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The β-cell glibenclamide receptor is an ADP-binding protein

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The effects of ADP on [^{3}H]glibenclamide binding to membranes and whole cells, the activity of the ATP-sensitive K^+ channel (K-ATP channel), intracellular Ca^{2+} concentration and insulin secretion were studied in a hamster pancreatic β-cell line, HIT T15. ADP dose-dependently inhibited [^{3}H]glibenclamide binding to membranes and to whole cells in a competitive manner. ADP–agarose also inhibited the binding to whole cells. The activity of the K-ATP channel was assayed by measuring ^{86}Rb efflux from whole cells. ADP inhibited the ^{86}Rb efflux elicited either by diazoxide or by ATP depletion. In the presence, but not in the absence, of extracellular Ca^{2+}, ADP evoked a rapid and sustained increase in intracellular Ca^{2+} concentration as estimated with the fluorescent dye quin 2. Insulin release from HIT cells was also increased by 0.5–2 mM-ADP in the presence of 0.5 mM-glucose. These effects of ADP on glibenclamide binding, K-ATP channel activity and insulin release were specific for ADP, and were not reproduced by any other nucleotide so far tested. The present findings strongly suggest that ADP and sulphonylureas have common binding sites on the extracellular side of β-cell plasma membranes, where they inhibit the activity of the K-ATP channel, resulting in an increase in intracellular Ca^{2+} concentration and insulin release.

INTRODUCTION

Sulphonylureas, such as glibenclamide, have been used to treat non-insulin-dependent (Type II) diabetes mellitus. These drugs increase insulin release from the pancreatic β-cell via inhibition of the activity of ATP-sensitive K^+ channels (K-ATP channels) in the plasma membrane (for review see Ashcroft, 1988). Closure of K-ATP channels depolarizes the plasma membrane and opens voltage-dependent Ca^{2+} channels, resulting in an increase in intracellular free Ca^{2+} concentration. The presence of a specific sulphonylurea-binding protein has been reported in the pancreatic β-cell (Gaines *et al.*, 1988; Schmid-Antomarchi *et al.*, 1987; Niki *et al.*, 1989*a*), cardiac muscle (Fosset *et al.*, 1988) and brain (Lupo & Bataille, 1987; Bernadi *et al.*, 1988). There is strong evidence (Bernadi *et al.*, 1988; Niki *et al.*, 1989*a*) that the sulphonylurea-binding protein may be the K-ATP channel or channel-associated protein. Competitive inhibition of [^{3}H]glibenclamide binding to β-cell membranes by ADP was first reported by Niki *et al.* (1989*a*). ADP has been reported to change the sensitivity of K-ATP channels to ATP (Kakei *et al.*, 1986; Dunne & Petersen, 1986) or to tolbutamide (Zünkler *et al.*, 1988) in electrophysiological studies. However, in these studies the nucleotide has always been applied from the intracellular space. In the present study, we have examined the effect of ADP on sulphonylurea binding to membranes and whole cells, ^{86}Rb efflux, intracellular Ca^{2+} concentration and insulin release in a hamster pancreatic β-cell line, HIT T15.

MATERIALS AND METHODS

Materials

Glibenclamide and [^{3}H]glibenclamide were gifts from Hoechst (Frankfurt, Germany). Diazoxide was a gift from Glaxo (Greenford, Middx., U.K.). Rat insulin was a gift from Novo (Copenhagen, Denmark). ATP, AMP, GTP, GDP, $NADP^+$, NADPH, NADH, adenosine, guanosine, NAD^+, adenosine 5′-[γ-thio]triphosphate (ATP[S]), adenosine 5′-[β,γ-methylene]-triphosphate (p[CH_2]ppA) and BSA (Fraction V) were purchased from Boehringer (Lewes, East Sussex, U.K.). Oligomycin, 2-deoxyglucose, Sepharose 4B-200, ADP–agarose (attached through ribose hydroxyl groups), ADP and GMP (sodium salts) were from Sigma (Poole, Dorset, U.K.). ^{86}Rb and ^{125}I-insulin were purchased from Amersham International (Amersham, Bucks., U.K.). RPMI 1640 tissue-culture medium (without glutamine) and L-glutamine were from Imperial Laboratories (Andover, Hants., U.K.). Foetal-calf serum, trypsin/EDTA, antibiotic and antimycotic solution, phosphate-buffered saline (Dulbecco's), flasks for tissue culture and Multiwells were purchased from Gibco (Uxbridge, Middx., U.K.). Glass-fibre filters were obtained from Whatman (Maidstone, Kent, U.K.). Quin 2-AM was from Aldrich (Gillingham, Dorset, U.K.). Luciferin/luciferase (ATP-monitoring agent) was obtained from Pharmacia LKB (Uppsala, Sweden). Optifluor was purchased from Packard Instruments (Downers Grove, IL, U.S.A.). Other agents used here were from BDH (Poole, Dorset, U.K.) and were of the purest grade available.

Cell culture

HIT T15 β-cells (passage numbers 75–90) were cultured in RPMI 1640 tissue-culture medium containing penicillin (100 units/ml), streptomycin (0.1 mg/ml), fungizone (0.25 μg/ml) and foetal-calf serum (10%, w/v) at 37 °C in an atmosphere of humidified air/CO_2 (19:1) as previously described (Ashcroft *et al.*, 1986). Cells were passaged weekly and harvested by using trypsin/EDTA. HIT cells were seeded in Multiwell plates at a density of 5×10^5 cells/well (for ^{86}Rb-efflux and insulin-secretion studies) and in culture flasks at 3×10^7 cells/flask (for whole-cell binding and quin 2 studies). The cells were cultured for 3–6 days before experiments or membrane preparation.

Membrane preparation

HIT cell membranes were prepared essentially as described by

Abbreviations used: K-ATP channel, ATP-sensitive K^+ channel; ATP[S], adenosine 5′-[γ-thio]triphosphate; p[CH_2]ppA, adenosine 5′-[β,γ-methylene]triphosphate.

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Gaines *et al.* (1988). In brief, after washing with 2 × 10 ml of phosphate-buffered saline, trypsin-treated cells were resuspended in 10 ml of 5 mM-Tris buffer (pH 8.0 with HCl) supplemented with protease inhibitors [1 mM-phenylmethanesulphonyl fluoride, soybean trypsin inhibitor (10 μg/ml), 10 μM-leupeptin and 1 mM-iodoacetamide], left for 40 min on ice and then homogenized. The homogenate was centrifuged for 10 min at 900 *g*. Then the supernatant was further centrifuged for 30 min at 96000 *g*. The pellets were resuspended at a concentration of 1–2 mg of protein/ml in buffer containing 20 mM-Mops (pH 7.4 with NaOH) and the protease inhibitors described above. Collected membranes were frozen immediately in liquid N_2 and stored at −70 °C.

[^{3}H]Glibenclamide binding to isolated membranes

Samples of HIT-cell membranes (100–200 μg of protein/ml) were incubated for 2 h at room temperature with [^{3}H]glibenclamide (25.3 d.p.m./fmol) in 0.4 ml (final volume) of 0.1 mM-$CaCl_2$/50 mM-Mops (pH 7.4 with NaOH) in the presence or absence of test substances. Bound radioactivity was separated from free by rapid filtration on Whatman GF/F filters soaked in the same buffer as that used for the incubation; the filters were washed with 5 × 5 ml of distilled water at 4 °C. They were then placed in 10 ml of Optifluor and left overnight. Radioactivity was determined by liquid-scintillation spectrometry by using an external standard. Non-specific binding was determined in parallel samples which contained 1 μM unlabelled glibenclamide. Protein concentration was determined (Bradford, 1976) with BSA as standard. The specific bound radioactivity was 0.4–2.6% and the non-specific was 0.1–0.2% of the total radioactivity added.

[^{3}H]Glibenclamide binding to intact β-cells

HIT cells (approx. 5×10^7) were trypsin-treated and washed with 2 × 10 ml of Hepes–Krebs buffer, containing (mM): NaCl 119, KCl 4.75, $NaHCO_3$ 5, $CaCl_2$ 2.54, $MgSO_4$ 1.2, Hepes 20 (pH 7.4 with NaOH). The cells were resuspended at a density of 1.5×10^7/ml in Hepes–Krebs buffer and then incubated for 2 h at 4 °C with [^{3}H]glibenclamide at a density of 1.5×10^6 cells/assay tube in 0.4 ml of Hepes–Krebs buffer in the absence or presence of various concentrations of ADP or 1 μM-glibenclamide (for non-specific binding). ADP–agarose, when used, was washed with 3 × 20 ml of Hepes–Krebs buffer and added to the assay mixture at a final concentration of approx. 0.6 mM-ADP. For these experiments, a beaded agarose (Sepharose 4B-200) washed similarly with Hepes–Krebs buffer in the presence (non-specific binding) or absence (total binding) of 1 μM-glibenclamide was used as a control. Bound radioactivity was separated from free by rapid filtration on Whatman GF/C filters soaked in Hepes–Krebs buffer; the filters were washed with 4 × 5 ml of Hepes–Krebs buffer at 4 °C. Radioactivity was measured and non-specific binding was determined as described in the membrane-binding study. Of the total radioactivity added, 0.8–3.6% was bound specifically and 0.3–0.5% non-specifically.

^{86}Rb-efflux measurements

HIT cells were seeded in Multiwell plates at a density of 5×10^5 cells/well and, after culture for 3–6 days, were loaded with ^{86}RbCl (1–8 mCi/mg; 0.1 μCi/well) overnight. Culture medium was then removed and replaced with solution A, containing (mM): NaCl 124, $CaCl_2$ 1.8, $MgCl_2$ 0.8, KCl 10, Hepes (pH 7.5 with NaOH) 20. In some experiments, cells were incubated for 20 min at 37 °C in solution A plus ^{86}RbCl (0.1 μCi/ml) in the absence or presence of 1 mM-2-deoxyglucose and oligomycin (1.2 μg/ml) to deplete the cells of ATP. After incubation, the medium was replaced with solution A and incubation was continued for a further 10 min at 37 °C in the absence or presence of various test substances. When extracellular Ca^{2+} was omitted from solution A, the cation was replaced with Mg^{2+} iso-osmotically. To determine the cellular content of ^{86}Rb, cells were washed twice with solution A supplemented with 1 μM-glibenclamide and extracted with 1 ml of 0.1 M-boric acid (pH 8 with NaOH)/0.5 M-NaCl. The extract was counted for radioactivity by Čerenkov counting. Stock solutions of glibenclamide (1 mM) and oligomycin (2.4 mg/ml) were prepared in dimethyl sulphoxide. Diazoxide was prepared as a stock solution (120 mM) in 0.5 M-NaOH.

ATP assay

To measure intracellular ATP concentration in HIT cells in the ^{86}Rb-efflux study, cells incubated under parallel conditions were extracted in 0.5 ml of 4% (w/v) $HClO_4$. ATP in the extracts was quantified with a luciferase assay and a commercial luminometer (LKB 1250). Intracellular concentrations of ATP were calculated on the basis of a HIT-cell intracellular volume of 1 pl/cell (Ashcroft & Stubbs, 1987). Since these flux experiments were performed in the absence of exogenous fuel substances, a decrease in ATP content of up to 20% in non-ATP-depleted cells and 40% in ATP-depleted cells was observed during the 10 min incubation. Therefore intracellular ATP concentrations were expressed as the mean of the values at the start and end of the incubation.

Measurement of intracellular Ca^{2+}

HIT cells (approx. 5×10^7) were detached with trypsin/EDTA, washed in 20 ml of Hepes–Krebs buffer containing 5 mg of BSA/ml and resuspended in 4 ml of the same buffer. Quin 2/AM was added from a 100 mM stock solution in dimethyl sulphoxide to a final concentration of 50 μM, and the cells were incubated for 20 min at 37 °C. At the end of the incubation, HIT cells were diluted 4-fold in the medium and incubated for a further 20 min at 37 °C. They were collected by centrifugation (190 *g* for 5 min), resuspended in Ca^{2+}-free (supplemented with 0.1 mM-EGTA) or Ca^{2+}-containing (2.54 mM) Hepes–Krebs buffer with 5 mg of BSA/ml at a density of 2.5×10^7 cells/ml and stored on ice before use. HIT cells (2.5×10^6/ml) were transferred in a final volume of 2 ml to a Perkin–Elmer LS5 luminescence spectrometer and preincubated for 10 min with continuous stirring before a challenge with ADP. After addition of 1 mM-ADP, the incubation was continued for a further 20 min. The intracellular free Ca^{2+} concentration was calculated as described by Hughes & Ashcroft (1988).

Insulin secretion

After preincubation for 1 h at 37 °C in 1 ml (per well) of Hepes–Krebs buffer (without glucose) containing BSA (5 mg/ml), HIT cells were further incubated for 90 min in 0.5 ml of BSA-containing Hepes–Krebs buffer supplemented with test substances as described. At the end of the incubation, a sample was collected, centrifuged briefly to sediment any detached cells and stored at −20 °C until assayed. Insulin release was measured by radioimmunoassay, with rat insulin as standard (Ashcroft & Crossley, 1975).

Miscellaneous

The nucleotides used here were sodium salts, except NAD^+, adenosine and guanosine (free acid) and ATP[S] and p[CH_2]ppA (tetralithium salts). All data are given as means ± S.E.M. for the numbers of observations indicated. The significance of observed differences was assessed by Student's *t* test.

RESULTS

Effects of ADP and other nucleotides on [^{3}H]glibenclamide binding to HIT-cell membranes

Fig. 1(*a*) shows the concentration-dependence of specific [^{3}H]glibenclamide binding to HIT-cell membranes. As previously reported (Niki *et al.*, 1989*a*), Scatchard plots were curvilinear, suggesting the presence of both high- and low-affinity binding sites; these had K_d values of 1.2 and 211 nM and $B_{max.}$ values of 384 and 863 fmol/mg of protein respectively. ADP (1 mM) competitively inhibited high-affinity [^{3}H]glibenclamide binding, whereas 1 mM-ATP had no significant effect. The following nucleotides were also without effect at a concentration of 1 mM on [^{3}H]glibenclamide binding: AMP, adenosine, GTP, GDP, GMP, guanosine, NAD^+, NADH, $NADP^+$ and NADPH. The dose-dependence for the inhibitory effect of ADP on [^{3}H]glibenclamide binding is shown in Fig. 1(*b*). Displacement of 50% of the specific binding was found with approx. 1.5 mM-ADP.

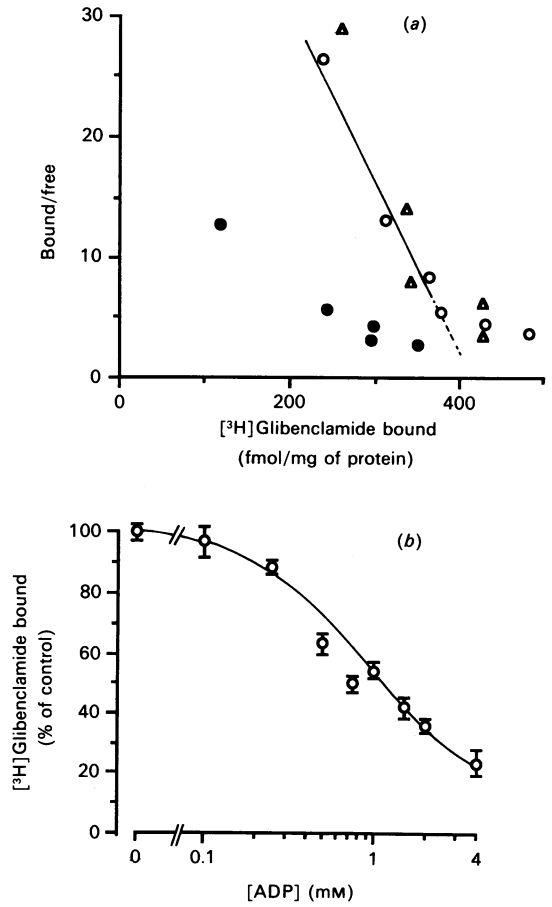

Fig. 1. [^{3}H]Glibenclamide binding to HIT-cell membranes: effects of ADP and ATP

(*a*) Samples of HIT-cell membranes (186 μg of protein/ml) were incubated with [^{3}H]glibenclamide (1.7–24.6 nM) as described in the Materials and methods section in the absence (○) or presence of 1 mM-ADP (●) or -ATP (△). Specific binding was determined by subtracting the non-specific binding (radioactivity remaining in the presence of 1 μM unlabelled glibenclamide) from the total radioactivity bound. Data are presented as Scatchard plots; each point is the mean of duplicate determinations. (*b*) Dose-dependence of the inhibition by ADP of [^{3}H]glibenclamide (3.74 nM) binding to HIT-cell membranes.

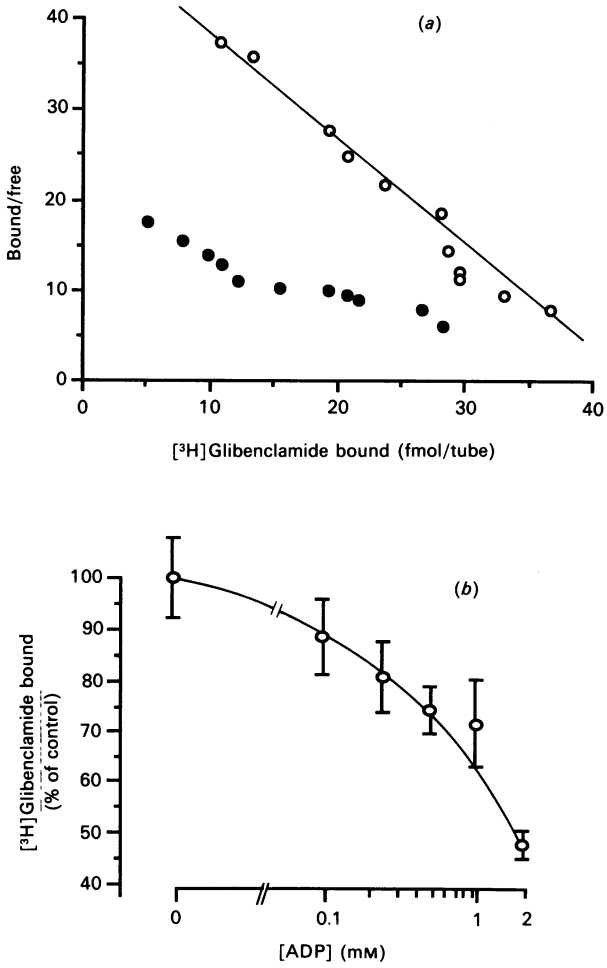

Fig. 2. [^{3}H]Glibenclamide binding to intact HIT cells: effects of ADP

(*a*) Effects of 1 mM-ADP on the dose-dependent binding of [^{3}H]glibenclamide (1.1–13.3 nM) to intact HIT cells. Each tube contained 1.5×10^6 cells. Specific binding to the cells in the absence (○) or presence (●) of 1 mM-ADP is presented as a Scatchard plot; each point is the mean of duplicate determinations. (*b*) Dose-dependence of the inhibition by ADP of [^{3}H]glibenclamide (2.58 nM) binding to intact HIT cells.

Table 1. Effects of various adenine nucleotides on ^{86}Rb efflux from intact HIT cells evoked by diazoxide

^{86}Rb efflux over 10 min is expressed as a percentage of the ^{86}Rb content at the start of the incubation. Diazoxide (300 μM) and adenine nucleotides (1 mM) were added during the 10 min incubation as described in the Materials and methods section. Data are given as means ± S.E.M. for four observations: * significantly less than control (no addition) ($P < 0.001$).

Addition (1 mM)	^{86}Rb efflux (%/10 min)
None	58.4 ± 0.6
ATP	49.1 ± 1.1
ADP	30.1 ± 0.6*
AMP	55.1 ± 1.2
Adenosine	51.2 ± 1.4
p[CH_2]ppA	54.0 ± 0.9
ATP[S]	54.1 ± 0.5

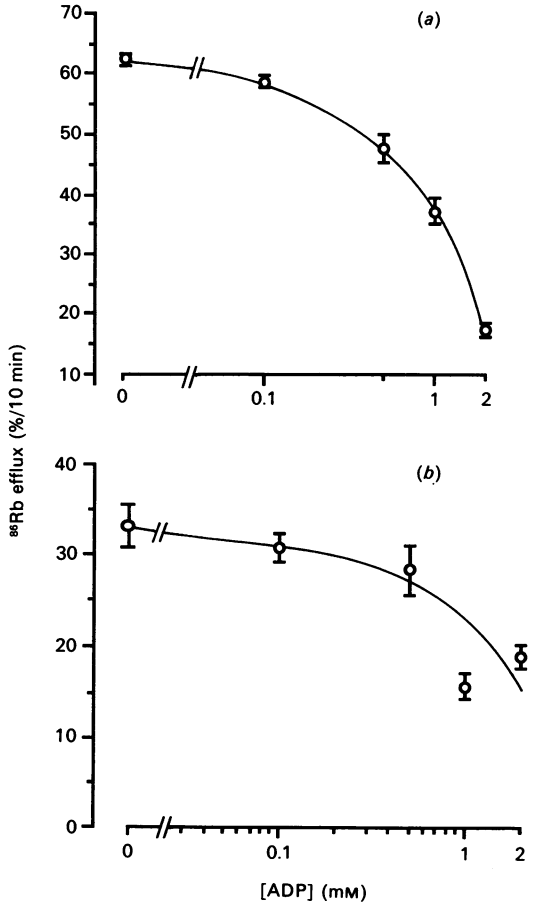

Fig. 3. Effects of ADP on ^{86}Rb efflux from intact HIT cells evoked by (*a*) diazoxide or (*b*) ATP depletion

(*a*) ^{86}Rb efflux over 10 min was expressed as a percentage of the ^{86}Rb content at the start of the incubation; 300 μM-diazoxide with or without various concentrations of ADP was added during the 10 min incubation as described in the Materials and methods section. (*b*) ATP depletion was carried out by exposure of the cells to oligomycin (1.2 μg/ml) and 1 mM-2-deoxyglucose during a 20 min preincubation. Data are given as means $\pm$ S.E.M. for four observations.

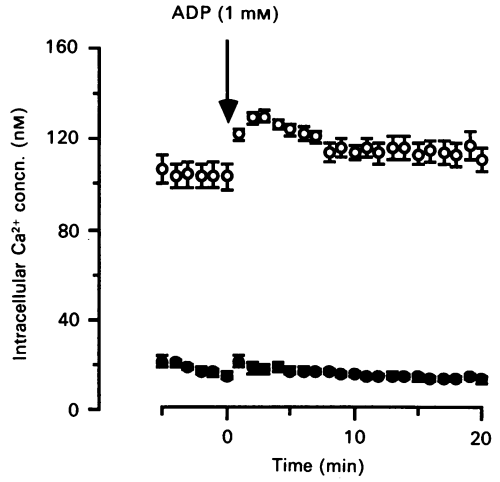

Fig. 4. Effect of ADP on the intracellular Ca^{2+} concentration in HIT cells

Intracellular Ca^{2+} concentrations were measured with quin 2. After quin 2/AM loading, the cells were resuspended and incubated in Hepes–Krebs buffer containing 2.54 mM-Ca^{2+} (○, $n = 6$) or in Ca^{2+}-free buffer supplemented with 0.1 mM-EGTA (●, $n = 3$). ADP (1 mM) was added to the media at 0 min. The incubation was continued for a further 20 min. Data are given as means $\pm$ S.E.M.

Effects of ADP on [^{3}H]glibenclamide binding to intact HIT β-cells

[^{3}H]Glibenclamide showed saturable and high-affinity binding to whole cells. Non-specific binding was approx. 20% of the specific binding. Scatchard plots (Fig. 2*a*) were linear and gave a K_d of 3.59 nM and $B_{max.}$ of 18700 binding sites/cell. ADP (1 mM) competitively inhibited the binding. The concentration-dependence of this effect of ADP is shown in Fig. 2(*b*). Displacement of 50% of the specific binding required approx. 1.8 mM-ADP. ADP–agarose (equivalent to 0.6 mM-ADP) also significantly inhibited the specific binding (100 ± 2.9% for controls with 20% agarose versus 82.9 ± 3.4% for ADP-agarose; $n = 9$, $P < 0.01$).

Effects of ADP and other nucleotides on ^{86}Rb efflux from HIT β-cells

During a 10 min incubation, the ^{86}Rb content of control cells decreased by 19.8 ± 1.5% ($n = 5$). Addition of 300 μM-diazoxide increased ^{86}Rb efflux to 58.4 ± 0.6% ($n = 6$). Table 1 shows that, of several adenine nucleotides tested at 1 mM, only ADP markedly decreased (by 49%) the ^{86}Rb efflux evoked by diazoxide. This effect of ADP was also observed when Ca^{2+} was omitted from the solution; ^{86}Rb efflux was 53.1 ± 0.9% (with 300 μM-diazoxide) versus 25.7 ± 2.3% (with 1 mM-ADP and 300 μM-diazoxide) ($P < 0.01$, $n = 4$).

Fig. 3(*a*) shows the dose-dependence of the effect of ADP on ^{86}Rb efflux from HIT cells evoked by 300 μM-diazoxide. ADP (0.1–2 mM) progressively inhibited diazoxide-evoked ^{86}Rb efflux, with a half-maximal effect at approx. 1.2 mM-ADP. In the presence of 2 mM-ADP, ^{86}Rb efflux (17.3 ± 1.2%, $n = 5$) was not significantly different from that in the absence of diazoxide.

^{86}Rb efflux elicited by ATP depletion was also inhibited by increasing concentrations of ADP (Fig. 3*b*). Intracellular ATP concentration was 5.19 ± 0.22 mM ($n = 5$) in the non-ATP-depleted cells and 0.30 ± 0.03 mM ($n = 5$) in the ATP-depleted cells. Addition of diazoxide did not change the intracellular ATP concentration in non-ATP-depleted cells (5.21 ± 0.79 mM; $n = 3$).

Effect of ADP on intracellular Ca^{2+} concentration

As shown in Fig. 4, 1 mM-ADP caused a rapid and sustained rise in the intracellular Ca^{2+} concentration in HIT cells incubated in Hepes–Krebs buffer containing 2.54 mM-Ca^{2+}. At 3 min after addition of ADP, the intracellular Ca^{2+} concentration reached a peak (130 ± 3 nM at 3 min versus 103 ± 6 nM at 0 min). The intracellular Ca^{2+} concentration then gradually decreased, but the concentration was still above the control value at 7 min after the ADP addition. In the absence of extracellular Ca^{2+} this effect was not observed; the small transient increase seen in intracellular Ca^{2+} concentration did not reach statistical significance (21.3 ± 2.5 nM at 1 min after ADP addition versus 15.1 ± 1.7 nM at 0 min).

Effects of ADP and other nucleotides on insulin release

In normal Hepes–Krebs buffer ([Ca^{2+}] = 2.54 mM), 1 mM-ADP increased insulin release in the presence of 0.5 mM-glucose (19.4 ± 1.2 μ-units/well per 90 min in the absence of ADP versus 42.3 ± 7.2 μ-units/well per 90 min with 1 mM-ADP; $n = 6$, $P < 0.01$); this stimulatory effect of ADP was not observed in the absence of glucose (results not shown). In high-Ca^{2+} (5 mM)

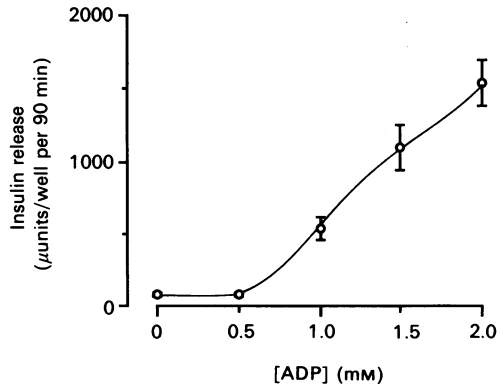

Fig. 5. Effect of ADP on insulin release from HIT cells

After a 60 min preincubation in the absence of glucose, HIT cells (approx. 10^6 cells/well) were incubated for 90 min with modified Hepes–Krebs buffer containing 5 mM-Ca^{2+} and supplemented with 0.5 mM-glucose and various concentrations of ADP. Rates of insulin release (μ-units/well per 90 min) are given as means ± S.E.M. for 12–24 observations.

Table 2. Effects of adenine nucleotides on insulin release from HIT cells

The experimental design is described in the legend to Fig. 5. All additions were present at a concentration of 1 mM. Data are given as means ± S.E.M. for 16–24 observations: * significantly greater than control (no addition) ($P < 0.001$); † significantly less than control (no addition) ($P < 0.05$).

Addition (1 mM)	Insulin release (μ-units/well per 90 min)
None	644 ± 17
ATP	972 ± 94
ADP	1802 ± 146*
AMP	219 ± 17†
Adenosine	764 ± 79
p[CH_2]ppA	339 ± 15†
ATP[S]	729 ± 25

Hepes–Krebs buffer, insulin release in the presence of 0.5 mM-glucose was significantly increased (110 ± 19 μ-units/well per 90 min; $n = 6$, $P < 0.01$). The stimulatory effect of ADP was more pronounced at 5 mM-Ca^{2+} (305 ± 32 μ-units/well per 90 min; $n = 6$, $P < 0.001$). Therefore, we examined the dose–response of insulin release for ADP and the effects of other adenine nucleotides, using high-Ca^{2+} Hepes–Krebs buffer (Fig. 5 and Table 2). As shown in Fig. 5, 1–2 mM-ADP increased insulin release from HIT cells in a dose-dependent manner. When 1 mM concentrations of various adenine nucleotides were added to the incubation media, only ADP increased insulin release. ATP, ATP[S] and adenosine failed to affect insulin release, whereas AMP and p[CH_2]ppA significantly inhibited release (Table 2).

DISCUSSION

A K^+ channel sensitive to ATP was discovered in cardiac-muscle cells by Noma (1983). K-ATP channels were also found in the pancreatic β-cell (Cook & Hales, 1984), and were shown to be responsible for the membrane-potential changes when the pancreatic β-cell was stimulated with glucose (Ashcroft *et al.*, 1984). The β-cell K-ATP channel was subsequently shown to be specifically inhibited by sulphonylureas, and high-affinity binding sites were demonstrated on membranes from β-cells and brain (Schmid-Antomarchi *et al.*, 1987; Fosset *et al.*, 1988; Bernadi *et al.*, 1988; Niki *et al.*, 1989*a*). The K_d of 3.59 nM from the present whole-cell binding study is comparable with values obtained for high-affinity binding by using HIT-cell membranes [0.7 nM (Gaines *et al.*, 1988); 1.1 nM (Niki *et al.*, 1989*a*); 1.2 nM (the present study)]. The estimate for $B_{max.}$ is approx. 4 times that for the high-affinity binding sites reported for membranes from the same cell line (Niki *et al.*, 1989*a*; the present study). This difference may result from degradation of the binding protein during membrane preparation, since the low-affinity binding sites observed in membrane binding studies on brain (Lupo & Bataille, 1987) and HIT cells (Niki *et al.*, 1989*a*; the present study) are not obvious in the binding study with whole cells.

Although 1 mM-ATP had no effect on [^{3}H]glibenclamide binding to membranes, ADP at the same concentration inhibited the binding, suggesting that ATP binds to different sites from those for sulphonylureas or ADP. Among a wide variety of nucleotides, only ADP inhibited the binding of [^{3}H]glibenclamide to HIT-cell membranes. ADP (1 mM) increased the apparent K_d for [^{3}H]glibenclamide without changing the $B_{max.}$ in both isolated-membrane and also whole-cell binding studies, suggesting that ADP inhibited glibenclamide binding competitively.

The location of sulphonylurea-binding sites has not been established. Since sulphonylureas are known to be highly lipid-soluble, it is considered that these agents are easily permeant across plasma membranes. That is why tolbutamide is equally effective in inhibiting the K-ATP channel in patch-clamp methods irrespective of whether the sulphonylurea is applied from the intracellular or the extracellular space (Sturgess *et al.*, 1988). The present finding that ADP also inhibited [^{3}H]glibenclamide binding to intact HIT cells suggested that the binding sites exist on the extracellular side of plasma membranes, since the nucleotide is quite unlikely to reach the intracellular space because of its high polarity. The finding that ADP–agarose also inhibited the binding to whole cells supports this idea. A similar conclusion was reached by Bowen & Lazarus (1974) from studies using dextran-linked sulphonylurea.

The activity of the K-ATP channel in whole cells was assayed as glibenclamide-blockable ^{86}Rb efflux in HIT cells that had been exposed to diazoxide or depleted of ATP (Niki *et al.*, 1989*a*). The present finding that extracellular ADP inhibited the activity of the K-ATP channel suggests that ADP acts on this channel as an inhibitor via sharing common binding sites with sulphonylureas. The ADP-specific inhibition of ^{86}Rb efflux was not due to the chelating effect of ADP on Ca^{2+}, since the inhibition was also observed in the absence of extracellular Ca^{2+}. Although extracellularly added ATP also slightly inhibited the efflux, this probably results from concomitant conversion of ATP into ADP by ecto-ATPase at the plasma membrane, since p[CH_2]ppA and ATP[S], non-hydrolysable analogues of ATP (Yount, 1975), had less effect on the efflux than ATP did. One of the reasons why extracellular ADP had a less inhibitory effect on ^{86}Rb efflux elicited by ATP depletion than on diazoxide-evoked ^{86}Rb efflux may be that progressive ATP depletion allows dephosphorylation of the channel protein and consequent 'run-down' of channel activity (Ohno-Shosaku *et al.*, 1987).

A 'spare channel' theory, in which the resting β-cell membrane potential is determined essentially by the last few channels to be closed by high concentrations of ATP, has been suggested to explain the discrepancy between effective concentrations of ATP in whole-cell studies and in isolated patches (Cook *et al.*, 1988). This model predicts that significant changes in membrane potential and hence insulin release require concentrations of a channel blocker some 100-fold higher than the $K_{0.5}$ of the

channel for the blocker. However, in the present study we observed that the concentration of ADP that gave 50% displacement of glibenclamide binding and inhibition of ^{86}Rb efflux was capable of eliciting marked stimulation of insulin release. This suggests that other mechanism(s), such as the participation of additional cytoplasmic factor(s) or the intracellular distribution of ATP (Niki *et al.*, 1989*b*), may be more responsible for the apparent discrepancy.

The observed increase in intracellular Ca^{2+} concentration evoked by ADP is likely to result from Ca^{2+} influx across the plasma membrane via voltage-dependent Ca channels, consistent with the view that ADP inhibits the K-ATP channel, since only a small and statistically non-significant increase in intracellular Ca^{2+} concentration was elicited by ADP in Ca^{2+}-free buffer. We consider that these effects of ADP on ^{86}Rb efflux or insulin release from HIT cells are unlikely to be mediated by P2-purinergic receptors (Bertrand *et al.*, 1986), since these receptors have a similar affinity for ADP and ATP, in contrast with the marked specificity for ADP reported here. Interestingly, these effects of ADP on intracellular Ca^{2+} in HIT cells are similar to those observed in quin 2-loaded platelets (Hallam & Rink, 1985).

These data indicate that extracellular ADP can increase insulin release via inhibition of the K-ATP channel, and raise the possibility that extracellular ADP as well as intracellular ATP may be a physiological modulator of the channel. A possible source of extracellular ADP in the vicinity of the β-cell is the parasympathetic release of ATP from cholinergic neurones. Since β-cell plasma membranes have a high activity of ecto-ATPase (S. J. H. Ashcroft, unpublished work), it is likely that extracellular ADP would be formed from ATP and could therefore function at the β-cell plasma membrane to augment the potentiatory effect on insulin release of the acetylcholine itself. Such a synergistic effect between ADP and acetylcholine on insulin release has been demonstrated by Bertrand *et al.* (1986), using perfused pancreas, although they reported that ATP as well as non-metabolized analogues of ATP could also increase insulin release potentiated by acetylcholine.

The present findings obtained from sulphonylurea binding, ionic flux, quin 2 and insulin-secretion studies together strongly suggest that sulphonylureas and ADP have common binding sites on the extracellular side of β-cell plasma membranes which are inhibitory to the K-ATP channel.

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