Stimulatory Effect of Veratridine on Lysophosphatidylethanolamine Formation in Rat Brain Synaptosomes

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Abstract—[3H]-Arachidonic acid incorporation into phospholipids of synaptosomal lysates prepared from veratridine-treated synaptosomes was examined. Synaptosomal lysates were shown to acylate exogenously added lysophosphatidylcholine, lysophosphatidylinositol, and lysophosphatidylethanolamine, when incubated with [3H]-arachidonic acid, ATP, CoA and MgCl2, yielding the respective phospholipids. Preincubation of synaptosomes with veratridine for 30 sec gave rise to an increase in [3H]-arachidonic acid incorporation into phosphatidylethanolamine, but not phosphatidylcholine nor phosphatidylinositol, indicating that lysophosphatidylethanolamine might be produced by veratridine. This increase of radioactivity in phosphatidylethanolamine caused by veratridine was completely inhibited by 1 μM tetrodotoxin or in calcium-free condition. These observations show that lysophosphatidylethanolamine was formed in a calcium-dependent manner and accumulated in synaptosomes treated with veratridine, which may relate to its action on the sodium channel and enhanced calcium influx.

Recent studies have indicated that arachidonic acid release from phospholipids is increased within depolarized synaptosomes and within chromaffin cells when stimulated by nicotinic agonists or elevated K+ (1–3). These findings suggest that increase of phospholipid turnover may be involved in the process of the transmitter release. In these studies, however, it is not clear whether arachidonic acid is released by the activation of phospholipase A2 or activation of phospholipase C and the subsequent action of diacylglycerol lipase: two pathways known to produce arachidonic acid from phospholipids, respectively. This problem may be solved by making sure of the formation of lysophospholipids or monoacylglycerol, the products of these pathways.

The formation of phospholipid from lysophospholipid has been demonstrated in synaptosomes (4, 5). In the presence of labeled fatty acid, radioactivity in phospholipids was increased by increasing the amount of lysophospholipids added (6, 7). We thought that it might be possible to assay the level of lysophospholipids in synaptosomes by determining the change in phospholipids that are formed by the acylation of the target compounds, lysophospholipids. For this purpose, the rate of [3H]-arachidonic acid incorporation into phospholipids was measured, and the results are described in this report.

Materials and Methods

The sources of materials used in this work were as follows: veratridine, arachidonic acid, adenosine-5'-triphosphate (ATP), essentially fatty acid-free bovine serum albumin (FA-free BSA), lysophosphatidylcholine (lysoPC), lysophosphatidylethanolamine (lysoPE) from Sigma; lysophosphatidylinositol (lysoPI) from Serdany Research Laboratory, Inc.; tetrodotoxin from Sankyo Co., Ltd.; coenzyme A (CoA) from Kojin Co.; [3H]-arachidonic acid ([3H]-AA, 135 mCi/mmol) from Amersham International plc.

Krebs-Ringer bicarbonate buffer (KR) was prepared as described elsewhere (8).

Synaptosomes were isolated from male
Sprague-Dawley rat cerebral cortices by differential and sucrose-density gradient centrifugation using the method of Gurd et al. (9), as modified by Matsuda and Cooper (10). The synaptosomal fraction at the 1.0 M–1.2 M sucrose interface was removed, diluted with 3 vol. of 0.1 mM EDTA/1 mM sodium phosphate, pH 7.4, and centrifuged at 20,000 g for 20 min. The synaptosomes were resuspended in KR and preincubated at 37°C for 30 min, and then they were centrifuged at 15,000 g for 10 min. The synaptosomes were suspended with KR containing 0.5% FA-free BSA at 4°C for 15 min. After the incubation, the synaptosome suspensions were centrifuged and washed twice with KR.

Synaptosome suspensions (250 μl) containing about 1 mg protein were incubated at 37°C for 10 min. Then 250 μl KR containing 100 μM veratridine was added. The mixtures were further incubated for 30 sec unless otherwise indicated. After incubation, the mixtures in the reaction tubes were dipped in cooled acetone (−60°C) to terminate the reaction. Then the frozen synaptosomes were thawed and disrupted by sonication at 4°C for 10 sec to avoid the influence of the depolarizing effect of veratridine on the acylation step. The second incubation for acylation was started by addition of the mixture (250 μl) containing 0.1 μCi [3H]-AA, 10 nmol cold arachidonic acid, 2.5 μmol ATP, 50 nmol CoA, 5 μmol MgCl2, and 1 μmol ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). After 5 min incubation at 37°C, the reaction was terminated by adding 4 ml of chloroform/methanol (2:1, v/v) containing 0.05% tert-butylhydroxytoluene with immediate mixing. The extracted total lipids were separated by thin layer chromatography on silica gel plates, which were developed in chloroform/methanol/acetic acid/water (25: 15:4:2, v/v). Phospholipid spots were visualized by brief exposure to iodine vapor, and the radioactivity in the spots was counted by a liquid scintillation counter. The change of [3H]-AA incorporation into phospholipids was taken as the difference between the incorporation by synaptosomal lysates of nonpreincubated synaptosomes and that of preincubated ones. Calcium-free KR was prepared by omitting CaCl2 and adding 1 mM EGTA.

Protein was measured by the method of Lowry et al. (11).

Results

Synaptosomal lysates were incubated in the presence of exogenously added lysoPC, lysoPI, and lysoPE with [3H]-AA and cofactors; the incorporation of the radioactivity was recognized in phosphatidyli-
choline (PC), phosphatidylinositol (PI), and phosphatidylethanolamine (PE), respectively. Figure 1 shows that the incorporation of the radioactivity into PC and PE was approximately linear with increasing amount of lysoPC and lysoPE in the range of 1 to 10 nmol per assay with, however, some deviation from linearity above that concentration. Incorporation into PI was larger than those into PC and PE, but it did not show a clear linearity.

[3H]-AA incorporation into phospholipids of synaptosomal lysates prepared from the synaptosomes, which were preincubated with or without veratridine, was examined in the absence of exogenous lysophospholipid. As shown in Table 1, 30 sec preincubation of synaptosomes with 50 μM veratridine, which is enough to cause transmitter release and calcium influx into synaptosomes (12, 13), markedly enhanced [3H]-AA incorporation into PE of synaptosomal lysates, but not into PC nor PI.

Veratridine is known to depolarize synaptosomes by opening the sodium channel and subsequently stimulating the influx of calcium ion (12–15). In order to check the correlation between the effect of veratridine on [3H]-AA incorporation into PE and its action as a depolarizing agent, we examined the effect of tetrodotoxin, a specific sodium channel blocker, and the effect of removing calcium ion from the incubation medium (calcium-free KR). Figure 2 shows that 1 μM tetrodotoxin, which is known to be enough to block veratridine-induced depolarization (12, 13), and the calcium-free condition prevented the increase in [3H]-AA incorporation into PE.

![Fig. 2. Effects of the presence of tetrodotoxin or the calcium-free condition during the preincubation of synaptosomes on [3H]-arachidonic acid incorporation into phosphatidylethanolamine of synaptosomal lysates. Synaptosomes were preincubated with (■■■) or without (□□□) 50 μM veratridine in normal KR (None), KR containing 1 μM tetrodotoxin (1 μM TTX), or calcium-free KR (Ca2+ (-)) at 37°C for 30 sec. [3H]-Arachidonic acid incorporation was determined as described under "Materials and Methods". Values are means±S.E.M. of 3 experiments.](image)

### Table 1. Effect of preincubation of synaptosomes with veratridine on [3H]-AA arachidonic acid incorporation into phospholipids of synaptosomal lysates

| Phospholipids             | [3H]-Arachidonic acid incorporation (dpm/mg protein) | Veratridine |
|---------------------------|------------------------------------------------------|-------------|
|                           |                                                      | (-)         | (+)         |
| Phosphatidylcholine       | 693±67                                               | 720±80      |
| Phosphatidylinositol      | 586±112                                              | 480±107     |
| Phosphatidylethanolamine  | 640±128                                              | 1760±168*   |

Synaptosomes were incubated with or without 50 μM veratridine at 37°C. Thirty sec after the addition, the synaptosomes were frozen at −60°C, and then they were thawed and disrupted by sonic treatment. The synaptosomal lysates were further incubated with [3H]-AA and cofactors at 37°C for 5 min as described under "Materials and Methods". Values are means±S.E.M of 3 experiments. *: P<0.01 compared with the control.
Fig. 3. Effect of preincubation with veratridine for various time intervals on [3H]-arachidonic acid incorporation into phosphatidylethanolamine of synaptosomal lysates. Synaptosomes were incubated for various time intervals with (○) or without (▲) 50 μM veratridine. [3H]-Arachidonic acid incorporation was determined as described under “Materials and Methods”. Values are means ± S.E.M. of 3 experiments.

[3H]-AA incorporation into the PE of synaptosomal lysates was determined from 15 to 120 sec of preincubation of synaptosomes with or without veratridine (Fig. 3). [3H]-AA incorporation into PE was increased within the first 30 sec and then decreased gradually. After 90 sec of the addition of veratridine, [3H]-AA incorporation into PE reached the level of the control.

**Discussion**

When lysophospholipids were exogenously added to the synaptosomal lysates, radioactivity in the phospholipids was increased. These results indicate that synaptosomal lysates have an active acylation system with less specificity to lysophospholipid species. Majewaska and Sun (16) and Strosznajder et al. (7) showed that labeled arachidonic acid was preferentially transferred to lysoPC and lysoPI using intact synaptosomes. However, the present study shows that not only lysoPC and lysoPI but also lysoPE were acylated by synaptosomal lysates. This discrepancy may be explained by the possibility that sonic treatment may release intracellular membrane fragments, which contain high activity to transfer fatty acid to lysophospholipid (4). [3H]-AA incorporation into PC and PE showed a linear increase by increasing amounts of added lysoPC and lysoPE, respectively, but that into PI did not. It was reported that lysoPI was a fairly good substrate for acyltransferase in synaptosomes (4, 6, 7). Therefore, [3H]-AA incorporation into PI may reach maximum at a low concentration of lysoPI.

The data in this paper have shown that preincubation of synaptosomes with veratridine enhanced [3H]-AA incorporation into PE but not into PC nor PI. This might be explained by the following considerations: LysoPE, a substrate for acyltransferase, is generated in synaptosomes by veratridine, while either lysoPC or lysoPI is not. Then this increase of lysoPE leads to an enhancement of [3H]-AA incorporation into PE. Furthermore, when synaptosomes were incubated with veratridine in the presence of tetrodotoxin or under a calcium ion-free condition, the effect of veratridine on the [3H]-AA incorporation was completely inhibited. These findings suggest that the increase of lysoPE in synaptosomes is closely related to the action of veratridine on the sodium channel and subsequent calcium influx into synaptosomes.

The [3H]-AA incorporation was at its maximum at 30 sec after the addition of veratridine and then gradually decreased. This means that veratridine causes a rapid increase of lysoPE, and then the level of lysoPE returned to the original level. These results suggest that rapid formation of lysoPE and subsequent reacylation or further degradation of the formed lysoPE could occur in synaptosomes.

It is demonstrated in this paper that preincubation of synaptosomes with veratridine enhanced [3H]-AA incorporation into PE of synaptosomal lysates, depending on extracellular calcium ion, suggesting that the formation of lysoPE was stimulated by calcium ion influx induced by veratridine, a depolarizing agent. Lysophospholipids have been reported to have lytic activity to mem-
branes and have been postulated to play an important role in the stimulation-secretion coupling (17–19). However, more data will be needed before it becomes clear whether lysoPE formation in synaptosomes by veratridine is implicated in the process of neurotransmitter release.

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