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Biochimica et Biophysica Acta 1284 (1996) 119–121

BBA Biochimica et Biophysica Acta

Rapid report

Ammonium inhibition of Ca^{2+}-dependent inwardly rectifying K^+ currents in HeLa cells

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Received 9 May 1996; revised 22 July 1996; accepted 24 July 1996

Abstract

The patch-clamp technique was used to examine the effect of ammonium on inwardly rectifying Ca^{2+}-dependent K^+ channels of HeLa cells. Ammonium added extracellularly blocked macroscopic K^+ currents with an IC_{50} of 1.6 mM. Inhibition was fully reversible and voltage-independent and is not likely to be secondary to an increase in intracellular pH as this would have the opposite effect. In these experiments ammonium appeared to have an intracellular site of action. Intracellular ammonium rapidly and reversibly inhibits the activity of single Ca^{2+}-dependent K^+ channels underlying the macroscopic currents. This compound is often employed to manipulate intracellular pH but its use would not be indicated when full activity of the type of channels studied here needs to be unaltered.

Keywords: HeLa cell; Patch clamp; Ammonium inhibition; Calcium ion dependence; Potassium ion current

K^+ channels play important roles in the maintenance of cell membrane potential, response to agonists, cell proliferation and regulation of cell volume in numerous excitable and non-excitable cells. We have recently studied in HeLa cells currents showing inward rectification, dependence upon intracellular Ca^{2+} concentration, little voltage dependence and sensitivity to the scorpion venom toxin charybdotoxin [1]. Single channels corresponding to the currents referred to above have been reported in HeLa cells [2]. In addition to intracellular Ca^{2+} several other types of regulatory signals are known to modulate the activity of K^+ channels. Among these is intracellular pH. A convenient way to study the effect of intracellular pH is by incubation with ammonium that will enter the cell in the uncharged form to get subsequently protonated and therefore alkalinise the cytosol (see for instance, [3]). Addition of ammonium, however, inhibited inwardly rectifying, Ca^{2+}-dependent K^+ currents in HeLa cells, an effect that is opposite to that expected for cell alkalinization and appears to be due to inhibition of the currents by ammonium.

The details of the cell culture procedure have been described elsewhere [4]. Briefly, HeLa cells were grown at 37°C in DMEM supplemented with 10% (v/v) foetal calf serum, 80 000 I.U./l penicillin and 0.1 g/l streptomycin. Cells were maintained in a 5% CO_2/95% air atmosphere and used 2–3 days after being subcultured in 35 mm plastic dishes. Standard whole-cell and single-channel patch-clamp recordings [5] were performed as described previously [1]. Patch-pipettes were fabricated from thin-walled borosilicate (hard) glass capillary tubing of outside diameter 1.5 mm (Clark Electromedical, Reading, UK) and had resistances of 2–4 (10 for excised patches) MΩ when filled with K^+-rich solutions. Currents were recorded using a List EPC-7 amplifier (List Medical, Germany). Osmolality of the bathing and intracellular (pipette) solutions were 290 and 285 mosmol/l, respectively, as measured with a freezing-point depression osmometer (Advanced Instruments, USA). In whole-cell recording experiments, pipette solution was made slightly hypotonic to avoid activation of volume-regulated Cl^- currents. Free Ca^{2+} concentrations in mixtures of EGTA or BAPTA and $CaCl_2$ were calculated using a multiple equilibrium program (EQCAL, Elsevier, Cambridge, UK). Voltage and current signals from the amplifier together with synchronising pulses were

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PII S0005-2736(96)00139-3

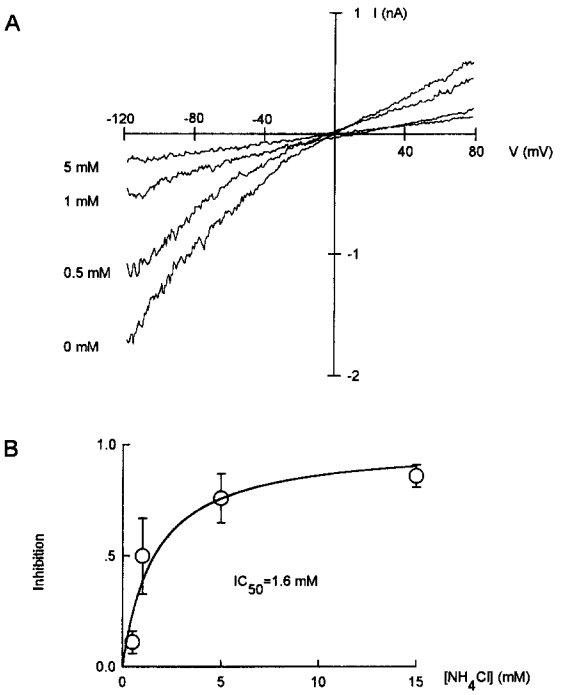

Fig. 1. Effect of ammonium on whole-cell K^+ currents in HeLa cells. In (A) currents were elicited by 1 s voltage ramps from −120 mV to +80 mV from a holding potential of 0 mV. Intracellular solution was (in mM) 145 KCl, 1.2 $MgCl_2$, 0.65 $CaCl_2$, 1 EGTA, 2 Na_2ATP, 10 Hepes (pH 7.4). The extracellular solution was 145 KCl, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes (pH 7.4). (B) Concentration-dependence of ammonium inhibition measured at −120 mV. Means ± S.E.M. of four separate determinations.

recorded on digital audio tape (Biologic, France). The signals were digitised using a Beltron Turbo-AT computer equipped with a PC-Lab$^+$ interface card (National Instruments, USA) at a sampling rate of 700 Hz (2 kHz in single-channel recordings). The voltage pulse generator and the analysis programs were written by J. Dempster (University of Strathclyde, Glasgow, Scotland). Whole-cell currents were not corrected for the presence of leak. All errors quoted are standard errors of the mean.

Recordings of whole cell currents were routinely started with an intracellular solution rich in KCl and a NaCl-rich extracellular solution. Under these conditions small inward and large outward currents were seen, with a reversal potential between −50 and −75 mV. Replacing extracellular Na^+ with K^+ markedly increased inward currents and shifted the reversal potential to around 0 mV. This indicated that currents being measured were carried by K^+. Currents elicited by voltage ramps in HeLa cells measured in symmetrical K^+ concentrations at an intracellular free Ca^{2+} concentration of 100 nM are shown in Fig. 1A. The current observed had the characteristic slight inward rectification seen previously for these Ca^{2+}-dependent K^+ currents. Addition of 0.5 mM ammonium chloride to the medium led to an inhibition of the current by about 15%. The inhibition was not instantaneous and for the case illustrated it occurred with a rate constant of 0.3 min^{-1} (data not shown). The currents shown were taken after an interval of exposure allowing for full effect. Increasing the concentration of ammonium chloride resulted in an increase in the degree of inhibition which reached about 80% at 5 mM. The inhibition was independent of the membrane potential at all concentrations tested. The slow onset of the inhibition by ammonium of Ca^{2+}-dependent K^+ currents could be explained if this ion was exerting its effect after crossing the plasma membrane in the uncharged form and being protonated in the intracellular milieu, thus increasing intracellular pH. This is unlikely to be the mechanism of inhibition in the present experiments because the effect occurred at low concentrations of ammonium which are not expected to cause important shifts in pH. In addition, increasing intracellular pH to 8.2 (with BAPTA as Ca^{2+} buffer) had a small stimulating effect on the Ca^{2+}-dependent K^+ currents (not shown) suggesting that the ammonium effect observed here cannot be caused by intracellular alkalinization. The concentration dependence of the effect measured in experiments like those in Fig. 1A, is shown in Fig. 1B. The effect was well described by a rectangular hyperbola with a concentration giving 50% inhibition of 1.6 mM ammonium.

An alternative explanation to a shift in intracellular pH to account for the effect of ammonium reported above is that this compound has to enter the cell in order to inhibit the channel at an intracellular site. This possibility was explored by including ammonium in the intracellular (pipette) solution. Inclusion of ammonium in the pipette, intracellular solution even at low concentration abolished the activity of Ca^{2+}-activated K^+ currents from the onset of the experiment. The effect, however, appeared to be transient, as currents developed with time. As cells in these experiments were superfused continuously with solution devoid of ammonium, it is conceivable that the compound might permeate and be washed away thus decreasing its effective intracellular concentration. That this might be the case is consistent with the fact that the recovery of the currents was prevented by addition of extracellular ammonium to cancel the outwardly-directed concentration gradient. The effect of intracellular ammonium was maintained when the higher concentration of 1 mM was used (Fig. 2B).

To confirm the inhibitory effect of intracellular ammonium excised inside-out patches were used. As discussed in a previous report [1] a single channel activity corresponding to the currents measured here has been reported before in HeLa cells [2]. A very similar channel has been shown to be present in T84 cells of intestinal origin and is thought to be responsible for their basolateral membrane conductance to K^+ [6–8]. In addition a channel sharing similar characteristics of rectification and Ca^{2+} dependence has been shown to be involved in cell volume regulation in Ehrlich cells [9]. We have used membrane patches and observed abundant Ca^{2+}-dependent K^+ single channel activity in HeLa cells. An example of this is

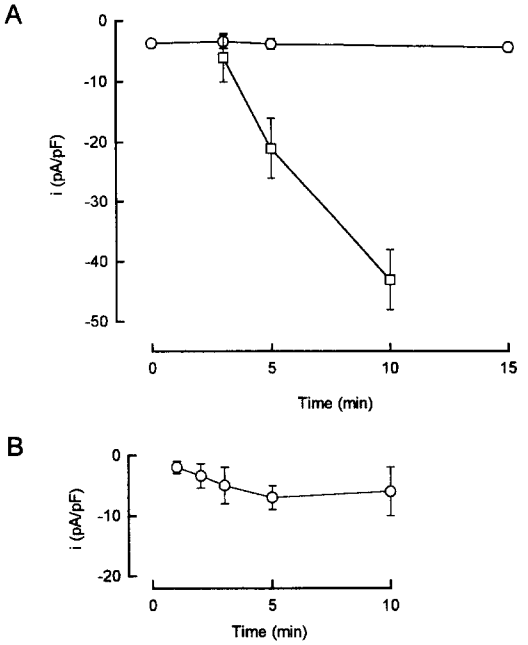

Fig. 2. Effect of intracellular ammonium on whole-cell K^+ currents in HeLa cells. Currents were elicited by 500 ms square voltage pulses to −100 mV from a holding potential of 0 mV. Solutions were as described in the legend to Fig. 1. Currents measured with pipette solutions containing 1 mM (circles) or 0.1 mM (squares) ammonium are shown in (A). Results obtained with 0.1 mM ammonium both in the pipette and the bath solution are plotted in (B). Means ± S.E.M. of three experiments for each curve shown.

shown in Fig. 3 where channel activity has been measured in symmetrical 145 mM KCl at an intracellular Ca^{2+} concentration of 0.1 mM (similar results were obtained at 100 nM Ca^{2+}). Under these symmetrical 145 mM K^+ conditions, single channel conductances were (all results not shown, unless otherwise indicated) 31 and 14 pS at −90 and 70 mV, respectively. The channel was selective for K^+ over Na^+ and its open probability was independent of voltage in the −90 to 70 mV range. Open probability, however, was affected by intracellular Ca^{2+} in manner consistent with these single channels being responsible for the macroscopic currents described before [1] and those illustrated in Fig. 1A. As shown in Fig. 3, addition of ammonium at the intracellular aspect of the membrane did not alter the single channel current but reduced the open probability by 72%. The effect was immediate and there was a prompt recovery after wash. Similar results were obtained in three separate experiments.

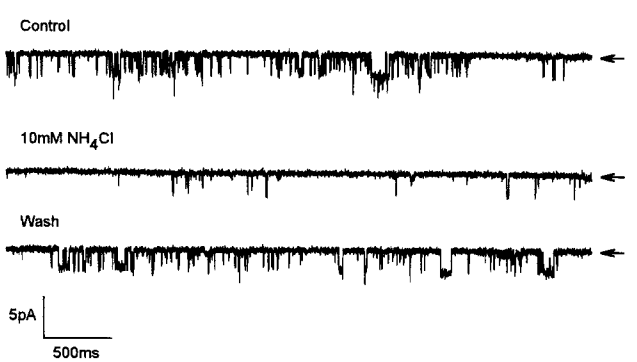

Fig. 3. Effect of intracellular ammonium on single K^+ channel activity in HeLa cells. Single channel recordings were taken in an excised inside-out membrane patch from a HeLa cell. The pipette solution had the same composition as the extracellular solution in Fig. 1A. The solution bathing the intracellular aspect of the patch was similar to the pipette solution in Fig. 1A but contained 100 μM $CaCl_2$ and no EGTA. The potential was −70 mV. Arrows indicate the closed current level.

It is concluded that ammonium inhibits the inwardly-rectifying, Ca^{2+}-dependent K^+ channels of HeLa cells from the intracellular aspect of the membrane. The mechanism by which ammonium causes blockade has not been explored. The lack of voltage-dependence of the inhibition, however, suggests that it either inhibits in the uncharged form or that its site of action is not within the field of the membrane. More work will be needed to elucidate its mode of action. Ammonium is often used to manipulate intracellular pH [3], but this use would not be indicated when full activity of the type of channels studied here needs to be unaltered.

This work was supported by grant No. 1940408 from Fondecyt (Chile). Institutional support to CECS from a group of Chilean private companies (Empresas CMPC, CGE, COPEC, Minera Escondida, Novagas, Business Design Ass., ENERSIS, Xerox de Chile) is also acknowledged. We are grateful to John Dempster (University of Strathclyde, Scotland) who kindly supplied the electrophysiological software and to Ana-Luisa Eguiguren and Andrés Stutzin for their assistance and advice throughout this study.

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