Biochemical Pharmacology, Vol. 38, No. 6, pp. 915–922, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

DIRECT INHIBITION OF CONTRACTILE APPARATUS BY ANALOGUES OF AMILORIDE IN THE SMOOTH MUSCLE OF GUINEA-PIG TAENIA CAECUM AND CHICKEN GIZZARD

HIROSHI OZAKI, TAKAHIRO MORIYAMA, HIDEAKI KARAKI, KAZUHIRO KOHAMA* and EDWARD J. CRAGOE JR†

Department of Veterinary Pharmacology, Faculty of Agriculture and *Department of Pharmacology, Faculty of Medicine, The University of Tokyo, Bunkyo-ku, Tokyo, Japan and †2211 Oak Terrace Drive, Lansdale, PA 19446, U.S.A.

(*Received* 22 *July* 1988; *accepted* 4 *October* 1988)

Abstract—The relaxant effects of amiloride and its analogues, benzamil, 5-(*N*,*N*-diethyl)-amiloride (DEAM) and 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIAM), were investigated using smooth muscle of guinea-pig taenia caeci and chicken gizzard. High K^+-induced contractions of intact taenia and gizzard were inhibited by these compounds (1–100 μM) with the order of potency; benzamil ≥ EIAM > DEAM > amiloride. Contractions of permealized taenia and gizzard were also inhibited by these compounds at concentrations 8–35 times higher than those needed to inhibit the contractions of intact tissues. These compounds inhibited 20 K myosin light chain (MLC) phosphorylation at the concentrations needed to inhibit the contraction in the permealized muscles. Calmodulin (CaM) activity, as monitored by erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase and phosphodiesterase activities, was inhibited by DEAM and EIAM at similar concentrations as those to inhibit the MLC phosphorylation. Benzamil also inhibited CaM activity at concentrations 4–8 times higher than those required to inhibit MLC phosphorylation. However, amiloride failed to inhibit CaM activity. Among these compounds, amiloride and benzamil inhibited Ca^{2+}/CaM-independent MLC phosphorylation due to trypsin-treated MLC kinase. Taenia tissue gradually accumulated these compounds and the tissue/medium ratio exceeded 3.5–17 after a 3-hr incubation period. These results indicate that amiloride and its analogues inhibit smooth muscle contraction mainly by the direct inhibition of MLC phosphorylation. The inhibitory effect of amiloride may be attributable to the inhibition of MLC kinase, whereas the inhibitory effect of DEAM and EIAM may largely be attributable to the inhibition of CaM. Benzamil may inhibit contraction by the inhibition of both MLC kinase and CaM. Differences in the drug-sensitivity between intact and permealized tissues may be attributable to the difference in drug accumulation by the cell.

Amiloride, a K^+-sparing diuretic, has widely been used to study the mechanisms of transmembrane Na^+ movements. At low concentrations ($IC_{50} < 1$ μM), amiloride inhibits conductive Na^+ channels, and at higher concentrations ($IC_{50} > 1$ μM), amiloride inhibits exchange pathways such as Na^+–H^+ exchange and Na^+–Ca^{2+} exchange mechanisms [1]. Recently, some analogues have been reported to be more potent and specific inhibitors of Na^+–H^+ exchange [2–4] and Na^+–Ca^{2+} exchange mechanisms [5, 6]. These include benzamil, 5(*N*,*N*-diethyl)-amiloride (DEAM)† and 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIAM).

† Abbreviations used: DEAM, 5(*N*,*N*-diethyl)-amiloride; EIAM, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; CaM, calmodulin; MLC, myosin light chain; ATP, adenosine-5′-triphosphate; cyclic AMP, adenosine-3′:5′-monophosphoric acid; ATPγS, adenosin-5′-*O*(3-thiotriphosphate); Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene-glycol-bis(beta-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTE, dithioerythritol; DTT, dithiothreitol; DFP, diisopropyl-fluorophosphate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide.

In smooth muscles, amiloride has been shown to inhibit the contraction by inhibiting the norepinephrine release from adrenergic nerve terminals [7, 8], inhibiting the Na^+–Ca^{2+} exchange mechanism [9] and inhibiting MLC kinase [10]. In the present study, we have examined the effects of amiloride, benzamil, DEAM and EIAM on the contractile systems of guinea-pig taenia caeci and chicken gizzard smooth muscles. The results show that the amiloride and its analogues inhibit MLC kinase and/or CaM.

MATERIALS AND METHODS

Intact taenia and gizzard. Guinea-pig taenia caeci and chicken gizzard were removed after the animals were stunned by a blow on the neck and bled. Smooth muscle tissues were cut into small pieces (2 mm width and 7 mm length). Physiological salt solution contained (mM); NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5 for taenia, and NaCl 118.9, KCl 4.5, $CaCl_2$ 2.5, $MgSO_4$ 2.4, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 11.9 for gizzard. High K^+ solution was made by replacing

NaCl with equimolar KCl. Na^+ free, high K^+ solution was made by replacing NaCl with equimolar tris(hydroxymethyl)aminomethane (Tris)–HCl or choline–Cl (with 2 μM atropine). Pyruvate (10 mM) was added to the above solution to avoid the possible substrate depletion through the inhibition of Na^+–glucose co-transport [11]. The solution was saturated with 95% O_2–5% CO_2 at 37° and pH 7.4.

Permealized tissue. Methods for chemical skinning of smooth muscle tissue have been described by Sparrow *et al.* [12]. A thin bundle of taenia or gizzard (0.2 mm in width and 1.5 mm in length) was prepared and soaked for 30 min in a solution containing 20 mM imidazole (pH 7.4), 5 mM ethyleneglycolbis-(beta-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), 50 mM KCl and 150 mM sucrose. Then, 1% (v/v) Triton X-100 and 0.5 mM dithioerythritol (DTE) were added to this solution and muscle was incubated for 0.5–4 hr at 4°. After rinsing for 15 min with the solution without Triton X-100, muscle was stored in 20 mM imidazole, 4 mM EGTA, 10 mM $MgCl_2$, 7.5 mM adenosine-5'-triphosphate (ATP), 1 mM NaN_3 and 0.5 mM DTE with 50% glycerol at −20° for up to 10 days. Relaxing solution contained 20 mM imidazole, 50 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 1 mM NaN_3, 0.1 μM CaM (isolated from bovine testis) and 2 mM EGTA at pH 6.8 and 23–24°. Ca^{2+} concentrations were changed by adding an appropriate amount of $CaCl_2$ to EGTA. The apparent binding constant of EGTA for Ca^{2+} was considered to be 10^{-6} M at pH 6.8 [13, 14].

Preparation of native actomyosin. Native actomyosin (myosin B), containing CaM, MLC kinase, phosphatase, tropomyosin, actin and myosin, was prepared from chicken gizzard as described previously [10, 15]. Muscle tissue (approximately 30 g) was homogenized and blended with four volumes of solution containing 0.4 M KCl, 20 mM Tris–HCl (pH 7.5), 10 mM ATP, 1 mM NaN_3, 0.5 mg/ml dithiothreitol (DTT) and 0.05 mM diisopropylfluorophosphate (DFP). The material was centrifuged at 10,000 *g* for 5 min. ATP (10 mM) was added to the supernatant and was recentrifuged at 80,000 *g* for 30 min. The supernatant fraction was then dialyzed against a nine-fold volume of solution containing 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 1 mM NaN_3, 0.5 mg/ml DTT and 0.05 mM DFP for 6–12 hr. Dialyzed material was centrifuged at 10,000 *g* for 5 min. Precipitated native actomyosin was washed two times with 50 mM KCl, 1 mM $MgCl_2$ and 1 mM $NaHCO_3$ and then resuspended in 1 mM $NaHCO_3$, 1 mM NaN_3 and 0.05 mM DFP.

Preparation of myosin and MLC kinase. Myosin was prepared from chicken gizzard by modifying the method of Ebashi [16]. MLC kinase was also prepared from chicken gizzard by modifying the method of Adelstein and Klee [17] as described by Nakamura and Nonomura [18]. The Ca^{2+}/CaM-independent MLC kinase was prepared by the partial proteolysis of MLC kinase [19]; MLC kinase was digested with TPCK–trypsin at the protein/enzyme ratio of 100/1 in 0.5 M NaCl, 1 mM EGTA and 20 mM Tris–HCl (pH 7.5) at 25° for 10 min. The digestion was terminated by the addition of 4-fold excess of trypsin inhibitor over TPCK–trypsin.

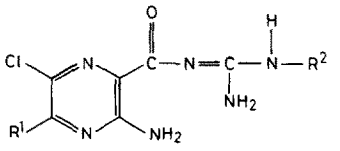
Phosphorylation of MLC. Native actomyosin (1 mg/ml) was incubated in 1 mM ATP, 50 mM KCl, 8 mM $MgCl_2$, 20 mM Tris–maleate (pH 6.8) and 2 mM EGTA–Ca^{2+} buffer in the presence of amiloride and its analogues at 25° for 30 sec. The reaction was stopped by the addition of solid urea in the final concentration of 8 M. MLC phosphorylation was analyzed by urea polyacrylamide gel electrophoresis (urea–PAGE) [20]. The patterns of protein band on the gel were visualized by means of silver-stain unless otherwise specified. The extent of the phosphorylation was measured by microdensitometer (Bio-Rad Laboratories, Richmond, CA).

Thiophosphorylation of MLC. Purified myosin (2.4 mg/ml) was thiophosphorylated with the trypsin-treated MLC kinase (0.1 mg/ml) in 70 mM NaCl, 20 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 0.1 mM EGTA and 1 mM adenosin-5'-*O*-(3-thiotriphosphate) ($ATP_\gamma S$) in the presence of 1 mM amiloride or its analogues at 25° for 10 min. Thiophosphorylation was analyzed with urea–PAGE as described above.

$(Ca^{2+} + Mg^{2+})$-ATPase activity. $(Ca^{2+} + Mg^{2+})$-ATPase activity of erythrocyte membranes was measured by modifying the methods of Gopinath and Vincenzi [21]. Rabbit blood anticoagulated by sodium citrate was washed three times with 170 mM Tris–HCl at pH 7.4. The buffy coat was carefully removed. Erythrocytes were lysed in 10 vol. of 17 mM Tris–HCl and 1 mM EGTA solution at pH 7.4. The ghosts were pelleted at 15,000 *g* for 40 min and washed three times with lysing buffer. The $(Ca^{2+} + Mg^{2+})$-ATPase assay was performed at 25° in a medium containing 0.5 mg/ml erythrocyte membranes, 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 20 mM Tris–maleate (pH 6.8), 0.1 mM ouabain, 60 nM CaM and 2 mM EGTA–Ca^{2+} buffer. The reaction was started by the addition of 2 mM ATP and terminated by the addition of trichloroacetic acid. The amount of inorganic phosphate liberated during a 30 min incubation was determined as described by Martin and Doty [22].

Phosphodiesterase activity. Phosphodiesterase activity was measured by modifying the methods of Toe *et al.* [23]. Calmodulin-free nucleotide phosphodiesterase (extracted from bovine heart) was purchased from Boehringer Mannheim (F.R.G.). Enzyme activity was measured in 20 mM Tris–maleate (pH 6.8), 10 mM $MgCl_2$, 20 mM imidazole, 0.05 U/ml phosphodiesterase, 20 nM CaM, 0.5 U/ml 5'-nucleotidase and 2 mM EGTA–Ca^{2+} buffer. The reaction was started by the addition of 2 mM adenosin-3':5'-monophosphoric acid (cyclic AMP) and terminated by the addition of trichloroacetic acid. The amount of inorganic phosphate liberated during a 30 min was determined by the Martin–Doty method.

Chemicals. Amiloride, benzamil, DEAM and EIAM were synthesized by the methods as described previously [24]. Chemical structures of these compounds are shown in Fig. 1. These compounds were dissolved in 100% dimethyl sulfoxide (DMSO) to make 10 mM stock solution. Final volume of DMSO in the test solutions was 0.1–1% for the experiments in intact tissue and 1% for the experiments in permealized tissue or biochemical studies and each concentration of DMSO was added to a vehicle control.



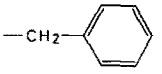
	R^1	R^2
Amiloride	H_2N—	—H
Benzamil	H_2N—	—CH_2— 
Diethyl-amiloride	$(C_2H_5)_2N$—	—H
Ethylisopropyl-amiloride	$(C_2H_5)(H)(H_3C)(CH_3)C$—N—	—H

Fig. 1. Chemical structures of amiloride and its analogues used in the present study.

Atropine and choline-Cl were purchased from Tokyo Kasei (Tokyo, Japan), EGTA from Dojindo Laboratories (Kumamoto, Japan), CaM from Pharmacia Japan (Tokyo, Japan), ATP, cyclic AMP, DFP, Triton X-100, Tris, 5'-nucleotidase, and trypsin inhibitor from Sigma Chemicals (St. Louis, MO), phosphodiesterase and $ATP_\gamma S$ from Boehringer Mannheim (FRG), imidazole and glycerol from Wako Pure Chemicals (Osaka, Japan), TPCK-trypsin from Worshinton Biochemicals (NJ) and DTT, DTE and pyruvate from Nakarai Chemicals (Kyoto, Japan).

RESULTS

Intact taenia and gizzard

A high K^+ (45.4 mM) solution induced a rapid and transient contraction followed by a slow and sustained contraction in the guinea-pig taenia caeci. Amiloride (100 μM), benzamil (30 μM), DEAM (30 μM) and EIAM (30 μM) inhibited both transient and sustained contractions to the same extent as shown in Fig. 2. The maximum effect was obtained

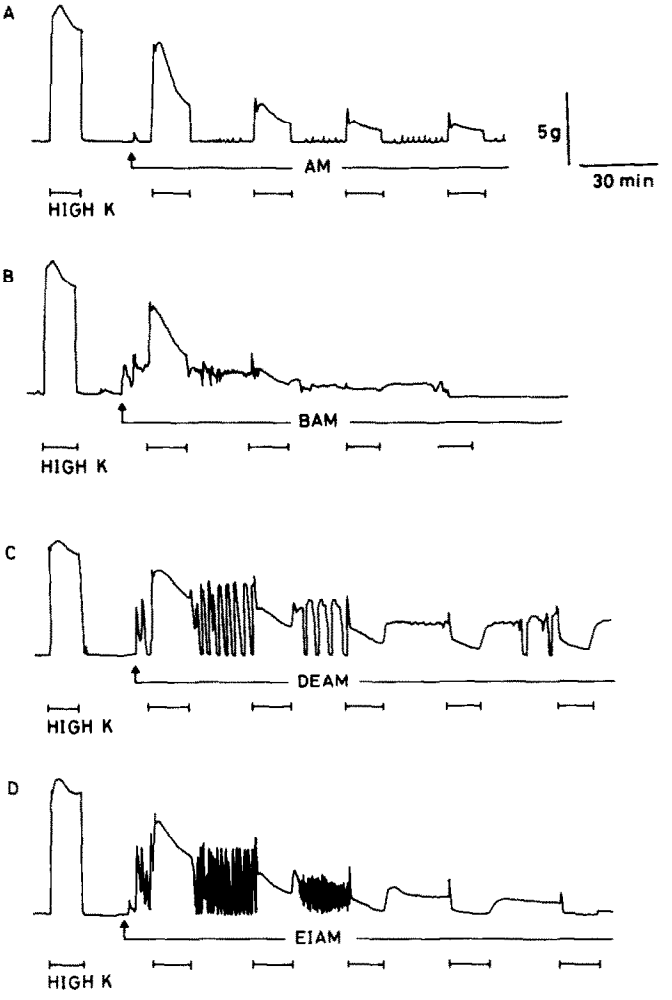


Fig. 2. Effect of amiloride (AM, 100 μM) (A), benzamil (BAM, 30 μM) (B), DEAM (30 μM) (C) and EIAM (30 μM) (D) on the contraction induced by high K^+ (45.4 mM) in guinea-pig taenia caeci. High K^+ was applied repeatedly in the presence of these compounds.

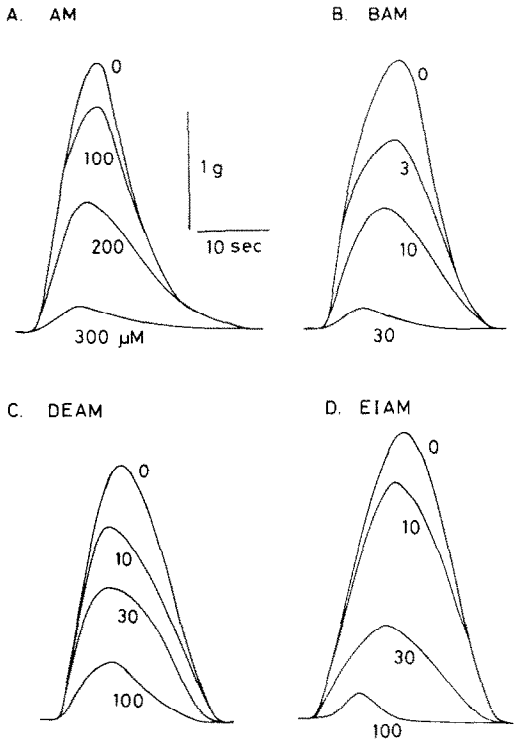


Fig. 3. Effect of amiloride (100–300 μM) (A), benzamil (3–30 μM) (B), DEAM (10–100 μM) (C) and EIAM (10–100 μM) (D) on the contraction induced by high K^+ (123.5 mM) in chicken gizzard smooth muscle strip. Compounds were added 60 min before the addition of high K^+.

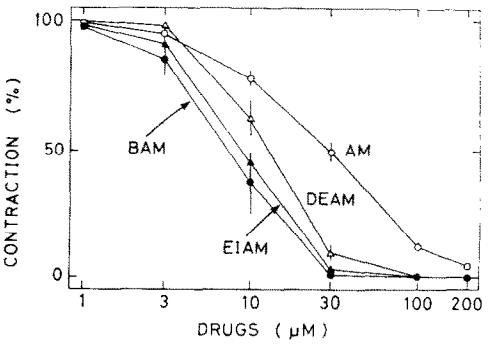


Fig. 4. Effect of amiloride (○), benzamil (●), DEAM (△) and EIAM (▲) on the high K^+-induced sustained contraction of taenia. High K^+ (45.4 mM) was added to induced sustained contraction. These compounds were cumulatively added during the sustained contraction induced by high K^+. Approximately 60 min was needed to obtain a steady inhibiting effect after the addition of the compounds. Mean values (±SE) for amiloride (N = 6), benzamil (N = 4), DEAM (N = 6) and EIAM (N = 4) are shown.

after about 60 min incubation. In chicken gizzard smooth muscle, high K^+ (123.5 mM) solution caused only a transient contraction which lasted about 20 sec. When the muscle was pretreated with amiloride (100–300 μM), benzamil (3–30 μM), DEAM (10–100 μM) or EIAM (10–100 μM) for 60 min, the high K^+-induced contraction was inhibited in a concentration-dependent manner (Fig. 3). Cumulative addition of these compounds during the high K^+-induced sustained contraction in the taenia inhibited the contraction. Concentration–inhibition curves are shown in Fig. 4 and concentrations for producing half-maximum inhibition (IC_{50}) are listed in Table 1.

After the taenia was treated with 45.4 mM K^+, external Na^+ (96.8 mM) was replaced with equimolar choline–Cl or Tris–HCl. The Na^+ removal decreased the tension by approximately 40%. Amiloride and its analogues, added after the Na^+ removal, decreased the remaining tension to the resting tension level and the IC_{50} values were similar to those in the presence of external Na^+ (Table 1). NH_4Cl (10–20 mM), added during the contraction induced by 45.4 mM K^+ solution with Na^+, decreased the sustained contraction by 30–40%. Amiloride and its analogues also decreased the contraction in the presence of NH_4Cl to the resting tension level.

As shown in Fig. 2, amiloride and its analogues increased the frequency of the spontaneous contractions in taenia. Similarly, the replacement of

Table 1. IC_{50} values of amiloride and its analogues on the K^+-induced contraction of intact taenia, Ca^{2+}-induced contraction of permealized taenia and phosphorylation of MLC of chicken gizzard native actomyosin and phosphodiesterase and erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase activities

	Amiloride	Benzamil	DEAM	EIAM
		(μM)		
Intact taenia				
High K^+ (45.4 mM) with 120.6 mM NaCl	29	7.2	12.8	8.8
High K^+ (45.4 mM) with 23.8 mM NaCl	35	5.7	6.1	6.4
Permealized taenia				
Ca^{2+} (10 μM)	240	115	430	310
Phosphorylation of MLC				
Ca^{2+} (10 μM)	280	120	680	530
(Ca^{2+} + Mg^{2+})-ATPase				
Ca^{2+} (10 μM)	≫1000	940	640	910
Phosphodiesterase				
Ca^{2+} (10 μM)	≫1000	440	400	240

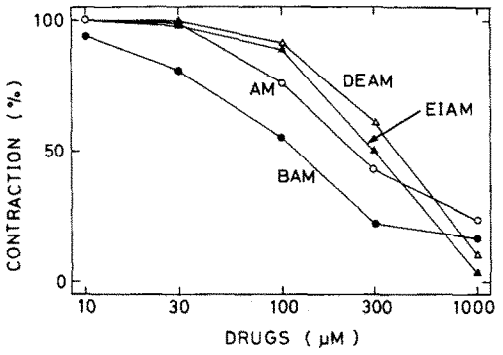


Fig. 5. Effect of amiloride (○), benzamil (●), DEAM (△) and EIAM (▲) on contraction of permealized taenia induced by 10 μM Ca^{2+}. Each concentration of compounds was cumulatively added after the tension reached the steady levels. Each point represents the mean of 4 experiments.

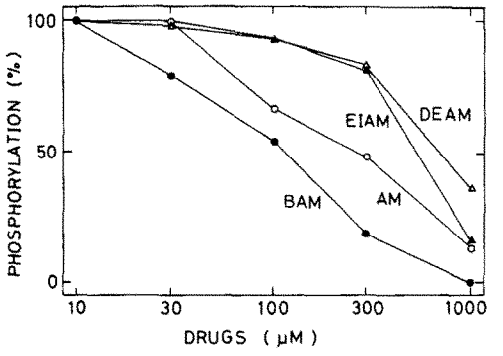


Fig. 7. Effect of amiloride (○), benzamil (●), DEAM (△) and EIAM (▲) on the phosphorylation of MLC in native actomyosin preparation. Reaction was started by application of 10 μM Ca^{2+} in the presence or absence of the compounds. The sample was collected 30 sec after the addition of Ca^{2+}.

external Na^+ with Tris or choline or the addition of NH_4Cl (10 mM) increased the spontaneous activity (data not shown).

Permealized taenia and gizzard

Ca^{2+} (10 μM) induced sustained contraction in the permeabilized taenia and gizzard. Cumulative addition of amiloride, benzamil, DEAM and EIAM inhibited this contraction. Concentration-inhibition curves obtained in taenia are shown in Fig. 5 and IC_{50} values are listed in Table 1. Concentrations needed to inhibit the contraction of permealized taenia were 8–35-fold greater than those obtained in the intact taenia.

Uptake of the compounds by taenia

Since amiloride, benzamil and EIAM are highly fluorescent, the uptakes of these compounds were determined with fluorophotometry. The taenia accumulated these compounds. The tissue/medium ratio measured 3 hr after the incubation was 3.5, 17.0 and 13.5 times for amiloride, benzamil and EIAM, respectively (Fig. 6).

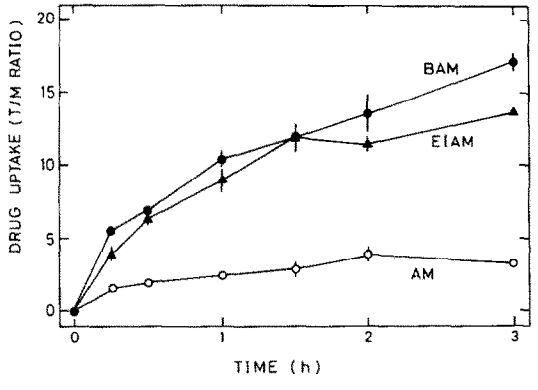


Fig. 6. Uptake of amiloride, benzamil and EIAM by taenia. Tissues were treated with a solution containing 30 μM amiloride (○), benzamil (●) or EIAM (▲) for 0–180 min. Tissues were then transferred to a 50% methanol solution for 48 hr to extract the compounds. Concentration of the compounds in the medium was measured fluorometrically. Values are expressed as tissue/medium ratio ± SE (N = 4 each).

MLC phosphorylation

Native actomyosin, which contained a CaM/MLC kinase system, was used to examine the effects of these compounds on MLC phosphorylation. In the absence of Ca^{2+}, almost none of the 20 Kdaltons MLC was phosphorylated. Addition of 10 μM Ca^{2+} in the presence of 1 mM ATP changed MLC to the phosphorylated form. Amiloride, benzamil, DEAM and EIAM inhibited the phosphorylation of MLC in a concentration-dependent manner (Fig. 7 and Table 1). Figure 8 shows the inhibitory effect of benzamil in the presence of different concentrations of ATP (0.1, 0.5, 1 and 5 mM). The inhibitory effect of benzamil was augmented by decreasing the ATP concentration from 1 to 0.5 or 0.1 mM, whereas the inhibitory effect was decreased by increasing the ATP concentration to 5 mM. These results were essentially similar to those reported with amiloride [10].

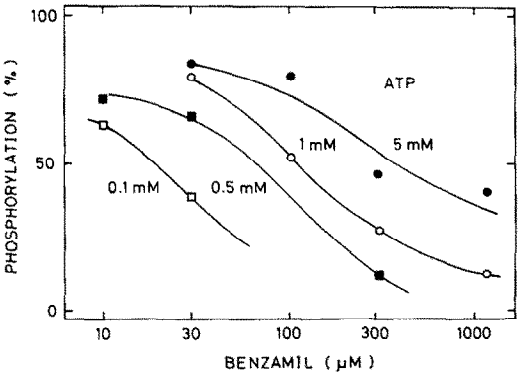


Fig. 8. Inhibitory effect of benzamil on the phosphorylation of MLC in the presence of 0.1 mM (□), 0.5 mM (■), 1 mM (○) and 5 mM (●) ATP. Experimental conditions were the same as described in Fig. 7.

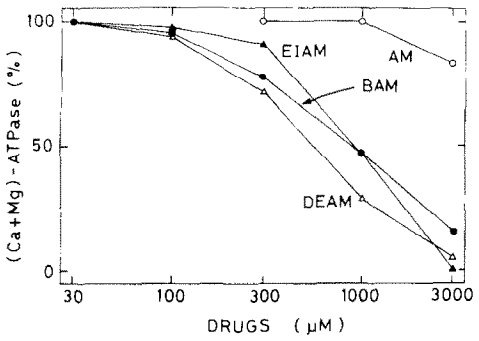


Fig. 9. Effect of amiloride (○), benzamil (●), DEAM (△) and EIAM (▲) on (Ca^{2+} + Mg^{2+})-ATPase activity. Reaction was started by adding 2 mM AMP in the presence or absence of the compounds. The (Ca^{2+} + Mg^{2+})-ATPase activity was increased by 11-fold by the addition of Ca^{2+} and CaM. The activity in the absence of Ca^{2+} and calmodulin was subtracted from the control. These compounds did not inhibit the ATPase activity in the absence of Ca^{2+}.

Effect on phosphatase activity

After MLC had been phosphorylated by adding 10 μM Ca^{2+}, subsequent removal of Ca^{2+} decreased the amount of phosphorylated MLC by endogenous phosphatase in a time-dependent manner. These compounds (1 mM) did not affect the decrease, suggesting that these compounds have no effect on phosphatase activity.

CaM activity

Effect of these compounds on CaM activity were examined using erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase activity and cyclic nucleotide phosphodiesterase activity. In the presence of 10 μM Ca^{2+}, these enzyme activities increased by about 11 times. DEAM and EIAM inhibited the (Ca^{2+} + Mg^{2+})-ATPase and phosphodiesterase activities at similar concentrations to inhibit the contraction of permealized tissue or MLC phosphorylation. Benzamil also inhibited (Ca^{2+} + Mg^{2+})-ATPase and phosphodiesterase activities although its IC_{50} values were four to eight times greater than those for permealized tissue or MLC phosphorylation. In contrast, amiloride (<1 mM) failed to inhibit the (Ca^{2+} + Mg^{2+})-ATPase and phosphodiesterase activities. Benzamil and EIAM (0.1–1 mM) did not inhibit erythrocyte membrane ATPase and phosphodiesterase activities in the absence of Ca^{2+} (in the presence of 2 mM EGTA). Concentration–inhibition curves for Ca^{2+}/CaM-dependent enzyme activities are shown in Fig. 9 and IC_{50} values are listed in Table 1.

Assay with purified myosin and MLC kinase

To examine the direct effect of amiloride and its analogues on MLC kinase, thiophosphorylation with Ca^{2+}/CaM-independent MLC kinase was adopted in place of phosphorylation avoiding the possible complication due to phosphatase activity in the myosin preparation. As shown in Fig. 10, trypsin-treated MLC kinase phosphorylated MLC in the absence of Ca^{2+}/CaM. Amiloride and benzamil at 1 mM were effective in inhibiting the Ca^{2+}/CaM-independent MLC kinase activity. However, DEAM and EIAM were ineffective.

DISCUSSION

Amiloride, benzamil, DEAM and EIAM inhibited the contraction of intact guinea-pig taenia and chicken gizzard. In the taenia, high K^+ (45.4 mM) solution caused a phasic contraction followed by a sustained contraction. These contractions are due to the release of stored Ca^{2+} and to Ca^{2+} influx through Ca^{2+} channels, respectively [25, 26]. Amiloride and its analogues non-selectively inhibited these contractions. Similarly, these compounds inhibited the transient contraction in chicken gizzard elicited by high K^+ (123.5 mM) solution.

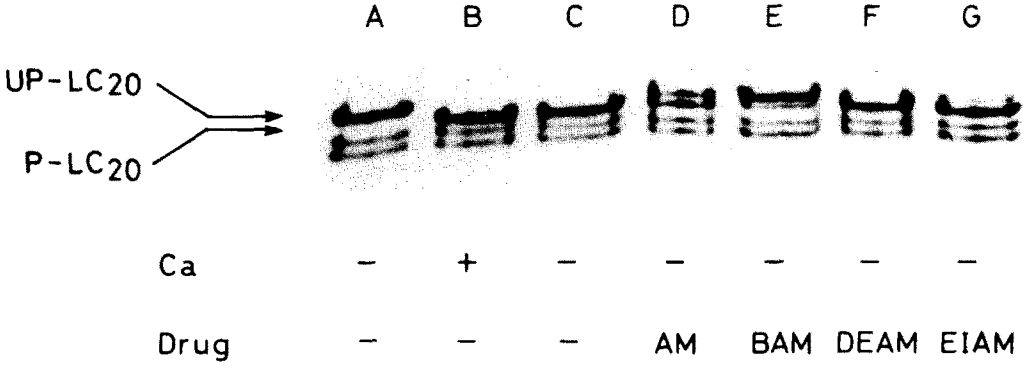


Fig. 10. Thiophosphorylation of myosin by MLC kinase. Myosin was thiophosphorylated by MLC kinase (0.1 mg/ml)–CaM (0.1 mg/ml) system (A, B) or by Ca^{2+}/CaM-independent MLC kinase (0.1 mg/ml) (C–G) and subjected to urea–PAGE followed by Coomassie Brilliant Blue staining. Conditions for thiophosphorylation: A, 0.1 mM EGTA; B, 0.1 mM Ca^{2+}; C, 0.1 mM EGTA; D, 0.1 mM EGTA + 1 mM amiloride; E, 0.1 mM EGTA + 1 mM benzamil; F, 0.1 mM EGTA + 1 mM DEAM; G, 0.1 mM EGTA + 1 mM EIAM.

Amiloride and its analogues may decrease intracellular pH by inhibiting Na^+–H^+ exchange and this may be the mechanism of action of these compounds. To evaluate this possibility, Na^+–H^+ exchange was inhibited by replacing external Na^+ with Tris or choline and it was found that the high K^+-induced contraction was inhibited by 30–40%. NH_4Cl has been shown to decrease intracellular pH in various tissues including smooth muscle [27]. NH_4Cl (10 mM) also inhibited the high K^+-induced contraction by approximately 40%. These results suggest that the possible decrease in intracellular pH induced by amiloride and its analogues is at least partly responsible for their inhibitory effect. As demonstrated in Fig. 2, amiloride analogues increased the spontaneous activity in taenia. The removal of external Na^+ or the addition of NH_4Cl showed similar effects. These observations suggest that, beside the relaxant effect, the decrease in intracellular pH may stimulate the spontaneous spike discharge in the taenia.

Amiloride and its analogues, however, inhibited the contraction in the muscle in which the Na^+–H^+ exchange was previously inhibited by Na^+ removal. Further, the IC_{50} values in the Na^+-deficient solution are almost the same as those obtained in the presence of external Na^+. Since these compounds inhibited the Ca^{2+}-induced contraction of permealized taenia and gizzard, these compounds may have an additional effect to directly inhibit the contractile elements.

Smooth muscle contraction has been shown to be due to the Ca^{2+}/CaM-dependent phosphorylation of MLC [28, 29]. Amiloride and its analogues inhibited the MLC phosphorylation in the native actomyosin preparation. The inhibition of MLC phosphorylation correlated with the inhibition of the contraction in permealized muscles. DEAM and EIAM inhibited CaM activity at the same concentration ranges as those that inhibit MLC phosphorylation. Benzamil also inhibited CaM activity, although the IC_{50} was four to eight times greater than that to inhibit MLC phosphorylation. In contrast to these analogues, amiloride (<1 mM) did not inhibit the CaM activity. These results suggest that amiloride may inhibit MLC kinase, DEAM and EIAM may inhibit CaM and benzamil may inhibit both MLC kinase and CaM. With a reconstituted system containing purified myosin and Ca^{2+}/CaM-independent MLC kinase, amiloride and benzamil were effective in inhibiting the MLC kinase activity, whereas DEAM and EIAM were ineffective. These data support the idea that the site of action of amiloride and benzamil is MLC kinase, whereas that of DEAM and EIAM is CaM.

The order of potency to inhibit the sustained contraction of intact taenia was benzamil ≥ EIAM > DEAM > amiloride, which was not consistent with the results obtained from the permealized muscles. Further, the effective concentration ranges of these inhibitors in permealized tissue are approximately 10 times greater than those in intact tissue. In our studies, intact taenia accumulated benzamil and EIAM and the tissue/medium ratio reached 17.0 and 13.5, respectively, after a 3 hr incubation period. Considering the extracellular space (30–40%) and the solid space (15–20%) of taenia, these compounds seem to be concentrated in cell water to the level 27–34 times higher than that in the medium. Amiloride also seems to be concentrated by about sevenfold. The difference in the order of sensitivity between intact and permealized tissues to amiloride and the analogues may partially be explained by the differences in drug accumulation in the cell which may depend on the lipid solubility of the agents (the analogues are more hydrophobic than amiloride). It has been reported that, when whole cell or intact tissue is incubated with a micromolar order of radiolabeled amiloride, it accumulates amiloride to an intracellular concentration of a millimolar order [30, 31].

It has been reported that amiloride inhibits protein kinase activities, such as cyclic AMP- and cyclic GMP-dependent kinases and tyrosine kinase [31, 32]. The present study indicated that the inhibitory effect of benzamil on MLC phosphorylation was competitively antagonized by ATP. A similar result has been reported with amiloride [10]. The nonselective inhibitory effect of this compound on protein kinases can be explained by the direct competition with ATP. Amino-group at C_5 position (R^1 in Fig. 1) seems to be essential for the inhibition of MLC kinase.

In summary, the present study demonstrated a correlation between the inhibition of contraction and MLC phosphorylation for four different amiloride derivatives. The inhibition by amiloride may be attributable to the inhibition of MLC kinase and that by DEAM and EIAM may be attributable to CaM. Benzamil may inhibit the contraction through the inhibition of both MLC kinase and CaM.

Acknowledgements—This work was supported by Research Grant from Ministry of Education, Science and Culture, Japan, the Naito Foundation, Daiwa Health Foundation, and Mochida Memorial Foundation for Medical and Pharmaceutical Research.

REFERENCES

1. Benos DJ, Amiloride: a molecular probe of sodium transport in tissue and cells. *Am J Physiol* **242**: C131–C145, 1982.
2. Vigne P, Frelin C, Cragoe Jr EJ and Lazdunski M, Structure–activity relationships of amiloride and certain of its analogues in relation to the blockade of the Na^+/H^+ exchange system. *Mol Pharmacol* **25**: 131–136, 1984.
3. Frelin C, Vigne P and Lazdunski M, The role of the Na^+/H^+ exchange system in cardiac cells in relation to the control of the internal Na^+ concentration. *J Biol Chem* **259**: 8880–8885, 1984.
4. Besterman JM, Statford-May Jr W, LeVine III H, Cragoe Jr EJ and Cuatrecasa P, Amiloride inhibits phorbol ester-stimulated Na^+/H^+ exchange and protein kinase C. *J Biol Chem* **260**: 1155–1159, 1985.
5. Siegel PKS, Cragoe Jr EJ, Trumble MJ and Kaczorowski GJ, Inhibition of Na^+/Ca^{2+} exchange in membrane vesicle and papillary muscle preparations from guinea pig heart by analogues of amiloride. *Proc Natl Acad Sci U.S.A.* **81**: 3238–3242, 1984.
6. Schellenberg GD, Anderson L, Cragoe Jr EJ and Swanson PD, Inhibition of synaptosomal membrane Na^+–Ca^{2+} exchange transport by amiloride and amiloride analogues. *Mol Pharmacol* **27**: 537–543, 1985.

7. Akhtar-Khavari F, Khoyi MA and Rezaei E, Effects of amiloride on contraction and the release of tritium from rat vas deferens preloaded with [^{3}H]noradrenaline. *Br J Pharmacol* **74**: 123–127, 1981.
8. Palaty V, Amiloride acts as an *alpha*-adrenergic antagonist in the isolated rat tail artery. *Can J Physiol Pharmacol* **64**: 931–933, 1985.
9. Moreland RS, Major TC and Webb C, Contractile response to ouabain and K^+-free solution in aorta from hypertensive rats. *Am J Physiol* **250**: H612–619, 1986.
10. Ozaki H, Kojima T, Moriyama T, Karaki H, Urakawa N, Kohama K and Nonomura Y, Inhibition by amiloride of contractile elements in smooth muscle of guinea-pig taenia caecum and chicken gizzard. *J Pharmacol Exp Ther* **243**: 370–377, 1987.
11. Suzuki T, Karaki H and Urakawa N, Mechanism of inhibition of contraction by high K, Na deficient solution in smooth muscle of guinea-pig taenia coli. *Arch int Pharmacodyn Ther* **248**: 43–49, 1980.
12. Sparrow MP, Mrwa U, Hofmann F and Ruegg JC, Calmodulin is essential for smooth muscle contraction. *FEBS Lett* **125**: 141–145, 1981.
13. Saida K and Nonomura Y, Characteristics of Ca^{2+}- and Mg^{2+}-induced tension development in chemically skinned smooth muscle fibers. *J Gen Physiol* **72**: 1–14, 1978.
14. Harafuji H and Ogawa Y, Re-examination of the apparent binding constant of ethylene glycol bis(beta-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid with calcium around neutral pH. *J Biochem (Tokyo)* **87**: 1305–1312, 1980.
15. Ozaki H, Ishihara H, Kohama K, Nonomura Y, Shibata S and Karaki H, Calcium-independent phosphorylation of smooth muscle myosin light chain by okadaic acid isolated from black sponge (*Halichondria okadai*). *J Pharmacol Exp Ther* **243**: 1167–1173, 1987.
16. Ebashi S, Calcium binding activity of vesicular relaxing factor. *J Biochem (Tokyo)* **50**: 236–244, 1961.
17. Adelstein RS and Klee CB, Purification and characterization of smooth muscle myosin light chain kinase. *J Biol Chem* **256**: 7501–7509, 1981.
18. Nakamura S and Nonomura Y, A simple and rapid method to remove light chain phosphatase from chicken gizzard myosin. *J Biochem (Tokyo)* **96**: 575–578, 1984.
19. Walsh MP, Bridenbaug R, Kerrick WGL and Hartshorne DJ, Gizzard Ca^{2+}-independent myosin light chain kinase: evidence in favor of the phosphorylation theory. *Fed Proc* **42**: 45–50, 1983.
20. Pires E, Perry SV and Thomas MAW, Myosin light chain kinase, a new enzyme from striated muscle. *FEBS Lett* **41**: 292–296, 1974.
21. Gopinath RM and Vincenzi FF, Phosphodiesterase protein activator mimics red blood cell cytoplasmic activator of (Ca^{2+} + Mg^{2+})ATPase. *Biochem Biophys Res Commun* **77**: 1203–1209, 1977.
22. Martin JB and Doty DM, Determination of inorganic phosphate, modification of isobutyl alcohol procedure. *Anal Chem* **21**: 965–967, 1949.
23. Teo TS, Wang TH and Wang JH, Purification and properties of the protein activator of bovine heart cyclic adenosine 3′,5′-monophosphate phosphodiesterase. *J Biol Chem* **248**: 588–595, 1973.
24. Cragoe Jr EJ, Woltersdorf Jr OW, Bicking JB, Kwong SF and Jones JH, Pyrazine diuretics. II. *N*-Amidino-3-amino-5-substituted-6-halopyrazinecarboxamides. *J Med Chem* **10**: 66–75, 1967.
25. Urakawa N and Holland WC, Ca^{45} uptake and tissue calcium in K-induced phasic and tonic contraction in taenia coli. *Am J Physiol* **207**: 873–876, 1964.
26. Karaki H and Weiss GB, Calcium release in smooth muscle. *Life Science* **42**: 111–122, 1988.
27. Karaki H and Weiss GB, Effect of transmembrane pH gradient changes on potassium-induced relaxation in vascular smooth muscle. *Blood Vessels* **18**: 36–44, 1981.
28. Adelstein RS and Eisenberg E, Regulation and kinetics of the actin myosin interaction. *Ann Rev Biochem* **49**: 921–956, 1980.
29. Hartshorne D J and Mrwa U, Regulation of smooth muscle actomyosin. *Blood Vessels* **19**: 1–18, 1982.
30. Smith RL, Macara IG, Levenson R, Housman D and Cantley L, Evidence that a Na^+/Ca^{2+} antiport system regulates murine erythroleukemia cell differentiation. *J Biol Chem* **257**: 773–780, 1982.
31. Davis RL and Czeck MP, Amiloride directly inhibits growth factor receptor tyrosine kinase. *J Biol Chem* **260**: 2543–2551, 1985.
32. Ralph RK, Smart J, Wojcik SJ and MaQuillan J, Inhibition of mouse mastcytoma protein kinases by amiloride. *Biochem Biophys Res Commun* **104**: 1054–1059, 1982.