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STIMULATION OF THE K_{ATP} CHANNEL BY ADP AND DIAZOXIDE REQUIRES NUCLEOTIDE HYDROLYSIS IN MOUSE PANCREATIC β-CELLS

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SUMMARY

1. The mechanisms by which ADP and the hyperglycaemic compound diazoxide stimulate the activity of the ATP-regulated K^+ channel (K_{ATP} channel) were studied using inside-out patches isolated from mouse pancreatic β-cells maintained in tissue culture.

2. The ability of diazoxide and ADP to increase K_{ATP} channel activity declined with time following patch excision and no stimulation was observed after 15–40 min.

3. Activation of K_{ATP} channels by ADP required the presence of intracellular Mg^{2+}. The stimulatory effect of ADP was mimicked by AMP but only in the presence of ATP. Replacement of ATP with the non-hydrolysable analogue β,γ-methylene ATP did not interfere with the ability of ADP to stimulate K_{ATP} channel activity. By contrast, enhancement of K_{ATP} channel activity was critically dependent on hydrolysable ADP and no stimulation was observed after substitution of α,β-methylene ADP for standard ADP.

4. The ability of diazoxide to enhance K_{ATP} channel activity was dependent on the presence of both internal Mg^{2+} and ATP. Diazoxide stimulation of K_{ATP} channel activity was not observed after substitution of β,γ-methylene ATP for ATP. However, in the presence of ADP, at a concentration which in itself had no stimulatory action (10 μM), diazoxide was stimulatory also in the presence of the stable ATP analogue.

5. The stimulatory action of diazoxide on K_{ATP} channel activity in the presence of ATP was markedly enhanced by intracellular ADP. This potentiating effect of ADP was not reproduced by the stable analogue α,β-methylene ADP and was conditional on the presence of intracellular Mg^{2+}. A similar enhancement of channel activity was also observed with AMP (0·1 mM). In the absence of ATP, diazoxide was still capable of stimulating channel activity provided ADP was present. This effect was not reproduced by AMP.

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6. In both nucleotide-free solution and in the presence of 0·1 mM ATP, the distribution of the K_{ATP} channel open times were described by a single exponential with a time constant of ≈ 20 ms. Addition of ADP or diazoxide resulted in the appearance of a second component with a time constant of > 100 ms which comprised 40–70% of the total number of events. Under the latter experimental conditions, the open probability of the channel increased more than fivefold relative to that observed in the presence of ATP alone.

7. We propose that ADP- and diazoxide-induced stimulation of K_{ATP} channel activity reflects the same basal mechanism and involves a diffusible cytoplasmic regulatory component which is not part of the K_{ATP} channel itself. The effect of diazoxide is dependent on the presence of Mg-ADP and may result from amplification of the ADP action, culminating in the induction of a novel kinetic state characterized by openings of long duration.

INTRODUCTION

The ATP-regulated K^+ channel (K_{ATP} channel) constitutes the resting conductance of the insulin-secreting pancreatic β-cell (reviewed by Ashcroft, 1988; Ashcroft & Rorsman, 1989; Rorsman & Trube, 1990). Glucose produces a concentration-dependent inhibition of the K_{ATP} channel and at insulin-releasing glucose concentrations, channel activity is inhibited by $> 95\%$. Glucose metabolism is currently believed to exert its inhibitory action by increasing the cytoplasmic ATP/ADP ratio, and varying it in a physiological range mimics the inhibitory action of glucose on the K_{ATP} channel (Kakei, Kelly, Ashcroft & Ashcroft, 1986; Dunne, West-Jordan, Abraham, Edwards & Petersen, 1988). The effects of ATP and ADP on channel activity appear to be mediated by two separate processes/receptors: one stimulatory and one inhibitory (Dunne & Petersen, 1986; Findlay, 1987; Bokvist, Ämmälä, Ashcroft, Berggren, Larsson & Rorsman, 1991). However, the mechanisms are not well understood.

The hyperglycaemic sulphonamide diazoxide inhibits glucose-stimulated insulin secretion and electrical activity (Henquin, Charles, Nenquin, Mathot & Tamagawa, 1982; Henquin & Meissner, 1982). Patch-clamp experiments have shown that diazoxide antagonizes the inhibitory effect of ATP on the K_{ATP} channel (Trube, Rorsman & Ohno-Shosaku, 1986; Dunne, Illot & Petersen, 1987; Gillis, Gee, Hammoud, McDaniel, Falke & Misler, 1989; Kozlowski, Hales & Ashford, 1989). The mechanisms by which diazoxide stimulates channel activity have not been elucidated. There is evidence from tumoural insulin-secreting cell lines suggesting the involvement of protein phosphorylation. In support of this idea, diazoxide has been reported to be without effect or even inhibitory in the absence of intracellular Mg^{2+} (Kozlowski *et al.* 1989; but compare Dunne *et al.* 1987) or after replacement of ATP with non-hydrolysable analogues (Dunne, 1989; Kozlowski *et al.* 1989).

In the present study we have compared the mechanisms by which ADP and diazoxide exert their respective stimulatory actions on the K_{ATP} channel in mouse pancreatic β-cells. We show that both diazoxide and ADP induce a distinct open state, characterized by openings of long duration, by a mechanism which may involve nucleotide hydrolysis.

METHODS

Preparation of cells

Mouse pancreatic islets were isolated by collagenase digestion and dispersed into single cells by treatment with trypsin or shaking in low-Ca^{2+} solution as a previously described (Rorsman & Trube, 1986; Arkhammar, Nilsson, Rorsman & Berggren, 1987). Cells were plated on Corning Petri dishes (Corning Glass Works, Corning, N.Y.) and maintained for 1–4 days in RPMI 1640 tissue culture medium containing 5 mM glucose and supplemented with fetal calf serum (10% v/v; Flow Laboratories, Irvine, UK), 100 μg/ml streptomycin and 100 i.u./ml penicillin (both supplied by Northumbria Biologicals Ltd, Cramlington, UK). In this preparation the vast majority of the cells produce action potentials in the presence of glucose and can therefore be assumed to represent insulin-secreting β-cells (Rorsman & Trube, 1986).

Electrophysiology

We used the inside-out configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). This recording mode permits free access to the cytoplasmic aspect of the plasma membrane making it easy to vary the 'intracellular' composition. Moreover, diazoxide has been reported to be considerably more effective as an activator of the K_{ATP} channel when applied to the intracellular rather than to the extracellular side of the membrane (Kozlowski *et al.* 1989). Pipettes were pulled from alumino- or borosilicate glass (Hilgenberg, Malsfeld, FRG), coated with Sylgard near the tips and fire-polished. Both types of electrodes had resistances of 2–3 MΩ when filled with standard extracellular medium. Currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG). During the experiment the current signal was stored, pending analysis, on either magnetic tape using an FM tape recorder (Racal Store 4DS, Hythe, England) or on videotape employing a VCR (Sony-2000, Sony, Tokyo, Japan) and a modified digital audio processor (PCM-F1, Sony, Tokyo, Japan). Channel activity was measured at 0 mV membrane potential. With the solutions used currents will be outward (i.e. into the pipette) and channel records are displayed according to the convention with upward deflections denoting outward currents. K_{ATP} channel activity was identified on the basis of the sensitivity to ATP and the unitary amplitude (1·5–2 pA). The zero current potential of the pipette was adjusted with the electrode in the bath before establishment of the seal.

Data analysis

Records were filtered at 200 Hz (−3 dB value) by using an 8-pole Bessel filter (Frequency Devices, Haverhill, MA, USA), digitized at 500 Hz using a Labmaster ADC (Scientific Solutions, Solon, OH, USA) and stored in a computer (Compaq 386/20, Houston, TX, USA). The degree of channel activity under the respective experimental conditions was assessed by using in-house software by digitizing segments of the current records (30–60 s long) and forming histograms of baseline and open-level data points. The mean current (I) was then calculated according to the equation:

$$\bar{I} = \sum_{j=1}^{N} \frac{(I_j - I_B)}{N}, \tag{1}$$

where N is the number of samples, I_j is the current observed in sample j and I_B is the value of a user-defined baseline. Unless otherwise indicated, each experimental condition was tested with identical results in at least five (usually more) different patches.

The analysis of the distribution of K_{ATP} channel open times was restricted to segments of the experimental record containing only one or two active channels using the digitized data (see above). Events were identified using a 50% amplitude criterion and an idealized record was constructed. The kinetic constants were derived by approximation of the data to exponential functions by the method of maximum likelihood (Colquhoun & Sigworth, 1983).

The open probability (P_{open}) was estimated by approximating the observed probability (P_x) of finding x simultaneously active channels out of total number of N channels to the binomial

$$P_x = \frac{N!}{x!(N-x)!} P_{open}{}^{x} (1 - P_{open})^{(N-x)}. \tag{2}$$

For display, representative segments were selected from the experimental record under steady-state conditions.

Solutions

The pipettes were filled with standard extracellular medium containing (mM): 138 NaCl, 5·6 KCl, 1·2 $MgCl_2$, 2·6 $CaCl_2$ and 5 Hepes-NaOH (pH 7·40). The bath solution (i.e. the 'intracellular' solution) consisted of (mM): 125 KCl, 1 $MgCl_2$, 10 EGTA, 30 KOH and 5 Hepes-KOH (pH 7·15). ATP, ADP and AMP and their analogues ATP-α-S (Boehringer Mannheim), ATP-γ-S (Boehringer-Mannheim), β,γ-methylene ATP (Sigma) and α,β-methylene ADP (Sigma) were added to the intracellular medium as indicated in the text and figures. Diazoxide was prepared as a concentrated stock solution in dimethyl sulphoxide (DMSO; final concentration of DMSO: $< 0{\cdot}1\%$). The nucleotides were added as their Mg^{2+}, Li^+ or Na^+ salts. In the two latter cases, an equal amount of Mg^{2+} was added to maintain an excess of Mg^{2+}. In some experiments, Mg^{2+} was omitted from the intracellular medium as indicated in the text and the concentration of ATP and ADP lowered to 0·03 mM to compensate for the greater blocking efficacies of ATP^{4-} and ADP^{3-} as compared with the Mg^{2+} salts (Ashcroft & Kakei, 1989). Patches were excised into nucleotide-free solution and ATP was first applied to test for channel inhibition. ATP was then removed and the patches were subsequently exposed to the test solutions indicated in the text or figures. The records shown are, unless otherwise stated, obtained shortly after patch excision. It was ascertained that ADP or diazoxide exerted their expected effects under control conditions both before and after any test situation. Mg-ATP (0·1 mM) was present in the intracellular solution for most of the time to reduce 'run-down' of K_{ATP} channels (Ohno-Shosaku, Zünkler & Trube, 1987). Despite this precaution some run-down still took place. This might be due to the continuous presence of Mg^{2+} leading to the activation of Mg^{2+}-dependent phosphatases with resulting channel dephosphorylation and run-down (cf. Kozlowski & Ashford, 1990). The bath had a volume of 0·5 ml and was perfused at a rate of 4 ml/min. The test compounds were added either to the perfusion medium or applied locally by means of a puffer pipette (experiments with ATP-α-S). To facilitate the comparison of the effects, the nucleotides and diazoxide were generally applied at a concentration of 0·1 mM. It should be emphasized, however, that different results might have been observed had the experiments been conducted with other concentrations of the nucleotides (cf. Dunne *et al.* 1987).

RESULTS

The effects of diazoxide depend on time after patch isolation

The stimulatory action of diazoxide observed shortly after excision is shown in Fig. 1*A*. It is apparent that diazoxide evokes a current which, in fact, is larger than that observed in the nucleotide-free solution. This contrasts with the results obtained 15 min later (Fig. 1*B*). It is clear that although K_{ATP} channel activity had declined (run-down), ATP-remained an effective inhibitor of the channel. By contrast, diazoxide was without stimulatory action. This suggests that the effects of diazoxide are exerted by mechanisms other than direct interaction with the K_{ATP} channel. Consistent with previous reports on insulin-secreting cell lines (Dunne *et al.* 1987; Kozlowski *et al.* 1989), no effect of diazoxide was observed in nucleotide-free solution (data not shown). Moreover, the stimulatory action of diazoxide depended on the presence of intracellular Mg^{2+}, at least at the ATP concentration tested (cf. Dunne *et al.* 1987; data not shown).

Stimulatory action of ADP depends on nucleotide hydrolysis

In agreement with earlier studies (Kakei *et al.* 1986; Dunne & Petersen, 1986; Misler, Falke, Gillis & McDaniel, 1986), ADP was able to counteract the inhibitory effect of ATP and the current observed in the simultaneous presence of both nucleotides often exceeded that which was observed in the absence of both

nucleotides. The stimulatory effect of ADP on K_{ATP} channel activity was not reproduced by the metabolically stable analogue α,β-methylene ADP (Fig. 2*A*). However, as illustrated in Fig. 2*B*, there is no requirement of metabolizable ATP, and the stimulatory action persisted after replacement of ATP with β,γ-methylene ATP.

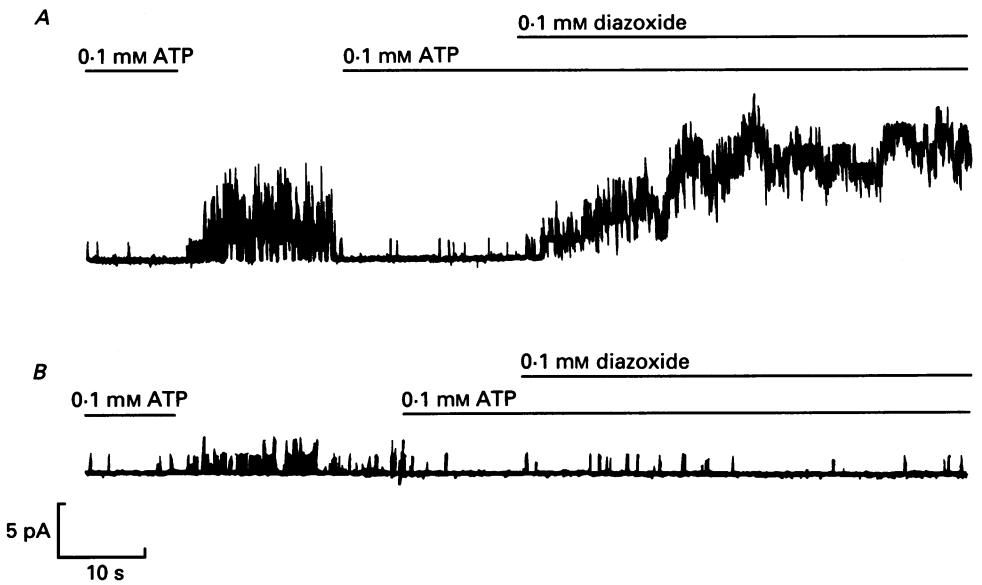

Fig. 1. Time-dependent decline of diazoxide effect on K_{ATP} channel activity. *A*, the effects of diazoxide on K_{ATP} channel activity in an inside-out patch shortly after patch isolation. The mean current in the absence of ATP was 3·2 pA which was reduced to 0·04 pA in the presence of 0·1 mM ATP and subsequently increased to 8·6 pA in the presence of diazoxide. *B*, lack of stimulatory action of diazoxide in the same patch 15 min later.

As already reported for both rat insulinoma cells (Dunne & Petersen, 1986; Findlay 1987) and mouse pancreatic β-cells (Bokvist *et al.* 1991), ADP also increased channel activity when applied in the absence of ATP (Fig. 2*C*). Consistent with the previous observations of Findlay (1987), this effect of ADP was not observed following replacement of ADP with a stable ADP analogue (α,β-methylene ADP). The stimulatory effect of ADP required the presence of internal Mg^{2+}. In agreement with the findings of Findlay (1987), ADP (30 μM) was, in fact, inhibitory when applied in the absence of this ion (data not shown).

Stimulatory action of diazoxide requires metabolizable ATP

In rat insulinoma cells, stimulation of K_{ATP} channel activity has been shown to require hydrolysable ATP and was not observable in the presence of stable analogues. Contrary to the observations made in RINm5F-cells (Dunne, 1989), stimulation by diazoxide of channel activity in mouse pancreatic β-cells was seen in

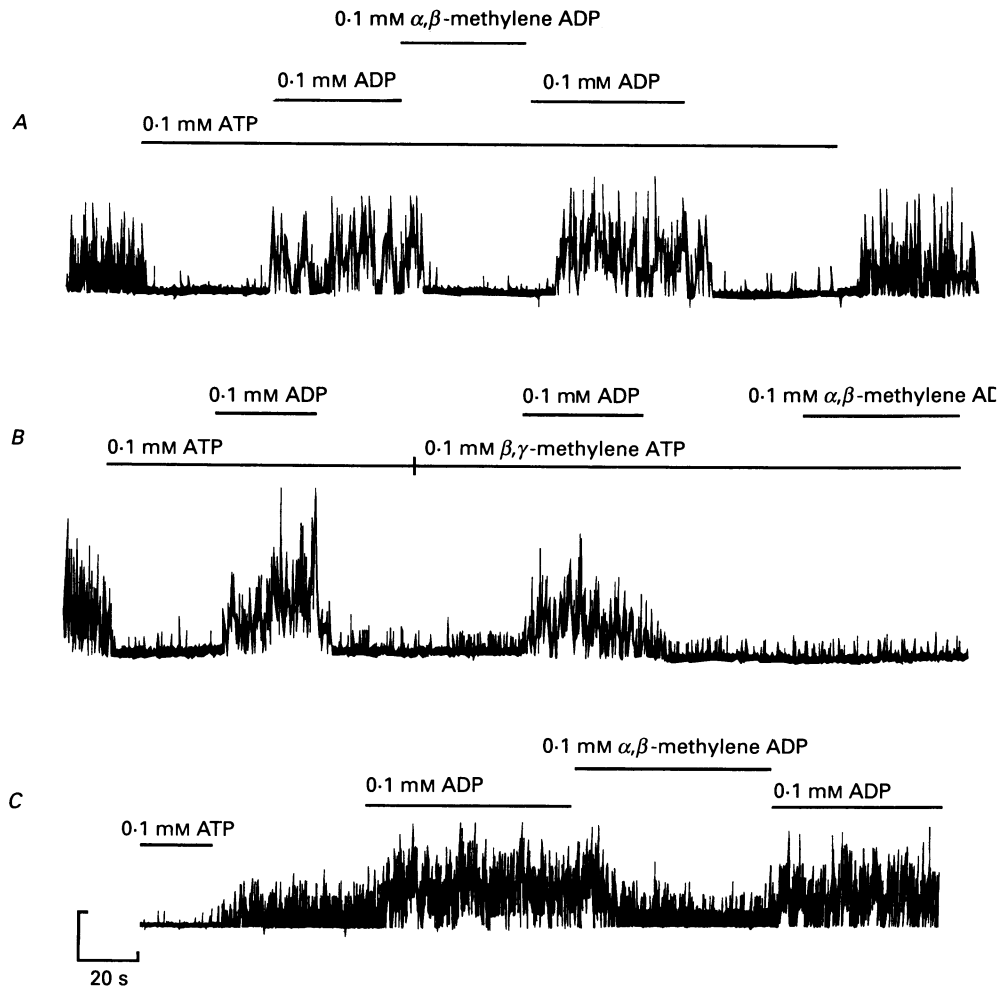

Fig. 2. The ability of ADP to reactivate K_{ATP} channels inhibited by ATP is dependent on hydrolysable ADP but not ATP. *A*, channel activity in the absence of ATP amounted to 1·0 pA which was reduced to < 0·01 pA after inclusion of 0·1 mM ATP in the intracellular solution. Addition of ADP produced pronounced stimulation of K_{ATP} channel activity and a mean current of 1·9 pA was observed. Substituting the stable analogue α,β-methylene ADP for standard ADP resulted in a reduction of channel activity to that observed in the presence of ATP alone (mean current 0·04 pA). Returning to standard ADP was associated with reappearance of channel activity (mean current 2·7 pA). *B*, a mean current of 3·1 pA was observed in the nucleotide-free solution which was reduced to 0·07 pA by addition of 0·1 mM ATP. Inclusion of ADP in the intracellular solution increased the mean current to 3·6 pA. In the presence of the stable ATP analogue β,γ-methylene ATP the mean current fell to 0·11 pA which subsequently increased to 2·9 pA after addition of standard ADP but was unaffected by the stable ADP analogue α,β-methylene ADP. Same patch as in *A*. *C*, ability of ADP to stimulate channel activity is not mimicked by a non-hydrolysable analogue. Channel activity in the nucleotide-free solution was 1·2 pA. This increased to 6·2 pA after application of ADP. Replacement of ADP with the stable analogue α,β-methylene ADP abolished this effect and mean current fell to 0·84 pA. The vertical calibration bar is 2.5 pA in *A* and *B* and 5 pA in *C*.

the presence of ATP-γ-S (Fig. 3*A*). An increase in channel activity by diazoxide was also produced when ATP was replaced by ATP-α-S (Fig. 3*B*). However, no enhancement of channel activity was observed when ATP was replaced with β,γ-methylene ATP (Fig. 3*C*).

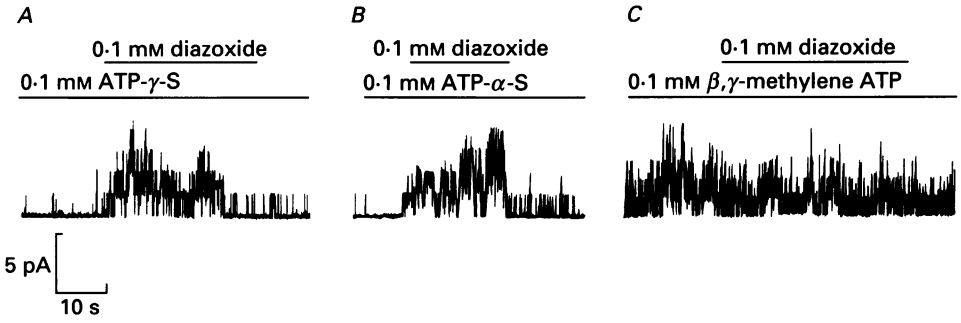

Fig. 3. The stimulatory action of diazoxide on K_{ATP} channel requires ATP hydrolysis. *A*, stimulation by diazoxide of K_{ATP} channel activity in the presence of 0·1 mM ATP-γ-S. Channel activity increased > 30-fold from 0·07 to 2·5 pA. In the same patch diazoxide exerted a comparable effect in the presence of standard ATP and a 24-fold stimulation was observed. *B*, diazoxide increased channel activity 25-fold in the presence of 0·1 mM ATP-α-S and mean current rose from 0·09 to 2·22 pA; larger than the 10-fold stimulation observed in the presence of standard ATP. Identical effects were observed in three out of three patches. *C*, failure of diazoxide to stimulate channel activity in the presence of the stable analogue β,γ-methylene ATP. The mean current in the presence of the nucleotide alone was 2·0 pA and decreased to 1·4 pA after adding diazoxide. Note that although β,γ-methylene ATP was somewhat less effective than standard ATP in blocking the K_{ATP} channel, inhibition in the presence of this nucleotide nevertheless amounted to ≈ 70 %. In the same patch, diazoxide produced a 40-fold stimulation of channel activity when applied in the presence of standard ATP.

Interactions between ADP and diazoxide

The observation that the ability of diazoxide to stimulate K_{ATP} channel activity required the presence of hydrolysable ATP may indicate that a metabolic product of the nucleotide mediates the effect. Figure 4*A* shows a recording from a patch initially exposed to 0·1 mM ATP in which addition of ADP to the bath solution (in the continued presence of ATP) exerted only a moderate stimulatory action. It can be seen that diazoxide, under these experimental conditions, produced a pronounced increase in the K_{ATP} channel activity. This effect was far greater than that observed when diazoxide was applied in the absence of ADP (Fig. 4*B*).

Diazoxide was also able to increase channel activity in the absence of ATP provided ADP was present. Figure 5*A* shows the strong 4·5-fold stimulation observed when diazoxide was applied in the presence of ADP (0·1 mM). The ability of ADP to potentiate the effect of diazoxide was neither mimicked by a stable analogue (α,β-methylene ADP; Fig. 5*B*), nor could it be observed in the absence of intracellular Mg^{2+} (data not shown).

Interactions between AMP and diazoxide

Since the potentiating action of ADP was not reproduced by a stable analogue, we subsequently studied the effect of some of its metabolites. Figure 6*A* shows that AMP shares the capacity of ADP to counteract ATP-induced inhibition of the

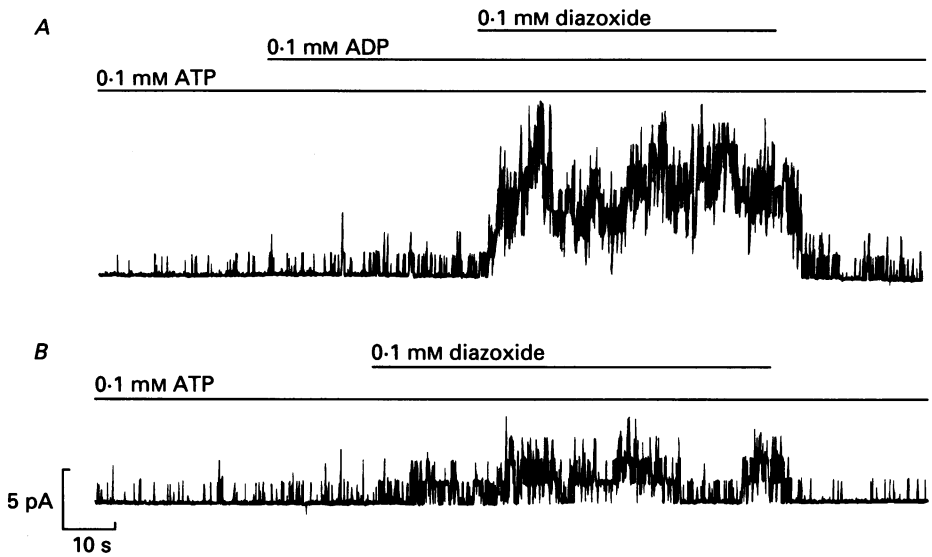

Fig. 4. ADP enhances stimulatory action of diazoxide on K_{ATP} channel activity in the presence of ATP. *A*, addition of 0·1 mM ADP to a patch in which channel activity was inhibited by ATP increased channel activity 7-fold, from 0·03 pA to 0·20 pA. Subsequent addition of 0·1 mM diazoxide resulted in ≈ 35-fold stimulation of channel activity and a mean current of 6·9 pA was observed. *B*, when diazoxide was subsequently added to the same patch as in *A*, but in the absence of ADP it had a smaller effect on channel activity and the mean current rose from 0·09 to 1·5 pA.

K_{ATP} channel. The inclusion of diazoxide into the perfusion medium under these experimental conditions resulted in a strong enhancement of channel activity. The current induced by diazoxide in the presence of AMP (Fig. 6*A*) exceeded that recorded in its absence (Fig. 6*B*). AMP had no effects when added to a nucleotide-free solution and diazoxide was without stimulatory effect under these latter conditions (Fig. 6*C*). IMP (inosine monophosphate), the deamination product of AMP, was likewise without effect irrespective of whether it was applied alone or in combination with ATP or diazoxide (not shown).

Addition of a low concentration of ADP restores the stimulatory effect of diazoxide in the presence of a non-hydrolysable ATP analogue

The data of Figs 4–6 suggest that a metabolic product of ATP hydrolysis mediates the stimulatory effect of diazoxide. The results shown in Fig. 7 indicate that the presence or generation of Mg-ADP is critical. As shown in Fig. 7*A*, addition of Mg-

ADP at a concentration which itself has little stimulatory effect (Fig. 7*B*) restored the ability of diazoxide to increase channel activity in a medium in which β,γ-methylene ATP was substituted for ATP. It should be noted that diazoxide, when added alone, failed to enhance channel activity in the presence of this stable ATP

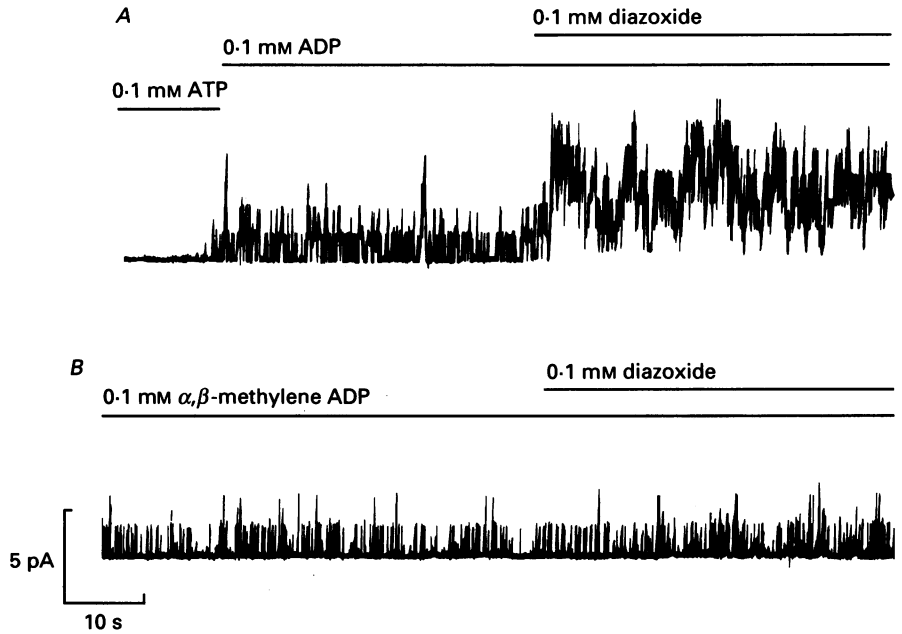

Fig. 5. Diazoxide stimulation of channel activity in the presence of ADP. *A*, channel activity in an inside-out patch in the presence of 0·1 mM ADP alone and a mean current of 0·90 pA was observed. Addition of diazoxide increased the mean current to 4·2 pA. *B*, no stimulation of K_{ATP} channel activity in the presence of the stable analogue α,β-methylene ADP. *A*, and *B* were obtained from the same patch.

analogue (Figs 3*C* and 7*A*). Although β,γ-methylene ATP was somewhat less effective than ATP as an inhibitor of the K_{ATP} channel, it still produced > 70% reduction of channel activity (Fig. 7*A*).

Effects of ATP, ADP and diazoxide on K_{ATP} channel kinetics

Figure 8*A* shows the changes in channel activity observed following the additions of ATP and diazoxide to an inside-out patch containing only two to three active channels. It is clear that the openings observed in the presence of diazoxide are much longer than those observed in the presence of ATP alone. In Fig. 8*B–E* we have attempted to quantify this effect by analysing the distribution of the channel openings in nucleotide-free solution (Fig. 8*B*), in the presence of ATP alone (Fig. 8*C*), in the simultaneous presence of ATP and ADP (Fig. 8*D*) and in the combined presence of ATP and diazoxide (Fig. 8*E*). The insets show examples of the channel openings under the respective experimental conditions on an expanded time base. In

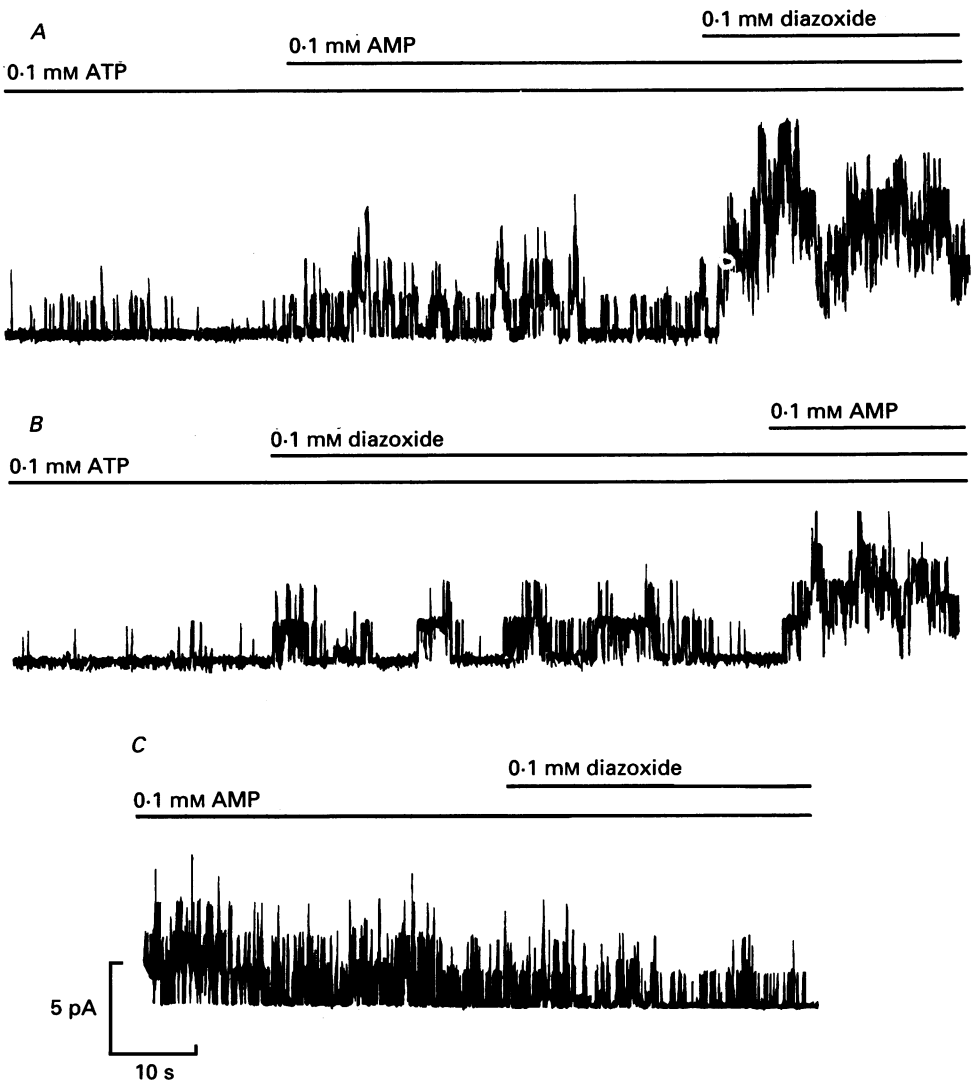

Fig. 6. AMP potentiates diazoxide action in the presence of ATP. *A*, addition of AMP reactivates channels inhibited by ATP and increases the mean current from 0·11 to 0·66 pA. A further 7-fold enhancement was obtained by adding diazoxide resulting in a mean current of 4·4 pA. *B*, effects of diazoxide and AMP when added in opposite order. Diazoxide, when added alone, increased channel activity from 0·02 to 0·62 pA which was increased to 2·1 pA after inclusion of AMP into the medium. *C*, lack of K_{ATP} channel stimulation by diazoxide in the presence of AMP alone. The gradual decline in channel activity in panel *C* is probably unrelated to the additions of AMP and diazoxide and may reflect 'run-down' of K_{ATP} channel activity, which occurs in the absence of ATP (Ohno-Shosaku *et al.* 1987). All records obtained from the same patch. Similar effects were obtained in another 4 out of 5 patches.

the absence of any added nucleotides, channel activity consisted of relatively short openings. The distribution of the openings was described by a single exponential with a time constant (τ) of 20 ± 2 ms ($n = 6$). The mean duration of the openings ($\bar{t}$), was

26 ± 4 ms. After addition of ATP, channel activity was markedly decreased but the distribution of the remaining openings was not much affected and could be described by a single exponential with a time constant of 26 ± 2 ms ($n = 5$). The value of $\bar{t}$ amounted to 28 ± 4 ms. This indicates that the principal action of ATP on K_{ATP}

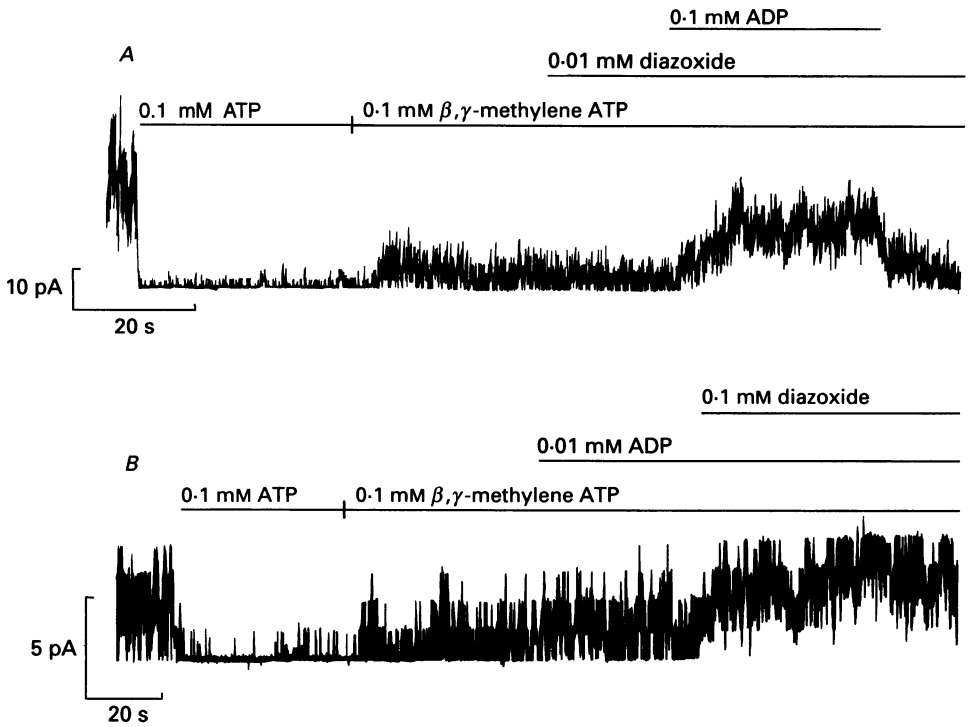

Fig. 7. A low concentration of ADP restores the ability of diazoxide to increase channel activity in the presence of a stable ATP analogue. *A*, addition of 0·01 mM ADP restored the stimulatory action of diazoxide (0·1 mM) in the presence of β,γ-methylene ATP (0·1 mM). Note that diazoxide had no stimulatory effect in the presence of the stable ATP analogue alone. *B*, ADP, when applied at the low concentration used in *A* (but in a different patch), exerted a weak stimulatory effect on channel activity. Inclusion of 0·1 mM diazoxide in the continued presence of β,γ-methylene ATP and ADP led to a pronounced stimulation of channel activity.

channel kinetics is to prolong the duration of the closed times. In the presence of both ATP and ADP, channel activity consisted of two types of openings: both short openings, similar to those observed in the presence of ATP alone, and long openings which occasionally lasted several hundred milliseconds (Fig. 8*D*). Accordingly, the distribution of the openings was best described as the sum of two exponentials with τ values of 20 ± 9 and 183 ± 9 ms ($n = 6$). The *slow* component comprised 68 ± 9% of the events. Under these experimental conditions the value of $\bar{t}$ increased to 220 ± 69 ms. A similar type of channel activity was observed in the presence of diazoxide (Fig. 8*E*) although less pronounced than that observed in the presence of ATP and ADP. In the presence of the K_{ATP} channel opener, the distribution of the

openings was described as the sum of two exponentials with τ values of 24 ± 9 and 127 ± 37 ms ($n = 5$). The *rapid* component comprised 59 ± 7 % of the total number of events. The value of $\bar{t}$ increased fivefold and averaged 94 ± 23 ms.

To further quantify the effects on channel kinetics we performed binomial analysis of the amplitude distribution histograms. In the absence of any nucleotides,

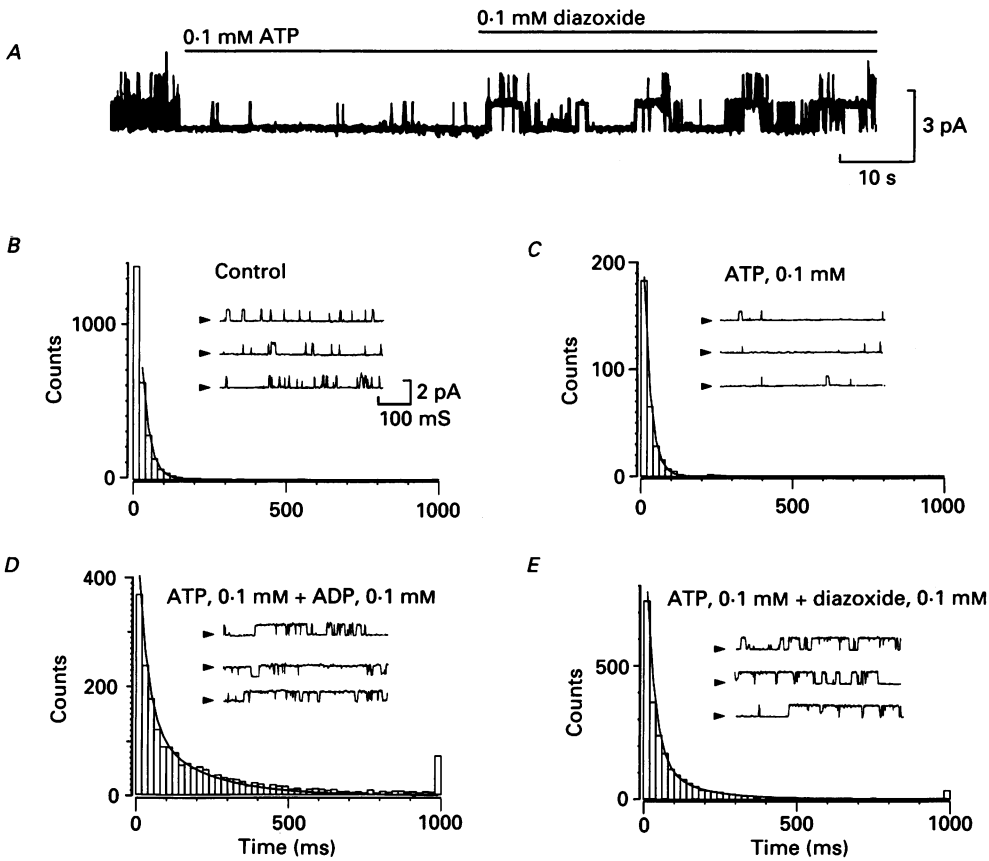

Fig. 8. Effects of ATP, ADP and diazoxide on K_{ATP} channel kinetics. *A*, effects of ATP and diazoxide on K_{ATP} channel activity in an inside-out patch. *B*, frequency *versus* lifetime histogram of channel openings under control conditions (nucleotide-free solution). The distribution of channel lifetimes could be described by a single exponential with a time constant $\tau = 26$ ms. A total number of 2627 events were analysed. *C*, distribution of open times in the presence of 0·1 mM ATP. A total of 309 events were analysed and could be fitted by a single exponential with $\tau = 22$ ms. *D*, the distribution of open times in the simultaneous presence of both 0·1 mM ATP and 0·1 mM ADP. The distribution was best described as the sum of two exponentials with $\tau_1 = 20$ and $\tau_2 = 183$ ms. A total number of 1929 events were analysed of which 68 % of the integrated events belonged to the slow component. *E*, distribution of open times in the presence of 0·1 mM ATP and 0·1 mM diazoxide. The distribution was described by two exponentials with $\tau_1 = 22$ and $\tau_2 = 116$ ms. A total of 2222 events were analysed of which 52 % belonged to the slow component. Insets show the K_{ATP} channel activity observed under the respective experimental conditions on an expanded time base. Arrowheads indicate current level when the channel is closed.

the open probability (P_{open}) averaged $0{\cdot}06 \pm 0{\cdot}02$ ($n = 6$). This was reduced to $0{\cdot}02 \pm 0{\cdot}01$ ($n = 5$) by addition of 0·1 mM ATP ($P < 0{\cdot}01$). Subsequent inclusion of ADP or diazoxide (in the continued presence of ATP) increased P_{open} to $0{\cdot}27 \pm 0{\cdot}05$ ($n = 6$; $P < 0{\cdot}001$) and $0{\cdot}12 \pm 0{\cdot}05$ ($n = 5$; $P < 0{\cdot}05$), respectively. In addition to the effects on the open probability, the number of active channels in the patch (N in eqn (2)) also appeared to be affected in a way similar to that reported by Kozlowski *et al.* (1989). However, this was not analysed in detail since the degree of channel activity varied from one patch to another and not all compounds were applied to the same patch.

DISCUSSION

Stimulatory effect of ADP on K_{ATP} channel activity requires hydrolysable ADP both in the presence and absence of ATP

Earlier studies on a rat insulin-secreting cell line (RINm5F) have indicated that the effects of ADP in the absence and presence of ATP differ with regard to the requirement of hydrolysable ADP, indicating they may depend on separate processes (Dunne & Petersen, 1986). The present study failed to observe such a difference. Our data are rather compatible with the idea that the two effects reflect the same mechanism. In fact, the present observations that stable analogues are not able to mimic the action of ADP and that its stimulatory effect requires the presence of internal Mg^{2+}, whereas the inhibitory effect is produced by both stable analogues and free ADP^{3-}, adds to previous arguments that K_{ATP} channel inhibition and stimulation involve separate mechanisms (Findlay, 1987; Bokvist *et al.* 1991).

The inability of ADP^{3-} and α,β-methylene ADP to mimic the stimulatory effect of Mg-ADP can be interpreted in terms of ADP serving as a substrate in an enzymatic reaction resulting in, for example, the phosphorylation of the K_{ATP} channel protein. Although we cannot exclude this possibility, there are a few (if any) examples of such reactions. Another explanation is that ADP^{3-} and α,β-methylene ADP are not recognized at the stimulatory nucleotide receptor site of the K_{ATP} channel (cf. Bokvist *et al.* 1991). This possibility is suggested by analogy with the situation in blood platelets where α,β-methylene ADP is unable to substitute for Mg-ADP in binding studies (Haslam & Cusack, 1981).

Effects of ADP and diazoxide on K_{ATP} channel kinetics

Kozlowski *et al.* (1989) have previously concluded, based on binomial analysis of single-channel data, that diazoxide increases K_{ATP} channel activity in tumoural CRI-G1 cells by increasing the open state probability. Our data indicate that this is also true for the K_{ATP} channel in mouse β-cells. With regard to the effects of diazoxide on K_{ATP} channel kinetics, Gillis *et al.* (1989) present *qualitative* data suggesting that the burst duration (recorded as inward currents with symmetrical 150 mM K^+ solutions) is increased, whereas the duration of the interburst closures is reduced. We now demonstrate that when measured with physiological ionic gradients, both ADP and diazoxide increase K_{ATP} channel activity by promoting a long-lasting open state which accounts for as much as 40–70% of the total number of events. From the observations that the changes in the mean open time and the open probability induced by ADP and diazoxide occur in parallel, it appears that the increase in channel activity evoked by these compounds can be explained by their

action on the distribution of the open times and it is therefore not necessary to postulate an effect on the distribution of closed times.

It is worth pointing out that the effect we now describe for the action of diazoxide on the K_{ATP} channel kinetics is reminiscent of that previously reported for the effects

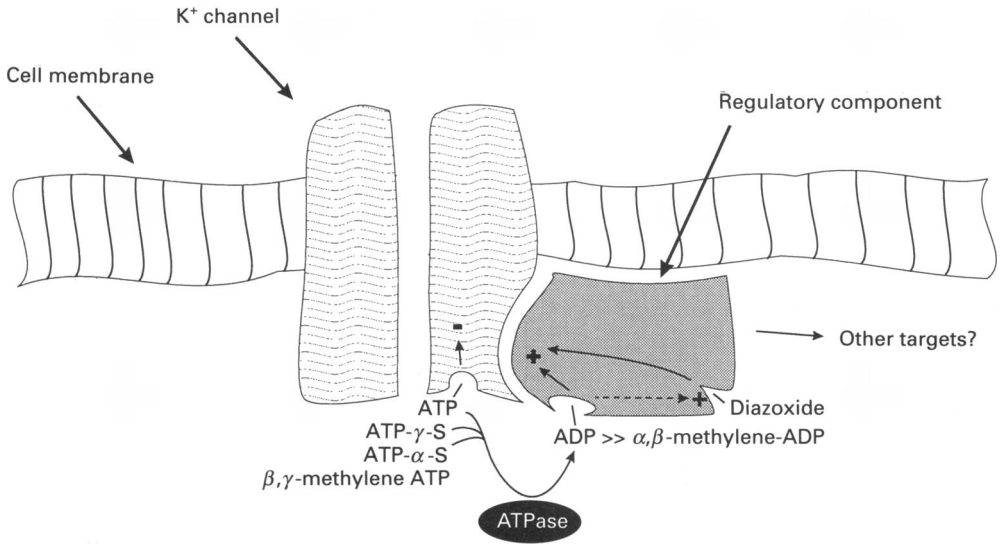

Fig. 9. Model for the regulation of the ATP-regulated K^+ channel by ATP, ADP and diazoxide. The model is explained in the text.

of the dihydropyridine agonist Bay K 8644 on L-type Ca^{2+} channels. Armstrong & Eckert (1987) have suggested that the agonists stabilize a phosphorylated state of the channel. Recently, intracellular application of ATP has been demonstrated to evoke long openings of rat brain Ca^{2+}-activated K^+ channels incorporated into planar bilayers (Chung, Reinhart, Martin, Brautigan & Levitan, 1991), an effect which was attributed to activation of a kinase closely associated with the K^+ channel. It is clear that the actions we report here for ADP and diazoxide on the K_{ATP} channel kinetics are similar to these effects.

Mechanisms of ADP and diazoxide stimulation of K_{ATP} channel activity

The possibility that diazoxide acts as a competitive ATP antagonist and simply displaces ATP from the inhibitory site can be discounted in view of the observations that whereas inhibition of the K_{ATP} channel is produced both by Mg-ATP and ATP^{4-}, stimulation of channel activity by diazoxide is observed only in the presence of the Mg^{2+} salt. A similar dependence on Mg^{2+} has previously been reported by Kozlowski *et al.* (1989). This could indicate that hydrolysis of ATP is part of the mechanisms by which diazoxide acts (Dunne, 1989). However, it has also been demonstrated that provided the ATP concentration is lowered concomitantly with the Mg^{2+} removal, diazoxide remains stimulatory even in the absence of Mg^{2+} (Dunne *et al.* 1987). This argues that the interactions between Mg^{2+}, the adenine nucleotides and diazoxide may be more complex.

As already discussed, ADP stimulates K_{ATP} channel activity by a mechanism distinct from that utilized for channel inhibition and that is mediated by a diffusible cytoplasmic constituent (Findlay, 1987; Bokvist *et al.* 1991). Our data indicate that diazoxide stimulation also results from mechanisms other than direct interaction with the K_{ATP} channel protein. This hypothesis rests on the observation that the stimulatory effect of diazoxide in inside-out patches declines more rapidly than can be accounted for by channel run-down (Fig. 1). This suggests that the stimulatory effect is mediated by a diffusible cytosolic component which is not part of the K_{ATP} channel and that washes out gradually following patch excision. A tentative model for the regulation of the K_{ATP} channel by ADP and diazoxide is presented in Fig. 9.

We believe the observations that the effects of diazoxide on channel activity and kinetics are similar to those of Mg-ADP and represent an important clue to the mechanism of diazoxide action and we propose that the stimulatory action of diazoxide is dependent on the binding of Mg-ADP to a cytosolic regulatory constituent. Although we have no information concerning the nature of this component, a regulatory protein is the most likely candidate. Binding of Mg-ADP (but not ADP^{3-} or α,β-methylene ADP) to the regulatory protein/molecule produces a conformational change. This leads to the association with the K_{ATP} channel and promotion of channel opening. When the regulatory protein has bound Mg-ADP, its affinity to diazoxide is increased. The simultaneous binding of both Mg-ADP and diazoxide stabilizes and/or amplifies the conformational change of the protein thus resulting in a more pronounced stimulation of channel activity. The model implies continuous ATPase activity within the patch. This could be due to intrinsic enzymatic activity of the regulatory protein leading to the generation of a small local ADP concentration sufficient to activate some of the ADP binding sites. The addition of exogenous Mg-ADP at a relatively high concentration ($\geqslant 10\ \mu M$) leads to the binding of more Mg-ADP with resulting further stimulation of channel activity. The model implies that diazoxide will be able to enhance K_{ATP} channel activity in the presence of any nucleotide which can form Mg-ADP. This can be formed from ATP-γ-S, in which the chemical modification is restricted to the γ-phosphate group (cf. Chung *et al.* 1991), but is unlikely to be formed from β,γ-methylene ATP, in which the bond between the β- and γ-phosphate group has been changed. Our model is also consistent with the observation that a low concentration of ADP restores the capacity of diazoxide to promote K_{ATP} channel opening in the presence of a stable ATP analogue. Finally, the observation that AMP potentiates the action of diazoxide can also be explained by this model since ADP may be formed by adenylate kinase from ATP and AMP. In fact, the observation that AMP was only effective in the presence of ATP supports this idea. It is of interest that Shen, Tung, Machulda & Kurachi (1991) have reported a similar requirement of nucleotide diphosphate, including ADP, for the stimulation of the cardiac K_{ATP} channel by nicorandil. It may therefore be speculated that different K_{ATP} channel activators utilize the same fundamental molecular mechanisms to produce K_{ATP} channel stimulation in various tissues.

The ability of Mg-ADP to enhance the action of diazoxide is similar to the previous reported ability of this nucleotide to potentiate the inhibitory action of the sulphonylurea tolbutamide (Zünkler, Lins, Ohno-Shosaku, Trube & Panten, 1988; Panten, Heipel, Rosenberger, Scheffer, Zünkler & Schwanstecher, 1990) suggesting

that the ADP binding component is also involved in this process. In fact, the participation of this regulatory protein is not necessarily restricted to the control of ion channel activity but may include other cellular processes. For example, recent experiments have suggested that tolbutamide and diazoxide may modulate insulin secretion by direct interference with the secretory machinery (Shibier, Flatt, Efendić & Berggren, 1991).

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REFERENCES

ARKHAMMAR, P., NILSSON, T., RORSMAN, P. & BERGGREN, P.-O. (1987). Inhibition of ATP-regulated K^+-channels precedes depolarization-induced increased in cytoplasmic free Ca^{2+} concentration in mouse pancreatic β-cells. *Journal of Biological Chemistry* **262**, 5448–5454.

ARMSTRONG, D. & ECKERT, R. (1987). Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proceedings of the National Academy of Sciences of the USA* **84**, 2518–2522.

ASHCROFT, F. M. (1988). Adenosine 5′-triphosphate-sensitive potassium channels. *Annual Review of Neuroscience* **11**, 97–118.

ASHCROFT, F. M. & KAKEI, M. (1989). ATP-sensitive K^+ channels in rat pancreatic β-cells: modulation by ATP and Mg^{2+} ions. *Journal of Physiology* **416**, 349–367.

ASHCROFT, F. M. & RORSMAN, P. (1989). Electrophysiology of the pancreatic β-cell. *Progress in Biophysics and Molecular Biology* **54**, 87–143.

BOKVIST, K., ÄMMÄLÄ, C., ASHCROFT, F. M., BERGGREN, P.-O., LARSSON, O. & RORSMAN, P. (1991). Separate processes mediate nucleotide-induced inhibition and stimulation of the ATP-regulated K^+-channels in mouse pancreatic β-cells. *Proceedings of the Royal Society* B **243**, 139–144.

CHUNG, S., REINHART, P. H., MARTIN, B. L., BRAUTIGAN, D. & LEVITAN, I. B. (1991). Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. *Science* **253**, 560–562.

COLQUHOUN, D. & SIGWORTH, F. J. (1983). Fitting and statistical analysis of single-channel records. In *Single-Channel Recording*, ed. SAKMANN, B. & NEHER, E., pp. 191–263, Plenum Press, New York.

DUNNE, M. J. (1989). Protein phosphorylation is required for diazoxide to open ATP-sensitive potassium channels in insulin (RINm5F) secreting cells. *FEBS Letters* **250**, 262–266.

DUNNE, M. J., ILLOT, M. C. & PETERSEN, O. H. (1987). Interaction of diazoxide, tolbutamide and ATP^{4-} on nucleotide-dependent K^+ channels in an insulin-secreting cell line. *Journal of Membrane Biology* **99**, 215–224.

DUNNE, M. J. & PETERSEN, O. H. (1986). Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell line. *FEBS Letters* **208**, 59–62.

DUNNE, M. J., WEST-JORDAN, J. A., ABRAHAM, R. J., EDWARDS, R. H. T. & PETERSEN, O. H. (1988). 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. *Journal of Membrane Biology* **104**, 165–177.

FINDLAY, I. (1987). The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *Journal of Physiology* **391**, 611–629.

GILLIS, K. D., GEE, W. M., HAMMOUD, A., MCDANIEL, M. L., FALKE, L. C. & MISLER, S. (1989). Effects of sulfonamides on a metabolite-regulated ATP_i-sensitive K^+ channel in rat pancreatic B-cells. *American Journal of Physiology* **257**, C1119–1127.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp for high-resolution current recordings from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HASLAM, R. J. & CUSACK, N. J. (1981). Blood platelet receptors for ADP and for adenosine. In *Purinergic Receptors*, ed. BURNSTOCK, G., pp. 221–285. Chapman and Hall, London.

HENQUIN, J. C. & MEISSNER, H. P. (1982). Opposite effects of tolbutamide and diazoxide on ^{86}Rb fluxes and membrane potential in pancreatic β-cells. *Biochemical Pharmacology* **31**, 1407–1415.

HENQUIN, J. C., CHARLES, S., NENQUIN, C. M., MATHOT, F. & TAMAGAWA, T. (1982). Diazoxide and D-600 inhibition of insulin release. Distinct mechanisms explain the specificity for different stimuli. *Diabetes* **31**, 776–783.

KAKEI, M., KELLY, R. P., ASHCROFT, S. J. H. & ASHCROFT, F. M. (1986). The ATP-sensitivity of K^+-channels in rat pancreatic B-cells is modulated by ADP. *FEBS Letters* **208**, 63–66.

KOZLOWSKI, R. Z. & ASHFORD, M. L. J. (1990). ATP-sensitive K-channel rundown is Mg-dependent. *Proceedings of the Royal Society* B **240**, 397–410.

KOZLOWSKI, R. Z., HALES, C. N. & ASHFORD, M. L. J. (1989). Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line. *British Journal of Pharmacology* **97**, 1039–1050.

MISLER, S. L., FALKE, L. C., GILLIS, K. & MCDANIEL, M. L. (1986). A metabolite-regulated potassium channel in rat pancreatic B-cells. *Proceedings of the National Academy of Sciences of the USA* **83**, 7119–7123.

OHNO-SHOSAKU, T., ZÜNKLER, B. J. & TRUBE, G. (1987). Dual effects of ATP on K^+-currents of mouse pancreatic β-cells. *Pflügers Archiv* **408**, 133–138.

PANTEN, U., HEIPEL, C., ROSENBERGER, F., SCHEFFER, K., ZÜNKLER, B. J. & SCHWANSTECHER, C. (1990). Tolbutamide-sensitivity of the adenosine 5′-triphosphate-dependent K^+-channel in mouse pancreatic B-cells. *Naunyn-Schmiedeberg's Archives of Pharmacology* **342**, 566–574.

RORSMAN, P. & TRUBE, G. (1986). Calcium and delayed potassium currents in mouse pancreatic β-cells under voltage-clamp conditions. *Journal of Physiology* **374**, 531–550.

RORSMAN, P. & TRUBE, G. (1990). Biophysics and physiology of ATP-regulated K^+ channels (K_{ATP}). In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential*, ed. COOK, N. S., pp. 96–116. Ellis Horwood. Chichester.

SHEN, W. K., TUNG, R. T., MACHULDA, M. M. & KURACHI, Y. (1991). Essential role of nucleotide diphosphates in nicorandil-mediated activation of cardiac ATP-sensitive K^+ channel. *Circulation Research* **69**, 1152–1158.

SHIBIER, O., FLATT, P. R., EFENDIĆ, S. & BERGGREN, P.-O. (1991). Intracellular action of sulfonylureas in the stimulation of insulin release. *Diabetologia* **34**, A91.

TRUBE, G., RORSMAN, P. & OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in mouse pancreatic β-cells. *Pflügers Archiv* **407**, 493–499.

ZÜNKLER, B. J., LINS, S., OHNO-SHOSAKU, T., TRUBE, G. & PANTEN, U. (1988). Cytosolic ADP enhances the sensitivity to tolbutamide of ATP-dependent K^+-channels from pancreatic B-cells. *FEBS Letters* **239**, 241–244.