
DETERMINATION AND REGULATION OF THE CALPAIN ACTIVITY IN SUBCELLULAR FRACTIONS OF THE RAT BRAIN

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Introduction

Despite the considerable progress in the understanding of the molecular mechanisms of neurosecretion achieved last years, many aspects of this problem remain unsolved. At present, release of neurotransmitter from the synaptic terminal (exocytosis) is considered as a complex multistep calcium-dependent process; the main steps of this process are "docking", i.e. close rapprochement of the synaptic

vesicles with the cytoplasmic side of the membrane of the synaptic terminal, preparation of the vesicles for fusion, and fusion itself, the unification of the synaptic vesicle membranes and presynaptic membrane with pore formation, followed by mediator efflux to the synaptic cleft (Sollner et al., 1993; Sudhof, 1995). It is known (Sudhof, 1995; Hilfiker et al., 1999) that many proteins, membrane or cytoplasmic, are involved in all stages of the neurosecretory processes. Various enzymes, including protein kinases, phosphatases, phospholipases and proteinases (De Camilli, 1995; Kondo et al., 1997) play significant roles in the regulation of exocytosis.

Fusion of synaptic vesicles with the presynaptic membrane is the final and very important stage of the process of neurosecretion. The results of investigations of synaptic membrane fusion in a model system (Tsyvkin et al., 2002) showed that its character changes in the presence of enzymes (proteinases, phospholipases) providing modification of the proteins and lipids involved in the membrane composition.

Calpain is one of the most widespread calcium-dependent neutral cysteine proteinases. Apparently, this enzyme takes an active part in mediating calcium signals in the cells of different tissues. Changes in the activity of this enzyme seem to be significant factors in various pathologies of the nervous system such as Alzheimer's disease, multiple sclerosis, neurotoxicosis, cerebral ischemia, *etc.* (Goll et al., 2003; Vosler et al., 2008). It is known (Chan, Mattson, 1999; Goll et al., 2003) that the calpain substrates are rather diverse: these are proteins of the cytoskeleton, enzymes (kinases and phosphatases), membrane receptors, and transporters. Calpain is suggested to regulate cell membrane fusion by splitting proteins taking part in this process (Zimmerman et al., 1999).

There are different opinions about the mechanism of calpain activation. It is known that natural calpain inhibitor, calpastatin, is present permanently in the cell and connection with this very specific inhibitor provides an inactive state of calpain in the absence of the calcium signal (Tullio et al., 1999). Calpain was supposed to be activated after binding of the enzyme to the membrane structures stimulated by the entry of calcium ions (Johnson, Guttman, 1997), and conformational changes in the enzyme molecule lead to the opening of the hydrophobic sites important for the calpain interaction with the membrane (Fernandez-Montalvan et al., 2006). However, it was shown by other authors that calpain is active in the soluble state (Takeuchi et al., 1992) and can bind with the membrane in the model system even without calcium ions presence (Garret et al., 1988).

It is obvious that the activity of calpain is determined by the complex interaction of a number of different factors: it depends in many respects on the state (soluble or membrane-associated) of the given enzyme. Thus, investigation of the distribution and modulation of the calpain activity at the subcellular level can refine our knowledge of the mechanisms of its functioning, explain the reasons for a number of pathologies of the nervous system and help in the search for

methods of their corrections (there are findings that intracellular localization of this enzyme changes in some pathological states) (Nixon, 1989).

Membrane lipids are one of the important factors under investigation in connection with calpain activity. However, the data concerning lipid influence on this enzyme activity are rather contradictory. Up to date only phosphatidylinositol from all phospholipids studied is marked as a positive factor for calpain activity by different authors (Pontremoli et al., 1985; Saido et al., 1992).

Microdomains of the biological membranes containing mainly cholesterol and sphingolipids (lipid rafts) appear to be involved in the complex network of cell functioning (Brown, London, 2000). There are few and rather controversial works about calpain possible localization in these structures (Babiychuk et al., 2002; Morford et al, 2002; Goudeneque et al., 2007).

We have decided to study subcellular calpain localization in the rat brain, trying to provide conditions close to those in a nerve cell in the normal state. We also study the influence of lipids on calpain enzyme activity in different cell fractions.

Activity of calpain in subcellular fractions of the rat brain

We used casein stained with a fluorescent label (FITC-labeled casein) as a substrate in the measurements of the calpain activity. The calpain activity was calculated as a calcium-dependent increase in the quantity of products of hydrolysis as compared with probe containing EDTA.

It follows from Table 1, where the values of specific calpain activity of various fractions from rat brain tissue are presented, that soluble proteins display largest activity in the investigated preparations. In the membrane fractions the value of calpain specific activity was approximately one order smaller than those in the soluble one. Earlier, it was shown (Chakrabarti et al., 1990) that after myelin fraction purification the activity of calpain in this fraction was few times higher than that in the cytoplasmic fraction. In our experiments, myelin fraction, isolated by analogous method, was characterized by a very small calpain activity (specific activity was approximately two times lower than that in coarse mitochondrial fraction and 20 times lower than that in cytoplasmic one).

Considering that the natural calpain inhibitor calpastatin is present in the cells, it was interesting to investigate how calpain activity changes upon removal of this inhibitor from preparations. This series of experiments was carried out with supernatant fluid S2 obtained after centrifugation at 12 000 g of the brain tissue homogenate. Calpastatin was removed from the preparation on DEAE-Servacel column. Comparing the calpain activity before and after of the procedure, we can see that the enzyme activity in the purified fraction is considerably higher. So, before purification the specific activity of calpain in the fraction was about 1000 units; after purification it reached nearly 7000 units. Thus, noticeable calpain activity can be registered both in cytoplasm and membrane fractions of

rat brain under conditions close to physiological standard, i.e., when natural inhibitor of calpain, calpastatin, is present in the cells. At the same time, the removal of calpastatin considerably increases the specific activity of the enzyme present in the preparations. This fact proves the existence of an essential blocking influence of calpastatin under experimental conditions.

The influence of the calpain inhibitor I on the enzyme activity of preparations isolated from rat brain was studied as well. The concentration dependence of the effect of the inhibitor on the calpain activity in brain cytoplasmic fraction shows that calpain inhibitor I at a concentration of 10^{-6} M practically completely inhibits enzyme activity ($K_{0.5}$ is about 10^{-7} M). Our experiments also showed that the calpain activity in the coarse mitochondrial fraction of the brain was hindered completely in the presence of 10^{-6} M of calpain inhibitor I.

From Table 2, where data on the protein content and the distribution of the calpain activity among subcellular fractions isolated from rat brain tissue are presented, we can see that an overwhelming part of the enzyme activity is detected in the cytoplasmic fraction. At the same time, a noticeable calpain activity is found in the membrane fractions as well. Thus, about 13% of the total activity is determined in the coarse mitochondrial fraction. A very small (less than 1%), but clearly detectable calpain activity was found in the microsomal fraction.

This way, in our experiments we measured the protein content and specific activity of the calcium-dependent proteinase calpain in the subcellular fractions of rat brain. It should be mentioned that despite the great number of studies devoted to calcium-dependent proteinases, only a few quantitative data on the distribution and specific activity of these enzymes in different subcellular fractions can be found in the literature, and the available information is rather contradictory.

Thus, some authors (Banik et al., 1992) investigating calpain activity in different tissues, showed that 60% of the enzyme activity in the brain tissue is dis-

Table 1. Specific activity of calpain (units of fluorescence/mg of protein/hour) in subcellular fractions from rat brain

Fraction	Specific activity
Supernatant fluid S2	1078.6
Cytoplasm	977.3
S2 after purification on DEAE-Servacel	7201.0
Coarse mitochondrial	88.8
Myelin	45.9
Microsomes	57.5

Table 2. Distribution of protein and calpain activity among subcellular fractions of rat brain. Total amount of protein and total enzyme activity, respectively, in the supernatant fluid and pellet after centrifugation of homogenate at 12 000 g were taken as 100%

Fraction	Protein content, %	Activity of calpain, %
Cytoplasm	36.6	87.1
Microsome	2.4	0.2
Coarse mitochondrial	61.0	12.7

covered in membrane fractions and 30% in cytosolic ones, while in the tissues of other organs (liver, kidney, heart muscles), the proportions are opposite: only about 10% of the enzyme activity is revealed in the membranes, and about 88% — in cytosol. Other authors found 95% of the enzyme activity in the soluble brain fraction (Takeuchi et al, 1992), which is close to our results (87% of the total enzyme activity was observed in the cytosolic fraction of the brain with the protein amount in this fraction being about 37% of the total protein in the homogenate; see Table 2).

Lipids and calpain activity in subcellular fractions of rat brain

In order to study the role of cholesterol in regulation of the calpain activity, we used methyl- β -cyclodextrin. This substance is known to bind cholesterol and extract it from the membrane. It is known that the structure of membrane rafts is disturbed after extraction, and the changes in membrane proteins functioning are shown to be a result of this process (Yancey et al., 1996).

We used 15 and 30 mM methyl- β -cyclodextrin for cholesterol extraction from membrane fraction of the rat brain. Extraction up to 23% of the membrane cholesterol was established not to influence the calpain specific activity in the membrane fraction. So, one can suppose that in such experimental conditions decrease in the cholesterol concentration in the membrane is not critical for calpain activity.

As shown before, calpain of the smooth muscle is localized in the membrane sites where rafts are absent (Babiychuk et al., 2002). However, in some other types of cells, calpain molecules were observed in the rafts only (Nuzzi et al., 2007). It is possible that calpain localization in the membrane rafts is not stable and depends on the type and state of the cell (Haim et al., 2006; Nuzzi et al., 2007).

We studied the influence of negative charged phospholipids (cardiolipin and phosphatidylserine), neutral phosphatidylcholine and their mixtures on the calpain activity in cytoplasmic fraction of the rat brain. It was shown that calpain activity increases insignificantly only in the presence of phosphatidylcholine and 0.25 mM calcium (this concentration is submaximal for the determination of the calpain activity). Cardiolipin and phosphatidylserine were shown to decrease enzyme activity. One can suppose that interaction of phosphatidylcholine vesicles with hydrophobic part of calpain molecule occurs in the presence of calcium ions in submaximal concentrations and can be essential for calpain activation *in vivo* (Fernandez-Montalvan et al., 2006).

Acknowledgements. We are greatly indebted to Prof. P. Kostyuk whose constant interest in the role of calcium ions in a nerve cell had inspired us to work with calcium-dependent proteinases. We also thank Drs. I. Triakash and V. Gumenyuk for their help in the experiments with lipids.

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