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The Gating of Nucleotide-Sensitive K^+ Channels in Insulin-Secreting Cells Can Be Modulated by Changes in the Ratio ATP^{4-}/ADP^{3-} and by Nonhydrolyzable Derivatives of Both ATP and ADP

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Summary. The ^{31}P-NMR technique has been used to assess the intracellular ratios and concentrations of mobile ATP and ADP and the intracellular pH in an insulin-secreting cell line, RINm5F. The single-channel current-recording technique has been used to investigate the effects of changes in the concentrations of ATP and ADP on the gating of nucleotide-dependent K^+ channels. Adding ATP to the membrane inside closes these channels. However, in the continued presence of ATP adding ADP invariably leads to the reactivation of ATP-inhibited K^+ channels, even at ATP^{4-}/ADP^{3-} concentration ratios greater than 7 : 1. Interactions between ATP^{4-} and ADP^{3-} seem competitive. An increase in the concentration ratio ATP^{4-}/ADP^{3-} consistently evoked a decrease in the open-state probability of K^+ channels; conversely, a decrease in ATP^{4-}/ADP^{3-} increased the frequency of K^+ channel opening events. Channel gating was also influenced by changes in the absolute concentrations of ATP^{4-} and ADP^{3-}, at constant free concentration ratios. ADP-evoked stimulation of ATP-inhibited channels did not result from phosphorylation of the channel, as ADP-β-S, a nonhydrolyzable analog of ADP, not only stimulated but enhanced ADP-induced activation of K^+ channels, in the presence of ATP. Similarly, ADP was able to activate K^+ channels in the presence of two nonhydrolyzable derivatives of ATP, AMP-PNP and $\beta\gamma$methylene ATP.

Key Words K^+ channel · ATP · ATP^{4-} · ADP^{3-} · RINm5F cell

Introduction

The secretion of insulin from pancreatic B-cells is the single most important process governing the maintenance of a homeostatic level of glucose in the blood. It has now been firmly established that a prerequisite for insulin release is the metabolism, by the B-cells, of glucose (Ashcroft, 1980; Hedeskov, 1980; Meglasson & Matschinsky, 1986; Wollheim & Biden, 1987). When directly challenged with glucose or other carbohydrate secretagogues the membranes of insulin-releasing cells undergo complex changes in their electrical pattern of activity, consisting of oscillatory waves of depolarization followed by repolarization (Dean & Matthews, 1970), fluctuations that are mediated by changes in the permeability of the membranes to K^+ ions (Meissner, 1976; Henquin & Meissner, 1984). The functional significance of these changes in the K^+ ion permeability, is to mobilize calcium, the key intracellular regulator of insulin release, from the outside to the inside of the cell, through the opening of voltage-gated calcium channels (Matthews & Sakamoto, 1975; Petersen & Findlay, 1987).

Recent single-channel current-recording experiments have demonstrated that carbohydrate-induced changes in the K^+ ion permeability, the trigger mechanism for insulin secretion, are mediated through the closure of a specific class of potassium-selective pore, the adenosine 5′ triphosphate (ATP)-sensitive K^+ channel (Ashcroft, Harrison & Ashcroft, 1984; Rorsman & Trube, 1985; Dunne et al., 1986; Misler et al., 1986; Petersen & Findlay, 1987).

A number of other nucleotides apart from ATP (Cook & Hales, 1984; Findlay, Dunne & Petersen, 1985*a*; Findlay & Dunne, 1986) are able to influence the gating of these channels, including adenosine 5′ diphosphate (ADP) (Dunne & Petersen, 1986*a*; Kakei et al., 1986) and the guanosine- (Dunne & Petersen, 1986*b*) and pyridine- (Dunne, Findlay & Petersen, 1988) based nucleotides.

The nucleotide-dependent K^+ channel is, therefore, at the heart of stimulus-secretion coupling in insulin-secreting cells.

How are changes in the K^+ ion permeability of insulin-secreting cell membranes coupled to the metabolism of carbohydrates in the cytoplasm? A number of proposals have been made to explain the link between these two discrete events. Coupling

Table 1. Relationship between the total concentrations of ATP and ADP added to the K^+-rich solution and the calculated free ATP^{4-} and ADP^{3-} concentrations

	Total concentrations (mM)		Calculated free concentrations (mM)	
	ATP	ADP	ATP^{4-}	ADP^{3-}
1.	5.1	2.3	0.84	1.23
2.	2.8	0.7	0.47	0.38
3.	5.6	1.4	0.94	0.76
4.	3.1	0.4	0.57	0.23
5.	6.2	0.8	1.17	0.45
6.	6.4	0.5	1.21	0.29
7.	6.7	0.3	1.39	0.18

may occur via the availability of high-energy phosphate intermediates or reducing equivalents (Malaisse et al., 1978, 1979) or via the availability of protons (Lebrun et al., 1986). A further possibility, and the one under investigation in this study, is that the coupling factor consists of changes in the cytosolic level of ATP and/or changes in the cytosolic ATP/ADP ratio.

Biochemical studies have indicated that both the intracellular concentration of ATP and the cytosolic ATP/ADP ratio increase upon stimulation of insulin-secreting cells (Ashcroft, Weerasinghe & Randle, 1973; Malaisse et al., 1979; Malaisse & Sener, 1987), and from patch-clamp studies it has been shown that ADP is indeed able to modulate the activity of ATP-sensitive K^+ channels in the presence of ATP, by increasing the concentration of ATP required to evoke channel closure (Dunne & Petersen, 1986*a*; Kakei et al., 1986; Misler et al., 1986; Ribalet & Ciani, 1987; Stanfield, 1987).

Recent experiments, however, have shown that the inhibitory effects of ATP on K^+ channels in insulin-secreting cells do not require Mg^{2+}, but are in fact inhibited by Mg^{2+}, indicating that ATP influences channels through the availability of the free ATP ion, ATP^{4-} (Ashcroft & Kakei, 1987; Dunne, Ilott & Petersen, 1987).

It is, therefore, the purpose of this investigation to study how K^+ channel gating is influenced: (i) by changes in the concentration ratio ATP^{4-}/ADP^{3-}, (ii) by changes in the concentrations of both ATP^{4-} and ADP^{3-}, at a constant ATP^{4-}/ADP^{3-} ratio, and (iii) to investigate whether the ADP-evoked openings of ATP-inhibited K^+ channels are dependent on protein phosphorylation, through the use of non-hydrolyzable derivatives of both ADP, adenosine 5′-O-(2-thiodiphosphate) (ADP-β-S) and ATP, adenylyl-imidodiphosphate (AMP-PNP) and methyleneadenosine 5′-triphosphate ($\beta\gamma$methylene ATP).

In order to carry out these experiments we have used the single-channel current-recording technique applied to the open-cell preparation of the clonal insulin-secreting cells, RINm5F.

^{31}P-nuclear magnetic resonance has for the first time been applied to insulin-secreting cells. Its use has enabled us to assess the intracellular concentrations of both ATP and ADP, to determine the relative intracellular ratios of the nucleotides and to measure the intracellular pH of the RINm5F cells.

Materials and Methods

Cell Isolation and Maintenance

All experiments were carried out on the clonal insulin-secreting cell line RINm5F (Halban, Praz & Wollheim, 1983) maintained as previously described (Dunne et al., 1986).

Media

Patch-clamp recording pipettes (Type 101-PB, Ceebe Glass, Denmark), coated with a sylgard resin (Dow Corning Corp., USA) had a final resistance of around 5 MΩ when filled with a Na^+-rich solution containing (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 glucose, 10 HEPES and 1 EGTA; no calcium was added and the pH set at 7.2 (NaOH). The Na^+-rich solution, with 2 mM $CaCl_2$ added, was used initially in the cell bath following the removal of tissue culture media. Prior to experimentation, however, this solution was removed and the cells bathed in a K^+-rich solution of the following composition (mM): 140 KCl, 2.5 glucose, 10 HEPES and 1 EGTA; no calcium was added and the pH set at 7.2 (KOH). The osmolality of all solutions was 290 ± 5 mosmol/kg.

All experiments were carried out at room temperature, 22–25°C.

Using an iterative procedure on a BBC microcomputer (Findlay, Dunne & Petersen, 1985*b*; Squire & Petersen, 1987) the compositions of a series of test solutions containing a range of ATP^{4-} and ADP^{3-} concentrations were worked out taking into account the stability constants for all reactions of Ca, Mg, H and the various forms of ATP, ADP and EGTA (Sillen & Martell, 1971; Martell & Smith, 1974) as shown in Table 1. 5.5 mM $MgCl_2$ was added to solutions 1, 3, 5, 6 and 7 (*see* Table 1), 3 mM $MgCl_2$ to solutions 2 and 4 (*see* Table 1) and 0.5 mM $MgCl_2$ to the nucleotide-free control K^+-rich solution. The free Mg^{2+} concentration was in this way kept relatively constant (0.4–0.5 mM).

Single-Channel Current Recording and Analysis

Single-channel current records were obtained, using the principles described by Hamill et al. (1981) from RINm5F permeabilized or open-cell preparations. The generation of the open-cell recording configuration and the arrangement of the superfusion system used to exchange control with test solutions has been described in detail (Dunne et al., 1986, 1987).

Analysis of data to determine the apparent channel open-

state probability was performed on stretches of continuous current records (filtered at 1 kHz, low pass) lasting between 10 and 20 sec. Open-state probability (P) determinations were restricted to records where at the time of analysis no more than six coincident K^+ channel open-events were observed.

All data were digitized at 8 kHz (CED 1401, Cambridge, U.K.) and analyzed using a Tandon microcomputer in conjunction with a software package supplied by CED Cambridge, U.K. Idealized current records were obtained from computerized threshold analysis.

The apparent open-state probability values were determined based on the maximal number of K^+ channel opening events, i.e. the number of channel openings recorded initially, seconds after cell permeabilization, before the inherent run-down of K^+ channels had taken place. From these values it was therefore possible to determine the average K^+ATP channel current in a particular open-cell and to express this as a fraction of the theoretically maximum membrane patch current.

The pipette voltage in all cases was held at 0 mV throughout the experiment. In all single-channel current records illustrated, upward deflections represent outward current flow (i.e. from the inside of the cell to the outside). All current traces shown, photographed directly from the oscilloscope screen, have been filtered at 400 Hz (low pass).

Nuclear Magnetic Resonance (NMR) Measurements

Prior to experimentation RPMI 1640 tissue culture medium was removed, the cells washed and resuspended in the Na^+-rich bathing medium, containing 2 mM $CaCl_2$.

Cell suspensions were then placed in 5 mm NMR tubes and lightly spun down to achieve a pellet containing approximately 10^6 cells. ^{31}P-NMR spectra (101 MHz) were recorded on a Bruker WM250 spectrometer. The shimming of the field homogeneity was achieved, without any deuterium lock by optimizing the water-free induction decay (FID) signal. Probe tuning was checked on a 50 mM sample of KH_2PO_4.

Typically, 500 FID's were accumulated in 4K data points. A 45° pulse angle was used with a relaxation delay of 1.5 sec and an acquisition time of 0.51 sec. 10 Hz of line broadening was applied before fourier transformation.

Chemical shifts were measured in parts per million (ppm) with respect to phosphocreatine (PC) as the internal reference at 0 ppm.

For absolute measurements of the metabolic concentrations of ATP, ADP and P_i in RINm5F cells it is necessary to directly relate the ^{31}P-NMR spectra recorded from the cells with that of the spectra obtained from a standard phosphate-containing solution (50 mM KH_2PO_4).

This was carried out using a reference microcapillary tube, containing 200 mM methylenediphosphonate (MDP) (in D_2O), which resonates at −16 ppm.

The MDP probe was firstly placed in a 5 mm NMR tube containing the standard 50 mM phosphate solution. Once the ^{31}P-NMR spectra had been obtained, the sample tube was removed and added to a suspension of the RINm5F cells. These were then lightly centrifuged and analyzed for their ^{31}P-content, in the presence of the MDP, as described.

The integrated peak areas of the 50 mM KH_2PO_4 and the 200 mM MDP solutions, obtained in the absence of cells, were then measured and the ratio of KH_2PO_4/MDP evaluated. This coupling factor, 1.04, was then used to standardize the MDP peak

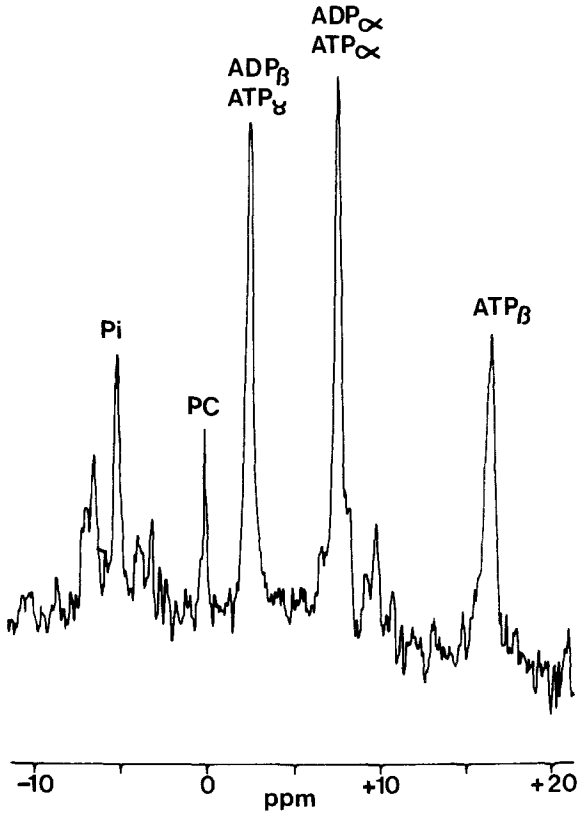

Fig. 1. ^{31}P-nuclear magnetic resonance spectra of RINm5F cells. ^{31}P-NMR (101 MHz) analysis was carried out on approximately 10^6 cells using a Bruker WM250 spectrometer. Peak assignments for adenosine triphosphate α-, β- and γ-phosphate (ATP), adenosine diphosphate α- and β-phosphate (ADP), inorganic phosphate (P_i) and phosphocreatine (PC) were made from the chemical shift values (ppm)

observed in ^{31}P-NMR spectra recorded from intact RINm5F cells to 50 mM.

Using this type of analysis it was, therefore, possible to quantify the integrated area of a peak that a 50 mM phosphate sample would give rise to, and thereby allow us to evaluate the relative metabolic concentrations of ATP, ADP and P_i in the RINm5F cells.

Results

Measurement of the Intracellular Concentration Ratio ATP/ADP

Figure 1 shows a typical ^{31}P-NMR spectrum of the RINm5F cells. Peak identification was made from the chemical shift value in ppm with respect to PC.

When studying the ^{31}P-NMR spectra of intact cells it is generally found that the peaks corresponding to the two ADP-phosphate residues, ADP α- and ADP β-phosphate, respectively, are not resolved, but rather overlap with two of the three

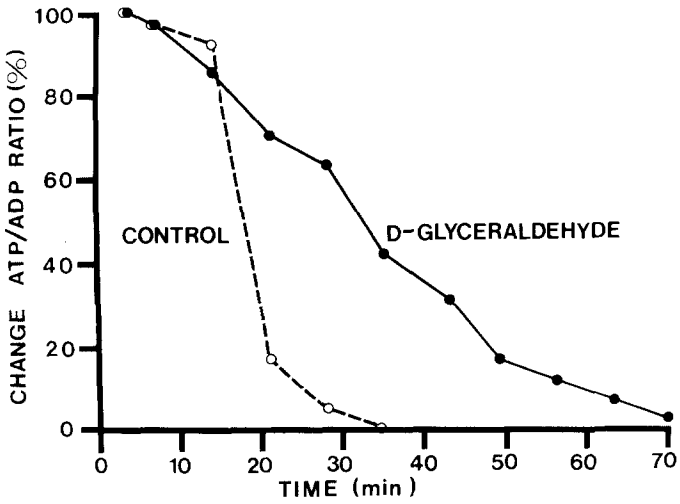

Fig. 2. Effect of D-glyceraldehyde on the ATP/ADP of RINm5F cells. Changes in the concentrations of the nucleotides have been expressed as a percentage of the initial ATP/ADP ratio

Table 2. Assessment of the intracellular ATP/ADP and intracellular concentrations of ATP, ADP and inorganic phosphate (P_i), in RINm5F cells, using ^{31}P-NMR

	ATP/ADP	ATP (mM)	ADP (mM)	P_i (mM)
Experiment 1	3.0	5.5	1.83	9.84
Experiment 2	2.4	7.59	3.16	6.96
Experiment 3	3.7	4.16	1.12	3.85
Average	3.03	5.75	2.03	6.83

ATP-phosphate peaks, ATP α- and ATP γ-phosphate, respectively. As the integrated peak areas of NMR signals are directly proportional to the concentrations of metabolites that give rise to them, the ratio ATP/ADP can therefore be assessed from measurements of these areas.

In order to assess the contribution made by ADP to the ATP resonances, the integrated area of the well-resolved ATP β-phosphate peak at 16.4 ppm is subtracted from the integrated area of the 2.4 ppm terminal ATP-phosphate peak. Using this type of analysis the ratio ATP/ADP can be determined.

In the record shown (Fig. 1), the ratio ATP/ADP is 2.7.

This value was typical of four further experiments, and the average value of ATP/ADP was found to be 2.7 ± 0.2 (SE) (n = 5).

In a separate series of experiments the MDP probe was used to assess the intracellular concentrations of ATP, ADP and inorganic phosphate (P_i). The results of this analysis are shown in Table 2.

In this particular series of experiments the ratio of ATP/ADP was found to be 3.0 ± 0.4 mM (n = 3).

Measurement of the Intracellular pH

Assessment of the intracellular pH (Gadian, 1982) of the RINm5F cells was made using the equation:

$$pH = 6.75 + \log_{10}[(d - 3.27)/(5.69 - d)]$$

where d is the chemical shift value, in ppm, of the inorganic phosphate resonance, with respect to the phosphocreatine resonance.

From analysis of five different ^{31}P-NMR spectra the average value of d was found to be 5.14 ± 0.02 (n = 5) ppm (5.1 in Fig. 1) indicating that on average the intracellular pH of these cells is 7.28.

The Effect of D-Glyceraldehyde on the Intracellular Concentration Ratio ATP/ADP

Figure 2 shows the result from an experiment in which the effects of 10 mM D-glyceraldehyde on the ATP/ADP ratio of the RINm5F cells were tested using ^{31}P-NMR data. The changes in the ratio ATP/ADP have been expressed as a percentage of the initial ATP/ADP value.

The experiments have been carried out on the centrifuged pellet of RINm5F cells. Under these conditions the cells become hypoxic over a period of time. In the absence of carbohydrate the ratio of ATP/ADP invariably fell dramatically with only about 20% of the initial ATP/ADP value remaining 20 min after beginning the experiment. ATP/ADP was zero after 35 min (n = 8).

However, when ^{31}P-NMR spectra were obtained in the presence of 10 mM D-glyceraldehyde the ATP/ADP value was found to be maintained for a substantially longer period of time. Under these

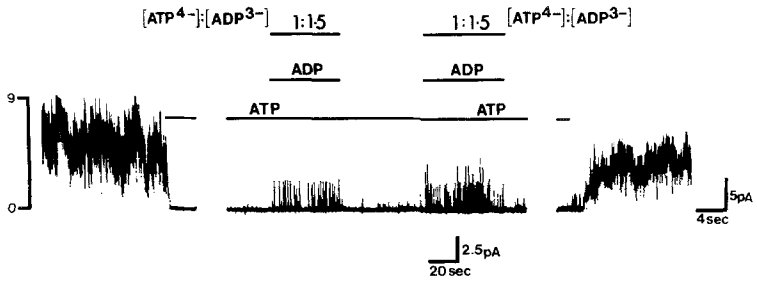

Fig. 3. Stimulation of ATP-inhibited K^+ channels by ADP. When 0.75 mM ATP^{4-} (5 mM ATP) is added to the membrane inside complete inhibition of K^+ channels results. ADP ($[ADP^{3-}]$ = 1.23 mM) added in the presence of ATP ($[ATP^{4-}]$ is now 0.84 mM) (solution 1 in Table 1) reactivates ATP-inhibited channels. When ADP is removed complete inhibition of K^+ channels results, and is followed, upon readdition of ADP, by channel activation. Cellular permeabilization (0.05% saponin) yielded an initial patch current of 20 pA, the record beginning 10 sec after this event. The scale bar on the left-hand side of the current trace represents the number of coincident K^+ channel openings. Note that the vertical scale has been magnified during the test period

conditions, 20 min after beginning the experiment, 80% of the initial ATP/ADP value was retained.

Effects of Different Concentration Ratios of ATP^{4-}/ADP^{3-} on the Gating of K^+ Channels

Figure 3 shows the result of an experiment in which ATP-inhibited K^+ channels are activated by ADP in the continued presence of ATP.

The current recording begins 10 sec after permeabilization of an intact RINm5F cell with 0.05% saponin. The resulting open-cell preparation had an initial patch current of 20 pA, with coincident openings from at least nine K^+ channels being observed. Channel openings were abolished when 0.75 mM ATP^{4-} (5 mM ATP) was added to the inside of the membrane.

In the continued presence of ATP, ADP (solution 1 in Table 1) was able to evoke a clear and sustained activation of K^+ channels to on average 1.2% of the maximal patch current. Removal of ADP from the membrane once again closed the K^+ channels, lowering the average K^+_{ATP} channel current to 0.005% of the control value, which was subsequently increased to 1.7% upon readmission of ADP. Finally, removal of all nucleotides from the membrane inside resulted in a recovery of channel activity to around 80% of the maximal prestimulus level of activity, indicating that during the period of the experiment some channel run-down had occurred.

The series of experiments represented by Figs. 4–6 show how different ratios of ATP^{4-}/ADP^{3-}, ranging from 0.7 (1 : 1.5) to 7.7 (7.7 : 1) (Table 1), influence the behavior of the K^+ channels.

Figure 4(*A*) shows that 0.76 mM ADP^{3-} is able to activate K^+_{ATP} channels inhibited by 0.94 mM ATP^{4-} (solution 3), increasing the membrane patch current from 0 to 0.12% of the maximal value. Under these test conditions, where the ratio of the free ions is 1.2 the ratio of the total nucleotide concentrations (ATP/ADP) is 4.

ADP-evoked stimulation of K^+ channels is further enhanced when the ratio ATP^{4-}/ADP^{3-} is switched from 1.2 to 0.7 (solution 1) increasing the patch current from 0.12 to 1.3%, which is subsequently reduced to 0.12% of the maximal value upon return to the 1.2 test solution.

Finally, complete inhibition of K^+ channels, is once again seen when ADP is removed from the membrane inside.

In the series of experiments represented by Fig. 4(*B*), K^+ channel openings evoked by the addition of a 2.5 ratio of ATP^{4-}/ADP^{3-} (solution 5) to the inner membrane are further enhanced when the solution is exchanged for one containing more ADP^{3-} than ATP^{4-} (solution 1), resulting in an increase in the patch current from 0.31 to 2.4% of the maximal value. The number of channel open events was subsequently lowered when the ATP^{4-}/ADP^{3-} ratio of 2.5 was re-established, evoking a reduction in the patch current from 2.4 to 0.5% of the maximal value.

The degree of K^+ channel activation in Fig. 4(*B*) is greater than that observed for Fig. 4(*A*), even though ATP^{4-}/ADP^{3-} is lower in record *A*. The reason for this difference is that the individual records come from separate open-cell preparations in which not only is the initial number of K^+ channels different, but also the relative rates of channel run-down.

Figure 5 shows a somewhat more detailed experiment, where the influence of changes in the ratio of ATP^{4-}/ADP^{3-} were studied using solutions

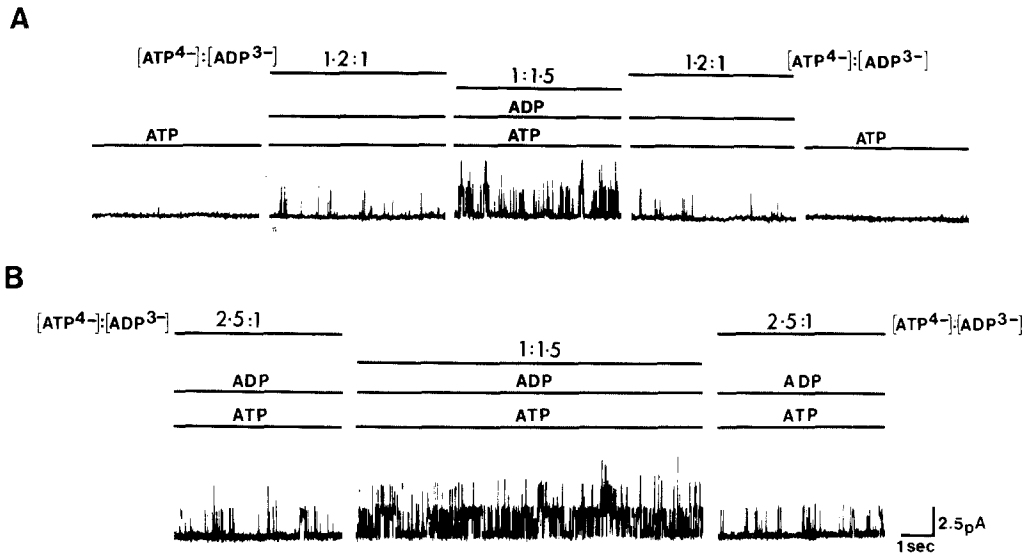

Fig. 4. K^+ channel activation is dependent upon the ratio of ATP^{4-}/ADP^{3-}. Panel *A* shows how K^+ channel gating is influenced by changes in the ratio of ATP^{4-}/ADP^{3-} from 1.2 : 1 (1.2) (solution 3) to 1 : 1.5 (0.7) (solution 1) and back to 1.2 : 1, and Panel *B* shows how changes in the ratio from 2.5 : 1 (2.5) (solution 5) to 1 : 1.5 (solution 1) and back to 2.5 : 1 affect K^+ channels. Records *A* and *B* come from two separate open-cells. Record *A* begins 420 sec after permeabilization of the cell, which evoked an initial patch current of 20 pA (this record comes from the same cell as that shown in Fig. 3). Record *B* begins 320 sec after permeabilization and had an initial patch current of 27 pA

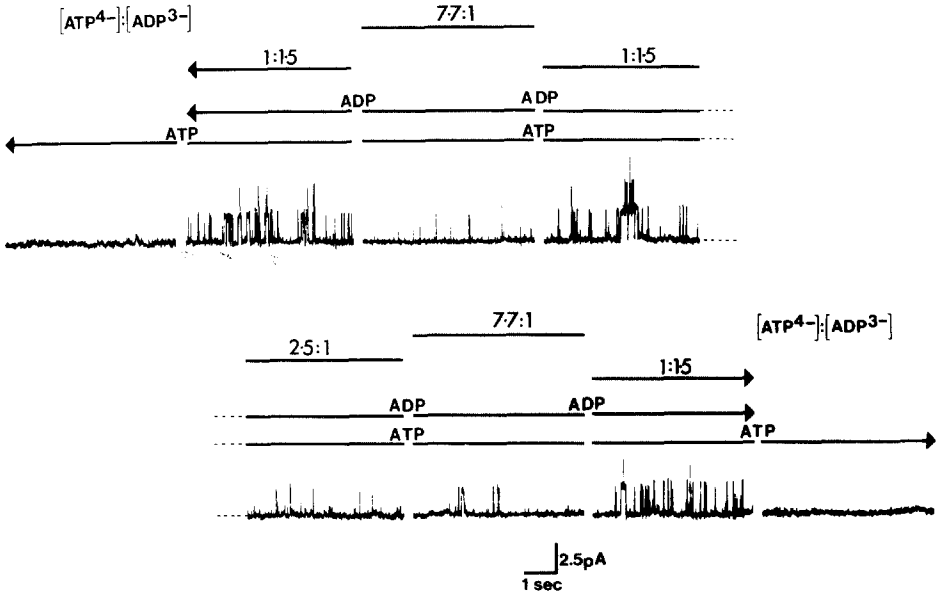

Fig. 5. The effects of 1 : 1.5 (0.7) (solution 1), 2.5 : 1 (2.5) (solution 5) and a 7.7 : 1 (7.7) (solution 7) ratio of ATP^{4-}/ADP^{3-} on the gating of K^+ channels. The record shown is taken from the same patch as that of Figs. 3 and 4(*A*), beginning 670 sec after permeabilization of the cell

that contained 0.7 (solution 1), 2.5 (solution 5) and 7.7 (solution 7) ratios of the free ions.

Figure 6 shows graphically the relationship between the ATP^{4-}/ADP^{3-}, ATP/ADP and the K^+_{ATP} current values analyzed in 58 separate RINm5F open-cell membrane patches. Included in this Figure is the average patch current recorded in the absence of any nucleotides, i.e. the prestimulus level of activity, and the average K^+_{ATP} current recorded in the presence of ATP alone.

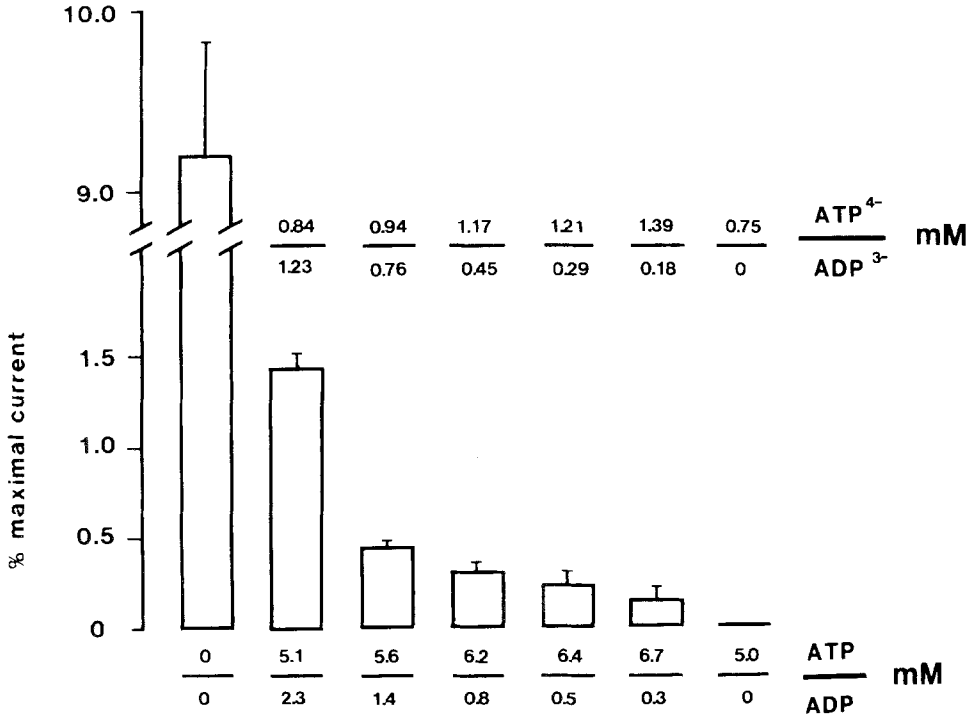

Fig. 6. Relationship between ATP^{4-}/ADP^{3-}, ATP/ADP and the K^+ATP current recorded in RINm5F open-cell membrane patches. The K^+_{ATP} channel current has been expressed as a percentage of the theoretically maximal current; (i) in the total absence of nucleotides, i.e., the prestimulus level of activity (n = 8), (ii) in the presence of a variety of ATP^{4-}/ADP^{3-} test solutions as indicated: 0.7 (n = 7 separate open cells), 1.2 (n = 5), 2.5 (n = 6), 4.2 (n = 8), 7.7 (n = 4), and (iii) in the presence of ATP alone (n = 19). Mean ± SEM values are shown

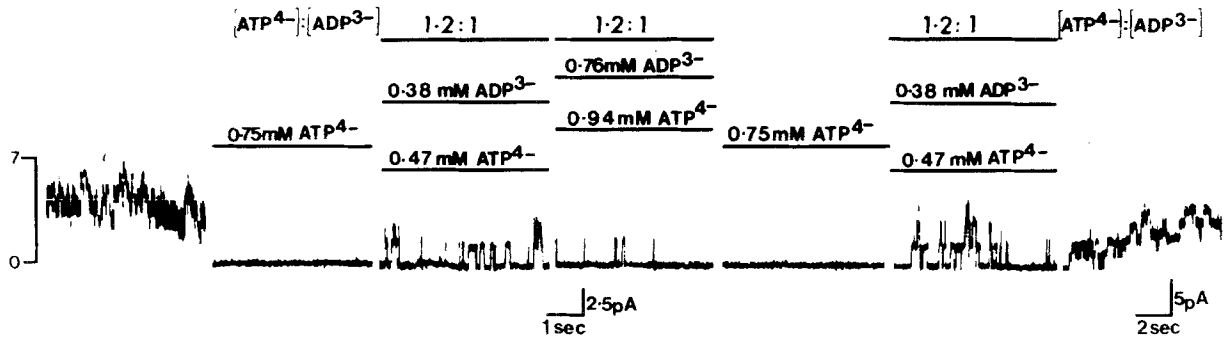

Fig. 7. K^+ channel gating is dependent upon changes in the concentrations of ATP^{4-} and ADP^{3-} at a constant 1.2 : 1 ratio of the free ions ATP^{4-}/ADP^{3-} (solutions 2 and 3). The scale bar on the left-hand side of the current trace corresponds to the number of coincident K^+ channel openings. Note that in the continual presence of ATP removal of ADP results in a clear total inhibition of K^+ channels. The vertical scale has been magnified during the test period. The current trace begins 10 sec after permeabilization and the initial patch current was 22 pA

Effects of Changes in the Concentrations of ATP^{4-} and ADP^{3-} on the Gating of K^+ Channels at a Constant ATP^{4-}/ADP^{3-}

Figure 7 shows the effects of changes in the concentrations of ATP^{4-} and ADP^{3-} while maintaining a constant 1.2 ratio of the free ions (solutions 2 and 3).

ATP completely inhibited the K^+_{ATP} current activated upon permeabilization of an intact RINm5F cell, lowering the patch current from 9.8% of the maximal value to 0. Adding a solution that contained a 1.2 ratio of ATP^{4-}/ADP^{3-} (solution 2) reactivated ATP-inhibited K^+ channels and increased the patch current from 0 to 3.1%. A subsequent increase in the concentrations of ATP and ADP,

Table 3. Effect of changes in the concentrations of both ATP^{4-} and ADP^{3-} at a constant ratio of ATP^{4-}/ADP^{3-}, on the K^+_{ATP} channel current recorded in RINm5F open cells[a]

			% Maximal current
(*A*) $n = 3$	"low"	1.2	0.94 ± 0.07
	"high"	1.2	0.41 ± 0.06
	"low"	1.2	0.96 ± 0.1
(*B*) $n = 3$	"low"	2.5	0.52 ± 0.02
	"high"	2.5	0.36 ± 0.01
	"low"	2.5	0.42 ± 0.02

[a] In (*A*) the ratio is maintained at 1.2 and in (*B*) at 2.5. All current values have been expressed as a percentage of the maximal current value (*see* Materials and Methods). The number of separate open-cell recordings has been indicated.

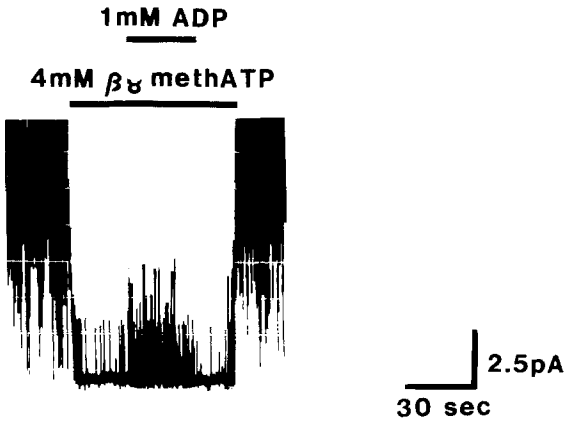

Fig. 8. Stimulation of K^+ channels by ADP in the presence of $\beta\gamma$ methylene-ATP. K^+ channels inhibited by 4 mM $\beta\gamma$methylene $\beta\gamma$ ATP are stimulated in the continued presence of the ATP analog by ADP. The record starts 250 sec after opening the cell and the initial patch current was 37 pA

while maintaining the ratio of the free ions (solution 3), lowered the patch current from 3.1 to 0.93% of the maximal control value.

When ADP was removed from the solution bathing the inside of the membrane, in the continued presence of ATP, complete inhibition of K^+ channels resulted, which was followed after reintroduction of ADP, in the form of the "low 1.2" solution (solution 2), by a clear increase in the patch current to 2.5% of the maximal value. Finally, removal of all nucleotides from the membrane inside resulted in a further increase in the patch current to 9.4% of the maximal value.

Quantitative analysis of these experiments is presented in Table 3*A*.

In a similar series of experiments increases in the concentrations of the free nucleotides, while maintaining a free concentration ratio of around 2.5 (solutions 4 and 5), decreased the frequency of channels openings. Whereas, decreasing the concentrations increased the activity of K^+ channels. The quantitative analysis of these experiments is presented in Table 3*B*.

Effects of ADP and ADP-β-S on ATP-, AMP-PNP- and βγMethylene ATP-Inhibited K^+ Channels

The results of a series of experiments, which show the effects of nonhydrolyzable analogs of both ATP and ADP on the gating of ADP-reactivated and ATP-inhibited K^+ channels, respectively, are presented in Figs. 8 and 9, and in Table 4.

Figure 8 shows the result of an experiment in which the inner membrane of a RINm5F open-cell is exposed to a 4 mM concentration of the ATP analog $\beta\gamma$methylene ATP, which results in a clear suppression, but not complete inhibition of K^+ channels, to 0.3% of the maximal patch current value. Adding ADP in the continued presence of $\beta\gamma$methylene ATP evoked a pronounced and sustained stimulation to 1.7% of the maximal current. The effect was reversible. Removing ADP lowered the patch current to 0.2% of the maximal value, which was increased to 7% upon removal of all nucleotides from the bathing solution.

Both this and similar experiments indicate that ADP is able to activate K^+ channels in the presence of a nonhydrolyzable analogs of ATP.

In Fig. 9(*A*) complete channel closure evoked by 4 mM ATP is typically followed by reactivation of channels when 1 mM ADP is added, increasing the K^+_{ATP} patch current from 0 to 0.13% of the maximal value. The reactivation of K^+ channels is further enhanced when ATP is removed and 4 mM AMP-PNP added, in the continued presence of ADP, increasing the patch current to 1% of the maximal value. The effects are reversible. Removing AMP-PNP and adding back ATP reduced the patch current to 0.2% of the maximal value, which is followed by complete inhibition of K^+ channels when ADP is finally removed from the inner membrane.

Figure 9(*B*) shows that K^+ channels closed by ATP, are activated by ADP in the presence of ATP, resulting in an increase in the patch current to 0.18% of the maximal control level of activity. This is further increased to 4.9% when ATP is removed and replaced by $\beta\gamma$methylene ATP. Returning the cell to the 4 mM ATP/1 mM ADP solution, decreased the K^+_{ATP} current to 0.25% of the maximum.

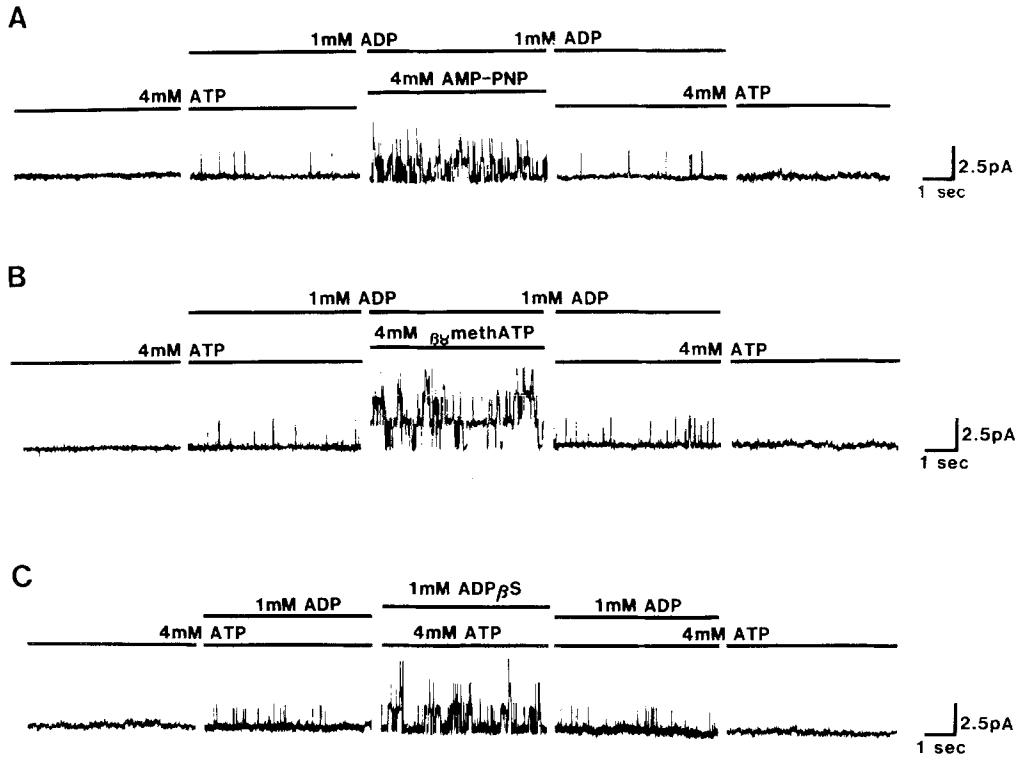

Fig. 9. Effects of ADP and ADP-β-S on ATP-, AMP-PNP and βγmethylene ATP-inhibited K^+ channels. Records *A*, *B* and *C* come from three separate membrane patches beginning 10, 310 and 15 sec, respectively, after permeabilization of the cell which yielded initial patch currents of 27, 30 and 20 pA for *A*, *B* and *C*, respectively. Panel *A* shows that ADP-induced stimulation of ATP-inhibited K^+ channels is enhanced by removing ATP and adding AMP-PNP in the continual presence of ADP. Panel *B* shows a similar experiment where βγmethylene ATP is used as the nonhydrolyzable ATP analog. Panel *C* shows that changing ADP-β-S for ADP, in the continual presence of ATP, enhances the ADP-evoked activation of K^+ channels

Finally, when the ADP is removed the membrane patch current was reduced to zero. Quantification of these results is presented in Table 4*A* and 4*B*, respectively.

Figure 9(*C*) shows that K^+ channels activated by ADP, increasing the patch current from 0 to 0.08% of the maximal value, are further stimulated when ADP is replaced with ADP-β-S, increasing the membrane patch current to 2.5%. Returning to the ATP/ADP solution lowered this value to 0.12%, until finally, removing ADP closed all the K^+ channels and decreased the K^+_{ATP} patch current to zero.

The quantitative analysis of three separate open-cells where this type of experiment was carried out is presented in Table 4*C*.

Discussion

The spectrum shown in Fig. 1, and the subsequent determinations of the intracellular concentrations of ATP and ADP and the ratio ATP/ADP represent the first applications of the ^{31}P-NMR technique to insulin-secreting cells.

The average values for the intracellular concentrations of ATP and ADP, 5.75 ± 1 mM and 2.03 ± 0.6 mM, respectively, are comparable to previous biochemical estimations of total ATP and ADP in insulin-secreting cells giving values of about 4 and 1.5 mM, respectively (Ashcroft et al., 1973; Malaisse et al., 1979, 1982; Kakei et al., 1986; Ashcroft, Ashcroft & Harrison, 1987). In the RINm5F cells, recent measurements with the firefly luciferase luminescence assay give ATP concentration values of about 4 mM (Wollheim et al., 1988), only slightly lower than the NMR values obtained in this study.

The average value of 2.8 ± 0.17 (n = 8) for the ratio ATP/ADP obtained in our NMR studies appears to be in good agreement with ATP/ADP ratios previously measured in unstimulated pancreatic B-cells, by a variety of biochemical techniques. Using the firefly lantern luciferase assay, values of 2.4 (Malaisse et al., 1979), 2.68 (Malaisse et al., 1982), 2.85 (Kakei et al., 1986) and 2.16 (Malaisse &

Table 4. Effects of AMP-PNP (*A*) and βγmethylene ATP (*B*), and ADP-β-S (*C*) on the K^+_{ATP} current recorded from ADP-reactivated and ATP-inhibited K^+ channels, respectively, in RINm5F open-cell recordings[a]

	% Maximal current
(*A*) $n = 4$	
ATP	0
ATP + ADP	0.24 ± 0.06
AMP-PNP + ADP	1.4 ± 0.3
ATP + ADP	0.17 ± 0.02
ATP	0
(*B*) $n = 4$	
ATP	$5 \times 10^{-5} \pm 1 \times 10^{-3}$
ATP + ADP	0.3 ± 0.05
βγmethATP + ADP	3.5 ± 0.07
ATP + ADP	0.26 ± 0.04
ATP	$1.3 \times 10^{-5} \pm 1 \times 10^{-3}$
(*C*) $n = 3$	
ATP	0
ATP + ADP	0.18 ± 0.01
ATP + ADP-β-S	1.5 ± 0.08
ATP + ADP	0.16 ± 0.07
ATP	0

[a] All current values have been expressed as a percentage of the maximal current value (*see* Materials and Methods). The number of separate open-cell recordings has been indicated. Concentrations of nucleotides as in Fig. 9.

Sener, 1987) have been obtained, and using HPLC values of 2.78 (Hoenig & Matschinsky, 1987) and 1.35 (Ribalet & Ciani, 1987) have been reported.

Quantification of metabolic concentrations using ^{31}P-NMR is based on the fact that the NMR signal is directly proportional to the total amount of mobile species to be detected. The fact that there is such a close correlation between the intracellular concentrations of ATP and ADP determined by NMR with the concentrations determined by biochemical techniques, implies that in the insulin-secreting cells NMR is detecting the full intracellular content of the nucleotides (cytosolic and mitochondria (concentrations), or alternatively, at least the same population of ATP and ADP as that detected by biochemical assays. This finding is similar to that observed in frog sartorii and perfused heart, where NMR is thought to detect within 20% of the total content of the metabolites (Gadian & Radda, 1981).

In a number of systems the ADP contents measured by NMR are somewhat discordant with the concentrations measured by other analytical techniques (Gadian & Radda, 1981). These discrepancies may be explained by the fact that a large proportion of ADP is tightly bound to proteins or myofilaments and is therefore, rendered too immobile to generate a detectable signal. This does not, however, appear to be the case in insulin-secreting cells where both ATP and ADP are relatively mobile entities.

The intracellular pH (pH_i) value obtained in this study is also in agreement with cited values of pH_i from mammalian pancreatic B-cells. Using the 5,5-dimethyloxazolidine-2,4-dione method values of 7.05 (Hellman, Sehlin & Täljedal, 1972), 7.1 (Malaisse et al., 1979; Hutton et al., 1980) and 7.2 (Sener et al., 1978) have been reported.

In the absence of D-glyceraldehyde ATP/ADP invariable decreased with time (Fig. 2). If, however, the cells were incubated with D-glyceraldehyde the ATP/ADP value was maintained for a substantially longer period of time, since D-glyceraldehyde metabolism generates ATP. Ashcroft et al. (1973) have previously shown that glucose increases the ATP content of mouse pancreatic islets.

Two recent single-channel current-recording studies (Ashcroft & Kakei, 1987; Dunne et al., 1987) have suggested that the inhibitory effects of ATP on K^+ channels does not require the presence of Mg^{2+}, but rather depends on the concentration of the free ATP^{4-} ion. We have therefore studied the influence that interactions between ATP^{4-} and ADP^{3-} have on the gating of K^+ channels. This has been carried out using a number of different test solutions of ATP^{4-}/ADP^{3-}, ranging from 1 : 1.5 to 7.7 : 1. The total concentration ratios of the added nucleotides, ATP/ADP, in each of the test solutions ranged from 2.2 to 22.5 : 1.

In the continued presence of ATP^{4-}, ADP^{3-} invariably led to the stimulation of K^+ channels. The interactions between the two free ions appear to be competitive (*see* Figs. 3–6). Whenever the ratio of ATP^{4-}/ADP^{3-} is increased a reduction in the K^+_{ATP} channel current results. Conversely when the ratio was decreased, a corresponding increase in this value was invariably seen. When the ratio of ATP^{4-}/ADP^{3-} was held constant, increasing the concentrations of both the free ions decreased the number of channel opening events, with corresponding decreases in the concentrations evoking increases in channel activity (*see* Fig. 7 and Table 3).

Consistent with the finding that ATP^{4-} and ADP^{3-} compete for control of channel gating is the finding that ADP-induced stimulation of K^+ channels is not dependent on protein phosphorylation (*see* Figs. 8 and 9), since both ADP and ADP-β-S were consistently found to stimulate K^+ channels in the presence of a number of nonhydrolyzable analogs of ATP or ATP itself, respectively. Surprisingly, the degree of channel activation evoked by ADP in the presence of either AMP-PNP or βγmethylene ATP, or indeed by ADP-β-S in the

presence of ATP, appeared to be greater than that evoked by ADP in the continued presence of ATP (*see* Table 4).

In the case of the K^+ channels stimulated by ADP in the presence of nonhydrolyzable analogs of ATP, the enhanced degree of channel activation might be thought to indicate that the ATP analogs do not evoke the same degree of channel inhibition as ATP. However, at the concentrations of ATP derivatives used—4 mM in all experiments—almost complete inhibition of channel activity would be expected (Fig. 8 and Ohno-Shosaku, Zünkler & Trube, 1987). An enhanced stimulation of K^+ channels could nevertheless be explained if the ATP analogs bind to the ATP-inhibitory binding site on the K^+ channel with a lower affinity for the site than ATP. This would make it easier for ADP to displace the molecule, and result in a greater degree of K^+ channel activation.

The finding that 1 mM ADP is able to stimulate K^+ channels in the presence of a 4 mM concentration of ATP, is interesting, as in the absence of ATP a 1 mM concentration of ADP evokes inhibition of K^+ channels (Dunne & Petersen, 1986*a*; Kakei et al., 1986; Dunne et al., 1988). Even more surprising is the finding that 1 mM ADP-β-S is able to evoke any kind of stimulation of K^+ channels, especially in the presence of ATP, as in ATP-free solutions ADP-β-S always resulted in complete closure of K^+ channels at concentrations of 100 μM and above (Dunne & Petersen, 1986*a*; Findlay, 1987). One possible explanation for this finding could be that ADP-β-S is able to compete more readily for the ATP-inhibitory binding-site than ADP. As neither ADP nor ADP-β-S are as potent inhibitors of K^+ channels as ATP, displacement of ATP would lead to relief of K^+ channel inhibition. If ADP-β-S is able to compete more readily than ADP, an enhanced stimulation would be seen when ADP is replaced by ADP-β-S.

The findings presented in this study, that channel activation is not dependent on phosphorylation (Figs. 8 and 9) and that ATP and ADP compete for the gating of the K^+_{ATP} channels is in agreement with the conclusions of Ohno-Shosaku et al. (1987) and Findlay (1987), both of whom demonstrate that stimulatory effects of nucleotides on the gating of ATP-sensitive K^+ channels in insulin-secreting cells do not depend on phosphorylation of the K^+ channel. Although the overall effects of how changes in ATP^{4-}/ADP^{3-} and nonhydrolyzable analog-ATP/ADP interactions influence the gating of the K^+_{ATP} channels appear to be of a competitive nature, we cannot dismiss from these experiments the possibility that (i) the ATP-sensitivity of the channels is modified allosterically by ADP and (ii) that interactions with ATP^{3-}, $MgATP^{2-}$ and ADP^{2-} and $MgADP^-$ are also important.

An interesting effect of ADP on ATP-inhibited K^+ channels is demonstrated in Fig. 3, and is worthy of comment. In the continued presence of ATP a second application of ADP results in a greater degree of K^+ channel stimulation than that observed after the first application of ADP. A similar effect was also noted in a study with the oral hyperglycemic sulfonamide diazoxide, a potent activator of K^+ channels in the presence of ATP (Dunne et al., 1987). Repeated applications of the drug in the continued presence of ATP (Fig. 3 from Dunne et al., 1987) was also found to enhance the degree of channel activation seen. Two explanations are possible. These effects may be due to a build-up in concentration of either ADP or diazoxide at the stimulatory site, through the repeated administration of the drugs. Alternatively, as long-term exposure of K^+ channels to ATP results in both a maintenance of channel integrity and a refreshment of run-down channels (Findlay & Dunne, 1986), the longer ATP is present at the inner membrane the greater will be the number of available K^+ channels.

It has been proposed that the carbohydrate-evoked closure of K^+ channels in insulin-secreting cells could be mediated by an increase in the ATP and a decrease in the ADP concentrations (Dunne & Petersen, 1986*a*; Kakei et al., 1986). The results presented here give information about the changes in internal ATP and ADP concentrations that are needed in order to obtain measurable changes in K^+ channel open-state probability. From the values for ATP and ADP concentrations obtained in our NMR experiments (5.8 and 2.0 mM, respectively) it would appear that the first solution listed in Table 1 is relatively close to being physiological, as the total ATP concentration is 5.1 mM and the ADP concentration 2.3 mM. In Fig. 3 it can be seen that channel openings do occur when this solution is used, although the K^+_{ATP} channel current is very much lower than in the absence of any nucleotides. This is in agreement with our previous findings in RINm5F cells where K^+ channel openings are certainly tonically inhibited by the ATP present intracellularly and where marked channel activation occurs when the cell membrane is permeabilized (outside the isolated patch area from which recording is made) and the intracellular nucleotides washed away (Dunne et al., 1986). Since, as shown in Fig. 3, there were no channel openings when ATP was present alone, it is clear that ADP exerts a physiologically important role in allowing a certain degree of K^+ channel opening and thereby preventing membrane depolarization in the resting unstimulated cell. An increase in the internal ATP concentration from 5.1 to 5.6

coupled with a decrease in the ADP concentration from 2.3 to 1.4 mM (Table 1 and Fig. 4) evokes a clear decrease in the K^+_{ATP} channel current. The observation that such relatively modest changes in ATP and ADP concentrations can produce clear changes in K^+ channel gating (*see* Fig. 6) is compatible with the hypothesis that carbohydrate-evoked depolarization could be explained by K^+ channel closure mediated by changes in intracellular nucleotide concentrations. The exact relationship between changes in total and free nucleotide concentrations obviously depends on the exact value of the intracellular Mg^{2+} concentration which is unknown in these cells and which might very well change during the transition from rest to secretion (Henquin et al., 1983). The effect of changes in plasma glucose concentration, within a range relevant for physiological control of insulin secretion on free ATP^{4-} and ADP^{3-} concentrations in the cytosol is unknown and it is, therefore, at present not possible to state with confidence that carbohydrate-evoked K^+ channel closure is in fact mediated by changes in ATP^{4-} and ADP^{3-} concentrations, but this does remain a possible hypothesis. What is clear from the data presented here is that relatively small fluctuations in ATP and ADP concentrations can have very considerable influence on K^+ channel gating.

With regard to the question of stimulus-secretion coupling it should be borne in mind that there is at least one other potential powerful regulator of K^+ channel gating, namely diacylglycerol-mediated phosphorylation. In the RINm5F cells it has been shown that glyceraldehyde stimulation increases the diacylglycerol concentration by *de novo* synthesis and an active phorbol ester and a cell-permeable diacylglycerol analog mimic the effect of glyceraldehyde by evoking K^+ channel closure (Dunne & Petersen, 1987; Wollheim et al., 1988).

A quantitative evaluation of the effects of ATP and ADP on K^+ channels is also complicated by the fact that several other nucleotides have important influences on K^+ channel gating in insulin-secreting cells. From previous studies of the RINm5F cells it is known that GTP, GDP as well as NAD, NADH, NADP and NADPH can have various effects depending on the exact concentrations used (Dunne & Petersen, 1986*b*; Dunne et al., 1988).

The control of the ATP-sensitive K^+ channels in insulin-secreting cells is clearly very complex. In the absence of precise knowledge of the cytosolic levels of the many relevant free nucleotide concentrations at the critical site near the inner surface of the plasma membrane in the period following stimulation of secretion it is not possible to make definitive statements about which of the possible intracellular coupling mechanisms are of the greatest physiological importance.

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