Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 329 (2005) 733–737
BBRC
www.elsevier.com/locate/ybbrc

Protein disulphide isomerase binds ammodytoxin strongly: Possible implications for toxin trafficking

Jernej Šribar [a], Gregor Anderluh [b], Jay W. Fox [c], Igor Križaj [a,*]

[a] Department of Biochemistry and Molecular Biology, Jožef Stefan Institute, Ljubljana, Slovenia
[b] Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia
[c] W.M. Keck Biomedical Mass Spectrometry Laboratory, The University of Virginia Biomedical Research Facility, University of Virginia Medical School, Charlottesville, VA 22908, USA

Received 31 January 2005

Abstract

Ammodytoxin, a group IIA secreted phospholipase A_2 from the venom of the long-nosed viper (*Vipera ammodytes ammodytes*), is a potent presynaptically acting neurotoxin. It blocks the secretion of neurotransmitter from the nerve cell, thus hindering the communication with the neighbouring neuron or muscle cell. To express the neurotoxicity, ammodytoxin should interact with specific receptors in the axon terminal and express phospholipase activity. Our previous results indicate that, following the association with a receptor on the external side of the presynaptic membrane, the toxin penetrates into the cytosol of the nerve cell. Here, we show that the toxin associates specifically with protein disulphide isomerase, a protein in the lumen of endoplasmic reticulum, which may be crucial for the retention and concentration of the toxin in this cellular compartment and for its subsequent transport across the membrane of endoplasmic reticulum into the cytosol of the nerve cell.

Keywords: Secreted phospholipase A_2; Ammodytoxin; Snake venom; *Vipera ammodytes ammodytes*; Neurotoxicity; Protein disulphide isomerase; Mechanism of action

Secretions of venomous animals, toxic plants, and microorganisms are rich sources of compounds with different pharmacological activities. The use of such a compound in medicine and cell biology as a drug or tool requires, however, precise knowledge of its molecular basis of action.

One of the so far unexploited groups of proteins, abundantly present in venoms, are the secreted phospholipases A_2 (sPLA$_2$) [1,2]. They exhibit a wide variety of (patho)physiological effects [1,3]. Particularly interesting in terms of their potential of use are sPLA$_2$s that exhibit presynaptic neurotoxicity (β-neurotoxins). These toxins block the release of acetylcholine from the motoneuron into the synaptic cleft, terminating its communication with the muscle [4]. The victim dies within 1 or 2 h due to paralysis of the respiratory muscles. β-Neurotoxins are toxic not only to motoneurons in peripheral nervous system but, if they cross the blood–brain barrier, also to neurons in the central nervous system [5,6]. Electron microscopy studies of a β-neurotoxin-paralysed neuro-muscular junction reveals a depletion of the neurotransmitter-containing synaptic vesicles (SV) in the nerve terminal. The axolemma displays numerous clathrin-coated invaginations, suggesting that the retrieval of SV from the plasma membrane is impaired. Formation of larger vesicles in nerve terminals and increased release of neurotransmitter in the first phase of intoxication [4] may be due to increased fusogenicity of some membranes in the nerve ending caused by the phospholipase activity of the toxin [7]. A further

* Corresponding author. Fax: +386 1 257 35 94.
E-mail address: igor.krizaj@ijs.si (I. Križaj).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.02.036

characteristic are damaged mitochondria which, in some cases, completely lose the internal structure of cristae [8]. The phospholipase activity is not enough to cause these effects—β-neurotoxins are able to interact specifically with certain components in the nerve ending [9].

To study the molecular mechanism of action of β-neurotoxins, we have been using ammodytoxin (isoforms A and C) from the venom of the long-nosed viper (*Vipera ammodytes ammodytes*). One extracellular and several intracellular binding proteins for ammodytoxin (Atx) have been identified as being potentially implicated in the process of neurotoxicity: neuronal M-type $sPLA_2$ receptor [10,11] in the plasma membrane, R25 in mitochondria [12,13], and calmodulin (CaM) [8], and 14-3-3 proteins [15] in the cytosol of the nerve cell. Fluorescence-labelled AtxA can penetrate into the cytosol of the rat hippocampal neuron [16]. This multi-disulphide-bridged toxin has been proven to be enzymatically active in the cytosol, despite the relatively reducing nature of this cellular compartment and low micromolar concentration of the Ca^{2+} cofactor. Based on these results we proposed that Atx and related neurotoxic $sPLA_2$s exert their toxic action inside nerve cells [16].

The issue that remained unanswered was, however, how β-neurotoxins are translocated into the cytosol of the neuron to act. Here we show that Atx binds with high affinity to protein disulphide isomerase (PDI), a protein in the lumen of endoplasmic reticulum (ER). As in the case of some other toxins, the interaction with PDI may be crucial for reverse transport of Atx and related $sPLA_2$-neurotoxins from the ER into the cytosol of the nerve cell.

Materials and methods

Identification of Atx-binding proteins in porcine cerebral cortex. A demyelinated mitochondrial/synaptosomal P2 fraction of porcine cerebral cortex was prepared and the protein content in the membrane preparation was determined as described previously [10]. P2 membranes (5 mg membrane protein/ml) were extracted for 1 h by gentle agitation at 4 °C in 75 mM Hepes, pH 8.2, containing 150 mM NaCl, 2.5 mM $CaCl_2$, and 1.5% (w/v) Triton X-100 (Roche Molecular Biochemicals). The extract was centrifuged at 106,200*g* for 1 h, and cold deionized water was added to the supernatant to give a final detergent concentration of 0.15% (w/v).

AtxC was purified from *V. a. ammodytes* venom [5] and attached to activated CH-Sepharose (CHS) as described [14]. The gel was washed, equilibrated with 75 mM Hepes, pH 8.2, 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% (w/v) Triton X-100, and stored at 4 °C. A control gel was prepared in the same way, the active groups being derivatized with ethanolamine (EA) instead of AtxC. The gel was equilibrated with 100 ml of 75 mM Hepes, pH 8.2, containing 150 mM NaCl, 2.5 mM $CaCl_2$, and 0.1% (w/v) Triton X-100. The P2 detergent extract (100 ml) was incubated with 5 ml of the gel at 4 °C for 4 h with gentle agitation. The gel was transferred to a column and washed extensively with the equilibration buffer. The AtxC-binding proteins were eluted at 10 ml/h with 140 mM Mes, pH 5.0, containing 200 mM NaCl, 4 mM $CaCl_2$, and 0.2% (w/v) Triton X-100. One millilitre fractions were collected directly into 0.4 ml of 0.5 M triethanolamine, pH 8.2, 150 mM NaCl, and concentrated in Centricon YM-10 concentrators (Millipore, USA). Their protein composition was analysed by SDS–PAGE (12.5% acrylamide gels) [17] under reducing conditions (0.5% (m/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 30 mM Tris–HCl, pH 6.8), followed by silver staining. Protein molecular mass standards used were from Bio-Rad.

Mass spectrometry. About 1 μg of each sample from AtxC-CHS and EA-CHS affinity chromatography was separated on SDS–PAGE (12.5% acrylamide gels) and the proteins were silver stained. Protein bands were excised from the gels and reduced with dithiothreitol (DTT), alkylated with iodoacetamide, and digested with Promega modified trypsin for 16 h. The peptides were extracted from gel pieces with 50% (v/v) acetonitrile/5% (v/v) formic acid and concentrated for LC–MS and MS/MS analysis on a Finnigan LCQ ion trap mass spectrometre [15]. Spectra were analysed by database searching using the Sequest algorithm against the NCBI non-redundant database.

Isolation of PDI from porcine cerebral cortex. PDI was isolated following the method of Lambert and Freedman [18] with slight modifications in the ammonium sulphate precipitation procedure of proteins to obtain a higher yield.

Surface plasmon resonance. AtxC was immobilized on flow cell 1 of a CM5 sensor chip (Biacore AB, Sweden) as described [15]. Flow cell 2 was mock-immobilized and used as a reference surface. Surface plasmon resonance (SPR) experiments were performed in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$ (buffer A) at 10 μl/min, and 25 °C, using a Biacore X system (Biacore AB, Sweden). The interaction between PDI and the chip-immobilized AtxC was studied by injecting solutions of PDI (concentrations 35–175 nM) in buffer A over the chip for 2 min. Between consecutive injections, the chip was regenerated three times for 1 min with 10 mM HCl, followed by 5 mM NaOH and 1 M NaCl. The kinetic constants, k_{on} and k_{off}, for the interaction of $sPLA_2$s with immobilized PDI were determined by using the Biaevaluation 3.2 software.

Results and discussion

To get new insight into the molecular basis of the action of β-neurotoxins, we searched for additional molecules in the nerve tissue that specifically associated with AtxC. A Triton X-100 extract of a larger quantity of demyelinated P2 membranes (25 mg membrane protein) from porcine cerebral cortex was incubated with 5 ml AtxC-CHS. The affinity-gel was washed extensively and the AtxC-retained proteins were eluted by lowering the pH of the mobile phase from 8.2 to 5.0. The eluted proteins were analysed on SDS–PAGE and visualized by silver staining (AtxC-CHS in Fig. 1). The same procedure was carried out simultaneously on the EA-CHS (Fig. 1) to spot the proteins which were specifically retained by AtxC-CHS. Besides the already known AtxC-binding proteins CaM, M-types $sPLA_2R$, and 14-3-3 proteins, a protein with apparent molecular mass of 70 kDa (R70) was present in AtxC-CHS but not in the EA-CHS eluate (Fig. 1). Unlike most of the above AtxC-binding proteins, this protein escaped the identification using affinity-labelling with [^{125}I]AtxC and cross-linker disuccinimidyl suberate, as did the 14-3-3 proteins, probably due to the absence of reactive amino groups on appropriate positions in the molecule [15].

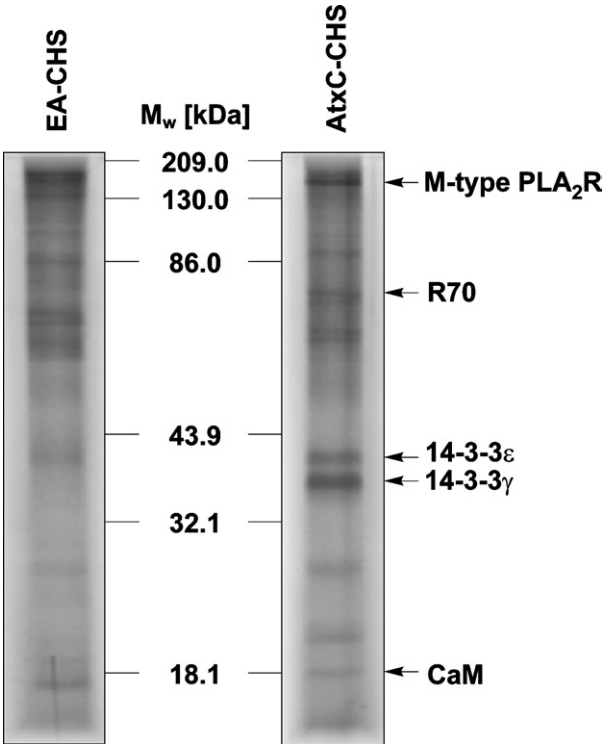

Fig. 1. Specific retention of porcine cerebral cortex R70 protein on AtxC-CH-Sepharose. Triton X-100 extract of P2 membranes was incubated at pH 8.2 with AtxC- and with ethanolamine (EA)-derivatized CH-Sepharose (CHS). The resins were thoroughly washed and the bound material was eluted at pH 5.0. The proteins specifically retained by gel-immobilized AtxC (arrows) were detected by comparing the SDS–PAGE protein patterns of samples obtained from AtxC- and EA-derivatized gels. Besides CaM, the M-type PLA_2R and 14-3-3 proteins, already known as AtxC-binding proteins, a new AtxC-binding protein with an apparent molecular mass of 70 kDa (R70) was present. For experimental details see Materials and methods.

The R70 protein band was reduced, alkylated, and digested with trypsin in the gel. The resulting peptides were extracted from the gel and their amino acid sequences were determined by LC–MS/MS. Nine peptides were sequenced and identified as parts of PDI (Fig. 2). The structurally identified R70-peptides constitute 21.0% of the PDI sequence.

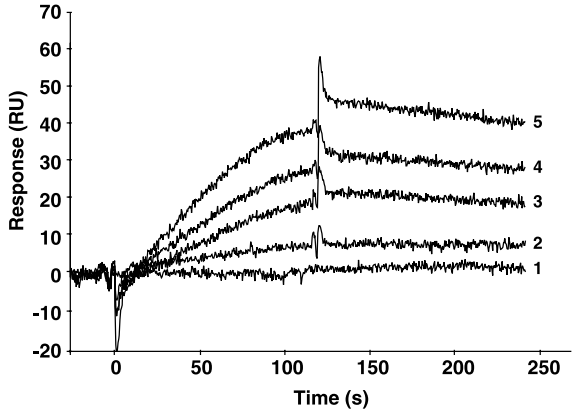

Fig. 3. AtxC and PDI interact directly. AtxC was covalently immobilized on the surface of a CM5 sensor chip. PDI in concentrations ranging from 35 to 175 nM was injected for 2 min over AtxC-immobilized and control flow cells of the sensor chip. Between the injections the surface was regenerated with 1 min pulses of 10 mM HCl, 5 mM NaOH, and 1 M NaCl. The signals were subtracted and expressed in resonance units (RU) as a function of time. In the figure, the traces (1–5) obtained with 0, 35, 88, 117, and 175 nM PDI, respectively, are overlaid. From the traces, k_{on} and k_{off} were derived, giving a K_d^{app} of 1.27 ± 0.05 μM.

To verify the specificity of the interaction between PDI and AtxC, we prepared pure PDI from porcine cerebral cortex. Direct binding of PDI to AtxC was confirmed by surface plasmon resonance (SPR) technique, which gave an apparent dissociation constant (K_d^{app}) of 1.27 ± 0.05 μM for the interaction (Fig. 3).

PDI is an enzyme that catalyses disulphide bond formation and isomerization (oxido-reductase or isomerase activity) and also a chaperone that inhibits aggregation of proteins [19]. It is found predominantly in the lumen of the ER, where its concentration approaches the millimolar range. The K_d in micromolar range indicates that a tight association between the two proteins is possible in the ER, in spite of the potentially very low concentration of $sPLA_2$-toxin.

The interaction between Atx and PDI may be important for intracellular trafficking of the toxin. PDI has been demonstrated to play an important role in the retrograde transport of cholera toxin (CT) during the

GAPDEEDHVL VLHK**GNFDEA** **LAAHK**YLLVE FYAPWCGHCK ALAPEYAKAA
GKLKAEGSEI RLAK**VDATEE** **SDLAQQYGVR** GYPTIKFFKN GDTASPKEYT
AGR**EADDIVN** **WLK**KRTGPAA STLSDGAAAE ALVESSEVAV IGFFKDMESD
SAKQFFLAAE VIDDIPFGIT SNSDVFSK**YQ** **LDKDGVVLFK** KFDEGRNNFE
GEVTKEKLLD FIK**HNQLPLV** **IEFTEQTAPK** IFGGEIKTHI LLFLPKSVSD
YEGKLSNFKK AAESFKGKIL FIFIDSDHTD NQR**ILEFFGL** **K**KEECPAVR**L**
ITLEEEMTKY KPESDELTAE KITEFCHRFL EGKIKPHLMS QELPDDWDKQ
PVKVLVGKNF EEVAFDEKKN VFVEFYAPWC GHCKQLAPIW DKLGETYKDH
ENIVIAKMD**S** **TANEVEAVK**V HSFPTLKFFP ASADR**TVIDY** **NGER**TLDGFK
KFLESGGQDG AGDDDDLEDL EEAEEPDLEE DDDQKAVKDE L

Fig. 2. R70 is protein disulphide isomerase (PDI). The sequence of bovine PDI is shown. Using ESI-MS/MS, nine tryptic peptides from R70 were sequenced. Their structures are shown in bold and constitute 21.0% of the PDI sequence.

infection of target cells [20]. CT invades cells by binding to lipid raft-associated ganglioside GM1 with subsequent translocation into the lumen of the ER [21]. The lipid raft pathway may also be used by $sPLA_2$-neurotoxins to reach reticular structures in nerve endings. Caveolae, a special type of lipid raft, have already been shown to mediate the translocation of the structurally very similar mouse group IIA $sPLA_2$ into the perinuclear area, a specific part of the ER, in human embryonic kidney 293 cells [22]. Caveolae are not present in neurons but other types of lipid rafts which accumulate in the axonal plasma membrane can serve the same purpose [23]. An alternative mode of translocation, internalization of $sPLA_2$ neurotoxins via endosomes, is questionable since bafilomycin A1, an inhibitor of V-type ATPase, did not prevent the intoxication of NM preparation by $sPLA_2$ neurotoxin although, under the same conditions, it prevented the toxic action of clostridial neurotoxins [24].

The C-terminal KDEL-sequence is known to be responsible for retrieving ER-proteins that escape their resident compartment. CT possesses this sequence in its structure and, when the toxin enters the ER, this motif ensures its retention and concentration there. Atx does not have such a sequence at its C-terminus but, in the complex with PDI which has it, could acquire the characteristics of KDEL-containing proteins. A similar assumption has been made in the case of a plant protein toxin, ricin, which also lacks the C-terminal KDEL or related sequence. Its Golgi-to-ER transport, ER-retention, and ER-concentration functions were suggested to be the result of its association with reticular KDEL-containing protein calreticulin [25].

Two β-neurotoxin-binding proteins with ER localization have already been discovered. Taipoxin, a presynaptically acting $sPLA_2$ from the venom of the Australian taipan (*Oxyuranus scutellatus scutellatus*), binds to a taipoxin-associated calcium binding protein of 49 kDa (TCBP-49) [26] and crotoxin, a presynaptically acting $sPLA_2$ from the venom of South American rattlesnake (*Crotalus durissus terrificus*), binds to crocalbin [27]. Both these $sPLA_2$-binding proteins have KDEL-related sequences at their C-termini and may be used by these toxins to persist and concentrate in the lumen of ER, in the same way as proposed for the PDI–Atx couple.

The toxin must pass the membrane of ER to reach the cytosol of the nerve cell and express its toxicity. In the case of CT, it was suggested that the translocation occurs through a protein translocator, the Sec61p channel, with the assistance of PDI [28]. PDI, acting as a redox-dependent chaperone, is supposed to unfold the toxin and present it to the protein-conducting channel [20]. A similar scenario can be imagined also for Atx and related $sPLA_2$-neurotoxins, especially as protein unfolding is not an absolute prerequisite for ER-to-cytosol dislocation [29]. Moreover, as we demonstrated for AtxA inside yeast cells, the refolding of the toxin back to the active conformation on the cytosolic side should not be a problem [16].

In conclusion, Atx binds with high affinity to PDI, a major protein in the lumen of the ER. The observed interaction could be essential for retention and concentration of the toxin inside the lumen of ER as well as for its transport across the ER membrane into the cytosol.

Acknowledgments

This work was supported by the Slovenian Ministry of Higher Education, Science and Technology (Grants P0-0501-0106 and SLO-US-2001/43) and by a grant from the University of Virginia Pratt Committee. For technical assistance we thank Jani Božič. Many thanks to Dr. Uroš Petrovič and Dr. Roger H. Pain for critical reading of the manuscript.

References

[1] R.M. Kini (Ed.), Venom Phospholipase A_2 Enzymes: Structure, Function and Mechanism, Wiley, Chichester, 1997.
[2] M.H. Gelb, E. Valentin, F. Ghomashchi, M. Lazdunski, G. Lambeau, Cloning and recombinant expression of a structurally novel human secreted phospholipase A_2, J. Biol. Chem. 275 (2000) 39823–39826.
[3] I. Kudo, M. Murakami, Phospholipase A_2 enzymes, Prostagladins Other Lipid Mediat. 68-69 (2002) 3–58.
[4] C.C. Chang, Neurotoxins with phospholipase A_2 activity in snake venoms, Proc. Natl. Sci. Counc. B. ROC 9 (1985) 126–142.
[5] F. Gubenšek, A. Ritonja, J. Zupan, V. Turk, Basic proteins of *Vipera ammodytes* venom: studies of the structure and function, Period. Biol. 82 (1980) 443–447.
[6] F. Dorandeu, I. Pernot-Marino, J. Veyret, C. Perrichon, G. Lallement, Secreted phospholipase A_2-induced neurotoxicity and epileptic seizures after intracerebral administration: an unexplained heterogeneity as emphasized with paradoxin and crotoxin, J. Neurosci. Res. 54 (1998) 848–862.
[7] C. Montecucco, O. Rossetto, How do presynaptic PLA_2 neurotoxins block nerve terminals?, Trends Biochem. Sci. 25 (2000) 266–270.
[8] R.W. Dixon, J.B. Harris, Nerve terminal damage by β-bungarotoxin: its clinical significance, Am. J. Pathol. 154 (1999) 447–455.
[9] I. Križaj, F. Gubenšek, Neuronal receptors for phospholipases A_2 and β-neurotoxicity, Biochemie 82 (2000) 807–814.
[10] A. Čopič, N. Vučemilo, F. Gubenšek, I. Križaj, Identification and purification of a novel receptor for secretory phospholipase A_2 in porcine cerebral cortex, J. Biol. Chem. 274 (1999) 26315–26320.
[11] N. Vardjan, N.E. Sherman, J. Pungerčar, J.W. Fox, F. Gubenšek, I. Križaj, High-molecular-mass receptors for ammodytoxin in pig are tissue-specific isoforms of M-type phospholipase A_2 receptor, Biochem. Biophys. Res. Commun. 289 (2001) 143–149.
[12] N. Vučemilo, A. Čopič, F. Gubenšek, I. Križaj, Identification of a new high-affinity binding protein for neurotoxic phospholipases A_2, Biochem. Biophys. Res. Commun. 251 (1998) 209–212.

[13] J. Šribar, A. Čopič, M. Poljšak-Prijatelj, J. Kuret, U. Logonder, F. Gubenšek, I. Križaj, R25 is an intracellular membrane receptor for a snake venom secretory phospholipase A_2, FEBS Lett. 553 (2003) 309–314.
[14] J. Šribar, A. Čopič, A. Pariš, N.E. Sherman, F. Gubenšek, J.W. Fox, I. Križaj, A high affinity acceptor for phospholipase A_2 with neurotoxic activity is a calmodulin, J. Biol. Chem. 276 (2001) 12493–12496.
[15] J. Šribar, N.E. Sherman, P. Prijatelj, G. Faure, F. Gubenšek, J.W. Fox, A. Aitken, J. Pungerčar, I. Križaj, The neurotoxic phospholipase A_2 associates, through a non-phosphorylated binding motif, with 14-3-3 protein and isoforms, Biochem. Biophys. Res. Commun. 302 (2003) 691–696.
[16] U. Petrovič, J. Šribar, A. Pariš, M. Rupnik, M. Kržan, N. Vardjan, F. Gubenšek, R. Zorec, I. Križaj, Ammodytoxin, a neurotoxic secreted phospholipase A_2, can act in the cytosol of the nerve cell, Biochem. Biophys. Res. Commun. 324 (2004) 981–985.
[17] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[18] N. Lambert, R.B. Freedman, Structural properties of homogeneous protein disulphide-isomerase from bovine liver purified by a rapid high-yielding procedure, Biochem. J. 213 (1983) 225–234.
[19] B. Wilkinson, H.F. Gilbert, Protein disulphide isomerase, Biochim. Biophys. Acta 1699 (2004) 35–44.
[20] B. Tsai, Y. Ye, T.A. Rapoport, Retro-translocation of proteins from the endoplasmic reticulum into the cytosol, Nat. Rev. Mol. Cell Biol. 3 (2002) 246–255.
[21] W.I. Lencer, B. Tsai, The intracellular voyage of cholera toxin: going retro, Trends Biochem. Sci. 28 (2003) 639–645.
[22] M. Murakami, T. Kambe, S. Shimbara, S. Yamamoto, H. Kuwata, I. Kudo, Functional association of type IIA secretory phospholipase A_2 with the glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan in the cyclooxygenase-2-mediated delayed prostanoid-biosynthetic pathway, J. Biol. Chem. 274 (1999) 29927–29936.
[23] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–40.
[24] L.L. Simpson, J.A. Coffield, N. Bakry, Inhibition of vacuolar adenosine triphosphatase antagonizes the effects of clostridial neurotoxins but not phospholipase A_2 neurotoxins, J. Pharmacol. Exp. Ther. 269 (1994) 256–262.
[25] P.J. Day, S.R. Owens, J. Wesche, S. Olsnes, L.M. Roberts, J.M. Lord, An interaction between ricin and calreticulin that may have implications for toxin trafficking, J. Biol. Chem. 276 (2001) 7202–7208.
[26] D. Dodds, A.K. Schlimgen, S.-Y. Lu, M.S. Perin, Novel reticular calcium binding protein is purified on taipoxin columns, J. Neurochem. 64 (1995) 2339–2344.
[27] M.J. Hseu, C.-Y. Yen, M.-C. Tzeng, Crocalbin: a new calcium-binding protein that is also a binding protein for crotoxin, a neurotoxic phospholipase A_2, FEBS Lett. 445 (1999) 440–444.
[28] A. Schmitz, H. Herrgen, A. Winkeler, V. Herzog, Cholera toxin is exported from microsomes by the Sec61p complex, J. Cell Biol. 148 (2000) 1203–1212.
[29] B. Tirosh, M.H. Furman, D. Tortorella, H.L. Ploegh, Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation, J. Biol. Chem. 278 (2003) 6664–6672.