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Inhibition of L-type calcium channels by internal GTP [γS] in mouse pancreatic β cells

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Abstract. Pretreatment of pancreatic β cells with pertussis toxin resulted in a 30% increase in peak whole-cell Ca^{2+} currents recorded in the absence of exogenous intracellular guanine nucleotides. Intracellular application of 90 μM GTP[γS], by liberation from a caged precursor, resulted in 40% reduction of the peak Ca^{2+} current irrespective of whether the current was carried by Ca^{2+} or Ba^{2+}. Effects on the delayed outward K^+ current were small and restricted to a transient Ca^{2+}-dependent K^+ current component. Inhibition by GTP[γS] of the Ca^{2+} current was not mimicked by standard GTP and could not be prevented either by pretreatment with pertussis toxin or by inclusion of GDP[βS] or cyclic AMP in the intracellular medium. The inhibitory effect of GTP[γS] could be counteracted by a prepulse to a large depolarizing voltage. A similar effect of a depolarizing prepulse was observed in control cells with no exogenous guanine nucleotides. These observations indicate that inhibition of β cell Ca^{2+} current by G protein activation results from direct interaction with the channel and does not involve second-messenger systems. Our findings also suggest that the β cell Ca^{2+} current is subject to resting inhibition by G proteins.

Key words: Insulin secretion – Pancreatic β cells – Ca^{2+} channels – G proteins

Introduction

Voltage-activated L-type Ca^{2+} channels play an important role in glucose-stimulated insulin secretion (for reviews: [3, 28]). Glucose promotes Ca^{2+} entry through voltage-dependent Ca^{2+} channels by two mechanisms. First, by inhibiting ATP-regulated K^+ channels, glucose depolarizes the β cells and thus activates the Ca^{2+} channels [4, 7]. Second, glucose metabolism exerts a direct stimulatory action on the Ca^{2+} channel, shifting the activation curve to more negative membrane potentials [36, 39]. Substances such as adrenaline, galanin and somatostatin inhibit insulin secretion and lower the cytoplasmic free Ca^{2+} concentration by pertussis-toxin (PTX)-sensitive mechanisms [14, 26]. This suggests that activation of GTP-binding proteins (G proteins) may result in suppression of the Ca^{2+} current. Indeed, in clonal insulin-secreting HIT cells, adrenaline and somatostatin have been demonstrated to inhibit the Ca^{2+} current by a G-protein-dependent mechanism [22, 24]. Here we demonstrate that L-type Ca^{2+} channels in mouse pancreatic β cells are also subject to regulation by G proteins, opening the possibility that such mechanisms participate in the modulation of insulin secretion in response to nutrients, hormones and neurotransmitters.

Materials and methods

Preparation of cells. Pancreatic islets were isolated from NMRI or ob/ob mice by collagenase digestion and dispersed into single cells by shaking in Ca^{2+}-free medium or treatment with trypsin as previously described [30]. The cells were plated in Corming petri dishes and maintained for 1–4 days in RPMI-1640 tissue-culture medium, containing 5 mM glucose and supplemented with 10% (v/v) foetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Electrophysiological recordings and analysis. Whole-cell Ca^{2+} currents were recorded with the patch-clamp method [16]. Pipettes were pulled from aluminosilicate or borosilicate glass (Hilgenberg GmbH, Malsfeld, FRG), coated with Sylgard near their tips and fire-polished. They had resistance of 1–3 MΩ when measured with the standard pipette solution. Membrane currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG) and the hard- and software Pclamp (Axon Instruments, Foster City, Calif., USA). For analysis, the current signal was filtered at 1–3 kHz, digitized at 2–8 kHz and stored in a computer. Unless otherwise indicated, leak currents and capacitive transients were removed on-line by subtracting the summed response to seven hyperpolarizing pulses with an amplitude one-seventh of the test

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pulse. The holding potential was −80 mV and voltage pulses were applied at a frequency of less than 0.5 Hz. Analysis was performed using a computer and in-house software or the program Pclamp. The reference potential for all measurements was the zero-current potential of the pipette obtained immediately before seal establishment. Data are expressed as mean values ± SEM of the indicated number of experiments (= cells) and statistical significances were evaluated using Student's *t*-test.

Solutions. The standard extracellular solution contained (in mM) 138 NaCl, 5.6 KCl, 10.2 $CaCl_2$ (or $BaCl_2$), 1.2 $MgCl_2$, 10 tetraethylammonium chloride, 5 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)/NaOH (pH 7.4) and 0.1 μg/ml tetrodotoxin. The standard pipette solution used for the recording of Ca^{2+} currents was composed of (in mM) 150 *N*-methyl-D-glucamine, 110 HCl, 10 [ethylenebis(oxonitrilo)]tetraacetic acid (EGTA), 2 $CaCl_2$, 1 $MgCl_2$, 3 MgATP and 5 HEPES/HCl (pH 7.15). When K^+ currents were recorded this solution was replaced by a medium containing (in mM) 125 KCl, 30 KOH, 1 $MgCl_2$, 2 $CaCl_2$, 5 HEPES/KOH (pH 7.15), 10 EGTA and 3 MgATP. Nucleotides other than ATP were added at the concentrations indicated in the legends to the figures. Whenever nucleotides were added as Na^+ or Li^+ salts, an equal concentration of $MgCl_2$ was added to maintain an excess of Mg^{2+}. Caged GTP (NPE-caged, Na^+ salt) and caged GTP[γS] (NPE-caged guanosine 5′-[γ-thiotriphosphate], Li salt) were purchased from Sigma (St. Louis, Mo,) and Molecular Probes (Eugene, Or.), respectively, and added at a concentration of 100 μM. Photolysis of the caged precursor was effected by 2 s ultraviolet irradiation (300–450 nm; irradiance 20 mW/mm²). The efficiency of liberation was assumed to be the same as for caged ATP [2], giving a final concentration of GTP and GTP[γS] of around 90 μM. The details of the photorelease apparatus are described elsewhere [2]. In a few experiments the β cells were pretreated with 100 ng/ml pertussis toxin (Sigma) at + 37°C for more than 4 h.

Results

Effects of pretreatment with pertussis toxin on β cell Ca^{2+} currents

Figure 1A shows Ca^{2+} currents recorded from β cells under control conditions. Inward Ca^{2+} currents were first observed during a depolarization to −50 mV and reached a maximum at a membrane potential of about 0 mV. The Ca^{2+} currents were typically biphasic, consisting of a sustained and an inactivating component. The currents observed after pretreatment of the cells with PTX are shown in Fig. 1B. The Ca^{2+} currents recorded from control cells (upper trace) and PTX-pretreated cells (lower trace) during a depolarization to 0 mV are compared in Fig. 1C. It is clear that PTX pretreatment increased the mean current amplitude but did not detectably interfere with the activation/inactivation properties. The effects of PTX are summarized in the current-voltage relationship in Fig. 1D. PTX produced a small (30%) increase of the peak Ca^{2+} current amplitude, which was most pronounced at membrane potentials of about 0 mV.

Effects of intracellular application of GTP[γS] and GTP on Ca^{2+} current

Figure 2A shows whole-cell Ca^{2+} currents evoked by depolarizations going to 0 mV in a single β cell 2.5 min

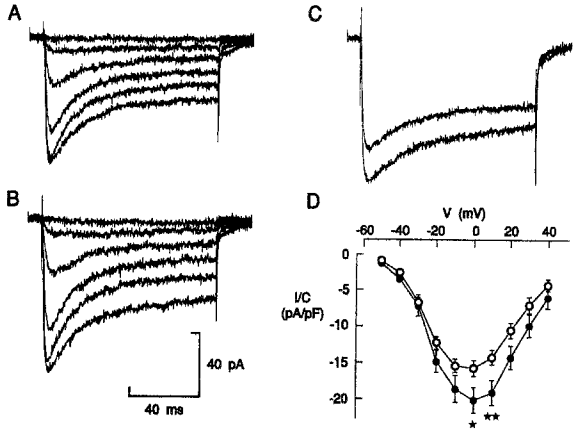

Fig. 1A–D. Effect of pertussis toxin on β cell Ca^{2+} currents. **A** Whole-cell Ca^{2+} currents recorded from control cells during depolarization to membrane potentials between −50 mV (*top*) and −10 mV (*bottom*). **B** Whole-cell Ca^{2+} currents recorded from cells pretreated for more than 4 h with pertussis toxin (PTX) (100 ng/ml) during depolarizations to membrane potentials between −50 mV and −10 mV. **C** Comparison of current responses obtained during depolarizations to 0 mV in untreated cells (*upper trace*) and cells pretreated with PTX (*lower trace*). **D** Current density (*I/C*; current amplitude divided by cell capacitance-voltage (*V*) relationship of the current recorded from control cells (○, 11 observations) and cells treated with PTX (●, 9 experiments). * $P < 0.05$; ** $P < 0.025$. Same results obtained in two different experimental series

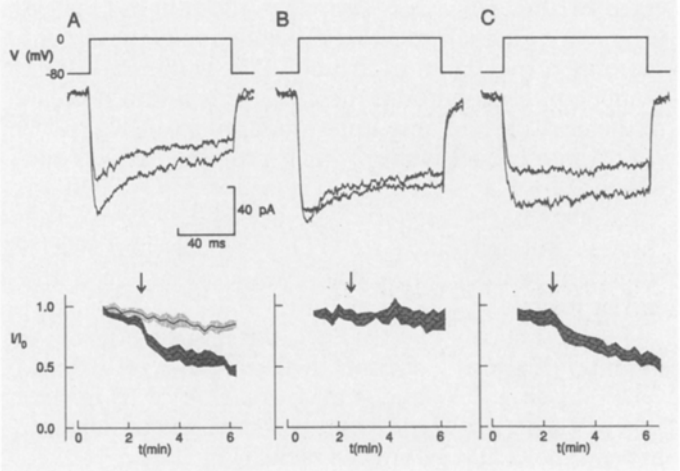

Fig. 2A–C. GTP[γS]-induced inhibition of β cell Ca^{2+} currents. **A** (*Top*) whole-cell Ca^{2+} currents recorded immediately before (*lower trace*) and 70 s after liberation of 90 μM GTP[γS] (*upper trace*). (*Below*) time course of GTP[γS]-induced inhibition. GTP[γS] was added as indicated by the *arrow*. The *upper curve* shows the change in Ca^{2+} current under control conditions: when caged GTP[γS] was present but not liberated, when caged GTP[γS] was absent but the cell was subjected to the UV plus or when neither UV light nor GTP[γS] was applied; data are pooled from all tree conditions and represent mean values (*continuous line*) ± SEM (*shaded area*) of a total of 12 experiments. **B** Effect of applying 90 μM GTP. **C** Effect of adding GTP[γS] when currents were carried by Ba^{2+}. Data are presented as mean values (*continuous line*) ± SEM (*shaded area*) of 7, 6 and 6 separate experiments in **A–C** respectively. The currents are expressed relative that observed during the first pulse and are plotted against the time elapsed after establishing the whole-cell configuration

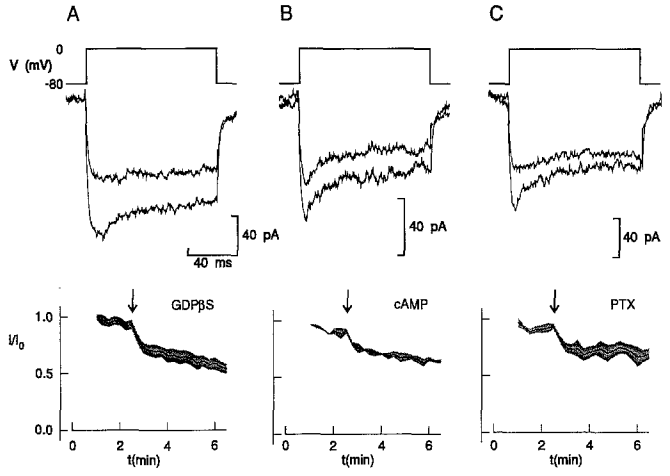

Fig. 3. A–C Failure of GDP[βS] (0.5 mM), cyclic AMP (1 mM) and pretreatment with pertussis toxin to prevent Ca^{2+} current inhibition by GTP[γS]. Data are mean values ± SEM of 6 (**A**), 4 (**B**) and 6 (**C**) experiments. In each panel the *upper part* shows the current before and 70 s after addition of GTP[γS] and the *lower part* the time course of the Ca^{2+} current changes

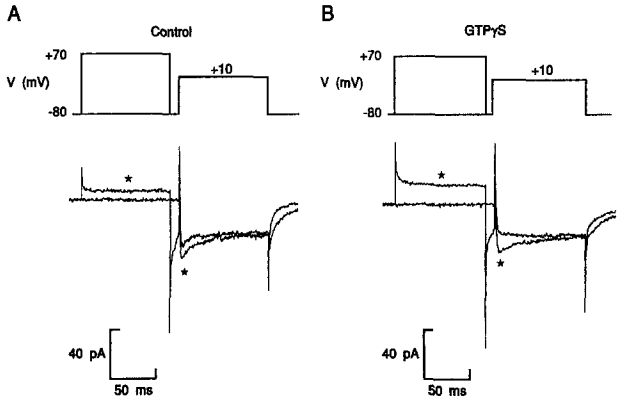

Fig. 4A, B. The effect of depolarizing prepulses on G-protein-induced Ca^{2+} channel inhibition. **A** Ca^{2+} currents recorded from a β cell dialysed with standard intracellular solution during a depolarization to + 10 mV with (*) or without a prepulse going to + 70 mV. **B** Ca^{2+} currents recorded from a cell approximately 5 min after release of 90 μM GTP[γS] with (*) or without a depolarizing prepulse to + 70 mV. Leak currents and capacitive currents have not been removed

after establishment of the whole-cell configuration but before the addition of GTP[γS] (lower trace) and approximately 70 s after releasing about 90 μM GTP[γS] from its caged precursor (upper trace). The time-dependent changes in the Ca^{2+} current in seven different cells are summarized below. GTP[γS] was applied at the time indicated by the arrow. It is clear that addition of GTP[γS] produces a gradually developing reduction of the current, reaching a maximum of around 35% within 1–2 min. Under control conditions there was only a slow decrease of the peak current amplitude amounting to 10%–15% over 6 min (upper curve), which probably reflects run-down of the Ca^{2+} channels. The action of GTP[γS] was equal at voltages between −50 mV and + 50 mV (not shown), not mimicked by GTP (Fig. 2B) and was of similar magnitude when Ba^{2+} was used as the charge carrier instead of Ca^{2+} (Fig. 2C).

Figure 3 shows that GTP[γS] was equally efficient in inhibiting the Ca^{2+} channel in the presence of 0.5 mM GDP[βS] (Fig. 3A) and 1 mM cyclic AMP (Fig. 3B). Likewise, pretreatment with pertussis toxin failed to prevent the GTP[γS]-induced block (Fig. 3C).

Inhibition of Ca^{2+} currents by G proteins is voltage-dependent

Dolphin and co-workers [10, 35] have proposed that the slow activation of the Ca^{2+} currents observed when the regulatory G protein is activated by GTP[γS] is due to gradual recovery from a modified closed state from which the Ca^{2+} channel is unable to open during depolarization. We have investigated whether this also applies to the G-protein-induced inhibition of the β cell Ca^{2+} current using the two-pulse voltage protocol of Grassi and Lux [15] and Scott and Dolphin [35]. As shown in Fig. 4B, a prepulse going to + 70 mV ending 10 ms before the subsequent test pulse to + 10 mV, accelerated Ca^{2+} current activation and increased the peak current amplitude when the channels were partially blocked by GTP[γS]. In eight different cells the prepulse increased Ca^{2+} current amplitude by 52 ± 3% ($P < 0.001$).

Interestingly, a similar effect of prepulsing was also observed under control conditions in the absence of added GTP[γS] (Fig. 4A) and a mean increase of 21 ± 5% ($n = 4$; $P < 0.025$) was observed following the depolarizing prepulse. In both the presence and absence of GTP[γS], a slowly deactivating inward tail current was observed. This current showed inwardly rectifying properties and reversed at about −10 mV (data not shown). Consequently, it did not contribute to the *inward* current amplitude observed during the test pulse going to + 10 mV.

Effects of GTP[γS] on delayed outward K^+ current

We also investigated the effects of GTP[γS] on the whole-cell delayed outward current [30, 37]. As shown in Fig. 5A, application of 90 μM GTP[γS]had only a small inhibitory effect (−15 ± 2%; $P < 0.01$) on the delayed outward current. The difference current, obtained by subtracting the current response measured 70 s after liberation of GTP[γS] (○) from that recorded immediately before the ultraviolet flash (●), is shown in Fig. 5B. An effect of GTP[γS] was only observable in cells possessing a detectable inward Ca^{2+} current. In two cells lacking Ca^{2+} currents, GTP[γS] was without effect.

Discussion

We demonstrate that the Ca^{2+} current in mouse pancreatic β cells is modulated by G proteins, as indicated by

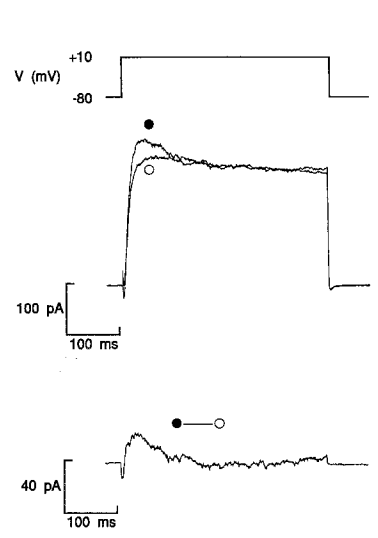

Fig. 5A, B. Effects of GTP[γS] on voltage-dependent delayed outward K^+ current. **A** Delayed outward K^+current evoked by depolarization from −80 mV to +10 mV immediately before (●) and about 70 s after release of approximately 90 μM GTP[γS] (○). Note that the effect of GTP[γS] is restricted to the beginning of the pulse. **B** Current inhibited by GTP[γS] obtained by subtracting the response after addition of GTP[γS] from that observed before addition. Currents shown are averages obtained from four different cells

the effects of pretreatment with PTX and intracellular addition of GTP[γS]. The action of G proteins was characterized by a reduction of an inactivating current component and slowed time course of activation. These effects are qualitatively and quantitatively similar to those reported in other cells (reviews: [9, 10, 34]).

In our experiments we took advantage of the technique of photoliberating GTP[γS] from a caged (inactive) precursor, which allows virtually instantaneous addition of the nucleotide. However, the inhibition obtained after releasing GTP[γS] developed gradually over a few minutes. A similar slow time-course for the development of the GTP[γS] effect on voltage-dependent Ca^{2+} channels has also been observed by others in both nerve [12, 38] and endocrine cells [25]. The dissociation of GDP from the guanine-nucleotide-binding site is very low in purified G proteins and this has been suggested to account for the slow development of the GTP[γS]-induced inhibition of the Ca^{2+} current [10]. It is therefore somewhat surprising that inclusion of GDP[βS] in the pipette did not detectably influence the rate by which the GTP[γS]-induced inhibition developed. In this context it is also worthy of note that GTP did not mimic the action of GTP[γS] on the β cell Ca^{2+} currents. Similar observations have been made in nerve cells and then interpreted in terms of hydrolysis of GTP by the GTPase activity of the G protein [38].

Since activation of G proteins is known to inhibit adenylate cyclase in a variety of tissues including the pancreatic β cells [28], it was important to exclude the possibility that inhibition of the β cell Ca^{2+} current is secondary to a reduction of the cytoplasmic cyclic AMP concentration. The observation that GTP[γS] blocked the Ca^{2+} current to the same extent in the presence of 1 mM cyclic AMP suggests that this is not the mechanism by which GTP[γS] blocks the Ca^{2+} current in the β cell.

In a few cells application of GTP[γS] resulted in a slowed time course of activation (cf. Fig. 4B). However, this was not consistently observed and in most cells the time constant of activation in the presence of GTP[γS] remained roughly the same as under control conditions. Dolphin [10] has proposed that this results from a portion of the Ca^{2+} channels not being associated with activated G protein and therefore inaccessible for inhibition. A more consistent feature of the GTP[γS] effect was the preferential inhibition of the inactivating Ca^{2+} current component with the sustained part being relatively more resistant. In mouse β cells it is unlikely that this results from a selective inhibition of a certain class of Ca^{2+} channels (i.e. T-type). Although rat β cells are equipped with both L- and T-type Ca^{2+} channels [5, 19, 33], the available evidence suggests that the voltage-gated Ca^{2+} channels in mouse β cells are predominantly of the L-type [31, 36; but cf 21]. Furthermore, inactivation of the Ca^{2+} current in mouse β cells is Ca^{2+}-dependent [27]. The idea that GTP[γS] inhibits L-type Ca^{2+} channels is supported by our observation that when Ca^{2+} was replaced by Ba^{2+} as the charge carrier, Ca^{2+} current inactivation was abolished but GTP[γS] remained equally effective in reducing the current amplitude. We therefore propose that the reduction of the Ca^{2+} current amplitude induced by GTP[γS] results in smaller intracellular Ca^{2+} transients in the vicinity of the Ca^{2+} channels, which then leads to less Ca^{2+}-dependent inactivation.

The failure of PTX to prevent the inhibitory effect of GTP[γS] is perhaps surprising since this compound has been demonstrated to counteract the inhibitory effect of GTP[γS] on the Ca^{2+} current in both hippocampal [38] and sensory neurons [11, 12]. However, PTX is believed to prevent the interaction between the receptor and G protein [29], but not GTP[γS]-induced activation [10]. A failure of PTX to prevent the action of GTP[γS] has also been observed in neuroblastoma × glioma cells [17, 34] and is analogous to the observation that GTP[γS] is able to inhibit adenylate cyclase even in the presence of PTX [18, 23].

The effects of G protein activation on the voltage-dependent currents in the β cell seem restricted to the Ca^{2+} channels. The small decrease in the delayed outward current has the amplitude and time course expected for the Ca^{2+}-activated K^+ current [37]. It is therefore reasonable to conclude that this effect is secondary to the suppression of the Ca^{2+} current. This conclusion is reinforced by the observation that the action of GTP[γS] was only observed in cells containing Ca^{2+} currents.

The effects of both pretreatment with PTX and the two-pulse voltage protocol suggest that the Ca^{2+} current is subject to partial (20% − 30%) tonic inhibition in the intact β cell. The physiological significance of this observation is not clear. A similar effect has also been observed in sensory neurons [35]. The effects of GTP[γS] are generally believed to mimic those of an extracellular agonist. Indeed, hormones and neurotransmitters exerting an inhibitory effect on glucose-stimulated insulin secretion are

known to reduce the cytoplasmic Ca^{2+} concentration and repolarize the cell by a mechanism involving a PTX-sensitive G protein [26]. It would therefore seem rational that inhibition of the Ca^{2+} current constitutes one of the mechanisms by which these agonists repolarize the β cell, inhibit electrical activity and insulin secretion. In accordance with this, an inhibitory effect of adrenaline and somatostatin on the Ca^{2+} currents has been demonstrated in the clonal insulin-secreting cell line HIT [22, 24]. However, to date no such inhibitory action of galanin [1] or adrenaline [6] has been observed in tissue-cultured mouse β cells. In fact, the inhibitory action of adrenaline, galanin and somatostatin on the β cell electrical activity [8, 13] can fully be accounted for by activation of a low-conductance G-protein-dependent K^+ channel in the β cell [32]. It remains possible that there are other inhibitory compounds that utilize G-protein-dependent reduction of the Ca^{2+} current to decrease insulin secretion. Finally, we point out that glucose stimulation of pancreatic β cells is associated with marked changes in the intracellular GTP/GDP ratio [20], which may, via interaction with G proteins, be translated into variations of the Ca^{2+} channel activity.

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