Cell. Mol. Life Sci. 65 (2008) 2897 – 2912
1420-682X/08/182897-16
DOI 10.1007/s00018-008-8113-3
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Cellular and Molecular Life Sciences

Review

Cellular pathology induced by snake venom phospholipase A_2 myotoxins and neurotoxins: common aspects of their mechanisms of action

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Received 05 March 2008; received after revision 08 April 2008; accepted 29 April 2008
Online First 20 June 2008

Abstract. A large variety of snake toxins evolved from PLA_2 digestive enzymes through a process of 'accelerated evolution'. These toxins have different tissue targets, membrane receptors and mechanisms of alteration of the cell plasma membrane. Two of the most commonly induced effects by venom PLA_2s are neurotoxicity and myotoxicity. Here, we will discuss how these snake toxins achieve a similar cellular lesion, which is evolutionarily highly conserved, despite the differences listed above. They cause an initial plasma membrane perturbation which promotes a large increase of the cytosolic Ca^{2+} concentration leading to cell degeneration, following modes that we discuss in detail for muscle cells and for the neuromuscular junction. The different systemic pathophysiological consequences caused by these toxins are not due to different mechanisms of cell toxicity, but to the intrinsic anatomical and physiological properties of the targeted tissues and cells.

Keywords. Snake venoms, PLA_2, neurotoxins, myotoxins, Ca^{2+}.

Introduction

Hundreds of species of venomous snakes with a wide variety of prey produce venoms which contain varying proportions of toxins endowed with phospholipase A_2 (PLA_2) activity. These proteins have evolved from an ancestral PLA_2 with digestive function. They fold very similarly and display a range of enzymatic turnover values from zero to the high values typical of digestive enzymes such as those found in pancreatic secretions. These toxins are produced by the snake venom gland and, when injected in the prey (usually in large muscles or subcutaneously), cause a range of pathological alterations. Some PLA_2 toxins act specifically on the peripheral nervous system and paralyse the prey within very short time periods; others target both the nervous and the muscular systems, and a large number act mainly on skeletal muscles causing extensive damage (myotoxins); still others exert various effects, such as anticoagulation, or affect platelet aggregation, hypotension, hemolysis and edema [1].

Neurotoxins produced by elapid snakes (β-bungarotoxin, taipoxin, textilotoxin etc.) are the main, or the sole, responsible for the death of the prey. These neurotoxins exert their action at peripheral nerve terminals [2]. Death usually follows respiratory paralysis with little or no damage to other organs [3,4]. However, if the patient's respiration is mechanically

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Table 1. Some properties of selected[a] snake PLA_2 and PLA_2-homologue toxins.

Toxin name	Snake species	Structural group	Number of subunits	Sequence code	PDB code	Lethal potency[b]	PLA_2 activity[c]	References
Neurotoxic PLA_2s								
ammodytoxin A	*Vipera ammodytes*	IIa	1	P00626	n.d.	21 iv	280	[138]
β-bungarotoxin	*Bungarus multicinctus*	Ia	2	P00617/P00989	2NBT	19 ip	61	[139–141]
taipoxin	*Oxyuranus scutellatus*	Ia	3	P00614/-15/-16	n.d	2 iv	0.4	[142]
textilotoxin	*Pseudonaja textilis*	Ia	5	P23026/-27/-28/P30811	n.d.	1 iv	3.2	[143]
Neurotoxic and myotoxic PLA_2s								
notexin	*Notechis scutatus*	Ia	1	P00608	1AE7	17 iv	1390	[144–145]
crotoxin	*Crotalus d. terrificus*	IIa	2	P08878/P62022	2QOG	110 iv	85	[146–147]
Myotoxic D49 PLA_2s								
myotoxin III	*Bothrops asper*	IIa	2	P20474	n.d.	470 iv	750	[148]
bothropstoxin II	*Bothrops jararacussu*	IIa	2	n.a.	n.d.	n.d.	27	[149]
Myotoxic PLA_2 homologues								
AppK49	*Agkistrodon p. piscivorus*	IIa	1	P04361	1PPA	25000 iv	- K49 [d]	[150–152]
ACL myotoxin	*A. contortrix laticinctus*	IIa	1	AAC59887	1S8G	n.d.	- K49	[96,153,154]
myotoxin II	*Atropoides nummifer*	IIa	2	P82950	2AOZ	n.d.	- K49	[155–157]
myotoxin II	*Bothrops asper*	IIa	2	P24605	1CLP	n.d.	- K49	[158–160]
bothropstoxin I	*Bothrops jararacussu*	IIa	2	Q90249	2H8I	4800 iv	- K49	[161–163]
myotoxin II	*Bothrops moojeni*	IIa	2	Q9I834	1XXS	7600 ip	- K49	[164–166]
ammodytin L	*Vipera ammodytes*	IIa	1	P17935	n.d.	3600 ip	- S49	[47, 167]

[a] A few representatives out of the large number of snake PLA_2 toxins were selected on the basis of most available structural and functional information.
[b] Mouse LD_{50} in μg/kg, by intravenous (iv) or intraperitoneal (ip) routes.
[c] μmol/min/mg toxin.
[d] All PLA_2 homologues are here considered to be enzymatically inactive, residue at position 49 is indicated by single letter.
n.d.: not determined; n.a.: not available.

supported, very frequently there is a complete recovery. Venom PLA_2s may also induce 'central neurotoxicity' when administered by intracerebroventricular injection [5]. However, since PLA_2s do not cross the blood-brain barrier, this effect is unlikely to be pathologically relevant and, therefore, only peripheral neurotoxicity will be discussed in this review.

Some neurotoxic PLA_2s also affect the skeletal muscles, as in the case of notexin and crotoxin [2] (and Table 1). In general, they cause extensive and irreversible damage to skeletal muscle. These toxins usually induce a systemic myotoxicity, *i.e.* rhabdomyolysis, with impressive increments in plasma creatine kinase (CK) and other muscle-derived enzymes, and with myoglobinuria, often leading to acute renal failure and hyperkalemia [6]. The ability of these PLA_2s to induce systemic myotoxicity is directly related to their high selectivity for targets in muscle cells and, in some cases, in nerve terminals. Accordingly, these toxins do not induce cytotoxicity when incubated with other cell types or with immature muscle cell precursors, *i.e.* myoblasts [7]. This is in contrast with other PLA_2s that induce mostly local myotoxicity, and are capable of lysing many different cell types [8, 9] (see below). Invariably, all the myotoxic PLA_2s cause an extensive inflammatory reaction which develops following necrosis itself and as a consequence of their direct action on inflammatory cells; they are also able to induce prominent pain, *i.e.* hyperalgesia and allodynia [10]. In the case of viperid snake venoms, the local pathology induced by PLA_2s is further complicated by the action of other toxic components, mostly zinc-dependent metalloproteinases, which cause haemorrhages, blistering and other alterations responsible for extensive tissue damage and sequelae due to poor tissue regeneration. Moreover, toxin-induced tissue damage favours infection by bacteria [11], which further complicates the local pathology and may end up in gangrene and tissue loss requiring amputation of the affected limb [6].

The ensemble of these PLA_2 toxins is intensively studied because envenomings constitute a major health problem in many countries [12]. On the other hand, toxins are also studied because they may help to reveal unknown aspects of tissue and cell physiology [13]. This review will not provide an extensive coverage of the vast literature on these snake toxins, as this was recently done for the myotoxins [14] and for the neurotoxins [15, 16]. Rather, we intend to review and discuss the shared features in the structure and function of the snake PLA_2 toxins responsible for such diverse effects, in order to identify common steps in their molecular and cellular mode of action and to discuss them in relation to the specific pathologies they induce.

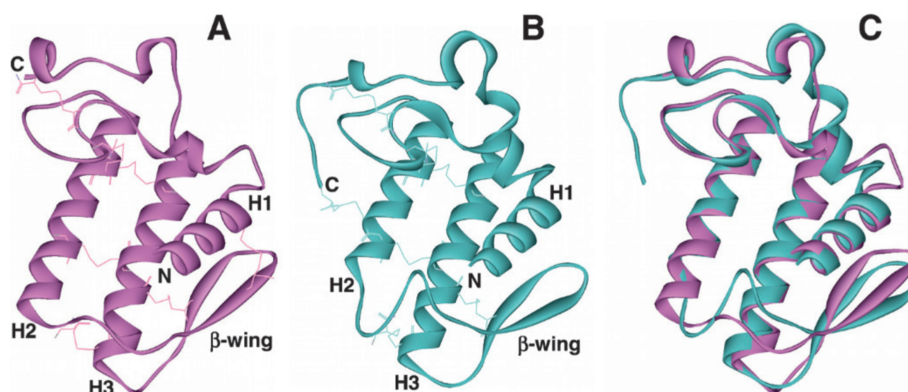

Figure 1. Three-dimensional structures of snake venom PLA_2s. Ribbon representations of (*A*) notexin (1AE7), a group I phospholipase A_2 from *Notechis scutatus scutatus* venom [145] and (*B*) D49 basic PLA_2 (1VAP), a group II enzyme from *Agkistrodon piscivorus piscivorus* venom [168]. Disulfide bonds are shown in stick representation. Superposition of both structures (*C*) illustrates the conservation in the calcium-binding loop and the three major helices (H1, H2, H3), despite evident deviations in the other interconnecting loops and the β-wing. Note the extended C-terminal in the group II enzymes (*B*) in comparison to group I (*A*). N- and C-termini are indicated by N and C, respectively.

Structure of snake PLA_2 toxins

The crystallographic structure of several snake PLA_2, toxins has been determined (structures at Protein Data Bank: http://www.rcsb.org/pdb/). Secreted PLA_2s fold very similarly in a characteristic scaffold consisting of four main helices held together by seven intrachain disulfide bonds (Fig. 1). These proteins vary in length (119–134 amino acids) and are flattened ellipsoids of approximate dimensions $45 \times 30 \times 20$ Å. Two long antiparallel and disulfide-linked α-helices define an elongated and water-shielded 'hydrophobic channel', which leads the phospholipid substrate to the catalytic site; this is characterized by the presence of four key residues: His48, Asp49, Tyr52 and Asp99. The His residue hydrogen-binds the water molecule used for hydrolysis, whilst Asp49 coordinates and positions the Ca^{2+} ion which binds and polarizes both the phosphate and the *sn*-2 carbonyl groups of the phospholipid molecule during hydrolysis. Another highly conserved region in PLA_2 is the 'calcium-binding loop', comprising residues Tyr28, Gly30 and Gly32 which, in combination with Asp49, binds the Ca^{2+} ion required for catalysis. In addition, there is an 'interfacial binding surface', which mediates the adsorption of the enzyme onto the lipid-water interface of the phospholipid membrane bilayer, strongly promoted by anionic amphipatic molecules such as fatty acids (FAs) [17]. These general features are common to both group I and group II venom PLA_2s, which mainly differ in the position of one of their seven disulfide bonds, by the presence of the 'pancreatic loop' in some of group I enzymes and by an extended C-terminal loop in the group II counterparts [17].

A distinct subgroup of PLA_2 homologue toxins present the replacement of the key Asp49 residue with a Lys, Arg, Ser Gln, or Asn residue, among other substitutions, with the consequent loss of Ca^{2+}-binding and enzymatic activity. The most abundant of these catalytically inert proteins correspond to the myotoxic Lys49 PLA_2 homologues [18]. The major toxicity-determining site in these PLA_2 homologues encompasses residues 115–129 in the C-terminal region, which includes a variable combination of positively charged and hydrophobic/aromatic residues, with the ability to alter the bilayer membrane integrity [18, 19]. The widespread occurrence of these PLA_2 homologues in viperid venoms is intriguing from the molecular evolutionary standpoint, since evidently they diverged from a gene encoding catalytically active variants and must play a relevant adaptive role, as judged by their abundance in many venoms. The nature of this role remains, however, poorly understood.

Many myotoxic and neurotoxic PLA_2s are monomers, although oligomerization, particularly dimerization, is not uncommon in these proteins (Table 1). Several neurotoxins include additional subunits, such as a disulfide-linked K^+ channel-binding subunit in the case of β-bungarotoxin [20]. Taipoxin contains two additional PLA_2 subunits with no catalytic activity, and textilotoxin consists of five subunits, only one of which has PLA_2 activity [1]. Crotoxin is a heterodimer of a toxic PLA_2 subunit and of a non-toxic smaller subunit termed crotapotin or subunit A, which results from the proteolytic processing of a PLA_2 chain, and has been described as an enhancer of the toxicity of the PLA_2 subunit [21]. Alternatively, it has been suggested that oligomerization is an evolutionary strategy to increase the binding affinity and specificity

of a toxin for a defined target such as the motoneuron endplate [22].

Steps involved in the cellular toxicity of snake PLA_2 toxins

Distribution

The available experimental data indicate that cell intoxication by snake PLA_2 myotoxins and neurotoxins can be divided into several steps. The distribution of different toxins within the injected animal body appears to vary substantially from one toxin to the other, and two groups of toxins can be broadly identified. The systemically-acting group, which includes the neurotoxins and systemic myotoxins, and the locally-acting myotoxins, which induce only moderate elevations of plasma CK activity and do not cause myoglobinuria and systemic myonecrosis [23–25]. Although part of the action of the first group of toxins is displayed close to the site of injection, on adjacent skeletal muscles and neuromuscular junctions (NMJs), clinical reports indicate that the neurotoxins are also capable of reaching distant anatomical loci, such as the respiratory muscles or facial muscles, after having been injected in a leg or foot [3,4]. Likewise, crotoxin, notexin and other systemically-acting PLA_2s are capable of inducing necrosis of distant muscles [26, 27]. However, PLA_2s toxins are unable to cross the blood-brain barrier and therefore do not reach the central nervous system. The molecular and physiological basis of this rapid toxin redistribution within the body of the prey is not known, though its evolutionary advantage is evident. At variance, the group of locally-acting toxins may do so because of their rapid binding to various components of the tissues where they are injected, thereby precluding their systemic distribution and restricting their action to the local scenario [25].

Cell binding

A first essential step in cell pathology is toxin binding to the target cells (Fig. 2). As crucial as this step is, it is the least understood in molecular terms. Notwithstanding the large amount of work done, for none of these snake toxins is the molecular identity of their functional receptors is known, except for β-bungarotoxin which binds to K^+ channels of the presynaptic membrane of peripheral nerves [15, 16, 28, 29]. Whilst both skeletal muscle cell lines and isolated muscle fibers are available for experimental studies, this is not the case of the peripheral motoneurons, which are the main targets of neurotoxins. Most research has been thus performed with brain homogenates and synaptosomes, and with primary cultures of neurons isolated from the embryonic central nervous system; thus, the possibility that they are only partially relevant models for what happens *in vivo* should be considered. Indeed, β-bungarotoxin is rather specific for motor nerve terminals, whilst sensory discharges are not affected for several hours of incubation with the toxin. At variance, in the central nervous system this neurotoxin appears to bind more specifically to cholinergic and GABAergic terminals [30, 31]. Despite this limitation, these studies have indicated the presence of a limited number of high-affinity binding sites, mainly located at presynaptic nerve terminals [28, 31–33]. No competition among the different neurotoxins for binding to the presynaptic membrane was evidenced, indicating that they bind to different receptors.

Even less is known with respect to the receptors of myotoxic PLA_2s, although they appear to be expressed predominantly on the cell surface of differentiated myotubes and adult muscle fibers, but not in undifferentiated myoblasts [34, 35] because the former cells are toxin-sensitive, whilst the latter ones are more resistant to the action of these toxins, especially in the case of systemically-acting PLA_2s [7]. The selectivity for certain muscle cells is further evidenced by the preference of some systemically-acting myotoxins, like notexin and crotoxin, for type I muscle fibers [36, 37]. The binding of notexin to muscle cell plasma membrane has been documented by immunoelectron microscopy [37]. Furthermore, the ability of cyclosporin A, an inhibitor of calcineurin, to suppress the myotoxic action of crotoxin can be interpreted on the basis of the calcineurin-induced shift of muscle fiber type to crotoxin-susceptible type I, slow oxidative fibers [38]. However, the identity of receptors remains elusive. M-type PLA_2 receptor does not seem to be relevant for myotoxicity in the case of OS_2, an elapid PLA_2, and the myotoxic Lys49 PLA_2s do not bind to this receptor [39]. The recent finding of strong and specific binding of Lys49 PLA_2s to the VEGF receptor is intriguing, but the possible significance of this interaction in myotoxicity is unkown [40]. It is noteworthy, however, that binding of Lys49 PLA_2s to this receptor occurs through the C-terminal region, known to play a key role in plasma membrane perturbation [41]. It has been observed that negatively-charged lipids in the plasma membrane may play a role in the binding of some locally-acting myotoxic PLA_2s [42]. Thus, the nature and identity of myotoxic PLA_2 receptors in target cells remain as open questions.

The ensemble of these observations, together with the rapidity of the degenerative events occurring in muscle and nerves (within the range of seconds to

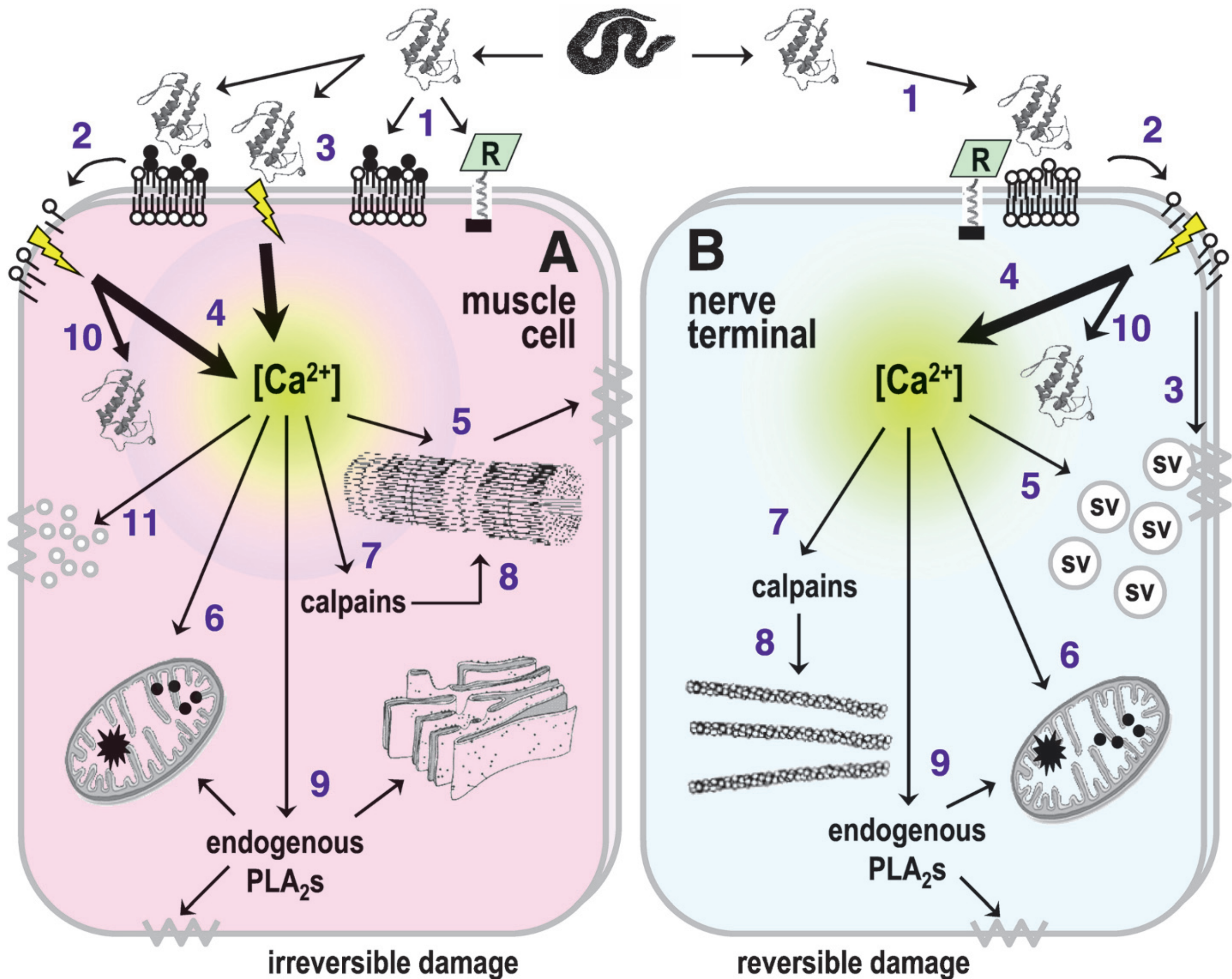

Figure 2. Schematic representation of the action of myotoxic and neurotoxic snake PLA_2s and PLA_2 homologues in skeletal muscle cells (*A*) and motor nerve terminals (*B*). (*A*) Myotoxic PLA_2s can be Asp49 catalytically-active variants or PLA_2 homologues devoid of enzymatic activity. Step (1) represents the myotoxins (left panel) or neurotoxins (right panel) binding to either to high-affinity specific protein receptors (R) or to low-affinity lipid domains of the plasma membrane. Step 2 is the hydrolysis of phospholipids that takes place for both neurotoxins (right panel) and PLA_2 active myotoxins (left panel). The enzymatically-inactive myotoxins act via direct perturbation of the membrane (left panel, step 3). Step 2 is in any case followed by alteration of the membrane structure. Steps 2 (both panels) and 3 (left panel) represent a perturbed plasma membrane which becomes permeable to ions, thus losing its membrane potential and allowing a large influx of Ca^{2+} from the extracellular medium. Such altered membrane promotes the exocytosis of ready-to-release synaptic vesicles (SV) at the nerve terminal (step 3, right panel). Step 4 in both panels represents a large increment in cytosolic Ca^{2+} concentration, which induces hypercontraction of myofilaments (step 5, left panel) and, in turn, may cause mechanical damage to the plasma membrane. In addition, membrane repair processes, depending on dysferlin, may take place by vesicle fusion to damaged membrane segments (arrow 11, left panel). The large increment of cytosolic Ca^{2+} causes the exocytosis of the reserve pool of synaptic vesicles (step 5, right panel). Ca^{2+} uptake by mitochondria, through the uniporter, results in mitochondrial swelling, disorganization of cristae, formation of hydroxyapatite crystals and flocculent densities, and opening of the permeability transition pore, thus resulting in a severe impairment of mitochondrial function (step 6, both panels). Ca^{2+}-dependent proteinases (calpains) are activated (step 7, both panels) and degrade cytoskeletal components (step 8, both panels), further affecting the mechanical integration of the cell. Ca^{2+}-dependent cytosolic PLA_2s are activated and promote further hydrolysis of intracellular membranes and plasma membrane (step 9, both panels). Plasma membrane disruption allows the entry of neurotoxins and, possibly, myotoxic PLA_2s (step 10, both panels), which hydrolyze and damage intracellular membrane systems, until inactivated by yet undetermined molecular events.

minutes), strongly suggest that the primary site of action of these PLA_2 toxins is the plasma membrane, and not an intracellular target. This does not exclude the possibility, reviewed recently [15], that at least some of these toxins enter the cell, further contributing to cell pathology with an intracellular action (Fig. 2). However, this entry is likely to occur after an initial action on the plasma membrane which marks the onset of degenerative events. The present situation calls for more experimental efforts in order to identify the toxin receptors, which, in all likelihood, are physiologically-important molecules in the membranes of nerve terminals and muscle fibers, as they do not seem to be negatively selected by the evolu-

tionary pressure exerted by the toxin-producing snakes on their prey. That such evolution takes place is well illustrated by the development of resistance to α-bungarotoxin by mutation of its receptor, the nicotinic ACh receptor, in different animals [43, 44].

Membrane damage

Since the discovery of snake PLA_2 toxins, it was obvious to consider the possibility that they act via the hydrolysis of phospholipids of the plasma membrane, and a long list of studies were dedicated to test this possibility. Almost invariably, experimental designs have been directed to determine the consequences on toxicity of inactivating the enzymatic activity. Sr^{2+} and other divalent cations, which do not support the PLA_2 catalytic activity, replaced Ca^{2+} in the medium of isolated NMJ preparations or cells in culture [1, 15, 28]. In addition, chemical modifications of PLA_2 specific residues were used in other studies, and more recently this issue was approached by site-directed mutagenesis [39, 45–48]. In general, the reduction or abolition of PLA_2 activity of neurotoxins and myotoxins was correlated to a varying extent with loss of toxicity, but no conclusive evidence about the involvement of the PLA_2 activity in toxicity was obtained.

Direct approaches involving measurement of PLA_2 hydrolysis products in neurons and use of the hydrolysis products themselves were attempted only recently [49–51]. As this literature has been analysed in detail recently elsewhere [1, 15, 16], we would like to make here only a general, but substantial, summary, and then discuss the consequences of these findings. Clearly, PLA_2 activity cannot be directly involved in the action of the Lys49 myotoxins, and their mechanism of action will be discussed afterwards.

For the snake venom myotoxins and neurotoxins which are enzymatically active, the PLA_2-catalysed production of lysophospholipids (LysoPLs) and FAs is involved in membrane damage. However, this PLA_2 activity is not sufficient by itself, as toxicity is strictly associated with the precise site and mode of binding of each toxin to the membrane. In other words, it is the specific binding properties of each toxin that determine the localization and extent of the membrane damage caused by PLA_2 activity [16, 52, 53]. This knowledge helps to explain a puzzling aspect of the action of these neurotoxins, *i.e.* that there is no correlation between their PLA_2 activities *in vitro* and their toxicities [16] (Table 1). The two most potent PLA_2 neurotoxins are taipoxin and textilotoxin, which have the lowest *in vitro* PLA_2 activity (Table 1). However, if one assumes that they bind with high affinity and specificity to selected targets of the presynaptic membrane, then their damaging activity is maximized as the phospholipid hydrolysis occurs at toxicology-relevant sites [52, 53]. These neurotoxins have been suggested to bind to the so-called active zones, *i.e.* those microdomains of the presynaptic membrane where synaptic vesicle neurotransmitter release takes place [16, 50, 53]. An alternative suggestion has been that they act on the internal membrane layer of synaptic vesicles, once they become exposed to the medium following exocytosis [54]. This strict connection between PLA_2 activity, specificity and affinity of binding also appears to be valid for the PLA_2 myotoxins that could bind to microdomains of the sarcolemma particularly sensitive to the presence of LysoPLs and FAs, or where their activity is maximized and causes relevant derangement to the plasma membrane organization.

What are the consequences of the toxin-catalysed conversion of phospholipids into LysoPLs and FAs to the membrane bilayer organization? This question can be answered on the basis of the molecular properties of LysoPLs and FAs with respect to those of phospholipids. In general, without going into the details of the different shapes of the various classes of phospholipids, they have a rather cylindrical shape with a balance between the hydrophilic head group and the hydrophobic fatty acid chains, as to be compatible with the formation of a bilayer membrane structure. LysoPLs have an inverted cone shape and a lower hydrophobic portion with respect to phospholipids; they spontaneously form spherical micelles in polar solvents. When they are comprised within bilayer biological membranes, they induce a positive curvature of the membrane layer where they are located; in the case of the toxins, this is the outer monolayer of the plasma membrane. Long-chain FAs are cone-shaped and hydrophobic, and at variance with LysoPLs, they have a high mobility such that they can rapidly move from one membrane layer to the other. This high flip-flop rate allows their rapid equilibration in the two layers of biological membranes. The presence of LysoPLs and FAs within the plasma membrane has three major consequences: a) it favours the membrane fusion of synaptic vesicles and inhibits the opposite process of endocytosis; b) it increases the membrane permeability to ions; and c) FAs may partition into intracellular organelles and alter their function, as depicted in Figure 2.

Why does the presence of LysoPLs on the monolayer trans, and of FAs in cis, with respect to the site of membrane fusion, result in the induction of exocytosis and inhibition of endocytosis? This is better understood on the basis of the molecular mechanism of membrane fusion, which envisages the existence of a stable lipid hemifusion intermediate which requires energy to proceed to pore formation [55–57]. The

membrane change induced by snake PLA_2s lowers this energy barrier and thus induces exocytosis and inhibits the reverse process of membrane fission, involved in endocytosis, for the very same reason [49, 53]. This rationale explains well the finding that crotoxin injected into the cytosol inhibited exocytosis [58], because this generates the opposite topological situation of LysoPLs on the cytosolic leaflet, which is anti-fusogenic.

The consequences of snake PLA_2 action are clearly different depending on the target membrane. At the motor endplate it causes the release of the ready-to-release pool of synaptic vesicles, and very likely also of the recycling pool of vesicles, and inhibits their retrieval [49, 59–61]. The PLA_2 myotoxins are expected to promote exocytosis and inhibit endocytosis at the sarcolemmal level, but no information about this possibility is yet available.

The second important consequence of the presence of LysoPLs and FAs within the membrane is that they increase its permeability to ions [62–66], leading to loss of membrane potential due to the re-equilibration of Na^+, K^+ and Cl^- ions [67]. If one considers that the highest transmembrane concentration gradient present at the plasma membrane is that of Ca^{2+}, estimated to be of four orders of magnitude, from the millimolar outside to the 0.1 μM in the cytosol, it is not surprising that snake PLA_2 neurotoxins, as well as the simple addition of a LysoPL/FA equimolar mixture, cause a rapid increase of $[Ca^{2+}]$ within the nerve terminals [68] (Fig. 2, right panel). In principle, Ca^{2+} can enter directly through membrane lesions caused by LysoPLs/FAs and/or through voltage-gated Ca^{2+} channels, activated by membrane depolarization. The channels inhibited by ω-conotoxin MVIIC and nimodipine were reported to contribute little, if any, to this Ca^{2+} influx in primary cultures of neurons [68]. On the other hand, inhibitors of voltage-gated Ca^{2+} channels were found to substantially reduce the damage caused by β-bungarotoxin on the isolated rat phrenic nerve/diaphragm [54]. At variance, in the mouse hemidiaphragm, the paralysis time induced by different neurotoxins was not affected by the presence of specific inhibitors of the Ca^{2+} channels known to operate at the NMJ [68; Caccin et al, unpublished observations]. Whatever the exact mode of Ca^{2+} entry, this cytosolic $[Ca^{2+}]$ rise by itself causes the exocytosis of all the reserve pool of synaptic vesicles (Fig. 2), as was clearly documented by studies employing α-latrotoxin [61, 69, 70].

The PLA_2 activity of presynaptic snake neurotoxins is not FA specific. Eicosopentanoic acid and arachidonic acid were recently found to promote exocytosis as deduced from increased membrane capacitance in PC-12 cells [71]. Moreover, arachidonic acid, and some detergents, were found to stimulate the release of Munc18 from syntaxin with ensuing formation of SNARE complexes, a passage which is expected to increase exocytosis [72, 73]. Moreover, omega-3 and omega-6 polyunsaturated FAs were found to stimulate membrane expansion at the nerve growth cone by a mechanism requiring syntaxin 3 [74]. Accordingly, it is possible that these FAs contribute to the increased neuroexocytosis stimulated by the snake neurotoxins, mentioned above. The possibility that the FAs generated by the snake PLA_2 toxins may partition into intracellular organelles should also be considered. FA are known uncouplers of mitochondrial respiration, with inhibition of ATP production [75], and their involvement in the action of snake PLA_2 neurotoxins was suggested long ago [76]. However, this is not supported experimentally, as FAs alone do not inhibit neurotransmitter release at the NMJ [49]. Moreover, the effect of the neurotoxins at the NMJ and on synaptosomes is rapidly reversed by albumin washing, which removes FAs [50, 67], and this should not be the case if FAs are inside mitochondria. Myotoxins were shown to induce the production of FAs in primary human myoblasts and mouse NB41A3 neuroblastoma cells [77, 78] and *in vivo* after myotoxin injection in muscle tissue [79]. LysoPLs were not measured at the same time. The ability of LysoPLs to induce membrane damage in muscle fibers has been described [80]. At the present time, it cannot be excluded that FAs could play a role in myotoxin intoxication of muscle cells, through uncoupling of mitochondria, since they are highly dependent on the activity of these organelles.

The Lys49 PLA_2 myotoxins are devoid of enzymatic activity [17, 46, 81], and therefore their membrane action has to be explained on grounds different from direct hydrolysis of phospholipids. These PLA_2 homologues have a highly cationic face [82–84]. Accordingly, they preferentially disrupt the integrity of negatively-charged liposomes [85, 86], and the addition of phosphatidylserine and phosphatidic acid to erythrocytes renders these otherwise resistant cells susceptible to the lytic effect of these toxins [42]. Thus, it is possible that negatively-charged microdomains in cell membranes constitute 'acceptor' sites for this group of basic myotoxins. The preferential cytotoxicity of Lys49 PLA_2s for differentiated muscle cells may depend on the appearance of protein receptors or anionic lipidic microdomains following the fusion of myoblasts to form myotubes and fully differentiated muscle fibers. The alternative explanation that myotubes are more susceptible to any type of membrane-perturbing agent has been discarded [34].

A large set of experimental evidence indicates that a cationic-hydrophobic stretch of residues present within the C-terminal region of the Lys49 myotoxins is responsible for membrane damage. Synthetic peptides corresponding to this region are cytotoxic in eukaryotes and prokaryotes [87–91], and the higher susceptibility of myotubes in comparison to myoblasts is also reproduced by such synthetic peptides [34]. The role of this molecular region in myotoxicity and liposome disruption is also supported by site-directed mutagenesis experiments [19, 46, 92]. Moreover, cytotoxicity and myotoxicity can be mimicked by the same C-terminal peptides made with D-amino acids, arguing against a specific protein-protein interaction [91]. This region is able to disorganize lipid bilayers owing to its cationic-hydrophobic property. The dimeric character of many Lys49 PLA_2s may contribute to their mechanism of action by allowing a change in quaternary structure that favours membrane contact and penetration [93, 94]. In addition, the dimeric character of these toxins may enhance their membrane-damaging effects by increasing binding affinity owing to the divalent interaction, as opposed to the monovalent binding of monomers; the latter ones are still capable of exerting myotoxicity and cytotoxicity, though with a lower potency than the dimers [22, 95]. An FA molecule bound to the inactive catalytic site has been observed in the crystal structure of some of these myotoxins, and proposed to induce the exposure of a 'hydrophobic knuckle' involving the side chains of hydrophobic residues that may contribute to bilayer disorganization [96]. This hypothesis might explain the strict conservation of the 'active site' in these enzymatically-inactive molecules [96]. It might also explain the puzzling observation that alkylation of the His48 of the 'catalytic centre' of Lys49 myotoxins reduces their toxicity [97, 98], as this chemical modification might interfere with the binding of the FA molecule, thus precluding the conformational change that exposes the hydrophobic knuckle.

The membrane perturbation induced by the Lys49 myotoxins increases the permeability of liposomes to entrapped probes, and of myotubes to Ca^{2+} [35, 86]. The entry of Ca^{2+} ions is very large and is compatible with the identification by scanning electron microscopy of large lesions of the sarcolemma [25, 37], as well as with the immediate (within 10–60 s) muscle fiber contractions observed intravitally upon exposure to a Lys49 myotoxin [99]. A combined effect of both LysoPLs/FAs and toxin-induced membrane perturbation seems to be involved in the action of the catalytically-active Asp49 myotoxic PLA_2s because, in the absence of catalysis, there is a residual myotoxic effect [8, 85, 98]. This mixed type of membrane-perturbing activity is also supported by experiments employing mutants of a Ser49 PLA_2 homologue in which enzymatic activity was restored by substituting three residues at the Ca^{2+}-binding loop, in addition to the Ser49/Asp49 substitution [47]. Microscopic observations clearly indicate that membrane damage does not occur by osmotic lysis, but instead by a direct perturbation of membrane integrity leading to disorganization and disruption [99].

Muscle fiber plasma membrane damage, provoked either by catalytically-dependent or -independent mechanisms, triggers a rapid sequence of degenerative events that bring the muscle cells beyond the 'point of no return' within minutes. Ultrastructural and intravital observations document such rapid membrane damage with rapid depolarization of muscle fibers [37, 99–106]. This is accompanied by a prominent influx of Ca^{2+} [35, 79], which is the most significant consequence of plasma membrane disruption, since it promotes a complex series of cellular derangements (see below).

Injection of myotoxins in a muscle generates a concentration gradient of PLA_2s, from a high concentration spot at the injection site to lower concentrations in the neighbouring muscle regions. Such a gradient implies that the extent of damage to the sarcolemma might vary in different muscle fascicles. Some fibers close to the injection site would be drastically and irreversible damaged, without any possibility of repair. However, in regions distant from the central injection area there might be muscle fibers suffering more limited membrane damage. It is suggested that some of these fibers might be able to repair such limited membrane perturbation, by a Ca^{2+}-dependent repair mechanism that involves the fusion of membrane vesicles, with the participation of dysferlin, calpains and annexins [107]. Thus, the concept of thresholds of muscle damage, which depend on the extent of membrane perturbation, is an issue that needs to be investigated in PLA_2-induced myotoxicity.

The general conclusion that can be drawn at this point is that, whatever membrane perturbation is caused by the snake neurotoxins and myotoxins, the major event in cellular intoxication is the same: an increase of ion permeability with alteration of membrane potential, and what appears to be more important, a large increase in the cytosolic $[Ca^{2+}]$, predominantly due to an influx from the extracellular space.

Calcium entry and cellular degenerative events

Ca^{2+}-mediated signalling is one of the major intracellular signalling mechanisms in all cells, and it is certainly a major regulator of nerve and muscle physiology [108, 109]. The large increase of cytosolic

[Ca^{2+}] caused by the snake PLA_2 myotoxins and neurotoxins triggers in muscles and nerve terminals the physiological events controlled by Ca^{2+} signalling, but in an excessive, pathological way (Fig. 2). Nerve terminals release all their content of synaptic vesicles [61], whereas the myofilaments of skeletal muscle hypercontract, causing further mechanical damage to the sarcolemma [37, 99, 104]. As the increase of cytosolic [Ca^{2+}] is persistent in time, these initial events are followed by a series of pathological consequences known to be linked to Ca^{2+} overload, leading muscle cells to a 'point of no return', *i.e.* irreversible damage and necrosis (Fig. 2).

Mitochondrial alteration is a common electron microscopy observation in animals injected with neurotoxins or myotoxins. Mitochondria are swollen with altered crystae, sometimes with internal vacuoles and disrupted membranes [104, 110–113] and may contain hydroxyapatite crystals in the matrix [102] as well as flocculent amorphous densities [104] (Fig. 2). In addition, *in vivo* observation of mitochondria in neurotoxin-treated neurons with membrane potential fluorescent indicators showed a progressive loss of membrane potential with parallel change of morphology from a spaghetti-like shape to an inactive rounded one [68]. Different factors may contribute to the mitochondrial impairment caused by snake PLA_2 toxins: a) the entry of Ca^{2+} in the cytosol activates the mitochondrial Ca^{2+} uniporter transport that leads to an accumulation of Ca^{2+} inside the mitochondrial matrix driven by the electrochemical potential; this eventually results in the precipitation of calcium phosphate and in mitochondrial damage. Clearly, the electrochemical energy used to pump Ca^{2+} inside goes at the detriment of the production of ATP. b) In the case of catalytically-active PLA_2 toxins, one should also consider that FAs are produced and that they very rapidly partition into intracellular organelles; they can thus act as mitochondrial uncouplers, particularly in muscles [75]. This latter effect would by itself cause a reduction of ATP production, which in turn contributes to the increase in cytosolic [Ca^{2+}] because of inactivation of Ca^{2+}-ATPase and of Ca^{2+} exchangers at the plasma membrane and endoplasmic/sarcoplasmic reticulum. c) An important consequence in muscles is that the damaged mitochondria release cell death mediators through the opening of the permeability transition pore [114]. This is suggested by the ability of cyclosporin A to abrogate myotoxicity induced by crotoxin [38] because cyclosporin A inhibits the opening of the permeability transition pore.

If Ca^{2+}-induced mitochondrial alterations and loss of function are relatively simple to detect *in vivo*, this may not be the case for other less-prominent intracellular organelles. But it is very likely that they do occur as well. The available knowledge of cellular biochemistry suggests that a variety of cytosolic hydrolases will be activated very rapidly by Ca^{2+}. They include: a) Ca^{2+}-dependent proteinases, such as calpains, which hydrolyze key structural components including desmin, titin and α-actinin, further compromising the mechanical integration of the contractile machinery in muscles [25, 115]; b) activation of intracellular Ca^{2+}-dependent PLA_2s, which cause further hydrolysis of phospholipids in the plasma and intracellular membranes; and c) activation of other intracellular hydrolases. This will lead to a rapid degradation of several essential cell components, including the observed degeneration of neurofilaments at nerve terminals [54] and of the sarcomeres in muscle cells [25, 116, 117].

Cell necrosis and the release of contents from necrotic cells is accompanied by the onset of a prominent inflammatory reaction, with the synthesis and release of mediators that promote increments in vascular permeability leading to edema and recruitment of neutrophils and macrophages [104]. This opens the possibility that these inflammatory cells contribute to further tissue damage, although experiments made on the role of neutrophils in myonecrosis induced by a PLA_2 do not support this hypothesis [118]. However, the potential detrimental role of other inflammatory cells and mediators remains to be elucidated.

In addition to all these events, plasma membrane disruption may allow the entry of the PLA_2 toxins themselves, which are active in the Ca^{2+}-enriched cytosol, causing further damage to intracellular membranes (Fig. 2). These proteins may enter because of their particular structural features or following the phospholipid hydrolysis and membrane perturbation they cause. There is evidence of toxins inside cells *in vitro* and *in vivo* [119–121]. Some PLA_2 toxins are known to affect mitochondria *in vitro* in Ca^{2+}-containing media [72, 122, 123]. Nevertheless, it seems likely that such intracellular action is secondary to the primary disturbance to the integrity of plasma membrane.

The cellular and tissue consequences of these molecular pathogenic processes are clearly different in the case of neurotoxins and myotoxins, owing to the different anatomy of the targeted cells. In fact, the endplate of motoneurons is in most cases very distant from the cell body, which resides in the spinal cord (central nervous system) and is protected by the blood-brain barrier. Following the inactivation of the toxin, the damaged nerve terminal shrinks and is encircled by Schwann cells which phagocytose cell debris, while the innervated muscle becomes slightly atrophic [54, 124]. A very similar pattern of events takes place at the NMJ in latrotoxin-injected animals

[125]. This toxin is known to bind to the presynaptic membrane and to promote a large increase in cytosolic [Ca^{2+}] [69, 70]. The strong similarities between the morphological alterations of nerve terminals induced by snake neurotoxins and latrotoxin [30, 37, 54, 112, 113, 125] strongly supports the role of Ca^{2+} overload in nerve terminal as the major degenerative event in the action of snake presynaptic PLA_2 neurotoxins.
At variance, the Ca^{2+} overload induced by myotoxic PLA_2s in muscle fibers occurs throughout the length of these cells [35], causing widespread hypercontraction [104] and cell death through a variety of Ca^{2+}-dependent degenerative events [25]. In the case of the Lys49 PLA_2 myotoxins, the tissue degenerates around the site of toxin injection, whilst a systemic rhabdomyolysis develops with systemic toxins such as crotoxin and various elapid PLA_2s [25, 84]. However, regardless of the local vs. systemic nature of the myotoxic effect, the mechanisms of cellular damage are similar for the two types of myotoxins.

Toxin inactivation, tissue repair and regeneration
Little is known about the processes involved in the inactivation of snake PLA_2 toxins *in vivo*, though it appears that they have to be inactivated before any repair/regeneration process can develop. There appears to be little doubt that toxins are eventually degraded and eliminated, but the kinetics and exact mechanisms of inactivation are ill-known. This knowledge should be particularly important as it may have therapeutic relevance. Ammodytoxin A has been show to resist the reducing effect of glutathione [121]. PLA_2-binding proteins, such as the M-type receptor described in skeletal muscle [126], may contribute to toxin sequestration and inhibition, since a recombinant soluble form of this receptor inhibits the enzymatic activity of PLA_2s [127]. An interesting, albeit speculative, possibility for an inactivating mechanism occurring *in vivo* relates to the observation that myotoxic PLA_2s can induce the degranulation of mast cells, with the consequent release of their mediators [128, 129]. Among these, heparin could play an important inactivating role as myotoxic PLA_2 are highly basic proteins, and heparin inhibits the myotoxic effect of many PLA_2s [130, 131]. Thus heparin, if rapidly released by mast cells upon exposure to PLA_2 myotoxins, may constitute a host protective factor that would limit the spread of myonecrosis. This possibility would need to be evaluated experimentally. Likewise, anionic domains abundant in extracellular matrix glycosaminoglycans may inhibit cationic myotoxins. A protective role of mast cell-derived mediators during bee (*Apis mellifera*) and snake (*Atractaspis engaddensis*) venom poisoning was shown in a mouse model [132]. Other possible mechanisms, such as proteolytic degradation, denaturation by reducing agents or complex formation with plasma components known to inhibit PLA_2s [133], await to be investigated.
Within a few days/weeks, the nerve terminal, damaged by the snake PLA_2 presynaptic neurotoxins, regenerates, and the innervated muscle reacquire their structure, size and functional activity [134]. Presynaptic Schwann cells appear to play a major role in NMJ regeneration after nerve terminal crushing [135], and it is likely that they also play an important role in the case of neurotoxin-induced damage. In the case of PLA_2-induced myotoxicity, an orderly and successful regeneration of muscle fibers occurs [134]. This proceeds by a stereotyped sequence of events characterized by phagocytosis and removal of necrotic material, followed by activation of myogenic satellite cells, which are present in the periphery of muscle fibers and are resistant to the action of these toxins [134]. Upon their activation to myoblasts, there is a replicative phase, followed by the fusion of myoblasts to form myotubes, with the appearance of a number of proteins characteristic of differentiated muscle fibers, and then the development of mature fibers which conserve centrally-located nuclei, a characteristic feature of regenerating muscle [134, 136]. Myotoxic PLA_2s such as notexin have become highly useful tools to investigate the cellular mechanisms involved in muscle regeneration [134]. When muscles are injected with crude viperid venoms, which contain hemorrhagic metalloproteinases and other toxic proteins in addition to myotoxic PLA_2s, a poor regenerative response has been described [137]. This is likely to depend on the effects exerted by venom components to the microvasculature, jeopardizing blood supply to regenerating tissue. Moreover, damage to peripheral nerve terminals and incomplete removal of necrotic debris after injection of crude venoms are also likely to contribute to poor regeneration [136, 137]. Thus, PLA_2 myotoxins and neurotoxins are relevant tools not only to investigate the cellular mechanisms of nerve and muscle degeneration, but also the processes associated with repair and regeneration.

Conclusions and future lines of investigation

Hundreds of species of venomous snakes, classified in the families Elapidae and Viperidae, have evolved a very large variety of PLA_2 toxins with an amazing diversification in their toxic profiles, some of them even lacking PLA_2 activity. These toxins display different modes of binding to cells and different mechanisms of plasma membrane damage. As it

emerges from the present discussion, the initial phases of the intoxication process are ill-known for several groups of toxins, and this clearly calls for further investigations. The identification of the receptors of PLA_2 s in nerve and muscle is a subject that demands attention. Besides the relevance for a better understanding of their basic mechanism of action, such knowledge is also important considering the major human health impact that snakebite envenomings cause around the world [12].

Whatever the basis for cell specificity of toxic PLA_2 s, it clearly emerges that the different initial molecular pathogenic processes converge into a major event which plays the central role: the entry of Ca^{2+}, mainly from the extracellular medium, into the cell cytosol. The initial consequences of increased cytosolic $[Ca^{2+}]$ are different in nerve terminals and muscle cells, as synaptic vesicle fusion and insertion in nerve terminal plasmalemma takes place in the former cells, whereas hypercontraction of sarcomeres occurs in the latter. However, many accompanying and ensuing events are common for the two cell types. Mitochondria collapse following an overwhelming calcium uptake, and possibly due to FA uncoupling, with progressive loss of ATP, which is essential for pumping Ca^{2+} out of the cell and for repair reactions. Ca^{2+} activates a variety of hydrolytic enzymes which contribute to degeneration of nerve terminals and muscle necrosis, depending on the toxicological profile of each toxin.

On the basis of the present discussion, we would like to propose to focus research efforts on increased cytosolic $[Ca^{2+}]$ as the central pathogenic event, which triggers a cascade of cellular degenerative processes (Fig. 2). Several techniques are available to image intracellular calcium in a time- and space-resolved manner, and their use will undoubtedly contribute to further insights in the pathogenic events induced by the snake PLA_2 toxins in nerve terminals and muscle cells. On these grounds, the potential role of the various components of the Ca^{2+}-signalling kit in modulating such an increment in Ca^{2+} in the cytosol also deserves further investigation.

Acknowledgements. Work in the authors' laboratories is supported by Vicerrectoría de Investigación, Universidad de Costa Rica, and by research grants from Telethon-Italia GP0272Y01, the University of Padova and FIRBRBNE01RHZM.

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