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Inhibitory Effects of Calcium Antagonists on Mitochondrial Swelling Induced by Lipid Peroxidation or Arachidonic Acid in the Rat Brain In Vitro

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Inhibitory effects of calcium antagonists, efonidipine (NZ-105), nicardipine, nifedipine, nimodipine and flunarizine, on mitochondrial swelling induced by lipid peroxidation or arachidonic acid in the rat brain in vitro were investigated. Mitochondrial swelling and lipid peroxidation induced by $FeSO_4$ and ascorbic acid system showed a close and significant relationship. Mitochondrial swelling and lipid peroxidation induced by $FeSO_4$ and ascorbic acid were inhibited by all of calcium antagonists tested. The order of inhibition was: flunarizine > nicardipine > efonidipine > nimodipine > nifedipine. This result suggests that calcium antagonists tested have anti-peroxidant activities resulting in protection of mitochondrial membrane damage and that each moiety of these structures would play an important role in appearance of anti-peroxidant activities. Furthermore, flunarizine and efonidipine inhibited mitochondrial swelling induced by arachidonic acid, which is not associated with lipid peroxidation. In contrast, nicardipine, nifedipine, and nimodipine did not inhibited this swelling. It is possible that flunarizine and efonidipine could directly interact with mitochondrial membrane. In conclusion, it is capable that calcium antagonists tested may protect from the membrane damage induced by lipid peroxidation and that flunarizine and efonidipine could stabilize the membrane, which is attributed to a direct interaction with the membrane.

KEY WORDS: Calcium antagonists; efonidipine hydrochloride(NZ-105); brain mitochondria; swelling; lipid peroxidation; arachidonic acid.

INTRODUCTION

It has been authorized that free radicals attack polyunsaturated fatty acids in membrane and produce lipid peroxides, resulting in membrane damage and physiological disorders (1-3). Especially, brain has more contents of lipids than other organs, so, it is thought that brain is susceptible to injury by free radicals and lipid peroxidation (4) and mitochondria have been recognized to an important source of free radicals in mammalian tissues (5).

On the other hand, calcium antagonists have beneficial pharmaco-activities and are clinically used for the treatment of hypertension, cardiac angina, and amelioration of cerebral circulation (6,7). A main mechanism of them is recognized to antagonize voltage-operated calcium channel (L-type calcium channel).

Recently, effects of them on the ischemia-reoxygenation model of myocardium have been reported (8,9). Cell damage in this model is in part related with free

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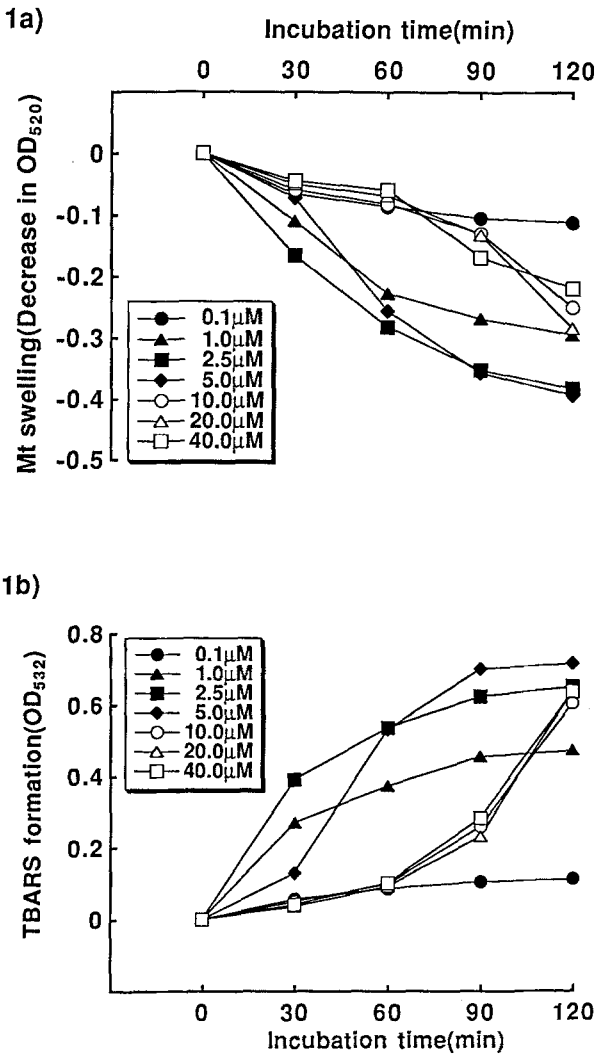

Fig. 1. Changes in mitochondrial swelling and lipid peroxidation at various concentrations of $FeSO_4$ and ascorbic acid (50 μM) in the rat brain. Swelling and lipid peroxidation were started by addition of $FeSO_4$ (final concentration of 0.1-40.0 μM) to mitochondrial fraction. After incubation for 30, 60, 90, and 120 min, turbidity at 520 nm was measured as a marker of mitochondrial swelling. Decrease in turbidity at 520 nm was calculated by subtracting the absorbance at each time from at time 0. After measurement of turbidity, 1 ml of 20% (w/v) trichloroacetic acid was added to reaction mixture. After centrifugation, 1 ml of 0.5% (w/v) of 2-thiobarbituric acid was added to 1 ml of resultant supernatant and mixture was boiled for 20 min. After cooling, the absorbance at 532 nm (TBARS) was measured as a marker of lipid peroxidation. Each point indicates the mean of 3 experiments for duplicate samples. 1a) Mitochondrial swelling, 1b) Lipid peroxidation.

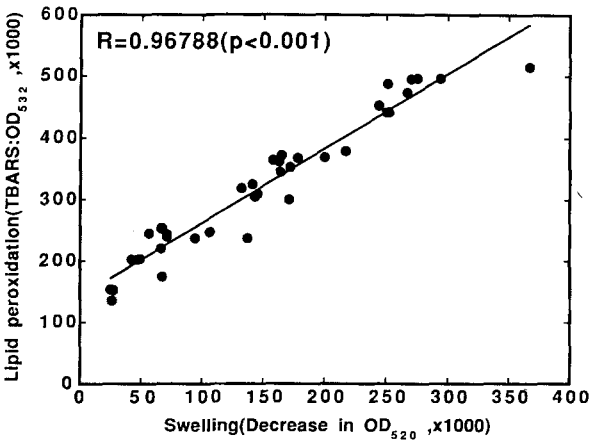

Fig. 2. Relationship between mitochondrial swelling and lipid peroxidation in the rat brain. Turbidity at 520 nm was measured as a marker of swelling. Lipid peroxidation was determined by measurement of absorbance at 532 nm (TBARS). n=44.

radicals and lipid peroxidation (10,11), so antioxidant activities of them have been studied by using myocardiac membrane in vitro (12,13). However, studies on effects of them on mitochondrial membrane damage induced by lipid peroxidation in the brain are not sufficient and protective activities of them against membrane damage induced by different mechanism from lipid peroxidation is not clear.

In this paper, inhibitory effects of calcium antagonists, efonidipine (NZ-105), nicardipine, nifedipine, nimodipine, and flunarizine, on mitochondrial swelling induced by lipid peroxidation using $FeSO_4$ and ascorbic acid system in the rat brain were examined. Furthermore, effects of them on mitochondrial swelling induced by arachidonic acid, which is not associated with lipid peroxidation, were investigated for the purpose of clarifying a direct interaction with membrane.

EXPERIMENTAL PROCEDURE

Preparation of a Crude Mitochondrial Fraction in the Rat Brain. Male Wistar rats, weighing 200-220 g, were purchased from Charles River Japan Inc., Japan. Rats were taken feds and water ad libitum until use. Rats were killed by decapitation. Brain was immediately removed and cerebral cortex and hippocampus were separated on ice according to the method described by Glowinski and Iversen (14). Separated cortex and hippocampus were placed in ice-cold 0.3 M mannitol (pH 7.2) containing 0.1 mM EDTA and homogenized by teflon-glass homogenizer (1500 rpm, 3 strokes) at 4°C. A crude mitochondrial fraction was prepared using the method described by Ozawa et al. (15). Briefly, the homogenate was centrifuged at 600 *g* for 8 min at 4°C and supernatant was centrifuged at 10,000 *g* for 10 min. Then pellet was suspended with 25 mM Tris-HCl buffer (pH 7.2) containing 175 mM KCl and was centrifuged at 5,000 *g* for 10 min for washing. Washed pellet was recentrifuged at 10,000 *g* for 10 min. Resultant pellet was finally resuspended with 25 mM Tris-HCl buffer (pH 7.2) containing 175 mM KCl. This suspension was used as a

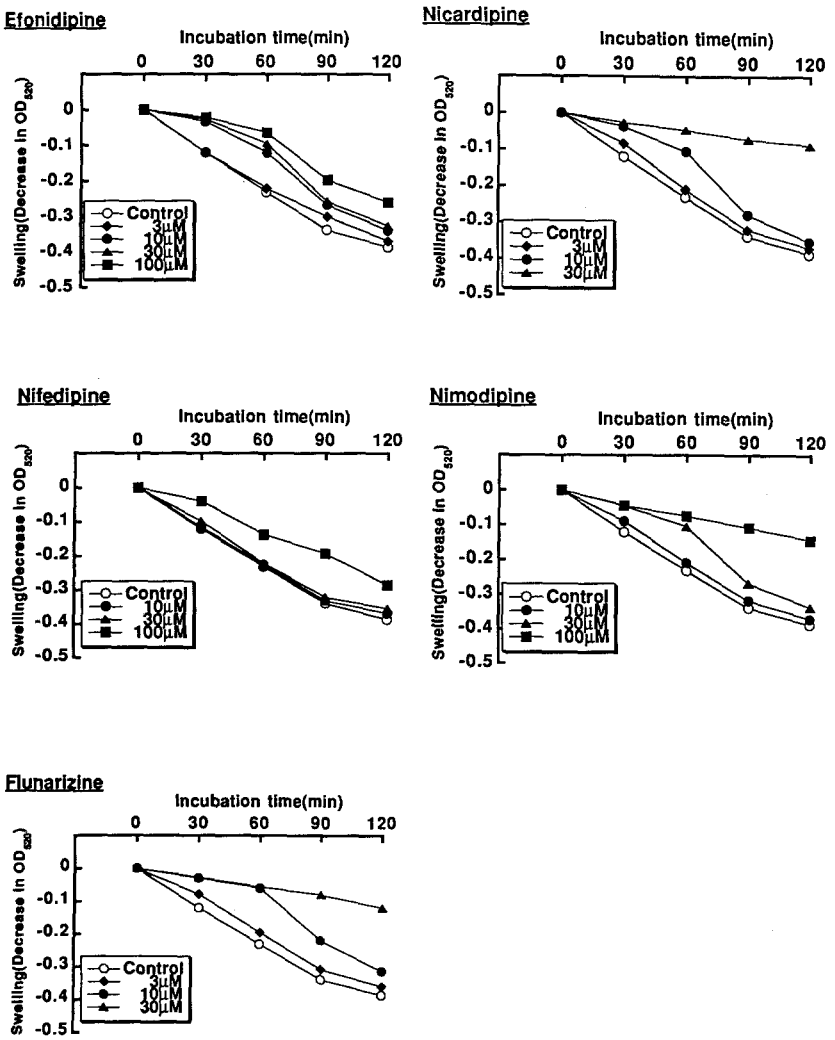

Fig. 3. Effects of calcium antagonists on mitochondrial swelling induced by $FeSO_4$ (2.5 μM) and ascorbic acid (50.0 μM) in the rat brain. Efonidipine was added at final concentrations of 3, 10, 30, and 100 μM. Nifedipine and nimodipine were added at final concentrations of 10, 30, and 100 μM. Nicardipine and flunarizine were added at final concentrations of 3, 10, and 30 μM. Mitochondrial swelling was determined by the same procedure described legend to Fig. 1. Each point indicates the mean of 3 experiments for duplicate samples.

crude mitochondrial preparation. A crude mitochondrial preparation was stored at 4°C until experiments.

Mitochondrial Swelling Induced by Lipid Peroxidation Using $FeSO_4$ and Ascorbic Acid System in the Rat Brain. Mitochondrial swelling induced by lipid peroxidation using $FeSO_4$ and ascorbic acid system was carried out according to the method described by Kubo et al. (16). Briefly, reaction mixture, in a final volume of 1.00 ml, contained as below at final concentrations: 25 mM Tris-HCl buffer (pH 7.4), mitochondrial fraction (0.3 mg protein/ml), 50.0 μM ascorbic acid and drug solution. Swelling and lipid peroxidation were initiated by addition of $FeSO_4$ (final concentrations of 0.1-40.0 μM) at room temperature. After incubation for 30, 60, 90, and 120 min, changes in turbidity at 520 nm of reaction mixture were measured using spectrophotometer (UV-200, Shimazu Co. Ltd., Tokyo, Japan) as a marker of swelling (17). Lipid peroxidation was terminated by 1.00 ml of 20% (w/v) trichloroacetic acid in an ice-cold water bath. Mixture was vortexed and centrifuged at 3,000 rpm for 15 min. 1.00 ml of resultant supernatant was mixed with 1.00 ml of 0.5% (w/v) 2-thiobarbituric acid and incubated for 20 min in a boiling bath. After cooling, the absorbance at 532 nm (Thiobarbituric acid reactive substances: TBARS) was measured using spectrophotometer as a marker of lipid peroxidation. Drugs were dissolved in dimethylsulfoxide at a final concentration of 1% (v/v), which was not affected the mitochondrial swelling and lipid peroxidation.

Mitochondrial Swelling Induced by Arachidonic Acid in the Rat Brain. Mitochondrial swelling induced by arachidonic acid was carried out according to the method described by Hillered and Chan (18). Reaction mixture, in a final volume of 1.00 ml, containing as below at final concentrations: 25 mM Tris-HCl buffer (pH 7.4), a crude mitochondrial preparation (0.25 mg protein/ml), 0.2 mM arachidonic acid and drug solution. Mitochondrial swelling was started by addition of arachidonic acid. After incubation for 10, 20, and 30 min at room

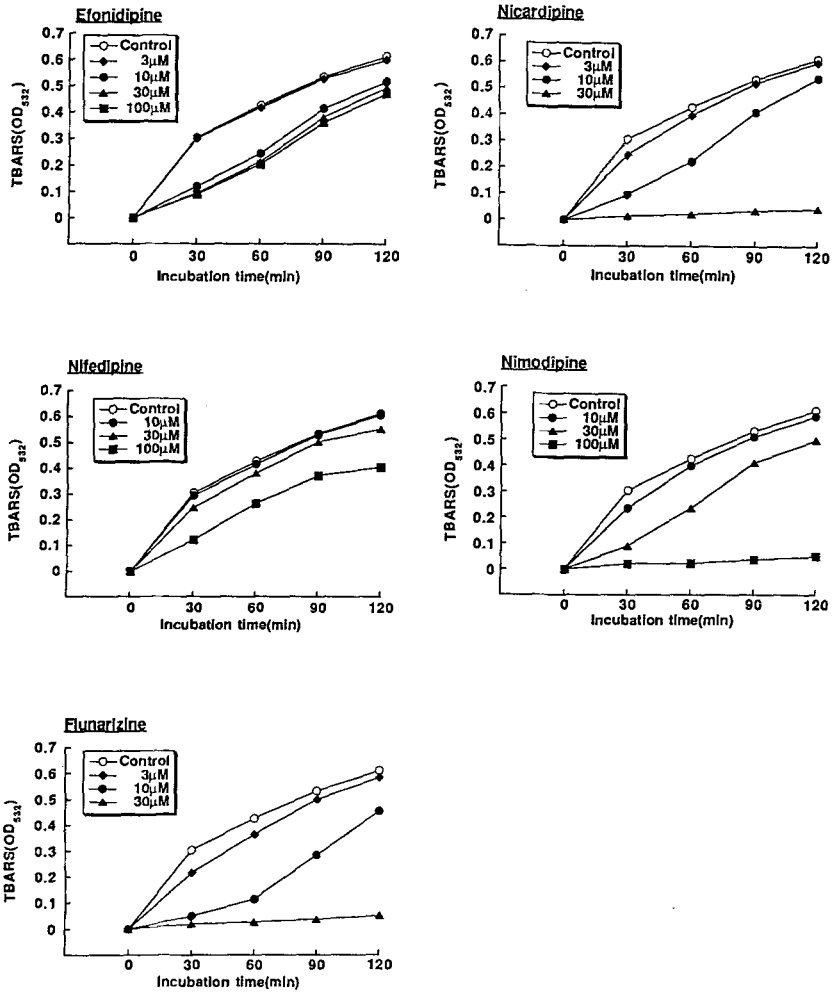

Fig. 4. Effects of calcium antagonists on mitochondrial lipid peroxidation induced by $FeSO_4$ (2.5 μM) and ascorbic acid (50.0 μM) in the rat brain. Concentrations of calcium antagonists were the same as described legend to Fig. 3. Each point indicates the mean of 3 experiments for duplicate samples.

temperature, turbidity of reaction mixture was measured at 520 nm as a marker of swelling. Drugs were dissolved in dimethylsulfoxide and arachidonic acid was dissolved in ethanol at a final concentration of 1% (v/v). TBARS was measured by the same procedure described above.

Protein Assay. Protein content of a crude mitochondrial preparation was quantified by BCA protein assay kit (PIERCE, Illinois, USA) using bovine serum albumin as a standard.

Chemicals. Efonidipine hydrochloride (NZ-105, efonidipine) and nimodipine were synthesized in Nissan Chemical Industries Co., LTD. (Tokyo, Japan). Nicardipine hydrochloride (nicardipine), nifedipine, flunarizine dihydrochloride (flunarizine), and arachidonic acid (sodium salt) were obtained from SIGMA Chemical Co. (St. Louis, MO). 2-Thiobarbituric acid (sodium salt) and ascorbic acid were purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were the highest grade available.

Statistical Analysis. Statistical significance was determined by using Dunnett's multiple range test with one-way analysis of variance (ANOVA) and p value of less than 0.05 was considered significant.

RESULTS

Changes in Mitochondrial Swelling Induced by Lipid Peroxidation Using $FeSO_4$ and Ascorbic Acid in the Rat Brain. Mitochondrial swelling and lipid peroxidation induced by $FeSO_4$ at final concentrations of 0.1-40.0 μM and ascorbic acid at a final concentration of 50.0 μM were shown in Fig. 1a and 1b, respectively. Mitochondrial swelling and lipid peroxidation were apparently observed from 1.0 μM of $FeSO_4$. The highest swelling and lipid peroxidation were resulted from inducing by $FeSO_4$

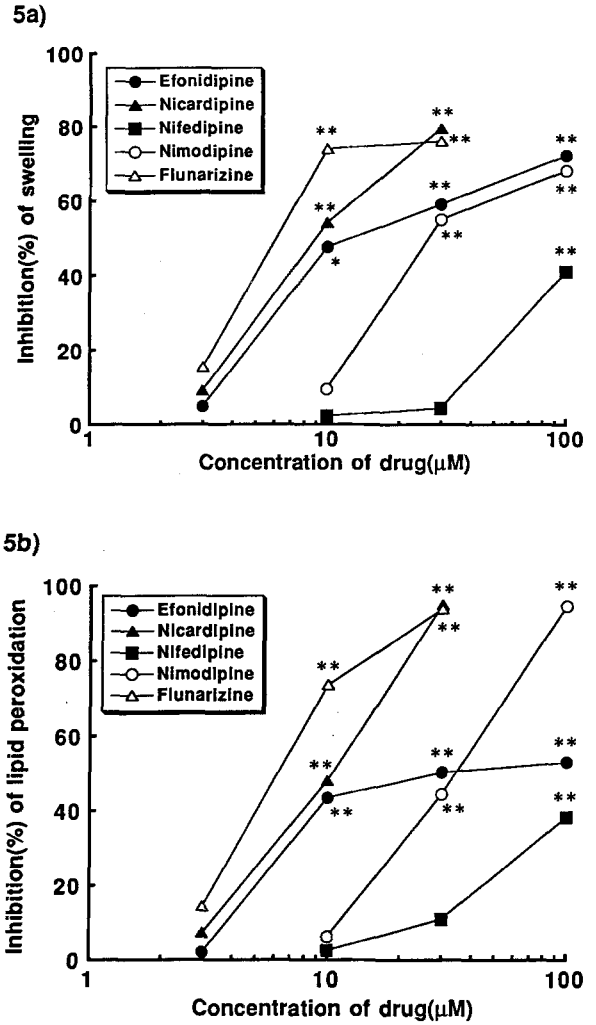

Fig. 5. Inhibition of mitochondrial swelling and lipid peroxidation by calcium antagonists at incubation for 60 min. Each point indicates the mean of 3 experiments for duplicate samples. 5a) Mitochondrial swelling, 5b) Lipid peroxidation. *, $p<0.05$; **, $p<0.01$; Significantly different from control value.

at a concentration of 2.5 μM until incubation for 60 min. But $FeSO_4$ at final concentrations of 10.0, 20.0, and 40 μM, mitochondrial swelling was clearly observed at only 120 min. Mitochondrial swelling and lipid peroxidation showed a close and significant relationship (Fig. 2). Consequently, when inhibitory effects of calcium antagonists on mitochondrial swelling induced by lipid peroxidation were evaluated, 2.5 μM of $FeSO_4$ and 50 μM of ascorbic acid were used.

Inhibitory Effects of Calcium Antagonists on Mitochondrial Swelling Induced by Lipid Peroxidation Using $FeSO_4$ and Ascorbic Acid System in the Rat Brain. Effects of calcium antagonists on mitochondrial swelling and lipid peroxidation induced by $FeSO_4$ (2.5 μM) and ascorbic acid (50.0 μM) system were shown in Fig. 3 and Fig. 4. They inhibited mitochondrial swelling and lipid peroxidation in a dose-dependent manner. Nicardipine and flunarizine inhibited from 3 to 30 μM and efonidipine and nimodipine from 10 to 100 μM. Nifedipine inhibited only at 100 μM. Fig. 5a and 5b showed the inhibition of mitochondrial swelling and lipid peroxidation by calcium antagonists at incubation for 60 min. Flunarizine showed the strongest inhibition of mitochondrial swelling and lipid peroxidation. The order of inhibition was: flunarizine > nicardipine > efonidipine > nimodipine > nifedipine. Concentration required for 50% inhibition (IC_{50}) against swelling and lipid peroxidation were indicated in Table I. IC_{50}s against swelling were equal to these against lipid peroxidation except for efonidipine. IC_{50} of efonidipine against lipid peroxidation was greater by 2 fold than that against swelling.

Table I. IC_{50} of Calcium Antagonists Against Mitochondrial Swelling and Lipid Peroxidation

Drug	$FeSO_4$ + AsA		AA
	swelling	TBARS	swelling
Efonidipine	12.7	40.1	105.9
Nicardipine	10.5	9.6	>100
Nifedipine	156.8	214.9	>100
Nimodipine	38.4	32.4	>100
Flunarizine	8.3	7.3	28.7

IC_{50} (μM) was calculated by Probit method.
AsA, ascorbic acid; TBARS, thiobarbituric acid reactive substances; AA, arachidonic acid.

Inhibitory Effects of Calcium Antagonists on Mitochondrial Swelling Induced by Arachidonic Acid in the Rat Brain. Effects of calcium antagonists on mitochondrial swelling induced by arachidonic acid in the rat brain were shown in Fig. 6. Flunarizine and efonidipine inhibited this swelling. Fig. 7 showed the inhibition of mitochondrial swelling induced by arachidonic acid at incubation for 30 min by calcium antagonists. Flunarizine inhibited significantly at 30 and 100 μM and efonidipine inhibited weakly but significantly at 100 μM. Nicardipine, nifedipine, and nimodipine did not show apparent inhibition. IC_{50}s of efonidipine and flunarizine were 105.9 μM and 28.7 μM, respectively (Table I). Increases in TBARS were not detected through the incubation (data not shown).

DISCUSSION

It has been reported that lipid peroxidation and edema are induced by an intracortical injection of iron salts (19)

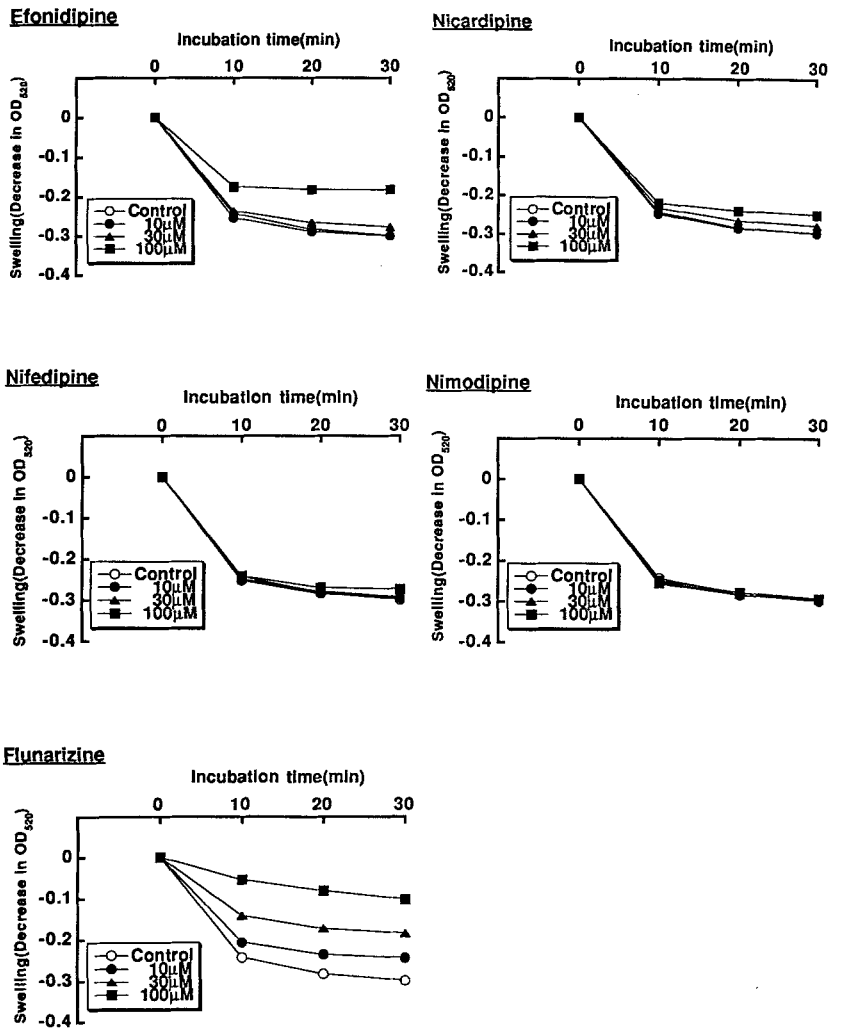

Fig. 6. Effects of calcium antagonists on mitochondrial swelling induced by arachidonic acid in the rat brain. Arachidonic acid was added at a final concentration of 0.2 mM. Calcium antagonists were added at final concentrations of 10, 30, and 100 μM. Mitochondrial swelling was determined by the same procedure described legend to Fig. 1. Each point indicates the mean of 3 experiments for duplicate samples.

and that arachidonic acid is a potent inducer of brain edema (20). Lipid peroxidation is implicated in cerebral injury by ischemia and reoxygenation (21).

Mitochondria are an important organella which are involved in tricarboxylic acid cycle, oxidative phosphorylation, etc., therefore, its damage causes irreversible physiological disorders. Furthermore, it has been appreciated that mitochondria are one of origins which produce reactive oxygens (5) and that mitochondrial respiration is inhibited by free radicals (22).

On the other hand, main action of calcium antagonists is thought to block the calcium-dependent slow action potentials, so, they are useful for many diseases related with calcium channel. Recently, other pharmacological activities of them have been noted, i.e., antiperoxidant activities (23-25).

In the present study, inhibitory effects of calcium antagonists on mitochondrial swelling induced by lipid peroxidation of arachidonic acid in the rat brain were investigated. It is indicated that mitochondrial swelling induced by $FeSO_4$ and ascorbic acid system was arised for lipid peroxidation, because the magnitude of swelling was closely related to that of lipid peroxidation. However, the effects of $FeSO_4$ and ascorbic acid were exhibited the bell-shaped dose response curve. It is thought that the phenomenon is general in this system and that the ratio of Fe^{3+}/Fe^{2+} is an important factor for iron-induced lipid peroxidation (26). Calcium antagonists in-

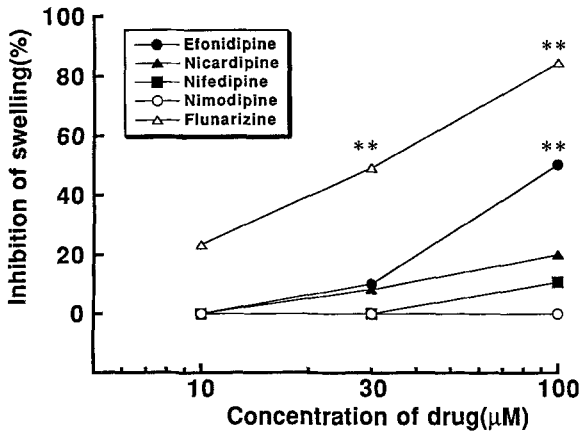

Fig. 7. Inhibition of arachidonic acid-induced mitochondrial swelling by calcium antagonists at incubation for 30 min by calcium antagonists. Each point indicates the mean of 3 experiments for duplicate samples. **, $P<0.01$; Significantly different from control value.

hibited the mitochondrial swelling and lipid peroxidation in a dose-dependent manner. IC_{50}s of them against swelling correlated with these of them against lipid peroxidation except for efonidipine. Effect of efonidipine was broad from 10 to 100 μM. IC_{50} of efonidipine against lipid peroxidation was greater than that against swelling. It is possible that efonidipine could prevent from membrane damage, in part, by the different mechanism from anti-peroxidant activity. Kubo (16) reported that flunarizine inhibited the lipid peroxidation and swelling in mitochondria. Our data in this study were in accord with their findings. Antiperoxidant activity of nifedipine was reported by several laboratories. Mak (27) reported that nifedipine inhibited the sarcolemnal membrane-lipid peroxidation. Goncalves (28) showed that nifedipine had the anti-peroxidant activity against microsomal membrane. Their data indicated high potency of nifedipine against lipid peroxidation. However, our data showed that nifedipine inhibited lipid peroxidation, but weaker than other calcium antagonists. This difference is probably attributed to source of lipid peroxidation or organella and organs. Suno (29) investigated that the inhibition of ferric chloride-induced mitochondrial swelling by idebenone, in result, idebenone inhibited mitochondrial swelling and lipid peroxidation at 37 μM and 53 μM giving 50%-inhibition, respectively. Idebenone is emphasized of anti-oxidant activity (30) and amelioration of dysfunction with cerebral ischemia (31). We examined that the effect of idebenone on mitochondrial swelling induced by $FeSO_4$ and ascorbic acid and obtained the result that idebenone inhibited the swelling at 25 μM giving 50%-inhibition (unpublished data). From our results in this paper and studies on idebenone, it is possible that calcium antagonists could ameliorate cerebral dysfunction-induced lipid peroxidation under ischemia and reoxygenation, which are relevant to anti-peroxidant activities, since they inhibited mitochondrial swelling induced by lipid peroxidation at same or lower concentrations than idebenone.

On the other hand, effects of calcium antagonists on mitochondrial swelling induced by arachidonic acid in the rat brain were investigated. Arachidonic acid is a major polyunsaturated fatty acids of phospholipid in membrane and are liberated by stimulation of phospholipase A_2 during ischemia (32,33), resulting in membrane damage. Mitochondrial swelling induced by arachidonic acid was not derived from lipid peroxidation because increase in TBARS was not detected during incubation. Flunarizine and efonidipine inhibited this swelling. In contrast, nicardipine, nifedipine, and nimodipine were not inhibited. Furthermore higher concentrations of flunarizine and efonidipine were required for inhibition of this swelling than these of them required for mitochondrial swelling induced by lipid peroxidation. It is supposed of different actions of flunarizine and efonidipine against mitochondrial swelling induced by arachidonic acid from their anti-peroxidant activity. Hillered (18) suggested that mitochondrial swelling induced by arachidonic acid was mediated by a mechanism dependent on energy failure, or detergent action of arachidonic acid. From these points of view, it is possible that flunarizine and efonidipine might stabilize mitochondrial membrane, which is ascribably a direct interaction with membrane.

In conclusion, these results obtained in this study suggested that all of calcium antagonists tested may protect from the membrane damage induced by lipid peroxidation and that each moiety of chemical structures would play an important role in appearance of anti-peroxidant activities. Interestingly, flunarizine and efonidipine may stabilize mitochondrial membrane, which is probably attributed to a direct interaction with membrane. Calcium antagonists may be effective for physiological disorders associated with lipid peroxidation, in part, by the inhibition of membrane damage arised from lipid peroxidation. In particular, flunarizine and efonidipine could be effective against membrane damage causing by different mechanism from lipid peroxidation.

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