The Caveolin Scaffolding Domain Modifies 2-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate Receptor Binding Properties by Inhibiting Phospholipase A2 Activity*  

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Activation of the enzyme phospholipase (PLA2) has been proposed to be part of the molecular mechanism involved in the alteration of 2-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) glutamate receptor responsiveness during long term changes in synaptic plasticity (long term potentiation). This study assesses the effect of the caveolin-1 scaffolding domain (CSD) on the activity of the regulatory enzyme PLA2. Caveolin-1 is a 22-kDa cholesterol-binding membrane protein known to inhibit the activity of most of its interacting partners. Our results show that the calcium-dependent cytosolic form of PLA2 (cPLA2) and caveolin-1 co-localized in mouse primary hippocampal neuron cultures and that they were co-immunoprecipitated from mouse hippocampal homogenates. A peptide corresponding to the scaffolding domain of caveolin-1 (Cav-(82–101)) dramatically inhibited cPLA2 activity in purified hippocampal synaptosomes. Activation of endogenous PLA2 activity with KCl or melittin increased the binding of [3H]AMPA to its receptor. This effect was almost completely abolished by the addition of the CSD peptide to these preparations. Moreover, we demonstrated that the inhibitory action of the CSD peptide on AMPA receptor binding properties is specific (because a scrambled version of this peptide failed to have any effect) and that it is mediated by an inhibition of PLA2 enzymatic activity (because the CSD peptide failed to have an effect in membrane preparations lacking endogenous PLA2 activity). These results raised the possibility that caveolin-1, via the inhibition of cPLA2 enzymatic activity, may interfere with synaptic facilitation and long term potentiation formation in the hippocampus.

Phospholipase (PLA2) belongs to a superfamily of enzymes that play a central role in the regulation of arachidonic acid (AA) release from membrane phospholipids and catalyze the production of various metabolites. PLA2 activity has been postulated to play an important role in key metabolic pathways. In addition to its involvement in signal transduction, membrane repair, neurodegeneration, and apoptosis (1), there is a growing body of evidence suggesting a role in the modulation of neurotransmitter release and long term potentiation (LTP) (2–4). Changes in synaptic function observed with LTP are thought to be the result of modifications of postsynaptic currents mediated by the 2-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) subtype of glutamate receptors (5, 6). PLA2 activity was shown to be part of the molecular mechanisms regulating AMPA receptor function during long term changes in synaptic operation (7–9). It has been proposed that during high frequency stimulation a large entry of calcium mediated by N-methyl-D-aspartate (NMDA) glutamate receptor activation might cause an increase in PLA2 activity. The enzyme would augment AMPA receptor affinity by changing the lipid environment of AMPA receptors, thereby producing LTP (10). Recently, it has been reported that detergent-insoluble caveolin-rich membrane domains are markedly enriched in AMPA-type glutamate receptors (11), AA and PLA2 (12, 13), suggesting a possible role for PLA2 in caveolin-dependent cellular functions.

Caveolae are small (50–100 nm) flask-shaped invaginations of the plasma membrane (14, 15). The main molecular features of caveolae are the presence of caveolin, an integral membrane protein (21–24 kDa) (16, 17), and its distinct lipid composition (enrichment of cholesterol and glycosphingolipids) (18, 19). Three caveolin family members have recently been cloned and were designated caveolin-1, caveolin-2, and caveolin-3 (16, 20, 21). Caveolin-1 and -2 are ubiquitously expressed (16, 20, 21). Caveolin-1 and -2 are ubiquitously expressed (22, 23) whereas caveolin-3 is almost exclusively found in muscles (21, 24).

Caveolae have been implicated in the sequestration of many signaling molecules (14). Caveolin is thought to function as a scaffolding protein within these membrane microdomains where it interacts with several signaling proteins (14, 25, 26). A short (20 amino acids residues 82–101) cytosolic N-terminal region of caveolin, termed the caveolin scaffolding domain (CSD), is involved in the formation of caveolin oligomers and mediates the interaction with signaling molecules, which generally results in the inactivation of signaling (27). Most caveolin-interacting proteins identified so far contain a caveolin-binding motif located within their enzymatically active catalytic domain (28). This caveolin-binding motif is also present within the catalytic domain of the cytosolic form of PLA2 (cPLA2); yet, virtually nothing is known about the potential relationship of caveolin with cPLA2.

In this article, we report evidence demonstrating the presence of the cytosolic (85-kDa) PLA2 in caveolin-enriched mem-
brane fractions isolated from hippocampal preparations. We also show that a CSD peptide can regulate the enzymatic activity of cPLA2 in these preparations. Finally, we explored the effect of the CSD fragment on PLA2-mediated modulation of AMPA receptor binding properties.

EXPERIMENTAL PROCEDURES

Co-immunoprecipitation of cPLA2 with Caveolin-1

Co-immunoprecipitation was performed using antibodies directed against the N-terminal domain of the scaffold protein. The immunoprecipitates were separated by SDS-PAGE and probed with cPLA2 antibodies to resolve caveolae-enriched subcellular fractions (29). As shown in Fig. 1A, almost all the endogenously expressed caveolin-1 (Cat-1) is recovered in the caveolae fractions 2, 3, and 4, at the 5−30% sucrose interface. By contrast, most of the total cellular protein is distributed in the denser sucrose fractions (fractions 8−12) (not shown). Importantly, a fraction of the total endogenous cPLA2 protein (with the expected molecular mass of 110 kDa (34)) was recovered in caveolae fractions (Fig. 1A), demonstrating the presence of the cPLA2 protein in caveolin-enriched fractions from mice hippocampi.

To determine whether caveolin-1 forms a stable complex with cPLA2, hippocampal homogenates were subjected to immunoprecipitation using antibodies directed against the N-terminal domain of caveolin-1 (residues 2−21) or cPLA2 protein. The immunoprecipitates were separated by SDS-PAGE and probed with cPLA2, hippocampal homogenates were subjected to immunoprecipitation using antibodies directed against the N-terminal domain of caveolin-1 (residues 2−21) or cPLA2 protein. The immunoprecipitates were separated by SDS-PAGE.
and subjected to immunoblot analysis, with antibodies directed against either caveolin-1 or cPLA2. As shown in Fig. 1B, caveolin-1 protein was found in immunoprecipitates of cPLA2, and, conversely, a small amount of cPLA2 protein was detected in immunoprecipitates of caveolin-1. To further confirm these biochemical findings, the localization of cPLA2 and caveolin-1 in cultured hippocampal neurons was assessed. Although cPLA2 and caveolin-1 were located over the entire neuronal cell surface, both proteins localized more substantially to the cell body, and in many cells, high levels of expression were also focally enriched on growth cones (Fig. 2). These results strongly support an association between cPLA2 and caveolin-1.

Inhibition of cPLA2 Activity by the CSD Peptide—Caveolin-1 has been shown to down-regulate the activity of several signaling molecules (27). This observation, together with the results described above, led us to examine the possible reduction of cPLA2 activity in synaptoneurosomes treated with the CSD peptide. Synaptoneurosomes, obtained by low speed centrifugation and filtration of brain homogenates, are a suspension of synaptic plasma membranes that contains synaptosomes with attached postsynaptic densities. The presence of both caveolin-1 and cPLA2 has been confirmed by Western blots carried out on hippocampal synaptoneurosome preparations (Fig. 1A). Incubation of synaptoneurosomes for 1 h in the presence of \[^{3}H\]arachidonate resulted in a significant lipid incorporation of AA (123 ± 15 × 10^5 dpm/mg of protein) and a basal release corresponding to approximately 2% of total \[^{3}H\]arachidonate incorporation. Purified CSD peptide produced a concentration-dependent inhibition of \[^{3}H\]arachidonate release on PLA2 stimulation by melittin (Fig. 3). A highly significant effect of CSD peptide treatment was observed at 5 nM, with a 38.9% reduction of arachidonate release when compared with nontreated controls. A 70.9% reduction was reached with a concentration of only 50 nM of the purified CSD peptide.

To confirm specifically that the inhibition of AA release observed is cPLA2-dependent, the effect of the CSD peptide was tested on synaptoneurosomes pretreated with the cPLA2 inhibitor, AACOCF3. Fig. 4A shows that the CSD peptide completely lost its effect on melittin-stimulated release of AA in the presence of the cPLA2 inhibitor. To further identify specifically the cPLA2 as a target of the CSD peptide, we also investigated the inhibitory action of the CSD peptide on recombinant cPLA2 activity. As shown in Fig. 4B, the CSD peptide completely...
Inhibition of the PLA2-induced Modulation of AMPA Binding

Experimental Procedures.

A.

Because several studies indicate that calcium-dependent PLA2 participates in selective changes in AMPA receptor properties (33, 35), the previous results prompted us to examine the possibility that the inhibition of cPLA2 activity could interfere with AMPA receptor binding. Double immunofluorescence was first used to examine whether caveolin-1 and AMPA receptors are co-localized at the cellular level. Fig. 5 shows double labeling of cultured hippocampal neurons with a mouse monoclonal antibody directed against the GluR2 subunit of glutamate AMPA receptor and a rabbit polyclonal antipeptide antibody generated against caveolin-1. Note that GluR2 immunostaining appears primarily at the cell body and as puncta localized to areas of cellular outgrowth. Although caveolin-1 exhibited a more diffuse fluorescence on projections, the merged image shows a general co-localization of GluR2 and caveolin-1 in hippocampal neurons.

It has been previously shown that potassium-induced depolarization of synaptoneurosomes increased [3H]AMPA binding to membrane fractions (36, 37). It has also been reported that treatment of synaptoneurosomes with melittin, a potent activator of endogenous phospholipases, increases [3H]AMPA binding (8). Fig. 6 illustrates the effect of melittin and KCl in the presence of different concentrations of CSD peptide. Preincubation of synaptoneurosomes with CSD peptide produced a marked reduction of the melittin- and KCl-induced increase in [3H]AMPA binding to membrane fractions. However, the inactive CSD-X peptide at the highest concentration (50 nM) did not affect AMPA binding in both situations (not shown).

As shown in Fig. 7, the CSD peptide also reduced the basal [3H]AMPA binding to its receptor. To eliminate the possibility that the CSD peptide directly interacts with AMPA receptors, [3H]AMPA binding was evaluated on purified membranes, which, unlike synaptoneurosome preparations, do not contain...
PLA₂ activity (not shown). Membrane preparations were obtained by a low speed centrifugation of hippocampal homogenates (3500 rpm) followed by two ultracentrifugations (180,000 rpm) of the supernatants. Our results showed no CSD-induced decrease of AMPA receptor binding in these membrane preparations (Fig. 7A). Similarly, the inhibitory effect of CSD peptide was completely abolished when the binding experiments with synaptoneurosomes were carried out at 0 °C instead of 33 °C (Fig. 7B). In these low temperature conditions, no phospholipase activity is detectable (not shown), indicating again that the CSD peptide modulation of [³H]AMPA binding on synaptoneurosomes reflects an inhibition of PLA₂ enzymatic activity by the peptide rather than a direct action of the compound on AMPA receptors.

**DISCUSSION**

In the present study, we confirm the presence of both caveolin-1 and cPLA₂ in mouse hippocampal neurons. Moreover, subcellular fractionation experiments revealed that a significant fraction of cPLA₂ is found in caveolin-rich membrane microdomains from hippocampal preparations. Co-immunoprecipitation experiments also indicated that caveolin-1 and cPLA₂ associate with each other, suggesting that caveolin-1 may functionally interfere with cPLA₂ enzymatic activity. In support of this assumption, we found that the peptide corresponding to the CSD peptide dramatically inhibited cPLA₂-mediated release of arachidonic acid. In addition, we showed that the inhibition of cPLA₂ activity by the CSD peptide modified [³H]AMPA binding in synaptoneurosomes. Taken together, these results suggest that the inhibition of cPLA₂ by caveolin-1 may be an important and previously unrecognized mechanism for modulating neuronal AMPA receptor binding properties.

Caveolin-1 is an integral membrane protein, and one of its proposed roles is to regulate the activity of signaling proteins that reside in caveolae. Numerous signaling molecules such as Ha-RAS, c-Src, and eNOS (28) have been shown to be functionally associated with caveolin-1. It was recently reported that caveolin-1 is enriched in AA (12), the release of which is regulated by cPLA₂, sPLA₂ (secretory), and iPLA₂ (Ca²⁺-independent) and platelet-activating factor (PAF) acetylhydrolase (38). Although there are a number of types of PLA₂ in the brain, the 85-kDa cPLA₂ (type IV) is unique because it selectively releases AA from phospholipids (39). In resting cells, cPLA₂ is localized to the cytoplasm, but in response to increases in cytosolic free calcium, the enzyme translocates to the plasma membrane and the nuclear envelope (40). Based on our findings, cPLA₂ appears to be, in part, localized in the caveolae. However, in this context it is important to note that the presence of caveolin-1 in cPLA₂ immunoprecipitation (Fig. 1) suggests that caveolin-1 detectable in cPLA₂ immunoprecipitation (Fig. 1).

Among the proteins that functionally associate with caveolin-1, a common denominator seems to be the presence of a...
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motif that interacts with the scaffolding domain of caveolin-1 (CSD) (amino acids 82–101), which is a juxtaembrane region in caveolin-1 containing numerous aromatic residues. In our study, a peptide derived from the scaffolding domain of caveolin-1 was used to examine the putative functional relationship between caveolin-1 and the activity of the regulatory enzyme cPLA2. Synaptoneurosumes, which are pinched-off nerve terminals associated with released post-synaptic structures, are useful tools to study mechanisms of transmitter release, regulation of transmitter receptors and second messenger pathways. We found that the CSD peptide dramatically inhibits melittin-induced AA release from synaptoneurosum preparation. Melittin is a strong activator of endogenous PLA2s. With the use of the cPLA2 inhibitor, AACOCF3, we showed that the major proportion of melittin-induced AA release is cPLA2-dependent. The absence of any CSD peptide effect on synaptoneurosumes pretreated with the cPLA2 inhibitor and the complete inhibition of the recombinant cPLA2 activity by the CSD peptide specifically identified cPLA2 as a target of the CSD peptide. Moreover, the scrambled version of the CSD peptide (CSD-X) did not affect melittin-induced AA release, showing the specificity of the observed effect of the CSD fragment on endogenous cPLA2. It should be noted here that in synaptoneurosum preparations, 50% of the formed vesicles are likely to be inside-out, the remaining being right-side-out. This explains how the CSD peptide we used, which is a cell-impermeable peptide, can interact with intracellular cPLA2.

How might caveolin-1 inhibit the activity of cPLA2? One possible physiological mechanism for the CSD inhibition of cPLA2 activity is that, following the enzyme activation, cPLA2 translocation to the cell membrane (40) may promote its physical interaction with caveolin-1. Another hypothesis is that cPLA2 clustering with other signaling molecules inside caveolae may indirectly inactivate cPLA2 activity. For instance, cPLA2 can be stimulated by protein kinases such as protein kinase C (41) and mitogen-activated protein kinase (42), which are known to be inhibited by caveolin-1 (43).

Although the exact mechanism for the putative inhibition of cPLA2 activity by caveolin-1 is still unknown, the modulatory effect observed may have an important homeostatic role in brain cells. For example, it has recently become apparent that activation of the calcium-dependent PLA2 could be part of the molecular mechanisms involved in alterations of AMPA receptor properties during long term changes in synaptic operation (LTP) (7–9). In accordance with a recent study providing biochemical evidence for a localization of AMPA-type glutamate receptors to caveola-like structures (11), our study shows the co-immunolocalization of the GluR2 subunit of AMPA receptors with caveolin-1 in primary hippocampal neuron cultures.

Increased PLA2 activity has previously been shown to upregulate [3H]AMP binding to AMPA receptor (44, 45). In accordance with these findings, the activation of endogenous PLA2 activity with KCl or melittin increased AMPA binding to its receptor. This effect was almost completely abolished by the addition of the CSD peptide to synaptoneurosum preparations. It is not excluded that a small fraction of the effect of CSD peptide on AMPA binding might have been mediated by the inhibition of PLA2α other than the cPLA2. However, as we showed previously with the cPLA2 inhibitor, the effect of melittin on synaptoneurosumes is mostly cPLA2-dependent. This indicates that the inhibition of the cPLA2 isoform of the enzyme might be principally involved in the reduction of AMPA binding, which is consistent with our experiments showing the highly potent and efficient inhibition of the purified recombinant cPLA2 by the CSD peptide. We also demonstrated that the inhibitory action of the CSD peptide on AMPA binding depends on its modulation of PLA2 activity by carrying out experiments in conditions where PLA2 enzymatic activity is absent. Interestingly, biochemical evidence has shown that the [3H]AMP binding is augmented after LTP induction (46–48). Moreover, several reports suggest that different compounds that inhibit PLA2 activity interfere with LTP (4, 49, 50). Thus, we could hypothesize that an up-regulated physiological level of caveolin-1 expression in the brain, such as seen in aging (51–53) or in Alzheimer disease (53), might negatively interfere with synaptic function.

In conclusion, we show for the first time the regulation of cPLA2 activity and the resulting modulation of AMPA receptor binding properties by the scaffolding domain of caveolin-1. These results raise the possibility that caveolin-1 may interfere with phospholipid-derived metabolite production, synaptic facilitation, and LTP formation. In this regard, future investigations into this area should include experiments dedicated to evaluating the potential involvement of the caveolin scaffolding domain in regulating changes of AMPA receptor properties in synaptic plasticity and LTP formation in vivo.

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