FINAL REPORT FOR PD104344

CONTROL OF THE GROWTH ASSOCIATED PROTEIN 43 – CALMODULIN INTERACTION BY THE SIGNALLING LIPIDS SPHINGOSINE AND LYSOPHOSPHATIDIC ACID

The brain-abundant phosphoprotein, the growth-associated protein 43 (GAP43) is considered a crucial component in axon guidance, synaptic plasticity and neuroregeneration, its action is under the control of Ca^{2+} signals. Calmodulin (CaM), the ubiquitous Ca^{2+} sensor of eukaryotic cells, is involved in a wide variety of signalling events, regulating the activity of numerous proteins in a Ca^{2+}-dependent manner. Their interaction was verified both *in vitro* [1] and *in vivo* [2], and the GAP43-CaM complex is suggested to be bound to the membrane.

We hypothesized that the molecular state, and therefore, the activity of both GAP43 and CaM can be regulated by the signalling-induced local accumulation of lipid mediators.

First we determined the selectivity of the CaM/GAP43–lipid mediator interactions, and characterized the lipid-protein affinities quantitatively.

Utilizing fluorescence spectroscopy and protein cross-linking, we have shown that GAP43 binds selectively to lysophosphatidic acid (LPA), while CaM to sphingosine (Sph). We have also found that in both interactions the lipids are in an associated form, resembling lipid clusters which might easily occur in natural membranes. It is worth to note, that Sph binds to both apo- and calcium-bound CaM (Ca^{2+}CaM), that is in the absence and the presence of calcium ions. GAP43 binding to LPA was obviously also affected by the Ca^{2+} level, however, this effect can be attributed to the binding of Ca^{2+} ions to the phosphate moiety of the lipid headgroup, which shields the phosphate negative charge thereby the interaction area.

The lipid-protein affinities for the CaM-Sph interaction are in the nM-range as indicated by isothermal titration calorimetry (ITC, Fig. 1). This technique revealed two binding processes in the CaM-Sph interaction. According to the stoichiometry obtained, at lower protein to lipid ratio CaM could bind to Sph micelle surface, while a higher CaM access could lead to micelle disintegration. In the absence of Ca^{2+}, mixed protein-lipid micelles might form, and in the presence of Ca^{2+}, several Sph monomers might bind to a CaM molecule. A micelle disintegration process has been detected by fluorescence studies using ANS, a lipid micelle indicator dye.

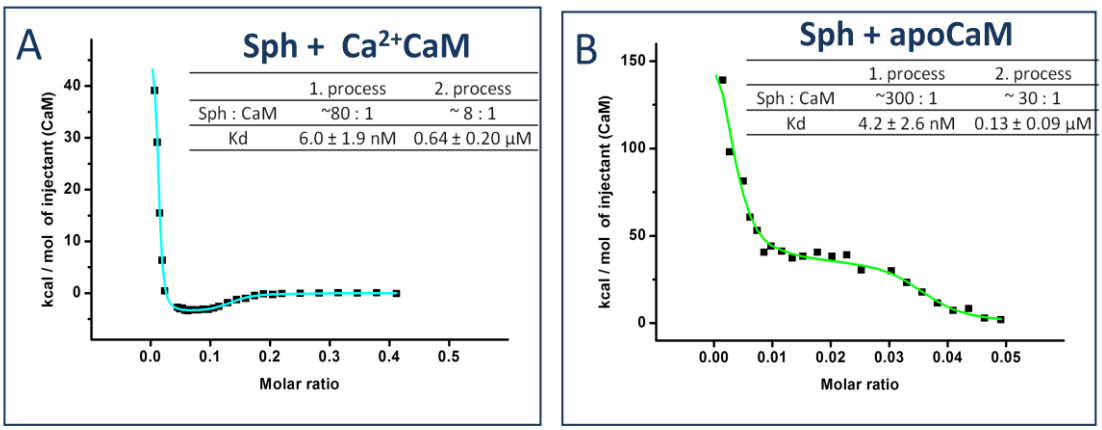

Fig. 1: The stoichiometry and affinity of the CaM-Sph interaction determined by ITC

The crystal structure of the Ca^{2+}CaM-Sph complex (Fig. 2), successfully determined in collaboration with the Protein Crystallography Laboratory of HAS-ELTE, showed an evidence that CaM might bind not only to a Sph surface, but it can also accommodate a few Sph monomers as suggested by the ITC titration. In our x-ray structure, the CaM is in an overall collapsed conformation, characteristic of the CAM-target peptid complexes, while wrapping around several Sph molecules occupying the target peptid binding site (Fig. 2B). This arrangement allows an inhibition of CaM by Sph of a competitive type. The lipids showed lower electron densities so that not all parts of the molecules could be determined (Fig. 2A), which was also seen for another CaM-lipid, the Ca^{2+}CaM-SPC structure [3], and indicates higher flexibilities of the lipid moiety in the complex.

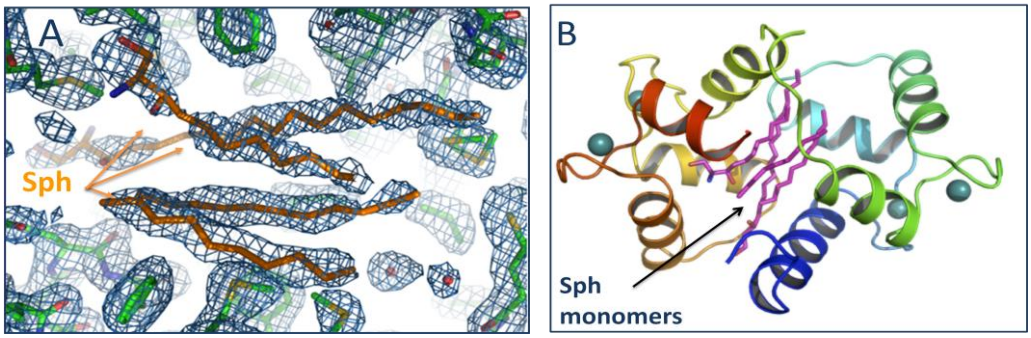

Fig. 2: The crystal structure of the Ca^{2+}CaM-Sph complex

Our results obtained by *in vitro* functional assays performed with several CaM-dependent enzymes showed that CaM binding to the Sph in its associated form can also inhibit CaM function (Fig. 3). We have shown that the presence of Sph micelles could inhibit

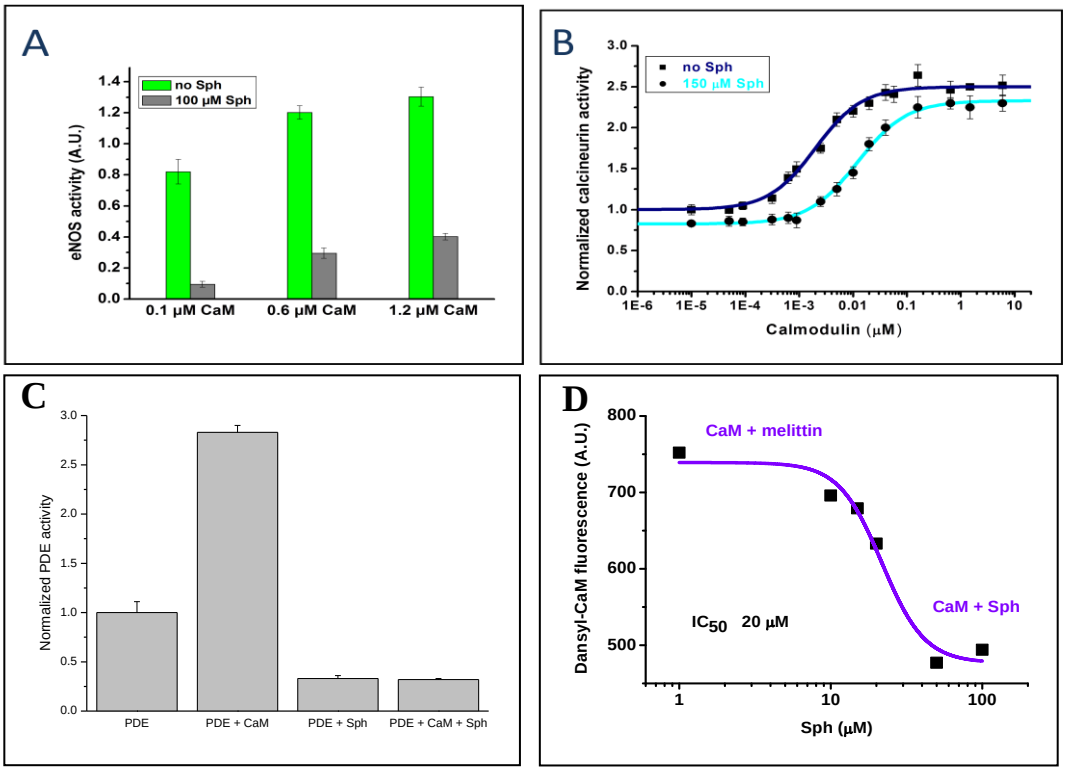

Fig. 3: The effect of Sph to the CaM action: *in vitro* functional assays

the calcineurin-, phosphodiesterase (PDE)-, and endothelial nitric oxide synthase (eNOS)-activating ability of CaM (Fig 3A-C), suggesting an inhibitory role for Sph above its CMC (critical micelle concentration) value. The latter *in vitro* finding was confirmed by *ex vivo* results provided by our collaborators at the Semmelweis University suggesting the inhibition of the NO-mediated (eNOS-dependent) vasorelaxation of mouse thoracic aorta by Sph (Fig. 11). Our result that clustered Sph was able to displace melittin, the CaM-binding domain model peptide, from CaM (Fig. 3D), strengthens the idea that Sph might be a potent CaM-antagonist.

In the case of GAP43, the presence of the LPA micelles causes oligomerization of the protein as suggested by the increased protein size detected by protein cross-linking (Fig. 5) and dynamic light scattering (DLS, Fig. 4A). The size enhancement measured is consistent with the formation of round-shaped oligomers reported earlier for the protein in the presence of SDS [4]. The size of the oligomers was depending on the protein to lipid ratio applied. At higher LPA concentrations providing higher area to the interaction, oligomers containing fewer GAP43 molecules formed (Fig. 4C). Utilizing circular dichroism (CD) spectroscopy, some structure rearrangement of the intrinsically disordered (IUP) GAP43 protein could be detected (Fig. 4B). Similar spectrum change was reported for the GAP43 in the presence of other lipids bearing negative charge in the headgroup, but the effect was not attributed to the protein oligomerization [5]. The small but reproducible and characteristic change in the CD spectrum can be referred as a disorder-to-order transition of a part of the protein upon oligomerization. Regarding CaM, no structural change upon binding to Sph could be seen utilizing CD.

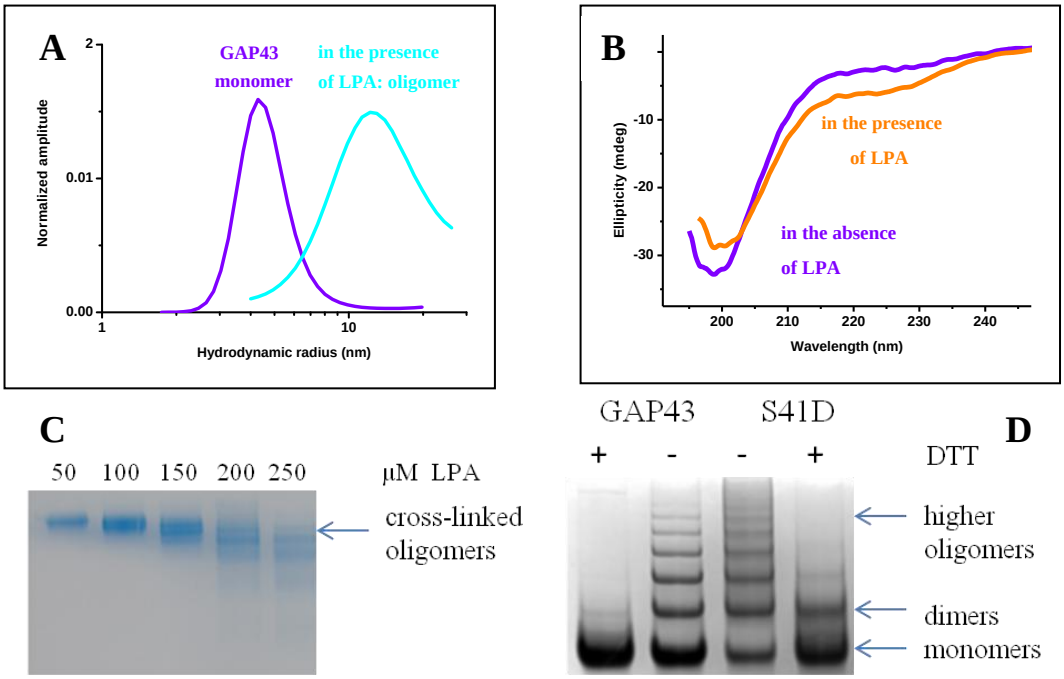

Fig. 4: Structure and oligomerization of GAP43 studied by (A) DLS, (B) CD spectroscopy, (C) SDS PAGE, and (D) native PAGE.

It was also observed that GAP43 is prone to oligomerize upon longer storage as detected by native gelelectrophoresis (PAGE, Fig. 4D). The oligomers might be stabilised by disulfide bridges since the monomeric (dimeric) state could be regained from the higher oligomeric state upon addition of reducing agents like DTT.

It is known that the action of GAP43 is affected by its phosphorylation on Ser41, since only the phosphorylated protein favors filopodial extensions [6]. All the experiments were performed with the non-phosphorylated GAP43 (recombinantly expressed "wild-type", wt.) as well as with the Ser41Asp variant, mimicking the permanently phosphorylated protein. The variants behaved similarly in both structural and lipid binding assays. Their binding to LPA was of similar strength as determined by protein cross-linking (Fig. 5) and ITC (Fig. 8A). Concretely, we could evaluate apparent binding constants in the low micromolar range (~2.5 and ~6 µM for the wild-type and the mutant protein, respectively). This suggests that the lipid binding properties of GAP43 might not change dramatically upon phosphorylation.

After describing the lipid-protein interactions, we have characterized the CaM-GAP43 interaction in the presence of the lipid mediators using *in vitro* binding and functional assays. According to the considerations above, all experiments were performed i) in the presence or the absence of lipid micelles or liposomes containing Sph or LPA, ii) with the non-phosphorylated GAP43 as well as with the Ser41Asp variant, mimicking the permanently phosphorylated protein, and iii) in the presence and the absence of Ca^{2+} ions. In addition, we also utilized the synthesized CaM-binding motif (IQ domain) of GAP43, and its Ser41-phosphorylated form (pIQ) to study further the effect of phosphorylation since the GAP43 S41D variant bearing a negative charge on the site of phorphorylation only mimicks the phosphorylated protein while the sizes of the modified groups are clearly different.

Utilizing protein cross-linking, we have shown that addition of CaM did not significantly affect the oligomerization of GAP43 in the presence of LPA micelles, and the

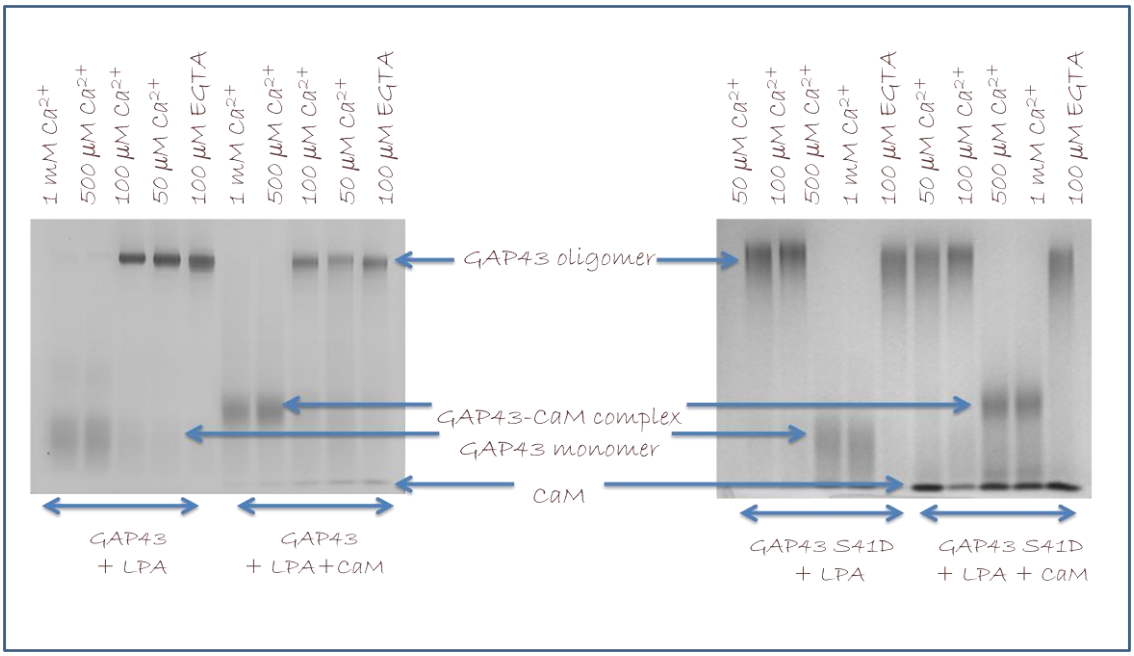

Fig. 5: Oligomerization of GAP43 in the presence of LPA and CaM (protein cross-linking)

CaM-GAP43 complex formation is effaced in the presence of the lipid (Fig. 5, left panel). Similar behaviour was observed for the phosphorylation-mimicking S41D mutant suggesting again that the lipid binding properties are similar for the two variants (Fig. 5, right panel). We have also found that high amounts of Ca^{2+} can bind to and bridge the LPA micelles and, thereby, decrease the free micelle surface, which in turn seemingly favours the protein-protein interaction (Fig. 5). Since Sph bears a reactive primer amine group, mixtures containing Sph are not suitable for cross-linking reactions *via* amine-coupling used here.

Utilizing fluorescence spectroscopy, the apparent dissociation constant (K_d) for the protein-protein interaction was determined by titration of the in-house dansyl-labelled CaM (dCaM) with GAP43. This technique is very sensitive to the conformation of CaM, the dCaM fluorescence changes easily upon addition of Ca^{2+}, the lipids Sph and LPA, and the protein binding partner GAP43. Evaluation of the titration curves allowed the following observations:

- using Ca^{2+}-saturated CaM (Fig. 6A and 6C),

i) addition of GAP43 causes a spectrum change similar to that of binding of the model target peptide melittin or the lipid Sph,

ii) the GAP43–CaM affinity is in the high nanomolar range (~0.5 µM),

iii) the K_d is slightly lower with the non-phosphorylated GAP43 variant (~2-3-fold),

iv) the K_d is similar with the non-phosphorylated GAP43 full protein and their IQ domain,

v) the K_d is significantly lower with the pIQ than with the IQ motif (~0.5 vs. ~15 µM),

vi) the fluorescence change upon binding is dependent on the oligomeric state of GAP43, the oligomers give higher signal enhancement and somewhat different K_d values.

- using apo-CaM (Fig. 6B and 6D)

i) practically no change in the dCaM fluorescence was detected when titrating with monomeric (dimeric) GAP43 variants so that no K_d values could be determined

ii) titration with the IQ motif also resulted in no signal change

iii) GAP43 samples containing higher amounts of oligomers gave higher signals yielding K_d values of ~10 µM.

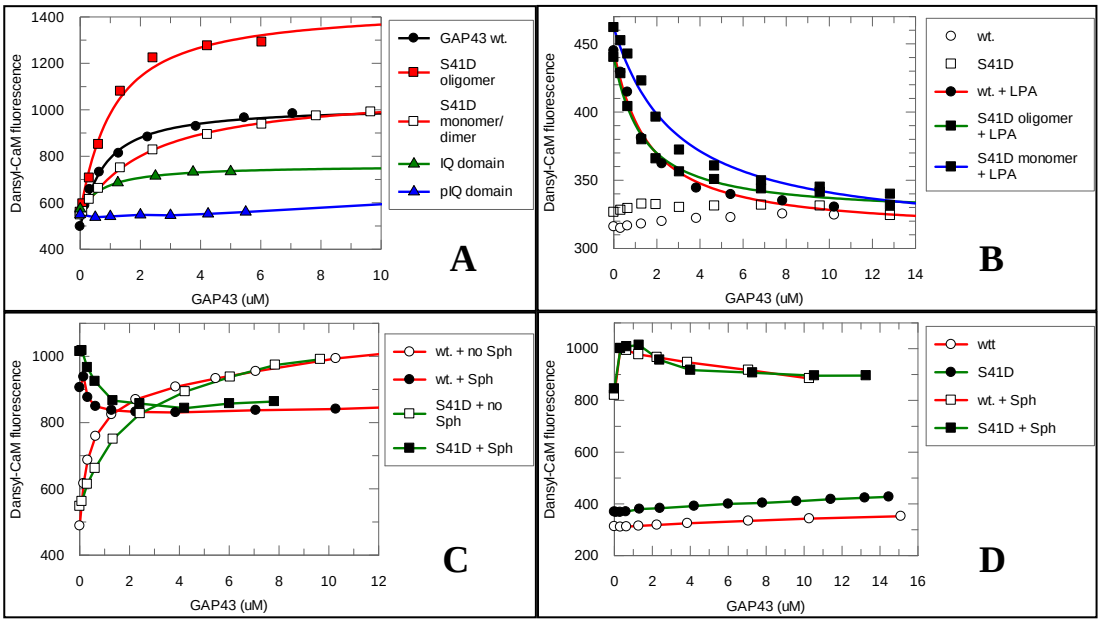

Fig. 6: Titration of dansyl-CaM with GAP43 in the presence and absence of LPA and Sph

Testing the effect of the lipid mediators LPA and Sph, both lipids were shown to influence the binding of GAP43 to dansyl-CaM. We have found that

i) some binding of LPA to apo-CaM was indicated by the increased dCaM fluorescence, and the bound LPA was obviously displaced by GAP43 (Fig. 6B). The data could be fitted to a saturation curve yielding K_d values of ~2 μM, which is in good agreement with the values obtained for the GAP43-LPA interaction by ITC (Fig. 8A),

ii) using Ca^{2+}CaM, LPA could affect the binding of GAP43 to CaM only when Ca^{2+} ions at concentrations lower than that of LPA were added as also seen using SDS PAGE,

iii) titrations with GAP43 in the presence of Sph using Ca^{2+}CaM or apo-CaM (Fig. 6C and 6D) only slightly changed the dCaM fluorescence elevated upon addition of Sph, which is consistent with a binding affinity higher for Sph over GAP43,

iv) the two GAP43 variants behaved similarly.

Probing GAP43 binding to immobilized CaM in real time using quartz crystal microbalance (QCM), we could detect a complex binding pattern (Fig. 7A). Fitting to a model assuming two sites of binding, we could evaluate somewhat different relative affinities compared to those obtained by fluorescence spectroscopy. Concretely, the K_d values estimated from the QCM sensorgrams indicated i) similar affinities in the absence and the presence of calcium ions for both GAP43 variants, and ii) approx. 4- and 10-fold higher affinities for the wild type GAP43 over the S41D mutant for Ca^{2+}-saturated CaM and apo-CaM, respectively. Moreover, the K_d values were approx. 4-fold lower than those calculated from the fluorescence titrations suggesting that immobilisation of CaM might facilitate GAP43 binding. We have also observed different binding affinities and kinetics for the monomeric/dimeric and the higher oligomeric GAP43 forms (Fig. 7A). Oligomers showed slower association and dissociation to and from the CaM surface.

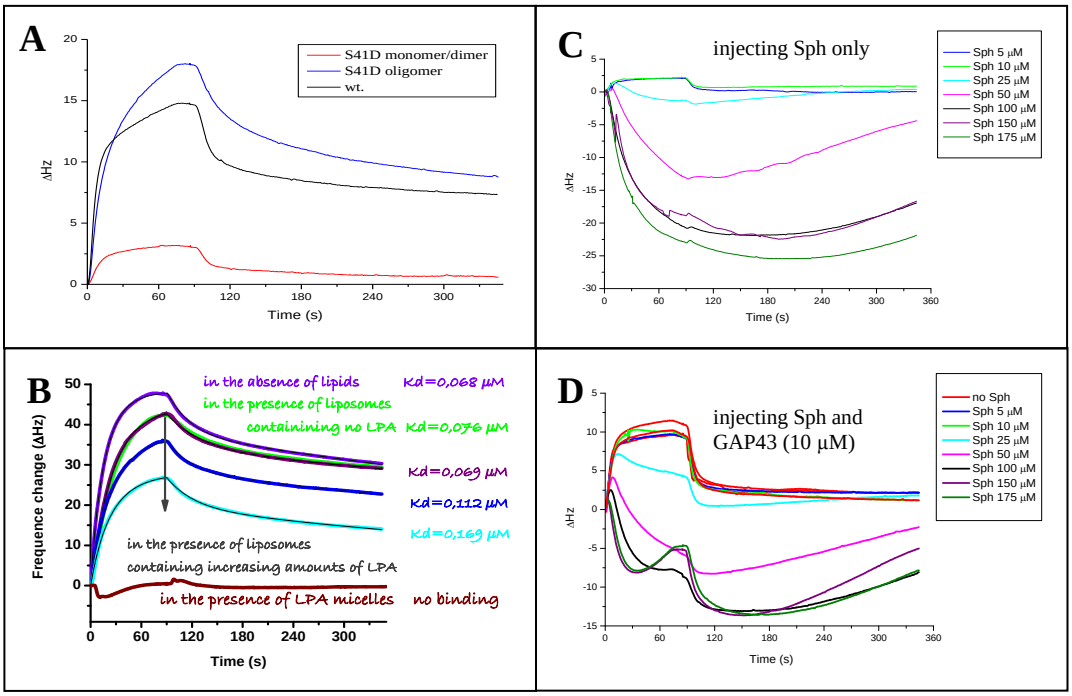

Fig. 7: GAP43 and lipid binding to immobilised CaM studied by QCM

Testing GAP43 binding in the presence of LPA or Sph, preincubation of GAP43 with LPA micelles could abolish GAP43 binding to the immobilised CaM (Fig. 7B). Similarly, the presence of LPA incorporated in liposomes mimicking membrane rafts decreased GAP43 binding (Fig. 7B). Sph alone showed a special binding pattern on the QCM chip (Fig. 7C), which might be explained by decreased viscosity upon addition of Sph micelles. Injecting GAP43 and Sph micelles together resulted in curves which are seemingly the combination of those of Sph and GAP43 alone suggesting that Sph binding to CaM compete with GAP43 binding (Fig. 7D). These curves indicated several binding events of clearly different affinities and kinetics, and are consistent with a scenario as follows: first might bind one of the GAP43 forms, then Sph might bind somewhat slower but stronger, thereafter another GAP43 form might bind. It was also seen that Sph at concentrations below its CMC (critical micelle formation concentration, ~10-15 µM for Sph) showed no detectable binding to CaM (Fig. 7C), and did not disturb GAP43 binding to CaM (Fig. 7D).

Isothermal titration calorimetry is useful to determine the stoichiometry and thermodynamic parameters of interactions. However, titration of GAP43 with CaM or titration of CaM with GAP43 resulted in no curves suitable for evaluation when using the MicroCal iTC200 instrument available in our institute. Most possibly, the heat produced upon the binding is small, which is common among IUP interactions, where the binding is entropically driven. Indeed, the reported enthalpy values for the CaM-GAP43 interaction [7] is fairly low to be studied with our instrument. However, the binding affinities in the three-component systems could be investigated in competitive experiments, studying the effect of the protein binding partner for the characteristic ITC curves obtained for both the CaM-Sph (Fig. 1) and the GAP43-LPA interactions (Fig. 8A). The competitive titration of the apo-CaM-LPA mixture with GAP43 resulted in a curve very similar to that of titration of LPA only with GAP43 suggesting the dominance of the lipid-protein interaction over the protein-protein binding again (Fig. 8B).

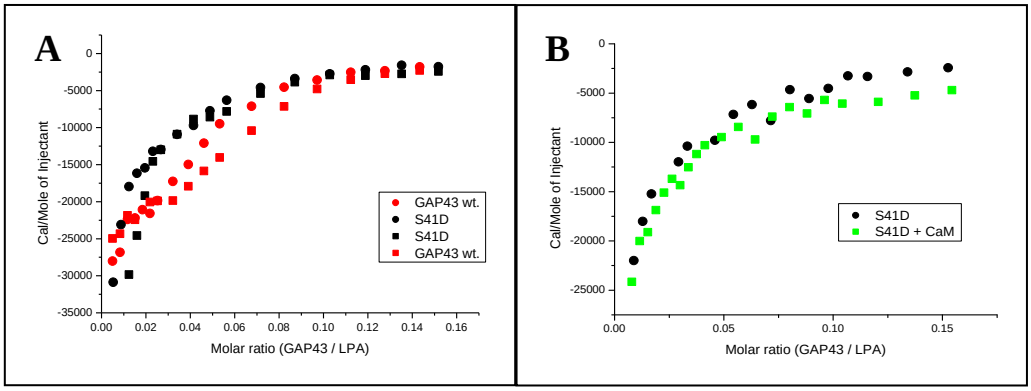

Fig. 8: Affinities in the CaM-GAP43-LPA system studied by ITC

In our institute is now available a novel instrument for studying biomolecular interactions utilizing microscale thermophoresis (MST). Since this method is very sensitive to the presence of aggregates or oligomers, thus the possible different behaviour of the various oligomeric forms of GAP43 could also be studied. For these measurements we labelled CaM and GAP43 with a red fluorescent dye. The presence of the oligomers were clearly seen in the

thermophoresis curves (Fig. 9A), however, as the titrations resulted in change in fluorescence of the labelled protein as well, the fluorescence signals were used for the evaluation (Fig. 9B). Using labelled CaM, we obtained very similar curves yielding in turn very similar K_d values in the high nanomolar range for the GAP43-CaM interaction, obviously independently of which GAP43 variant, and the apo- or the Ca^{2+}-saturated CaM was present. Experiments with the labelled GAP43 resulted in curves yielding K_d values in the low micromolar range, approx. one order of magnitude higher than those obtained with labelled CaM. This indicates that several lysines are in the vicinity of the CaM-binding region of GAP43, the labelling of which could interfere with CaM binding resulting in apparent weaker binding. We have also performed a couple of experiments in the three-component systems (in the presence of the lipids Sph and LPA), however, the data are not consistent to interpret yet. The method requires handling with small volumes of diluted samples, the protein and lipid content of which can easily adsorb to the plastic surfaces, thus it is not clear yet whether it is a real binding event what we see in the titration experiments.

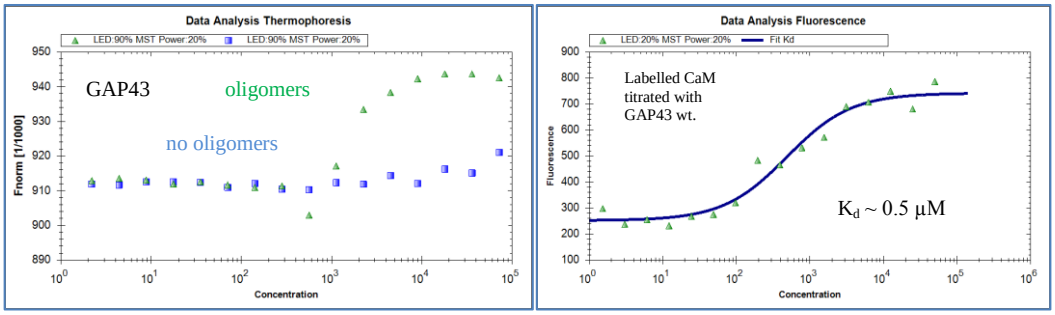

Fig. 9: Affinities for the CaM-GAP43 interaction studied by MST

It is worth to mention that various affinity values have been reported for the GAP43-CaM interaction. GAP43 was believed to bind to CaM with a higher affinity in the absence of calcium ions, in contrast to the majority of the proteins containing IQ motifs. However, similar K_d values of ~0.3 µM were also reported for its interaction with the apo- and the Ca^{2+}CaM [8]. Our findings are rather consistent with the latter data. As for the phosphorylation, GAP43 was believed to release from CaM when phosphorylated. In contrast, our results with the phosphorylation-mimicking Ser41Asp variant suggested only moderate decrease in affinity to CaM compared to the non-phosphorylated GAP43. However, the phosphorylated IQ domain showed appreciably lower affinity than the non-phosphorylated one. This might suppose that the Ser→Asp change was not the right construct to be investigated in this case although this replacement is widely used to mimic phosphorylation, even to study GAP43 function [9]. However, these results can also be interpreted with the different calcium-binding properties of the carboxyl or phosphate groups rather than the affinity to the CaM-binding partner.

To probe the biological relevance of our results obtained in *in vitro* binding assays, we performed experiments using cell lines of neuronal origin, expressing CaM and GAP43 endogenously. After testing several cell lines (SK-N-MC, PC-12, Neuro-2a, SH-SY5Y) we worked with the mouse neural Neuro-2a (N2a) cell line that has been extensively used to study axonal growth and signalling pathways. The co-localisation in differentiated cells

growing neurites were studied using antibodies recognising the phosphorylated ($GAP43_p$), non-phosphorylated ($GAP43_{nonp}$) or both GAP43 forms, and CaM. $GAP43_{nonp}$ was shown to be distributed both in the cytosol and at the plasma membrane (Fig. 10A) while $GAP43_p$ was rather membrane-associated (Fig. 10B). We were not able to detect a strong co-localisation between CaM and GAP43 in non-treated N2a cells, although partly co-localisation of the two proteins could be seen along the plasma membrane, and the neurites (Fig. 10C).

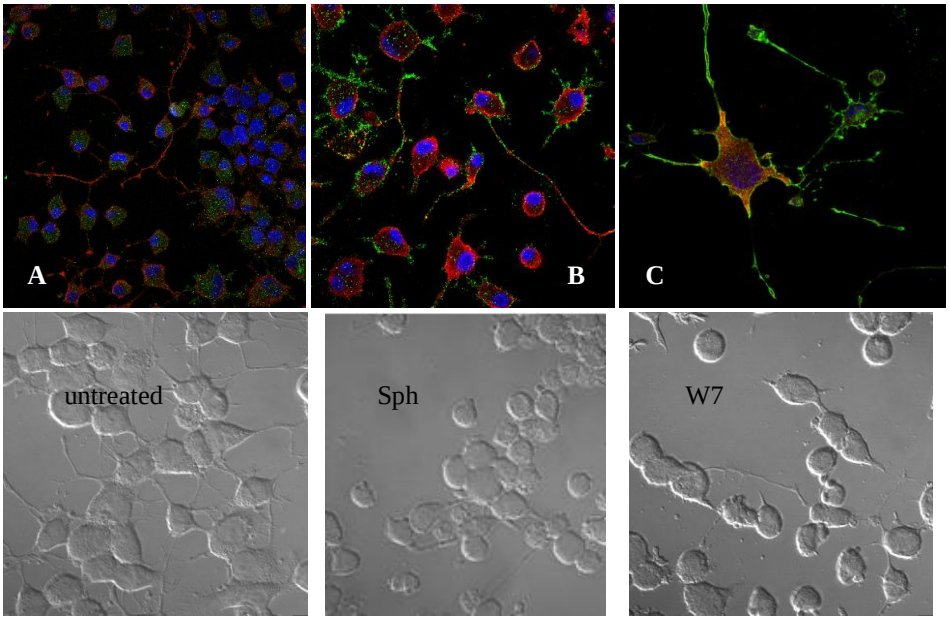

Fig. 10: Co-localisation of GAP43 (green) and CaM (red) (upper panel), and the effect of Sph and W7 on the morphology of N2a cells (lower panel)

The effect upon treatment with lysolipids was also investigated. Sph added outside the cells is able to penetrate the membrane and flip-flop between the leaflets, so it easily accumulates in the inner leaflet of the plasma membrane, the possible site of the interactions to be investigated. Upon addition of Sph, we have found that Sph caused neurite retraction in N2a cells (Fig. 10). Inhibition of neurit outgrowth by Sph was already reported for PC-12 cells differentiated with NGF (nerve growth factor) [10]. Sph was suggested to act *via* the inhibition of the protein kinase C (PKC) [10]. However, we hypothesized that Sph might act *via* its binding to CaM. Therefore we investigated the effect upon treatment of known small molecule CaM-antagonists like W7 and TFP, which mainly hydrophobic compounds can also accumulate in or travel through the cell membrane. We could observe the same effect of neurite retraction as seen with Sph, however, to a lesser extent, which can be attributed to their lower affinity to CaM (the K_d values for Sph, W7, and TFP are <1 µM, ~5-15 µM, and ~1-5 µM, respectively) and/or their weaker ability to go across the membrane compared to Sph. Nevertheless, these results are consistent with a mechanism, in which the effect of Sph might be mediated *via* its CaM-antagonist property.

Neurite retraction was also reported for another lysolipids like LPA and S1P (sphingosine-1-phosphate) using PC-12 cell lines [11, 12]. The effect of the latter two phospholipids that are not able to go across the membrane was shown to be receptor-

mediated. Adding LPA and S1P exogenously, we could reproduce the phenomenon. To study the effect of the accumulation of LPA in the inner leaflet of the membrane, the putative interaction site, activation of the LPA producing enzymes acting on the membrane lipids is needed. The development of such a model system is in progress in our research group.

As mentioned above, we also have *ex vivo* results on regulation of the activity of CaM by Sph. In a collaboration, we investigated the acetylcholine-induced eNOS-mediated relaxation before and during incubation of intact mouse aorta vessels with Sph. We have found that treatment of the vessels with Sph induced significant rightward shift of the acetylcholine dose-response curve, while Sph also showed a tendency to decrease the maximal relaxing effect (Fig. 11). No significant effect of Sph on the direct NO-donor SNP-mediated vasorelaxation could be detected, which indicated that the inhibitory effect of Sph is not due to the decreased NO sensitivity of the vascular smooth muscle. NO under physiologic conditions is a vasorelaxant and attenuates inflammation, while its increased production leads to the initiation and progression of inflammation. Our results support the idea that the vascular effects of sphingosine might be due to its influence on the activity of Ca^{2+}-calmodulin-dependent eNOS enzyme *via* binding to calmodulin thereby preventing eNOS activation.

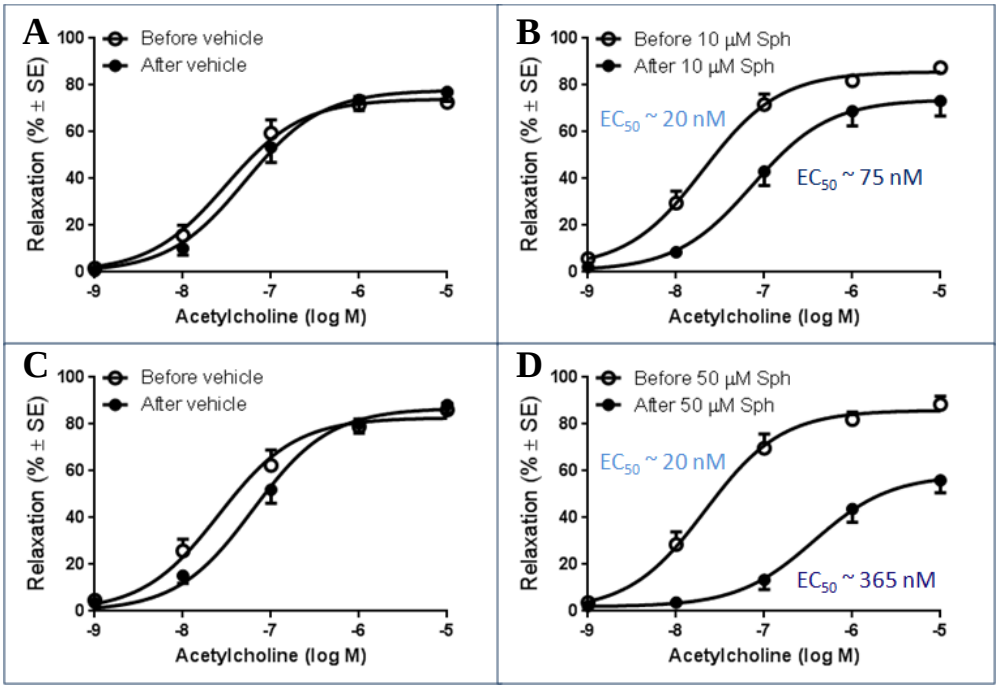

Fig. 11: Effect of Sph on the vasorelaxation of isolated mouse aorta vessels

In summary, we have characterized the GAP43-LPA, and the CaM-Sph protein-lipid interactions as well as the affinities in the four-component system in *in vitro* binding and functional assays, and tested the biological relevance of these protein-lipid interactions in cell lines and in *ex vivo* conditions. We have demonstrated that the lipid mediators LPA and Sph can influence the GAP43-CaM interaction. Our results show a promising way of regulation of GAP43 and CaM by the lipid mediators LPA and Sph, respectively, and can contribute to the understanding of complexity in cellular signalling as well as of protein-lipid interactions.

The results of the research were presented as poster presentations at national and international conferences as follows:

- Magyar Biokémiai Egyesület Jelátvitel Konferenciája, Esztergom, 2012
- 43. és 45. Membrán-transzport Konferencia, Sümeg, 2013 és 2015
- Sphingolipid Club Annual Meeting, Assisi, Italy, 2013
- FASEB Conference: Lysophospholipid and Other Related Mediators - From Bench to Clinic, Sapporo, Japan, 2013
- International Ceramide Conference and Sphingolipid Club Joint Meeting, Izmir, Turkey, 2015
- Bioactive Lipids in Cancer, Inflammation, and Related Diseases Conference, Budapest, 2015

The results concerning the CaM-Sph interaction are ready to publish. Results characterizing the CaM-GAP4 interaction in the presence of the lipid mediators LPA and Sph are near to publish.

In addition to the main project of this OTKA project, we joined a research seeking for novel drugs against malaria. Calmodulin, the key protein of our research was suggested a potencial target of some phenotypically active compounds of the Malaria Box assembled by MMV, the Medicines for Malaria Venture foundation. To test the *in silico* prediction of our collabolators at the Institute IMIM in Barcelona, we have tested ~ 40 MMV compounds in *in vitro* functional assays for inhibiting the calmodulin from Plasmodium falciparum (Pf), the causative agent of the most dangerous form of malaria. For these assays, we recombinantly expressed, purified, and characterized Pf CaM for the first time. Among others, we have found a potent antagonist, the interaction of which with Pf CaM was characterized utilizing fluorescence spectroscopy, functional assays with several CaM-dependent enzymes, and ITC. A crystal structure of the Pf CaM, especially complexed with an antagonist could also help drug design against malaria. Crystallization trials of the CaM-antagonist complexes (in collaboration with the Protein Crystallography Laboratory of HAS-ELTE) yielded small crystals, unfortunately, they are not suitable for structure determination yet. Nevertheless, the results of the binding and functional assays were satisfactory enough for publication together with the results obtained by our collabolators in Barcelona. However; the laboratories collecting data on the MMV malaria project decided to publish all their findings in a review-like paper, which would give the perspective of the mechanism of action of the potent anti-malarial MMV compounds. The assembling of this paper is in progress.

On the other hand, we solved the structure of Pf CaM complexed with the CaM-binding model peptide melittin, revealing several binding modes of the peptide to the protein (Fig. 12A). The human CaM-melittin complex, also not been reported yet, could also be crystallized, showing difference in the conformation of both the protein domains and the bound peptide, compared to the Pf CaM complex. In addition, we performed a comparative study using several biophysical and biochemical methods, and have shown that the bovine/human and Pf CaMs share several features involving inhibitor, and lipid binding to CaM, and enzyme activation by CaM (Fig. 12B). After the structure determination of the

human CaM-melittin complex, the results characterizing Pf CaM compared to mammalian CaMs are ready to publish.

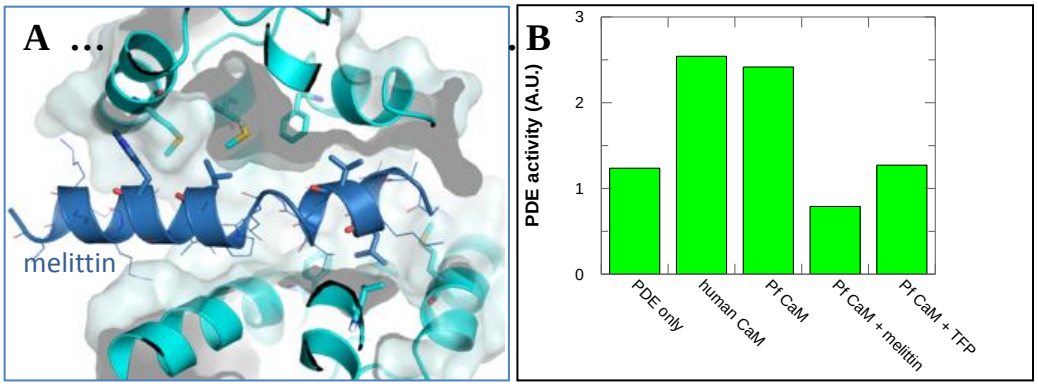

Fig. 12: A) The crystal structure of the Pf CaM-melittin complex, and B) effect of known CaM-antagonists on the activation of PDE by Pf CaM.

All the findings obtained by the support of the OTKA are expected to be published within the next year, and will contribute to the success of this proposal.

References:

1. Andreasen, T.J., et al., *Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes.* Biochemistry, 1983. **22**(20): p. 4615-8.
2. Gamby, C., et al., *Growth-associated protein-43 (GAP-43) facilitates peptide hormone secretion in mouse anterior pituitary AtT-20 cells.* J Biol Chem, 1996. **271**(17): p. 10023-8.
3. Kovacs, E., et al., *Structure and mechanism of calmodulin binding to a signaling sphingolipid reveal new aspects of lipid-protein interactions.* Faseb J, 2010. **24**(10): p. 3829-39.
4. Zakharov, V.V. and M.I. Mosevitsky, *Oligomeric structure of brain abundant proteins GAP-43 and BASP1.* J Struct Biol. **170**(3): p. 470-83.
5. Hayashi, N., et al., *Circular dichroism and 1H nuclear magnetic resonance studies on the solution and membrane structures of GAP-43 calmodulin-binding domain.* J Biol Chem, 1997. **272**(12): p. 7639-45.
6. Mosevitsky, M.I., *Nerve ending "signal" proteins GAP-43, MARCKS, and BASP1.* Int Rev Cytol, 2005. **245**: p. 245-325.
7. Kumar, V., et al., *Structural basis for the interaction of unstructured neuron specific substrates neuromodulin and neurogranin with Calmodulin.* Sci Rep. **3**: p. 1392.
8. Alexander, K.A., et al., *Regulation of calmodulin binding to P-57. A neurospecific calmodulin binding protein.* J Biol Chem, 1987. **262**(13): p. 6108-13.
9. Gauthier-Kemper, A., et al., *Interplay between phosphorylation and palmitoylation mediates plasma membrane targeting and sorting of GAP43.* Mol Biol Cell. **25**(21): p. 3284-99.
10. Hall, F.L., et al., *Suppression of nerve growth factor-directed neurite outgrowth in PC12 cells by sphingosine, an inhibitor of protein kinase C.* J Biol Chem, 1988. **263**(9): p. 4460-6.
11. Tigyi, G., et al., *Lysophosphatidic acid-induced neurite retraction in PC12 cells: control by phosphoinositide-Ca2+ signaling and Rho.* J Neurochem, 1996. **66**(2): p. 537-48.
12. Sato, K., et al., *Exogenous sphingosine 1-phosphate induces neurite retraction possibly through a cell surface receptor in PC12 cells.* Biochem Biophys Res Commun, 1997. **240**(2): p. 329-34.