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High-Molecular-Mass Receptors for Ammodytoxin in Pig Are Tissue-Specific Isoforms of M-Type Phospholipase A_2 Receptor

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Studying the molecular basis of presynaptic neurotoxicity of ammodytoxin C, a secretory phospholipase A_2 from the venom of *Vipera a. ammodytes* snake, we demonstrated the existence of two high-molecular-mass ammodytoxin C-binding proteins in porcine tissues, one in cerebral cortex and the other in liver. These proteins differ considerably in stability and Western blotting properties. However, as shown by immunological analysis and tandem mass spectrometry sequencing of several internal peptides derived from the purified receptors, both belong to secretory phospholipase A_2 receptors of the M type, which are Ca^{2+}-dependent multilectins homologous to the macrophage mannose receptor. Based on Southern blot analysis of genomic DNA and deglycosylation of the receptors, the difference between the two proteins most likely stems from the different posttranscriptional and posttranslational modifications of a single gene product. Our findings raise the possibility that the M-type receptors for secretory phospholipases A_2 may display different physiological properties in different tissues.

Key Words: **ammodytoxin; snake venom; *Vipera ammodytes ammodytes;* secretory phospholipase A_2; presynaptic neurotoxicity; M-type phospholipase A_2 receptor; C-type multilectin.**

Secretory phospholipases A_2 (sPLA$_2$s) form a large family of structurally related enzymes that catalyze hydrolysis of the *sn*-2 ester bond of glycerophospholipids, generating free fatty acids and lysophospholipids (1, 2). These low molecular mass (13–18 kDa), disulfide-rich, Ca^{2+}-dependent enzymes are found in different mammalian tissues and animal venoms. Apart from their role in phospholipid digestion, sPLA$_2$s are involved in many other physiological and pathological processes (3–5). Some effects produced by sPLA$_2$s are not just a consequence of their enzymatic activity but also of their specific interaction with particular proteins in target cells such as the voltage-dependent K^+ channel, pentraxins, reticulocalbins, Ca^{2+}-dependent (C-type) multilectins, factor Xa, glypican-1 (6, 7), and, recently described, v-Src oncoprotein (8) and calmodulin (9).

Using the presynaptically neurotoxic sPLA$_2$ from the *Oxyranus s. scutellatus* snake venom, the M-type sPLA$_2$ receptor (sPLA$_2$R) in rabbit skeletal muscle was the first to have been partially characterized (10, 11). Later, cDNAs encoding the M-type receptors have been cloned in different species (11–14) and it has been found that these receptors constitute a new group inside the C-type multilectin mannose receptor family (15). Rat, rabbit and human M-type sPLA$_2$Rs have been shown to be capable of endocytosis (14, 16, 17), presumably a general feature of this protein family. It has been suggested that the physiological role of the M-type sPLA$_2$R is to internalize and deliver sPLA$_2$ to specific compartments within the cell where the enzyme then exerts its activity. Depending on the cell type and sPLA$_2$ isoform (18), the latter could generate a variety of biological responses such as cell proliferation (19), cell migration (20), eicosanoid production (21, 22), stimulation of extracellular matrix invasion by normal and cancer cells (23), and signal transduction events, leading to cytosolic PLA$_2$ activation (24). The binding of sPLA$_2$ to M-type sPLA$_2$R also plays an important role in the production of inflammatory cytokines during endotoxic shock (25).

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In the course of our study on the molecular mechanism of presynaptic neurotoxicity of ammodytoxin C (AtxC), a group IIA $sPLA_2$ from *Vipera a. ammodytes* snake venom (26, 27), we identified a 180 kDa membrane acceptor (R180) in porcine cerebral cortex. It shares some similarity (high molecular mass, exogenous ligand binding characteristics) with the M-type $sPLA_2$Rs; however, in terms of its relatively high abundance in brain, very high stability, and Western blot analysis, it is substantially different (28). Along with the suggestion that the M-type $sPLA_2$R is a single-copy gene product (13), simultaneous discovery of a canonical 200-kDa M-type $sPLA_2$R in the liver of the same animal (L200) strengthened the belief that R180 is a novel type of $sPLA_2$R (28).

To address the problem as to whether two different types of high molecular mass $sPLA_2$Rs or several isoforms of the M-type $sPLA_2$R exist in the same species, we purified and structurally characterized both receptors. Amino acid sequencing confirmed that both porcine $sPLA_2$Rs, R180, and L200, are M-type receptors, demonstrating for the first time that more than one M-type $sPLA_2$R exists on the protein level in the same species. The differences in biochemical properties of these $sPLA_2$Rs, which are probably manifested also on the physiological level, are the result of diverse tissue-specific posttranslational and likely also posttranscriptional modifications of $sPLA_2$Rs as revealed by Southern blot and deglycosylation analyzes.

MATERIALS AND METHODS

Materials. AtxC was isolated from *Vipera a. ammodytes* venom (26, 29). Affi-Gel and protein molecular mass standards were obtained from Bio-Rad (Hercules, CA). Wheat germ lectin–Sepharose was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Na-^{125}I (carrier free) was from NEN Life Science Products (Boston, MA) and disuccinimidyl suberate from Pierce (Rockford, IL). Guinea pig polyclonal antibodies (pAb) against rabbit M-type $sPLA_2$R, able also to recognize mouse, rat and human M-type $sPLA_2$Rs, were a gift from Dr. Gerard Lambeau, Institute de Pharmacologie Moleculaire et Cellulaire, CNRS, Valbonne, France. Peroxidase-conjugated goat anti-guinea pig IgGs were obtained from Cappel Research Products (ICN Biomedicals, Irvine, CA). Triton X-100 and peptide *N*-glycosidase F (PNGF) were supplied by Roche Diagnostics (Indianapolis, IN). Porcine genomic DNA was a gift from Dr. Dušan Kordiš, Jožef Stefan Institute, Ljubljana, Slovenia. Restriction endonucleases were obtained from New England Bio-Labs (Beverly, MA) and 1 kb DNA size standards from MBI Fermentas (Hanover, MD). Oligonucleotides were from MWG-Biotech AG (Ebersberg, Germany). All other chemicals used were of analytical grade.

Purification of AtxC-binding proteins. Demyelinated P2 fraction of porcine cerebral cortex and P2/P3 fraction of porcine liver were prepared as described (28). Membranes 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 $CaCl_2$, 3% (w/v) Triton X-100 and afterwards centrifuged at 100,200*g* for 1 h. Detergent extracts were diluted 2-fold with cold deionized water before further purification on wheat germ lectin–Sepharose (WGA) and AtxC-affinity chromatography (28). Fractions from AtxC-affinity chromatography containing AtxC-binding proteins were concentrated on Centricon YM-100 (Millipore, Bedford, MA).

Mass spectrometry. 1-2 μg of each AtxC-binding protein were separated on SDS–PAGE (7% polyacrylamide gels) and the gels were silver stained. The protein band was excised and transferred to a siliconized tube. The piece of gel was destained, then reduced in dithiothreitol, alkylated in iodoacetamide and digested with Promega modified trypsin. The resulting peptides were extracted from the gel with 50% acetonitrile/5% formic acid and analyzed by LC/MS/MS using a Finnigan LCQ ion trap Mass spectrometer (Finnigan Mat, San Jose, CA). Amino acid sequence data from the isolated peptide was analyzed by database searching using the Sequest search algorithm against the NCBI nonredundant data base.

Radioiodination of AtxC and affinity-labeling. Radioiodinated AtxC (^{125}I-AtxC) was prepared and tested as described (30). The specific radioactivity of the preparation was 300 Ci/mmol. ^{125}I-AtxC was cross-linked to AtxC-binding proteins as reported (28).

Deglycosylation of AtxC-binding proteins. Native AtxC-binding proteins were treated with 3 U of PNGF in 0.25 M Na_2HPO_4, pH 7.5, containing 10 mM EDTA (deglycosylation buffer) at 37°C for 24 h. In some experiments receptors were denatured by boiling for 5 min in deglycosylation buffer supplemented with 0.5% (w/v) SDS and 10 mM 2-mercaptoethanol. Prior to addition of PNGF to these samples Triton X-100 was added to 0.75% (w/v).

Amplification and characterization of the DNA fragment encoding part of the porcine M-type $sPLA_2$R. The DNA fragment corresponding to the exon 15 in porcine M-type $sPLA_2$R was amplified from porcine genomic DNA by PCR using an upstream primer, 5′-GGA ATT CGG TTG TCT CTT CGT TTT TAG ACA A-3′, with *Eco*RI restriction site, and a downstream primer, 5′-CGG ATC CTC TTG GGA TTT TGC ATA TCC A-3′, with *Bam*HI restriction site. The primers were designed on the basis of the sequence of the bovine and human M-type $sPLA_2$R genes. PCR was performed in a 100 μl reaction mixture containing 1 μg of porcine genomic DNA, PCR II reaction buffer (Perkin–Elmer Life Sciences, Boston, MA; Promega, Madison, WI), 1.5 mM $MgCl_2$, 0.2 mM each dNTP and 0.4 μM each primer with 2.5 U AmpliTaq (Perkin–Elmer Life Sciences, Boston, MA; Promega, Madison, WI). Amplification included 30 cycles of 30 s at 94°C, 30 s at 50°C and 30 s at 74°C with the final 5 min extension at 74°C. The PCR fragment was digested with endonucleases *Eco*RI and *Bam*HI, cloned into pUC19 and sequenced by dideoxynucleotide sequencing (31) on an ABI Prism 310 Genetic Analyzer (Perkin–Elmer Applied Biosystems, Foster City, CA).

Southern blot analysis. Samples of porcine genomic DNA were completely digested with *Eco*RI, *Bam*HI and *Pst*I, respectively, separated by electrophoresis on 0.7% agarose gel and transferred to a Hybond-N membrane according to manufacturer's instructions (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Hybridization was performed at 42°C for 36 h in a hybridization buffer (900 mM NaCl, 90 mM Na citrate, pH 7.0 (i.e., 6× SSC), 5× Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured, fragmented herring sperm DNA, and 50% deionized formamide (32) containing the ^{32}P-labeled probe (1.14×10^9 cpm/μg). The hybridization probe was a 147-bp DNA fragment corresponding to exon 15 of porcine M-type $sPLA_2$R gene, labeled with [α-^{32}P]dATP (3000 Ci/mmol) by the standard procedure (32) using the PCR primers described above. The membrane was washed at room temperature in 2× SSC/0.1% SDS and 1× SSC/0.1% SDS, for 30 min each, and afterwards at 37°C in 1× SSC/0.1% SDS and 0.1× SSC/0.1% SDS, for 10 min each. Signals were detected with autoradiography using X-Omat AR film (Eastman Kodak Co, Rochester, NY).

RESULTS

Purification of R180 from porcine cerebral cortex and L200 from porcine liver. The high-molecular-mass AtxC-binding proteins, R180 and L200, were purified from porcine tissues using the procedure described (28)

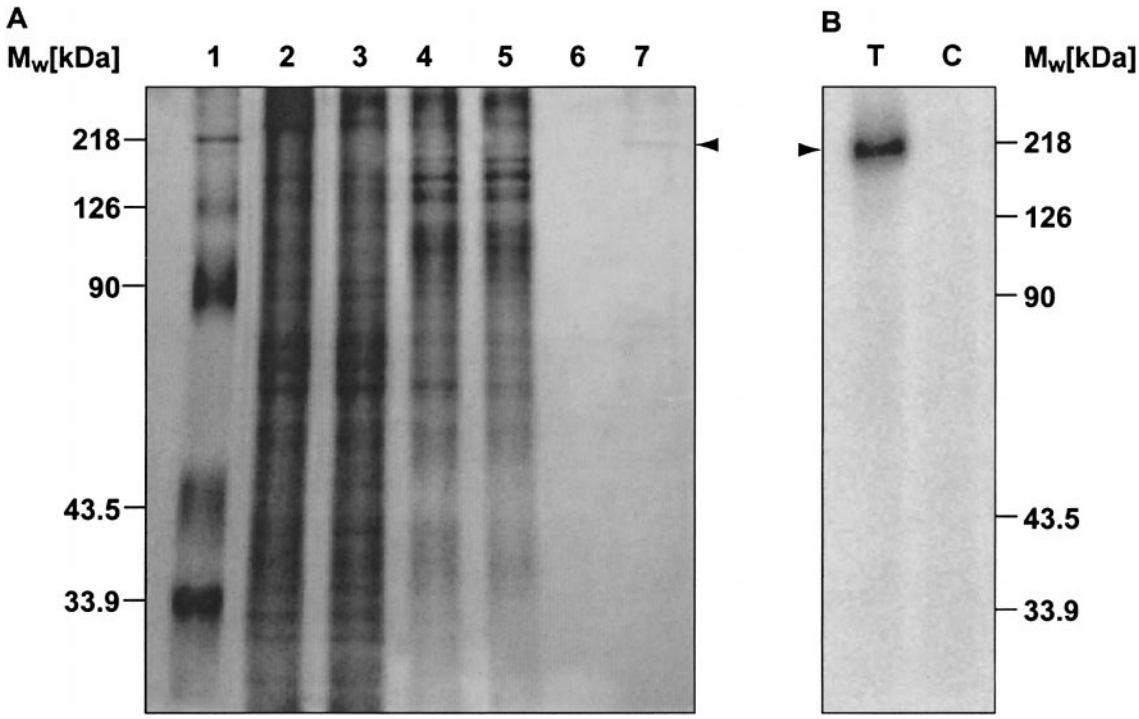

FIG. 1. Purification of L200 from porcine liver. (A) Samples obtained at different steps of the purification procedure were analyzed by 10% SDS–PAGE under nonreducing conditions. The gel was silver-stained. Lane 1, molecular mass standards; lane 2, crude detergent extract of P2/P3 membrane fraction, 2.6 μg of protein; lane 3, breakthrough from wheat germ lectin–Sepharose 6MB, 2.2 μg of protein; lane 4, eluate from wheat germ lectin–Sepharose 6MB, 1 μl from 8 ml; lane 5, breakthrough from AtxC-Affi-Gel 10, 1 μl from 8 ml; lane 6, AtxC-Affi-Gel 10 Triton X-100 (0.3% (w/v)) washing, 5 μl from 40 ml; lane 7, eluate from AtxC-Affi-Gel 10, 21 μl from 7 ml. The arrow indicates the position of pure L200 in lane 7. (B) The final product (lane 7) specifically reacted with ^{125}I-AtxC, shown by incubation with ^{125}I-AtxC in the absence (T) or presence (C) of 200-fold excess of unlabeled AtxC over the labeled toxin.

with slight modifications. Using the published protocol the brain receptor retained its toxin-binding activity throughout the isolation, while the liver receptor activity was completely lost. The critical step was elution of the receptor from the AtxC-affinity resin achieved by temporarily decreasing pH from 7.4 to 5.0. In the case of L200, however, it was irreversibly inactivated. Replacement of Sr^{2+}/EGTA, as originally used in the extraction buffer, by Ca^{2+} increased the stability of L200 to low pH significantly, allowing it to be isolated in the pure and active form (Fig. 1). Determined by semiquantitative densitometric analysis of the silver stained SDS–PAGE band, about 20 μg of pure L200 were obtained from the P2/P3 membrane fraction from porcine liver containing 6.9 mg of membrane protein.

Molecular characterization of AtxC high-molecular-mass receptors. It was observed that R180 is much more difficult than L200 to transfer from an SDS–PAGE gel to nitrocellulose membrane (data not shown) suggesting that the apparent difference in immunoreactivity to rabbit skeletal muscle M-type $sPLA_2R$ pAb may be the result of the different blotting characteristics of these molecules. To avoid the influence of blotting on immunodetection we tested the direct binding of Ab to receptors in ^{125}I-AtxC affinity labeling experiments with R180 and L200 in the presence of excess anti M-type $sPLA_2R$ specific pAb. The results in Fig. 2 show that the M-type $sPLA_2R$ specific pAb substantially reduced the cross-linking of ^{125}I-AtxC to both porcine $sPLA_2Rs$, R180 and L200. On the contrary, the same amount of non-specific pAb (goat anti-guinea pig IgGs) used in control experiments had no effect on binding of ^{125}I-AtxC to the receptors, strongly suggesting that the recognition of both porcine $sPLA_2Rs$ by the M-type $sPLA_2R$ specific pAb is specific. The identity of the porcine high molecular mass AtxC-binding proteins was confirmed by sequence analysis using tandem mass spectrometry. The sequences of peptides derived from R180 and L200 by in-gel tryptic digestion are identical to the corresponding stretches in the sequence of bovine M-type $sPLA_2R$ (12) (Table 1) demonstrating that both R180 and L200 are $sPLA_2Rs$ of the M-type.

Deglycosylation of R180 and L200. Since it has been shown that porcine $sPLA_2Rs$ are the members of the M-type receptors, the next question was why these two proteins display such diverse biochemical properties. It has been demonstrated in our previous study

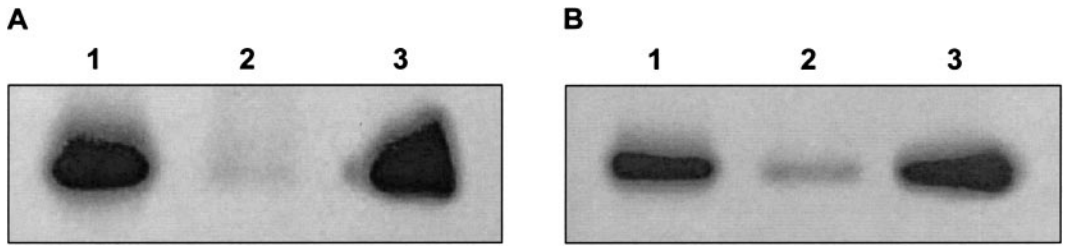

FIG. 2. Inhibition of ^{125}I-AtxC binding to R180 and L200 by M-type sPLA$_2$R antibodies. (A) Autoradiogram of the SDS–PAGE gel of the purified R180, incubated with ^{125}I-AtxC in the absence (lane 1) and presence of either antibodies to M-type sPLA$_2$R (lane 2) or anti-guinea pig IgGs (lane 3). The antibodies were added to the purified R180 in excess 24 h before the cross-linking procedure (for details see Materials and Methods). (B) The same experiment described under A was carried out with purified L200.

that R180 binds to certain lectins (28) and L200 is here shown to be retained by wheat germ lectin–Sepharose. Both receptors are therefore glycoproteins. To investigate whether or not the porcine receptors are glycoforms of the same protein or isoforms on the protein level, we treated purified R180 and L200 with PNGF, which releases N-linked glycans from glycoproteins. SDS–PAGE analysis of the samples before and after deglycosylation showed a reduction in their molecular masses but not to the same level (Fig. 3). The apparent molecular mass of the brain receptor R180 decreased by about 20 kDa, while the liver receptor L200 decreased by about 35 kDa. These results strongly suggest that both sPLA$_2$Rs differ in their N-glycosylation. If the deglycosylation was performed on the native receptors the deglycosylated forms were still able to bind ^{125}I-AtxC.

Genomic DNA blot analysis for the sPLA$_2$R. The possibility that the two M-type sPLA$_2$Rs in pig are isoforms on the protein level was addressed by determining whether the receptors are encoded by the same gene or by two different but related genes. Southern blot analysis of porcine genomic DNA was carried out using a single exon probe (exon 15) encoding a part of the carbohydrate recognition domain 4 (CRD-4) of the porcine M-type sPLA$_2$R. We used this probe, since the CRD-4 is the most conserved region among M-type sPLA$_2$Rs (13). The results of DNA blot analysis are shown in Fig. 4. Under the hybridization condition used, only one positive band was detected in each lane of alternatively restricted porcine genomic DNA. This result indicates that porcine M-type sPLA$_2$Rs are encoded by a single-copy gene.

DISCUSSION

To characterize the high molecular mass AtxC-binding proteins, we isolated and purified both receptors, R180 and L200, to homogeneity. The biologically active form of L200 could only be obtained from porcine liver when the previously described procedure for isolating R180 (28) was modified by replacing Sr^{2+}/EGTA in the extraction buffer with Ca^{2+}. The presence of Ca^{2+} ions, specifically during its extraction from the biological membrane, proved to be essential for stabilizing L200, preventing its irreversible inactivation at pH 5, and the AtxC-binding

TABLE 1

Peptide Sequence Analysis of R180 and L200 by Tandem Mass Spectrometry

AtxC receptor	Peptides[a]		
R180	870	DGSPVIYQNWDK	881
	1339	IPEGVWQLSSCQDK	1353
L200	353	YYATHCEPGWNPHNR	367
	825	SDILTIHSAHEQEFIHSK	842
	870	DGSPVIYQNWDK	881
	923	VWVIEK	928
	1308	WFDGTPTDQSNWGIR	1322
	1339	IPEGVWQLSSCQDKK	1353

[a] Two tryptic peptides from R180 and six from L200 were sequenced. Peptides were found identical to the corresponding parts in the sequence of bovine M-type sPLA$_2$R (12).

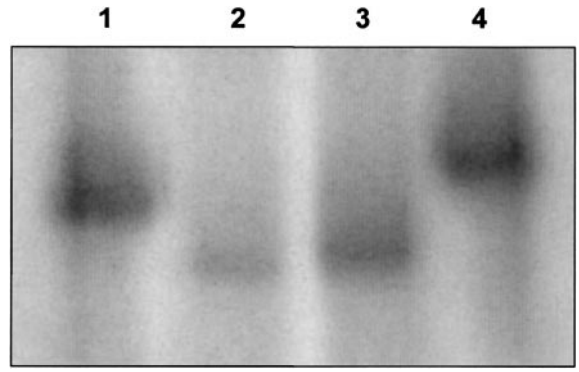

FIG. 3. Effect of peptide *N*-glycosidase F (PNGF) treatment on R180 and L200. The purified sPLA$_2$Rs were treated with PNGF (lanes 2 and 3) and analyzed on SDS–PAGE in comparison to PNGF-untreated samples (for details see Materials and Methods). Lanes 1 and 2 contain R180 while lanes 3 and 4 contain L200.

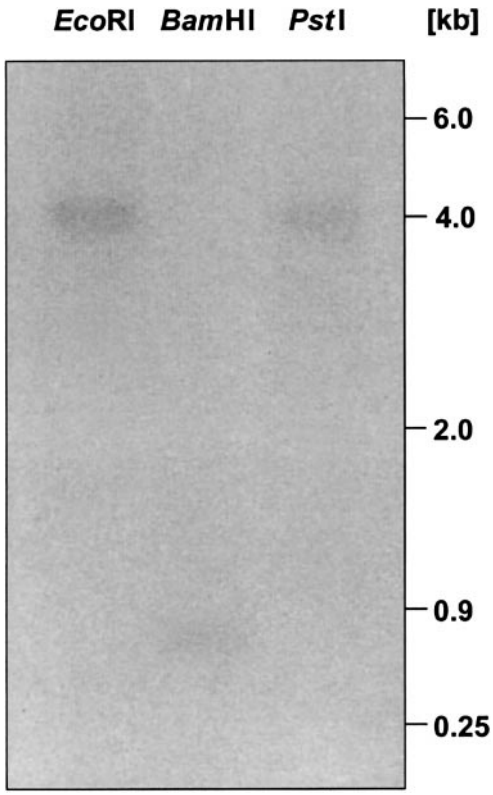

FIG. 4. Southern blot analysis of the porcine M-type $sPLA_2R$ gene. Porcine genomic DNA was digested with restriction endonucleases *Eco*RI, *Bam*HI, and *Pst*I and afterward analyzed by hybridization with the probe corresponding to exon 15 in the porcine M-type $sPLA_2R$ gene. The positions of DNA size standards (in kb) are shown on the right side of the autoradiogram.

activity of L200 was largely restored following its low-pH elution from toxin-affinity chromatography. Since the binding of $sPLA_2$s to L200 was shown to be independent of the presence of Ca^{2+} ions (28), the necessity of this ion in solubilizing the receptor indicates its importance in stabilizing and maintaining the structure that L200 adopts in the membrane and also in solution. On the contrary, R180 did not require Ca^{2+} during solubilization. In addition, we observed that R180 is much more difficult to blot from an SDS–PAGE gel to nitrocellulose membrane than L200, which could account for the absence of a positive signal in Western blot analysis using M-type $sPLA_2R$ specific pAb in the case of R180 (28). The inhibition of ^{125}I-AtxC binding to R180 and L200 by antibody to M-type $sPLA_2R$ but not by non-specific Ab (Fig. 2), and the partial sequencing of the $sPLA_2Rs$ confirmed conclusively that both are of the M-type (Table 1). The possibility of the existence of two different genes, encoding closely related proteins in the porcine genome, is discounted by the absence of closely related genes, as detected by genomic DNA blot analysis, suggesting that porcine M-type $sPLA_2Rs$, like human (13) and cattle (12) are the product of a single-copy gene. The M-type $sPLA_2Rs$ are glycoproteins that contain from 14 to 16 potential N-glycosylation sites in their extracellular domains (11, 12, 14). Using peptide *N*-glycosidase F, R180 and L200 were found to differ in N-glycosylation, explaining at least some of the observed differences. Protein glycosylation has been often demonstrated to be physiologically very important (33, 34). Although we did not observe any difference in the $sPLA_2$ binding properties of R180 and L200 (28), the interaction between $sPLA_2$ and an M-type $sPLA_2R$ was found to be influenced by the carbohydrate moiety of the receptor (35, 36). Newly discovered endogenous $sPLA_2$ (2, 8), which are potential physiological ligands for M-type $sPLA_2R$, should be analyzed for their affinity toward diverse isoforms of M-type $sPLA_2R$ to reveal the true biological role of the latter.

The N-deglycosylated receptors still did not display identical molecular masses, suggesting further structural differences between the two. O-glycosylation is not likely to be the cause since it has not been detected among the M-type $sPLA_2Rs$. Additionally, using "NetOglyc," the O-glycosylation site prediction program (37), no potential O-glycosylation site was found in the closely related bovine M-type $sPLA_2R$. A more plausible explanation of the additional structural difference between R180 and L200 would be the alternative splicing of porcine M-type $sPLA_2R$ mRNA. Multiple forms of M-type $sPLA_2R$ mRNA have been observed in rabbit, cattle and human (11, 12, 14). Furthermore, tissue specific RNA processing was observed by RNA blot analysis of bovine M-type $sPLA_2R$ mRNA (12). Poly(A)$^+$ RNA isolated from brain thus contained different mRNA species from the one isolated from kidney, which is consistent with our discovery of tissue specific M-type $sPLA_2R$ isoforms on the protein level. Additional post-transcriptional modification may also have a significant influence on the physiological role of the protein, best illustrated by the finding of an alternatively processed transcript of a human M-type $sPLA_2R$, encoding a secreted soluble form of M-type $sPLA_2R$ (14).

In the present work we showed that both high molecular mass membrane receptors for $sPLA_2$ in pig are M-type $sPLA_2Rs$. They are very likely encoded by a single-copy gene and, depending on the tissue, post-transcriptionally and posttranslationally processed in different ways. As such modifications could play an important role in modulating the protein function, it is reasonable to expect that the tissue specific isoforms of M-type $sPLA_2R$ display different physiological properties. Functional diversity associated with the M-type $sPLA_2R$ (18, 38) is becoming understood in terms of interplay between a variety of mammalian $sPLA_2$s and apparently also the receptor itself.

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