LIFE PROJECT
AFTER CU

LIFE12 ENV/IT/000336

"Anti-infective environmental friendly molecules
against plant pathogenic bacteria for reducing Cu"

ANNEX 7

DELIVERABLE ACTION C3

Monitoring of the absence of side effects for the anti-virulence peptides on common targets of any living organism at laboratory level

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1. INTRODUCTION

The absence of any side effect of the AFTER Cu anti-virulence peptides was here monitored on a common and universally conserved target of any living organism, using copper as a positive control and to optimize both the investigation protocol and the experimental procedures. As foreseen, the target chosen to this purpose are a complex of proteins, belonging to the so-called P-type ATPases, a large group of ubiquitous evolutionarily related molecular pumps highly conserved in both eukaryotes and prokaryotes, and which are constantly present into their cellular membranes (Kühlbrandt, 2004; Bublitz *et al.*, 2011). The most part of P-type ATPases are transporters with a high level of specificity for pumping into the cell a wide array of ions, mostly cations. Actually, there are also some ATPases involved in the maintenance of the asymmetric nature of the biomembranes, and more generally in many transport processes in virtually all living organisms. As far as bacteria are concerned, they have got several ATPases shown to be essential for basic homeostasis and for their protection from several stresses. For instance, recently a P-type ATPase involved in iron transport was demonstrated essential for the virulence of *Listeria monocytogenes*, as well as for its resistance to haem and haemin-mediated toxicity (McLaughlin *et al.*, 2012). Similar data were already known for many P-type ATPases in *M. tuberculosis* (Schaible & Kaufmann, 2004) and *S. aureus* (Stauff *et al.*, 2008). A P-type ATPase from the basidiomycete *Trametes versicolor* was identify and characterised, as a copper-trafficking enzyme effective in protecting this fungus under elevated copper conditions (Uldschmid *et al.*, 2003).

The P-type ATPases are targets for many toxic compounds, such as heavy metals and even biotic toxins from several organisms and microrganisms, thus having a dramatic impact on their cell homeostasis. For this reason P-type ATPases are an ideal and universal target to develop and perform wide spectrum toxicity tests. Here copper toxicity was tested at subcellular and molecular levels, by studying the effects of copper ions (Cu^{2+}) on the activity of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), which belongs to the P-type ATPase family. In particular, SERCA hydrolyzes one molecule of ATP to transport two Ca^{2+} ions against their electrochemical potential gradient from the cytoplasm to the lumen of sarcoplasmic reticulum (SR) (Brini & Carafoli, 2009). The SERCA transport activity plays a major role in cell Ca^{2+} signaling and homeostasis (Brini & Carafoli, 2009). Some studies revealed that various heavy metal ions (*e.g.* Cd^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}) may affect SERCA activity in different types models thus acting as strong SERCA inhibitors (Gramigni *et al.*, 2009 and references therein). Such inhibition typically results into a dramatic elevation of cytosolic calcium concentration, endoplasmic reticulum stress, and eventual cell death through apoptosis. The effects obtained on SERCA activity by copper were here also compared with those caused by the AFTER Cu peptides.

2. DELIVERABLE ACTION C3

2.1 Experimental procedures and results

To demonstrate a possible inhibition of SERCA by copper ions, we employed a novel electrical method that makes use of a solid supported membrane (SSM). The SSM consists of a hybrid alkanethiol/phospholipid bilayer supported by a gold electrode (Figure 1), and represents a convenient model system for a biological membrane (Pintschovius & Fendler, 1999; Tadini-Buoninsegni *et al*. 2006). Membrane fragments or vesicles incorporating the transport protein of interest are adsorbed on the SSM surface (Figure 1). The protein is then activated by a concentration jump of an appropriate substrate through rapid solution exchange. If at least one electrogenic step, *i.e*. a net charge movement across the membrane generated by the protein, is involved in the relaxation process following protein activation, a current transient can be recorded due to the capacitive coupling between SSM and membrane fragment/vesicle (Tadini-Buoninsegni *et al.*, 2008a). Charge transfer in P-type ATPases has been investigated by the SSM-based technique to obtain insight into the ion transport mechanism (Tadini-Buoninsegni *et al.*, 2008a).

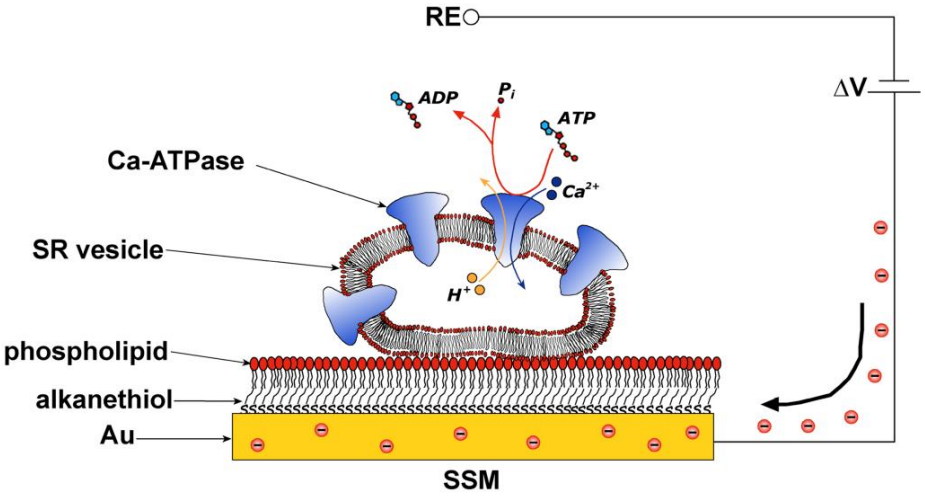

Fig. 1 Schematic diagram of a SR vesicle containing SERCA adsorbed to a SSM and subjected to an ATP concentration jump (not drawn to scale). RE is the reference electrode. If the ATP jump induces net charge displacement, a transient current is measured along the external circuit (the red spheres are electrons). For simplicity, only four Ca^{2+}-ATPase molecules are shown in the vesicle.

To gain information on the interaction of Cu^{2+} ions or of the AFTER Cu peptides with SERCA and its possible inhibition, we performed current measurements on sarcoplasmic (SR) vesicles containing SERCA. SR vescicles were adsorbed on the SSM surface during an incubation time of 60 min. SERCA Ca2+-ATPase was then activated by the rapid injection of a solution containing ATP. In particular, a 100μM ATP concentration jump was carried out in the presence of $CaCl_2$ (10 μM). If at least one electrogenic step, *i.e*. a net charge movement across the vesicular membrane generated by

SERCA, is involved in the relaxation process that follows protein activation, a current signal is recorded due to the capacitive coupling between vesicle membrane and SSM [Schulz *et al*. 2008; Tadini-Buoninsegni *et al*. 2008]. It should be pointed out that the current amplitude is related to the number of adsorbed ATPase molecules that are activated after the ATP concentration jump, and the associated charge, which is obtained by numerical integration of the current signal, corresponds to the overall amount of Ca^{2+} ions translocated by the proteins following their activation. In ATP concentration-jump experiments, two buffered solutions were employed, the "non-activating" and the "activating" solution: the non-activating solution contained 100 mM KCl, 25 mM MOPS (pH 7.0), 1 mM MgCl2 and 10 µM CaCl2; the activating solution contained, in addition, 100 µM ATP.

To prevent Ca2+ accumulation into the vesicles, 1 µM calcium ionophore A23187 (calcimycin) was used. The concentration jump experiments were performed by the $SURFE^2R^{One}$ device (Nanion Technologies). The temperature was maintained at 22-23 °C for all the experiments.

To verify the reproducibility of the current signals on the same SSM, each single measurement was repeated 6 times and then averaged to improve the signal to noise ratio. Standard deviations did not exceed 5%. Moreover, each set of measurements was usually reproduced using two-three different SSM sensors.

The current transient observed following the ATP jump was taken as a control measurement (Figure 2).

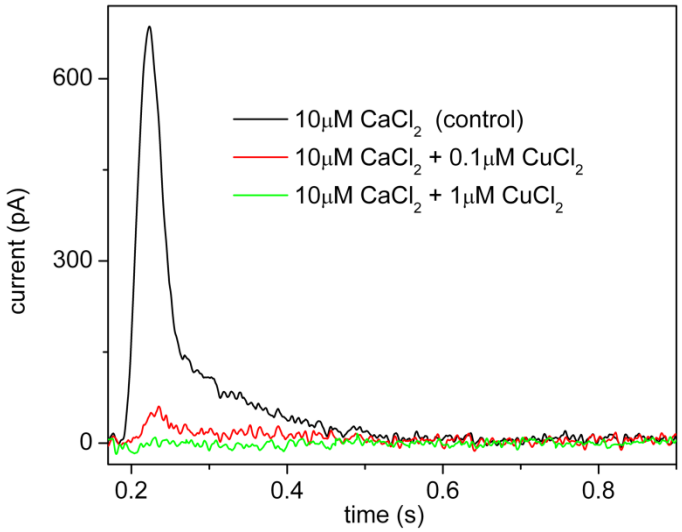

Fig. 2 Current transients obtained after 100 µM ATP concentration jumps on SERCA in the presence of 10 µM $CaCl_2$, without (control) or with $CuCl_2$ (0.1 or 1µM).

It is worth mentioning that the charge obtained by numerical integration of the ATP-dependent current transient is attributed to an electrogenic event corresponding to translocation of bound Ca^{2+} through

the ATPase after utilization of ATP (Tadini-Buoninsegni *et al.*, 2008b). To gain information on the interaction of Cu^{2+} ions with SERCA and its possible inhibition, the ATP concentration jump was then performed at 10 μM $CaCl_2$ and in the presence of $CuCl_2$ (0.1 or 1 μM) and the corresponding ATP-induced current transient was compared to the control measurement (Figure 2).

Remarkably, we demonstrated that Cu^{2+} ions (both at 0.1 and 1 μM concentrations) practically abolish the ATP-induced current signal generated by the ATPase. Therefore, although Cu^{2+} accumulation in the apoplast and in the cytoplasm of plant cells is still unknown, we may conclude that even sub-micromolar copper exerts a strong inhibitory effect on SERCA by interfering with ATP-dependent Ca^{2+} translocation through the enzyme.

The effects of the peptides AP17, Li27 and Psa21 on current signals generated by Ca^{2+}-ATPase were then evaluated, by adding several concentrations to both the non-activating and activating solutions.

The ATP-induced current signal observed in the presence of each of the AFTER Cu peptides, generated by the P-type ATPase following 100μM ATP concentration jumps, was compared to the control measurement obtained in the absence of these substances.

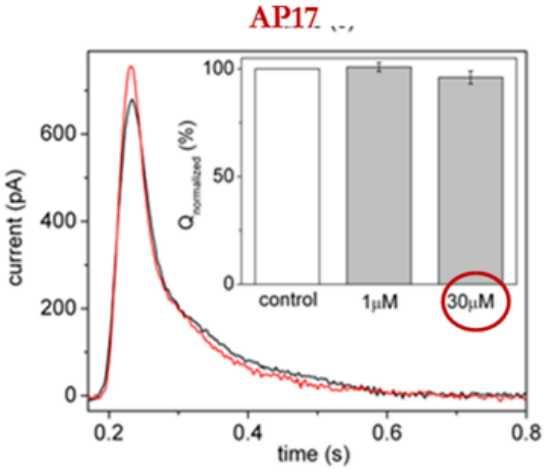

Fig. 3 Current transients obtained after 100 μM ATP concentration jumps on SERCA in the presence of 10 μM $CaCl_2$, without (control) or with AP17 peptide (1 or 30 μM, in red into inset).

As shown in Fig. 3 taking as an example AP17, we found no effect of the AFTER Cu peptides on the ATP-induced current signal and related charge over a concentration range from 1 to 30μM. Thus, this result indicates that the peptides AP17, Li27 and Psa21 do not affect ATP-dependent translocation of calcium ions by Ca^{2+}-ATPase.

3. CONCLUSIONS

In human and animal medicine, as well in plant protection, the compounds aiming to be used for the control of diseases have to be carefully tested to exclude unexpected and damaging side effects. Therefore, the AFTER CU anti-virulence peptides have to be also assayed in this sense. We planned to monitor at laboratory level this property using as targets those biological systems, present and very conserved structurally and functionally in any living organism.

Ca^{2+} is a commonly employed signal that cellules use to regulate many different cellular processes by increasing intracellular Ca^{2+}concentrations ($[Ca^{2+}]_i$) levels (Berridge *et al.*, 1998). Wrong and/or prolonged increased intracellular Ca^{2+}concentrations may lead to damaging effects. Therefore $[Ca^{2+}]_i$ levels must be strictly controlled by a variety of selective Ca^{2+}transporters, belonging the P-type ATPase family. This highly conserved family includes the sarcoplasmic–endoplasmic reticulum Ca^{2+} ATPase (SERCA) Ca^{2+} pumps, which controls the correct cytosolic $[Ca^{2+}]$ levels. Any agent having inhibitory effects on these Ca^{2+} pumps will be toxic to cells and have harmful consequences to cell life.

A number of SERCA inhibitors have been found so far, very often represented by widespread environmental contaminants which are able to bio-accumulate within any living organism, including man. The detrimental effects of this SERCA inhibitors have been shown even at low micromolar concentrations. Since the inhibitory effect on SERCA, such as on other members of the P-type ATPase family, can be measured quite rapidly, we decided that this system could be a very useful tool to exclude the presence of any side and toxic effect caused by the AFTER CU anti-virulence peptides. The screening test was so optimised using copper as challenging compound. Moreover, in the general frame of the AFTER CU project, it is important to undelined that the results obtained by these *in vitro* assays showed for copper a very high toxicity, even at submicromolar concentrations.

4. REFERENCES

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