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COMPETITIVE INHIBITION OF TRITIUM-LABELED PLATELET-ACTIVATING FACTOR BINDING TO RABBIT PLATELET MEMBRANES BY AMILORIDE AND AMILORIDE ANALOGS

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Amiloride and its structural analogs, ethylisopropyl amiloride, benzamil, and dichlorobenzamil, inhibit both the specific [^{3}H]C_{18}-PAF binding to rabbit platelet membranes and PAF-induced aggregation of gel-filtered rabbit platelets. Detailed analysis of binding inhibitions demonstrate that ethylisopropyl amiloride is a competitive inhibitor with an equilibrium dissociation constant (K_B) of 23 μM. The concentration of amiloride and its analogs needed to inhibit the PAF-induced aggregation is high and there exists no correlation between their inhibitory activities of platelet aggregation and those of Na^+/H^+ antiporter. However, the inhibitory effects on the PAF-induced aggregation are parallel to those on the specific [^{3}H]C_{18}-PAF binding. The inhibitory effects of amiloride and its analogs on the activation of platelets are at the PAF-receptor binding step, rather than at the Na^+/H^+ antiporter.

Platelet-activating factor (PAF), 1-O-alkyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine (1,2), is a lipid mediator produced by various types of cells including neutrophils, eosinophils, mast cells, and vascular endothelial cells upon chemical and immune stimulation (3). PAF has a wide spectrum of biological activity (3). The action of its biological effects in several defined systems is due to the interaction of PAF with its specific receptors and a guanine nucleotide-regulatory protein seems to be involved in the signal transduction process (4,5). However, the information on the effector systems is still missing.

Involvement of Na^+/H^+ antiporter has been recently reported in activation of platelets by epinephrine (6,7), PAF (8) and other stimuli (7,9-11) and the activation of sodium-proton exchange has been proposed to be a prerequisite for Ca^{2+} mobilization in human platelets (11,12). Here, we examined the inhibitory effects of PAF-induced rabbit platelet aggregation by amiloride and several selected amiloride analogs. The concentration of amiloride or its analogs required to inhibit the PAF-induced rabbit platelet aggregation is about 100 times higher than that to inhibit the Na^+/H^+ antiporter in the well-defined systems (13). Also, there exists no correlation on the inhibitions of PAF-induced platelet aggregation and their Na^+/H^+ antiporter activities, but the inhibitory effects on the PAF-induced

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aggregation are parallel to those on the specific [^{3}H]PAF binding to isolated rabbit platelet membranes. Therefore, the inhibitory effects of amiloride and its analogs on PAF-induced rabbit platelet aggregation seems to be not related to their activities of Na^+/H^+ antiporter but at the PAF-receptor binding step.

MATERIALS AND METHODS

Materials

The tritium-labeled 1-O-octadecyl (9,10-^{3}H)-2-O-acetyl-sn-glyceryl-3-phosphorylcholine ([^{3}H]C_{18}-PAF) was purchased from New England Nuclear (Boston, MA) with a specific activity of 126.9 Ci/mmol. The unlabeled 1-O-hexadecyl-2-O-acetyl-sn-glyceryl-3-phosphorylcholine (C_{16}-PAF) was purchased from BACHEM (Torrance, CA), and used without further purification. Amiloride, ethylisopropyl amiloride, benzamil, and dichlorobenzamil were synthesized at Merck Sharp & Dohme Research Laboratories (West Point, PA).

Methods

Preparations of Rabbit Platelet Membranes - Rabbit platelet membranes were prepared as previously described (4,14). Membrane fraction B contains more receptor sites than membrane fraction A (14). Membrane fraction B was the major portion of the membrane preparation and also no detectable differences were shown in the PAF receptor between membrane fraction A and B in terms of the equilibrium dissociation constant (K_D) of the ^{3}H-labeled PAF or PAF receptor antagonists or the equilibrium inhibition constant (K_I) of several selected antagonists in inhibiting the [^{3}H] PAF binding to the prepared membranes (15). Membrane fraction B was therefore used throughout the experiments.

Inhibition of [^{3}H]C_{18}-PAF Binding - Inhibition of [^{3}H]C_{18}-PAF binding to rabbit platelet membranes by amiloride or amiloride analogs was performed as previously described (15) except 0.3 nM final concentration of [^{3}H]C_{18}-PAF was used. To determine the competitive inhibition of ethylisopropyl amiloride, 0.05 to 10 nM [^{3}H]-C_{18}-PAF was used to perform the saturation binding studies either with or without the addition of ethylisopropyl amiloride. The nonspecific binding for each concentration of [^{3}H]C_{18}-PAF was determined in the presence of excess (1000-fold) unlabeled C_{16}-PAF. The apparent dissociation constant (K_D', in the presence of ethylisopropyl amiloride), the equilibrium dissociation constant (K_D, in the absence of inhibitor) and the maximal detectable receptor number (B_{max}) were calculated by using programs of EBDA and LIGAND from Elsevier-Biosoft, Cambridge, UK on an IBM-AT computer. The Schild plot was obtained as previously described (16).

Preparation of Gel-Filtered Rabbit Platelets and Monitoring of Rabbit Platelet Aggregation - Gel-filtered rabbit platelets were prepared as described previously (17). Aggregation of rabbit platelets was monitored with a dual aggregometer (Chrono-Log Corp.) as reported before (17).

RESULTS

Inhibition of [^{3}H]C_{18}-PAF Binding to Rabbit Platelet Membranes - Fig. 1 shows the normalized inhibition of [^{3}H]C_{18}-PAF (0.3 nM) receptor binding in rabbit platelet membranes by amiloride, ethylisopropyl amiloride, benzamil and dichlorobenzamil. Ethylisopropyl amiloride was the most potent one with an IC_{50} value of 20 uM, which is about 100X more potent than amiloride itself.

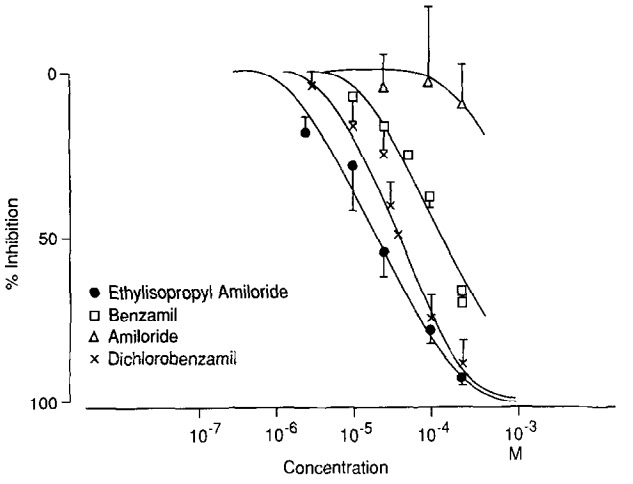

Figure 1. Inhibition of specific [^{3}H]C_{18}-PAF binding to rabbit platelet membranes. [^{3}H]C_{18}-PAF (0.3 nM) was incubated in a rabbit platelet membrane (100 ug protein) suspension in the presence of increasing concentrations of amiloride or its structural analogs. The assay medium was 10 mM $MgCl_2$, 10 mM Tris and 0.25% BSA at pH 7.0. The data point and error bar are the mean and standard deviation of three separate competition assays. Triplicate determinations were performed in each assay.

Dichlorobenzamil, a blocker for Na^+/Ca^{2+} exchanger, and benzamil, a Na^+ channel blocker, which show activities on the inhibition of Na^+/H^+ antiporter far less than amiloride, showed inhibitory activities in between with IC_{50} of 40 and 145 uM respectively.

Competitive Inhibition of [^{3}H]C_{18}-PAF Binding to Rabbit Platelet Membranes by Ethylisopropyl Amiloride - As demonstrated in Fig. 2, ethylisopropyl amiloride was found to be a competitive receptor antagonist. It altered the apparent dissociation constant (K_D') of [^{3}H]C_{18}-PAF binding to its receptor in rabbit platelet membranes, but not the maximal number of detectable receptor sites (Fig. 2 and Table I). Here, the K_D value of [^{3}H]C_{18}-PAF binding to rabbit platelet membranes is 1.99 $\pm$ 0.08 nM, which is roughly the same as the equilibrium inhibition constant (K_I) we reported for the unlabeled C_{18}-PAF (K_I = 1.06 ($\pm$0.14) nM) (15). In the Schild plot, by plotting logarithm of [(K_D'/K_D)-1] vs logarithm of the concentration of ethylisopropyl amiloride, a unit slope was obtained (slope = 1.06) (Fig. 2B). The equilibrium dissociation constant (K_B) of ethylisopropyl amiloride to the PAF receptor site can thus be obtained indirectly from the concentration of ethylisopropyl amiloride to inhibit the [^{3}H]C_{18}-PAF binding to have [(K_D'/K_D)-1] equal to one and is 23 uM (Fig. 2B). Ethylisopropyl amiloride is therefore a PAF competitive receptor antagonist.

Inhibition of PAF-Induced Aggregation of Gel-Filtered Rabbit Platelets - PAF potently induced aggregation of gel-filtered rabbit platelets with ED_{50}

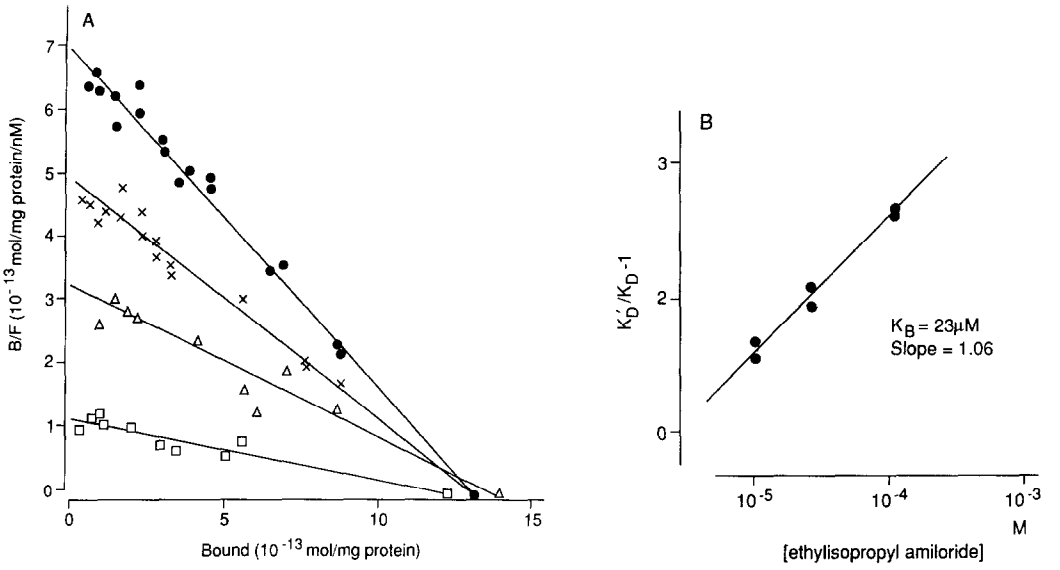

Figure 2A. Scatchard plots of [^{3}H]C_{18}-PAF binding to rabbit platelet membranes without (●) and with ethylisopropyl amiloride at a concentration of 10 (X), 25 (△), and 100 uM (□). The assay medium was 10 mM $MgCl_2$, 10 mM Tris and 0.25% BSA at pH 7.0. Straight lines were drawn following the linear regression using programs of EBDA and LIGAND on an IBM-AT computer. The data points are the mean of the triplicate determinations.

Figure 2B. Schild analysis by plotting log (K_D'/K_D/1) vs. logarithm of the concentration of ethylisopropyl amiloride. Straight line and slope were drawn and calculated following the linear regression with a hand calculator. K_B = 23 uM, slope - 1.06.

values close to 1 x 10^{-10} M (17). Amiloride and its analogs inhibited the PAF-induced aggregation (Fig. 3). The ED_{50} values for amiloride and ethylisopropyl amiloride to inhibit aggregation induced by PAF at 3.6 x 10^{-10} M are 0.4 nM and 20 uM respectively. Benzamil, a Na^+ channel blocker, and dichlorobenzamil, a blocker of Na^+/Ca^{2+} antiporter, also inhibited PAF-induced rabbit platelet aggregation with ED_{50} of 50 uM and 10 uM respectively. There

TABLE 1

Effects of Ethylisopropyl Amiloride on the Apparent Dissociation Constant (K_D') and the Maximal Number of Receptor Sites (B_{max}) in [^{3}H]C_{18}-PAF Receptor Binding to Rabbit Platelet Membranes

Ethylisopropyl Amiloride conc.	K_D	B_{max}
uM	nM	10^{-12} mol/mg protein
0 (n=3)	1.99 ± 0.088	1.351 ± 0.031
10 (n=2)	2.81 ± 0.043	1.333 ± 0.031
25 (n=2)	4.21 ± 0.62	1.395 ± 0.015
100 (n=2)	11.15 ± 0.42	1.304 ± 0.114

Values are mean ± S.D.

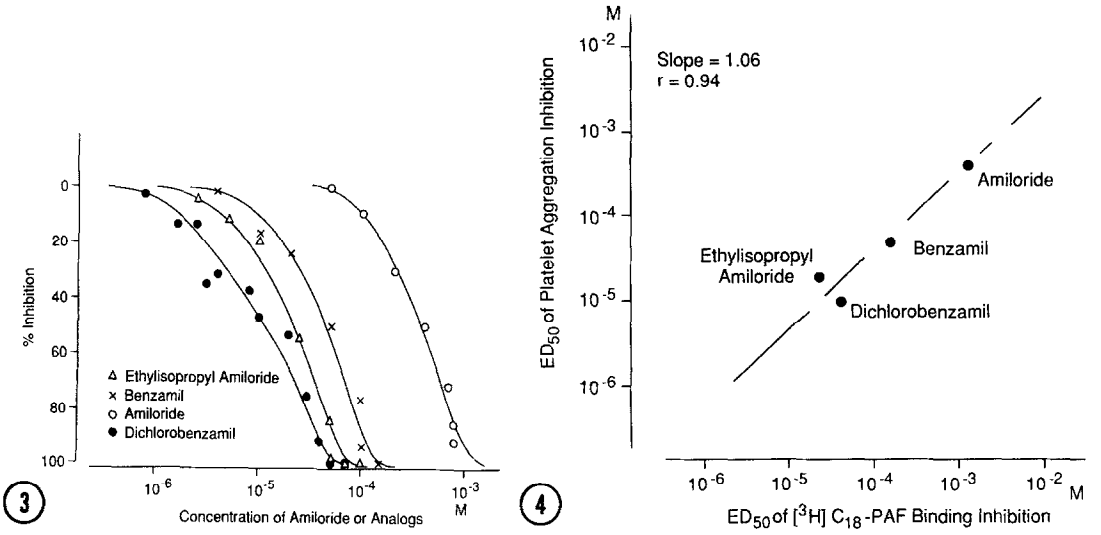

Figure 3. Inhibition of PAF-induced aggregation of gel-filtered rabbit platelets by amiloride or analogs. 3.6×10^{-10} M PAF was used to induced platelet aggregation. The percent inhibition was expressed as the ratio of platelet aggregation induced by PAF in the presence of amiloride (○), ethylisopropyl amiloride (△), benzamil (X), or dichlorobenzamil (●) to that from the control experiments.

Figure 4. Plot of ED_{50} values of $[^3H]C_{18}$-PAF binding displacement curves (from Fig. 1) against those of inhibition of PAF-induced rabbit platelet aggregation (from Fig. 3). The straight line and slope were drawn and calculated following the linear regression with a hand calculator. Slope = 1.06, correlation coefficient = 0.94.

exists a linear correlation between the potencies of these compounds in displacing $[^3H]C_{18}$-PAF from its binding sites and in inhibiting PAF-induced rabbit platelet aggregation (Fig. 4) with a strong correlation (γ=0.94; slope = 1.06).

DISCUSSION

Here, we have demonstrated that amiloride and its structural analogs, ethylisopropyl amiloride, benzamil, dichlorobenzamil inhibit both the specific $[^3H]$PAF binding to isolated rabbit platelet membranes and the aggregation of rabbit platelets induced by PAF. Detailed analysis of binding inhibition of $[^3H]C_{18}$-PAF to rabbit platelet membranes demonstrate that ethylisopropyl amiloride is a competitive inhibitor with a K_B value of 23 uM. The potencies of these compounds in competing $[^3H]C_{18}$-PAF binding from its binding correlate very well with those in inhibiting PAF-induced rabbit platelet aggregation. These results suggest that inhibitory effects on PAF-induced rabbit platelet aggregation by amiloride and/or its analogs is at the PAF-receptor binding step.

Sweatt et al (8) have reported that the addition of ethylisopropyl amiloride (40 uM, Fig. 1 of ref. 8) leads to an inhibition of PAF-induced serotonin release and thromboxane B_2 production in human platelets and thus concluded that the Na^+-H^+ exchanger plays an important role in activation of human platelets by PAF. However, the concentration of ethylisopropyl amiloride used in their studies (8) are very high. Also, as demonstrated here, the ED_{50} values for amiloride and ethylisopropyl amiloride to inhibit aggregation by PAF are 0.4 mM and 20 uM, which are about 100 times higher than the concentration needed to inhibit Na^+/H^+ antiport of MDCK cells (13). Benzamil and dichlorobenzamil, which show no activities on the Na^+-H^+ antiporter, also show activities in inhibiting the PAF-induced rabbit platelet aggregation. These results suggest that activation of platelets by PAF may not be related to the activation of the Na^+/H^+ antiporter. Also, benzamil inhibits PAF-induced rabbit platelet aggregation with an ED_{50} of 50 uM, which is again about 100 times higher concentration than that required to block the Na^+ channel from pig kidney outer cortex membrane vesicles (13). The activation of platelets by PAF may not be involved in the benzamil-sensitive Na^+ channel. However, the potency of dichlorobenzamil, a blocker of Na^+/Ca^{2+} antiporter, in inhibiting the PAF-induced rabbit platelet aggregation is higher than that of ethylisopropyl amiloride, whereas, ethylisopropyl amiloride is more potent than dichlorobenzamil in inhibiting the specific $[^3H]C_{18}$-PAF binding to rabbit platelet membranes. These results suggest that Na^+/Ca^{2+} exchanger may possibly be involved in the activation of platelets induced by PAF.

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