Effect of Lipids on the Activity of Calpain in Subcellular Fractions Obtained from the Rat Brain

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We studied the effect of lipids on the activity of a neutral cysteine proteinase, calpain, in subcellular fractions obtained from the rat brain. Extraction of nearly 23% of membrane cholesterol from the coarse mitochondrial fraction did not result in modifications of specific activity of calpain in this fraction. Detergents (digitonin or Triton X-100) used in 0.3% concentration enhanced the activity of calpain in the coarse mitochondrial fraction. Examination of the effects of preparations of different phospholipids on the activity of calpain in the cytoplasm demonstrated that only phosphatidylcholine, but not phosphatidylserine and/or cardiolipin, insignificantly increased the activity of calpain (independently of the size and structure of phospholipid vesicles). We hypothesize that the mechanisms underlying interaction between calpain and lipids are not universal; in native cells and model experiments, they can differ noticeably from each other and are modified depending on the corresponding conditions.

Keywords: calpain, cholesterol, phospholipids, activation of an enzyme.

INTRODUCTION

Calpains belong to the familiy of widely distributed neutral cysteine proteinases; these enzymes are, in most cases, represented by two main subgroups (μ- and m-calpains). For optimal activity of the enzymes belonging to these subgroups, the corresponding micro- or millimolar concentrations of calcium are necessary. Many studies demonstrated that calpains play important roles in mediation of calcium signalling in cells of various tissues [1-3]. Proteins of cytoskeleton, different enzymes (kinases, phosphatases, and phospholipases), and also proteins of membrane receptors and transporters [4] are substrates for the above enzymes. Calpains can be found in both cytoplasmic and membrane cellular fractions, but data on their amount and ratios in these fractions are contradictory; it seems probable that these indices in various tissues are different [5-7]. Calpains are involved in numerous processes that are realized in cells of the nerve tissues under normal conditions (e.g., structural and functional reorganization of synapses related to long-term potentiation of synaptic transmission and the control of neurotransmitter release) [8, 9]. Modifications of the activity of calpains are observed in different pathologies of the nervous system (Alzheimer's disease, multiple sclerosis, neurotoxicoses, and cerebral ischemia) [2, 10-12]. In recent years, studies are concentrated on the possibility of using inhibitors of calpains for correction of a number of pathological states, including neurological diseases [13, 14]. It should, however, be recognized that the mechanisms underlying the action of proteolytic enzymes of this group remain unstudied in detail.

It is known that calcium concentrations necessary for activation of both main forms of calpain (3 to 50 and 400 to 800 μM for half-maximum activation of μ- and m-calpains, respectively) are many times higher than the concentration of this ion in the cell cytoplasm, even at moments of maximum increases in this index, e.g., during the neurotransmitter release [4, 15]. Taking into account that a protein inhibitor of calpain, calpastatin, is constantly present in the cells, it can be hypothesized that activation of calpain is a

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rather complex process. Most likely, the corresponding pathway should include dissociation (permanent or transient) of the enzyme and the inhibitor in the cells. Either the presence of some compartments with an increased calcium concentration sufficient for activation of the enzyme in such a cell or a temporary increase in its affinity for calcium are necessary. The exact molecular mechanism of activation of calpain remains unclear.

It was initially hypothesized that calpain usually exists in the cytoplasm in an inactive state and is transformed in the functional state only after autolysis (as a result, the concentration of calcium necessary for activation of the enzyme decreases significantly [16]) and/or after binding of calpain to the cell membrane structures [15, 17]. However, other studies showed [5, 18] that calpain in the soluble state is also active. m-Calpain extracted from skeletal muscles binds to phospholipid vesicles in the absence of calcium [19], while binding of μ-calpain to the membranes does not result in a drop in the calcium concentration necessary for its activation [20]. It is supposed that binding of calpain to the membranes and its activation are realized at the expense of modifications of the conformation of enzyme molecules due to which hydrophobic compartments in the active center become more accessible for interaction with the membranes [20].

In searching for factors capable of influencing the affinity of the enzyme for calcium, it was hypothesized that lipids present in cellular membranes represent one of such factors [4, 15]. However, examination of the effects of different phospholipids (main components of biological membranes) on the activity of calpain did not allow one to propose unambiguous conclusions. According to the data obtained by some authors, the calcium concentration necessary for activation of calpain in the presence of phosphatidylinositol, phosphatidylcholine, and phosphatidylserine decreased [21], while other researchers observed the effect of an increase in the affinity of enzyme for calcium only in the presence of phosphatidylinositol [22], and polyphosphoinositides [23]. As some authors showed, multicomponent lipid mixtures including gangliosides and phosphatidylinositol and capable of stimulating the activity of calpain at calcium concentrations close to normal intracellular ones can be rather effective from this aspect [24]. Recently, it was reported that the activity of calpain in the cell is controlled by binding of phosphoinositides to cytoskeletal proteins that are substrates for the above enzyme [25].

It is known that separate cholesterol- and sphingolipid-enriched microdomains, which are insoluble in nonionic detergents, the so-called rafts, are present in the molecular structure of biological membranes [26]. The presence of such domains is very important; perhaps, this phenomenon is responsible for selective localization of certain cell proteins in the membrane, as well as for spatial dissociation and regulation of different processes realized in the cell, including processes of cellular signalling, membrane transport, and exocytosis. It was demonstrated that m-calpain is localized in cholesterol-enriched microdomains of the membranes of T-cells [27] and in caveolae of the muscle cells [28]; it appears in such parts of the membranes during chemotaxis [29]. At the same time, it was found [30] that calpain splits annexins of smooth muscles in those sites of the plasma membrane where microdomains are absent. Therefore, information on the role of membrane cholesterol in the functioning of calpain and the data on phospholipid-induced activation of this enzyme are rather contradictory.

Earlier, we studied the distribution of activity of calpain in subcellular fractions obtained from the rat brain tissue under conditions close to those existing in nerve cells in the norm, i.e., with no separation of calpain from calpastatin [18]. We demonstrated that the main part (87%) of the total enzymatic activity of calpain was found in the cytoplasmic fraction. At the same time, an appreciable activity of this enzyme was also observed in the studied membrane fractions (coarse mitochondrial fraction, microsomes, and myelin). The highest specific activity of calpain, which increased significantly after its separation from calpastatin and was suppressed in the presence of an inhibitor of calpain I, was observed in the cytoplasmic fraction. It was of obvious interest to elucidate whether the activity of calpain in the above fraction is influenced by the presence of phospholipids in the medium for estimation of this activity. In this study, we also examined the effects of detergents on the activity of calpain and investigated the effects of extraction of cholesterol from the membrane (and, as a consequence, destruction of lipid rafts) on the activity of calpain in the membrane fraction (coarse mitochondrial one) obtained from the rat brain tissue.

METHODS

Isolation of Subcellular Fractions from the Rat Cerebral Tissue. Adult male rats were decapitated; the brains were isolated and placed on ice. All further procedures were performed at 4°C. The cerebral

tissue was chopped by a scalpel and homogenized in solution A (1 g of tissue in 10 ml of solution) using a glass homogenizer with a Teflon pestle. The composition of solution A was the following (mM): sucrose, 320; Tris-Cl, 20 (pH 7.4); ethylenediaminetetraacetic acid (EDTA), 2; dithiothreitol (DTT), 2; and phenylmethylsulfonyl fluoride, 0.1.

The homogenate was centrifuged for 10 min at 750g; the sediment was removed, and the supernatant liquid H1 was centrifuged for 20 min at 12,000g. Then, the obtained supernatant liquid H2 was centrifuged for 60 min at 100,000g, to obtain the cytoplasm fraction. To obtain the so-called membrane fraction, the coarse mitochondrial fraction sediment O2 obtained after centrifugation at 12,000g was suspended in the solution A. Extraction of calpastatin and isolation of a purified preparation of m-calpain from the soluble fraction was performed using the technique of Takeuchi et al. [5]. The content of protein in the obtained preparations was estimated using the Lowry technique [31].

Estimation of the Activity of Calpain in Subcellular Fractions. The activity of calpain was estimated using casein with a fluorescent label (FITC) as a substrate. Casein was labeled according to the earlier described technique [32], by elution of the labeled substrate from a Sephadex G-50 column with a solution of the following composition (mM): Tris-Cl, 10 (pH 7.0) and NaCl, 150. The activity of calpain in subcellular fractions was estimated in 100-µl samples containing a substrate solution of the following composition (mM): Tris-Cl, 50 (pH 7.4); dithiothreitol, 5; $CaCl_2$, 5 (or EDTA, 5), as well as 0.2% FITC-casein. The reaction was initiated by the addition of 10 to 500 µg of a protein preparation, and after incubation at 30°C (except for specially indicated cases) it was stopped by the addition of 50 µl of bovine serum albumin (5 mg/ml) and 50 µl of cooled 12% trichloroacetic acid to the sample. The samples were kept on ice for 20 min and centrifuged at 5,000g for 8 min. Then, 100 µl of the supernatant liquid containing fluorescent products of hydrolysis of the substrate were added to 2 ml of 0.2 M Tris-Cl (pH 8.5). The intensity of fluorescence of the samples was measured at 490-nm (excitation) and 525-nm (emission) wavelengths. The activity of calpain was estimated as a calcium-dependent increment of fluorescence, which is the difference between the value of fluorescence of the calcium-containing sample and the value of fluorescence of the EDTA-containing sample.

Phospholipid Vesicles (Liposomes). Solutions of phospholipids (3 mg of phosphatidylcholine, phosphatidylserine, or cardiolipin) in chloroform-methanol were dried in a nitrogen stream up to complete removal of the solvent. The obtained film of phospholipids was suspended in 1 ml of the buffer solution containing 50 mM Tris-HCl (pH 7.4). Large multilayer liposomes of different diameters were obtained using mechanical shaking of the phospholipid suspension for 3 min, while monolayer liposomes were obtained by means of pressings the phospholipid suspension through a polycarbonate filter with 100-nm pores.

Extraction of Cholesterol from the Membrane Fraction of the Rat Brain Tissue. The solution of methyl-β-cyclodextrin was added to 100 µl of suspension of the coarse mitochondrial fraction O2 (4 mg protein/ml), up to a final concentration of 15 mM or 30 mM. Distilled water was added to the control sampling. The samples were incubated for 30 min at 37°C with permanent shaking and then centrifuged at 3,000g for 5 min; the obtained sediment was washed out with 1 ml of the solution containing (mM): sucrose, 320 and Tris-Cl, 10 (pH 7.5). The amount of cholesterol in the obtained sediment was estimated by the technique of Zlatkis et al. [33].

RESULTS AND DISCUSSION

Estimation of the activity of calpain according to splitting of the fluorescent substrate by preparations of the subcellular fractions (where this enzyme was not separated from ballast proteins and, moreover, it was not separated from a specific protein inhibitor of calpain, calpastatin, permanently present in the cell) requires long-lasting (during several hours) incubation of the enzyme together with the substrate in the presence of millimolar concentrations of calcium in the medium. It is known that such long-lasting incubation of calpain in the presence of calcium ions can result in noticeable inactivation of the enzyme [4]. When studying the temperature dependence of the activity of calpain, we found that incubation at 30°C is optimal under our experimental conditions. For example, as can be seen from Fig. 1A, the amount of fluorescent products of splitting of FITC-casein by preparations of the cytoplasm from the rat cerebral tissue during 1 h at 37 and 30°C was nearly identical, but when we used a longer incubation, the rate of formation of the products of hydrolysis at 37°C decreased, as compared with that observed at 30°C. A similar trend was found in the course of estimation of the activity in the membrane fraction from the rat cerebral cells (B). In these preparations, the amount of fluorescent

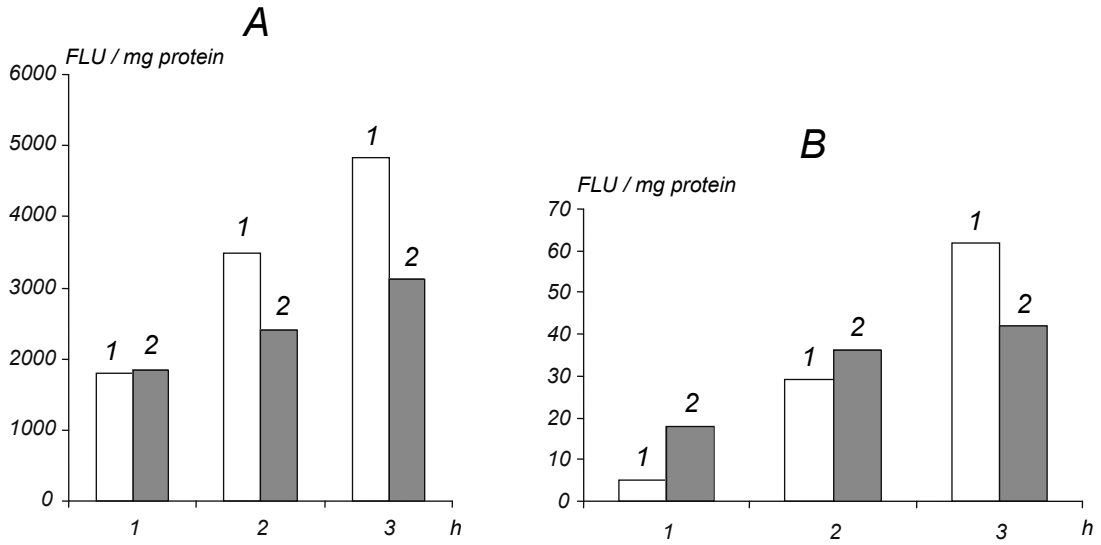

Fig. 1. Effect of temperature on the activity of calpain in the cytoplasmic (A) and membrane (B) fractions from the rat brain. Abscissa) Time for estimation of the activity of calpain, h; ordinate) normalized intensity of fluorescence, fluorescent units (FLU) per 1 mg protein. 1 and 2) At temperatures of 30 and 37°C, respectively.

products formed during 1-h-long incubation at 30°C was smaller than that at 37°C and practically the same at both tested temperatures under conditions of incubation for 2 h. If we used 3-h-long incubation, the amount of fluorescent products formed at 37°C was significantly smaller than that at 30°C. Therefore, estimation of the activity of calpain in preparations of the subcellular fraction under conditions of long-lasting incubation should be performed with a maximum correctness at 30°C.

Our studies of the distribution of the activity of calpain in subcellular fractions from the rat brain demonstrated that the specific activity of calpain in a soluble cerebral fraction exceeds by nearly two orders of magnitude the specific activity of this enzyme in all the cerebral membrane fractions studied in our experiments earlier [18]. As was found [6], the activity of calpain in the presence of Triton X-100 increases only in the nerve tissue and remains unchanged in all other rat tissues under study. In our experiments, Triton X-100 and digitonin in concentrations of 0.3% did not influence the activity of calpain in the rat cytoplasmic cerebral fraction and intensified such an activity (Triton X-100 by 23%, and digitonin by 20%) in the membrane fraction. It seems probable that the studied nonionic detergents in relatively small amounts exert no inhibitory effect on the enzyme (this is indicative of the absence of suppression of the activity of the latter in the cytoplasmic fraction). It is probable, however, that the active center of the calpain molecule in the presence of "soft" detergents becomes more accessible for the substrate.

To elucidate the role of cholesterol in the control of the activity of calpain, we used an agent such as methyl-β-cyclodextrin (MCD) that binds cholesterol and extracts it from the membrane. It is known that such extraction results in damage to the structure of microdomains of the membrane. This, in turn, leads to dysfunction of membrane proteins [34]. To remove cholesterol from the rat cerebral membrane fraction, we used 15 or 30 mM MCD. Table 1 shows data on the amount of cholesterol and the specific activity of calpain in the cerebral membrane fraction treated with the above-mentioned agent in different concentrations. It can be seen that the amount of removed cholesterol increases with increase in the concentration of MCD; correspondingly, its concentration in the membranes decreases. These data indicate that the removal of about 23% of membrane cholesterol did not influence the value of the specific activity of calpain in the membranes of nerve cells; it practically remained unchanged, as compared with the corresponding value in control preparations. Therefore, it can be hypothesized that damage to the structure and loss of integrity of lipid rafts is not a crucial event influencing the activity of calpain in the membranes of nerve endings.

It was demonstrated earlier [30] that calpain is localized at the membrane sites of smooth muscles where cholesterol-enriched microdomains are absent. In some other cells, calpain was found exclusively in these microdomains [27-29, 35]. It seems probable that localization of calpain in cholesterol-enriched microdomains of the membrane is not constant; such

TABLE 1. Effect of Removal of Cholesterol on the Activity of Calpain in the Membrane (Coarse Mitochondrial) Fraction Obtained from the Rat Brain

Concentration of methyl-β-cyclodextrin (MCD), mM	Amount of cholesterol		Specific activity of calpain	
	μM/mg protein	% with respect to the control	FLU/mg protein · h	% with respect to the control
0 (Control)	0.519	100	31.0	100
15	0.440	85	28.3	91
30	0.400	77	32.9	106

Footnotes. Cholesterol was removed from the coarse mitochondrial fraction obtained from the rat brain using MCD (for details, see Methods). The activity of calpain was estimated in samples incubated for 3 h at 30°C. Specific activity was calculated as a calcium-dependent increment of fluorescence per 1 mg of protein during 1 h of incubation. Amount of cholesterol and specific activity of calpain in the samples untreated with MCD were taken, respectively, as 100%.

TABLE 2. Effects of Phospholipids on the Activity of Calpain in Cytoplasmic Fraction Obtained from the Rat Brain

Preparation	Concentration of calpain, mM	Lipid/lipid mixture added	Relative activity of calpain, %
Calpain in cytoplasmic fraction	0.25	–	68
–"–	1.0	–	89
–"–	4.5	–	100
–"–	0.25	phosphatidylcholine	90
–"–	1.0	–"–	113
–"–	4.5	–"–	90
–"–	0.25	cardiolipin	37
–"–	1.0	phosphatidylcholine + cardiolipin	56
–"–	4.5	cardiolipin	87
–"–	0.25	phosphatidylserine	81
Purified m-calpain	1.0	–	80
–"–	4.5	–	100
–"–	1.0	phosphatidylcholine	105
–"–	4.5	–"–	80

Footnotes. Samples were incubated during 2 h at 30°C with addition of the corresponding phospholipids (mass ratio of protein/lipid in a sample was 1:3) in the presence of different concentrations of calcium (in this table, the concentration of free calcium calculated using CaBuf software is shown). The activity of calpain in the sample containing 4.5 mM Ca^{2+}_{free} with no addition of phospholipids was taken as 100%.

localization depends on the type, function, and state of the cell [29, 36].

Phospholipids are considered one of the probable factors responsible for activation of calpain *in vivo* [4, 15]. Nevertheless, data on the effects of some phospholipids and their mixtures on the activity of the enzyme *in vitro* are scarce and contradictory [21-24]. We studied the effects of negatively charged phospholipids (cardiolipin and phosphatidylserine), a neutral lipid (phosphatidylcholine), and their mixtures on the activity of calpain in the cytoplasmic fraction of nerve cells from the rat brain. Phospholipids were used as suspensions of multilayer and/or monolayer (diameter up to 100 nm) liposomes.

The data obtained in our experiments are shown in Table 2; they indicate that the activity of calpain increased insignificantly only in the presence of phosphatidylcholine and only in samplings containing calcium in submaximum concentrations. In the presence of calcium in maximum amounts necessary for the action of the enzyme, phosphatidylcholine exerted a mild inhibitory effect. Cardiolipin and phosphatidylserine did not intensify the activity of calpain in the rat cerebral cytoplasmic fraction; in contrast, they suppressed this activity. All the observed effects did not depend on the technique of isolation of phospholipid vesicles and (according to preliminary findings) did not differ from each other whether we used monolayer (up to 100 nm) or multilayer large liposomes. It can be hypothesized that interaction between phosphatidylcholine liposomes and a hydrophobic part of the protein calpain molecule resulting in its activation occurs in the presence of submaximum calcium concentrations. Such interaction can be very significant for activation of calpain in the native cells [15, 20].

Using ion-exchange chromatography, we can isolate fractions containing predominantly μ-calpain and calpastatin in 0.15 M KCl medium or m-calpain in 0.3 M KCl medium [5]. We demonstrated earlier [18] that such differentiation increases several times the activity of calpain in the cytoplasmic fraction of nerve cells. In our study, we found that only phosphatidylcholine, among all the studied phospholipids in purified preparations of m-calpain, increased (only insignificantly) the activity of the enzyme at submaximum calcium concentrations (Table 2). The effect of other phospholipids on the activity of m-calpain was similar to that described for cytoplasmic preparations (not illustrated).

Therefore, our findings do not support the earlier-proposed opinion that negatively charged phospholipids are the most effective activators of calpain [22, 23]. At the same time, it is reasonable to believe that activation of calpain by phospholipids *in vitro* can be realized in different ways, depending on the split substrate [37]. At present, it becomes more and more probable that the wide distribution of this enzyme and the diversity of its functions are related to the existence of various specific mechanisms of its activation. These mechanisms, most likely, are not universal ones; they depend on both the type of tissue and localization of calpain in one subcellular structure fulfilling a specific function in the cell or another.

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