0026-895X/93/020393-06$03.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, 44:393-398

MOL PHARM

Inhibition of Na-K-Cl Cotransport by Amiloride Analogues Is Associated with Stimulation of Cyclic AMP-Dependent Protein Kinase

ROSS D. FELDMAN and S. JEFFREY DIXON

Departments of Medicine and Pharmacology and Toxicology (R.D.F.) and Division of Oral Biology and Department of Physiology (S.J.D.), The University of Western Ontario, London, Ontario, Canada N6A 5A5

Received October 28, 1992; Accepted May 20, 1993

SUMMARY

Analogues of amiloride are widely used as pharmacological probes for inhibition of sodium-hydrogen counter-transport. In Jurkat cells, a leukemic T lymphocyte cell line, analogues of amiloride are also potent inhibitors of Na-K-Cl cotransport. The effects of these agents are not additive with those of β-adrenoceptor agonists (which inhibit Na-K-Cl cotransport presumably by stimulation of adenylyl cyclase). Further, analogues of amiloride potently stimulate cAMP-dependent protein kinase activity. The present studies indicate that β-adrenoceptor agonists and analogues of amiloride both act to inhibit Na-K-Cl cotransport and both stimulate cAMP-dependent protein kinase activity. Furthermore, these studies demonstrate a novel mechanism by which amiloride analogues may mediate effects separately from inhibition of sodium-hydrogen exchange.

Sodium-potassium-chloride cotransport has been extensively studied in many cell models, where it appears to play an important role in volume regulation (1). In human lymphocytes, Na-K-Cl cotransport is an important mechanism for potassium influx (2).

Regulation of Na-K-Cl cotransport is complex. Cotransport activity is directly modified by substrate concentration (1). Furthermore, it may be regulated indirectly by signal transduction systems. Elevations in intracellular cAMP have variable effects on Na-K-Cl cotransport and, depending on the system, may be either inhibitory (3–5) or stimulatory (6–9). In human lymphocytes, we have recently demonstrated that agents that increase intracellular cAMP inhibit Na-K-Cl cotransport (2). The explanation for this variability (cAMP-mediated stimulation versus inhibition) is unknown, but this suggests that the relationship between intracellular cAMP and regulation of Na-K-Cl cotransport may be indirect, perhaps mediated through effects on other ion transport mechanisms.

Consequently, we initiated studies to examine effects on Na-K-Cl cotransport of agents thought to selectively modulate other ionic transport mechanisms. The present studies demonstrate that analogues of amiloride (previously thought to be relatively selective inhibitors of Na-H exchange) inhibit Na-K-Cl cotransport and stimulate PKA.

These studies were supported by grants from the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario. R.D.F. is supported as a Career Investigator of the Ontario Heart and Stroke Foundation. S.J.D. is supported by a Development Grant from the Medical Research Council of Canada.

Materials and Methods

Cell culture. Jurkat cells, a leukemic T lymphocyte line, were used. These cells have been useful in the study of ion transport in lymphoid cells (2, 10). Jurkat cells were grown in suspension under 5% CO_2 in 75-cm^2 flasks, in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 1 mg/ml gentamicin sulfate. Cells were grown to densities ranging from 0.75 to 1.5 × 10^6 cells/ml at the time of the study.

^{86}Rb uptake studies. Rubidium uptake was assayed as described previously (2), using methods modified from those of Grinstein *et al.* (11). Cells were taken from culture, sedimented for 15 min at 300 × *g*, and resuspended in Dulbecco's modified Eagle's medium with 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4$, 110 mM NaCl, 0.9 mM NaH_2PO_4, 2.5 μM $Fe(NO_3)_3$ (GIBCO BRL), and 10 mM HEPES, pH 7.4. Assays were performed at a final concentration of 5–10 × 10^6 cells/ml. ^{86}Rb was added at a concentration of 1–2 μCi/ml. Assays were stopped by aliquoting 100 μl of the reaction mixture into chilled microcentrifuge tubes filled with 100 μl of a solution of $BaCl_2$ (3 mM) and ouabain (0.1 mM) layered over 100 μl of a 3:1 Mazola oil/*n*-butylphthalate mixture. Cells were rapidly sedimented through the oil interface (1 min at 15,000 × *g*). Centrifuge tube tips containing the pellets were clipped and deposited in vials and radioactivity was determined by scintillation counting (Beckman LS 6000). Na-K-Cl cotransport was assessed by the extent of bumetanide (10 μM) inhibition of total ^{86}Rb uptake, as described previously (2).

ABBREVIATIONS: PKA, cAMP-dependent protein kinase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BCECF, bis(carboxyethyl)-5,6-carboxyfluorescein; HMA, *N,N*-hexamethyleneamiloride; DDA, dideoxyadenosine.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Assays of adenylyl cyclase activity and cAMP accumulation. Assays of adenylyl cyclase activity were performed in digitonin-permeabilized Jurkat cells according to our previously published methods (2, 12). Jurkat cells were centrifuged at 300 × *g* for 15 min and were resuspended in Hanks' balanced salt solution without calcium but with 0.5 mM EDTA, 2 mM $MgSO_4$, and 1 mg/100 ml digitonin. Cells were incubated for 15 min at 4°, washed twice with Hanks' solution without digitonin as described above, and finally suspended at a density of 10–20 × 10^6 cells/ml for assay. Adenylyl cyclase activity was assessed by the conversion of [α-^{32}P]ATP to [^{32}P]cAMP, by our modifications (12) of the methods of Salomon *et al.* (13). The ^{32}P-labeled nucleotides were separated by sequential Dowex and alumina chromatography.

Assays of cAMP accumulation were performed according to our previously published methods (14). Intact cells were resuspended in Dulbecco's phosphate-buffered saline (GIBCO BRL), pH 7.4 at 37°. Assays were performed in the presence of 100 μM isobutylmethylxanthine and 100 μM Ro 20–1724 to inhibit cyclic nucleotide phosphodiesterase. After a 15-min incubation, the reactions were terminated by placing the tubes in boiling water for 5 min. cAMP content was assayed using a competitive binding protein method (cAMP assay system; Amersham Life Sciences, Oakville, Canada).

Assessment of PKA activity. PKA activity was determined in permeabilized cells as described previously (2, 12). To assess PKA activity in cytosolic preparations, Jurkat cells resuspended in Hanks' balanced salt solution, as described above, were disrupted by nitrogen cavitation (600 psi for 15 min, Parr Bomb). After sedimentation at 300 × *g* for 5 min, the supernatant was centrifuged at 60,000 × *g*. The resulting supernatant was assayed for PKA activity.

PKA activity was assessed by ^{32}P-phosphorylation of synthetic substrate (Kemptide; Sigma Chemical Co.) using [γ-^{32}P]ATP (1–2 μCi/assay tube). Permeabilized cells or supernatants were incubated for 10 min at 30° with 1 mM Kemptide, a phosphodiesterase inhibitor (0.5 mM isobutylmethylxanthine), 0.8 mM ATP, and 100 μM GTP, in a final volume of 100 μl. Phosphorylated substrate was separated by adherence to phosphocellulose (Whatman P81). After six washes with phosphoric acid (75 mM), radioactivity on the phosphocellulose strips was assessed by scintillation counting.

Measurement of cytosolic pH. The cytosolic pH of cells loaded with the fluorescent pH-sensitive dye BCECF was monitored directly by fluorescence spectrophotometry as described previously (15). The Na-H exchanger was activated by acid loading or osmotic shrinking.

Cells were loaded by incubation with BCECF/acetoxymethyl ester (Molecular Probes, Junction City, OR) and acidified by exposure to 1 μM nigericin in Na^+- and K^+-free medium containing 140 mM *N*-methyl-D-glucamine chloride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, titrated to pH 7.30 ± 0.02 with *N*-methyl-D-glucamine at 37° and adjusted to 290 ± 5 mOsM (15). Alternatively, Na-H exchange was activated by allowing cells to equilibrate in hypotonic medium (50% RPMI 1640 medium/50% water) for 15 min, followed by resuspension in isotonic assay buffer containing 123 mM NaCl, 12 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, equilibrated with 5% CO_2 at 37°, titrated to pH 7.30 ± 0.02 with NaOH, and adjusted to 290 ± 5 mOsM (16).

Fluorescence measurements were made at 37° with continuous magnetic stirring and in the presence of 5% CO_2/95% air. Calibration of cytosolic pH versus fluorescence was obtained using the K^+-nigericin method (16).

Results and Discussion

Effects of amiloride analogues on Na-K-Cl cotransport. The effects of 5-amino-substituted analogues of amiloride on Na-K-Cl cotransport were determined by assessing bumetanide-sensitive ^{86}Rb uptake. HMA (1 μM) induced a 22 ± 8% reduction in Na-K-Cl cotransport. The effect of HMA was potent, with an EC_{50} of 5.2 nM (log EC_{50} = −8.3 ± 1.1, three experiments). Similarly, the potent amiloride analogues isobutylmethylamiloride (1 μM) and dimethylamiloride (1 μM) caused a 22 ± 5% and 18 ± 5% inhibition of Na-K-Cl cotransport, respectively (four experiments, $p < 0.05$) (Fig. 1). In contrast, benzamil (1 μM), an analogue of amiloride that has a relatively low potency for inhibition of Na-H exchange (17), did not significantly inhibit Na-K-Cl cotransport (4 ± 7% inhibition, $p > 0.2$). In addition, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (500 μM), a potent inhibitor of chloride-bicarbonate transport, had no significant effect on bumetanide-sensitive rubidium uptake (104 ± 11% of control, six experiments). This pattern of effects is consistent with a mechanism acting via inhibition of Na-H exchange (17). However, similar potencies of 5-amino-substituted amiloride analogues have also been reported for other effects not clearly linked to the Na-H antiport (e.g., binding of amiloride analogues to α_2-adrenoceptors) (18).

We previously demonstrated that agents that increase intracellular cAMP (e.g., the β-adrenergic receptor agonist isoproterenol) inhibit Na-K-Cl cotransport (2). To determine whether the effects of amiloride analogues and β-adrenergic agonists are additive, bumetanide-sensitive ^{86}Rb uptake was assessed in the presence of isoproterenol (10 μM), HMA (1 μM), or both agents combined (Fig. 2). Isoproterenol caused a 37 ± 6% inhibition of Na-K-Cl cotransport (nine experiments), consistent with our recent studies demonstrating β-adrenoceptor-mediated inhibition of Na-K-Cl cotransport. HMA alone caused a 24 ± 6% inhibition of Na-K-Cl cotransport. When HMA and isoproterenol were added together, inhibition of Na-K-Cl cotransport was no greater than that seen with isoproterenol alone (38 ± 6%). The nonadditive effects of isoproterenol and HMA are consistent with a common mechanism for their effects.

Effects of amiloride analogues and β-adrenergic agonists on cytosolic pH. The studies outlined above indicated that β-adrenergic agonists and those analogues of amiloride thought to selectively inhibit Na-H exchange both acted to attenuate Na-K-Cl cotransport. In some systems, agents that increase cAMP inhibit Na-H exchange (19, 20). Thus, our initial hypothesis was that these two classes of agents might act via a common mechanism, namely, alterations in intracellular pH. To assess this directly, β-adrenergic agonist- and amiloride analogue-mediated alterations in intracellular pH

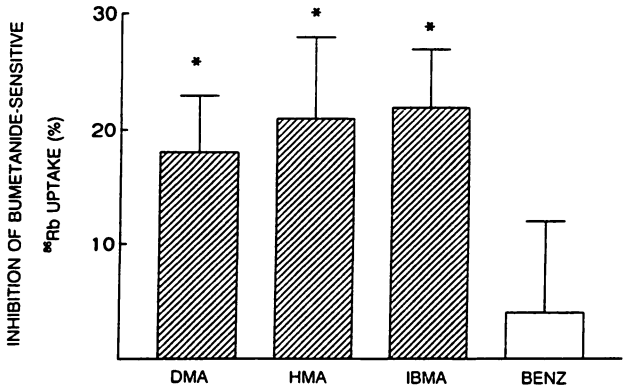

Fig. 1. Inhibition of Na-K-Cl cotransport by amiloride analogues. The effects of dimethylamiloride (*DMA*) (1 μM), HMA (1 μM), isobutylmethylamiloride (*IBMA*) (1 μM), and benzamil (*BENZ*) (1 μM) on bumetanide-sensitive ^{86}Rb uptake are depicted. Data represent the mean ± standard error from four experiments performed separately under identical conditions. *, $p < 0.05$ for a significant inhibition of uptake.

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

aspet

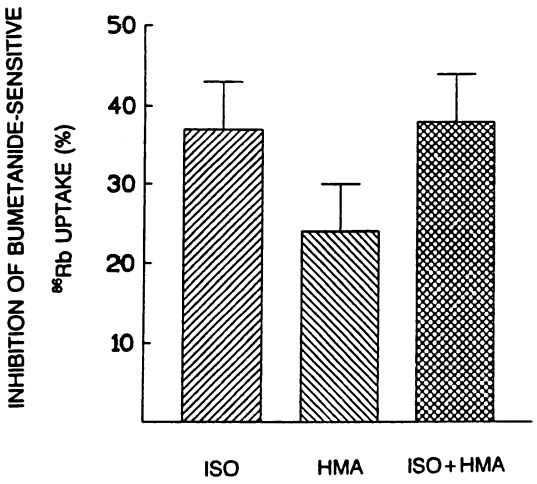

Fig. 2. Effect of isoproterenol (*ISO*) (10 μM) and HMA (1 μM), alone and together, on Na-K-Cl cotransport. Data represent the mean ± standard error from nine experiments performed separately under identical conditions.

TABLE 1

Effects of isoproterenol and HMA on alkalinization associated with regulatory volume increase

Data represent the mean ± standard error from 11 experiments.

Treatment	Initial rate of alkalinization
	pH units/min
Control	0.111 ± 0.019
Isoproterenol (10 μM)	0.108 ± 0.014
HMA (1 μM)	0.004 ± 0.003

were assessed fluorometrically. However, when assayed under the conditions used in the assessment of ^{86}Rb uptake, neither isoproterenol (10 μM) nor HMA (1 μM) had a significant effect on cytosolic pH (threshold sensitivity, <0.01 pH units/min). The lack of effect of HMA suggested that Na-H exchange is not active under basal conditions.

The effects of β-adrenergic agonists on Na-H exchange also were assessed when the antiport was activated by osmotically shrinking the cells. Under control conditions, this regulatory volume increase protocol was associated with significant alkalinization (Table 1; Fig. 3), consistent with the osmotic activation of Na-H exchange, as described previously (16). As expected, alkalinization was blocked by the addition of HMA (1 μM) (Table 1; Fig. 3). In contrast, isoproterenol (10 μM) had no significant effect on the rate of alkalinization due to regulatory volume increase (Table 1). As an alternate approach, Na-H antiport activity was assessed during recovery from acid loading. Again, isoproterenol (10 μM) had no significant effect on the initial rate of alkalinization (control, 0.29 ± 0.03 pH units/min; isoproterenol, 0.25 ± 0.04 pH units/min; seven experiments).

These studies indicated that, in Jurkat cells, β-adrenergic agonists did not inhibit the Na-H antiporter. Furthermore, under the same conditions in which isoproterenol and amiloride analogues inhibited ^{86}Rb uptake they had no detectable effects on intracellular pH. Thus, if analogues of amiloride and β-adrenergic agonists inhibited Na-K-Cl cotransport by a common mechanism, that mechanism could not be inhibition of Na-H exchange.

Effects of amiloride analogues on adenylyl cyclase activity and PKA. To explore an alternative hypothesis, i.e.,

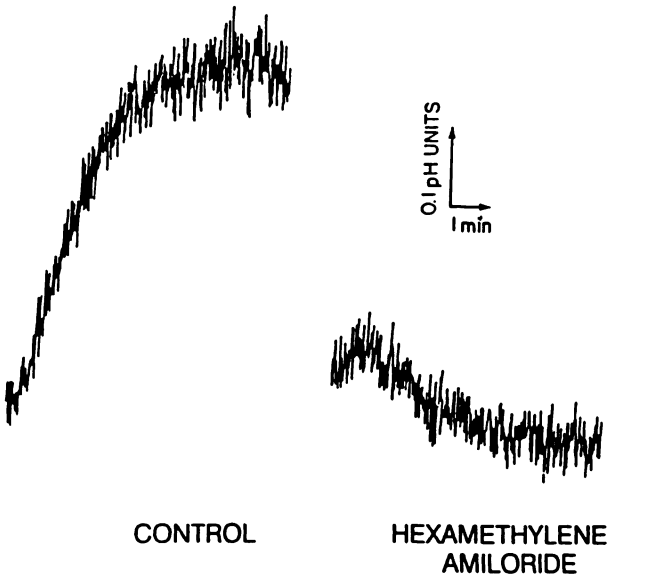

Fig. 3. Alterations in cytosolic pH with regulatory volume increase and the effect of HMA. Data represent a typical tracing from one of 11 experiments performed separately under identical conditions. After incubation in a hypotonic solution of 50% water/50% RPMI 1640 medium, BCECF-loaded cells were resuspended in isotonic assay buffer and the increase in cytosolic pH was assessed fluorometrically (*left*). The addition of HMA (1 μM) blocked alkalinization associated with osmotic cell shrinkage (*right*).

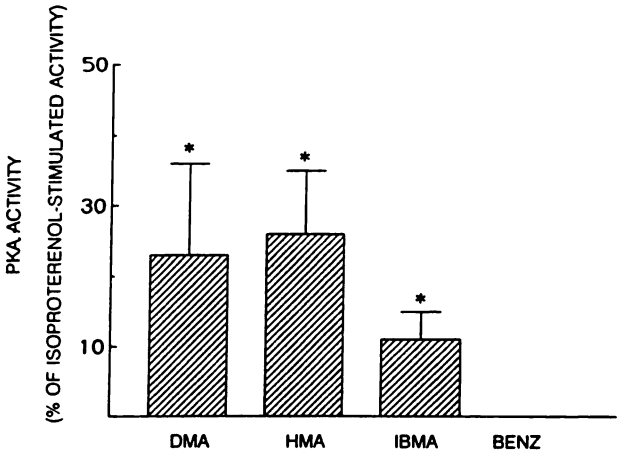

Fig. 4. Effect of amiloride analogues on PKA activity. The maximal effects of dimethylamiloride (*DMA*) (1 μM), HMA (1 μM), isobutylmethylamiloride (*IBMA*) (1 μM), and benzamil (*BENZ*) (1 μM) are expressed as a percentage of isoproterenol (100 μM)-stimulated PKA activity (39 ± 9 pmol of phosphoprotein/min/10^6 cells). Data are expressed as the mean ± standard error from four experiments. *, $p < 0.05$ versus basal activity.

β-adrenergic agonists and amiloride analogues both act by stimulating the adenylyl cyclase/PKA signal transduction system, we first assessed PKA activity in permeabilized cells. At concentrations associated with maximal inhibition of Na-K-Cl cotransport, the 5-amino-substituted amiloride analogues HMA, isobutylmethylamiloride, and dimethylamiloride all stimulated PKA activity to a maximum of approximately 20–30% of that produced by isoproterenol (Fig. 4). HMA was potent, with an EC_{50} of 62 nM (log EC_{50} = −7.2 ± 0.2, three experiments), comparable to the EC_{50} for HMA inhibition of Na-K-Cl cotransport. In contrast, benzamil, which did not inhibit Na-K-Cl cotransport, had no effect on PKA activity. It

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

should be noted that the effects of these amiloride analogues were independent of changes in the concentrations of either sodium or hydrogen ions, because assays were performed in a cell preparation permeable to small molecules.

To rule out a direct effect of amiloride analogues on PKA, the PKA activity was assessed in a cytosolic fraction. In this cell-free preparation cAMP stimulated the activity of PKA to 822 ± 34 pmol of phosphoprotein/min/mg of protein (three experiments). In contrast, neither isoproterenol nor HMA stimulated PKA above basal levels (HMA, 30 ± 9 pmol of phosphoprotein/min/mg protein; isoproterenol, 27 ± 6; basal, 26 ± 12; three experiments).

To further investigate the site of action of the amiloride analogues, assays of adenylyl cyclase activity were performed in permeabilized cells. The effect of HMA (1 μM) was compared with that of the β-adrenergic agonist isoproterenol (10 μM, with 100 μM GTP). As demonstrated previously (2), β-adrenergic receptor stimulation is associated with a significant increase in adenylyl cyclase activity. In contrast, HMA did not cause a detectable increase in adenylyl cyclase activity either alone or in combination with GTP (100 μM) (Fig. 5).

To determine whether amiloride analogues might mediate a detectable increase in adenylyl cyclase activity in an intact cell system, cAMP accumulation was assessed under basal conditions and with the addition of isoproterenol (10 μM) or HMA (1 μM). Isoproterenol increased cAMP accumulation to 238 ± 36% of control (three experiments). In contrast, HMA did not detectably increase cAMP accumulation (basal, 0.63 ± 0.20 pmol/10^6 cells; isoproterenol, 1.53 ± 0.64 pmol/10^6 cells; HMA, 0.37 ± 0.15 pmol/10^6 cells).

By direct assay, we were unable to demonstrate that HMA stimulated adenylyl cyclase activity. However, we realized that partial agonists that act via elevations in intracellular cAMP and stimulation of PKA activity may not necessarily mediate detectable increases in adenylyl cyclase activity. This phenomenon is due to the coupling between adenylyl cyclase activation, stimulation of PKA, and the ultimate functional effect (in this case, inhibition of Na-K-Cl cotransport). We demonstrated previously that in Jurkat cells submaximal inhibition of Na-K-Cl cotransport occurs at concentrations of isoproterenol that mediate <50% of maximal stimulation of PKA activity and barely detectable increments in adenylyl cyclase activity (2). Thus, analogues of amiloride could stimulate adenylyl cyclase undetectably but still activate PKA via a cAMP-dependent mechanism.

As an alternative indirect approach to elucidate how amiloride analogues stimulate PKA, we assessed the additivity of HMA and isoproterenol. Even at maximal concentrations, isoproterenol-stimulated adenylyl cyclase activity is associated with only submaximal PKA stimulation [as defined by cAMP (1 mM)-stimulated PKA activity]. Thus, if HMA acted directly on PKA (and thus its effect was independent of elevations in intracellular cAMP), it would be anticipated that the effects of isoproterenol and HMA would be additive. However, at maximal concentrations of isoproterenol and HMA (10 μM and 1 μM, respectively), no additive effect on PKA activity was seen. This finding is consistent with a common mechanism for these agents to stimulate PKA via cAMP elevation (Fig. 6).

DDA, a P-site inhibitor of adenylyl cyclase, has been useful in delineating the role of adenylyl cyclase stimulation in other hormone-mediated effects (21–23). To determine whether HMA might act via an adenylyl cyclase-dependent mechanism, PKA activity was assessed in the presence and in the absence of DDA (100 μM). cAMP-stimulated PKA activity was not affected by DDA coincubation (Fig. 7), consistent with a DDA effect proximal to PKA. In contrast, isoproterenol-stimulated PKA activity was inhibited by >50% by coincubation with DDA. These data are consistent with an effect of DDA to inhibit PKA stimulation by those agents that stimulate adenylyl cyclase. Similarly, HMA-stimulated PKA activity was inhibited by DDA, consistent with HMA stimulation of PKA via adenylyl cyclase activation.

Therefore, despite our inability to detect an HMA-mediated increase in adenylyl cyclase activity by direct assays, three lines of evidence suggest that amiloride analogues stimulate PKA by increasing cAMP. They are 1) the lack of a direct effect of HMA on PKA in cell-free preparations, 2) the lack of any additive effect of HMA and isoproterenol in stimulating PKA, and 3) the selective inhibition of HMA-stimulated PKA activity by DDA.

Conclusions

Amiloride analogues were found to inhibit Na-K-Cl cotransport. This effect is potent and common to those agents that

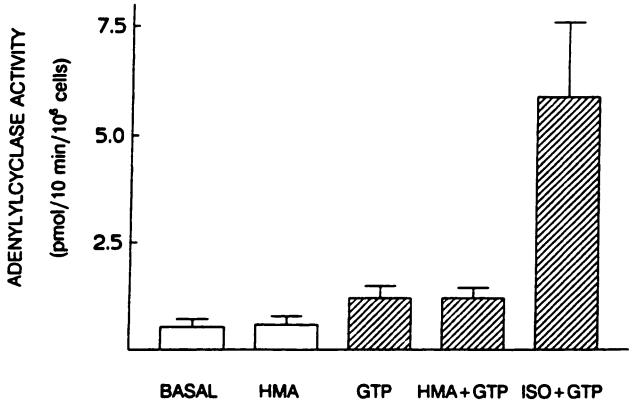

Fig. 5. Effect of HMA (1 μM), either alone or with GTP (100 μM), on adenylyl cyclase activity. β-Adrenoceptor stimulation of adenylyl cyclase is illustrated by the effect of isoproterenol (10 μM) with GTP (100 μM) (ISO + GTP). Data represent the mean ± standard error from six experiments performed separately under identical conditions.

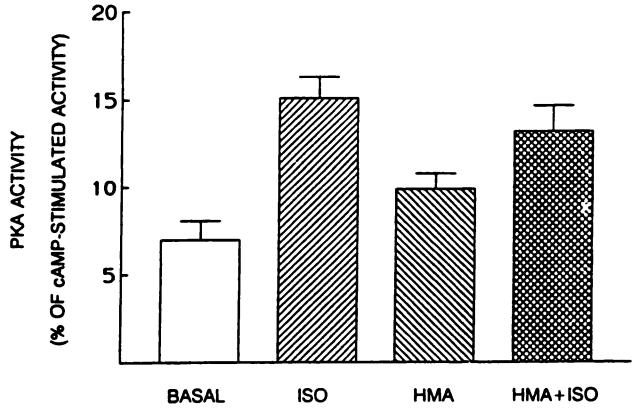

Fig. 6. Effects of isoproterenol (*ISO*) (10 μM) and HMA (1 μM), alone and together, on PKA activity. The data are expressed as a percentage of cAMP (1 mM)-stimulated PKA activity (156 ± 16 pmol of phosphoprotein/min/10^6 cells). Data represent the mean ± standard error from four experiments performed separately under identical conditions.

Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

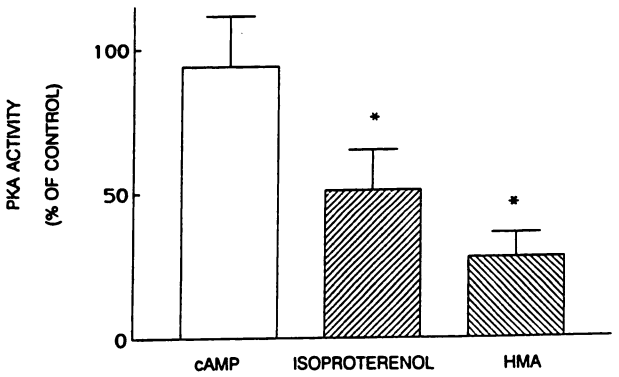

Fig. 7. Effect of direct adenylyl cyclase inhibition on HMA (1 μM)-stimulated PKA activity. Adenylyl cyclase was inhibited by DDA (100 μM). Data are expressed as a percentage of control PKA activity (in the absence of DDA). The effect of DDA on HMA-stimulated PKA activity is compared with the effect of DDA on isoproterenol (100 μM)-stimulated PKA activity and its lack of effect on cAMP (1 mM)-stimulated PKA activity. Basal activity was not decreased by DDA (109 ± 21% of control). Data represent the mean ± standard error from three experiments performed separately under identical conditions. *, $p < 0.05$ versus control.

inhibit sodium-hydrogen exchange. The effects of β-adrenergic agonists and amiloride analogues on Na-K-Cl cotransport are not additive, suggesting a common mechanism of inhibition.

In other cell systems, elevations in cAMP may have variable effects on the Na-H antiporter (19, 20, 24). Whether these effects are direct or indirect is unclear. Previous studies in lymphoma cells (and enteric endocrine cells) have suggested β-adrenergic receptor-mediated stimulation of the Na-H antiporter via a cAMP-independent mechanism (24). In contrast, β-adrenergic agonists do not alter Na-H exchange in Jurkat cells, as assessed by monitoring cytosolic pH using fluorescence techniques. Thus, inhibition of Na-H exchange cannot represent a common mechanism by which isoproterenol and amiloride analogues inhibit Na-K-Cl cotransport. In fact, it is unlikely that inhibition of Na-H exchange could contribute appreciably to the effects of amiloride analogues on Na-K-Cl cotransport, because the Na-H antiport appears to be inactive under the conditions used in our ^{86}Rb uptake studies.

Amino-substituted analogues of amiloride potently stimulated PKA activity, although this effect was clearly smaller than that of isoproterenol. Based on the coupling relationship between stimulation of PKA activity and inhibition of Na-K-Cl cotransport by isoproterenol (2), the extent of HMA-stimulated PKA activity would predict the significant but submaximal inhibition of Na-K-Cl cotransport seen in the present studies (i.e., submaximal, compared with the effects of isoproterenol).

Analogues of amiloride have been demonstrated to have multiple effects on adenylyl cyclase-linked signal transduction systems. Previous studies have focused on their allosteric interactions with receptors. In other cell systems, analogues of amiloride inhibit α_2-adrenoceptor agonist binding (25–27). Comparable effects in β-adrenergic receptor systems have also been demonstrated (25, 27). In contrast to these inhibitory effects, the present studies indicate a novel mechanism of action of these agents in stimulating PKA.

Beyond the effects of amiloride analogues on the inhibition of Na-K-Cl cotransport, these studies may have important implications for those investigators who use these agents as selective probes of Na-H exchange. The EC_{50} of HMA for stimulation of PKA corresponds closely to that reported for inhibition of Na-H exchange (17). Thus, effects of these agents previously ascribed solely to inhibition of Na-H exchange might also be related to stimulation of PKA.

Acknowledgments

The authors gratefully acknowledge the expert technical assistance of Mr. William DeYoung, Ms. Julie Patterson, and Ms. Ruth Renaud in the performance of these studies and the help of Mrs. Glenda Patterson in the preparation of the manuscript.

References

1. O'Grady, S. M., H. C. Palfrey, and M. Field. Characteristics and functions of Na-K-Cl cotransport in epithelial tissues. *Am. J. Physiol.* **253**:C177-C192 (1987).
2. Feldman, R. D. *beta*-Adrenergic inhibition of Na-K-Cl cotransport in lymphocytes. *Am. J. Physiol.* **263**:C1015-C1020 (1992).
3. Brock, T. A., C. Brugnara, M. Canessa, and M. A. Gimbrone. Bradykinin and vasopressin stimulate Na^+-K^+-Cl^- cotransport in cultured endothelial cells. *Am. J. Physiol.* **250**:C888-C895 (1986).
4. Smith, J. B., and L. Smith. $Na^+/K^+/Cl^-$ cotransport in cultured vascular smooth muscle cells: stimulation by angiotensin II and calcium ionophores, inhibition by cyclic AMP and calmodulin antagonists. *J. Membr. Biol.* **99**:51–63 (1987).
5. Garay, R. P. Inhibition of the Na^+/K^+ cotransport system by cyclic AMP and intracellular Ca^{2+} in human red cells. *Biochim. Biophys. Acta* **688**:786–792 (1982).
6. Silva, P., K. Spokes, J. A. Epstein, A. Stevens, and F. H. Epstein. Cyclic AMP stimulates ouabain-insensitive ion movement in shark rectal gland. *J. Comp. Physiol. B Metab. Transp. Funct.* **154**:139–144 (1984).
7. Haas, M., L. G. Johnson, and R. C. Boucher. Regulation of Na-K-Cl cotransport in cultured canine airway epithelia: a [^{3}H]bumetanide binding study. *Am. J. Physiol.* **259**:C557-C569 (1990).
8. Pewitt, E. B., R. S. Hegde, M. Haas, and H. C. Palfrey. The regulation of Na/K/2Cl cotransport and bumetanide binding in avian erythrocytes by protein phosphorylation and dephosphorylation. *J. Biol. Chem.* **265**:20747–20756 (1990).
9. Paulais, M., and R. J. Turner. β-Adrenergic up-regulation of the Na^+-K^+-$2Cl^-$ cotransporter in rat parotid acinar cells. *J. Clin. Invest.* **89**:1142–1147 (1992).
10. Maldonado, D., M. Schumann, P. Nghiem, Y. Dong, and P. Gardner. Prostaglandin E_1 activates a chloride current in Jurkat T lymphocytes via cAMP-dependent protein kinase. *FASEB J.* **5**:2965–2970 (1991).
11. Grinstein, S., C. A. Clarke, A. Dupre, and A. Rothstein. Volume-induced increase of anion permeability in human lymphocytes. *J. Gen. Physiol.* **80**:801–823 (1982).
12. Feldman, R. D. β-Adrenergic desensitization reduced the sensitivity of adenylate cyclase for magnesium in permeabilized lymphocytes. *Mol. Pharmacol.* **35**:304–310 (1988).
13. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
14. Motulsky, H. J., D. Smith, B. I. Terman, and R. D. Feldman. Regulation of hormone-stimulated cyclic AMP accumulation in intact human mononuclear leukocytes by blood plasma. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**:329–343 (1987).
15. Grinstein, S., S. Cohen, and A. Rothstein. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* **83**:341–369 (1984).
16. Grinstein, S., C. A. Clarke, and A. Rothstein. Activation of Na^+/H^+ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *J. Gen. Physiol.* **82**:619–638 (1983).
17. Simchowitz, L., and E. J. Cragoe. Inhibition of chemotactic factor-activated Na^+/H^+ exchange in human neutrophils by analogues of amiloride: structure-activity relationships in the amiloride series. *Mol. Pharmacol.* **30**:112–120 (1986).
18. Garritsen, A., A. P. Ijzerman, M. T. M. Tulp, E. J. Cragoe, and W. Soudijn. Receptor binding profiles of amiloride analogues provide no evidence for a link between receptors and the Na^+/H^+ exchanger but indicate a common structure on receptor proteins. *J. Recept. Res.* **11**:891–907 (1991).
19. Felder, C. C., T. Campbell, F. Albrecht, and P. A. Jose. Dopamine inhibits Na^+-H^+ exchanger activity in renal BBMV by stimulation of adenylate cyclase. *Am. J. Physiol.* **259**:F297-F303 (1990).
20. Weinman, E. J., S. Shenolikar, and A. M. Kahn. cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney brush-border membranes. *Am. J. Physiol.* **252**:F19-F25 (1987).
21. Siegl, A. M., and J. W. Daly. Receptor (norepinephrine), P-site (2′,5′-dideoxyadenosine), and calcium-mediated inhibition of prostaglandin and forskolin-activated cyclic AMP-generating systems in human platelets. *J. Cyclic Nucleotide Protein Phosphorylation Res.* **10**:229–246 (1985).
22. Themmen, A. P. N., J. W. Hoogerbrugge, F. F. G. Rommerts, and H. J. van der Molen. Is cAMP the obligatory second messenger in the action of lutropin

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012

on Leydig cell steroidogenesis? *Biochem. Biophys. Res. Commun.* **128**:1164–1172 (1985).
23. Reid, J. R., C. Lowe, J. Cornish, D. H. Gray, and S. J. M. Skinner. Adenylate cyclase blockers dissociate PTH-stimulated bone resorption from cAMP production. *Am. J. Physiol.* **258**:E708-E714 (1990).
24. Barber, D. L., M. E. McGuire, and M. B. Ganz. β-Adrenergic and somatostatin receptors regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **264**:21038–21042 (1989).
25. Wilson, A. L., K. Seibert, S. Brandon, E. J. Cragoe, and L. E. Limbird. Monovalent cation and amiloride analog modulation of adrenergic ligand binding to the unglycosylated α_{2B}-adrenergic receptor subtype. *Mol. Pharmacol.* **39**:481–486 (1991).
26. Howard, M. J., R. J. Hughes, H. J. Motulsky, M. D. Mullen, and P. A. Insel. Interactions of amiloride with α- and β-adrenergic receptors: amiloride reveals an allosteric site on α_2-adrenergic receptors. *Mol. Pharmacol.* **32**:53–58 (1987).
27. Howard, M. J., M. D. Mullen, and P. A. Insel. Amiloride interacts with renal α- and β-adrenergic receptors. *Am. J. Physiol.* **253**:F21-F25 (1987).

Send reprint requests to: Ross D. Feldman, Department of Medicine, Room 6-OF11, University Hospital, P.O. Box 5339, London, Ontario, Canada N6A 5A5.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Thammasart University on December 3, 2012