Volume 293, number 1,2, 29–33 FEBS 10376 November 1991

Identification of different receptor types for toxic phospholipases A2 in rabbit skeletal muscle

Gérard Lambeau, Jacques Barhanin and Michel Lazdunski

Institut de Pharmacologie Moléculaire et Cellulaire, 660 route des Lucioles, Sophia Antipolis 06560 Valbonne, France

Received 20 september 1991

Oxyuranus scutellatus scutellatus toxins 1 (OS_1) and 2 (OS_2) are two phospholipase A_2s (PLA_2) isolated from the venom of the Australian Taipan snake. Their iodinated derivatives have been used to characterize PLA_2 binding sites on rabbit skeletal muscle. Competition and cross-linking experiments indicate that ^{125}I-labelled OS_2 binding sites in rabbit skeletal muscle in vivo are distributed into two classes of receptors. One class binds OS_2 and OS_1 and is insensitive to the bee venom PLA_2. It is composed of a 180 kDa binding protein. This class of PLA_2 receptor is expressed at a high level in rabbit myotube membranes. The other class of PLA_2 receptor identified with ^{125}I-OS_2 also binds with high affinity the bee venom PLA_2 but not OS_1 and is composed of major polypeptides of 34, 48 and 82 kDa. This second class of receptor is similar to the one found in brain membranes. The density of the two classes of receptors varies during muscle development.

Skeletal muscle; Neurotoxin; Myotoxin; Phospholipase A_2; Snake venom

1. INTRODUCTION

Snake venom phospholipase A_2 (PLA_2s), EC 3.1.1.4, form a large family of enzymes exerting a variety of toxic effects in mammals [1], which may be neurotoxic [2], cardiotoxic [3], and/or myotoxic [4]. Many of them also have anticoagulant [5] and hemolytic [6] effects. The neurotoxicity of these presynaptically active neurotoxins does not seem to correlate with their phospholipase activity [7]. In fact, the different types of toxicity of the different venom PLA_2s may be due to the presence of different specific binding sites which we have recently identified for these toxic enzymes in different tissues. Specific binding sites for neurotoxic PLA_2s have been characterized on rat brain synaptosomes with a radio-iodinated derivative of a neurotoxic PLA_2 isolated from the venom of the Australian taipan snake *Oxyuranus scutellatus scutellatus* called OS_2 [8]. These binding sites are of very high affinity for the toxin and are protein in nature. High affinity PLA_2 binding sites for OS_2 have also been identified in cultured rabbit muscle cells and purified to homogeneity from this biological source [9]. Affinity labeling and purification studies have shown that myotube and neuronal OS_2 binding sites are, surprisingly, composed of different protein subunits.

The work reported here extends our previous results on PLA_2 binding sites and definitely shows that there exist two types (at least) of PLA_2 receptors as it shows several types of receptors for most hormones and neurotransmitters. Both neuronal and myotube types of OS_2 receptors can in fact be found in skeletal muscle. The respective proportion of thes two types of receptors varies with development.

Correspondence address: M. Lazdunski, Institut de Pharmacologie Moléculaire et Cellulaire, 660 route des Lucioles, Sophia Antipolis 06560 Valbonne, France. Fax: (33) (93) 957704.

2. MATERIALS AND METHODS

2.1. *Materials*

OS_1 and OS_2 from *Oxyuranus scutellatus scutellatus* and PLA_2 from *Apis mellifera* venoms were purified as previously described [8,10]. Na ^{125}I (IMS30) was from Amersham Corp. All other reagents were of analytical grade.

2.2. *Iodination of OS_2 and OS_1*

The iodination procedure was described previously [8,9]. The specific radioactivity was routinely 3000–3500 cpm/fmol of toxin.

2.3. *Membrane preparations*

Crude microsomal fractions of skeletal muscle and membrane fractions of myotubes were prepared as previously described [9,11]. Protein concentrations were determined according to Bradford [12] after digestion of membranes in 0.1 N NaOH and using bovine serum albumin as a standard.

2.4. *Binding studies*

All binding experiments were performed ad 20°C in a buffer consisting of 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 0.1 mM $CaCl_2$, and 0.1% bovine serum albumin. Membranes were incubated with the radiolabeled ligand in the absence or presence of unlabeled competitor for 1 h before filtration [8]. Routinely, and except where otherwise specified, the incubation volumes were 0.5 and 1 ml for ^{125}I-OS_1 and ^{125}I-OS_2 binding, respectively.

2.5. *Cross-linking experiments*

Membranes (0.3 mg/ml) were incubated in 1 ml of a 20 mM HEPES buffer, pH 7.4, containing 140 mM NaCl, 0.1 mM $CaCl_2$, and 0.1% bovine serum albumin with 300 pM ^{125}I-OS_2 or 250 pM ^{125}I-OS_1 in the absence or the presence of varying concentrations of unlabeled toxin.

After 45 min at 20°C, incubation mixtures were centrifuged at 12 000 × g for 10 min and resuspended in 1 ml of 20 mM HEPES, pH 8.2, 140 mM NaCl, 0.1 mM $CaCl_2$. Following the addition of 50 μM suberic acid bis-N-hydroxysuccinimide ester (DSS, Sigma,freshly dissolved in Me_2SO at 5 mM) for 5 min at 20°C, the reaction was stopped by addition of 100 μl of 1 M Tris-HCl, pH 8.0, and centrifugation at 12 000 × g for 10 min. Resulting pellets were solubilized with an SDS sample buffer [13] under reducing conditions (4% ß-mercaptoethanol) and analyzed by SDS polyacrylamide gel electrophoresis [13]. Gels were stained with Coomassie brilliant blue, dried, and autoradiographed at −70°C using Kodak X Omat AR films and intensifying screen (Du Pont Cronex Hi-plus).

3. RESULTS AND DISCUSSION

OS_2 and OS_1 are two different toxic phospholipases from taipan venom [8]. It has been shown previously that ^{125}I-OS_1 shares the same binding sites as ^{125}I-OS_2 in rabbit myotube membranes but that it does not bind with high affinity to rat brain membranes [8,9].

Fig. 1 illustrates equilibrium binding experiments performed on a microsomal preparation from adult rabbit skeletal muscle using ^{125}I-OS_2 (Fig. 1A) and ^{125}I-OS_1 (Fig. 1B) as ligands. Maximal binding capacities (B_{max}) were 8 ± 3 (n = 3) and 2 ± 1.4 (n = 4) fmol/mg of proteins for ^{125}I-OS_2 and ^{125}I-OS_1, respectively. These low binding capacities were determined with an acceptable accuracy because of the very high specific radioactivities of the two toxins (55 TBq/mmol) and of the low level of non-specific binding.

The binding properties of these PLA_2s to adult skeletal muscle membranes were very different from those preciously described for membranes from skeletal muscle cells in culture, for which the B_{max} values for ^{125}I-OS_2 and ^{125}I-OS_1 were considerably higher (1800–2200 fmol/mg of protein) and essentially identical for the two toxins [9]. Scatchard plots obtained for ^{125}I-OS_2 binding to membranes of embryonic (18-day-old embryos) rabbit skeletal muscle are presented in Fig. 1C. In this case specific ^{125}I-OS_2 binding was best fitted with a model involving the presence of 2 families of binding sites. The very high affinity component has a Kd of 2± 1.5 pM and a B_{max} of 67 ± 15 fmol/mg of protein (n = 3); the lower affinity component has a K_d of 33 ± 9 pM and a B_{max} of 203 ± 35 fmol/mg of protein. When ^{125}I-OS_1 was used as ligand, only one family of binding sites was detected with a K_d of 48 ± 10 pM and a B_{max} of 40 ± 12 fmol/mg of protein (n = 3) (Fig. 1D). Fig. 2 shows that specific ^{125}I-OS_1 binding was totally inhibited by both unlabeled OS_1 and OS_2 with $K_{0.5}$ values of 32 and 18 pM, respectively, whereas Fig. 3 shows that unlabeled OS_1 only partially (~20%) inhibited ^{125}I-OS_2 bind-

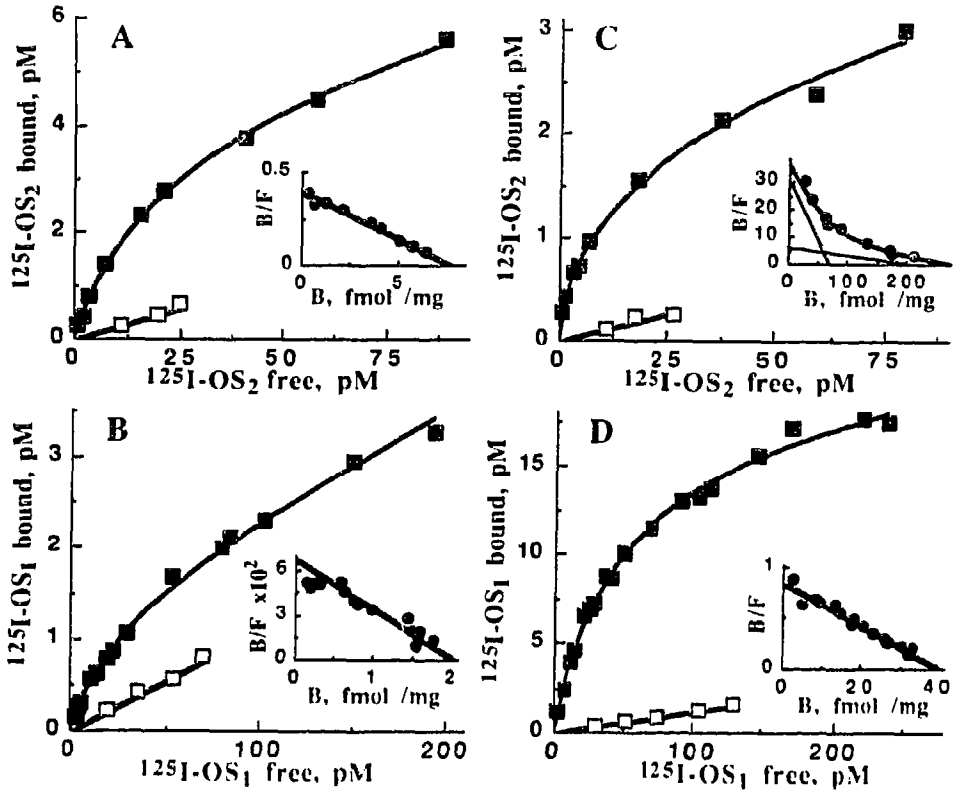

Fig. 1. Equilibrium binding of ^{125}I-OS_2 (panel A and C) or ^{125}I-OS_1 (panel B and D) to rabbit skeletal muscle microsomal membranes of adult (panel A and B) or 18-day-old embryos (panel C and D). Main panels: membranes (550, 740, 10 and 450 μg of protein/ml in A, B, C and D, respectively) were incubated with increasing concentrations of radiolabeled toxin in the absence (■, total binding) or the presence (□, non-specific binding) of 2 nM unlabeled OS_2 (A and C) or 30 nM unlabeled OS_1 (B and D). Insets: Scatchard plot of specific binding components for ^{125}I-OS_2 and ^{125}I-OS_1, respectively. B, Bound ^{125}I-toxin in pmol/mg of protein; F, free ^{125}I-toxin in nM. The straight lines represented in inset C were derived from a fitting of the data based on the presence of two families of independent binding sites.

ing with a $K_{0.5}$ value of 41 pM. This suggests that ^{125}I-OS_1 binding sites represent only a part of the total population of ^{125}I-OS_2 binding sites.

All binding parameters for ^{125}I-OS_2 and ^{125}I-OS_1 determined on membranes of skeletal muscle cells at the embryonic or at the adult stage and on membranes of cultured myotubes are listed in Table I. Results from experiments performed on muscle from young (4-day-old) rabbits have also been included. The first observation is that the overall density of ^{125}I-OS_2 binding sites decreases drastically from 1900 ± 200 in muscle cells in culture to 8 ± 3 fmol/mg of protein in adult muscle tissue. The OS_1 binding site density also decreases dramatically from 1820 ± 150 to 2 ± 1.4 fmol/mg of protein. Moreover while the B_{max} values for ^{125}I-OS_2 and ^{125}I-OS_1 binding are nearly identical in cultured cells, they are in an ~6:1 ratio in the three other muscle membrane preparations. The easiest interpretation of these results is that rabbit muscle in vivo expresses 2 types (at least) of PLA_2 binding sites, one of which binds both OS_2 and OS_1 and will be referred to as the 'OS_2/OS_1' binding site. It is the main type of site in skeletal myotubes in culture and is then very highly expressed. The other type of site is much more selective for OS_2 and it will be referred to as the 'OS_2' binding site. This site has apparently the same properties as the neuronal OS_2 binding site but are not expressed in skeletal muscle myotube in culture [8]. Such an interpretation is strongly supported by the data in Fig. 3 showing that only about 20% of the total ^{125}I-OS_2 binding is displaceable by OS_1 in skeletal muscle membranes from 18-day-old embryos, whereas 100% of this binding is inhibited by OS_1 in membranes from myotubes in culture [9] with similar $K_{0.5}$ values (48 pM v. 34 pM).

The OS_2/OS_1 receptor found in membranes of cultured skeletal muscle cells is completely insensitive to the bee venom PLA_2 while the brain OS_2 receptor has

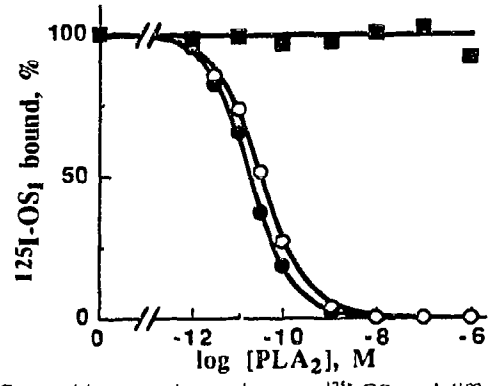

Fig. 2. Competition experiments between ^{125}I-OS_1 and different unlabeled PLA_2s for the binding to skeletal muscle membranes of 18-day-old rabbit embryos. Membranes (200 μg of protein/ml) were incubated in 0.5 ml with 30 pM ^{125}I-OS_1 and increasing concentrations of unlabeled OS_1 (○), OS_2 (●) or bee venom PLA_2 (■). Results are expressed as percentage of the maximal specific binding measured in the absence of competitor. 100% corresponds to 3 pM of ^{125}I-OS_1 specifically bound. Non-specific binding was measured in the presence of 30 nM OS_1 and accounted for 10% of the total binding.

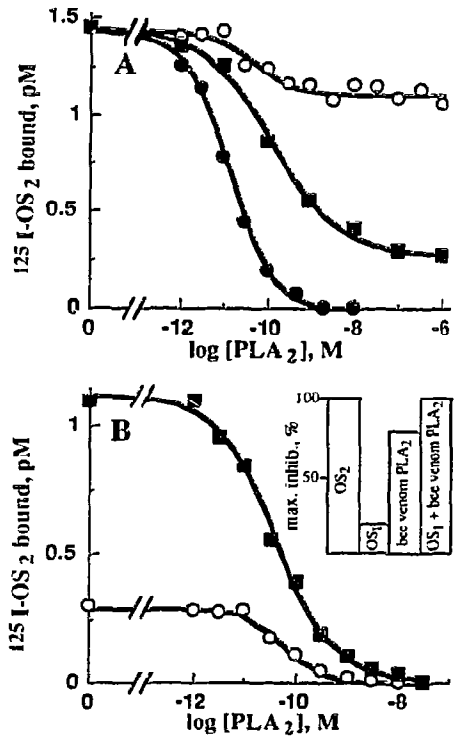

Fig. 3. Competition experiments between ^{125}I-OS_2 and different unlabeled PLA_2s for the binding to skeletal muscle membranes of 18-day-old rabbit embryos. (A) Membranes (10 μg of protein/ml) were incubated in 1 ml with 22 pM ^{125}I-OS_2 and increasing concentrations of unlabeled OS_1 (○), OS_2 (●) or bee venom PLA_2 (■). (B) As in (A), except that competition experiments were done in 2 ml with ^{125}I-OS_2 and unlabeled OS_1 (○) in the presence of 300 nM bee venom PLA_2 or with ^{125}I-OS_2 and unlabeled bee venom PLA_2 (■) in the presence of 300 nM OS_1. Results are expressed in pM of ^{125}I-OS_2 specifically bound. Non-specific binding was measured in the presence of 2 nM unlabeled OS_2 and accounted for less than 30% of the total binding. The inset in panel B indicates the maximal inhibition observed with the different unlabeled toxins.

a high affinity for this neurotoxin [8,9]. This suggests that of the two classes of binding sites for OS_2, the OS_2/OS_2 binding site and the OS_2 binding site, only the OS_2 binding site is recognized by the bee venom PLA_2. If such an hypothesis is correct, then one would expect that when OS_1 provides a ~20% inhibition of ^{125}I-OS_2 binding (Fig. 3), the bee venom PLA_2 would provide an 80% inhibition. The bee venom PLA_2 indeed inhibits ^{125}I-OS_2 binding by ~80% with a $K_{0.5}$ value of 120 pM (Fig. 3A). Moreover, as expected, a mixture of OS_1 and bee venom PLA_2 completely inhibits ^{125}I-OS_2 binding (Fig. 3B) with $K_{0.5}$ values of 58 pM and 36 pM, respectively. Competition experiments with unlabeled OS_2 resulted in a 100% inhibition with a $K_{0.5}$ value of 12 pM (Fig. 3).

The protein compositions of the neuronal (OS_2 type) and myotube (OS_2/OS_1 type) PLA_2 receptors are very different. The OS_2 type of receptor is made of major components of mol. wt. 82, 48 and 34 kDa [8,9]. The OS_1/OS_2 type is made up of only component of 180 kDa

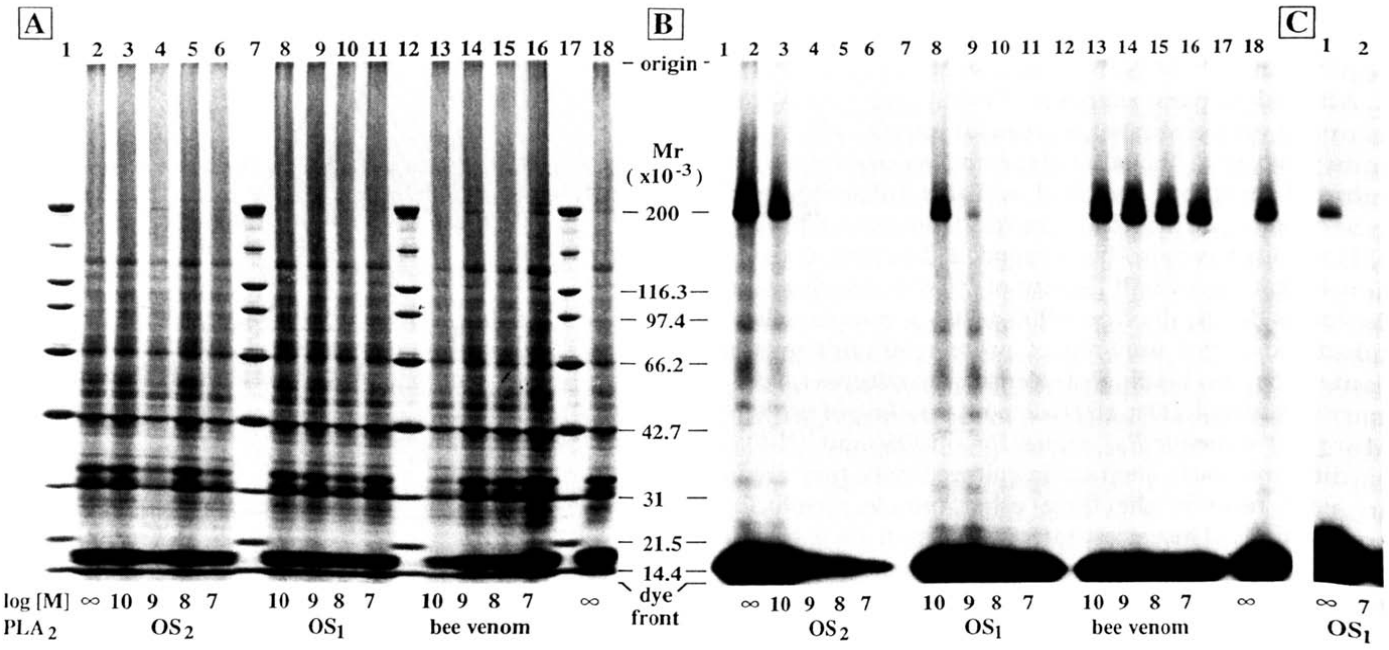

Fig. 4. Cross-linking experiments of ^{125}I-OS$_2$ and ^{125}I-OS$_1$ to skeletal muscle membranes of 18-day-old rabbit embryos. (A and B) Membranes (300 μg of protein/ml) were incubated with 300 pM ^{125}I-OS$_2$ in the absence (lanes 2,18) or the presence of increasing concentrations of OS$_2$ (lanes 3–6), OS$_1$ (lanes 8–11) or bee venom PLA$_2$ (lanes 13–16), cross-linked with 50 μM DSS and loaded onto a 4–14% SDS-polyacrylamide gel under reducing conditions. Coomassie blue staining (A) and autoradiogram patterns (B) of the gel are shown. (C) Membranes (300 μg of protein/ml) were incubated with 250 pM ^{125}I-OS$_1$ in the absence (lane 1) or the presence (lane 2) of 30 nM OS$_1$, cross-linked with 50 μM DSS and loaded onto a 4–14% SDS-polyacrylamide gel under reducing conditions. Only the autoradiogram patterns are represented. In all panels, 100 μg of proteins were loaded in each track. The position of the M_r markers (lanes 1, 7, 12 and 17) are indicated (myosin, 200 000; β-galactosidase, 116 200; phosphorylase B, 97 400; bovine serum albumin, 66 200; ovalbumin, 42 700; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; cytochrome c, 14 400; from Bio-Rad). Gels were exposed on Kodak X Omat AR film for 4 days (panels A and B) or 7 days (C).

[9]. Cross-linking experiments have been performed on membranes of embryonic muscle using both ^{125}I-OS$_1$ and ^{125}I-OS$_2$ as markers. All the experiments were carried out with DSS at a concentration of 50 μM which does not produce detectable changes in the electrophoretic pattern of membrane proteins (Fig. 4A). The label-

Table I

Binding properties of ^{125}I-OS$_2$ and ^{125}I-OS$_1$ on different skeletal muscle membrane preparations

Development stage	Direct binding experiments							Competition experiments			
	^{125}I-CS$_2$					^{125}I-OS$_1$		^{125}I-OS$_2$/OS$_1$		^{125}I-OS$_1$/OS$_2$	
	K_d^a (pM)	K_d^a (pM)	B_{max}^a fmol/mg protein)	B_{max}^b (fmol/mg protein)	$B_{max}^a+B_{max}^b$ fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	$K_{0.5}$ (pM)	Inhibition (%)	$K_{0.5}$ (pM)	Inhibition (%)
Primary cultured cells	7.4±0.9	-	1900±200	-	1900±200	34±8	1820±150	34±6	100	10±4	100
Embryos, 18-day-old	2±1.5	33±9	67±15	203±35	271±50	48±10	40±12	41±5	20	18±5	100
Neonates, 4-day-old	4±2.3	46±12	29±8.5	86±13	115±21.5	44±9	18±7	39±7	23	20±4	100
Adult	17±5	-	8±3	-	8±3	30±12	2±1.4	63±10	25	15±3	100

A membrane preparation of differentiated myotubes or crude microsomal fractions of rabbit skeletal muscle removed at several stages of development were incubated with increasing ^{125}I-toxin concentrations for direct binding experiments, or a fixed ^{125}I-toxin concentration and various concentrations of unlabeled toxin, for competition experiments as defined in Figs. 1 or 2 and 3, respectively. The protein concentrations used in primary cultured cells and 4-day-old neonates binding experiments were 2 and 20 µg of protein/ml for ^{125}I-OS$_2$ or 5 and 600 µg of protein/ml for ^{125}I-OS$_1$, respectively. Each value is the mean ± SE of at least two independent experiments performed in duplicate.

[a] High affinity component binding site
[b] Low affinity component binding site

ing pattern obtained with ^{125}I-OS$_2$ as ligand was complex (Fig. 4B). The major labeled polypeptide components had mol. wts. of 180, 82, 48 and 34 kDa (after subtraction for toxin contribution assuming a 1:1 stoichiometry and a toxin molecular mass of 14 kDa). The specificity of this labeling pattern was demonstrated by the concentration-dependent protection observed with OS$_2$ (Fig. 4B, lanes 3–6) consistent with its $K_{0.5}$ value (370 pM) found under the same experimental conditions (not shown). Components of mol.wt. of 82, 48 and 34 kDa were of the same size as those previously identified in rabbit brain membranes [9]. The component of mol.wt. 180 kDa is present in very low amounts in brain membranes but is the only one labeled in cultured muscle cells [9].

When covalent labeling with ^{125}I-OS$_2$ was protected by unlabeled OS$_1$ instead of OS$_2$, only the component of 180 kDa disappeared (Fig. 4B, lanes 8–11). Conversely, protection by the bee venom PLA$_2$ only protected the other bands, leaving unchanged the labeling of the 180 kDa polypeptide (Fig. 4B, lanes 13–16). Finally, in affinity labeling experiments performed with ^{125}I-OS$_1$ as marker, only the 180 kDa band was detected (Fig. 4C). Taken all together, these cross-linking experiments clearly indicate again that both types of PLA$_2$ receptors, the myotube type OS$_2$/OS$_1$ binding sites (180 kDa) and the neuronal-like type OS$_2$ binding sites, co-exist in rabbit skeletal muscle and that they can be discriminated by OS$_1$ and by the bee venom PLA$_2$.

The relative densities of the different types of PLA$_2$ receptors vary during rabbit skeletal muscle development. The amount of the OS$_2$/OS$_1$ receptor (180 kDa) is extremely high in aneurally cultured rabbit skeletal muscle cells and decreases throughout the in vivo development (Table I). The neuronal OS$_2$ type of PLA$_2$ receptors is not expressed in culture but is highly expressed in vivo at the embryonic stage. The expression of these PLA$_2$ receptors is then developmentally regulated and probably greatly influenced by factors such as innervation, growth factors, hormones and/or muscle contractile activity.

The exact physiological function of the different types of PLA$_2$ binding sites remains to be elucidated.

Acknowledgements: We thank M.-M. Larroque, C. Roulinat and F. Aguila for expert technical assistance. This work was supported by the Centre National de la Recherche Scientifique, the Association des Myopathes de France, and Ministère de la Défense Nationale (Grant DRET 90/192).

REFERENCES

[1] Broad, A.J., Sutherland, S.K. and Coulter, A.R. (1979) Toxicon 17, 661–664.
[2] Howard, B.D. and Gundersen, C.B. (1980) Annu. Rec. Pharmacol. Toxicol. 20, 307–336.
[3] Lee, C.Y., Ho, C.L. and Eaker, D. (1977) Toxicon 18, 443–454.
[4] Gopalakrishnakone, P., Dempster, D.W., Howgood, B.J. and Elder, H.Y. (1984) Toxicon 22, 85–98.
[5] Verheij, H.M., Boffa, M.C., Rothen, C., Bryckaert, M.C., Verger, R. and De Hass, G.H. (1980) Eur. J. Biochem. 112, 25–32.
[6] Condrea, E., Fletcher, J.E., Rapuano, B.E., Yang, C.C. and Rosenberg, P. (1981) Toxicon 19, 705–720.
[7] Strong, P.N. (1987) in: Cellular and Mulecular Basis of Cholinergic Function (Dowdall M.J. and Howthorne J.N. eds.) pp. 534–549, Elles Horwoods, Chichester, U.K.
[8] Lambeau, G., Barhanin, J., Schweitz, H., Qar, J. and Lazdunski, M. (1989) J. Biol. Chem. 264, 11503–11510.
[9] Lambeau, G., Schmid-Alliana, A., Lazdunski, M. and Barhanin, J. (1990) J. Biol. Chem. 265, 9526–9532.
[10] Shipolini, R.A., Callewaert, G.L., Cottrell, R.C., Doonan, S., Vernon, C.A. and Banks, B.E.C. (1971) Eur. J. Biochem. 20, 459–468.
[11] Lambeau, G., Lazdunski, M. and Barhanin, J. (1991) Neurochem. Res. 16, 651–658.
[12] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.