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5-HPETE Is a Potent Inhibitor of Neuronal Na^+, K^+-ATPase Activity

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The effects of 1 uM concentrations of arachidonic acid hydroperoxide (HPETES) products of 5-, 12- and 15-lipoxygenase on Na^+, K^+-ATPase activity were investigated in synaptosomal membrane preparations from rat cerebral cortex. 5-HPETE inhibited Na^+, K^+-ATPase activity by up to 67 %. In contrast, 12-HPETE and 15-HPETE did not inhibit Na^+, K^+-ATPase activity. In addition, neither 5-HETE or LTA_4 inhibited Na^+, K^+-ATPase activity. Dose-response studies indicated that 5-HPETE was a potent ($IC_{25} = 10^{-8}$ M) inhibitor of Na^+, K^+-ATPase activity. These findings indicate that 5-HPETE inhibits Na^+, K^+-ATPase activity by a mechanism that is dependent on the hydroperoxide position and independent of further metabolism by 5-lipoxygenase. It is proposed that 5-HPETE production by 5-lipoxygenase and subsequent inhibition of neuronal Na^+, K^+-ATPase activity may be a mechansim for modulating synaptic transmission.

Lipoxygenases are dioxygenases that catalyze a stereo- (S-configuration) and position selective oxidation of arachidonic (eicosatetraenoic) acid to hydroperoxyeicosatetraenoic acids (HPETES) (see reference 1 for reveiw). Lipoxygenases that produce hydroperoxides predominantly at the 5, 8, 12 and 15 positions of arachidonic acid have been identified (2). In addition to the oxidation reactions, lipoxygenases may also catalyze the further metabolism of HPETES to hydroxy (HETES) and epoxy (leukotriene A_4) acids (1). Despite an understanding of the lipoxygenase reaction mechanisms (1), knowledge of the biological function of lipoxygenase products is limited (see reference 2 for review).

Na^+, K^+-ATPase is the plasma membrane cation pump that generates the Na^+ and K^+ gradients required for the general ion and metabolite homeostasis of all mammalian cells (see references 3 and 4 for reviews). In neurons, Na^+, K^+-ATPase activity is important for the maintenance of excitability and regulates synaptic transmission by providing the Na^+-electrochemical gradient that drives the uptake of neurotransmitters from the synapse and the extrusion of intraneuronal Ca^{2+} (5). Na^+, K^+-ATPase activity has been reported to be inhibited by free fatty acids (6-8) as well as oxidized metabolites of arachidonic acid produced by cyclooxygenase (9-11) and P450-monooxygenase (11,12). However, the effect of lipoxygenase products on Na^+, K^+-ATPase activity is largely unknown. The present study has investigated the effect of arachidonic acid metabolites of 5-, 12-, and 15-lipoxygenase on Na^+, K^+-ATPase activity in synaptosomal (nerve-ending) plasma membranes from rat cerebral cortex.

EXPERIMENTAL PROCEDURES

Preparation of synaptosomal membranes. Male Sprague-Dawley rats (12-14 weeks old) (Taconic Farms, Germantown, NY) were sacrificed by decapitation. Cerebral cortices were rapidly dissected on ice followed by homogenization in 15 ml of ice-cold 0.32 M sucrose containing 10 mM Tris (pH 7.0) and 1 mM EDTA. A crude synaptosomal pellet was obtained as described previously (13). The crude synaptosomal pellet was washed three times by repeated resuspension in the homogenization buffer and recentrifugation for 20 minutes at 12,000 × g. The washed pellet was subjected to ultracentrifugation (SW41Ti rotor) on a discontinuous Ficoll (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) density gradient for 30 minutes at 100,000 × g as described by Booth and Clark (14). The synaptosome fraction was collected at the 7.5 % and 12 % Ficoll interface, diluted with homogenization buffer and isolated by centrifugation for 20 minutes at 20,000 × g. A synaptosomal membrane fraction was obtained by osmotic lysis of the synaptosomes. Specifically, the synaptosomes were resuspendend in 20 volumes of 5 mM Tris (pH 8.1) containing 1 mM EDTA and incubated on ice for one hour as described earlier (15). Following three strokes in a hand-held glass/Teflon homogenizer, the synaptosomal membrane fraction was isolated by centrifugation of the lysate for 20 minutes at 20,000 × g. The synaptosomal membranes were resuspended in a minimal volume of the lysis buffer and 30 ul aliquots (containing about 300-400 ug protein) were frozen a minimum of 2 hours at −20° C prior to resuspension in assay buffers. Synaptosomal membrane samples were utilized within 24 hours of preparation for ATP hydrolysis studies and within 3 hours of preparation for the PGE_2 assay.

ATP hydrolysis. Synaptosomal membranes (32-48 ug protein/ml final) were preincubated for 15 minutes in 50 mM Tris buffer (pH 7.4) containing 5 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl. Within each experiment, synaptosomal membranes were also preincubated with 1 mM ouabain for the determination of Na^+, K^+-ATPase-depen-

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dent ATP hydrolysis. ATP hydrolysis was initiated by the addition of 25 ul of a solution containing ATP (5 mM final) and the arachidonic acid metabolites under investigation. All reactions were performed at 21-23° C and allowed to proceed for 4 minutes. Reactions were terminated by the addition of 2 ml of a phosphate-sensitive color reagent (0.36% w/v ammonium molybdate, 0.03% w/v malachite green, 0.0315% w/v Triton X-405 in 0.5 M HCl) and phosphate release was determined colorimetrically as described by Doughty (16). Na^+, K^+-ATPase-dependent ATP hydrolysis was defined as the 1 mM ouabain-inhibitable phosphate release.

Preparation of arachidonic acid metabolites. Solutions of HPETES and 5-HETE in ethanol (Biomol; Plymouth Meeting, PA) were evaporated to dryness under nitrogen and redissolved in acetone. A solution of the methyl ester of LTA_4 in hexane was evaporated to dryness under nitrogen. The methyl ester was hydrolyzed by incubation in 1 ml of methanol: 50 % NAOH (9:1) for 3 hours at 4° C. The alkaline $LTA_4 \cdot Na$ was diluted with 10 volumes of H_2O and applied to a reverse phase C-18 cartridge. The cartridge was washed with 10 volumes of H_2O and $LTA_4 \cdot Na$ was eluted with 1 ml methanol. The solution of $LTA_4 \cdot Na$ in methanol was evaporated to dryness under nitrogen and redissolved in acetone. The acetone solutions of HPETES, 5-HETE and LTA_4 were diluted with H_2O prior to assay. The final concentration of acetone in the assay was 0.01% and did not affect Na^+, K^+-ATPase activity.

Protein assay. Protein was quantified by the DC protein assay (Bio-Rad Laboratories, Richmond, CA), a procedure based on the method of Lowry et al. (17). Bovine serum albumin was used as standard.

RESULTS

The effect of 1 uM concentrations of the S isomers of 5-, 12- and 15-HPETE on Na^+, K^+-ATPase-dependent

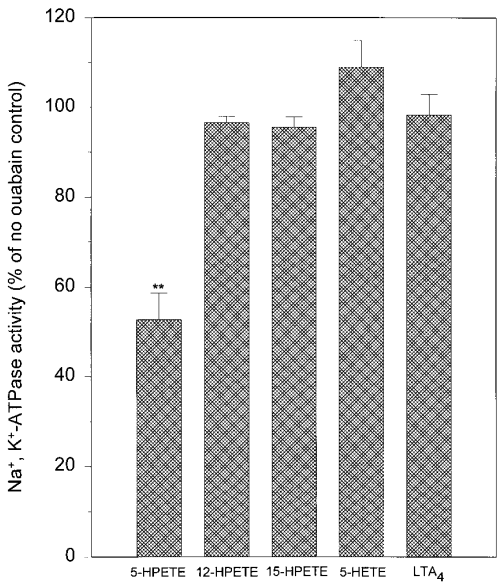

FIG. 1. The effect of 1 uM concentrations of lipoxygenase metabolites of arachidonic acid on Na^+, K^+-ATPase activity (1 mM ouabain-inhibitable Pi release) following a 4 minute incubation of the synaptosomal membranes with 5 mM ATP. Data are the mean ±s.e.m. of 4 experiments. Basal Na^+, K^+-ATPase activity ranged from 184 to 228 nmoles Pi/mg/min. **$P < 0.01$ for comparison of Na^+, K^+-ATPase activity in the absence and presence of 5-HPETE.

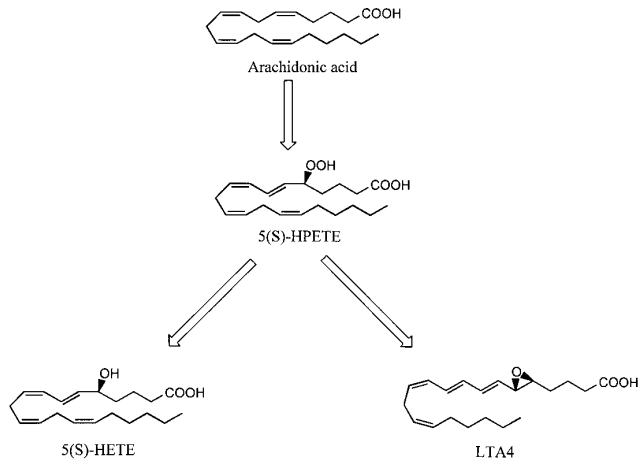

FIG. 2. Arachidonic acid metabolism by 5-lipoxygenase.

(1 mM ouabain-inhibitable) ATP hydrolysis (Pi release) in the synaptosomal membranes was determined following a 4 minute incubation period. 5-HPETE inhibited Na^+, K^+-ATPase activity up to 67 % (figure 1). In contrast, 12- and 15-HPETE did not significantly affect Na^+, K^+-ATPase activity (figure 1).

Further metabolism of 5-HPETE by 5-lipoxygenase produces the hydroxy (5-HETE) or the epoxy (leukotriene A_4) acid (1) (figure 2). To determine whether the inhibitory effect of 5-HPETE on Na^+, K^+-ATPase activity might be due to further metabolism of 5-HPETE by 5-lipoxygenase, the effects of 1 uM concentrations of 5-HETE and leukotriene (LT) A_4 on Na^+, K^+-ATPase activity were examined. Neither 5-HETE or LTA_4 inhibited Na^+, K^+-ATPase activity (figure 1).

Finally, the sensitivity of Na^+, K^+-ATPase activity to inhibition by a range of concentrations (10^{-10}-10^{-5} M) of 5-HPETE was determined. Figure 3 shows that 5-HPETE was a potent inhibitor of Na^+, K^+-ATPase activity. 25 % and 50 % inhibitions of Na^+, K^+-ATPase activity were produced by about 10^{-8} M and 10^{-6} M 5-HPETE, respectively. Based on the established molecular activity (about 10,000 ATP/min/ enzyme) of Na^+, K^+-ATPase activity (see reference 18 for review), the extent of inhibition (about 50 nmoles ATP/min/mg) of ATP hydrolysis produced by 10^{-8} M 5-HPETE was equivalent to a minimal inhibition of about 1 enzyme per 100 molecules of 5-HPETE.

DISCUSSION

Na^+, K^+-ATPase activity has been reported to be inhibited by free fatty acids (6-9) as well as by cyclooxygenase (9-11) and P450 monooxygenase (11,12)-derived metabolites of arachidonic acid. However, the relevance of lipoxygenase-derived metabolites of arachidonic acid as possible regulators of Na^+, K^+-ATPase activity has not been determined.

The present findings demonstrate that 5-HPETE, the

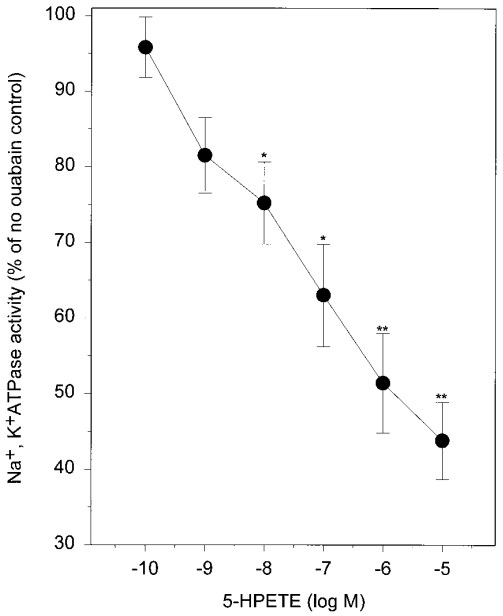

FIG. 3. The response of Na^+, K^+-ATPase activity (1 mM ouabain-inhibitable Pi release) to 5-HPETE following a 4 minute incubation of the synaptosomal membranes with 5 mM ATP. Data are the mean ± s.e.m. of 4 experiments. *P < 0.05 and **P < 0.01 for comparison of Na^+, K^+-ATPase activity in the absence and presence of 5-HPETE.

predominant arachidonic acid hydroperoxide product of 5-lipoxygenase (1), inhibited synaptosomal membrane Na^+, K^+-ATPase activity. The potency of inhibition (IC_{25} = 10^{-8} M) of Na^+, K^+-ATPase activity by 5-HPETE supports the possibility that 5-HPETE may function as a regulator of neuronal Na^+, K^+-ATPase activity in vivo. The putative physiologic significance of the inhibition of Na^+, K^+-ATPase activity by 5-HPETE is further supported by the lack of inhibition produced by of 12-HPETE or 15-HPETE. This result clearly indicates that inhibition by 5-HPETE was dependent on the position of the hydroperoxide and argue against a nonspecific mechanism of action. Importantly, the lack of inhibition of Na^+, K^+-ATPase activity produced by 5-HETE or LTA_4 indicates that the inhibition by 5-HPETE was independent of further 5-lipoxygenase-dependent metabolism of 5-HPETE. Thus, while a requirement for further metabolism of 5-HPETE by a non-5-lipoxygenase enzyme cannot be excluded, this result is consistent with the possibility that inhibition of Na^+, K^+-ATPase activity by 5-HPETE may have resulted from a direct action of 5-HPETE either on 1) Na^+, K^+-ATPase or 2) a regulator (e.g., protein kinase) of Na^+, K^+-ATPase.

Importantly, 5-lipoxygenase has been identified in brain (19,20). Increased brain levels of 5-lipoxygenase products have been associated with aberrant conditions (e.g., ischaemia, epileptic seizures) (see reference 21 for review). However, knowledge of the physiological function of these products in brain is limited. While one study has demonstrated that leukotriene C_4 increased the activity of muscarine-inactivated M-K^+ channels in hippocampal neurons (22), the present findings are the first to demonstrate a functional signficance of 5-HPETE.

The Na^+ and K^+ gradients generated by Na^+, K^+-ATPase activity are important for the "resting" membrane potential. In addition, the inward Na^+ gradient helps to maintain (and restore following depolarization) low levels of synaptic neurotransmitters by driving the removal of intracellular Ca^{2+} by Na^+/Ca^{2+} exchange and the reuptake of neurotransmitters (4,5). Thus, it is conceivable that the inhibition of neuronal Na^+, K^+-ATPase activity by 5-HPETE may increase 1) neuronal excitability by decreasing the threshold for depolarization and 2) neurotransmission by increasing synaptic levels of neurotransmitters. This puative neuroexcitatory effect of 5-HPETE may be relevant to mechanisms of both neuromodulation and excitotoxicity.

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