# Mechanism for reactivation of the ATP-sensitive $K^+$ channel by MgATP complexes in guinea-pig ventricular myocytes

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1. A mechanism underlying reactivation of the adenosine 5′-triphosphate-sensitive $K^+$ ($K^+_{ATP}$) channels by MgATP complexes after run-down was examined in guinea-pig ventricular myocytes using the patch-clamp technique with inside-out patch configuration.
2. After run-down was induced by exposure of the intracellular side of the membrane patch to $Ca^{2+}$ (1 mM), channel activity was reactivated by exposure and subsequent wash-out of MgATP (2 mM). Addition of inhibitors of various serine/threonine protein kinases to the MgATP solution did not suppress reactivation of the run-down channels.
3. Non- or poorly hydrolysable ATP analogues were unable to reactivate run-down channels.
4. The degree of channel recovery was dependent upon the duration of MgATP exposure. The apparent half-activation value ($K_{½}$) of MgATP for reactivation was decreased with increasing exposure time.
5. Various products of ATP hydrolysis were unable to reactivate run-down channels except a relatively low concentration (100 μM) of ADP exposure.
6. Other nucleotide triphosphates, in the presence of $Mg^{2+}$, were unable to reactivate run-down channels.
7. Fluorescein 5-isothiocyanate (50 μM), which interacts with lysine residues of the nucleotide-binding site on various ATPases, inhibited $K^+_{ATP}$ channel activity. After wash-out, channel activity recovered only slightly.
8. These data suggest that the hydrolysis of ATP is important for reactivation of run-down $K^+_{ATP}$ channels but that protein phosphorylation by serine/threonine protein kinases may not be involved. Since no products of ATP hydrolysis could reproduce MgATP-induced channel reactivation and since the degree of channel recovery was dependent upon the duration of MgATP application, the hydrolysis energy appears to be utilized for channel reactivation.

Since the ATP-sensitive $K^+$ channel ($K^+_{ATP}$ channel) is distributed widely in various tissues and has known physiological importance, increasing efforts have been made to clarify the functions and regulatory mechanisms of this channel (see Ashcroft, 1988). In these studies, however, characterization of this channel has been complicated by a major problem, namely, loss of channel activity with time after patch excision in the single channel recordings or cell dialysis during whole-cell recordings. This phenomenon, usually called 'run-down', has been reported for $K^+_{ATP}$ channels from β-cells (Findlay, Dunne & Petersen, 1985*a*; Ohno-Shosaku, Zünkler & Trube, 1987), insulin-secreting cell lines (Findlay, Dunne, Ullrich, Wollheim & Petersen, 1985*b*; Sturgess, Kozlowski, Carrington, Hales & Ashford, 1988), mammalian cardiac cells (Trube & Hescheler, 1984; Kakei, Noma & Shibasaki, 1985), and amphibian skeletal muscle (Spruce, Standen & Stanfield, 1987).

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In view of the practical and perhaps pathophysiological importance of run-down, several studies addressed the subcellular mechanism for run-down and reactivation of the $K^+_{ATP}$ channel, but the exact mechanism has not been defined. Since run-down channels can be reactivated by ATP in the presence of $Mg^{2+}$, and because non-hydrolysable ATP analogues, such as adenosine 5′-($\beta$,$\gamma$-methylene) triphosphate (AMP-PCP) and adenosine 5′-($\beta$,$\gamma$-imino) triphosphate (AMP-PNP) cannot be substituted for ATP, it has been suggested that phosphorylation of this channel protein or an associated regulatory subunit may be necessary to maintain this channel in an activated state (Ohno-Shosaku *et al.* 1987; Takano, Qin & Noma, 1990). However, recent studies have raised the question of whether or not run-down and the MgATP-induced reactivation of the $K^+_{ATP}$ channels occurs via a mechanism of a reversible phosphorylation–dephosphorylation process (Findlay, 1988; Kozlowski & Ashford, 1990; Albitz, Kammermeier & Nilius, 1990; de Weille, Müller & Lazdunski, 1992). For example, the finding that the free energy of the ATP hydrolytic reaction (phosphorylation potential) does not have any influence on the activity of the $K^+_{ATP}$ channel may point against the involvement of a phosphorylation process on channel reactivation (Albitz *et al.* 1990). In the present study, therefore, we performed experiments to examine the mechanism underlying the reactivation of this channel by MgATP complexes after run-down was induced by $Ca^{2+}$. The results suggest that the reactivation of $K^+_{ATP}$ channels occurs via a unique mechanism that is different from a reversible phosphorylation process of a target protein.

## METHODS

### Preparation

Enzymatic dissociation of single ventricular myocytes from guinea-pig hearts was essentially the same as previously reported from our laboratory (Furukawa, Fan, Sawanobori & Hiraoka, 1993). Briefly, the animals were anaesthetized with pentobarbitone sodium (40–50 mg $kg^{-1}$) after heparin administration (300 i.u. $kg^{-1}$). The chest was opened under artificial respiration and the aorta was cannulated before removal of the heart. Using a Langendorff apparatus, the excised heart was first perfused retrogradely via the aorta with Tyrode solution followed by perfusion with nominally $Ca^{2+}$-free Tyrode solution until the heart stopped contraction. Enzymatic digestion was achieved by recirculating the perfusion apparatus with 0·04% (w/v) collagenase (Type I, Sigma Chemical Co., St Louis, MO, USA) dissolved in nominally $Ca^{2+}$-free Tyrode solution for 15–20 min, after which the collagenase was washed out with high-$K^+$, low-$Cl^-$ solution. The temperature and the rate of all perfusates were kept at 36–37 °C and at 10–15 ml $min^{-1}$, respectively, during coronary perfusion. All perfusates were equilibrated with 100% $O_2$. Thereafter, the atria were trimmed away, and small pieces of the ventricular tissues were dissected. After the tissues had been incubated for 5 min in a beaker containing high-$K^+$, low-$Cl^-$ solution at 37 °C, single cells were separated from tissue pieces by passing them through a 300 $\mu$m mesh. The filtrate was centrifuged at 19 *g* for 5 min, and the pellet was finally resuspended and stored in the high-$K^+$, low-$Cl^-$ solution at 4 °C for a minimum of 60 min. Cells were studied within 12 h of the isolation.

### Materials and solutions

Tyrode solution contained (mM): NaCl, 143; KCl, 4; $MgCl_2$, 0·5; $CaCl_2$, 1·8; $NaH_2PO_4$, 0·33; glucose, 5·5; *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (Hepes), 5; the pH was adjusted to 7·4 with NaOH. Nominally $Ca^{2+}$-free Tyrode solution was made by removing $CaCl_2$ from Tyrode solution, leaving the other components the same as in the Tyrode solution. High-$K^+$, low-$Cl^-$ solution contained (mM): glutamic acid, 70; taurine, 15; KCl, 30; $KH_2PO_4$, 10; Hepes, 10; $MgCl_2$ 0·5; glucose, 11; ethyleneglycol-bis-($\beta$-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 0·5; the pH was adjusted to 7·4 with KOH. We used nominally $Ca^{2+}$-free, $Mg^{2+}$-free solution for the bathing solution (artificial intracellular medium, ATP free) which contained (mM): KCl, 142; Hepes, 5; EGTA, 2; glucose, 5·5; the pH was adjusted to 7·4 with KOH. Since the contamination of $Ca^{2+}$ from various reagents in the bathing solution was at most 1·44 $\mu$M, the concentration of free $Ca^{2+}$ was estimated to be less than $1 \times 10^{-8}$ M from the apparent dissociation constants. In the case of $Ca^{2+}$-containing solution, 1 mM $CaCl_2$ was added to, and EGTA was omitted from, the bathing solution. Nucleotides were dissolved in the bathing solution as required immediately before each experiment. ATP (Sigma Chemical Co.) was used as either a dipotassium or magnesium salt. When $K_2ATP$ was added to the solution, the final $K^+$ concentration was maintained constant at 142 mM by varying KCl. Adenosine 5′-($\beta$,$\gamma$-methylene) triphosphate (AMP-PCP; lithium salt), adenosine 5′-*O*-(3-thiotriphosphate) (ATP$\gamma$S; lithium salt), adenosine 5′-($\alpha$,$\beta$-methylene) triphosphate (AMP-CPP; lithium salt), adenosine 5′-diphosphate (ADP; potassium salt), adenosine 5′-monophosphate (AMP; sodium salt), adenosine 5′-*O*-(2-thiodiphosphate) (ADP$\beta$S;trilithium salt), adenosine 3′,5′-cyclic monophosphate (cAMP; sodium salt), guanosine 5′-triphosphate (GTP; sodium salt), cytidine 5′-triphosphate (CTP, sodium salt), uridine 5′-triphosphate (UTP; sodium salt), inosine 5′-triphosphate (ITP; sodium salt), and 3′-deoxythymidine 5′-triphosphate (dTTP; sodium salt) were purchased from Sigma Chemical Co. Protein kinase inhibitor (PKI; Sigma Chemical Co.), H7, H8, KN62, W7 (all from Seikagaku Kogyo Co., Tokyo, Japan) and KT5720 (Kyowa Medex Co., Tokyo, Japan) were added to the bathing solution at final concentrations as described in the text from stock solutions in distilled water. Staurosporine (Sigma Chemical Co.) was prepared as a 1 mM stock solution in 100% (w/v) dimethyl sulphoxide (DMSO), stored at 4 °C until needed, and was added to the bathing solution at a final concentration of 5 $\mu$M. Fluorescein 5-isothiocyanate (FITC; isomer I, Sigma Chemical Co.) was prepared as a 100 mM solution in 100% (w/v) DMSO on each experimental day and was dissolved in the bathing solution at a concentration of 50 $\mu$M. FITC was protected from light with aluminum foil and used within 3 h, due to its photolability. The final concentration of DMSO contained in each solution was less than 0·05%. The catalytic subunit of cAMP-dependent protein kinase (PKA) was kindly supplied by Dr Tanaka (Mie University, Tsu City, Japan). It was purified from fresh porcine cardiac muscle by the method of Beavo & Krebs (1974). The catalytic subunit was eluted with a linear gradient of 30–300 mM potassium phosphate buffer containing 0·1 mM EGTA. Protein concentration was determined by protein

assay (Bio-Rad Laboratories, Richmond, CA, USA). The pipette solution (extracellular medium) contained (mM): KCl, 142; $CaCl_2$, 1·8; $MgCl_2$, 0·53; Hepes, 5; glucose, 5·5; the pH was adjusted to 7·4 with KOH.

### Electrophysiology

The single channel current was recorded in the inside-out configuration of the patch-clamp technique, using a patch-clamp amplifier (Axopatch, Axon Instruments Inc., Foster City, CA, USA). The temperature of the bathing solution was at a room temperature of 22–24 °C in most of the experiments, and was at 33–35 °C in some experiments, as indicated in the text. The current signals were amplified to 0·2 V $pA^{-1}$ and then stored on a video cassette recorder (HR-S 7000, Victor Co., Tokyo, Japan) via a PCM converter system (RP-882, NF Inst., Yokohama, Japan) at a conversion rate of 40 kHz. The recorded signals were filtered off-line through an 8-pole Bessel low-pass filter (48 dB $octave^{-1}$, FV-665, NF Inst.) at a −3 dB frequency ($f_c$) and digitized at 1–10 kHz onto the disk of a computer (IBM-PC/AT) using an analog-to-digital converter (CED 502, Cambridge Electronic Design, Cambridge, UK).

### Data analysis

A '50% threshold' criterion was used to detect events with the help of manual confirmation. The open probability ($P_o$) was calculated using the equation:

$$P_o = (\sum_{j=1}^{N} t_j j)/(T_d N), \quad (1)$$

where $t_j$ is the time spent at current levels corresponding to $j = 0,1,2,....N$ channels in the open state. $T_d$ is the duration of the recording and $N$ is the number of channels active in the patch. Recordings of 30–60 s were required for measurement of $P_o$.

Data are presented as means ± S.D. Student's unpaired $t$ test was used to calculate statistical significance. A $P$ value less than 0·05 was considered significant.

## RESULTS

### Protein kinase is not involved in channel reactivation

Representative tracings demonstrating spontaneous (*A*) and $Ca^{2+}$-induced run-down (*B*) are shown in Fig. 1. When the intracellular side of the membrane was exposed to the ATP-free internal solution, channel activity declined both spontaneously and gradually with time (Fig. 1*A*). When 1 mM $Ca^{2+}$ was applied to the intracellular side of the membrane, channel activity ran down quickly and did not recover upon wash-out of $Ca^{2+}$ (Fig. 1*B*). In both cases, channel activity was reactivated when the intracellular side of the membrane was superfused with a solution containing ATP (2 mM) in the presence of $Mg^{2+}$ for 3 min, and thereafter MgATP was washed out. The rate and magnitude of spontaneous run-down varied considerably among experiments, and even on the same experimental day among patches, while application of 1 mM $Ca^{2+}$ induced complete run-down within 20–30 s in every trial. Thus, most of the following analyses were performed using run-down induced by 1 mM $Ca^{2+}$.

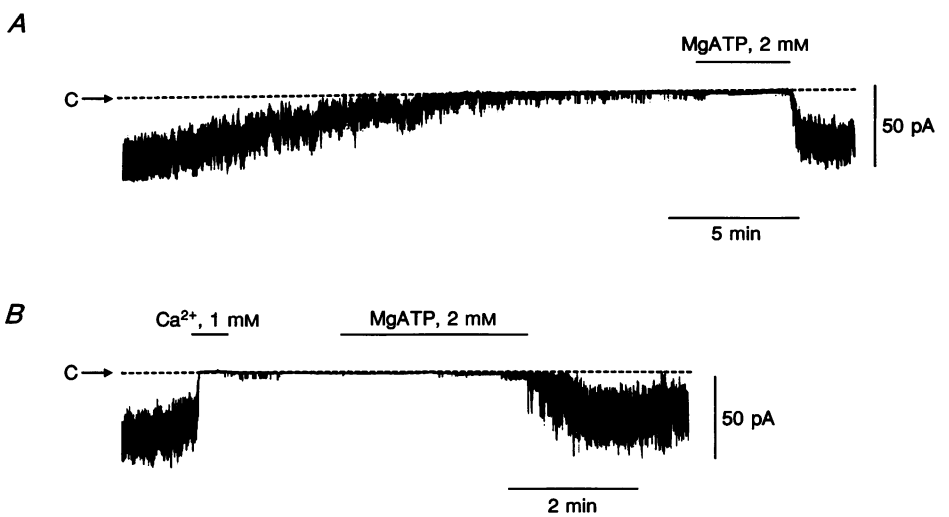


**Figure 1. Spontaneous and $Ca^{2+}$-induced run-down, and reactivation by MgATP**
Trace *A* illustrates spontaneous run-down of the $K^+_{ATP}$ channel activity and its reactivation by transient (3 min) exposure to MgATP (2 mM). Trace *B* illustrates run-down of the $K^+_{ATP}$ channel activity induced by exposure of the intracellular side of the membrane to 1 mM $Ca^{2+}$ and its reactivation by exposure to MgATP (2 mM). Records were made from cell-free inside-out membrane patches. In this and following figures, the concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-8}$ M, except during perfusion with solution containing 1 mM free $Ca^{2+}$; the membrane potential was held at −50 mV; C represents closed level for the $K^+_{ATP}$ channels; an inward current is downward; the current records were filtered at $f_c = 1$ kHz for display.

It has been suggested that the spontaneous and $Ca^{2+}$-induced run-down and MgATP-induced reactivation of the $K^+_{ATP}$ channel are due to reversible phosphorylation–dephosphorylation of the $K^+_{ATP}$ channel itself, or of an associated regulatory protein (Ohno-Shosaku *et al.* 1987; Takano *et al.* 1990). In order to test this hypothesis further, we performed a series of experiments. First, to determine whether or not a protein kinase mediates the effect of MgATP on reactivating run-down channels, we tested the effects of several serine-/threonine-type protein kinase inhibitors on reactivation of channel activity by MgATP. Figure 2*A* shows representative experiments studying the effects of PKI (1 μM), H7 (20 μM), KN62 (5 μM), and staurosporine (5 μM). After channel activity had run down completely as a result of exposure to 1 mM $Ca^{2+}$, the intracellular side of the membrane was transiently (3 min) exposed to the solution containing MgATP (2 mM) as well as each of these protein kinase inhibitors. As shown in Fig. 2*A*, channel activity could be reactivated by a transient exposure to MgATP despite the presence of these protein kinase inhibitors. In order to make a quantitative analysis, the $P_o$ values were measured between 2 and 3 min after wash-out of MgATP in the presence or absence of protein kinase inhibitors, and were normalized to the value before run-down (panel *a* in Fig. 2*B*). In the absence of a protein kinase inhibitor, $P_o$

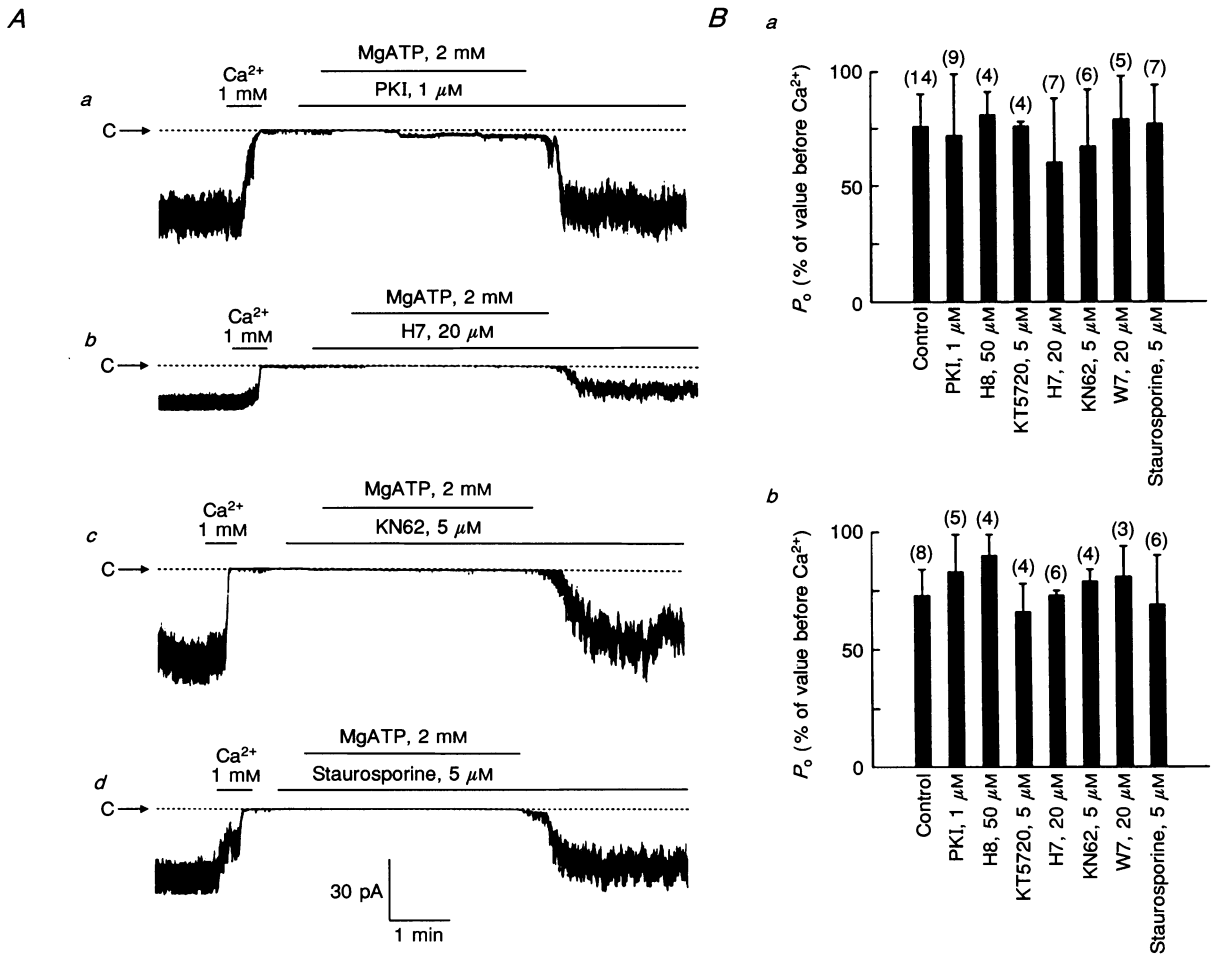


**Figure 2. Effects of inhibitors of various classes of serine/threonine protein kinases on MgATP-induced reactivation of run-down $K^+_{ATP}$ channels**

*A*, after complete run-down of channel activity was induced by exposure to 1 mM $Ca^{2+}$ for 30 s, MgATP (2 mM) was applied to the intracellular side of the membrane in the presence of 1 μM PKI (trace *a*), 20 μM H7 (trace *b*), 5 μM KN62 (trace *c*), or 5 μM staurosporine (trace *d*). Each of these protein kinase inhibitors was applied from 30 s before application of MgATP to the end of the experiment. None of these protein kinase inhibitors inhibited reactivation of the run-down channels by MgATP. *B*, the $P_o$ was measured after wash-out of MgATP (2 mM) applied in the presence of each of the protein kinase inhibitors, and was expressed as a percentage of the value before application of $Ca^{2+}$. The numbers in parentheses on top of each bar indicate the number of experiments. Panel *a* displays data obtained at a temperature of 22–24 °C, and panel *b* at a temperature of 33–35 °C.

after wash-out of 2 mM MgATP was 76 ± 14% of the value before run-down. This value was not significantly different from those when MgATP was perfused with each of the protein kinase inhibitors (1 μM PKI, 50 μM H8, 5 μM KT5720, 20 μM H7, 5 μM KN62, 20 μM W7 and 5 μM staurosporine). In order to test the possibility that the relatively low temperature (22–24 °C) of these experiments might have attenuated the effects of these protein kinase inhibitors, we also performed similar experiments at a temperature of 33–35 °C (panel *b* in Fig. 2*B*). At this higher temperature $P_o$, relative to the control value, was also not significantly affected by the absence or presence of any of the classes of protein kinase inhibitors.

We also examined an effect of the catalytic subunit of PKA on run-down channels (Fig. 3*A*). After complete run-down had been induced by exposure to 1 mM $Ca^{2+}$, the intracellular side of the membrane was superfused with a solution containing 0·25 mM MgATP in the absence or presence of the catalytic subunit of PKA (1 μg ml$^{-1}$). The addition of the catalytic subunit of PKA to the MgATP solution did not enhance the magnitude of reactivation of run-down channels. We performed similar experiments using various concentrations of MgATP, and compared the magnitude of recovery of channel activity in the absence and presence of the catalytic subunit of PKA (Fig. 3*B*). The presence of the catalytic subunit of PKA

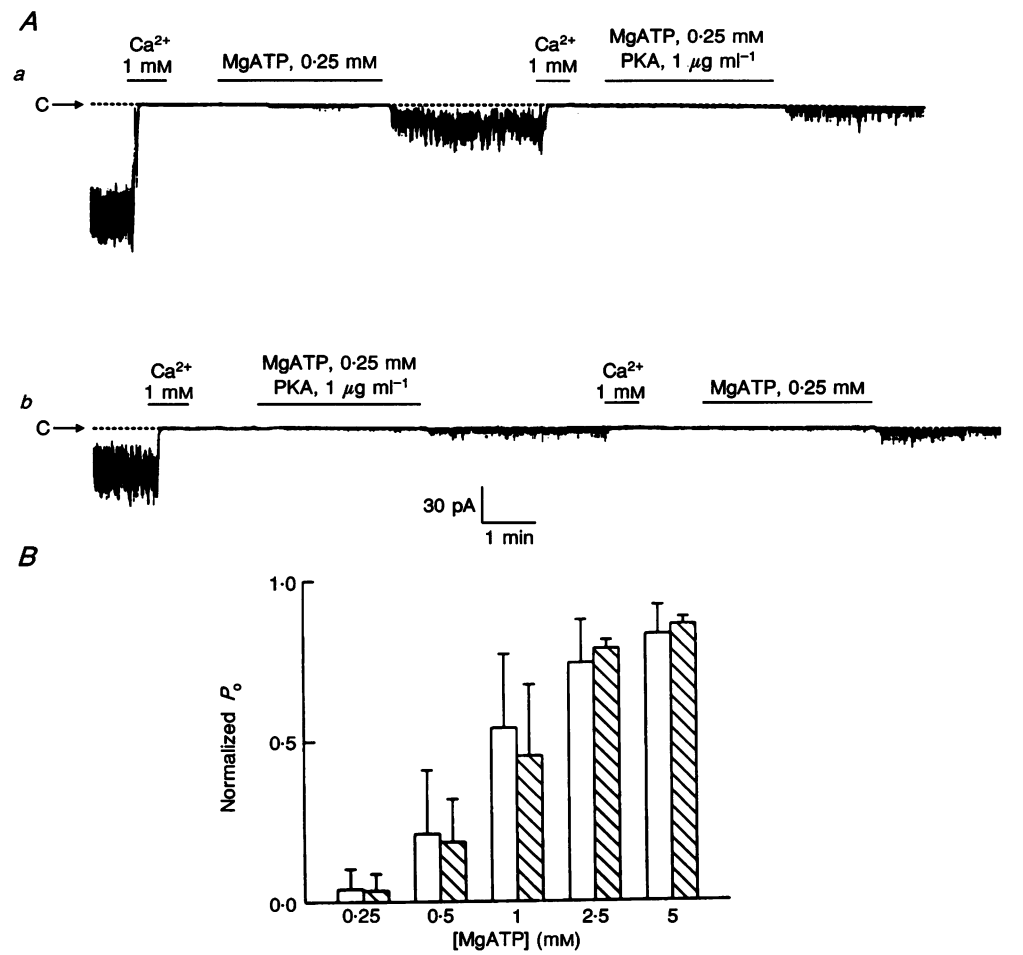


**Figure 3. Effects of the catalytic subunit of PKA on the reactivation of the run-down $K^+_{ATP}$ channels**

*A*, after complete run-down was induced by exposure to 1 mM $Ca^{2+}$, 0·25 mM MgATP in the absence or presence of the catalytic subunit of PKA (1 μg ml$^{-1}$) was applied to the intracellular side of the membrane for 3 min, and was then washed out. In panel *a*, MgATP was applied in the absence of the catalytic subunit of PKA first, and thereafter in its presence. In panel *b*, the order of the experiment was reversed. In either case, the presence of the catalytic subunit of PKA did not enhance the magnitude of reactivation of the run-down channels by 0·25 mM MgATP. *B*, a dose–response relationship for $P_o$ and the MgATP concentration for reactivating run-down channels in the absence (□) and the presence (▨) of the catalytic subunit of PKA. $P_o$ after wash-out of MgATP normalized to the value before exposure to $Ca^{2+}$ was compared in the absence and the presence of the catalytic subunit of PKA in the various concentrations of MgATP.

did not significantly affect the degree of channel recovery at any concentration of MgATP. It has previously been reported from our laboratory that the catalytic subunit of PKA harvested from porcine cardiac muscle inhibits sodium channel activity in isolated guinea-pig ventricular myocytes (Sunami *et al.* 1991). Thus, the inability of the catalytic subunit of PKA to enhance the reactivation of run-down $K^+_{ATP}$ channels in guinea-pig ventricular myocytes appears not to be due to tissue specificity of this enzyme. Furthermore, in order to exclude the possibility that the catalytic subunit of PKA had deteriorated, we confirmed its activity by showing that it still inhibited the sodium channel on each experimental day.

## Effects of ATP analogues on channel reactivation

We also tested whether or not non- or poorly hydrolysable ATP analogues could reactivate run-down channels. AMP-PCP contains a methyl group in place of two oxygens between the $\beta$ and $\gamma$ phosphate; this substitution inhibits hydrolysis. On the other hand, ATP$\gamma$S can serve as a substitute for protein kinases in the phosphorylation of target proteins, but the transferred thiophosphate is not readily removed by protein phosphatases (Eckstein, 1985). As shown in Fig. 4*A*, 2 mM AMP-PCP in the presence of $Mg^{2+}$ did not reactivate run-down channels. This finding was similar to previous reports (Ohno-Shosaku *et al.* 1987; Takano *et al.* 1990). After a transient exposure of the intracellular side of the membrane to the solution containing ATP$\gamma$S (2 mM) and $Mg^{2+}$, run-down channels were not reactivated. Inability to reactivate run-down channels was confirmed in all seven experiments for AMP-PCP and in all eleven experiments for ATP$\gamma$S.

AMP-CPP contains a methyl group between the $\alpha$ and $\beta$ phosphates. AMP-CPP can potentially act as a high-energy phosphate donor in some enzymatic reactions, as it appears to do in protein kinase reactions, while this compound is also a good non-substrate competitive inhibitor of some enzymes due to the resistance of its $\alpha,\beta$-methylene bridge to hydrolysis. As shown in Fig. 4*C*, 2 mM AMP-CPP in the presence of $Mg^{2+}$ did not substantially reactivate the run-down channels. Similar

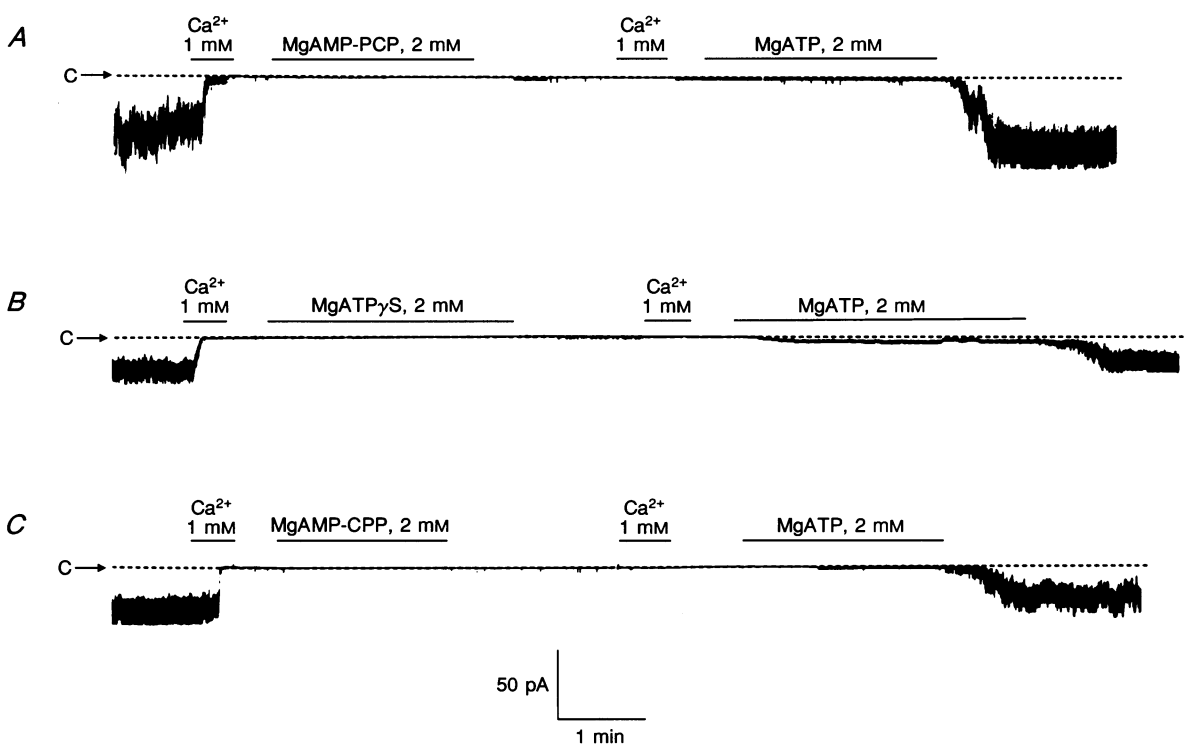


**Figure 4. Effects of various ATP analogues on reactivation of the run-down of $K^+_{ATP}$ channels**
After complete run-down was induced by exposure to 1 mM $Ca^{2+}$, 2 mM AMP-PCP (trace *A*), ATP$\gamma$S (trace *B*), or AMP-CPP (trace *C*) was applied to the intracellular side of the membrane patch in the presence of $Mg^{2+}$ for 3 min, and then washed out. None of these ATP analogues could reactivate the run-down channels. When 2 mM MgATP was applied to the patches for 3 min after failure of reactivation by these ATP analogues, MgATP could still reactivate run-down channels almost completely.

findings were observed in all eight experiments tested. AMP-PCP, ATPγS and AMP-CPP are commercially available only as the lithium salt, and $Li^+$ affects the general structure of the plasma membrane. Thus, to test whether the non-specific effect of $Li^+$ might have masked the effects of AMP-PCP, ATPγS and AMP-CPP, we examined the action of MgATP on reactivating run-down channels in the presence of 2 mM $Li^+$. Even in the presence of 2 mM $Li^+$, MgATP (2 mM) could still reactivate the run-down channels with a similar magnitude to that by 2 mM MgATP alone (data not shown).

## Time dependence of MgATP-induced channel reactivation

Data shown above appear to provide evidence, although indirect, suggesting that the MgATP must be hydrolysed to reactivate the run-down $K^+_{ATP}$ channel. If this assumption is correct, then the degree of reactivation should be a function of duration of application of MgATP. In order to test whether or not this assumption is correct, after channels had been run down by exposure to 1 mM $Ca^{2+}$, MgATP at a concentration of 0·5, 1, 2 or 5 mM was applied for various durations and the degree of reactivation was plotted against the duration of MgATP exposure (Fig. 5*A*). At any concentration of MgATP, the run-down channels recovered to a greater degree when the exposure duration was longer. Channel recovery by 2 or 5 mM MgATP appeared to reach its steady state within 3 min of MgATP application, while that by 0·5 or 1 mM MgATP appeared not to reach a steady-state level within 5 min of MgATP application.

We also constructed dose-dependent curves of channel reactivation with MgATP application of 1, 2, 3 or 5 min (Fig. 5*B*), in which $P_o$, normalized to the value before run-down, was plotted against the logarithm of the concentration of MgATP for 1, 2, 3 or 5 min exposure. The data were fitted by a least-squares analysis according to the Hill equation:

$$\text{Normalized } P_o = a - a/\{1 + ([\text{MgATP}]/K_{½})^n\}, \quad (2)$$

where $a$ is the maximum recovery of the run-down channel, $K_{½}$ is the [MgATP] causing half-maximal reactivation, and $n$ is the Hill coefficient. The $K_{½}$ was greater when the duration of MgATP exposure was shorter; it was 2·33, 1·12, 0·78 and 0·69 mM for a 1, 2, 3 and 5 min exposure, respectively. The $n$ value appeared to be smaller when the exposure time was shorter; it was 1·60, 2·0, 2·4 and 2·3 for a 1, 2, 3 and 5 min exposure, respectively. Thus, the degree of channel reactivation by MgATP appears to be a function of the duration of MgATP exposure, providing further indirect evidence suggesting that the MgATP must be hydrolysed. It may be worth noting that the maximum recovery of the run-down channel (the value $a$) was less than 1 ($a = 0·83$ for

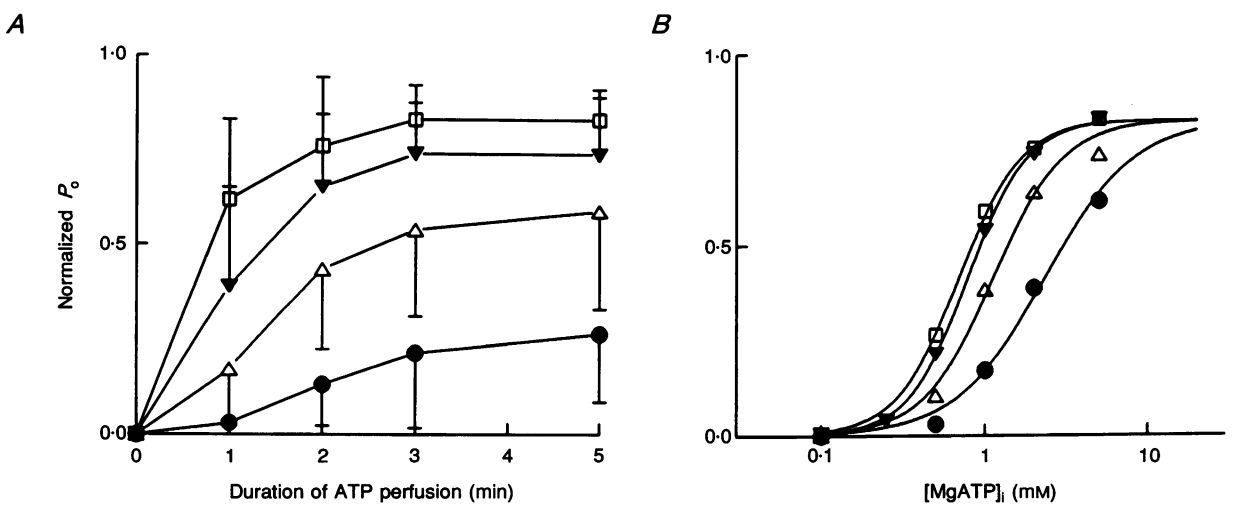


**Figure 5. Effects of changes in the duration of MgATP application on the degree of channel recovery**

*A*, the application of MgATP (at ATP concentrations of 0·5 (●), 1 (△), 2 (▼) and 5 (□) mM) was terminated at 1, 2, 3 or 5 min and the $P_o$ after wash-out of MgATP, normalized to the value before run-down, was plotted against the duration of MgATP application. At any concentration of MgATP, the run-down channel recovered to a greater degree when the duration of MgATP application was longer. *B*, dose–response curves of channel reactivation constructed by 1 (●), 2 (△), 3 (▼) or 5 (□) min exposure of MgATP. The $K_{½}$ appeared to be greater and the Hill coefficient was smaller when the duration of MgATP application was shorter.

5 mM ATP, for example). This appears to indicate that there are some other mechanisms of run-down, probably excision-induced run-down, in addition to the MgATP-dependent one.

## Products of ATP hydrolysis on reactivation of channel activity

Since hydrolysis of ATP appears to be required for channel reactivation, we next tested if products of ATP hydrolysis could reactivate the run-down $K^+_{ATP}$ channels. After complete run-down was induced by exposure to 1 mM $Ca^{2+}$ for 30 s, various combinations of products of ATP hydrolysis, $P_i$, $PP_i$, ADP and AMP, were applied to the intracellular side of patches (Fig. 6). Panel *A* displays some representative tracings. $P_i$ (trace *a*) or $PP_i$ (data not shown) did not affect channel activity during their perfusion or after wash-out. Furthermore, neither $P_i$ nor $PP_i$, when perfused with ADP and/or AMP, affected reactivation. Thus, data on reactivation in the presence of $P_i$ and/or $PP_i$ in combination with nucleotides are not shown. Application of MgADP at a concentration of 2 mM activated run-down channels only slightly during its perfusion (trace *b*). Since it has been reported that ADP at relatively low concentrations (50–500 μM) could activate run-down channels, but at high concentrations inhibited the channel (Dunne & Petersen, 1986; Kakei, Kelly, Ashcroft & Ashcroft, 1986; Lederer & Nichols, 1989; Tung & Kurachi, 1991), we also tested the effects of 100 μM

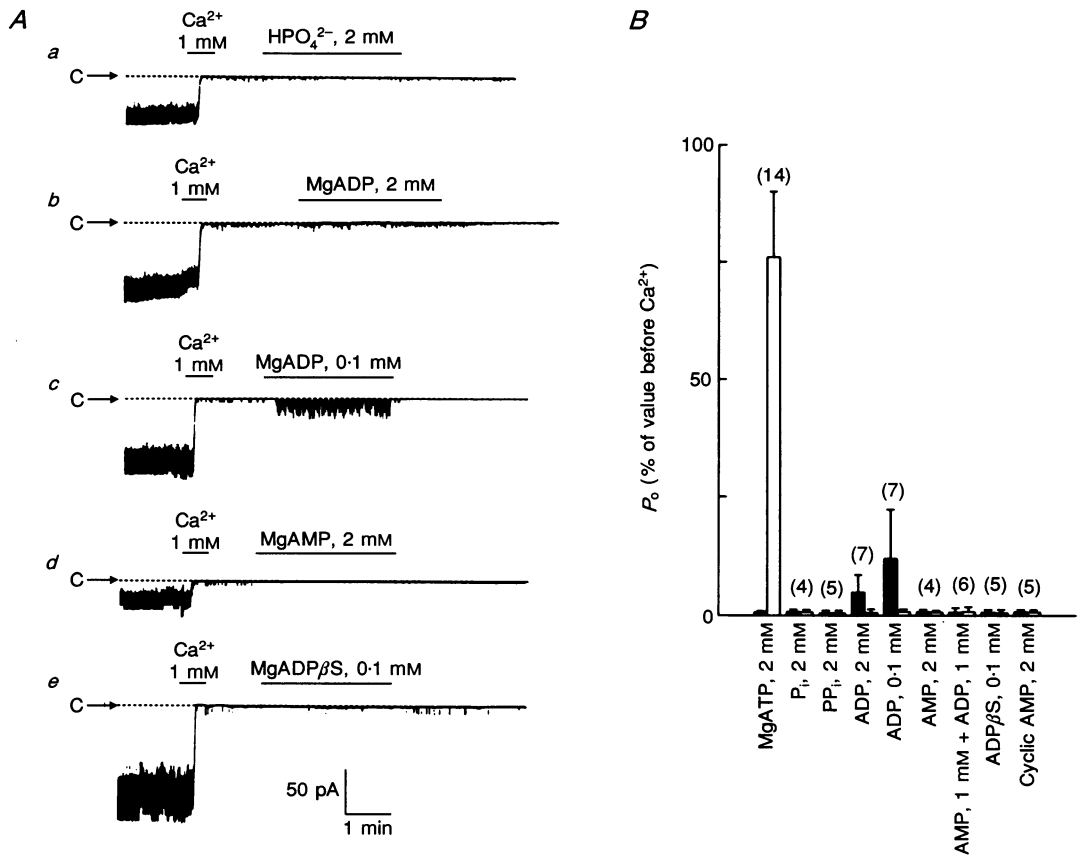


**Figure 6. Effects of various products of ATP hydrolysis on reactivation of the run-down $K^+_{ATP}$ channels**

*A*, after complete run-down was induced by exposure to 1 mM $Ca^{2+}$, various products of ATP hydrolysis were applied to the intracellular side of the membrane patch for 3 min, and were then washed out. A representative tracing for 2 mM $HPO_4^{2-}$ (trace *a*), 2 mM MgADP (trace *b*), 0·1 mM MgADP (trace *c*), 2 mM MgAMP (trace *d*), or 2 mM MgADPβS (trace *e*) is shown. Only MgADP at a concentration of 0·1 mM activated the run-down channels during its perfusion, but channel activity vanished upon wash-out of ADP. *B*, the $P_o$ was measured during perfusion with various products of ATP hydrolysis (■) and after their wash-out (□), and was expressed as a percentage of the value before exposure to $Ca^{2+}$. The numbers in parentheses on top of each bar indicate the number of experiments.

MgADP. MgADP at 100 $\mu$M activated run-down channels to a greater degree than at high concentration (2 mM); however, its activation was transient and quickly disappeared upon wash-out of MgADP (trace *c*). Application of MgAMP did not reactivate run-down channels (trace *d*). In order to test whether hydrolysis of ADP was required for the activation effect, we tested the effect of a poorly hydrolysable ADP analogue, ADP$\beta$S (100 $\mu$M), in the presence of $Mg^{2+}$. As shown in trace *e*, in contrast to ADP (trace *c*), ADP$\beta$S did not reactivate run-down channels even during its perfusion. In order to make a more quantitative analysis, we performed four to seven similar experiments for each of the ATP hydrolysis products. The mean $P_o$ values during perfusion and after wash-out of various ATP hydrolysis products were normalized to the value before run-down, and are displayed in Fig. 6*B*. Only MgADP at a concentration of 100 $\mu$M activated run-down channels during its perfusion. Although we showed that cAMP-dependent protein kinase was unlikely to mediate MgATP-induced reactivation of run-down channels, it is still possible that cAMP synthesized from ATP by the plasma membrane-bound enzyme adenylate kinase is responsible for reactivation of run-down channels independently of PKA activation. However, 2 mM cAMP in the presence of $Mg^{2+}$ did not reactivate the run-down channels during its perfusion or after its wash-out.

## Effect of other nucleotide triphosphates (NTPs)

PKA (Lemaire, Labrie & Gauthier, 1974), the insulin-like growth factor I (IGFI) receptor (Sasaki, Rees-Jones, Zick, Nissley & Rechler, 1985), and the $Na^+$,$K^+$-ATPase (Glynn & Hoffman, 1971) have a high specificity for ATP, whereas the ATP-binding cassette (ABC) superfamily (cystic fibrosis transmembrane regulator $Cl^-$ channel) has a broad nucleotide specificity with the nucleotide selectivity sequence of ATP > GTP > ITP $\approx$ UTP > CTP (Anderson, Berger, Rich, Gregory, Smith & Welsh, 1991). We also tested the nucleotide selectivity for reactivation of the run-down $K^+_{ATP}$ channels (Fig. 7). After complete run-down was induced by application of 1 mM $Ca^{2+}$ for 30 s, the intracellular side of the membrane was transiently (3 min) superfused with a solution containing 2 mM GTP, CTP, UTP, ITP or dTTP in the presence of 2 mM $Mg^{2+}$ (Fig. 7*A*). None of these NTPs could reactivate the run-down channels. In order to make a quantitative analysis, $P_o$, after wash-out of each of the MgNTPs, was normalized to the value before run-down (Fig. 7*B*). $P_o$ after wash-out of MgNTP was 1·7 $\pm$ 2·1% for GTP, 1·9 $\pm$ 2·3% for CTP, 5·9 $\pm$ 6·9% for UTP, 1·9 $\pm$ 2·1% for ITP and 4·3 $\pm$ 5·1% for dTTP. Therefore, the $K^+_{ATP}$ channel also has a high specificity for ATP for its reactivation effect.

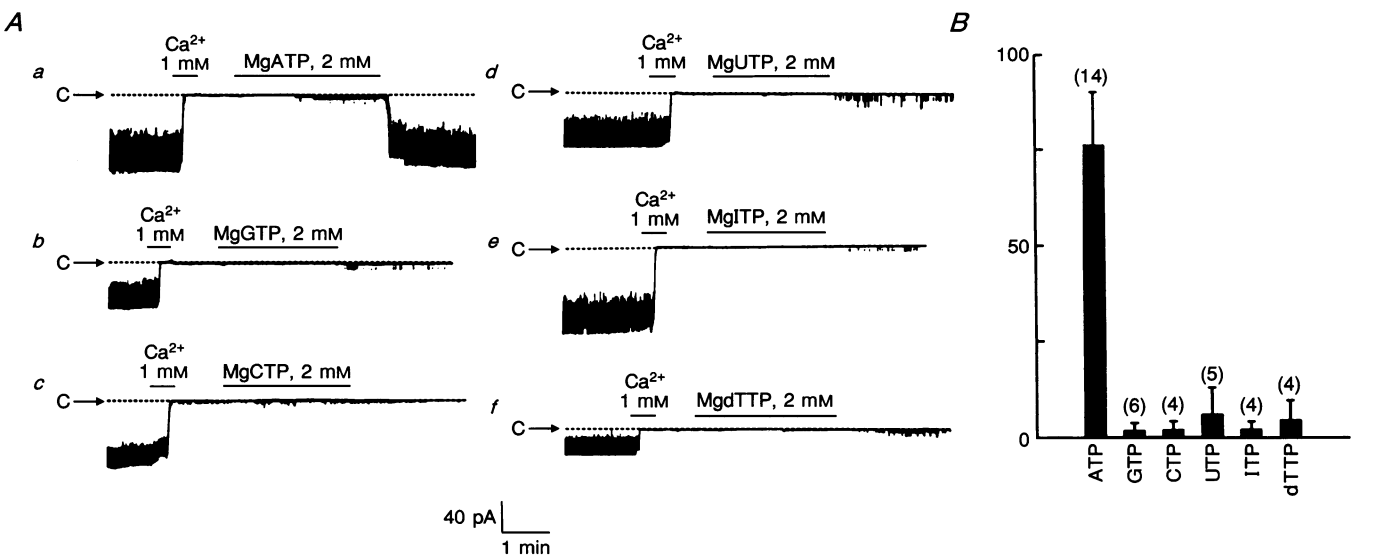


**Figure 7. Various NTPs failed to reactivate the run-down $K^+_{ATP}$ channels**
*A*, after complete run-down was induced by exposure to 1 mM $Ca^{2+}$, 2 mM ATP (trace *a*), GTP (trace *b*), CTP (trace *c*), UTP (trace *d*), ITP (trace *e*), or dTTP (trace *f*) in the presence of $Mg^{2+}$ was applied to the intracellular side of the membrane patch for 3 min, and was then washed out. None of these NTPs, other than ATP, could reactivate run-down channels. *B*, $P_o$ was measured after wash-out of each of the NTPs, and was expressed as a percentage of the value before exposure to $Ca^{2+}$. The numbers in parentheses on top of each bar indicate the number of experiments.

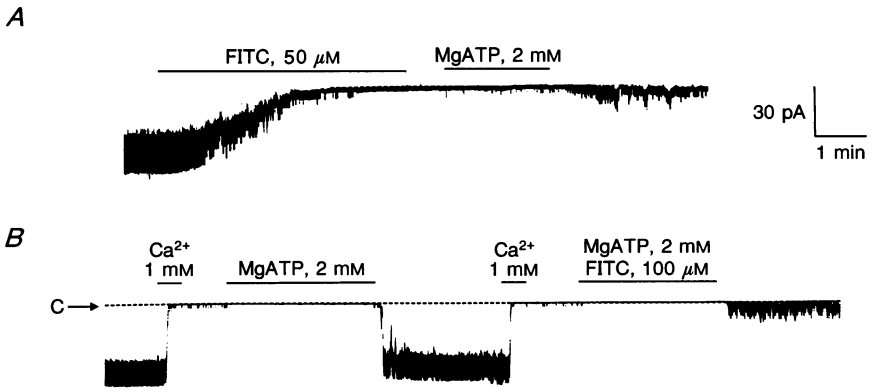


**Figure 8. FITC induced run-down of the $K^+_{ATP}$ channels and inhibited MgATP-induced reactivation**

*A*, when 50 μM FITC was applied to the intracellular side of the membrane patch containing fully activated $K^+_{ATP}$ channels, channel activity declined quickly, and did not recover on wash-out of FITC or treatment with 2 mM MgATP. *B*, when the intracellular side of the membrane was superfused with solution containing 2 mM MgATP in the presence of 50 μM FITC after channel run-down was induced by 1 mM $Ca^{2+}$, recovery of channel activity was much less than that found with MgATP in the absence of FITC.

## Effects of FITC

FITC is known to react with the nucleotide-binding sites on various ATPases with a high specificity for ATP (Muallem & Karlish, 1983; Scott, 1985; Farley & Faller, 1985; Abbott, Amler & Ball, 1991). Since reactivation of the $K^+_{ATP}$ channel also showed a high specificity to ATP (Fig. 7), we studied the effects of FITC on $K^+_{ATP}$ channel activity. As shown in Fig. 8*A*, FITC (50 μM), when applied to the intracellular side of $K^+_{ATP}$ channels before run-down, induced channel inactivation, and the rate of run-down was apparently much faster than the spontaneous run-down. Channel activity did not recover after wash-out of FITC, and recovered only slightly by treatment with MgATP (2 mM) for 3 min. These findings were observed in eleven of the fourteen patches tested. When FITC was applied to the run-down channels with 2 mM MgATP, reactivation by MgATP was suppressed (trace *a* in Fig. 8*B*). In the presence of 50 μM FITC, an average reactivation by 2 mM MgATP in eleven patches (23 ± 19%) was significantly less than that by 2 mM MgATP in the absence of FITC (76 ± 13%; $P < 0.01$).

## DISCUSSION

This study provides data elucidating a possible mechanism underlying the reactivation of the $K^+_{ATP}$ channels by MgATP complexes after run-down was induced by exposure to $Ca^{2+}$. None of the inhibitors of serine/threonine protein kinases suppressed the reactivation effect of MgATP. The degree of channel recovery was dependent on the duration of MgATP exposure, and the non- or poorly hydrolysable ATP analogues could not reactivate the run-down channels. Furthermore, various products of ATP hydrolysis could not reactivate the run-down channels as MgATP complexes did. Thus, the hydrolysis of ATP, probably the free energy produced by ATP hydrolysis, appears to be important for reactivation of the run-down channels.

In the present study, the run-down of the $K^+_{ATP}$ channel was exclusively caused by the application of $Ca^{2+}$, and then it was reversed by MgATP complexes. In some experiments, we also tested for spontaneous run-down, and found a similar tendency between $Ca^{2+}$-induced and spontaneous run-down. For example, none of the protein kinase inhibitors suppressed MgATP-induced channel recovery from spontaneous run-down. However, as suggested by the finding that reactivation of run-down channels by MgATP was less than complete for both spontaneous and $Ca^{2+}$-induced run-down, there appears to be some other mechanism of run-down in addition to the MgATP-dependent one; at least an excision-dependent run-down suggested by Takano *et al.* (1990) might be involved. Since complete spontaneous run-down is a lengthy process, it should be influenced by the excision-dependent run-down to a greater degree than the $Ca^{2+}$-induced one would be. Thus, in the present study, we exclusively utilized the $Ca^{2+}$-induced run-down to study the mechanism of the MgATP-dependent channel recovery. We have shown previously that a protein phosphatase inhibitor, okadaic acid, did not inhibit spontaneous or $Ca^{2+}$-induced run-down of this channel (Furukawa *et al.* 1993), which appears to argue against the idea that $Ca^{2+}$ causes run-down through a dephosphorylation process. It has been reported that most divalent cations can induce channel

run-down, with $Ca^{2+}$ being most potent, followed by $Mg^{2+}$ and $Co^{2+}$ (Findlay, 1988; Kozlowski & Ashford, 1990). No known protein phosphatases are activated by divalent cations, further supporting the idea that the dephosphorylation process may not be involved in run-down of the $K^+_{ATP}$ channel. It may be more reasonable to assume that the binding of divalent cations to the $K^+_{ATP}$ channel itself or to the regulatory protein(s) induces a conformational change, resulting in the inactivation of the channel, and that the binding affinity with the protein differs among divalent cations.

If a conformational change was induced by $Ca^{2+}$, then how can it be reversed by MgATP? It was previously shown that the degree of reactivation was a function of the duration of MgATP application (Takano *et al.* 1990). The present study also demonstrates that the degree of channel reactivation is very dependent upon the duration of MgATP exposure. At any concentration of ATP examined, the run-down channel recovered to a greater degree when the duration of MgATP application was longer. At a concentration of 2 or 5 mM, the time course of channel recovery appears to follow an exponential function, as suggested by Takano *et al.* (1990). The apparent $K_{½}$ and the Hill coefficient also varied with the duration of MgATP application. These findings appear to be in accordance with the hypothesis that the hydrolysis of ATP is required for removal of the $Ca^{2+}$ inhibition. AMP-PCP and AMP-CPP, ATP analogues that cannot be hydrolysed at the oxygens between the $\beta$ and $\gamma$ phosphates and between the $\alpha$ and $\beta$ phosphates, were not able to substitute for ATP in channel reactivation. These findings are still only indirect evidence for a requirement of ATP hydrolysis because the insertion of a methyl group at these sites could sterically affect the binding of non-hydrolysed ATP. Nonetheless, if one combines these observations with the findings that the degree of reactivation was dependent upon the duration of ATP exposure, it may be likely that the hydrolysis of ATP at the oxygens between the $\beta$ and $\gamma$ phosphates and between the $\alpha$ and $\beta$ phosphates is important in channel reactivation.

The possible involvement of ATP hydrolysis in channel recovery suggests at least three possible models of channel recovery for consideration: (i) one or more ATP hydrolysis products are directly responsible for channel recovery; (ii) hydrolysis energy might cause conformational change of the protein, resulting in the removal of the $Ca^{2+}$-induced channel inhibition; or (iii) the phosphorylation of a target protein is responsible for the removal of the $Ca^{2+}$ inhibition. We tested the effects of various ATP hydrolysis products alone or in combination on the run-down $K^+_{ATP}$ channels, and failed to reproduce MgATP-induced channel reactivation. The possible involvement of the third hypothesis was evaluated from various viewpoints. All of the inhibitors of the serine/threonine protein kinases examined failed to block the MgATP-induced recovery of the run-down channel. The exogenously applied catalytic subunit of PKA did not affect the magnitude of reactivation. ATP$\gamma$S, a good substrate for serine/threonine protein kinases, could not replace ATP in channel reactivation. These data did not completely exclude the possibility of the phosphorylation of a target protein as a mechanism of channel reactivation because it is impossible to show conclusively that the protein kinase inhibitors or the exogenously applied catalytic subunit of PKA gains access to the necessary sites in the membrane. Furthermore, although it is generally suggested that ATP$\gamma$S can serve as a substrate for most serine/threonine protein kinases in the phosphorylation of target proteins (Eckstein, 1985), it is also possible that the presence of a thiol side chain at the $\gamma$-position could change the affinity for the protein kinase. Nevertheless, since all the findings are incompatible with this hypothesis, it is unlikely that the phosphorylation of a target protein by known serine/threonine protein kinases is responsible for the reactivation of run-down $K^+_{ATP}$ channels. Therefore, the second hypothetical model may be most likely. In this model, ATP is hydrolysed, producing free energy; this hydrolysis energy is then utilized to cause a conformational change of channel proteins, and a new thermo-kinetically unfavourable form of the protein could then activate the $K^+_{ATP}$ channel. Albitz *et al.* (1990) previously failed to demonstrate any influence of the Gibbs free energy (enthalpy) of ATP hydrolysis on the steady-state activity of the $K^+_{ATP}$ channels. However, the effect of the reactivating MgATP is presumably not in a steady state, unless one assumes very slow unbinding kinetics, since reactivation is observed following removal of the reactivating MgATP. Thus, the data by Albitz *et al.* do not necessarily argue against the importance of hydrolysis energy for the reactivation effect of MgATP.

FITC reacts with the lysine residue of the nucleotide-binding sites on various ATPases with high specificity for ATP over other NTPs, such as $Ca^{2+}$-ATPases in the sarcoplasmic reticulum (Scott, 1985) or erythrocyte (Muallem & Karlish, 1983), $Na^+,K^+$-ATPases (Abbott *et al.* 1991), and $H^+,K^+$-ATPase (Farley & Faller, 1985), inhibiting ATP binding to these sites and their enzyme activity. Since reactivation of the run-down $K^+_{ATP}$ channels also showed a high specificity for ATP (Fig. 7), we tested the effect of FITC on this channel. FITC induced irreversible inactivation of the $K^+_{ATP}$ channel and inhibited the reactivation effect of MgATP complexes. An inwardly rectifying potassium channel has recently been cloned from rat kidney, which contained a Walker A-motif, a common motif for the ATP-binding site, and recovered from run-down on application of MgATP (Ho *et al.* 1993). The Walker A-motif contains a lysine residue in its structure and it is suggested that this lysine residue plays a key role in electrostatic bonds between the negatively charged ATP and the positively charged

lysine. For MgATP-induced reactivation of the $K^+_{ATP}$ channel, it may also be possible that the binding of MgATP to a site that is not related to the channel closure by ATP ('allosteric interaction') may be necessary and that a lysine residue plays a key role in this allosteric interaction. However, although it is suggested that FITC reacts with the lysine residue of the ATP-binding sites of many ATP-binding proteins, no direct evidence was provided for the possibility that FITC induced inactivation of the $K^+_{ATP}$ channel by interacting with the lysine residue of the ATP-binding site. More direct evidence, probably full sequencing of the $K^+_{ATP}$ channel and identification of a possible ATP-binding site using a mutation technique, is needed to justify the importance of the allosteric interaction of MgATP to the channel protein for channel reactivation.

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