Nilvadipine Attenuates Ischemic Degradation of Gerbil Brain Cytoskeletal Proteins

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We have previously demonstrated that transient cerebral ischemia induces marked decreases in concentrations of cytoskeletal proteins and have suggested putative involvement of calpain in the decrease of microtubule-associated protein 2 (MAP2) content. We examine the effect of nilvadipine, a new calcium channel blocker, on protein degradation in gerbil brains after 5 minutes of bilateral carotid artery occlusion and compare this effect with those of nimodipine and nicardipine. By densitometric quantification of the electrophoretically separated soluble proteins, mean±SEM MAP2 content in the hippocampus (14.4±1.8 μg/mg protein) was depleted (5.4±0.5 μg/mg, $p<0.01$) 4 days after ischemia; this depletion was significantly inhibited by 1 or 10 mg nilvadipine/kg/day. MAP2 content was also depleted in vitro when normal nonischemic brain extract was incubated with calcium, but this degradation was not inhibited by the calcium channel blockers. Our results suggest that calcium channel blockers do not act directly on calpain but act at the calcium channels of neurons and may suppress activation of the enzyme and attenuate ischemic degradation of cytoskeletal protein. We found nilvadipine to be the most potent drug among those studied, and we believe it could be useful for the treatment of cerebral ischemia. (*Stroke* 1989;20:78-83)

Accumulating evidence shows that calcium channel blockers have protective effects against cerebral ischemia,[1] and this protection has been attributed not only to the vascular effects of these drugs[2] but also to their direct effects on nervous tissue.[3] Nilvadipine,[4] a new dihydropyridine-derived calcium channel blocker, possesses advantages over known blockers in having a selective relaxant effect on the basilar artery and prolonged facilitation of blood flow. A previous study has shown that nilvadipine was effective in reducing ischemia in models of chronic coronary artery occlusion in dogs.[5]

Recently, one of us demonstrated[6,7] that transient cerebral ischemia induced marked decreases in concentrations of microtubule-associated protein 2 (MAP2) and calspectin in gerbil brains. These cytoskeleton-related proteins are presumed to play a critical role in neuronal activity, such as membrane excitation and axonal flow.[8] Therefore, investigations concerning the degradation of these proteins in ischemia will promote further understanding of the mechanisms of ischemic brain damage. Moreover, since MAP2 exists almost exclusively in dendrites,[9] it can be used as a specific marker of damage to neural cells; other biochemical indicators such as ATP, fatty acids, and protein synthesis are not cell type–specific. We have also suggested the participation of the calcium-activated neutral protease calpain in this decrease of MAP2 and calspectin contents since in vitro MAP2 and calspectin are good substrates for the enzyme,[10,11] which is present in the cytoplasm of various types of cells.[12]

We examined the effects of the calcium channel blocker dihydropyridine derivatives nilvadipine, nimodipine, and nicardipine on the degradation of proteins in vivo and in vitro with special regard to MAP2 in gerbil brains after 5 minutes of cerebral ischemia.

Materials and Methods

Male Mongolian gerbils (*Meriones unguiculatus*) weighing 52–82 g obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan) were restrained, and 20–30 mg lidocaine (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was sprayed onto the surface of the

neck and subcutaneously after incision of the skin. Local anesthesia was chosen because general anesthetics per se have a protective effect against ischemic injury.[13] Both common carotid arteries were exposed and temporarily clamped with microclips (Sugita aneurysm clip, Mizuho Ika Kogyo Co., Ltd., Tokyo, Japan) for 5 minutes, and recirculation after release of the clips was confirmed microscopically.

Nilvadipine (5-isopropyl-3-methyl-2-cyano-1,4-dihydro-6-methyl-4-(m-nitrophenyl)-3,5-pyridinedicarboxylate), nimodipine, and nicardipine were synthesized in the Research Laboratories of Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan) and were dissolved in polyethylene glycol 400 (solvent). All drugs were given subcutaneously on the basis of a preliminary study of the time course of the concentration of nilvadipine in plasma, which showed long-lasting delivery via this administration route.

The gerbils were divided into groups; in Group 1 ($n=56$), 0, 0.01, 0.1, or 1 mg/kg of the three calcium channel blockers were administered 1 hour before occlusion of the carotid arteries, and the gerbils were decapitated 1 hour after occlusion. In Group 2 ($n=51$), 0, 0.1, 1, or 10 mg/kg of the calcium channel blockers were given 1 hour before occlusion and daily for 4 days, and the gerbils were killed 1 hour

after the final dosing. Fourteen sham-operated gerbils were subjected to only exposure of the carotid arteries. Gerbils dying during occlusion of the carotid arteries were discarded.

In all groups, the brains were quickly removed onto ice-cooled plates and sectioned into the frontal cortex, the striatum, and the hippocampus. Tissue samples were homogenized in four volumes (vol:wt) of 0.01 M phosphate buffer (pH 7.2) and centrifuged at 100,000g for 30 minutes at 4° C. The supernatant was boiled with the same volume of sample buffer (3.2% sodium dodecylsulfate [SDS], 16% glycerol, and 0.12 M Tris-HCl at pH 6.8) for 5 minutes and stored at −80° C until used.

To measure the content of cytoskeletal proteins, equal volumes (10 μl) of the supernatants (20–30 μg protein) was loaded onto SDS-5% polyacrylamide gel for electrophoresis (SDS-PAGE) according to the method of Laemmli.[14] The separated proteins were stained with Coomassie brilliant blue R-250 (Nakarai Chemicals Co., Ltd., Kyoto, Japan) and quantified[15] with a densitometer (CS-930, Shimazu Seisakusho, Kyoto, Japan). The proteins were identified by the immunoblotting technique.[6,7] In brief, MAP2,[16] calspectin,[17] and clathrin[18] were prepared from bovine brain and used to immunize rabbits.
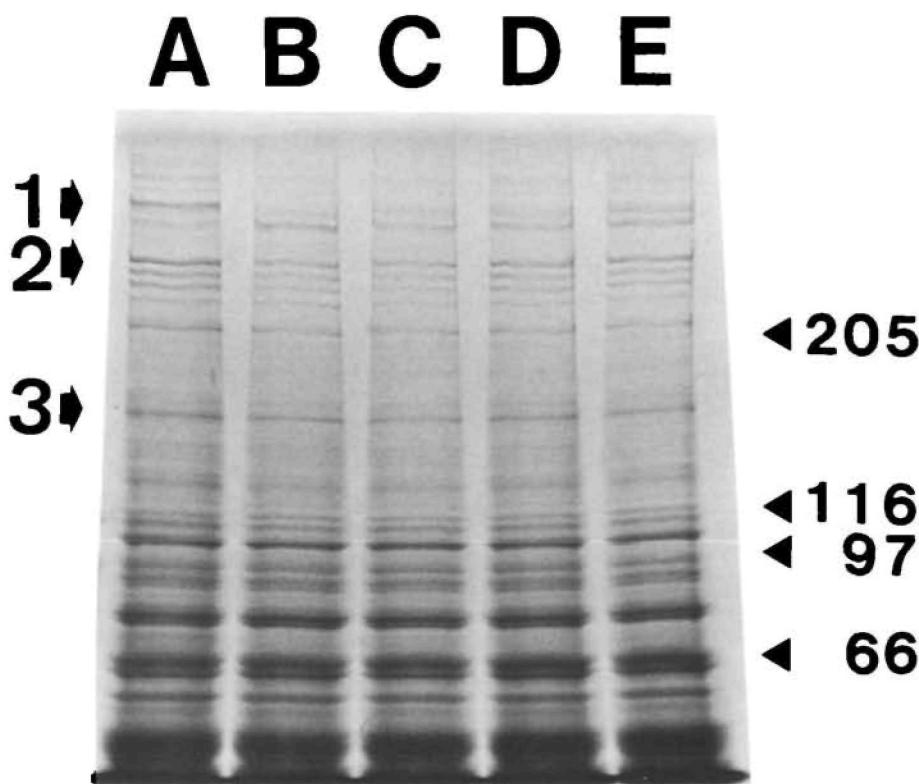

FIGURE 1. *Sodium dodecylsulfate-5% polyacrylamide gel electrophoresis patterns of hippocampal proteins of sham-operated gerbil and gerbils subjected to 5 minutes of cerebral ischemia with subsequent reperfusion for 4 days (Lanes B–E). Equal volumes were applied to each lane. Lane A, sham-operated, nonischemic brain; Lane B, gerbil treated with polyethylene glycol 400 solvent; Lane C, gerbil treated with 0.1 mg nilvadipine/kg/day; Lane D, gerbil treated with 1 mg nilvadipine/kg/day; Lane E, gerbil treated with 10 mg nilvadipine/kg/day. Arrows on left indicate bands: 1, microtubule-associated protein 2 (MAP2); 2, calspectin; 3, clathrin. Note that bands 1, 2, and 3 in Lane A were depleted after ischemia (Lane B), while low-molecular-weight bands were well preserved. Molecular weights of standard proteins are indicated as kilodaltons by triangles on right (205, myosin; 116, β-galactosidase; 97, phosphorylase b; 66, bovine serum albumin).*

Electrophoretically separated gerbil brain proteins were transferred onto nitrocellulose sheets,[19] which were incubated with the rabbit antisera. Bound antibodies were demonstrated with peroxidase-labeled second antibodies. Protein content was determined by the method of Lowry et al[20] using bovine serum albumin as a standard.

In vitro degradation of MAP2, calspectin, and clathrin was assayed using a crude extract containing both calpain and substrates of unoperated gerbil whole brain prepared as described above and stored at −80° C until used. A 50-μl volume of protein solution was incubated at 37° C for 60 minutes with 1.4 mM ethyleneglycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Nakarai), 2 mM $CaCl_2$, or 2 mM $CaCl_2$ plus various protease inhibitors or calcium channel blockers (0.01 mM N-ethylmaleimide [NEM, Nakarai], 1 mM phenylmethylsulfonyl fluoride [PMSF, Nakarai], 0.1 mM [L-3-*trans*-carboxyoxyran-2-carbonyl]-L-Leu-agmatin [E-64, Peptide Institute, Minoo, Japan], 0.1 mM leupeptin [Sigma Chemical Co., St. Louis, Missouri], 1 mM pepstatin A [Peptide Institute], or 0.01 mM nilvadipine, nimodipine, or nicardipine). Sample solutions were then subjected to SDS-PAGE and analyzed as described. Two separately prepared cerebral extracts were used in the in vitro assay.

To compare the sham-operated and solvent-treated (doses of 0 mg/kg calcium channel blockers) groups we used Wilcoxon's U test. To assess drug effects in vivo, we used analysis of variance followed by the multiple comparison method of Dunnett,[21] with the solvent group serving as the control. We analyzed results of the in vitro assay using Wilcoxon's U test.

Results

The contents of three soluble proteins of the gerbil brain (band 1 [260 kDa], band 2 [230–240 kDa], and band 3 [180 kDa]) were depleted after ischemia (Figures 1 and 2). These three proteins were identified as MAP2, calspectin, and clathrin, respectively, by the immunochemical method (not shown). The majority of the other proteins were electrophoresed beneath the gel bottom in 5% polyacrylamide gel (Figure 1), and any change in their concentrations could not be detected in relation to ischemia, even when analyzed in 10% polyacrylamide gel. The relations between optical density of these three proteins and total peak area were linear for 10- to 40-μg samples. For sham-operated gerbils, 1-mg samples contained 11.8–14.4 μg MAP2, 11.6–15.0 μg calspectin, and 13.8–16.1 μg clathrin; no significant regional differences in protein content were seen (Table 1). At 1 hour and 4 days after ischemia (solvent treatment), protein contents were drastically decreased to approximately one half or one third except in the frontal cortex, where only slight decreases were seen 1 hour after ischemia.

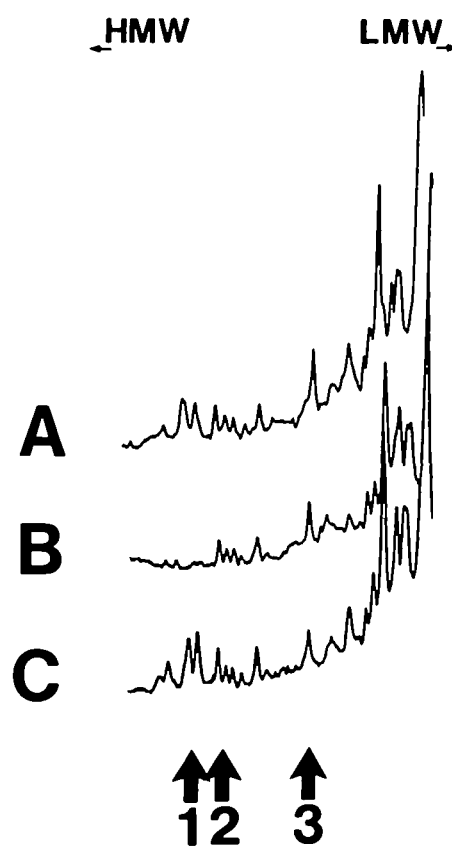Nilvadipine dose-dependently and significantly ($p < 0.05$ or 0.01) suppressed the depletion of MAP2

FIGURE 2. *Densitometric scannings of band stained in electrophoresis show molecular weight range around ≥ 100 kDa. Samples were hippocampal proteins of* (A) *sham-operated,* (B) *solvent-treated, and* (C) *10 mg nilvadipine/kg/day-treated gerbils subjected to 5 minutes of cerebral ischemia with subsequent reperfusion for 4 days. Arrows indicate microtubule-associated protein 2 (band 1), calspectin (band 2), and clathrin (band 3).*

in all three brain regions 1 hour and 4 days after ischemia (Table 1). Nimodipine and nicardipine also significantly suppressed the depletion of MAP2, though their effects varied across regions (nimodipine was effective in the striatum and hippocampus, whereas nicardipine was effective in the frontal cortex and striatum). Effective doses of the three drugs were 1–10 mg/kg except in the striatum, where doses even as low as 0.01 mg/kg were effective in suppressing MAP2 depletion 1 hour after ischemia.

Nilvadipine also significantly suppressed the depletion of calspectin in the hippocampus 1 hour and 4 days after ischemia and in the frontal cortex 4 days after ischemia; nimodipine and nicardipine had similar but nonsignificant ($p > 0.05$) effects. No calcium blocker had any effect on the decrease of clathrin content in any brain region.

MAP2 and calspectin were broken down calcium-dependently in vitro (Table 2). On the other hand, clathrin content did not change at all in vitro (data not shown), although it was also depleted after ischemia. Known inhibitors of calpain (NEM, E-64, and leupeptin) completely inhibited the breakdown

TABLE 1. Effects of Calcium Channel Blockers on Ischemia-Induced Degradation of MAP2, Calspectin, and Clathrin After 5 Minutes of Cerebral Ischemia With Subsequent Reperfusion for 1 Hour or 4 Days

Treatment (mg/kg)	n	Frontal cortex			Striatum			Hippocampus		
		MAP2	Calspectin	Clathrin	MAP2	Calspectin	Clathrin	MAP2	Calspectin	Clathrin
1 hour reperfusion										
Sham operation	9	13.4±0.9	13.0±0.7	16.1±0.9	13.3±1.8	13.1±1.3	15.9±2.2	13.5±0.8	11.6±0.7	13.8±1.8
Solvent	11	9.7±0.2*	12.4±0.7	12.0±0.9*	4.4±0.6*	6.4±1.0*	7.0±0.6*	6.6±0.3*	8.3±0.6*	6.5±0.6*
Nilvadipine										
0.01	4	12.7±1.8	14.3±1.3	12.6±1.8	9.6±2.5†	9.5±2.3	6.4±1.0	8.4±1.2	10.7±1.7	8.1±3.2
0.1	5	12.2±0.5	13.1±0.5	12.2±0.7	10.0±2.0‡	7.8±1.1	9.0±2.9	8.9±0.6	9.9±1.0	7.2±2.0
1	6	15.3±1.3‡	13.4±1.1	12.5±0.6	10.6±0.8‡	9.9±1.0	7.8±1.2	10.5±1.0‡	12.5±1.6†	6.9±0.6
Nimodipine										
0.01	5	8.8±1.2	10.9±1.2	12.8±0.9	7.7±0.8†	8.2±1.2	10.9±3.3	6.5±0.4	7.9±0.6	8.4±0.5
0.1	5	9.7±1.7	9.8±2.0	12.8±1.0	7.7±1.1†	10.7±1.9	10.7±1.8	7.0±0.9	10.5±1.7	9.1±1.1
1	5	10.3±1.0	11.7±0.9	13.5±0.6	8.1±1.0†	10.1±2.8	10.8±2.0	9.7±1.6†	11.3±2.4	9.9±2.0
Nicardipine										
0.01	5	10.7±1.2	11.8±0.7	10.9±0.9	7.6±0.8†	8.9±0.7	8.2±1.4	7.1±1.0	10.5±1.6	8.5±1.0
0.1	5	12.3±1.1	12.9±0.7	10.4±0.8	8.1±1.3†	10.0±0.9	7.5±0.6	7.3±0.4	9.4±0.7	7.9±0.5
1	5	13.5±1.5†	13.0±0.8	10.5±0.6	7.8±0.6†	8.6±0.9	10.0±1.0	7.9±0.7	8.7±1.2	9.2±1.2
4 days reperfusion										
Sham operation	5	13.7±1.3	12.8±0.5	14.8±0.9	11.8±1.0	12.8±2.4	14.0±1.3	14.4±1.8	15.0±0.6	14.6±0.8
Solvent	10	5.8±0.4*	8.1±0.6*	8.4±0.6*	4.6±0.5*	6.7±0.9	7.7±0.9*	5.4±0.5*	7.3±0.5*	8.1±0.9*
Nilvadipine										
0.1	5	5.6±0.8	9.6±0.4	7.4±0.1	5.3±0.8	5.8±0.8	7.3±0.6	6.0±1.0	8.6±1.1	8.5±1.0
1	4	11.0±0.7‡	11.8±1.0‡	7.7±0.8	10.2±1.7‡	8.7±1.1	6.6±0.5	10.2±1.0‡	11.0±0.6‡	8.5±0.9
10	5	10.3±1.3‡	11.4±0.8‡	7.4±0.5	8.3±0.6‡	8.9±0.4	6.9±0.9	11.3±0.8‡	11.7±0.6‡	8.8±0.5
Nimodipine										
0.1	5	7.3±1.9	6.6±1.3	8.2±1.5	5.3±1.0	7.3±1.5	8.3±1.7	5.6±0.8	9.3±1.5	7.5±0.7
1	4	5.6±2.2	6.7±2.2	8.8±1.3	9.3±0.4‡	9.3±0.7	9.0±0.8	8.6±1.5	8.4±1.9	8.3±1.7
10	4	6.6±2.6	6.5±1.9	8.6±0.9	9.6±1.5‡	9.3±0.8	9.6±1.6	8.9±2.0	9.3±0.9	10.4±2.4
Nicardipine										
0.1	4	6.6±0.9	8.0±1.2	10.2±1.0	6.4±0.4	8.8±1.2	8.0±1.1	7.0±0.7	7.7±0.6	7.8±1.4
1	5	8.0±1.0	7.6±1.5	8.7±0.4	7.3±1.0‡	9.4±0.7	9.3±1.1	6.1±1.0	8.0±0.9	8.1±0.8
10	5	8.8±1.0†	9.5±1.2	8.6±0.3	8.1±0.3‡	8.9±1.0	8.6±1.1	7.8±0.7	8.2±1.2	8.1±0.8

Data are mean±SEM μg/mg brain protein. MAP2, microtubule-associated protein 2; solvent, polyethylene glycol 400.
*$p<0.01$ vs. sham-operated group (Wilcoxon's U test).
†‡$p<0.05$, 0.01 vs. solvent-treated group (analysis of variance and Dunnett's test).

of MAP2 and calspectin, but PMSF, an inhibitor of serine proteases, only moderately inhibited the breakdown of calspectin. Pepstatin A, an inhibitor of aspartic proteases, did not inhibit the breakdown of either protein. In contrast to the results in vivo, no calcium channel blocker had any effect on the breakdown of MAP2 and calspectin in vitro.

Discussion

Some calcium channel blockers are known to increase cerebral blood flow,[22] even though they cause systemic hypotension, and are reported to have beneficial effects against neurologic, electroencephalographic, and cellular damage associated with cerebral ischemia.[23-25] The molecular mechanisms of the effect of calcium channel blockers are still obscure, and the effect of these drugs on energy metabolism is disputed.[3,24] We have demonstrated in the present experiments that calcium channel blockers have protective effects against the degradation of MAP2, a neuron-specific protein. In addition, the depletion of MAP2, calspectin, and clathrin concentrations after cerebral ischemia agreed with the early reports of Nishimura et al[6] and

Yamashita.[7] Concentrations of other cytoskeletal proteins and neurofilaments (MW 200, 150, or 70 kDa) are also assumed to decrease in ischemia,[26] but, in this experiment, we did not observe any decrease in the contents of proteins smaller than 200 kDa except for clathrin. Perhaps this is because neurofilament proteins are rather stable and therefore were not solubilized under our experimental conditions. Concerning tubulin, which is the main protein constituent of microtubules, some investigators[27] have reported that ischemia induced disintegration of microtubules. Although we did not observe any decrease in tubulin content, the observed decrease in MAP2 content might be related to microtubule disintegration since MAP2 is reported[16] to promote the assembly of microtubules. MAP2 was rather more easily degraded in ischemia than were the other two proteins (Table 1), and since MAP2 exists almost exclusively in the cytosol of dendrites and the cell bodies[9,28] of neurons, measurement of MAP2 content after ischemia may be a good way to assess damage to neural cells.

Calspectin is also present in neurons, and a large proportion of this protein is included in the mem-

TABLE 2. Effects of Protease Inhibitors and Calcium Channel Blockers on Calcium-Induced MAP2 and Calspectin Degradation in Vitro

Addition	n	% of unincubated control	
		MAP2	Calspectin
Extract I			
1.4 mM EGTA	11	98.2±8.8	113.8±5.0
2 mM CaCl₂			
+None	11	24.6±5.9	41.4±6.7
+0.01 mM NEM	5	83.2±5.8*	91.5±9.9*
+1 mM PMSF	5	22.6±7.0	66.2±8.2†
Extract II			
2 mM CaCl₂			
+Protease inhibitors			
None	4	33.4±4.3	43.3±9.2
0.1 mM E-64	4	96.0±15.1†	116.1±9.2†
0.1 mM leupeptin	4	86.1±19.1†	90.9±3.3†
1 mM pepstatin A	4	32.0±4.4	41.3±3.9
+Calcium channel blockers			
Solvent	4	31.7±11.0	48.7±8.1
0.01 mM nilvadipine	4	36.2±4.5	40.6±1.1
0.01 mM nimodipine	4	38.4±5.7	54.7±7.0
0.01 mM nicardipine	4	45.8±8.1	56.5±5.0

Two separately prepared cerebral extracts were used. Values are mean±SEM. EGTA, ethyleneglycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.
*†$p<0.01$, 0.05 vs. CaCl₂+none.

brane fraction,[29] which sediments during centrifugation. However, it seems unlikely that the observed depletion of calspectin merely reflects change of the protein from a cytosolic form to a membrane-bound one since when pellets were resolubilized in SDS after centrifugation and quantified after SDS-PAGE, there was no difference between band staining of the samples from sham-operated and ischemic gerbil brains (not shown). The above are cytoskeleton-related proteins and are presumed to play a critical role in neuronal activity. Therefore, their degradation in ischemia might induce critical damage to neuronal activity and finally to neuronal survival, but it is uncertain now whether this degradation induces damage or only reflects the results of damage.

Nilvadipine showed clear effects in all three brain regions studied, but nimodipine and nicardipine were effective in only two regions, the striatum being the most sensitive (Table 1). The reasons for this regional difference may be different regional distributions of MAP2, calspectin, and calpain and/or different regional sensitivities to the drugs. The first possibility seems rather unlikely because differences in regional distribution of MAP2,[30] calspectin,[29] and calpain[29] are reported to be slight, at least in the hippocampus, striatum, and frontal cortex. Supavilai et al[31] have reported that PN200-110, one of the dihydropyridine derivatives, is bound to brain tissues in the rank order of hippocampus> cortex>striatum in vitro. This uneven distribution of the dihydropyridine receptors seems to be related to regional differences in drug potency. Addition-

ally, the extent of interaction of dihydropyridine calcium channel blockers with muscarinic and adrenergic receptors is reported to depend on the lipophilicity of the drugs.[32] Such differences in lipophilicity or heterogeneous delivery of the drugs into brain regions might affect their potency, but no confirming studies have been done.

The protective effects of the drugs were similar at 1 hour and 4 days after ischemia (Table 1), which suggests that protein degradation was induced during and in the early stages after ischemia and that blocking of the calcium channels in these stages is an important protective step, though slow accumulation of calcium in the hippocampus has also been reported.[33] In the cortex, relatively slow and progressive protein depletion was seen (Table 1, solvent treatment), which may bear some relation to the observation of Pulsinelli et al[34] of progressive cortical cell death after ischemia.

As to the mechanisms of protein depletion, it is again presumed that MAP2 and calspectin are broken down by the calcium-activated neutral protease calpain as we have suggested.[6,7] In an in vitro study, MAP2 and calspectin were broken down calcium-dependently (Table 2), but clathrin was not. The calpain inhibitors[35] NEM, E-64, and leupeptin inhibited the degradation of MAP2 and calspectin, whereas pepstatin A, an inhibitor of cathepsin D (which is known to degrade cytoskeletal proteins[36]), had no such effect. The three calcium channel blockers were also ineffective when used at concentrations corresponding to the 10 mg/kg p.o. injection of nilvadipine used in an in vivo study with rat brain.[37] This finding suggests that the degradation of MAP2 and calspectin in ischemia is also related to the activation of calpain and that the protective effect of calcium channel blockers on the degradation of MAP2 in vivo is induced at sites of the calcium channels of neurons. Indeed, dihydropyridine-sensitive L-type calcium channels are thought to be distributed mainly at dendrites[38] where MAP2 is most abundant.[9] Furthermore, no calcium channel blocker had any effect on the degradation of clathrin, which further supports our findings that clathrin degradation is independent of calcium or calpain. Of course, our observation does not rule out the possibility that attenuated degradation was a secondary effect of the drug's vascular effect. For nilvadipine, however, unpublished data from our laboratories indicate no depressive effect, such as hypothermia or a decrease in spontaneous activity, on metabolism.

In conclusion, we have shown that nilvadipine, nimodipine, and nicardipine successfully protected against the depletion of MAP2 in neurons induced by brief cerebral ischemia. This effect may be due to these drugs' abilities to block the calcium channels of neurons and hence to suppress the activation of calpain. Thus, the beneficial effects of calcium channel blockers in cerebral ischemia may reflect their effects on neurons as well as that on the cerebral vasculature. Nilvad-

ipine had the most potent activity in our study, suggesting that this drug could be the more useful for the treatment of cerebral ischemia.

Acknowledgments

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