R25 is an intracellular membrane receptor for a snake venom secretory phospholipase A_2[1]

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Abstract **Ammodytoxin is a presynaptically neurotoxic (β-neurotoxic) snake venom secretory phospholipase A_2 ($sPLA_2$). We detected a 25 kDa protein which binds the toxin with very high affinity (R25) in porcine cerebral cortex [Vučemilo et al., Biochem. Biophys. Res. Commun. 251 (1998) 209–212]. Here we show that R25 is an integral membrane protein with intracellular localisation. It is the first $sPLA_2$ receptor known to date that localises to intracellular membranes. Centrifugation on sucrose gradients was used to fractionate porcine cerebral cortex. The subcellular composition of the fractions was determined by following the distribution of organelle-specific markers. The distribution of R25 in the fractions matched the distribution of the mitochondrial marker succinate dehydrogenase, but not the markers for plasma membrane, lysosomes, endoplasmic reticulum, synaptic and secretory vesicles. R25 most likely resides in mitochondria, which are known to be targets for $sPLA_2$ neurotoxins in the nerve ending and are potentially implicated in the process of β-neurotoxicity.**

Key words: Ammodytoxin; Snake venom; Secretory phospholipase A_2; Presynaptic neurotoxicity; Intracellular membrane receptor; Mitochondrion; *Vipera ammodytes ammodytes*

1. Introduction

Secretory phospholipases A_2 ($sPLA_2$s) catalyse the hydrolysis of the ester bond at the *sn*-2 position of glycerophospholipids, liberating free fatty acids and lysophospholipids. To be catalytically active, these enzymes require Ca^{2+} ions. They form a family of structurally related, 13–18 kDa disulphide-rich enzymes which further divides into eight groups (G), including GI, GII, GIII, GV, GIX, GX, GXI and GXII [1,2]. $sPLA_2$s are widely spread enzymes. They have been found in different mammalian tissues [3] and as components of many animal venoms [4]. Besides being involved in the digestion of phospholipids in dietary food and the production of diverse lipid mediators, $sPLA_2$s have been found to play an important role in the antibacterial defence, exocytosis, anticoagulation, inflammation, ischemia, atherosclerosis and cancer [3]. In addition, some venom $sPLA_2$s evolved neurotoxic, myotoxic, cardiotoxic and other pharmacological effects [4]. Many of these effects cannot be explained by the phospholipase activity of $sPLA_2$ alone, but rather involve the interaction of the enzyme with specific binding proteins [5]. $sPLA_2$-binding proteins have been discovered in different tissues, and their number is gradually increasing. They include the plasma membrane (PM)-localised M-type $sPLA_2$ receptors ($sPLA_2$Rs) [6,7], glypican-I [8] and the voltage-dependent K^+ channel [9]. Pentraxins are soluble extracellular $sPLA_2$-binding proteins [10], whereas crocalbin and TCBP-49 reside in the lumen of the endoplasmic reticulum (ER) [11,12]. Additionally, the cytosolic calmodulin (CaM) and 14-3-3 proteins were also found to bind to some $sPLA_2$s [13,14]. $sPLA_2$s can therefore act extracellularly, or they can be internalised to reach their intracellular targets. $sPLA_2$s were found to be able to enter a variety of cells. The distribution of $sPLA_2$ in particular cell types was demonstrated to be group-dependent, implying the functional non-redundancy of $sPLA_2$s [15]. Depending on the type of cell and the group of $sPLA_2$s, they were detected in the perinuclear area, caveolae or lysosomes [8,16–23]. Externally added GIB $sPLA_2$ was translocated to the nucleus of proliferative U_{III} cells [24]. The mitochondrial localisation of $sPLA_2$ is controversial. GIIA $sPLA_2$ was purified from rat liver mitochondria [25], where it should reside at the contact sites between the outer and the inner membrane [26]. Also, mitochondria seem to be the target of GIIA $sPLA_2$ at least in mitochondrial permeability transition [27], cell death during chemical hypoxia [28] and in the process of β-neurotoxicity [29].

Ammodytoxin C (AtxC) is a presynaptically or β-neurotoxic GIIA $sPLA_2$ from the venom of *Vipera ammodytes ammodytes*. Investigating the molecular basis of its action, we have detected a 25 kDa protein (R25) in porcine cerebral cortex which binds AtxC with a high affinity [30]. As we report in this communication, R25 appears to be the first integral membrane $sPLA_2$-binding protein located intracellularly. Our data show that it co-localises with mitochondria.

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[1] This work is dedicated to the memory of Jošt Kuret.

Abbreviations: Atx, ammodytoxin; AtxC, ammodytoxin C; CaM, calmodulin; DSS, disuccinimidyl suberate; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; G, group; K_d, dissociation constant; PI-PLC, phosphatidylinositol-phospholipase C; PM, plasma membrane; R25 and R180, receptors for AtxC in porcine cerebral cortex of 25 and 180 kDa, respectively; $sPLA_2$, secretory PLA_2; $sPLA_2$R, $sPLA_2$ receptor

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2. Materials and methods

2.1. Materials

AtxC was isolated as described in [31]. Protein molecular mass standards were from Bio-Rad, $Na^{125}I$ (carrier-free) from New England Nuclear, Triton X-100 from Roche Molecular Biochemicals, Brij 35 and Lubrol 17A17 from Serva, *p*-iodonitrotetrazolium violet from Sigma and Z-Phe-Arg-AMC·HCl from Bachem. All other reagents and chemicals were of analytical grade.

2.2. Radioiodination of AtxC and crotoxin

AtxC and crotoxin isoform CA_2CB_d were radioiodinated as described in [32,33]. Radioactive toxins were identical to the native toxins in enzymatic, neurotoxic and immunological properties and were kept at 4°C.

2.3. Fractionation of porcine cerebral cortex

Fresh porcine brains were obtained from a local slaughterhouse. Until dissecting the cortex, the brains were kept on ice. The demyelinated crude mitochondrial–synaptosomal fraction P2, which was used in some experiments, was prepared as described in [34].

Fractionation of porcine cerebral cortex was performed essentially following the procedure of Casado et al. [35]. Fresh porcine cerebral cortex (10 g) was immersed in 100 ml buffer A (7 mM imidazole, pH 7.4, 0.32 M sucrose, 2 mM EDTA) to which protease inhibitors (31 μg/ml benzamidine, 25 μg/ml bacitracin, 2 μg/ml soybean trypsin inhibitor, 1.4 μg/ml pepstatin, 1 μg/ml leupeptin, 0.1 mM PMSF) were added immediately before use. The tissue was homogenised with a Glas-Col Potter homogeniser (500 rpm, five strokes). The homogenate was centrifuged for 10 min at $1000\times g$. The resulting pellet was resuspended in 40 ml buffer A and centrifuged for 12 min at $1000\times g$. The pellet was resuspended in 38 ml buffer A and is referred to as the nuclear fraction, P1. Both supernatants were pooled and centrifuged for 20 min at $12\,000\times g$, the pellet resuspended in 40 ml buffer A and centrifuged again using the same conditions. The pellet was finally resuspended in 20 ml buffer A to obtain the crude mitochondrial–synaptosomal fraction, P2. To isolate the microsomal fraction, P3, the two supernatants were pooled and ultracentrifuged for 90 min at $105\,000\times g$ and the pellet resuspended in 22 ml buffer A.

Fractions P1, P2 and P3 were further fractionated on three different discontinuous sucrose density gradients in a swinging bucket rotor (TST 28.38, Sorvall) for 120 min at $55\,000\times g$. Fraction P1 (19 ml) was layered on 19 ml of 0.8 M sucrose. After the centrifugation two fractions were obtained, P1a at the interphase and P1b as pellet. Fraction P2 (10 ml) was layered on four 7 ml layers of 0.8, 1.0, 1.2 and 1.4 M sucrose (top to bottom). Five fractions were collected, four at the interphases (P2a–P2d) and the pellet (P2e). Fraction P3 (11 ml) was layered on three 9 ml layers of 0.8, 1.0 and 1.2 M sucrose (top to bottom). Following the centrifugation three fractions were obtained at the interphases (P3a–P3c) and the pellet (P3d).

2.4. Determination of protein concentration in fractions

Protein concentration was measured by the method of Markwell et al. [36] using bovine serum albumin as a standard.

2.5. Determination of enzyme activities in fractions

NADPH: cytochrome *c* reductase (EC 1.6.2.4) activity was assayed using the method of Masters et al. [37]. Cathepsin L (EC 3.4.22.15) activity was determined by the method of Barret and Kirschke [38]. Succinate dehydrogenase (EC 1.3.99.1) activity was assayed by the method of Morre [39] and K^+-(*p*-nitrophenyl phosphatase) (EC 3.1.3.41) activity by the method of Marcheselli et al. [40].

2.6. Solubilisation of membrane proteins

Fractions of porcine cerebral cortex were extracted for 1 h at 4°C by gentle agitation in 75 mM HEPES, pH 8.2, containing 150 mM NaCl, 2.5 mM $CaCl_2$, and 2% (w/v) Triton X-100. The resulting extracts were centrifuged for 1 h at 4°C at 14 000 rpm in an Eppendorf centrifuge (5417 R) and the supernatants collected. Different concentrations of Triton X-100 and NaCl were tested for their ability to solubilise the AtxC-binding proteins.

2.7. Covalent binding of ^{125}I-AtxC to solubilised AtxC-binding proteins – affinity labeling

Extracts (2 mg protein/ml) were incubated for 30 min at room temperature with 10 nM ^{125}I-AtxC or 2.5 nM ^{125}I-crotoxin in the presence or absence of a 200-fold excess of unlabeled AtxC or crotoxin, respectively. Disuccinimidyl suberate (DSS) was added to 100 μM final concentration and after 5 min the cross-linking reaction was stopped by the addition of SDS–PAGE sample buffer. Samples were analysed by SDS–PAGE, gels dried and autoradiographed at −70°C using Kodak X-Omat AR films [34]. Autoradiographs were quantified using QuantiScan (Biosoft).

2.8. Electron microscopy

Suspensions of membranes from the fractions were fixed with 1% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 200 mM phosphate buffer (pH 7.4) for 30 min, and postfixed with 0.1% (w/v) OsO_4 for 10 min at room temperature. The membranes were centrifuged for 5 min and then embedded in 2% (w/v) Agar Noble (Difco, USA). After solidation the embedded membranes were cut to small pieces and transferred into Eppendorf tubes. All the following steps were done on an ice bath. The membranes were dehydrated in a

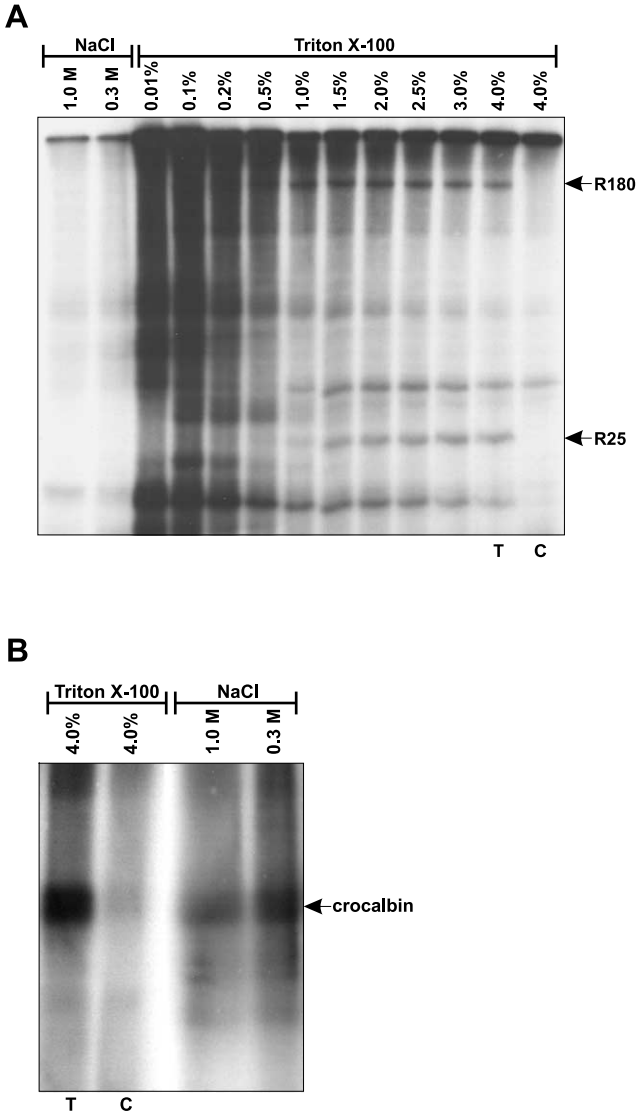

Fig. 1. AtxC-binding proteins in porcine cerebral cortex are integral membrane proteins. The demyelinated P2 membranes of porcine cerebral cortex were extracted in high-ionic-strength buffers and buffers containing the noted concentration of detergent Triton X-100. The extracts were affinity-labeled using (A) ^{125}I-AtxC or (B) ^{125}I-crotoxin. Samples (100 μg of protein/lane) were analysed by SDS–PAGE, gels dried and autoradiographed. The positions of the specific adducts are indicated on both autoradiograms. For experimental details, see Section 2.

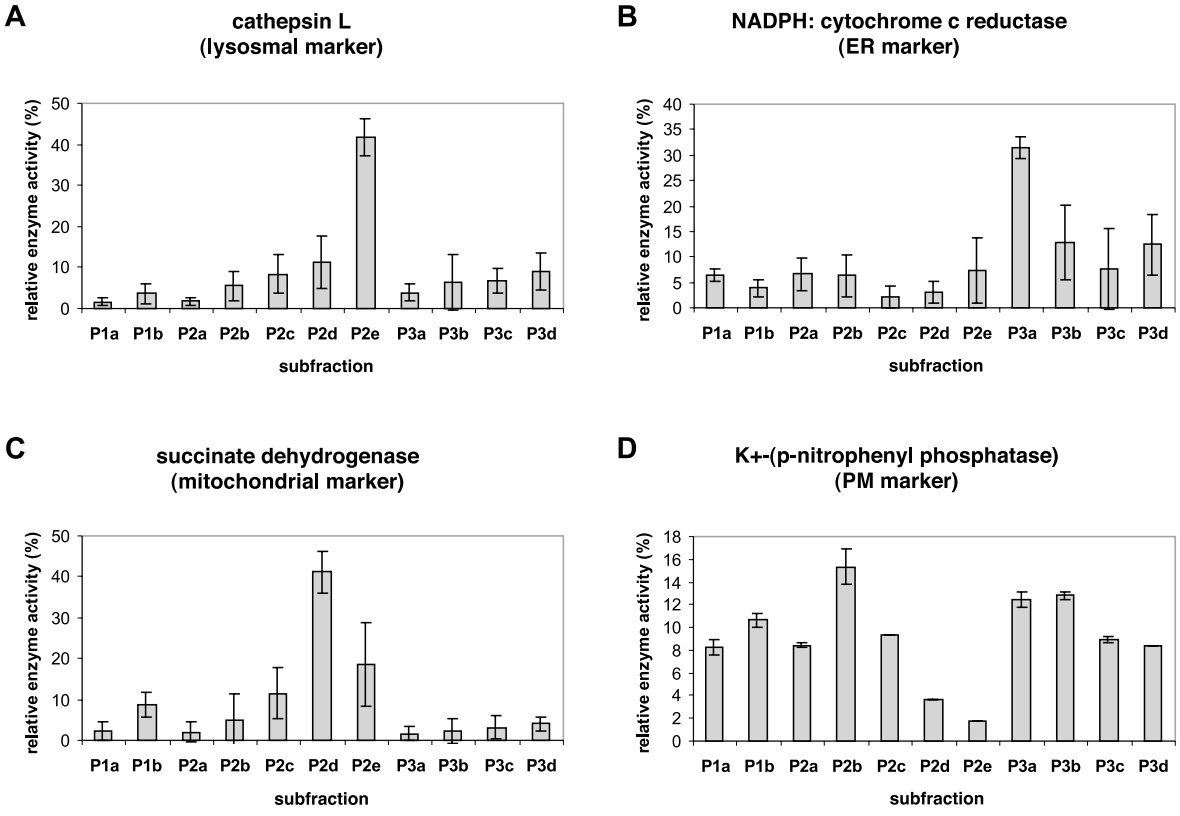

Fig. 2. The distribution of organelle-specific marker enzymes in fractions of porcine cerebral cortex. Each fraction was tested for the activity of different marker enzymes. The value in a particular fraction is expressed relative to the sum of the enzyme activity in all fractions. Results are means ± S.E.M. from eight experiments. For experimental details, see Section 2.

graded series of ethanol (30, 50, 70, 90, 100% (v/v) twice; 5 min each step), transferred into a mixture of the acrylic resin LR White (SPI, USA) and ethanol (100%) (v/v 1:1) for 30 min, followed by an incubation with pure resin for another 30 min. After an additional change the membranes were left in the resin overnight, transferred to the capsules and polymerised at 50°C for 2 days. Ultrathin sections were cut with a diamond knife and mounted on copper grids with formvar as supporting film, stained with uranyl acetate and lead citrate and examined in an electron microscope (Jeol JEM 1200EXII).

3. Results

3.1. R25 is an integral membrane protein

As shown in Fig. 1A, neither 0.3 M NaCl nor 1 M NaCl in the extraction buffer released the AtxC-binding protein of 25 kDa (R25) from the demyelinated P2 fraction of the porcine cerebral cortex. The same was true for R180, which is an M-type $sPLA_2R$ that resides in the plasma membrane [7]. Using the same extraction procedure, crocalbin, a crotoxin-binding protein in the lumen of ER, was released from the P2 membrane preparation already with 0.3 M NaCl (Fig. 1B). Increasing the concentration of the detergent Triton X-100 in the extraction buffer, the lowest concentration to fully solubilise R25 from the membranes was determined to be 1.5% (w/v), two times higher than that sufficient to optimally solubilise the membrane spanning R180. According to its extraction characteristics, we conclude that R25 is an integral membrane protein. To check the possibility of glycosylphosphatidylinositol (GPI) anchoring of R25 into the PM, the P2 membranes were treated with phosphatidylinositol-phospholipase C (PI-PLC), which cuts the GPI-proteins free from their membrane anchors. R25 was not removed from the membranes by this treatment (data not shown).

3.2. Subcellular fractionation of the porcine cerebral cortex and characterisation of the fractions

The porcine cerebral cortex homogenate was separated by differential centrifugation into three crude fractions: P1 (nuclear fraction), P2 (crude mitochondrial–synaptosomal fraction) and P3 (microsomal fraction). Using discontinuous sucrose gradients, these crude fractions were further fractionated to obtain 11 fractions [35]. The bulk of the proteins was found in the fraction P1b (54% of the total), followed by the fractions P2c and P1a (21% and 11% of the total protein, respectively).

To determine their subcellular composition, the organelle-specific markers were quantified in each of the isolated fractions. The activities of the following enzymes were measured: cathepsin L as a lysosome-specific enzyme (Fig. 2A), NADPH:cytochrome *c* reductase as an ER-specific enzyme (Fig. 2B), succinate dehydrogenase as a mitochondrion-specific enzyme (Fig. 2C), and K^+-(*p*-nitrophenyl phosphatase) as a PM-specific enzyme (Fig. 2D). The presence of PM in the fractions was also quantified by ^{125}I-AtxC affinity labeling of R180 (Figs. 3A). The distribution of synaptotagmin I (p65), a protein specifically located in synaptic and secretory vesicles, was determined by immunoblotting.

Lysosomes were highly enriched in the fraction P2e (Fig. 2A). The largest part of ER membranes was found in the crude microsomal fraction, specifically in the fraction P3a (Fig. 2B). The distribution of crocalbin, an ER-residing cro-

toxin-binding protein [12], was revealed by ^{125}I-crotoxin affinity labeling (data not shown). It confirmed the data obtained by following the activity of NADPH:cytochrome *c* reductase. Mitochondria concentrated mostly in the fraction P2d (Fig. 2C). The highest content of PM was found in the fraction P2b, followed by the microsomal fractions P3a and P3b (Figs. 2D and 3A,B). Synaptic vesicles and secretory granules

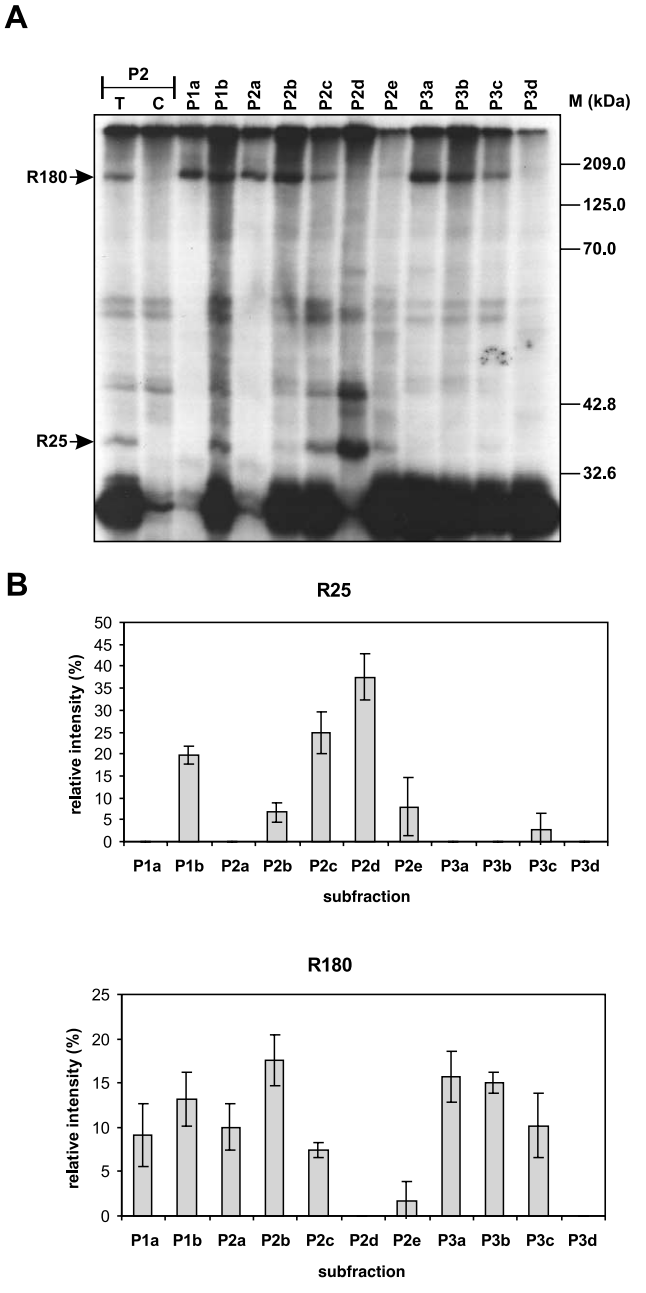

Fig. 3. The distribution of R180 and R25 in fractions of porcine cerebral cortex. A: The fractions were solubilised and affinity-labeled with ^{125}I-AtxC. The two experiments, in absence (T) and in presence (C) of an excess of unlabeled AtxC over ^{125}I-AtxC, to determine the positions of specific adducts, were performed on the total P2 fraction. Samples (100 μg of protein/lane) were analysed by SDS–PAGE. An autoradiogram of the gel is shown with the positions of the specific adducts indicated by arrows. For experimental details, see Section 2. B: The intensities of the specific bands were quantified and represented relative to the sum of intensities in all fractions.

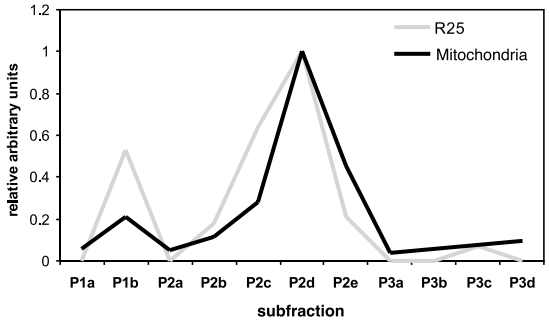

Fig. 4. The distribution of R25 and the mitochondrial marker succinate dehydrogenase in fractions of porcine cerebral cortex are highly correlated. The distributions of succinate dehydrogenase activity (black line) and R25 (grey line) in fractions of porcine cerebral cortex are given in arbitrary units, the ratio between the quantity in a particular fraction and the quantity in the most enriched one.

were mostly present in the P2b fraction followed by P2a and P2c, while in P2d only a very slight signal for synaptotagmin I was detected (data not shown).

3.3. The distribution of AtxC-binding proteins in fractions

The distribution of specific receptors for AtxC in the subcellular fractions was determined using ^{125}I-AtxC affinity labeling. Two specific adducts with the apparent molecular masses of around 200 and 39 kDa were followed (Fig. 3A), corresponding to two AtxC-receptors, R180 and R25, respectively [30,34]. The quantification of the intensity of the specific bands is shown in Fig. 3B. R180, an M-type sPLA$_2$R [7], was found to be present in fractions enriched in PM (Fig. 2D), as expected [41]. R25, however, fractionated in a completely different way, displaying a maximal enrichment in the fraction P2d (Fig. 3). The comparison of the R25 distribution (Fig. 3) with the marker enzyme profiles (Fig. 2A–E) shows a very good match with the distribution of the mitochondrial marker (Fig. 4) but not with the other markers, suggesting mitochondrial localisation of R25.

The fractions P2b and P2d, which contained the highest amounts of R180 and R25, respectively, were also examined under the electron microscope (Fig. 5). This morphological analysis additionally confirmed that P2b contained mainly PM fragments and that P2d contained mainly mitochondria.

4. Discussion

Studying the molecular mechanism of presynaptic neurotoxicity of Atx, a snake venom GIIA sPLA$_2$, we detected a 25 kDa protein (R25) in porcine cerebral cortex which may be involved in this process [30]. R25 binds AtxC and other related Atxs with a very high affinity (dissociation constants K_d in the nM range) [42–45]. The binding depends on the presence of Ca^{2+} or some other divalent ion, e.g. Sr^{2+}. There have been some indications favouring the idea that R25 is an oligomeric protein with CaM as a toxin-binding subunit [13], but other data suggest that the receptor is a monomeric protein [44]. Supporting the latter, we demonstrate in this work that R25 could not be released from the membranes under the conditions which allow the release of peripherally bound proteins. R25 therefore behaves as an integral membrane protein. A preliminary study has shown that R25 does not co-localise with a well-characterised receptor from the same tissue, an

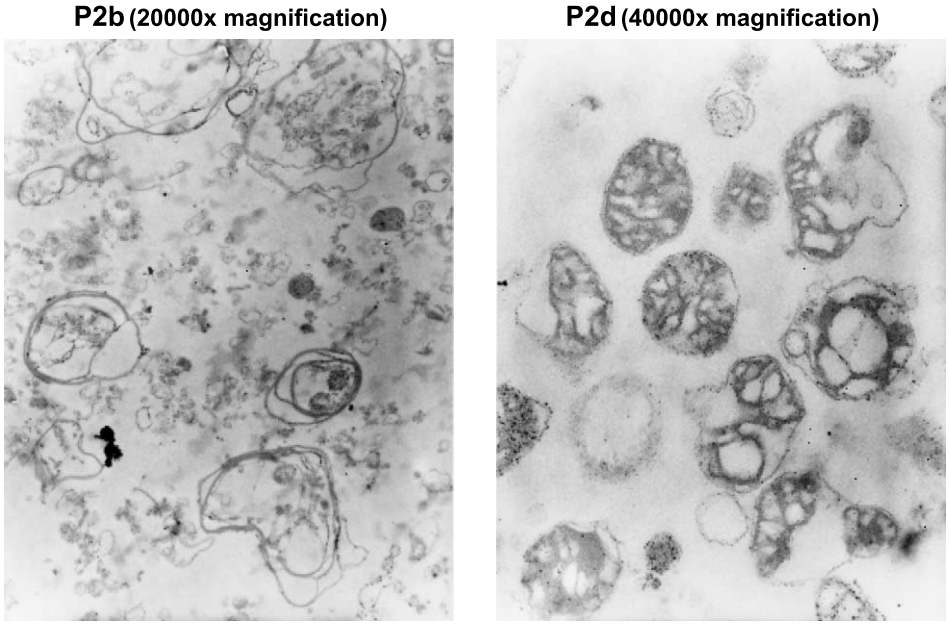

Fig. 5. The ultrastructure of the porcine cerebral cortex fractions P2b and P2d. The electron microghraphs of the fractions with the highest content of AtxC-receptors, R180 and R25, are magnified 20 000- and 40 000-fold, respectively.

M-type $sPLA_2R$, R180, which is a PM-spanning protein [46], suggesting that R25 resides in some other part of the cell. The detailed analysis of 11 subcellular fractions of the porcine cerebral cortex homogenate in the present work confirms that R25 is not a PM-residing protein and thus represents the first intracellular integral membrane receptor for $sPLA_2$. R25 was mainly absent also from the fractions enriched in lysosomes, ER, synaptic and secretory vesicles, dismissing the possibility of its localisation in any of these membrane compartments. However, it exhibited a distribution in the fractions that is practically identical to the distribution of mitochondria. The connection between mitochondria and $sPLA_2$s is intriguing. It has been observed that the PLA_2 inhibitor trifluoroperazine inhibits a mitochondrial permeability transition, the state when normally impermeant solutes cross the inner mitochondrial membrane at significant rates [27]. The effect was ascribed to activation of phospholipolytic activity of $sPLA_2$ in mitochondria. Almost simultaneously, Aarsman et al. reported the isolation of GIIA $sPLA_2$ from the rat mitochondria [25]. The enzyme was localised to the contact sites between mitochondrial inner and outer membrane [26]. Indication about the existence of a high-affinity receptor for GIIA $sPLA_2$ in mitochondria that we present in this work strengthens the belief that the link between GIIA $sPLA_2$ and mitochondria is physiologically relevant. The interaction of GIIA $sPLA_2$ with the integral membrane R25 may properly position the enzyme in the organelle for its subsequent enzymatic action. The direct targeting of de novo synthesised GIIA $sPLA_2$ to mitochondria is questionable. Namely, following the expression in BHK cells, the fusion between EGFP (enhanced green fluorescent protein) and pro-GIIA $sPLA_2$ displayed a distribution pattern typical for a secretory protein and the construct was not detected in mitochondria [47]. The pathway from the outside of the cell into the mitochondria is even more difficult to imagine; however, the fact is that mitochondria are severely affected upon intoxication of the neurone by the presynaptically neurotoxic $sPLA_2$s [29]. In the case of Atx, the morphological changes of this organelle, such as swelling and loss of the architecture of internal cristae [48], resulting in mitochondrial uncoupling, may be related to its interaction with R25.

In summary, we have demonstrated that a 25 kDa receptor for Atx from porcine cerebral cortex represents (as far as we know) the first intracellular integral membrane receptor for GIIA $sPLA_2$. R25 may be directing the distribution of the $sPLA_2$ enzyme in the target nerve cell. The receptor is most likely located in mitochondria, the organelles that are strongly disturbed by the action of $sPLA_2$ neurotoxins, implying the involvement of R25 in the process of β-neurotoxicity.

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