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EFFECTS OF ALAMETHICIN ON HORMONAL ACTIVATION OF RENAL ADENYLATE CYCLASE

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Abstract—The effects of the peptide antibiotic, alamethicin, on hormonally stimulated adenylate cyclase were studied in the rat kidney. In the medullary 100,000 *g* fraction, antidiuretic hormone-stimulated adenylate cyclase activity was observed only in the presence of alamethicin. Alamethicin augmented the stimulatory effect of parathyroid hormone in the cortical 100,000 *g* fraction. Lubrol PX solubilized adenylate cyclase activity but, in contrast to alamethicin, did not increase PTH stimulation of adenylate cyclase. Alamethicin had little effect in 1,000 *g* fractions. Therefore, the effects of this antibiotic do not appear to be due to direct stimulation of adenylate cyclase. In addition, the results cannot be explained by inhibition of phosphodiesterase by alamethicin. Alamethicin is known to increase ionic conductance in membranes by the formation of channels. The effects of alamethicin on adenylate cyclase in the 100,000 *g* fraction could be due to the formation of channels which increase the permeability of vesicles and thereby increase accessibility to substrate and/or hormones. Different physiochemical properties of renal cortical and medullary plasma membranes are suggested by differences observed in the responsiveness of adenylate cyclase to hormones and to alamethicin. The presence of latent antidiuretic hormone-stimulated adenylate cyclase was observed in the outer medullary 100,000 *g* fraction.

Alamethicin, a peptide antibiotic, has been studied extensively with respect to its ability to increase ionic conductance in lipid membranes by the formation of channels [1–3]. Studies of lecithin bilayer vesicles demonstrated that alamethicin produced channels and permitted the outward passage of europium ions in the presence of a potassium gradient [4]. Although the majority of studies on alamethicin have been on model lipid membrane systems, alamethicin has been reported to increase calcium permeability in sarcoplasmic reticulum vesicles [5]. In addition to increasing ionic permeability, alamethicin has been demonstrated recently to uncover latent basal and sodium fluoride adenylate cyclase activity in vesicles of sarcoplasmic reticulum [6].

Adenylate cyclase is thought to be a vectoral plasma membrane enzyme, with hormonal receptors located on the outside surface and the catalytic unit on the inside surface of the membrane. If membranes were in an impermeable vesicular conformation, alamethicin could render substrate (ATP) or stimulator (fluoride) more accessible to critical sites, thus enhancing adenylate cyclase activity [6]. In addition, hormonal activation of adenylate cyclase should depend upon both the accessibility of the hormone to the receptor site and of substrate to the catalytic unit. Vesicles have been observed in electron micrographs of renal cortical and medullary membrane preparations [7–10]. The presence of adenylate cyclase within impermeable renal membrane vesicles could alter the interpretation of experimental results. However, a channel-forming antibiotic like alamethicin might be expected to uncover latent hormonally stimulated adenylate cyclase activity by increasing vesicular permeability. The present study was designed to evaluate the effects of alamethicin on hormonal activation of adenylate cyclase in the 1,000 *g* and 100,000 *g* fractions of the renal cortex and medulla.

MATERIALS AND METHODS

Cyclic [^{3}H—G]AMP (38.9 Ci/mmole) was obtained from New England Nuclear, Boston, Mass. [α—^{32}P]ATP (25 Ci/mmole) was obtained from the International Chemical and Nuclear Corp., Irvine, CA. Synthetic arginine vasopressin [antidiuretic hormone (ADH), 100 I.U./ml], parathyroid hormone (PTH, 218 units/mg), Dowex 50W-X4 (200–400 mesh), neutral alumina, Lubrol PX, ATP, GTP and cyclic AMP were obtained from the Sigma Chemical Co., St. Louis, MO. Scintillation fluid (ACS) was purchased from Amersham-Searle, Arlington Heights, IL. PGE_2 and alamethicin were gifts from Dr. John Pike and Dr. George Whitfield, respectively, The Upjohn Co., Kalamazoo, MI. PGE_2 was dissolved in absolute ethanol, 20 mg/ml, and stored at −20°. PGE_2 solutions were prepared by diluting the stock with 0.02% Na_2CO_3. Alamethicin was dissolved immediately prior to use in absolute ethanol and diluted 1:10 with 0.02% Na_2CO_3. All other chemicals were of the highest grade available. Male Sprague–Dawley rats, weighing 300–350 g, were obtained from Eldridge Laboratory Animals, Barnhart, MO.

Preparation of renal fractions. Rats were anesthetized with ether, and the kidneys were immediately removed, bisected, and sliced with a Stadie–Riggs microtome. Tissue slices (0.5 mm thick) from cortex and outer medulla were collected separately,

minced, and homogenized. Homogenization was accomplished with six strokes from a Teflon–glass homogenizer at 1400 rev/min in buffer containing 10 mM Tris–HCl (pH 7.6), 1 mM EDTA, 3 mM $MgSO_4$, and 0.25 M sucrose (0.1 g tissue/ml). Homogenates were filtered through gauze and centrifuged at 1,000 *g* for 10 min. The resultant supernatant fraction was then centrifuged at 40,000 *g*. The supernatant fraction from the 40,000 *g* centrifugation was centrifuged at 100,000 *g* for 1 hr, and the pellet was homogenized in 10% of the starting volume and quick-frozen in aliquots. The original 1,000 *g* pellet was washed twice in 40% of the original volume of buffer without sucrose and centrifuged each time at 1,000 *g* for 10 min. The final pellets were resuspended in 30% of the original volume of buffer and the suspensions were quick-frozen in aliquots, and stored at −70°. No significant change in enzyme activity was observed during several months of storage [11].

Assay of renal adenylate cyclase activity. The standard reaction mixture for determining adenylate cyclase activity contained 2 mM ATP (Lot no. 125C–7250), 4–8 cpm/pmole [α—^{32}P]ATP, 1.3 mM cyclic AMP, 40,000 cpm [^{3}H]cyclic AMP, 8 μM GTP, 5 mM $MgSO_4$, 20 mM caffeine, 20 mM creatine phosphate, 93 units/ml creatine phosphokinase, 0.4 mg/ml bovine serum albumin. Previous studies have determined the dose–response curves for PGE_2, PTH and ADH in the renal cortical and outer medullary 1,000 *g* fractions [12–14]. Additional studies have shown that 10 units/ml PTH and 8×10^{-4} M PGE_2 are also maximally stimulatory concentrations in the 100,000 *g* fractions. The maximal stimulatory concentration of ADH (10^{-7} M) in the outer medullary 1,000 *g* fraction was 300 times the lowest effective concentration [14]. The final assay volume was 0.075 ml. Lubrol PX and alamethicin concentrations were expressed on a weight basis relative to the protein concentration or as per cent of solution (w/v). The incubation was for 5 min at 30°, unless otherwise indicated, and was initiated by the addition of the homogenate and terminated by the addition of 0.020 ml of 0.5 N HCl and heating for 60 sec. Adenylate cyclase activity was linear with time between 5 and 15 min of incubation at the protein concentrations used in these experiments (120–135 μg). The cyclic [^{32}P]AMP product was isolated using a two-step column chromatographic procedure [15]. Recoveries of cyclic AMP ranged from 80 to 90 per cent, with the ^{32}P blank usually undetectable. Adenylate cyclase activity was corrected for recovery and expressed as pmoles cyclic AMP produced/5 min/mg of protein. Basal (non-stimulated) adenylate cyclase activity was determined in the presence of the diluent control for alamethicin or PGE_2 (ethanol). The diluent did not affect the basal activity. Using methods described previously [16], there was no measurable cyclic nucleotide phosphodiesterase activity with the standard reaction mixture for adenylate cyclase. Membrane protein concentrations were estimated by the method of Lowry *et al.* [17], using bovine serum albumin as a standard. Differences between mean values of three determinations from representative experiments were analyzed for significance using Student's *t*-test for unpaired values ($P < 0.05$).

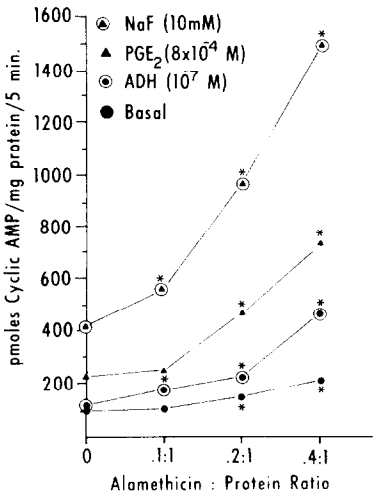

Fig. 1. Effects of alamethicin on 100,000 *g* outer medullary adenylate cyclase activity. The per cent of alamethicin in the 0.4:1 solution was 0.064%. An asterisk (*) indicates $P < 0.05$, compared with the corresponding control value in the absence of alamethicin.

Solubilization experiments. Cortical 100,000 *g* membranes were incubated with diluent (control), Lubrol PX or alamethicin for 5 min at 30° in a total volume of 0.5 ml. The membranes were then centrifuged for 60 min at 100,000 *g* in 0.4-ml microfuge tubes placed in water-filled tubes of a SW 50.1 rotor. The pellets were resuspended in 0.4 ml of buffer. The pellets and supernatant fractions were analyzed directly for adenylate cyclase activity.

RESULTS

The presence of ADH sensitive adenylate cyclase activity in the 100,000 *g* outer medullary fraction was revealed by alamethicin (Fig. 1). When alamethicin to protein ratios were increased from 0.1:1 to 0.4:1, ADH (10^{-7} M)-stimulated activity increased. NaF (10 mM) activity was similarly increased. In contrast, neither PGE_2 (8×10^{-4} M)-stimulated nor basal activities increased until the alamethicin to protein ratio was 0.2:1. In the presence of alamethicin (0.4:1), maximal stimulatory concentrations of PGE_2 and ADH were additive (940 ± 38 pmoles cyclic AMP/5 min/mg of protein). At this alamethicin concentration, hormonal and NaF-stimulated adenylate cyclase activities were increased more than 3-fold. The medullary 100,000 *g* fraction contained approximately 41–55 per cent of the total corresponding basal, PGE_2, and NaF activities in the 1,000 *g* fraction.

In the 100,000 *g* cortical fraction, PTH, basal, and NaF-stimulated adenylate cyclase activities all increased with alamethicin to protein ratios of 0.1:1 to 0.3:1 (Fig. 2). Higher ratios of alamethicin abolished hormonal activation but continued to increase NaF-stimulated activity. The basal, PTH, and NaF-stimulated adenylate cyclase activities recovered in the cortical 100,000 *g* fraction represented approximately 30–36 per cent of the total corresponding activities in the 1,000 *g* fraction.

The effects of Lubrol PX on basal, NaF and hor-

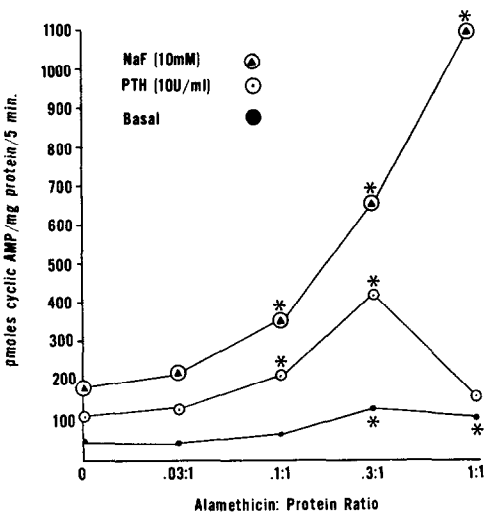

Fig. 2. Effects of alamethicin on 100,000 *g* cortical adenylate cyclase activity. The per cent of alamethicin in the 1:1 solution was 0.18%. An asterisk (*) indicates $P < 0.05$, compared with the corresponding control value in the absence of alamethicin.

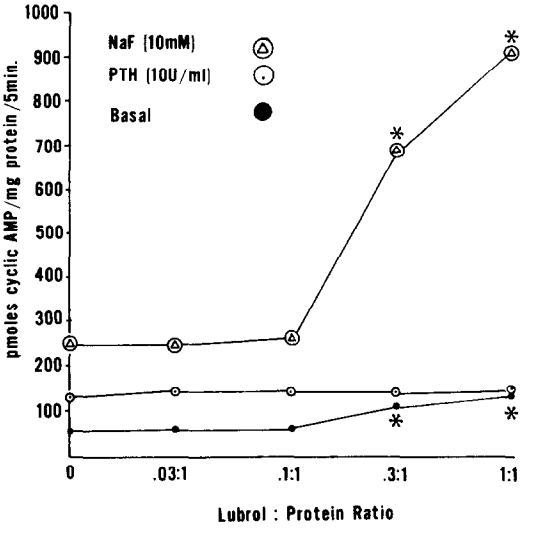

Fig. 3. Effects of Lubrol PX on 100,000 *g* cortical adenylate cyclase activity. The per cent of Lubrol PX in the 1:1 solution was 0.18%. An asterisk (*) indicates $P < 0.05$, compared with the corresponding control value in the absence of Lubrol PX.

monal stimulation were compared to those of alamethicin (Fig. 3). Lubrol PX increased both basal and NaF-stimulated activities. However, PTH (10 units/ml) stimulation was not increased by low concentrations of Lubrol PX and was abolished at high concentrations (1:1).

The capacity of Lubrol PX and alamethicin to solubilize adenylate cyclase was examined (Table 1). In control membranes, all of the adenylate cyclase activity remained in the 100,000 *g* pellet. This enzyme was stimulated by PTH and NaF. After centrifugation of alamethicin-treated membranes (0.3:1 and 1:1), all the detectable adenylate cyclase activity remained in the pellet, a finding similar to that noted with control membranes. With 0.3:1 alamethicin, membranes demonstrated enhanced basal, PTH and NAF-stimulated activities, while membranes incubated with the higher ratio of alamethicin to protein were not stimulated by PTH. Addition of Lubrol PX resulted in the appearance of basal and

Table 1. Effects of treatment with alamethicin or Lubrol PX on adenylate cyclase activities in the cortical 100,000 *g* fraction*

	Pellet (pmoles cyclic AMP/5 min/mg protein)	Supernatant fraction (pmoles cyclic AMP/5 min/mg protein)
Control		
Basal	33 ± 7	ND†
PTH	94 ± 3	ND
NaF	111 ± 14	ND
0.3:1 Alamethicin		
Basal	74 ± 6	ND
PTH	185 ± 8	ND
NaF	406 ± 26	ND
1:1 Alamethicin		
Basal	107 ± 11	ND
PTH	102 ± 1	ND
NaF	629 ± 29	ND
1:1 Lubrol PX		
Basal	54 ± 4	92 ± 13
PTH	72 ± 2	74 ± 3
NaF	467 ± 26	199 ± 2

* Membranes were incubated with alamethicin diluent (control), alamethicin, or Lubrol PX for 5 min. The membranes were centrifuged at 100,000 *g* for 1 hr. Pellets were resuspended in their original volume of buffer. The pellets and supernatant fractions were analyzed directly for adenylate cyclase as described in Materials and Methods. Concentrations used were: PTH, 10 units/ml, and NaF, 10 mM. The per cent of alamethicin and Lubrol PX in solution was 0.3:1 alamethicin, 0.05%; 1:1 alamethicin, 0.18%; and 1:1 Lubrol PX, 0.18%. Values are expressed as means ± S.E.

† ND = not detected.

Table 2. Effects of alamethicin on 1,000 *g* adenylate cyclase activities*

	Cyclic AMP (pmoles/5 min/mg protein)		
		Alamethicin: Protein ratio	
	Control	0.1:1	0.3:1
Cortex			
Basal	127 ± 9	117 ± 10	107 ± 10
PTH	344 ± 24	351 ± 8	285 ± 23
NaF	329 ± 29	333 ± 21	275 ± 11
Outer medulla	Control	0.1:1	0.2:1
Basal	204 ± 10	216 ± 24	234 ± 15
ADH	692 ± 53	777 ± 34	884 ± 46†
PGE_2	769 ± 58	806 ± 47	770 ± 28
ADH and PGE_2	943 ± 31	1121 ± 16†	1155 ± 69†
NaF	610 ± 12	679 ± 44	927 ± 24†

* Concentrations used were: PTH, 10 units/ml; NaF, 10 mM; ADH, 10^{-7} M; and PGE_2, 8×10^{-4}M. The per cent of alamethicin in solution was: cortex 0.1:1, 0.018% and 0.3:1, 0.054% while in the medulla 0.1:1, 0.0164% and 0.2:1, 0.032%.
† Significantly different ($P < 0.05$) than the corresponding control value.

NaF-stimulated adenylate cyclase activity in the supernatant fraction. No stimulation by PTH was observed in the supernatant fraction. Lubrol PX-solubilized basal and NaF-stimulated activity represented 63 and 30 per cent, respectively, of the corresponding activity present in control.

The effect of alamethicin on 1,000 *g* cortical and outer medullary adenylate cyclase is shown in Table 2. Alamethicin did not alter cortical activities. Alamethicin (0.2:1) increased ADH (30 per cent) and NaF (50 per cent) stimulation in medullary fractions, but had no effect upon either basal or PGE_2-stimulated activities. ADH and PGE_2-stimulated adenylate cyclase activities were additive in control and alamethicin-treated membranes.

DISCUSSION

These results demonstrate that, in the outer medulla, expression of ADH-stimulated adenylate cyclase activity in the 100,000 *g* fraction (homogenate which sediments between 40,000 and 100,000 *g*) was observed only in the presence of alamethicin. Prostaglandin E_2 stimulation could be observed in the absence of alamethicin. In the cortex, PTH stimulated adenylate cyclase in 100,000 *g* fractions in the absence of alamethicin. Alamethicin and the detergent, Lubrol PX, increased both basal and NaF-stimulated activities. In contrast to alamethicin, treatment with Lubrol PX abolished PTH stimulation and solubilized adenylate cyclase. Lubrol PX has also been shown to abolish ADH stimulation and to solubilize adenylate cyclase in rat renal medulla [18, 19]. Cyclic nucleotide phosphodiesterase activity is not measurable using these assay conditions, i.e. inclusion of (1) 20 mM caffeine, (2) 1.3 mM cyclic AMP, and (3) [^{3}H]cyclic AMP in the reaction mixture. Therefore, these results cannot be explained on the basis of inhibition of phosphodiesterase by alamethicin. A separate effect of alamethicin on phosphodiesterase cannot, however, be excluded. The lack of effect of alamethicin on 1,000 *g* adenylate cyclase activities suggests that alamethicin is not acting as a general stimulator or potentiator (i.e. NaF) of adenylate cyclase. Previous studies suggest that both the 100,000 *g* and the 1,000 *g* fraction adenylate cyclase activities are associated with plasma membranes [20]. Electron micrographs of renal cortical and medullary membrane preparations have demonstrated the presence of vesicles [7–10]. Membranous vesicles observed in renal cortical microsome preparations are thought to be the site of PTH-stimulated calcium uptake [21].

Effects of alamethicin could be explained by the presence of relatively impermeable vesicles in the 100,000 *g* fractions. The permeability of these vesicles may be increased by alamethicin, facilitating the accessibility of the hormone to the receptor site or of ATP to the catalytic unit, and resulting in increased adenylate cyclase activity. This interpretation is consistent with the known ability of alamethicin to form channels in membranes [1–4] and the reported increased permeability produced by alamethicin in sarcoplasmic reticulum vesicles [5, 6]. Low concentrations of alamethicin caused a preferential increase in ADH compared to PGE_2-stimulated adenylate cyclase activity. Furthermore, in the presence of alamethicin (0.4:1), ADH and PGE_2-stimulated adenylate cyclase activities were additive. These results suggest that ADH and PGE_2 may be stimulating distinct adenylate cyclases. In the case of ADH, all the 100,000 *g* vesicles appear to be impermeable or unresponsive. By contrast, medullary PGE_2 and cortical PTH-responsive adenylate cyclases appear associated with both permeable (responsive) and impermeable vesicles. In the medullary 1,000 *g* fraction, alamethicin elicited a preferential increase in ADH compared to PGE_2-stimulated adenylate cyclase activity. However, the 30 per cent increase in ADH stimulation was quite modest compared to the 3-fold increase seen in the 100,000 g fraction. These results suggest that the adenylate

cyclase systems in the 1,000 *g* fractions, particularly cortical, are more accessible to both hormone and substrate.

The presence of adenylate cyclase within impermeable membrane vesicles may explain results observed in previous experiments. Homogenization of red blood cells in buffers with different concentrations of cations results in orientation of ghosts which can be right-side-out or inside-out [22]. In addition, homogenization of tissues with different buffers has resulted in the localization of adenylate cyclase into different subcellular fractions [23]. Certain cations have also been shown to alter adenylate cyclase activity [24, 25]. Furthermore, adenylate cyclase has been shown to be altered in certain disease states [26, 27]. It is possible that these changes in adenylate cyclase activity could be explained by membrane alterations resulting in differences in membrane vesicularization *in vitro*. Alamethicin is a potential agent for testing the presence of impermeable vesicles which maintain hormonal responsiveness.

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