Volume 250, number 2, 262-266 FEB 07266 July 1989

Protein phosphorylation is required for diazoxide to open ATP-sensitive potassium channels in insulin (RINm5F) secreting cells

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Received 20 April 1989

The patch-clamp open-cell recording configuration has been used to investigate the effects of non-hydrolyzable analogues of ATP on the diazoxide-activation of K_{ATP} channels in the insulin-secreting cell line RINm5F. K^+ channels inhibited by 0.1, 0.5 and 1.0 mM ATP were consistently activated by 200 μM diazoxide. During sustained activation of channels, exchange of ATP for either AMP-PNP, AMP-PCP or ATPγS abolished the effects of diazoxide. If diazoxide was added to the membrane in the continued presence of AMP-PNP, AMP-PCP or ATPγS either no effects were observed or alternatively a small transient activation of channels occurred. This study suggests that protein phosphorylation is necessary for diazoxide to activate ATP-sensitive potassium channels in insulin-secreting cells.

Patch clamp; K^+ channel; Diazoxide; ATP; (RINm5F cell)

1. INTRODUCTION

Control of the gating of ATP-sensitive potassium channels (K_{ATP} channels) in insulin-secreting cells plays a fundamental role in the regulation of insulin release [1,2]. Carbohydrate-induced closure of channels results in the initiation of a depolarization of the membrane [3,4]. This depolarization is required for the opening of voltage-gated Ca^{2+} channels, which leads to a rise in the intracellular Ca^{2+} concentration, the key intracellular regulator of insulin secretion [5,6].

Potassium channel modulator compounds, especially the novel family of K^+ channel openers, are becoming increasingly important in the pharmacological control of physiological disorders [7]. The sulphonamide diazoxide causes hyperglycemia in humans, and has therefore been used to treat certain forms of pancreatic insulinomas [8]. Diazoxide has the opposite effect to glucose; hyperpolarizing the cell membrane, thereby abolishing the glucose-induced release of insulin [9]. Through the use of the patch-clamp technique it has been shown that diazoxide mediates its effects through the selective activation of K_{ATP} channels [10,11]. However, the mechanism of activation is, to some degree unknown, but appears to depend in a complex manner upon the presence of cytosolic ATP [11].

The present study was undertaken to investigate the possibility that protein phosphorylation may be important for diazoxide to activate K_{ATP} channels in insulin-secreting cells.

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Abbreviations: ATP, adenosine 5'-triphosphate; AMP-PNP, adenylyl-imidodiphosphate; AMP-PCP, methyleneadenosine 5'-triphosphate; ATPγS, adenosine 5'-*O*-(3-thiotriphosphate)

2. METHODS AND MATERIALS

All experiments were carried out upon the clonal insulinoma cell line RINm5F, cultured and prepared as previously described [4,12]. Single-channel currents [13] from ATP-sensitive K^+ channels were recorded using the open- or saponin-permeabilized cell technique, in order to deter the inherent run-down of these channels [11,14]. The patch-clamp recording pipette was filled with a Na^+-rich extracellular type solution containing (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 glucose,

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00145793/89/$3.50

10 Hepes and 1 EGTA, $CaCl_2$ was not added and the pH was maintained at 7.2 (NaOH). The bath was filled with a K^+-rich intracellular type solution containing (mM): 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 2.5 glucose, 10 Hepes and 0.5 EGTA, $CaCl_2$ was not added and the pH was maintained at 7.2 (KOH). Stock solutions of diazoxide (Glaxo Research Group, England) were prepared in dimethyl sulphoxide (DMSO), which had no effects upon K^+ channels at concentrations up to 1% [11]. The osmolality of all solutions was 290 ± 5 mOsmolal/kg. All experiments were carried out at room temperature (22–25°C).

Figures showing single-channel current records were obtained from pen-recorder traces of single-channel current data stored on F.M. tapes (Racel 4DS recorder). Upward deflections represent outward current flow (i.e. from the inside to the outside of the membrane patch). Channel open probability has been expressed as a percentage of the control or pretest level of activity. This method of quantification was preferred to that of expressing an absolute value of open-state probability since the definitive number of operational channels in a particular patch of membrane is often unknown [12].

3. RESULTS

Fig.1 shows a typical RINm5F open-cell recording, containing openings from several large and small ATP-sensitive K^+ channels [15]. Openings from these channels were abolished when 0.5 mM ATP is added to the bath solution, which is in contact inside of the plasma membrane. In the continued presence of ATP, diazoxide (200 μM) evokes a marked and sustained activation of ATP-inhibited channels. This effect of diazoxide has been quantified in 22 separate open-cell recordings in which the inside of the plasma membrane had been exposed to either 1, 0.5 or 0.1 mM ATP, summarised in fig.2. 0.1 mM ATP was added to 4 separate patches and found to close K^+ channels to varying degrees on all 12 occasions (12/12), 0.5 mM ATP was added to 14 patches (29/29) and 1 mM ATP to 4 patches (9/9). In the continued presence of ATP, diazoxide always produced a marked and sustained activation of channels; with 0.1 mM ATP 14/14 applications, 7 of which were found suitable for analysis (fig.2A), 49/49 times with 0.5 mM ATP (42 of which were analysed) (fig.2B) and 24/24 times with 1 mM ATP (10 of which were analysed (fig.2C). In agreement with our previous study [11], the action of diazoxide was more pronounced in the presence of lower (fig.2A) rather than higher (fig.2C) concentrations of ATP.

In contrast to the action of diazoxide on ATP-inhibited K^+ channels, replacing ATP with ATPγS in the continued presence of diazoxide, inhibited the response (fig.1). Adding diazoxide in the presence of ATPγS had no effect on the channels. Only when ATPγS was removed and replaced with ATP could diazoxide activate channels (fig.1). Finally when ATP is removed the level of activity recovers to the pretest value.

In 9 other open cells, where the same experiment was carried out, ATPγS always closed diazoxide-activated K^+ channels (20/20 applications). Similar effects were also obtained for AMP-PNP in 4 other patches (12/13 applications), the analysis of 6 of these applications is illustrated in fig.3A.

Adding diazoxide (200 μM) to either AMP-PNP (4 patches), AMP-PCP (4 patches) or ATPγS (14 patches)-inhibited channels had two effects. Either the sulphonamide had no significant influence upon the channels inhibited by the ATP analogue; AMP-PNP (5/12 applications), AMP-PCP (4/4 applications) or ATPγS (9/16 applications), shown for ATPγS in fig.3C. Alternatively diazoxide brought about a brief transient activation of K_{ATP} channels; AMP-PNP (7/12 applications) or

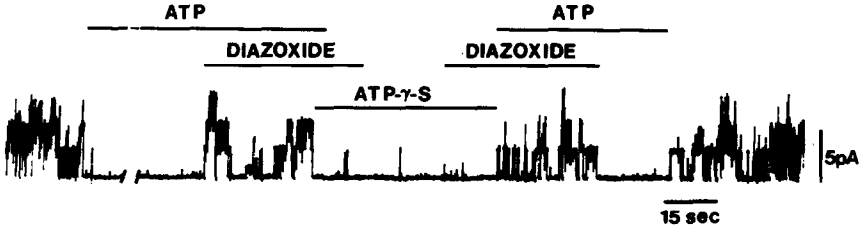

Fig.1. The effects of 0.5 mM ATP and 0.5 mM ATPγS upon 200 μM diazoxide-activated K_{ATP} channels in insulin-secreting cells. The record shown is taken from a RINm5F open cell, permeabilized with 0.05% saponin, applied to the bath solution, outside the area from which the recording is being made [4]. The break in the record corresponds to a 25 s period, during which time the membrane was continuously exposed to 0.5 mM ATP.

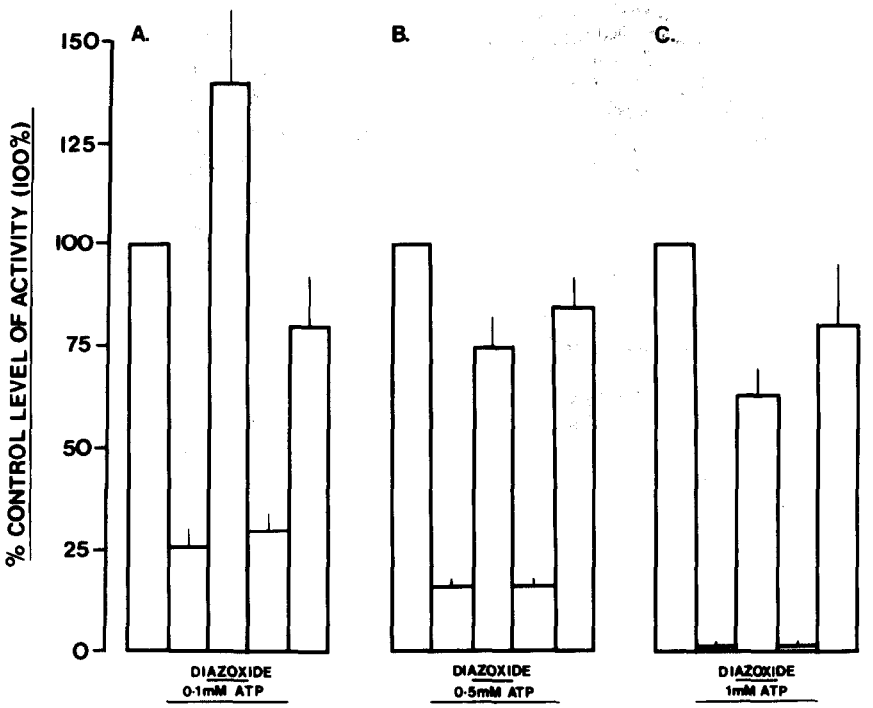

Fig.2. Comparison between the effects of diazoxide upon 0.1 mM (A), 0.5 mM (B) and 1 mM (C) ATP-inhibited K^+ channels. Changes in the activities of channels have been expressed as a percentage of the pre-control level of activity (100%). Average values have been plotted as means ± SE from 7 (A), 42 (B) and 10 (C) experiments.

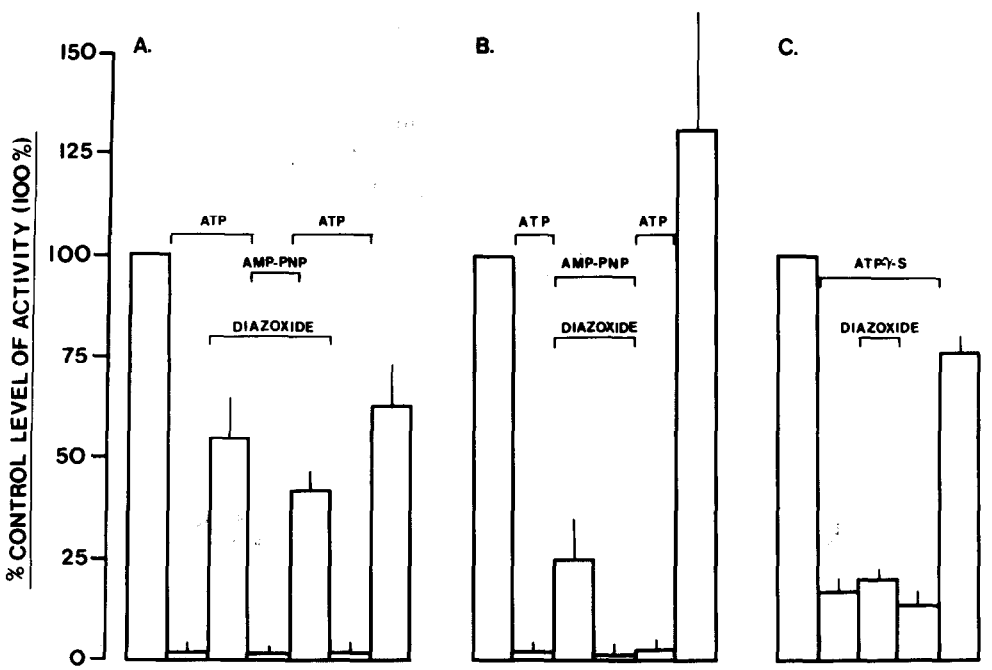

Fig.3. The effects of 1 mM AMP-PNP and 0.5 mM ATPγS on diazoxide-activated K_{ATP} channels. Average values have been plotted as means ± SE from 6 (A), 4 (B) and 9 (C) experiments.

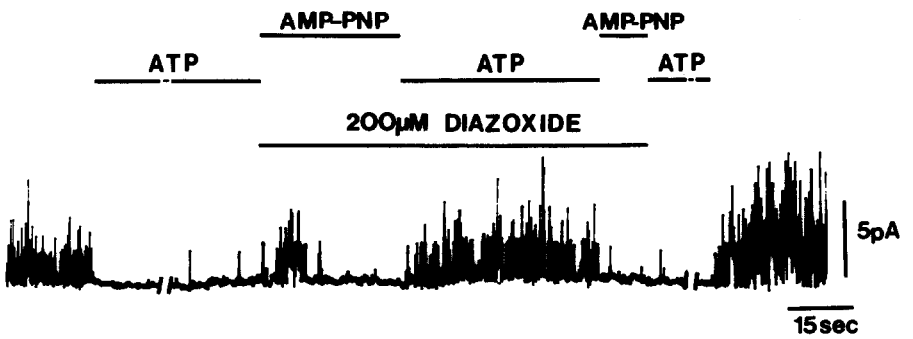

Fig.4. The action of 1 mM AMP-PNP upon 0.5 mM ATP-sensitive K^+ channels activated by 200 μM diazoxide. The breaks in the record correspond to a 20 and a 30 s period, respectively, during which time the membrane was continuously exposed to 0.5 mM ATP.

ATPγS (7/16 applications), as shown for AMP-PNP in fig.3B, where 4 of these experiments were found suitable for analysis. This transient activation of channels, appeared only to follow a period of prolonged exposure of the membrane to ATP, since as illustrated in fig.4, a second application of AMP-PNP (or ATPγS) to the membrane closed channels in a manner similar to that seen for ATPγS in fig.1.

4. DISCUSSION

The experiments presented in this study of the mechanism of activation of K_{ATP} channels by diazoxide suggest that protein phosphorylation must take place in order for the sulphonamide to open channels. In previous experiments we have shown that ATP, although required by diazoxide, will close diazoxide-activated channels [11], this finding has been confirmed (fig.2). In the continued presence of non-hydrolyzable analogues of ATP (AMP-PNP, AMP-PCP and ATPγS), diazoxide has no significant effects upon K^+ channels. However, following prolonged exposure of the membrane to ATP, a very brief transient activation of channels was sometimes observed. One possible explanation for this could be that the transient effects reflect the time delay in exchanging the analogue for ATP in the nucleotide-binding domain of the channel. Once ATP has been removed, channels are inhibited.

In conclusion these findings would tend to suggest that either (i) the binding site accessed by ATP enabling diazoxide to activate K^+ channels, cannot be reached by the analogues AMP-PNP, AMP-PCP or ATPγS or (ii) that phosphorylation of either the K_{ATP} channel or its control units is required, in order for diazoxide to bind and open the channels. Alternatively, it could be argued that since interactions between ATP and diazoxide are complex, AMP-PNP, AMP-PCP and ATPγS are more potent at inhibiting channels and therefore close diazoxide-activated channels. However, since in both these (figs 2 and 3) and in previous experiments [16], we have shown that ATP and its analogues have very similar efficacies of inhibition, this possibility can be dismissed.

Acknowledgements: I wish to thank Professor Petersen for his extremely valuable discussions and criticisms of this work. Dr B.C. Ross (Glaxo Research Group, England) for his kind donation of diazoxide. Tim Underwood for his assistance in maintaining the RINm5F cells, and Mark Houghton and Alan Higgins for their technical assistance.

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