Evidence that amiloride antagonises insulin-stimulated protein phosphorylation by inhibiting protein kinase activity

Ross Holland, James R. Woodgett and D. Grahame Hardie+

Biochemistry Department, Dundee University, Medical Sciences Institute, Dundee DD1 4HN, Scotland

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The diuretic drug amiloride antagonises the insulin-dependent increase in phosphorylation of ATP-citrate lyase in hepatocytes isolated from rats that had been starved and refed a fat-free diet. Studies with a range of protein kinases and protein phosphatases that have been shown to phosphorylate or dephosphorylate purified ATP-citrate lyase in vitro revealed that amiloride was a non-specific inhibitor of all protein kinases tested, but did not significantly affect any of the protein phosphatases. These results cast doubt on previous claims that growth factors stimulate phosphorylation of ribosomal protein S6 by activating an amiloride-sensitive Na^+/H^+ exchange system, and that insulin inhibits a protein phosphatase that is activated by amiloride.

Insulin *Protein phosphorylation* *Amiloride* *Protein kinase*

1. INTRODUCTION

Insulin increases the phosphorylation of several proteins in isolated cells or cell culture systems, including ATP-citrate lyase [1,2], ribosomal protein S6 [3–5] and an unidentified rat liver polypeptide of M_r 46000 [6,7]. Recently it has been reported that the diuretic drug amiloride* opposes the effects of serum or insulin on ribosomal protein S6 in CC139 cells [8] and on the M_r 46000 polypeptide in isolated hepatocytes [9]. These effects have been interpreted as showing that growth-factor activation of S6 phosphorylation in CC139 cells is triggered by stimulation of Na^+/H^+ exchange in the plasma membrane, and that insulin inhibits an amiloride-stimulated protein phosphatase in isolated hepatocytes. To investigate the action of amiloride further, we studied the phosphorylation of ATP-citrate lyase, since both the protein kinases [10–12] and protein phosphatases [13] acting on this protein are well characterised. Our results show that amiloride is a non-specific inhibitor of protein kinase activity and cast doubt on the interpretation of the previous studies discussed above.

+ To whom correspondence should be addressed

* Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide) is a potassium-sparing diuretic, its diuretic effect being thought to be due to inhibition of Na^+ uptake via Na^+/H^+ exchange in the kidney tubule

2. METHODS

Collagenase (type 1) casein and ovalbumin were from Sigma Chemicals; pyruvate kinase and ATP were from Boehringer; bovine serum albumin (fraction V) was from BDH Chemicals and was defatted as in [14]; [γ-^{32}P]ATP was from Amersham International; rat mammary acetyl-CoA carboxylase [15] and ATP-citrate lyase [11] were purified as described. Rabbit skeletal muscle glycogen synthase was purified as in [16].

Protein phosphatases 1, 2A and 2C were assayed using [^{32}P]phosphorylase *a* or [^{32}P]phosphorylase kinase as in [17]. Protein phosphatases 1 and 2A were purified to homogeneity from rabbit skeletal muscle [T. Resink, B.A. Hemmings, H.Y. Lim Tung and P. Cohen (1983) submitted] and protein phosphatase 2C purified as far as the Sephadex

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G-200 step from rat liver as in [13]. Cyclic AMP-dependent protein kinase, phosphorylase kinase, calmodulin-dependent glycogen synthase kinase, and glycogen synthase kinases -3, -4 and -5 were isolated from rabbit skeletal muscle and assayed as in [16]. Casein kinase-1 was purified from rat mammary gland by ammonium sulphate precipitation and chromatography on phosphocellulose and Sephadex G-200 [M.R. Munday and D.G.H. (1983) in preparation]. It was assayed in 50 μl final vol. using casein (2 mg/ml), EGTA (0.1 mM), EDTA (2 mM), Mg acetate (6 mM), sodium glycerophosphate (20 mM), [γ-^{32}P]ATP (0.2 mM; 28000 cpm/nmol) (pH 7.0). Incorporation of radioactivity into protein was determined as in [11].

Hepatocytes were isolated as in [18] from male Sprague–Dawley rats (150–250 g) which had been starved for 72 h and refed for 48 h (fat-free diet, ICN Nutritional Biochemicals). Cells were incubated in minimal essential medium with additions as in [6] at ~10^7 cells/ml. Where added, insulin was at 10^{-8} M and amiloride at 1 mM. In ^{32}P-labelling experiments cells were incubated in non-radioactive medium (1.2 mM phosphate) for 30 min and were then harvested and resuspended in medium containing [^{32}P]phosphate (0.2 mM; 0.625 Ci/mmol) and incubation continued for 60 min before addition of insulin or amiloride. Cells used for protein phosphatase assays were pre-incubated for 90 min as for ^{32}P-labelling experiments except that unlabelled phosphate (1.2 mM) was used throughout. Cells were homogenised using 40 strokes of a Dounce homogeniser in 0.25 ml/ml cell suspension of either: (a) 50 mM Tris–Cl$^-$ (pH 7.5), 2 mM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaF, 0.25 M sucrose, plus protease inhibitors as in [15] (for experiment shown in fig.1); or (b) 50 mM Tris–Cl$^-$ (pH 7.0), 4 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 0.25 M sucrose (for experiments involving protein phosphatase assays or studies of dephosphorylation).

3. RESULTS

3.1. *Effect of amiloride on ^{32}P-labelling of cytosolic proteins in isolated hepatocytes*

To examine the effects of amiloride on phosphorylation of ATP-citrate lyase, hepatocytes were isolated from rats which had been starved for 72 h and then refed a fat-free diet, a procedure which causes a large increase in the amount of ATP-citrate lyase protein [1]. If amiloride was added at the beginning of the 60 min ^{32}P-labelling period, it substantially reduced the labelling of all cytosolic proteins (fig.1). If cells were labelled for 60 min and then resuspended in non-radioactive medium with or without insulin (10^{-8} M) or amiloride (1 mM), insulin caused an increase, and amiloride a decrease, in the labelling of polypeptides of M_r 116000 and 46000 (fig.1). The M_r

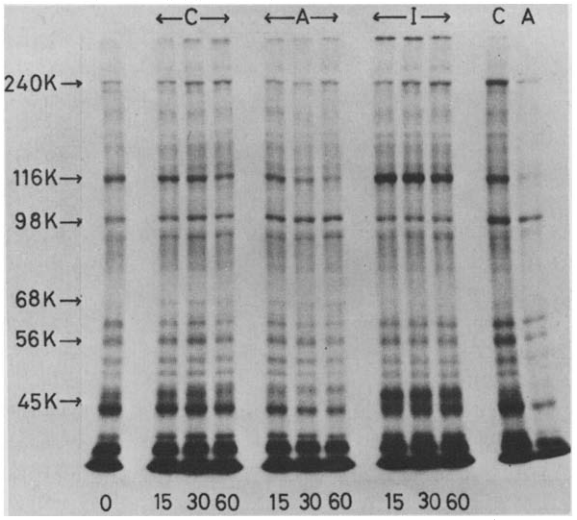

Fig.1. Effects of insulin and amiloride on ^{32}P-labelling of cytosolic proteins in isolated hepatocytes during a chase with non-radioactive medium. Cells were ^{32}P-labelled for 60 min as in section 2. The photograph shows an autoradiogram after electrophoresis of supernatant fractions (100000 × *g*; 60 min) in an SDS–7% polyacrylamide gel [20]. The left-hand track shows a sample of cells homogenised at the end of the labelling period. The 9 central tracks show samples homogenised at the times indicated (minutes) after resuspension in non-radioactive medium in the presence of: (C) no additions; (A) amiloride; (I) insulin. The right-hand 2 tracks show the effect of including amiloride during the 60 min labelling period: (C) no addition; (A) amiloride. Arrows at left mark the migration positions of marker proteins: rat mammary acetyl-CoA carboxylase (240000, [15]), rat mammary ATP-citrate lyase (116000, [10]), rabbit muscle phosphorylase *b* (98000, [21]), bovine serum albumin (68000, [22]), rabbit muscle pyruvate kinase (56000, [23]), and hen egg ovalbumin (45000, [24]).

Table 1

Effect of amiloride and insulin on protein phosphatases 1, 2A and 2C

Type of preparation	Protein phosphatase	Amiloride (1 mM) (% control)	Amiloride (0.1 mM) (% control)	Insulin (10^{-8} M) (% control)
Purified phosphatase	1	84 ± 4(2)	104 ± 4(2)	–
	2A	90 ± 4(2)	102 ± 3(2)	–
	2C	87 ± 1(2)	95 ± 1(2)	–
Liver homogenate	1	104(1)	–	–
	2A	97(1)	–	–
Intact isolated hepatocytes	1	98 ± 20(4)	–	104 ± 18(13)
	2A	91 ± 4(11)	–	114 ± 7(25)
	2C	97 ± 5(5)	–	91 ± 10(5)

Results are expressed as mean ± standard error of the mean for the number of paired experiments shown in brackets. In cell-free experiments the enzyme sample was preincubated with amiloride for 5 min at 30°C before starting the reaction with [^{32}P]substrate. In intact cell experiments cells were treated with or without amiloride or insulin for 15 min and then homogenates were prepared for protein phosphatase assays

116000 polypeptide comigrates with the subunit of purified rat mammary ATP-citrate lyase (fig.1), is greatly elevated on starving and refeeding, and has been identified as ATP-citrate lyase in several laboratories [1,2].

3.2. *Effects of amiloride and insulin on protein phosphatase activities*

Protein phosphatase activity dephosphorylating [^{32}P]ATP-citrate lyase in rat liver cytosol is accounted for by 3 enzymes: protein phosphatase 1, 2A and 2C [13]. Therefore, we tested the effects of amiloride on the activity of these 3 protein phosphatases in a variety of experimental systems (table 1). The only significant effect observed was a slight inhibition of the purified enzymes by 1 mM amiloride. Addition of 1 mM amiloride to a liver homogenate also had no significant effect on the dephosphorylation of ATP-citrate lyase, the M_r 46000 phosphoprotein or any other phosphoprotein detectable by autoradiography after SDS–polyacrylamide gel electrophoresis (not shown). Neither insulin nor amiloride, when added directly to isolated hepatocytes, had any significant effect on any of the protein phosphatases as judged by assays in extracts prepared from the cells (table 1).

Table 2

Effect of amiloride on protein kinase activities

Protein kinase	Amiloride (mM)	Activity (% control)
1. Cyclic AMP-dependent protein kinase	0.1	40
2. Calmodulin-dependent glycogen synthase kinase	0.1	47
3. Phosphorylase kinase	0.1	45
4. Glycogen synthase kinase-3	0.1	47
5. Glycogen synthase kinase-4	0.1	60
6. Glycogen synthase kinase-5	0.1	75
7. Casein kinase I	1.0	56

Protein kinases 1–6 were assayed using glycogen synthase as substrate (0.4 mg/ml) and 0.1 mM ATP as in [16]; casein kinase I was assayed as in section 2

3.3. *Effect of amiloride on protein kinase activities in vitro*

The effect of amiloride on a wide range of homogeneous or partially purified protein kinases is shown in table 2. All were inhibited by amiloride. Experiments with the catalytic subunit of cyclic AMP-dependent protein kinase showed that the inhibition was competitive with respect to ATP, being relieved if ATP concentration was increased (not shown).

4. DISCUSSION

These results show that the reported antagonism between amiloride and insulin also extends to ATP-citrate lyase. On the basis of experiments with hepatocytes isolated from rats fed ad libitum, it was proposed [9] that amiloride activates an insulin-sensitive protein phosphatase. Our data do not support this hypothesis. The conclusions [9] were based on the observation that the effects of insulin and amiloride were preserved in cells that had been chased with non-radioactive medium. However, this result can only rule out the possibility that insulin and/or amiloride act on protein kinase activity if the specific radioactivity of the γ-phosphate of intracellular ATP decreases very rapidly during the chase. The results of our pulse–chase experiment suggest that this is not the case. There is very little loss of ^{32}P from cytosolic proteins during a 60 min chase and in the presence of insulin the ^{32}P-labelling of ATP-citrate lyase and the M_r 46000 polypeptide actually increase during the chase (fig.1).

In isolated hepatocytes ATP-citrate lyase is phosphorylated exclusively at the same site phosphorylated on the purified enzyme by cyclic AMP-dependent protein kinase in vitro [12,19]. The protein phosphatase activity dephosphorylating this site in rat liver cytosol is accounted for by protein phosphatases 1, 2A and 2C [13]. This gave us the opportunity to test directly the hypothesis [9] that amiloride activates a protein phosphatase. No evidence could be found for activation of any protein phosphatase which acts on ATP-citrate lyase (table 1).

An alternative explanation for the data in fig.1 is that amiloride inhibits ATP-citrate lyase kinase activity. While the protein kinase(s) responsible for phosphorylation of ATP-citrate lyase under basal conditions or in the presence of insulin are not identified, all protein kinases tested were inhibited by amiloride. Of those tested, cyclic AMP-dependent protein kinase, the calmodulin-dependent glycogen synthase kinase, glycogen synthase kinase-5 and casein kinase-1 all phosphorylate purified rat mammary ATP-citrate lyase in vitro [10; P.S. Guy, D.G.H. and J.R.W. (1983) unpublished]. In at least one case (cyclic AMP-dependent protein kinase) the inhibition was shown to be competitive with respect to ATP. Examination of the structure of amiloride (fig.2) reveals that it has a heterocyclic ring structure not unlike that of purines and pyrimidines. Amiloride may therefore inhibit protein kinases by competing at the ATP binding site.

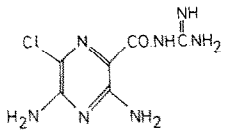

Fig.2. Structure of amiloride.

Our results also cast doubt on conclusions drawn from experiments with amiloride on CC139 cells [8]. It was claimed that the inhibition of phosphorylation of ribosomal protein S6 by amiloride was due to a change in intracellular pH brought about by inhibition of Na^+/H^+ exchange at the plasma membrane. However, inspection of their data suggests that > 0.1 mM amiloride is required to produce significant inhibition of S6 phosphorylation, whereas the ID_{50} for the effect of amiloride on Na^+-dependent proton extrusion was 3 μM [8]. In isolated hepatocytes the ID_{50} for amiloride on phosphorylation of cytosolic proteins ranges from 2–6 mM [9]. Our data suggest that the inhibition of phosphorylation of ribosomal protein S6 in CC139 cells by amiloride could be a direct effect on protein kinase activity and may be independent of the effects of the drug on Na^+/H^+ exchange.

Although we were unable to detect an effect of insulin treatment of isolated hepatocytes on the activity of protein phosphatases 1, 2A or 2C measured in cell extracts, we cannot rule out the possibility that an effect of insulin on one of these protein phosphatases is lost on preparation of the extract. Whether insulin stimulates phosphorylation of ATP-citrate lyase and the M_r 46000 polypeptide by activating a protein kinase or inhibiting a protein phosphatase remains in our view an open question.

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