Phentolamine Inhibits Exocytosis of Glucagon by G_{i2}
Protein-dependent Activation of Calcineurin in
Rat Pancreatic \( \alpha \)-Cells*

Marianne Høy, Krister Bokvist, Weng Xiao-Gang, John Hansen, Kirstine Juhl,
Per-Olof Berggren†, Karsten Buschard§, and Jesper Gromada¶

From the Laboratory of Islet Cell Physiology, Novo Nordisk A/S, Novo Alle, DK-2880 Bagsvaerd, §Bartholin Institutet,
Kommunehospitalet, Øster Farimagsgade 5, DK-1353 Copenhagen, Denmark and ¶The Rolf Luft Center for Diabetes
Research, Department of Molecular Medicine, Karolinska Institutet, Karolinska Hospital L1:02,
S-171 76 Stockholm, Sweden

Capacitance measurements were used to investigate the molecular mechanisms by which imidazoline compounds
inhibit glucagon release in rat pancreatic \( \alpha \)-cells. The imidazoline compound phentolamine reversibly decreased
depolarization-evoked exocytosis >80% without affecting the whole-cell Ca\(^{2+} \) current. During intracellular
application through the recording pipette, phentolamine produced a concentration-dependent decrease in the rate of exocytosis (IC\(_{50} \) = 9.7 \( \mu \)M). Another imidazoline compound, RX871024, exhibited similar effects on exocytosis (IC\(_{50} \) = 13 \( \mu \)M). These
actions were dependent on activation of pertussis toxin-
sensitive G\(_{i2} \) proteins but were not associated with stim-
ulation of ATP-sensitive K\(^{+} \) channels or adenylyl cyclase
activity. The inhibitory effect of phentolamine on exocytosis on
exocytosis resulted from activation of the protein phos-
phatase calcineurin and was abolished by cyclosporin A
and deltameptrin. Exocytosis was not affected by intra-
cellular application of specific \( \alpha_{2} \), I\(_{1} \), and I\(_{2} \) ligands.
Phentolamine reduced glucagon release (IC\(_{50} \) = 1.2 \( \mu \)M)
from intact islets by 40%, an effect abolished by pertussis
toxin, cyclosporin A, and deltameptrin. These data
suggest that imidazoline compounds inhibit glucagon
secretion via G\(_{i2} \)-dependent activation of calcineurin in
the pancreatic \( \alpha \)-cell. The imidazoline binding site is
likely to be localized intracellularly and probably
-closely associated with the secretory granules.

Thirty years has elapsed since the initial demonstration that
the imidazoline compound phentolamine stimulated glucose-
induced insulin release in humans (1–3). Good evidence exists
that the insulinotropic effects of imidazoline compounds do not
result from antagonism of \( \alpha_{2} \)-adrenergic receptors but rather
from inhibition of ATP-sensitive K\(^{+} \) (\( K_{ATP} \))1 channels in the
\( \beta \)-cell plasma membrane (6–9), resulting in membrane depo-
larization, stimulation of Ca\(^{2+} \) influx, and exocytosis. In addition,
imidazoline compounds also stimulate insulin release by a
direct interaction with the exocytotic machinery (10).

Recent evidence suggests that imidazoline compounds stim-
ulate not only insulin release but also somatostatin release
while suppressing glucagon secretion (11). The mechanism un-
derlying the inhibitory action of imidazoline compounds on
glucagon release is not clear but may involve either a direct or
a paracrine effect on the \( \alpha \)-cells (11–13). Here we have com-
bined the patch clamp technique with capacitance measure-
ments of exocytosis to explore the effects of different imidazo-
line compounds on exocytosis in single rat pancreatic \( \alpha \)-cells. We
thereby provide the first direct evidence that imidazoline
compounds inhibit Ca\(^{2+} \)-dependent exocytosis of glucagon via
G\(_{i2} \)-dependent activation of the serine/threonine protein
phosphatase calcineurin.

EXPERIMENTAL PROCEDURES

Preparation of Islets and \( \alpha \)-Cells—Male Lewis rats (250–300 g; Mal-
legaard, Lille Skensved, Denmark) were anesthetized by pentobarbital
(100 mg/kg intraperitoneally), and the pancreas was removed. The
experimental procedures were approved by the local ethical committee.
Islets were isolated by collagenase digestion and dispersed into single
cells using dispase. Pancreatic \( \alpha \)-cells were separated by fluoresceine-
activated cell sorting as described elsewhere (14). Based on the hor-
monte contents and their glucose sensitivity, we estimate that the pre-
parations contain >80% \( \alpha \)-cells and <3% \( \beta \)-cells (14, 15). The cell
suspension was plated on 35-mm diameter Petri dishes and incubated in a
humidified atmosphere for up to 3 days in RPMI 1640 tissue culture
medium (Life Technologies Ltd., Paisley, United Kingdom) supple-
mented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml
penicillin, and 100 \( \mu \)l/ml streptomycin.

Electrophysiology—Pipettes were pulled from borosilicate glass,
coated with Sylgard near their tips, and fire-polished. When filled with
pipette solutions, the electrodes had a resistance of 5–4 megohms. The
whole-cell \( K_{ATP} \) conductance was estimated by applying 10-mV hyper-
deralizing and depolarizing voltage pulses (duration, 200 ms; pulse interval, 2 s)
from a holding potential of −70 mV using either the perforated patch or
standard whole-cell configuration. The currents were recorded using an
Axopatch 200B patch clamp amplifier, digitized, and stored in a
computer using the Digidata AD converter and the software pClamp (Axon
Instruments, Foster City, CA). The \( \alpha \)-cell membrane potential was
recorded using the perforated patch whole-cell configuration. Exocyt-
osis was measured as increases in cell capacitance using, except for Fig.
3, an EPC-9 patch clamp amplifier and the Pulse software (version 8.30; HEKA
Elektronik, Lamprocht/Pfalz, Germany). The interval between two
successive points was 0.2 s. All measurements of cell capacitance,
except those in Fig. 3 in which the perforated patch whole-cell config-
uration was used, have been performed using the standard whole-cell
recording mode. In Fig. 3, changes in cell capacitance were elicited by
500-ms voltage clamp depolarizations to 0 mV from a holding potential
of −70 mV using an EPC-7 patch clamp amplifier (List Elektronik,
Darmstadt, Germany) and in-house software written in AxoBasic (Axon

* This study was supported by grants from the Swedish Diabetes
Association, the Novo Nordisk Foundation, the Nordic Insulin Founda-
tion committee, and the Swedish Medical Research Council Grants
72X-00034, 72XS-12708, and 72X-09890 to P.-O. B.). The costs of pub-
lication of this article were defrayed in part by the payment of page
charges. This article must therefore be hereby marked "advertisement"
in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed:
Lilly Research Laboratories, Lilly Forschung GmbH, Essener Strasse
93, D-22419 Hamburg, Germany. Tel.: 49-40-5-27-24-323; Fax: 49-40-
5-27-24-615; E-mail: Gromada_Jesper@lilly.com.

Pipettes were pulled from borosilicate glass,
coated with Sylgard near their tips, and fire-polished. When filled with
pipette solutions, the electrodes had a resistance of 5–4 megohms. The
whole-cell \( K_{ATP} \) conductance was estimated by applying 10-mV hyper-
deralizing and depolarizing voltage pulses (duration, 200 ms; pulse interval, 2 s)
from a holding potential of −70 mV using either the perforated patch or
standard whole-cell configuration. The currents were recorded using an
Axopatch 200B patch clamp amplifier, digitized, and stored in a
computer using the Digidata AD converter and the software pClamp (Axon
Instruments, Foster City, CA). The \( \alpha \)-cell membrane potential was
recorded using the perforated patch whole-cell configuration. Exocyt-
osis was measured as increases in cell capacitance using, except for Fig.
3, an EPC-9 patch clamp amplifier and the Pulse software (version 8.30; HEKA
Elektronik, Lamprocht/Pfalz, Germany). The interval between two
successive points was 0.2 s. All measurements of cell capacitance,
except those in Fig. 3 in which the perforated patch whole-cell config-
uration was used, have been performed using the standard whole-cell
recording mode. In Fig. 3, changes in cell capacitance were elicited by
500-ms voltage clamp depolarizations to 0 mV from a holding potential
of −70 mV using an EPC-7 patch clamp amplifier (List Elektronik,
Darmstadt, Germany) and in-house software written in AxoBasic (Axon

1 The abbreviations used are: \( K_{ATP} \), ATP-sensitive K\(^{+} \) channels; nS,
nanosiemens; \( I_{f} \), fentorafadrs; RRP, readily releasable pool; TEA, tet-
raethylammonium; GDP\(_{iS} \), guanosine 5′-O-2-thiodiphosphate.
In incubation. Groups of 10 size-matched rat islets were preincubated for 60 min with KOH. For measurements of exocytosis using voltage clamp depolarization, the extracellular solution used for measurements of cell capacitance evoked by voltage clamp depolarizations contained 118 mM NaCl, 20 mM tetraethylammonium chloride, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 5 mM HEPES (pH 7.40 with NaOH), and 0 or 5 mM d-glucose. The extracellular solution used for measurements of cell capacitance evoked by voltage clamp depolarizations contained 125 mM potassium glutamate, 10 mM KCl, 1 mM MgCl2, 0.3 mM Mg-ATP, 10 mM EGTA, and 5 mM HEPES (pH 7.15 with KOH). The free Ca2+ concentration of the resulting buffer was 0.87 mM using the binding constants of Martell and Smith (18). The pipette solution used for measurements of membrane potential and KATP channel activity, using the perforated patch configuration, was composed of 76 mM K2SO4, 10 mM KCl, 10 mM NaCl, 1 mM MgCl2, 5 mM Mg-ATP, 10 mM EGTA, and 5 mM HEPES (pH 7.15 with KOH). The whole-cell capacitance evoked by voltage clamp was considered satisfactory when the cell capacitance averaged 0.3 nS. In a series of five experiments, the input resistance varied from 1.5–2 MΩ (Millipore) and discarded. The islets were resuspended in 200 µl of extracellular solution in the absence and presence of test compounds and the indicated glucose concentration. At the end of the test incubation (1 h), the medium was aspirated and assayed immediately for glucagon using a glucagon radioimmune assay kit (GL-32K; Linco Research, St. Charles, MO).

Data Analysis—In the infusion experiments, the exocytotic rate is presented as the increase in cell capacitance occurring during the first 60 s following establishment of the whole-cell configuration, excluding any rapid changes occurring during the initial ~10 s required for equilibration of the pipette solution with cytosol. Results are presented as mean values ± S.E. for the indicated number of experiments. Statistical significance was evaluated using Student's t test for paired or unpaired observations or Dunn’s test for multiple comparisons with a single control.

**RESULTS**

**Effects of Phentolamine on Electrical Activity and KATP Channels in Rat a-Cells**—Fig. 1 illustrates electrical activity recorded from a single rat a-cell using the perforated patch whole-cell configuration in the absence of glucose. Spontaneous electrical activity was observed in >80% of the tested cells (n > 80 cells), as expected for an a-cell-rich preparation. The application of phentolamine (0.1 mM) did not affect the ability of the a-cells to fire action potentials (Fig. 1A), whereas the subsequent addition of diazoxide (0.1 mM), which activates KATP channels in rat a-cells, was associated with a reversible inhibition of electrical activity (Fig. 1B).

Fig. 2A shows measurements of the whole-cell KATP current from an intact a-cell using the perforated patch whole-cell configuration. In the absence of glucose, the 10-mV voltage steps applied from a holding potential of −70 mV elicited currents with amplitudes of 2 pA, corresponding to an input conductance of 2 nS. In a series of five experiments, the input conductance averaged 0.3 ± 0.1 nS. The application of phentolamine or the sulfonylurea tolbutamide (both 0.1 mM) did not affect the current amplitude (phentolamine: 0.4 ± 0.1 nS, n = 5; tolbutamide: 0.4 ± 0.2 nS, n = 5), whereas the KATP channel opener diazoxide (0.1 mM) produced a 500% increase in the membrane current, and a specific conductance of 0.2 nS. In subsequent addition of diazoxide (0.1 mM), which activates KATP channels in rat a-cells, was associated with a reversible inhibition of electrical activity (Fig. 1B).

**Fig. 1. Effects of phentolamine and diazoxide on electrical activity.** Spontaneous electrical activity recorded from an individual rat pancreatic a-cell in the absence of glucose. Phentolamine and diazoxide (both 0.1 mM) were added to the bath solution during the periods indicated by the bars. The asterisks in B indicate a period of 1 min. The recording is continuous and representative of five cells.

Instruments, Foster City, CA) as detailed elsewhere (16). The volume of the recording chamber was 0.4 ml, and the solution entering the bath (1.5–2 ml/min) was maintained at 33 °C.

**Solutions**—The extracellular medium consisted of 138 mM NaCl, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 5 mM HEPES (pH 7.4 with NaOH), and 0 or 5 mM d-glucose. The extracellular solution used for measurements of cell capacitance evoked by voltage clamp depolarizations contained 118 mM NaCl, 20 mM tetraethylammonium chloride, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 5 mM HEPES (pH 7.40 with NaOH), and 5 mM glucose. Tetraethylammonium chloride was included in the medium to block the outward delayed rectifying K+ current, which otherwise obscures the smaller Ca2+ current (17). The pipette solution used for the infusion experiments consisted of 125 mM potassium glutamate, 10 mM KCl, 1 mM MgCl2, 0.3 mM CaCl2, 3 mM Mg-ATP, 10 mM EGTA, and 5 mM HEPES (pH 7.15 with KOH). The free Ca2+ concentration of the resulting buffer was 0.87 mM using the binding constants of Martell and Smith (18). The pipette solution used for measurements of membrane potential and KATP channel activity, using the perforated patch configuration, was composed of 76 mM K2SO4, 10 mM KCl, 10 mM NaCl, 1 mM MgCl2, 5 mM HEPES (pH 7.35 with KOH). For measurements of exocytosis using voltage clamp depolarizations, K2SO4 was replaced with CaSO4 in the pipette solution. Electrical contact was established by adding 0.24 mg/ml amphotericin B to the pipette solution (16). Perforation required a few minutes, and the voltage clamp was considered satisfactory when the Gmax was stable and >35–40 nS. The pipette solution used for recording of KATP channel activity using the standard whole-cell configuration contained 125 mM KCl, 10 mM K2SO4, 10 mM EGTA, 1 mM MgCl2, 5 mM HEPES, 0.3 mM Mg-ATP, and 0.3 mM K-ADP (pH 7.15). Pertussis toxin was obtained from RBI (Natick, MA). Deltamethrin and its inactive analog permethrin were from Alomone Labs (Jerusalem, Israel). All other chemicals were purchased from Sigma.

**Antisense and Sense Oligonucleotides**—Single a-cells were incubated for 24 h with a 20 μM concentration of the following antisense and sense oligonucleotides, obtained from TAG Copenhagen (Copenhagen, Denmark): antisense-G9, 5′-CATGTTGGGACGAGTCGGCCTCGTCGCGGCGGGCGCCGC-3′ (this sequence is based on the 5′-noncoding sequence upstream of the initiation codon of the rat G9, cDNA (19)); antisense-G9, 5′-CATCCTCTGGCCTCAGCGCGCCCGCGCGCGCGCCGCGGCGCAGGCAGACGGATG-3′ (from the leader sequence just before the rat G9, cDNA sequence; this sequence is common for G9, and G9a (19, 21)).

**Glucagon Release**—Glucagon release was measured at 37 °C in static incubation. Groups of 10 size-matched rat islets were preincubated for 30 min in 200 µl of extracellular solution consisting of 138 NaCl, 5.6 mM KCl, 2.6 mM CaCl2, 1.2 mM MgCl2, 5 mM HEPES (pH 7.4 with NaOH) and 0–20 mM d-glucose in 96-well Durapore membrane plates (Millipore, Molsheim, France). The medium was aspirated using a vacuum control pump (Millipore) and discarded. The islets were resuspended in 200 µl of extracellular solution in the absence and presence of test compounds and the indicated glucose concentration. At the end of the test incubation (1 h), the medium was aspirated and assayed immediately for glucagon using a glucagon radioimmunoassay kit (GL-32K; Linco Research, St. Charles, MO).

**RESULTS**

**Effects of Phentolamine on Electrical Activity and KATP Channels in Rat a-Cells**—Fig. 1 illustrates electrical activity recorded from a single rat a-cell using the perforated patch whole-cell configuration in the absence of glucose. Spontaneous electrical activity was observed in >80% of the tested cells (n > 80 cells), as expected for an a-cell-rich preparation. The application of phentolamine (0.1 mM) did not affect the ability of the a-cells to fire action potentials (Fig. 1A), whereas the subsequent addition of diazoxide (0.1 mM), which activates KATP channels in rat a-cells, was associated with a reversible inhibition of electrical activity (Fig. 1B).
channels expressed in rat α-cells are sensitive to phentolamine. Fig. 2B shows that phentolamine (0.1 mM) reduced the whole-cell $K_{ATP}$ current by 60%. The inhibitory effect of phentolamine on the whole-cell $K_{ATP}$ current was prompt and amounted on average to 55 ± 5% ($p < 0.001; n = 5$). The subsequent addition of 0.1 mM tolbutamide caused a complete but reversible block of the whole-cell $K^+$ conductance ($97 ± 1%$ inhibition; $p < 0.001; n = 5$).

**Effects of Phentolamine on Depolarization-evoked Exocytosis**—Fig. 3A illustrates whole-cell $Ca^{2+}$ currents and the associated changes in cell capacitance elicited by 500-ms depolarizations from −70 mV to 0 mV in an intact rat α-cell using the perforated patch configuration. In the presence of forskolin, which elevates cytoplasmic cAMP levels, the integrated $Ca^{2+}$ current amounted to 4.8 picocoulombs, and a capacitance increase of 87 fF was evoked. Two minutes after inclusion of 0.1 mM phentolamine in the bathing solution, the same membrane depolarization produced an integrated $Ca^{2+}$ current of 4.7 picocoulombs and a capacitance increase of 16 fF (82% inhibition). The depolarizations and increases in cell capacitance were not associated with any changes in cell conductance, and the capacitance measurements are accordingly likely to report exocytosis. On average (Fig. 3B), phentolamine produced $89 ± 16%$ ($p < 0.01; n = 5$) reversible inhibition of exocytosis, which was not associated with a change of the integrated $Ca^{2+}$ current (Fig. 3C).

**Phentolamine Inhibits Exocytosis Evoked by Intracellular$Ca^{2+}$**—The effects of phentolamine on exocytosis were further investigated in standard whole-cell experiments in which secretion was evoked by intracellular dialysis with a $Ca^{2+}$-EGTA buffer with a free $Ca^{2+}$ concentration of 0.87 μM. Following establishment of the whole-cell configuration, exocytosis was observed as a gradual capacitance increase (Fig. 4A, control). In general, cell capacitance reached a new steady-state level within 3–5 min. It is clear that inclusion of 0.1 mM phentolamine in the pipette solution exerted a strong inhibitory effect on exocytosis (Fig. 4A, phentolamine). On average, phentolamine evoked a $83%$ inhibition of the rate of capacitance increase measured over the first 60 s (excluding the first −10 s) after the establishment of the whole-cell configuration ($p < 0.01; n = 10$ (control) and $n = 4$ (phentolamine)). The effect of phentolamine on exocytosis was dependent on dose (Fig. 4B). No inhibition of exocytosis was observed at ≤3 μM. At higher concentrations, phentolamine decreased the rate of capacitance increase by 42–78%. Approximating the average data points of the inhibitory effect of phentolamine on exocytosis to the Hill equation yielded values of the half-maximal inhibitory concentration (IC$_{50}$) and cooperativity factor of 9 μM and 3, respectively. The maximal effects were seen at phentolamine concentrations of ≥100 μM (Fig. 4B).
by >70% for both compounds (p < 0.01; n = 5). The inhibitory effect of imidazoline compounds on exocytosis does not result from α2-adrenergic antagonistic activity or binding to I1 or I2 receptors, since clonidine (α2-adrenergic agonist), AGN 192403 (I1 ligand), or BU-224 (I2 ligand) failed to affect exocytosis (Table I). Furthermore, an irreversible blockade of either α2-adrenergic receptors with benextramine or I2 receptors with clonidine did not affect the ability of phentolamine to inhibit exocytosis (Table I). These data suggest that the inhibitory action of phentolamine on exocytosis does not involve α2-adrenergic, I1, or I2 receptors.

Table II shows that the inhibitory action of phentolamine on exocytosis was associated with suppression of glucagon release from batches of 10 size-matched rat islets. Phentolamine reduced glucagon release independently of the ambient glucose concentration, with the most pronounced effect at 2.5 mM glucose (54% inhibition). At this glucose concentration, phentolamine reduced glucagon release dose-dependently (Table III) with an IC50 at 1.2 μM, which is in fair agreement with that observed for inhibition of exocytosis.

**Phentolamine Evisits G12 Protein-dependent Inhibition of Exocytosis**—We explored whether the ability of phentolamine to inhibit exocytosis involved activation of GTP binding proteins. Fig. 5A shows that inclusion of a 1 mM concentration of the stable GDP analog GDPβS in the pipette solution abolished the inhibitory effect of phentolamine (0.1 mM) on exocytosis, and the exocytotic response amounted to 90% (n = 5) of the control level. The effect of phentolamine on exocytosis was probably mediated by activation of inhibitory G proteins of the G12/G13 type, since pretreatment of the α-cells with pertussis toxin (100 ng/ml for >20 h) abolished the inhibitory action of phentolamine (Fig. 5B).

To determine the type of pertussis toxin-sensitive G protein, we used antisense oligonucleotides against Gα12 and Gα13. Fig. 5C shows that α-cells pretreated for 24 h with antisense oligonucleotides against Gα12, Gα13, or Gαo did not affect the ability of phentolamine to inhibit exocytosis. In contrast, phentolamine did not suppress exocytosis in cells pretreated with antisense oligonucleotides against Gαo. In cells treated with sense oligonucleotides against Gαo, phentolamine decreased the rate of capacitance compared with that observed in control cells (Fig. 5C). This suggests that Gαo2 proteins mediate the inhibitory action of phentolamine on α-cell exocytosis.

**Inhibitory Effect of Phentolamine on Exocytosis Involves Activation of Calcineurin**—Dephosphorylation catalyzed by the serine/threonine protein phosphatase calcineurin (PP2B) underlies inhibition of exocytosis produced by adrenaline, somatostatin, and ATP in pancreatic β-cells (22, 23). As illustrated...
Phentolamine produces dose-dependent inhibition of glucagon release from rat islets

Glucagon release was measured from freshly isolated batches of 10 size-matched islets exposed to the indicated phentolamine concentration for 1 h in an extracellular medium with 2.5 mM glucose.

| Condition                  | Glucagon release (pg/10 islets/h) | n |
|----------------------------|----------------------------------|---|
| Control                    | 1347 ± 125                       | 15 |
| 0.1 μM phentolamine        | 1319 ± 240                       | 5  |
| 1 μM phentolamine          | 914 ± 123                        | 5  |
| 10 μM phentolamine         | 856 ± 62                         | 4  |
| 30 μM phentolamine         | 848 ± 124                        | 4  |
| 100 μM phentolamine        | 653 ± 74                         | 11 |
| 300 μM phentolamine        | 639 ± 71                         | 4  |

**Fig. 5.** Phentolamine produces $G_{i2}$ protein-dependent inhibition of exocytosis in rat $\alpha$-cells. Changes in cell capacitance were elicited by intracellular dialysis of single cells with 0.87 mM free $\text{Ca}^{2+}$ as described in the legend to Fig. 4. A (left), increases in cell capacitance in the absence and presence of 0.1 mM phentolamine using a pipette solution supplemented with 1 mM GDP/βS. B (left), effects of phentolamine in cells pretreated with pertussis toxin (PTX; 100 ng/ml for >20 h). Histograms (right) show average rates of increase in cell capacitance ($\Delta C_v/\Delta t$) measured over the first 60 s after establishment of the whole-cell configuration ± S.E. of five or six different experiments. C, histogram depicting average rates of increase in cell capacitance ($\Delta C_v/\Delta t$) measured over the first 60 s after establishment of the whole-cell configuration under control conditions and in the presence of 0.1 mM phentolamine in untreated cells and in cells pretreated with 20 μM of the indicated antisense or sense oligonucleotides for 24 h. Data are mean values ± S.E. of five different cells. * $p < 0.05$; ** $p < 0.001$.

In Fig. 6A, this may also apply with regard to phentolamine, since the immunosuppressant cyclosporin A, an inhibitor of calcineurin, abolished the inhibitory action of this imidazoline compound on exocytosis. A similar abolition of phentolamine-evoked inhibition of exocytosis was observed with the calcineurin inhibitor deltamethrin (Fig. 6B) but not in the presence of its inactive analogue permethrin (Fig. 6C). On the contrary, okadaic acid (an inhibitor of type 1, 2A, and 3 serine/threonine protein phosphatases) failed to counteract the inhibitory action of phentolamine (Fig. 6D). On average, phentolamine reduced the exocytotic response by 81% ($p < 0.05$; $n = 5$), similar to that observed in the absence of okadaic acid.

To ascertain that the decrease in exocytosis evoked by phentolamine infusion indeed reflects activation of calcineurin, we measured glucagon release from islets pretreated with inhibitors of this protein phosphatase. Table IV clearly demonstrates that deltamethrin and cyclosporin A prevented the inhibitory action of phentolamine on glucagon release. Under these conditions, glucagon release in the presence of phentolamine amounted to 95% (deltamethrin) and 93% (cyclosporin A) of the control level. On the contrary, phentolamine reduced glucagon release by >50% ($p > 0.01$; $n = 5$) in islets pretreated with either permethrin or okadaic acid (Table IV). Finally, no inhibition of glucagon release was observed in islets pretreated overnight with pertussis toxin.

**DISCUSSION**

Imidazoline compounds have been shown not only to stimulate insulin release but also to improve insulin sensitivity (10, 24), which constitutes two main defects underlying glucose intolerance in type 2 diabetic patients. Since patients with type 2 diabetes also exhibit exaggerated glucagon secretion, our present finding that phentolamine inhibits exocytosis of glucagon may constitute the basis for an additional target for the antidiabetogenic action of this class of compounds. The inhibitory action of phentolamine on exocytosis was not associated with a change in the activity of plasma membrane $K_{ATP}$ channels, the activity of voltage-gated $\text{Ca}^{2+}$ channels, or changes in cytoplasmic free $\text{Ca}^{2+}$ levels (data not shown) but results from a direct interference with the exocytotic machinery, an effect mediated by the protein phosphatase calcineurin.

In this study, we extend previous observations in $\beta$-cells (6, 7, 25) by showing that phentolamine blocked $K_{ATP}$ channel activity in standard whole-cell patch clamp experiments. This is consistent with the observation that rat $\alpha$-cells are equipped with $K_{ATP}$ channels identical to those expressed in $\beta$-cells (26, 27). The $\alpha$- and $\beta$-cell $K_{ATP}$ channel is a complex of two proteins: a pore-forming subunit, Kir6.2, and the sulfonylurea receptor, SUR1 (26, 28, 29). It has recently been demonstrated that phentolamine block of $K_{ATP}$ channels is mediated by Kir6.2 and results from a voltage-independent reduction in channel activity (9). Kir6.2 is also expressed in the heart, which may explain why native cardiac and $\beta$-cell $K_{ATP}$ channels share a similar sensitivity to phentolamine (6, 30).

In keeping with previous observations (15, 26), we found rat $\alpha$-cells to be spontaneously active in the absence of glucose. Exposure of the $\alpha$-cells to phentolamine in the absence of glucose was not associated with increased electrical activity. The failure of phentolamine to affect electrical activity is consistent with the inability of the imidazoline compound to reduce $K_{ATP}$ channel activity in metabolically intact cells. This suggests that the $K_{ATP}$ channels are already maximally inhibited in the absence of glucose and is consistent with the observation that tolbutamide failed to reduce channel activity under these conditions. Little information is available on how glucose inhibits glucagon secretion in rat $\alpha$-cells, except that inhibition is mediated by glucose metabolism (31).

Our data suggest that dephosphorylation of components regulating exocytosis underlies the inhibitory action of phentolamine on glucagon secretion from intact islets and the perfused
Calcineurin mediates the inhibitory effect of phentolamine on glucagon release from rat islets

Glucagon release was measured from batches of 10 size-matched -cells by intracellular dialysis with a pipette solution with free Ca\(^2+\) concentration of 0.87 \(\mu\)M as described in the legend to Fig. 4. Effects of phentolamine (0.1 mM) or okadaic acid (100 nM for 30 min), and pertussis toxin (100 ng/ml for 1 h) are shown in Table IV. Measurements of adenine nucleotide content in purified rat -cells have revealed that they have a high ATP/ADP ratio already at 1 mM glucose and that it does not change significantly during glucose stimulation (33). This contrasts with the situation in the \(\beta\)-cells, where the ATP/ADP ratio increases severalfold following an elevation in the glucose concentration. The constant high ATP/ADP ratio in rat -cells is likely to provide the energy to maintain the cells in a phosphorylated state and consequently to enable phentolamine to inhibit exocytosis in a glucose-independent manner.

Our results show that G\(_{i2}\) proteins mediate the inhibition of exocytosis by phentolamine in rat -cells. This is indeed consistent with the observation that G\(_{i2}\) proteins have been identified in rat pancreatic islets (34). However, it remains to be established whether calcineurin activity is controlled by direct interaction of the G\(_{i2}\) protein or whether intermediate proteins are responsible for signal transduction. Recent studies have revealed that G\(_{i}\) and G\(_{o}\) proteins are involved in the regulation of intracellular transport processes, adding novel targets to the list of effectors for these versatile molecular switches. Heterotrimeric G\(_{i}\) and G\(_{o}\) proteins have been found on chromaffin granules and small vesicles from rodent and bovine brain (35, 36). Interestingly, these heterotrimeric G-proteins differ in their composition of \(\alpha\)-subunits. G\(_{o1}\), G\(_{o2}\), G\(_{a1}\), and G\(_{a2}\)
were detected on small synaptic vesicles, whereas chromaffin granules only contain G_{o2} (36–38). These G proteins are in an ideal position for controlling transport processes across the granular membrane and the priming and fusion steps regulating exocytosis. Indeed, G_{o} proteins are involved in the exocytotic priming step in chromaffin cells (38), whereas G_{i3} proteins regulate swelling of zymogen granules, a potentially important prerequisite for granule fusion (39). These considerations raise the interesting possibility that phentolamine inhibits glucagon exocytosis by interfering with granular associated G_{i2} proteins.

REFERENCES

1. Cerasi, E., Efendic, S., and Luft, R. (1969) Lancet ii, 301–302.
2. Buse, M. G., Johnson, A. H., Kuperminc, D., and Buse, J. (1970) Metab. Clin. Ex. 19, 219–225.
3. Mishin, R. I., Edgar, P. J., and Lockwood, H. D. (1970) Diabetes 19, 688–693.
4. Efendic, S., Cerasi, E., and Luft, R. (1975) Diabetologia 11, 407–411.
5. Garrino, M. G., and Henquin, J. C. (1990) Diabetologia 33, 145–147.
6. Dunne, M. J. (1991) Br. J. Pharmacol. 103, 1847–1850.
7. Plant, T. D., and Henquin, J. C. (1990) Br. J. Pharmacol. 101, 115–120.
8. Jonas, J. C., Plant, T. D., and Henquin, J. C. (1992) Br. J. Pharmacol. 107, 8–14.
9. Proks P., and Ashcroft F. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11716–11720.
10. Zaitsev, S. V., Efanov, A. M., Efanova, I. B., Larsson, O., Östenson, C. G., Gold, G., Berggren, P. O., and Efendic, S. (1996) Diabetes 45, 1610–1618.
11. Efanova, I. B., Zaitsev, S. V., Efanov, A. M., Östenson, C. G., Raap, A., Mest, H. J., Berggren, P-O., and Efendic, S. (1998) Biochim. Cell Biol. 252, 162–165.
12. Mourtada, M., Smith, S. A., Morgan, N. G. (1997) Biochim. Cell Biol. 236, 162–166.
13. Rorsman, P., Berggren, P-O., Bokvist, K., Ericson, H., Möhler, H., Österson, C-G., Smith, P. A. (1989) Nature 341, 233–236.
14. Josefsen, J., Stenvang, J. P., Kindmark, H., Berggren, P. O., Horn, T., Kjær, T., and Buschard, K. (1996) J. Endocrinol. 149, 145–154.
15. Gromada, J., Bokvist, K., Ding, W. G., Bokvist, K., Renstrom, E., and Rorsman, P. (1997) J. Gen. Physiol. 110, 217–228.
16. Ammalía, C., Eliason, L., Bokvist, K., Larsson, O., Ashcroft, F. M., and Rorsman, P. (1993) J. Physiol. 4, 665–688.
17. Rorsman, P., and Trube, G. (1988) J. Physiol. 374, 51–550.
18. Martell, A. E., and Smith, B. M. (1971) Critical Stability Constants, Vols. 1 and 2, Plenum Press, New York.
19. Takano, K., Yasufuku-Takano, J., Kozasa, T., Nakajima, S., and Nakajima, Y. (1997) J. Biol. Chem. 272, 559–567.
20. Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M., and Kaziro, Y. (1988) J. Biol. Chem. 263, 665–6664.
21. Jones, D. T., and Reed, R. R. (1987) J. Biol. Chem. 262, 14241–14249.
22. Renstrom, E., Ding, W. D., Bokvist, K., and Rorsman, P. (1996) Neuron 17, 513–522.
23. Poulsen, C. R., Bokvist, K., Olsen, H. L., Hay, M., Capito, K., Gilon, P., and Gromada, J. (1999) Diabetes 48, 2171–2181.
24. Kashiwagi, A., Harano, Y., Suzuki, M., Kojima, H., Harada, M., Nishio, Y., and Shigeta, Y. (1986) Diabetes 35, 1085–1089.
25. Dunne, M. J., Harding, E. A., Jaggar, J. H., Squires, P. E., Liang, R., Kane, C., James, R. P. L., and London, N. J. M. (1995) Annu. N. Y. Acad. Sci. 763, 242–261.
26. Bokvist, K., Olsen H. L., Hey, M., Gorfdeelsen C. F., Holmes, W. F., Buschard, K., Rorsman, P., and Gromada, J. (1999) Pflügers Arch. Eur. J. Physiol. 438, 428–436.
27. Suzuki, M., Fujikura, K., Kotake, K., Inagaki, N., Seino, S., and Takata, K. (1999) Diabetologia 42, 1204–1211.
28. Inagaki, N., Gonoi, T., Clement, J. P., IV, Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995) Science 263, 1166–1170.
29. Sakura, N., Ammalía, C., Smith, P. A., Gribble, F. M., and Ashcroft, F. M. (1995) FEBS Lett. 377, 228–244.
30. Lee, K., Groh, W. J., Blair, T. A., Maylie, J. G., and Adelman, J. P. (1995) Eur. J. Pharmacol. 285, 309–312.
31. Heinberg, H., De Vos, A., Pipeleers, D., Thorens, B., and Schuit, F. (1995) J. Biol. Chem. 270, 8971–8975.
32. Gagliardino J. J., Krinks M. H., and Gagliardino E. E. (1991) Biochem. Biophys. Acta 1091, 370–373.
33. Ditimary, P., Dejamingh, S., Ling, Z., Pipeleers, D., Schuit, F., Henquin, J-C. (1998) J. Biol. Chem. 273, 33905–33908.
34. Berrow, N. S., Milligan, G., and Morgan, N. G. (1992) J. Mol. Endocrinol. 8, 103–108.
35. Aronin, N., and DiFiglia, M. (1992) J. Neurosci. 12, 3435–3444.
36. Ahnert-Hilger, G., Schafer, T., Spicher, K., Grund, C., Schultz, G., and Wiedenmann, B. (1994) Eur. J. Cell Biol. 65, 26–38.
37. Ahnert-Hilger, G., Nürnberg, B., Exner, T., Schafer, T., and Jahn, R. (1998) EMBO J. 17, 406–413.
38. Vitale, N., Deloulme, J. C., Thierry, D., Aunis, D., and Bader, M. F. (1994) J. Biol. Chem. 269, 30293–30298.
39. Jena, B. P., Schneider, S. W., Geibel, J. P., Webster, P., Oberleithner, H., and Sritharan, K. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13317–13322.
Phentolamine Inhibits Exocytosis of Glucagon by $G_{i2}$ Protein-dependent Activation of Calcineurin in Rat Pancreatic $\alpha$-Cells
Marianne Høy, Krister Bokvist, Weng Xiao-Gang, John Hansen, Kirstine Juhl, Per-Olof Berggren, Karsten Buschard and Jesper Gromada

*J. Biol. Chem.* 2001, 276:924-930.
doi: 10.1074/jbc.M007562200 originally published online September 19, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007562200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 17 of which can be accessed free at http://www.jbc.org/content/276/2/924.full.html#ref-list-1