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Inhibition of Ca^{2+}/calmodulin-dependent protein kinase II by arachidonic acid and its metabolites

(protein phosphorylation/icosanoids/neurotransmitter release)

DANIELE PIOMELLI*, JAMES K. T. WANG, TALVINDER S. SIHRA, ANGUS C. NAIRN, ANDREW J. CZERNIK, AND PAUL GREENGARD

Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **A variety of evidence indicates that activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) in nerve terminals leads to enhanced neurotransmitter release. Arachidonic acid and its 12-lipoxygenase metabolite, 12-hydroperoxyicosatetraenoic acid (12-HPETE), have been suggested to act as second messengers mediating presynaptic inhibition of neurotransmitter release. In the present study it was found that CaM-kinase II, purified from rat brain cortex, was inhibited both by arachidonic acid (IC_{50} = 24 μM) and by 12-HPETE (IC_{50} = 0.7 μM). Neither substance inhibited CaM-kinase I or III, protein kinase C, or the catalytic subunit of cAMP-dependent protein kinase. Specific inhibition of Ca^{2+}/calmodulin-dependent protein phosphorylation by arachidonic acid was also demonstrated in intact synaptic terminals (synaptosomes) isolated from rat forebrain. These results suggest that arachidonate and its metabolites may modulate synaptic function through the inhibition of CaM-kinase II-dependent protein phosphorylation.**

Within the presynaptic nerve terminal, stimulation of either of two Ca^{2+}-dependent protein kinases, Ca^{2+}/calmodulin-dependent kinase II (CaM-kinase II) or protein kinase C (PKC), leads to enhanced neurotransmitter release (for a review, see ref. 1). CaM-kinase II, a protein kinase with broad substrate specificity, is present throughout the mammalian nervous system (2). In nerve terminals, CaM-kinase II catalyzes the phosphorylation of several proteins, including synapsin I, a protein associated with synaptic vesicles (2). Phosphorylation of synapsin I by CaM-kinase II is thought to participate in presynaptic modulation of neurotransmitter release (3).

In neurons, several neurotransmitters, including histamine, norepinephrine, glutamate, and bradykinin, stimulate the formation of free arachidonic acid (Δ_4Ach) and of its lipoxygenase metabolites (4–7). Furthermore, Δ_4Ach and its 12-lipoxygenase product, 12-hydroperoxy-5,8,10,14-icosatetraenoic acid (12-HPETE), modulate ion conductances and produce presynaptic inhibition of neurotransmitter release in identified neurons of the mollusk *Aplysia californica* (8, 9). Moreover, modulation of gap junctions and ion channels by Δ_4Ach and other fatty acids has been demonstrated in non-neuronal tissues (10–14).

We have now investigated the actions of Δ_4Ach and of its lipoxygenase-derived products on CaM-kinase II as one test of the possibility that these lipids achieve certain of their physiological effects through actions on this multifunctional protein kinase.

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MATERIALS AND METHODS

Materials. [^{32}P]Orthophosphate and [γ-^{32}P]ATP were from New England Nuclear. P81 phosphocellulose paper was from Whatman. Δ_4Ach was obtained from Nu Check Prep (Elysian, MN) and all Δ_4Ach metabolites from Cayman (Ann Arbor, MI). Δ_4Ach solutions were prepared from a dimethyl sulfoxide (DMSO) stock solution (0.1 M, stored at −20°C) by dilution with water and sonication for 1 min. Δ_4Ach metabolites, stored in ethanol at −70°C, were dried under reduced pressure, dissolved in DMSO (5 μl), diluted with water, and sonicated. DMSO was added to control samples to a final concentration of 0.2% (vol/vol). Histone HF2B was from Worthington, histone III-S from Sigma, and *Staphylococcus aureus* V8 protease from Miles.

Purification of Proteins. CaM-kinase II was purified from rat forebrain (15), calmodulin from rabbit brain (16), synapsin I from bovine brain (17), CaM-kinase I from rat brain (18), and CaM-kinase III and its 100-kDa substrate (elongation factor 2) from rat pancreas (19). Rat brain calcineurin (20), rat brain PKC (21), bovine heart catalytic subunit of cAMP-dependent protein kinase (PKA) (22), and bovine caudate 32-kDa dopamine- and cAMP-regulated phosphoprotein (DARPP-32) (23) were purified and phosphorylated, when appropriate, as described, with minor modifications.

Protein Kinase Assays. Standard CaM-kinase II assays were performed essentially as described (15), but without bovine serum albumin (BSA), using [γ-^{32}P]ATP (50 μM, 200–500 cpm/pmol), calmodulin (0.75 μM), and as substrate a synthetic peptide (prepared by the Yale University Protein and Nucleic Acid Chemistry Facility) based on the sequence of the α subunit of rat brain CaM-kinase II (2) [MHRQET(*P*)VDCLK-NH_2; CaM-kinase II-(281–291)-undecapeptide amide, 25 μM]. In some experiments a synthetic peptide (prepared by Meng Ho, The Rockefeller University) containing phosphorylation site 3 of bovine synapsin I [YRQGPPQLPPGPAGPTRQAS(*P*)QAGP-NH_2] (24) or purified synapsin I was used. For kinetic experiments, various concentrations of calmodulin, ATP, and substrate [CaM-kinase II-(281–291)] were used.

CaM-kinase I and CaM-kinase III assays were performed under the conditions described for CaM-kinase II, using as substrates synapsin I (1.5 μM) and elongation factor 2 (0.1 μM), respectively. Samples were analyzed by $NaDodSO_4$/PAGE. The ^{32}P-labeled bands were excised from the dried gel and radioactivity was measured by Cerenkov counting. Ac-

Abbreviations: Δ_4Ach, arachidonic acid; BSA, bovine serum albumin; CaM-kinase, Ca^{2+}/calmodulin-dependent protein kinase; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; HETE, hydroxyicosatetraenoic acid; HPETE, hydroperoxyicosatetraenoic acid; PKA, catalytic subunit of cAMP-dependent protein kinase; PKC, protein kinase C.

*To whom reprint requests should be addressed.

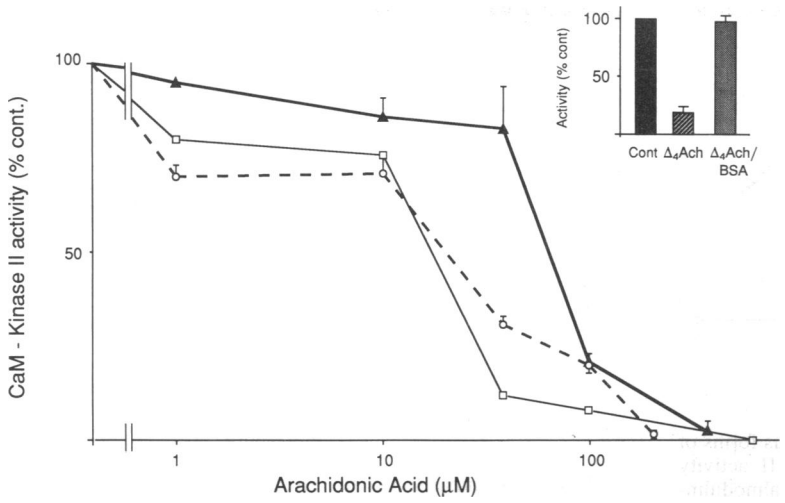

FIG. 1. Inhibition of CaM-kinase II activity by Δ_4Ach. Activity of purified CaM-kinase II was measured using the following substrates: CaM-kinase II-(281–291) peptide (○); α subunit (autophosphorylation) (□); synapsin I peptide (▲). (*Inset*) Reversibility of CaM-kinase II inhibition by Δ_4Ach. Kinase was incubated with Δ_4Ach (50 μM) for 2 min prior to the addition of BSA (150 μM). CaM-kinase II activity was measured using the CaM-kinase II-(281–291) peptide. All results are averages of 3–6 determinations. Error bars indicate SEM.

tivities of PKC and PKA were measured using the assay conditions described for CaM-kinase II, except that $CaCl_2$ and calmodulin were omitted. The substrates used were histone HF2B (10 μg/0.1 ml) for PKA and histone III-S (10 μg/0.1 ml) for PKC. PKC activity was measured in the absence of Ca^{2+} and phosphatidylserine.

To measure CaM-kinase II autophosphorylation, the assay was carried out in the absence of substrate and with a 10-fold higher enzyme concentration. Reactions were stopped with a $NaDodSO_4$ solution, and samples were heated for 2 min and subjected to $NaDodSO_4$/PAGE. The ^{32}P-labeled α subunit of CaM-kinase II was localized by autoradiography and excised from the gel, and radioactivity was measured.

Phosphatase Assay. Calcineurin phosphatase assays were carried out in a solution (final volume 40 μl) containing Tris buffer (50 mM, pH 7.0), $CaCl_2$ (1.5 mM), EGTA (1 mM), calmodulin (1 μM), and Brij-35 (0.01%, vol/vol). After a 1-min preincubation, samples were incubated at 30°C for 5 min with [^{32}P]DARPP as substrate. Reactions were stopped by the addition of ice-cold trichloroacetic acid (20% wt/vol, 0.1 ml) and BSA (6 mg/ml, 0.1 ml). After centrifugation, [^{32}P]phosphate was measured in the supernatant.

Preparation of Ca^{2+}/Calmodulin-Independent CaM-Kinase II. The Ca^{2+}/calmodulin-independent form of CaM-kinase II was prepared by autophosphorylation, essentially as described (25). In brief, CaM-kinase II was incubated in a reaction mixture containing $CaCl_2$ (0.5 mM), calmodulin (0.75 μM) and nonradioactive ATP (3 μM) for 15 sec at 0°C. The reaction was stopped with EDTA (9 mM) plus EGTA (0.5 mM). Ca^{2+}/calmodulin-independent activity was determined under standard conditions (see above), except that $CaCl_2$ was omitted and EGTA (1 mM) was present.

Proteolysis of CaM-Kinase II. CaM-kinase II was autophosphorylated for 5 min at 0°C in standard kinase buffer containing 0.5 mM ATP (26). Autophosphorylation was terminated by the addition of EDTA (25 mM). Samples of the reaction mixture were then incubated at 0°C for 30 min with chymotrypsin (substrate/protease weight ratio, 1:1). Reactions were stopped by the addition of aprotinin (1000 units/ml). Ca^{2+}/calmodulin-independent activity was measured as described above. The extent of proteolysis was determined by $NaDodSO_4$/PAGE as previously reported (26, 27); chymotrypsin digestion of autophosphorylated CaM-kinase II resulted in cleavage of both α and β subunits of the kinase, generating a prominent fragment with an apparent molecular mass of 32 kDa. The Ca^{2+}/calmodulin-independent activity of this proteolyzed CaM-kinase II was 79.4 ± 5.6% (mean ± SEM, n = 5) of that of the autophosphorylated kinase used as starting material.

Measurement of CaM-Kinase II Activity in Synaptosomes. Preparation of synaptosomes and incubation conditions were as described (28). When synaptosomes are incubated in the presence of a high concentration of KCl (40 mM), the resulting Ca^{2+} influx stimulates CaM-kinase II autophosphorylation and promotes the generation of a Ca^{2+}-independent form of the enzyme (29). Generation of this activity was determined as follows. Synaptosomes were incubated for 5 sec at 37°C in a Hepes-buffered solution (28) containing either

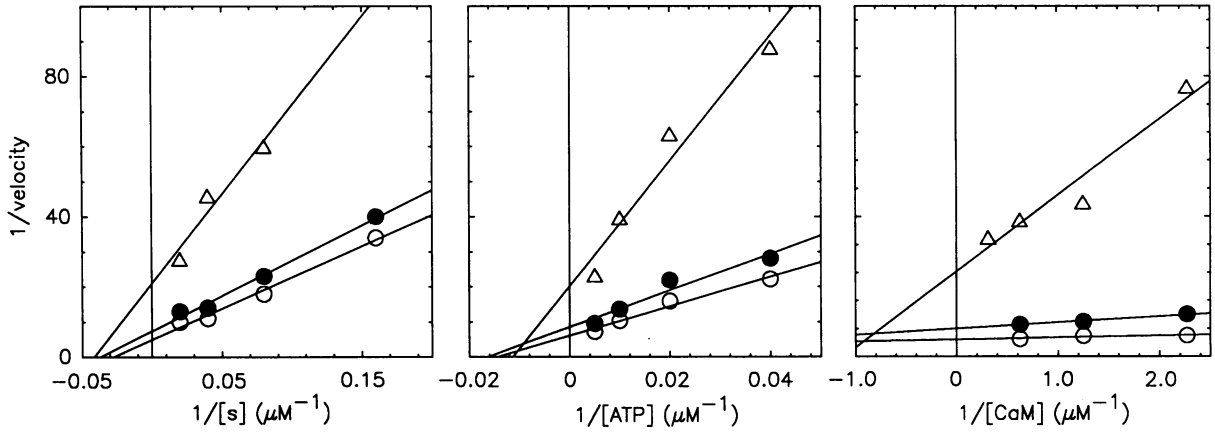

FIG. 2. Kinetic analysis of CaM-kinase II inhibition by Δ_4Ach. Shown are double-reciprocal plots of CaM-kinase II activity (velocity, [^{32}P]orthophosphate cpm incorporated per min per μg; reciprocal value multiplied by 10^5 for clarity) at various concentrations of substrate [s, CaM-kinase II-(281–291)], ATP, or calmodulin (CaM) in the presence of 0 (○), 25 (●), or 50 (△) μM Δ_4Ach. Each assay point represents the average of duplicate determinations. The results shown are typical of 5–10 experiments.

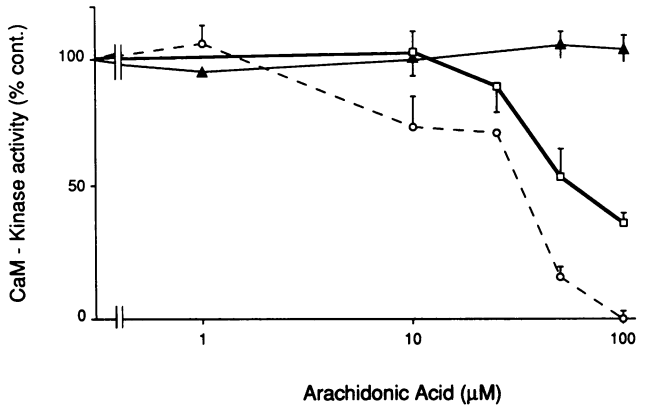

FIG. 3. Comparison of the effects of Δ_4Ach on various forms of CaM-kinase II. ○, Native enzyme, total CaM-kinase II activity (Ca^{2+}-independent plus Ca^{2+}-dependent); □, Ca^{2+}/calmodulin-independent activity of autophosphorylated CaM-kinase II; ▲, Ca^{2+}/calmodulin-independent activity of proteolyzed CaM-kinase II. Bars indicate SEM (n = 3–6).

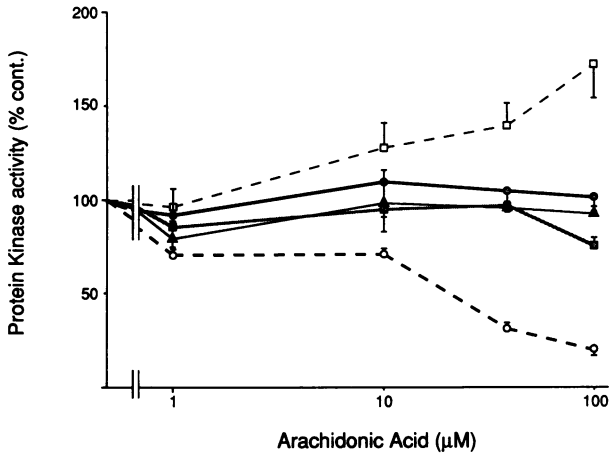

FIG. 4. Comparison of the effects of Δ_4Ach on the activities of CaM-kinase II (○), CaM-kinase I (▣), CaM-kinase III (▲), PKA (●), and PKC (□). Bars indicate SEM (n = 3–6).

low (5 mM) or high (40 mM) KCl in the presence or absence of Δ_4Ach. Incubations were stopped by the addition of 10 volumes of a lysis buffer (29) and Ca^{2+}/calmodulin-independent kinase activity was determined as described above.

Analysis of Endogenous Protein Phosphorylation in Synaptosomes. Synaptosomes were prelabeled with [^{32}P]orthophosphate and incubated in control or depolarizing medium as described (28). Synapsin I and the 87-kDa protein were immunoprecipitated (28) and isolated by NaDodSO$_4$/PAGE, and radioactivity was measured in the excised gel bands. Immunoprecipitated synapsin I was subjected to partial proteolysis with *S. aureus* V8 protease followed by one-dimensional peptide mapping (28).

RESULTS AND DISCUSSION

Inhibition of CaM-Kinase II by Δ_4Ach. Δ_4Ach inhibited the activity of purified CaM-kinase II in a concentration-dependent manner (Fig. 1). When the peptide CaM-kinase II-(281–291) was used as substrate, the Δ_4Ach concentration that produced half-maximal inhibition (IC_{50}) was 24 μM (Fig. 1). The CaM-kinase II holoenzyme is composed of two distinct subunits, α and β, which undergo Ca^{2+}/calmodulin-dependent autophosphorylation (2, 29). Autophosphorylation on a threonine residue (Thr-286 of the α subunit, Thr-287 of the β subunit) relieves the kinase of its requirement for Ca^{2+}/calmodulin, resulting in an autonomous enzyme (2). Δ_4Ach effectively inhibited autophosphorylation of CaM-kinase II (IC_{50} = 18 μM; Fig. 1) but was less potent in inhibiting phosphorylation of the synapsin I peptide (IC_{50} = 70 μM; Fig. 1) or synapsin I (IC_{50} = 70 μM; data not shown). The reasons for this apparent substrate dependence of the inhibitory action of Δ_4Ach are not known.

The reversibility of the effect of Δ_4Ach on CaM-kinase II was demonstrated by taking advantage of the capacity of BSA to bind fatty acids. CaM-kinase II was incubated for 2 min with Δ_4Ach before activity was assayed in the presence or absence of BSA. Addition of BSA completely reversed the inhibition of kinase activity by Δ_4Ach (Fig. 1 *Inset*).

Kinetic Studies. Several isoquinoline sulfonamide derivatives, such as H-7, inhibit protein kinases by competing with ATP for its binding site within the catalytic domain of the kinase (30). In contrast, kinetic studies demonstrated that CaM-kinase II inhibition by Δ_4Ach is noncompetitive with respect to either ATP or substrate (Fig. 2).

Certain lipophilic agents that inhibit PKC (31) also bind to Ca^{2+}/calmodulin and inhibit Ca^{2+}/calmodulin-dependent enzymes (32). To test whether Δ_4Ach acts through a similar mechanism, its inhibitory effects on CaM-kinase II were compared at various concentrations of calmodulin in the

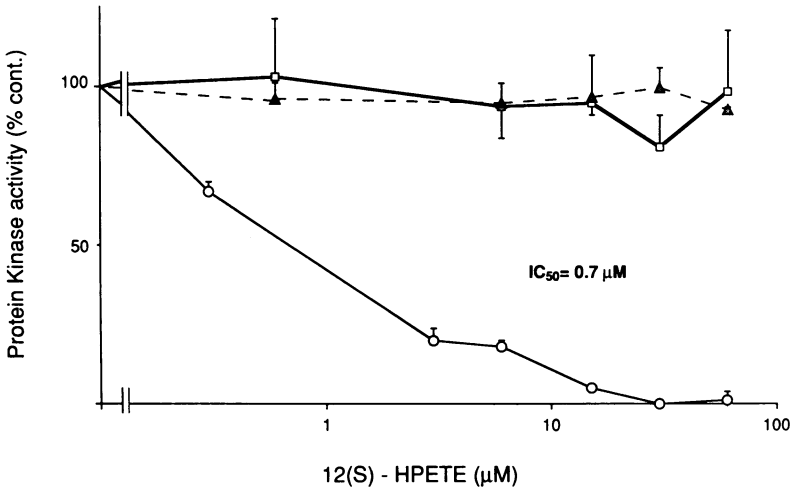

FIG. 5. Comparison of the effects of 12(S)-HPETE on the activities of CaM-kinase II (○), PKA (▲), and PKC (□). Bars indicate SEM (n = 3–6).

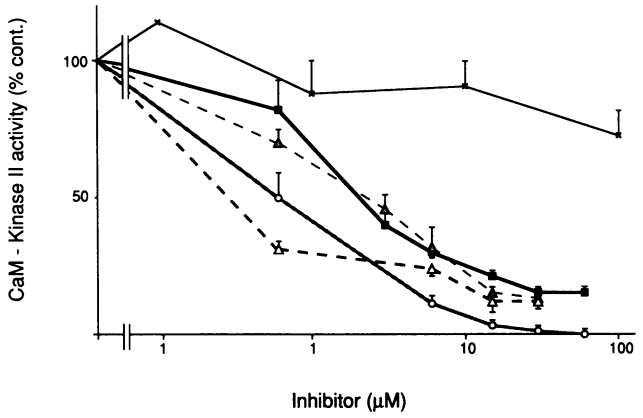

FIG. 6. Inhibition of CaM-kinase II activity by (12*S*)-12-hydroxyicosatetraenoic acid [12(*S*)-HETE] (■), 8(*S*)-HETE (▲), 12(*R*)-HETE (○), 8(*R*)-HETE (△), and Δ_4Ach methyl ester (x). Bars indicate SEM (n = 3).

presence of fixed concentrations of ATP and substrate. The inhibition produced by Δ_4Ach could be overcome by increasing the concentration of calmodulin (Fig. 2). However, double-reciprocal plot analysis yielded lines of best fit that did not intersect at the y axis, indicating that Δ_4Ach is not competitive with calmodulin.

Effects of Δ_4Ach on the Autophosphorylated and Proteolyzed Forms of CaM-Kinase II. Consistent with a direct action of the fatty acid on CaM-kinase II, Δ_4Ach inhibited the autophosphorylated, Ca^{2+}/calmodulin-independent form of CaM-kinase II [IC_{50} = 60 μM with CaM-kinase II-(281–291) as substrate; Fig. 3], although less effectively than it did the Ca^{2+}/calmodulin-dependent form (IC_{50} = 24 μM).

In a model of CaM-kinase II regulation, the catalytic site of the kinase, which resides in the NH_2-terminal portion of the protein, is proposed to bind to a regulatory region that contains calmodulin-binding and inhibitory sites (2, 26). This interaction is proposed to maintain the kinase in an inactive, closed conformation. Ca^{2+}/calmodulin binding is thought to produce a conformational change that results in displacement of the inhibitory subdomain and activation of the kinase. Limited proteolysis of autophosphorylated CaM-kinase II generates a fragment that lacks the kinase regulatory region and is independent of Ca^{2+}/calmodulin for activity (26, 27). This constitutively active fragment of the kinase was not inhibited by Δ_4Ach (Fig. 3).

These results suggest that the inhibitory effects of Δ_4Ach on CaM-kinase II are exerted by reversible binding of the fatty acid to the regulatory region of the kinase. Alternatively, Δ_4Ach may bind elsewhere on the enzyme molecule and favor its inactive, closed conformation.

Specificity of CaM-Kinase II Inhibition by Δ_4Ach. The inhibition of CaM-kinase II activity by Δ_4Ach appeared to be specific. The fatty acid had no inhibitory effect on PKA or on PKC (Fig. 4). In fact, PKC activity was stimulated (Fig. 4), as shown previously (33, 34). Furthermore, Δ_4Ach inhibited CaM-kinase I only very slightly and had no significant effect on CaM-kinase III (Fig. 4).

Table 1. Inhibition of CaM-kinase II by Δ_4Ach metabolites

Compound	IC_{50}, μM
12(*R*)-HETE	0.4
12(*S*)-HPETE	0.7
12(*S*)-HETE	2.0
12(*S*)-HHT*	9.0
8(*R*)-HETE	0.2
8(*S*)-HETE	2.0
5(*S*)-HETE	9.0
Leukotriene D_4	2.0
Leukotriene C_4	30.0
Δ_4Ach	24.0
Δ_4Ach methyl ester	>100.0

Concentrations that produced half-maximal inhibition (IC_{50}) of CaM-kinase II activity were determined from concentration–inhibition plots using six concentrations of inhibitor (ranging from 0.3 to 60 μM) in at least three separate experiments. Assays were carried out under standard conditions.
*(12*S*)-12-Hydroxyheptadecatrienoic acid, a cycloxygenase metabolite of Δ_4Ach.

CaM-Kinase II Inhibition by Lipoxygenase Products. The primary 12-lipoxygenase metabolite, 12(*S*)-HPETE, inhibited CaM-kinase II activity much more effectively than did Δ_4Ach, while it had no effect on PKA or PKC (Fig. 5) and inhibited only very weakly CaM-kinase III (at 180 μM, activity was 75 ± 4% of control, n = 6) and calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase (at 120 μM, activity was 66 ± 6% of control, n = 6). The kinetics of inhibition of CaM-kinase II by 12(*S*)-HPETE were similar to those produced by Δ_4Ach (data not shown). Other metabolites of Δ_4Ach were also found to be effective in inhibiting CaM-kinase II (Fig. 6 and Table 1). These structure–activity studies suggest the following order to potency: (*S*)-hydroperoxy acid = (*R*)-hydroxy acids > (*S*)-hydroxy acids > Δ_4Ach >>> Δ_4Ach methyl ester.

Although CaM-kinase II can be inhibited by several fatty acid derivatives, the greater potency of the hydroperoxy acids and hydroxy acids suggests that these metabolites may act as physiological modulators. 12-HETE, presumably formed through the short-lived intermediate 12-HPETE, is a major Δ_4Ach metabolite produced by mammalian brain tissue (35, 36), although the identity of the biological enantiomer (*R* or *S*) has not been established. In spite of their difference in potency, both enantiomers are effective inhibitors of the enzyme (Table 1).

Inhibition of CaM-Kinase II by Δ_4Ach in Isolated Synaptic Terminals. K^+-induced depolarization stimulated CaM-kinase II autoactivation about 6-fold over control (n = 3). Δ_4Ach inhibited this K^+-evoked autoactivation (Table 2).

In synaptosomes prelabeled with [^{32}P]phosphate, the Ca^{2+} influx evoked by high K^+ results in increased phosphoryla-

Table 2. Effects of Δ_4Ach on CaM-kinase II activity and on endogenous protein phosphorylation in isolated nerve terminals

KCl, mM	Δ_4Ach, μM	CaM-kinase II activity, % control	$^{32}P_i$ incorporation, % control: synapsin I, CaM-kinase II site	synapsin I, CaM-kinase I site	87-kDa
5	0	100	100	100	100
5	50	70 ± 8	98 ± 9	91 ± 4	114 ± 2
40	0	583 ± 69	298 ± 5	165 ± 3	175 ± 2
40	50	263 ± 116	213 ± 7	121 ± 8	179 ± 2

Data are presented as mean ± SEM (n = 3).

tion of several protein kinase substrates. These include synapsin I, a substrate for both CaM-kinase I and CaM-kinase II, and the 87-kDa protein, a prominent PKC substrate (28). Ca^{2+}-dependent phosphorylation of synapsin I by CaM-kinase I and CaM-kinase II occurs on distinct sites, which are located in different domains of the protein and can be analyzed by limited proteolysis (37). Δ_4Ach reduced the [^{32}P]phosphate content in both domains (Table 2). Since phosphorylation of synapsin I by purified CaM-kinase I was not significantly inhibited by Δ_4Ach (Fig. 4), the inhibition of the CaM-kinase I site seen in intact synaptosomes presumably occurred through an indirect action (e.g., an action of CaM-kinase II on CaM-kinase I or an effect of Δ_4Ach on Ca^{2+} influx). The Ca^{2+}-dependent increase in phosphorylation of the 87-kDa protein was not affected by Δ_4Ach, showing that PKC activity was not altered by the fatty acid under depolarizing conditions.

Many neurotransmitters present in the brain act by regulating protein kinases, which, in turn, affect the state of phosphorylation of specific protein substrates within neurons (38). The present study shows that products of the Δ_4Ach cascade may act in synaptic terminals through the inhibition of CaM-kinase II-dependent protein phosphorylation. This effect may contribute to the presynaptic regulation of neurotransmitter release.

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