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Block of Ca^{2+}-channels by alpha-endosulphine inhibits insulin release

*,[1]Anne Virsolvy, [2]Paul Smith, [3]Gyslaine Bertrand, [1]Laurent Gros, [1]Lisa Héron, [1]Guillermo Salazar, [3]Raymond Puech & [1]Dominique Bataille

[1]Institut National de la Santé et de la Recherche Médicale U376, CHU Arnaud de Villeneuve, 371 Avenue du Doyen Gaston Giraud, 34295 Montpellier Cédex 05, France; [2]Institute of Cell Signalling, Medical School, Queen's Medical Centre, Nottingham NG8 2UH and [3]Centre National de le Recherche Scientifique UPR 9023, Rue de La Cardonille, 34094 Montpellier, France

1 α-Endosulphine, isolated as an endogenous equivalent for sulphonylureas, is a 121-amino acids protein of 19 kDa apparent molecular mass, member of a cyclic AMP-regulated phosphoprotein family. We have previously shown that α-endosulphine inhibits sulphonylurea binding and K_{ATP} channel activity, thereby stimulating basal insulin secretion.

2 We now describe that in the perfused rat pancreas, no stimulation was detected and that α-endosulphine inhibited glucose stimulated insulin release. This inhibition was dose-dependent and affected both phases of insulin secretion.

3 This inhibitory effect of α-endosulphine also occurred on MIN6 β-cells when insulin release was stimulated either by glucose, sulphonylureas or a high K^+ depolarization. Inhibition was concentration-dependent with a half-maximal inhibition at 0.5 μM and was mirrored by inhibition of calcium influx.

4 Electrophysiological experiments demonstrated, in comparison to the effects of the sulphonylurea tolbutamide, that these inhibitory effects were linked to a direct inhibition of L-type Ca^{2+}-channels and were independent from a regulation of K_{ATP} channels.

5 Although α-endosulphine is able to stimulate insulin release under specific conditions acting *via* modulation of K_{ATP} channel activity, the present study suggests that, under physiological conditions, the peptide mainly acts to block voltage-gated Ca^{2+}-channels. This block leads to the inhibition of calcium influx and triggers inhibition of insulin release.

6 We conclude that α-endosulphine is not exclusively an endogenous equivalent for sulphonylureas and not solely a K_{ATP} channel regulator.

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Keywords: α-Endosulphine; insulin release; K_{ATP} channels; Ca^{2+}-channels; sulphonylurea receptor; cyclic AMP-regulated phosphoprotein

Abbreviations: ARPP, cyclic AMP-regulated phosphoprotein; K_{ATP}, ATP-dependent potassium channels; PKA, protein kinase A; PTX, pertussis toxin; SUR, sulphonylurea receptor

Introduction

α-Endosulphine was isolated as an endogenous equivalent for hypoglycemic sulphonylureas (Virsolvy-Vergine *et al*., 1992; 1996). These drugs, commonly used in the treatment of type II diabetic subjects (Loubatières, 1946), stimulate insulin secretion by a direct interaction with sulphonylurea receptors present in β-cells (Ashcroft & Ashcroft, 1990b). The sulphonylurea receptor, SUR (Aguilar-Bryan *et al*., 1995), is physically associated to a pore-forming subunit (Kir 6.2) to constitute the ATP-dependent potassium channel (K_{ATP}, Inagaki *et al*., 1995; 1996; Clement *et al*., 1997). K_{ATP} channels were first discovered in the heart (Noma, 1983) and were later found in many other tissues, especially the β-cells of the islet of Langerhans (Ashcroft & Ashcroft, 1990a). These channels couple the cell metabolism to electrical activity and thus, are implicated in many cellular functions such as hormone secretion, excitability of skeletal muscle (Spruce *et al*., 1985) and neurons, contraction of vascular smooth muscle (Standen *et al*., 1989) and cardiac cytoprotection (Zhenhai & Gross, 1994). They are regulated by nucleotides, essentially variations of intracellular ATP/ADP ratio, and pharmacological agents such as sulphonylureas or diazoxide (Miki *et al*., 1999). In β-cells, the regulation of their activity modulates insulin release: the closure of these channels leads to a membrane depolarization which, in turn, activates voltage-gated L-type Ca^{2+}-channels, the resulting calcium influx triggering insulin release (Ashcroft & Ashcroft, 1990a). α-endosulphine has been purified from porcine brain as a 121 amino acids protein of 19 kDa apparent molecular mass. Its primary structure revealed close similarities with cyclic AMP-regulated phosphoproteins ARPP-16 and ARPP-19 (Peyrollier *et al*., 1996). α-endosulphine is a new member of this phosphoprotein family, encoded by a specific gene (Héron *et al*., 1999). ARPP-16 and ARPP-19 are two substrates of protein kinase A (PKA), differing by a 16 amino acids N-terminus extension for ARPP-19 (Horiuchi *et*

*Author for correspondence; E-mail: virsolvy@montp.inserm.fr

al., 1990). ARPP-16 is only present in central nervous system whereas ARPP-19 is more ubiquitously distributed in all tissues and cell lines (Girault *et al*., 1990). The amino acid sequences of these three proteins revealed the presence of a highly conserved phosphorylation site. α-endosulphine and ARPP-16/19 are phosphorylated in response to activation of PKA (Dulubova *et al*., 2001). The function of these prominent PKA substrates is still unknown but they represent a highly conserved group of proteins that are likely to mediate actions of PKA in a variety of cell types. However, α-endosulphine has been previously shown to inhibit sulphonylurea binding and K_{ATP} channel activity. As a consequence of these actions, the peptide stimulates insulin release (Héron *et al*., 1998). The present study was undertaken to further characterize the insulinotropic properties of α-endosulphine. Thus, we explored the effectiveness of the peptide in the isolated perfused rat pancreas, an integrated model for insulin secretion. The results obtained on that *ex vivo* model led us to analyse the effects of the peptide under various glucose concentrations in the MIN6 β-cell line previously used for the characterization of α-endosulphine properties, studying its mode of action on insulin release *via* changes in membrane potential and calcium currents.

Methods

Chemicals

The substances used in this study were as follows: glibenclamide (Guidotti, Italy), somatostatin (Néosystem, France), forskolin, diazoxide and pertussis toxin (Sigma, U.S.A.). Human recombinant α-endosulphine protein used in this study was produced in a bacterial expression system as previously described (Héron *et al*., 1998).

Isolated perfused rat pancreas

Experiments were performed with male Wistar rats fed *ad libitum* and weighing 320–350 g. The surgical procedure for total isolation of the pancreas from all neighbouring tissues was conducted as previously described (Bertrand *et al*., 1986). The isolated pancreas was then perfused through its own arterial system with a Krebs–Ringer bicarbonate (KRB) buffer containing 0.2% BSA, glucose and the test substances. Media were continuously bubbled with 95% O_2–5% CO_2 and maintained at 37.5°C. A constant pressure was selected to give a flow rate of 2.5 ml min^{-1}. Any change in pancreatic vascular bed resistance induced change in pancreatic effluent output. The first sample was taken after a 30-min stabilization period, the test substances were infused from time 45 min. One-minute fractions were collected for flow rate measurement and then were immediately frozen until insulin determination.

Cell culture

MIN6 β-cells were kindly provided by Dr Ishihara (Third Department of Internal Medicine, University of Tokyo, Japan). They were grown as previously described (Ishihara *et al*., 1993) in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, U.S.A.) containing 25 mM glucose and supplemented with 15% foetal calf serum (Life Technologies), 100 u ml^{-1} penicillin (Life Technologies), 100 μg ml^{-1} streptomycin (Life Technologies) and 75 μM β-mercaptoethanol (Sigma), at 37°C in a 5% CO_2 atmosphere. Cells were seeded 5 days before each series of experiments, in 24-well plates at a density of 5.10^5 cells per well and were used between passages 15 and 25.

Insulin release

Eighteen hours before the experiment, the culture medium was renewed. On the day of the experiment, the cells were washed twice with KRB buffer, pH 7.5 containing 0.1% BSA (KRB–BSA) (Le Brigand *et al*., 1999). They were then preincubated for 1 h in KRB–BSA containing 1 mM glucose at 37°C, 5%CO_2 and incubated for 2 h in KRB–BSA containing various concentrations of glucose or other effectors. Incubations with forskolin and carbachol were performed in the presence of 3 mM glucose. After incubation, the medium was collected, centrifuged at $600 \times g$ for 5 min and stored at −20°C until insulin assay.

Insulin radio-immunoassay

Insulin release was determined in the different samples by radio-immunoassay using ^{125}I-porcine insulin (Cis bio International, France), rat insulin as standard (Novo, Denmark), an anti-porcine insulin guinea-pig antiserum (Kervran *et al*., 1976) and charcoal separation (Herbert *et al*., 1965).

Measurement of $^{45}Ca^{2+}$ influx

Eighteen hours before the experiment, the culture medium was changed. On the day of the experiment, cells were preincubated for 20 min at 37°C in KRB–BSA. The incubation was performed during 20 min at 37°C, in 250 μl KRB–BSA containing 8 μCi ml^{-1} $^{45}CaCl_2$ (Amersham, U.K.) and the test agents (Le Brigand *et al*., 1999). The reaction was stopped by rapid removal of the medium and two washes with 1 ml ice-cold buffer pH 7.4 (in mM: NaCl 140, Tris 10, $LaCl_3$ 1). Cells were then solubilized in 0.1% Triton X 100, $^{45}Ca^{2+}$ contained in the solution was then measured by β counting after addition of liquid scintillation medium (ACS II, Amersham).

Electrophysiology

For electrophysiology, MIN6 cells in culture that were round and apparently single were chosen. The cells used were confirmed to be single on the basis of their whole-cell capacitance (mean 4.4 ± 0.4 pF, $n = 16$), which is close to the theoretical expectation of 4.5 pF for a single β-cell of ~ 12 μM diameter, and the fact that the Ca^{2+}-currents lacked the phasic behaviour expected for electrically coupled but poorly voltage-clamped neighbours. In order to maintain cell metabolism and second-messenger systems intact, membrane currents and potential were recorded using the perforated-patch whole-cell technique as previously described (Smith *et al*., 1993; 2001). Ca^{2+}-currents were measured at 32°C, using voltage-clamp with a bath solution that contained (in mM): NaCl 108, TEACl 30, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6 HEPES 10 (pH 7.4 with NaOH), and glucose

10. The pipette solution contained (in mM): Cs_2SO_4 76, sucrose 55, KCl 10, $MgCl_2$ 1, HEPES 10 (pH 7.2 with CsOH). Perforation was obtained by the addition of 200 μg ml^{-1} amphotericin B to the pipette solution and was considered adequate when the series resistance was <30 MΩ (mean 16 ± 1.5 MΩ, $n=16$). Inward Ca^{2+}-currents were elicited by pulsing the membrane to potentials positive from -60 mV to $+60$ mV in incremental steps, 10 mV in amplitude and 250 ms duration applied at a frequency of 0.2 Hz. The holding potential was -70 mV. Currents flowing due to leak conductances and uncompensated capacitance were removed by subtracting the scaled average of currents elicited by voltage steps to -60, -80, and -90 mV. Membrane potential was monitored using current-clamp with the same solutions used for the measurement of Ca^{2+}-currents except that equimolar NaCl replaced TEACl in the bath and Cs^+ in the pipette was replaced by equimolar K^+. Glucose was added to the bath solution as required.

Results

Effect of α-endosulphine on insulin released by isolated perfused rat pancreas

As it was previously reported that α-endosulphine inhibits K_{ATP} channels and stimulates insulin release (Héron *et al*., 1998), we first investigated the effect of the peptide on the isolated perfused pancreas (Figure 1). In the presence of 8.3 mM glucose, insulin release was stimulated and averaged 18.5 ± 4.4 ng min^{-1} at 45 min (reference value for Figure 1a) compared to 0.4 ± 0.05 ng min^{-1}, the basal insulin level at 1 mM glucose. Addition of either 0.1 or 1 μM α-endosulphine induced a rapid and biphasic inhibition of glucose-induced insulin secretion. This inhibition was maintained during the peptide perfusion and was reversed upon its removal. Figure 1b shows the concentration-dependency of this inhibitory effect 2 min after the addition of α-endosulphine, the time at which maximal inhibition occurs. α-endosulphine at 0.01 μM and 1 μM significantly reduced insulin release by $13\pm3\%$ ($P<0.001$) and $46\pm8\%$ ($P<0.0001$) respectively.

The stimulatory effect of the peptide originally observed with MIN6 β-cells (Héron *et al*., 1998) was investigated using the isolated perfused pancreas model. In pancreata perfused with 1 mM glucose, α-endosulphine did not stimulate basal insulin release (Figure 2). On the other hand, when the glucose concentration was raised to 16.7 mM, the peptide clearly inhibited the biphasic insulin response. Inhibition occurred on both phases of secretion and was only partially reversed upon removal of α-endosulphine. The first peak of insulin release was reduced by 60% and the second phase by 80%. Furthermore, no stimulatory effect of α-endosulphine was recorded when insulin secretion was inhibited by 100 μM diazoxide, a K_{ATP} channel opener (not shown).

Effects of α-endosulphine on insulin secretion and calcium fluxes in MIN6 β-cells

Figure 3 represents the effects of α-endosulphine on insulin secretion *in vitro* from MIN6 β-cells. As previously described (Héron *et al*., 1998), α-endosulphine stimulated insulin release at a non-stimulatory glucose concentration (1 mM). This stimulatory effect was concentration-dependent between 0.5 and 2.2 μM, the maximal dose used in these experiments and

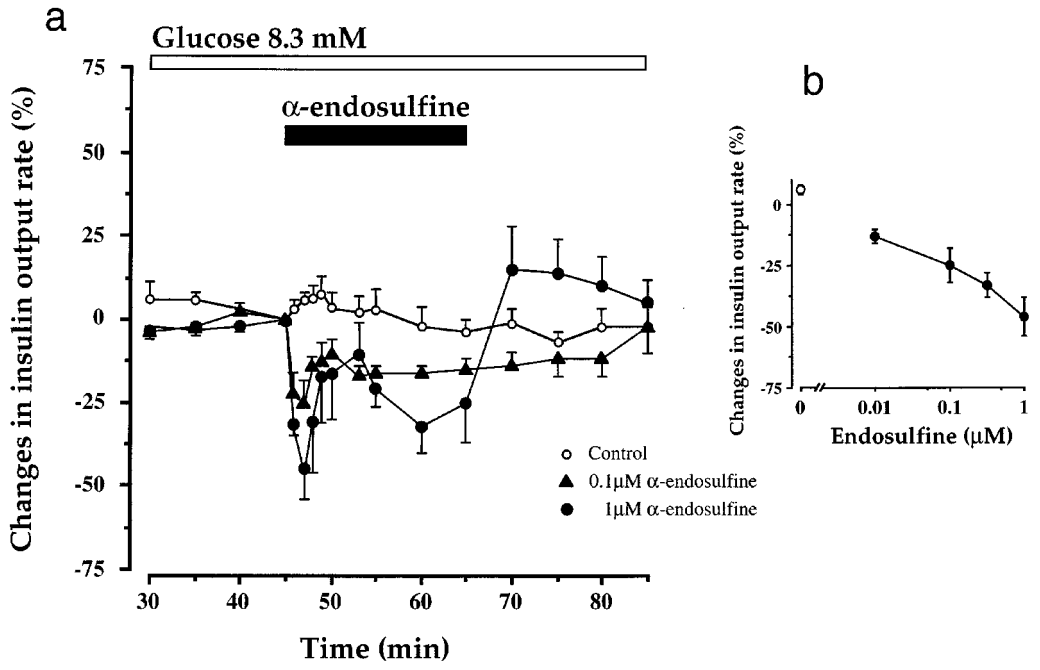

Figure 1 Inhibitory effect of α-endosulphine on glucose-stimulated insulin secretion from isolated perfused rat pancreas. (a) Effect of α-endosulphine on insulin secretion from pancreata perfused with 8.3 mM glucose: 0.1 μM; 1 μM and control. (b) Concentration-response curve for the inhibitory effect of α-endosulphine on glucose stimulated insulin release taken at 2 min after α-endosulphine perfusion *i.e.* at time 47 min. Data are expressed as changes in insulin output in relation to the value obtained after the equilibration period (45 min) taken as 0%, each point representing the mean$\pm$s.e.mean of 4–6 determinations.

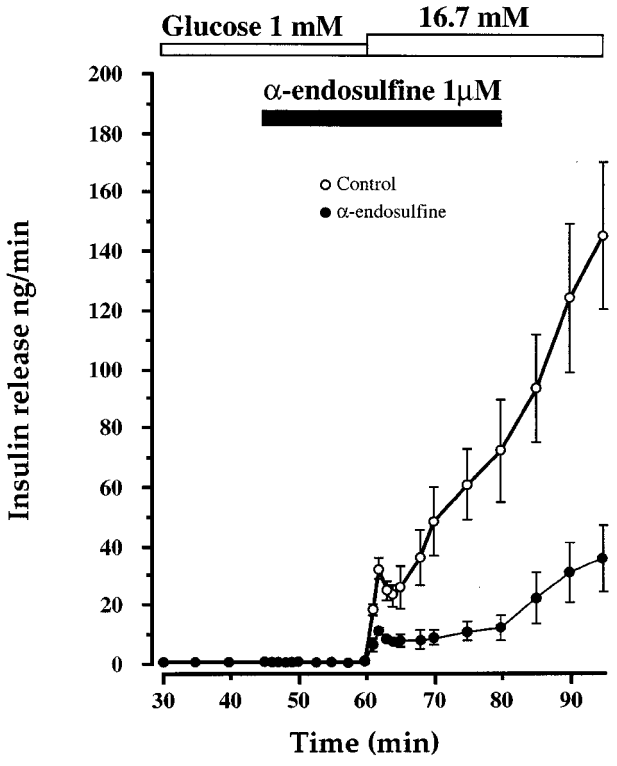

Figure 2 Effect of α-endosulphine on basal and glucose-induced biphasic insulin release from the isolated perfused rat pancreas. One μM α-endosulphine was applied in the presence of 1 mM glucose, 15 min before the glucose increase to 16.7 mM. Results were compared to the control without α-endosulphine. Data are expressed as mean values ± s.e.mean obtained from four experiments.

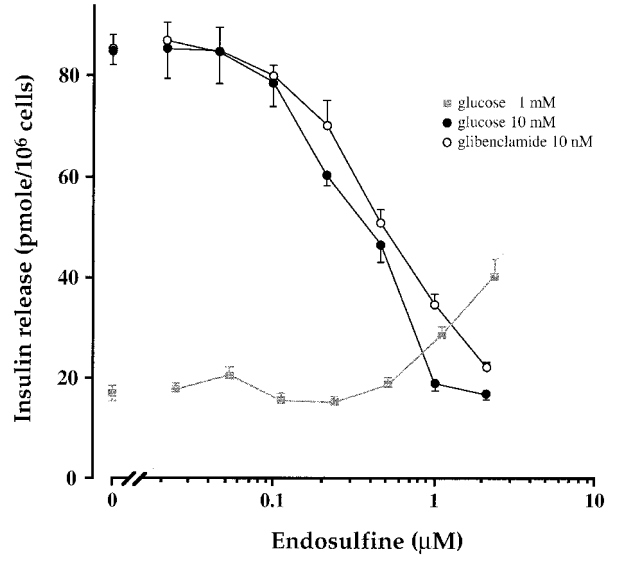

Figure 3 Concentration-response curves for the effect of α-endosulphine on insulin release from MIN6 cells in the presence of 10 mM glucose, 10 nM glibenclamide or 1 mM non-stimulatory glucose concentration. Data represent the mean values ± s.e.mean of three experiments each performed in triplicate.

which led to a 2 fold stimulation of release over basal. In the presence of 10 mM glucose, α-endosulphine inhibited the sugar-induced stimulation of insulin release in a concentration-dependent manner with an EC_{50} of 0.5 μM and a maximal effect at 1 μM. When 1 μM α-endosulphine was added, glucose was unable to stimulate insulin secretion which remained at basal values. The same inhibitory effect of the peptide was observed when 10 nM glibenclamide was substituted for glucose as the stimulus (Figure 3). The inhibitory effect of 1 μM α-endosulphine was also observed when the stimulant was changed to forskolin (100 μM) or to a membrane depolarization induced by 30 mM KCl (Figure 4a). Moreover, the inhibitory effect of α-endosulphine was still observed under stimulatory conditions known to bypass the regulatory role of K_{ATP} channels, namely membrane depolarization induced by high K^+ in the presence of high glucose and diazoxide (Gembal *et al*., 1992). On the other hand, when insulin release was triggered by carbachol, known to act predominantly by mobilizing intracellular Ca^{2+} stores (Wolf *et al*., 1988), α-endosulphine was without effect (Figure 4a). When Ca^{2+} entry was measured in parallel experiments using $^{45}Ca^{2+}$ (Figure 4b), the modification of Ca^{2+} influx closely mirrored the effects observed for the peptide on insulin release for each condition tested, suggesting a link between the two phenomena.

Receptor-linked inhibitory effects of regulatory peptides are often coupled to activation of G_i/G_o protein (Sharp, 1996), an intracellular pathway sensitive to *Bordetella pertussis* toxin (PTX) treatment. The involvement of such a mechanism in the inhibitory effect of α-endosulphine was therefore investigated. MIN6 cells were incubated overnight with 200 ng ml^{-1} PTX (Dalle *et al*., 1999). The ability of the peptide to inhibit the release of insulin stimulated by 10 mM glucose was then tested. The inhibitory effect of 1 μM α-endosulphine on insulin release (19.96 ± 0.85 pmole/10^6 cells, $n=5$) was not significantly affected by the toxin treatment (19.26 ± 0.78 pmole/10^6 cells, $n=5$). In parallel control experiments, the inhibitory effect of 100 nM somatostatin was significantly reversed by PTX pretreatment (data not shown).

Effects of α-endosulphine on membrane potential and Ca^{2+}-currents

Since α-endosulphine directly blocks K_{ATP} channels, the effect of the peptide was investigated on the membrane potential and electrical activity induced by glucose. In the absence of glucose, MIN6 cells were electrically silent with a membrane potential mean value of −67 ± 1 mV ($n=16$). Under these conditions, the addition of 1 μM α-endosulphine resulted in a rapid depolarization of the membrane potential (Figure 5a). Coincident with the initial depolarization was a transient firing of action potentials clearly illustrated on temporal expansion of the response. On cessation of this transient electrical activity the membrane potential depolarized to a steady state value of −19 ± 6 mV ($n=3$) which was devoid of action potentials. On removal of the peptide, the membrane potential returned to its original resting value. Addition of 200 μM tolbutamide, under the same conditions, also induced a rapid depolarization but, unlike the situation observed with α-endosulphine, a sustained firing of action potentials was observed (Figure 5a).

In five of seven cells tested, the addition of 10 mM glucose depolarized the membrane potential to a mean

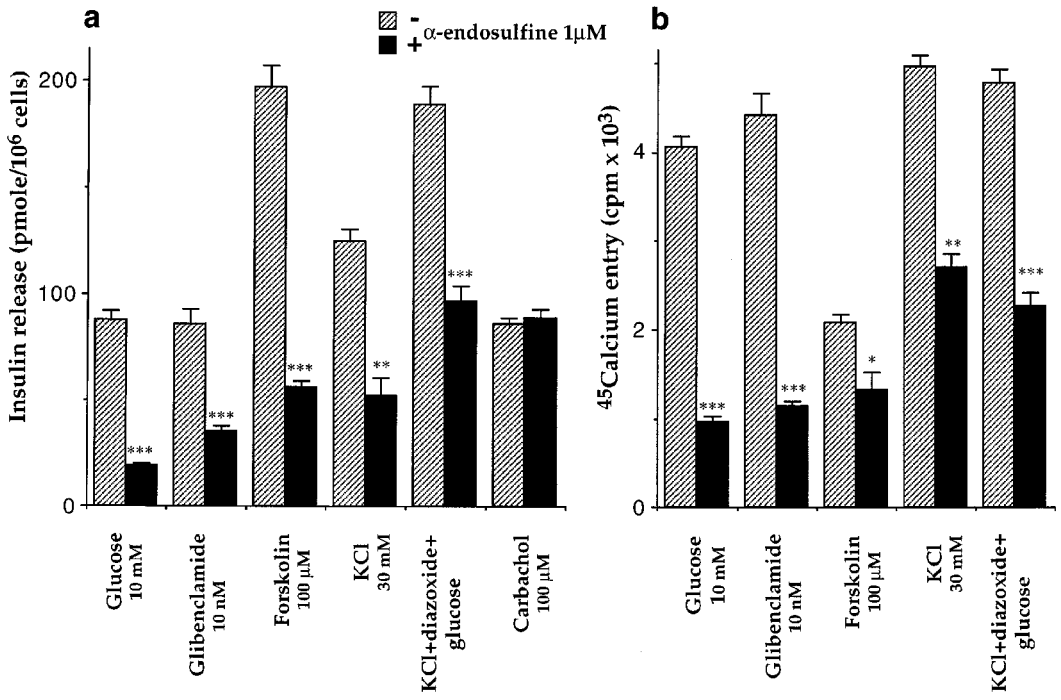

Figure 4 (a) Effect of 1 μM α-endosulphine on insulin release from MIN6 β-cells stimulated by different agents: glucose 10 mM, glibenclamide 10 nM, forskolin 100 μM, KCl 30 mM, diazoxide 100 μM and carbachol 100 μM. Incubations were realized in the presence of 5 mM glucose when the concentration was not specified. (b) Effect of 1 μM α-endosulphine on $^{45}Ca^{2+}$ influx from MIN6 cells stimulated under the same conditions as for insulin secretion. Data represent the mean values ± s.e.mean of three experiments performed in quadruplicate. Statistical significance was determined by comparing the values obtained in the presence and in the absence of α-endosulphine, using Student's *t*-test analysis. $*P<0.05$; $**P<0.01$; $***P<0.001$.

value of -51 ± 2 mV and induced action potential firing (data not shown). Subsequent addition of 1 μM α-endosulphine induced a further rapid and reversible depolarization (Figure 5b). This was associated with an initial increased rate of action potential firing which ceased as the cell continued to depolarize to a steady-state of -20 ± 3.9 mV ($n=7$). The depolarization was maintained for the duration of peptide application. The effect of the peptide was unaffected by the length of time for which it was applied: similar results being observed for applications of 30 and 300 s duration (data not shown). The effects of the peptide were reversible on its removal. In the presence of 10 mM glucose, addition of 200 μM tolbutamide also resulted in a small and reversible depolarization but which was associated with a maintained action potentials firing (Figure 5b). Application of 20 μM nifedipine to cells already firing action potentials in response to 10 mM glucose, produced a small depolarization that was associated with abolition of the action potentials (Figure 5b). The combination of 200 μM tolbutamide and 20 μM nifedipine produced results similar to those obtained with α-endosulphine: depolarization, block of action potentials and increased input resistance (Figure 5b).

Nifedipine directly blocks L-type Ca^{2+}-channels, since the effect of the dihydropyridine mimics part of the effects of α-endosulphine, it is possible that the peptide has a similar mode of action. To investigate this idea and to determine the mechanism by which α-endosulphine decreased Ca^{2+} influx (Figure 4a), Ca^{2+}-currents were measured directly in MIN6 using the perforated-patch whole-cell voltage-clamp method. Inward Ca^{2+}-currents were elicited on step depolarization of the cell to potentials positive to -60 mV. As shown in Figure 6a, under control conditions, currents rapidly peaked within 5 ms and then inactivated more slowly to a steady-state level. Both the peak and steady-state current-voltage relationships were bell shaped (Figure 6b). Currents reached a maximum around $+10$ mV and reversed at potentials positive to $+40$ mV. The currents were abolished by 20 μM nifedipine (data not shown). These electrophysiological characteristics are typical for whole-cell currents flowing through L-type voltage-gated Ca^{2+}-channels (McCleskey, 1994), the only type of functional voltage-gated Ca^{2+}-channel found in β-cells derived from pancreas of the mouse (Rorsman *et al.*, 1988). α-endosulphine at 1 μM, a concentration that maximally inhibited stimulated insulin secretion, blocked Ca^{2+}-currents at all voltages tested (Figure 6a). The block developed during the duration of the Ca^{2+}-current, occurred at all voltages tested and was concentration-dependent with an ED_{50} of 0.5–0.8 μM (Figure 6c). Maximal block was obtained at 10 μM. The peak current was inhibited less than the steady-state (Figure 6c), at $+10$ mV, 1 μM α-endosulphine blocked the peak current by $40\pm2.3\%$ whereas the steady-state currents were blocked by $85\pm4\%$ ($n=4$). Consistent with these findings, the block of Ca^{2+}-currents by α-endosulphine seems to be use dependent (data not shown) an idea compatible with the increased rate of inactivation. These data indicate that the peptide acts as a direct open channel blocker.

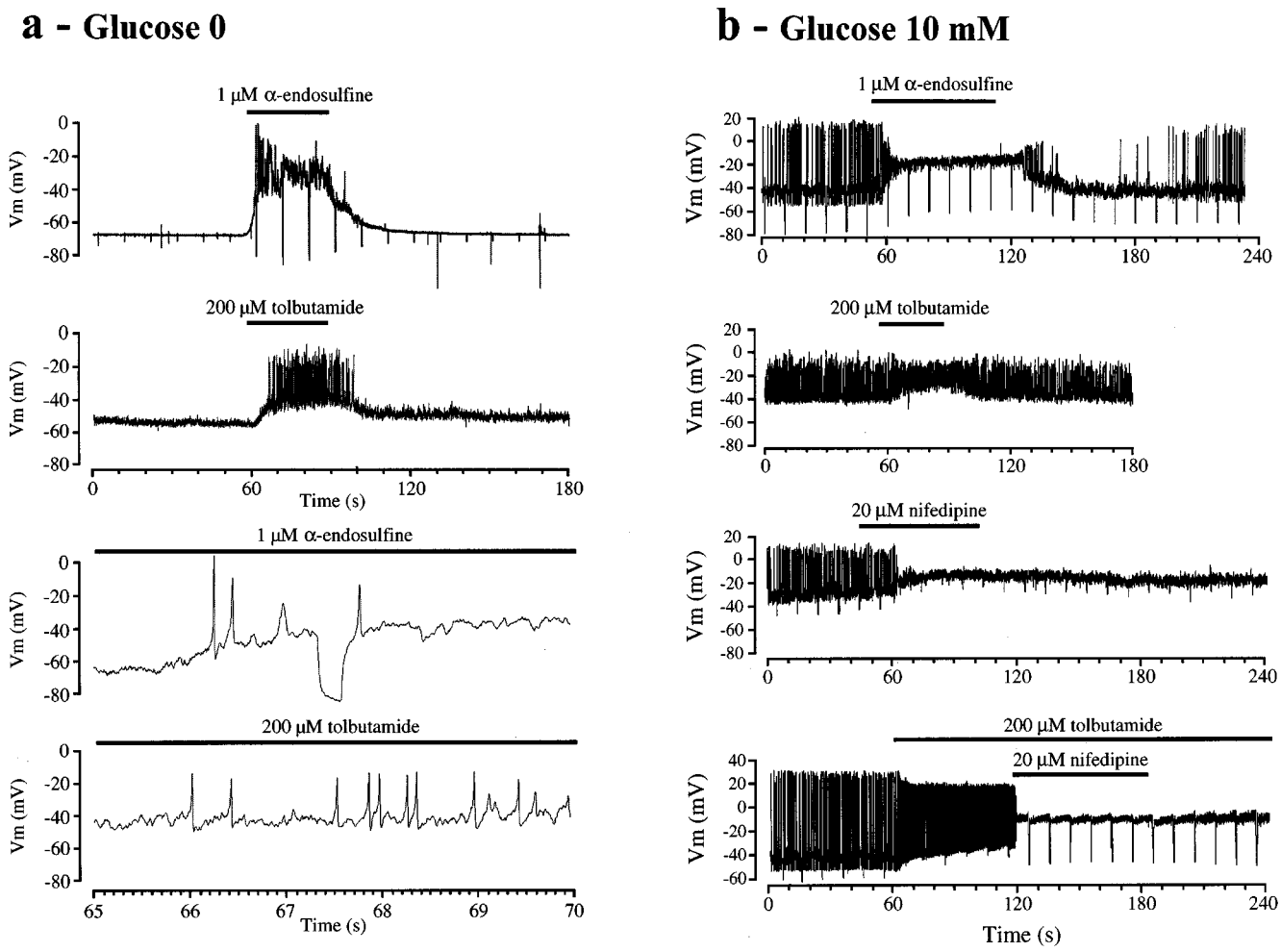

Figure 5 Representative perforated-patch current-clamp recordings of the membrane potential of a MIN6 cell. (a) Responses to 1 μM α-endosulphine in the absence of glucose, compared to those of 200 μM tolbutamide in a different cell displayed on two different time scales. (b) Response to 1 μM α-endosulphine of a cell firing of action potentials in the presence of 10 mM glucose compared to the responses obtained with 200 μM tolbutamide, 20 μM nifedipine and a combination of tolbutamide and nifedipine recorded in the same conditions. Negative downward deflections are the voltage responses to −10 pA current injections applied at 0.1 Hz which allow monitoring of the input-resistance of the cell.

Discussion

α-Endosulphine was originally described as an endogenous equivalent for sulphonylureas and a regulator of K_{ATP} channel activity (Héron *et al*., 1998). Using the MIN6 β-cell line, we had shown that α-endosulphine inhibited K_{ATP} channel activity and stimulated insulin release under non-stimulating glucose conditions. In order to substantiate these previous findings, we have investigated the effect of α-endosulphine under different conditions and models. This study presents the unexpected opposite effect of α-endosulphine in a more integrated model, the isolated perfused rat pancreas as well as on MIN6 β-cells in culture. Indeed, when the glucose concentration was increased to stimulate release, the induced insulin secretion was inhibited by α-endosulphine. In isolated perfused rat pancreas, the inhibition with α-endosulphine was slowly reversible and occurred on both phases of insulin stimulation. No stimulatory effect of α-endosulphine was observed in the perfused pancreas, even with drastically low glucose concentrations (1 mM) conditions under which such stimulation was originally described on MIN6 β-cells. In these cells, under stimulatory conditions, the inhibitory properties of the peptide were accompanied by a decrease in Ca^{2+} uptake, suggestive of a specific mechanism related to Ca^{2+} entry. This was confirmed by the following findings: (i) no inhibitory effect of α-endosulphine was observed when insulin release was stimulated by carbachol, known to release Ca^{2+} from intracellular stores (Wolf *et al*., 1988). (ii) α-endosulphine abolished the release induced by secretagogues (sulphonylurea, forskolin or K^+ depolarization) that require extracellular Ca^{2+} entry. The inhibitory effects of the peptide were not modified by conditions that bypass the regulation of secretion by K_{ATP} channels (Gembal *et al*., 1992) indicating that these channels were not involved in the inhibitory process. The inhibition was also independent of a *pertussis* toxin-sensitive G_i/G_o protein, which suggests a mechanism of action that differs from the inhibitory receptor-mediated pathway already characterized for other endogenous peptides (Sharp, 1996; Ullrich *et al*., 1990).

In both the presence and absence of glucose, α-endosulphine depolarized the membrane potential and transiently

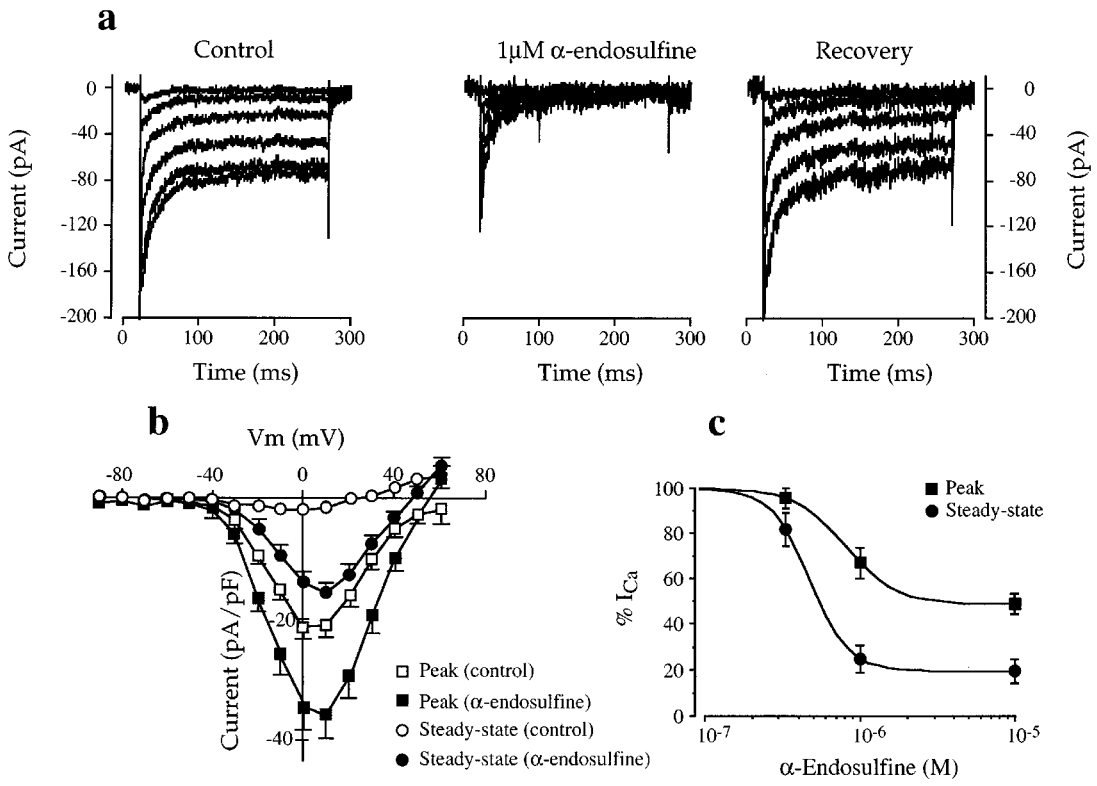

Figure 6 (a) Representative perforated-patch whole-cell recordings of Ca^{2+}-currents from the same MIN6 cell. Superimposed tracings were obtained in response to voltage steps between −40 (smallest currents) to +10 mV (largest currents), in 10 mV increments from a holding potential of −70 mV. Currents were elicited by 250 ms pulses under control conditions (left traces), in the presence of 1 μM α-endosulphine (middle traces) and for the recovery after a 3 min wash (right traces). (b) Mean current-voltage relationships for peak and steady-state recorded for control and with 1 μM α-endosulphine. Data are the mean±s.e.mean recorded from four cells. (c) Concentration dependence for the peak and steady-state Ca^{2+}-current (I_{Ca}) plotted as a percentage that remains in the presence of α-endosulphine. Currents recorded at +10 mV. Data are the mean±s.e.mean recorded from four cells.

elicited electrical activity indicative of K_{ATP} channel blockade, data that confirm previous findings (Héron *et al*., 1998). However, in the presence of stimulatory glucose concentrations, the ability of α-endosulphine to produce continued membrane depolarization devoid of action potentials on prolonged incubation is not attributable to sole block of K_{ATP} as this effect is not mimicked by sulphonylureas. On the other hand, nifedipine, either alone or in combination with tolbutamide, mimics the actions of the peptide on the membrane properties: depolarization, block of action potentials and increase in input resistance. These data suggest the hypothesis that α-endosulphine directly affects the activity of Ca^{2+}-channels and Ca^{2+} entry, an idea which was confirmed by electrophysiological measurement of the Ca^{2+}-currents. Thus, α-endosulphine can reduce Ca^{2+}-influx, and consequently insulin secretion, by a direct action on L-type Ca^{2+}-channels, probably by an open channel block mechanism where the rate of peptide association is slower than the rate of channel activation. This appears to be an original mode of action for an endogenous peptide, as only peptide toxins present in venom have been shown so far to display such features (Zamponi, 1997; Stotz *et al*., 2000).

According to the membrane potential recordings, the primary event which occurs upon α-endosulphine addition to the β-cell, is membrane depolarization related to K_{ATP} channel closure. In the absence of glucose, the depolarization activates L-type voltage gated Ca^{2+}-channels. The consecutive burst of calcium entry is sufficient to trigger insulin release in excess of basal. The process then comes to an end by the subsequent block by α-endosulphine of open Ca^{2+}-channels, which terminates insulin secretion. In the presence of 10 mM glucose, the cell membrane is depolarized and voltage-gated Ca^{2+}-channels are activated and the β-cell secretes insulin. Under these conditions, however, the further closure of K_{ATP} channels by α-endosulphine has little consequence on the stimulation of insulin release. Since the peptide directly blocks the open Ca^{2+}-channels, Ca^{2+} cannot enter and insulin secretion is globally reduced. Furthermore any small increase in insulin secretion that may arise due to the initial depolarization and temporary increase in action potential firing rate will be transient and far outweighed by the subsequent termination of calcium entry.

In the perfused pancreas, only the inhibitory effect was revealed in the presence of α-endosulphine. This discrepancy with the stimulatory effect observed in MIN6 β-cells could be explained by the fact that this cell line represents a homogenous population of pure β-cells in which it is possible to observe phenomena that are masked by compensatory mechanisms present in integrated models. It is likely that the 'basal' insulin secretion observed in the perfused pancreas already correspond to a slightly stimulated state as compared to the authentically basal secretion observable in MIN6 cells. In such conditions, only the inhibitory effect of α-endosulphine may be observed.

The physiological relevance of these activities on ionic channels *in vivo* under specific circumstances (e.g. extreme physiological or pathological conditions) remains to be established. Nevertheless, according to the present data, α-endosulphine is likely to play a role in the negative control of insulin release. It must be recalled that the endosulphine concept is made of two molecules, α-endosulphine and β-endosulphine (Virsolvy-Vergine *et al*., 1992) and that, as stated elsewhere (Bataille *et al*., 1999), the latter, for which the exact chemical nature is unknown, is likely to represent the real endogenous equivalent of sulphonylureas, while another role should be designated to α-endosulphine. Besides this role in the negative control of insulin secretion, it is possible that the peptide regulates other excitable cells such as neurons, vascular and cardiac cells which also expressed K_{ATP} channels and Ca^{2+}-voltage gated channels. A recent study indicates that the brain is a major location of α-endosulphine and that the brain levels of the peptide are considerably decreased in patients with Alzheimer's disease (Kim & Lubec, 2001) suggesting a neurobiological function of the molecule.

In conclusion, α-endosulphine does not appear solely as a regulator of K_{ATP} channels as initially thought, rather it is likely to play a role in the control of the electrical activity of the cell *via* a block of voltage-gated calcium channels in the β-cells and in other organs such as the brain.

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References

AGUILAR-BRYAN, L., NICHOLS, C.G., WECHSLER, S.W., CLEMENT, IV., J.P., BOYD, A.E., GONZALEZ, G., HERRERA-SOSA, H., NGUY, K., BRYAN, J. & NELSON, D.A. (1995). Cloning of the β-cell high affinity sulfonylurea receptor: a regulator of insulin secretion. *Science,* **268,** 423 – 425.

ASHCROFT, F.M. & ASHCROFT, S.J.H. (1990a). Properties and function of ATP-sensitive K-channels. *Cell Signal,* **2,** 197 – 214.

ASHCROFT, F.M. & ASHCROFT, S.J.H. (1990b). The sulfonylurea receptor. *Biochem. Biophys. Acta.,* **1175,** 45 – 59.

BATAILLE, D., HÉRON, L., VIRSOLVY, A., PEYROLLIER, K., LECAM, A., GROS, L. & BLACHE, P. (1999). α-Endosulphine, a new entity in the control of insulin secretion. *Cell. Mol. Life Sci.,* **56,** 78 – 84.

BERTRAND, G., CHAPAL, J., LOUBATIÈRES-MARIANI, M.M. (1986). Potentiating synergism between adenosine diphosphate or triphosphate and acetylcholine on insulin secretion. *Am. J. Physiol.,* **251,** 416 – 421.

CLEMENT, IV., J.P., KUNJILWAR, K., GONZALEZ, G., SCHWANSTECHER, M., PANTEN, U., AGUILAR-BRYAN, L. & BRYAN, J. (1997). Association and stoichiometry of K_{ATP} channel subunits. *Neuron.,* **18,** 827 – 833.

DALLE, S., SMITH, P., BLACHE, P., LE-NGUYEN, D., LE BRIGAND, L., BERGERON, F., ASHCROFT, F.M. & BATAILLE, D. (1999). Miniglucagon (glucagon 19 – 29): a potent and efficient inhibitor of secretagogue-induced insulin release through a Ca^{2+} pathway. *J. Biol. Chem.,* **274,** 10869 – 10876.

DULUBOVA, I., HORIUCHI, A., SNYDER, G.L., GIRAULT, J.A., CZERNIK, A.J., SHAO, L., RAMABHADRAN, R., GREENGARD, P. & NAIRN, A. (2001). ARPP-16/ARPP-19: a highly conserved family of cAMP-regulated phosphoproteins. *Neurochem.,* **77,** 229 – 238.

GEMBAL, M., GILON, P. & HENQUIN, J.C. (1992). Evidence that glucose can control insulin release independently from its action on ATP-sensitive K^+ channels in mouse β cells. *J. Clin. Invest.,* **89,** 1288 – 1295.

GIRAULT, A., HORIUCHI, A., GUSTAFSON, E.L., ROSEN, N.L. & GREENGARD, P. (1990). Differential expression of ARPP-16 and ARPP-19, two highly related cAMP-regulated phosphoproteins, one of which is specifically associated with dopamine-innervated brain regions. *J. Neurosci.,* **10,** 1124 – 1133.

HERBERT, V., LAW, K.S., GOTTLIEB, C.W. & BLEICHER, S.J. (1965). Coated charcoal immunoassay of insulin. *J. Clin. Endocrinol.,* **25,** 1375 – 1384.

HÉRON, L., VIRSOLVY, A., APIOU, F., LE CAM, A. & BATAILLE, D. (1999). Isolation, characterization and chromosomal localization of the human ENSA gene that encodes α-endosulphine, a regulator of β-cells K_{ATP} channels. *Diabetes,* **48,** 1873 – 1876.

HÉRON, L., VIRSOLVY, A., PEYROLLIER, K., GRIBBLE, F.M., LE CAM, A., ASHCROFT, F.M. & BATAILLE, D. (1998). Human α-endosulphine a possible regulator of sulfonylurea-sensitive K_{ATP} channel: molecular cloning, expression and biological properties. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 8387 – 8391.

HORIUCHI, A., WILLIAMS, K.R., KURIHARA, T., NAIRN, A.C. & GREENGARD, P. (1990). Purification and cDNA cloning of ARPP-16, a cAMP-regulated phosphoprotein enriched in basal ganglia, and of a related phosphoprotein ARPP-19. *J. Biol. Chem.,* **265,** 9476 – 9484.

INAGAKI, N., GONOI, T., CLEMENT, IV., J.P., NAMBA, N., INAZAWA, J., GONZALEZ, G., AGUILAR-BRYAN, L., SEINO, S. & BRYAN, J. (1995). Reconstitution of I_{KATP} inward rectifier subunit plus the sulfonylurea receptor. *Science,* **270,** 1166 – 1170.

INAGAKI, N., GONOI, T., CLEMENT, IV., J.P., WANG, C.Z., AGUILAR-BRYAN, L., BRYAN, J. & SEINO, S. (1996). A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive K^+ channels. *Neuron.,* **16,** 1011 – 1017.

ISHIHARA, H., ASANO, T., TSUKUDA, K., KATAGARI, H., INUKAI, K., ANAI, M., KIKUCHI, M., YAZAKI, Y., MIYAZAKI, J.I. & OKA, Y. (1993). Pancreatic beta cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia,* **36,** 1139 – 1145.

KERVRAN, A., RIEUTORD, M. & GUILLAUME, M. (1976). A simultaneous radioimmunoassay for growth hormone and insulin in the plasma of rats and rabbits. *Diab. Métabol.,* **2,** 67 – 72.

KIM, S.H. & LUBEC, G. (2001). Brain α-endosulphine is manifold decreased in brains from patients with Alzheimer's disease: a tentative maker and drug target? *Neurosc. Lett.,* **310,** 77 – 80.

LE BRIGAND, L., VIRSOLVY, A., MANECHEZ, D., GODFROID, J.J., GUARDIOLA-LEMAÎTRE, B., GRIBBLE, F.M., ASHCROFT, F.M. & BATAILLE, D. (1999). *In vitro* mechanism of action of insulin release of S-22068, a new putative antidiabetic compound. *Br. J. Pharmacol.,* **128,** 1021 – 1026.

LOUBATIÈRES, A. (1946). Etudes physiologiques et pharmacodynamiques de certains dérivés sulfamides hypoglycémiants. *Arch. Inter. Physiol.,* **54,** 174 – 177.

MCCLESKEY, E.W. (1994). Calcium channels: cellular roles and molecular mechanisms. *Cur. Opin. Neurobiol.,* **4,** 304 – 312.

MIKI, T., NAGASHIMA, K. & SEINO, S. (1999). The structure and function of the ATP-sensitive K^+ channel in insulin-secreting pancreatic beta-cells. *J. Mol. Endocrinol.,* **22,** 113 – 123.

NOMA, A. (1983). ATP-regulated K^+ channels in cardiac muscle. *Nature,* **305,** 147 – 148.

PEYROLLIER, K., HÉRON, L., VIRSOLVY, A., LE CAM, A. & BATAILLE, D. (1996). α-Endosulphine is a novel molecule structurally related to a family of phosphoproteins. *Biochem. Biophys. Res. Com.,* **223,** 583 – 586.

RORSMAN, P., ASHCROFT, F.M. & TRUBE, G. (1988). Single channel currents in mouse pancreatic B-cells. *Pflügers Arch.,* **412,** 597 – 603.

SHARP, G.W.G. (1996). Mechanisms of inhibition of insulin release. *Am. J. Physiol.,* **271,** 1781 – 1799.

SMITH, P.A., ASHCROFT, F.M. & FEWTRELL, C.M.S. (1993). Permeation and gating properties of the L-type calcium channel in mouse pancreatic beta cells. *J. Gen. Physiol.,* **101,** 767 – 797.

SMITH, P.A., SELLERS, L.A. & HUMPHREY, P.P.A. (2001). Somatostatin activates two types of inwardly rectifying K^+ channels in MIN6 cells. *J. Physiol.,* **532,** 127 – 142.

SPRUCE, A.E., STANDEN, N.B. & STANFIELD, P.R. (1985). Voltage-dependent ATP-sensitive potassium channels of skeletal muscle membrane. *Nature,* **316,** 736 – 738.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science,* **245,** 177 – 180.

STOTZ, S.C., SPAETGENS, R.L. & ZAMPONI, G.W.J. (2000). Block of voltage-dependent calcium channel by the green mamba toxin calcicludine. *J. Membr. Biol.,* **174,** 157 – 165.

ULLRICH, S., PRENTKI, M. & WOLLHEIM, C.B. (1990). Somatostatin inhibition of Ca^{2+}-induced insulin secretion in permeabilized HIT-T15 cells. *Biochem. J.,* **270,** 273 – 276.

VIRSOLVY-VERGINE, A., LERAY, H., KUROKI, S., LUPO, B. & BATAILLE, D. (1992). Endosulphine, an endogenous peptidic ligand for the sulfonylurea receptor: purification and partial characterization from ovine brain. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 6629 – 6633.

VIRSOLVY-VERGINE, A., SALAZAR, G., SILLARD, R., DENOROY, L., MUTT, V. & BATAILLE, D. (1996). Endosulphine, endogenous ligand for the sulfonylurea receptor: isolation from porcine brain and partial structural determination of the α-form. *Diabetologia,* **39,** 135 – 141.

WOLF, B.A., FLORHOLMEN, J., TURK, J. & MCDANIEL, M.L. (1988). Studies of the Ca^{2+} requirements for glucose- and carbachol-induced augmentation of inositol trisphosphate and inositol tetrakisphosphate accumulation in digitonin-permeabilized islets. Evidence for a glucose recognition site in insulin secretion. *J. Biol. Chem.,* **263,** 3565 – 3575.

ZAMPONI, G.W.J. (1997). Antagonist sites of voltage-dependent calcium channels. *Drug Devel. Res.,* **42,** 131 – 143.

ZHENHAI, Y. & GROSS, G.J. (1994). The ATP-dependent potassium channel: an endogenous cardioprotective mechanism. *J. Cardiovasc. Pharmacol.,* **24** (Suppl 4), 28 – 34.

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