ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 3, pp. 281-287. © Pleiades Publishing, Ltd., 2009.
Published in Russian in Biokhimiya, 2009, Vol. 74, No. 3, pp. 346-354.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-202, December 21, 2008.

A Secreted Caspase-3-Substrate-Cleaving Activity at Low pH Belongs to Cathepsin B: a Study on Primary Brain Cell Cultures

M. V. Onufriev[1], A. A. Yakovlev[1,2], A. A. Lyzhin[3], M. Yu. Stepanichev[1], L. G. Khaspekov[3], and N. V. Gulyaeva[1]*

[1]*Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences, ul. Butlerova 5a, 117485 Moscow, Russia; fax: (495) 952-4007; E-mail: nata_gul@pisem.net*
[2]*Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, ul. Institutskaya 3, 142290 Pushchino, Moscow Region, Russia*
[3]*Neurology Research Center, Russian Academy of Medical Sciences, Volokolamskoe Shosse 80, 125367 Moscow, Russia*

Received June 11, 2008
Revision received August 5, 2008

Abstract—The cysteine proteases caspase-3 and cathepsins are involved in both neuronal plasticity and neuropathology. Using primary neuroglial and glial cerebellar cultures, the pH dependence of cleavage of a synthetic caspase-3 substrate, Ac-DEVD-AMC, was studied. At acidic pH, cathepsin B cleaved Ac-DEVD, this activity being significantly higher than that of caspase-3 at pH 7.4. This activity is blocked by peptide inhibitors of both caspase-3 and cathepsin B. Substitution of culture medium for balanced salt solution stimulated cathepsin B secretion in both types of cultures. Ischemia (oxygen–glucose deprivation) significantly decreased secretion of cathepsin B activities into the culture medium.

DOI: 10.1134/S0006297909030067

Key words: cathepsin B, caspase-3, ischemia/reoxygenation, neurons, glia

Cathepsins are acid endopeptidases from the family of papain proteinases [1]. Cathepsins are primarily localized in lysosomes, where they participate in degradation of proteins to amino acids at acidic pH [2]. Cathepsins B and L are expressed in both neurons and glia and represent the main lysosomal cysteine proteases in the central nervous system [3]. Cathepsins are released into the cytoplasm under various pathological conditions, including cerebral ischemia, while excessive activity of these proteinases results in cell damage and death [4].

Cathepsin B participates in cell death of different types, in particular, by influencing activities of other cysteine proteases, including proinflammatory and effector caspases; e.g. at early stages of focal ischemia, cathepsin B can activate proinflammatory caspases 1 and 11 [5]. Culture medium from chromogranin A stimulated primary microglial cultures induced apoptosis and caspase-3 activation in neuronal cultures, this effect being blocked by an inhibitor of cathepsins, Z-FA-fmk, or antibodies to cathepsin B [6]. CA074-Me, a selective cathepsin B inhibitor, fully blocked processing of procaspase-3 in cerebellar granular cells undergoing apoptosis induced by serum deprivation and low K^+. In this situation, early stages of apoptosis were accompanied by an increase in the number of autophagosomes and lysosomes, while cathepsin B, initially a part of lysosomal–endosomal cellular compartment, was released into the cytosol [7].

Inhibitory analysis, though frequently used for exploring the involvement of definite proteinases in mechanisms of cell death, as a rule, does not provide doubtless results. Obviously, this is a result of both broad substrate specificity of proteinases and the fact that inhibitors are usually substrate analogs. For instance, synthetic inhibitors of cysteine proteinases, though initially targeted to this class specifically, possess a much broader inhibitory spectrum. Specific inhibitors of a neutral cysteine proteinase, calpain, interact with other cysteine proteinases, including cathepsin B [8]. Some caspase inhibitors, like Z-VAD-fmk and Z-DEVD-fmk, directly block calpain and prevent necrotic cell death induced by traumatic brain injury [9, 10]. Studies of a caspase inhibitor, Ac-YVAD-cmk, not only revealed its neuroprotective properties in models of oxidative stress and

Abbreviations: AMC, 7-amino-4-methylcoumarin; CA074, N-[L-3-trans-(propylcarbamoyl)-oxyran-2-carbonyl]-L-isoleucyl-L-proline; LDH, lactate hehydrogenase.

* To whom correspondence should be addressed.

ischemia (oxygen–glucose deprivation) *in vitro*, but also specified cathepsin B as a target of this inhibitor [11]. Caspase-3 inhibitor Z-DEVD-fmk co-applied with cathepsin B prevented the death of HT22 cells [6]. These data raised a question about the ability of cysteine proteases of different classes to cleave common substrates, the above-mentioned inhibitors being structural analogs of these substrates. This problem remains poorly studied, while the point about possible functional role of this phenomenon in the brain remains fully obscure. Recently, we have shown that an enzyme capable of cleaving a specific peptide substrate of caspase-3, Ac-DEVD-AMC, at low pH exists in rat brain [12]. It was cathepsin B that was apparently the enzyme possessing this "acid" DEVDase activity in the brain. We hypothesized that in certain situations (particularly, under hypoxia) cathepsin B might participate in cleavage of capsase-3 substrates in brain cells.

Another quite relevant but insufficiently explored problem is the question about the existence of secreted proteases in the brain. Cathepsins, in particular cathepsin B, participate in decomposition of extracellular matrix proteins [13], and, thus, cathepsin secretion contributes to cell migration [14]. Microglial cells are able to secrete cathepsin B [6, 15]. It remains unknown whether astrocytes and neurons can secrete cathepsin B. Nevertheless, intracellular localization of cathepsin B in hippocampal neurons demonstrates its non-lysosomal distribution in cell body and dendrites, predominantly in synapses, and, partially, in postsynaptic space [16]. Moreover, neuronal secretory vesicles contain cathepsins B and L, enzymes participating in processing of neuropeptides and β-amyloid [17].

The aim of this study was to investigate the ability of cathepsin B to cleave a synthetic substrate of caspase-3 and explore possible secretion of cathepsin B by primary neuroglial and glial cultures of rat cerebellum, in particular under unfavorable conditions like ischemia/reoxygenation.

MATERIALS AND METHODS

Culture conditions. Dissociated cerebellar granular cells and dissociated glial cultures from cerebella of 7-day-old Wistar rat pups were cultivated as described previously [18] in a CO_2-incubator (95% air, 5% CO_2, 35.5°C) for 7-8 days or 13-14 days, respectively.

Cell culture phenotyping. To define cellular content of cultures, the cell were fixed and incubated in a solution containing antibodies to neuron-specific protein NeuN (Chemicon, USA) or glial fibrillar acidic protein (GFAP) (Dako, Denmark). Immunohistochemical staining of the two culture types revealed that neuroglial cultures contained 92.1 ± 0.6% of neurons, while glia-enriched cultures contained 81.4 ± 2.2% of GFAP-positive cells. Though astrocytes were the predominant cell type in glial cultures, some neuronal conglomerates remaining after the incubation of cultures in low K^+ media were still present.

Substitution of culture medium. Neuroglial or glial cultures were transferred from the cultural medium to a balanced salt solution containing 10 mM glucose, 143.4 mM NaCl, 25 mM KCl, 2 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 5 mM Hepes, pH 7.4, and kept in the CO_2-incubator for 10 h at 35.5°C.

Ischemia–reoxygenation. Neuroglial and glial cultures were transferred from culture medium to a deoxygenated salt solution without glucose and were kept in a hermetic box with argon for 1.5-2 h at 35.5°C. Control cultures were simultaneously incubated in normoxic condition during same time period and in same solution with 10 mM glucose added. After the incubation, all cultures were transferred to the control salt solution and kept in the CO_2-incubator for 5 h [19].

Assays of proteolytic activities. Activities of proteolytic cleavage of caspase-3 substrate Ac-DEVD-AMC (Biomol, USA) and cathepsin B substrate Z-R-R-AMC (Biomol) were assayed in salt solution and cell lysates subjected to the above conditions [20]. The cleavage of the substrates was detected fluorometrically in the pH range of 3.0-7.4. In selected experiments, a specific inhibitor of caspase-3, Ac-DEVD-CHO (Biomol), or a specific inhibitor of cathepsin B, CA074 (N-[L-3-trans-(propylcarbamoyl)-oxyran-2-carbonyl]-L-isoleucyl-L-proline) (Biomol), was added into the incubation medium. The fluorescence of AMC (7-amino-4-methylcoumarin) generated as a result of proteolysis was assessed using a Hitachi F-3000 fluorometer at excitation and emission wavelengths of 400 and 488 nm, respectively. AMC (Sigma, USA) was used as a fluorescent standard for calculation of proteolytic activity.

Assay of lactate dehydrogenase. Lactate dehydrogenase (LDH) activity was assessed in salt solution or cell lysates using a fluorometric method [21] based on monitoring of NAD^+ accumulation in the reaction of pyruvate reduction. NAD^+ fluorescence was measured at excitation and emission wavelengths of 360 and 460 nm, respectively. LDH activity was expressed in nmol NADH/min.

Western blot. Samples of salt solution concentrated using Amicon filters, and cell lysate, or particulate fraction of neuroglial cultures were subjected to electrophoresis under denaturing conditions on 4-12% SDS-polyacrylamide gels using a Mini-Protean II device (Bio-Rad, USA). The proteins were then transferred from gels to polyvinyl difluoride membrane, incubated with primary polyclonal antibodies to cathepsin B (FL-339) (Santa-Cruz Biotechnology, USA), and stained bands corresponding to cathepsin B using a chemiluminescence method. Protein concentration in salt solution or cell lysates was determined using the Bradford method [22].

Statistical analysis. In each experiment, 5-8 independent cultures were used. Data are presented as mean ± SEM. The between-group difference was assessed using Student's *t*-test.

RESULTS

The cleavage of caspase-3 substrate Ac-DEVD-AMC by lysates of neuroglial cultures significantly increased with the decrease of incubation medium pH, reaching a maximum at pH 4.0 (Fig. 1a). The specific inhibitor of caspase-3, Ac-DEVD-CHO, effectively blocked the cleavage of the substrate in the pH range of 7.4-4.0. The cleavage of Ac-DEVD-AMC at pH 7.4 in the salt solution from neuroglial cultures increased with the duration of cell monolayer incubation (Fig. 1b, curve *1*). At pH 4.5, the cleavage of the substrate was more rapid and proceeded virtually at the same rate beginning from the first hour of incubation of cultures in the salt solution (Fig. 1b, curve *2*). Cell death monitored by the release of LDH to the salt solution was about 2% of the potentially possible value (estimated after lysis of cells by 1% Nonidet) within the period of 0.5-5 h, while cell death clearly increased at 10 h (Fig. 1b, curve *3*). The Ac-DEVD-AMC-cleaving activity at pH 7.4 in the salt solution from glial cultures was maximal at 1 h and 3 h of incubation (Fig. 2, curve *1*). The substrate-cleaving activity at pH 4.5 was higher, but changed in a similar way (Fig. 2, curve *2*). The release of LDH into the salt solution did not change during 10 h of incubation (Fig. 2, curve *3*). Since the intracellular Ac-DEVD-cleaving activity of both neuroglial and glial cultures did not change significantly during 10 h of incubation in the salt solution, the data are not presented. A comparison of DEVD-cleaving activity of neuroglial cultures at pH 4.5 after 1 h incubation in salt solution revealed a significantly higher activity in this solution as compared with cell lysate, while in glial cultures the specific extra- and intracellular activities were similar (table).

A 24-h incubation of neuroglial cultures with cell-permeable caspase-3 inhibitor FK010 (Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-FMK) within the concentration range of 2-50 μM inhibited the DEVDase activity of cell lysates at pH 7.4 by 60.2-70.9% (Fig. 3a), while at pH 4.5 the dose-dependence is more expressed (inhibition by 32.0-86.8%) (Fig. 3b). FK029 (Z-Phe-Ala-FMK), a cathepsin inhibitor with a predominant affinity to cathepsin B, just moderately decreased DEVDase activity of cell lysates at pH 7.4 (by 18.6-40.8%) (Fig. 3a), while at pH 4.5 it fully blocked the cleavage of Ac-DEVD (Fig. 3b). The caspase-3 inhibitor Ac-DEVD-CHO (5 μM) equally effectively blocked the cleavage of Ac-DEVD-AMC (50 μM) by salt solution from glial cultures at pH 7.4 and 4.5, while cathepsin B inhibitor CA074 (5 μM)

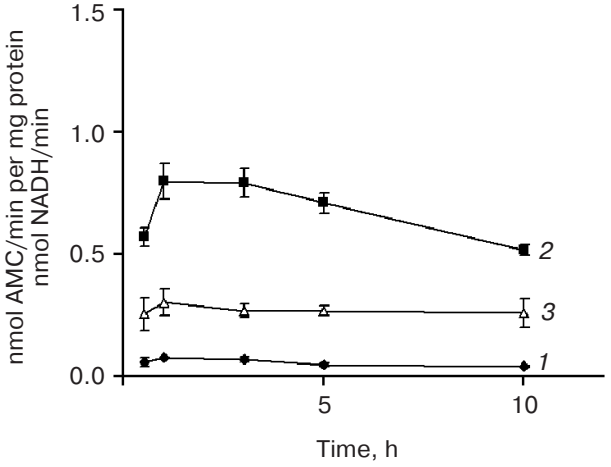

Fig. 2. Ac-DEVD-cleaving activity in salt solution from neuroglial cultures at pH 7.4 (*1*) and 4.5 (*2*) and LDH activity (*3*).

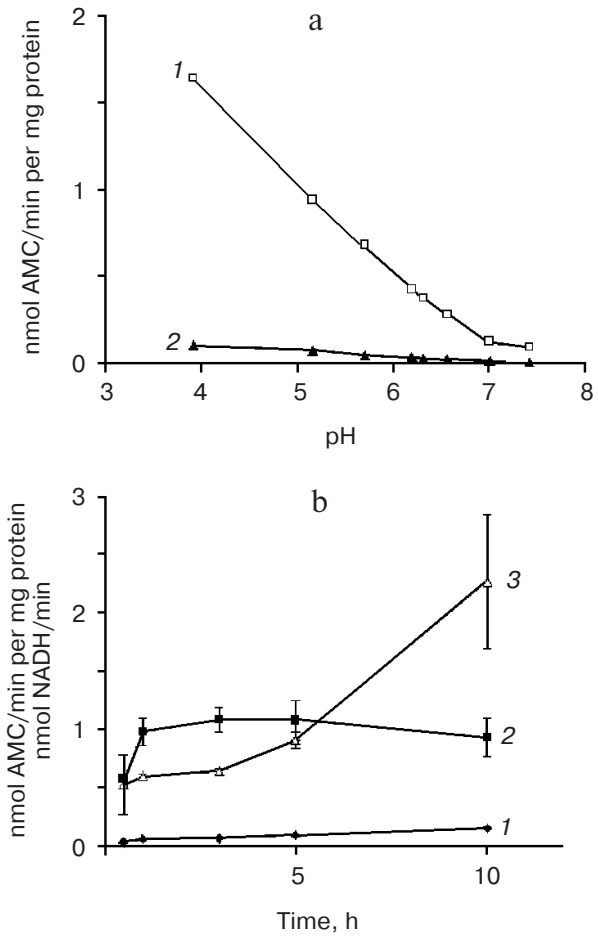

Fig. 1. A pH profile of Ac-DEVD-AMC cleavage by lysates of neuroglial cultures (a) without inhibitor (*1*) and in the presence of caspase-3 inhibitor Ac-DEVD-CHO (*2*) and Ac-DEVD-cleaving activity in the salt solution from neuroglial cultures (b) at pH 7.4 (*1*) and 4.5 (*2*) and lactate dehydrogenase activity (curve *3*).

Ac-DEVD-AMC-cleaving activity (nmol AMC/min per mg) of balanced salt solution (BSS) and lysates from neuroglial and glial cultures 1 h after incubation in the salt solution

pH of incubation medium	Neuroglial cultures		Glial cultures	
	BSS	lysate	BSS	lysate
7.4	0.07 ± 0.005	0.04 ± 0.004	0.05 ± 0.006	0.04 ± 0.003
4.5	0.83 ± 0.062	0.66 ± 0.052*	0.59 ± 0.057	0.52 ± 0.045

* Difference lysate versus BSS is significant at $p < 0.05$.

significantly inhibited the AC-DEVD-cleaving activity only at pH 4.5 (Fig. 4a). The cleavage of cathepsin B substrate Z-RR-AMC (50 μM) was more active at pH 7.4 as compared with pH 4.5 and did not depend on the presence of Ac-DEVD-CHO (5 μM) at pH 7.4, while it could be fully blocked by Ac-DEVD-CHO at pH 4.5. CA074 (5 μM) effectively blocked the cleavage of cathepsin B substrate Z-RR-AMC both at pH 7.4 and 4.5 (Fig. 4b).

The rate of cleavage of caspase-3 substrate Ac-DEVD-AMC by the active form of cathepsin B was similar at pH 7.4 and 6.0 (Fig. 5a). However, decreasing pH to 4.5 increased the activity more than twofold. Caspase-3 inhibitor Ac-DEVD-CHO did not affect Ac-DEVD-AMC cleavage at pH 7.4 and 6.0, but fully suppressed it at pH 4.5. A significant decrease of Ac-DEVD-AMC in the presence of cathepsin B inhibitor CA074 was evident at pH 6.0 and 4.5. The proteolysis of cathepsin B substrate Z-RR-AMC by purified cathepsin B at pH 7.4 and 6.0 was an order a magnitude higher than the cleavage of Ac-DEVD-AMC (Fig. 5b) with a maximum at pH 6.0. The efficacy of Ac-DEVD-CHO blocking activity significantly increased from pH 6.0, while cathepsin B inhibitor CA074 significantly prevented the substrate cleavage at all pH values studied.

Western blots of glial cell lysates (lane *1*), particulate fraction of glial cells (lane *2*), and salt solution (lane *3*)

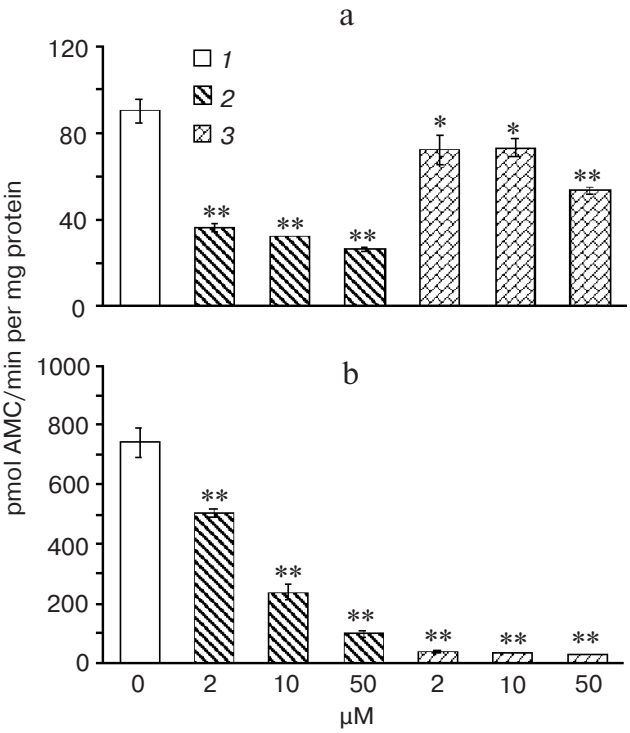

Fig. 3. Ac-DEVD-cleaving activity of cell lysates at pH 7.4 (a) and 4.5 (b) after 24 h incubation of neuroglial cultures in the presence of caspase-3 inhibitor FK010 (Z-DEVD-fmk) (*2*) and cathepsin inhibitor FK029 (Z-FA-fmk) (*3*). *, ** Difference from control without inhibitors (*1*) at $p < 0.05$ and $p < 0.01$, respectively.

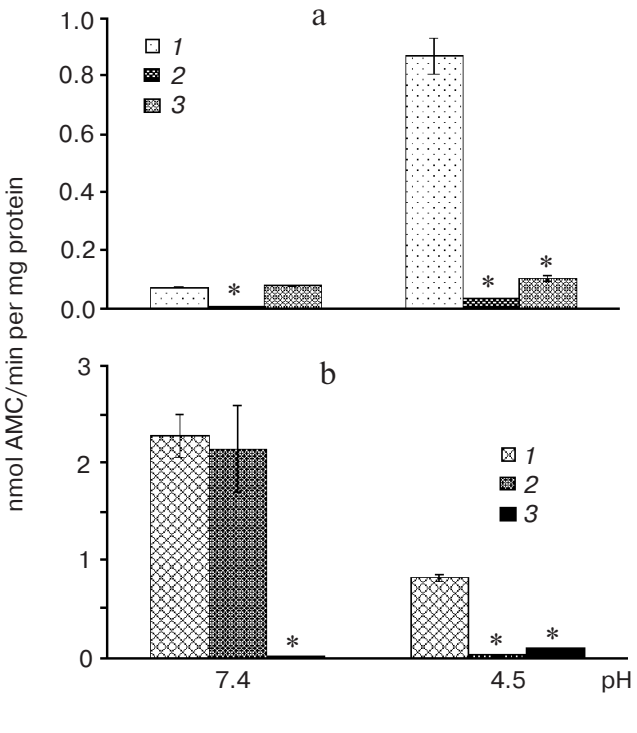

Fig. 4. Effects of caspase-3 inhibitor Ac-DEVD-CHO or cathepsin B inhibitor CA074 on the cleavage of caspase-3 substrate Ac-DEVD-AMC (a) and cathepsin B substrate Z-RR-AMC (b) by salt solution from glial cultures at different pH values: *1*) without inhibitors; *2*) Ac-DEVD-CHO; *3*) CA074. * Difference from control without inhibitors at $p < 0.01$.

revealed a band near 40 kDa corresponding to procathepsin B (Fig. 6). A band in the 30 kDa region that is present both in cell lysate (lane *1*) and in salt solution (lane *3*) belongs to the single chain form of cathepsin B [23]. Protein bands revealed in salt solution in the region of 25 kDa (27, 24, and 23 kDa), are, most probably, results of processing the single chain form of cathepsin B and may correspond to heavy chains of cathepsin B double chain form [24]. In addition, a 21 kDa band was revealed in cell lysate that might correspond to the heavy chain of active cathepsin B form. As a result of oxygen–glucose deprivation of neuroglial cultures, the DEVD-cleaving activity in the salt solution at pH 7.4 was significantly (by 23.3 ± 7.9%) higher than control (Fig. 7, curve *1*), while at pH 4.5 it decreased significantly (by 30.6 ± 2.1%; Fig. 7, curve *2*). A similar decrease was evident at pH 6.0 when cathepsin B substrate Z-RR-AMC was used (Fig. 7, curve *3*). During reoxygenation, a significant decrease in proteolytic activity could be demonstrated only when Ac-DEVD-AMC was used as a substrate; after 3 h of reoxygenation the activity was 68.5 ± 10.2% of that in the salt solution from control cells. The release of LDH into salt solution increased up to 9.5 ± 1.0% from intracellular activity under ischemia; after 3 and 5 h of reoxygenation, it was significantly higher than control (by 5.8 ± 1.2 and 3.5 ± 0.5%, respectively) (Fig. 7, curve *4*).

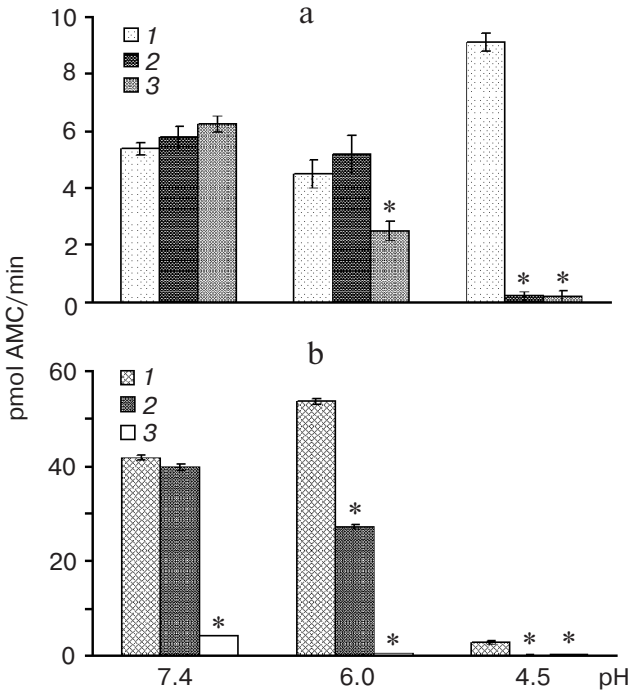

Fig. 5. Effects of caspase-3 inhibitor Ac-DEVD-CHO or cathepsin B inhibitor CA074 on the cleavage of caspase-3 substrate Ac-DEVD-AMC (a) and cathepsin B substrate Z-RR-AMC (b) by the active form of cathepsin B from bovine spleen at different pH values: *1*) without inhibitors; *2*) Ac-DEVD-CHO; *3*) CA074. * Difference from control without inhibitors at $p < 0.01$.

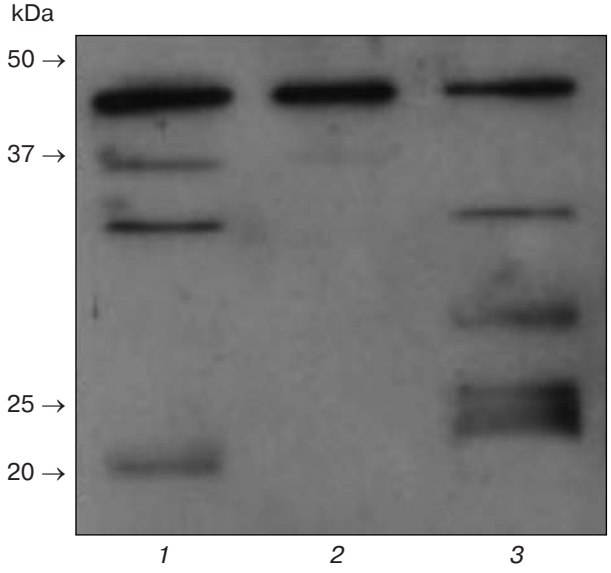

Fig. 6. Identification of cathepsin B by immunoblotting. Lanes: *1*) lysate of glial cells; *2*) particulate fraction from glial cells; *3*) concentrated salt solution after 1 h incubation of glial cultures.

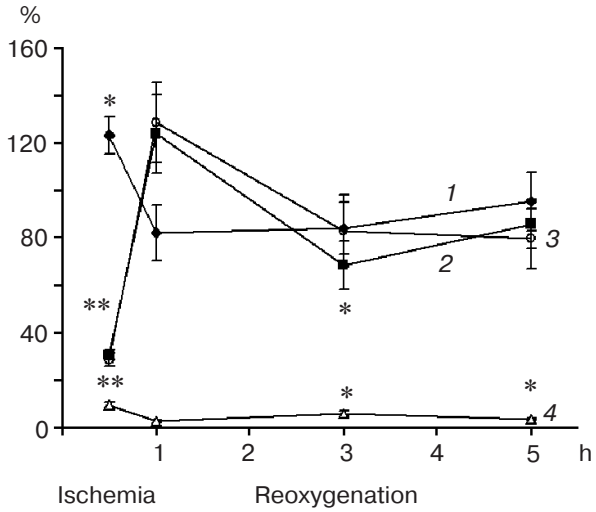

Fig. 7. Effects of ischemia/reoxygenation of neuroglial cultures on activities of proteolytic enzymes in the salt solution: *1*) cleavage of caspase-3 substrate Ac-DEVD-AMC at pH 7.4; *2*) cleavage of caspase-3 substrate Ac-DEVD-AMC at pH 4.5; *3*) cleavage of cathepsin B substrate Z-RR-AMC at pH 6.0; *4*) release of LDH into salt solution. *, ** Differences from control at $p < 0.05$ and $p < 0.01$, respectively.

DISCUSSION

The following main facts have been ascertained in this study. First, cathepsin B cleaves a synthetic substrate of caspase-3 at acidic pH values *in vitro* in cerebellar cell cultures (earlier we discovered a similar proteolytic activity in rat brain [8]). Second, a caspase-3 inhibitor blocks

the cleavage of a synthetic caspase-3 substrate by cathepsin B at acidic pH *in vitro* and when it is applied to cell cultures. Third, substitution of culture medium with salt solution induces secretion of cathepsin B from neuroglial and glial culture. Fourth, the secreted cathepsin B activity in neuroglial cultures decreases under ischemia.

Cathepsin B possesses both endopeptidase and exopeptidase activities; it is able to bind a dipeptide substrate containing basic amino acids in P1 and P2 positions (arginine in P1 and arginine or phenylalanine in P2) [25]. A detailed analysis of the pH dependence of cathepsin B dependent hydrolysis demonstrated that at least seven amino acid residues dissociating in the range of pH 3-9 determine substrate binding and/or conversion [26]. Our results give evidence of the ability of cathepsin B to cleave the bond not only after arginine residue in cathepsin B substrate Z-RR-AMC, but also the peptide bond after arginine residue in caspase-3 substrate Ac-DEVD-AMC at acidic pH *in vitro*.

Analogs of caspase-3 synthetic substrate, an irreversible caspase-3 inhibitor FK010 (Z-DEVD-fmk) and a reversible inhibitor of caspase-3 Ac-DEVD-CHO, block Ac-DEVD-cleaving activity of cathepsin B at acidic pH *in vitro*. The proteolysis by cathepsin B of its conventional substrate is also inhibited by Ac-DEVD-CHO at acidic pH. The amino acid sequence of caspase-3 synthetic substrate Ac-DEVD-AMC corresponds to the cleavage site of poly(ADP-riboso)polymerase (PARP1), an enzyme participating in DNA repair. During apoptosis, PARP, localized in the nucleus, undergoes proteolysis by caspase-3 and caspase-7 to yield the fragments of 89 and 24 kDa [27-29]. During necrotic cell death, PARP is also cleaved but fragments of 50 kDa appear [30, 31]. In experiments *in vivo* and *in vitro* Gobeil et al. [32] revealed the involvement of lysosomal proteinases, including cathepsin B, in PARP degradation during necrosis induction in Jurkat T cells, PARP proteolysis by purified cathepsin B proceeding at pH 7.4. Thus, cathepsin B and caspase-3 possesses common physiological and synthetic substrates, and changes in extra- and intracellular pH can, apparently, modify the specificity of proteolytic activity of cathepsin B.

Cathepsin B functions not only in lysosomes. In our study, substitution of culture medium with salt solution induced a release of cathepsin B from neuroglial and glial cultures, the time course of the release being not related to cell death, unlike the release of caspase-3. The ratio of cathepsin B-specific activity in salt solution and cell lysates suggest that the enzyme is secreted. Identification of cathepsin B using immunoblotting confirms the presence of both the proenzyme (40 kDa) and activated forms of cathepsin B (30, 27, 24, and 23 kDa) in the salt solution. Stimulation of microglial cells BV2 by lipopolysaccharide resulted in secretion of procathepsin B and the single chain form of cathepsin B (30-32 kDa) into the culture medium [33]. We suggest that substitution of the medium with salt solution induces additional secretion of other proteinases participating in the processing of procathepsin B.

Most lysosomal cysteine proteinases, including cathepsin B, are unstable and possess low activity at neutral pH values, optimal pH values lying in the acidic range (pH 5.5) [1]. Our results suggest that in situations of decreased extra- and/or intracellular pH, a broadening of cathepsin B proteolytic activity spectrum takes place in the brain. Let us speculate about possible functional significance of such situations *in vivo*. Maintaining stable pH is necessary for normal functioning of neurons. Under physiological conditions, the extracellular pH in the brain is 7.3, while the intracellular pH is 7.0 [34, 35]. Cerebral ischemia is accompanied by a significant decrease in pH since oxygen deficit resulting from decrease in cerebral blood flow induces an increase in glycolysis and lactate accumulation [36, 37]. Under ischemia, brain pH usually decreases to 6.5 in normoglycemia, but under severe ischemia or hyperglycemia pH may decrease to 6.0 or even lower values [38, 39], the degree and trend of changes in extra- and intracellular pH being not necessarily similar. In focal brain ischemia in rats, the predominant decrease of extracellular pH proceeded faster than the decrease of intracellular pH in the peri-infarct zone [39]. The release of cathepsin B from lysosomes during acute focal ischemia without reoxygenation suggests possible involvement of cathepsin B at early stages of infarct zone formation [5]. In our experiments, ischemia (oxygen–glucose deprivation) of neuroglial cultures induced a decrease in the activity and/or release of cathepsin B to the salt solution before the onset of reoxygenation and was accompanied by an increase of caspase-3 activity in the salt solution, which, most probably, was related to cell damage and LDH release. The decrease of cathepsin B activity in the salt solution during ischemia might be related to the release of inhibitors of lysosomal cysteine protease of the cystatin family from damaged cells [40].

This work was supported by the Russian Foundation for Basic Research (grant No. 08-04-00870).

REFERENCES

1. Turk, B., Turk, V., and Turk, D. (1997) *Biol. Chem.*, **378**, 141-150.
2. Kirschke, H., Langner, J., Riemann, S., Wiederanders, B., Ansorge, S., and Bohley, P. (1980) in *Protein Degradation in Health and Disease*, Ciba Foundation Symposium (Evered, D., and Whelan, J., eds.) Excerpta Medica, Amsterdam, pp. 5-35.
3. Yamashima, T. (2000) *Prog. Neurobiol.*, **62**, 273-295.
4. Boya, P., Andreau, K., Poncet, D., Zamzani, N., Perfettini, J. L., Metivier, D., Ojcius, D. M., Jaattela, M., and Kroemer, G. (2003) *J. Exp. Med.*, **197**, 1323-1334.

5. Benchoua, A., Braudeau, J., Reis, A., Couriaud, C., and Onteniente, B. (2004) *J. Cerebr. Blood Flow Metab.*, **24**, 1272-1279.
6. Kingham, P. J., and Pocock, J. M. (2001) *J. Neurochem.*, **76**, 1475-1484.
7. Canu, N., Tufi, R., Serafino, A. L., Amadoro, G., Ciotti, M. T., and Calissano, P. (2005) *J. Neurochem.*, **92**, 1228-1242.
8. Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) *Cell*, **78**, 761-771.
9. Knoblach, S. M., Alroy, D. A., Nikolaeva, M., Cernak, I., Stoica, B. A., and Faden, A. I. (2004) *J. Cerebr. Blood Flow Metab.*, **24**, 1119-1132.
10. Waterhouse, N. J., Finucane, D. M., Green, D. R., Elce, J. S., Kumar, S., Alnemri, E. S., Litwack, G., Khanna, K., Lavin, M. F., and Watters, D. J. (1998) *Cell Death Differ.*, **5**, 1051-1061.
11. Gray, J., Haran, M. M., Schneider, K., Vesce, S., Ray, A. M., Owen, D., White, I. R., Cutler, P., and Davis, J. B. (2001) *J. Biol. Chem.*, **276**, 32750-32755.
12. Yakovlev, A. A., Gorokhvatsky, A. Y., Onufriev, M. V., Beletsky, I. P., and Gulyaeva, N. V. (2008) *Biochemistry* (Moscow), **73**, 332-336.
13. Buck, M. R., Karustis, D. G., Day, N. A., Honn, K. V., and Sloane, B. F. (1992) *Biochem. J.*, **282**, 273-278.
14. Kawada, A., Hara, K., Kominami, E., Hiruma, M., Akiyama, M., Ishibashi, A., Abe, H., Ichikawa, E., Nakamura, Y., Watanabe, S., Yamamoto, T., Umeda, T., and Nishioka, K. (1997) *Br. J. Dermatol.*, **136**, 361-366.
15. Ryan, R. E., Sloane, B. F., Sameni, M., and Wood, P. L. (1995) *J. Neurochem.*, **65**, 1035-1045.
16. Graber, S., Maiti, S., and Halpain, S. (2004) *Neuropharmacology*, **47**, 706-713.
17. Hook, V. Y. (2006) *Cell. Mol. Neurobiol.*, **26**, 449-469.
18. Andreeva, N., Khodorov, B., Stelmashook, E., Cragoe, E., and Victorov, I. (1991) *Brain Res.*, **548**, 322-325.
19. Khaspekov, L. G., Onufriev, M. V., Lyzhin, A. A., Victorov, I. V., and Gulyaeva, N. V. (2002) *Neurochemistry*, **19**, 37-40.
20. Yakovlev, A. A., Onufriev, M. V., Stepanichev, M. Y., Braun, K., and Gulyaeva, N. V. (2001) *Neurochemistry*, **18**, 41-43.
21. Kosenko, E., Montoliu, C., and Giordano, G. (2004) *J. Neurochem.*, **89**, 1101-1110.
22. Bradford, M. M. (1976) *Anal. Biochem.*, **72**, 248-254.
23. Nishimura, Y., Kawabata, T., and Kato, K. (1988) *Arch. Biochem. Biophys.*, **261**, 64-71.
24. Mort, J. S. (1998) in *Handbook of Proteolytic Enzymes* (Woessner, J., ed.) Academic Press, London, pp. 609-617.
25. Khouri, H. E., Plouffe, C., Hasnain, S., Hirama, N., Storer, A. C., and Menard, R. (1991) *Biochem. J.*, **275**, 751-757.
26. Hasnain, S., Hirama, T., Tam, A., and Mort, J. S. (1992) *J. Biol. Chem.*, **267**, 4713-4721.
27. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995) *Nature*, **376**, 37-43.
28. Lazebnik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G., and Earnshaw, W. C. (1994) *Nature*, **371**, 346-347.
29. Germain, M., Affar, E. B., D'Amours, D., Dixit, V. M., Salvesen, G. S., and Poirier, G. G. (1999) *J. Biol. Chem.*, **274**, 28379-28384.
30. Shah, G. M., Shah, R. G., and Poirier, G. G. (1996) *Biochem. Biophys. Res. Commun.*, **229**, 838-844.
31. Casiano, C. A., Ochs, R. L., and Tan, E. M. (1998) *Cell Death Differ.*, **5**, 183-190.
32. Gobeil, S., Boucher, C. C., Nadeau, D., and Poirier, G. G. (2001) *Cell Death Differ.*, **8**, 588-594.
33. Ryan, R. E., Sloane, B. F., Sameni, M., and Wood, P. L. (1995) *J. Neurochem.*, **65**, 1035-1045.
34. Chesler, M. (1990) *Prog. Neurobiol.*, **34**, 401-427.
35. Back, T., Hoehn, M., Mies, G., Busch, E., Schmitz, B., Kohno, K., and Hossmann, K. A. (2000) *Ann. Neurol.*, **47**, 485-492.
36. Siesjo, B. K., Katsura, K., and Kristian, T. (1996) *Adv. Neurol.*, **71**, 209-233.
37. Tombaugh, G. C., and Sapolsky, R. M. (1993) *J. Neurochem.*, **61**, 793-803.
38. Kraig, R. P., Pulsinelli, W. A., and Plum, F. (1985) *Brain Res.*, **342**, 281-290.
39. Nedergaard, M., Kraig, R. P., Tanabe, J., and Pulsinelli, W. A. (1991) *Am. J. Physiol.*, **260**, R581-R588.
40. Chapman, H. A., Riese, R. J., and Shi, G.-P. (1997) *Annu. Rev. Physiol.*, **59**, 63-88.