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# Interaction of Diazoxide, Tolbutamide and $ATP^{4-}$ on Nucleotide-Dependent $K^+$ Channels in an Insulin-Secreting Cell Line

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**Summary.** The single-channel current recording technique has been used to study the effects of diazoxide, tolbutamide and ATP, separately and combined, on the gating of nucleotide-regulated $K^+$ channels in the insulin-secreting cell line RINm5F. The effects of diazoxide, tolbutamide and $ATP^{4-}$ were studied at the intracellular membrane surface, using the open-cell membrane patch configuration. Alone diazoxide was found only inconsistently to evoke channel stimulation, 57% of all applications of the drug (72 times in 48 separate patches) having no effect at concentrations between 0.02 and 0.4 mM. In the presence of ATP, however, diazoxide consistently evoked channel activation (seen 87 times in 49 patches, 95% of all applications). The interactions of diazoxide and ATP seemed competitive. Stimulation of channels by diazoxide in the presence of 1 mM ATP was suppressed if the concentration of ATP was elevated to 2 or 5 mM. In solutions in which $Mg^{2+}$ had been chelated with EDTA, diazoxide failed to activate channels closed by 1 mM ATP; however, this was not due to a direct effect on the channels caused by the absence of $Mg^{2+}$, but could be explained by the enhanced $ATP^{4-}$ concentration after $Mg^{2+}$ removal. When the total ATP concentration was lowered to give the same $[ATP^{4-}]$ in the absence of $Mg^{2+}$ to that present in the control experiments, diazoxide was able to evoke full activation. Channel inhibition evoked by tolbutamide, 0.01 to 1.0 mM, did not require the presence of either ATP or $Mg^{2+}$. In the presence of ATP tolbutamide further reduced the number of channel openings. Diazoxide was able to compete with tolbutamide for control of channel activity, an effect that was augmented by the presence of ATP. In the presence of 0.1 mM tolbutamide, diazoxide was unable to stimulate channel openings; however, if the dose of tolbutamide was lowered or ATP made available to the inside of the membrane, channel stimulation occurred.

**Key Words** $K^+$ channel · ATP · diazoxide · tolbutamide · RINm5F cell

## Introduction

$K^+$ channels regulated by the nucleotides adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), guanosine 5′-triphosphate (GTP) and guanosine 5′-diphosphate (GDP) acting from the inside of the plasma membrane dominate the electrical characteristics of resting insulin-secreting cells (Cook & Hales, 1984; Findlay, Dunne & Petersen, 1985*b*; Rorsman & Trube, 1985; Dunne et al., 1986; Dunne & Petersen, 1986*a*,*b*; Kakei et al., 1986; Misler et al., 1986; Petersen & Findlay, 1987; Ribalet & Ciani, 1987).

The nucleotides have complex actions on the $K^+$ channels. Thus ATP, which was originally thought simply to block $K^+$ channel openings in both heart (Noma, 1983) and pancreatic insulin-secreting cells (Cook & Hales, 1984), also reactivates the channels after run-down (Findlay & Dunne, 1986), and this effect, unlike the ATP-evoked inhibition, appears to be $Mg^{2+}$ dependent (Misler et al., 1986; Findlay, 1987; Ohno-Shosaku, Zunkler & Trube, 1987). ADP can activate or inhibit the ATP-sensitive $K^+$ channels dependent on the ADP dose and the state of the channels (Dunne & Petersen, 1986*a*), whereas GTP and GDP are activators of run-down ATP-sensitive channels (Dunne & Petersen, 1986*b*). Findlay (1987) concludes that the activating effects of all the nucleotides is $Mg^{2+}$-dependent. Since the reactivating effect of ATP, in contrast to the inhibitory effect, cannot be mimicked by several nonhydrolyzable ATP analogues (Ohno-Shosaku et al., 1987) and the ADP activation cannot be mimicked by the nonhydrolyzable ADP analogue adenosine 5′-O-(2-thiodiphosphate) (ADP-$\beta$S) (Dunne & Petersen, 1986*a*) it could be suggested that channel activation occurs as a consequence of phosphorylation (Ohno-Shosaku et al., 1987). However, nonhydrolyzable GTP and GDP analogues can reactivate channels (Dunne & Petersen, 1986*b*).

The ATP-sensitive $K^+$ channels can also be regulated by various sulphonamide drugs. The sulphonylurea tolbutamide, which evokes insulin secretion, closes the $K^+$ channels (Sturgess et al., 1985; Trube, Rorsman & Ohno-Shosaku, 1986) whereas diazoxide opens the same channels (Trube, Rorsman & Ohno-Shosaku, 1986). It seems possible

that there may be similarities in mechanisms of action between the inhibitory and activating effects of the various nucleotides and the sulphonamides.

Very little is known about the mechanism by which diazoxide opens $K^+$ channels, and we therefore decided to investigate this further with specific emphasis on the possibility that all channel activators might be $Mg^{2+}$ dependent. In patch-clamp experiments using the open cell-attached configuration (Maruyama & Petersen, 1984; Dunne et al., 1986) we found that diazoxide (0.4 mM), in contrast to tolbutamide, had inconsistent effects in the absence of ATP, but consistently activated channels inhibited by 1 mM Mg-ATP. Although ATP was needed in order for diazoxide activation to be observed, high ATP concentrations (5 mM) completely suppressed this effect. In $Mg^{2+}$-free or low $Mg^{2+}$ solutions, 0.4 mM diazoxide totally failed to activate channels in the presence of 1 mM ATP; however, this was not due to an effect on the channels evoked by the absence of $Mg^{2+}$, but could be explained by the enhanced $ATP^{4-}$ concentration after $Mg^{2+}$ removal. When the total ATP concentration was lowered to give the same $ATP^{4-}$ concentration in the absence of $Mg^{2+}$ to that existing in the experiments with 1 mM MgATP, the activating effect of diazoxide was fully expressed. Diazoxide appears not to be a genuine channel activator, but is able to counteract ATP-evoked channel inhibition. This effect of diazoxide is not $Mg^{2+}$ dependent.

## Materials and Methods

All experiments were carried out on the insulin-secreting cell-line RINm5F (Halban, Praz & Wollheim, 1983; Praz et al., 1983) maintained as previously described (Dunne et al., 1986). Single-channel current recordings were obtained using standard patch-clamp techniques (Hamill et al., 1981). The details of the procedures used in our laboratory have recently been described. Figure 1 illustrates the arrangement of the pipette system and the series of piped outlets (Yellen, 1982) used for permeabilization of the plasma membrane and rapid exchange of bath solution in the vicinity of the open cell. The battery of eight piped outlets was constructed from polythene tubing (Portex, England (i.d., = 0.5 mm)) and reduced manually to have a final internal bore diameter of around 150 $\mu$m. Cell permeabilization, outside the isolated patch from which single-channel recordings were made, was carried out using a solution of 0.05% saponin (wt/vol) applied from a pipette (Fig. 1) as recently described by Dunne et al. (1986).

The recording patch pipettes were filled with a $Na^+$-rich solution of the following composition (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 glucose and 10 HEPES with the pH set at 7.2. EGTA was present in a concentration of 0.5 mM. The bath solution contained (mM): 140 KCl, 10 NaCl, 2.5 glucose and 10 HEPES. No $Ca^{2+}$ was added and 1–2 mM EGTA added. In most experiments 1.13 mM $MgCl_2$ was present, but in experiments where low ($\mu$M) $Mg^{2+}$ concentrations were required either no $MgCl_2$ was present and 1 mM EGTA added or 2 mM EDTA was added. $Na_2$ ATP was used in varying concentrations as indicated in the text. The free concentrations of $Mg^{2+}$ and $ATP^{4-}$ were calculated using an iterative procedure on a BBC (model B) microcomputer using the stability constants for all the reactions between Ca, Mg, H and the various forms of ATP and EGTA or EDTA (Findlay, Dunne & Petersen, 1985*a*: Squire & Petersen, 1987). Stock solutions of tolbutamide (Sigma) or diazoxide (Glaxo) were prepared from 0.1 mM KOH or dimethylsulphoxide (DMSO). The pH of the final solutions was readjusted to 7.2 if necessary. The maximal DMSO concentration in the final solution used for the experiments was 1%. 10 experiments on 10 separate membrane patches showed that neither 0.1 or 1% DMSO had any effect on channel currents.

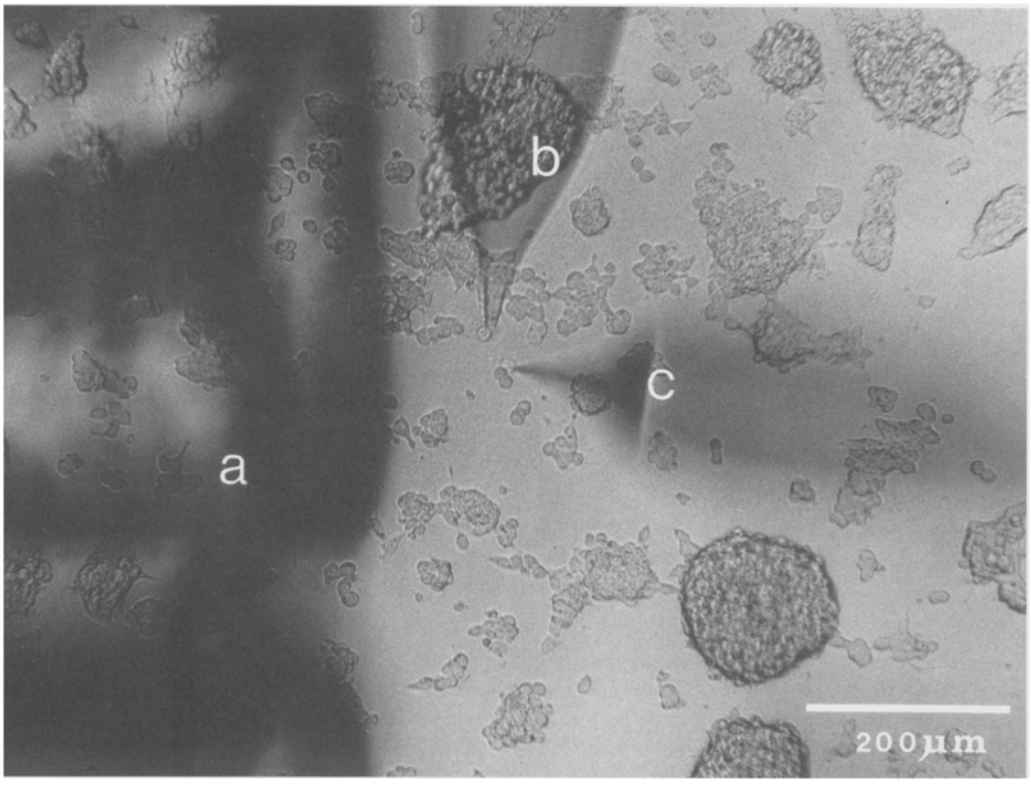
## Results

### The Effect of Diazoxide Alone

As described in a previous paper from our laboratory (Dunne et al., 1986), permeabilization of the plasma membrane, outside the isolated patch area from which single-channel recording is made, results in a large increase in $K^+$ current across the patch membrane due to wash-out of intracellular ATP, and this current can be completely blocked by adding ATP to the bath. In the absence of ATP in the bathing solution this current will gradually run down with a time course varying considerably from cell to cell. When diazoxide (0.4 mM) was added to the bath variable results were obtained. In some cases a clear increase in outward $K^+$ channel currents were seen (31 times in 22 patches), but in most cases there was no effect of the drug (41 times in 26 patches). We therefore proceeded to investigate whether more consistent effects could be obtained when the channels had first been inhibited by ATP.

### Diazoxide Activation of ATP-Inhibited $K^+$ Channels

Figure 2*A* shows the result of an experiment in which 1 mM ATP caused a very marked inhibition of the outward $K^+$ currents. When 0.02 mM diazoxide was added a clear increase in the number of channel openings was observed with a further increase following an enhancement of the diazoxide concentration to 0.4 mM. Reduction of the drug level back to 0.02 mM caused a decline in the open-state probability of the channels, and after removal of diazoxide the ATP inhibition was again fully expressed. Finally, ATP removal caused a marked in-

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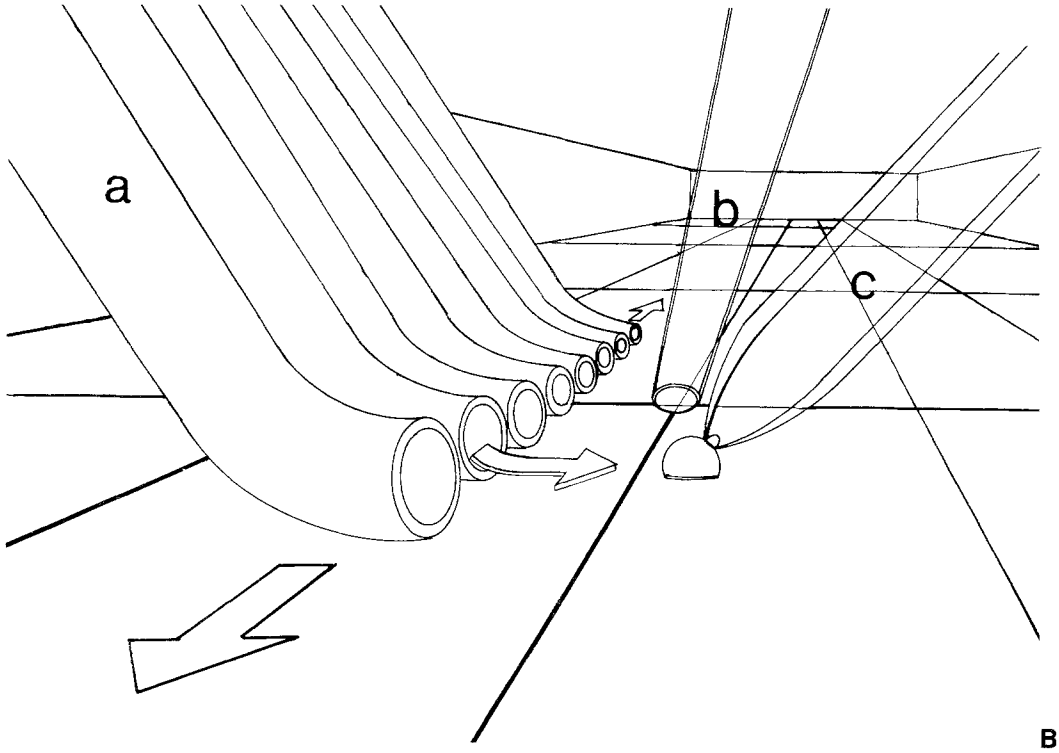


B

**Fig. 1.** Photomicrographic (*A*) and schematic (*B*) illustrations of the arrangement of the superfusion system (*a*), saponin-containing pipette (*b*) and the patch-clamp pipette (*c*) from which open-cell single-channel current events can be recorded

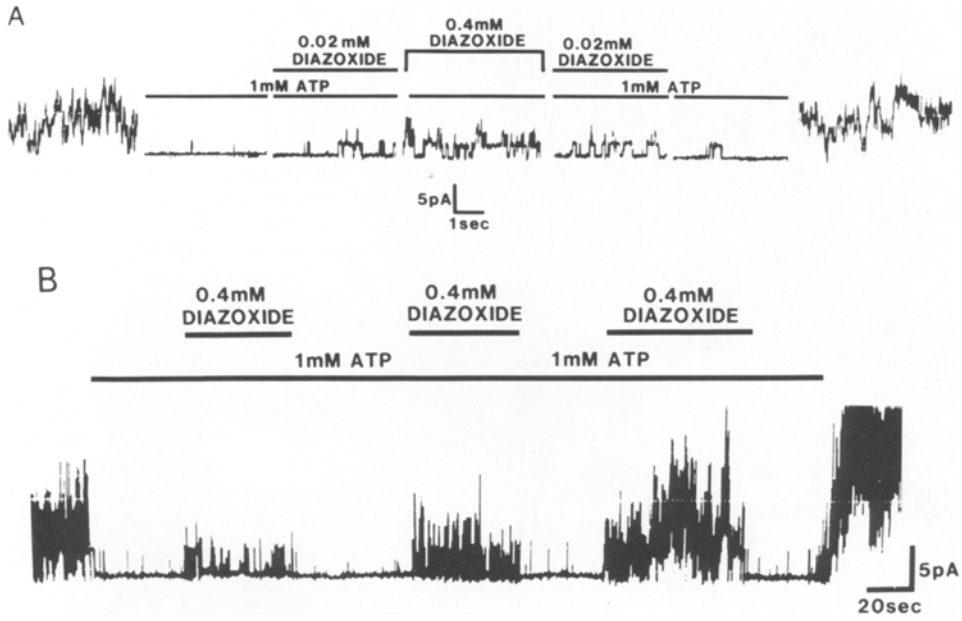


**Fig. 2.** Stimulation of ATP-inhibited potassium channels by diazoxide. Open-cell patch-clamp current recordings were obtained from two different RINm5F cells. Panel *A* shows concentration-dependent stimulation of ATP-sensitive $K^-$ channels by diazoxide in the continual presence of 1 mM ATP. The current records begin some 450 sec after permeabilization of the cell with 0.05% saponin, which yielded an initial patch current of 28 pA, subsequently decaying to around 19 pA. Interruptions in both current traces and application bars correspond to periods of 3.2, 1.2, 4.5, 4.6, 4.2 and 3.9 sec, respectively. Panel *B* shows how channel stimulation is enhanced by successive applications of diazoxide in the continual presence of ATP. The continuous current recording begins 20 min after the initial permeabilization of the cell. Initial patch current was 60 pA. In these as well as all the other experiments illustrated in subsequent figures the pipette voltage was clamped at 0 mV and the current recordings were filtered at 400 Hz (low pass). The continuous current record shown in panel *B* is taken from the same open cell as those shown in Figs. 3 and 5

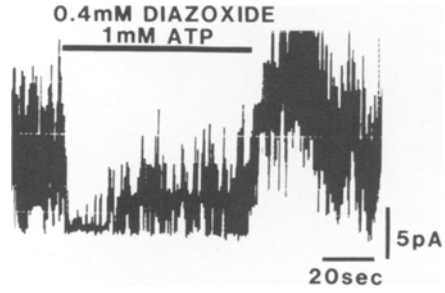


**Fig. 3.** The dual effect of ATP and diazoxide on ATP-sensitive $K^+$ channels. The record begins 28 min after permeabilization and illustrates the biphasic effect that the simultaneous application of 0.4 mM diazoxide and 1.0 mM ATP has on the $K^+$-channel currents. Initial inhibition of channel activity is followed within 20 sec by clear channel stimulation

crease in outward channel current. Dose-dependent stimulation of channels was observed in each of five applications of the drug to five separate membrane patches. In contrast to the inconsistent action of diazoxide when tested in the absence of ATP, application of 0.4 mM diazoxide in the presence of 1 mM ATP ($[ATP^{4-}] = 230\ \mu M$) caused a clear and reversible increase in channel activity 87 out of 92 times in the 49 separate membrane patches investigated. The maximal membrane patch current in a series of 10 experiments on 10 separate patches was on average reduced from 100% (in the control situation) to 12 ± 4% by 1 mM ATP and subsequently enhanced, in the continual presence of ATP, to 80 ± 8% of the control value by 0.4 mM diazoxide. The action of diazoxide was fully reversible, removal of the drug decreasing the patch-current to 11 ± 3%, a value which was subsequently increased to 100 ± 9% of the original control level of activity by the removal of ATP. In only five out of the 92 cases when diazoxide (0.4 mM) was applied were no effects observed.

Figure 2*B* shows the result of an experiment in which diazoxide was repeatedly applied after brief intervals to the same membrane patch in the continued presence of 1 mM ATP. Each successive diazoxide administration resulted in a larger increase in the outward channel currents, the maximal membrane patch current observed being increased to 41.9, 83.9 and 152.7%, respectively, of

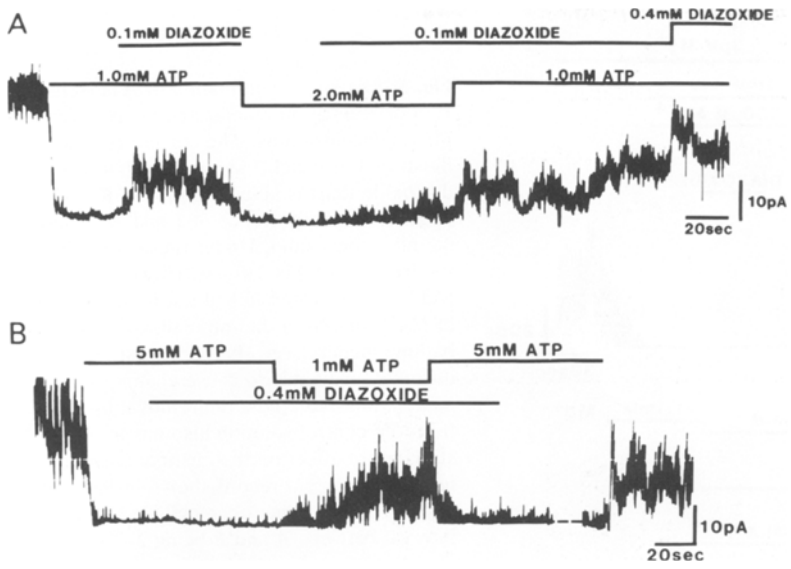


**Fig. 4.** Competitive interactions between diazoxide and ATP. Panel *A* shows competition for channel activity between 0.1 and 0.4 mM diazoxide and 1 and 2 mM ATP and Panel *B* the interaction between 0.4 mM diazoxide and 1 and 5 mM ATP. The current records shown in *A* and *B* are taken from two separate open cells beginning 5 and 600 sec after permeabilization of each cell, respectively, which evoked initial patch currents of 37 and 25 pA, respectively. In Panel *B* the period represented by the dotted line corresponds to an interval of some 50 sec during which time the cell was exposed to 5 mM ATP while the bath was drained; this part of the record has been omitted due to artifacts generated

the maximal current before ATP application (100%). Similar effects were observed in a further two separate membrane patches (four times).

Simultaneous application of ATP and diazoxide resulted in a biphasic response, consisting of an initial drastic inhibition of channel activity followed by an increase in the number of channel openings observed. Removal of the two substances caused a further marked increase in the outward current (Fig. 3). This pattern was observed in all the four experiments of this type carried out on four separate membrane patches.

Although diazoxide had more consistent activating effects on the channel in the presence of ATP than in its absence, higher concentrations of ATP reduced or abolished the diazoxide-evoked stimulation (Fig. 4). Figure 4*A* shows that 0.1 mM diazoxide evoked a much smaller degree of channel activation in the presence of 2 mM ATP than when it was applied in a period where the membrane patch was exposed to 1 mM ATP. Reduction of the ATP concentration from 2 to 1 mM in the continued presence of 0.1 mM diazoxide caused an immediate increase in the outward channel currents. Finally, a further transient increase in channel activity was observed when the diazoxide concentration was stepped from 0.1 to 0.4 mM in the presence of 1 mM ATP. In Fig. 4*B* it is seen that 0.4 mM diazoxide had no stimulatory effect in the presence of 5 mM ATP, but as soon as the ATP concentration had been reduced to 1 mM in the continued presence of the sulphonamide the usual channel activation was seen which could then subsequently be abolished by re-establishing the high (5 mM) ATP level. These effects were consistent and observed seven times in seven separate membrane patches.

## The Effect of Changing the $Mg^{2+}$ Concentration on the Diazoxide-Evoked Activation

Figure 5*A* shows the result of an experiment in which the diazoxide activation in the presence of ATP is first observed at the usual millimolar $Mg^{2+}$ concentration. Thereafter $Mg^{2+}$ is chelated by EDTA and diazoxide fails to reactivate ATP-inhibited $K^+$ channels. Later in the same experiment (Fig. 5*B*) diazoxide activation is observed again at the control $Mg^{2+}$ level, but EDTA application during continued diazoxide stimulation reversibly abolishes the diazoxide-evoked channel activation. In solutions containing 1 mM ATP and having a low ionized $Mg^{2+}$ concentration (either by EDTA chelation or omission of $MgCl_2$ and EGTA chelation) diazoxide (0.4 mM) consistently failed to evoke any increase in outward channel currents ($n$ = 20, 11 separate patches).

When ATP and diazoxide were introduced together in an EDTA-containing solution with a low ($\mu$M) $Mg^{2+}$ concentration, the ATP-evoked channel inhibition was observed alone (Fig. 6). The diazoxide-induced activation could only be brought into operation by augmenting [$Mg^{2+}$] through removal of EDTA (Fig. 6). Reducing [$Mg^{2+}$] again removed the diazoxide-evoked channel activation (Fig. 6).

The results of experiment of the type shown in Figs. 5 and 6 might indicate that the diazoxide-

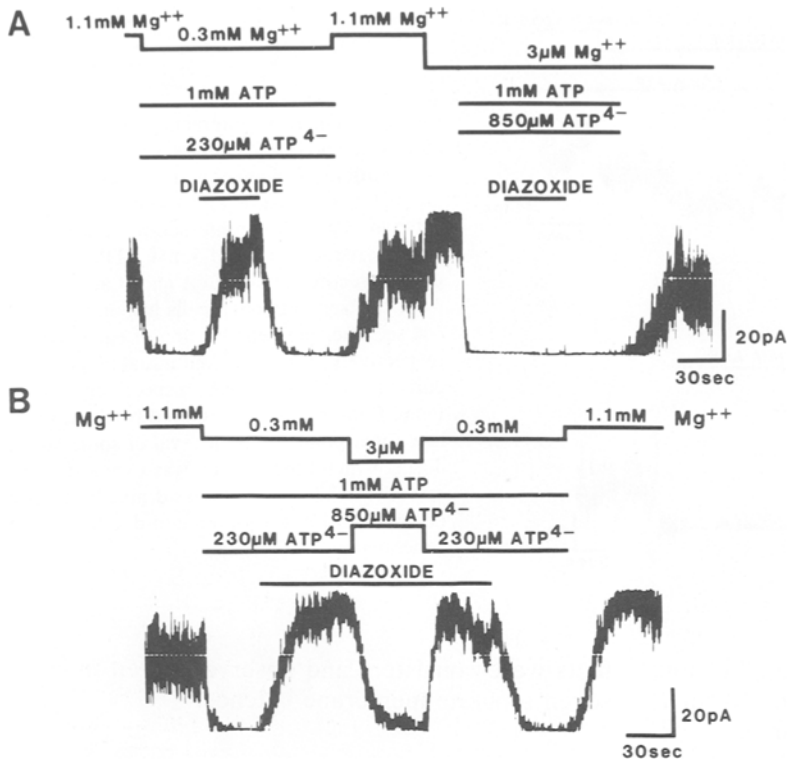


**Fig. 5.** Diazoxide stimulation of ATP-sensitive $K^+$ channels at millimolar and micromolar $Mg^{2+}$ concentrations. The current record illustrated in Panel *A* shows how 0.4 mM diazoxide (bar) is able to stimulate $K^+$-ATP channels in the presence of 1 mM ATP and 0.3 mM magnesium. Under these conditions the free $[ATP^{4-}]$ is 230 $\mu$M. However, when $[ATP^{4-}]$ is elevated to 850 $\mu$M by the removal of $Mg^{2+}$ ions from the intracellular-type bathing media (free $[Mg^{2+}]$ = 3 $\mu$M), 0.4 mM diazoxide is unable to cause channel stimulation. Note how the removal of $Mg^{2+}$ from the control solution also has a stimulatory effect on the channel currents (*see* text). The current record shown in Panel *B* is taken from the same open cell as *A*, the interval between *A* and *B* being 2–3 sec, with record *A* beginning 10 sec after permeabilization. Channel activation evoked by 0.4 mM diazoxide (bar) is only seen when $[ATP^{4-}]$ is around 230 $\mu$M, reversible inhibition of channel activity resulting if the concentration is raised to 850 $\mu$M, even in the continual presence of diazoxide

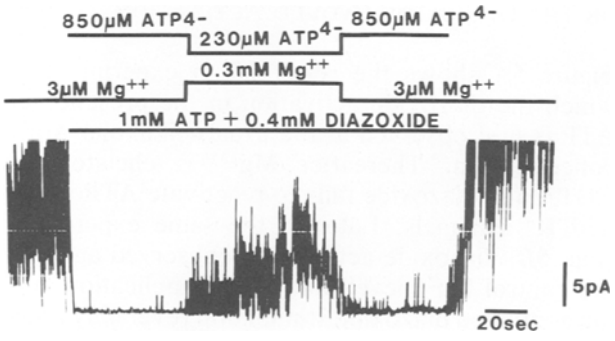


**Fig. 6.** Continuous membrane patch-current record obtained from an open-cell 150 sec after permeabilization, with an initial outward current of 32 pA. In the presence of 850 $\mu$M $ATP^{4-}$ the simultaneous application of diazoxide and ATP is unable to evoke the biphasic effects seen at lower $ATP^{4-}$ concentrations (*see* Fig. 3). Reducing $[ATP^{4-}]$ in the presence of diazoxide, via a change in the magnesium ion concentration (*see* bar) evokes reversible stimulation of channel currents

evoked activation of ATP-inhibited $K^+$ channels is $Mg^{2+}$ dependent. On the other hand, results of the type shown in Fig. 4 had shown that elevated ATP concentrations could also inhibit the diazoxide effects. As indicated by the labeling of the traces shown in Figs. 5 and 6 the free $ATP^{4-}$ concentration is substantially higher (850 $\mu$M) when $Mg^{2+}$ is chelated by EDTA than in the control situation (230 $\mu$M). It was therefore necessary to test whether diazoxide could activate channels in a low $Mg^{2+}$ solution at an $ATP^{4-}$ concentration of 230 $\mu$M.

Figure 7*A* shows that lowering $[Mg^{2+}]$ during diazoxide-evoked channel activation in such a way that $[ATP^{4-}]$ stays constant does not inhibit the diazoxide-induced effect, but in fact augments it. Later in the same experiment (Fig. 7*B*) diazoxide is seen to cause its usual activating effect, increasing the average patch current to 94% of the level of activity observed before ATP inhibition in the presence of a low $Mg^{2+}$ concentration at $[ATP^{4-}]$ = 230 $\mu$M. This effect was consistently observed in all the 14 patches investigated ($n$ = 32).

The increase in patch current following reduction in $[Mg^{2+}]$ during diazoxide stimulation (Fig. 7*A*) is also seen in the absence of diazoxide (Fig. 5*A*) and is due to an increase in single-channel conductance as well as an increase in the open-state probability (Findlay, 1987; Ashcroft & Kakei, 1987).

The results shown in Fig. 7 indicate that the diazoxide-evoked activation of ATP-inhibited $K^+$ channels is not $Mg^{2+}$ dependent. $Mg^{2+}$, however, appears to have an important indirect effect on the ability of diazoxide to induce channel activation via its influence on the free $ATP^{4-}$ concentration (Figs. 5 and 6). The already substantial literature on ATP-sensitive $K^+$ channels does not deal with the possi-

bility that it is the [$ATP^{4-}$] concentration rather than the total ATP concentration which determines the channel activity, except for a recent abstract (Ashcroft & Kakei, 1987) in which this hypothesis is supported.

Figure 8*A* shows the result of an experiment in which the total ATP concentration is varied in the presence of a constant total $Mg^{2+}$ concentration, whereas Fig. 8*B* represents the converse experiment in which the total ATP concentration is held constant (after the initial 30 sec), whereas the free $Mg^{2+}$ concentration is changed by removal and readmission of EDTA. In this typical experiment the peak patch current was reduced to 9% of the control value by 230 $\mu$M $ATP^{4-}$ and the current subsequently increased to 49 and 70% of the control value by lowering [$ATP^{4-}$] to 10 and 1 $\mu$M, respectively. In Fig. 8*B* the level of activity is increased from 17 to 64% of the control value when [$ATP^{4-}$] is lowered from 85 to 10 $\mu$M by increasing the free $Mg^{2+}$ concentration. Experiments of the type shown in Fig. 8*B*, indicating that it is not the total ATP but the $ATP^{4-}$ concentration that determines the degree of channel inhibition, were consistently observed in all the nine patches investigated ($n$ = 45).

### The Effect of Tolbutamide and Diazoxide Together

It has already been shown that tolbutamide closes ATP-sensitive $K^+$ channels (Trube et al., 1986), and this was confirmed in the present series of experiments (Fig. 9*A*). These effects were seen both in the presence of 1.1 mM $Mg^{2+}$ as well as after reduction of [$Mg^{2+}$] to about 3 $\mu$M in all the 25 patches investigated ($n$ = 63). Inhibitory effects were seen at concentrations as low as 0.01 mM (Fig. 9*C*) with virtually complete channel closure evoked by 1 mM of the drug. Although 0.1 mM tolbutamide evoked a clear but not complete inhibition of channel activity, 0.4 mM diazoxide consistently failed to reactivate channels in this situation in all the 14 patches investigated ($n$ = 14) (Fig. 9*B*). However, at a lower tolbutamide concentration (0.01 mM) diazoxide (0.4 mM) was able to cause reactivation (Fig. 9*C*).

Diazoxide-evoked activation of tolbutamide-inhibited $K^+$ channels was promoted by the presence of ATP. Figure 10*A* shows the result of an experiment in which 0.05 mM tolbutamide caused a marked inhibition of the outward patch current. 0.4 mM diazoxide failed to cause any reactivation, but addition of 0.5 mM ATP evoked a biphasic response consisting of an initial inhibition followed by reactivation. Figure 10*B* shows that when diazoxide (0.4 mM) is added in the combined presence of ATP (0.5 mM) and tolbutamide (0.05 mM) marked channel activation is observed. These effects were consistently seen in each of seven separate open cells tested ($n$ = 7).

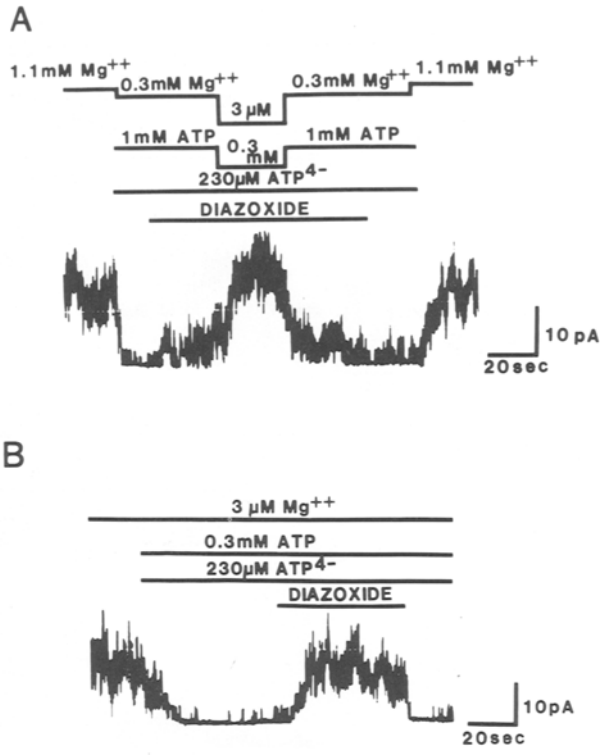


**Fig. 7.** Diazoxide stimulation in the presence of 230 $\mu$M $ATP^{4-}$. Panel *A* shows how 0.4 mM diazoxide is able to stimulate channel currents in the presence of a constant concentration of 230 $\mu$M $ATP^{4-}$. [$ATP^{4-}$] is maintained at 230 $\mu$M by either 0.3 mM $Mg^{2+}$ and 1 mM ATP or 0.3 mM ATP and low $Mg^{2+}$ (3 $\mu$M) media (*see* bars). Panel *B* shows how 0.4 mM diazoxide is able to evoke channel activation in the low $Mg^{2+}$ solution (3 $\mu$M) with [$ATP^{4-}$] = 230 $\mu$M. Both the continuous current records were obtained from the same open-cell. The start of *A* comes 200 sec after permeabilization, evoking an initial patch current of 32 pA. The interval between *A* and *B* is 30 sec

## Discussion

The oral hypoglycemic sulphonylurea tolbutamide evokes insulin release and depolarization of insulin-secreting cells (Matthews, 1985), whereas the hyperglycemic sulphonamide diazoxide reduces insulin secretion (Henquin et al., 1982) and hyperpolarizes the plasma membrane of pancreatic beta cells (Henquin & Meissner, 1982). Henquin and Meissner (1982) showed that these two drugs act by changing the membrane $K^+$ permeability, tolbutamide evoking a decrease and diazoxide causing an increase. Sturgess et al. (1985) were the first to

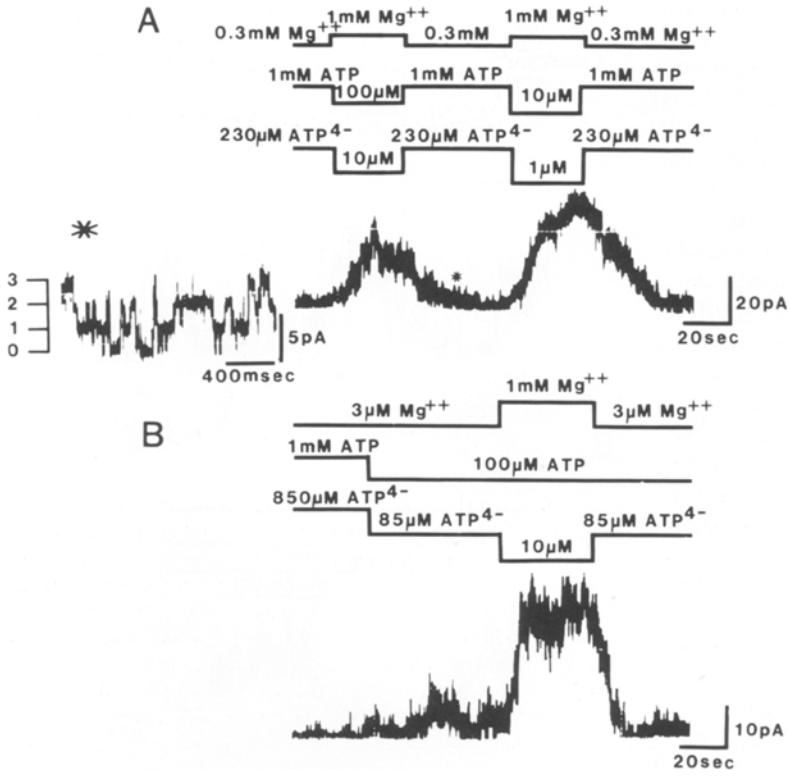


**Fig. 8.** Modulation of channel activity by changes in the concentration of free $ATP^{4-}$ ions. Panel *A* shows that channel activity can be influenced by changes in $[ATP^{4-}]$, brought about by changing the absolute concentration of ATP while maintaining millimolar concentrations of magnesium. A concentration-dependent activation of channel currents is shown when the absolute concentration of ATP is reduced from 1 mM to 100 $\mu$M and 10 $\mu$M, resulting in a decrease in $[ATP^{4-}]$ from 230 $\mu$M to 10 $\mu$M and 1 $\mu$M, respectively (*see* bars). In Panel *B* the concentration of ATP is maintained at 100 $\mu$M after the initial 30 sec and free $[ATP^{4-}]$ is varied by the addition of $Mg^{2+}$ to the bathing media (*see* bars). The resulting decrease in $[ATP^{4-}]$ from 85 to 10 $\mu$M evokes channel stimulation. Both records *A* and *B* come from the same open-cell, which had an initial patch current of 88 pA. The interval between *A* and *B* is 200 sec

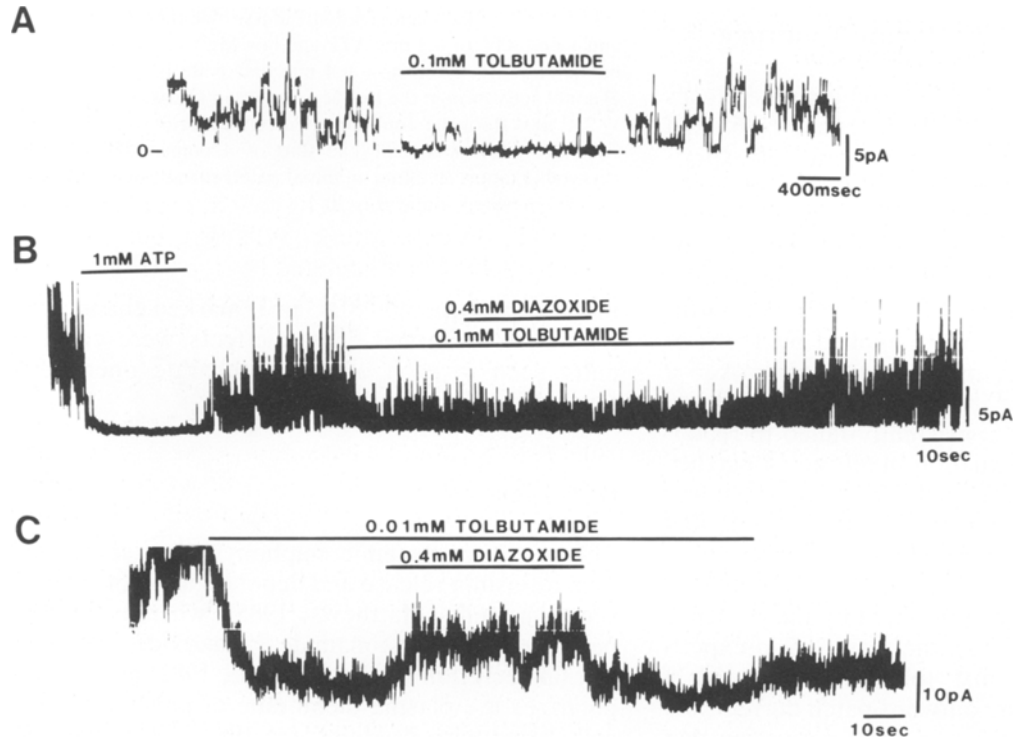


**Fig. 9.** Competitive interactions between tolbutamide and diazoxide. Records *A–C* are taken from three separate open-cell membrane patches. Record *A* shows that 0.1 mM tolbutamide is able reversibly to inhibit channel currents when applied to the intracellular surface of the membrane. The continuous traces shown in Panels *B* and *C* show that diazoxide is only able to stimulate channels when the concentration of tolbutamide is reduced from 0.1 to 0.01 mM. In record *C* the patch has undergone extensive run-down during the experiment with only around 50% of the control level of activity being redeemable upon removal of the test solutions

show that tolbutamide decreased the fractional open time of ATP-sensitive $K^+$ channels in the insulin-producing cell line CRI-Gl, a finding that was confirmed in the more elaborate study of Trube et al. (1986) on mouse pancreatic beta cells in which it was also shown for the first time that diazoxide activates ATP-sensitive $K^+$ channels.

Our study confirms that tolbutamide acts directly to close ATP-sensitive $K^+$ channels since the inhibitory effect is seen in permeabilized cells and is independent of the presence of nucleotides or $Mg^{2+}$. In contrast, diazoxide failed to consistently activate the ATP-sensitive $K^+$ channels in the absence of ATP. However, diazoxide was effective in reactivating channels that had been inhibited by ATP (Fig. 2). While ATP was required in order for the diazoxide effect to be expressed, too much ATP suppressed the response (Fig. 4). The simplest hypothesis to account for this finding might be that diazoxide acts as a competitive inhibitor of ATP, binding to a site on or near the channel, but much more work remains to be done in order to determine whether this is the correct explanation.

It has been suggested that activation of the ATP-sensitive $K^+$ channel by ADP, GTP and GDP (Findlay, 1987) as well as the rebound activation following ATP-evoked inhibition (Misler et al., 1986; Findlay, 1987; Ohno-Shosaku et al., 1987) is $Mg^{2+}$ dependent. The reactivating ATP effect could not be mimicked by nonhydrolysable ATP-analogues, and Ohno-Shosaku et al. (1987) have therefore suggested that hydrolysis of ATP may be necessary to keep the channels in a state available for opening. In relation to these findings it was therefore of interest to observe that the diazoxide-evoked reactivation of ATP-inhibited channels appeared to be $Mg^{2+}$ dependent (Figs. 5 and 6). This might seem to fit with the view that Mg-ATP is required for channel activation to occur. However, at a constant total ATP concentration removal of $Mg^{2+}$, or EDTA chelation of $Mg^{2+}$ present in the solution, causes a very substantial increase in $[ATP^{4-}]$ (Figs. 5 and 6). In view of the experiments showing a degree of competition between ATP and diazoxide (Fig. 4), a simpler hypothesis to account for the results represented by Fig. 5 would be that in the low $Mg^{2+}$ solution, $[ATP^{4-}]$ was so high that it totally suppressed the ability of diazoxide to reactivate the channels. In fact, our results show that Mg-ATP is not required in order for diazoxide to reactivate channels since stimulation of channel activity was consistently observed at very low $Mg^{2+}$ concentrations when the total ATP concentration was kept at a sufficiently low level (0.3 mM) (Fig. 7).

We have previously suggested that the ATP-binding site on the inside of the plasma membrane associated with $K^+$ channel inhibition is similar to

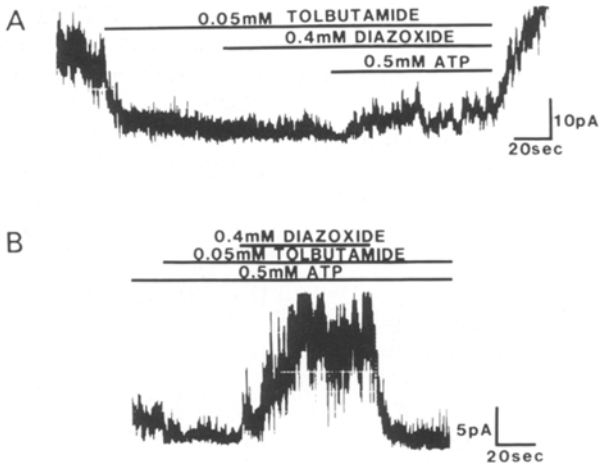


**Fig. 10.** ATP requirement for stimulation of channel currents by diazoxide in the presence of 0.05 mM tolbutamide. Panel *A* shows that tolbutamide-inhibited channels are unaffected by the presence of 0.4 mM diazoxide. Only when 0.5 mM ATP is added, in the continual presence of diazoxide and tolbutamide, does activation result. Note that ATP evokes a biphasic response, inhibition followed by stimulation, similar to that seen in Fig. 3. In Panel *B* the activity of ATP-inhibited channels is further suppressed by 0.05 mM tolbutamide. In the presence of both these agents, diazoxide evokes a clear and sustained stimulation of potassium channels. Both *A* and *B* are traces from separate open-cell membrane patches and begin 300 and 30 sec, respectively, after permeabilization—the initial patch-current recorded in each case was 40 and 34 pA, respectively

that of $P_2$ purinergic receptors present on the outside of the plasma membranes of many different cell types (Petersen et al., 1986). The effects of external ATP on these different cell types are not induced by Mg-ATP, the substrate for ATPases, but are mediated by $ATP^{4-}$ and inhibited by $Mg^{2+}$ (Dahlquist & Diamant, 1974; Cockroft & Gomperts, 1980; Steinberg & Silverstein, 1987). Our results (Fig. 8) and those recently presented in an abstract by Ashcroft & Kakei (1987) indicate that the ATP-evoked $K^+$ channel inhibition is also mediated by $ATP^{4-}$ and inhibited by $Mg^{2+}$. These findings give a new perspective to the conclusions of Misler et al. (1986), Ohno-Shosaku et al. (1987) and Findlay (1987) that $Mg^{2+}$ is required for channel reactivation by ATP and several other nucleotides, since in their experiments effects investigated in the absence and presence of $Mg^{2+}$ were not tested at the same free nucleotide concentrations.

It was a surprise to find that diazoxide was relatively inefficient in counteracting the inhibitory effect of tolbutamide in the permeabilized cells (Figs. 9 and 10), since it works very well in this respect in intact cells (Henquin & Meissner, 1982; Trube et al., 1986). Our results make it clear that it must be the presence of ATP in the intact cells that accounts

for this difference, as the presence of ATP markedly augmented the diazoxide-induced reactivation of tolbutamide-inhibited channels in the permeabilized cells (Fig. 10).

It would appear that diazoxide is not a real activator of ATP-sensitive $K^+$ channels in the sense that, for example, BK8644 is for $Ca^{2+}$ channels of the L-type (Hess, Lanman & Tsien, 1984). Diazoxide interferes directly or indirectly with the inhibitory effect of $ATP^{4-}$, which is always very marked in intact cells (Findlay et al., 1985*b*; Dunne et al., 1986) and in this way promotes channel opening.

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