Scientific paper

A Secreted Phospholipase A_2 Binds to Calmodulin at Sub-micromolar Concentrations of Calcium

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Abstract

To determine the possibility that calmodulin, a cytosolic regulatory protein, and ammodytoxin, a neurotoxic secreted phospholipase A_2, interact *in vivo*, we studied the dependence of their association on Ca^{2+}. The interaction between the two proteins was positively dependent on Ca^{2+}, and greatest at millimolar concentrations of this ion. Importantly, they interacted already in the presence of sub-micromolar concentrations of Ca^{2+}, as demonstrated by affinity labelling, laser tweezers and surface plasmon resonance. Tight binding of the secreted phospholipase A_2 to calmodulin is therefore expected on depolarization of the axolemma, when the concentration of free Ca^{2+} at active zones rises to 100 μM. These results strengthen the proposal that binding of ammodytoxin to calmodulin is a step in the process of presynaptic toxicity of this and related phospholipases A_2. Moreover, they suggest that some other (patho)physiological effects induced by endogenous secreted phospholipases A_2 could be also due to their interaction with calmodulin in the cytosol of cells.

Keywords: Secreted phospholipase A_2, Ammodytoxin, Calmodulin, Neurotoxicity, Ca^{2+}-dependence, Cytosol

1. Introduction

Secreted phospholipases A_2 (sPLA$_2$s; EC 3.1.1.4) hydrolyse the ester bond at the *sn*-2 position of 1,2-diacyl-*sn*-3-phosphoglycerides in a Ca^{2+} dependent manner. In spite of considerable structural similarity, these enzymes display a remarkable variety of physiological and pathological activities.[1,2] Some sPLA$_2$s are potent presynaptic neurotoxins (β-neurotoxins). Abundantly present in snake venoms, they inhibit the communication between nerves and muscles. The most obvious effects that β-neurotoxins induce in the nerve terminal are a reduction in the number of synaptic vesicles, appearance of Ω-shaped invaginations in the presynaptic membrane, damage of mitochondria, inhibition of synaptosomal protein phosphorylation and influence on certain terminal ion currents.[3] Due to this complexity, the molecular basis of β-neurotoxicity is still largely unknown. It is clear however that the phospholipase activity and the specific binding of β-neurotoxins at the nerve ending are both essential for β-neurotoxicity.[4] We demonstrated that ammodytoxin (Atx), a β-neurotoxin from *Vipera a. ammodytes* venom, enters the cytosol of rat hippocampal neurons and that this multi-disulfide-bridged toxin is stable under cytosolic conditions.[5,6] These findings strengthen the proposal that β-neurotoxins act inside nerve cells and that the binding of sPLA$_2$s to certain intracellular proteins[3] is physiologically relevant. Calmodulin (CaM) is one of the most interesting of these proteins. Present in the cytosol of eukaryotic cells, it occupies a central position in the regulation of many cellular processes, including neurotoxic sPLA$_2$-affected processes such as exocytosis, endocytosis, protein phosphorylation and ion channel conductivity.[7] We have observed that all the

Atx mutants expressing high neurotoxicity also show high binding affinity for CaM,[8] suggesting that binding of Atx to CaM is important for the β-neurotoxicity.

CaM and $sPLA_2$s bind Ca^{2+} and their activities and binding properties are influenced by its concentration. The association between Atx and CaM was established in the presence of millimolar concentration of Ca^{2+}.[9] The intracellular concentration of Ca^{2+} ($[Ca^{2+}]$), where the interaction would have to take place physiologically, is however ~10^4-fold lower. We therefore decided to determine whether the $[Ca^{2+}]$ that exists in the cytosol of a eukaryotic cell could support the binding of Atx to CaM.

2. Materials and Methods

2. 1. Materials

Ammodytoxin (Atx) was purified from *Vipera a. ammodytes* venom.[10] Native and recombinant vertebrate CaM were used in our experiments – human brain CaM was purchased from Calbiochem (USA) while mouse CaM was prepared in our laboratory by recombinant expression in *E. coli* as described.[11] All other reagents and chemicals used were of analytical grade.

2. 2. Determination of $[Ca^{2+}]$

$[Ca^{2+}]$ in buffers was determined using flame atomic absorption spectrometry (FAAS) on a Varian Spectra AA 110 (Mulgrave, Australia) atomic absorption spectrometer, as described.[12]

2. 3. Radioiodination of Atx

Atx was radioiodinated to a specific activity of 52 Ci/mmol as described.[13] ^{125}I-Atx was shown to be identical to the native Atx in enzymatic, neurotoxic and immunological properties.

2. 4. Cross-linking of ^{125}I-Atx to CaM

Vertebrate CaM was incubated at 20 nM for 30 min at room temperature with 10 nM ^{125}I-Atx in the labelling buffer (75 mM Hepes, pH 7.4, 150 mM NaCl and 0.15% (w/v) Triton X-100). The $[Ca^{2+}]$ in the buffer was either zero (no addition of Ca^{2+}), 50 μM, 100 μM, 1 mM or 10 mM. One experiment was performed in the labelling buffer containing 1 mM EGTA, which complexed both free and protein bound Ca^{2+}. The control experiment was performed in the presence of a 500-fold molar excess of unlabelled Atx over the labelled toxin in the labelling buffer containing 1 mM Ca^{2+}. Freshly dissolved disuccinimidyl suberate (DSS) (Pierce, USA) in DMSO was added to all samples to a final concentration of 100 μM and, after 5 min, the cross-linking reaction was stopped by the addition of SDS-PAGE sample buffer. Samples were analyzed on 12.5% (w/v) polyacrylamide gels, which were dried and autoradiographed at –70 °C using Kodak BioMax Light Film (Sigma-Aldrich, USA).

2. 5. Immobilization of Proteins on Polystyrene Microspheres

Amino-modified polystyrene microspheres (IDC, USA), having a diameter of 0.97 μm, were coated with Atx and those with a diameter of 1.80 μm with CaM. The 4% (w/v) stock suspension of microspheres was diluted 10-fold and incubated with 10% (w/v) glutaraldehyde for 80 min with gentle mixing (120 rpm) at room temperature. Microspheres were rinsed twice with doubly distilled deionised water supplemented with 1 mM Ca^{2+} and resuspended in 150 μl of this solution. Optimal surface density of each attached ligand on the spheres was found empirically. It was achieved when 1% of the amount of each protein calculated to completely cover the microspheres was added to suspensions of the appropriate microspheres, which were then incubated overnight at 4 °C with constant stirring (120 rpm). The microspheres were rinsed three times with 150 μl of Ca^{2+}/water and resuspended. BSA was added to the suspension to a final concentration of 5 mg/ml. Samples were stirred (120 rpm) for 45 minutes at room temperature to completely react the remaining reactive groups. Samples of each size of microsphere were finally rinsed three times with 150 μl of 50 mM Tris-HCl, pH 8.4, resuspended in 150 μl of buffer and then mixed together. Derivatized microspheres were stored at 4 °C for a maximum of one week.

2. 6. Laser Tweezers

Laser tweezers enable microscopic dielectric particles to be brought and held together – "trapping" – by using tightly focused laser beams. The trapping mechanism relies on a strong electric field gradient in a focal region of tightly focused laser beam that attracts dielectric particles if their refractive index is higher than that of surrounding medium.[14–16] The maximum laser trap holding force is proportional to the laser beam intensity.

Laser tweezers were built around an inverted microscope Axiovert 200M (Zeiss, Germany). A continuous wave infrared laser Compass 2500MN with a wavelength of 1064 nm (Coherent, USA) was introduced into the microscope through the bottom port and was tightly focused on the sample by a water immersion microscope objective Acroplan 63W – NA 0.9 (Zeiss, Germany). Because of the low water absorption at 1064 nm, the sample temperature in the trap centre rose by less than 1°C. The position and intensity of the trap were precisely controlled at subnanometer resolution by acousto-optic deflectors DTD 274HA6 (Intraaction, USA) driven by a dedicated beam-steering controller BSC160 (Aresis, Slovenia). Two laser traps were generated by time-multiplexing the laser beam

between the traps with a 50 kHz switching rate. Bright field sample images were recorded with a high resolution CMOS camera PL A741 (PixeLink, Canada).

The affinity between Atx and CaM was measured using polystyrene beads of two different sizes, the smaller coated with Atx and the larger with CaM. The beads served as handles for laser tweezers. Coated beads of each type were mixed together in buffer and introduced into the sample cell, made of a microscopic slide and a cover slip glued together with UV-curing glue. All glass surfaces, as well as the polystyrene beads, were coated with a layer of BSA to prevent the beads sticking to the glass and to each other. The interaction between Atx and CaM molecules was established by bringing and keeping two corresponding beads together for a few seconds. The formation of the non-covalent bond was checked by switching off the laser tweezers and observing whether the beads drifted apart due to Brownian motion or not.

2. 7. Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore X system (Biacore AB, Sweden). Atx was covalently immobilized on the surface of flow cell 1 located on a Sensor Chip CM5 from Biacore. Flow cell 2 was mock-immobilized with ethanolamine and used as a reference surface. HBS-EP buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) was used to immobilize approximately 750 response units (R.U.) of protein. SPR experiments were performed at 25 °C in 50 mM Tris-HCl buffer, pH 8.4 or 7.4, and different [Ca^{2+}]s. The interaction between CaM and the chip-immobilized Atx was studied by injecting CaM (0.5, 1, 5, 10, 20 and 30 μM) over the chip at different [Ca^{2+}]s (0, 0.1, 1 and 10 mM). Association and dissociation were followed at a flow-rate of 30 μl/min for 1 min and 3 min respectively. The chip was regenerated between successive experiments with 30 μl of 10 mM NaOH. The experimental curves were fitted according to a 1:1 interaction model using the Biacore BIAevaluation 3.2 software.

3. Results

3. 1. [Ca^{2+}]-dependence of Association Between ^{125}I-Atx and CaM Detected by Chemical Cross-linking

Using chemical cross-linking, the complex between Atx and CaM was formed only when Ca^{2+} ions were present in the incubation mixture (Figure 1, lanes 1 to 5). Removal of free Ca^{2+} ions by EGTA (Figure 1, lane 6) prevented its formation. Using this technique, we scanned the [Ca^{2+}]-dependence of binding between Atx and CaM over the range of 50 μM to 10 mM Ca^{2+}. The amount of the complex increased from 50 μM to 10 mM Ca^{2+}. Atx and CaM contain one and up to four bound Ca^{2+} ions, respectively. To verify whether just structurally bound Ca^{2+} suffices for complex formation or not 20 nM CaM and 10 nM Atx were mixed in the absence of externally added Ca^{2+}, meaning that at most 90 nM Ca^{2+} (20 × 4 nM on CaM and 10 × 1 nM on Atx) was present during the incubation. Following cross-linking the adduct formation was clearly visible (Figure 1, lane 7) demonstrating that sub-micromolar concentration of free Ca^{2+} is enough for both molecules to interact. The possibility that the variation of [Ca^{2+}] affected the yield of the cross-linking reaction and not only the affinity between Atx and CaM was excluded by the laser tweezers and SPR experiments described below.

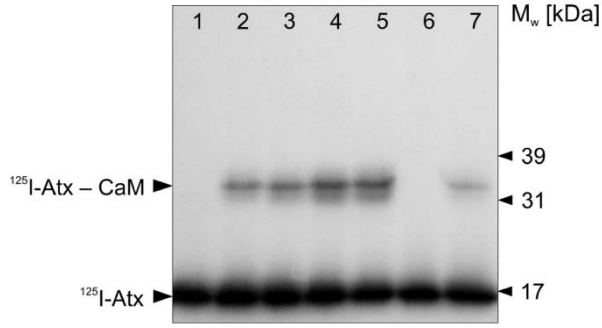

Figure 1: ^{125}I-Atx affinity labelling of CaM as a function of Ca^{2+} concentration. CaM was affinity labelled with ^{125}I-Atx as detailed under Materials and methods in the presence of different [Ca^{2+}]s (lane 1: 1 mM, lane 2: 50 μM, lane 3: 100 μM, lane 4: 1 mM, lane 5: 10 mM, lane 7: 0 M) or EGTA (lane 6: 1 mM). In lane 1, a 500-fold molar excess of Atx over ^{125}I-Atx was present during the incubation to define the specific signals. On autoradiogram, positions of the specific adduct and unbound ^{125}I-Atx are shown by arrowheads.

3. 2. [Ca^{2+}]-dependence of Association Between Atx and CaM Determined by Laser Tweezers

After the interaction between Atx and CaM was ascertained (see under *laser tweezers* in Methods) we measured its strength by laser tweezers. This was done by holding two beads linked by non-covalent Atx – CaM bond in two separate optical traps while the separation between the trap centres was slowly increased. The laser power was kept constant during the measurement. If the link between the beads did not break, another measurement with a higher laser power was performed until the link broke or the maximum laser power was reached. Non-breaking of the link was attributed to the formation of multiple non-covalent Atx – CaM bonds and the experiment was discarded. The laser power at which the bond broke was taken as a quantitative measure of the bond strength. The experiments were repeated at different [Ca^{2+}] in solution while keeping other conditions constant.

Bond strengths were measured for [Ca^{2+}] of 0 μM (0.15, $P < 0.05$), 1 μM (0.33, $P < 0.05$), 10 μM (0.44, $P = 0.079$) and 100 μM (0.54, $P = 0.079$). At least 20 measurements were made at each concentration of calcium. In Figure 2, the values are expressed relative to the value obtained in the absence of externally added Ca^{2+} (of 0 μM). The bond strength at 100 μM Ca^{2+} was 3.7 times greater than that at 0 μM Ca^{2+}. The laser tweezers experiments thus also demonstrated that Atx and CaM interact already when only the protein-bound Ca^{2+} is present. Adding additional Ca^{2+} to the solution led to stronger non-covalent Atx – CaM bond. Reliable measurements could not be obtained above 100 μM Ca^{2+}, the most probable reason being that the bond strength was out of the range of our measurement capabilities. Those results were therefore discarded.

Due to methodological reasons the laser tweezers experiments were carried out at pH 8.4. The SPR measurements presented below showed that the complex between Atx and CaM is equally stable at pH 7.4 and pH 8.4.

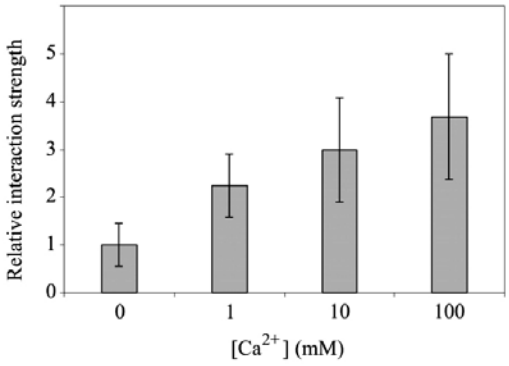

Figure 2: [Ca^{2+}]-dependence of the relative bond strength between Atx and CaM molecules as measured with laser tweezers. Two beads linked together by a non-covalent Atx – CaM bond were pulled apart with laser tweezers and the force to break the interaction was measured. Experiments were performed at four different [Ca^{2+}]s (0, 1 μM, 10 μM, 100 μM) while keeping other parameters constant. At each [Ca^{2+}] at least 20 measurements were made and the SD is shown by error bars.

3. 3. [Ca^{2+}]-dependence of Association Between Atx and CaM Determined by SPR

An SPR sensor chip was prepared with Atx immobilized on the surface. High concentration of 20 μM CaM, which is however in the range of the physiological concentration of CaM in the cytosol of a mammalian cell,[17] was needed to observe the interaction clearly at lower [Ca^{2+}]s. The [Ca^{2+}]-dependence of association between Atx on the chip and CaM is shown in Figure 3. Most importantly, interaction between the two proteins is seen in the absence of added Ca^{2+}. The extent of complex formation increased with increasing [Ca^{2+}], in agreement with the affinity labelling and laser tweezers results. Within the limits of experimental error, the results were identical using the binding buffer with the pH of 7.4 or 8.4. Association rate constant (k_a) for the interaction between Atx and CaM increased about 10-fold in the range from 0 to 10 mM Ca^{2+} in the buffer. On the other hand the dissociation rate constant (k_d) did not change by more than 10% over the same range of [Ca^{2+}]. Accordingly, the dissociation constant K_d for the interaction was about 10-fold higher at sub-micromolar [Ca^{2+}] than at millimolar Ca^{2+}.[18]

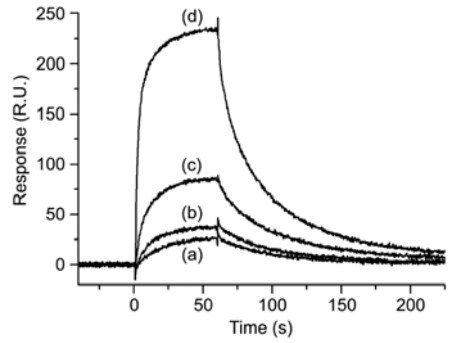

Figure 3: The [Ca^{2+}]-dependence of interaction between Atx and CaM measured by surface plasmon resonance. Atx was covalently immobilized on the sensor Chip CM5. Sensorgrams were recorded using 20 μM human CaM in 50 mM Tris-HCl containing, from (a) to (d), 0 mM, 0.1 mM, 1 mM and 10 mM Ca^{2+}. The association was followed for 1 min and the dissociation for three minutes.

4. Discussion

Three techniques, affinity labelling, laser tweezers and SPR, were employed to determine the [Ca^{2+}] dependence of Atx – CaM binding. The combined results showed that a complex (1 : 1 according to native electrophoresis performed at pH 7.4 and 8.4, results not shown) is formed as long as the structurally bound Ca^{2+} ions are present. The extent of complex formation depended on the [Ca^{2+}] in solution, as is evident from the affinity labelling and SPR experiments (Figures 1 and 3). In the same way, as indicated by the laser tweezers and SPR results, the binding of Atx to CaM increased with increasing [Ca^{2+}]. In the absence of added Ca^{2+}, the K_d for binding of Atx to CaM was sub-micromolar, which would ensure interaction with CaM in the mammalian cytosol, where its concentration can be as high as 10 μM.[17]

The most obvious general effect of presynaptically toxic $sPLA_2$s is impairment of synaptic vesicle cycling. It appears that these toxins promote Ca^{2+}-regulated neurosecretion and block the retrieval of synaptic vesicles from the plasma membrane of the motoneuron.[3] The first evidence that CaM is required in neurosecretion came from functional dissection of exocytosis in model systems such as permeabilized bovine chromaffin cells and in rat PC12 cells.[19,20] Later,

it was found that CaM may provide one of the links between Ca^{2+}-signalling and SNARE assembly. The binding site on the v-SNARE synaptobrevin for Ca^{2+}-loaded CaM (Ca^{2+}/CaM) was identified.[21] Precisely the same site binds to acidic phospholipids, and lipids can be competitively displaced by Ca^{2+}/CaM. Approaches using both mimetic peptide injection and directed mutagenesis in neuroendocrine cells indicated that CaM and/or phospholipid interactions with synaptobrevin are required for Ca^{2+}-dependent exocytosis.[17,22,23] In neurons some evidence points also to regulation by CaM of the refilling of readily releasable vesicle pool[24] while, in adrenal chromaffin cells, it was found that CaM is the Ca^{2+} sensor for rapid endocytosis.[25] In addition, presynaptically toxic $sPLA_2$s inhibit protein phosphorylation[26] and reduce terminal K^+-currents in motoneurons[27], both processes being regulated by CaM in neuronal cells.[7,28,29]

On depolarization of the presynaptic membrane, the intracellular free [Ca^{2+}] increases locally up to 100 μM. According to our results this is the calcium ion concentration at which Atx and CaM form a tight complex. It is thus possible that, by binding to CaM, Atx affects exo-/endocytosis, protein phosphorylation and the conductivity of certain ion channels in motoneurons. Conversely, since CaM is known as the modulator of activity of various enzymes[7], it could also be expected to influence the phospholipase activity of $sPLA_2$s.

Atx enters the cytosol, as demonstrated in hippocampal neurons.[5] Neurotoxic $sPLA_2$s are members of group IA, IIA and IIIA $sPLA_2$s[3] and are structurally closely related to mammalian group I, II and III $sPLA_2$s.[30] Endogenous $sPLA_2$s have been detected in cellular compartments on the way into which they should pass the cytosol. For example, group I $sPLA_2$ was translocated from outside the normal rat uterus stromal cell (U_{III} cell line) to its nucleus[31], group IIA $sPLA_2$ was detected in rat brain cortex mitochondria[32] and group V $sPLA_2$ was found in nuclei of PC12 and U251 astrocytoma cells.[33] Interaction between CaM and mammalian endogenous $sPLA_2$s has not been evaluated, but some of the various effects that these enzymes produce in different types of cells[2] may well be the result of their interaction with CaM in the cytosol of these cells.

In this study we demonstrated that already sub-micromolar [Ca^{2+}]s support the interaction between CaM and the snake venom $sPLA_2$. The presented results envisage the existence of CaM-mediated (patho)physiological/pharmacological effects of $sPLA_2$s, among them also presynaptic toxicity.

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Povzetek

Z namenom demonstracije, da se amoditoksin, nevrotoksična sekretorna fosfolipaza A_2, lahko veže na kalmodulin, regulatorni protein v citosolu, tudi *in vivo*, smo raziskali odvisnost tvorbe kompleksa med tema dvema proteinoma od Ca^{2+}. Ugotovili smo, da je interakcija med proteinoma pozitivno odvisna od koncentracije teh ionov, afiniteta vezave pa najvišja v območju milimolarnih koncentracij. Ključnega pomena je ugotovitev, da interakcija med kalmodulinom in fosfolipazo A_2 obstaja že v prisotnosti sub-mikromolarne koncentracije Ca^{2+}, kar smo demonstrirali s pomočjo afinitetnega označevanja, z lasersko pinceto in tehniko površinske plazmonske rezonance. Ob depolarizaciji aksoleme se lokalno, na področju aktivnih con, intracelularna koncentracija prostega Ca^{2+} dvigne do 100 μM, kar zagotavlja nastanek stabilnega kompleksa med kalmodulinom in nevrotoksično fosfolipazo A_2. Predstavljeni rezultati pritrjujejo hipotezi, da je vezava amoditoksina na kalmodulin korak v procesu presinaptične nevrotoksičnosti te in sorodnih nevrotoksičnih sekretornih fosfolipaz A_2. Kažejo pa tudi na možnost, da so nekateri, mehanistično še nepojasnjeni (pato)fiziološki procesi, ki jih povzročajo endogene sekretorne fosfolipaze A_2, posledica interakcije teh encimov s kalmodulinom v celičnem citosolu.