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A new photoprobe for studying biological activities of secreted phospholipases A_2

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Abstract

Ammodytoxin (Atx) is a snake venom phospholipase A_2 ($sPLA_2$s) with presynaptic toxicity, anticoagulant activity and the ability to influence cell cycle progression. These multiple physiological activities make this molecule a promising tool for studying processes influenced by the highly homologous mammalian $sPLA_2$s—for example cell proliferation and apoptosis. Secreted PLA_2s can act on cells as enzymes or as ligands for cellular receptors. To further characterize the $sPLA_2$-binding molecules in cells we have developed a new method based on AtxC and a biotin-containing cross-linking reagent sulfo-SBED which possesses both an amine-reactive and a photo-reactive site, together with a biotin moiety that enables specific detection and affinity-based concentration. The biological activity of the AtxC derivatized by sulfo-SBED was demonstrated by biotin-tagging of calmodulin and R25, both known AtxC targets, but not of other proteins. In addition, using the new protocol we specifically labelled 14-3-3 proteins, protein disulfide isomerase and two unknown proteins of 45 and 46 kDa in the mitochondrial–synaptosomal fraction of porcine cerebral cortex, none of which could be tagged by the previously used methods. The new methodology, which can be used for any $sPLA_2$, constitutes a novel approach to discovering and purifying $sPLA_2$-binding proteins, to studying the topology of their respective complexes and to following $sPLA_2$s in different biological systems.

Keywords: Ammodytoxin; Snake venom; Sulfo-SBED; Secreted phospholipase A_2; Binding proteins; Mechanisms of action

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1. Introduction

Secreted PLA_2s are enzymes that catalyze hydrolysis of the *sn*-2 ester bond in 1,2-diacyl-*sn*-3-phosphoglycerides. They are relatively small, 13–19 kDa, proteins, very similar in structure no matter whether they originate from a snake venom (sv) or from a mammalian cell [1]. Based on structural features, such as the number and position of disulfide bonds, low molecular mass $sPLA_2$s have been assigned to seven groups (G), comprising altogether 17 distinct isoenzymes, of which 12 are from mammals [2,3]. Secreted PLA_2s are very interesting proteins to study structure–function relations since, in spite of close structural resemblance, they display a remarkable variety of physiological and pathological activities [4–6]. These include lipid digestion, host defence, and lipid mediator production during normal and inflammatory processes. They can also affect cell migration and cell proliferation, and produce various pharmacological effects; they can be pre- or post-synaptically neurotoxic, myotoxic, cardiotoxic, cytotoxic, anticoagulant, convulsant, hypotensive and pro-inflammatory [4,7,8]. Some $sPLA_2$s are bactericidal [9] and display potent inhibitory effects against HIV-1 and *Plasmodium falciparum*, the most deadly malaria parasite [10,11]. The phospholipase activity is essential for some biological activities of $sPLA_2$ molecules, but not for all. In some processes $sPLA_2$s function as ligands and the so called ''pharmacological sites'' on surfaces of $sPLA_2$s allow them to bind specifically to membrane receptors or soluble proteins that participate in their action [12]. Several $sPLA_2$-binding proteins have been detected but only a few characterized to the extent that their role in the physiology of $sPLA_2$s is clear. Key tools for identifying the majority of $sPLA_2$-binding proteins have been sv $sPLA_2$s. For example, the M-type $sPLA_2$ receptor ($sPLA_2R$), which is one of the most thoroughly characterized $sPLA_2$-binding proteins, was discovered using the neurotoxic $sPLA_2$ OS_2 from the Australian Taipan *Oxyuranus s. scutellatus* [13]. Subsequently, it was found that the M-type $sPLA_2R$ is a high-affinity receptor for endogenous $sPLA_2$s, such as GIB and GX in mouse, and that it mediates the biological functions of these $sPLA_2$s such as cell proliferation, cell migration, hormone release, lipid mediator and cytokine production [14,15]. Ammodytoxin[1] (Atx—A, B and C isoforms) is another sv $sPLA_2$, from *Vipera a. ammodytes*, that was successfully used in the discovery of $sPLA_2$-binding molecules. It is a multifunctional protein expressing presynaptic neurotoxicity [16], anticoagulant activity [17] and interference with the cell cycle [18]. Using Atx-affinity chromatography or ^{125}I-Atx-affinity labelling and chemical cross-linking we identified several proteins that bind $sPLA_2$s with high-affinity: a neuronal isoform of M-type $sPLA_2R$ [19], calmodulin (CaM) [20], 14-3-3 proteins [21], R25 [22], protein disulfide isomerase (PDI) [23] and FXa [17].

The $sPLA_2$s are extraordinarily versatile in terms of their currently recognized biological effects, but not even the action of the majority of $sPLA_2$ isoenzymes is known, so it is clear that many more $sPLA_2$ acceptors must remain to be discovered. In addition, the (patho)physiological relevance of nearly all the identified $sPLA_2$–protein interactions

[1] *Abbreviations used:* Atx, ammodytoxin; CaM, calmodulin; DSS, disuccinimidyl suberate; m.p., membrane protein; ECL, enhanced chemiluminescence; G, group; NHS, *N*-hydroxysuccinimide; PDI, protein disulfide isomerase; PLA_2, phospholipase A_2; PyrPG, 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol; R25, receptor for Atx in porcine cerebral cortex of 25 kDa; $sPLA_2$, secreted PLA_2; $sPLA_2R$, $sPLA_2$ receptor; SA-HRP, streptavidin-linked horseradish peroxidase; sv, snake venom; sulfo-SBED, sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3′-dithiopropionate).

remains to be clarified. Both facts demand novel approaches for identifying and characterizing $sPLA_2$s-binding proteins.

Here, we describe the development of a new labelling procedure for $sPLA_2$ acceptors, based on sulfosuccinimidyl-2-[6-(biotinamido)-2-(*p*-azidobenzamido)-hexanoamido]ethyl-1,3′-dithiopropionate (sulfo-SBED) and AtxC. We show the synthesis of a sulfo-SBED-AtxC photoprobe and its characterization, and demonstrate its functionality.

2. Materials and methods

2.1. Materials

AtxC was purified from *Vipera a. ammodytes* venom as described [24]. Sulfo-SBED, disuccinimidyl suberate (DSS), monomeric avidin gel and streptavidin linked to horseradish peroxidase (SA-HRP) were from Pierce (USA). The substrate to measure the phospholipase activity was prepared from 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol (PyrPG) (Molecular Probes, USA). $Na^{125}I$ was from Perkin-Elmer Life Sciences (USA). 14-3-3 proteins were isolated as described [21]. Recombinant yeast PDI was a gift from Dr. William J. Lennarz, State University of New York at Stony Brook, USA [25]. Hog brain CaM was from Boehringer Mannheim (Germany). Anti-CaM antibodies were from Upstate Biotechnology (USA). BM Chemiluminescence Western Blotting Kit was from Roche Diagnostics (Switzerland).

2.2. Synthesis of sulfo-SBED-AtxC

Sulfo-SBED is a hetero bifunctional cross-linker that possesses a sulfonated *N*-hydroxysuccinimide (sulfo-NHS)—an amino-reactive group—and a photoactivatable aryl azide site, which forms upon photolysis a short-lived nitrene that reacts non-specifically or undergoes ring expansion and reacts with nucleophiles, especially amines [26] (Fig. 1). In addition, sulfo-SBED possesses two other characteristics: (i) a biotin moiety, which allows specific avidin/streptavidin-based purification or detection of labelled proteins and peptides [27] and (ii) a cleavable disulfide bond in the linkage between the two reactive groups, which allows label transfer between proteins in a complex [28].

One milligram of sulfo-SBED was dissolved in 25 μL DMSO immediately before use. AtxC was dissolved in water at 1 mg/mL and mixed with the sulfo-SBED at room temperature in molar ratios AtxC to sulfo-SBED of 1:0.5, 1:1 and 1:3. Reaction mixtures were stirred for 30 min and then dialyzed overnight against 50 mM Hepes/HCl, pH 7.3, 150 mM NaCl to remove excess sulfo-SBED and small molecular mass side products (10 kDa cut off). The resulting solution was aliquoted and frozen at −80 °C. All manipulations were performed in the dark.

2.3. Mass spectrometry

Aliquots of sulfo-SBED-AtxC were incubated in 100 mM Tris/HCl, pH 8.2, and irradiated with long-wave UV radiation (312 nm, 5×15 W) from a distance of 5 cm, for 10 min. Non-biotinylated toxin was separated from the biotinylated toxin on a monomeric avidin–Sepharose column. After applying the sample, the column was washed thoroughly with water (20–30 column volumes), then eluted with 100 mM glycine/HCl, pH 2.0.

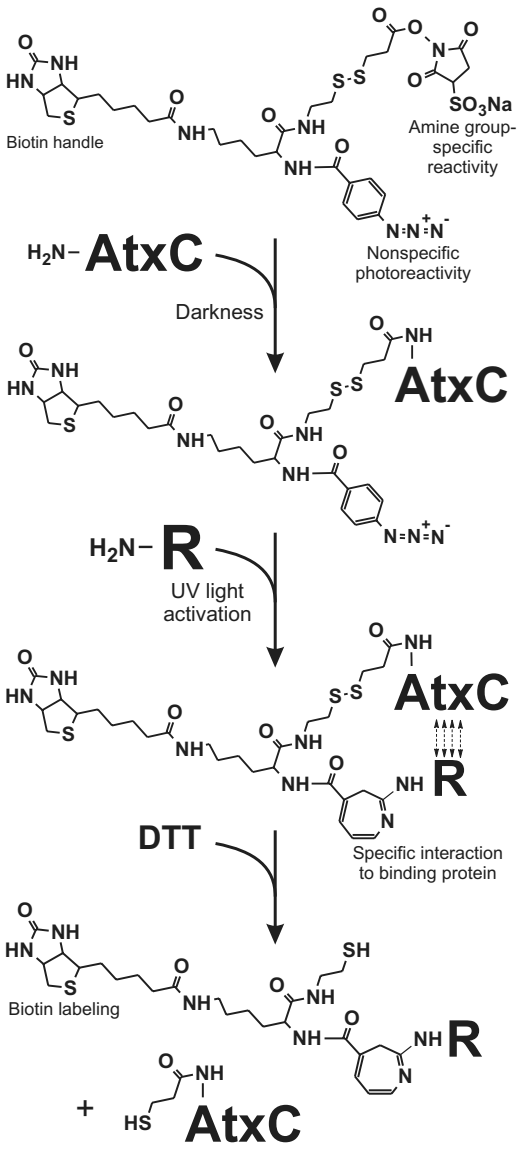

Fig. 1. Protocol for AtxC-mediated biotin transfer to the target proteins with sulfo-SBED reagent. Sulfo-SBED reacts first with an amino-group in AtxC to form a photoactivatable, nucleophile-reactive reagent. The cross-linker contains a biotin moiety that allows affinity purification and specific detection. The linkage between the two reactive sites in sulfo-SBED contains a disulfide bond, which, on reduction, enables transfer of the biotin label from the AtxC derivative to a protein in close contact (R).

Protein-containing fractions were collected, dialyzed against water and analyzed on an electrospray ionization, high resolution magnetic-sector AutospecQ mass spectrometer (Micromass, UK).

2.4. Phospholipase A_2 activity

The phospholipase A_2 activities of AtxC and biotinylated-AtxC were compared using the modified method of Radvanyi et al. [29]. Activity was assayed in 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 135 mM KCl, 2 mM $CaCl_2$, supplemented with 0.06% (w/v) fatty-acid free bovine serum albumin, using fluorogenic PyrPG phospholipid vesicles (1.4 μM final concentration) as substrate. Fluorescence was measured in 96-well plates on a SAFIRE microplate monochromator reader (Tecan, Austria) in 10 kinetic cycles at λ_{ex} 342 nm, λ_{em} 395 nm with 10 flashes and an integration time of 40 μs. All measurements were performed in triplicate. Background fluorescence was measured in control experiments lacking AtxC and subtracted from the experimental curves.

2.5. Labelling of soluble AtxC-binding proteins with sulfo-SBED-AtxC

Sulfo-SBED-AtxC was mixed with CaM, PDI and 14-3-3, each in the label-transfer buffer (75 mM Hepes/HCl, pH 8.2, 150 mM NaCl and 2 mM $CaCl_2$—final volume 100 μL) and incubated at room temperature for 60 min in the dark in the presence or absence of 100-fold excess of AtxC over sulfo-SBED-AtxC. The molar ratios between the photoprobe preparation and CaM and PDI were 1:1, while the 1:0.2 ratio was used for 14-3-3 proteins. After incubation, the mixtures were placed on ice and irradiated by five 15 W UV lamps at 312 nm at a distance of 5 cm. Five and 10 min exposures to UV radiation were tested. Labelled samples were analyzed as described below.

2.6. Labelling of CaM with ^{125}I-AtxC and DSS

AtxC was radioiodinated to a specific radioactivity of 52 Ci/mmol, as described [30]. Hog brain CaM (20 nM) in the label-transfer buffer was incubated for 30 min at room temperature with ^{125}I-AtxC (10 nM final concentration) in the presence or absence of 100-fold excess of AtxC. 2.6 mM DSS, in dimethyl sulfoxide, was added to a final concentration of 100 μM. The cross-linking reaction was stopped by adding SDS sample buffer to the sample in a ratio of 1:3. Samples were analyzed by SDS–PAGE under reducing conditions. Gels were dried and autoradiographed at −70 °C using Kodak BioMax Light Film (Sigma–Aldrich, USA).

2.7. Preparation of porcine cerebral cortex membranes

Fresh porcine brains were obtained from a local slaughterhouse and kept on ice until dissection. Demyelinated crude mitochondrial–synaptosomal fraction P2 and its sub-fractions were prepared as described [22]. The final preparations of membranes were suspended in water, their membrane protein (m.p.) content estimated [31], adjusted to 6–8 mg m.p./mL, and stored at −70 °C.

2.8. Labelling of porcine cerebral cortex membranes with sulfo-SBED-AtxC

Three μL of sulfo-SBED-AtxC stock solution were added to 500 μL of P2d membrane suspension (1.4 mg m.p./mL) in the label-transfer buffer in which $CaCl_2$ was substituted with 0.5 mM EGTA and 10 mM $SrCl_2$. The mixture was vortexed continuously at room

temperature in the dark. After 60 min, it was cooled on ice and exposed to five 15 W 312 nm UV lamps for 10 min at a distance of 5 cm. To differentiate between specifically and non-specifically labelled proteins, a parallel experiment was performed in which a 100-fold molar excess of AtxC over sulfo-SBED-AtxC was present. Labelled membranes were solubilized in SDS–PAGE sample buffer and analyzed as described below.

2.9. Analysis of conjugates

Sulfo-SBED-AtxC-labelled samples were analyzed on SDS–PAGE (12.5 or 15% acrylamide gels) under both reducing and non-reducing conditions. Gels were Western-blotted in a tank for 90 min at 200 mA. Samples with purified CaM and 14-3-3 proteins, as well as proteins in the P2d fraction, were transferred to PVDF membrane in 12.2 mM K_2HPO_4, 7.8 mM KH_2PO_4, pH 7, buffer. The PDI sample was transferred to nitrocellulose membrane in Towbin buffer (25 mM Tris/HCl, 192 mM glycine, 0.1% (w/v) SDS and 20% (v/v) MeOH). On the blots, the biotinylated proteins were detected using an SA-HRP and BM Chemiluminescence Western blotting detection system according to the manufacturer's instructions. The position of CaM on the blots was detected immunologically with mouse monoclonal anti-CaM antibodies at 1 μg/mL and located using the BM Chemiluminescence Western blotting detection system, as specified by the manufacturer, with Kodak BioMax Light Film (Sigma–Aldrich, USA).

3. Results and discussion

3.1. Synthesis and characterization of sulfo-SBED-AtxC

Sulfo-SBED is a commercially available trifunctional cross-linker that possesses one amine-reactive and one photo-reactive site and a biotin moiety that allows affinity-based enrichment of cross-linker containing species. AtxC is a basic protein and contains 10 primary amino groups. To avoid over-modification of the toxin, usually reflected in loss of biological activity, reaction conditions were sought under which the molecule of AtxC was modified only once. Optimal labelling was achieved when equimolar amounts of sulfo-SBED and AtxC were reacted. Under these conditions 44% of AtxC molecules were derivatized and no influence on phospholipase activity was detected. In agreement with this, no modification of the N-terminal α-amino group of AtxC, which has to be free for full enzymatic activity, was detected. For mass spectrometric analysis of the sulfo-SBED-AtxC, an aliquot was photolyzed in the presence of Tris, a primary amino group-containing compound, and purified on an avidin-affinity column. Three intense peaks were obtained in the ESI-MS spectra. The one at 13.741 Da corresponded to AtxC, which was evidently not completely removed in the biotin-affinity step. The most plausible explanation for its partial retention on avidin–Sepharose is formation of a complex between AtxC and biotinylated-AtxC. It is known that ammodytoxins are prone to form dimers. The signal at 14.394 Da represented a side product, a derivative of AtxC with sulfo-SBED via its aryl azide-reactive site and with a hydrolyzed sulfo-NHS group. The peak at 14.497 Da indicated the desired product, an adduct between sulfo-SBED-AtxC and Tris. The intensity of this signal depended on the preparative procedure. High yields of the photoprobe were achieved when labelling was performed as much as possible in complete darkness. No ESI-MS signals were observed on positions corresponding to

doubly or multiply sulfo-SBED modified toxin, provided a 1:1 or lower molar ratio of sulfo-SBED to AtxC was used.

CaM and ^{125}I-AtxC can be linked by DSS to give a covalent adduct of 31 kDa (Fig. 2a). The biological activity of the sulfo-SBED-AtxC derivative was assessed on the basis of its ability to tag CaM. Following incubation in the dark at room temperature, the reaction mixture was irradiated and analyzed on SDS–PAGE under reducing and non-reducing conditions. The proteins were Western-blotted on PVDF membranes and the biotin moiety of sulfo-SBED detected by SA-HRP and enhanced chemiluminescence (ECL). Under non-reducing conditions (Fig. 2b), a specific band at an apparent molecular mass of 31 kDa was observed. The presence of CaM in the biotin-containing band at 31 kDa was confirmed, using anti-CaM antibodies (Fig. 2c). Analysis under reducing conditions resulted in splitting the disulfide bond in the spacer arm of sulfo-SBED and in transferring the biotin label from sulfo-SBED-AtxC to CaM in the complex, resulting in a shift of the SA-HRP-detectable band from 31 to 17 kDa (Fig. 2d). The specificity of the procedure was further confirmed by the absence of labelling of proteins having no affinity for AtxC–bovine serum albumin, soybean trypsin inhibitor and non-Atx-specific antibodies. Optimal labelling of CaM with sulfo-SBED-AtxC was achieved with 0.7 μM sulfo-SBED-AtxC preparation in the reaction mixture and 10 min photo-excitation. Higher concentrations of sulfo-SBED-AtxC and higher molar ratios of photoprobe to CaM resulted in toxin auto-labelling and increased background. Lower concentrations or molar ratios, or shorter photo-activation times (e.g. 5 min), gave less intense labelling.

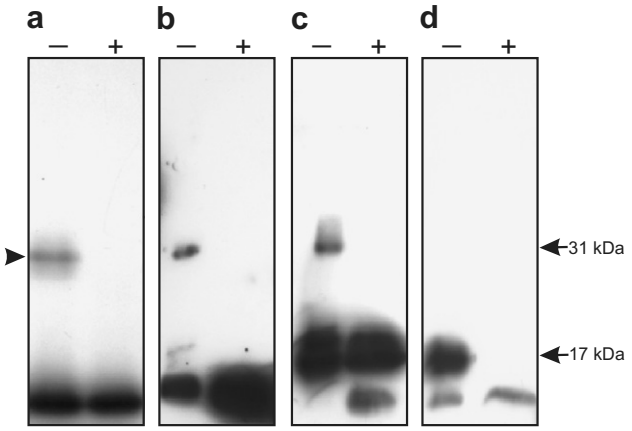

Fig. 2. The sulfo-SBED-AtxC derivative exhibits the biological activity of AtxC. The functionality of sulfo-SBED-AtxC was tested by its ability to bind CaM. (a) The position of the specific adduct between CaM and AtxC on 15% SDS–PAGE gel (arrowhead), was defined by incubation of CaM with ^{125}I-AtxC, followed by cross-linking with DSS, gel electrophoresis and autoradiography. (b–d) Sulfo-SBED-AtxC (0.7 μM) was incubated with an equimolar amount of CaM in the dark and then irradiated with 312 nm UV radiation for 10 min. SDS–PAGE analysis was performed under non-reductive (b and c) and reductive (d) conditions, proteins were electroblotted on PVDF membranes and the positions of biotin-containing proteins revealed by SA-HRP and ECL detection (b and d). (c) Using monoclonal anti-CaM antibodies, CaM was confirmed as a constituent of the 31 kDa band in (b) and 17 kDa band in (d). Incubations in the presence of 100-fold molar excess of AtxC over ^{125}I- or sulfo-SBED-labelled AtxC are designated by (+) and without it by (−). For experimental details see Section 2.

3.2. Labelling by sulfo-SBED-AtxC of proteins known to bind Atx specifically

14-3-3 proteins and PDI, proteins that are specifically complexed with gel-immobilized AtxC, escaped detection with the ^{125}I-AtxC and DSS procedure, explained by the absence of reactive amino groups on appropriate positions in these molecules [21,23]. The photoactivatable aryl azide group of sulfo-SBED is less specific than the amino-reactive NHS groups of DSS, so sulfo-SBED-AtxC would be predicted to have a broader ability to detect Atx-binding proteins than ^{125}I-AtxC/DSS. In keeping with this expectation, PDI and 14-3-3 proteins were clearly detected by the sulfo-SBED-AtxC photoprobe (Fig. 3). The optimal conditions for labelling and biotin-transfer established in the CaM system were also the most effective for PDI. Due to the low amount of 14-3-3 proteins available, a 5-fold molar excess of the photoreagent over 14-3-3 was used, which resulted in higher background and transfer of the biotin also to AtxC (Fig. 3b).

3.3. Labelling of porcine cerebral cortex membranes by sulfo-SBED-AtxC

The sulfo-SBED-AtxC photoprobe was then used to label the sub-fraction P2d of porcine cerebral cortex mitochondrial–synaptosomal membranes, the sub-fraction in which the still functionally uncharacterized AtxC receptor R25 is most abundantly present [22]. The biotin-label transfer analysis is shown in Fig. 4. Known Atx-binding proteins in the extract, CaM, R25 and 14-3-3 proteins, were clearly detected. PDI, which resides in the endoplasmic reticulum (P3, microsomal fraction), should not be present in the

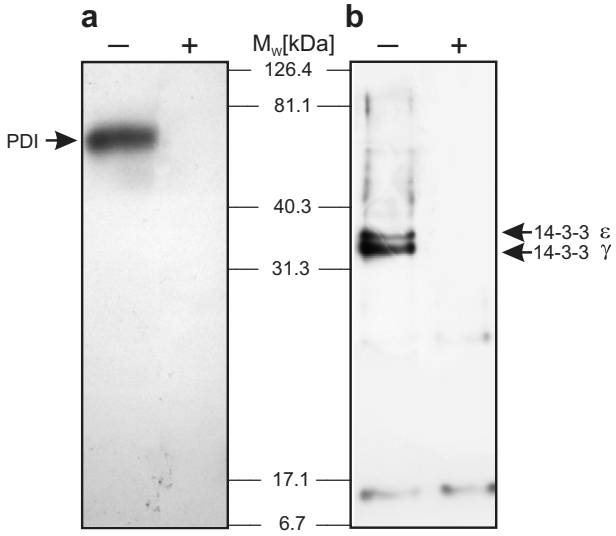

Fig. 3. Sulfo-SBED-AtxC labelling of PDI and 14-3-3, Atx-binding proteins that cannot be affinity-labelled with ^{125}I-AtxC and DSS. PDI (0.7 μM) and 14-3-3 proteins (0.14 μM) were incubated with sulfo-SBED-AtxC (0.7 μM) in the dark. The cross-linking reaction proceeded for 10 min under UV radiation (312 nm). SDS–PAGE analysis (12.5% acrylamide gels) under reducing conditions was followed by Western blotting of the samples and SA-HRP/ECL detection on the membranes. Incubations in the presence or absence of 100-fold molar excess of the native AtxC over sulfo-SBED-AtxC are designated by (+) and (−). For experimental details see Section 2.

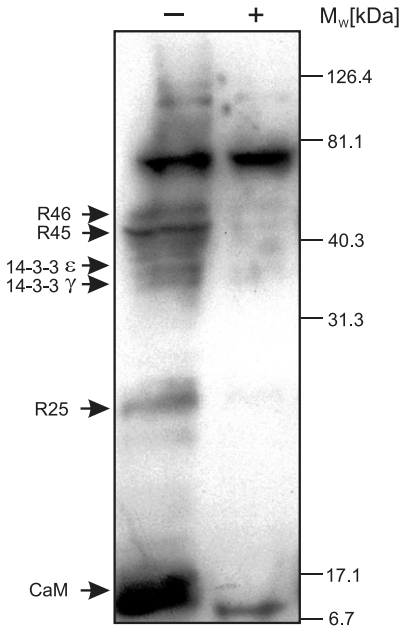

Fig. 4. Labelling of porcine cerebral cortex mitochondrial–synaptosomal membrane sub-fraction P2d with sulfo-SBED-AtxC. The suspension of membranes (1.4 mg m.p./ml) was incubated with 0.4 μM sulfo-SBED-AtxC in the dark and then exposed to UV radiation. The sample was dissolved in reductive SDS–PAGE sample buffer. Following SDS–PAGE electrophoresis (12.5% acrylamide gels) and Western blotting to PVDF membrane, the biotin-containing bands were visualized by the SA-HRP/ECL detection system. The two experiments were performed, in the absence (−) and presence (+) of an excess of unlabelled over labelled AtxC, to determine the positions of specific bands. For experimental details see Section 2.

sub-fraction analyzed, and, indeed, no signal at the molecular mass of PDI was observed. Most interestingly, using the new photoprobe, two additional proteins, R45 and R46, with apparent molecular masses of 45 and 46 kDa, were specifically labelled. The identity and physiological roles of these two $sPLA_2$-binding proteins remain to be determined. The best labelling results on membranes were obtained using 0.4 μM sulfo-SBED-AtxC, a slightly lower concentration of the photoreagent than that used for optimal labelling of binding proteins in solution.

4. Conclusions

By reacting AtxC, a model $sPLA_2$ from snake venom, with the trifunctional cross-linking reagent, sulfo-SBED, we have obtained a biologically active photo-reactive derivative that biotin-tags proteins with which AtxC comes into close contact. Its specificity has been demonstrated by labelling CaM, 14-3-3 proteins and PDI, all known targets of AtxC. The new labelling method will simplify mapping interaction interfaces between AtxC and interacting proteins to allow the rational design of mutants for use in further physiological studies. The technique can also provide an alternative means for affinity-based purification of AtxC receptors, as exemplified with the mitochondrial receptor for AtxC, R25, and two novel brain receptors, R45 and R46. Last, but not least, the

photoprobe opens up the possibility of pulse-chasing $sPLA_2$ in biological systems, providing a new approach to studying cellular internalization and localization of ammodytoxins.

The method was developed using AtxC as a model $sPLA_2$ but, given the very high structural stability of $sPLA_2$s, it can be expected to be generally applicable, thus opening up an effective new approach to studying the (patho)physiological activities of this important family of enzymes on the molecular level.

Acknowledgments

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