The Journal of Neuroscience, October 1993, 13(10): 4361–4371

Activation of Protein Kinase C by Arachidonic Acid Selectively Enhances the Phosphorylation of GAP-43 in Nerve Terminal Membranes

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Arachidonic acid (AA), a *cis*-unsaturated fatty acid that activates certain subspecies of protein kinase C (PKC), has been proposed to act as a retrograde messenger in modifying the efficacy of synapses during long-term potentiation (LTP). One prominent PKC substrate of the nerve terminal membrane, GAP-43 (F1, B-50, neuromodulin), shows an increase in phosphorylation that correlates with the persistence of LTP. The present study investigated whether AA might exert its effects on presynaptic endings by modulating the phosphorylation of GAP-43 and other membrane-bound proteins. Using synaptosomal membranes from the rat cerebrocortex, in which *in vivo* relationships between protein kinases and their native substrates are likely to be preserved, we found that in the absence of Ca^{2+}, AA exerted a modest effect on the phosphorylation of GAP-43 and several other proteins; however, when AA was applied in conjunction with Ca^{2+}, GAP-43 showed a particularly striking response: at Ca^{2+} levels likely to exist at the nerve terminal membrane during synaptic activity (10^{-7} to 10^{-5} M), AA (50 μM) increased the sensitivity of GAP-43 phosphorylation to Ca^{2+} by an order of magnitude, and increased its maximal level of phosphorylation by 50%. At resting Ca^{2+} levels, AA potentiated the stimulation in GAP-43 phosphorylation produced by 4β-phorbol 12,13-dibutyrate, a diacylglycerol (DAG) analog. The stimulatory effect of AA and its synergistic interaction with Ca^{2+} were found to be mediated by PKC, since they were blocked by a specific peptide inhibitor of PKC, $[Ala^{25}]PKC_{(19-31)}$, but were unaffected by an inhibitor of protein phosphatase activity or by scavengers of free radicals. Since GAP-43 has been implicated in the development and plasticity of synaptic relationships, the synergistic effects of AA and the intracellular signals Ca^{2+} and DAG on the phosphorylation of GAP-43 may serve as an AND gate to modify presynaptic function and/or structure in response to coincident pre- and postsynaptic activity.

[Key words: GAP-43 (B-50, F1, neuromodulin), arachidonic acid, protein kinase C, presynaptic phosphoproteins, long-term potentiation, plasticity, retrograde messenger]

The rise in intracellular Ca^{2+} that occurs with activation of NMDA receptors causes the release of arachidonic acid (AA) from membrane phospholipids by activating Ca^{2+}-dependent phospholipases (Dumuis et al., 1988; Sanfeliu et al., 1990). AA, a *cis*-unsaturated fatty acid, diffuses readily across cellular membranes and can modify a number of neuronal functions, including amino acid uptake (Chan et al., 1983; Volterra et al., 1992), transmitter release (Lynch and Voss, 1990; Herrero et al., 1991, 1992), and ion channel activity (Anderson and Welsh, 1990; Keyser and Alger, 1990). These properties suggest that AA may transmit information retrogradely across a synapse, modifying the function of presynaptic elements in response to postsynaptic activation. Such retrograde signaling has been proposed to be necessary for long-term potentiation (LTP) (Williams and Bliss, 1988). The basis of this proposal is that although LTP is initiated postsynaptically at synapses of the dentate gyrus and hippocampal CA1 field (see Gustafsson and Wigstrom, 1988), at least part of the enduring physiologic change is presynaptic (Bekkers and Stevens, 1990; Malinow and Tsien, 1990). Retrograde signaling may also be important during the development of the brain's circuitry, since NMDA receptors appear to be involved in integrating information impinging on postsynaptic cells to stabilize selective presynaptic contacts (Kleinschmidt et al., 1987; Constantine-Paton et al., 1990).

The possibility that AA may serve as a retrograde messenger in LTP is based in part on the findings that (1) the extracellular concentration of AA rises with LTP (Lynch et al., 1989; Clements et al., 1991), (2) LTP is blocked by drugs that inhibit the liberation of AA from membrane phospholipids (Linden et al., 1987; Williams and Bliss, 1988; Lynch et al., 1989), and (3) exogenous AA applied to the hippocampus in conjunction with low-frequency stimulation produces a persistent enhancement in synaptic efficacy (Williams et al., 1989). Besides AA, substantial evidence has accumulated to suggest that nitric oxide, a membrane-diffusible free radical that can also be generated upon NMDA receptor activation, may be a retrograde messenger in LTP (Bohme et al., 1991; O'Dell et al., 1991; Schuman and Madison, 1991). To date, it is unclear whether AA, nitric oxide, or another molecule serves as a necessary and sufficient retrograde signal in LTP, or whether effects of these putative signals interact with one another to induce persistent synaptic changes.

One possible target of the retrograde signal in LTP is GAP-

Received Oct. 15, 1992; revised Mar. 12, 1993; accepted Apr. 15, 1993.

We are grateful for the support of the National Institutes of Health (EY 05690 to L.I.B. and Training Grant 5T32MH14275-16 to J.D.S.) and The Boston Neurosurgical Foundation. We thank Michael Belliveau for assistance on graphics and Dr. Paul Rosenberg for helpful discussions.

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43, a major phosphoprotein of the presynaptic membrane that has been implicated in the development and plasticity of synaptic connections (see reviews of Benowitz and Routtenberg, 1987; Skene, 1989). During development, high levels of GAP-43 are found in growth cones of actively growing axons and in immature presynaptic endings (Skene and Willard, 1981; Perrone-Bizzozero et al., 1986; Dani et al., 1991). Although in most parts of the brain, levels of GAP-43 decline precipitously after synaptic connections have matured, certain neurons, particularly those in limbic and associative regions, continue to express high levels throughout life (Benowitz et al., 1988, 1989). The possibility that GAP-43 may contribute to ongoing structural and/or functional changes of these nerve endings is supported by the findings that enhanced phosphorylation of GAP-43 is correlated with the persistence of LTP (Lovinger et al., 1985; Gianotti et al., 1991) and with the potentiation of transmitter release (Dekker et al., 1989a,b).

The phosphorylation of GAP-43 is mediated by protein kinase (PKC) (Aloyo et al., 1983), and among the Ca^{2+}-dependent subspecies of PKC, the purified β-subspecies has been found to most effectively phosphorylate GAP-43 (Sheu et al., 1990). However, multiple PKC subspecies exist in the nerve terminal *in vivo* (Shearman et al., 1991b), and their activity is influenced by the lipid environment (Takai et al., 1979a,b; Kishimoto et al., 1980) and by the relative levels of kinase to substrate (Buday and Farago, 1990). Furthermore, in *in vitro* assays using purified lipid and protein components, the Ca^{2+}-dependent PKC subspecies are differentially sensitive to AA when the fatty acid is added to the reaction mixture alone or in combination with Ca^{2+} and/or diacylglycerol (DAG) (Sekiguchi et al., 1987; Shearman et al., 1991a; Shinomura et al., 1991). Therefore, it is unclear whether the PKC subspecies that is responsible for the phosphorylation of GAP-43 *in situ* is responsive to AA, and whether there is a differential sensitivity among membrane-bound presynaptic phosphoproteins to signals likely to be generated upon coincident activation of pre- and postsynaptic elements. The present study used nerve terminal membranes, in which the relationships between protein kinases and the substrates with which they are associated *in vivo* are likely to be preserved, to examine the effect of AA on the phosphorylation of GAP-43 and other membrane-bound proteins, and the possible interactive effects of this fatty acid with Ca^{2+} and DAG, intracellular signals generated with presynaptic depolarization.

The data demonstrate that among the phosphoproteins native to the nerve terminal membrane, GAP-43 appears to be affected most markedly by AA working synergistically with Ca^{2+} and DAG: AA enhanced GAP-43 phosphorylation and this effect was strongly potentiated by phorbol dibutyrate, a DAG analog, or by elevating Ca^{2+} within the physiologic range. This targeting of AA to the phosphorylation of GAP-43 may be important for structural and functional modifications of presynaptic elements that result from coordinated pre- and post-synaptic activity.

Materials and Methods

Materials. Rats were purchased from Charles River (Wilmington, MA). Lipids, phorbol esters, (+)-α-tocopherol, superoxide dismutase, catalase, diaminobenzidine, and *Staphylococcus aureus V8* protease were obtained from Sigma (St. Louis, MO). Horseradish peroxidase–conjugated rabbit anti-sheep IgG was obtained from Cappel (Durham, NC). Peptide [Ala^{25}]$PKC_{(19-31)}$ was purchased from UBI (Lake Placid, NY). Okadaic acid was purchased from LC Services (Woburn, MA). All electrophoresis reagents and PVDF membrane were purchased from Bio-Rad (Richmond, CA). γ-^{32}P-ATP (3000 μCi/mmol) was obtained from Dupont/New England Nuclear (Boston, MA).

Preparation of synaptosomal membranes. Synaptosomal membranes were isolated essentially by the method of Whittaker et al. (1964). Male Sprague–Dawley rats (~125 gm) were decapitated and their brains removed; cerebrocortices were dissected and placed in ice-chilled 0.32 M sucrose containing 1 mM Tris-HCl, pH 7.3. All subsequent procedures were performed at 0–4°C. Tissue was homogenized in 10 vol of buffered sucrose using a glass–Teflon system (1000 rpm, 12 strokes, 0.25 mm clearance). The homogenate was centrifuged at 1000 × *g* for 10 min, and the resulting supernatant was centrifuged at 10,000 × *g* for 20 min. This pellet (P_2) was washed by resuspension in buffered sucrose, recentrifuged at 10,000 × *g* for 20 min, and exposed for 45 min with stirring to a hypoosmotic buffer (1 mM Tris-HCl, pH 7.3; 5 mM $MgCl_2$; 0.1 mM $CaCl_2$; 40 μM leupeptin; 1 mM phenylmethylsulfonyl fluoride) in order to lyse the synaptosomes. The lysate was then layered on top of a discontinuous sucrose gradient (1.2 M and 0.8 M sucrose containing 1 mM Tris-HCl, pH 7.3) and centrifuged at 53,000 × *g* for 2 hr. Synaptosomal membranes were collected at the 0.8 M/1.2 M sucrose interface. In some experiments, the mitochondrial pellet and the myelin fragments floating on top of the 0.8 M sucrose layer were also used. Each of these fractions was diluted and washed with 1 mM Tris-HCl, pH 7.3/5 mM $MgCl_2$ and then pelleted by centrifugation. Synaptosomal membrane pellets were resuspended in the Tris/Mg^{2+} solution and stored at −80°C in small aliquots. Just prior to use, aliquots were thawed, washed, and resuspended in phosphorylation buffer (see below). In one experiment, phosphorylation assays were performed using freshly prepared membranes that had not undergone freezing. Protein determination was made by the method of Lowry et al. (1951) using BSA as the standard.

Phosphorylation of synaptosomal membranes. Synaptosomal membranes (20 μg of protein per sample) were incubated at 30°C for 5 min in a reaction mixture (100 μm final volume) containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA; concentrations of $CaCl_2$, lipids, and drugs varied with the experiment. In one experiment, the incubation times were varied from 0 to 300 sec. The concentration of $CaCl_2$ required to yield the desired free Ca^{2+} concentration in a 1 mM EGTA–containing reaction mixture was determined using a computer program based on that described by Caldwell (1970). In the cases in which lipids and drugs were added with organic solvents, appropriate vehicle controls were used (see below). Reactions were initiated by adding 10 μM ATP containing 3–5 μCi γ-^{32}P-ATP, and allowed to proceed for 15 sec before being terminated by adding 100 μl of twice-concentrated Laemmli sample buffer (final concentrations: 125 mM Tris-HCl, pH 6.8; 1% SDS; 10% glycerol; 5% β-mercaptoethanol; see Laemmli, 1970) and freezing on dry ice.

Lipid and drug treatment. Stock solutions of the fatty acids AA, oleic acid, and elaidic acid were prepared in ethanol, and that of arachidic acid in chloroform. AA stock solutions were stored at −80°C under N_2; the other fatty acid stock solutions were prepared fresh. Working solutions of the free fatty acids were prepared by drying an aliquot of the stock solution under a stream of N_2 and then resuspending in 20 mM Tris-HCl (pH 7.5) by vortex mixing and sonicating. AA was bath sonicated (3 × 1 min at 0–4°C; Branson model 1200) for all experiments except for ones designed to test the relative effectiveness of various fatty acids on GAP-43 phosphorylation. For the latter experiments, each fatty acid was probe sonicated (Heat Systems Ultrasonics model W-220F) at the lowest setting until it was visibly dispersed. Working solutions of the free fatty acids were kept in the dark until use. Stock solutions of phosphatidylserine (PS) were prepared in 95% chloroform/5% methanol; its working solution was prepared by drying under a stream of N_2 and then resuspending in 20 mM Tris-HCl (pH 7.5) by vortex mixing and probe sonicating. Stock solutions of phorbol esters, DAG, and okadaic acid were prepared in dimethyl sulfoxide (DMSO); the working solutions were prepared by diluting in 20 mM Tris-HCl (pH 7.5) and vortex mixing. The final concentration of DMSO in these samples was 0.01% for phorbol esters, 0.05% for DAG, and 0.1% for okadaic acid. In control experiments, these concentrations of DMSO were found not to affect protein phosphorylation. A stock solution of (+)-α-tocopherol was prepared in ethanol, and diluted in 20 mM Tris-HCl (pH 7.5) to the desired concentration for the working solution. The final concentration of ethanol in the sample was 0.05%, a concentration that did not affect protein phosphorylation in control experiments.

Polyacrylamide gel electrophoresis and autoradiography. Samples (8 μg of protein) in SDS-containing sample buffer were heated at 95°C for 10 min before being resolved on 10.5% polyacrylamide gels (24 cm in

height, 1.5 mm in thickness) as described by Laemmli (1970). SDS-PAGE molecular weight standards were run in parallel with the samples. Electrophoresis was performed in a Hoeffer SE-600 gel apparatus at 25 mA per gel for 17–20 hr. These electrophoretic conditions were applied based on preliminary experiments showing consistently good separation of GAP-43 from other closely migrating phosphoproteins; under these conditions, proteins migrating faster than carbonic anhydrase (31 kDa) were run off the gel. Gels were fixed and stained with Coomassie brilliant blue (1 mg/ml) in a solution of 50% methanol/10% acetic acid, and then destained, vacuum dried, and set against preflashed Kodak X-OMAT AR film at −80°C. Quantitation of phospho–GAP-43 was achieved by subjecting the resulting autoradiogram to laser densitometric scanning using an LKB Ultrascan system. Multiple exposures were often taken so that all phospho–GAP-43 bands were within the linear range of sensitivity.

Western blot analysis of GAP-43. Electrophoretically separated proteins were transferred to PVDF membrane under conditions described by Towbin et al. (1979) in a Hoeffer TE52 transfer apparatus (0.5 A, 3.5 hr). Membranes were washed in Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.5; 150 mM NaCl) and then incubated in a blocking solution of 5% fetal bovine serum, 5% BSA in TBS containing 0.1% Tween-20 for 2 hr at room temperature. Blocked membranes were incubated overnight at 0–4°C with either affinity-purified sheep anti–GAP-43 IgG (Benowitz et al., 1988) or normal sheep IgG at a dilution of 1:1000 in blocking solution that contained 300 mM NaCl, rather than 150 mM NaCl, in order to reduce nonspecific binding of IgG. Membranes were washed in TBS containing 300 mM NaCl and 0.1% Tween-20, and incubated for 2 hr at room temperature with horseradish peroxidase–conjugated rabbit anti-sheep IgG at a dilution of 1:2000 in blocking solution. Antibody binding was visualized with diaminobenzidine (0.05% in TBS) in the presence of $NiCl_2$ (0.25%) and H_2O_2.

Proteolytic digestion of GAP-43. The ^{32}P-labeled protein (from 10 μg of synaptosomal membrane protein) migrating with an apparent molecular weight of 48 kDa was excised from a dried 10.5% polyacrylamide gel. The gel slab was homogenized in Laemmli sample buffer into which was added *Staphylococcus aureus V8* protease (1 ng/μl). The sample was incubated for 1 hr at room temperature and then loaded onto a 15% polyacrylamide gel; molecular weight standards were run in parallel. The gel was run at 12 mA until the dye front reached approximately 5 cm from the bottom. The gel was then fixed, stained, dried, and set against film to visualize the migration positions of the radiolabeled peptide products resulting from the proteolytic digestion.

Results

AA modulates GAP-43 phosphorylation

The effect of AA (10–500 μM) on GAP-43 phosphorylation was initially tested in the presence of 1 μM free Ca^{2+} and in its absence (1 mM EGTA). Under both conditions, AA affected GAP-43 phosphorylation in a dose-dependent and biphasic manner (Fig. 1*A,B*): GAP-43 phosphorylation was increased linearly in response to AA at concentrations between 0 and 50 μM; doses higher than 50 μM produced less of an enhancement in GAP-43 phosphorylation, and at the highest AA concentration tested (500 μM) GAP-43 phosphorylation was depressed below the baseline level. In the absence of Ca^{2+}, 50 μM AA nearly doubled the level of GAP-43 phosphorylation [81 ± 10 (±SEM) percent increase, $N = 14$]. The addition of Ca^{2+} alone elevated its phosphorylation approximately threefold (306 ± 49% increase, $N = 14$), and this was further doubled by the addition of 50 μM AA. Thus, a sixfold change (616 ± 109% increase, $N = 14$) in GAP-43 phosphorylation was produced through the interactive effects of Ca^{2+} and AA. Though Figure 1 shows changes in GAP-43 phosphorylation that are more pronounced than the mean changes, this experiment qualitatively represents 14 out of 14 independent experiments in demonstrating synergistic effects of AA (50 μM) and Ca^{2+} (1 μM). We tested whether the observed effects of AA and Ca^{2+} might be influenced by freezing the membranes before use, since it is possible that PKC activity may be altered by this process. However, we found that the changes in GAP-43 phosphorylation produced by AA and Ca^{2+} were very similar in freshly prepared membranes that had not been frozen before use and membranes prepared in parallel but frozen and thawed before the phosphorylation assay (data not shown).

The magnitude of change in GAP-43 phosphorylation produced by AA and Ca^{2+} was greater than for any other nerve terminal membrane phosphoprotein visualized on our gels (Fig. 1*A*). Another group of proteins that migrated with apparent molecular weights of 70–80 kDa were maximally stimulated 3.5-fold over baseline by 250 μM AA in the presence of Ca^{2+}. There were also low-molecular-weight proteins (apparent molecular weights between 10 and 21 kDa) whose phosphorylation was modulated by AA; however, these were not synaptosomal membrane proteins since they were found to be enriched in either the mitochondrial or myelin fraction (data not shown).

Verification that the 48 kDa phosphoprotein is GAP-43

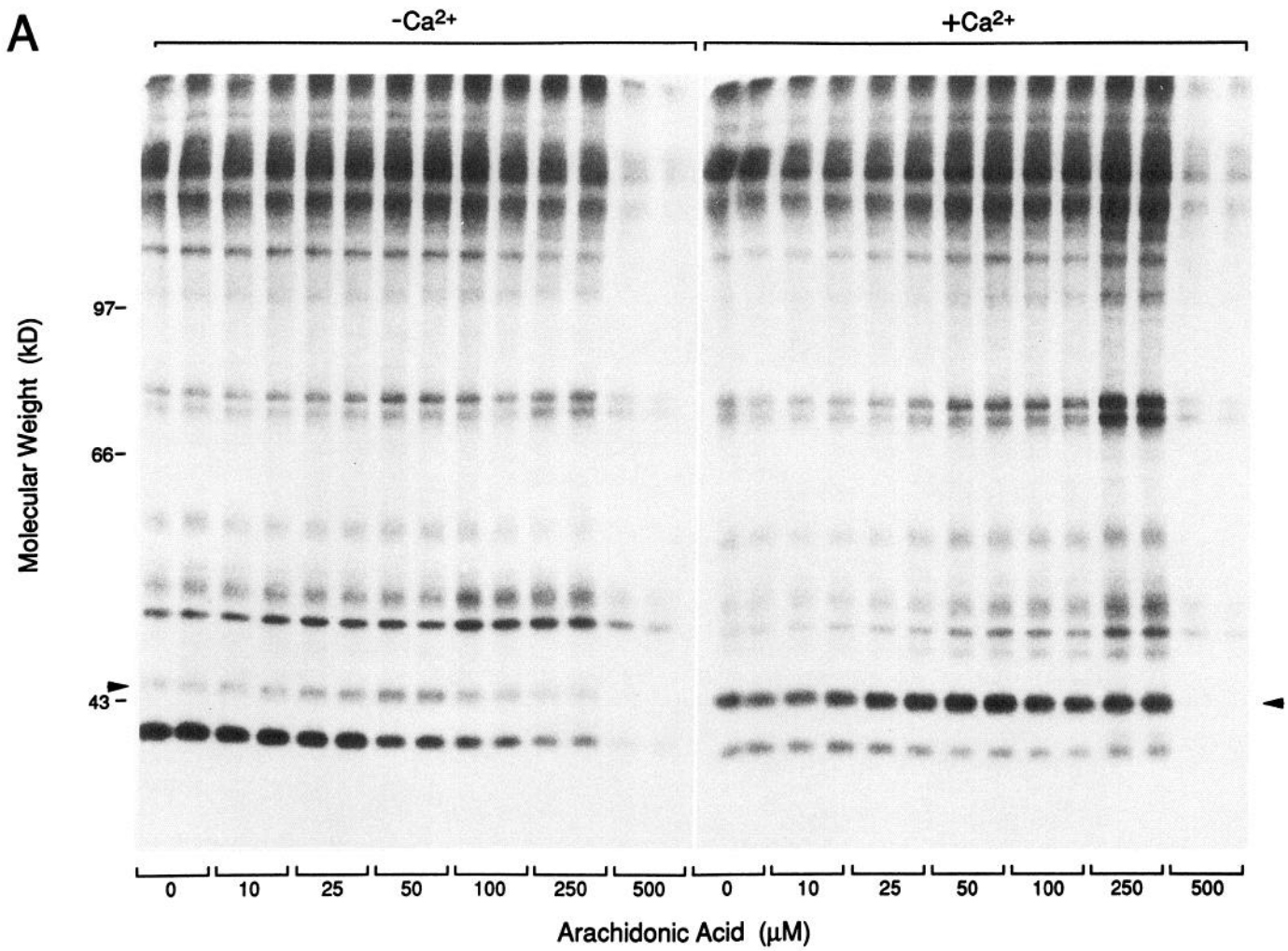
To demonstrate that the phosphoprotein migrating with an apparent molecular weight of 48 kDa was actually GAP-43, we applied Western blot analysis using anti–GAP-43 antibodies and phosphopeptide analysis after proteolytic digestion by *Staphylococcus aureus V8* protease. An autoradiogram of the [^{32}P]-labeled phosphoproteins from the synaptosomal membrane fraction (10 μg of protein) shows a prominent 48 kDa phosphoprotein (Fig. 2, lane 3) that was below that level of detection by Coomassie blue staining (100 μg of protein; lane 2). Western blotting of the synaptosomal membrane proteins (40 μg) using affinity-purified sheep anti–GAP-43 IgG revealed several immunoreactive isoforms of GAP-43 migrating at the same position as the 48 kDa phosphoprotein (lane 4); no staining was visualized using IgG from nonimmunized sheep (lane 5). Partial proteolytic digestion of the 48 kDa phosphoprotein with *Staphylococcus aureus V8* yielded phosphopeptides with apparent molecular weights of 27 and 13 kDa (lane 6), corresponding to the identified partial proteolytic digestion products of GAP-43 produced by *V8* (Oestreicher et al., 1989). The fact that no other phosphopeptides were generated by this partial digestion suggests that the radiolabeled 48 kDa band is GAP-43 exclusively.

Fatty acid specificity

To test whether the observed effects of AA on GAP-43 phosphorylation were specific, we compared the effects of AA to those of other structurally related lipids. Whereas AA (20:4) stimulated GAP-43 phosphorylation at 50 μM and inhibited its phosphorylation at 500 μM, the saturated 20-carbon fatty acid (arachidic acid; 20:0) suppressed GAP-43 phosphorylation at 50 μM and caused no change from control at 500 μM (Fig. 3). Fifty micromolar *cis*-unsaturated fatty acid oleic acid (18:1*c*) stimulated GAP-43 phosphorylation to a level comparable to that produced by AA; 500 μM oleic acid produced no change from control levels. The *trans*-isomer of oleic acid, elaidic acid (18:1*t*), caused no change from control at either 50 or 500 μM.

Effect of AA on GAP-43 phosphorylation is mediated by PKC

To determine whether the effects of AA on GAP-43 phosphorylation were mediated by changes in PKC activity, we used a specific peptide inhibitor (R^{19}FARKGALRQKNV^{31}) that corresponds to the pseudosubstrate region of PKC except for the substitution of Ala^{25} for the serine phospho-accepting site. Fig-



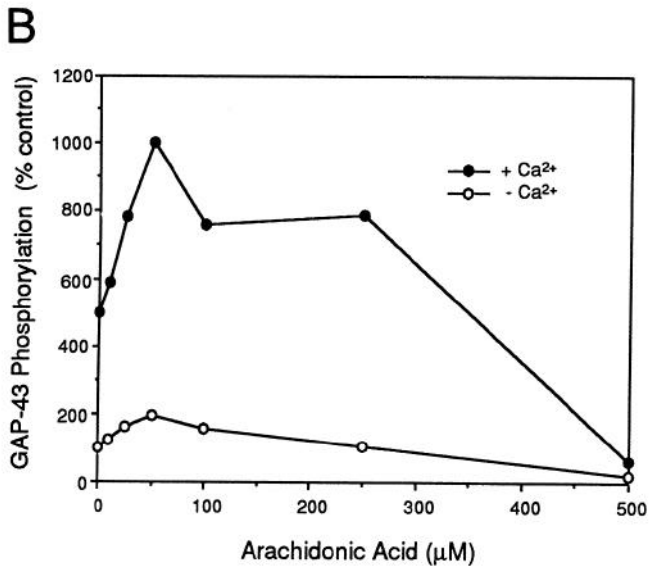


Figure 1. AA modulates the phosphorylation of GAP-43 in nerve terminal membranes. The incorporation of ^{32}P into synaptosomal membrane proteins was examined over a range of AA concentrations in the presence of free Ca^{2+} (1 μM) or in its absence (1 mM EGTA). ^{32}P-labeled proteins were separated by SDS-PAGE and visualized by exposing the dried polyacrylamide gel to X-OMAT AR film. The autoradiogram (*A*) was subjected to densitometric scanning to quantify levels of phospho–GAP-43 (*arrowheads*). Each point of the resultant plot (*B*) is the mean of duplicate samples. These results are representative of two complete dose–response series and at least 12 limited series.

ure 4 shows that 10 μM [Ala^{25}]PKC$_{(19-31)}$ blocked the stimulation of GAP-43 phosphorylation induced by 50 μM AA in both the absence and presence of Ca^{2+}. The peptide inhibitor also suppressed GAP-43 phosphorylation in the absence of exogenous AA under both Ca^{2+} conditions, and reduced the magnitude with which 500 μM AA inhibited the phosphorylation of GAP-43. The phosphorylation of several other proteins was also suppressed by this PKC inhibitor, most noticeably that of the proteins that migrated with apparent molecular weights of 70–80 kDa.

In addition to activating PKC, it is possible that AA could affect GAP-43 phosphorylation by altering protein phosphatase activity. To examine this possibility, nerve terminal membranes were treated with AA in the presence and absence of the protein phosphatase inhibitor okadaic acid at 1 μM. At this concentration, protein phosphatases 1 and 2A are inhibited specifically (Cohen et al., 1990) and the dephosphorylation of GAP-43 in synaptosomal membranes is almost completely blocked (Han and Dokas, 1991). We found that okadaic acid did not change either the direction or the magnitude of change in GAP-43 phosphorylation caused by 50 or 500 μM AA (data not shown). As an indication that the drug was active in this system, an elevation in the overall level of protein phosphorylation was observed.

Additionally, it is possible that the effects of AA on GAP-43 phosphorylation may be attributable to free radicals that can be generated by the metabolism of AA (Kukreja et al., 1986), and that have been shown to affect many biologically active molecules (see Halliwell and Gutteridge, 1985). We first tested whether the effects of AA on GAP-43 phosphorylation were altered by the presence of a lipid peroxide scavenger, (+)-α-tocopherol. (+)-α-tocopherol (50 μM) did not affect the ability of AA to modify GAP-43 phosphorylation (Fig. 5*A*). As evidence that (+)-α-tocopherol was active in this system, it suppressed the 500 μM AA-induced inhibition of phosphorylation of proteins that migrated with apparent molecular weights of 70–80 kDa. Second, a potential involvement of superoxide radicals was tested by comparing the effects of AA on GAP-43 phosphorylation in the presence and absence of a superoxide scavenging system, superoxide dismutase, and catalase. The presence of this scavenging system (each enzyme at 100 U/ml) did not alter the effects of AA on GAP-43 phosphorylation (Fig. 5*B*); however, as was observed with (+)-α-tocopherol, the 500 μM AA–induced inhibition in the phosphorylation of 70–80 kDa proteins was blocked by adding these enzymes to the reaction mixture (data not shown).

Taken together, these experiments suggest that the stimulatory effects of AA and Ca^{2+} on GAP-43 phosphorylation do not result from an inhibition of protein phosphatase activity or the effects of free radicals, but are very likely mediated by an activation of PKC. The fact that the peptide inhibitor only partially blocked the inhibitory effect of 500 μM AA on GAP-43 phosphorylation suggests that this latter effect is only partially mediated by PKC. The non-PKC-mediated component appears to be due not to alterations in protein phosphatase activity or effects of free radicals, but to some other undetermined mechanism, perhaps a perturbation of the lipid environment.

Effects of AA and Ca^{2+} on GAP-43 phosphorylation are synergistic

Interactions between Ca^{2+} and AA on GAP-43 phosphorylation were examined in more detail by exposing nerve terminal membranes to Ca^{2+} concentrations in the range of 10^{-9} M to 10^{-4} M in the presence and absence of exogenous AA (50 μM). Between 10^{-9} M and 10^{-7} M free Ca^{2+}, the effects of AA and Ca^{2+} were additive (Fig. 6*A,B*): GAP-43 phosphorylation was slightly enhanced by increasing the Ca^{2+} concentration within this range, and AA produced a relatively constant incremental change above that produced by Ca^{2+} alone. However, when the Ca^{2+} concentration was elevated to between 10^{-7} M and 10^{-5} M, levels that are likely to exist at the submembrane of nerve endings during

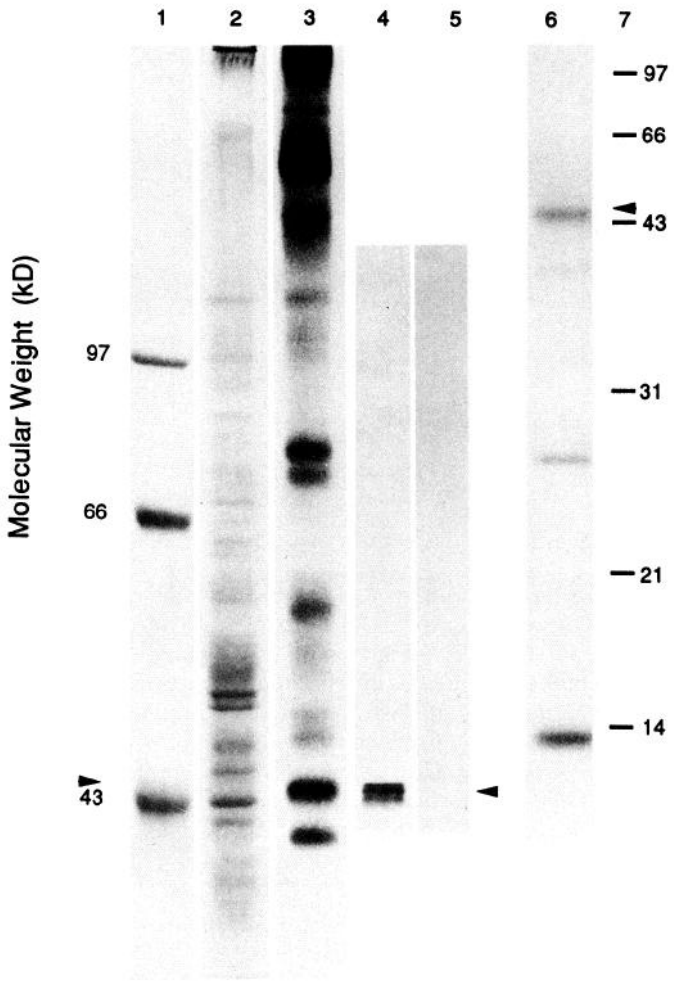


Figure 2. Verification that the 48kDa phosphoprotein is GAP-43. Synaptosomal membranes were phosphorylated and processed as described in Figure 1. *Lane 1,* Coomassie brilliant blue staining of molecular weight standards run in parallel with membrane proteins. *Lane 2,* Coomassie staining of proteins in the synaptosomal membrane fraction (100 μg of protein). *Lane 3,* Autoradiogram of phosphoproteins from the synaptosomal membrane fraction (10 μg of protein); note prominent band at 48 kDa. *Lane 4,* Western blot of synaptosomal membrane proteins (40 μg) reacted with affinity-purified sheep anti–GAP-43 IgG (Benowitz et al., 1988) reveals several isoforms migrating at the same position as the 48 kDa phosphoprotein. *Lane 5,* Control Western blot using affinity-purified IgG from nonimmunized sheep. *Lane 6,* Autoradiogram of partial proteolytic digest, using *Staphylococcus aureus V8* protease (1 ng/μl), of the 48 kDa phosphoprotein (from 10 μg of synaptosomal membrane protein) that was excised from a 10.5% polyacrylamide gel and rerun on a 15% polyacrylamide gel after digestion. ^{32}P-labeled phosphopeptides with apparent molecular weights of 48, 27, and 13 kDa correspond to the identified *V8* partial digestion products of GAP-43 (Oestreicher et al., 1989). *Lane 7,* Molecular weight standards run on a 15% polyacrylamide gel.

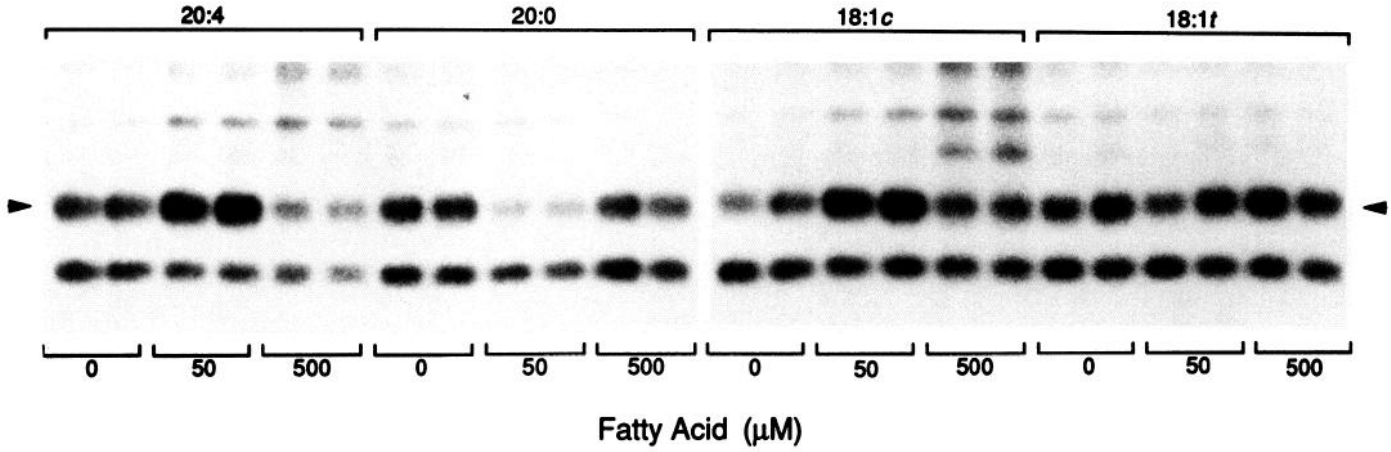


Figure 3. *Cis*-unsaturated, but not *trans*-unsaturated or saturated, fatty acids enhance GAP-43 phosphorylation. Synaptosomal membranes were treated as described in Figure 1 in the presence of AA (*20:4*), arachidic acid (*20:0*), oleic acid (*18:1c*), or elaidic acid (*18:1t*). The free Ca^{2+} concentration was 1 μM. *Arrowheads* indicate the migration position of GAP-43.

synaptic activity (Fogelson and Zucker, 1985), the effects of AA and Ca^{2+} were synergistic: GAP-43 phosphorylation was stimulated approximately 2.5-fold over this Ca^{2+} concentration range, and AA produced a greater than additive change, most strongly amplifying the effect of Ca^{2+} at 10^{-6} M. Within this Ca^{2+} concentration range, AA increased the sensitivity of GAP-43 phosphorylation to Ca^{2+} by over an order of magnitude (e.g., the level of GAP-43 phosphorylation obtained at 10^{-6} M Ca^{2+} in the presence of AA was higher than that achieved at 10^{-5} M Ca^{2+} in the absence of AA), and increased by over 50% the maximal level of GAP-43 phosphorylation achieved in response to Ca^{2+} alone. The time course of responsiveness of GAP-43 phosphorylation to Ca^{2+} (10^{-6} M) and AA (50 μM) was examined. We found that by 15 sec, the earliest time point tested, the effects of Ca^{2+} and AA acting independently and in conjunction were qualitatively similar, though of a somewhat lower magnitude, to those observed at 5 min.

In contrast to GAP-43 phosphorylation, the phosphorylation of the 70–80 kDa proteins did not respond synergistically to these effectors (Fig. 6*A*). Further elevating the Ca^{2+} concentration to 10^{-4} M produced no change in GAP-43 phosphorylation relative to that observed at 10^{-5} M Ca^{2+}, and no stimulatory effect of AA was observed. In one set of duplicate samples, the effects of free Ca^{2+} concentrations intermediate to those depicted in Figure 6 (i.e., $10^{-6.5}$, $10^{-5.5}$, $10^{-4.5}$) were tested in the absence and presence of AA, and were found to produce changes that closely follow the curve shown.

Relative efficacy of AA as compared to conventional PKC lipid activators

PS and DAG are cofactors for PKC activation, though the different subspecies of PKC have varying degrees of dependency on these two lipids (Huang et al., 1986; Jaken and Kiley, 1987). The effects upon GAP-43 phosphorylation of equimolar con-

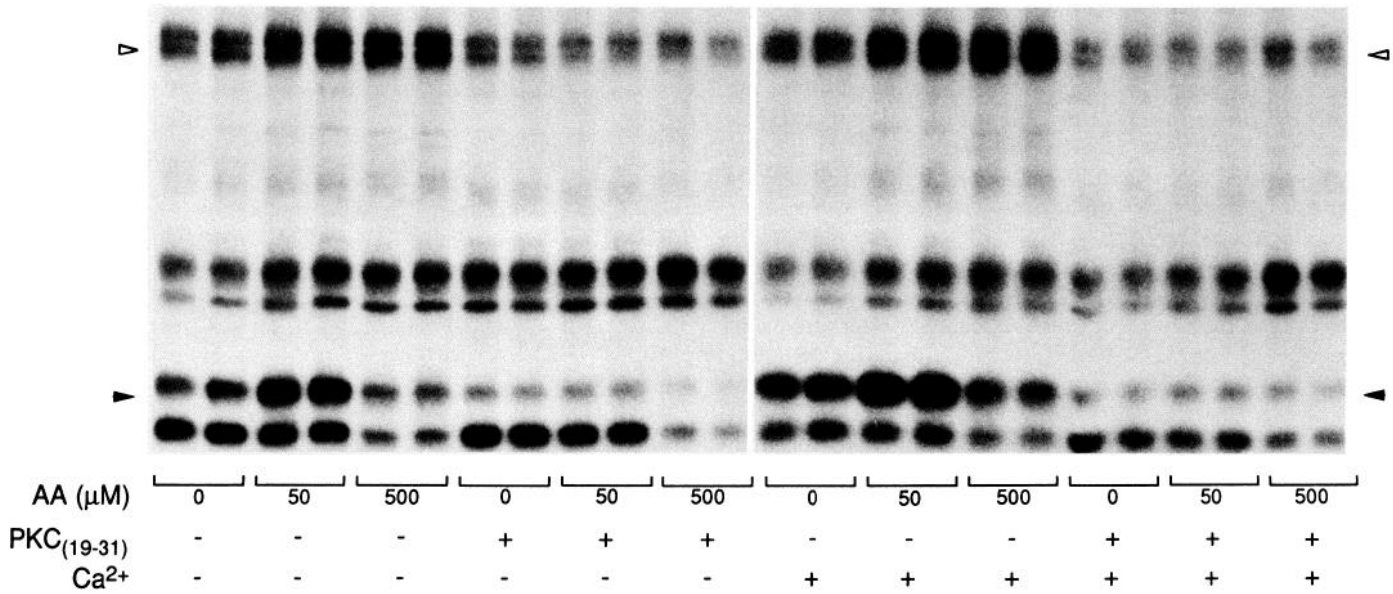


Figure 4. The AA-induced stimulation of GAP-43 phosphorylation is blocked by a specific inhibitor of PKC. Synaptosomal membranes were incubated with AA in the presence or absence of the pseudosubstrate inhibitor peptide of PKC, $PKC_{(19-31)}$ (10 μM); free Ca^{2+} was either 1 μM or was chelated with 1 mM EGTA. *Solid arrowheads* indicate the migration position of GAP-43; *open arrowheads* indicate proteins whose phosphorylation was also suppressed by the inhibitor.

centrations of AA, PS, and synthetic DAG (1,2-dioctanoyl-*sn*-glycerol) were tested in order to assess the potency of AA relative to the conventional PKC lipid activators. At 0.1 μM free Ca^{2+}, AA was slightly less effective in stimulating GAP-43 phosphorylation than DAG, but somewhat more effective than PS (Fig. 7). When the free Ca^{2+} concentration was elevated to 1 μM, AA stimulated GAP-43 phosphorylation as effectively as DAG, whereas PS suppressed the Ca^{2+}-induced stimulation of GAP-43 phosphorylation.

Effects of AA and DAG on GAP-43 phosphorylation are synergistic

The presence of DAG has been reported to potentiate the activation of α-, β-, and γ-subspecies of PKC by AA in *in vitro* assays using purified lipid and protein components (Shearman et al., 1991a; Shinomura et al., 1991) and in cellular assays of PKC activity (Lester et al., 1991; Larsson et al., 1992). The present study examined whether there is a synergistic interaction between the abilities of DAG and AA to stimulate the phosphorylation of GAP-43 in its native nerve terminal membrane. At 0.1 μM free Ca^{2+}, the effect of AA on GAP-43 phosphorylation was markedly potentiated by the addition of 4β-phorbol 12,13-dibutyrate (PDB), a specific PKC activator at the DAG binding site (Castagna et al., 1982) (Fig. 8): whereas AA (50 μM) alone caused a 1.5-fold increase in GAP-43 phosphorylation, this effect was amplified 3.5-fold in the presence of PDB (1 μM). At 1 μM free Ca^{2+}, AA enhanced GAP-43 phosphorylation to the same degree as PDB, and adding both activators together did not further increase its level of phosphorylation. The stimulatory effect of PDB was mediated by PKC, since adding to the reaction mixture 4α-phorbol (1 μM), a phorbol that does not activate PKC, did not change GAP-43 phosphorylation (data not shown). In contrast, phosphorylation of the 70–80 kDa proteins did not respond synergistically to AA and PDB; under both Ca^{2+} conditions, adding these two PKC effectors to the reaction mixture produced a less than additive effect on their phosphorylation.

Discussion

These results demonstrate that AA stimulates the phosphorylation of GAP-43 in its native nerve terminal membrane by activating PKC (Fig. 4); this effect cannot be attributed to changes in protein phosphatase activity or to the generation of free radicals (Fig. 5). The ability of AA to stimulate GAP-43 phosphorylation is synergistic with those of Ca^{2+} and DAG (Figs. 6, 8): AA increases the sensitivity of GAP-43 phosphorylation to Ca^{2+}, elevates the protein's maximal level of phosphorylation, and at resting Ca^{2+} levels potentiates the stimulation in GAP-43 phosphorylation caused by an analog of DAG (PDB). Extracellular AA levels have been reported to rise within a few minutes after the induction of LTP and to be elevated for many hours thereafter, probably as a result of the free fatty acid being hydrolyzed from postsynaptic membrane phospholipids upon activation of phospholipases (Clements et al., 1991). The present findings support the possibility that this lipid-diffusible molecule could act on presynaptic endings to potentiate increases in GAP-43 phosphorylation that would be expected to occur with elevations in intracellular Ca^{2+} and/or membrane-associated DAG secondary to presynaptic activation. The synergistic effects of AA and Ca^{2+}/DAG on GAP-43 phosphorylation might therefore act as a molecular AND gate to alter presynaptic structure and/or function in response to coordinated pre- and post-

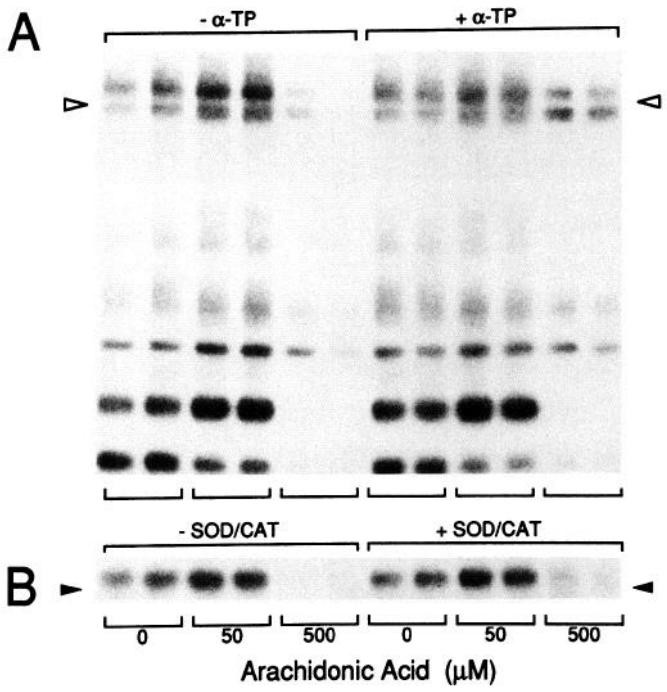


Figure 5. Effects of AA on GAP-43 phosphorylation are not mediated by free radicals. Synaptosomal membranes were incubated with AA in the presence or absence of the free radical scavenger (+)-α-tocopherol (*α-TP*), at 50 μM (*A*), or superoxide dismutase (*SOD*) and catalase (*CAT*), each at 100 U/ml (*B*). The free Ca^{2+} concentration was 1 μM. *Solid arrowheads* indicate migration position of GAP-43; *open arrowheads* indicate proteins whose level of phosphorylation in response to 500 μM AA was altered by *α-TP* (the same proteins were responsive to *SOD/CAT*; data not shown).

synaptic activity. The possibility that a diffusible retrograde messenger interacts with a presynaptic signal to induce LTP is supported by the finding that potentiation spreads to synapses of activated nerve terminals that neighbor the CA1 cell to which a depolarizing stimulus had been applied (Bonhoeffer et al., 1989). Although the precise transduction mechanisms by which changes in GAP-43 phosphorylation might alter presynaptic physiology are unknown, the protein's proposed involvement in phospholipid metabolism (Jolles et al., 1980), G_o protein activation (Strittmatter et al., 1990), calmodulin binding (Alexander et al., 1987), and membrane skeleton dynamics (Meiri et al., 1986) provides clues as to possible intermediate steps.

In vitro studies, using artificial lipid membrane and purified protein components, had previously demonstrated that AA affects the activity of the α-, β-, γ-, and δ-subspecies of PKC (Sekiguchi et al., 1987, 1988; Shinomura et al., 1991; Ogita et al., 1992), and in one of these studies, GAP-43 was used as a substrate for the β-subspecies (Shinomura et al., 1991). Using nerve terminal membranes in which the relationships between protein kinases and their substrates that occur *in vivo* are likely to be preserved, the present study allowed us to identify GAP-43 as a target of the synergistic effects of AA and Ca^{2+}/DAG. The magnitude of change that AA produced in GAP-43 phosphorylation was likely to have been influenced by the presence of PS and low levels of *cis*-unsaturated fatty acids and DAG endogenous to the biologic membrane (see Shinomura et al., 1991), and this may reflect the physiologic relationship between free AA and the phosphorylation of membrane-associated protein kinase substrates more closely than can be estimated by *in*

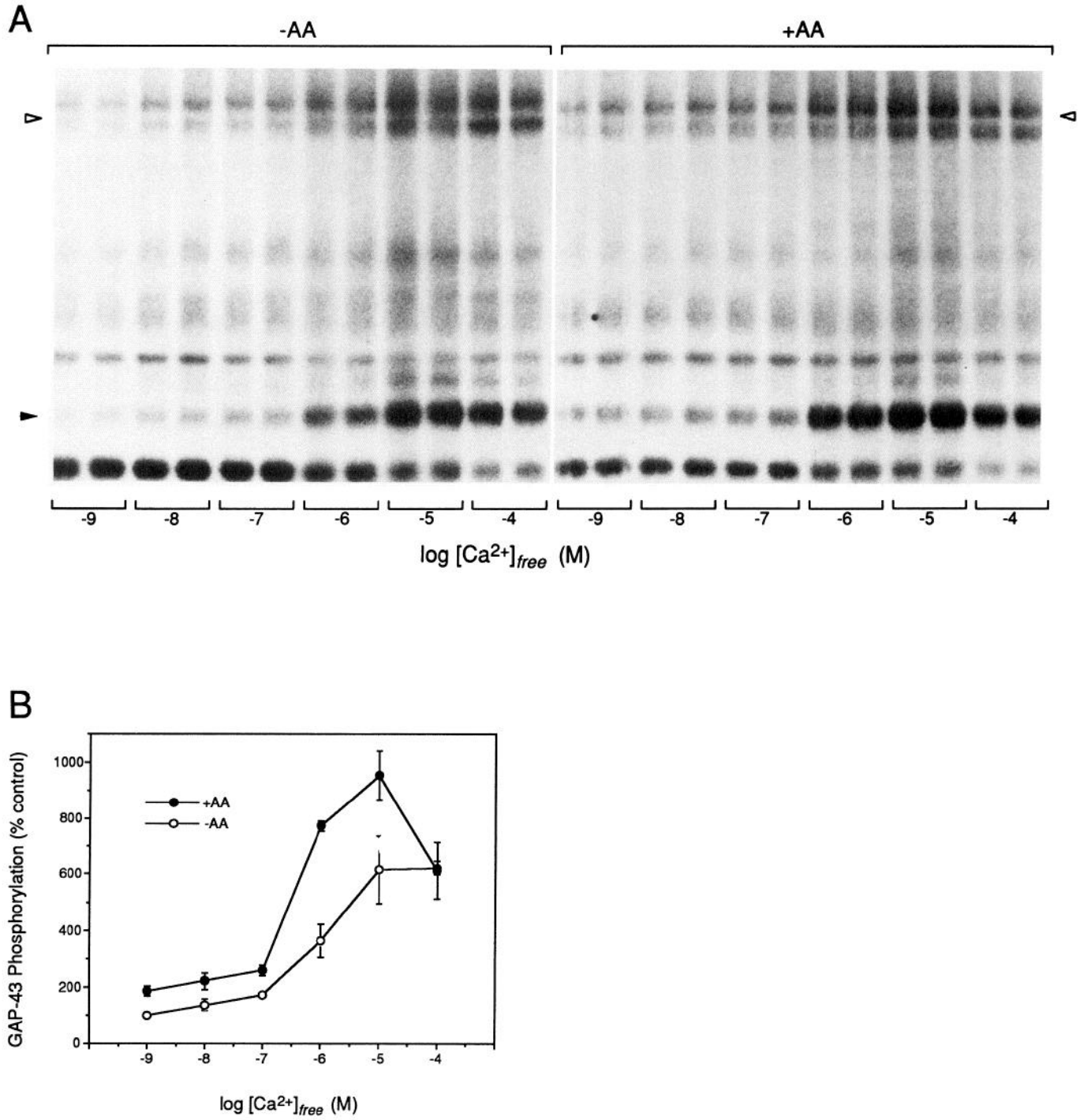


Figure 6. The effects of AA and Ca^{2+} on GAP-43 phosphorylation are synergistic. Synaptosomal membranes were phosphorylated in the presence or absence of AA (50 μM) under varying levels of free Ca^{2+}. A representative autoradiogram shows the response of GAP-43 phosphorylation (*solid arrowheads*) and that of other likely PKC substrates (*open arrowheads*) (*A*). The *plotted data* (*B*) are the mean ± SEM of three independent experiments.

vitro assays using isolated components. AA also affects, though to a lesser degree than GAP-43, the phosphorylation of proteins that migrated with apparent molecular weights of 70–80 kDa. These latter proteins are, at least in part, PKC substrates since their phosphorylation was inhibited by a specific PKC inhibitor (Fig. 4). Although the one-dimensional gel system employed in this study did not allow us to identify these proteins definitively, preliminary proteolytic digest experiments suggest that they could include MARCKS, PKC itself (both well-established PKC substrates in this molecular weight range), and synapsin.

Another *cis*-unsaturated fatty acid, oleic acid, shared with AA the ability to enhance GAP-43 phosphorylation at 50 μM (Fig. 3), consistent with the observation that oleic acid can activate PKC *in vitro* (Murakami and Routtenberg, 1985). However, following NMDA receptor stimulation (Pellerin and Wolfe, 1991) or LTP induction (Clements et al., 1991), there is a selective

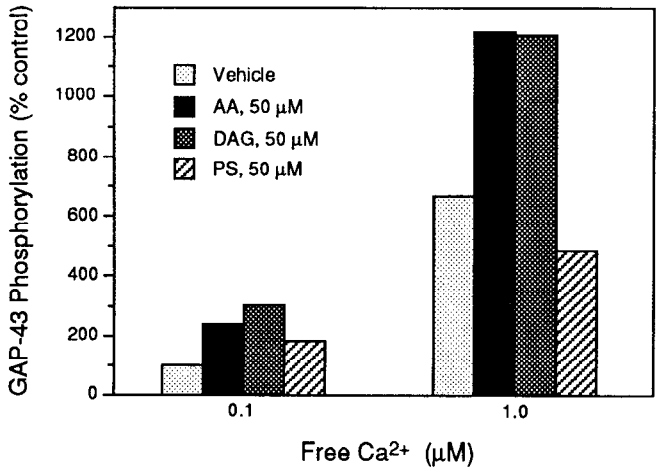


Figure 7. Relative efficacy of PKC activators in stimulating GAP-43 phosphorylation. Exogenous AA, synthetic DAG (1,2-dioctanoyl-*sn*-glycerol), or PS were each added at 50 μM. The free Ca^{2+} concentration was either 0.1 or 1.0 μM. Each bar is the mean of duplicate samples and is representative of two independent experiments.

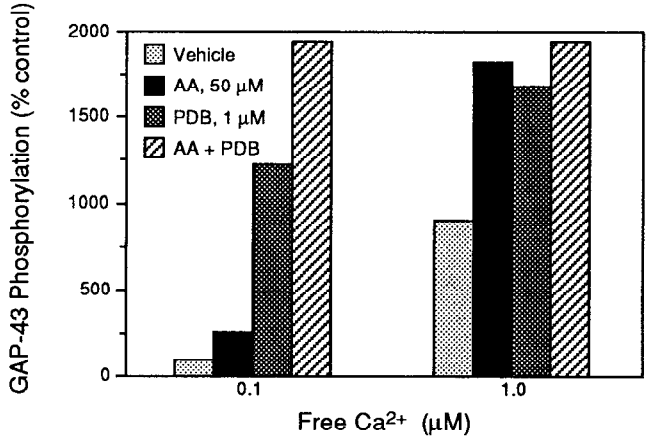


Figure 8. The effects of AA and DAG on GAP-43 phosphorylation are synergistic. Synaptosomal membranes were phosphorylated in the presence of exogenous AA (50 μM), the diacylglycerol analog PDB (1 μM), or both. The free Ca^{2+} concentration was either 0.1 or 1.0 μM. As a control, 4α-phorbol (1 μM) did not modify GAP-43 phosphorylation (not shown). Each bar is the mean of duplicate samples and is representative of three independent experiments.

increase in the extracellular levels of AA, suggesting that AA is more likely to be a physiologic retrograde messenger *in vivo* than other unsaturated or saturated fatty acids. Unlike AA, oleic acid is not metabolized by either lipoxygenase or cyclooxygenase, and thus it is the fatty acid itself, and not a metabolite, which is the active modulator of PKC activity; by extension, the effect of AA on GAP-43 phosphorylation is also likely to be mediated by the unmetabolized fatty acid. The effects of these two *cis*-unsaturated fatty acids on GAP-43 phosphorylation are not shared by the *trans*-isomer of oleic acid, elaidic acid, or the saturated 20-carbon fatty acid, arachidic acid. These findings are consistent with other reports of *trans*-unsaturated and saturated fatty acids being either less effective or completely lacking the effect of their *cis*-unsaturated fatty acid counterparts in a number of cellular systems (see Karnovsky et al., 1982; Barbour et al., 1989; Koda et al., 1989).

AA was found to affect GAP-43 phosphorylation in a biphasic manner, with concentrations from 0 to 50 μM linearly increasing its phosphorylation and higher concentrations producing less of an effect (Fig. 1). Although the mechanism underlying this inverted U-shaped dose–response curve is unknown, similar biphasic effects of AA on PKC activity have been described for purified γ-subspecies using histone as substrate (Sekiguchi et al., 1987, 1988), and for purified β-subspecies when either the concentration of histone in the reaction mixture was relatively low (Buday and Farago, 1990) or when purified GAP-43 was used as the substrate (Shinomura et al., 1991). The optimal AA concentration for stimulating PKC activity in these reports was 20–100 μM, which is in good agreement with the present results. In hippocampal slices maintained under low-Mg^{2+} conditions, AA induces LTP in a similar biphasic fashion, with effectiveness diminishing at concentrations greater than 100 μM (Kato et al., 1991). Thus, the inverted U-shaped dose–response curve we observed might reflect either an inherent kinetic property of the active PKC subspecies or some interaction with its membrane environment.

A question arising from the present study is the identity of the PKC subspecies responsible for phosphorylating GAP-43 in our membrane preparation and *in vivo*. Our findings that GAP-43 phosphorylation is Ca^{2+} sensitive and responds biphasically to AA are compatible with the previously described kinetic properties of both the purified β- and γ-PKC subspecies (Sekiguchi et al., 1988; Shearman et al., 1989; Sheu et al., 1990; Shinomura et al., 1991). Both the β- and γ-subspecies have been detected in rat cerebrocortical nerve terminals (Shearman et al., 1991b) and in our preparation (using subspecies-specific antibodies kindly provided by Dr. Tsunao Saitoh, UC San Diego; data not shown). However, direct comparisons between results from the present PKC assays, using biologic membrane containing its native complement of lipids, and those from *in vitro* assays of individual PKC subspecies in artificial lipid membrane cannot be made because the kinetic properties of PKC are influenced by the lipid environment within which the enzyme resides (Takai et al., 1979a,b; Kishimoto et al., 1980). Another confounding factor is that membranes from lysed synaptosomes may contain, in addition to PKC subspecies that are associated with the membrane *in vivo*, subspecies that may have translocated from the cytosol to the membrane (see Kraft and Anderson, 1983; Wolf et al., 1985). The variability in basal and stimulated PKC activity that we found from one preparation to another may have been due to differences in the endogenous lipid environment and in the degree to which the subspecies insert into the membrane (see Huang, 1989). Nevertheless, our data suggest that even if more than one PKC subspecies is involved in GAP-43 phosphorylation, the dominant kinase is a common target of AA, Ca^{2+}, and DAG, since their effects are synergistic and not simply additive.

In conclusion, the enhancement of GAP-43 phosphorylation by AA may be important in allowing synaptically related neurons to communicate bidirectionally. AA can be released from an activated postsynaptic neuron and may diffuse retrogradely to potentiate an increase in the level of GAP-43 phosphorylation initiated by the coincident activity of its presynaptic partner. An elevation in GAP-43 phosphorylation may in turn facilitate the release of transmitter from presynaptic endings, and thereby strengthen the communication back to its postsynaptic neuron in the anterograde direction. Such reciprocal interactions might be critical for the coordination of pre- and postsynaptic activity required for LTP and other synaptic modifications; during de-

velopment, these interactions may play a role in the activity-dependent tuning of synaptic relations between nerve terminals containing high levels of GAP-43 and postsynaptic neurons expressing NMDA receptors.

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