Biochimica et Biophysica, 642 (1981) 88—95

BBA 79150

THE EFFECT OF AMILORIDE ON HORMONAL REGULATION OF AMINO ACID TRANSPORT IN ISOLATED AND CULTURED ADULT RAT HEPATOCYTES

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(Received August 18th, 1980)

Key words: Amino acid transport; Glucagon; Insulin; Epidermal growth factor; Amiloride; (Rat hepatocyte)

Summary

Insulin and glucagon stimulate amino acid transport in isolated rat hepatocytes. Amiloride, a specific Na^+-influx inhibitor, completely inhibited the hormonal (glucagon or insulin) stimulation of α-aminoisobutyric acid influx by preventing the emergence of a high-affinity transport component.

The drug also inhibited [^{14}C]valine incorporation into hepatocyte protein. The half-maximal concentration of amiloride for inhibition of protein synthesis was similar to that required for inhibition of hormone-stimulated amino acid transport (approx. 0.1 mM). In primary cultured rat hepatocytes, amiloride markedly depressed the stimulation of α-aminoisobutyric acid transport by glucagon, or a mixture of glucagon, insulin and epidermal growth factor.

These results suggest that amiloride inhibits the hormonal stimulation of hepatocyte amino acid transport by preventing the synthesis of high-affinity transport proteins. They also suggest that the hormonal stimulation of hepatocyte amino acid transport is dependent, at least partly, on Na^+ influx.

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Introduction

An increase in the influx of Na^+ may represent an early signal in the initiation of cell proliferation [1—4]. A stimulation of Na^+ uptake in response to a mitogenic signal would be expected to modify rapidly the membrane potential and intracellular pH. Both of these parameters have indeed been shown to be altered in primary cultured rat hepatocytes following exposure to mitogenic media [2,3]. However, the nature of Na^+-dependent cellular events which might be implicated in the initiation of DNA synthesis is largely unknown. In hepatocytes, Na^+-dependent amino acid transport has been reported to increase in vitro after addition of growth-stimulating peptides [2,5], and in vivo during the prereplicative period that follows partial (67%) hepatectomy in the rat [2,6]. In the latter situation, the increase in amino acid transport observed in hepatocytes from the remnant was shown to result from the emergence of a high-affinity transport component, which possessed the properties of a pure A (alanine-preferring) system [6]. In isolated hepatocytes from normal rats, a high-affinity component, similar to that observed after partial hepatectomy, has been shown to be induced by glucagon added to the cells in vitro [5].

In the present studies we have investigated the importance of Na^+ fluxes in the hormonal regulation of hepatic amino acid transport. We used amiloride, a specific inhibitor of Na^+ influx in frog skin [7] and toad bladder [8]. This drug has recently been shown to inhibit the stimulation of Na^+ uptake, α-aminoisobutyric acid uptake, thymidine incorporation into DNA and cell growth in primary cultures of adult rat hepatocytes following exposure to serum-free media containing insulin, glucagon and epidermal growth factor [2—4].

Materials and Methods

Chemicals. Porcine monocomponent insulin and porcine glucagon were generously supplied by Novo (Copenhagen, Denmark), and by E. Lilly Co. (Indianapolis, IN, U.S.A.); EGF (epidermal growth factor) was a gift from D. Gospodarowicz (Cardiovascular Research Institute, University of California, San Francisco). α-Amino[1-^{14}C]isobutyric acid (specific activity 60 Ci/mol), and L-[^{14}C]valine (specific activity 283 Ci/mol) were purchased from the Radiochemical Centre, Amersham (U.K.). The unlabeled nonmetabolizable amino acid, α-aminoisobutyric acid, was obtained from Sigma. All other reagents were of the best grade commercially available. Amiloride hydrochloride was generously supplied by Merck, Sharp & Dohme (West Point, PA, U.S.A.; and Paris, France).

Preparation of isolated hepatocytes and incubation procedures. Hepatocytes were isolated from adult male Wistar rats by collagenase dissociation of the liver as previously described [5]. Cell viability, estimated by cell membrane refractility under phase contrast microscopy [5], was routinely 94—96%. In all experiments, hepatocyte suspensions were incubated in a Krebs-Ringer bicarbonate buffer (pH 7.4) containing 10 mg/ml bovine serum albumin (fraction V), 0.8 mg/ml bacitracin and 50 μg/ml gentamycin and gassed with a mixture of 5% CO_2 and 95% O_2.

Primary cultured hepatocytes. Adult rat hepatocytes were isolated, plated

and permitted to progress through one growth cycle as described elsewhere [2,9]. Detailed descriptions of sera, collagenase, buffer and arginine-free media preparations and of plastic ware have been published [10]. All media were supplemented with 0.4 mM L-ornithine.

Transport assays. (1) Isolated hepatocytes: freshly prepared (within 1 h after cell isolation) suspensions of hepatocytes ($2 \cdot 10^6$ cells/ml) were incubated for 2 h with or without hormone and with or without amiloride at 37° C. At the end of this incubation period, cells were collected by centrifugation ($2000 \times g$ for 5 s) and resuspended in the same buffer (without hormone) at a concentration of approx. $3 \cdot 10^6$ cells/ml.

Amino acid transport was assayed in 1.5 ml plastic conical microcentrifuge tubes (Eppendorf) as previously described [5].

(2) Cultured hepatocytes: 14-day-old cultures were used. Washed cultures (approx. $0.6 \cdot 10^6$ cells/dish) were given (zero time in Fig. 3) 2 ml fresh serum-free medium without or with 0.4 mM amiloride, or 2 ml serum-free medium containing varying concentrations of glucagon, or a mixture of insulin, glucagon and EGF (50 ng/ml of each peptide), without or with 0.4 mM amiloride. At the times indicated, media were aspirated and the dishes were washed twice with 2 ml buffer [2]; 2 ml fresh buffer containing 2 μM amino[^{14}C]isobutyric acid (approx. 0.2 μCi/dish) were then added. After 5 min, assays were terminated and analytical studies were performed as described elsewhere [2]. All experiments were done at 37° C.

Dose dependence of amiloride effects on aminoisobutyric acid transport and valine incorporation into protein in isolated hepatocytes. Cells ($3 \cdot 10^6$/ml) were incubated for 2 h at 37° C in plastic conical microcentrifuge tubes (200 μl/tube) in the presence of varying concentrations of amiloride. Aminoisobutyric acid uptake was measured by adding 50 μl of buffer containing 0.1 mM amino[^{14}C]isobutyric acid (0.125 μCi). The reaction was stopped 10 min later as indicated for transport assays.

For the determination of [^{14}C]valine incorporation into cell protein, hepatocytes were first incubated for 90 min with amiloride as described above; 10 mM [^{14}C]valine (0.25 μCi in 50 μl) was then added to the cells, and the reaction was terminated 30 min later by adding 1.0 ml of chilled buffer. Cells were collected by centrifugation (10 s at $2000 \times g$), resuspended in 1.0 ml of buffer containing 5 mM valine, centrifuged again and the cell pellet was resuspended in 1.0 ml of 0.3 M $HClO_4$. After 15 min on ice, the resulting precipitate was collected by centrifugation (20 s at $2000 \times g$), washed three times with 0.3 M $HClO_4$, and counted for ^{14}C radioactivity.

Results

Effect of amiloride on the hormonal stimulation of aminoisobutyric acid influx in isolated hepatocytes and cultured hepatocytes

Isolated hepatocytes. In these studies, the effect of amiloride on the induction of the high-affinity transport component by glucagon, insulin, or dibutyryl cyclic AMP was investigated by first incubating hepatocytes for 2 h with and without hormone (or cyclic AMP) in the absence or presence of amiloride; this duration (2 h) of hepatocyte incubation prior to measuring aminoisobutyric

acid influx has previously been shown to be required for the maximal expression of the hormonally induced high-affinity transport component [5]. The influx of aminoisobutyric acid at varying aminoisobutyric acid concentrations was then measured over 4-min periods, and the data were plotted as v against v/[aminoisobutyric acid] [5]. Neither amiloride nor hormone (or cyclic AMP) was present during the transport step.

In the absence of amiloride, glucagon, insulin, and dibutyryl cyclic AMP stimulated aminoisobutyric acid influx mainly at low (0.1—2.5 mM) aminoisobutyric acid concentrations, resulting in curvilinear plots (Fig. 1). This phenomenon has previously been characterized [5], and was shown to result from the synthesis of high-affinity transport proteins. In contrast, the presence of amiloride together with insulin or glucagon(or with dibutyryl cyclic AMP) inhibited the stimulatory effect of the hormone and of dibutyryl cyclic AMP. This effect led to plots which were almost linear and similar to that observed under basal (i.e., non-stimulated) conditions (Fig. 1).

To investigate the time dependence of the amiloride inhibition of glucagon-stimulated aminoisobutyric acid influx, the drug was added to incubation media at varying times during the 120 min exposure of hepatocytes to the hormone (Table I). When amiloride was present with the hormone at the beginning of the incubation (i.e., for 120 min), the glucagon-stimulated influx of 0.1 mM aminoisobutyric acid was inhibited by 80%. The residual 20% aminoisobutyric acid influx was similar to the basal influx measured after a 2 h incubation of hepatocytes in the absence of hormone and amiloride (Fig. 1, upper left). The

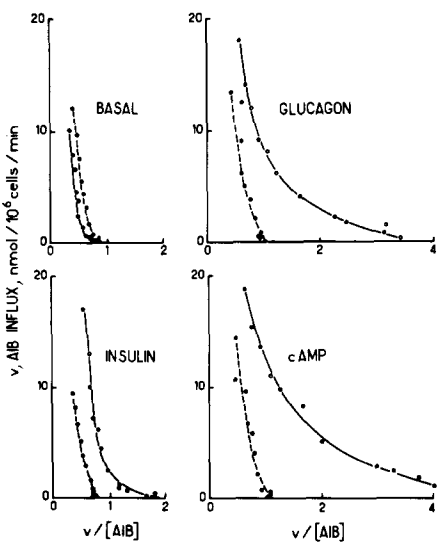

Fig. 1. Effect of amiloride on the hormonal stimulation of aminoisobutyric acid (AIB) influx in isolated hepatocytes. Cells were incubated for 2 h at 37°C in the absence (○———○) or presence (●- - - - -●) of 0.4 mM amiloride, and in the absence (basal) or presence of 100 nM insulin, 100 nM glucagon or 0.1 mM dibutyryl cyclic AMP (cAMP). Hepatocytes were then collected by centrifugation and resuspended in fresh buffer (in the absence of amiloride and hormone) containing varying aminoisobutyric acid concentrations (from 0.1 to 30 mM) and aminoisobutyric acid influx was measured over 4-min periods. The Na^+-dependent part of aminoisobutyric acid influx was plotted as v against v/[AIB]. The results are shown for a typical experiment. Similar results were obtained in three separate experiments.

TABLE I

EFFECT OF DURATION OF HEPATOCYTE EXPOSURE TO AMILORIDE ON GLUCAGON-STIMULATED AMINOISOBUTYRIC ACID INFLUX

Hepatocytes were incubated for 2 h at 37°C with 100 nM glucagon. Amiloride (0.4 mM) was added at the times indicated. Amino[^{14}C]isobutyric acid (0.1 mM) influx was measured in 4-min assays. Values are means ± S.E. of triplicate determinations.

Duration of exposure to amiloride (min)	Aminoisobutyric acid influx (pmol/10^6 cells per min)
0	476.6 ± 4.9
30	396.3 ± 4.2
60	189.1 ± 3.3
90	152.2 ± 2.5
105	123.9 ± 4.2
120	93.3 ± 1.8

later the drug was added, the less it inhibited glucagon-stimulated transport; exposing hepatocytes to amiloride for the last 30 min of the 120 min incubation with glucagon resulted in a weak (17%) inhibition of hormone-stimulated aminoisobutyric acid influx (Table I).

When hepatocytes were incubated for 2 h with glucagon and increasing concentrations of amiloride, it was found that the drug inhibited hormone-stimulated aminoisobutyric acid transport in a dose-dependent fashion. Half-maximal inhibition was observed with amiloride at about 0.1 mM (Fig. 2).

Cultured rat hepatocytes. Fig. 3 shows the time course of changes in the rates of 2 μM amino[^{14}C]isobutyric acid uptake in 14-day-old, stationary-phase hepatocyte cultures [2], following exposure to fresh serum-free media (zero time on Fig. 3) containing varying concentrations of glucagon, or a mixture of

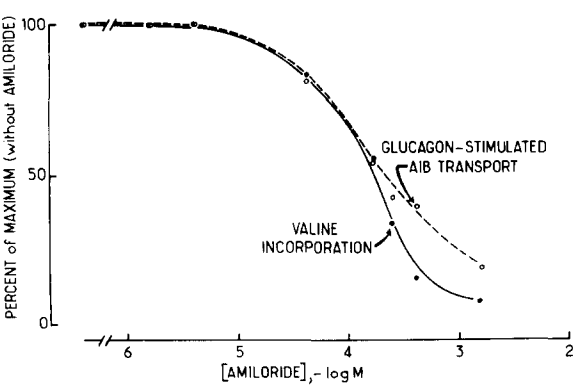

Fig. 2. Dose-response curves for the inhibition by amiloride of valine incorporation into protein and of glucagon-stimulated aminoisobutyric acid (AIB) transport in isolated hepatocytes. Cells were incubated for 2 h at 37° C with varying concentrations of amiloride in the absence (valine incorporation) or presence (aminoisobutyric acid transport) of 100 nM glucagon. Aminoisobutyric acid uptake was measured by adding 0.1 mM amino[^{14}C]isobutyric acid 10 min before the end of the incubation. Valine incorporation was measured by adding [^{14}C]valine (2 mM) 30 min before the end of the incubation. Valine incorporation into cell protein and aminoisobutyric acid uptake were determined. Results are expressed as the percentage of the maximal value observed in the absence of amiloride. Similar results were obtained in two other experiments.

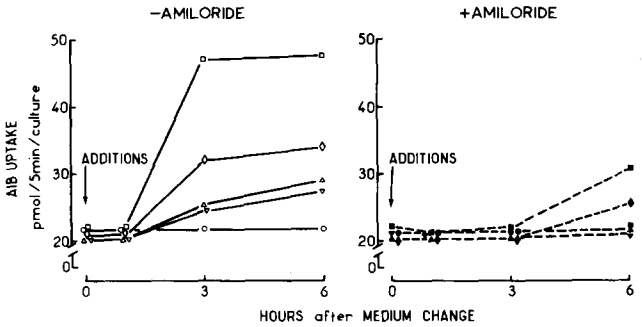

Fig. 3. Time course of change in rates of amino[^{14}C]isobutyric acid uptake in 14-day-old hepatocyte cultures following exposure to fresh serum-free media (○, ●); 1.4 nM glucagon (▽, ▼); 14 nM glucagon (△, ▲); 1.4 μM glucagon (◇, ◆); or a mixture of 14 nM glucagon, 8 nM insulin and 8 nM epidermal growth factor (□, ■), in the absence (open symbols, solid lines) or presence (closed symbols, dotted lines) of 0.4 mM amiloride. AIB, aminoisobutyric acid.

glucagon, insulin and epidermal growth factor. After a lag period of 1 h, the rate of aminoisobutyric acid uptake increased in cultures receiving glucagon or the peptide mixture. The glucagon stimulation was dose dependent, and required longer periods of time for the lower hormone concentrations. The peptide mixture was the most effective in stimulating aminoisobutyric acid uptake, with a maximum reached at 3 h. Amiloride (0.4 mM) virtually abolished the stimulatory effects of glucagon and peptide mixture observed at 3 h. However, the amiloride inhibition did not fully overcome the stimulatory effect of high glucagon concentrations (1.4 μM) of the peptide mixture at 6 h. Amiloride did not detectably alter the non-stimulated, basal rate of aminoisobutyric acid uptake (Fig. 3).

Effect of amiloride on [^{14}C]valine incorporation into protein in isolated hepatocytes

Protein synthesis rates were determined by measuring the incorporation of 2 mM [^{14}C]valine into hepatocyte protein. Under this condition it was found that in isolated hepatocytes, valine re-utilization is minimized and the valine precursor pool may be considered similar to the extracellular valine concentration (Fehlmann, M., Samson, M. and Freychet, P., unpublished data). Fig. 2 shows that amiloride markedly inhibited protein synthesis. The half-maximally effective concentration of amiloride for the inhibition of protein synthesis (approx. 0.15 mM) was very close to that observed for the inhibition of glucagon-stimulated aminoisobutyric acid transport (Fig. 2). This suggests that amiloride inhibits the hormonal stimulation of amino acid transport by preventing the synthesis of high-affinity transport proteins which is induced in isolated hepatocytes following exposure to glucagon (or dibutyryl cyclic AMP).

Discussion

Amiloride has recently been shown to inhibit not only Na^+ uptake but also thymidine incorporation into DNA and cell proliferation in primary cultured

rat hepatocytes stimulated to grow by serum-free, peptide-supplemented media containing a mixture of insulin, glucagon and epidermal growth factor [2—4]. Amiloride was also found to inhibit the stimulation of hepatic DNA synthesis and prereplicative amino[^{14}C]isobutyric acid uptake in vivo following partial (67%) hepatectomy in the rat [2]. These observations suggested that an increased Na^+ influx might represent an early signal for cell proliferation [1—4]. However, the nature of Na^+-dependent prereplicative event(s) that could regulate cell proliferation is largely unknown. Na^+-dependent amino acid transport may represent a limiting step with regard to subsequent protein synthesis rates, RNA synthesis and DNA synthesis initiation [2].

The present study has shown that amiloride inhibits the hormonal stimulation of aminoisobutyric acid transport in hepatocytes. In freshly isolated cells, the drug abolished the stimulatory effect of insulin or glucagon on aminoisobutyric acid influx by blocking the hormonal induction of a high-affinity transport component in a fashion similar to the effect of cycloheximide [5]. In cultured hepatocytes, amiloride also markedly diminished aminoisobutyric acid uptake stimulated by glucagon, or by a peptide mixture (insulin, glucagon and epidermal growth factor) previously shown to stimulate adult rat hepatocyte proliferation in primary cultures [2]. In the latter system, the hormonal stimulation was observed to 'escape' from amiloride inhibition as the duration of incubation increased. Whether this phenomenon is related to the delayed emergence of a short-lived protein possibly implicated in the general stimulatory effects of the peptide mixture, or is instead a consequence of amiloride metabolism and inactivation by the cells, or a combination of both, remains to be elucidated. Neither in cultured hepatocytes nor in freshly isolated cells was the basal, non-stimulated rate of aminoisobutyric acid transport affected by the drug.

Amiloride did not alter glucagon or insulin binding to their receptor sites in isolated hepatocytes (Fehlmann, M., unpublished data), or ^{125}I-labeled EGF binding to its receptor sites in cultured hepatocytes [2], indicating that the drug's inhibitory effect on the hormonal stimulation of amino acid transport occurs at a step, or steps, beyond the receptor.

This inhibition is in fact probably exerted at a rather distal step of hormone action (at least for glucagon), since the drug also inhibited the emergence of the high-affinity transport component induced by dibutyryl cyclic AMP.

In freshly isolated hepatocytes, the time dependence of amiloride inhibition was closely related to the time dependence of the emergence of the high-affinity transport component induced by insulin or glucagon [5], suggesting that the drug may interfere with the synthesis of these transport proteins. This hypothesis is further supported by the similarity between the dose dependence of the amiloride inhibition of [^{14}C]valine incorporation into protein and the dose dependence of the drug inhibition of amino[^{14}C]isobutyric acid transport. In isolated hepatocytes, amiloride does inhibit protein synthesis (Fehlmann, M., Samson, M. and Freychet, P., unpublished observations). Although part of this effect appears to be unrelated to the inhibition of Na^+ influx, the hormonal stimulation of amino acid transport and DNA synthesis initiation are dependent on the presence of Na^+ in the extracellular milieu (Ref. 2 and Fehlmann, M., unpublished data). In addition, a 2—3-fold difference exists between the

ID_{50} value [2] for amiloride inhibition of amino[^{14}C]isobutyric acid uptake in isolated (0.1 mM) and cultured hepatocytes (0.04 mM [3]) whereas, for cultured dog kidney cells, an apparent K_i of 0.017 mM for amiloride inhibition of Na^+ influx has been reported [11]. Because the latter K_i value agrees well with ID_{50} values for amiloride inhibition of DNA synthesis initiation and of 0.1 mM amino[^{14}C]isobutyric acid uptake (stimulated by mitogenic media) in the cultured hepatocyte system [2—4], we cannot yet exclude the possibility that this discrepancy reflects an artifact of experimental variables (e.g., conditions of hormone activation, cell shape, cell age, cell-cell interactions, etc.) that distinguish freshly isolated cells from those cultured as long-term 'monolayers'.

In conclusion, these studies have shown that amiloride, an inhibitor of Na^+ influx, abolishes or markedly depresses the stimulation of amino acid transport induced by glucagon or insulin in isolated hepatocytes, and by glucagon or a peptide mixture (insulin, glucagon and epidermal growth factor) in cultured hepatocytes. The data further suggest that the effect of amiloride is exerted through an inhibition of protein synthesis required for the appearance of a high-affinity component of Na^+-dependent neutral amino acid transport. The appearance of this component may constitute one of the Na^+-dependent prereplicative events that control cell growth initiation.

Acknowledgements

We thank A. Kowalski for technical assistance, G. Visciano for illustration work, and J. Duch for secretarial assistance. This work was supported in part by grant ATP 74.79.106 from the Institut National de la Santé et de la Recherche Médicale (I.N.S.E.R.M., France), research funds from the University of Nice (France), and the United States Public Health Service (U.S.P.H.S., CA 29540-01 and AM 28215).

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