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Effects of Chemical Modification of Amino and Sulfhydryl Groups on K_{ATP} Channel Function and Sulfonylurea Binding in CRI-G1 Insulin-secreting Cells

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Abstract. The effects of several group-specific chemical reagents were examined upon the activity of the ATP-sensitive potassium (K_{ATP}) channel in the CRI-G1 insulin-secreting cell line. Agents which interact with the sulfhydryl moiety (including 1 mM *N*-ethylmaleimide (NEM), 1 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DNTB) and 1 mM *o*-iodobenzoate) produced an irreversible inhibition of K_{ATP} channel activity when applied to the intracellular surface of excised inside-out patches. This inhibition was substantially reduced when attempts were made to eliminate Mg^{2+} from the intracellular compartment. ATP 50 μM and 100 μM tolbutamide were each shown to protect against the effects of these reagents. The membrane impermeable DNTB was significantly less effective when applied to the external surface of outside-out patches. Agents which interact with peptide terminal amine groups and ε amino groups of lysine [1 mM methyl acetimidate and 1 mM trinitrobenzene sulfonic acid (TNBS)] and also the guanido group of arginine (1 mM methyl glyoxal) produced a Mg^{2+}-dependent irreversible inhibition of K_{ATP} channel activity which could be prevented by ATP but not tolbutamide. The irreversible activation of the K_{ATP} channel produced by the proteolytic enzyme trypsin was prevented only when methyl glyoxal and methyl acetimidate were used in combination to inhibit channel activity. Radioligand binding studies showed that the binding of ^3H glibenclamide was unaffected by any of the above agents with the exception of TNBS which completely inhibited binding with a EC_{50} of 307 ± 6 μM.

These results provide evidence for the presence of essential sulfhydryl (possibly cysteine), and basic amino acid (possibly lysine and arginine) residues associated with the normal functioning of the K_{ATP} channel. Furthermore, we believe that the sulfhydryl group in question is situated at the internal surface of the membrane, possibly near to the channel pore.

Key words: K_{ATP} channels — Chemical modification — Sulfhydryl group — Basic amino acids — Pancreatic β-cells

Introduction

In insulin-secreting cells, the ATP-sensitive K^+ (K_{ATP}) channel is a major influence on the membrane potential, and inhibition or activation of this channel results in depolarization and hyperpolarization, respectively. Consequently, the activity of the K_{ATP} channel is a determining factor in the response of the pancreatic β-cell to alterations in nutrient levels, although an incompletely characterized counterbalancing depolarizing current is also present (Ashford, 1990).

The pivotal role of the K_{ATP} channel in the process of insulin secretion has made it an important target for therapeutic modulation. The anti-diabetic compounds, the sulfonylureas, are specific inhibitors of the K_{ATP} channel in insulin-secreting cells (Sturgess et al., 1985) while diazoxide has been shown to activate this channel (Trube, Rorsman & Ohno Shosaku, 1986; Kozlowski, Hales & Ashford, 1989) and these actions re-

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sult in stimulation and inhibition, respectively, of insulin secretion (Sturgess et al., 1988). Other inhibitors of K_{ATP} channel activity, including barbiturates (Kozlowski & Ashford, 1991) and imidazolines (Plant & Henquin, 1990), have also been demonstrated to increase insulin secretion. Another agent which has for a long time been known capable of eliciting insulin secretion is the impermeable sulfhydryl reagent *para*-chloromercuribenzenesulfonate (pCMBS), and this has been interpreted to suggest that relatively superficial sulfhydryl groups exist in the β-cell membrane which are important in the regulation of insulin secretion (Bloom et al., 1972).

Recently, an important sulfhydryl group associated with the ATP binding site of the K_{ATP} channel of skeletal muscle (Weik & Neumcke, 1989) has been identified. Radioligand binding studies with ^{3}H-glibenclamide have also shown that the presence of the disulfide reducing agent, dithiothreitol (DTT) is required in the incubation medium for nucleotide-induced displacement of this radioligand (Gopalakrishnan et al., 1991). In addition, it has recently been shown that intracellular application of agents which modify cysteine residues via redox reactions can alter the inactivation behavior of mammalian $I_{K(A)}$ channels (Ruppersberg et al., 1992).

In view of these observations, we have used a variety of group-specific reagents to investigate the significance of the sulfhydryl group to the activity of the K_{ATP} channel in the CRI-G1 insulin-secreting cell line. The importance of the basic amino acid residues lysine and arginine has also been examined using both chemical reagents and the proteolytic enzyme trypsin, in view of reports that these residues have critical roles in the normal functioning of both K^+ (Frace & Eaton, 1992; Spires & Begenisich, 1992) and Na^+ channels (Eaton et al., 1978). This study also examines the effects of these reagents on [^{3}H]-glibenclamide binding to membranes from this cell line to assess the relationships between these chemical groups, the sulfonylurea binding site and the K_{ATP} channel.

Materials and Methods

Cell Culture

Cells from the insulin-secreting cell line (CRI-G1) were cultured and passaged at 2–5 day intervals as previously described (Carrington et al., 1986). Cells used for patch clamp recording were plated onto 3.5 cm petri dishes (Falcon 3001) and used 2–4 days (inclusive) after plating.

Electrical Recording and Analysis

This study has used both the cell-free and whole cell configurations of the patch clamp recording technique as described by Hamill et al. (1981). 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 patch clamp amplifier and were stored on digital audio tape. 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 (Dempster, 1988) and run on a microcomputer. Data segments between 30 and 90 sec duration were replayed at the recorded speed and filtered at 1.0 kHz (−3 dB; 8-pole Bessel) 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 (Kozlowski et al., 1989). Changes in $N_f \cdot P_o$ as a result of the action of the chemical modifiers or drugs are expressed as percentage of control. To determine statistically whether intracellular Mg^{2+} altered the inhibitory effectiveness of the various agents, the compound in question was perfused into the bath 1 min after patch excision until complete solution exchange was achieved (approximately 1 min). The agent was left in contact with the patch for 1 min and the $N_f \cdot P_o$ value was determined immediately prior to washout. The percentage inhibition produced in the presence and absence of Mg^{2+} was assessed for significant differences using one way analysis of variance (ANOVA). To assess the effectiveness of ATP and tolbutamide in protecting channel activity from chemical modification, the protecting agent (ATP or tolbutamide) was applied to the bath until complete solution exchange was achieved (30 to 90 sec). The chemical modifier was then applied in the presence of the protecting agent for the same period of time before both agents were washed out simultaneously. The $N_f \cdot P_o$ value following washout of both agents was expressed as a percentage of the control $N_f \cdot P_o$ value obtained prior to the addition of either agent. The recovery of channel activity was then compared using the above statistical test.

To determine accurately whether the agents used inhibited the K_{ATP} channel in a Mg^{2+}-dependent manner, the whole-cell configuration was used. To obtain whole cell currents, the cell was voltage-clamped at a holding potential of −70 mV and alternate ± 10 mV pulses of 200 msec duration were elicited at 2 sec intervals as described previously (Sturgess et al., 1988). Once the peak K_{ATP} current had been attained for 4 min, the inhibitory agent was applied for a period of 2 min before being washed out. This protocol was used in the presence (solution B) and absence (solution C) of Mg^{2+}-containing solutions in the electrode. To quantify the extent of K_{ATP} channel inhibition, the amplitude of the current response was measured 6 min after the application of the inhibitory agent (at which time the full effect of the modifying agent had been achieved) and was expressed as a percentage of the control K_{ATP} current amplitude. To quantify the rate at which the modifying agent produced its effect, the K_{ATP} current amplitude was measured every 2 min (I_t) after the peak current (I_p) was attained and any change expressed as the natural logarithm of the normalized ratio (I'):

$$I' = \ln(I_t/I_p \times 100)$$

By plotting I' against time, the effects of the inhibitory agents could be defined by the rate constants associated with the loss of channel activity. By comparing these rate constants, it was possible to dis-

tinguish the inhibitory effects of these agents from the normal Mg^{2+}-dependent rundown of K_{ATP} channel activity. Data are presented in the text as the arithmetic mean ± standard error associated with the mean.

Membrane Preparation and Binding

CRI-G1 cells were grown to 70–80% confluence in Falcon 3027 805 cm^2-roller bottles (approx. 2.5×10^7 cells), harvested by scraping with a rubber policeman into Hanks physiological saline which consisted of (mM): NaCl 137.0, KCl 5.4, $MgSO_4$ 1.67, $CaCl_2$ 4.0, Na_2HPO_4 0.34, $NaHCO_3$ 4.2, pH 7.4 and centrifuged for 10 min at $220 \times g$ (MSE, Crawley, UK). 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°C. The cell suspension was homogenized in a 40 ml capacity glass tube homogenizer (Jencons Scientific, Leighton Buzzard, Beds., UK) using 10 strokes of a Teflon pestle driven at 600 rev × min^{-1}, then sonicated for 10 sec at 30 W (MSE Sonifier). The homogenate was centrifuged for 15 min at $500 \times g$ to remove unbroken cells and nuclei and the resulting supernatant was centrifuged for 30 min at $70{,}000 \times g$ (OTD65B Sorvall Ultracentrifuge, Du Pont, Stevenage, UK) 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 E64 and stored under liquid nitrogen prior to use.

CRI-G1 membranes (50 μg protein) were incubated for 2 hr at room temperature in 0.5 ml assay buffer, 50 mM Tris/HCl (pH 7.4) containing 0.2 nM [^{3}H]-glibenclamide (50.9 Ci $mmol^{-1}$, Dupont, UK) and 0.04–10 mM chemical modifying reagent. Incubations were terminated by addition of 2 ml ice-cold buffer and bound ligand was collected by rapid vacuum filtration onto 2.5 cm diameter GF/B filter discs (Whatman International, Maidstone, UK). Filters were washed with 4 × 2 ml of buffer, and the bound radioactivity was determined by liquid scintillation counting using OptiPhase HiSafe II (LKB Scintillation Products, Loughborough, UK) and a Packard liquid-scintillation spectrometer (Canberra Packard, Berks, UK).

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, pH 7.2 with NaOH. In experiments with inside-out patches, the bath solution was (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, K-EGTA 10.0, HEPES 10.0, pH 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 conducted in the absence of intracellular Mg^{2+}, solution B was replaced by solution C which contained (mM) KCl 140.0, $CaCl_2$ 4.6, K-EDTA 10.0, HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and <3.0 nM, respectively. The pipette solution in these studies contained (mM) KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH (solution D). In experiments on outside-out patches, the bathing solution was D and the pipette solution B. In whole-cell voltage clamp studies, the cells were bathed in solution A and the pipette contained either solution B (Mg^{2+} present) or solution C (Mg^{2+} absent). The concentration of free divalent cations was determined by using a program for calculating metal ion/ligand binding "METLIG" (England and Denton, University of Bristol).

Channel blockers and protein modifying agents were dissolved in the appropriate bath solution immediately prior to the experiment, and the pH was readjusted to 7.2 with KOH. All compounds tested were obtained from Sigma (Poole, Dorset, UK) and included; tolbutamide, glibenclamide, K_2ATP, NEM, *o*-iodobenzoate, iodoacetate, hydrogen peroxide, DNTB, methyl acetimidate, methyl glyoxal, TNBS, DTT, glutathione, sodium borohydride and trypsin (type XI from bovine pancreas). Tolbutamide, *o*-iodobenzoate and NEM were made up as 500 mM stock solutions in 1 M KOH while glibenclamide was made up as a 5 mM stock solution in methanol. All other reagents were water soluble.

Drugs were applied to membrane patches or whole cells by superfusing the bath by a gravity feed system, at a rate of approximately 6 ml min^{-1} which allowed complete solution exchange within 30–60 sec. All experiments were performed at room temperature, 22–25°C.

Results

Effects of Sulfhydryl Reagents on K_{ATP} Channel Activity

Figure 1*a* shows the effect of 1 mM NEM on K_{ATP} channel activity when applied to an inside-out patch, at a membrane potential of +40 mV, isolated from a CRI-G1 insulin-secreting cell. At this concentration, NEM did not alter the single channel current amplitude or the rectification induced by the presence of Mg^{2+} at the cytoplasmic surface but produced a substantial and irreversible inhibition of channel activity (Table 1). In some patches, the large conductance Ca^{2+}-activated potassium channel was present (Sturgess et al., 1986) and the activity of this channel was not altered by NEM (Fig. 1*a*) or any of the other sulfhydryl reagents used in this study, as reported previously for skeletal muscle (Weik & Neumcke, 1989). The inhibition of K_{ATP} channel activity by NEM was independent of membrane holding potential (−50 to +50 mV) both in the presence (solution B, $n = 5$) and absence (solution C, $n = 5$) of Mg^{2+} at the intracellular surface. Under the conditions of temperature and pH used for these experiments, NEM is considered to be a highly specific reagent for sulfhydryl groups (Means & Feeney, 1971). However, to ensure that sulfhydryl group modification is entirely responsible for the inhibition of K_{ATP} channel activity, we have investigated the actions of other chemical reagents reported to be more or less specific for the sulfhydryl group moiety (Torchinsky, 1977). Iodoacetate can modify a number of functional groupings in amino acids, but under these experimental conditions (i.e., pH 7.2 and room temperature), like NEM, it causes an irreversible alkylation of sulfhydryl groups. When tested on inside-out membrane patches, 5 mM iodoacetate also induced a substantial and irreversible inhibition of K_{ATP} channel activity (Table 1). Reagents that act to oxidize sulfhydryl groups also caused irreversible inhibition of K_{ATP} channel activity. For example, *o*-iodobenzoate is a highly specific oxidizer of

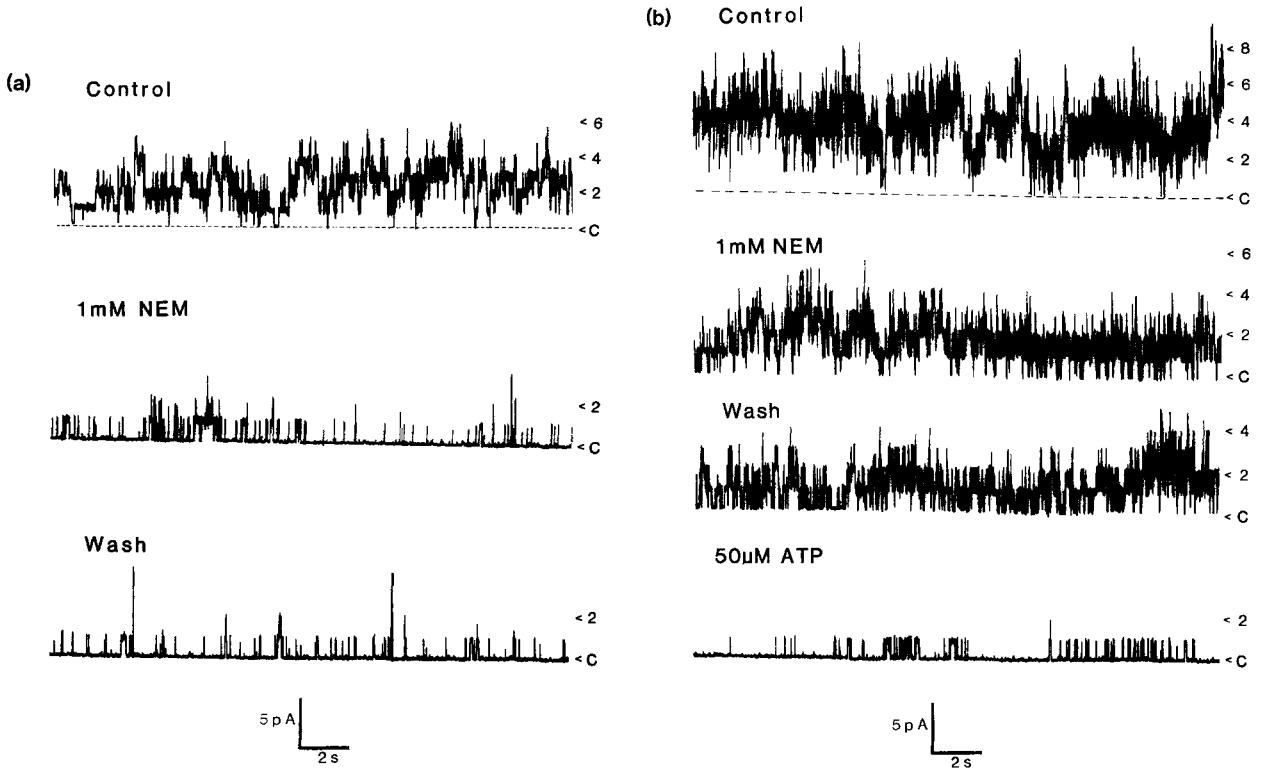

Fig. 1. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of +40 mV. Single channel openings are denoted by upward deflections (outward currents). (*a*) 1 mM NEM in the presence of 0.65 mM Mg^{2+} inhibited the activity of the K_{ATP} channel in an irreversible manner. A large conductance calcium-activated potassium channel was also present in this particular patch, but was not affected by the application of NEM. The values for $N_f P_o$ were as follows: control 2.535; NEM 0.145; wash 0.122. (*b*) 1 mM NEM in the absence of Mg^{2+} produced a much weaker inhibition of K_{ATP} channel activity. However, 50 μM ATP retained its inhibitory effectiveness. The values for $N_f P_o$ were as follows: control 4.856; NEM 2.356; wash 2.015; ATP 0.126. Note the much greater level of activity in the absence of Mg^{2+} before drug application.

Table 1. The effect of various sulfhydryl reagents on K_{ATP} channel activity in the insulin-secreting cell-line CRI-G1 in the presence and absence of intracellular Mg^{2+}

Chemical	% Inhibition	
	+0.65 mM Mg^{2+}	Mg^{2+} Free
Iodoacetate (5 mM)	84.6 ± 9.1 (n = 19)	52.5 ± 4.1 (n = 14)*
o-Iodobenzoate (1 mM)	95.0 ± 5.7 (n = 10)	56.8 ± 8.6 (n = 13)*
N-ethylmaleimide (1 mM)	94.5 ± 5.8 (n = 12)	55.7 ± 8.6 (n = 11)*
Hydrogen peroxide (50 mM)	74.2 ± 11.3 (n = 9)	36.5 ± 7.1 (n = 10)*

% inhibition refers to the reduction in the K_{ATP} channels open probability ($N_f P_o$) as a percentage of the control activity. This inhibition was independent of the membrane holding potential (−50 to +50 mV).
Values are means ± SEM; the number of experiments performed is shown in parentheses.
* Significantly ($P < 0.05$) different with respect to inhibition produced in the presence of 0.65 mM Mg^{2+}.

sulfhydryl groups (Torchinsky, 1977) and application of 1 mM of this reagent almost completely blocked channel activity (Table 1). The disulfide DNTB is considered a completely specific oxidant of the sulfhydryl function. As illustrated in Fig. 2*a*, a potent irreversible inhibition of K_{ATP} channel activity was also produced by this compound. Hydrogen peroxide is a strong oxidant which attacks the sulfur-containing amino acids cysteine and methionine. As reported by Weik and Neumcke (1989), this agent also inhibited K_{ATP} channel activity although much higher concentrations were necessary to achieve effects quantitatively similar to the other reagents used (Table 1).

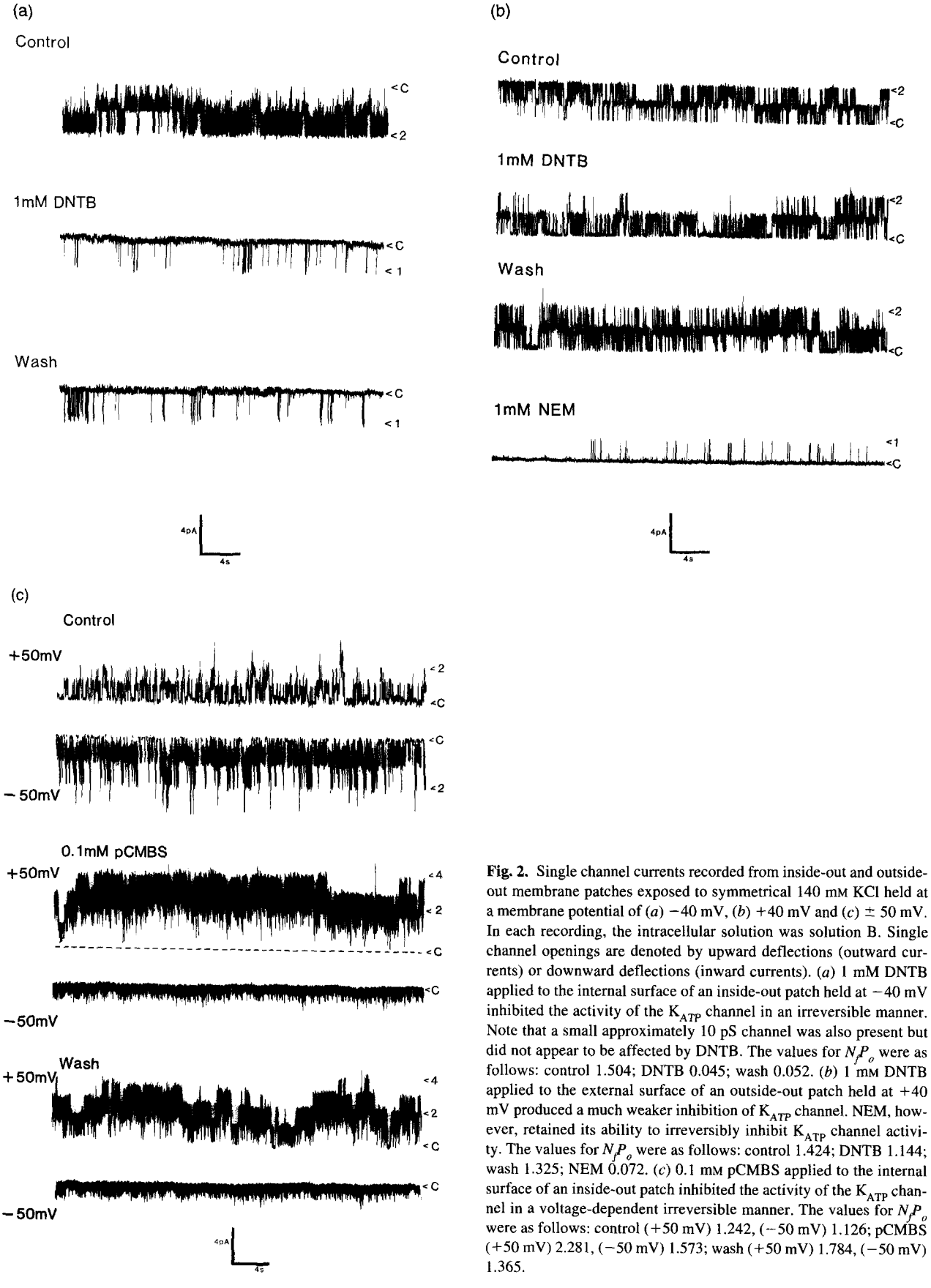

Fig. 2. Single channel currents recorded from inside-out and outside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of (*a*) −40 mV, (*b*) +40 mV and (*c*) ± 50 mV. In each recording, the intracellular solution was solution B. Single channel openings are denoted by upward deflections (outward currents) or downward deflections (inward currents). (*a*) 1 mM DNTB applied to the internal surface of an inside-out patch held at −40 mV inhibited the activity of the K_{ATP} channel in an irreversible manner. Note that a small approximately 10 pS channel was also present but did not appear to be affected by DNTB. The values for $N_f P_o$ were as follows: control 1.504; DNTB 0.045; wash 0.052. (*b*) 1 mM DNTB applied to the external surface of an outside-out patch held at +40 mV produced a much weaker inhibition of K_{ATP} channel. NEM, however, retained its ability to irreversibly inhibit K_{ATP} channel activity. The values for $N_f P_o$ were as follows: control 1.424; DNTB 1.144; wash 1.325; NEM 0.072. (*c*) 0.1 mM pCMBS applied to the internal surface of an inside-out patch inhibited the activity of the K_{ATP} channel in a voltage-dependent irreversible manner. The values for $N_f P_o$ were as follows: control (+50 mV) 1.242, (−50 mV) 1.126; pCMBS (+50 mV) 2.281, (−50 mV) 1.573; wash (+50 mV) 1.784, (−50 mV) 1.365.

Because the inhibition of channel activity by *o*-iodobenzoate is through oxidation of sulfhydryl groups, attempts were made to reverse this inhibition by addition of reducing agents. None of the reagents tested, glutathione (5 mM), DTT (5 mM) or sodium borohydride (5 mM) reversed the *o*-iodobenzoate-induced inhibition of the channel in the presence of intracellular Mg^{2+} (*data not shown*). Indeed, both DTT and glutathione *per se* induced considerable inhibition of channel activity (by 69.8 ± 17.6%, $n = 5$ and 93.8 ± 3.9%, $n = 10$, respectively).

Protection against NEM-induced K_{ATP} Channel Modification

It has been reported that there is an important sulfhydryl group associated with the nucleotide binding site both in murine skeletal muscle and rat cerebral cortex (Weik & Neumcke, 1989; Gopalakrishnan et al., 1991). Therefore, we examined the possibility that ATP could modify the inhibitory effects of the sulfhydryl reagent NEM when applied to isolated inside-out patches in CRI-G1 insulin-secreting cells. Figure 3*a* shows that on application of 50 μM ATP to an inside-out membrane patch there was a 89.5 ± 4.9% ($n = 5$) inhibition of channel activity, and following subsequent addition of 1 mM NEM in the continued presence of ATP, the level of channel activity was 94.0 ± 3.1% ($n = 5$). After the simultaneous washout of both inhibitory agents, there was a return in K_{ATP} channel activity to 38.0 ± 1.8% ($n = 5$) of the control channel activity. This contrasts with the effect of 1 mM NEM applied in the complete absence of ATP; under such conditions the level of activity following washout of NEM was 5.4 ± 2.8% ($n = 12$) of the control channel activity. Thus, the ability of 1 mM NEM to induce an irreversible inhibition of K_{ATP} channel activity in inside-out membrane patches is significantly ($P < 0.05$) reduced by the concurrent presence of 50 μM ATP. Figure 3*a* also illustrates that once ATP was removed from the patch subsequent addition of NEM produced an irreversible block of channel activity. Identical results were obtained in five separate experiments.

Weik and Neumcke (1989) also found in murine skeletal muscle that the presence of intracellular ATP protected against the irreversible inhibition of K_{ATP} channels by NEM. However, in their experiments no Mg^{2+} was present in the intracellular bath solution. On repeating the experiments with solutions containing 10 mM EDTA and no added Mg^{2+}, it was found that in the absence of ATP all the sulfhydryl reagents were significantly less effective inhibitors of K_{ATP} channel activity (Table 1). This reduction in inhibitory efficacy is illustrated for NEM in Fig. 1*b*.

Since removal of intracellular Mg^{2+} has previously been shown to reduce the rate of K_{ATP} channel rundown (Kozlowski & Ashford, 1990), it is possible that a component of the inhibition seen in the presence of Mg^{2+} may have arisen due to this process. To examine the Mg^{2+} dependence of K_{ATP} channel inhibition in closer detail, therefore, the whole-cell voltage-clamp recording configuration was used. Using this technique, there is a gradual increase in K_{ATP} current with time, presumably due to washout of intracellular ATP by dialysis with the pipette solution (Sturgess et al., 1988). After several minutes, the current amplitude peaks and subsequently proceeds to decline with time due to rundown. Figure 4*c* demonstrates the Mg^{2+} dependence of this decline in K_{ATP} current with time. In agreement with previous reports (Kozlowski & Ashford, 1990), the present study has found that in the presence of Mg^{2+} the loss of K_{ATP} current can be characterized by a biphasic time course with rate constants of 1.71 ± 0.11 (α phase) and 8.13 ± 0.64 (β phase; Table 2) hr^{-1}. In contrast, in the absence of Mg^{2+}, rundown could be characterized by a single rate constant of 0.45 ± 0.03 hr^{-1}. To examine the effect of sulfhydryl group modification upon whole cell K_{ATP} currents, 5 mM NEM was applied to the cell for a two-minute period once the peak K_{ATP} current had been attained. When this procedure was performed, NEM was seen to produce an irreversible reduction in the size of the K_{ATP} current both in the presence and absence of intracellular Mg^{2+}. As demonstrated in Fig. 4, the onset of the inhibitory effect was similar both in the presence and absence of this cation. However, the magnitude of the inhibition was Mg^{2+} dependent. In the presence of Mg^{2+}, 5 mM NEM produced 91.0 ± 4.2% ($n = 4$) inhibition while in the absence of this cation, the inhibition produced was significantly less (43.5 ± 3.6%, $n = 5$; $P < 0.05$). The K_{ATP} channel currents which remained after the addition of NEM in the absence of intracellular Mg^{2+} were not affected by the subsequent reapplication of this agent ($n = 3$). NEM was also shown to produce a dramatic change in the rate of loss of K_{ATP} activity both in the presence and absence of Mg^{2+} (Table 2). In the presence of Mg^{2+}, the slow α phase of rundown was no longer observed and the loss of activity was characterized by a single rate constant of 11.04 ± 0.78 hr^{-1}. In contrast, in the absence of Mg^{2+}, the rate of loss of channel activity was characterized by a rapid initial phase (3.91 ± 0.37 hr^{-1}) followed by a slower phase (0.36 ± 0.05 logarithmic units hr^{-1}).

Recently, we have reported that inhibition of K_{ATP} channel activity by the sulfonylureas in CRI-G1 cells is also Mg^{2+} dependent (Lee & Ashford, 1993), and it has been shown that there is an interaction between DTT, adenine nucleotides and the glibenclamide binding site (Gopalakrishnan et al., 1991). Therefore, we examined the possibility that the presence of a sulfonylurea could also protect K_{ATP} channels against inhibition by

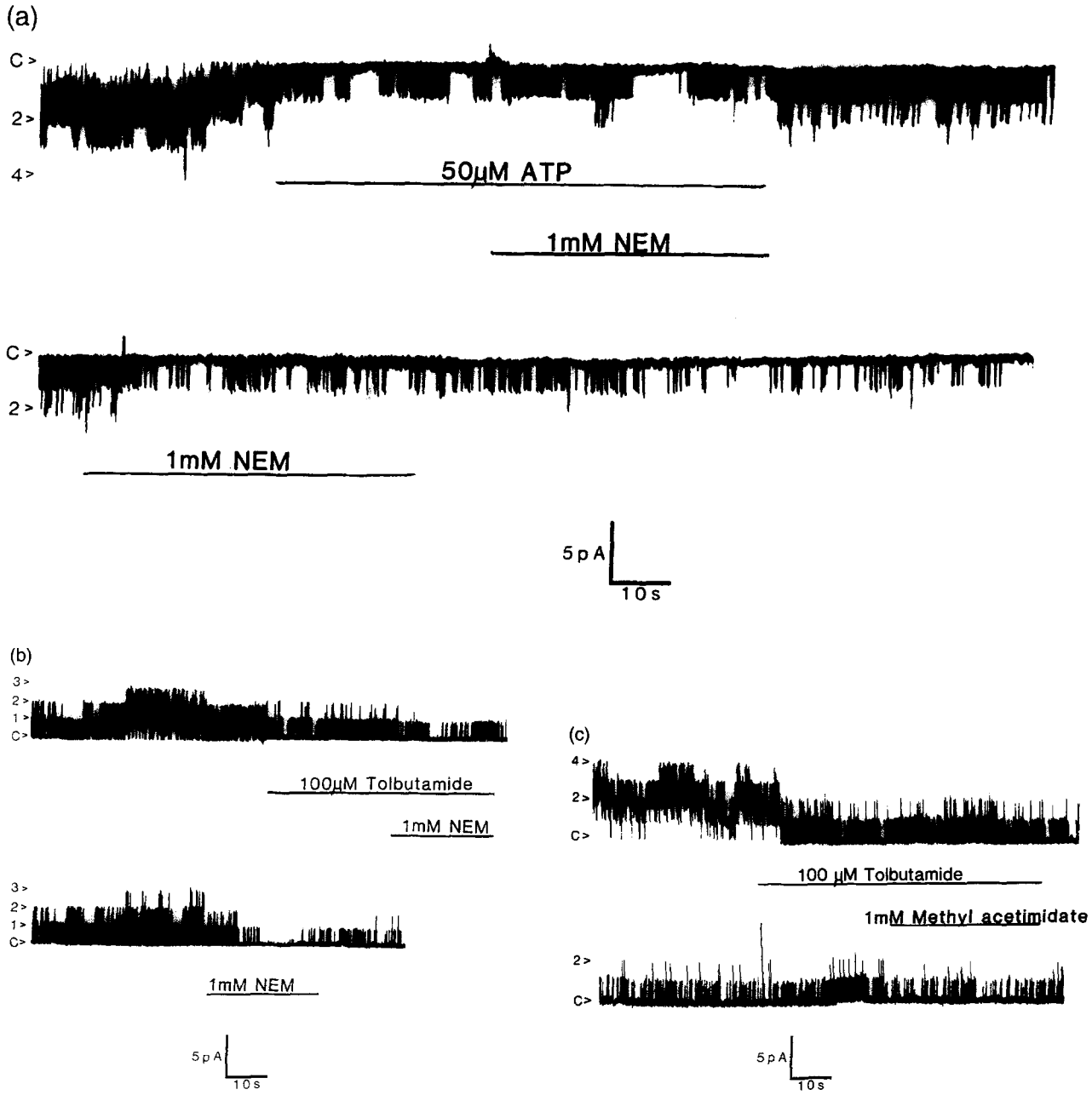

Fig. 3. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of (*a*) −40 mV or (*b* and *c*) +40 mV. Each continuous recording was perfused throughout the course of the experiment with solution B, to which additions were made as denoted in each respective figure. Single channel openings are denoted by upward deflections (outward currents) or downward deflections (inward currents). (*a*) The preapplication of 50 μM ATP protected K_{ATP} channel activity from the irreversible inhibitory effects of 1 mM NEM. The values for $N_f P_o$ were as follows: control 2.784, ATP 0.312; ATP + NEM 0.297; wash 1.042; NEM 0.104; wash 0.044. (*b*) The preapplication of 100 μM tolbutamide protected K_{ATP} channel activity from the irreversible effects of 1 mM NEM. The values for $N_f P_o$ were as follows; control 1.987; tolbutamide 0.413; tobutamide + NEM 0.178; wash 0.976; NEM 0.034; wash 0.065. (*c*) The preapplication of 100 μM tolbutamide failed to protect K_{ATP} channel activity from the irreversible effects of 1 mM methyl acetimidate. The values for $N_f P_o$ were as follows: control 2.359; tolbutamide 0.264; tolbutamide + methyl acetimidate 0.156; wash 0.103.

NEM. Tolbutamide was chosen in preference to glibenclamide since the former agent inhibits the channel in a reversible manner (Sturgess et al., 1985). Application of 100 μM tolbutamide to inside-out membrane patches produced an 89.2 ± 4.3% ($n = 5$) inhibition of K_{ATP} channel activity. When 1 mM NEM was introduced in the presence of this concentration of tolbutamide, the level of channel inhibition was 94.2 ± 2.4%. Howev-

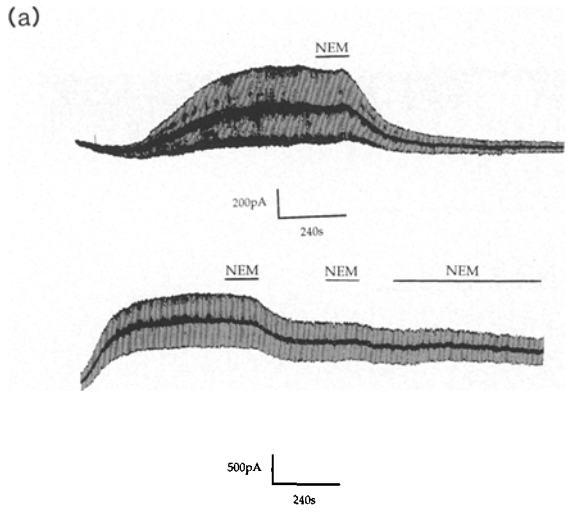

Fig. 4. The effect of NEM upon whole cell K_{ATP} channel currents in the presence and absence of intracellular Mg^{2+} recorded from voltage-clamped CRI-G1 cells. The cell membrane was voltage-clamped at −70 mV and alternate ± 10 mV pulses were applied every 2 sec. The current responses are denoted by the vertical lines. (*a*) In these recordings, the external bathing solution was A while the pipette contained solution B in the upper part and solution C in the lower part of the figure. The 2 min application of 5 mM NEM (indicated by the bar), 4 min after obtaining peak K_{ATP} currents produced an irreversible inhibition of channel currents. As shown in the upper part of the figure, in the presence of Mg^{2+}, the inhibition produced by NEM increased with time until virtually no K_{ATP} current remained. In contrast, the lower part of the figure shows that in the absence of Mg^{2+}, a proportion of the K_{ATP} current was not affected by the addition of NEM nor was it affected by its subsequent reapplication. (*b*) Change in whole cell K_{ATP} channel current amplitude with time after obtaining the peak current. The data are expressed as the natural logarithm of the normalized ratio (I') of the currents at time $t(I_t)$ with respect to the peak current (I_p(*see* Materials and Methods)). Filled symbols indicate data obtained in the presence of 0.65 mM Mg^{2+} intracellularly (solution B) while open symbols were obtained in the absence of intracellular Mg^{2+} (solution C). Squares represent control data while circles represent data obtained following the 2 min application of 5 mM NEM indicated in the figure.

er, simultaneous removal of these agents led to a return of channel activity to 43.8 ± 7.2% of the control value which is also significantly ($P < 0.05$) different from the recovery following exposure to this concentration of NEM *per se*. An example of the protection against NEM afforded by the presence of tolbutamide is shown in Fig. 3*b*. When tolbutamide was subsequently removed and NEM reapplied, a slight reversal of NEM inhibition was occasionally seen upon washout. In contrast, a similar reversal of NEM inhibition was never seen when NEM was reapplied following protection with ATP (Fig. 3*a*). This effect may be related to the greater lipophilic nature of tolbutamide which could allow it to be retained within the membrane lipid to provide some continued protection.

Site of Sulfhydryl Modification

Although the data presented above are indicative of an internal site for the sulfhydryl moiety, most of the reagents used are all considered to be sufficiently membrane permeable that there is some uncertainty at which side of the membrane interaction with the K_{ATP} channel occurs. In an attempt to resolve this issue, we decided to use the agent DNTB, a highly specific reagent for the sulfhydryl group (Torchinsky, 1977) which is relatively membrane impermeable. Application of 1 mM DNTB to the external surface of an outside-out patch produced little inhibition of K_{ATP} channel activity (16.3 ± 7.5%, $n = 5$; Fig. 2*b*) even after 5 min exposure. However, when the same concentration of DNTB was applied to the internal surface of an inside-out patch (Fig. 2*a*) there was an almost instantaneous irreversible reduction in channel activity (93.4 ± 4.5%, $n = 6$). pCMBS is a membrane impermeant sulfhydryl reagent (Vansteveninck, Weed & Rothstein, 1965) which is capable of initiating insulin secretion (Bloom et al., 1972). Like DNTB, 0.1 mM pCMBS produced only a small inhibition of K_{ATP} channel activity when applied to the external surface of the outside-out patch (32.8 ± 7.6, $n = 6$; *not shown*). However, unlike the other sulfhydryl modifying reagents, pCMBS induces a voltage-dependent form of block when applied to the intracellular surface. This is shown in Fig. 2*c* where at hyperpolarized potentials channel current amplitude was markedly diminished while at depolarized potentials, a flickery type of block was observed which also could not be reversed by washout ($n = 6$). Although pCMBS produced this flickery form of block, no significant change in the open state probability was observed at either membrane potential (116.3 ± 15.6%, $n = 6$ and 112.2 ± 14.3%, $n = 5$) activity compared to control at depolarized and hyperpolarized potentials, respectively). In contrast, the membrane permeable reagent NEM produced 92.4

± 4.6% (n = 5) inhibition of K_{ATP} channel activity when applied to the external surface (Fig. 2*b*). This was not significantly different from the inhibition produced by the same concentration of this agent applied to the internal surface (Fig. 1*a;* Table 1).

Importance of Lysine and Arginine Residues for K_{ATP} Function

The K_{ATP} channel of mammalian skeletal muscle is reported to be unaffected by the ε and α amino group reagents TNBS and pyridoxal-5′-phosphate (Weik & Neumcke, 1989). However, it is unclear from this study whether these agents were tested in the presence or absence of intracellular Mg^{2+} and so we have examined the effects of TNBS on K_{ATP} channels of insulin-secreting cells. Application of 1 mM TNBS to inside-out membrane patches in the presence of 0.65 mM intracellular Mg^{2+} produced a voltage-independent, irreversible inhibition of K_{ATP} channel activity by 84.7 ± 4.1% (n = 4). Although under the conditions used, this agent would be expected to react predominantly with amino groups, it is conceivable that the inhibition observed could be due to sulfhydryl modification (Means & Feeney, 1971). Thus, to determine the importance of amino residues in K_{ATP} channel function, we examined the actions of methyl acetimidate which is reputedly almost totally specific for the amino moiety. As shown in Fig. 5*a,* 1 mM methyl acetimidate in the presence of intracellular Mg^{2+} produced an irreversible inhibition of K_{ATP} channel activity by 82.7 ± 4.6% (n = 8). However, in the absence of intracellular Mg^{2+}, the inhibitory efficacy of this compound was significantly ($P < 0.05$) reduced (Fig. 5*b;* 55.6 ± 3.2%; n = 5). Similarly, the inhibitory effectiveness of 1 TNBS was significantly reduced by the removal of intracellular Mg^{2+} (59.3 ± 5.6%, n = 4; $P < 0.05$). Using the whole cell configuration, the external application of 5 mM methyl acetimidate produced 83.4 ± 4.3% (n = 3) inhibition of K_{ATP} channel currents in the presence of Mg^{2+}, but only 34.4 ± 3.2% (n = 3) inhibition in the absence of this cation. As seen for NEM, the introduction of methyl acetimidate was associated with an accelerated loss of K_{ATP} channel current. In the presence of intracellular Mg^{2+}, the rate of loss was characterized by a rate constant of 9.04 ± 0.59 (n = 3) hr^{-1}, while in the absence of this cation there was a rapid initial loss of K_{ATP} current (1.62 ± 0.21, n = 3) followed by a much slower decline (0.34 ± 0.05, n = 3; Table 2).

Since these chemical reagents show a similar Mg^{2+}-dependent K_{ATP} channel inhibitory behavior to the sulfhydryl reagents, the ability of ATP and tolbutamide to protect against the irreversible effects of these reagents was examined. ATP (50 μM) was seen to protect against the effects of 1 mM methyl acetimidate in experiments conducted in the inside-out configuration; application of 50 μM ATP produced 93.3 ± 4.4% (n = 5) inhibition of K_{ATP} channel activity and following the introduction of 1 mM methyl acetimidate the level of activity was 94.6 ± 3.4% (n = 5). After simultaneous washout of both inhibitory agents, there was an increase in K_{ATP} channel activity to 25.5 ± 6.3% (n = 5) of the control channel activity (*not shown*) which was significantly different from the effect of methyl acetimidate in the complete absence of ATP where only 5.8 ± 3.5% (n = 8) channel activity remained following washout ($P < 0.05$). When 100 μM tolbutamide was applied prior to methyl acetimidate there was a reduction in channel activity to 14.6 ± 6.7% (n = 6) of the control channel activity. The subsequent addition of 1 mM methyl acetimidate reduced channel activity to 9.3 ± 5.3% (n = 6) but this inhibition was not significantly reversed by the simultaneous washout of both agents (10.8 ± 5.4%, n = 6); Fig. 3*c*). Similar results were obtained for TNBS (*not shown*). Thus, it appears that ATP, but not tolbutamide, can protect against the effects of lysine group modification.

We have also examined the effects of methyl glyoxal, a highly specific modifier of the guanido group of arginine residues, on the activity of the K_{ATP} channel. This compound also inhibits K_{ATP} channel activity in an irreversible Mg^{2+}-dependent manner. In the presence of Mg^{2+}, 1 mM methyl glyoxal applied to inside-out membrane patches caused a 97.5 ± 1.1% (n = 5) inhibition of channel activity, whereas in the whole cell configuration, 5 mM methyl glyoxal applied to the external surface produced 84.5 ± 3.4% (n = 3) inhibition of K_{ATP} currents. In the absence of intracellular Mg^{2+}, 1 mM methyl glyoxal inhibited K_{ATP} channel activity by 41.3 ± 8.9% (n = 3) when applied to the intracellular surface of inside-out patches while 5 mM methyl glyoxal produced 23.2 ± 5.3% inhibition of whole cell K_{ATP} channel currents (n = 3). Methyl glyoxal was also seen to induce a change in the rate of loss of channel activity which was dependent upon the intracellular Mg^{2+} concentration (Table 2). The preapplication of 50 μM ATP was also seen to protect against the effects of this reagent. ATP (50 μM) produced 91.0 ± 6.3% (n = 5) inhibition of K_{ATP} channel activity and this was increased to 97.0 ± 1.7% (n = 5) when methyl glyoxal was added. Subsequent washout of both reagents together reduced the level of inhibition to 62.3 ± 3.6% (n = 5; *not shown*). In contrast, no return of channel activity was seen when methyl glyoxal was applied alone and subsequently washed out (98.3 ± 1.2%, (n = 5) inhibition remained; $P < 0.05$). In the presence of tolbutamide a small return in channel activity was seen [88.2 ± 1.1% (n = 5) inhibition remained] which was not significant.

Table 2. The effect of various group-specific chemical reagents on the rate of K_{ATP} current rundown in whole cell voltage-clamped CRI-G1 cells

Pipette solution	Chemical	Rate of current decay/hr^{-1}	
		α	β
Solution B ($+Mg^{2+}$)	Control	1.71 ± 0.11 (n = 5)	8.13 ± 0.64 (n = 5)
Solution C ($-Mg^{2+}$)	Control	0.45 ± 0.03 (n = 5)	
Solution B ($+Mg^{2+}$)	NEM (5 mM)	11.04 ± 0.78 (n = 4)	
Solution C ($-Mg^{2+}$)	NEM (5 mM)	3.91 ± 0.37 (n = 5)	0.36 ± 0.05 (n = 5)
Solution B ($+Mg^{2+}$)	Methyl acetimidate (5 mM)	9.04 ± 0.59 (n = 3)	
Solution C ($-Mg^{2+}$)	Methyl acetimidate (5 mM)	1.62 ± 0.21 (n = 3)	0.34 ± 0.05 (n = 3)
Solution B ($+Mg^{2+}$)	Methyl glyoxal (5 mM)	11.4 ± 0.29 (n = 3)	
Solution C ($-Mg^{2+}$)	Methyl glyoxal (5 mM)	1.44 ± 0.15 (n = 3)	0.29 ± 0.10 (n = 3)

Values are means ± SEM; the number of experiments performed is shown in parentheses.

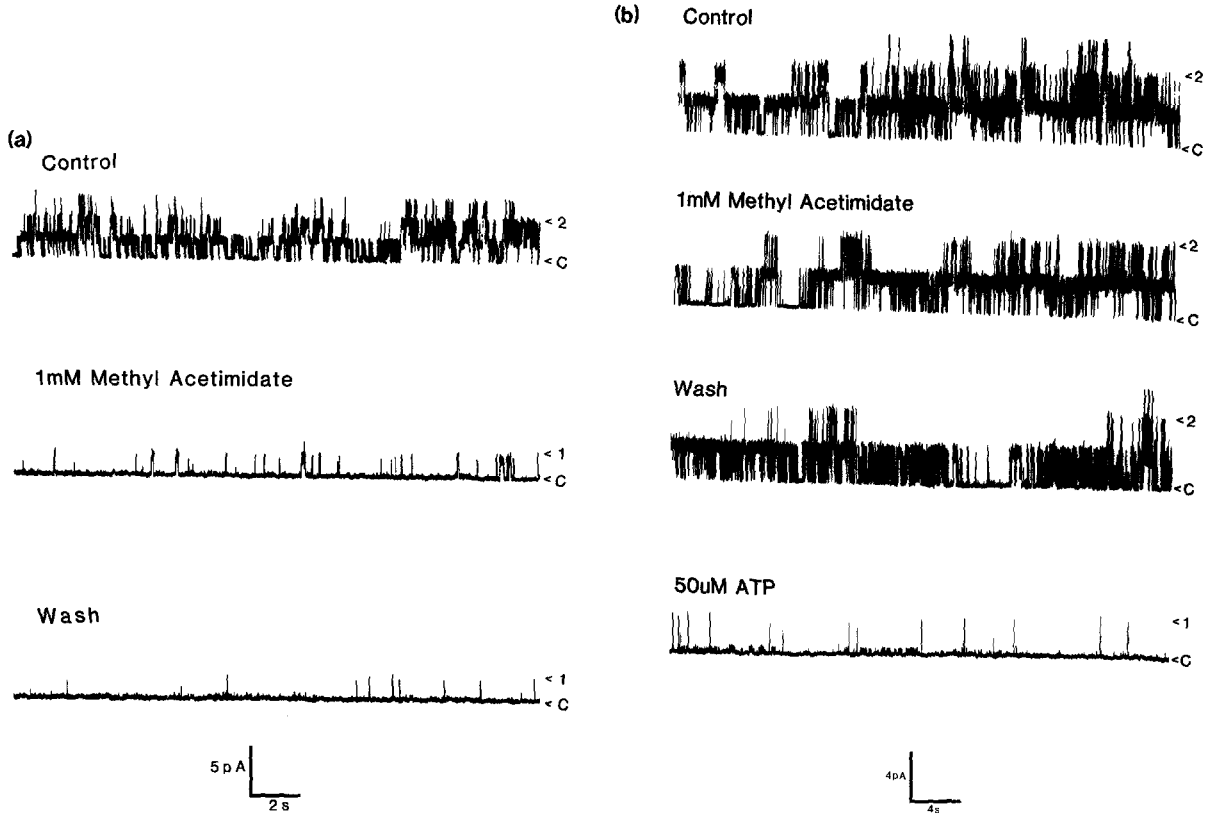

Fig. 5. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of (*a*) +30 mV or (*b*) +40 mV. Single channel openings are denoted by upward deflections (outward currents). (*a*) 1 mM methyl acetimidate in the presence of 0.65 mM Mg^{2+} inhibited the activity of the K_{ATP} channel in an irreversible manner. The values for $N_f P_o$ were as follows: control 0.953; methyl acetimidate 0.045; wash 0.022. (*b*) 1 mM methyl acetimidate in the absence of Mg^{2+} produced a much weaker inhibition of K_{ATP} channel activity. However, 50 μM ATP retained its inhibitory effectiveness. The values for $N_f P_o$ were as follows: control 1.355; methyl acetimidate 0.856; wash 0.715; ATP 0.076.

Effects of Amino and Sulfhydryl Modification on [^{3}H]-Glibenclamide Binding

Modification of sulfhydryl groups by iodoacetate or NEM had no significant effect on [^{3}H]-glibenclamide binding in CRI-G1 membranes even at concentrations up to 10 mM (Fig. 6*a*). Neither methyl acetimidate or methyl glyoxal, lysine and arginine modifying reagents, respectively, had any effect on [^{3}H]-glibenclamide binding (Fig. 6*b*). In contrast, the amino group reagent

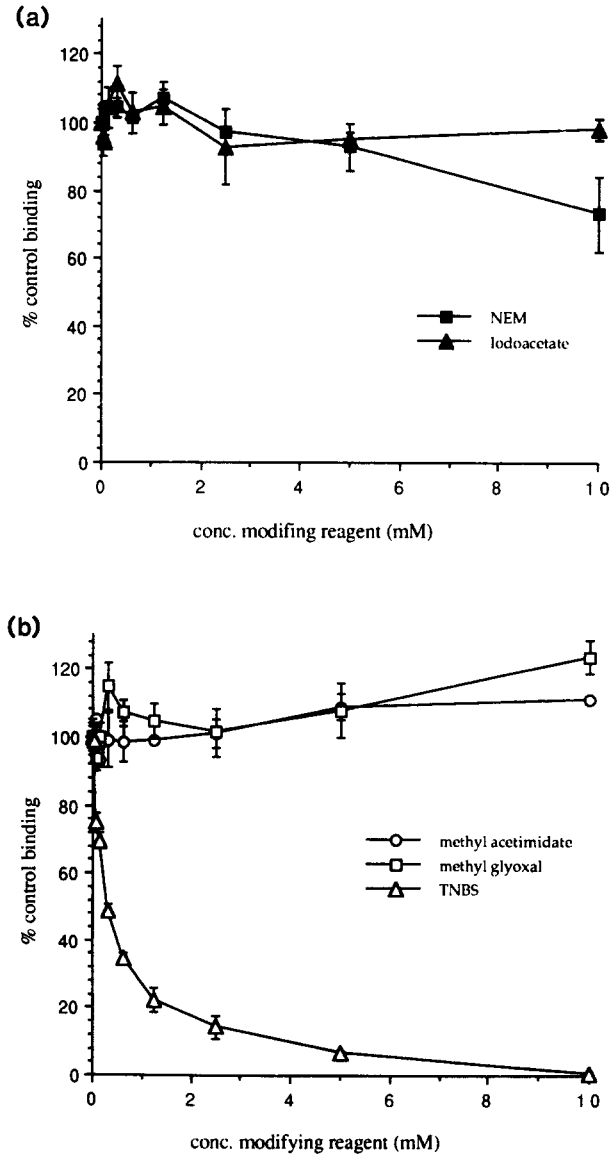

Fig. 6. Effect of chemical modifying reagents on [^{3}H]-glibenclamide binding to CRI-G1 membranes. Membranes were incubated with 0.2 nM [^{3}H]-glibenclamide and the indicated concentrations of chemical modifying reagent. The bound radioactivity was separated from the free by vacuum filtration onto GF/B discs and determined by liquid scintillation counting as described in Materials and Methods. Non-specific binding (NSB) was determined in the presence of 1 mM glibenclamide. Results are expressed as percentage of control binding (193.1 ± 7.5 fmol/mg protein) after subtraction of NSB and are mean ± SEM of three experiments, each performed in triplicate. (*a*) The sulfhydryl group-specific reagents NEM and iodoacetate did not inhibit the control level of ^{3}H-glibenclamide binding even at concentrations up to 10 mM. (*b*) The lysine-specific reagent methyl acetimidate and the arginine-specific reagent methyl glyoxal failed to reduce the control level of ^{3}H-glibenclamide binding even at concentrations up to 10 mM. However, the lysine reagent TNBS produced a complete inhibition of ^{3}H-glibenclamide binding with half-maximal inhibition at 307.0 ± 6.0 μM and a Hill coefficient of one.

TNBS (which modifies lysine residues) was shown to be a good inhibitor of [^{3}H]-glibenclamide binding in CRI-G1 membranes (Fig. 6*b*) with a half-maximal inhibition at 307.0 ± 6.0 μM (n = 3). Complete block of [^{3}H]-glibenclamide binding occurred at 10 mM TNBS.

Protection of K_{ATP} Channel from the Effects of Trypsin

Recently, it has been reported that intracellular application of the enzyme trypsin induces dramatic changes in the properties of the K_{ATP} channel in the murine β-cell (Proks & Ashcroft, 1993). Exposure to trypsin increases channel activity, apparently reverses the process of channel rundown and reduces the sensitivity of the channel to inhibition by ATP. This multiplicity of effects led the authors to suggest that the enzyme may be acting at more than one site. Trypsin cleaves peptide at both lysine and arginine residues (Keil, 1971), and following the results obtained with methyl glyoxal and methyl acetimidate above, we decided to investigate whether specific modification of one or both of these residues could protect the channel from all or some of the actions of trypsin. The effects of 100 μg/ml trypsin on K_{ATP} channel activity are demonstrated in Fig. 7*a*. Within 30 sec of application, trypsin induced a substantial and irreversible activation (354.5 ± 94.5, n = 9) of channel activity with no change in single channel conductance or Mg^{2+}-dependent rectification and no obvious rundown in activity following this treatment, even in the presence of intracellular Mg^{2+} (Kozlowski & Ashford, 1990). The trypsinized channel was found, in agreement with Proks and Ashcroft (1993), to be much less sensitive to ATP [50 μM now producing 18.6 ± 24.7% (n = 4) inhibition; Fig. 7*a;* compare this with the effects of 50 μM ATP above]. Tolbutamide was also found to be less effective as an inhibitor following trypsin treatment (100 μM producing only 9.3 ± 7.3% inhibition of the trypsinized channels; Fig. 7*a*). Application of the reagents methyl glyoxal or methyl acetimidate alone before addition of trypsin was unable to prevent its effects with activations of 164.0 ± 36.5% (n = 4) and 200.1 ± 20.2% (n = 3), respectively. However, when these reagents were applied together, trypsin was unable to reverse the inhibition produced (Fig. 7*b*) with 97.5 ± 1.2% (n = 5) inhibition before and after trypsin application. Furthermore, addition of the sulfhydryl reagent NEM either alone or in combination with either methyl glyoxal or methyl acetimidate failed to protect against the effects of trypsin (Fig. 7*c*).

Discussion

The data presented in the above study provide evidence to suggest that a sulfhydryl group in addition to lysine

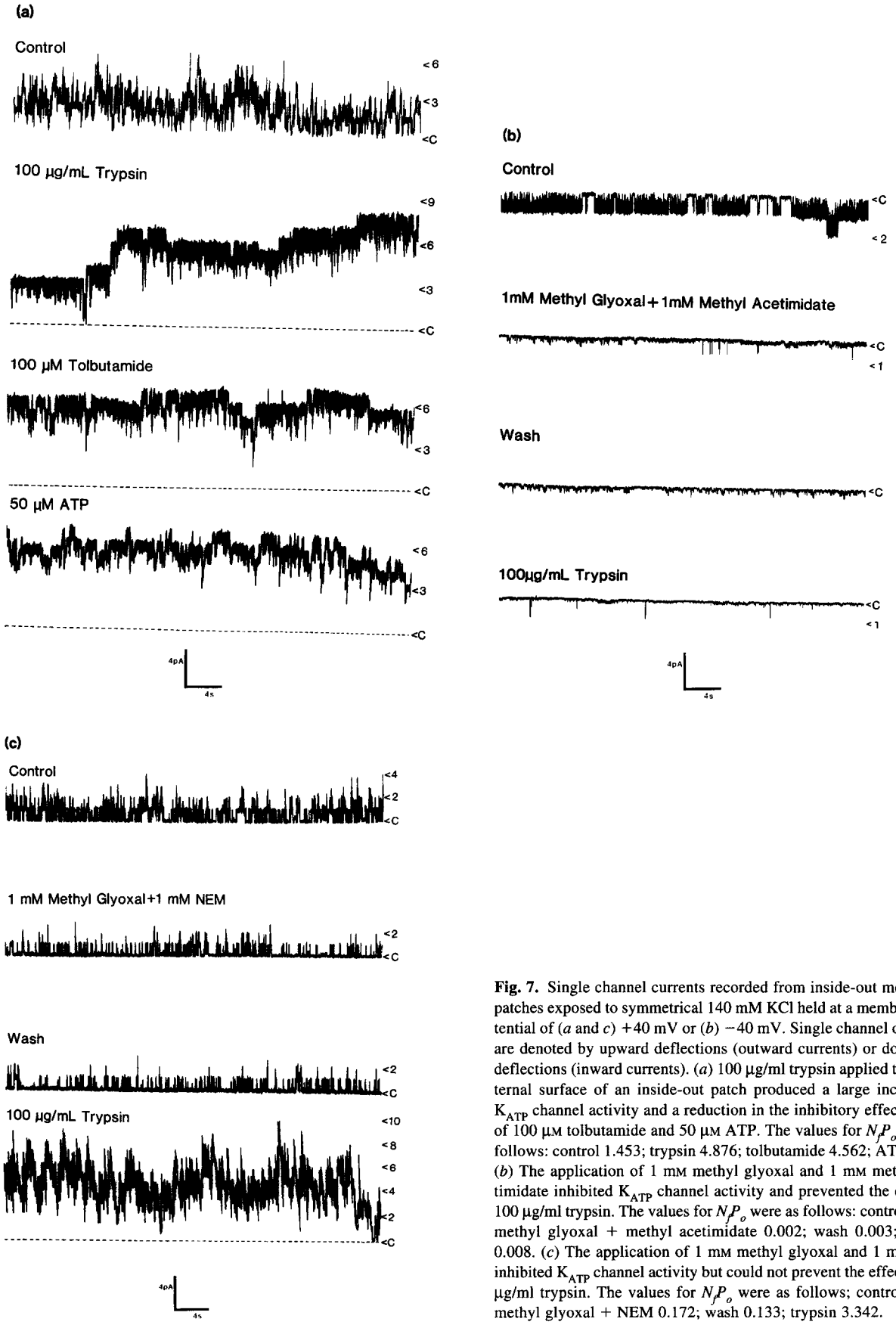

Fig. 7. Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of (*a* and *c*) +40 mV or (*b*) −40 mV. Single channel openings are denoted by upward deflections (outward currents) or downward deflections (inward currents). (*a*) 100 μg/ml trypsin applied to the internal surface of an inside-out patch produced a large increase in K_{ATP} channel activity and a reduction in the inhibitory effectiveness of 100 μM tolbutamide and 50 μM ATP. The values for $N_f P_o$ were as follows: control 1.453; trypsin 4.876; tolbutamide 4.562; ATP 4.137. (*b*) The application of 1 mM methyl glyoxal and 1 mM methyl acetimidate inhibited K_{ATP} channel activity and prevented the effect of 100 μg/ml trypsin. The values for $N_f P_o$ were as follows: control 0.857; methyl glyoxal + methyl acetimidate 0.002; wash 0.003; trypsin 0.008. (*c*) The application of 1 mM methyl glyoxal and 1 mM NEM inhibited K_{ATP} channel activity but could not prevent the effect of 100 μg/ml trypsin. The values for $N_f P_o$ were as follows; control 1.347; methyl glyoxal + NEM 0.172; wash 0.133; trypsin 3.342.

and arginine residues are all critically involved in the normal functioning of the K_{ATP} channel. Although none of the reagents we have used can be considered absolutely specific for the sulfhydryl moiety, the number of different reagents and their different side reactions makes it unlikely that any other chemical function could be responsible for the effects seen. The principal evidence for the involvement of both lysine and arginine residues in K_{ATP} channel function arises from the experiments conducted with methyl acetimidate, methyl glyoxal and trypsin. Trypsin cleaves peptides specifically at lysine or arginine residues (Keil, 1971), and it was found that both lysine-specific modification (via methyl acetimidate) and arginine-specific modification (via methyl glyoxal) were necessary for protection against this enzyme (Fig. 7*b*). Thus, this would suggest that there are at least two sites of trypsin-directed cleavage, one being lysine in nature and the other arginine. Furthermore, the failure of the sulfhydryl reagent NEM to protect against the trypsin effect both alone and in the presence of either a lysine-specific or arginine-specific reagent is further evidence that the three different types of reagent all interact with different chemical groups. It was shown that TNBS but not methyl acetimidate, both modifiers of lysine residues, can inhibit [^{3}H]-glibenclamide binding in CRI-G1 cell membranes. A plausible explanation for this finding is that TNBS modification adds a much bulkier group than methyl acetimidate to the lysine residue and that interaction with [^{3}H]-glibenclamide binding occurs through steric interference. This leads us to suggest that there is one, or more, crucial lysine residues adjacent to the [^{3}H]-glibenclamide binding site. Although we were unable to protect against the effects of lysine reagents with tolbutamide in our electrophysiological studies, this may have arisen because of the differences in chemical structure between tolbutamide and glibenclamide. Unfortunately, it was not feasible to use glibenclamide itself to test this theory since the effects of glibenclamide are themselves, essentially irreversible during the time course of these experiments. Alternatively, it may be that more than one essential lysine residue is associated with channel activity. Thus, although tolbutamide can protect the lysine residue associated with the glibenclamide binding site, other crucial residues are not protected and hence, channel inhibition is seen. In contrast to tolbutamide, ATP could protect against both lysine-specific (methyl acetimidate) and arginine-specific (methyl glyoxal) reagents. Several authors have suggested that more than one nucleotide binding site is associated with this channel (*see, for example,* Bokvist et al., 1991); thus ATP, in contrast to tolbutamide, might be expected to interact with the channel at several sites which may explain the greater protection afforded by this compound.

The lack of effect of the amino group reagent TNBS upon the skeletal muscle K_{ATP} channel (Weik & Neumcke, 1989) may have arisen if this modification was performed in the absence of intracellular Mg^{2+}, since it is unclear whether or not this cation was present in the above study. Alternatively, since the skeletal muscle channel displays certain features reminiscent of a trypsinized β-cell channel, e.g., lack of rundown, lower sensitivity to ATP and the sulfonylureas (Ashcroft & Ashcroft, 1990; Proks & Ashcroft, 1993), it may be that the lysine bearing portion of the protein which is cleaved off by trypsin in the β-cell is normally absent in skeletal muscle and that it is this portion of the molecule which is responsible for the differences in properties between the two channel types.

In the study conducted upon skeletal muscle, it was also shown that ATP apparently could protect against the effects of NEM. The same appears true of the present study, however, since not only ATP but also the sulfonylurea tolbutamide were found capable of producing this phenomenon. In both instances, a complete recovery of channel activity was not observed. This was also seen in the former study where it was suggested that even though 1 mM ATP was used, the channel was unlikely to be occupied by ATP all of the time and in the interim NEM might gain access to at least some of the channels and irreversibly modify them (Weik & Neumcke, 1989). The same problem is true of the present study with the added complication that these channels undergo irreversible rundown in activity at a much more rapid rate than do the channels in skeletal muscle.

These results are interesting in view of reports that cysteine and basic amino acid residues are associated with the ATP binding sites of many adenosine triphosphatases (Schuurmans Stekhoven & Bonting, 1981; Horisberger et al., 1991). Based on these findings, however, we cannot state conclusively whether these groups are situated near to the nucleotide binding site, nor can we say whether the sulfonylurea receptor and nucleotide binding site are very close in proximity. Further studies will be needed to provide conclusive evidence either for or against these possibilities. However, we believe these observations indicate that both ATP and tolbutamide induce similar conformational changes in the channel protein such that the essential sulfhydryl moiety is no longer so accessible to chemical modification but that only ATP can protect against modification of lysine and arginine residues.

The dependence on Mg^{2+} displayed by these chemical reagents is further evidence of the importance of this cation in K_{ATP} channel function. Other Mg^{2+}-dependent effects include an involvement in channel rundown (Kozlowski & Ashford, 1990), an involvement in the interaction between the sulfonylureas and the channel (Lee & Ashford, 1993), inward rectification of the channel (Horie, Irisawa & Noma, 1987) and a direct effect

upon the channel's open probability (Findlay, 1987). It is possible that Mg^{2+}-dependent rundown may be responsible for a component of the greater inhibition observed in the presence of these compounds. However, we do not believe that the greater inhibitory effectiveness of these agents in the presence of Mg^{2+} can be accounted for solely in terms of rundown. The whole cell studies presented demonstrate that, even when rundown is accounted for, these compounds inhibit K_{ATP} currents in a Mg^{2+}-dependent manner. In the presence of this cation, all the K_{ATP} channels are rapidly and irreversibly inhibited. In contrast, in the cation's absence, these compounds produce a rapid initial phase of channel inhibition followed by a second slower rate of channel loss similar to that normally seen in the absence of Mg^{2+}. These findings suggest that while all the K_{ATP} channels are susceptible to the effects of chemical modification in the presence of Mg^{2+}, in the absence of this cation only a small proportion of the channels can be modified. This suggestion is supported by the lack of effect produced by reapplication of these agents.

The observation that pCMBS and DNTB are ineffective from the external surface is suggestive of the sulfhydryl moiety being situated at the internal face of the channel protein. The presence of the heavy metal ion mercury in pCMBS may be the reason why this agent produces a different form of channel block to the other reagents which were used. It is possible to envisage the mercury ion interacting with the sulfhydryl function to produce a charged unit capable of sitting in the membrane itself but able to interact with the mouth of the channel to produce a voltage-dependent, rapid gating of the channel openings. If this is so, then it would suggest that the sulfhydryl group in question lies close to the pore of the channel. The small effects produced by pCMBS and DNTB upon the K_{ATP} channel when applied to the external surface of the cell makes it difficult to explain previous studies in which pCMBS produced a rapid and reversible stimulation of insulin secretion (Bloom et al., 1972). It may be that this agent achieves this stimulatory effect via other means. With regards to this assumption, it has been shown that chloromercuribenzene-*p*-sulfonic acid, a membrane impermeable sulfhydryl reagent related to pCMBS can enhance the Na^+ permeability of insulin-secreting cells and thereby achieve membrane depolarization (Hellman et al., 1975). Recently, Islam, Berggren and Larsson (1993) have also reported that an important sulfhydryl group is associated with the K_{ATP} channel in the pancreatic β-cell. Although these authors found, in contrast to the present study, that this inhibition was reversible, they also report that the sulfhydryl function is only accessible from the internal surface. The sites of action of methyl acetimidate and methyl glyoxal are less well defined since both agents can inhibit the K_{ATP} channels when applied to either the internal or the external surface. However, since these agents can prevent the proteolytic effects of trypsin which occur at the intracellular surface, it would appear that at least one vital lysine and arginine residue must lie on the intracellular side.

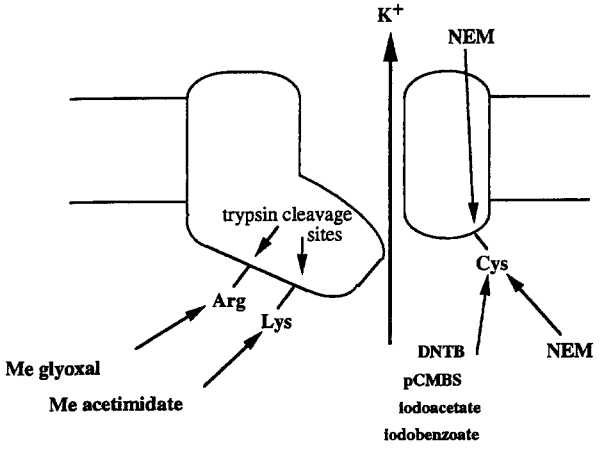

Fig. 8. Schematic representation of the relationship between the functional groups studied and the K_{ATP} channel activity. For simplicity, no attempt has been made to incorporate binding sites for the various pharmacological and intracellular modulators of the channel. Similarly, only one function has been drawn for each amino acid residue. *See Discussion* for further details.

In summary, this study has identified three amino acid residues which are apparently important for the normal functioning of the K_{ATP} channel, namely the sulfhydryl group most probably arising from a cysteine residue, an amino group possibly arising from the amino acid lysine or a terminal amine group and the guanido group probably arising from an arginine residue. A simple model which might explain the observations made in this report is illustrated in Fig. 8. In this model, all three functional groups are involved in the regulation of the activity of a hypothetical gating subunit which closes the channel in a Mg^{2+}-dependent manner. The chemical modification of any of these three functions leads to an irreversible closure of the gate with resultant loss of K_{ATP} channel activity. In contrast, trypsinization leads to proteolytic cleavage at lysine and arginine residues and the subsequent loss of the gating subunit activity. The inhibitory effects of these agents can be prevented by altering the protein conformation by removal of Mg^{2+} or possibly by the use of the inhibitory agents ATP and tolbutamide. Obviously, the data presented cannot distinguish between the presence of one or more of each of these important functional groups.

Previous studies have indicated that an important sulfhydryl group is associated with the K_{ATP} channel of skeletal muscle while there is no conclusive evidence of a sulfhydryl function being involved in sodium channels

(Rack et al., 1984). Amino groups involved in channel gating have been identified both in the Ca^{2+}-activated potassium channel from the GH_3 anterior pituitary cell line (Frace & Eaton, 1992) and in the delayed rectifier potassium channel of squid giant axons (Spires & Begenisich, 1992). However, in the former case, amino group modification led to an irreversible increase in channel open probability, while in the latter instance the amino group was thought to be positioned at an external site. It would appear, therefore, that the ATP-sensitive K^+ channel displays several unique structural characteristics which differentiates it from other cation channels and may contribute to the special properties it possesses.

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