Ca\(^{2+}\)/Calmodulin-independent Activation of Calcineurin from Dictyostelium by Unsaturated Long Chain Fatty Acids*

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This study describes a novel mode of activation for the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin. Using purified calcineurin from *Dictyostelium discoideum* we found a reversible, Ca\(^{2+}\)/calmodulin-independent activation by the long chain unsaturated fatty acids arachidonic acid, linoleic acid, and oleic acid, which was of the same magnitude as activation by Ca\(^{2+}\)/calmodulin. Half-maximal stimulation of calcineurin occurred at fatty acid concentrations of approximately 10 \(\mu M\) with either \(p\)-nitrophenyl phosphate or RII phosphopeptide as substrates. The methyl ester of arachidonic acid and the saturated fatty acids palmitic acid and arachidic acid did not activate calcineurin. The activation was shown to be independent of the regulatory subunit, calcineurin B. Activation by Ca\(^{2+}\)/calmodulin and fatty acids was not additive. In binding assays with immobilized calmodulin, arachidonic acid inhibited binding of calcineurin to calmodulin. Therefore fatty acids appear to mimic Ca\(^{2+}\)/calmodulin action by binding to the calmodulin-binding site.

The Ca\(^{2+}\)/calmodulin-stimulated protein Ser/Thr phosphatase (protein phosphatase 2B; calcineurin) plays a pivotal role in various cellular processes. Its function in T-cell activation including the dephosphorylation and mobilization of the transcription factor NF-AT (nuclear factor of activated T-cells) and the enhanced expression of interleukin-2 has been extensively studied (for review see Refs. 1 and 2). The structure of calcineurin has been conserved from yeast to man (3). The holoenzyme consists of a catalytic subunit (calcineurin A) and a regulatory subunit (calcineurin B). Calcineurin A varies in size from species to species (58–73 kDa) (4). Calcineurin B is a 19-kDa Ca\(^{2+}\)-binding protein homologous to calmodulin. High affinity binding of Ca\(^{2+}\) to calcineurin B at low Ca\(^{2+}\) concentrations (\(K_D < 10^{-8} M\)) leads to formation of the calcineurin A/B complex. Calcineurin B decreases the \(K_m\) of calcineurin A for its protein substrates (5).

The holoenzyme is tightly regulated by Ca\(^{2+}\)/calmodulin (for review see Refs. 6 and 7). Binding of Ca\(^{2+}\)/calmodulin to a basic amphipathic \(\alpha\)-helix (for review see Ref. 8) relieves inhibition by a C-terminal autoinhibitory domain and activates the enzyme by changing \(V_{max}\) (5). This suggests hydrophobic as well as ionic interactions between calcineurin A and Ca\(^{2+}\)/calmodulin (Ref. 6 and references therein). *Dictyostelium* calcineurin has previously been characterized in this laboratory (9, 10).

The enzyme has some unusual features as compared with its counterparts from other species, e.g. N-terminal and C-terminal extensions (9) resulting in its high molecular mass of 73 kDa. Expression of the single calcineurin A gene is developmentally regulated both at the mRNA and the protein level (9). Biochemical characterization of the protein purified from recombinant bacteria and an overexpressing *Dictyostelium* cell line revealed enzymatic properties that are fairly comparable with calcineurins from other sources (10). Pharmacological evidence suggests at least three distinct roles for *Dictyostelium* calcineurin. Using the calcineurin inhibitors FK 506 and cyclosporin A, Horn and Gross (11) have provided evidence that calcineurin is involved in both pathways of *Dictyostelium* differentiation. Moniakis et al. (12) have shown by using cyclosporin A that the Ca\(^{2+}\)-regulated expression of the Ca\(^{2+}\) ATPase, PAT1, is under the control of calcineurin. The calcineurin inhibitor deltamethrin, but not the inactive analog permethrin, was shown to inhibit chemotaxis toward the attractant cAMP, suggesting a role in chemotaxis for calcineurin (13).

Ca\(^{2+}\) signaling in *Dictyostelium* is essential for chemotaxis toward cAMP (for review see Ref. 14). The binding of cAMP to its cell surface receptors leads to Ca\(^{2+}\) influx across the plasma membrane and to an increase in the cytosolic Ca\(^{2+}\) concentration (15–17). It was shown that several inhibitors for phospholipase \(A_2\) blocked cAMP induced Ca\(^{2+}\) influx to a great extent, suggesting a prominent role for phospholipase \(A_2\) in the signal transduction pathway leading to Ca\(^{2+}\) uptake (18). Phospholipase \(A_2\) releases fatty acids from phospholipids. It was demonstrated that long chain fatty acids mimic the action of cAMP by bypassing both cAMP-receptor stimulation and activation of phospholipase \(A_2\), directly inducing Ca\(^{2+}\) influx (18). Moreover long chain fatty acids liberate Ca\(^{2+}\) from internal stores and thus induce capacitative Ca\(^{2+}\) entry (19).

*Dictyostelium* cells, which live as amoebae in the soil, are subject to large environmental changes, e.g. fluctuations in ion supply. Thus robust mechanisms supporting Ca\(^{2+}\) signaling are required to maintain chemotactic capability. One group of candidates for signaling molecules are long chain fatty acids (18, 19). In this study we have investigated direct effects of long chain fatty acids on *Dictyostelium* calcineurin activity and found a novel, Ca\(^{2+}\)/calmodulin-independent mode of activation for calcineurin.

**EXPERIMENTAL PROCEDURES**

*Materials and Purified Proteins—Arachidonic acid (free acid) was from Fluka (Buchs, Switzerland), arachidonic acid methyl ester, oleic acid, and bovine albumin (fatty acid-free) were from ICN (Eschwege, Germany). Arachidic acid and palmitic acid were from Sigma (Munch, Germany), and linoleic acid was from ICT (Bad Homburg, Germany). Calmodulin-Sepharose 4B was from Amersham Pharmacia Biotech (Freiburg, Germany). The Biomol Green reagent, the phosphate standard, and RII phosphopeptide were from Biomol (Hamburg, Germany). p-Nitrophenyl phosphate was from Merck (Darmstadt, Germany). Calmodulin (bovine brain) was from Alexis (San Diego, CA). Calcineurin A was from the Fakultät für Biologie, Universität Konstanz.*

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from Dictyostelium was purified from an overexpressing cell line as described previously (10). Purification and characterization of recombinant Dictyostelium calcineurin B will be described elsewhere.

Phosphatase Activity Assays—Phosphatase activity assays with p-nitrophenyl phosphate (pNPP) as substrate were performed routinely in duplicate or triplicate according to Hellstern et al. (10). Briefly, calcineurin A (final concentration, 140 nM) was preincubated in the absence or presence of calcineurin B (140 nM) and/or calmodulin (1 μM) in buffer A (50 mM Tris-HCl, 0.5 mM MnCl₂, 0.5 mM dithiothreitol, 0.2 mM CaCl₂, 20 mM MgCl₂, pH 7.8) for 1 h. Subsequently fatty acid solutions or 1% ethanol (vehicle) as a control were added 1 min prior to the addition of pNPP (20 mM). The reaction (total volume, 150 μl) was stopped after 1 h by adding an equal volume of 1 m Na₂CO₃, 20 mM EDTA, 2 mM EGTA, pH 8.0. All incubations were performed at 30 °C. The absorbance of the dye was measured at 405 nm and corrected for blank absorbance.

For determination of phosphatase activity with RII phosphopeptide as a substrate, the reaction was initiated by adding 55 μM of the phosphopeptide (total volume after addition, 50 μl). To terminate the reaction, 2 volumes of Biomol Green reagent were added after 40 min. The test was linear from 10 to 60 min. The dye was allowed to develop for 25 min before measuring the absorbance at 620 nm. In the experiments with calcineurin A alone, the final concentration of the phosphatase was 420 nM. In the presence of both calcineurin A and B, the concentration of each was 252 nM. The concentration of calmodulin was 1 μM throughout. 1 unit is defined as the release of 1 nmol phosphate min⁻¹ mg protein⁻¹.

Binding of Calcineurin A to Calmodulin-Sepharose 4B—Binding of calcineurin to calmodulin-Sepharose 4B in the absence or presence of arachidonic acid or arachidonic acid methyl ester was determined at 4 °C in a total volume of 250 μl containing 170 mM calcineurin A, arachidonic acid, or arachidonic acid methyl ester (20 μM each) or 1% ethanol (vehicle), and 100 μl of a calmodulin-Sepharose 4B slurry equilibrated in buffer B (50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, 1 mM EDTA, 0.5 mM dithiothreitol, pH 7.8). The mixture was incubated under constant agitation for 30 min. The Sepharose was pelleted by centrifugation in an Eppendorf centrifuge for 2 x 150-μl aliquots of the supernatant were withdrawn, and 100 μl was loaded onto SDS-polyacrylamide gels and subsequently subjected to Western blotting. For washing the resin the volume was adjusted to 250 μl with buffer B, resuspended, and recentrifuged. This procedure was repeated twice. After the second wash the supernatant was removed thoroughly. The protein was eluted by adjusting the volume to 250 μl with elution buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 20 mM EGTA, 0.5 mM dithiothreitol, pH 7.8), resuspending the resin, and incubation for 15 min. Elution was repeated once. Samples for SDS-polyacrylamide gel electrophoresis were withdrawn after each step as above.

To elute calcineurin A with arachidonic acid, the phosphatase was bound to calmodulin-Sepharose 4B in buffer B as above. The resin was washed twice with the same buffer. Subsequently buffer B containing 20 μM arachidonic acid or 20 μM arachidonic acid methyl ester or 1% ethanol was added, and the samples were incubated for 15 min. The gel was washed once with buffer B, and the final elution was carried out with elution buffer as above. Aliquots for SDS-polyacrylamide gel electrophoresis were taken as described above.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting—Proteins were chromatographed on SDS-polyacrylamide gels (20), blotted onto nitrocellulose membranes (BA85, Schleicher & Schüll) (21), and probed with a rabbit antiseraum (dilution, 1:5000) against recombinant Dictyostelium calcineurin A as described previously (9).

Other Methods—The critical micellar concentration of fatty acids was determined as described by Chattopadhyay and London (22). Ethanolic stock solutions of arachidonic acid, linoleic acid, and arachidonic acid methyl ester (100 mM each) were stored under nitrogen at ~80 °C. Diluted fatty acid solutions were prepared from stock aliquots immediately prior to the experiment and kept under nitrogen until use. All other fatty acid solutions were freshly prepared from solids or oils.

RESULTS

Effect of Arachidonic Acid on Calcineurin Activity—Fig. 1A shows a dose-response curve for activation of purified Dictyostelium calcineurin A by arachidonic acid (20:4) in the absence of calmodulin and calcineurin B. Half-maximal stimulation of the phosphatase activity with pNPP as substrate occurred between 5 and 10 μM of the fatty acid. Maximal stimulation was observed at 30 μM arachidonic acid and resulted in an approximately 4-fold activation. Arachidonic acid and calmodulin activated the phosphatase to the same extent. Calcineurin activities in the presence of 30 μM arachidonic acid and 1 μM calmodulin are not significantly different as determined by using the t test (p < 0.05). Basal activity amounted to 75.5 ± 6.9 and 117.3 ± 17.1 units for A and B, respectively.

Fig. 1. Dose-response relation for arachidonic acid-stimulated calcineurin activity. Arachidonic acid-stimulated (○) and Ca²⁺/calmodulin-stimulated (●) calcineurin activity was measured with pNPP (20 mM) as substrate as outlined under Experimental Procedures.* A, assays were performed in the presence of 140 nM calcineurin A, B, assays were performed in the presence of calcineurin A and calcineurin B (140 nM each). Increasing doses of arachidonic acid or the saturating concentration of 1 μM calmodulin were applied. Data represent the means ± S.E. of triplicates (arachidonic acid) or duplicates (calmodulin) from two independent experiments. Data are presented as the activity relative to basal activity measured in the absence of arachidonic acid or calmodulin. The activities in the presence of 30 μM arachidonic acid and 1 μM calmodulin are not significantly different as determined by using the t test (p < 0.05). Basal activity amounted to 75.5 ± 6.9 and 117.3 ± 17.1 units for A and B, respectively.

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2. The abbreviations used are: pNPP, p-nitrophenyl phosphate; TPR, tetratricopeptide repeats.
kinase (type II) is a model peptide substrate for calcineurin (23). As shown previously (10), the basal activity of Dictyostelium calcineurin A toward the RII peptide in the absence of the regulatory subunit calcineurin B (Fig. 2) is lower than toward the nonpeptide substrate pNPP (0.4 ± 0.3 unit versus 152 ± 32 units for RII peptide (n = 4) and pNPP (n = 25), respectively). Similar observations have been reported for recombinant Neurospora crassa calcineurin A (24).

Calcineurin B activates the catalytic subunit by lowering the $K_m$ (5). This is reflected by the rise in calcineurin activity upon addition of calcineurin B. In the absence of calcineurin B, Ca$^{2+}$/calmodulin activated calcineurin to approximately the same extent. Full activation was obtained in the presence of both calcineurin B and calmodulin. The activation by calmodulin in the absence of calcineurin B could be mimicked by the addition of 50 μM arachidonic acid. Addition of both calcineurin B and arachidonic acid again yielded full activation of the phosphatase. 50 μM arachidonic acid is a saturating dose for stimulation of the phosphatase activity. Half-maximal activity was obtained at 10 μM of the fatty acid (data not shown). Activation of calcineurin by arachidonic acid is therefore also valid for a peptide substrate.

**Effects of Other Unsaturated Fatty Acids on Calcineurin Activity**—The unsaturated long chain fatty acids linoleic acid (18:2) and oleic acid (18:1) exerted effects on calcineurin similar to those of arachidonic acid with respect to half-maximal and maximal stimulating concentrations (Fig. 3). The maximal stimulation by linoleic acid shown in Fig. 3 seems to be lower than that of oleic acid or arachidonic acid, but there is no significant difference between the maximal stimulation by linoleic acid and by Ca$^{2+}$/calmodulin (t test at the 0.05 level).

**Specificity of Stimulation by Unsaturated Fatty Acids**—To investigate the specificity of the effect of long chain unsaturated fatty acids on calcineurin and to obtain insight into the mechanism of stimulation, the methyl ester of arachidonic acid and the saturated fatty acids arachidic acid (20:0) and palmitic acid (16:0) were used. The methyl ester had no effect on the phosphatase activity (Table 1). Moreover, both of the saturated fatty acids neither stimulated basal activity nor influenced the Ca$^{2+}$/calmodulin-stimulated activity at concentrations that are saturating for the unsaturated fatty acids (50 μM). The data in Table I also show that the stimulation of the phosphatase activity by unsaturated fatty acids is not additive to Ca$^{2+}$/calmodulin stimulation because the stimulation in the presence of both unsaturated fatty acids and Ca$^{2+}$/calmodulin did not exceed the values in the presence of either compound. Taken together, these results suggest that both the carboxyl group and the unsaturated character of the fatty acids are required for stimulation of calcineurin. Calmodulin and unsaturated fatty acids may exert their effects through a similar mechanism, possibly by binding to the same site.

**Reversibility of Calcineurin Activation by Arachidonic Acid**—Next we analyzed whether the activation of calcineurin by arachidonic acid is reversible. Bovine serum albumin binds to arachidonic acid with high affinity ($K_a$, 62 nm) (25) and should therefore effectively abolish activation by the fatty acid. Activation of calcineurin by 30 μM arachidonic acid resulted in an approximately 3-fold stimulation over basal activity (Fig. 4).
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**FIG. 4.** Reversibility of the activation of calcineurin by arachidonic acid. Phosphatase assays were carried out in the presence of 140 nM calcineurin A. Left bar, calcineurin A was preincubated in buffer A for 50 min. Subsequently, 30 μM arachidonic acid (AA) was added and incubated for 10 min, and then 20 μM pNPP was applied to start the reaction. The reaction was stopped after 1 h as outlined under “Experimental Procedures.” Middle bar, same as above but 1 min before starting the reaction 15 μM bovine serum albumin (BSA) was added. Right bar, calcineurin A was preincubated for 1 or 10 min in the presence of 30 μM arachidonic acid. After this period bovine serum albumin was added, incubated for 1 min before the addition of calmodulin (CaM, 1 μM), and incubated for a further 60 or 50 min. Thereupon the reaction was started and finally stopped as above. Data are the means ± S.E. of duplicates from three independent experiments. Data are given as the activity relative to basal activity measured in the absence of arachidonic acid or calmodulin.

Addition of 15 μM fatty acid-free bovine serum albumin reduced the phosphatase activity to basal levels, indicating that complexing the fatty acid stops the activation process. The enzyme was thereafter susceptible to stimulation by Ca²⁺/calmodulin as shown in Fig. 4. Activation by Ca²⁺/calmodulin after capturing the fatty acid was of the same magnitude as the response to arachidonic acid, demonstrating that the structure of calcineurin had not been disrupted irreversibly by arachidonic acid.

**Inhibition of Calcineurin Binding to Immobilized Calmodulin by Arachidonic Acid**—Several lines of evidence suggested that unsaturated fatty acids and Ca²⁺/calmodulin exert their effects on calcineurin activity through similar mechanisms by binding to the same site. Therefore we tested whether arachidonic acid interferes with binding of purified Dictyostelium calcineurin to immobilized calmodulin. In a first set of experiments we examined binding of calcineurin to calmodulin in the presence of arachidonic acid (Fig. 5, A–C). Arachidonic acid inhibited binding of the phosphatase to calmodulin-Sepharose (Fig. 5B), whereas with equal concentrations of arachidonic acid methyl ester (Fig. 5C) most of the protein bound to the gel and could subsequently be eluted by buffer containing EGTA.

In a second set of experiments we investigated whether a buffer containing arachidonic acid was able to elute prebound calcineurin from calmodulin-Sepharose (Fig. 5, D–F). This is indeed the case, as shown in Fig. 5E. About half of the prebound protein could be displaced in a single elution step from calmodulin (Fig. 5E, lane E1), whereas all the protein remained bound to calmodulin in the presence of arachidonic acid methyl ester (Fig. 5F) and could only be eluted from calmodulin-Sepharose by incubation with EGTA.

**DISCUSSION**

We show here that the long chain unsaturated fatty acids arachidonic acid, linoleic acid, and oleic acid activate calcineurin from Dictyostelium. The activation is independent of Ca²⁺/calmodulin. Half-maximal activation occurred at a concentration (5–10 μM) well below the critical micellar concentration (20 μM), indicating that it is not merely an effect of calcineurin binding to micelles. Polito and King (26) have described activation of calcineurin from bovine brain by binding to acidic phospholipids. This effect is clearly distinct from the one reported here because phospholipids bind to the regulatory subunit of calcineurin B (27). Activation of Dictyostelium calcineurin by unsaturated fatty acids is independent of the presence of calcineurin B. Stimulation of enzymatic activity is specific for unsaturated fatty acids because saturated fatty acids were not active. Moreover, the negatively charged carboxy group is necessary for the activation process because the methyl ester derivative of arachidonic acid was inactive. Stimulation of calcineurin by fatty acids was demonstrated with chemically distinct substrates, the chromogenic substrate pNPP, and the RII phosphopeptide substrate corresponding to a phosphorylation site within the RII subunit of the cAMP-dependent protein kinase. The effect is readily reversible as shown by relieving the activation of calcineurin through capturing the fatty acids by albumin. Calcineurin activation by fatty acids could be a means for in vivo regulation. We suggest that stimulation by fatty acids or Ca²⁺/calmodulin are alternative mechanisms.

What are the structural determinants for fatty acid binding to calcineurin A? The most likely candidates are the hydrophobic binding sites for calcineurin B and Ca²⁺/calmodulin. The following data exclude the calcineurin B-binding site as a candidate: (i) arachidonic acid activates the phosphatase both in the absence and in the presence of calcineurin B (Fig. 1). Even in the presence of low Ca²⁺ concentrations the binding of calcineurin B to the catalytic subunit is nearly irreversible (5),...
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and the calcineurin A/B complex is only dissociated in the presence of urea, SDS, or guanidine hydrochloride (Ref. 6 and references therein) and (ii) if arachidonic acid disturbed calcineurin B binding to calcineurin A, then the phosphatase activity would not increase upon addition of calcineurin B with RII phosphopeptide as a substrate (Fig. 2). Our results suggest that Ca²⁺/calmodulin and unsaturated fatty acids share the same binding domain and exert their effects through similar mechanisms: (i) arachidonic acid inhibits binding of calcineurin A to calmodulin and displaces calcineurin from immobilized calmodulin (Fig. 5); (ii) activation by fatty acids and by Ca²⁺/calmodulin is not additive (Table I); (iii) not only the full stimulation by Ca²⁺/calmodulin of the RII phosphopeptide phosphatase activity in the presence of calcineurin B could be mimicked by arachidonic acid but also the partial stimulation in the absence of calcineurin B (Fig. 2); and (iv) maximal activation by unsaturated fatty acids and Ca²⁺/calmodulin is in the same range (Figs. 1 and 2).

Although these data argue strongly in favor of direct competition of Ca²⁺/calmodulin and arachidonic acid for binding to the same site, we cannot distinguish between fatty acid binding to the enzyme and to Ca²⁺/calmodulin in our competition experiments. Binding of Ca²⁺ to calmodulin results in exposure of hydrophobic and acidic amino acids on the protein surface that interact with a basic amphipathic α-helix within the target protein (28). Therefore hydrophobic and ionic interactions might be required for binding of fatty acids to the basic amphipathic α-helix, which forms the Ca²⁺/calmodulin-binding site (8). Accordingly, not only the unsaturated hydrophobic character of the lipids is essential for proper activation but also the additional negatively charged carboxy residue. Therefore, the acidity of Ca²⁺/calmodulin renders an interaction with acidic lipids unlikely.

Several calmodulin-binding proteins were found to be activated by long chain fatty acids. Human erythrocyte Ca²⁺-ATPase was activated by oleic and linoleic acid (29–31), bovine brain cyclic nucleotide phosphodiesterase was stimulated by oleic acid (31, 32), and porcine cyclic nucleotide phosphodiesterase was activated by both unsaturated and saturated fatty acids (33). Myosin light chain kinase from chicken gizzard was activated by unsaturated fatty acids (34, 35, 36) and type 2C (37). In both cases activation of fatty acids that activate calcineurin. Calcineurin might trigger liberation of Ca²⁺, which would be sufficient for the chemotactic response (39). In addition, Ca²⁺ release leads to capacitative Ca²⁺ entry at low extracellular Ca²⁺ concentrations (38). Taken together, this leads to an increase in the Ca²⁺/calmodulin concentration resulting in the alternative activation of calcineurin. As soon as the cytosolic Ca²⁺ concentration reaches resting levels through Ca²⁺ ATPase activity, calcineurin activation is relieved.

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