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# MODIFICATION OF THE ADENOSINE 5′-TRIPHOSPHATE-SENSITIVE $K^+$ CHANNEL BY TRYPSIN IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. The adenosine 5′-triphosphate (ATP)-sensitive $K^+$ channel current was recorded in guinea-pig ventricular myocytes using the patch clamp technique with inside-out patch configuration. Modification of the channel activity by intracellular application of an endoprotease trypsin was studied, and was related to a possible model of regulation of this channel.

2. Maximal ATP-sensitive $K^+$ channel activity was observed immediately upon formation of inside-out patches in the ATP-free internal solution, thereafter activity declined both spontaneously and gradually with time; a phenomenon known as run-down. When trypsin (1 mg/ml) was applied to the intracellular side of the membrane upon formation of inside-out patches, spontaneous run-down did not occur, and this trypsin action was irreversible. Neither trypsin (1 mg/ml) applied with trypsin inhibitor (0·25 mg/ml) nor heat-denatured trypsin (1 mg/ml) could mimic this effect. When trypsin was applied to the patches after run-down, channels were reactivated at $\sim$ 13 min.

3. Treatment with trypsin did not affect unitary current amplitude, channel gating kinetics, or sensitivity to intracellular ATP.

4. Intracellularly applied $Ca^{2+}$ induced run-down of channel activity in a dose-dependent manner. In membrane patches that were treated with trypsin (1 mg/ml) for 20 min, intracellularly applied $Ca^{2+}$ up to 1 mM did not induce run-down of channel activity.

5. Intracellular application of an exopeptidase, carboxypeptidase A (1 mg/ml), but not Leu-aminopeptidase (0·5 mg/ml), prevented spontaneous or $Ca^{2+}$-induced run-down of channel activity.

6. As postulated for several other channels, such as $Na^+$ and $Ca^{2+}$ channels, there may be a possible 'chemical gate' that is responsible for run-down of this channel activity. Application of trypsin might somehow modify this 'chemical gate', resulting in prevention of spontaneous or $Ca^{2+}$-induced run-down. This target site for trypsin may be situated on the carboxy-terminus of the channel proteins, or of associated regulatory units. Because ATP sensitivity remained intact after trypsin

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treatment, the trypsin-selective site for channel inhibition is not related physically to the ATP binding site.

## INTRODUCTION

Potassium-selective single ion channels that are inhibited by increased adenosine 5′-triphosphate (ATP) concentration in the internal surface of the cell membrane (ATP-sensitive $K^+$ channels) have been described in cardiac muscle cells (Noma, 1983; Trube & Hescheler, 1984), skeletal muscle cells (Spruce, Standen & Stanfield, 1985), pancreatic $\beta$-cells (Cook & Hales, 1984; Findlay, Dunne & Petersen, 1985), neurons (Ashford, Sturgess, Trout, Gardner & Hales, 1988), and recently in arterial smooth muscle cells (Standen, Quayle, Davies, Brayden, Huang & Nelson, 1989). Because of its wide distribution in various tissues and possible physiological importance (see Ashcroft, 1988), increasing efforts have been made to clarify the function and regulatory mechanism of this channel.

The amino acid sequences of several types of $K^+$ channels have been identified. In the *Shaker* $K^+$ channel, it was found that the channel protein contains one internal domain extending through the phospholipid bilayer, which consists of six segments (Tempel, Papazian, Schwarz, Jan & Jan, 1987). Other parts of the protein chain were situated at the outer or inner side of the membrane and may be responsible for the regulation of the channel activity. Although at present, the amino acid sequence of the ATP-sensitive $K^+$ channel has not been identified, a similar channel protein structure has been assumed for this channel. Thus, one might expect that intracellular perfusion with proteolytic agents would affect amino acid residues at the inner side of the membrane and thereby modify the regulation of the channels. Chemical modification using various proteolytic reagents has been used to elucidate structure–function relations of several sarcolemmal ion channels. For $Na^+$ or $Ca^{2+}$ channels, intracellular application of proteolytic agents, such as pronase and trypsin, has been reported to delay inactivation of $Na^+$ and $Ca^{2+}$ currents, possibly by removing a 'chemical' inactivation gate that is normally controlled by phosphorylation (Armstrong, Bezanilla & Rojas, 1973; Hescheler & Trautwein, 1988). Similar proteolytic modification has been reported for the *Shaker* $K^+$ channel (Hoshi, Zagotta & Aldrich, 1990), the $Ca^{2+}$-activated $K^+$ channel in paramecium (Kubalski, Martinac, Saimi & Kung, 1989), and the muscarinic $K^+$ channels in atrial myocytes (Kirsch & Brown, 1989). To characterize the properties of the ATP-sensitive $K^+$ channel modified by an endoprotease, trypsin, we performed patch-clamp experiments using cell-free membrane patches isolated from guinea-pig ventricular myocytes. The observed effects on the ATP-sensitive $K^+$ channel were related to a possible model of regulation of this channel.

## METHODS

### *Preparation*

Enzymatic dissociation of single ventricular myocytes from guinea-pig hearts was essentially the same as previously reported from our laboratory with minor modifications (Hirano & Hiraoka, 1988). Briefly, the animals were anaesthetized with pentobarbitone sodium (40–50 mg/kg) after heparin administration (300 i.u./kg). 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 until the coronary effluent

was free of blood, 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., Ltd, St Louis, MO, USA) dissolved in nominally $Ca^{2+}$-free Tyrode solution. Enzymatic perfusion was continued until the solution flowed freely (15–20 min), after which the collagenase was washed out with high-$K^+$, low-$Cl^-$ solution. The perfusate temperature was maintained at 36–37 °C and the coronary perfusion rate was maintained at 10–15 ml/min. 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 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 after the isolation.

*Solutions*

The 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. The nominally $Ca^{2+}$-free Tyrode solution was made by removing $CaCl_2$ from Tyrode solution, with other components the same as in the Tyrode solution. The 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; ethylene glycol-bis (*β*-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0·5; the pH was adjusted to 7·4 with KOH. The bathing solution (artificial intracellular medium, ATP free) contained (mM): KCl, 142; Hepes, 5; EGTA, 2; glucose, 5·5; the pH was adjusted to 7·4 with KOH. Concentration of free $Ca^{2+}$ in the bathing solution was estimated to be $1 \times 10^{-7}$ M and that of free $Mg^{2+}$ to be $1 \times 10^{-6}$ M from the apparent dissociation constants (Fabiato & Fabiato, 1979). Nucleotides were added to the bathing solution as required. ATP (Sigma Chemical Co., Ltd, St Louis, MO, USA) was used as either 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. For solutions which required $Ca^{2+}$ at a concentration of 1, 5 or 10 $\mu$M, mixtures of EGTA and $CaCl_2$ were used as calculated from the apparent dissociation constants (Fabiato & Fabiato, 1979). In the case of solution containing $Ca^{2+}$ at a concentration of 100 $\mu$M or 1 mM $Ca^{2+}$, 100 $\mu$M or 1 mM $CaCl_2$ was added to and EGTA was omitted from the bathing solution. Trypsin (from porcine pancreas, type II), trypsin inhibitor (type II-S), carboxypeptidase A (type I-PMSF), adenosine 5'-O-(3-thiotriphosphate) (ATP$\gamma$S) were obtained from Sigma Chemical Co., Ltd (St Louis, MO, USA). Leu-aminopeptidase was obtained from Koch-Light Ltd (Haverhill, Suffolk, UK). These were dissolved directly in the bathing solution on each experimental day to obtain a final concentration as described in the text. Okadaic acid (Wako Chemical, Osaka, Japan) was prepared as a 1 mM stock solution in dimethyl sulphoxide (DMSO), stored at 4 °C until needed, and was added to the bathing solution at a final concentration of 10 $\mu$M. The final concentration of DMSO contained in each solution was less than 0·05%. 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.

*Recording methods*

A few drops of the isolated single myocytes were introduced to a recording chamber (0·5 ml in volume) placed on the stage of an inverted phase-contrast microscope (Diaphot TMD, Nikon Co., Tokyo, Japan) and were allowed to settle to the bottom (5 min). The recording chamber was then continuously superfused with filtered bathing solution at a rate of 3 ml/min. In our system, the bath solution could be replaced completely within 1 min by switching from one solution to another. Single rod-shaped cells having smooth surfaces with explicit striations were selected for electrical measurements as described below. All the experiments were performed in the inside-out patch configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) at a room temperature of 22–24 °C. Single channel currents were recorded using a patch-clamp amplifier (Axopatch, Axon Inst. Inc., Burlingame, CA, USA). The current signals were amplified to 0·2 V/pA 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, FV-665, NF Inst., Yokohama, Japan) 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 Ltd, Cambridge, UK).

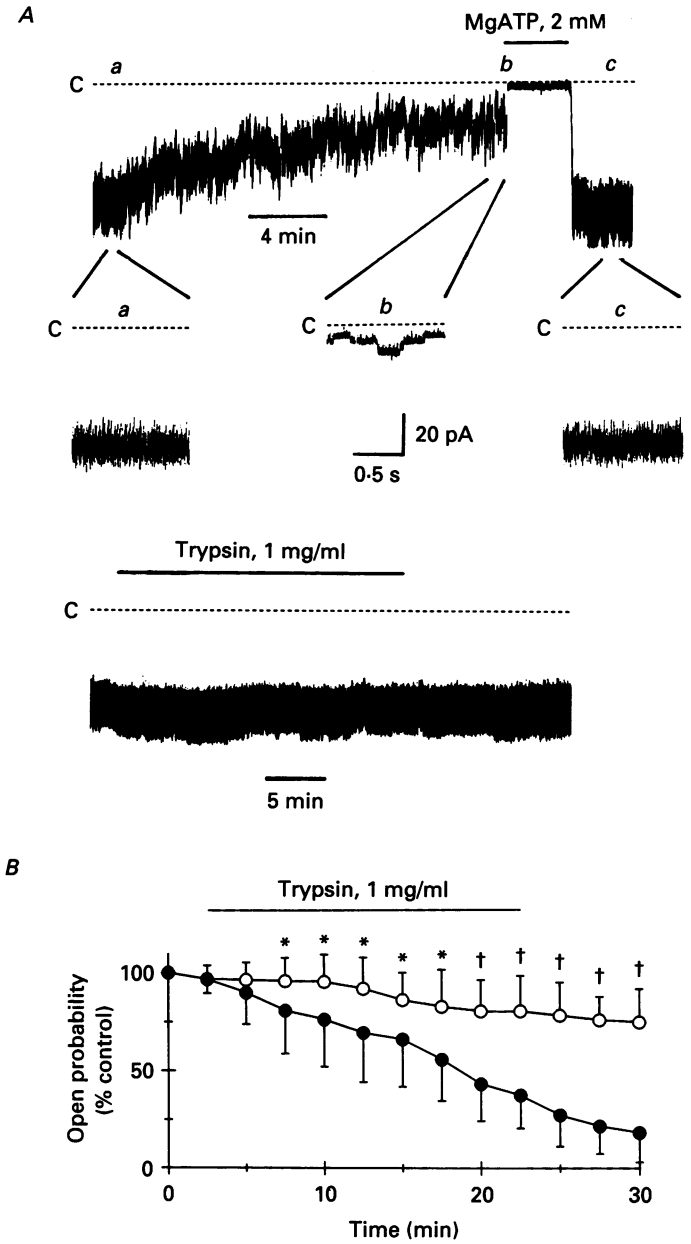


Fig. 1. Effect of intracellularly applied trypsin on spontaneous run-down. *A*, the top trace illustrates the run-down of ATP-sensitive $K^+$ channel activity and its reactivation by exposure to MgATP in patches in which the intracellular side of the membrane was superfused with bath solution containing no trypsin. Expansions of the records at the times marked by *a*, *b*, and *c* are shown in the middle panel. The bottom trace illustrates a continuous recording of ATP-sensitive $K^+$ channels during superfusion of the intracellular side of membrane with bath solution containing trypsin (1 mg/ml), and after wash-out of trypsin. No recognizable run-down of channel activity occurred during and after wash-out of trypsin. Records were made from a cell-free inside-out membrane patch. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M.

*Data analysis*

A '50% threshold' criterion was used to detect events with the help of manual confirmation. Open and closed times were measured from records where only a single channel was active. Each distribution histogram of open or closed time was formed from continuous recordings of more than 60 s. A simplex method of least-squares analysis (Nelder & Mead, 1965) was applied for fitting a probability function with a form of single or double exponentials of open or closed times. In order to increase the number of observations, an overall estimate of mean open time was also applied to the records from multiple-channel patches (Fenwick, Marty & Neher, 1982).

The open probability ($P_o$) was calculated using the equation derived by Spruce *et al.* (1985):

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

where $t_j$ is the time spent at current levels corresponding to $j = 0, 1, 2, \ldots N$ 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 $P_o$; longer recording times were required for greater $N$. In the early experiments, when more than one channel was active in a patch, we estimated $N$ by means of the maximum-likelihood method (Patlak & Horn, 1982). The estimated $N$ was not different from that derived from inspection of the record in ATP-free solution at positive potentials where $P_o$ was around 1·0.

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

## RESULTS

### *Trypsin prevented spontaneous run-down*

Application of trypsin at a concentration of 1 mg/ml to the intracellular side of membrane patch substantially delayed run-down of channel activity. Maximal ATP-sensitive $K^+$ channel activity was observed immediately upon formation of inside-out membrane patches in the ATP-free internal solution; thereafter activity declined both spontaneously and gradually with time, a phenomenon known as run-down. The rate of run-down varies considerably among studies, and even in the same study among different patches. Under experimental conditions in which no divalent cation was added to the intracellular solution and $Ca^{2+}$ was chelated with EGTA, most of the patches (around 80%) had a relatively consistent and gradual rate of run-down, while the rest of patches (around 20%) had a more rapid rate of run-down. This difference in rate of run-down became quite apparent 2·5 min after formation of inside-out patches. In 52/263 patches (19·8%), run-down of channel activity occurred rapidly as defined by $P_o$ being less than one-third of the control value after 2·5 min. In these patches channels generally lost all activity after 10 min. In the majority of patches (211/263, 80·2%) $P_o$ was greater than two-thirds of the control value after 2·5 min, and run-down was more gradual. In order to compare the rate

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The membrane potential was held at −50 mV. C, closed level for the ATP-sensitive $K^+$ channel in this and following figures. Inward current is downward. The current records were filtered at $f_c$ = 1 kHz for display. *B*, the probability of channel opening measured at a membrane potential of −50 mV every 2·5 min after excision of membrane patch. ○, control ($n$ = 7); ●, trypsin treated ($n$ = 7). Measurement was made from a recording of 30–60 s and the open probability is expressed as a percentage of the value immediately upon excision of membrane patch. The time period when trypsin (1 mg/ml) was superfused is indicated as a bar at the top of the graph. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. The bars associated with data points in this and following figures represent S.D. *$P < 0·05$ between control patches and trypsin-treated patches; †$P < 0·01$.

of run-down between control patches and patches treated with trypsin, only the patches whose $P_o$ at 2·5 min was more than two-thirds of the control value were used. A representative tracing showing run-down in these patches is shown at the top of Fig. 1*A*. Twenty minutes after the patch excision, the channel activity decreased to 31·2% of the level immediately after the patch excision. This loss of channel activity was almost completely reversed when the patch was superfused with 2 mM MgATP for 3 min followed by wash-out of the MgATP. In the bottom trace in Fig. 1*A*, exposure of the intracellular surface of another membrane patch to trypsin (1 mg/ml) was initiated 2·5 min after formation of the inside-out patch. There was very little decline of channel activity with time, and the activity remained almost constant up to the end of the 20 min superfusion with trypsin. After the superfusate was changed to that without trypsin, run-down of channel activity was not apparent for more than 20 min, suggesting that this effect of trypsin was irreversible. $P_o$ was measured at a membrane potential of −50 mV every 2·5 min after patch excision in patches with ($n = 7$) and without ($n = 7$) exposure to trypsin. $P_o$ was calculated from a 30–60 s recording period, and was normalized to the $P_o$ value calculated from a recording period of 30–60 s immediately after patch excision (Fig. 1*B*). In membrane patches without trypsin treatment, the $P_o$ 20 min after patch excision was 43·2 ± 19·1% of the control value; that in patches treated with trypsin was 80·2 ± 16·8% ($P < 0·01$). Trypsin at a concentration of 1 mg/ml was included in the pipette solution in another six experiments. The rate of decline of channel activity was not slowed (data not shown), suggesting no effect of trypsin when it was applied to the extracellular side of membrane. We also tested the effect of trypsin applied to the intracellular surface of the patches that had a rapid rate of run-down, that is those for which channel activity became less than one-third of the control value after 2·5 min. Trypsin, however, was not as effective in preventing run-down for these patches; for these patches the $P_o$ after 20 min was 0·4 ± 0·5% of the control value.

To examine whether the effect of trypsin in preventing spontaneous run-down of channel activity was due to proteolysis of the channel or some associated protein, we performed two types of control experiments. Firstly, we added a saturating concentration (0·25 mg/ml) of trypsin inhibitor, and the $P_o$ was measured every 2·5 min and was normalized to the $P_o$ obtained immediately after patch excision (Fig. 2*A*). Trypsin inhibitor by itself did not affect channel activity, and subsequent application of trypsin at 1 mg/ml in the presence of trypsin inhibitor did not prevent spontaneous run-down of channel activity. Secondly, trypsin was heat-denatured by boiling at a temperature of 100 °C for 10 min. As shown in Fig. 2*B*, channel activity declined with time during superfusion of the intracellular side of the membrane with denatured trypsin at a concentration of 1 mg/ml, and the rate of decline of channel activity was comparable to that observed in the absence of trypsin. The protective effect of trypsin against spontaneous run-down of channel activity, therefore, correlated with its enzymatic activity.

To test the question of whether treatment with trypsin could reactivate channels that had run down, trypsin at a concentration of 1 mg/ml was applied to the bath solution after the channel had run down spontaneously. Figure 3 shows a representative experiment in which the addition of trypsin to the bath solution of a run-down channel reactivated channel activity at ~ 13 min after application of

trypsin. A similar finding was observed in four of the seven patches, in which the effect of trypsin on the run-down channel was examined.

*Trypsin did not change unitary conductance*

Application of trypsin to the intracellular side of membrane patch did not affect the conductance of single channel current of the ATP-sensitive $K^+$ channel. The

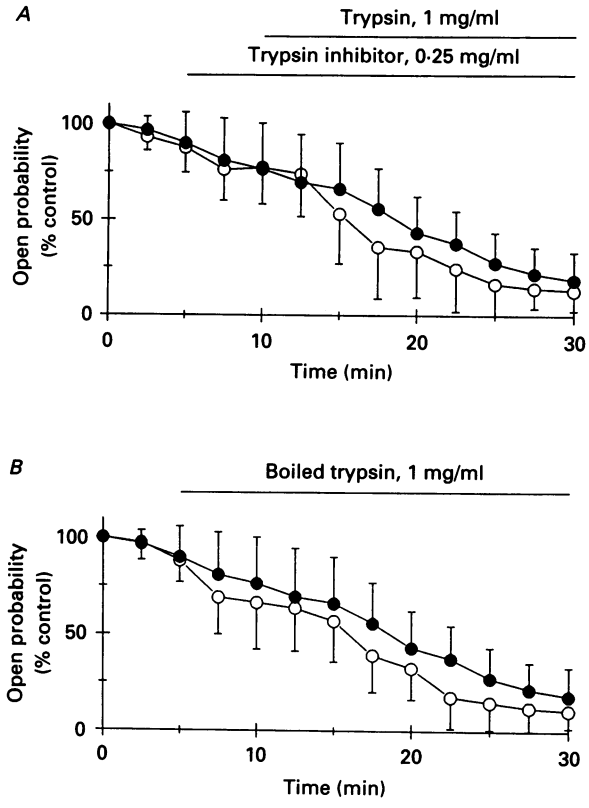


Fig. 2. Effects of trypsin applied with trypsin inhibitor (*A*) and heat-denatured trypsin (*B*). *A*, the probability of channel opening was measured at a membrane potential of −50 mV every 2·5 min after excision of the membrane patch. ●, control ($n = 7$); ○, trypsin inhibitor ($n = 4$). Measurement was made from a recording of 30 s duration and the open probability is expressed as a percentage of the value immediately upon excision of the membrane patch. The time periods when trypsin inhibitor (0·25 mg/ml) and trypsin (1 mg/ml) were superfused are indicated as bars at the top of the graph. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Application of trypsin (1 mg/ml) in the presence of trypsin inhibitor (0·25 mg/ml) did not significantly affect the time course of decline in open probability compared to that in control patches. *B*, the effect of boiled trypsin on time course of decline in open probability. The time period when heat-boiled trypsin (1 mg/ml) was superfused is indicated as a bar at the top of the graph. ●, control ($n = 7$); ○, boiled trypsin ($n = 3$). The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Application of boiled trypsin (1 mg/ml) did not significantly affect the time course of decline in open probability compared to that in control patches.

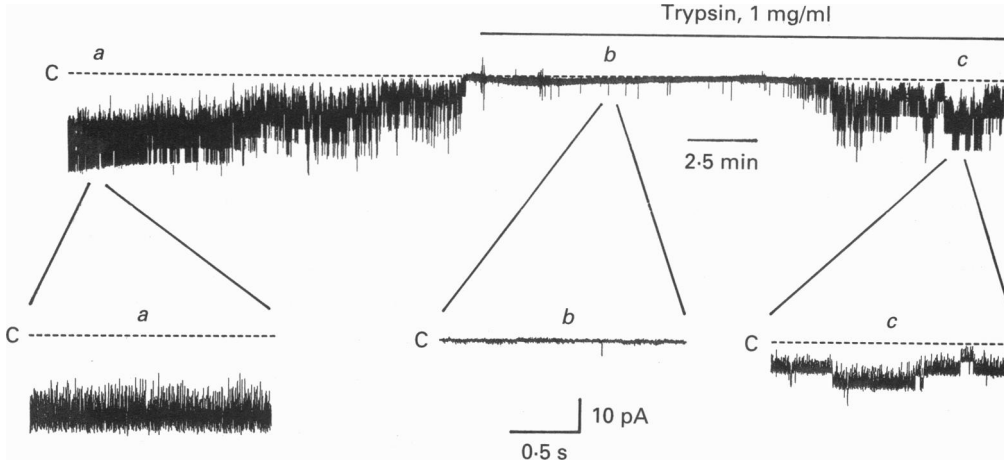


Fig. 3. Effect of trypsin applied to the run-down channels. Upper trace illustrates the effect of intracellularly applied trypsin (1 mg/ml) to the patches whose channel activity was run down after long exposure to ATP-free solution. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Trypsin reactivated the run-down channel activity in ~ 13 min. Expansions of the records at the times marked by *a*, *b*, and *c* are shown in the lower traces. Membrane potential was held at −50 mV. The current records were filtered at $f_c = 1$ kHz for display.

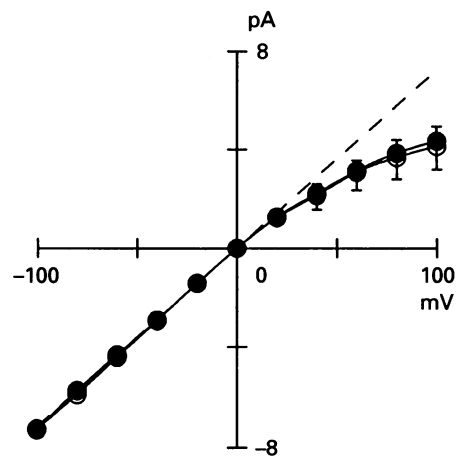


Fig. 4. Effect of trypsin on the *I–V* relationship. *I–V* curves were obtained before (●) and after (○) treatment with trypsin in seven patches. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Intracellularly applied trypsin did not affect the *I–V* relationships of the ATP-sensitive $K^+$ channel. Note that open and closed symbols are almost superimposable.

current–voltage (*I–V*) relationship obtained in seven membrane patches before and after treatment with trypsin for 20 min is shown in Fig. 4. The *I–V* curves before and after treatment with trypsin at negative membrane potentials displayed linear relationships with slope conductances of 73·2 ± 2·8 and 73·0 ± 2·7 pS (n.s.), respectively. At membrane potentials positive to +40 mV, the *I–V* curves displayed

inward rectification both before and after treatment with trypsin. The magnitude of inward rectification was not significantly different between the two conditions; the amplitude of single channel current at a potential of +100 mV was 4·4±0·6 pA before the treatment with trypsin and 4·2±1·0 pA after trypsin (n.s.).

*Trypsin did not affect channel kinetics*

The open and closed time distributions were analysed to assess the effect of trypsin on the kinetic properties of the ATP-sensitive $K^+$ channel current. In the absence of ATP, the distributions of open time and closed time were measured from records showing no overlaps of the unitary current throughout the entire period of recording, suggesting that the membrane patch contained only one ATP-sensitive $K^+$ channel. Both before and after trypsin treatment, the open time distribution could be fitted to a single exponential function, and the closed time distribution to a function with a sum of two exponentials. Table 1 lists time constants for these fits. Trypsin had no apparent effect on these time constants, indicating a lack of effect of trypsin on the gating kinetics of the ATP-sensitive $K^+$ channel.

TABLE 1. Effect of trypsin on open and closed time constants, $\tau_{open}$ and $\tau_{closed}$

| | $\tau_{open}$ (ms) | | $\tau_{closed}$ (ms) | |
|---|---|---|---|---|
| | Fast | Slow | Fast | Slow |
| Control | 0·24±0·08 | 1·23±0·26 | 0·29±0·10 | 1·60±0·30 |
| Trypsin | 0·26±0·12 | 1·30±0·30 | 0·26±0·12 | 1·56±0·23 |
| *P* value | n.s. | n.s. | n.s. | n.s. |

*Trypsin did not affect ATP sensitivity*

We next examined the sensitivity of trypsin-modulated channels to ATP at the intracellular membrane surface. After the control data were obtained, the intracellular surface of the inside-out membrane patch was superfused with bathing solution containing ATP at concentrations of 0·01, 0·025, 0·05, 0·1, 0·25, 0·5, 1 or 2·5 mM for 3 min. The blocking effect of ATP is known to occur almost instantaneously. Thus, in our experimental system, the effect of each concentration of ATP reached a plateau about 1·5 min after starting the superfusion with the ATP-containing solution and $P_o$ was calculated using the data recorded between 2 and 3 min after starting the ATP perfusion. The bathing solution was then returned to that containing no ATP. Thereafter, the next concentration of ATP was tested. When $P_o$ upon re-exposure to ATP-free solution was less than 80% of the control value, the data were not used for further analysis. Figure 5*A* displays an experimental result in which five different concentrations of ATP (0·05, 0·1, 0·25, 0·5 and 1 mM) were tested with only a small degree of run-down in a control patch (left panel) or in the same patch treated with trypsin (right panel). $P_o$ decreased as the ATP concentration was increased to a similar degree in both non-treated and trypsin-treated patches. Figure 5*B* displays a dose–response curve for control patches

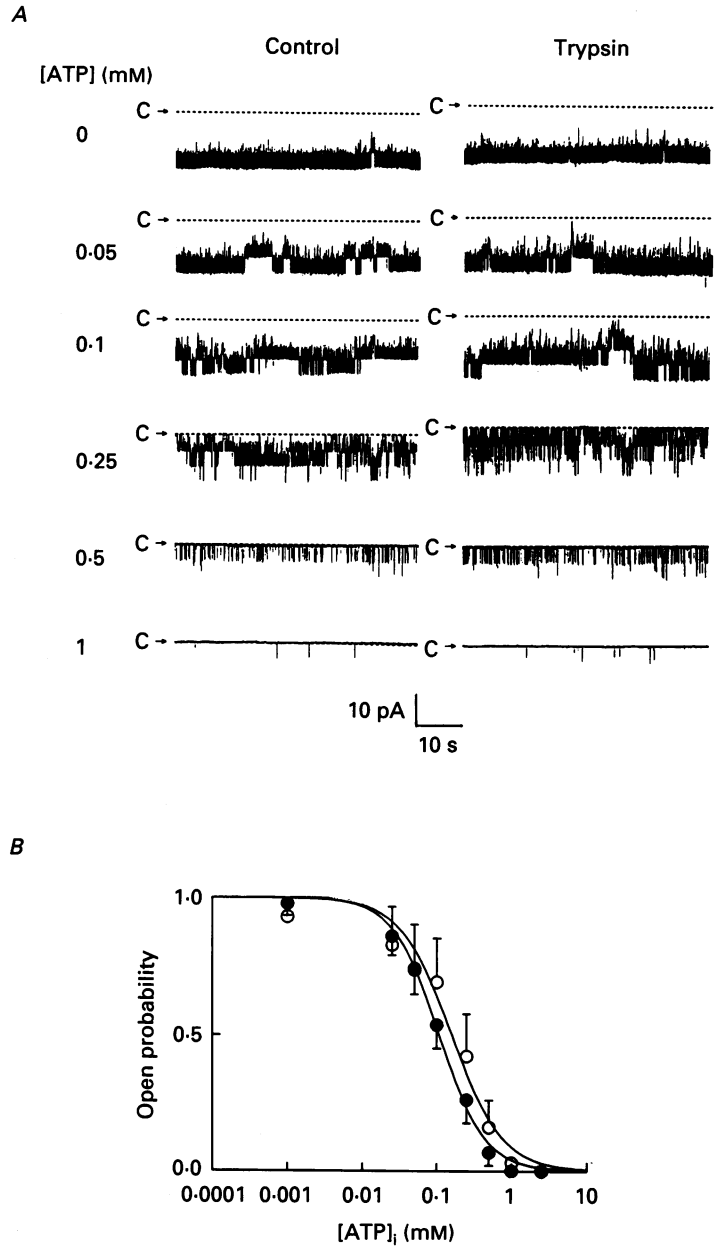


Fig. 5. Effect of trypsin on sensitivity of the channel to intracellular ATP concentration. *A*, representative tracings in which the five different concentrations of ATP (dipotassium salt) indicated in the left were tested with a small degree of run-down in a control patch (left panel) and in a patch treated with trypsin (1 mg/ml). The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. The channel activity decreased as the ATP concentration was increased to a similar degree in both non-treated and trypsin-treated patches. Membrane potential was held at $-50$ mV. The current records were filtered at $f_c = 1$ kHz for display. *B*, a dose–response relationship between open probability and the ATP concentration in non-treated (●) and trypsin-treated (○) patches. The open-state probability normalized to the control value was plotted against

($n = 6$–11 in each point) and for patches treated with trypsin ($n = 7$–11 in each point). In the graph, $P_o$ normalized to the control value was plotted against the logarithm of the concentration of ATP. The data were fitted by a least-squares analysis according to the Hill equation (eqn (2)):

$$\text{relative current} = 1/\{1 + ([\text{ATP}]/k)^n\}, \quad (2)$$

where $k$ is the [ATP] causing half-maximal inhibition, and $n$ is the Hill coefficient. There was no significant difference in $k$ or $n$ between non-treated and trypsin-treated patches: $k$ was $0{\cdot}11 \pm 0{\cdot}04$ mM in non-treated patches and $0{\cdot}16 \pm 0{\cdot}11$ mM in trypsin-treated patches (n.s.), $n$ was $1{\cdot}71 \pm 0{\cdot}22$ in non-treated patches and $1{\cdot}61 \pm 0{\cdot}77$ in trypsin-treated patches (n.s.).

*Trypsin also prevented $Ca^{2+}$-induced run-down*

Application of divalent cations, including $Ca^{2+}$, to the intracellular aspect of the membrane induces run-down of channel activity in a dose-dependent manner (Findlay, 1988; Kozlowski & Ashford, 1990). We tested various concentrations of $Ca^{2+}$ on run-down of channel activity (Fig. 6). Illustrated in Fig. 6*A* is the result of superfusing the intracellular aspect of membrane patch, containing multiple ATP-sensitive $K^+$ channels, with 1 mM $Ca^{2+}$ for 1 min, which induced complete run-down of channel activity in ~ 15 s. Although channel activity did not recover after wash-out of $Ca^{2+}$, it was almost completely restored when run-down channels were superfused with 2 mM MgATP for 3 min followed by MgATP wash-out. Superfusion of the intracellular side of a non-treated patch with 10 $\mu$M $Ca^{2+}$ for 1 min also induced run-down of channel activity, but not completely (Fig. 6*B*). After washing out the $Ca^{2+}$, channel activity recovered slightly compared to channel activity during perfusion with 10 $\mu$M $Ca^{2+}$, suggesting that suppression of channel activity by 1 min perfusion with 10 $\mu$M $Ca^{2+}$ consisted mainly of irreversible run-down, but that a reversible inhibitory effect also contributed slightly to the suppression of channel activity induced by this relatively short period of exposure to a relatively low concentration of $Ca^{2+}$. Channel activity was completely restored by treatment with 2 mM MgATP followed by wash-out of MgATP. After superfusion with trypsin (1 mg/ml) for 20 min, application of 1 mM $Ca^{2+}$ did not induce run-down of channel activity even though $Ca^{2+}$ was applied for more than 5 min (Fig. 6*C*). In Fig. 6*D*, we compared $Ca^{2+}$-induced run-down of channel activity between non-treated patches and trypsin-treated patches at various $Ca^{2+}$ concentrations. As shown in Fig. 6*B*, a relatively short (1 min) superfusion of a low concentration of $Ca^{2+}$ caused both reversible inhibition and irreversible run-down. In order to separate the reversible component of $Ca^{2+}$ inhibition from $Ca^{2+}$-induced run-down, $P_o$ was measured 40–60 s following the cessation of a 1 min perfusion with $Ca^{2+}$-containing solutions at various concentrations of $Ca^{2+}$. This point was chosen because by 40 s after $Ca^{2+}$ removal $P_o$ had reached a new steady state. The values were normalized to those before

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the logarithm of the ATP concentration. The continuous lines were obtained by fitting the Hill equation (eqn (2) in the text) to the data. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M.

application of $Ca^{2+}$ ($n = 4$–7 in each concentration of $Ca^{2+}$). In patches in which trypsin was not applied, application of $Ca^{2+}$ at a concentration of 10 $\mu$M or higher induced a significant decrease in the $P_o$ of the channel, while in patches treated with trypsin, application of any concentration of $Ca^{2+}$ did not significantly decrease $P_o$.

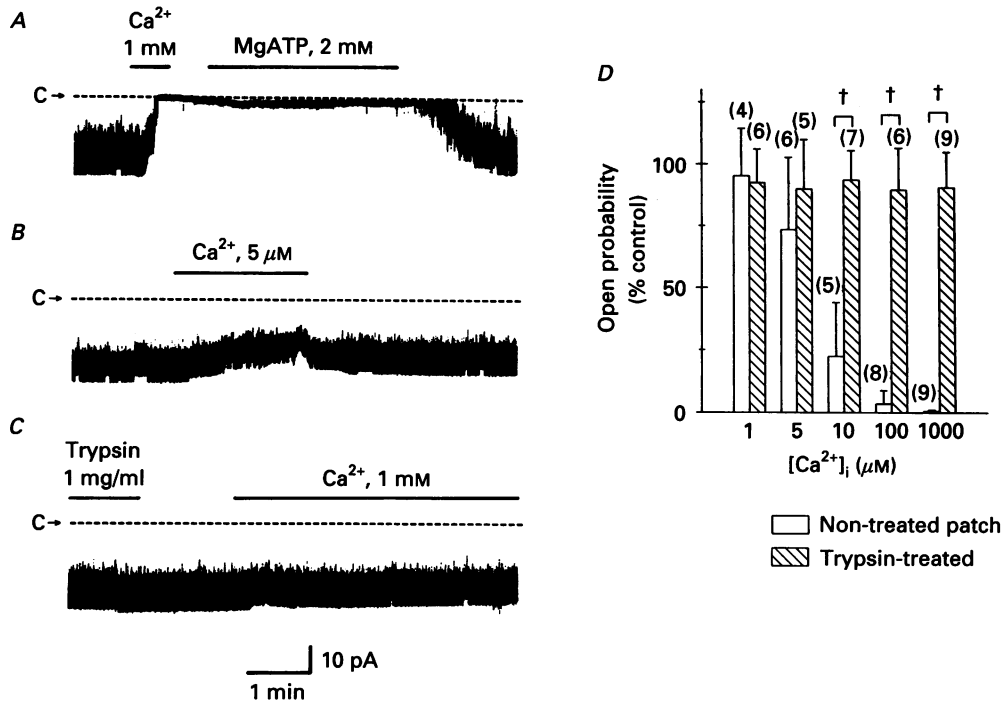


Fig. 6. $Ca^{2+}$-induced run-down of channel activity and the effect of trypsin. *A*, the run-down of channel activity during superfusion with a solution containing 1 mM $Ca^{2+}$, and its reactivation by exposure to MgATP (2 mM) in patches in which the intracellular side of the membrane was superfused with bath solution containing no trypsin. Membrane potential was held at −50 mV. The current records were filtered at $f_c$ = 1 kHz for display. *B*, superfusion with solution containing 10 $\mu$M $Ca^{2+}$ induced run-down of channel activity, but not completely. Washing out the $Ca^{2+}$ restored channel activity slightly, and channel activity was completely restored by treatment with 2 mM MgATP followed by wash-out of MgATP. Membrane potential was held at −50 mV. The current records were filtered at $f_c$ = 1 kHz for display. *C*, in patches which were pretreated with trypsin (1 mg/ml) for 20 min, application of 1 mM $Ca^{2+}$ did not show significant run-down of channel activity. Membrane potential was held at −50 mV. The current records were filtered at $f_c$ = 1 kHz for display. *D*, the probability of channel opening measured between 40 and 60 s after wash-out of $Ca^{2+}$ following a 1 min perfusion with various $Ca^{2+}$ concentrations. The open probability was measured at a membrane potential of −50 mV from a 10 s recording and was expressed as a percentage of that in the absence of $Ca^{2+}$. The number in parentheses on top of each bar indicates the number of experiments. Before exposure to trypsin (open bars), application of $Ca^{2+}$ induced run-down of channel activity in a dose-dependent manner, while after treatment with trypsin (1 mg/ml) for 20 min (hatched bars) application of any concentration of $Ca^{2+}$ did not induce run-down of channel activity. † $P < 0.01$ between, before and after application of $Ca^{2+}$.

*Carboxypeptidase A, but not Leu-aminopeptidase, mimicked the trypsin effect*

Figure 7*A* shows the effect upon spontaneous and $Ca^{2+}$-induced run-down of channel activity of Leu-aminopeptidase, an exopeptidase that catalyses proteolysis from amino-terminus (N-terminus). During superfusion of the intracellular surface of

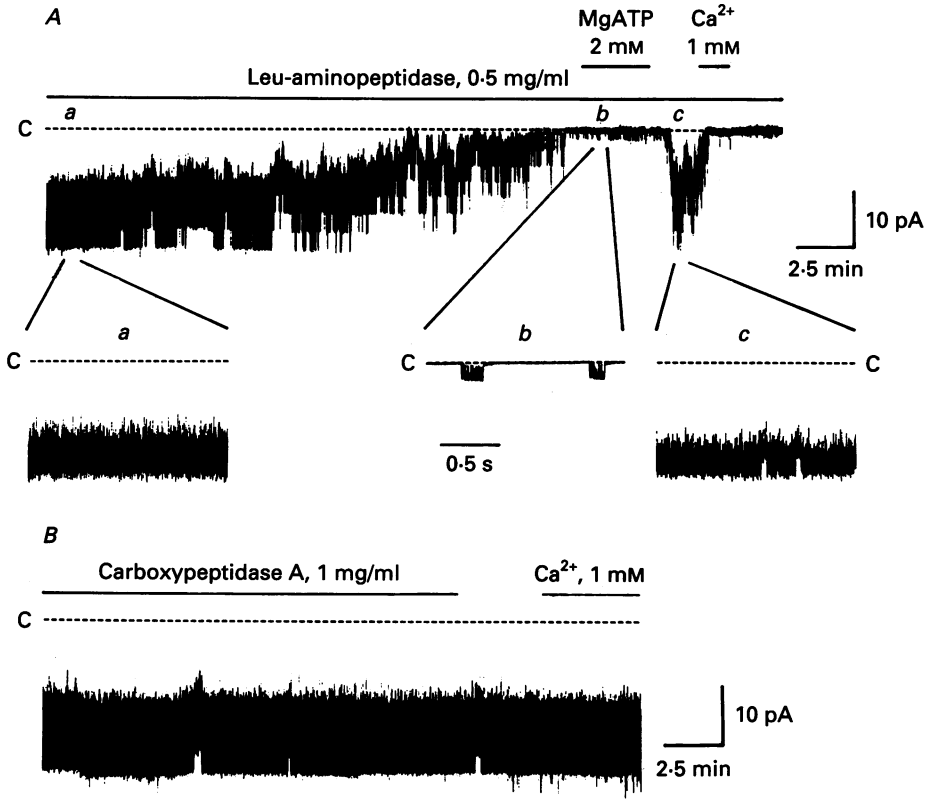


Fig. 7. Effect of exopeptidase on spontaneous and $Ca^{2+}$-induced run-down of channel activity. *A*, upper trace illustrates continuous recording during superfusion of the intracellular surface of the membrane patch with bath solution containing Leu-aminopeptidase (0·5 mg/ml). During application of Leu-aminopeptidase, the activity of the ATP-sensitive $K^+$ channel declined with time. After channel activity was restored by exposure to MgATP (2 mM) for 3 min, intracellularly applied $Ca^{2+}$ (1 mM) induced run-down of channel activity in ~ 15 s. Expansions of the records at the times marked by *a*, *b*, and *c* are shown in the lower panel. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M except during perfusion with 1 mM $Ca^{2+}$. *B*, continuous recording of the ATP-sensitive $K^+$ channel in the bath solution containing carboxypeptidase A (1 mg/ml). Intracellularly applied carboxypeptidase A prevented both spontaneous and $Ca^{2+}$-induced run-down of channel activity. Membrane potential was held at −50 mV. The current records were filtered at $f_c = 1$ kHz for display. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M except during perfusion with 1 mM $Ca^{2+}$.

the membrane patch with Leu-aminopeptidase (0·5 mg/ml), the activity of ATP-sensitive $K^+$ channels decreased spontaneously and the rate of run-down was comparable to those in the non-treated patches. After channel activity was restored

by exposure to MgATP (2 mM), 1 mM $Ca^{2+}$ induced complete run-down of channel activity in ~ 15 s. Figure 7*B* shows the effect of carboxypeptidase A, an exopeptidase which cleaves proteins from their carboxy-terminus (C-terminus). In contrast to Leu-aminopeptidase, during treatment with carboxypeptidase A (1 mg/ml), spontaneous

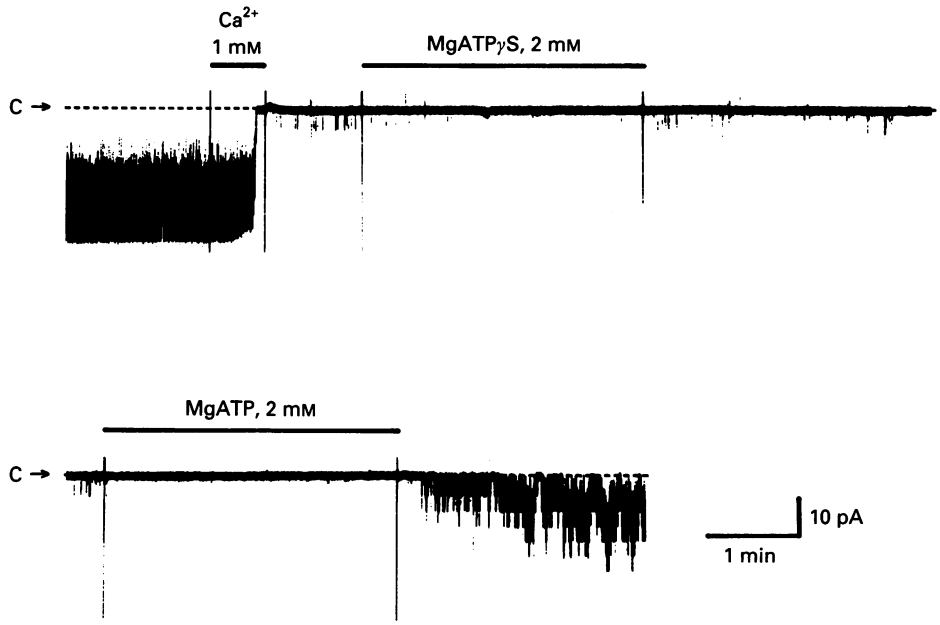


Fig. 8. Lack of reactivating effect of ATPγS. After superfusion with 1 mM $Ca^{2+}$ for 30 s induced complete run-down of channel activity, 2 mM ATPγS in the presence of $Mg^{2+}$ failed to reactivate channel activity, while 2 mM ATP in the presence of $Mg^{2+}$ reactivated channel activity. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Membrane potential was held at −50 mV. The current records were filtered at $f_c = 1$ kHz for display.

run-down was not seen over 20 min. After wash-out of carboxypeptidase A, neither spontaneous nor $Ca^{2+}$-induced run-down of channel activity was observed. Similar results were obtained in all three experiments for Leu-aminopeptidase and in three of the six experiments (50%) for carboxypeptidase A.

*ATPγS could not substitute for ATP in channel reactivation*

It has been suggested that the spontaneous $Ca^{2+}$-induced run-down of the ATP-sensitive $K^+$ channel is due to dephosphorylation of the ATP-sensitive $K^+$ channel itself, or of an associated regulatory protein (Findlay & Dunne, 1986; Ohno-Shosaku, Zünkler & Trube, 1987; Takano, Qin & Noma, 1990). In order to test further this hypothesis, we examined whether ATPγS in the presence of $Mg^{2+}$ could substitute for ATP in its action of reactivating run-down channels. ATPγS is a useful ATP analogue, because it can serve as a substrate 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. 8 ATPγS, when it was superfused to the intracellular surface of run-down channel for 3 min in the

presence of 2 mM $Mg^{2+}$, could not reactivate channel activity. We tested the effect of MgATPγS on reactivation of run-down channels in a total of six experiments, and in all of these six experiments, MgATPγS could not reactivate run-down channels.

*Effect of phosphatase inhibitors on run-down*

Finally, we studied the effect of inhibition of protein phosphatases that might be present in the excised patch on run-down of channel activity. Okadaic acid at a high

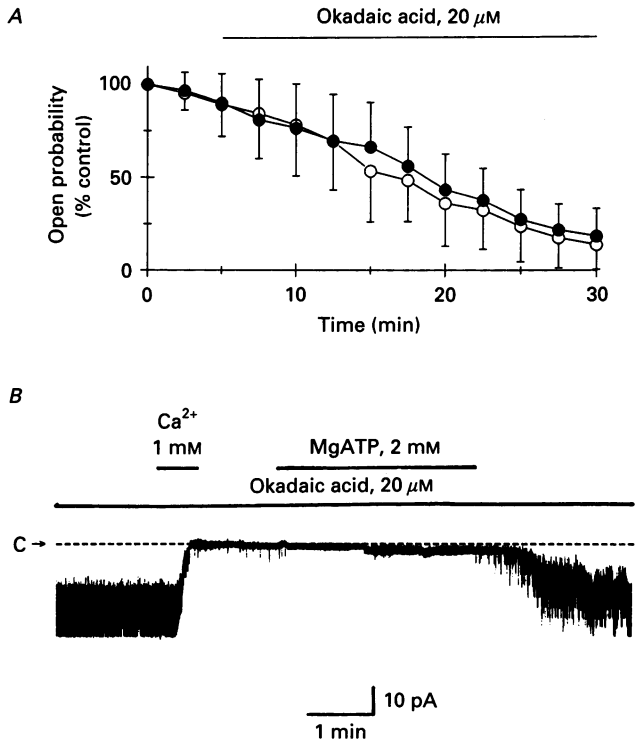


Fig. 9. Effect of a protein phosphatase inhibitor, okadaic acid, on spontaneous and $Ca^{2+}$-induced inactivation of channel activity. *A*, the probability of channel opening measured at a membrane potential of −50 mV every 2·5 min after excision of membrane patch. ●, control ($n = 7$); ○, with okadaic acid ($n = 4$). Measurement was made from a recording of 30 s and the open probability is expressed as a percentage of the value immediately upon excision of the membrane patch. The time period when okadaic acid (10 μM) was superfused is indicated as a bar on top of the graph. The concentration of free $Ca^{2+}$ in the intracellular solution was estimated to be $1 \times 10^{-7}$ M. Application of okadaic acid did not significantly affect the time course of decline in channel open probability compared to that in control patches. *B*, in the presence of okadaic acid (10 μM), superfusion with 1 mM $Ca^{2+}$ still induced complete run-down of channel activity in 15 s, and superfusion with 2 mM MgATP could restore channel activity. The concentration of free $Ca^{2+}$ in the intracellular solution was $1 \times 10^{-7}$ M except during perfusion with 1 mM $Ca^{2+}$.

concentration (10 μM) inhibits serine/threonine protein phosphatases 1, 2A, and 2B (Bialojan & Takai, 1988). As shown in Fig. 9*A*, the rate of run-down of channel activity was not different in the absence and presence of 10 μM okadaic acid. Furthermore, in the presence of 10 μM okadaic acid, application of 1 mM $Ca^{2+}$

induced a run-down of channel activity in ~ 15 s that was comparable to the rate of $Ca^{2+}$-induced run-down in the control (Figure 9*B*). Similar findings were observed in all five experiments in which we tested the effect of okadaic acid (10 $\mu$M) on $Ca^{2+}$-induced run-down.

## DISCUSSION

The major findings of the present study are as follows: (1) intracellular administration of the endoprotease trypsin prevented spontaneous and $Ca^{2+}$-induced run-down of the ATP-sensitive $K^+$ channel; (2) tryptic proteolysis did not affect other characteristics of this channel including unitary conductance, gating kinetics, or sensitivity to intracellular ATP; and (3) digestion with an exopeptidase, carboxypeptidase A, but not Leu-aminopeptidase, mimicked the effects of trypsin.

Chemical modification using various proteolytic reagents has been used to elucidate structure–function relations of several sarcolemmal ion channels. The presence of a 'chemical gate' as a susceptible site for proteolytic digestion has been suggested for several ionic channels. Trypsin has been shown to induce enhancement of $Ca^{2+}$ current (Hescheler & Trautwein, 1988). This effect was similar to the action of cyclic AMP-dependent protein kinase, and an exopeptidase, carboxypeptidase A, also reproduced this effect. Thus, it has been speculated that the unphosphorylated state of the protein chain between domain IV and the C-terminus might cause a blockage of the channel, possibly by a chemical gate, and that proteolytic cleavage of the carboxy-terminus as well as phosphorylation of this site might remove the blockage leading to an enhanced $Ca^{2+}$ current. Similar mechanisms have been suggested for proteolysis-induced removal of rapid inactivation of the *shaker* $K^+$ channels (Hoshi, Zagotta & Aldrich, 1990), $Ca^{2+}$-insensitive activation of the $Ca^{2+}$-dependent $K^+$ channels in *Paramecium* (Kubalski, Martinae, Saimi & Kung, 1989), and acetylcholine (ACh)-insensitive activation of the muscarinic $K^+$ channels in rat atrial myocytes (Kirsch & Brown, 1989). This hypothesis could also be applied to the action of trypsin on the ATP-sensitive $K^+$ channel. ATP-sensitive $K^+$ channels may enter an inactive state by closure of a postulated 'chemical gate', and trypsin may remove this blockage resulting in a loss of run-down. Trypsin could also reactivate the run-down channel; however, this effect of trypsin was observed in only four of the seven experiments (57%) after a delay of 10–15 min. This finding may suggest that the sensitivity or accessibility of trypsin to the channels appears to be different between active channels and run-down channels. In the case of the *shaker* $K^+$ channel, it has been demonstrated that trypsin cleaved about twenty amino acids from the N-terminus and prevented rapid inactivation (N-type inactivation) of this channel. To determine whether the tryptic action against run-down in the ATP-sensitive $K^+$ channel was due to cleavage of the N- or C-terminal portion of channel protein or an associated regulatory subunit, we tested the effect of the N-terminal-specific exopeptidase, Leu-aminopeptidase, and the C-terminal-specific exopeptidase, carboxypeptidase A. The finding that carboxypeptidase A, but not Leu-aminopeptidase, could mimic the effect of trypsin on prevention of run-down of channel activity suggests that the site sensitive to trypsin in the ATP-sensitive $K^+$ channel is on the C-terminal portion of channel protein or of an associated regulatory protein.

The final explanation, however, must await further elucidation of the molecular structure of this channel.

The finding that trypsin did not change unitary amplitude suggests that trypsin did not affect the ion-conducting pore of the ATP-sensitive $K^+$ channel. Inward rectification of the current–voltage relationships and fast flickering gating of this channel may be the result of binding of ions to the sites within the ion-conducting pore (Horie, Irisawa & Noma, 1987; Zilberter, Burnashev, Papin, Portnov & Khodorov, 1988). Thus, the observations that neither inward rectification nor fast flickering kinetics was affected by trypsin digestion support the idea that trypsin did not affect the ion-conducting pore. This finding is not unexpected because the ion-conducting pore-forming segments are necessarily transmembranous and would be inaccessible to proteolytic reagents (Catterall, 1988). Interestingly, trypsin did not affect the sensitivity of the channel to the ATP-blocking effect. Since ATP is hydrophilic and is effective when applied from the intracellular side, the binding site for this compound is assumed to be situated on the intracellular side. Thus, these sites, even though situated on the intracellular side, might not be accessed by trypsin, or, even though accessible by trypsin, might be resistant to tryptic digestion. We also tested the effects of trypsin treatment on pinacidil-induced activation of the ATP-sensitive $K^+$ channel and ADP-induced activation of channels partially inhibited by ATP. Trypsin did not affect these characteristics of ATP-sensitive $K^+$ channels either (data not shown).

Up to the present, several plausible explanations for the run-down mechanism of the ATP-sensitive $K^+$ channel have been proposed. One of the most attractive explanations is that dephosphorylation of the channel itself, or of associated regulatory proteins, results in channel run-down (Findlay & Dunne, 1986; Ohno-Shosaku *et al.* 1987; Takano *et al.* 1990). This hypothesis has come from several experimental observations: (1) the run-down in channel activity can be reversed by exposure to MgATP (Findlay & Dunne, 1986; Ohno-Shosaku *et al.* 1987); (2) $Mg^{2+}$ is required to restore the run-down in channel activity (Ohno-Shosaku *et al.* 1987); (3) non-hydrolysable ATP analogues such as adenylyl-imidodiphosphate (AMP-PNP) and adenylyl ($\beta,\gamma$-methylene)-diphosphonate (AMP-PCP) are unable to substitute for ATP in channel reactivation (Ohno-Shosaku *et al.* 1987). The data obtained in this study appear to be inconsistent with this reversible phosphorylation hypothesis. ATP$\gamma$S can serve as a substrate for protein kinases in the phosphorylation of target proteins, but the transferred thiophosphate is not readily removed by protein phosphatases (Eckstein, 1985). Therefore, we hypothesized that if run-down and reactivation of channel activity was due to reversible phosphorylation of a channel protein or of an associated regulatory subunit, ATP$\gamma$S in the presence of $Mg^{2+}$ could substitute for ATP in its reactivation effect on run-down of the channel, and the rate of run-down after reactivation with MgATP$\gamma$S should be delayed. Our experiment, however, showed that 2 mM MgATP$\gamma$S could not substitute for MgATP in its reactivation effect on run-down of the channel. Furthermore, an inhibitor of serine/threonine phosphatases, okadaic acid (10 $\mu$M), could not inhibit either spontaneous or $Ca^{2+}$-induced run-down, suggesting that dephosphorylation of the channel protein itself, or of the associated regulatory unit, might not be a main factor in run-down of this channel. Since the characteristic difference between

ATPγS and ATP is the slow rate of hydrolysis in the γ-phosphate in ATPγS, hydrolysis of ATP, rather than phosphorylation, may play some role in maintaining the channel in an active state. There are several other possible mechanisms proposed for run-down of this channel. One of these is that run-down may be mediated by $Mg^{2+}$

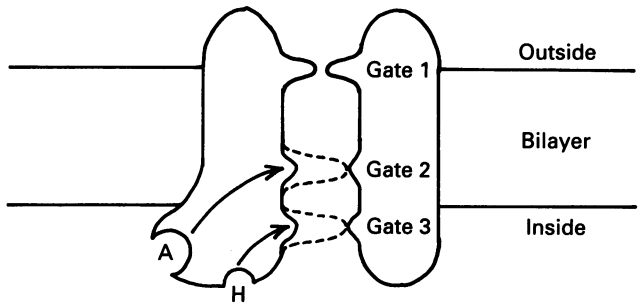


Fig. 10. A hypothetical model of the interaction of dual ATP action, inhibition and activation of ATP-sensitive $K^+$ channel activity. The presence of three channel gates is postulated. One gate (Gate 1) is within the electrical field of the membrane, and its opening and closing are regulated by the carrier flux. The other two gates (Gates 2 and 3) are outside the electrical field of the membrane. The opening and closing of Gate 2 are regulated by the signals from the ATP-binding site (A-site), while the opening and closing of Gate 3, possibly a 'chemical gate' are regulated by the signals from the site where hydrolysis of ATP occurs (H-site). These three signals may be independent, and only the H-site or the signal pathway from H-site to Gate 3 is trypsin-sensitive.

ions, and possibly other divalent cations, binding to the channel, or some modulatory protein, resulting in an allosteric change and entry into a long-lived closed state (Kozlowski & Ashford, 1990). It is likely that the run-down mechanism of this channel may be multifactorial. The observation by Kozlowski & Ashford (1990) that the run-down-inducing action of $Mg^{2+}$ over-rides the effects of chronic MgATP administration in insulin-secreting cells may be in line with the idea that divalent cations, including $Mg^{2+}$, and MgATP may play a different role in run-down and reactivation of this channel. Although many possible explanations for the mechanism of channel run-down have been proposed, an exact mechanism is still largely unknown. Further experiments are needed to determine whether hydrolysis of ATP may be involved in a multifactorial mechanism for channel run-down.

The relationships of dual ATP actions, inhibition and activation of channels, are currently unsettled. In rat ventricular cells, a model in which no apparent interaction is present between ATP-induced inhibition and activation of the channel has also been postulated (Lederer & Nichols, 1989). In guinea-pig ventricular cells, a functional unit has been postulated between the ATP-binding site and the channel gate, where the site related to run-down and reactivation of channel activity is present (Tung & Kurachi, 1991). Although our experimental data do not contradict these two hypotheses, we propose a somewhat modified view for the channel gating. It is generally believed that the ATP-sensitive $K^+$-channel has at least two gates: one gate (Gate 1 in Fig. 10) is located within the electrical field of the membrane and regulated by the carrier flux (Zilberter *et al.* 1988); another gate (Gate 2 in Fig. 10) is

outside the electrical field of the membrane and a target of ATP-induced inhibition. As shown in Fig. 10, an additional channel gate (Gate 3), possibly a 'chemical gate', may be present, and its gating kinetics appear to be regulated by hydrolysis of ATP (H-site). The kinetics of this gate (Gate 3) may be independent of the channel gate (Gate 2) whose gating kinetics are regulated by the signals from the ATP-binding site (A-site). Tryptic digestion appears to modify the proposed 'chemical gate' itself, or the regulatory subunit-containing H-site, keeping Gate 3 in an open state irreversibly.

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