activity and a $[Ca^{2+}]_i$ oscillatory activity in the presence of the sulphonylureas tolbutamide and glibenclamide [35]. Thus, an essential role for the K(ATP) channel as mediator of the β-cell bursting activity cannot be warranted.

Exposure to high Ca^{2+} levels is likely to enhance Ca^{2+} influx across the β-cell membrane. In the present work, we have assessed the possibility that other Ca^{2+} influx promoting agents [i. e. ionomycin, tetraethylammonium ions (TEA) and the L-type Ca^{2+} channel agonist Bay K 8644] might also hyperpolarize the β-cell membrane and trigger bursts of electrical activity in the presence of tolbutamide. Moreover, we have carried out a study aimed at assessing the pharmacological sensitivity of the ionic conductance underlying the bursts.

Materials and methods

Membrane potential recording. The membrane potential was recorded from microdissected mouse islets of Langerhans using a high impedance amplifier essentially as previously reported [4]. Briefly, 3- to 6-month-old female albino mice (Charles Rivers breeding) were killed by a blow to the head, followed by cervical dislodgement. Small pieces of pancreatic tissue containing well-exposed islets were pinned down to the plastic bottom of a fast perfusion chamber (volume, 40 µl) through which modified Krebs' solution flowed at a rate of approximately 2 ml/min. The solution had the following composition (mM): 125 NaCl, 5 KCl, 25 $NaHCO_3$, 2.56 $CaCl_2$, 1.1 $MgCl_2$ and 11 glucose. The solution was constantly gased with 95% O_2/5% CO_2 for a final pH of 7.4. To minimize bubble formation in the chamber, solutions were pre-heated to 38°C using a water bath. The solutions were then fed into a 4-way stop-cock valve located near the recording chamber. Although this valve was virtually dead-space free, the time required for complete solution exchange at the chamber level was approximately 4 s at a flow rate of 2 ml/min. Temperature control in the recording chamber was provided by a pre-heating stage inserted between the stop-cock valve and the chamber. Using this system, the temperature of circulating water from an external bath could be adjusted to give 37°C in the chamber.

Typical microelectrode resistances were 100−200 MΩ. The microelectrodes were pulled from borosilicate glass (OD = 2 mm, ID = 1 mm; Frederick and Haer, Maine, USA) using a microprocessor-controlled puller (model 763, Campden, UK) and filled with a 3M K-citrate/1 M KCl solution. The perfusion chamber was connected to ground through an agar bridge. The electrical signals were displayed on a storage oscilloscope, monitored on a fast chart recorder and permanently stored on digital tape via a DAT recorder (DTR 1200, Biologic, France). The data were transferred off-line at 1 kHz to a 386/33 computer via a D12-IEEE/N interface (Biologic). Data analysis was carried out using a program written in our laboratory by A. Abrunhosa. The plots were made using Sigma Plot (Jandel).

$[Ca^{2+}]_i$ recording from single islets of Langerhans. The $[Ca^{2+}]_i$ was recorded from single mouse islets of Langerhans using the Ca^{2+} indicator fura-2 [14] essentially as described previously [34, 35]. The islets were isolated by collagenase (type P, Boehringer Mannheim, Germany) digestion of the pancreas and kept incubating in HCO_3^-/CO_2-buffered modified Krebs' solution containing 3% bovine serum albumin (BSA; fraction V, protease-free, Sigma, St. Louis, Mo., USA) and 11 mM glucose for no less than 2 h. Groups of 6−12 islets were then incubated in the latter solution supplemented with 5 µM fura-2/AM for 45 min at 37°C, after which they were transferred to fura-2-free Krebs' solution (glucose concentration, 11 mM) at room temperature to minimize dye leakage.

Groups of 2−3 fura-2-loaded islets were transferred to a fast perfusion chamber placed on the stage of an inverted epifluorescence microscope (Nikon Diaphot, Japan). This chamber consisted of a 35-µl oval-shaped cavity drilled on a 2-mm wide Perspex plate. The bottom of the chamber consisted of a round glass cover slip (diameter, 16 mm) previously coated with poly-L-lysine to facilitate islet attachement. The solution leaving the recording chamber was driven into a second pool, where it was drained to waste by suction. Typically, the islets were allowed to adhere to the cover slip for approximately 5−10 min prior to starting the perfusion.

The experimental solutions were pre-heated to 38°C and fed into a 4-way stop-cock valve located near the chamber. The time required for complete solution exchange at the chamber level was approximately 5 s at a flow rate of approximately 2 ml/min, adjustable by gravity. Temperature in the chamber was 37°C.

A dual excitation microfluorescence system (Deltascan) supplied by Photon Technology International (Princeton, N. J., USA) was used to record single islet fluorescence as described previously [35]. Briefly, the islets were excited at 340 nm and 380 nm by alternately shifting the excitation light beam to one of two monochromators via a chopper wheel. The fluorescence was detected by a photomultiplier tube after passing through a band-pass interference filter centred at 510 nm. The fluorescence data were automatically corrected for background fluorescence and acquired at 10 Hz by a 386/SX 16 computer. The loss of fluorescence signal arising from photobleaching usually did not exceed 11%/h.

The fluorescence data were calibrated in vitro using the equation $[Ca^{2+}]_i = K_d\beta(R-R_{min})/(R_{max}-R)$, where the parameters have their usual meanings [14]. The fluorescences necessary for the calculation of R_{max}, R_{min} and β were obtained from the excitation spectra of fura-2 dissolved in calibration solution at $[Ca^{2+}] = 0$ and 2 mM. The calibration solutions had the following composition (mM), saturating Ca^{2+} solution: 100 KCl, 10 NaCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 3-(N-morpholino)propanesulphonic acid (MOPS) and 1−2 µM fura-2 (pH 7.0); 0 Ca^{2+} solution: 100 KCl, 10 NaCl, 1 $MgCl_2$, 2 ethylenebis(oxonitrilo)tetraacetate (EGTA), 10 MOPS, 10 tris(hydroxymethyl)aminomethane (TRIS) and 1−2 µM fura-2 (pH 7.6).

Materials. Ionomycin and Bay K 8644 were from Calbiochem (San Diego, Calif., USA). Fura-2/AM and fura-2 (acid) were from Molecular Probes (Eugene, Ore., USA). Charybdotoxin (CTX) was from Alomone Labs (Jerusalem, Israel). All other chemicals were from Sigma. Ionomycin, Bay K 8644 and fura-2/AM were added to the solutions from concentrated stocks in dimethylsulphoxide (DMSO; final DMSO concentration in the solutions, ≤ 0.1%).

Results

In the presence of 11 mM glucose, the membrane potential of islet cells alternated between hyperpolarized (silent) and depolarized (active) phases with superimposed action potentials. The cells exhibiting this pattern of electrical activity have been identified as β-cells throughout this work.

Bursts of electrical activity and $[Ca^{2+}]_i$ oscillations triggered by high external Ca^{2+} levels

Sulphonylurea drugs have long been known to evoke continuous electrical activity in the presence of 10 or 11 mM glucose [13, 15]. Raising $[Ca^{2+}]_o$ to 10.2 or

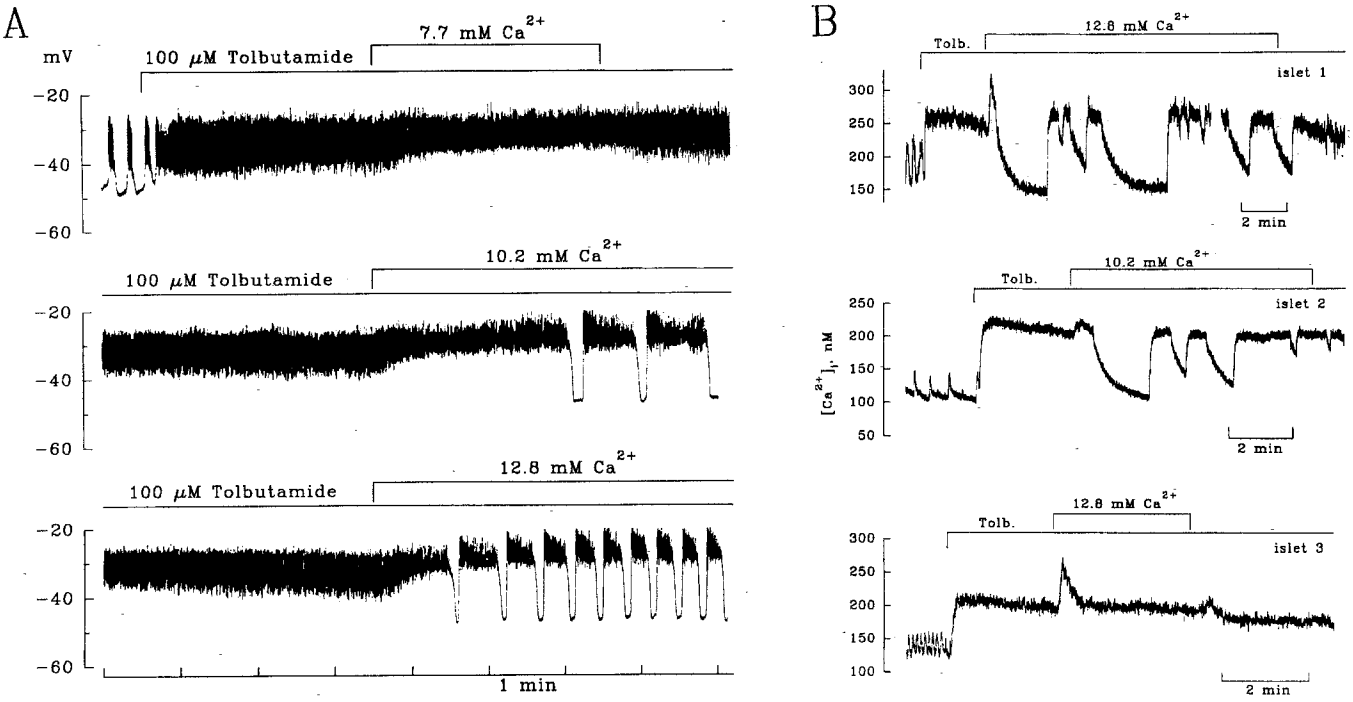

Fig. 1 A, B. Bursts of electrical activity and $[Ca^{2+}]_i$ oscillations triggered by high external Ca^{2+} levels. **A** The external Ca^{2+} concentration was raised from 2.56 mM to 7.7 mM (*upper panel*), 10.2 mM (*middle panel*) and 12.8 mM (*lower panel*) ind the presence of tolbutamide, as indicated. Same cell throughout. **B** Three different fura-2-loaded islets were challenged with 10.2 or 12.8 mM Ca^{2+} in the presence of tolbutamide (*Tolb*) as indicated. The *vertical scale* represents intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), as measured by a dual excitation epifluorescence procedure (see Materials and methods). The glucose concentration was 11 mM throughout (**A**, **B**)

12.8 mM in the presence of 11 mM glucose and 100 μM tolbutamide turned this continuous firing pattern into a bursting electrical activity (Fig. 1 A, middle and lower traces; see also [35]). In most of the experiments (17 out of 23 different islets), exposure to 10.2 or 12.8 mM Ca^{2+} triggered bursts of electrical activity in less than 50 s from the time of switching the stop-cock valve. However, occasionally the bursts were initiated with delays ranging from 1 to 3 min, an example of which is shown in the middle trace of Fig. 1 A. Exposure to 7.7 mM Ca^{2+} consistently failed to trigger a bursting activity (Fig. 1 A, upper trace; see also [35]).

In parallel experiments, we have measured the $[Ca^{2+}]_i$ from fura-2-loaded whole islets exposed to 11 mM glucose. These islets displayed fast $[Ca^{2+}]_i$ oscillations characterized by rise times of 1−2 s and relatively slower descending phases (Fig. 1 B), as previously reported [34, 35, 39]. Tolbutamide evoked a rapid $[Ca^{2+}]_i$ rise, which was typically followed by a slow decay towards a plateau. Furthermore, exposure to 10.2 or 12.8 mM Ca^{2+} in the presence of tolbutamide regenerated an oscillatory pattern, which in some experiments (e. g. those labelled "islet 1" and "islet 2" in Fig. 1 B) was noticeably irregular and of substantially larger amplitude than the oscillations recorded prior exposure to tolbutamide (compare with the regular oscillatory pattern depicted in Fig. 2 of [35]). It is also noteworthy that the $[Ca^{2+}]_i$ measured at the peak of the high-Ca^{2+}-induced oscillations overshot the extrapolated $[Ca^{2+}]_i$ recorded in tolbutamide. However, the extent of this effect was highly variable from islet to islet (e. g. compare the $[Ca^{2+}]_i$ overshoots in islets 1 and 2, Fig. 1 B).

The appearance of $[Ca^{2+}]_i$ oscillations during exposure to 10.2 or 12.8 mM Ca^{2+} was often preceded by a fast $[Ca^{2+}]_i$ transient, which was triggered as soon as the solution reached the islet and lasted for approximately 10−30 s (record labelled "islet 1" in Fig. 1 B; see also [35]). The maximal amplitude of the initial $[Ca^{2+}]_i$ transient was typically 50−150 nM. However, this amplitude was less than 30 nM in a fraction of the islets examined (3 out of 13 islets), as shown in Fig. 1 B ("islet 2"). It should be emphasized that the initial $[Ca^{2+}]_i$ rise was also observed in experiments where exposure to 12.8 mM Ca^{2+} failed to evoke $[Ca^{2+}]_i$ oscillations (e. g. "islet 3" in Fig. 1 B).

Effects of high Ca^{2+} in the presence of nifedipine

Exposure to high Ca^{2+} may evoke bursts of electrical activity by enhancing Ca^{2+} influx through L-type voltage-sensitive Ca^{2+} channels. To assess this possibility, the membrane potential was recorded while the islets were exposed to high Ca^{2+} in the presence and absence of the dihydropyridine antagonist nifedipine.

Figure 2 shows that exposure to 10 μM nifedipine in the presence of tolbutamide rapidly hyperpolarized the β-cell membrane and suppressed the spiking activity. A slower depolarization ensued, driving the membrane potential to a steady level close to the membrane potential

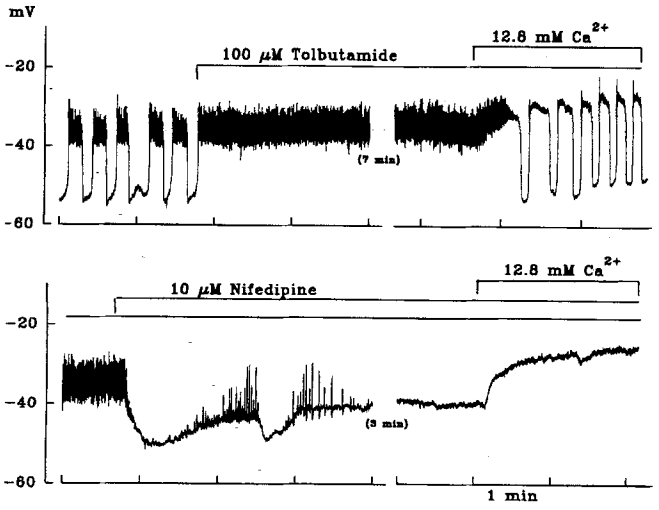

Fig. 2. Effect of high Ca^{2+} in the presence of nifedipine. The external Ca^{2+} concentration was raised from 2.56 to 12.8 mM in the presence of tolbutamide (*upper panel*) and tolbutamide + nifedipine (*lower panel*), as indicated. The *lower trace* is the direct continuation of the *upper trace*. The break in the upper trace corresponds to 7 min of continuous electrical activity. No electrical activity was recorded during the 3-min break in the lower trace. The glucose concentration was 11 mM throughout

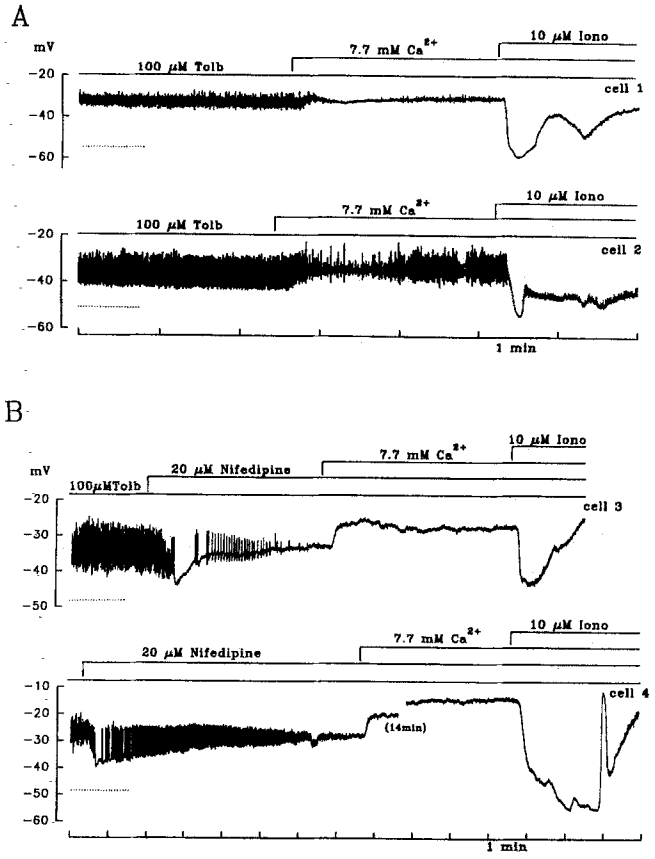

Fig. 3 A, B. Effect of ionomycin on tolbutamide-evoked electrical activity in the presence of nifedipine. **A** The external Ca^{2+} concentration was raised from 2.56 to 7.7 mM as indicated (100 μM tolbutamide throughout). Ionomycin (*Iono*) was added in the presence of 7.7 mM Ca^{2+} and tolbutamide as indicated. **B** Nifedipine was added in the presence of tolbutamide (*Tolb*) as indicated. The external Ca^{2+} concentration was raised from 2.56 to 7.7 mM in the presence of tolbutamide and nifedipine as indicated. Ionomycin was added in the presence of 7.7 mM Ca^{2+}, nifedipine and tolbutamide as indicated. The break in the *lower trace* corresponds to 14 min of continuous recording. The *dotted lines* represent the burst silent phases recorded in the presence of 11 mM glucose prior to tolbutamide exposure. The glucose concentration was 11 mM throughout. Each trace represents an experiment carried out on a different islet

at the feet of control spikes in approximately 3 min. It is noteworthy that the spiking activity reappeared soon after the membrane started to depolarize during exposure to nifedipine (Figs. 2, 3 B). However, the amplitude of the spikes gradually decayed throughout nifedipine exposure so that by the end of 3–5 min the spiking activity became suppressed.

Raising $[Ca^{2+}]_o$ to 10.2–12.8 mM in the presence of tolbutamide and nifedipine evoked a sustained membrane depolarization (Fig. 2), the maximal amplitude of which was variable from islet to islet and averaged 8.2 ± 5.2 mV (± SD; $n = 5$ islets). Since in these experiments the Ag/AgCl electrode used as reference was electrically connected to the recording chamber through an agar bridge (see Materials and methods), the membrane depolarization evoked by high Ca^{2+} cannot be ascribed to a junction potential arising at the electrode surface as a consequence of $CaCl_2$ addition.

Modulation of membrane potential by ionomycin

The simplest interpretation of the mechanisms underlying the burst requires a feed-back loop between $[Ca^{2+}]_i$ changes and a $[Ca^{2+}]_i$-sensitive conductance [12]. Thus, one might expect to mimic the inter-burst hyperpolarization by artificially raising the $[Ca^{2+}]_i$, for example, with the help of a Ca^{2+} ionophore. Indeed, we have recently shown that ionomycin evokes pronounced $[Ca^{2+}]_i$ rises in whole islets [35]. Furthermore, ionomycin drives the membrane potential to a level close to the silent phase potential, provided that the ionophore is applied in the presence of external Ca^{2+} levels greater than or equal to 5.1 mM [35]. We have now investigated whether the L-type Ca^{2+} conductance might be involved in the ionomycin-evoked hyperpolarization by examining the ionophore effect in the presence of nifedipine.

Extracellular space restrictions within the islet may cause liposoluble molecules, such as ionomycin, to reach the impaled cell at a lower concentration than in the bath. To minimize this possibility, only rather superficial impalements were used to characterize the ionophore effects. Figure 3 A shows that raising $[Ca^{2+}]_o$ to 7.7 mM in the presence of tolbutamide depolarized the β-cell membrane by approximately 3–5 mV (calculated at the foot of the spikes from expanded records; average 3.9 ± 1.7 mV, $n = 9$ islets) and attenuated the spiking activity. Exposure to 7.7 mM Ca^{2+} failed to evoke bursting activity, as previously reported [35]. Figure 3 A also shows that exposure to 10 μM ionomycin in the presence of tolbutamide and 7.7 mM Ca^{2+} evoked membrane hyperpolarizations similar to those previously observed in

5.1 mM Ca^{2+} [35]. It is noteworthy that, following ionomycin exposure, the membrane potential was driven to a level close to the silent phase potential recorded in control (dotted lines in Fig. 3).

In the experiments depicted in Fig. 3 B, the $[Ca^{2+}]_o$ was raised to 7.7 mM in the presence of 20 μM nifedipine after the drug had completely suppressed the spiking activity. This caused the β-cell membrane to depolarize by approximately 5−13 mV (average 7.4 ± 1.1 mV, $n = 7$ islets). Figure 3 B also shows that nifedipine failed to impair the ionomycin-evoked hyperpolarization observed in control.

Bursts of electrical activity and $[Ca^{2+}]_i$ oscillations triggered by Bay K 8644

The L-type Ca^{2+} channel agonist Bay K 8644 is known to increase the open probability of the Ca^{2+} channel [31]. Thus, Bay K 8644 might be expected to enhance Ca^{2+} influx and to exert a high-Ca^{2+}-like effect on the continuous electrical activity generated by tolbutamide.

This was indeed the case in two out of five experiments, as illustrated in Fig. 4 A. Thus, addition of 1 μM Bay K 8644 slowly hyperpolarized the membrane and greatly increased the voltage undershoots following each spike. Furthermore, bursts of electrical activity resumed approximately 3 min after exposure to the Ca^{2+} channel agonist. When compared with control bursts, the bursts observed in the steady-state of the Bay K 8644 effect were characterized by slightly hyperpolarized (approx. 3 mV) active phases and unmodified silent phase potentials. As a consequence, the plateau amplitude (difference between the active and the silent phase potentials) decreased by approximately 3 mV (see expanded bursts in Fig. 4 A, lower panel). In addition, Bay K 8644 increased the mean amplitude of the spikes. This is reminescent of the Bay K 8644 effects reported previously [22].

In the remaining three experiments, 1 μM Bay K 8644 did not appear to affect the amplitude of the spikes but slightly enhanced the voltage undershoots following each spike (Fig. 4 B, upper panel and expanded records). In these experiments, 1 μM Bay K 8644 failed to evoke bursts of electrical activity. Raising Bay K 8644 concentration to 10 μM greatly increased the amplitude of the spikes and slowly hyperpolarized the membrane in a manner similar to that depicted in Fig. 4 A for 1 μM Bay K 8644, but the spiking activity pattern remained essentially continuous (Fig. 4 B, lower panel).

The effects of Bay K 8644 on the $[Ca^{2+}]_i$ are illustrated in Fig. 5. Exposure to 1 μM Bay K 8644 in the presence of tolbutamide evoked fast $[Ca^{2+}]_i$ oscillations originating from levels above the extrapolated $[Ca^{2+}]_i$ recorded in tolbutamide. It is also apparent that the frequency of the $[Ca^{2+}]_i$ oscillations recorded in Bay K 8644 was approximately threefold higher than in control prior to tolbutamide. This is reminescent of the frequency characteristics of the Bay-K-8644-evoked bursting activity shown in Fig. 4 A (approx. 5 bursts/min versus 2−3 bursts/min observed in control prior to addition of tolbutamide).

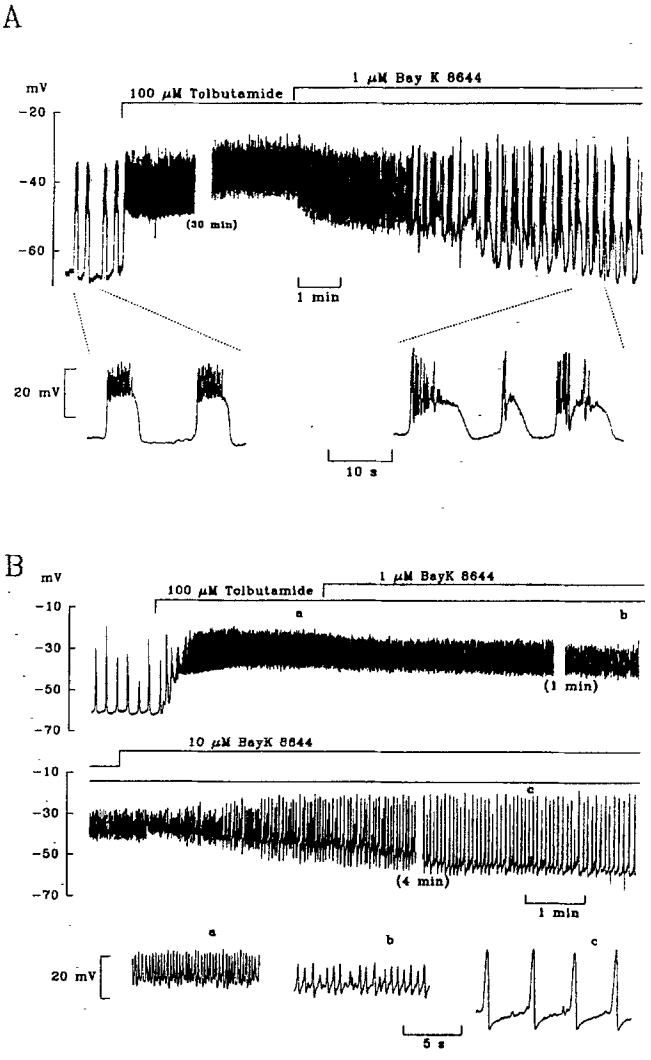

Fig. 4 A, B. Bursts of electrical activity triggered by Bay K 8644 in the presence of tolbutamide. **A** Bay K 8644 was added in the presence of tolbutamide and 11 mM glucose as indicated. The break in the trace corresponds to 30 min of continuous electrical activity. Details of electrical activity are shown in the *lower panel* on an expanded time scale (*left:* bursts recorded in 11 mM glucose; *right:* bursts recorded during Bay K 8644 exposure). **B** Experiment carried out on a different islet. The islet was sequentially exposed to 1 μM (*upper trace*) and 10 μM (*lower trace*) Bay K 8644 in the presence of tolbutamide and 11 mM glucose as indicated. The lower trace is the direct continuation of the upper trace. The breaks in the upper and lower traces correspond to 1 min and 4 min of continuous electrical activity respectively. Details of electrical activity (a−c) are shown on an expanded time scale

Effect of tetraethylammonium

Studies of the effects of TEA on the activity of the maxi K(Ca) channel indicate that these channels can be blocked with an apparent K_d of 0.14 mM [8]. In the following experiments, we have assessed the effect of high Ca^{2+} on tolbutamide-evoked electrical activity in the presence of 2 mM TEA.

Exposure to 2 mM TEA in the presence of tolbutamide turned the continuous electrical activity into a bursting pattern, as illustrated in Fig. 6. This effect is specially apparent in the experiment depicted in

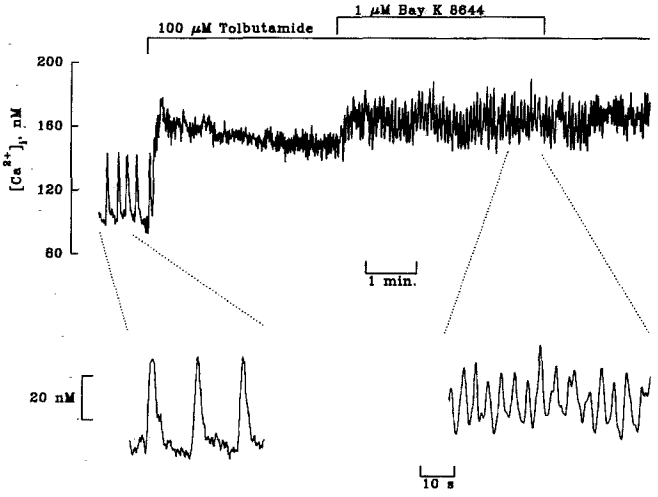

Fig. 5. $[Ca^{2+}]_i$ rises triggered by Bay K 8644 in the presence of tolbutamide. The islet was challenged with Bay K 8644 in the presence of tolbutamide as indicated. Shown below are $[Ca^{2+}]_i$ oscillations at an expanded time scale (*left:* oscillations recorded in 11 mM glucose; *right:* oscillations recorded during exposure to Bay K 8644)

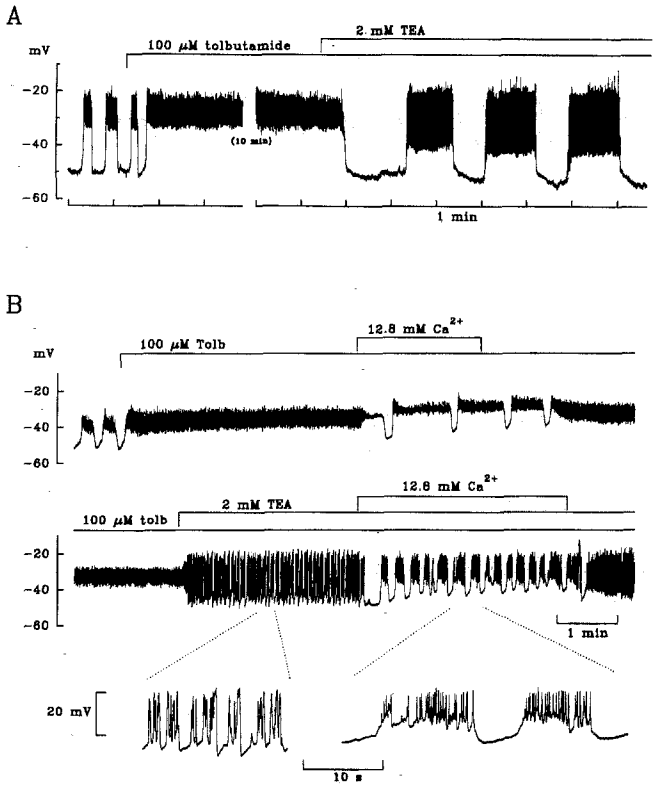

Fig. 6 A, B. Effects of high Ca^{2+} in the presence of 2 mM tetraethylammonium and tolbutamide. **A** Tetraethylammonium (*TEA*) was added in the presence of tolbutamide as indicated. The break in the trace corresponds to 10 min of continuous electrical activity. **B** Experiment carried out on a different islet. In the *upper trace,* the islet was exposed to 12.8 mM Ca^{2+} in the presence of tolbutamide as indicated. The *lower trace* is the direct continuation of the upper trace. In the lower trace, the islet was exposed to 12.8 mM Ca^{2+} in the presence of TEA and tolbutamide as indicated. Shown below are expanded records of bursting electrical activity (*left:* bursts recorded in the presence of TEA; *right:* bursts recorded in the presence of high Ca^{2+} and TEA). The glucose concentration was 11 mM throughout (**A**, **B**)

Fig. 6 A, where well-defined silent and active phases may be seen to occur in the presence of 2 mM TEA (representative of experiments performed on four out of seven islets). The frequency of the TEA-evoked bursts varied largely from islet to islet (for example, in the experiments depicted in Fig. 6 A and B the bursting frequency was approx. 0.5/min and 21/min, respectively). It should be emphasized that, whereas the membrane potential at the silent phase in TEA was similar to that recorded prior to tolbutamide, the active phase potential in TEA was somewhat more negative than in control (approx. 6 mV in Fig. 6 A). This led to a reduction in the plateau potential recorded in TEA, a phenomenon resembling the Bay K 8644 effect depicted in Fig. 4 A.

Figure 6 B shows that 2 mM TEA did not impair the bursting activity evoked by exposure to 12.8 mM Ca^{2+} ($n = 3$ experiments). The bursts recorded in TEA + 12.8 mM Ca^{2+} displayed lower plateau potentials and active phase durations (Fig. 6 B, lower panel) than the high-Ca^{2+}-evoked bursts recorded in the absence of TEA (Fig. 6 B, upper panel).

Lack of effect of CTX

CTX is a specific blocker of the TEA-sensitive maxi K(Ca) channel found in the pancreatic β-cell [21]. Using rather superficial impalements to minimize the possibility that CTX might reach the impaled cell at a lower concentration than in the bath, we found that 40–80 nM CTX failed to affect the bursting activity triggered by 12.8 mM Ca^{2+} ($n = 4$ different islets; data not shown).

Effect of quinine

The alkaloid quinine blocks several K^+ channel types in the β-cell, namely the K(ATP) channel, the delayed rectifier and the maxi K(Ca) channel [7, 8, 23]. In the following experiments, we have investigated whether quinine-sensitive K^+ channels might mediate the bursts by assessing the effect of high Ca^{2+} on tolbutamide-evoked electrical activity in the presence of 200 μM quinine.

Quinine (100–200 μM) has long been known to increase the amplitude of the spikes in the β-cell [3, 32], an effect likely to reflect blockade of the voltage-sensitive K^+ channels which mediate spike repolarization [i. e. the delayed rectifier, the maxi K(Ca) channel, or both]. We have confirmed that exposure to 200 μM quinine in the presence of tolbutamide increases the amplitude of the spikes along a time course of minutes (Fig. 7). However, long-term exposures to higher quinine concentrations (0.5 or 1 mM) had the opposite effect of reducing the amplitude of the spikes (data not shown). The latter effect resembles the previously described action of high concentrations of quinidine on glucose-induced electrical activity [28]. Because we suspected that this phenomenon might reflect non-specific blockade of the voltage-sensitive Ca^{2+} conductance, we have not used drug concentrations higher than 200 μM

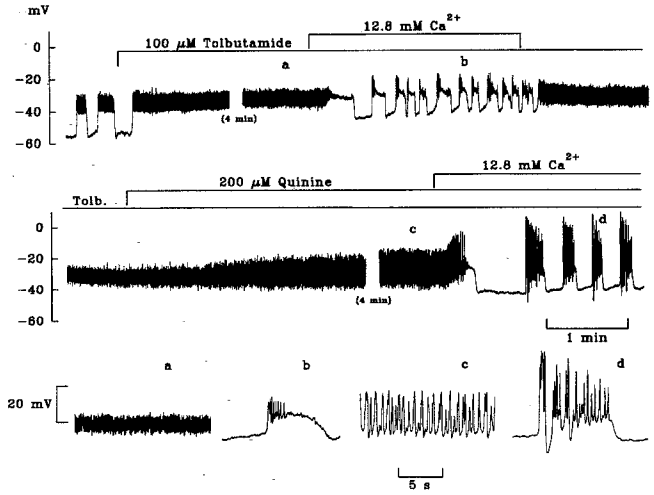

Fig. 7. Bursts of electrical activity triggered by high Ca^{2+} in the presence of quinine and tolbutamide. *Upper trace:* the islet was exposed to 12.8 mM Ca^{2+} in the presence of tolbutamide as indicated. *Lower trace:* direct continuation of the upper trace. The islet was exposed to 12.8 mM Ca^{2+} in the presence of quinine and tolbutamide (*Tolb*) as indicated. The breaks in the traces correspond to 4 min of continuous electrical activity. Shown below are selected records (a–d) at an expanded time scale. The glucose concentration was 11 mM throughout

in the experiments designed to investigate the sensitivity of high-Ca^{2+}-evoked bursts to quinine.

Figure 7 shows that 200 μM quinine did not impair the bursts of electrical activity triggered by high Ca^{2+} in the presence of tolbutamide ($n = 3$ experiments). It is also noteworthy that quinine augmented the amplitude of the spikes observed in high Ca^{2+}. In one experiment, quinine greatly increased the voltage undershoots following the action potentials, resulting in occasional hyperpolarizations to membrane potential levels below the burst silent phase potential (see expanded record d in Fig. 7).

Discussion

When applied in the presence of intermediate glucose concentrations, the sulphonylurea tolbutamide turns the regular bursting pattern of the pancreatic β-cell into a continuous firing pattern [15]. This phenomenon probably originates from the suppression of residual activity of K(ATP) channels, as previously hypothesized [1]. We now report that several drugs and/or conditions known to enhance Ca^{2+} influx across the plasma membrane (i. e. 1 μM Bay K 8644, 2 mM TEA and raising $[Ca^{2+}]_o$ to 10–13 mM) can restore the ability of tolbutamide-treated cells to display bursting electrical activity. The ability of the cells to raise $[Ca^{2+}]_i$ in response to these agents has been directly documented in the cases of high Ca^{2+} and Bay K 8644: while the high-Ca^{2+}-evoked $[Ca^{2+}]_i$ oscillations are typically preceded by a rapid $[Ca^{2+}]_i$ transient (Fig. 1 B), the major effect of Bay K 8644 is to trigger $[Ca^{2+}]_i$ oscillations distinctly above the tolbutamide baseline (Fig. 5). TEA is also known to enhance Ca^{2+} influx and to elicit large $[Ca^{2+}]_i$ transients associated with individual spikes [34], probably because the drug blocks the large-conductance maxi K(Ca) channel [8] and the delayed rectifier K^+ channel [7] and, thus, enhances the time the Ca^{2+} currents stay activated during each spike.

Nifedipine (10–20 μM) was found to suppress the electrical activity (Figs. 2, 3), suggesting that the L-type voltage-sensitive Ca^{2+} channel is the only Ca^{2+} channel type responsible for Ca^{2+} influx during the action potentials. Thus, enhanced Ca^{2+} influx through these channels may be considered as the ultimate trigger of the bursts of electrical activity evoked by high Ca^{2+}, Bay K 8644 and TEA in the presence of tolbutamide. While this is specially apparent in the case of the L-type Ca^{2+} channel agonist Bay K 8644, the possibility that Ca^{2+} influx through L-type Ca^{2+} channels might also mediate the appearance of high-Ca^{2+}-evoked bursts is emphasized by the observation that these bursts are impaired by the dihydropyridine nifedipine at 10 μM (Fig. 2).

$[Ca^{2+}]_i$-dependent processes of inactivation of the voltage-sensitive Ca^{2+} channel [27, 36] have been hypothesized to underlie the bursts of electrical activity exhibited by pancreatic β-cells [10] and *Aplysia* neurones [20]. In the β-cell, this concept has been disputed on the grounds that the $[Ca^{2+}]_i$-dependent inactivation process appears to be too fast to account for the termination of the burst [38]. Indeed, under whole-cell voltage-clamp recording conditions $[Ca^{2+}]_i$-dependent inactivation of Ca^{2+} current is normally over in less than 100 ms [36], whereas the burst active phase recorded in the presence of 11 mM glucose lasts normally for several seconds. Importantly, work carried out by Chad and Eckert [9] in *Helix aspersa* neurones has shown that Ca^{2+} currents undergo slower Ca^{2+}-dependent inactivation processes critically dependent upon the phosphorylation status of the Ca^{2+} channel, a mechanism that has never been systematically investigated in the β-cell. We have seen that the primary effect of nifedipine exposure in the presence of tolbutamide is to hyperpolarize the β-cell membrane and suppress the electrical activity (Figs. 2, 3). Thus, the L-type Ca^{2+} conductance appears to make a significant contribution to the inter-spike membrane potential, a conclusion that sets the ground to implicate Ca^{2+} current inactivation in physiological regulation of membrane potential. Based on our current experiments it is difficult to assess unambiguously whether $[Ca^{2+}]_i$-dependent inactivation of the Ca^{2+} currents might play a role in the termination of the burst depolarized phase. However, it should be emphasized that the spiking activity recorded along individual bursts triggered by Bay K 8644 tends to concentrate towards the beginning of the burst, a phenomenon that resembles the highly asymmetrical spike distribution along bursts modified by exposure to the L-type Ca^{2+} channel agonist CGP 28392 [18]. Moreover, the burst active phases triggered by high Ca^{2+} in the presence of tolbutamide display a reduced spiking activity, which often consists of a single low amplitude spike followed by several seconds of random voltage noise, clearly enhanced when compared with the burst silent phase (see Figs. 2, 7; see also Fig. 1 of [35]). While the attenuation of spiking activity along the burst

is likely to originate from $[Ca^{2+}]_i$-induced inactivation of Ca^{2+} currents, the non-gradative nature of such attenuation argues against the latter inactivation process being a crucial factor for the termination of the burst depolarized phase. We have shown that nifedipine does not impair the ionomycin-induced hyperpolarization observed in the presence of tolbutamide and 7.7 mM Ca^{2+} (Fig. 3 B). This observation indicates that a $[Ca^{2+}]_i$-sensitive process distinct from Ca^{2+}-induced inactivation of the L-type Ca^{2+} channel has the potential to hyperpolarize the β-cell membrane. In this respect, it should be borne in mind that the average $[Ca^{2+}]_i$ changes brought about by ionomycin [35] are normally much larger than the changes evoked by either Bay K 8644 or high Ca^{2+}. Thus, it is possible that the ionophore-evoked hyperpolarizations are mediated by a channel which might be relatively inactive at physiological $[Ca^{2+}]_i$ levels.

Our data do not support the hypothesis that the pacemaker current underlying the burst might be carried by the large conductance maxi K(Ca) channel. This is because: (1) delivering CTX to β-cells located in the islet outermost cell layers had no apparent effect on high-Ca^{2+}-evoked bursting electrical activity, an observation consistent with the reported lack of effect of the toxin on glucose-induced (11 mM) bursting electrical activity also recorded from intact islets [21]; (2) rather than producing a sustained depolarization of the β-cell, exposure to 2 mM TEA {a concentration that reportedly suppressed maxi K(Ca) channel activity in voltage-clamped patches of β-cell membranes [8]} triggered bursts in the presence of tolbutamide and failed to suppress the bursting activity evoked by high Ca^{2+} (Fig. 6); and, (3) 200 μM quinine[1] failed to block high-Ca^{2+}-evoked bursts (Fig. 7), in spite of the fact that the alkaloid turns the bursting electrical activity into a continuous firing pattern when applied in 11 mM glucose [3]. [Since quinine is also known to block the K(ATP) channel [8], the latter effect resembles the sulphonylurea action on the bursting activity and is likely to be accounted for by suppression of the residual activity of these channels. In keeping with our observations, Ribalet and Beigelman [28] have previously shown that the bursting electrical activity suppressed by 100 μM quinidine, a stereoisomer of quinine, can be restored by raising external Ca^{2+} to 17.5 mM.]

We have observed that high Ca^{2+} evokes a pronounced depolarizing effect in the presence of nifedipine (Fig. 2). Interestingly, exposure to high Ca^{2+} in the presence of another blocker of voltage-sensitive Ca^{2+} channels (D-600) evokes rapid $[Ca^{2+}]_i$ rises in mouse pancreatic β-cells [25]. Although this effect has been claimed to reflect primarily Ca^{2+} release from internal stores [25], it is possible that Ca^{2+} influx through dihydropyridine-insensitive Ca^{2+} channels might also contribute to the high-Ca^{2+}-evoked $[Ca^{2+}]_i$ rise. As noted above, no other functional voltage-sensitive Ca^{2+} channels besides the L-type channel appear to be present in adult mouse pancreatic β-cells [27, 31]. Thus, we hypothesize that a Ca^{2+}-conducting action channel (e. g. the "G-channel" recently described in human β-cells [30] or another as yet undocumented cation channel) might mediate the high-Ca^{2+}-evoked depolarization recorded in the presence of the nifedipine. If $[Ca^{2+}]_i$ sensitive, this putative cation channel would be a suitable candidate to carry the pacemaker current underlying the burst.

In conclusion, we have shown that enhanced Ca^{2+} influx through L-type Ca^{2+} channels has the potential to change a tonic firing pattern into a bursting pattern of electrical activity. This is probably because the channel underlying the burst is highly sensitive to $[Ca^{2+}]_i$ changes supported by Ca^{2+} influx. Furthermore, we have shown that the repolarization that terminates the bursts is highly unlikely to be provided either by activation of CTX- and TEA-sensitive large-conductance maxi K(Ca) channels or by the activation of K(ATP) channels. The burst is more likely to be mediated by distinct maxi K(Ca) channels, but the possible involvement of phosphorylation- and $[Ca^{2+}]_i$-dependent slow processes of inactivation of Ca^{2+} currents cannot be ruled out.

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[1] Quinine blocks the large conductance K(Ca) channel of mouse β-cells with an apparent IC_{50} of approximately 100 μM [8]

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