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Two Sites for Adenine-Nucleotide Regulation of ATP-Sensitive Potassium Channels in Mouse Pancreatic β-cells and HIT Cells

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Summary. ATP-inhibited potassium channels (K(ATP)) were studied in excised, inside-out patches from cultured adult mouse pancreatic β-cells and HIT cells. In the absence of ATP, ADP opened K(ATP) channels at concentrations as low as 10 μM and as high as 500 μM, with maximal activation between 10 and 100 μM ADP in mouse β-cell membrane patches. At concentrations greater than 500 μM, ADP inhibited K(ATP) channels while 10 mM virtually abolished channel activity. HIT cell channels had a similar biphasic response to ADP except that more than 1 mM ADP was required for inhibition. The channel opening effect of ADP required magnesium while channel inhibition did not. Using creatine/creatine phosphate solutions with creatine phosphokinase to fix ATP and ADP concentrations, we found substantially different K(ATP)-channel activity with solutions having the same ATP/ADP ratio but different absolute total nucleotide levels.

To account for ATP-ADP competition, we propose a new model of channel-nucleotide interactions with two kinds of ADP binding sites regulating the channel. One site specifically binds MgADP and increases channel opening. The other, the previously described ATP site, binds either ATP or ADP and decreases channel opening. This model very closely fits the ADP concentration-response curve and, when incorporated into a model of β-cell membrane potential, increasing ADP in the 10 and 100 μM range is predicted to compete very effectively with millimolar levels of ATP to hyperpolarize β-cells.

The results suggest that (i) K(ATP)-channel activity is not well predicted by the "ATP/ADP ratio," and (ii) ADP is a plausible regulator of K(ATP) channels even if its free cytoplasmic concentration is in the 10–100 μM range as suggested by biochemical studies.

Key Words potassium channels · pancreatic β-cells · ATP · ADP · ATP/ADP ratio

Introduction

During the past two decades researchers have sought the link between membrane electrical activity and fuel metabolism in the pancreatic β-cell (Dean & Matthews, 1970; Meissner & Schmelz, 1974). Various hypotheses have evolved to explain this relationship. A currently favored concept is that glucose stimulation increases the "ATP/ADP ratio" in the β-cell cytoplasm and this inhibits ATP-sensitive potassium (K(ATP))-channel activity (Dunne & Petersen, 1986; Misler et al., 1986; Ashcroft & Rorsman, 1990). Our studies have focused, therefore, on the receptor mechanism by which ADP operates on these channels.

When pancreatic β-cells are exposed to increasing concentrations of glucose, the activity of K(ATP) channels decreases (Ashcroft, Harrison & Ashcroft, 1984; Rorsman & Trube, 1985). This results in the depolarization of the membrane potential, calcium-dependent bursting electrical activity (Cook, Satin & Hopkins, 1991) and insulin secretion (Dean & Matthews, 1970; Meissner & Schmelz, 1974; Ribalet & Beigelman, 1980; Wollheim & Sharp, 1981; Wollheim & Pozzan, 1984). Although glucose-dependent changes in ATP levels may mediate the effects of glucose, it is a concern that there is a large discrepancy between the sensitivity of K(ATP)-channel activity to ATP (half-maximal inhibitory concentration ≈ 12 μM) as measured in cell-free membrane patches (Cook & Hales, 1984; Rorsman & Trube, 1985) and intracellular ATP concentrations (>2 mM) (Ashcroft, Weerasinghe & Randle, 1973; Malaisse et al., 1979). While some of this apparent dose discrepancy may be explained by the existence of a large excess of K(ATP) channels (the "spare-channel" model; Cook et al., 1988) or the existence of ATP gradients in cells (Nichols & Lederer, 1990), it is also possible that other factors may regulate K(ATP) channels.

Evidence has accumulated that ADP modifies the sensitivity of K(ATP) channels to ATP, such that the concentration-inhibition curve for ATP is shifted to the right (Dunne & Petersen, 1986; Kakei

et al., 1986; Misler et al., 1986). This has led to the suggestion that the "ATP/ADP ratio," and not ATP alone, largely determines K(ATP)-channel activity (Dunne & Petersen, 1986; Misler et al., 1986; Dunne et al., 1988).

The mechanism for the apparent competition between ATP and ADP is not clear. According to one hypothesis (Misler et al., 1986; Dunne et al., 1988), ADP competes with ATP for the ATP binding site and thus blocks the channel response to ATP. This competition has been shown, however, only for levels of ADP greater than 0.3 mM (Kakei et al., 1986; Misler et al., 1986; Findlay, 1987; Dunne et al., 1988) while measurements in pancreatic β-cells (Ghosh et al., 1991; Ohta et al., 1991) and hepatocytes (Akerboom et al., 1978; Veech et al., 1979; Jacobus, Moreadith & Vandegaer, 1982; Koretsky et al., 1990) indicate that most ADP is protein bound and free levels of ADP are below 100 μM.

To determine how such low levels of ADP might affect K(ATP) channels, we used excised, inside-out patches from mouse pancreatic β-cells and HIT cells to determine concentration effects of ADP in the absence and presence of ATP. We present evidence that ADP can stimulate K(ATP)-channel activity in the absence of ATP at concentrations as low as 10 μM with maximal excitatory effects observed for concentrations of ADP between 10 and 100 μM. We also explicitly determine to what extent the "ATP/ADP ratio" predicts K(ATP)-channel activity over the presumed physiologic range of ATP and ADP concentrations. We found that substantially different levels of channel activity can be observed in solutions with the same ATP/ADP ratio but different absolute concentrations of ATP and ADP.

A mathematical model incorporating two ADP binding sites successfully predicted several features of our and other's data. With this model we predict that ADP concentrations in the range of 10 to 100 μM can have potent ATP-competitive effects on the β-cell membrane potential. Taken together, the results suggest that ADP may be capable of regulating K(ATP)-channel activity even if the cytoplasmic free ADP concentration is below 100 μM. A preliminary account of this work has appeared (Hopkins & Cook, 1991).

Materials and Methods

Cell Culturing

Islets were isolated from the pancreases of adult female Swiss-Webster mice using collagenase digestion. Single islet cells were mechanically dispersed from islets in a zero calcium buffer and plated on polylysine-coated glass coverslips. In some preparations, trypsin was used to facilitate cell dispersion. The cells were maintained for up to seven days in RPMI 1640 culture medium supplemented with fetal bovine serum (10%), L-glutamine (1%), penicillin/streptomycin (1%), and 11.1 mM glucose.

HIT cells (Santerre et al., 1981) were obtained from Dr. Robert Santerre of Eli Lilly at passage 47 and maintained in Ham's F12 medium supplemented with fetal bovine serum (15%) and horse serum (2.5%). Cells used were typically from passages 58–80.

Patch-Clamp Techniques

Single cells were observed using an inverted microscope, and following formation of a gigaohm seal, patches of membrane were excised in the inside-out configuration (Hamill et al., 1981) at room temperature (20–22°C). Single-channel currents were recorded with a Dagan 8900 patch-clamp amplifier (Dagan, Minneapolis, MN) using fire-polished, Sylgard®-coated soda glass pipettes (3–7 MΩ). The data were stored on videocasette tape using a digital data recorder (Neurocorder DR-866; Neuro Data, NY), and segments of data were filtered at 0.3 kHz by an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) and digitized at 1 kHz by a microcomputer data-acquisition system (MacADIOS II, GW Instruments, Cambridge, MA).

The pipette potential for all experiments was +40 mV, which with symmetrical potassium solutions on each side of the membrane resulted in inward current flow across the membrane (current flowing out of the pipette). Channel activity is depicted as downward deflections by convention. K(ATP) channels were identified on the basis of their virtually complete inhibition by 2 mM ATP and their single-channel current amplitude (about 2.5 pA under these recording conditions; Cook & Hales, 1984). For each 10–30 sec segment of channel data, amplitude histograms were constructed from which average channel currents were calculated by integrating the current and dividing by the segment's duration. Average channel activity was calculated by dividing average channel current by the single-channel current amplitude (Misler et al., 1986). For patches with one active channel (no multiple openings observed during the experiment), the channel open probability was obtained directly by this method.

Switching of the various solutions bathing the patch was accomplished with a "sewer pipe" apparatus suspended in the bath (Yellen, 1982). The effects of tested compounds were determined by normalizing the average K(ATP)-channel activity (or open probability) in the presence of the compound(s) to the mean of the control (before application) and wash (following washout) episode average channel activity. The normalized values are referred to as fractional activity. Data were analyzed statistically where appropriate with $P < 0.05$ chosen as the level of significance. Data values are always given as mean ± SEM.

Solutions

The pipette solution for all experiments contained (in mM): 140 KCl, 10 HEPES, 2 $MgCl_2$, and 1 $CaCl_2$, pH adjusted to 7.2 with KOH. The control solution bathing the cytoplasmic face of the membrane for all experiments contained (in mM): 140 KCl, 10 HEPES, 1 $MgCl_2$, and 1 EGTA, pH adjusted to 7.2 with KOH. ADP test solutions included ADP as the sodium salt. An ATP test solution (2 mM, as the magnesium salt) was also applied to the patch periodically to establish channel identity and to decrease the rate of K(ATP)-channel run-down (Findlay & Dunne, 1986;

Table 1. Concentrations (in mM) of the constituents of seven solutions with high, medium and low ATP/ADP ratios and different total nucleotide concentrations

"ATP/ADP"	Total added compound			Calculated		
	ATP	Creatine	Creatine-PO_4	ATP	ADP	ATP/ADP
High	2	5.178	6.822	1.98	0.009	220
	4	"	"	3.98	0.018	220
Medium	1	10.002	1.998	0.97	0.029	33
	2	"	"	1.94	0.058	33
	4	"	"	3.88	0.116	33
Low	2	11.517	0.483	1.75	0.250	7
	4	"	"	3.50	0.500	7

Ohno-Shosaku, Zünkler & Trube, 1987). In experiments to test ADP effects on K(ATP)-channel activity in the nominal absence of magnesium, magnesium was omitted from the bath solution. Free ADP concentrations were calculated for each experimental solution using the program ALEX 3.3 based on the work of Fabiato (1988) and written by Dr. Michel Vivaudou, Laboratoire de Biophysique Moleculaire et Cellulaire, CENG/DBMS/BMC, Grenoble, France.

To obtain a high, medium and low fixed ATP/ADP ratio with two (or three) total adenine-nucleotide concentrations, we used seven solutions containing ATP (as the sodium salt), creatine (*C*), creatine phosphate (*CP*) and creatine phosphokinase (Table 1). The creatine phosphokinase (Boehringer Mannheim, from rabbit muscle) concentration was 18 U/ml. We assumed equilibrium for the ATP + $C \rightleftarrows$ ADP + *CP* reaction and calculated free levels of ATP and ADP using

$$ADP/ATP = C/CP \cdot K_{eq} \quad (1)$$

where $K_{eq} = 0.00596 = 1/168$ (Corkey et al., 1988).

Results

Physiological Concentrations of ADP Activate K(ATP) Channels

Previous studies have shown that ADP, at concentrations of 100 μM or greater, can stimulate K(ATP) channels in the absence of ATP and effectively compete with ATP for control of K(ATP)-channel gating (Dunne & Petersen, 1986; Findlay, 1987; Bokvist et al., 1991). Figure 1 shows the effect of 50 μM ADP in the absence of ATP on K(ATP)-channel activity in two different patches in mouse β-cells. In the first patch (Fig. 1*A*), ADP reversibly increased K(ATP)-channel activity. Below are shown amplitude histograms for the same patch in control solution (no ADP) and in the presence of 50 μM ADP. The probability of channel opening was greatly augmented in the presence of ADP, such that the size and number

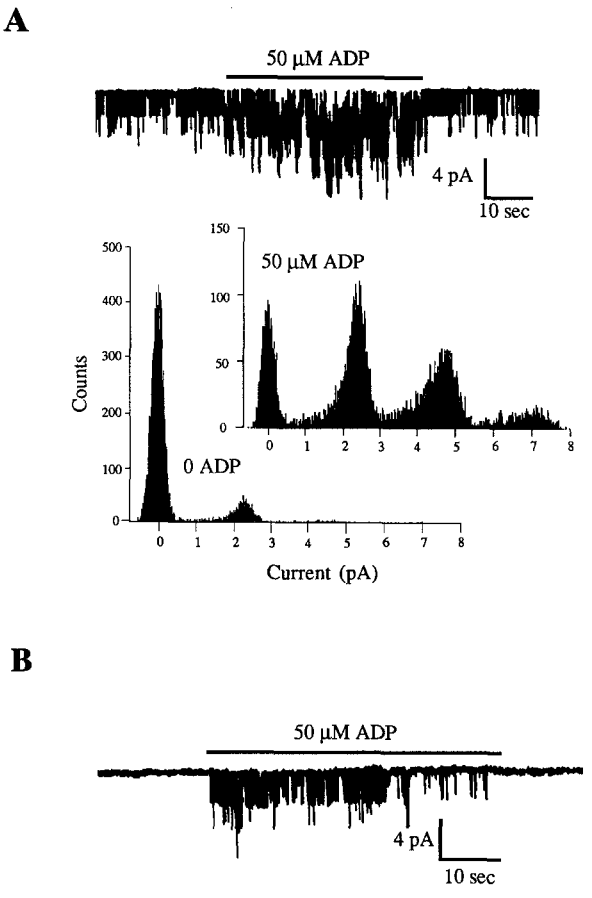

Fig. 1. (*A*) Data recorded from an inside-out patch from mouse pancreatic β-cell in the presence of 1 mM magnesium and the absence of ATP. Application of 50 μM ADP resulted in a reversible increase in K(ATP)-channel activity. Below are amplitude histograms for the patch shown above in the absence of ADP (left) and in the presence of 50 μM ADP (right). The peak at 0 pA in both histograms corresponds to the recording noise level. (*B*) Data from another patch demonstrating the ability of 50 μM ADP to stimulate K(ATP)-channel activity under conditions where the channel opening probability is apparently zero in control solution (no nucleotides present).

of peaks corresponding to open K(ATP) channels was increased. In a second patch (Fig. 1*B*), again in the absence of ATP, ADP elicited K(ATP)-channel activity in an otherwise silent record.

Figure 2 shows concentration-response data for ADP on K(ATP)-channel activity for mouse β-cells. The curve fit to the data is derived from the two-site binding model presented below. The concentrations referred to in the text correspond to total ADP, and the calculated free levels of ADP are also given in the figure. In patches from mouse β-cells, ADP activated K(ATP) channels at concentrations as low as 10 μM and activation gave way to net inhibition of channel activity at ADP levels beyond 500 μM. The peak of the excitatory effect was between 10 and 100

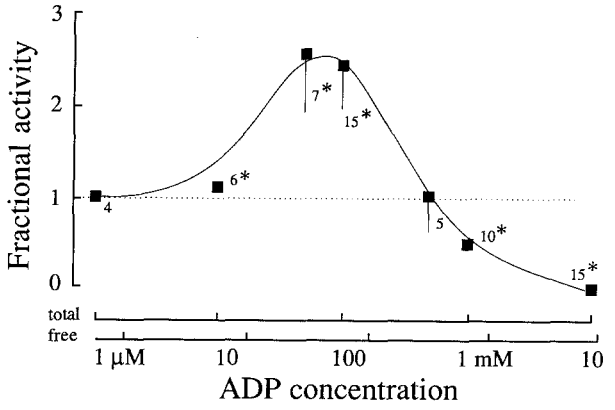

Fig. 2. Concentration-response curves for the effect of ADP on K(ATP)-channel activity in inside-out patches from mouse pancreatic β-cells. Fractional activity refers to the channel activity recorded in the presence of ADP normalized to the average of segments of control activity recorded before and after the application of ADP. A value of one would correspond to the same as control channel activity. The horizontal axes depict both the total and calculated free ADP concentration, and the numbers below the axes refer to the two closest tick marks on the total and free ADP axes. The numbers adjacent to the data points refer to the number of patches for a given ADP concentration. The error bars correspond to SEM and are smaller than the data symbols where they do not appear. The asterisks indicate that the adjacent data points are significantly different from the control level of activity (Wilcoxon matched-pairs signed-ranks test, $P < 0.05$). The total magnesium concentration was 1 mM for each ADP concentration.

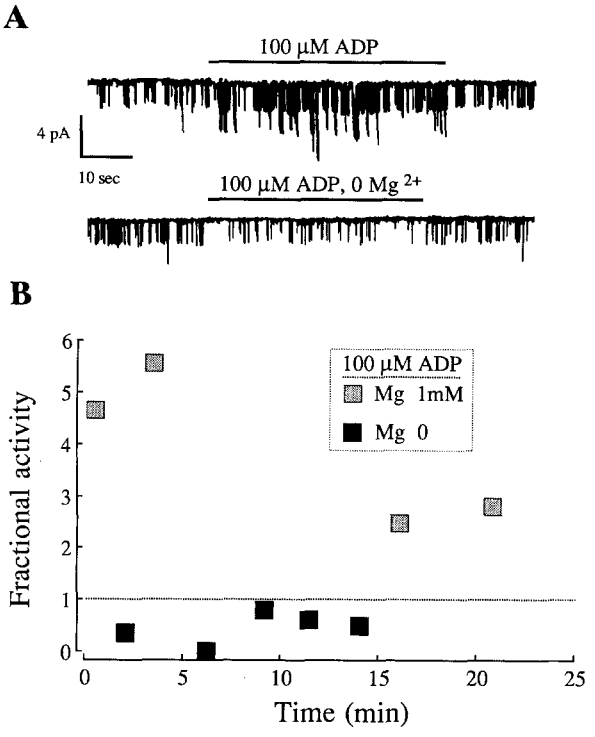

Fig. 3. ADP stimulation of K(ATP)-channel activity is magnesium dependent. (*A*) Data from a patch upon exposure to 100 μM ADP in the presence of 1 mM magnesium (top), and the same concentration of ADP in the nominal absence of magnesium (no added magnesium; bottom). The pipette solution contained 2 mM magnesium in each case. (*B*) Graph summarizing the results from an entire experiment from another patch. The symbols correspond to the fractional activity to 100 μM ADP in the presence (stippled squares) or absence (filled squares) of 1mM magnesium. The dotted horizontal line represents the control level of channel activity (fractional activity = 1). All data obtained from patches from mouse β-cells.

μM. At 10 mM ADP, an average of 96% of K(ATP)-channel activity was reversibly inhibited.

The ADP analogue, ADP-β-S (100 μM; with 1 mM magnesium) reversibly inhibited channel activity by 89 ± 5% in four patches but was unable to activate channels.

In patches obtained from HIT cells, excitatory ADP effects were apparent at higher ADP concentrations (1 mM ADP in two of three patches) than observed with mouse β-cells. As with mouse β-cells, 10 mM ADP inhibited about 96% of K(ATP)-channel activity in HIT cell patches. The mean fractional activity values for 100 μM, 1 mM and 10 mM ADP were 4.8 ± 1.3 (n = 6), 1.8 ± 0.9 (n = 3) and 0.035 ± 0.035 (n = 2), respectively. No attempt was made to model the HIT cell data.

Magnesium Is Required for ADP Stimulation of K(ATP)-Channel Activity

A previous study has shown that excitatory effects of nucleotides on K(ATP)-channel activity in RINm5F cells are magnesium dependent (Findlay, 1987). To test this in mouse cells, we measured K(ATP)-channel activity in the presence of 100 μM ADP and 1 mM magnesium or nominally magnesium-free solution. Figure 3*A* (top) shows the excitatory effect of 100 μM ADP plus 1 mM magnesium on K(ATP)-channel activity in a patch. In contrast, Fig. 3*A* (bottom) shows that 100 μM ADP decreased activity in the absence of added magnesium in the same patch.

Figure 3*B* summarizes the results from another patch with a single K(ATP) channel. In the presence of 1 mM magnesium, 100 μM ADP increased the channel open probability (normalized to the control open probability) nearly fourfold (3.87 ± 0.74 in four trials). In the absence of added magnesium, 100 μM ADP inhibited K(ATP)-channel activity by about half (0.45 ± 0.13 in five trials). The apparent inhibitory effect of ADP without added magnesium was not due to channel run-down, since the excitatory effect was present after return to 1 mM magnesium. In three patches, the average fractional activity in

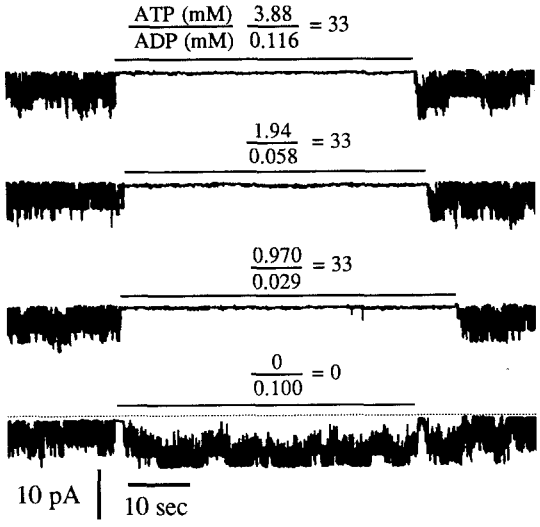

Fig. 4. Low levels of ADP did not effectively compete with ATP for control of channel gating. Creatine/creatine phosphate solutions were used to fix the ATP/ADP ratio at 33 for three solutions with ADP and ATP concentrations. K(ATP)-channel activity was substantially and reversibly blocked by each solution. The bottom trace demonstrates that 100 μM ADP was able, in the absence of ATP, to activate K(ATP) channels in the same patch. Data obtained from mouse β-cell patch.

the presence of 100 μM ADP with 1 mM magnesium was 3.78 ± 1.35 *versus* 0.29 ± 0.08 without magnesium. Magnesium, without ADP, at 0 to 1 mM does not alter channel activity, and its inhibitory effect at higher concentrations is in the wrong direction to account for the excitatory effect observed when 1 mM magnesium is present with 100 μM ADP (Ashcroft & Kakei, 1989).

Effect of ATP/ADP Ratio on K(ATP)-Channel Activity

To test the hypothesis that the ATP/ADP ratio primarily determines the level of K(ATP)-channel activity, creatine/creatine phosphate solutions were used to "clamp" ATP and ADP in their presumed physiologic range of concentrations (Table 1). Figure 4 shows that K(ATP)-channel activity was virtually abolished by 1, 2 and 4 mM total nucleotide solutions when the ATP/ADP ratio was 33 and the calculated ADP concentrations were 29, 58 and 116 μM, respectively. The bottom trace shows that ADP alone (100 μM) activated K(ATP) channels in the same patch, demonstrating that the channel block was not due to an inability of the channels to be activated by ADP. Table 2 summarizes the results from similar experiments in 11 patches. Measureable relief of K(ATP)-channel activity block by ATP was only observed for the low (=7) ATP/ADP ratio solutions, corresponding to ADP levels of 250 and 500 μM. The low ratio solution with 2 mM nucleotide yielded significantly more K(ATP)-channel activity than the solution containing 4 mM nucleotide with the same ATP/ADP ratio.

Table 2. Percentage of channel block (±SEM) due to three different ATP/ADP ratios at two (or three) total nucleotide concentrations[a]

ATP/ADP ratio	Total nucleotide concentration		
	1.0 mM	2.0 mM	4.0 mM
7	—	70.5 ± 3.3 (3)*	94.0 ± 0.7 (3)*
33	99.0 ± 1.0 (5)	99.8 ± 0.2 (5)	100.0 ± 0.0 (5)
220	—	100.0 ± 0.0 (3)	100.0 ± 0.0 (3)

[a] Channel block was calculated relative to control activity (i.e., no nucleotide) before and after application of the test solution. Asterisks mark values statistically different from 100% and from each other. The number of patches tested are in parentheses; data from a total of 11 patches.

A Model for ADP-ATP Competition for K(ATP)-Channel Gating

To account for our data, we propose a two-site model (*see* Fig. 5) as follows: (i) In the absence of nucleotides, the channel is open with a probability $P_o < 1.0$. (ii) ATP and ADP compete for binding to an "ATP site" which, when occupied, closes the channel (i.e., open probability $P = 0$). The inhibition constant for ATP acting at this site is fixed in the model at $K_{tt} = 12$ μM as previously measured (Cook & Hales, 1984). The inhibition constant for ADP at this site is K_{td}. (iii) Two MgADP bind to dual "MgADP sites" each with affinity = K_{dd}. When both sites are occupied by MgADP, the channel open probability increases from P_o to P_d. Dual, cooperative sites were required to reproduce the sharp upstroke of the channel activity *versus* ADP-concentration curve in the 10–100 μM range (Fig. 2). The curve represents a statistically adequate fit; other models, such as ones that incorporate higher orders of cooperativity for MgADP binding, may provide better fits. As discussed below, MgATP is thought not to activate channels at this site. (iv) For simplicity, we have assumed that MgADP binding to the MgADP site does not affect binding or action of ADP or ATP at the ATP site. This differs from a model proposed for cardiac (K(ATP) channels (Lederer & Nichols, 1989) where MgADP binding is proposed to reduce the affinity of the ATP site for its ligands.

To examine whether this model can account for the data, we derived equilibrium binding equations

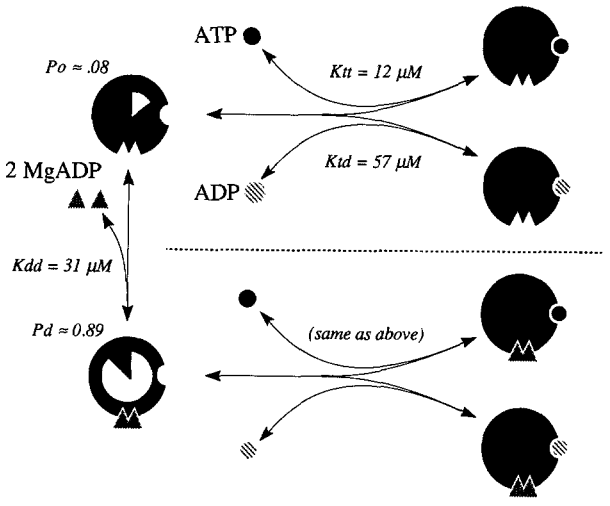

Fig. 5. Schematic representation of a binding model which may explain our observations on ATP-ADP competition for control of K(ATP)-channel gating in pancreatic β-cells. It is proposed that ATP (black circle) and ADP (hatched circle) compete for binding at an "ATP site" which, when occupied, closes the channel (indicated by the closure of the central "hole" in the figure). In addition, we propose a second (pair) of "MgADP sites" which bind MgADP (gray triangle), but not MgATP, and thereby increase the channel open probability from P_o to P_d. The binding of ADP and ATP at the ATP site is not affected by occupancy of the MgADP site (as shown below the dotted line). Italicized model parameters are defined and evaluated as described in Results.

(*see* Appendix) and used a least-squares fit to the data of Fig. 2 to determine free model parameters as follows:

$$P_o = 0.08, P_d = 0.89, K_{td} = 57\ \mu\text{M}, K_{dd} = 31\ \mu\text{M}. \quad (2)$$

In addition to quantitatively fitting the data in Fig. 2, we use this model in the Discussion to account for previously published data and to explore the implications for β-cell physiology.

Discussion

We studied the possibility that ADP competes with ATP in the physiological regulation of K(ATP)-channel activity in pancreatic β-cells. Towards this, we have (i) explicitly tested the idea that K(ATP) channels are regulated by the concentration ratio of ATP to ADP, (ii) determined the effects of low concentrations of ADP on control of K(ATP)-channel gating in the absence of ATP, and (iii) proposed a simple two-site model to account for our results. A key rationale for our approach is the suggestion that free ADP levels in β-cells are in the 10–100 μM range (Ghosh et al., 1991; Ohta et al., 1991).

The ATP/ADP Ratio Hypothesis

It is commonly expressed that K(ATP)-channel activity is determined by the "ATP/ADP ratio." By explicitly testing this, we found that absolute levels of adenine nucleotides (e.g., ADP and ATP) determine K(ATP)-channel activity independently of the ATP/ADP ratio. Table 2 shows that at a "low" ratio (ATP/ADP = 7), K(ATP)-channel activity was significantly changed by changes of total nucleotide concentration. These data and those of others (Dunne et al., 1988; Albitz, Kammermeier & Nilius, 1990) suggest that understanding the control of K(ATP)-channel activity requires knowing more than the ATP/ADP ratio, *per se*.

What is the Mechanism for ATP-ADP Competition?

It has been suggested that ATP-ADP competition results solely from ATP-ADP competition for a single binding site (Misler et al., 1986; Dunne et al., 1988); yet this is only plausible, however, if ADP is a partial agonist which binds to the ATP site, excluding ATP, but only partially inhibits channel opening. This possibility is ruled out by our finding that 10 mM ADP inhibits K(ATP)-channel activity by 96 ± 2% in both mouse β-cells and HIT cells.

Other studies showing that 100–500 μM ADP, in the absence of ATP, activates K(ATP) channels from insulin-secreting cells (Dunne & Petersen, 1986; Findlay, 1987; Bokvist et al., 1991) have suggestd a separate ADP site. We have extended the concentration range for this effect to ADP levels as low as 10μM (Fig. 2), following suggestions that cytoplasmic free ADP levels may be in the 10–100 μM range (Ghosh et al., 1991; Ohta et al., 1991). Channel activity peaked between 10 and 100 μM ADP while greater than 500 μM ADP (1 mM in HIT cells) inhibited channel activity as previously described (Cook & Hales, 1984). Because calculated free ADP levels were never less than 60% of the total ADP values (≤0.22 log units in Fig.2), qualitative conclusions from the data and model apply as well to free ADP levels.

A separate ADP site for channel activation is suggested by several observations. First, ADP activation occurs in the absence of ATP; i.e., competition with ATP is not required. Second, ADP activation requires magnesium while ADP inhibition does not (as in RINm5F cells; Findlay, 1987). Further-

more, Bokvist et al. (1991) found that ADP activation diminished over time while the inhibitory effects did not. Such observations suggest a distinct MgADP site on the K(ATP) channel, or associated protein, that increases channel opening probability.

To test this idea, we developed a simple two-site model of K(ATP) binding of ADP which closely predicts (Fig. 2) the ADP concentration-response curve although other activation models may do as well. Furthermore, neither this model, nor several simple modifications, were able to simultaneously fit the data of Fig. 2 (ATP-free) and the ATP-competitive effects for high ADP levels (250 and 500 μM; Table 2). This suggests that there may be an additional mechanism of ATP-ADP competition for ADP levels of 250 μM or more.

Is MgADP-Dependent Activation the Same as MgATP-Dependent "Refreshment"?

The rapidly reversible effects of MgADP suggest a mechanism unrelated to slow (minutes) cycles of channel "refreshment" and "run-down" (possibly involving protein phosphorylation; Findlay & Dunne, 1986; Ohno-Shosaku et al., 1987). It could be, however, that when dephosphorylated (i.e., run-down) channels bind MgATP, they are slowly phosphorylated to the highly activated, MgADP-bound state corresponding to the lower-left state in Fig. 5. As long as the channel remains phosphorylated, MgADP binding and unbinding reversibly switches the channels between the high and low activity states (left side of Fig. 5). Dephosphorylation then sends the channels back to the original run-down state. A similar mechanism has been suggested for cardiac K(ATP) channels (Tung & Kurachi, 1991).

Can K(ATP)-Channel Activation by Low Levels of ADP Compete Effectively with ATP?

ADP levels $\geq$250 μM competed with physiological levels of ATP (to 4 mM); competition by 10–100 μM ADP was not apparent (Fig. 4; Table 2). While this could indicate that low ADP levels may not be physiologically important, two factors must be considered. First, by the spare-channel hypothesis (Cook et al., 1988), less than 1% of β-cell K(ATP) channels need to be active for regulating the β-cell's membrane potential under physiological conditions. Second, such low channel activity ($P_o \leq 0.01$) is very difficult to detect in excised patches bearing only a few channels due to the brevity and rarity of channel openings (e.g., Fig. 4). This was the case in the original studies of ATP block of K(ATP)-channel activity (Cook & Hales, 1984).

The Model Predicts that Low Levels of ADP *Can* Hyperpolarize β-Cells

Given that such low levels of channel activity may be difficult to detect, we took a theoretical approach to asking whether low levels of ADP could be important for physiological channel regulation. We incorporated the two-site K(ATP)-channel model and its fit of our data (Fig. 2) into the previous "spare-channel" model of β-cell membrane potential (Cook et al., 1988). The spare-channel model combines an equation for ATP-induced K(ATP) channel closure with a membrane potential equation which balances K(ATP)-channel current with depolarizing "leak" currents. Three results from the model are important.

First, as pointed out above, the model accurately predicts the ADP concentration-effect curve of Fig. 2. Second, using the same parameters, the model reproduces (Fig. 6*A*) the right shift of the ATP concentration-inhibition curve that originally suggested the ATP/ADP ratio idea (Kakei et al., 1986). Third, incorporating the channel model into the spare-channel model predicts (Fig. 6*B*) that increasing ADP levels from 10 to 100 μM would effectively reverse the depolarizing effect of increasing ATP from 2 to 4 mM. Beyond 100 μM, ADP had little membrane potential effect, until at high levels (approaching 10 mM), ADP began to have ATP-mimetic effects. The qualitative conclusions of the model were unaffected by variations of the free parameters G_K, G_{leak}, E_L or E_K (*see* Appendix).

Summary and Conclusion: Implications for ADP Regulation of K(ATP) Channels

Our data show that low levels of ADP (10–100 μM) can regulate K(ATP)-channel activity in the absence of ATP and that a well-constrained model suggests that low levels of ADP can compete with ATP for the regulation of the β-cell membrane potential. Why is this important? Recent biochemical measures in β-cells (Ghosh et al., 1991; Ohta et al., 1991) indicate that most of the total cellular ADP, in the millimolar range (Kakei et al., 1986), is bound to cell proteins and is not "seen" by K(ATP) channels. It remains to be determined whether β-cell ATP and ADP levels are actually affected (Ohta et al., 1991) or not (Ghosh et al., 1991) by glucose stimulation. Our results do not rule out other metabolites as modulators of

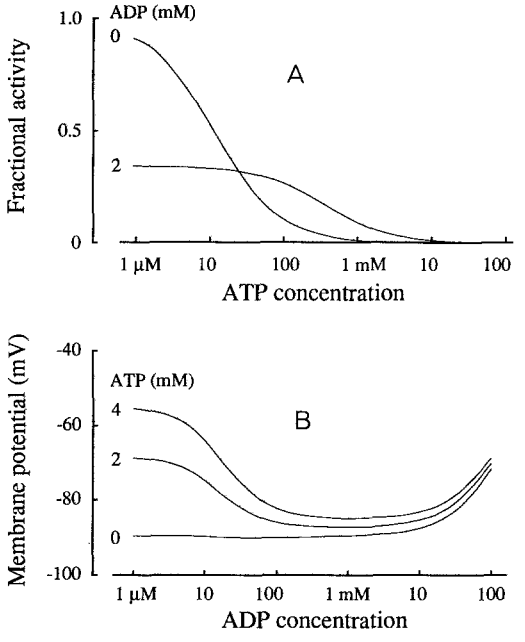

Fig. 6. Results of two-site K(ATP)-model calculations. (*A*) The model qualitatively reproduces the effects (Kakei et al., 1986) which originally suggested the ATP/ADP ratio idea. ADP (2 mM) inhibits K(ATP) activity and shifts the apparent inhibitory constant for ATP toward the millimolar range despite the fact that binding constants for ATP and ADP are both below 100 μM in the model. (*B*) As previously shown (Cook et al., 1988), in the absence of ADP, millimolar levels of ATP depolarize the β-cell through its operating membrane potential range (approximately −75 to −55 mV). Increasing the ADP level from 10 to 100 μM nearly completely reverses the ATP-induced depolarization. This effect saturates between 1 and 10 mM, and then the ATP-mimetic effect of ADP binding to the ATP site is apparent.

K(ATP) channels but do suggest a plausible mechanism for physiological regulation of K(ATP) channels by low levels of ADP.

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Appendix: Equations for the Two-Site Model in Fig. 5

Derive the Binding Equations for the Absence of ATP to Fit the Data of Fig. 2

First, calculate the fraction (F_{oo}) which are open because their ATP-site is not occupied by ADP:

$$F_{oo} = 1 - \mathrm{ADP}/(\mathrm{ADP} + K_{td}). \quad \text{(A1)}$$

Second, calculate the fraction (F_{od}) of these open channels which bind MgADP at the MgADP site. The fit to the data was considerably improved in the very steep region between 10 and 100 μM ADP by supposing that simultaneous binding of two MgADP are required for channel activation. This can be done by squaring the ADP/(ADP + K_{dd}) term in the equation for F_{od}:

$$F_{od} = F_{oo} \cdot [\mathrm{ADP}/(\mathrm{ADP} + K_{dd})]^2. \quad \text{(A2)}$$

Third, calculate the aggregate probability that the two populations of K(ATP) channels are open:

$$P = P_o \cdot (F_{oo} - F_{od}) + P_d \cdot F_{od}. \quad \text{(A3)}$$

Extend the Equations to Include ATP-ADP Binding Competition at the ATP site

First, account for ATP-ADP competition at the ATP site by modifying the binding constants:

$$K'_{tt} = K_{tt} \cdot (1 + \mathrm{ADP}/K_{td}) \quad \text{(A4)}$$

$$K'_{td} = K_{td} \cdot (1 + \mathrm{ATP}/K_{tt}). \quad \text{(A5)}$$

Then calculate the fraction of open channels (binding neither ATP nor ADP at the ATP site):

$$F_{oo} = 1 - \mathrm{ADP}/(\mathrm{ADP} + K'_{td}) - \mathrm{ATP}/(\mathrm{ATP} + K'_{tt}). \quad \text{(A6)}$$

Then, as above, get:

$$F_{od} = F_{oo} \cdot [\mathrm{ADP}/(\mathrm{ADP} + K_{dd})]^2 \quad \text{(A7)}$$

and finally:

$$P = P_o \cdot (F_{oo} - F_{od}) + P_d \cdot F_{od}. \quad \text{(A8)}$$

Use the Channel Opening Probability P in the Spare-Channel Model for Membrane Potential

Calculate the resting membrane potential resulting from outward potassium current through K(ATP) channels and inward current through a nonspecific leak pathway (Cook et al., 1988).

$$E_m = (GK \cdot EK + GL \cdot EL)/(GK + GL) \quad \text{(A9)}$$

where $GK = P \cdot G_{\mathrm{tot}} = P \cdot 100{,}000$ pS; GL = the leak conductance = 15 pS; EK = potassium reversal potential = −90 mV; and EL = the leak reversal potential = 0 mV.