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Imidazoline NNC77-0074 stimulates insulin secretion and inhibits glucagon release by control of Ca^{2+}-dependent exocytosis in pancreatic α- and β-cells

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Abstract

We have investigated the effects of the novel imidazoline compound (+)-2-(2-(4,5-dihydro-1*H*-imidazol-2-yl)-thiopene-2-yl-ethyl)-pyridine (NNC77-0074) on stimulus-secretion coupling in isolated pancreatic α- and β-cells. NNC77-0074 stimulated glucose-dependent insulin secretion in intact mouse pancreatic islets. No effect was observed at ≤2.5 mM glucose and maximal stimulation occurred at 10–15 mM glucose. NNC77-0074 produced a concentration-dependent stimulation of insulin secretion. Half-maximal (EC_{50}) stimulation was observed at 24 μM and at maximally stimulatory concentrations insulin release was doubled. The stimulatory action of NNC77-0074 on insulin secretion was not associated with membrane depolarisation or a change in the activity of ATP-sensitive K^+ channels. Using capacitance measurements, we found that NNC77-0074 stimulated depolarisation-induced exocytosis 2.6-fold without affecting the whole-cell Ca^{2+} current when applied via the extracellular medium. The concentration dependence of the stimulatory action was determined by intracellular application of NNC77-0074 through the recording pipette. NNC77-0074 stimulated exocytosis half-maximal at 44 nM and at maximally stimulatory concentrations the rate of exocytosis was increased twofold. NNC77-0074 stimulated depolarised-induced insulin secretion from islets exposed to diazoxide and high external KCl (EC_{50} = 0.45 μM). The stimulatory action of NNC77-0074 was dependent on protein kinase C activity. NNC77-0074 potently inhibited glucagon secretion from rat islets (EC_{50} = 11 nM). This was not associated with a change in spontaneous electrical activity and ATP-sensitive K^+ channel activity but resulted from a reduction of the rate of Ca^{2+}-dependent exocytosis in single rat α-cells (EC_{50} = 9 nM). Inhibition of exocytosis by NNC77-0074 was pertussis toxin-sensitive and mediated by activation of the protein phosphatase calcineurin. In rat somatotrophs, PC12 cells and mouse cortical neurons NNC77-0074 did not stimulate Ca^{2+}-evoked exocytosis, whereas the other imidazoline compounds phentolamine and efaroxan produced 2.5-fold stimulation of exocytosis. Our data suggest that the imidazoline compound NNC77-0074 constitutes a novel class of antidiabetic compounds that stimulates glucose-dependent insulin release while inhibiting glucagon secretion. These actions are exclusively exerted by modulation of exocytosis of the insulin- and glucagon-containing granules.

Keywords: Exocytosis; Glucagon; Imidazoline compound; Insulin; Pancreatic islet

1. Introduction

Imidazoline compounds have attracted considerable interest for more than a decade as possible therapeutic agents for the treatment of type-2 diabetes. This is based on the ability of imidazoline compounds to act as potent stimulators of insulin secretion (Plant and Henquin, 1990; Jonas et al., 1992; Chan, 1993). Many imidazoline compounds stimulate insulin secretion by closure of the ATP-sensitive K^+ channels (K_{ATP} channels) in the β-cell plasma membrane (Dunne et al., 1995; Proks and Ashcroft, 1997; Le Brigand et al., 1999), thereby causing membrane depolarisation, Ca^{2+} influx, and exocytosis of the insulin-containing granules. Compounds that block K_{ATP} channels stimulate insulin secretion at both

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low and high glucose concentrations. A strong insulinotropic effect at low glucose concentrations could lead to pronounced hypoglycaemia in patients treated with these drugs. Therefore, the discovery of a new class of imidazoline compounds that do not stimulate basal insulin secretion and lack effect on K_{ATP} channel activity but markedly potentiate glucose-induced insulin secretion has recently attracted much attention (Efanov et al., 2001).

Imidazoline compounds stimulate not only insulin release but also somatostatin release while inhibiting glucagon secretion (Efanova et al., 1998). These findings are particularly important when considering the antidiabetogenic action of imidazoline compounds since patients with type-2 diabetes often exhibit exaggerated glucagon secretion that aggravate the hyperglycaemia associated with the disease. We have recently reported that imidazoline compounds inhibit glucagon secretion by activation of the protein phosphatase calcineurin (Høy et al., 2001).

Here we demonstrate that the novel imidazoline compound (+)-2-(2-(4,5-dihydro-1*H*-imidazol-2-yl)-thiopene-2-yl-ethyl)-pyridine, hereafter referred to as NNC77-0074 (Fig. 1A, insert), potentiates glucose-induced insulin secretion without modulating K_{ATP} channel activity by stimulating insulin exocytosis. Furthermore, we demonstrate that NNC77-0074 inhibits glucagon secretion from pancreatic α-cells.

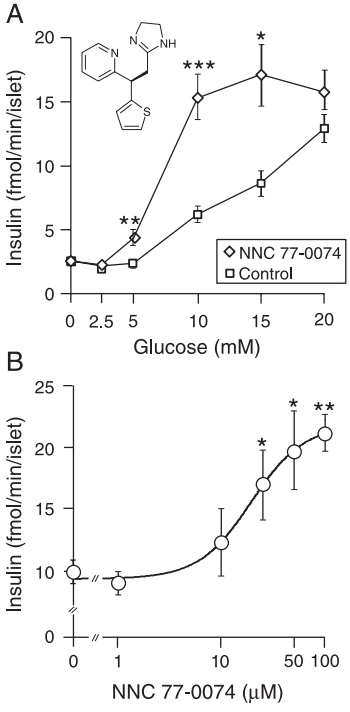

Fig. 1. NNC77-0074 stimulates glucose- and dose-dependent insulin secretion in mouse islets. (A) Batches of 10 size-matched islets, cultured overnight in RPMI-1640 medium, were exposed to the indicated concentrations of glucose alone (□) or in the presence of 100 μM NNC77-0074 (◇). Following 60-min incubation, the supernatant was removed and assayed for insulin. The inset shows structure of NNC77-0074. (B) As in (A), except that batches of 10 mouse islets were incubated at 10 mM glucose alone or in the presence of increasing concentrations of NNC77-0074 (0–100 μM). The line is the best fit of the average data points to the Hill equation. Data are mean values ± S.E.M. of five different experiments. *$P<0.05$; **$P<0.01$; ***$P<0.005$.

2. Methods

2.1. Preparation and culture of cells

Female NMRI mice (15–23 g; Bomholtgaard, Ry, Denmark) were stunned by a blow against the head and killed by cervical dislocation. The pancreas was quickly removed and islets of Langerhans were isolated by collagenase (type XI; Sigma) digestion. Single islet cells were prepared by shaking in a Ca^{2+}-free solution as described previously (Høy et al., 2002). Rat α- and β-cells were isolated and purified by fluorescence-activated cell sorting as described elsewhere (Josefsen et al., 1996). Rat pituitary somatotrophs were isolated as detailed previously (Gromada et al., 2002). Cultures of mouse neocortical neurons were prepared as described elsewhere (Varming, 1999). The procedures for scarifying the animals were approved by the Local Ethical Committee for Research on Animals in Copenhagen. For all the experiments, the cells were plated on Nunc Petri dishes and incubated in a humidified atmosphere with 5% CO_2 for up to 3 days in RPMI-1640 medium (Gibco, Life Technologies, UK) supplemented with 10% heat-inactivated foetal calf serum, 100 i.u./ml penicillin and 100 μg streptomycin.

2.2. Measurements of insulin secretion

All experiments were performed on mouse islets incubated overnight in RPMI-1640 tissue culture medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum, 100 i.u./ml penicillin and 100 μg streptomycin. Insulin secretion was measured in an extracellular medium containing (mM) 150 NaCl, 4.7 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES, 2 glutamine, 5 $NaHCO_3$, and 0.2% human serum albumin (pH 7.40 with NaOH) and 0–20 glucose. Islets were first incubated for 45 min in extracellular medium with 3.3 mM glucose at 37 °C. Batches of 10 size-matched islets were transferred to 0.2 ml of extracellular medium with indicated NNC77-0074 and glucose concentrations and incubated for 1 h. For measurements of insulin secretion in clamped islets (Fig. 3E), a modified extracellular medium was used containing (in mM) 129.7 NaCl, 25 KCl, 2.6 $CaCl_2$, 1.2 $MgSO_4$, 10 HEPES, 2 glutamine, 5 $NaHCO_3$, and 0.2% human serum albumin (pH 7.40 with NaOH), 10 glucose and 0.25 diazoxide. At the end of the experiment, samples were taken (100 μl) for hormone release measurements. Insulin was assayed using an enzyme-linked immunosorbant assay as described previously (Fuhlendorff et al., 1998).

2.3. Measurements of glucagon secretion

The release of glucagon was measured at 37 °C following static incubation of groups of 10 rat islets cultured overnight in RPMI-1640 medium as detailed previously (Høy et al., 2001).

2.4. Electrophysiology

Pipettes were pulled from borosilicate glass capillaries, coated with Sylgaard and fire-polished before use. The pipette resistance when filled with the pipette solutions was 2–4 MΩ. The zero-current potential was adjusted before establishment of the seal with the pipette in the recording bath. The membrane potential was recorded using the perforated patch whole-cell configuration. K_{ATP} current was monitored using the standard whole-cell configuration and was elicited by ± 10 mV voltage excursions (duration: 200 ms; pulse interval: 2 s) from a holding potential of − 70 mV. The recordings were carried out using an Axopatch 200B patch-clamp amplifier, a Digidata AD-converter (Axon Instruments; Foster City, CA, USA) and the software pClamp (version 6.0; Axon Instruments). All currents were filtered at 1 kHz using the internal filters of the amplifiers and acquired at a rate of 5 kHz. Exocytosis was measured as increases in cell membrane capacitance using, except in Fig. 3A, an EPC-9 patch clamp amplifier and the Pulse software (v. 8.01; HEKA Elektronik, Lamprecht/Pfalz, Germany). The interval between two successive points was 0.2 s and the measurements of cell capacitance were initiated < 5 s following establishment of the standard whole-cell configuration. In Fig. 3A, changes in cell capacitance were elicited by 500-ms voltage-clamp depolarisations 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 Instruments) as described previously (Ämmälä et al., 1993) using the perforated patch whole-cell configuration.

2.5. Solutions for electrophysiology

The extracellular solution was composed of (in mM) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4 with NaOH) and 5 or 20 D-glucose. The extracellular solution for recordings of cell capacitance using voltage-clamp depolarisation contained (in mM) 118 NaCl, 20 tetraethylammonium–Cl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 5 HEPES (pH 7.40 with NaOH). TEA was included in the medium to block the outward delayed rectifying K^+ current, which otherwise obscures the smaller Ca^{2+} current. The volume of the recording chamber was 0.4 ml and maintained at 33 °C. Solution entered the recording chamber at a rate of 1.5 ml/min. The pipette solution for measurements of whole-cell K_{ATP} channel activity consisted of (in mM) 125 KCl, 30 KOH, 10 EGTA, 5 HEPES, 1 $MgCl_2$, 0.3 Mg-ATP and 0.3 K-ADP (pH 7.15). For membrane potential recordings using the perforated patch technique, the pipette solution consisted of (in mM) 76 K_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.35 with KOH). A similar solution was used for the measurements of exocytosis triggered by voltage-clamp depolarisations with the exception that K_2SO_4 was replaced with an equimolar amount of Cs_2SO_4 and CsOH used for adjusting the pH. In the perforated patch measurements, electrical contact was established by adding 0.24 mg/ml amphotericin to the pipette solution. Perforation required a few minutes and the voltage-clamp was considered satisfactory when the series conductance was stable and >35–40 nS. When exocytosis was elicited by infusion of Ca^{2+}-EGTA buffers through the recording electrode, the pipette solution consisted of (in mM) 125 potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 10 EGTA, 5 or 9 $CaCl_2$ (pH 7.15 with KOH). The free Ca^{2+} concentrations of the resulting buffers were 0.22 or 2 μM using the binding constants of Martell and Smith (1971).

2.6. Measurements of cAMP

Cyclic AMP production was measured in batches of 30 freshly isolated mouse islets preincubated for 60 min in the presence of 1 mM of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine and subsequently stimulated with NNC77-0074 or the cAMP-elevating agent forskolin for 30 min. The reaction was terminated by the addition of HCl to a final concentration of 50 mM. The samples were neutralised with 50 mM NaOH and assayed for cAMP using a cAMP [^{125}I] scintillation proximity assay (Amersham, UK).

2.7. Materials

Bisindolylmaleimide, *N*-(2-[*p*-bromocinnamylamino]ethyl]-5-isoquinolinesulfoamide (H-89) and calphostin C were obtained from Calbiochem (La Jolla, Ca, USA). (+)-2-(2-(4,5-Dihydro-1*H*-imidazol-2-yl)-thiopene-2-yl-ethyl)-pyridine (NNC77-0074) was synthesised at Novo Nordisk (Måløv, Denmark). All other chemicals were purchased from Sigma. Compounds were prepared in stock solution either in water or dimethyl sulphoxide (DMSO). The final concentration of DMSO did not exceed 0.1%.

2.8. Data analysis

Results are presented as mean values ± S.E.M. for indicated number of experiments. The exocytotic rate ($\Delta C_m/\Delta t$) is presented as the increase in cell capacitance during the first 60 s following establishment of the whole-cell configuration, excluding any rapid changes during the initial 10 s required for equilibration of the pipette solution with cytosol. Statistical significances were evaluated using Dunnett's test for multiple comparisons with a single control and Student's *t*-test for either paired or unpaired observations.

3. Results

3.1. NNC77-0074 potentiates glucose-induced insulin secretion

Fig. 1A shows that NNC77-0074 (100 μM) stimulates insulin secretion from intact mouse islets in a glucose-dependent fashion. No stimulation was observed at ≤2.5 mM glucose, whereas NNC77-0074 enhanced insulin release at intermediate glucose concentrations (98–148% stimulation). The dose–response curve for the stimulatory action of NNC77-0074 is shown in Fig. 1B. At 10 mM glucose, half-maximal stimulation (EC_{50}) of insulin secretion by NNC77-0074 was observed at 24 μM and at maximally stimulatory concentrations of the compound (≥100 μM) produced a 2.2-fold enhancement.

3.2. Effect of NNC77-0074 on membrane potential and K_{ATP} channel activity

Stimulation of insulin secretion by NNC77-0074 (100 μM) was not associated with the induction of β-cell electrical activity in isolated β-cells (Fig. 2A). Subsequent elevation of the glucose level to 20 mM produced membrane depolarisation and initiation of electrical activity. The failure of NNC77-0074 to elicit electrical activity suggests that it does not close K_{ATP} channels. Indeed, NNC77-0074 (100 μM) did not affect the activity of the K_{ATP} current activated by intracellular dialysis of the β-cell with 0.3 mM ATP and 0.3 mM ADP (cf. Trube et al., 1986). In the presence of the compound, the whole-cell K_{ATP} current averaged 103 ± 5% ($n=5$) of that observed under control conditions. By contrast, subsequent application of the specific K_{ATP} channel antagonist tolbutamide (100 μM) produced 93 ± 3% ($P<0.001$; $n=5$) inhibition of the whole-cell K^+ current (Fig. 2B).

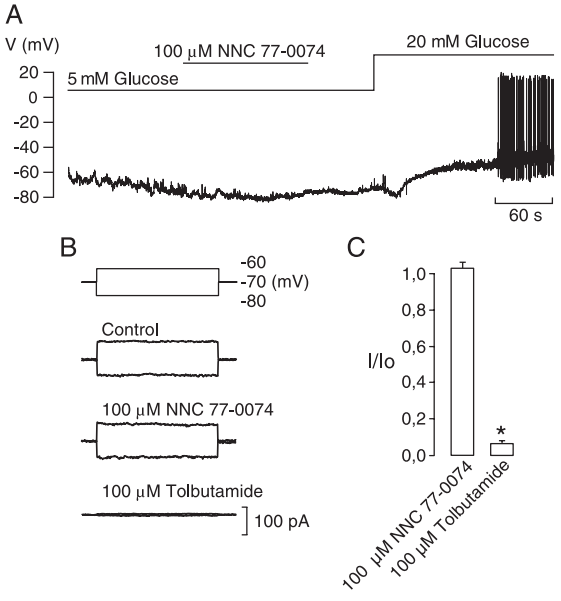

Fig. 2. NNC77-0074 does not affect membrane potential and K_{ATP} channel activity in mouse β-cells. (A) Representative membrane potential recording from an individual mouse pancreatic β-cell in the presence of 5 mM glucose. NNC77-0074 (100 μM) and 20 mM glucose were added to the extracellular solution during the periods indicated by the bars. The trace is representative of five experiments. (B) Whole-cell K_{ATP} currents were measured in response to ± 10 mV voltage excursions from a holding potential of −70 mV. The cells were dialysed with 0.3 mM ATP and 0.3 mM ADP in order to activate the K_{ATP} channels. Once steady-state had been attained (2–4 min; control), NNC77-0074 or tolbutamide (both 100 μM) was applied to the extracellular solution. (C) Histogram depicting average effects of NNC77-0074 (100 μM) and tolbutamide (100 μM) on whole-cell K_{ATP} channel activity. The current amplitudes in the presence of the compounds (I) are expressed relative to those obtained before drug application (I_o). The data are mean values ± S.E.M. of five experiments. *$P<0.001$.

3.3. NNC77-0074 potentiates Ca^{2+}-dependent exocytosis

Fig. 3A illustrates whole-cell Ca^{2+} currents and the associated changes in cell capacitance elicited by 500-ms depolarisations from −70 to 0 mV in an intact mouse β-cell using the perforated patch configuration. Under basal conditions, the integrated Ca^{2+} current amounted to 6.3 pC and a capacitance increase of 17 fF was evoked. The latter value corresponds to the discharge of six granules using a conversion factor of 3 fF per granule (Olofsson et al., 2002). Two minutes after inclusion of 1 μM NNC77-0074 in the bathing solution, the same membrane depolarisation produced a peak Ca^{2+} current of 6.5 pC and a capacitance increase of 73 fF (350% stimulation). On average (Fig. 3A), NNC77-0074 elicited a 269 ± 76% ($P<0.005$; $n=5$) stimulation of exocytosis (Fig. 3B), which was not associated with a change of the integrated Ca^{2+} current (Fig. 3C).

It seems likely that the effects of NNC77-0074 on exocytosis are exerted following its uptake into the β-cells. We have previously demonstrated that imidazoline-containing compounds remain potent stimulators of exocytosis when applied intracellularly through the patch electrode during standard whole-cell recordings (Høy et al., 2001). Subsequent experiments were therefore performed using the latter configuration. This recording method has the additional advantage that the diffusion barrier represented by the plasma membrane is removed. Following establishment of the whole-cell configuration and infusion of a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.22 μM, exocytosis was initiated and was observed as a gradual capacitance increase (Fig. 3D, inset). In general, the cell capacitance reached a new steady-state level within 3 min. It is clear that inclusion of 1 μM NNC77-0074 exerted a strong stimulation of the increase in cell capacitance. On average, NNC77-0074 stimulated exocytosis by 94% ($P<0.05$; $n=6$). No significant stimulation of exocytosis was observed at <1 nM but higher concentrations elicited a dose-dependent acceleration of secretion (Fig. 3D). Approximating the data points to the Hill equation yielded a half-maximal stimulatory effect at 44 nM and a co-operativity factor of 0.8. Maximal stimulation of exocytosis was seen at concentrations of NNC77-0074 ≥1 μM (Fig. 3D), at which exocytosis was increased ~2-

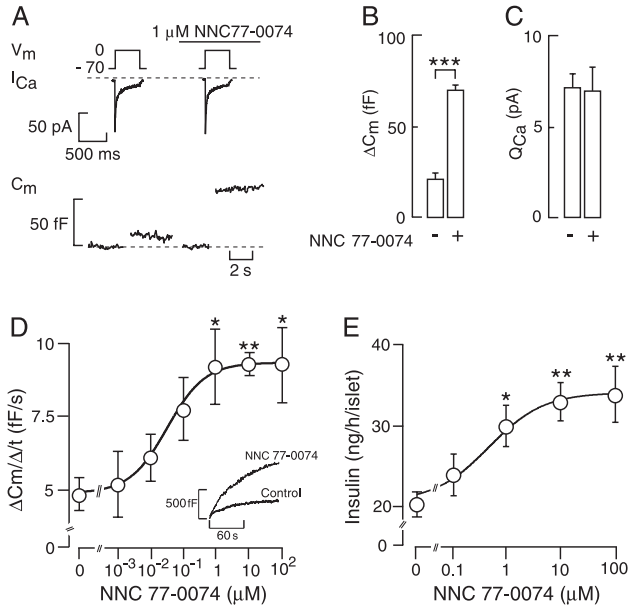

Fig. 3. NNC77-0074 stimulation of Ca^{2+}-evoked exocytosis in mouse β-cells. (A) Ca^{2+} current (I_{Ca}, middle) and membrane capacitance increases (C_m, bottom) evoked by 500-ms membrane depolarisations from −70 to 0 mV (V_m; top) before and 2 min after the addition of 1 μM NNC77-0074 using the perforated patch whole-cell configuration. Histograms summarising the increases in cell capacitance (ΔC_m; B) and integrated Ca^{2+} current (Q_{Ca}; C) in the absence (−) and presence (+) of 1 μM NNC77-0074. (D) The inset shows increases in cell capacitance observed during the first 2 min after establishment of the standard whole-cell configuration elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.2 μM in the absence (control) and presence of 1 μM NNC77-0074 in the pipette solution. Throughout the recording, the cell was voltage-clamped at −70 mV in order to avoid activation of the voltage-dependent Ca^{2+}-channels that would otherwise interfere with the measurement. The figure also shows the dose–response relationship for NNC77-0074-induced stimulation of exocytosis. The rate of capacitance increase ($\Delta C_m/\Delta t$) was measured over the first 60 s after establishment of the whole-cell configuration. The line is the best fit of the average data to the Hill equation. (E) Effects of NNC77-0074 on insulin secretion in islets exposed to 250 μM diazoxide, 25 mM KCl and 10 mM glucose. Insulin secretion was measured for 1 h in groups of 10 size-matched islets in the absence and presence of increasing concentrations of NNC77-0074. The line is the best fit of the average data to the Hill equation. Data are mean values ± S.E.M. of five to nine different experiments. *$P<0.05$; **$P<0.01$; ***$P<0.005$.

fold over the control rate of capacitance increase. Intracellular application of 1 μM NNC77-0074 to primary rat β-cells and the clonal insulinoma cell lines INS-1E and βTC-6 stimulated exocytosis to an extent comparable to that observed in mouse β-cells (data not shown).

The stimulatory action of NNC77-0074 on exocytosis was also observed in mouse islets exposed to 250 μM diazoxide, 25 mM KCl and 10 mM glucose (Fig. 3E). Under these experimental conditions, which reveal the K_{ATP} channel-independent stimulation of insulin secretion (Henquin, 2000), NNC77-0074 produced a dose-dependent enhancement of insulin release. Half-maximal stimulation was observed at 0.45 μM of the compound and a maximally stimulatory concentration of NNC77-0074 enhanced insulin secretion by 66% ($P<0.01$; $n=5$).

3.4. NNC77-0074 stimulates protein kinase C-dependent exocytosis

Imidazoline compounds have previously been reported to stimulate insulin secretion by a protein kinase C-dependent mechanism (Zaitsev et al., 1996). This also appears to be the case for NNC77-0074 as 1 μM of the compound failed to elicit exocytosis following inhibition of PKC by bisindolylmaleimide (4 μM for 20 min; Fig. 4A). Bisindo-

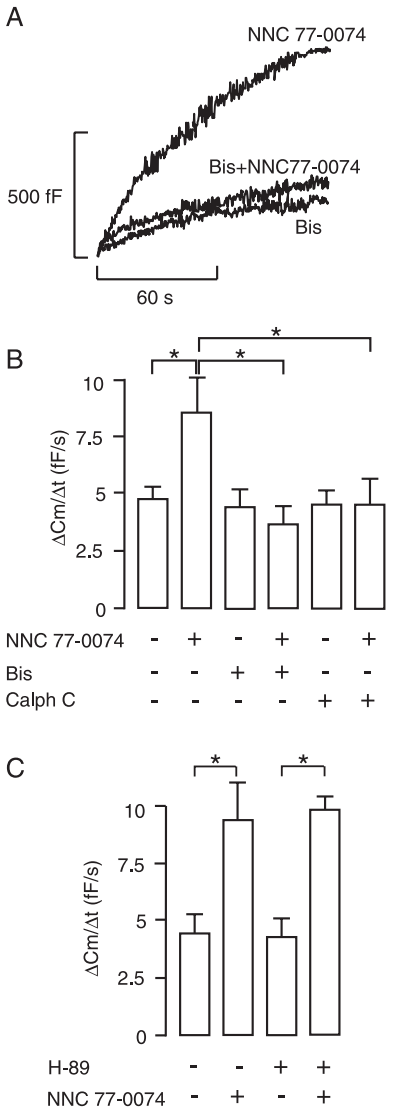

Fig. 4. NNC77-0074 stimulates PKC-dependent exocytosis. (A) Increases in cell capacitance elicited by 0.2 μM free Ca^{2+} in the absence and presence of NNC77-0074 (1 μM) and the PKC inhibitor bisindolylmaleimide (Bis; cells were pre-incubation >20 min with 4 μM Bis). (B) Histogram depicting mean rates of increase in cell capacitance ($\Delta C_m/\Delta t$) in the absence (−) and presence (+) of NNC77-0074 (1 μM) and the PKC inhibitors bisindolylmaleimide (Bis) and calphostin C (Calph C; cells pre-treated with 1.5 μM for >15 min). (C) As in (B), except that cell capacitance was recorded in the absence and presence of the protein kinase A inhibitor H-89 (10 μM for >20 min) and NNC77-0074 (1 μM). Data are mean ± S.E.M. of five different experiments. *$P<0.05$.

lylmaleimide did not affect exocytosis in the absence of NNC77-0074. Similar results were obtained in the presence of another PKC inhibitor calphostin C (Fig. 4B). By contrast, inhibition of protein kinase A (PKA) by H-89 did not reduce the ability of NNC77-0074 to stimulate an increase in cell capacitance (Fig. 4C). We can also exclude that the action of NNC77-0074 (100 μM) is secondary to increased intracellular levels of cAMP which averaged 694 ± 80 fmol/islet ($n = 5$) and 797 ± 73 fmol/islet ($n = 5$) in the absence and presence of NNC77-0074, respectively.

3.5. Effects of granular Cl^- channels on NNC77-0074-induced exocytosis

DIDS-sensitive ClC-3 chloride channels have been proposed to be present on β-cell secretory granules and to participate in sulfonylurea- and Ca^{2+}-dependent modulation of exocytosis (Barg et al., 1999, 2001). Intracellular application of the Cl^- channel blocker 4,4-diisothiocyantostilbene-2,2′-disulfonic acid (DIDS; 0.1 mM) abolished the ability of NNC77-0074 (1 μM) to stimulate exocytosis (Fig. 5A,B). DIDS itself did not affect the rate of capacitance increase measured during the first 60 s of intracellular dialysis with a Ca^{2+}-buffer with a free Ca^{2+} concentration of 0.2 μM (Fig. 5B).

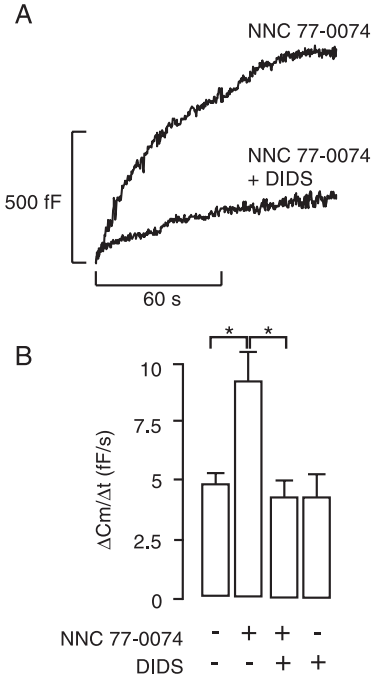

Fig. 5. DIDS inhibits NNC77-0074-induced exocytosis in mouse β-cells. (A) Increases in cell capacitance during the first 2 min after establishment of the standard whole-cell configuration. Exocytosis was elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.2 μM in the presence of 1 μM NNC77-0074 alone or together with 0.1 mM DIDS in the pipette solution. (B) Histogram depicting average rates of increase in cell capacitance ($\Delta C_m/\Delta t$) measured over the first 60 s after initiation of the experiment in the absence and presence of 1 μM NNC77-0074 and 0.1 mM DIDS. Data are mean ± S.E.M. of five different experiments. *$P < 0.05$.

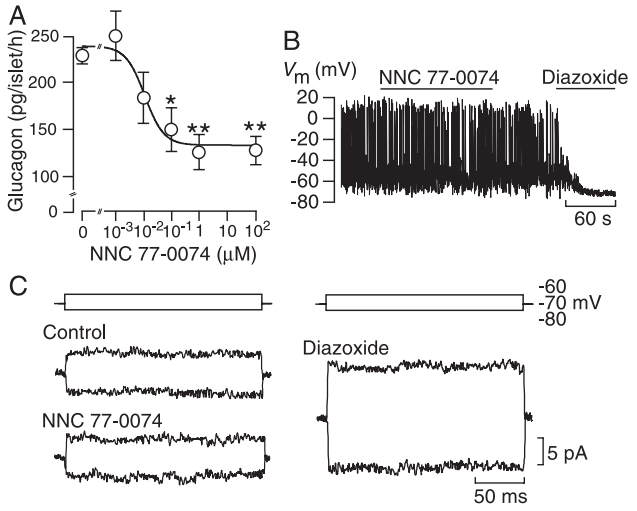

Fig. 6. Inhibition of glucagon secretion by NNC77-0074. (A) Effects of NNC77-0074 on glucagon release from batches of 10 size-matched rat islets. The islets were exposed for 1 h to the indicated NNC77-0074 concentrations (1 nM to 100 μM). The experiments were performed in an extracellular medium without glucose. The line is the best fit of the average data points to the Hill equation. Data are mean values ± S.E.M. of five to seven individual measurements. (B) Spontaneous electrical activity recorded from an individual rat α-cell in the absence of glucose using the perforated patch whole-cell configuration. NNC77-0074 (1 μM) and diazoxide (100 μM) were added to the bath solution during the periods indicated by the bars. The recording is representative of five cells. (C) Whole-cell K_{ATP} currents were measured in a single rat α-cell in response to 10-mV de- and repolarising voltage pulses from a holding potential of −70 mV using the perforated patch configuration. The traces were obtained under control conditions and 2 min after application of NNC77-0074 (1 μM) and diazoxide (100 μM). The traces are representative of five different cells.

3.6. NNC77-0074 inhibits glucagon secretion from rat islets

The ideal antidiabetic compound should stimulate insulin secretion and inhibit glucagon release. As illustrated in Fig. 6A, NNC77-0074 possesses this desirable property and dose-dependently inhibited glucagon secretion from intact rat islets; half-maximal inhibition (IC_{50}) of glucagon secretion by NNC77-0074 was observed at 11 nM and maximal inhibition seen at concentrations ≥ 1 μM where it amounted to 59% ($P < 0.05$; $n = 5$). As illustrated by Table 1, the inhibitory action of NNC77-0074 on glucagon secretion was dependent on the glucose concentration. Strong inhibition of glucagon release by NNC77-0074 was observed at ≤ 5 mM glucose, whereas no effect was observed at concentrations ≥ 10 mM.

Fig 6B,C demonstrates that the ability of NNC77-0074 to inhibit glucagon secretion is not due to suppression of α-cell electrical activity by activation of plasma membrane K_{ATP} channels. Under control conditions (0 mM glucose), spontaneous electrical activity consisting of overshooting action potentials was observed in >80% of the tested cells

Table 1
NNC77-0074 inhibits glucagon release from rat islets in a glucose-dependent manner

Condition	Glucagon release (pg/islet/h)	n
0 mM G	211 ± 17	8
0 mM G + NNC77-0074	147 ± 21[a]	8
2.5 mM G	186 ± 18	8
2.5 mM G + NNC77-0074	94 ± 21[b]	6
5 mM G	138 ± 14	8
5 mM G + NNC77-0074	93 ± 17[a]	8
10 mM G	101 ± 15	6
10 mM G + NNC77-0074	86 ± 19	6
20 mM G	89 ± 16	6
20 mM G + NNC77-0074	83 ± 12	6

Glucagon release was measured from batches of 10 size-matched freshly isolated islets exposed for 1 h to the indicated glucose (G) concentrations in the absence or presence of 1 μM NNC77-0074.
[a] $P < 0.05$ against control.
[b] $P < 0.01$ against control.

(Høy et al., 2001), as expected for an α-cell rich preparation. NNC77-0074 (1 μM) did not affect the ability of the α-cells to fire action potentials, whereas subsequent addition of the K_{ATP} channel opener diazoxide (100 μM) was associated with inhibition of electrical activity (Fig. 6B). The inhibitory action of diazoxide on electrical activity was reversible ($n = 5$; data not shown, but see Bokvist et al., 1999). As expected, the hyperpolarization produced by diazoxide was due to a 4.5-fold increase in whole-cell K_{ATP} conductance, whereas NNC77-0074 had no effect (Fig. 6C).

3.7. NNC77-0074 inhibits exocytosis in rat α-cells

We have previously reported that imidazoline compounds inhibit Ca^{2+}-dependent exocytosis in rat α-cells (Høy et al., 2001). Here we extend these observations and demonstrate that inclusion of 1 μM NNC77-0074 in the pipette solution inhibits exocytosis triggered by intracellular application of 2 μM Ca^{2+} (through the patch-pipette; Fig. 7A). On average, NNC77-0074 inhibited exocytosis measured over the first 60 s after establishment of the whole-cell configuration by 60% ($P < 0.01$; $n = 5$). The effect of NNC77-0074 on exocytosis was dependent on dose (Fig. 7B) and half-maximal inhibition was observed at 9 nM. Maximal inhibition was observed at ≥1 μM NNC77-0074 where it amounted to 64% (Fig. 7B). A similar strong inhibition of exocytosis was observed in αTC-1.9 glucagonoma cells (control: 17.3 ± 0.9 fF/s and NNC77-0074: 8.1 ± 0.7 fF/s; $P < 0.01$; $n = 5$; data not shown).

In agreement with our previous data (Høy et al., 2001), we demonstrate here that the inhibitory action of NNC77-0074 is mediated by pertussis toxin-sensitive G-proteins and was abolished in cells pre-treated for 20 h with 100 ng/ml pertussis toxin (Fig. 7C). Furthermore, our data suggest that the inhibitory action of NNC77-0074 on exocytosis involves activation of the protein phosphatase calcineurin and pre-treatment with cyclosporin A prevented inhibition by the compound (1 μM for >20 min; Fig. 7D). Calcineurin is expressed in rat α-cells (Gagliardino et al., 1991). It was ascertained separately that the effects on exocytosis measured by capacitance measurements have their counterpart in the action on glucagon release. After pre-treatment with the calcineurin inhibitor, glucagon release measured in the presence of NNC77-0074 amounted to 96 ± 5% ($n = 6$) of that seen under control conditions (data not shown).

3.8. NNC77-0074 does not affect exocytosis in neuroendocrine cell types

Finally, we explored whether NNC77-0074 affects Ca^{2+}-dependent exocytosis in non-pancreatic neuroendocrine cells (Fig. 8A). Interestingly, NNC77-0074 (100 μM) did not alter the rate of capacitance increase in rat pituitary cells, whereas both phentolamine and efaroxan (both 100 μM) more than doubled the rate of exocytosis (Fig. 8B). Similar

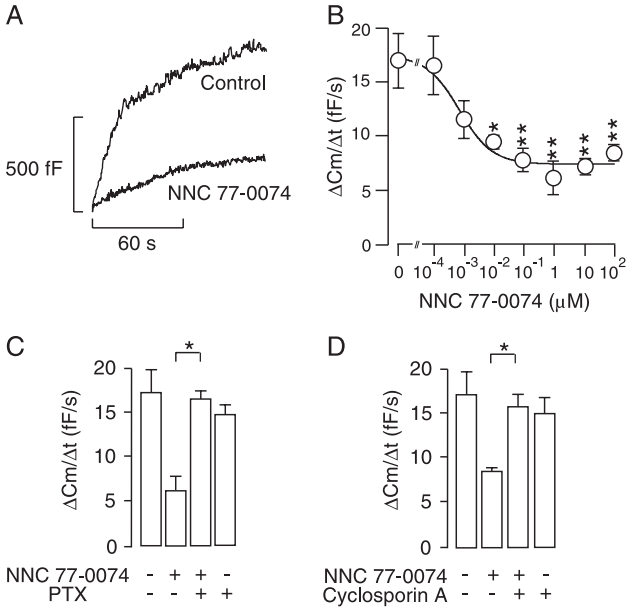

Fig. 7. Intracellular application of NNC77-0074 inhibits exocytosis in rat α-cells. (A) Increases in cell capacitance were elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 2 μM in the absence (control) and presence of 1 μM NNC77-0074 in the pipette solution observed during the first 2 min after establishment of the standard whole-cell configuration. (B) Dose-dependent inhibition of exocytosis by NNC77-0074. The rates of capacitance increase ($\Delta C_m/\Delta t$) were measured during the first 60 s after establishment of the whole-cell configuration. The line is the best fit of the average data points to the Hill equation. (C) Histogram showing average rates of increase in cell capacitance ($\Delta C_m/\Delta t$) using a pipette solution containing 2 μM free Ca^{2+} measured over the first 60 s after initiation of the experiment in the absence (−) and presence of 1 μM NNC77-0074 (+) in cells pretreated (+) or not (−) with pertussis toxin (PTX; 100 ng/ml for >20 h). (D) As in (C), except cells were pretreated with cyclosporin A (1 μM for >20 min). Data are mean ± S.E.M. of five different experiments. *$P < 0.05$; **$P < 0.01$.

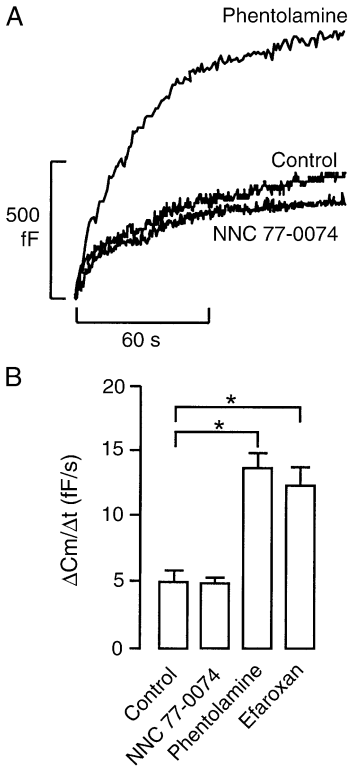

Fig. 8. NNC77-0074 does not affect exocytosis in rat pituitary cells. (A) Increases in cell capacitance were elicited by intracellular infusion with a Ca^{2+}-EGTA buffer with a free Ca^{2+} concentration of 0.2 μM in the absence (control) and presence of 100 μM NNC77-0074 or the classical imidazoline compound phentolamine (100 μM) in the pipette solution observed during the first 2 min after establishment of the standard whole-cell configuration. (B) Histogram shows average rates of increase in cell capacitance ($\Delta C_m/\Delta t$) measured over the first 60 s after initiation of the experiment in the absence (control) and presence of 100 μM NNC77-0074, 100 μM phentolamine or 100 μM of the imidazoline compound efaroxan. Data are mean values ± S.E.M. of five different experiments. *$P<0.05$.

results were observed in PC12 cells and mouse cortical neurons (data not shown).

4. Discussion

Our data demonstrate that the novel imidazoline compound NNC77-0074 controls pancreatic insulin and glucagon secretion via regulation of Ca^{2+}-dependent exocytosis by a mechanism independent of K_{ATP} channel blockade. Here we highlight a few particularly interesting observations of the present study.

K_{ATP} channels in the β-cell plasma membrane play an important function in coupling an elevation of the extracellular glucose concentration to initiation of insulin secretion. Closure of the K_{ATP} channels by pharmacological agents promotes insulin secretion by initiating the same series of cellular events as glucose. Consequently, many of the pharmacological agents currently used clinically to treat type-2 diabetes are effective already in the absence of glucose. On the contrary, agents stimulating exocytosis by a K_{ATP} channel-independent mechanism will only potentiate secretion when the normal stimulus-secretion pathway is stimulated by glucose, which will produce the increase in $[Ca^{2+}]_i$ required for initiating the process of exocytosis. Such a drug will not initiate insulin secretion per se, but rather work synergistically with an elevated glucose concentration and consequently minimise the risk for hypoglycaemia. Many compounds, which contain an imidazoline ring have been identified as inhibitors of K_{ATP} channels and therefore offer little advantage over the sulphonylurea drugs already used. Interestingly, the imidazoline 1*H*-indole, 2-(4,5-dihydro-1*H*-imidazol-2-yl)-1-phenyl-, monohydrochloride (RX871024) has been reported to exert a stimulatory effect on Ca^{2+}-dependent exocytosis (Zaitsev et al., 1996; Efanov et al., 2001). This observation encouraged the development of novel classes of imidazoline-containing compounds with direct stimulatory action on the secretory machinery. Here we demonstrate that NNC77-0074 both enhanced glucose-dependent insulin secretion and inhibited glucagon secretion. These effects did not involve closure of K_{ATP} channels but instead resulted from modulation of Ca^{2+}-dependent exocytosis. The above and previous data suggest the existence of at least two imidazoline binding sites in β-cells: one located on the Kir6.2 subunit of the K_{ATP} channel (Proks and Ashcroft, 1997), whereas the other is involved in the regulation of the exocytotic process. The latter binding site is distinct from the one controlling exocytosis in non-pancreatic secretory cells. This is supported by the observation that NNC77-0074 did not affect Ca^{2+}-induced exocytosis in rat pituitary cells, PC12 cells and mouse cortical neurons, whereas the classical imidazoline compounds phentolamine and efaroxan all enhanced secretion to a similar extent in these preparations as that observed in rat β-cells. It is pertinent that exocytosis in the glucagon-releasing α-cells and insulin-producing β-cells was oppositely regulated by NNC77-0074. This does not necessarily mean that the imidazoline-binding site in α-cells is distinct from that in β-cells. It may also indicate that it couples to a different effector system leading to stimulation and inhibition of exocytosis in β- and α-cells, respectively. Collectively, these observations raise the promising possibility that it may eventually be possible to develop a pancreas-selective imidazoline-containing antidiabetic agent that affect insulin and glucagon release reciprocally. Such a compound will have advantages over traditional K_{ATP} channel antidiabetic medication due to limited undesirable side-effects, including action on K_{ATP} channels in other tissues (Brady et al., 1998; Groop, 1992; Harrower, 2000).

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