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Self-Assembly of Okadaic Acid as a Pathway to the Cell

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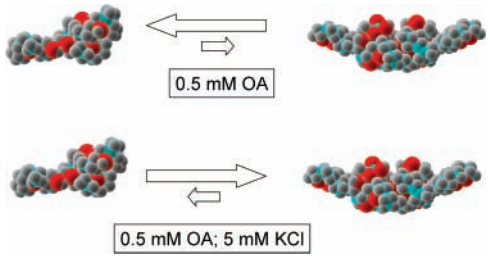
ABSTRACT



The polyether toxin okadaic acid (OA) inhibits several protein serine/threonine phosphatases that play central roles in the regulation of many essential cellular processes. The use of scanning tunneling microscopy (STM) shows that dimerization of such toxins is crucial to understand the mechanism of toxin transport across model membranes.

The marine polyether okadaic acid (OA) (Figure 1), produced by dinoflagellates of the genus *Prorocentrum* and *Dinophysis*, has had a dramatic impact upon human health, economy, and science.[1,2] The primary cellular targets of OA are several classes of protein serine/threonine phosphatases (PPPs) that play central roles in the regulation of many essential cellular processes, including metabolism, growth, division, and death.[3,4]

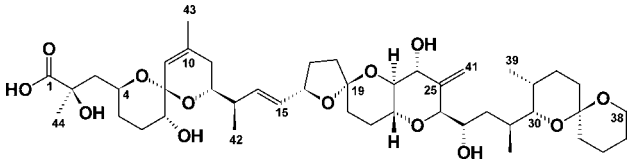


Figure 1. Okadaic acid.

Hence, OA has been a key laboratory tool employed in basic studies directed toward understanding the role of PPPs in diverse human disease related processes such as cancer, AIDS, inflammation, osteoporosis, Alzheimer's, and diabetes.

The existence of OA as a metal complex was first noted by NMR spectroscopy.[5] Its physiological importance was demonstrated in a later work where it was shown that the mechanical response of smooth muscle to OA is highly depressed in a potassium-free medium, and the complexed form of OA induces a virtually identical response indepen-

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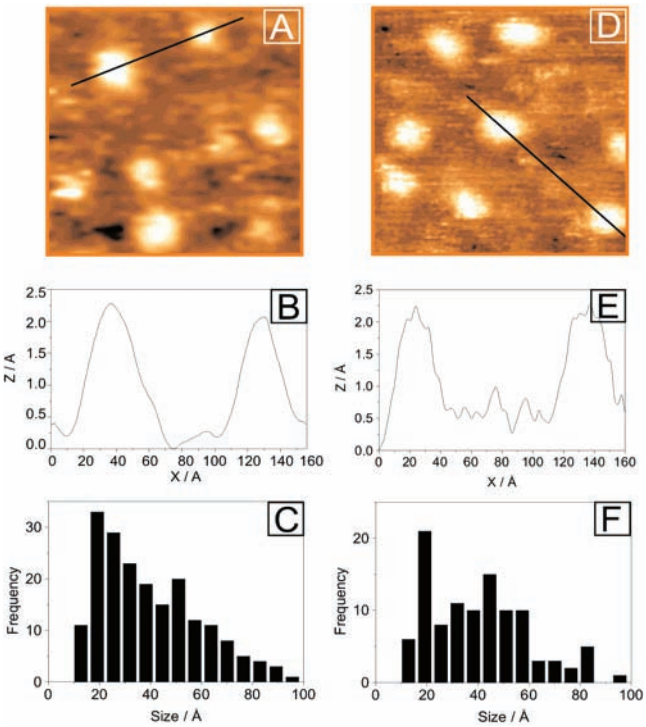

Figure 2. Representative STM images and their corresponding cross sections and histograms of size distributions. (A) STM image (20 nm × 20 nm); (B) the corresponding cross section showing (right) monomeric (20−25 Å) and (left) dimeric (40−45 Å) forms of okadaic acid; (C) size distribution histogram for the plate shown in (A); (D) STM image (20 nm × 20 nm); (E) the corresponding cross section obtained after dipping the gold plate into a toxin solution containing 0.5 mM KCl; and (F) the corresponding size distribution histogram for the plate shown in (D).

dently of the presence of this cation in the media.[6] These data prompted the authors to continue with the study, and subsequently, a model of the complex which consisted of two OA units and one potassium ion was proposed based on MS and NMR spectroscopy in combination with computational techniques. According to those results, potassium cations would be coordinated by two oxygens located at the spiro carbon C-8 and the oxygen present at C-7 giving a trigonal bipyramid binding geometry.[7] Deeper analysis about the dimerization process is beyond the scope of this work.

The proposed structure, which has a hydrophilic coordination site and a more hydrophobic surface exposed to the solvent, may be a means of improving the membrane permeability of OA, in accordance with the reported potassium dependence of OA activity in tissues. Such a model provides a rationalization, from a structural point of view, of the physiological importance of the potassium ion in the activity of OA.

However, little is known about how OA penetrates into the cells, i.e., about its interaction and transport across the

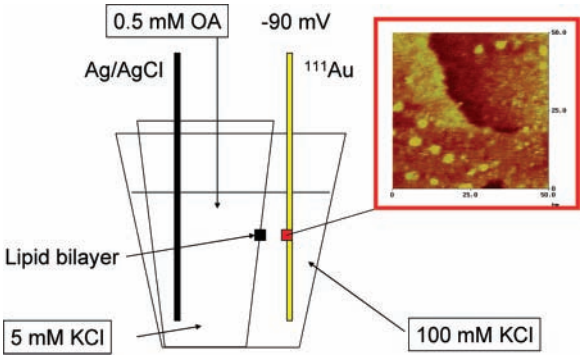

Figure 3. Scheme of the experimental setup utilized to monitor the flux of okadaic acid through a lipid bilayer. The photograph shows a section of the gold plate after 2 h, covered by molecules with a cross section of 40−45 Å approximately.

biological membranes. Here we report on the behavior of OA and its self-associated form with potassium ions when a model system that resembles a biological membrane is used. Nevertheless, the main problem to study this kind of process is to find a way to determine unequivocally the aggregation status of the molecule studied when it is interacting with membrane mimetics. In the present work, we propose the utilization of an experimental setup that makes use of scanning tunneling microscopy (STM) as a detection technique,[8] which indicates that the mechanism employed by this toxin to enter into the cells and consequently reach its molecular targets is a dimerization process involving a physiologically important cation (K^+). Experiments were planned only with potassium based on our previous results, where the presence of potassium ions in the media was determined necessary to observed a correct physiological response to okadaic acid.[6,7] Therefore, we did not perform experiments with other ions.

A necessary requirement to use STM as a detection technique is that OA molecules bind appropriately to the scanning surface.[9−10] For this reason, Au(111) plates were dipped into a 0.5 mM solution of OA, using immersion times ranging from 30 min to 48 h. Excellent results were obtained after 2 h, as can be seen in Figure 2A that shows a gold plate covered by particles around 20−25 Å (corresponding to the monomer size) and 40−45 Å (corresponding to the dimer size) in size. The corresponding histogram of size distribution (Figure 2C) is clearly shifted to the monomeric form of OA, in accordance with its molecular size (Figure 2B). With the aim of promoting the formation of OA dimers in solution, the experiment was repeated using the beforementioned conditions but now additionally containing 5 mM in KCl, i.e., the same K^+ concentration that can be found in the extracellular media. The result of this experiment was a gold surface (Figure 2D) now covered by a higher portion of OA molecules around 40−45 Å in size, confirming that

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the dimer/monomer ratio present in solution increases when K^+ is present (Figure 2F).

On the basis of the previous results, a simplified model system to study the flow and the agregation status of OA through lipid bilayers was designed as follows (Figure 3). The resting membrane potential of a cell is within the range of −50 to −90 mV; i.e., there is an excess of negative charge in the inner side of the plasmatic membrane. The main contributors to these potential are two cations, namely, Na^+ and K^+, as well as a Cl^- counterion. Taking into account the importance of the presence of potassium ions in the activity of OA, a gold plate (used as "detector") was put into a 100 mM KCl aqueous solution (equal to K^+ intracellular concentration), and inside of it, in a separated flask, a 5 mM KCl solution also containing 0.5 mM OA was placed. Both solutions were "connected" through a lipid bilayer.[11] In this way, the inner solution containing OA as well as K^+ and the outer one which only contained the electrolyte stayed separated and stable for hours. This was confirmed by placing a Ag/AgCl reference electrode inside of the okadaic acid containing solution to measure the open circuit potential of the system (E_{ocp} = −170 mV) which remained constant for hours. It should be noted that if no potential is applied the gold plate appears clean and free of OA molecules.[12] Afterward, by using a potentiostat, the gold plate was set at a potential 90 mV more negative than E_{ocp} for 2 h. Finally, the gold plate was observed using STM, and the results are shown in Figure 3. Clearly, when the potential is applied, OA molecules were able to pass through the lipid membrane and subsequently adsorbed onto the gold surface. Analysis of the cross section of most of the particles adsorbed onto the gold plate after the experiment (Figure 3 and see Supporting Information S8) revealed that they have the same size as those found in Figure 2E, that is, OA dimers. The open circuit potential after the experiment remained identical to that before, indicative of the stability of the membrane.

The results presented here show that in a 5 mM KCl aqueous solution (emulating the K^+ concentration in the extracellular space) OA is present in an equilibrium which involves species such as the monomer (OA free) and the dimer, the latter complexed with potassium ions (2OA-K^+). In addition, the STM images provide evidence that while the monomer is not able to cross lipid membranes the dimer (2OA-K^+) is capable of crossing them against a potassium gradient (from 5 to 100 mM) driven by the resting potential of the cell (−90 mV). From the previous results, we propose a simple mechanism by which the toxin penetrates into the cell, by dimerization, to enhance its lipophilicity. Afterward, once the dimer reaches the cytoplasm, we propose that the equilibrium is probably shifted to the monomeric species due to the strong interaction of the monomeric form of OA with protein phosphatases PP1 (K_i = 145 nM) and PP2A (K_i = 30 pM), as has been shown in pharmacological and X-ray studies where only the monomeric form of OA was observed bound to the proteins.[13−16] It is well-known that OA affects countless physiological processes, and its interactions with several second messengers have been investigated; however, no other cellular targets for it other than PP1 and PP2A have been identified to date. The results obtained in this work clearly support the idea that a passive transport mechanism driven by the membrane resting potential and the subsequent interaction with its molecular targets, that is, the inhibition of sensitive phosphatases (especially PP2A), may be responsible for many, if not all, of the observed cellular responses to OA (Figure 4).[17]

The strong adsorption of OA onto gold surfaces as well as the hability of scanning tunneling microscopy (STM) for

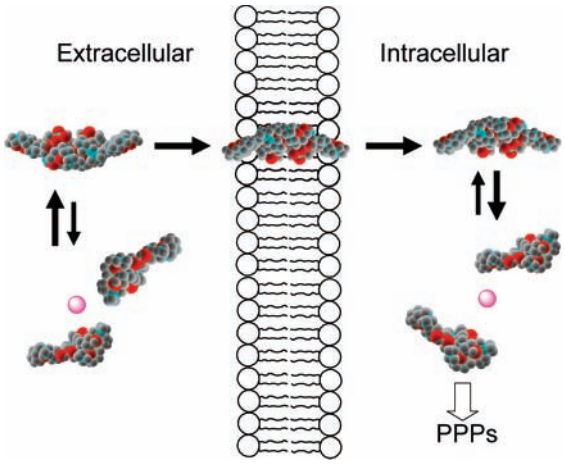

Figure 4. Cartoon of the proposed mechanism by which okadaic acid crosses the cell membrane to inhibit protein phosphatases (PPPs).[19] Pink spheres represent potassium ions.

(11) (a) A lipid bilayer was prepared using a solution of L-α-phosphatidylcholine (L-α-lecithin) from soybean (Sigma) in decane (Fluka). (b) Steinem, C.; Janshoff, A.; Ulrich, W. P.: Sieber, M.; Galla, H. J. *Biochim. Biophys. Acta* **1999**, *1279*, 169−180. (c) Favero, C.; Campanella, L.; D'Annibale, A.; Santicci, R.; Ferri, T. *Microchem. J.* **2003**, *74*, 141−148.

(12) We used such an experiment as our control to discard the possibility of OA leaking through the membrane. However, if we use a K^+ free inner solution, the system does not resemble by any means a physiological situation; therefore, OA dimers could not exist in that part of the system. In addition, in the proposed experiment, the inner solution would be a nonionic media (highly dielectrical), and in this way, the application of an electrical potential would promote a very important ohmic drop across the inner side of the system, preventing the acquisition of reliable results.

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(16) Clearly (Figure 4), OA is expected to remain dimeric upon entering cells (even to a higher extent than it is outside). The high affinity of PP1 (K_i = 145 nM) and PP2A (K_i = 30 pM) for OA suggests that the equilibrium would be shifted to the monomeric form of OA. It should be noted that X-ray structures of the complexes of OA with PP1 and PP2A showed only the monomeric form of OA bound to the proteins. However, as we do not have a value of the self-association constant for OA, we can not quantify how effectively PP1 or PP2A would alter such an equilibrium. We have no results about the effect of K^+ on the potency of okadaic acid against PP1 or PP2A, but it appears clear that its presence would not produce any significant modification, as in an in vitro assay the inhibitor would not have to cross the lipid barrier to reach its target, circumventing the need for a dimerization step.

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imaging single molecules make this technique a powerful tool for studying fascinating aspects in biology, such as the mechanism of toxin transport across model membranes.[18,20]

(19) This cartoon is only a proposal of the mechanism by which OA could enter into the cell. The existence of more extensive aggregation has been studied in our laboratory, but definitive results need further and detailed research.

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Supporting Information Available: Experimental details and scanning tunneling microscopy images. This material is available free of charge via the Internet at http://pubs.acs.org.

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