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The Effects of Trypsin on ATP-sensitive Potassium Channel Properties and Sulfonylurea Receptors in the CRI-G1 Insulin-secreting Cell Line

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SUMMARY

The effects of the proteolytic enzyme trypsin upon ATP-sensitive potassium (K_{ATP}) channel activity were examined in the CRI-G1 insulin-secreting cell line. Trypsin activated channels only when applied to the intracellular surface of the cell membrane. The activation could be prevented by the concomitant application of trypsin inhibitor or by heat inactivation of the enzyme. The trypsin-induced change in channel activity was accompanied by a reduction in the rate of channel rundown. However, trypsin did not affect the mean single channel conductance (55.2 pS), the ionic selectivity, or rectification of the K_{ATP} channel. Concentration response curves for various K_{ATP} channel inhibitors were constructed in the presence and absence of intracellular trypsin. The EC_{50} for tolbutamide was shifted from 30.0 ± 4.5 μM, with 100 μg/ml heat-inactivated trypsin present to 9.7 ± 1.0 mM with active trypsin in the intracellular solution. Treatment of the cells' external surface with 1 mg/ml trypsin did not alter the potency of tolbutamide. Intracellular trypsin also produced a significant fall in the potency of glibenclamide, meglitinide, and phentolamine but did not alter the effectiveness of thiopentone. Radioligand binding studies demonstrated a total loss of ^{3}H-labeled glibenclamide binding when the intracellular surface of the cells was exposed to trypsin. In contrast, ^{3}H-labeled glibenclamide binding was not affected when the enzyme was applied to the external surface. Trypsin treatment, therefore, alters a number of characteristics of K_{ATP} channel pharmacology, and we suggest that this is due to action at possibly more than one site but includes the functional cleavage of the sulfonylurea receptor from the K_{ATP} channel.

The K_{ATP} channel is present in a wide variety of tissues (for review, see Ref. 1) and appears to represent a novel class of ionic channels linked by their sensitivity to intracellular fluctuations in the concentrations of various nucleotides. In addition to this regulation, various pharmacological agents can also modulate K_{ATP} channel activity. For example, potassium channel openers such as diazoxide can potentiate channel activity (2), and numerous agents can act to reduce channel activity. These agents include the sulfonylureas (3), barbiturates (4), α adrenoceptor antagonists (5), and phenothiazines (6).

Of the agents known to inhibit K_{ATP} channel activity, the sulfonylureas appear most specific. Binding sites for these agents have been shown to have a very similar tissue distribution to the K_{ATP} channel itself, and this has fueled speculation that the sulfonylurea receptor is an integral part of this channel. Recent studies using various insulin-secreting cell lines have provided evidence both for and against this hypothesis (7, 8).

The site of action of the sulfonylureas is also presently a source of controversy. Initial studies suggest that these agents act from the external surface (9), although, more recently, an internal site of action has been proposed (10). These fundamental questions, like many of the uncertainties surrounding the properties of other ion channel proteins, awaits the elucidation of K_{ATP} channel structure before an answer can be obtained. However, enzymatic and chemical techniques have previously proven useful in aiding the molecular determination of channel structure and function. A good example of this approach has been the use of proteolytic enzymes to remove the inactivation of sodium, calcium, and potassium channels (11–13). These observations paved the way for the development of a "ball and chain hypothesis" for channel inactivation, which has subsequently been vindicated using molecular techniques (14).

Trypsin, which cleaves specifically at lysine and arginine residues (15), is capable of activating the K_{ATP} channel and reversing channel rundown when applied intracellularly to both cardiac and pancreatic β-cell membranes (16, 17). Coupled with this change in K_{ATP} channel activity is an apparent loss of sensitivity to diazoxide (19) and the sulfonylureas (20). Because we have recently shown that critical lysine and arginine residues are involved in the interaction between sulfonylureas and the K_{ATP} channel (18), the present study was undertaken in order to characterize the pharmacology of the trypsin-modified

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K_{ATP} channel and associated sulfonylurea receptor in greater detail.

Materials and Methods

Cell culture

Cells of the rat pancreatic islet cell line CRI-G1 were cultured and passaged at 2–5-day intervals as previously described (21). Cells used for patch-clamp experiments were plated onto 3.5-cm petri dishes (Falcon 3001) at a density of approximately 1.5×10^5 cells/dish. The cells were used 2–4 days (inclusive) after plating.

Electrical recording and analysis

This study employed both the cell-free and whole cell configurations of the patch-clamp recording technique, as described by Hamill *et al.* (22). Recording electrodes were pulled from borosilicate glass capillaries and, when filled with electrolyte, had resistances of 8–12 MΩ for isolated patch experiments, and 2–5 MΩ for whole cell recording. Single channel events were detected using a List EPC-7 or Axopatch 2D patch clamp amplifier and were stored on digital audio tape or magnetic tape (Racal 4DS). Records used for illustrative purposes were replayed into a chart recorder (Gould 2200), which filtered the data at 140 Hz. The potential across the membrane is described following the usual sign convention for membrane potential (i.e., inside negative). Outward current (defined as the current flowing from the intra- to the extracellular side of the membrane) is shown as upward deflections on all traces. The single channel current analysis was determined off-line using a program that incorporated a 50% threshold-crossing parameter to detect events (23) and run on an Apricot XEN-i286/45 microcomputer. Data segments between 30- and 90-s duration were replayed at the recorded speed and filtered at 1.0 kHz using an 8-pole Bessel filter and digitized at 5.0 kHz using a Data Translation 2801A interface. The average channel activity ($N_f \cdot P_o$) where N_f is the number of functional channels in the patch and P_o is the open state probability, was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded (24). Changes in $N_f \cdot P_o$ as a result of drug effects are expressed as a percentage of control. In order to determine statistically how channel modification by trypsin affected the inhibitory effectiveness of various agents, the percentage inhibition produced before and after trypsin application was assessed for significant differences using one-way analysis of variance (ANOVA). To obtain whole cell currents, the cell was voltage clamped at a holding potential of −70 mV, and alternate ±10-mV pulses of 200-ms duration were elicited at 2-s intervals as described previously (24, 25). Drug effects were quantified by measuring the amplitudes of the current responses (I) during drug exposure and comparing them with those observed under control conditions (I_c). Values for the controls were obtained by calculating the mean amplitude before and after the application of drug, which enabled the slow process of rundown to be taken into account as the experiment progressed. The concentration-inhibition curves were fitted by nonlinear regression to the following equation:

$$I/I_c = 1/(1 + (a/b)^n) \quad \text{(Eq. 1)}$$

where a = drug concentration, b = half-maximal inhibitory concentration, and n = Hill coefficient.

To quantify the extent of K_{ATP} channel rundown in the whole cell recording configuration, currents were recorded onto chart paper and the amplitudes measured by hand. The amplitudes of the current responses were measured at constant time intervals (I_t) after peak current was attained (I_p), and any change expressed as the natural logarithm of the normalized ratio (I') was as follows.

$$I' = \ln(I_t/I_p \times 100) \quad \text{(Eq. 2)}$$

By plotting I' against time, the rate constants for current rundown can be determined. The effect of trypsin upon the maximum current amplitude attained (taking cell size into account) was determined by measuring the peak current observed in response to the depolarizing pulses (taking into account the voltage error after series resistance compensation) and dividing by the measured capacitance of the cell. The resultant values in pA/pF represent the current densities/100 μm^2 (26).

Solutions

Before use, the cells were washed thoroughly with solution A, which consisted of (mM): NaCl (135.0), KCl (5.0), $CaCl_2$ (1.0), $MgCl_2$ (1.0), HEPES (10.0) with pH adjusted to 7.4 with NaOH. For whole cell voltage clamp studies, the cells were bathed in solution A, and the pipette contained (mM); KCl (140.0), $MgCl_2$ (1.0), $CaCl_2$ (2.0), K_2EGTA (10.0), HEPES (10.0) with pH adjusted to 7.2 with KOH, which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and 0.65 mM, respectively (solution B). In experiments using the inside-out configuration, the pipette contained solution A or (mM) KCl (140.0), $MgCl_2$ (1.0), $CaCl_2$ (1.0), HEPES (10.0) with pH adjusted to 7.2 with KOH (solution C), and the bath solution was solution B. In experiments on outside-out patches, the bath solution was solution C, and the pipette solution was solution B. The concentrations of free divalent cations were determined by using a program for calculating metal ion/ligand binding "METLIG" (P. England and R. Denton, University of Bristol).

Tolbutamide and thiopentone were each made up as 500 mM stock solutions in either 1 M KOH or 1 M NaOH. Meglitinide (HB699) and phentolamine were made up as 100 mM stock solutions in dimethyl sulfoxide, and glibenclamide stock solutions (5 mM) were made up in methanol. K_2ATP, in addition to trypsin (type XI; Sigma Chemical Co., St. Louis, MO) and trypsin inhibitor, were freshly dissolved into the respective solution immediately prior to the experiment. Trypsin was heat-inactivated by boiling at 90° for 15 min. Meglitinide was provided by Hoechst Aktiengesellshaft, Frankfurt, Germany. All other drugs listed above were obtained from Sigma (Poole, Dorset, United Kingdom).

Preparation of cells for binding

Membrane preparation. CRI-G1 cells were grown to 70–80% confluence in Falcon 3027 805-cm^2 roller bottles (~2.5×10^7 cells). Cells were harvested by scraping with a rubber policeman and centrifuged for 10 min at 220 × *g* (MSE; Crawley, United Kingdom). The supernatant was removed by aspiration, and cells were resuspended in ice-cold 10 mM HEPES pH 7.4 containing 200 mM D-mannitol and 65 mM sucrose. All subsequent procedures were performed at 4°. The cell suspension was homogenized in a 40-ml capacity glass tube homogenizer (Jencons Scientific, Leighton Buzzard, Beds., U. K.) using 10 strokes of a Teflon pestle driven at 600 rpm^{-1} and then sonicated for 10 s at 30 W (MSE Sonifier). The homogenate was centrifuged for 15 min at 500 × *g* to remove unbroken cells and nuclei, and the resulting supernatant was centrifuged for 30 min at 70,000 × *g* (OTD65B Sorvall ultracentrifuge; Du Pont Ltd., Stevenage, U. K.) to produce a crude membrane fraction. This was resuspended in 50 mM Tris/HCl (pH 7.4) and the latter centrifugation step repeated. The resulting pellet was resuspended in 50 mM Tris/HCl (pH 7.4) containing 100 μM phenylmethylsulfonyl fluoride, 10 μM Pepstatin A, and 20 μM trans-epoxysuccinyl-L-leucylamido(4-guanido)-butane (E64) and stored under liquid nitrogen prior to use.

Time course of trypsinization. Crude membrane fractions were prepared as described above. These were immediately incubated with 100 μg/ml trypsin for varying lengths of time before the addition of 200 μg/ml trypsin inhibitor. Reaction mixtures were then incubated for 2 hr at room temperature in 0.5 ml of assay buffer, 50 mM Tris/HCl (pH 7.4), 0.2 nM ^{3}H-labeled glibenclamide (50.9 Ci $mmol^{-1}$; Du Pont Ltd.) and 1 μM glibenclamide where appropriate. Incubations were terminated as described below.

Trypsinization of whole cells. Cells were grown to 70–80% confluence in 805 cm^2 roller bottles. The medium was removed by aspiration and cells incubated for 10 min at room temperature in Hanks physiological saline (137 mM NaCl, 5.4 mM KCl, 1.67 mM $MgSO_4$, 4

mM $CaCl_2$, 0.34 mM Na_2HPO_4, 4.2 mM $NaHCO_3$ (pH 7.4)) in the presence or absence of 100 μg/ml trypsin. Proteolytic action was stopped by the addition of 200 μg/ml trypsin inhibitor. Membranes were then prepared and stored as described above. Radioligand binding studies were then undertaken as indicated below.

^{3}H-Labeled glibenclamide binding. CRI-G1 membrane fractions (50 μg of protein) were incubated for 2 hr at room temperature in 0.5 ml of assay buffer, 50 mM Tris/HCl (pH 7.4) containing 0.04–5 nM ^{3}H-labeled glibenclamide (50.9 Ci $mmol^{-1}$; Du Pont Ltd.) and 1 μM glibenclamide where appropriate. Incubations were terminated by addition of 2 ml of ice-cold buffer, and bound ligand was collected by rapid vacuum filtration onto 2.5-cm diameter GF/B filter discs (Whatman International Ltd., Maidstone, U. K.). Filters were washed with 4 × 2 ml of buffer and the bound radioactivity determined by liquid scintillation counting using OptiPhase HiSafe II (LKB Scintillation Products, Loughborough, U. K.) and a Packard liquid-scintillation spectrometer (Canberra Packard, Berks., U. K.).

Results

Single channel studies

Initial experiments examined the effects of trypsin upon K_{ATP} channel activity when applied to either the internal or external surfaces of isolated membrane patches from the CRI-G1 insulin-secreting cell line. Exposure of the intracellular aspect of the patch to trypsin for approximately 30 s produced an increase in the level of channel activity (Fig. 1*a*). Analysis of the activation showed that trypsin at a concentration of 100 μg/ml provoked an increase in channel activity of 345.7 ± 34.3% (n = 15) over control values, which were measured immediately before enzyme application. This channel activation arose due to an increase in both the number of functional channels present in the patch (N_f) and their open probability (P_o) and was unaffected by the holding potential (±50 mV). Following trypsinization, the channels' sensitivity to ATP was reduced. In our studies, 50 μM ATP induced 87.4 ± 4.5% (n = 5) inhibition before trypsinization but only 18.7 ± 3.4% (n = 5) inhibition afterwards ($p < 0.05$) (Table 1). Although we have not examined the reduction in ATP's potency in great detail, after trypsinization, 500 μM ATP appeared to induce a similar level of K_{ATP} channel inhibition (90.4 ± 5.6%; n = 7) (see Fig. 4) as 50 μM ATP did before tryptic digestion (Fig. 1*a*). After trypsinization, K_{ATP} channel activity remained highly active for the duration of the patch lifetime with little or no rundown observed even after approximately 2 hr. In contrast, under similar conditions, the K_{ATP} channel in the absence of tryptic digestion underwent an irreversible loss of activity, which was complete within 30 min of patch excision. Exposure of the extracellular surface of membrane patches to 100 μg/ml trypsin did not alter the level of channel activity (n = 17; not shown). Furthermore concentrations of 500 μg/ml (n = 12) and 1 mg/ml (n = 5) trypsin consistently failed to activate K_{ATP} channels when applied to the external surface, though the higher dose did reduce patch stability.

Although the application of trypsin to the intracellular surface affected the level of channel activity, this treatment did not alter the channels' pore properties, which were examined pre- and post-trypsin in both symmetrical and asymmetrical potassium. The mean current-voltage relationships for the K_{ATP} channel under these conditions are displayed in Fig. 2. In symmetrical 140 mM potassium, the channel displayed a reversal potential of 0 mV and a mean slope conductance of 54.7 ± 5.8 pS (n = 17). The channel exhibited the property of inward rectification at depolarized potentials as expected in the presence of Mg^{2+} (27, 28). None of these characteristics were altered by trypsinization (n = 11). In asymmetric conditions, the extrapolated reversal potential was approximately −80 mV (n = 9), which is close to the calculated value of −84 mV for a potassium-selective conductance, and treatment of the isolated patch with trypsin again failed to affect the current-voltage relationship (n = 7). On no occasion was trypsin application observed to activate any other type of channel under the ionic conditions employed.

The nature of the K_{ATP} channel activation produced by trypsin was then examined to determine whether or not this was dependent on its enzymatic properties. The addition of the trypsin, in the presence of the same concentration of trypsin inhibitor (100 μg/ml), to the internal surface of an inside-out patch produced no change in the level of channel activity, and the gradual time-dependent fall in channel activity known as rundown was noted as previously characterized (n = 6) (26). Similarly, in four separate experiments, heat-inactivated trypsin (100 μg/ml) failed to affect channel activity.

Whole cell studies

The time-dependent loss of K_{ATP} channel activity termed rundown is not completely understood, although this process does appear to be Mg^{2+}-dependent in nature (26). Following trypsinization, it was noted that channel rundown was very much reduced in excised patch studies (Fig. 1*b*). In order to quantify the effects of trypsin upon the rate of channel rundown, the whole cell recording technique was employed. A typical whole cell recording illustrating rundown of K_{ATP} current is shown in Fig. 3*a*. In this experiment, the cell was dialyzed with solution B, which contained 0.65 mM Mg^{2+}. Upon formation of the whole cell voltage clamp, there is a gradual increase in the K_{ATP} current with time presumably due to washout of intracellular ATP with dialysis with the pipette solution (2). After several minutes, the current amplitude peaks and subsequently proceeds to decline with time (a phenomenon known as rundown. If the same procedure is repeated but with 100 μg/ml trypsin added to the pipette solution, a similar increase of K_{ATP} current occurs with time (Fig. 3*b*). However, in marked contrast to the experiment performed in the absence of trypsin, the current amplitude does not show a marked decline but remains at a level close to the peak amplitude for many minutes and then exhibits a very slow decrease.

The decrease in K_{ATP} current amplitude with time after the peak is illustrated graphically for both conditions in Fig. 3*c*. From this semi-logarithmic plot, it can be seen that in the presence of 0.65 mM Mg^{2+}, intracellularly, rundown occurs exponentially and exhibits two components as reported previously (26). In the present study, the initial slow α phase occurred at a rate 1.68 hr^{-1}, and the second more rapid β phase occurred at a rate of 7.44 ± 0.84 hr^{-1}. In contrast, when the pipette solution contained trypsin, the decline in K_{ATP} currents was much slower and could be characterized by a single rate constant of 0.47 ± 0.03 hr^{-1}.

The introduction of 100 μg/ml trypsin into the electrode solution was found to significantly increase the size of the whole cell conductance ($p < 0.05$). In the absence of trypsin, the whole cell current density was 16.32 ± 1.76 pA/pF (n = 42); however, when trypsin was added to the electrode solution,

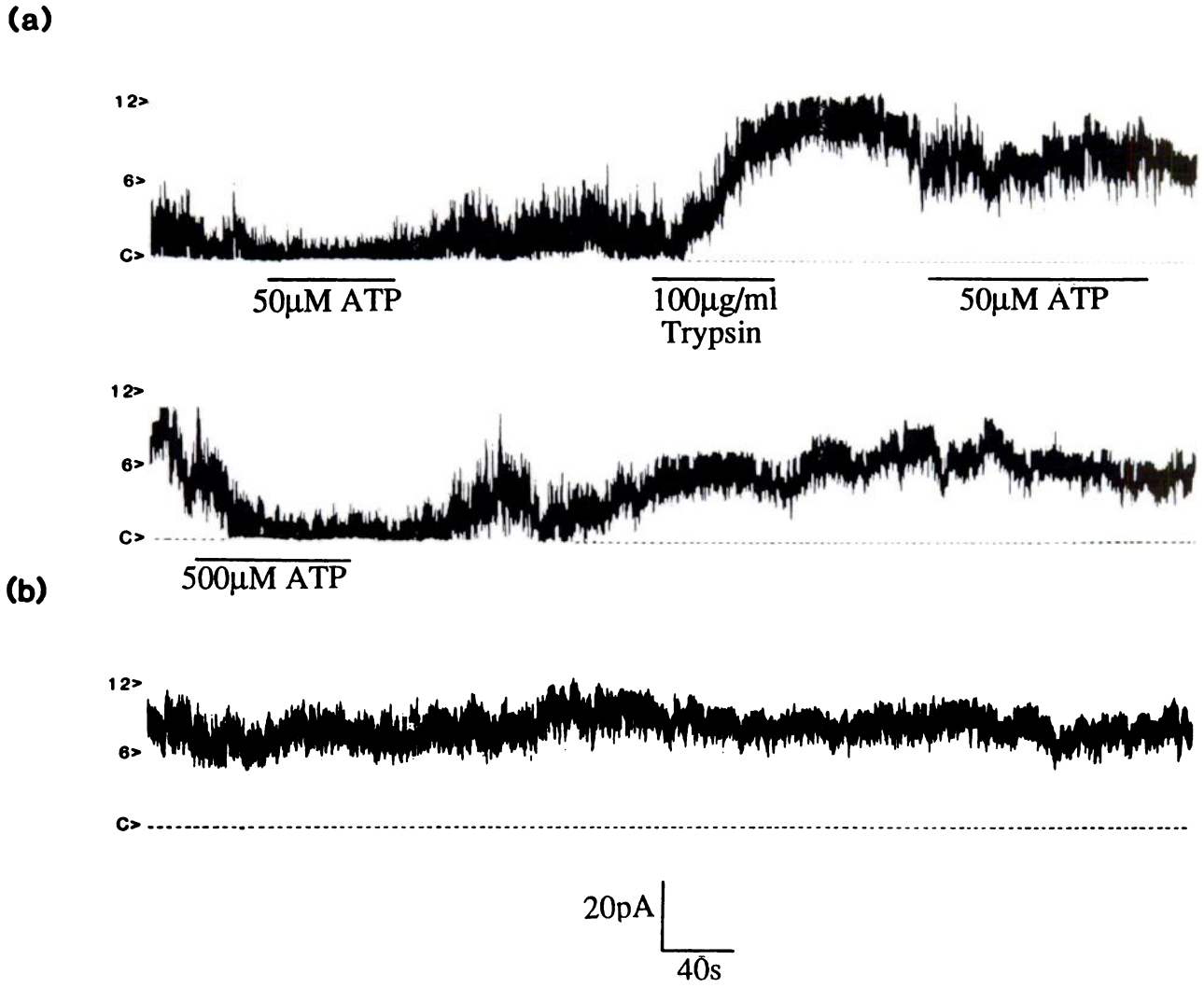

Fig. 1. Single channel currents recorded from an inside-out membrane patch exposed to symmetrical 140 mM KCl held at a membrane potential of +50 mV. The patch was continually perfused throughout the course of the experiment with solution A to which various additions were made as denoted by the *bars* beneath the recordings. *a*, prior to trypsinization, 50 μM ATP produces a reversible inhibition of K_{ATP} channel activity. The subsequent exposure of these channels to 100 μg/ml trypsin produces a large activation of channel activity, which is only slightly inhibited by 50 μM ATP. However, the application of 500 μM ATP is able to produce a large inhibition of channel activity. The values for $N_f \cdot P_o$ were as follows: control, 1.44; 50 μM ATP, 0.33; wash, 1.41; trypsin, 11.43; 50 μM ATP, 8.97; wash, 9.58; 500 μM ATP, 0.97; wash, 6.15. *b*, approximately 1 hr after the washout of 500 μM ATP, the K_{ATP} channel remained highly active with $N_f \cdot P_o$ of 9.15.

the whole cell current density was 25.47 ± 3.02 pA/pF (n = 28).

Pharmacology of the trypsinized K_{ATP}

Initial reports have shown that the intracellular application of trypsin dramatically reduces K_{ATP} channel sensitivity to tolbutamide in β-cells (20) and to tolbutamide and glibenclamide in cardiac myocytes (29). We therefore decided to examine the effect of trypsin on these sulfonylureas and other inhibitors of the K_{ATP} channel in CRI-G1 cells. A number of known K_{ATP} channel inhibitors were used at concentrations capable of inducing approximately 90% channel inhibition when applied to the internal surface of an inside-out patch in the absence of trypsin. The results obtained are summarized in Table 1. The inhibitory effectiveness of the sulfonylureas tolbutamide and glibenclamide are significantly reduced by pretreating the patch with 100 μg/ml enzyme trypsin (Table 1) ($p < 0.05$). Meglitinide is the nonsulfonylurea structural component of glibenclamide and is capable of inhibiting K_{ATP} channel activity and stimulating insulin secretion (25). In these studies, 10 μM meglitinide induced over 90% inhibition of K_{ATP} channel activity (Table 1). After trypsinization, however, the same concentration of meglitinide was significantly less effective, now producing less than 10% K_{ATP} channel inhibition ($p < 0.05$). An example of the lack of effect of glibenclamide following trypsinization is shown in Fig. 4.

The α_2 adrenoceptor antagonist phentolamine and the barbiturate thiopentone are also known to inhibit K_{ATP} channel activity (4, 5). As shown in Table 1, both agents produced a potent inhibition of channel activity prior to trypsin application. After trypsinization, the inhibitory effectiveness of phentolamine was significantly reduced (Table 1) ($p < 0.05$). However, the inhibitory effects of thiopentone were unaffected by this enzymatic digestion (Table 1).

TABLE 1

Summary of the effects of various K_{ATP} channel inhibitors when applied to the internal surface of excised inside-out patches before and after treatment with 100 μg/ml trypsin.

Membrane patches were held at holding potentials ranging between +50 and −50 mV under conditions of symmetrical 140 mM KCl. The electrode was filled with solution C, and the bath contained solution B. Percent inhibition refers to the reduction in the K_{ATP} channel open probability ($N_f \cdot P_o$) as a percentage of the control activity. The inhibition produced by all these compounds was independent of the membrane holding potential. Values are means ± SE; the number of experiments performed are shown in parentheses.

Agent	Inhibition	
	Before trypsin	After 100 μg/ml trypsin
	%	
100 μM Tolbutamide	95.2 ± 3.2 (n = 14)	8.4 ± 4.3 (n = 5)*
5 μM Glibenclamide	97.3 ± 5.7 (n = 4)	20.2 ± 11.3 (n = 4)*
50 μM ATP	87.4 ± 4.5 (n = 5)	18.7 ± 3.4 (n = 5)*
10 μM Meglitinide	92.3 ± 2.5 (n = 13)	6.3 ± 3.2 (n = 4)*
10 μM Phentolamine	81.34 ± 11.4 (n = 6)	53.8 ± 7.0 (n = 8)*
100 μM Thiopentone	92.2 ± 2.6 (n = 8)	90.3 ± 3.4 (n = 3)

* Significantly ($p < 0.05$) different with respect to inhibition produced prior to the application of trypsin.

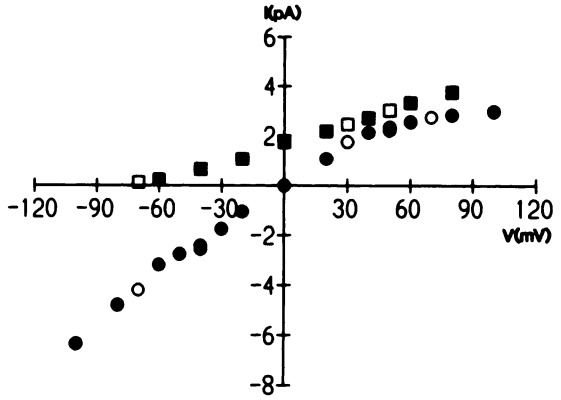

Fig. 2. Single channel current-voltage relationship of the K_{ATP} channel present in the CRI-G1 insulin-secreting cell line before and after application of 100 μg/ml trypsin to the intracellular surface of excised inside-out patches. *a*, current-voltage relationships for the K_{ATP} channel in symmetrical (*circles*) and asymmetrical (*squares*) conditions. *Filled symbols* indicate the values obtained for the trypsinized channel and directly overlie the values obtained for the normal channel (*open symbols*) except where additional points were obtained for the normal channel. Values given indicate the mean values of three experiments. In each case, the SE was within the size of the symbols. Note the pronounced inward rectification exhibited by the channel in both solution conditions.

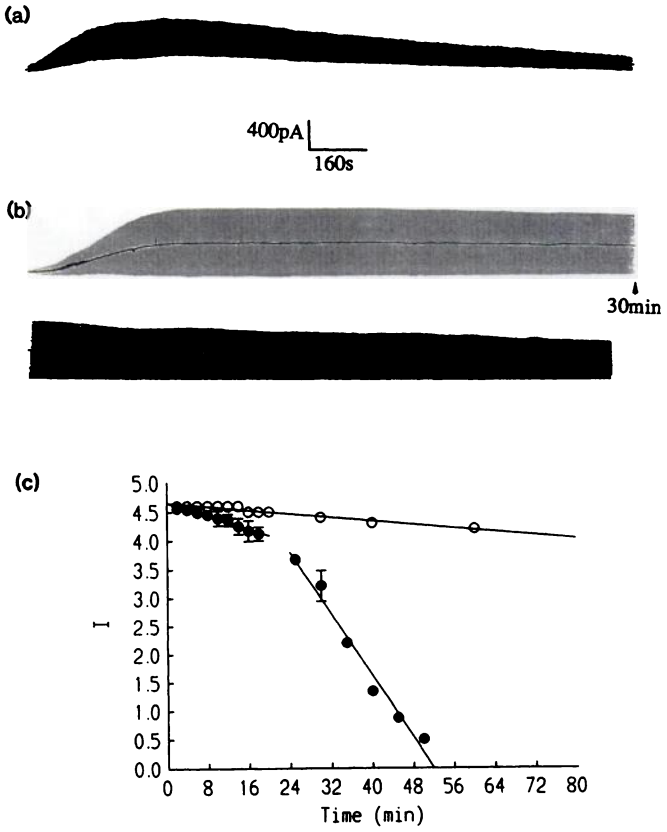

Fig. 3. Effects of 100 μg/ml trypsin on whole cell K_{ATP} currents recorded from voltage-clamped CRI-G1 cells. The cell membrane was voltage clamped at a holding potential between −70 and −75 mV, and alternate ±10 mV pulses, 200 ms in duration, were applied every 2 s. The resultant K_{ATP} current responses are denoted by the *vertical lines*. *a*, in this recording, the cell was bathed in solution A, and the electrode contained solution B. The cell exhibited the typical time course of activation and subsequent rundown of K_{ATP} currents. *b*, in this recording, the cell was bathed in solution A, and the electrode contained solution B to which 100 μg/ml trypsin was added. This resulted in a typical time course of activation, but, following the peak current, rundown was reduced substantially. *c*, change in whole cell K_{ATP} current amplitude with time after the peak current. The data are expressed as the natural logarithm of the normalized ratio (I') of the currents at a given time with respect to the peak current. The graphs were drawn from the data shown in *a* and *b* in the presence (○) and absence (●) of 100 μg/ml trypsin in the electrode solution.

The extracellular application of trypsin did not affect K_{ATP} channel activity *per se*, but it was necessary to test whether it could influence the pharmacological inhibition of the channel. When trypsin at 500 μg/ml was applied to the external surface of an outside-out patch, the inhibitory effectiveness of 100 μM tolbutamide applied to the external surface was not affected. In the absence of trypsin, this concentration of tolbutamide produced 83.6 ± 7.8% (n = 4) inhibition, and, following trypsinization, 75.8 ± 9.1% (n = 5) inhibition was achieved. Furthermore, the inhibitory effectiveness of both 10 μM phentolamine (80.4 ± 10.5% (n = 4) inhibition before and 73.3 ± 7.1% (n = 5) inhibition after trypsinization) and thiopentone (92.6 ± 4.8% (n = 5) inhibition before and 88.2 ± 7.2% (n = 5) inhibition after trypsinization) were similarly unaffected by trypsinization of the external surface of outside-out patches.

Detailed studies of the concentration dependence of K_{ATP} channel inhibitors cannot easily be performed on isolated membrane patches. The principal reasons for this are the wide variations in channel activity between patches and the phenomenon of channel rundown (30). A more accurate means by which to assess the concentration dependence of the above compounds is to use the whole cell configuration (2, 25).

Experiments were performed to examine tolbutamide inhibition of whole cell K_{ATP} currents following exposure of internal or external surfaces of the cell membrane to trypsin. In the absence of trypsin, from both the bath and pipette solutions, the application of extracellular tolbutamide produced inhibition of the whole cell current and an EC_{50} of 12.1 ± 2.2 μM and a Hill coefficient of 0.95, consistent with previous investigations (31). However, when tolbutamide was applied to cells that had been dialyzed with pipette solutions containing 100 μg/ml trypsin, there was a dramatic reduction in tolbutamide-dependent inhibition. For example, the initial application of an extremely high concentration of tolbutamide (1 mM) produced only a small reduction in the whole cell current, which slowly

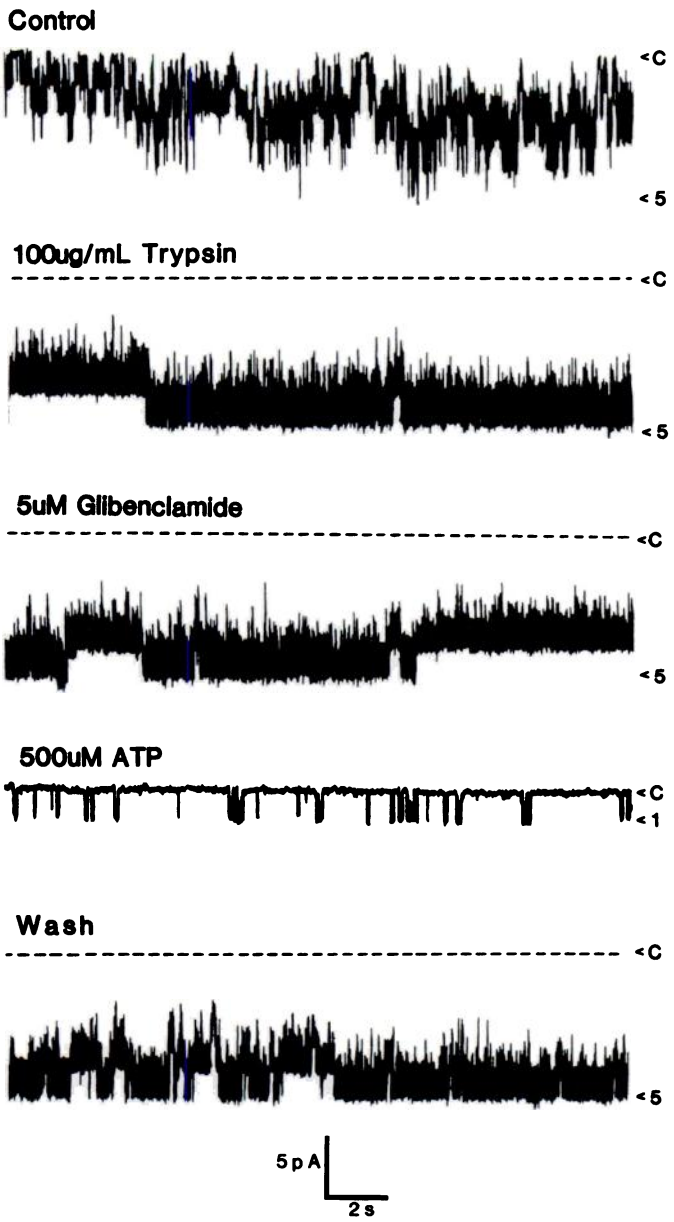

Fig. 4. Single channel currents recorded from an inside-out membrane patch exposed to symmetrical 140 mM KCl held at a membrane potential of −40 mV. The application of 100 μg/ml trypsin to the patches' intracellular surface induced a large activation of the K_{ATP} channel open probability. Once treated with trypsin, the sensitivity of the K_{ATP} channel to the sulfonylurea glibenclamide and also to ATP was reduced. The values for $N_f \cdot P_o$ were as follows: control, 2.11; trypsin, 4.45; glibenclamide, 4.11; ATP, 0.09; wash, 4.02.

reversed as the trypsin began to take full effect (Fig. 5*a*). Subsequent exposure of the cell to the same concentration of tolbutamide failed to produce any inhibition. Furthermore, 10 mM tolbutamide produced only approximately 50% inhibition, and 100 μM tolbutamide was ineffective. The EC_{50} for tolbutamide in the presence of 100 μg/ml trypsin in the electrode solution was found to be 9.7 ± 1.0 mM with a Hill coefficient of 1.9 (22 cells; Fig. 5*d*). To obtain these values, it was necessary to assume the same maximal effectiveness of tolbutamide as was observed in the absence of trypsin, where an EC_{50} of 12.1 ± 2.2 μM was obtained (31). As shown in Fig. 5, *b* and *c*, tolbutamide inhibition was only slightly affected by an electrode solution of 100 μg/ml heat-inactivated trypsin or by treatment of the cells' external surface with 1 mg/ml trypsin. The EC_{50} obtained for tolbutamide under such conditions were 30.0 ± 4.5 μM (14 cells) and 47.7 ± 12.2 μM (16 cells), respectively.

Similarly, the introduction of 100 μg/ml trypsin into the pipette solution dramatically reduced the sensitivity of the K_{ATP} whole cell currents to glibenclamide. The EC_{50} for glibenclamide under such circumstances was calculated to be 1.0 ± 0.2 mM (19 cells) with a Hill coefficient of 2.9. These values were obtained assuming the same maximal effectiveness of glibenclamide as obtained previously (31), where an EC_{50} of 2.1 nM and a Hill coefficient of 1.48 was derived.

The effect of trypsin on the nonsulfonylurea K_{ATP} channel inhibitors was also examined using the whole cell recording. Fig. 6 displays the effects of trypsin upon the ability of meglitinide and phentolamine to inhibit whole cell K_{ATP} currents. In the absence of trypsin, both agents produced a poorly reversible inhibition of K_{ATP} currents as demonstrated in Fig. 6, *a* and *b*. The calculated EC_{50} values under such conditions were 39.8 ± 8.9 nM for meglitinide (18 cells) and 0.79 ± 0.08 μM for phentolamine (21 cells). These values compare well with those reported previously (5, 32). However, when 100 μg/ml trypsin was introduced into the electrode solution, the inhibitory effectiveness of meglitinide was dramatically reduced (Fig. 6, *c* and *d*), and the effectiveness of phentolamine was also reduced though to a lesser extent. Under such conditions, the EC_{50} for meglitinide was 28.3 ± 25.2 mM (15 cells) and the EC_{50} for phentolamine was 69.2 ± 17.9 μM (24 cells). In contrast, the barbiturate thiopentone (100 μM) produced an inhibition of the whole cell currents in the presence of intracellular trypsin (Fig. 5*a*) with an EC_{50} of 36.8 ± 10.8 μM (18 cells), which was not significantly different from the value we have previously obtained in the absence of trypsin (69.5 ± 18.6 μM) (31).

Binding studies

In order to assess whether or not the effect of trypsin was to remove the sulfonylurea receptor or to disrupt the linkage between channel and receptor, the effects of trypsin upon the binding characteristics of glibenclamide were examined. In Fig. 7*a*, the effect of exposing 100 μg/ml trypsin to the membrane homogenate for varying lengths of time is shown. After a 10-min exposure to the enzyme, specific high affinity glibenclamide binding was completely lost. However, when the above concentration of trypsin was exposed to the external surface of intact cells for a period of 10 min and glibenclamide binding subsequently examined, no significant change in the characteristics of ^{3}H-labeled glibenclamide binding was observed (Fig. 7*b*) (K_d 1.23 ± 0.26 nM and B_{max} 1449 ± 77 fmol/mg for trypsin treated cells, K_d 0.97 ± 0.12 nM and B_{max} 1183 ± 115 fmol/mg for control conditions).

Discussion

The principal finding of this study is that in the CRI-G1 insulin-secreting cell line, the exposure of the intracellular surface of the plasma membrane to trypsin leads to an irreversible change in K_{ATP} channel characteristics. This action appears specific to the intracellular surface, because the enzyme is ineffective when applied to the external surface at 5 and 10 times the concentration used to produce effects at the internal surface. Trypsin cleaves peptides specifically at lysine and

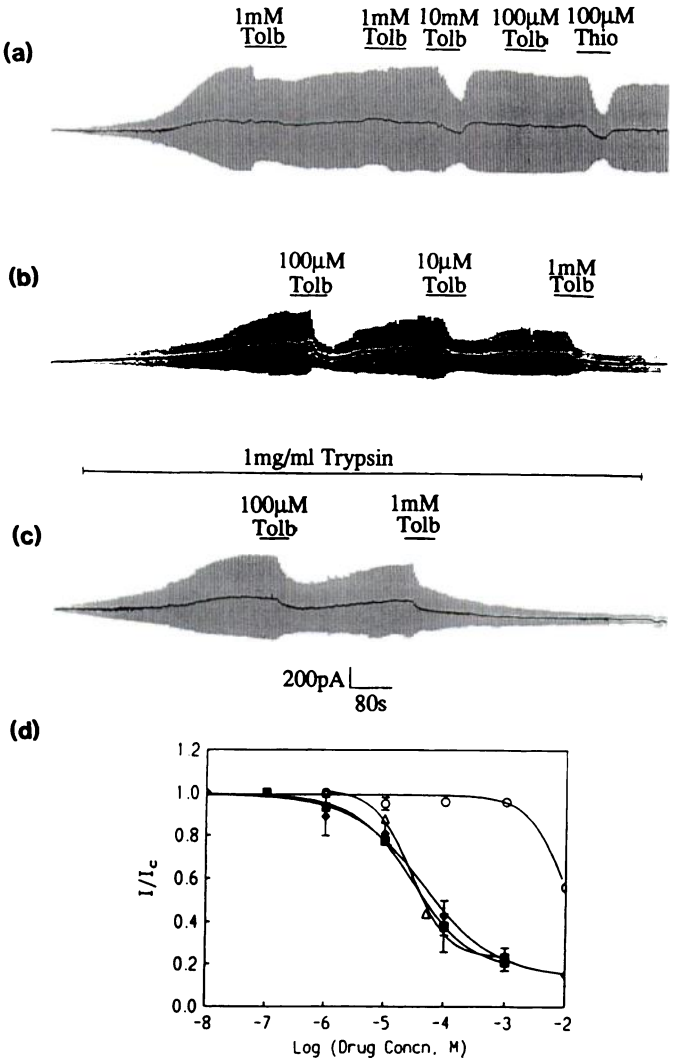

Fig. 5. Recordings of whole cell voltage-clamped K_{ATP} currents from single CRI-G1 cells. The extracellular solution was A, and the pipette solution was B. The cell membrane was clamped at −70 mV, and alternate voltage pulses of ±10 mV, 200 ms in duration, were applied at 2-s intervals. The current responses are denoted by the *vertical lines*. The bath was continuously perfused with solution A, and drug additions were made as denoted by the *bars*. The abbreviation *Tolb* represents tolbutamide, and *Thio* represents thiopentone. *a*, with 100 μg/ml trypsin present in the electrode solution, the inhibitory effectiveness of tolbutamide is small. The application of tolbutamide at high concentrations (1 mM) early in the course of the experiment produces an inhibitory effect that gradually reverses. The effects of tolbutamide are then lost, except for effects seen with extremely high concentrations (10 mM). However, the inhibitory action of thiopentone is not influenced by the presence of trypsin. *b*, in the presence of 100 μg/ml heat-inactivated trypsin, tolbutamide retains its normal K_{ATP} channel inhibitory properties. Compare the large irreversible inhibition produced by 1 mM tolbutamide here with the lack of effect of this concentration of tolbutamide in *a*. *c*, the application of 1 mg/ml trypsin to the cells' external surface does not affect the inhibitory properties of tolbutamide. 1 mM tolbutamide produces a large, irreversible inhibition of K_{ATP} currents. *d*, concentration-inhibition curves for tolbutamide and thiopentone upon K_{ATP} currents recorded using the whole cell configuration. Data are presented as fractions of control currents (I_c), taking rundown into consideration. All points are the means of between three and eight experiments; *vertical lines* show SE, and, where no vertical lines are shown, the SE was smaller than the symbol. Symbols are as follows: ■, concentration-inhibition curve for tolbutamide in the presence of heat-inactivated 100 μg/ml trypsin within the electrode solution; ○, concentration-inhibition curve for tolbutamide in the presence of 100 μg/ml trypsin within the electrode solution; ◆, concentration-inhibition curve for tolbutamide in the presence of 1 mg/ml trypsin in the external solution; △, concentration-inhibition curve for thiopentone in the presence of 100 μg/ml trypsin within the electrode solution. The values for EC_{50} were obtained by fitting the data using nonlinear regression to a modified Hill equation using GraphPAD InPlot (GraphPAD Software, San Diego, CA). In order to fit the curve for tolbutamide in the presence of 100 μg/ml trypsin in the electrode solution, the data were fit assuming the same maximal response as obtained with heat-inactivated trypsin in the electrode solution.

arginine residues (15), and it would appear that this proteolytic action is the mechanism by which trypsin achieves its effects, because both heat-inactivated trypsin and trypsin in the presence of trypsin inhibitor fail to produce similar effects.

The most obvious effects of trypsin are the increase in K_{ATP} channel activity and reduction in the rate of channel rundown. Thus, the trypsinized channel functions similarly to the K_{ATP} channel in the absence of intracellular Mg^{2+} (26). For example, in Mg^{2+}-free conditions, the reported whole cell current density is 25.4 ± 2.5 pA/pF, and rundown can be characterized by a single rate constant of 0.20 ± 0.08 hr^{-1}. These values compare well with the equivalent values obtained for the trypsinized channel in the present study. However, although the effects of Mg^{2+} removal are reversible, those of tryptic digestion are not.

In conjunction with the activation of K_{ATP} channel by trypsin, there is a reduction in the sensitivity of the channel to ATP (see Ref. 20) and an almost total loss of sulfonylurea sensitivity. Despite producing such dramatic changes in the susceptibility of the channel to modulation, the pore properties of the channel remain apparently unaffected by trypsinization with no measurable difference in its conductance or selectivity properties. Furthermore, trypsinization produces no apparent change in the channel rectification properties, supporting the evidence for the channel pore's being unchanged. Similar results have been reported by others (16, 20).

It has recently been suggested that the K_{ATP} channel is a modified dephosphorylated form of the delayed rectifier (33) and the possibility, therefore, arises that the changes we have observed upon the addition of trypsin are due to appearance of the delayed rectifier. However, we believe that the effects observed in the present study can be attributed to a change in the activation of the K_{ATP} channel alone. First, the data obtained from single channel recording experiments clearly indicate that the biophysical characteristics of the trypsin-induced K^+ channels, which include their conductance, rectification, and voltage insensitivity, are identical to those of the normal K_{ATP} channel observed in these cells (26, 28, 31). Additionally, although the ATP sensitivity of the trypsinized K_{ATP} channel was reduced, it was retained, with 500 μM ATP producing 90.4 ± 5.6% inhibition. This observation is important, as previous studies have shown that the voltage-activated potassium currents present in this cell line are not inhibited by millimolar concentrations of this nucleotide (34). In addition, the channels responsible for carrying the delayed rectifier current in β-cells have been identified (35) and have much smaller single channel conductance (5–10 pS) than the trypsin-activated channels we have observed. If the treatment of the excised membranes with trypsin had uncovered voltage-activated channels, we would also have expected to record differences in the level of channel activity at depolarizing and hyperpolarizing potentials, and no such changes were observed. In agreement with these findings, other workers have also failed to observe the activation of other channels by trypsin under similar conditions in both insulin-

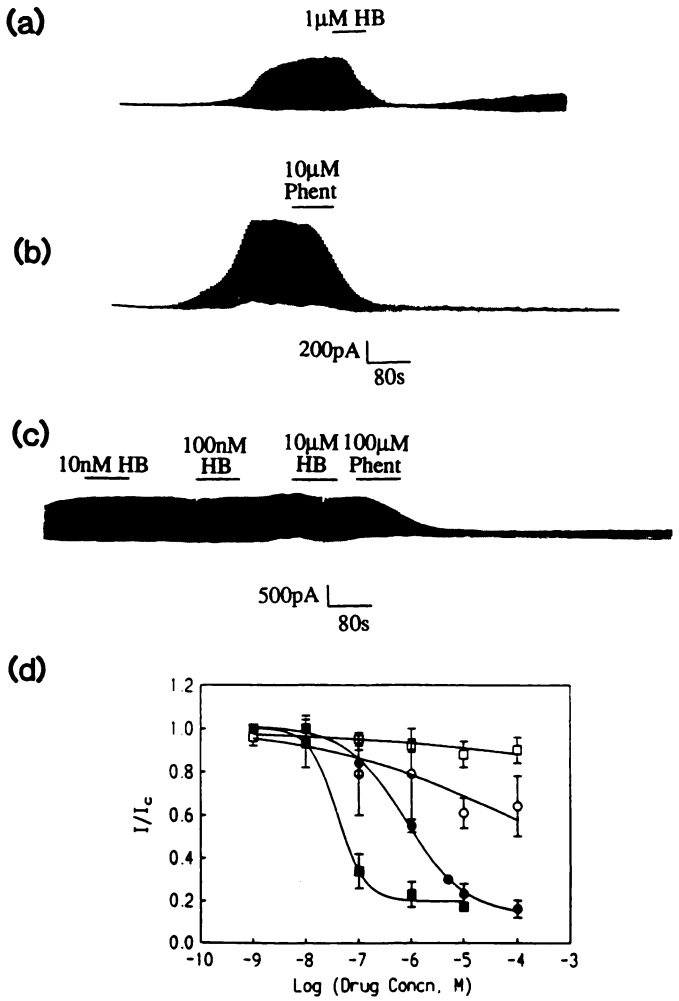

Fig. 6. Recordings of whole cell voltage-clamped K_{ATP} currents from single CRI-G1 cells. The extracellular solution was A, and the pipette solution was B. The cell membrane was clamped at −70 mV and alternate voltage pulses of ±10 mV, 200 ms in duration, were applied at 2-s intervals. The current responses are denoted by the *vertical lines*. The bath was continuously perfused with solution A, and drug additions were made as denoted by the *bars*. The abbreviation *HB* represents meglitinide, and *Phent* represents phentolamine. *a*, 1 μM meglitinide produced a potent inhibition of K_{ATP} currents, which was only poorly reversible on washout. *b*, 10 μM phentolamine also produced a substantial inhibition of K_{ATP} currents, which was poorly reversed on washout. *c*, the application of 100 μg/ml trypsin to the electrode solution markedly reduces the inhibitory potency of these agents. Under these conditions, meglitinide is without effect even at 10 μM, and the potency of phentolamine is also reduced. *d*, concentration-inhibition curves for meglitinide and phentolamine upon K_{ATP} currents recorded using the whole cell configuration. Data are presented as fractions of control currents (I_c), taking rundown into consideration. All points are the means of between three and eight experiments; *vertical lines* show SE, and, where no vertical lines are shown, the SE was smaller than the symbol. Symbols are as follows: ■, concentration-inhibition curve for meglitinide in the absence of 100 μg/ml trypsin within the electrode solution; □, concentration-inhibition curve for meglitinide in the presence of 100 μg/ml trypsin within the electrode solution; ●, concentration-inhibition curve for phentolamine in the absence of 100 μg/ml trypsin in the electrode solution; ○, concentration-inhibition curve for phentolamine in the presence of 100 μg/ml trypsin within the electrode solution. The values for EC_{50} were obtained by fitting the data using nonlinear regression to a modified Hill equation using GraphPAD InPlot (GraphPAD Software). In order to fit the curves for meglitinide and phentolamine in the presence of 100 μg/ml trypsin in the electrode solution, the data were fit assuming the same maximal response as obtained in the absence of this enzyme.

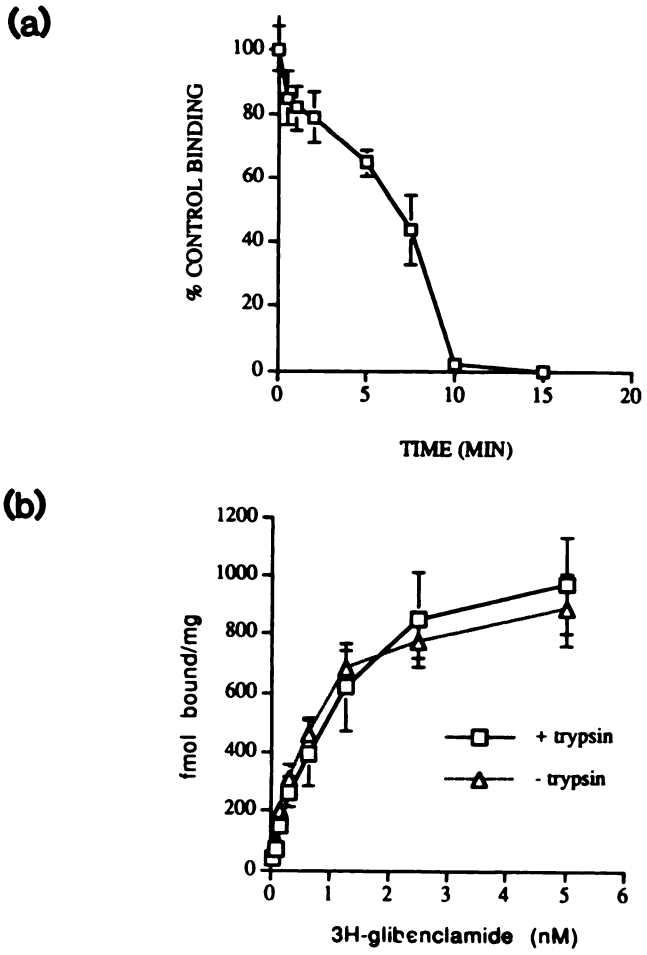

Fig. 7. The effects of trypsin upon the characteristics of ^{3}H-labeled glibenclamide binding to the sulfonylurea receptor in membranes of CRI-G1 insulin-secreting cells. *a*, the time course of loss of specific ^{3}H-labeled glibenclamide binding to CRI-G1 cell membranes incubated with 100 μg/ml trypsin. Note that specific binding is almost completely lost after a 10-min exposure to trypsin. Membranes were incubated with 100 μg/ml trypsin for the indicated times before the addition of 200 μg/ml trypsin inhibitor. Reaction mixtures were then incubated with 0.2 nM ^{3}H-labeled glibenclamide as described under Materials and Methods. Results are expressed as a percent of control binding (238 fmol/mg of protein), after subtraction of nonspecific binding, and are the mean ± SE of three experiments each performed in triplicate. *b*, the effect of trypsin upon the characteristics of ^{3}H-labeled glibenclamide binding when applied to the cells' external surface. Cells were preincubated with 100 μg/ml trypsin for 10 min before being homogenized and incubated with increasing concentrations of ^{3}H-labeled glibenclamide as described under Materials and Methods. Results are expressed as fmol ^{3}H-labeled glibenclamide bound/mg of protein after subtraction of nonspecific binding and are the mean ± SE of three experiments each performed in triplicate.

secreting cells (20) and cardiac myocytes (16), where the concentration of trypsin employed was 10 times that used in the present study.

Although the whole cell recording configuration provides much more limited information regarding the nature of the ionic conductance studied, we also believe that the potassium conductance observed in this study, both before and after trypsinization, is carried by K_{ATP} channels. In addition to the single channel data described above, it has previously been shown that the voltage-activated potassium channels present in this cell line are activated only when the membrane potential is increased above −20 mV (34, 35). Thus, under the conditions

employed in this study (i.e., holding potential −70 ± 10 mV), it is highly unlikely that these channels would contribute to the observed potassium conductance. This assumption is verified by the finding that the ability of the barbiturate thiopentone to inhibit whole cell K_{ATP} channel currents following trypsinization is unaltered. Previous studies have shown that voltage-dependent potassium channels present in this cell line are much less sensitive to thiopentone than are K_{ATP} channels (4). Thus, if trypsin were to activate other potassium channels, which then contributed to the whole cell potassium conductance, a marked reduction in the barbiturates' potency would be expected.

In agreement with the findings of the present study, Proks and Ashcroft (20) report that trypsinization reduced K_{ATP} channel sensitivity to ATP and ADP. In contrast, Furukawa *et al.* (16) report that this enzyme did not alter the channels' ATP sensitivity. These differences may be due to the fact that the present study and that of Proks and Ashcroft (20) used insulin-secreting cells, and the study by Furukawa *et al.* (16) used cardiac myocytes. This conclusion seems vindicated by the fact that a second study, also in cardiac myocytes, similarly reports no change in the ATP sensitivity of trypsinized K_{ATP} channel (29). Thus, it would appear that the K_{ATP} channel present in insulin-secreting cells differs from the K_{ATP} channel present in cardiac myocytes in that the inhibitory ATP binding site of the former channel is trypsin sensitive. It should be noted that in the studies performed on cardiac myocytes, much higher concentrations of trypsin have been used than in this study, and the former study in insulin-secreting cells (20). This may suggest that the enzyme produces its effects at different sites in the two types of tissues. With regard to this, in the study undertaken by Furukawa *et al.* (16), the effects of trypsinization could be mimicked by carboxypeptidase A. However, this enzyme was without significant effect upon insulin-secreting cells (20).

Despite these differences, there is agreement regarding the loss of K_{ATP} channel sensitivity to the sulfonylureas produced by trypsinization in both cardiac myocytes (29) and also insulin-secreting cells (Ref. 20 and present study). In the former studies, only glibenclamide and tolbutamide were used, and no attempt was made to construct concentration response curves for these agents. In the present study, we have examined the effects of trypsin over a wide range of concentrations and have shown that the loss of sulfonylurea inhibitory potency produced by application of this enzyme to the cytoplasmic aspect of the plasma membrane is almost complete. Although some inhibition of K_{ATP} channel activity is produced by extremely high concentrations of these drugs (e.g., 10 mM tolbutamide), it is likely that the inhibition produced by the sulfonylureas at these concentrations is of a nonspecific nature similar to that reported for other potassium channels (36, 37). The present study also demonstrates that trypsinization removes the K_{ATP} channel inhibitory effects of the nonsulfonylurea, meglitinide. Previous studies have suggested that this agent may act via the sulfonylurea receptor (38), and this theory may explain the present findings. Alternatively, it may be that trypsin prevents the sulfonylureas and meglitinide from inhibiting K_{ATP} channel activity by acting at different sites.

We have recently shown that removal of intracellular Mg^{2+} also reduces the inhibitory effectiveness of the sulfonylureas and meglitinide (31). However, since sulfonylurea binding was not affected by Mg^{2+} removal, this loss of potency was thought to be due to functional uncoupling of the receptor from the K_{ATP} channel. Diazoxide, like the sulfonylureas, influences K_{ATP} channel activity in a Mg^{2+}-dependent manner (24), such that removal of intracellular Mg^{2+} reduces its effectiveness. Similarly, intracellular trypsin abolishes K_{ATP} channel sensitivity to this agent (19). In addition to these observations, it has been reported that diazoxide can allosterically modify the binding of the sulfonylureas to their receptors (39). Thus, it is possible that both diazoxide and the sulfonylureas interact with the K_{ATP} channel via the same Mg^{2+}-dependent protein, which is removed by the application of trypsin.

The inhibitory effects of the barbiturate thiopentone (4) are unaffected by trypsinization, suggesting that its site of action is different from that of the sulfonylureas and also meglitinide. In support of this assumption, we have previously shown that the inhibitory effects of thiopentone are also Mg^{2+} independent (31). In contrast, the inhibitory action of the α_2 adrenoceptor antagonist phentolamine on K_{ATP} channel activity (5), although very variable following trypsinization, is reduced. It will be interesting to examine whether this compound inhibits the K_{ATP} channel in a Mg^{2+}-dependent manner.

From our ligand binding studies, we have demonstrated that trypsin is capable of totally removing specific glibenclamide binding within 10 min of enzyme exposure to the intracellular surface of insulin-secreting cell membranes. A similar finding was reported for the sulfonylurea receptor present in the rat cerebral cortex (40). However, the enzyme failed to affect high affinity receptor binding when exposed to the extracellular surface (Fig. 7*b*). These results further support the proposition that the sulfonylurea receptor is present at the intracellular aspect of the plasma membrane (10, 25).

At present, it is unclear whether the tryptic loss of effectiveness of ATP, the sulfonylureas, diazoxide, and also phentolamine is due to the removal of a single proteinacious fragment or whether the enzyme modifies the channel at multiple sites. It is also unclear whether the increase in channel activity and removal of channel rundown produced by trypsin are a result of the removal of further protein fragments or whether they arise due to the loss of the above residue(s). In a recent study, we have shown that both lysine and arginine groups are required to be available before trypsin can alter channel function (18). This evidence, in addition to the multiple effects produced by trypsin in the present study, may suggest that the enzyme has more than one site of action upon the K_{ATP} channel protein.

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References

1. Ashcroft, S. J. H., and F. M. Ashcroft. Properties and functions of ATP-sensitive K channels. *Cell. Signal.* **2**:197–214 (1990).
2. Trube, G., P. Rorsman, and T. Ohno-Shosaku. Opposite effects of tolbutamide and diazoxide on the ATP-dependent K^+ channel in pancreatic β-cells. *Pflugers Arch.* **407**:493–499 (1986).
3. Sturgess, N. C., M. L. J. Ashford, D. L. Cook, and C. N. Hales. The sulfonylurea receptor may be an ATP-sensitive potassium channel. *Lancet* **ii**:474–475 (1985).
4. Kozlowski, R. Z., and M. L. J. Ashford. Barbiturates inhibit ATP-K^+ channels and voltage-activated currents in CRI-G1 insulin-secreting cells. *Br. J. Pharmacol.* **103**:2021–2029 (1991).
5. Dunne, M. J. Block of ATP-regulated potassium channels by phentolamine and other α-adrenoceptor antagonists. *Br. J. Pharmacol.* **103**:1847–1850 (1991).
6. Muller, M., J. De Weille, and M. Lazdunski. Chlorpromazine and related phenothiazines inhibit the ATP-sensitive K^+ channel. *Eur. J. Pharmacol.* **198**:101–104 (1991).

7. Aguilar-Bryan, L., C. G. Nichols, A. S. Rajan, C. Parker, and J. Bryan. Co-expression of sulfonylurea receptors and K_{ATP} channels in hamster insulinoma tumor (HIT) cells. *J. Biol. Chem.* **267**:14934–14940 (1992).
8. Khan, R. N., C. N. Hales, S. E. Ozanne, A. A. Adogu, and M. L. J. Ashford. Dissociation of K_{ATP} channel and sulfonylurea receptor in the rat clonal insulin-secreting cell line, CRI-D11. *Proc. R. Soc. Lond. (Biol.)* **253**:225–231 (1993).
9. Bowen, V., and N. R. Lazarus. Insulin release from the perfused rat pancreas. *Biochem. J.* **142**:385–389 (1974).
10. Zunkler, B. J., G. Trube, and U. Panten. How do sulfonylureas approach their receptors in the β cell plasma membrane? *Naunyn Schmiedebergs Arch. Pharmacol.* **340**:328–332 (1989).
11. Armstrong, C. M., F. Bezanilla, and E. Rojas. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* **62**:375–391 (1973).
12. Hescheler, J., and W. Trautwein. Modification of L type calcium current by intracellularly applied trypsin in guinea pig ventricular myocytes. *J. Physiol.* **404**:259–274 (1988).
13. Hoshi, T., W. N. Zagotta, and R. W. Aldrich. Biophysical and molecular mechanisms of Shaker potassium channel inactivation. *Science* **250**:533–538 (1990).
14. Zagotta, W. N., T. Hoshi, and R. W. Aldrich. Restoration of inactivation in mutants of Shaker potassium channels by a peptide derived from ShB. *Science* **250**:568–571 (1990).
15. Keil, B. Trypsin, in *The enzymes* (P. D. Boyer, ed.) Vol. III. Academic Press, New York, 250–273 (1971).
16. Furukawa, T., Z. Fan, T. Sawanobori, and M. Hirasoka. Modification of the adenosine 5′ triphosphate-sensitive K^+ channel by trypsin in guinea pig ventricular myocytes. *J. Physiol.* **466**:707–726 (1993).
17. Trube, G., J. Hescheler, and K. Schoter. Regulation and function of ATP-dependent K^+ channels in pancreatic β-cells, in *Secretion and its control* (G. S. Oxford and C. M. Armstrong, eds.) Society of General Physiologists, Series 44. Rockefeller University Press, New York, 84–95 (1989).
18. Lee, K., S. E. Ozanne, C. N. Hales, and M. L. J. Ashford. Effects of chemical modification of amino and sulfydryl groups of K_{ATP} channel function and sulfonylurea binding in CRI G1 insulin-secreting cells. *J. Membr. Biol.* **139**:167–181 (1994).
19. Kozlowski, R. Z., and M. L. J. Ashford. Nucleotide-dependent activation of K_{ATP} channels by diazoxide in CRI G1 insulin-secreting cells. *Br. J. Pharmacol.* **107**:34–43 (1992).
20. Proks, P., and F. M. Ashcroft. Modification of K-ATP channels in pancreatic β-cells by trypsin. *Pflugers Arch.* **424**:63–72 (1993).
21. Carrington, C. A., E. D. Rubery, E. C. Pearson, and C. N. Hales. Five new insulin-producing cell lines with differing secretory properties. *J. Endocrinol.* **109**:193–200 (1986).
22. Hamill, O. P., A. Marty, E. Neher, B. Sakman, and F. J. Sigworth. Improved patch clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* **391**:85–100 (1981).
23. Dempster, J. Computer analysis of electrophysiological signals, in *Microcomputers in physiology: a practical approach* (P. J. Fraser, ed.) IRL Press, Oxford, 51–93 (1988).
24. Kozlowski, R. Z., C. N. Hales, and M. L. J. Ashford. Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line. *Br. J. Pharmacol.* **97**:1039–1050 (1989).
25. Sturgess, N. C., R. Z. Kozlowski, C. A. Carrington, C. N. Hales, and M. L. J. Ashford. Effects of sulfonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. *Br. J. Pharmacol.* **95**:83–94 (1988).
26. Kozlowski, R. Z., and M. L. J. Ashford. ATP-sensitive K^+ channel rundown is Mg^{2+} dependent. *Proc. R. Soc. Lond. (Biol.)* **240**:397–410 (1990).
27. Findlay, I. The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J. Physiol.* **391**:611–629 (1987).
28. Sturgess, N. C., M. L. J. Ashford, C. A. Carrington, and C. N. Hales. Single channel recordings of potassium currents in an insulin-secreting cell line. *J. Endocrinol.* **109**:201–207 (1986).
29. Nichols, C. G., and A. N. Lopatin. Trypsin and α chymotrypsin treatment abolishes glibenclamide sensitivity of K_{ATP} channels in rat ventricular myocytes. *Pflugers Arch.* **422**:617–619 (1993).
30. Findlay, I., and M. J. Dunne. ATP maintains ATP-inhibited K^+ channels in an operational state. *Pflugers Arch.* **407**:238–240 (1986).
31. Lee, K., S. E. Ozanne, C. N. Hales, and M. L. J. Ashford. Mg^{2+}-dependent inhibition of K_{ATP} activity by sulfonylureas in CRI-G1 insulin-secreting cells. *Br. J. Pharmacol.* **111**:632–640 (1994).
32. Zunkler, B. J., S. Lenzen, K. Manner, U. Panten, and G. Trube. Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K^+ currents in pancreatic β-cells. *Naunyn Schmiedebergs Arch. Pharmacol.* **337**:225–230 (1988).
33. Edwards, G., T. Ibbotson, and A. H. Weston. Levcromakalim may induce a voltage-independent K current in rat portal veins by modifying the gating properties of the delayed rectifier. *Br. J. Pharmacol.* **110**:1037–1048 (1993).
34. Kozlowski, R. Z., N. C. Sturgess, C. N. Hales, and M. L. J. Ashford. Voltage-activated currents in the CRI-G1 rat insulin-secreting cell line. *Comp. Biochem. Physiol.* **100A**:613–621 (1991).
35. Zunkler, B. J., G. Trube, and T. Ohno-Shosaku. Forskolin-induced block of delayed rectifying K^+ channels in pancreatic β-cells is not mediated by cAMP. *Pflugers Arch.* **411**:613–619 (1988).
36. Crepel, V., K. Krnjevic, and Y. Ben-Ari. Sulfonylureas reduce the slowly inactivating D type outward current in rat hippocampal neurons. *J. Physiol.* **466**:39–54 (1993).
37. Reeve, H. L., P. F. T. Vaughan, and C. Peers. Glibenclamide inhibits a voltage-gated K^+ current in the human neuroblastoma cell line SH-SY5Y. *Neurosci. Lett.* **135**:37–40 (1992).
38. Brown, G. R., and A. J. Foubister. Receptor binding sites of hypoglycemic sulfonylureas and related [(acylamino)alkyl] benzoic acids. *J. Med. Chem.* **27**:79–81 (1984).
39. Schwanstecher, M., S. Behrends, C. Brandt, and U. Panten. The binding properties of the solubilized sulfonylurea receptor from a pancreatic β cell line are modulated by the Mg^{2+} complex of ATP. *J. Pharmacol. Exp. Ther.* **262**:495–502 (1992).
40. Lupo, B., and D. Bataille. A binding site for ^{3}H-glipizide in the rat cerebral cortex. *Eur. J. Pharmacol.* **140**:157–169 (1987).

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