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**Studies of P2X₄ receptor dynamics upon agonist activation by High-Speed Atomic Force
Microscopy and Molecular Dynamics simulations**

Submitted for the Degree of Master of Science in Physics

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List of abbreviations

5BDBD	5-(3-Bromophenyl)-1,3-dihydro-2H-Benzofuro[3,2-e]-1,4-diazepin-2-one
AIC	Akaike information Criterion
ATP	Adenosine triphosphate
APO state	P2X4 receptor state that is closed and not bounded to ATP
cDNA	Complementary DNA
CSD	Cumulated Squared Displacement
DMSO	Dimethyl sulfoxide
DOPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
HBS	HEPES buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HS-AFM	High-Speed Atomic Force Microscopy
HOLO state	P2X4 receptor state that is open and bounded to 3 ATP molecules
HOLO(-) state	P2X4 receptor state that is open but not bounded to ATP
L α -PC	L- α -phosphatidylcholine
MD	Molecular Dynamics
MSD	Mean Squared Displacement
PMSF	Phenylmethane sulfonyl fluoride
POPC	1-palmitoyl-2-oleoyl-glycero-3-phosphocholine
NMR	Nuclear Magnetic Resonance
SD	Standard Deviation
STM	Scanning Tunneling Microscopy
TBS	Tris-buffered saline
vdW	van der Waals

Abstract

Membrane proteins are surrounded by a myriad of other biomolecules and hence most of the experiments conducted in native conditions are highly complex to interpret at the single molecule level. P2X membrane receptors, an ATP-triggered channel family, are involved in calcium signaling and inflammation. However, to date, there is limited information about its dynamical biophysical behavior in a well-known lipid environment. In particular, receptor diffusion and pore size should depend on agonist activation, lipid bilayer composition and vary throughout time. Suitable experimental methodologies to tackle them have just recently emerged, such as HS-AFM where topographic features of the membrane proteins can be recorded at second resolution. In addition, combining this with all-atom MD simulations, it would be possible to provide mechanistic understanding. Therefore, the goal of this thesis is to characterize the P2X4 receptor diffusion and pore dilation on reconstituted liposomes via HS-AFM imaging and MD simulations. Our HS-AFM results indicate that HOLO state of the P2X4 receptor decreased their mean displacements by 22% compared to the APO state and our MD results shows that interaction of protein-water and protein-lipid interaction fluctuate 9% and 43% higher in the APO state respectively. Also, the HOLO state showed a dilation of its extracellular domain compared to the APO state via HS-AFM imaging; however, MD showed us only a dilation of the internal profile of the P2X4 receptor but not an external broadening. This discrepancy may arise due to lipidic composition in MD which only had neutral lipids and experiments were carried with a proportion of 3:1 of neutral:negatively charged lipids. Moreover, each ATP molecule was calculated to have a high interaction with the two adjacent subunits that is in contact and therefore it may force the subunits to stay close at that point, working as a hinge. Taken together, combining HS-AFM and MD can lead to novel insights into the dynamical behavior of individual receptors. Since our experiments were performed on receptors supported on mica, future work could be to suspend lipid bilayers on porous alumina membranes to emulate the conditions observed in cell membranes where extra and intracellular sides are present.

Introduction

Membrane Proteins

Biological membranes consist of lipids, proteins and carbohydrates. The lipid components are amphipathic and include phospholipids, sphingolipids and sterols which are assembled in two layers and interact by their hydrophobic regions. Membrane proteins containing one or more transmembrane (TM) segments are embedded in this lipid bilayer and are grouped as integral or peripheral depending upon presence of extracellular and intracellular domains. In addition, some proteins can be structurally modified by linking to carbohydrates (Luckey, 2014).

Since lipid bilayer works intrinsically as a barrier between the extracellular environment and intracellular media, membrane proteins are key not only in sensing the extracellular environment but also in generating cell response to those changes. Moreover, membrane proteins are of pharmacological interest, 62% are drug-targeted and 38% of the disease-related proteins are linked to them (Yildirim, Goh, Cusick, Barabási, & Vidal, 2007).

Membrane proteins can be classified in different families: transporters, ion channels and receptors. Transporters are capable of moving different substrates away from or to towards the cell via changing their structural conformation. Ion channels allow the flux of ions to enter or leave the cell following an ion electrochemical gradient and receptors bind to specific ligands to trigger downstream signaling within the cell.

One of the most significant facts about biological membranes is how crowded these are (Fig. 1). For example, a typical plasma membrane is 50% dry weight in protein, this in turn has a meaning that each integral protein is surrounded by approximately 50 lipids (Alberts et al., 2008) and therefore leaving an average protein-protein distance of 30-35 Å (Luckey, 2014) and an average of 25% surface corresponding to integral proteins only (Grasberger, Minton, DeLisi, & Metzger, 1986). Therefore, membrane proteins are interacting tightly to both lipids and other proteins and more important, their function is regulated by these interactions.

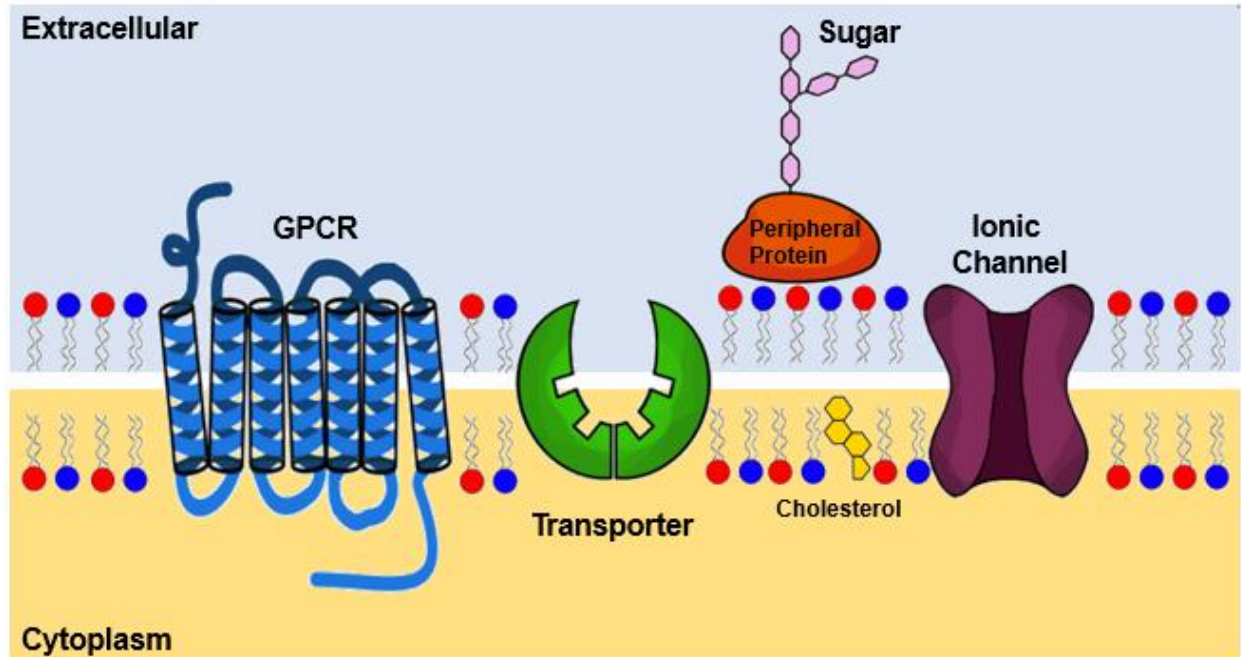


Figure 1. 2D scheme of the cell membrane. From left to right, it is shown two types of lipids (blue and red circles with tails), a G-protein coupled receptor with its seven transmembrane segments (blue), a transporter in their outward-facing conformation (green), a peripheral protein (orange) linked to a sugar molecule (violet), a cholesterol molecule (yellow) and an ion channel (purple).

Membrane protein interactions

By considering biological membranes as a mixture of proteins and lipids, we have three different interactions to look at: lipid-protein, protein-protein and lipid-lipid and any perturbation to one of these interactions will be reflected in the other two.

In the case of lipid-protein interactions, membrane proteins are surrounded by a shell of lipids molecules and because of proteins having not a regular surface, lipids can adopt a vast arrange of conformations around them, making small contacts up to being tightly anchored to the protein. Lipid-protein interactions can be classified depending on where the lipid is located. For example, if lipids are surrounding the TM segments like a solvent, they are called ‘annular lipids’ and is the most abundant case of lipid-protein interaction reported by crystallographic data. On the other hand, if lipids are interfacing between TM segments (mostly with alpha-helix secondary structure)

of the protein, these are called 'non-annular lipid' (Lee, 2003). Evidence coming from mass spectrometry pointed out that resistance to unfolding is correlated with specific lipid-binding events, i.e. a distinction between lipids that are just tightly interacting to those that affect membrane protein structure and/or modulate their function (Laganowsky et al., 2014). Two well studied examples of lipid-protein interaction are the KcsA channel and the nicotinic receptors, the first being K⁺ channel, that specifically needs to interact with the heads of negatively charged lipids for the gating process to occur; while for the second being a Na⁺/K⁺ channel, it needs the binding with neutral lipids such as cholesterol and negatively charged lipids like phosphatidylserine (PS).

In the case of protein-protein interactions, several protein regions can be responsible for such as TM segments, intracellular or extracellular domains, and non-integral proteins, namely peripheral and hydrosoluble proteins. A well-known example of protein-protein interactions occurs in the family of voltage-gated calcium channels (Ca_v), which consists of an α₁ subunit that comprises the conduction pore and bound accessory proteins that change drastically the Ca_v behavior. For example, the δ subunit increases about threefold the Ca_v current once coexpressed in heterologous systems . When this interaction is disrupted (altered) due to point mutations, it can lead to some major diseases like epilepsy and cerebellar ataxia (Davies et al., 2007).

Finally, lipid-lipid interactions can lead to a preferential lipid contact with respect to others, having the potential to form microscopic domains of over 300nm diameter called "lipid rafts". These domains have importance not only by introducing heterogeneity to the system, but also making segregation of specific protein and lipid elements that are likely to be abundant in there (Sezgin, Levental, Mayor, & Eggeling, 2017).

P2 receptors

Upon binding of ATP molecules, metabotropic P2Y and ionotropic P2X receptors can be activated. The ATP-triggered signaling produce responses lasting from milliseconds to minutes, and even longer time scales through changes in gene regulation via second messengers. Moreover, P2 receptor signaling is even more diverse due to the fact that ATP concentration effect ranges from nanomolar in P2Y receptors up to hundreds of micromolar in P2X7 receptors. (Khakh & North, 2012) .

The P2X receptors are a family of trimeric ion channels (Barrera, Ormond, Henderson, Murrell-Lagnado, & Edwardson, 2005) that upon activation conduct the Na⁺ and Ca²⁺ inward current and K⁺ outward current, which changes membrane potential and raises the intracellular Ca²⁺ concentration (Samways, Li, & Egan, 2014).

For a long time, much of the structural information of P2X receptor had been collected by using electrophysiology and scanning cysteine accessibility mutagenesis (SCAM). However, it was all indirect information. A breakthrough was made by Gouaux's group in 2009 (Kawate, Michel, Birdsong, & Gouaux, 2009) by solving the X-ray structure of zebrafish P2X4 receptor in closed (APO state) and ATP-bound open conformations (HOLO state), which lead to several important crystallographic insights into a variety of P2X isoforms later (Hattori & Gouaux, 2012; Karasawa & Kawate, 2016) .

From a physiological role, when P2X4 receptor expression is upregulated in microglia, it has a key role in the pathogenesis of neuropathic pain (Tsuda, Masuda, Tozaki-Saitoh, & Inoue, 2013), triggering inflammation and be associated with pathologies like post-ischemic inflammation, rheumatoid arthritis, airways inflammation in asthma. In addition, P2X4 receptor mRNA is expressed in a variety of human tissues, such as brain, spinal cord, sensory ganglia, superior cervical ganglion, lung, bronchial epithelium, thymus, bladder, acinar cells of the salivary gland, adrenal gland, testis and vas deferens (Burnstock & Verkhratsky, 2012).

P2X4 receptor structure

P2X4 is formed by three subunits, each one resembling a dolphin that is perpendicular to the lipid membrane with the fluke in the intracellular side, the peduncle in contact with the lipid membrane and the rest of the upper body (including the dorsal fin and flippers) standing up to 70 Å on the extracellular side. The peduncle is comprised of two alpha helices -making the trimmer to have six transmembrane spans- and the ATP binding site is down below the head and above a dorsal fin of one of the other two dolphins. Before X-ray structure was elucidated, P2X were thought to have an entrance through the top of the channel, but X-ray structure suggested that entrance was located on the sides of the channel (lateral fenestrations) (Fig. 2).

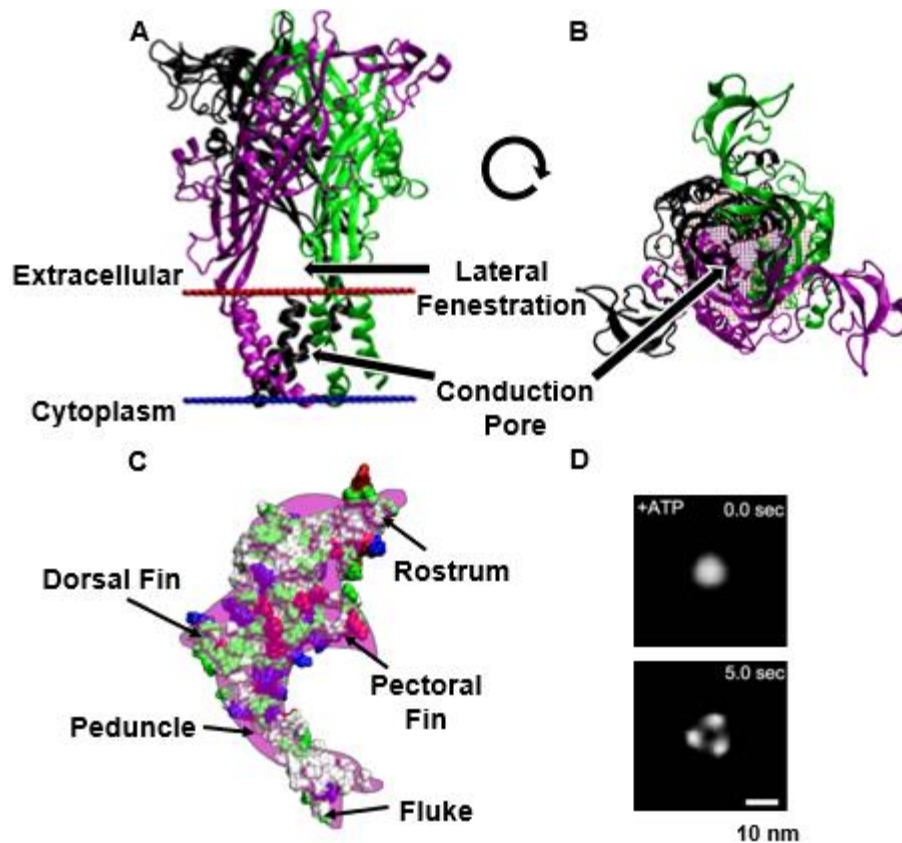


Figure 2. 3D structure of P2X4 on its open state & pore dilation upon activation. (A) Side view of P2X4 showing the three subunits (black, purple & green), the lateral fenestration and the conduction pore. (B) Top view of the receptor. PDB code: 4dw1. (C) Scheme of dolphin-like structure of a single subunit. (D) P2X4 receptor dilation of extracellular domain observed upon application of 1 mM ATP, image taken from Fig. 3 in (Shinozaki et al., 2009). Note: ATP is not displayed in Fig A & B.

Upon activation due to ATP binding, P2X4 receptor undergoes conformational changes that alter the interactions within the protein subunits; with the lipid bilayer and solvent. This rearrangement rapidly increases conductivity to ions, but if the stimulation is prolonged, P2X4 undergoes structural changes that allow the conduction of large cations such as NMDG, in this case, some P2X4 receptors are said to undergo “Pore Dilation” which is a broadening of the receptor (Li, Toombes, Silberberg, & Swartz, 2015). In addition, this P2X4 receptor activation process should also have strong implications at the interacting time with the environment, since P2X4 receptors expressed in living cells undergo a change in their diffusion coefficient (D) upon activation (Toulme & Khakh, 2012).

Diffusion

Due to thermal collisions, molecules are in perpetual movement and membrane proteins are not an exception. By studying membrane protein mobility, important information can be obtained about membrane structure, interactions between membrane components, and mechanism of membrane and/or cytoskeleton functions (Qian, Sheetz, & Elson, 1991). Moreover, by studying this movement at single molecule level, it can provide valuable information that is lost in techniques that report diffusion as an average of large ensembles.

A well-known example are neurons, they make interconnections via their nearer surface -called synapse- which needs receptors correctly retained over that area, meaning that neuron must have a strategy to prevent them from diffusing outside. Therefore, diffusion processes are implicated in the regulation of receptor number at synapses, and they are directly associated with synaptic plasticity (Choquet & Triller, 2003).

Diffusion is usually measured by recording a fluorescent molecule attached (labeled) to the protein of interest. This label is typically a fluorescent protein or quantum dot and the goal of these experiments consists in tracking the X-Y position of the fluorescence reporter over time and then characterize the movement. 2D movement can be characterized in terms of the covered surface and how fast the process occurs.

Depending on its behavior, a particle with the same diffusion coefficient may have four different types of motion. i) If a particle is restricted to move in the space as it would be locked, then it has a corralled type of motion. ii) If a particle has a movement composed of random displacements plus a directed movement it is said to have a diffusion with flow. iii) If the particle is allowed to cover the whole space, then it is said to have a normal diffusion and finally, iv) if the distances explored do not follow a linear relationship with time, and if it rather follows a power law and it does not fit in the other three cases, then it is said to have an anomalous diffusion.

The parameter used to characterize the different types of motion is the mean squared displacement (MSD):

$$\langle r^2(n\Delta t) \rangle = \frac{1}{N} \sum_{i=1}^N \Delta x_{i,n\Delta t}^2 + \Delta y_{i,n\Delta t}^2 \quad (eq. 1)$$

Where $\Delta x_{i,n\Delta t}$ and $\Delta y_{i,n\Delta t}$ are the displacement between two positions x and y of the trajectory separated by a time $n\Delta t$.

Depending upon the type of particle movement (Fig. 3) and the diffusion coefficient, MSD curves (eq. 1) will have different shapes. A parabolic shape represents a diffusion with flow (Fig. 3b blue curve), a curved shape with an asymptote represents a corralled type of motion (Fig. 3b black curve), a line means a free diffusion, and if the MSD follows a power law ($MSD \sim Dt^\alpha$, with $\alpha < 1$ for a subdiffusive process and $\alpha > 1$ for a superdiffusive process) then it is an anomalous type of diffusion (Fig. 3b red curve) (Saxton & Jacobson, 1997).

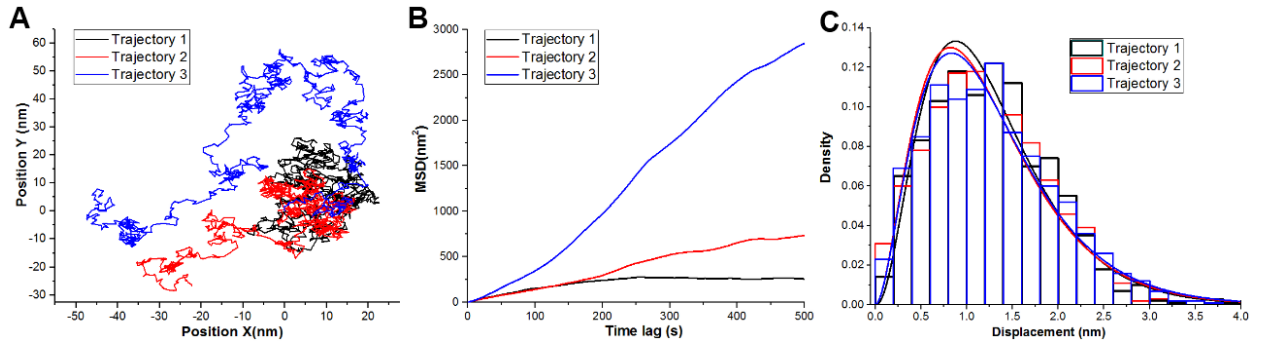


Figure 3. Simulation of free diffusing particles. (A) It is shown the trajectories of different particles that have velocities given by a Gaussian distribution. (B) MSD analysis of the particles showing different behavior, nevertheless all present the same diffusing coefficient (C).

Another way to look at the diffusion D is to use the velocity v (eq. 2) at which the particles move (Qian et al., 1991). While both methods should yield the same results, velocity measurements make short correlations whereas MSD plots make correlations through the whole history of the particle tracking giving more details. However the latter requires a higher number of measurements in order to numerically converge and as longer the temporal distance between correlations (i.e., higher $n\Delta t$ in eq. 1), the higher statistical error (Saxton & Jacobson, 1997).

$$\langle v^2 \rangle = \frac{4D}{\Delta T} \quad (eq. 2)$$

Why single-molecule experiments will provide new information?

Due to the high complexity and organization of biological membranes, to interpret membrane protein diffusion is a challenging task. For example, NMDA (N-methyl-D-aspartate) receptors are key components in the neuron excitability, but they interact with at least 77 different proteins, (Husi, Ward, Choudhary, Blackstock, & Grant, 2000) , which in consequence should regulate how these receptors diffuse across the plasma membrane. Therefore, it would be important to study the membrane protein diffusion process in isolated lipid bilayers without the participation of accessory proteins or cytoskeleton present in living cells to understand at a very elemental level how different components affect their behaviour.

Atomic Force Microscopy

Atomic force microscopy (AFM) was invented by Binnig in 1986 (Binnig, Quate, & Gerber, 1986) as a variant of scanning tunneling microscopy (STM) and was originally thought as a tool to visualize atoms on solids. Thirty years later it has evolved to be used as a primary tool in biophysical research and has been used to get insights in a broad number of topics like cell mechanics and morphology, protein structure and function, and molecular manipulation among others. This successfulness was due to some key advantages of AFM: it can be operated at physiological conditions and samples can be studied in a nearly native state.

The most basic setup for an AFM consists of a probe that makes contact with the sample, a laser and a photodiode that tracks the bending and vibrating information of the probe, and a feedback system that takes this information to give instruction to a X-Y-Z stage that allows to move the probe through the sample (Fig. 4).

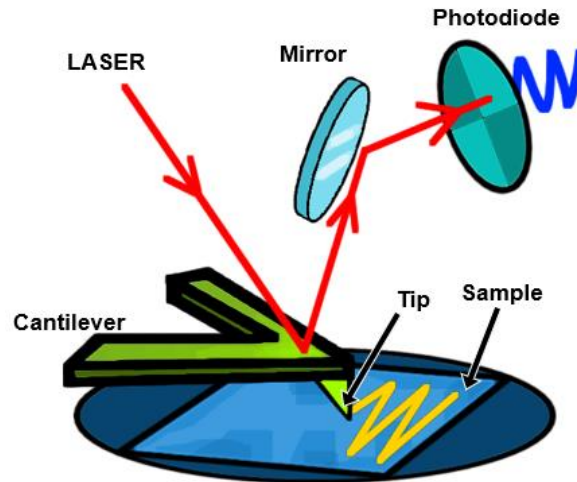


Figure 4. Simplified AFM setup. A Laser beam points towards to the end of the cantilever and it is reflected into a mirror and then into the photodiode that captures all the oscillations as the tip interacts closely with the sample.

As researchers noticed that AFM could be applied to other areas, the technique evolved to suit the needs and give birth to a very large number of variants. In the case of biology, one of the first modifications- due to the fragility of the samples - was to implement what is commonly known as tapping mode, consisting of the sample being intermittently hit by the probe in order to reduce friction forces exerted as scanning takes place. A second challenge to overcome due to inherent dynamics -time evolution- of different biological processes, was to modify tapping mode to achieve a higher temporal resolution, enabling resolution times up to 100ms. This was accomplished mainly by two facts: i) a development of faster feedback systems that were able to track amplitude changes faster and ii) smaller cantilevers with the regular stiffness but less mass, thus having a higher resonant frequency, allowing the system to oscillate faster and therefore to acquire more pixels per second and decrease the time interaction between sample and cantilever, which is a key point when scanning samples that are fragile. As a consequence, a new AFM imaging method was implemented, named High-Speed AFM (HS-AFM), that has become a complementary technique to X-ray crystallography, NMR and electron microscopy in the study of protein structure and dynamics (Casuso, Rico, & Scheuring, 2011).

In our context, AFM will be used to study how activation of P2X4 receptor upon agonist binding modifies both its mobility and extracellular domain shape (i.e., does P2X4 receptor present “pore dilation” in the outer domain?).

Classical Molecular Dynamics

Molecular dynamics (MD) consists in simulating *in silico* the conditions of the system of interest by providing a set of defined interactions which evolve as they would do experimentally. To date, MD has been used to study diverse areas related to physics, chemistry and biology (De Vivo, Masetti, Bottegoni, & Cavalli, 2016).

In Classical MD, the system moves according to Newtonian equations of motion:

$$m_{\alpha} \frac{d^2 \vec{r}_{\alpha}}{dt^2} = - \frac{\partial}{\partial \vec{r}_{\alpha}} U_{total}(\vec{r}_1, \vec{r}_2, \dots, \vec{r}_n)$$

Where m_{α} is mass of the atom α , U_{total} is the total potential energy that depends on all atomic positions ($r_1, r_2, \dots, r_n$) and is called force field of the simulation. The force field, such as CHARMM (Chemistry at HARvard Molecular Mechanics) (Table 1), is the most crucial part of simulation since to achieve realistic results it has to mimic as accurate as possible how atoms interact in reality (Phillips et al., 2005).

Among the different contributors of potential energy, they can be split into two categories, bonded and non-bonded. In the case of bonded interactions, there are four terms: bonding energy, angle energy, dihedral energy and improper dihedral energy. While for non-bonded energy, there are two terms: van der Waals forces and electrostatic interactions. These are defined as follow:

Bond Energy

$$U_{bond} = \sum_{bonds i} k_i^{bond} (r_i - r_{i0})^2$$

k_i^{bond} is the bond force constant and r_{i0} is the equilibrium distance between the two atoms.

Angle Energy

$$U_{bond} = \sum_{angle i} k_i^{angle} (\theta_i - \theta_{i0})^2$$

Angle represents the energy that is stored in three atoms ABC that bends around a central atom B. k_i^{angle} is the angle force constant and θ_{i0} is the equilibrium angle between AB and BC. A well-known example could be the angle present in water molecules, being 104.5° between the two OH segments.

Dihedral Energy

$$U_{dihedral} = \sum_{dihedrals i} k_i^{dihedral} [1 + \cos (n_i \chi_i - \delta_i)]$$

Dihedrals represent the energy of rotation around a covalent bond, which provides most of the conformational configurations to the system. This potential requires four consecutive bonded atoms. K_i dihedral is the dihedral force constant, n is the multiplicity, χ is the angle between the plane containing the first three atoms and the plane containing the last three, and δ is the equilibrium angle.

Improper Dihedral Energy

Improper represents the energy necessary to keep groups that are planar in the same way (e.g., aromatic groups) or to prevent molecules from making a transition to their specular images; this is to keep the chirality (Abraham, Hess, Spoel, & Lindahl, 2015). This potential requires three atoms connected to a central one,

$$U_{improper} = \sum_{improper i} k_i^{improper} (\varphi_i - \varphi_{i0})^2$$

van der Waals interactions

$$U_{vdw} = \sum_i \sum_{j>i} 4\epsilon_{ij} \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right]$$

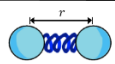
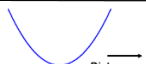
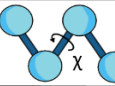
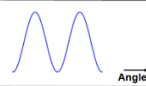
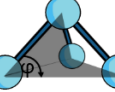
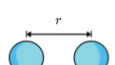
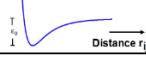
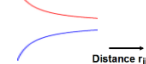
This type of interaction has origins on the dipole nature of atoms. van der Waals interactions are modeled as a 6-12 Lennard-Jones potential, where ϵ_{ij} is the minimum interaction energy between the pair ij and σ_{ij} is the distance for which the energy interaction of the pair ij is zero. Expression associated with r^{-12} describes Pauli repulsion at short ranges and the term r^{-6} correspond to attractive long-range interactions.

Coulomb Interaction

$$U_{Coulomb} = \sum_i \sum_{j>i} \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}}$$

This type of interaction occurs between two charged atoms and can be attractive or repulsive depending on the charge of the two atoms $-q_i$ and q_j -, the magnitude also depends on the distance r_{ij} . ϵ_0 is the electrical permittivity of the space.

Table 1. CHARMM Force Field. Light blue spheres represent atoms that are connected by bonds (stick or spring). In the case of improper dihedral two planes are depicted, they contain three atoms.

Type of Potential	Molecular Detail	Shape
Bond		
Angle		
Dihedral		
Improper Dihedral		
van der Waals		
Coulombic		

Note: Coulombic potential has an attractive and repulsive force owing to the interaction of the same (red line) or opposite charges (blue line).

Once the force is computed for each atom, equations of motion are integrated. For this integration, firstly, all velocities of particles are updated by knowing its acceleration. Secondly, all atoms are moved by knowing their current position and their new velocity. As a third step and by using an updated position, a new potential/force is calculated, and the cycle is repeated again, allowing the system to evolve in time solely by its own interactions.

Single molecule experiments are extremely useful at studying biophysical properties of systems, in particular referring to membrane proteins. However, they often lack information at the atomic structural level which is the one that dictates how the system behaves. In this framework, MD simulations could postulate mechanistic insights into membrane protein regulation.

In the case of ligand-activated receptors, upon activation due to a ligand/agonist, receptors are likely to change both their domain conformation and interaction with the lipid bilayer. The main goal of using MD simulations is to give an atomistic insight of what experiments are telling us. Firstly, we have two X-ray structures of zebrafish P2X4 receptor in a closed and open states that were used to generate, by homology modeling, the corresponding rat P2X4 isoform. Secondly, an *in-silico* system was designed, comprising rat P2X4 receptor model embedded in a lipid bilayer, surrounded by water molecules. Thirdly, MD simulations were carried out to study the temporal evolution and the main differences between both receptor states.

The use of single molecule techniques in artificial systems (i.e., reconstituted liposomes) gives a framework in which all components are known and therefore the results are less complex to understand than others performed on cells. In this context, HS-AFM arises as a powerful method that is compatible with physiological conditions and at the same time can be used in artificial conditions to modify the complexity of the system at will.

Hypothesis

P2X4 receptors reconstituted in liposomes change their diffusion and pore dilation properties upon agonist activation.

General goal

To characterize the P2X4 receptor diffusion and pore dilation on reconstituted liposomes via HS-AFM imaging and MD simulations.

Specific Goals

1. To evaluate the P2X4 receptor diffusion and pore dilation in reconstituted liposomes using HS-AFM.
2. To evaluate structural differences between the closed and open state of the P2X4 receptor, associated with protein diffusion and pore dilation, via MD simulations.

Materials & Methods

Cell Culture & Transfection

TsA201 cells (a subclone of HEK cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin and 100 units/ml penicillin in an atmosphere of 5% CO₂/air at 37°C. For transfection, 125 µg of rat P2X4 receptor cDNA with the human influenza hemagglutinin (HA) tag was used to transfect 5 x 162 cm² flask by using Polyethylenimine (PEI) into highly confluent (at least 80%) TsA201 cells during 48 hrs.

P2X4 receptor purification

P2X4 receptor purification was performed by immunoaffinity chromatography. First, transfected cells were removed using cell extraction buffer (EDTA 0.002M+HBS 1X). Extracted cells were pelleted by centrifugation at 2500 rpm for 5 min. The pelleted cells were then resuspended in solubilization buffer (9 ml of 25mM Tris, 150 mM NaCl, 10 mM EDTA at pH 7.5 mixed with 1ml % triton, 10 µl PMSF and 1 protease tablet) and incubated on a rotating wheel for 1hr at 4°C in order to precipitate DNA. The supernatant was then centrifuged at 35000 rpm. Supernatant was saved and incubated with 50 µL of anti-HA-agarose bio-beads (Sigma-Aldrich) for 3hrs at 4°C. Bio-Beads were washed with washing buffer (9ml solubilization buffer, 1ml 10% triton) and centrifuged at 10000 rpm three times. One last washing step was performed including a solution of 0.1% of CHAPS. Finally, proteins were eluted from bio-beads by incubating them with HA peptide (230 µL of 0.1% CHAPS with 6µL of 150 µL HA peptide).

Western blot and silver staining analyses

A fraction of purified protein was heated 95°C for 5 min and then run in a 10% SDS Polyacrylamide gel.

Silver staining analysis was carried out by sequentially treating the SDS gel with 50ml fixative solution (ethanol 40% V/V, acetic acid 10% V/V and water 50% V/V) for 1 hr. and 50ml incubation solution (ethanol 30% V/V, water 70% V/V dissolving 4.1g anhydrous sodium acetate, 127 mg anhydrous sodium thiosulphate and 260 ul of glutaraldehyde 4% V/V) for 1hr. After washing with

milli Q water, gel was incubated in 50ml silver solution (100 ml of water dissolving 100 mg AgNO₃ with 95 µl 4% para-formaldehyde) for 40 min followed by an incubation with 50ml developer solution (250 ml of water dissolving 6.25 mg of anhydrous sodium carbonate and 100 µl 4% para-formaldehyde) until bands are clearly seen in purification rail and then stopped by adding 50ml stop solution (100 ml of water dissolving 1.46 g sodium-EDTA • 2H₂O) for 10 min.

Western blot analysis was carried out by first transferring the proteins from the gel onto nitrocellulose membrane using a semi dry system. After this, nitrocellulose membrane was incubated in 50ml blocking buffer (TBS 1X, 1% V/V TWEEN and 5% W/V milk powder) for 1 hr. With non-specific interactions reduced, membrane was incubated overnight at 4°C with mouse Anti-HA antibody (BIO-RAD) at a dilution of 1:1000 in blocking buffer. The next day, membrane is washed with blocking buffer and then incubated for 1 hr. with secondary antibody (goat anti-mouse antibody conjugated with horseradish peroxidase) at a dilution of 1:1000. Membrane is once again washed and bands are visualized by using enhanced chemiluminescence kit.

Proteoliposome fabrication

36 µL of 25mg/ml L-α-Phosphatidylcholine (L-α PC, Avanti Lipids) and 30 µL of 10 mg/ml 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS, Avanti Lipids), both dissolved in chloroform, were put in a vial glass. After 30 min of drying them in a nitrogen stream, they were mixed with 0.012g of CHAPS and rehydrated with 370 µL of HEPES-Buffered Saline (HBS) 1X. The rehydrated lipids were mixed with 150 µL of purified P2X₄ receptor and separated into 3 equal volumes to perform dialysis with detergent-free medium for two days at 4°C. Proteoliposomes were suspended in imaging buffer with 30mM CaCl₂ for 15min. After this, 20 µL of them were incubated in a freshly cleaved mica for 15 min and rinsed gently 4-6 times with 200 µL of imaging buffer in order to remove non-adsorbed liposomes.

Porous alumina surface experiment

Porous alumina surface (provided by dr. Samuel Hevia's lab) was incubated with 1% poly-Lysine for 15min and then washed under a stream of N₂. After that, 20 µL of proteoliposomes were incubated for 15min and rinsed gently 4-6 times with 200 µl of imaging buffer (HBS 1X).

AFM Setup

A Dimension FastScan AFM located at Prof. Michael Edwardson's lab (University of Cambridge) was used to image P2X4 receptors.

A FastScanD probe was mounted and a microvolume cell was put on top of the head. Microvolume cell was filled with ~100 μ L of imaging buffer and head was lowered until a tight seal between sample and microvolume cell was made. Laser was aligned with the probe in order to maximize the sum and deflection was adjusted to zero.

Before engaging the sample, cantilever was tuned to its resonance frequency and a target amplitude of 5nm. Parameters were set for taking images of 1 μ m and a resolution of 2 nm while not caring for temporal resolution.

When engaging the sample, a region with bilayer and receptor (prominent white blobs) was looked for. In order to have a criterion for selecting blobs that were single P2X4 receptors pointing with the extracellular region upwards, they should have a height between 3 to 5 nm and a width of no more than 20 nm.

High-speed AFM imaging of P2X4 receptors

In order to achieve a temporal resolution of nearly 1 frame/second, an autotune was first performed 50 nm away from the surface with a target amplitude of 1 nm and the frequency of excitation adjusted near the frequency of resonance (typically ± 5 kHz around the frequency of resonance). After this, scanning parameters were set, using 250 nm of image size, 512 points per line, 64 lines, 62 Hz of scan frequency, 3 of integral gain, 2 of proportional gain and a setpoint typically between 80 to 90%. When receptor appear successfully imaged at this speed, integral gain, proportional gain, drive frequency and setpoint were varied slightly in order to improve image quality.

Images are continuously saved and after the first 40 images were captured, buffer is changed for another with different composition (e.g., first buffer containing HBS 1X and second containing

HBS 1X with 100 μ M ATP) and after a short period of equilibration, at least another 30 images are captured.

Analysis of AFM images

Images were transformed from Bruker format to .tiff format in grayscale by using Nanoscope v1.5. The Z range was set between 1 nm and 4.5 nm. The stack of images was opened with ImageJ and for each frame, Adrian's FWHM (an ImageJ plugin) was used to locate the center of mass and full width at half maximum of the receptor.

Once position for each frame was located, diffusion was calculated by a modification of formulae 10 in (Qian et al., 1991) (eq. 2 herein). For this, diffusion was calculated by taking the slope of cumulated squared displacement (CSD, eq. 3) vs time (number of frames). Ratio between diffusion before and after pharmacological stimulation was calculated.

$$\sum_{i=1}^N (r_i - r_{i-1})^2 = 4DN\Delta T \quad (eq.3)$$

Where r_i is the position at frame i , D is the "diffusion coefficient" and ΔT is the time between frames, and N the number of frames.

Statistical differences in receptor mobility across groups were assessed by using a Kruskal-Wallis analysis of variance. In the case of assessing the difference between groups, Mann-Whitney U test was performed.

P2X4 receptor homology modeling

Aminoacid sequence of P2X4 from rat was obtained from UNIPROT (entry 51577) and was aligned against the sequence of the zebrafish crystallized structures of P2X4 (Hattori & Gouaux, 2012) (PDB codes 4DW0 for the APO structure & 4DW1 for the HOLO structure) by using Multalin. 100 homology models for the APO and HOLO structures were generated by using Modeller 9.18 (Šali, 1993) and the one having lowest DOPE score for each case was selected and verified by using the web servers MolProbity (Chen et al., 2010) and ProSA (Wiederstein & Sippl, 2007) to evaluate both stereochemical and energetics of each model. From the two final

models, N and C-term tails were cut from MET 1 to VAL 28 and from LEU 358 to GLU 388 respectively.

ATP docking into P2X4 receptor

ATP structure was taken from zebra fish P2X4 (PDB 4DW1) and its electrostatic charge was optimized by using Spartan v 10 (Deppmeier et al., 2011) while structure was constrained in order to retain a similar shape to the crystalized one. Docking was prepared in AutoDock Tools 1.5.6 (Huey & Morris, 2003). Briefly, binding pockets had volumes of $22.5 \times 22.5 \times 22.5 \text{ \AA}^3$ and the center of each box was placed similar to where ligand is seen in the crystal structure. Docking was performed in AutoDock 4 and the 100 best conformations were saved and grouped in clusters of root mean square displacement (RMSD) less than 2 Å. Selected dockings for each binding pocket had negative binding energy and a similar spatial orientation as the crystallized bound ATP.

Preparation of the System for Molecular Dynamics

Homology model of P2X4 receptor was protonated accordingly to the physiological pH and later combined with the selected ATP conformation for each binding pocket. P2X4 receptor was inserted in an equilibrated POPC bilayer (of size $150 \times 150 \text{ \AA}^2$) created using the Visual Molecular Dynamics suite (VMD)(Humphrey, Dalke, & Schulten, 1996). In order to do this, both bilayer and receptor were aligned and all overlapped lipids (nearer than 0.8 Å) and water molecules (nearer than 3 Å) were deleted. The complex of receptor-bilayer was solvated by using TIP3 water model and NaCl was added to both neutralize and provide a physiological concentration of salt.

Molecular Dynamics simulations

All simulations were performed using NAMD 2.12 with temperature at 310 K and pressure at 1 atm with periodic boundary conditions (PBC). The first step was to minimize the energy by running a simulation of 10000 steps in the NVT ensemble (Constant number of particles, volume and temperature) and then simulated for 80 ns in the NPT ensemble (constant number of particles, pressure and temperature). In order to maintain temperature and pressure constant, Langevin dynamics and Nosé-Hoover Langevin piston methods were used for temperature and pressure

coupling. To calculate electrostatic interactions, Ewald sums were used with a grid density of 1 Å. Ligand parameterization was done using the SwissParam web server (Zoete, Cuendet, Grosdidier, & Michielin, 2011) and CHARMM27 forcefield was used for lipids and protein.

Analysis of Molecular Dynamics

Root mean squared fluctuations (RMSF) and RMSD were calculated with VMD's TimeLine plugin, after spatial alignment of the structures. In the case of Timeline plugin, average for the “equilibrium part” of the simulation was calculated for each residue with a custom code written in Python 2.7 (see appendix of codes).

RMSF per residue is defined as:

$$RMSF_{residue} = \sqrt{\frac{1}{T} \sum_{t=1}^T (v_t - \bar{v})^2} \text{ (eq. 4)}$$

Where T is the number of frames that were taken into account (i.e. from “equilibrium part” onwards), v_t is the position of the alpha carbon (C_α) of that residue at time t and $\bar{v}$ is the average position through the T frames. In essence, RMSF is the standard deviation of the C_α in that particular residue, giving insights in the flexibility.

RMSD per residue is defined as:

$$RMSD_{residue} = \sqrt{\frac{1}{T} \sum_{t=1}^T (v_t - v_{reference})^2} \text{ (eq. 5)}$$

Where T is the number of frames taken into account, v_t is the C_α position of that residue at time t and $v_{reference}$ is the C_α position of that residue in the reference structure (usually being the initial structure). In essence, RMSD is the average deviation of the C_α position with respect to the reference structure.

Interaction energies between segments of protein, protein and lipids, protein and water and protein and ATP were calculated with NAMD energy plugin. Fluctuation of energies was calculated as the standard deviation of the signal.

The number of water molecules and lipids surrounding the protein were calculated with custom code written in TCL via VMD interface (see appendix of codes).

Internal shape of the protein was calculated by using HOLE program (Smart, Neduvelil, Wang, Wallace, & Sansom, 1996).

Results & Discussion

P2X4 receptor purification

According to the western blot analysis, P2X4 receptor purification results (Fig. 5) are in agreement with the accepted value of 43.5k Da for each receptor subunit plus HA tag. In addition, the presence of a single band in the silver staining analysis of sample elution suggests that it is highly pure and so represents an excellent material for AFM experiments.

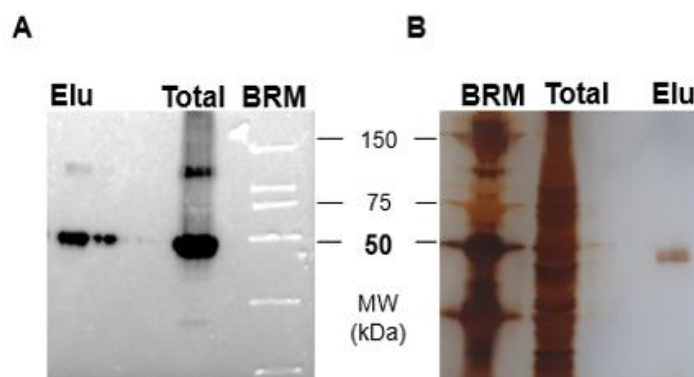


Figure 5. P2X4 receptor purification from TsA201 cells. (a) Western blot is showing a band in the fraction elution with a molecular weight lower than 50 kDa. Another band is observed between the 100 and 150 kDa bands corresponding to the receptor trimer (approx. 127 kDa). (b) Silver staining is showing the non-specific staining with a strong band below the 50 kDa band. ELU: elution, Total: total membrane fraction, BRM: broad range marker.

Imaging Proteoliposomes

Scanned receptors reported two different sizes (Fig. 6), corresponding to the receptor pointing its extracellular domain upwards and downwards. The corresponding height is lower than the given by the X-ray structure which might be due to a structure not completely resembling the physiological condition and/or to AFM tapping forces in the range of 100 pN or smaller (Casuso et al., 2012) with our scanning parameters. In the case of the receptor's width, it is necessary to

consider the convolution effect given by Fast Scan D probes (with a nominal radius of 5nm and up to 8nm). By performing a simulation of scanning and considering the receptor as a square step with 7 nm of height and 7 nm of width (Melcher, Hu, & Raman, 2008), the simulated width would be at least 15 nm (Fig. 7), which is in agreement with the measured one.

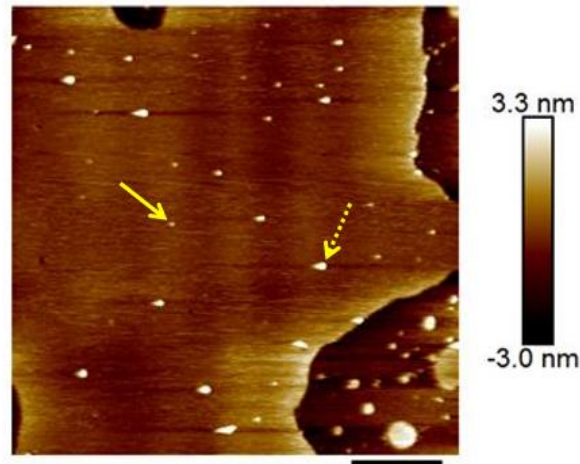


Figure 6. AFM topographic image of a proteoliposome supported on mica. A $1 \times 1 \mu\text{m}^2$ scan showing the mica, $\text{L}\alpha$ -PC/DOPS bilayer and P2X4 receptors (white blobs) on it. Blobs show two different sizes corresponding to the protein facing their extracellular domain upwards (dashed yellow arrow) and downwards (solid yellow arrow). Scale bar = 200 nm.

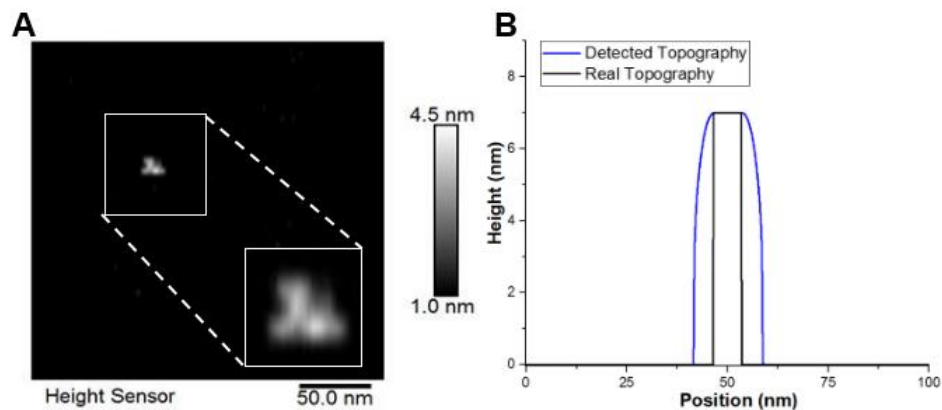


Figure 7. AFM topographic image of a single P2X4 receptor on a lipid bilayer. (A) A $250 \times 250 \text{ nm}^2$ image shows a P2X4 receptor composed of three blobs and a maximum height of about 4.5 nm.

(B) A simulation is showing the convolution effect on the detected topography produced by the probe on a feature of 7 nm height and 7 nm width (dimensions of crystallized zebrafish P2X4).

Tracking P2X4 receptor movement

After scanning of P2X4 receptors, the position XY of each particle is measured by using Adrian's FWHM (Fig. 8) and by doing this to every single frame, the trajectory for the receptor is drawn (Fig. 9). A typical membrane protein has a diffusion coefficient of about $5 \mu\text{m}^2/\text{s}$ (Ramadurai et al., 2009) which indicates a distance covered in one axis of a few hundreds of μm s in 30 seconds. However, the covered distance in the X and Y axes for P2X4 receptors was about 3 to 4 nm in 30s. This much lower membrane protein diffusion coefficient has been reported to be the case for supported lipid bilayers and is independent of the technique that is used. For example, the diffusion coefficient of $5\text{HT}_{3\text{A}}$ receptor was measured by quantum dots and it was $\sim 0.1 \text{ nm}^2/\text{s}$ (Poudel, Jones, & Brozik, 2013).

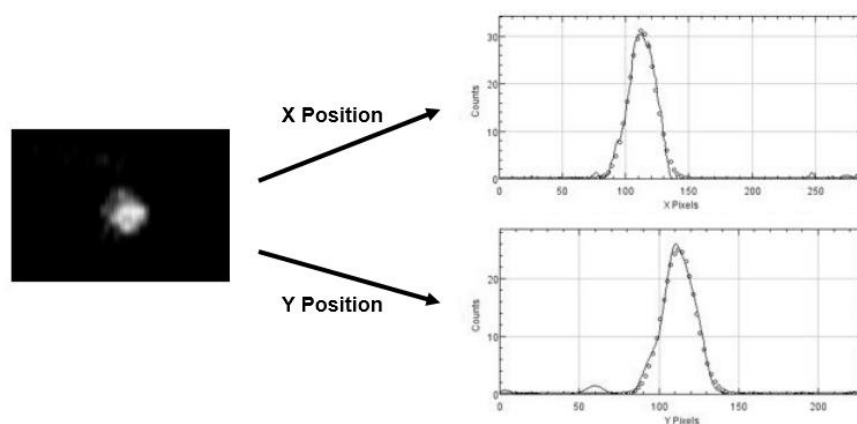


Figure 8. Determination of X and Y position of a single receptor in a single frame. (Left) A scanned P2X4 receptor is shown. (Right) The position in X and Y is measured by using Adrian's FWHM plugin. The continuous line reflects the average topography of both X and Y axes. Dotted curves represent fits of a Gaussian function.

It is important to highlight why CSD analysis of P2X4 receptors is better suited for AFM analysis rather than conventional MSD measurements (see details in appendix: Comparison of CSD and MSD). Membrane proteins and lipid bilayers formed by two types of lipids have different

conformations and an increased number of ways to interact each other. Moreover, given that AFM systems present thermal drift, which in most of the cases is not linear over time, long-time correlations (MSD) present more fluctuations, in particular when system stabilization is delayed due buffer exchange after pharmacological stimulation. Previous studies have developed an alternative type of analysis for a system having more than one diffusion coefficient based on analysis of quadratic displacement autocorrelations and densities of displacements with a fixed timestep (Matsuoka, Shibata, & Ueda, 2009). Therefore, CSD calculations (a simplified method of that mentioned previously), which are focused on characterizing long-term mobilities, could represent a better option than MSD, especially for systems that likely undergo conformational transitions and present a non-linear drift.

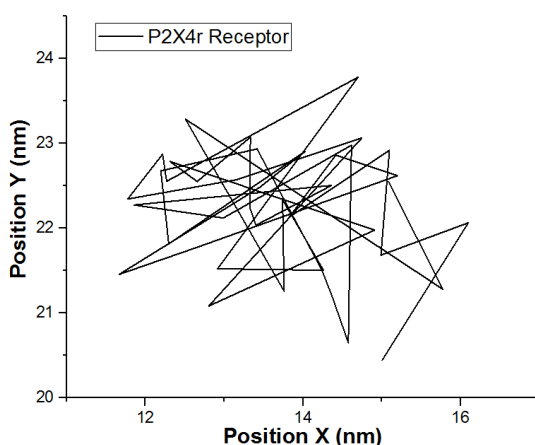


Figure 9. The P2X4 receptor trajectory during 30 seconds in a α -PC/DOPS bilayer supported on mica.

Pharmacological Stimulation of P2X4 receptors

Pharmacological stimulation of P2X4 receptors was performed with 100 μ M ATP which is around 100 times bigger than its EC_{50} (concentration triggering half of the maximal response, corresponding to 1.4 μ M for rat P2X4 receptor (Jones et al., 2000)) to facilitate their response. In addition, several controls were included such as those using only buffer, only the agonist at concentration below EC_{50} , and the P2X4 receptor antagonist 5-BDBD.

In order to compare the pharmacological stimulation from control conditions, two strategies were adopted: first, it was analyzed the receptor mean displacements between frames (Fig. 10), and second, it was analyzed the CSD values (Fig. 11).

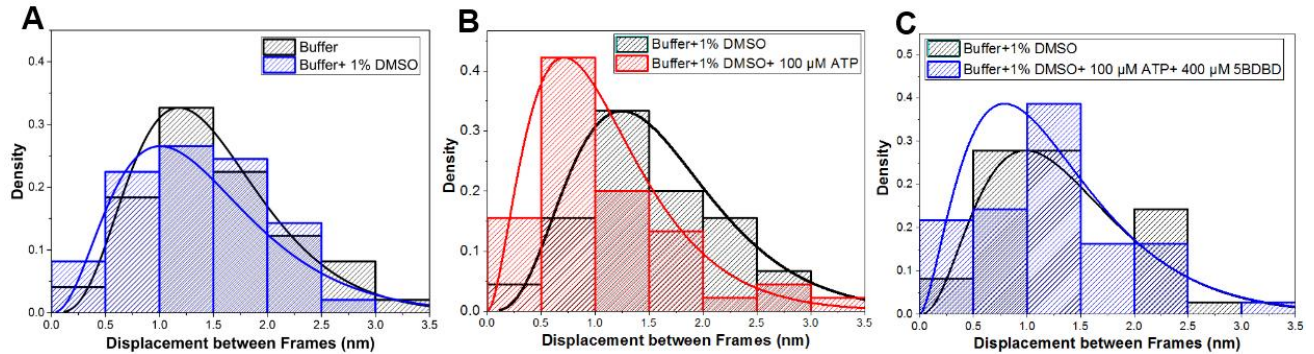


Figure 10. Distribution of displacements between two frames for P2X4 receptors at different pharmacological conditions. (A) Displacement histogram of P2X4 receptors in HBS buffer (black) and after (blue) buffer is exchanged for one containing 1% DMSO. (B) Displacement histogram of P2X4 receptors in HBS buffer containing 1% DMSO (black) and after (red) buffer is exchanged for HBS buffer containing 1% DMSO and 100 μ M ATP. (C) Displacement histogram of P2X4 receptors in HBS buffer containing 1% DMSO (black) and after the buffer is exchanged for HBS buffer containing 1% DMSO, 100 μ M ATP and 400 μ M 5BDBD (blue). Continuous line: Adjusted gamma distribution fit. Each graph represents a single experiment.

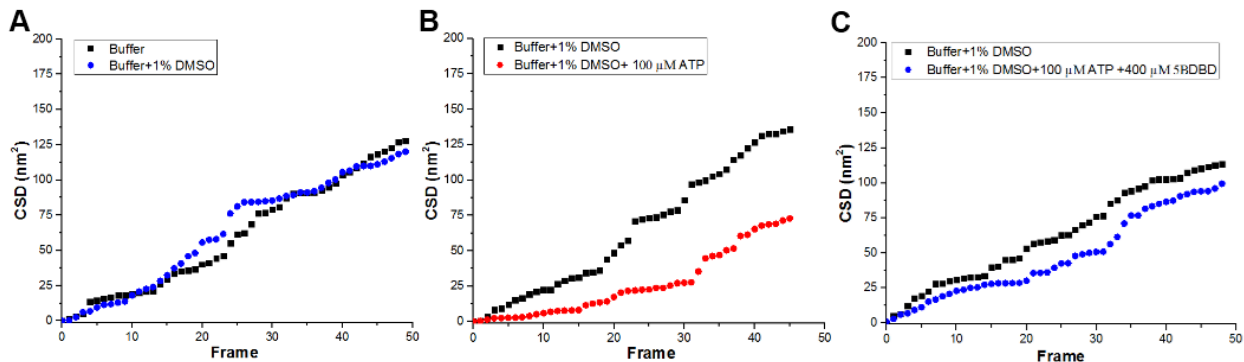


Figure 11. Cumulated squared displacements of P2X4 receptors at different pharmacological conditions. (A) Before (black) and after (blue) stimulation with HBS buffer containing 1% DMSO.

(B) Before (black) and after (red) stimulation with HBS buffer containing 1% DMSO and 100 μ M of ATP. (C) Before (black) and after (blue) stimulation with HBS buffer containing 1% DMSO, 100 μ M of ATP and 400 μ M of 5BDBD. 1 Frame=1 s.

Using the first approach, once P2X4 receptor is stimulated with ATP, its average displacement was significantly smaller (22% less) whereas receptor stimulated with ATP in the presence of a competitive antagonist or only with DMSO did not change its movement (Fig. 12A). Using the second approach, similar results were obtained where the decrease of receptor movement triggered by ATP was 38% (Fig. 12B).

Moreover, previous studies measured the diffusion of P2X4 and P2X2 receptors in microglia and HEK-293 cells respectively (Richler, Shigetomi, & Khakh, 2011; Toulme & Khakh, 2012) , and it turned out that activation increased the diffusion coefficient, but those experiments are not directly interpretable since cell has cytoskeleton, an extremely diverse lipid composition and a myriad of other proteins that P2X receptors can interact with. In addition, these are performed in labeled receptors that are not allowed to explore all their possible conformational states upon activation.

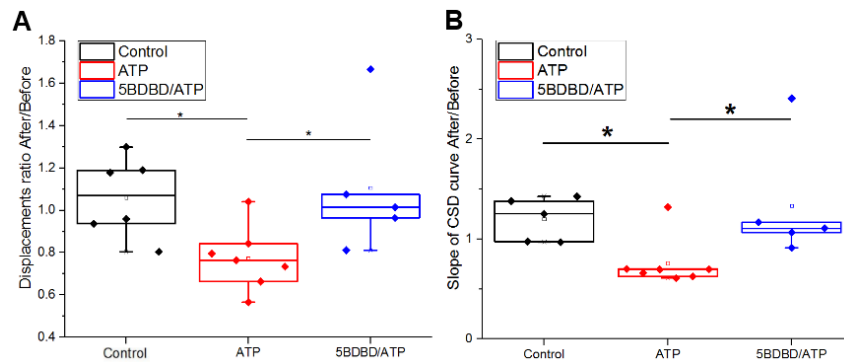


Figure 12. Statistical analysis of the P2X4 receptor movement during different pharmacological stimulation. (a) Each point denotes the ratio of the mean of the displacement histograms after and before pharmacological stimulation. (b) Each point denotes the ratio of the slope of the CSD curve vs. frame after and before buffer exchange (n=5 for DMSO and DMSO/5BDBD/ATP, n=7 for DMSO/ATP). * p<0.05. DMSO stands for HBS buffer exchanged by HBS 1x with 1% DMSO. ATP stands for HBS buffer with 1% DMSO exchanged by HBS buffer with 1% DMSO and 100 μ M ATP. ATP/5BDBD stands for HBS 1x with 1% DMSO exchanged by HBS 1x with 1% DMSO, 100 μ M ATP and 400 μ M 5BDBD.

The current HS-AFM experiments strongly suggest that this decrease in P2X4 receptor movement between two consecutive frames is due to agonist activation. This is further supported from 1 μ M ATP stimulation where this concentration was not enough to trigger displacements of P2X4 receptors (data not shown). As 5BDBD is a competitive inhibitor, it binds to the same ATP binding pocket and prevents receptor activation (Balázs et al., 2013).

The decrease of P2X4 receptor displacement after ATP stimulation could mean three different things: a) the receptor decreased its diffusion coefficient, b) the receptor did not change its diffusion coefficient but instead decreased the effective surface it can cover and c) changing both parameters already mentioned. These three different possibilities could represent different molecular mechanisms.

In the case of decreased diffusion coefficient, this could be explained by 1) a stabilization of protein-lipid/protein-water interaction and therefore a lower energy fluctuation over time, 2) stronger protein-lipid/protein-water interaction and therefore near lipids are likely to be anchored to the protein, increasing its effective mass and cross-section surface, 3) lipids surrounding the protein interact stronger between themselves, making a higher energy barrier that receptor has to surpass in order to move.

In the case of reducing the effective area of diffusion, there are few possibilities to interpret this result. On one hand, when membrane proteins are in cells, “corralled” diffusion may arise due to the interaction of the intracellular part of the receptor with the cytoskeleton and limiting the accessible surface of the receptors. However, our experiments were performed on mica, which is an “atomically flat” surface (with an RMS below 0.2 nm)(Lu, Wang, Faghihnejad, Zeng, & Liu, 2011) where small corrals should limit the protein movement. Therefore, if the protein stays in the same corral through the experiment, then the effective area should remain constant. Nevertheless, it cannot be discarded that after P2X4 receptor activation both the protein and bilayer adjacent to the mica might increase their interaction, although for this to occur there should be a considerable difference in the interaction area between APO and HOLO states of the protein.

To test some of the hypothesis mentioned above molecular dynamics simulations were performed. The main questions to address are the different types of interaction, namely: subunit-subunit, protein-water, protein-lipid and lipid-lipid interactions. This bioinformatic approach will provide molecular insights on the structural differences between the HOLO and APO states in contact with the lipid bilayer.

Homology modelling, molecular docking and molecular dynamics simulations of P2X4 receptors

All-atom molecular dynamics (MD) simulations were performed from the homology model of P2X4 receptor (Fig. 13 and 14), which was inserted on a POPC lipid bilayer. MD simulations were carried out for about 90 ns and all the systems and their different characteristics were analyzed starting from the time where RMSD was stable (Fig. 14). MD simulations are required from models based on crystallographic data since this not necessarily reflects the native contacts present on large membrane proteins (Heymann et al., 2013).

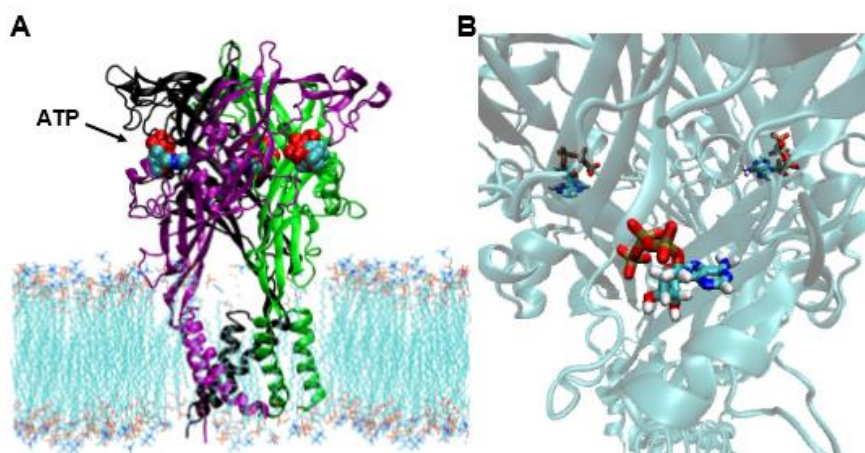


Figure 13. P2X4 receptor embedded in POPC bilayer and Molecular Docking of ATP molecules.

(A) Homology model of rat P2X4 receptor (Holo state), generated by using zebrafish P2X4 receptor (PDB 4DW1) as a template, that is embedded in POPC bilayer (water molecules are not shown). It also shows three docked ATP molecules. (B) Zoom at the binding pocket showing three docked ATP molecules, red: oxygen, green: phosphorus, white: hydrogen, gray: carbon, blue: nitrogen.

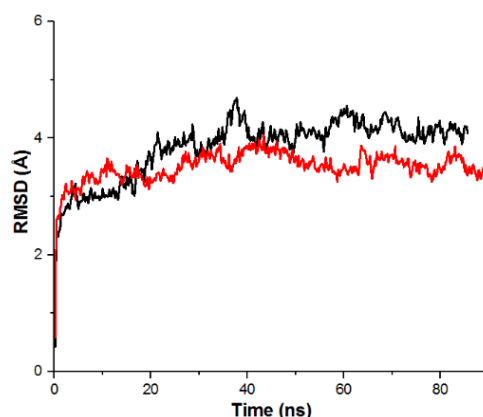


Figure 14. RMSD of the P2X4 receptor in the APO and HOLO states through simulation. (Red) RMSD of HOLO state has an average of 3.6 Å between 20 and 86 ns. (Black) RMSD of APO state has an average of 4.1 Å between 20 and 95 ns.

Structural Analysis of P2X4 receptor

Firstly, it is necessary to know how the receptor model evolved from the initial structure. To do that, RMSD per residue was calculated (eq. 5) (Fig. 15), which indicated that APO state changes substantially from the initial structure. Residues with an $\text{RMSD} > 5 \text{ Å}$ were in the TM segments (residues 29 to 39 and 352 to 357) and mostly in the “Dolphin rostrum” (residues from 115 to 185). These segments belong to the region depicted in black in Fig. 15C. In the case of HOLO state, RMSD per residue was high in the same regions as in APO state but with lower magnitude in the dolphin rostrum. One of the first facts observed through the simulation of the HOLO state is that the crystallized “dilated pore” at the transmembrane level was not stable, giving the possibility the open mechanism could need more than just ATP molecules docked to it, for this it has been postulated that drugs like ivermectin can modulate the activity of P2X4 by placing itself at the transmembrane subunit interface (Silberberg, Li, & Swartz, 2007). Consequently, a POPC molecule placed in the pore of the receptor remained stable through the whole simulation even it could be thought to be not thermodynamically favorable due to possible interactions of the tails with water molecules and polar residues (data not shown).

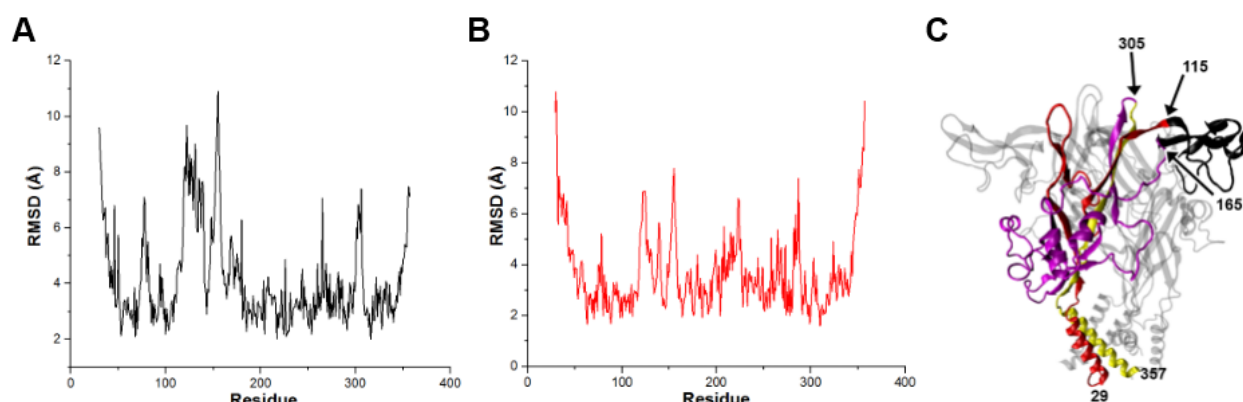


Figure 15. RMSD per residue in the APO and HOLO states of P2X4 receptors. (A) RMSD of APO structure shows a large difference from the initial structure at some regions and even larger than in HOLO state (B). (C) P2X4 receptor model indicating several segments, from residue 29 to 114 (red); 115 to 164 (black); 165 to 304 (purple); 305 to 357 (yellow).

The second point to look at regarding the structure of the P2X4 receptor is what segments were more flexible during the “equilibrated” part of the simulation. To test protein flexibility, the RMSF parameter was calculated (eq. 4) (Fig. 16A) and to visualize the differences easily, the difference between those two structures was calculated as well and expressed as a t-test. A highly positive t value means more flexibility for the APO structure and a highly negative value means that HOLO state is more flexible (Fig. 16B). APO had higher values at mostly all residues but specially at the ones in the binding pocket for ATP namely LYS 67, LYS 69, LYS313 and ARG295, while HOLO state had a higher flexibility in a few. The importance of testing residue flexibility is that it could give insights on what segments of the protein are prone to produce differences between two states and therefore how the conformational changes occur (e.g., unfolding)(Zhao, Zeng, & Massiah, 2015) upon agonist activation.

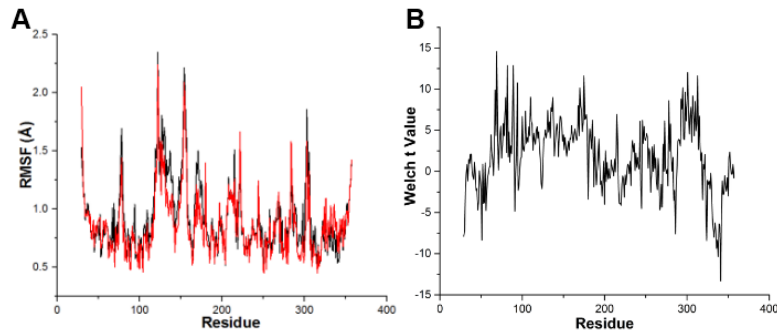


Figure 16. RMSF of the P2X4 receptor in the APO and HOLO states. (A) Average of RMSF per residue from 20 to 95 ns for the HOLO state (red) and from 20 to 86 ns for the APO structure. (B) statistical difference between the APO and HOLO states, Y-axis denotes the t value for Welch test

In the same context of residue flexibility, it was worth to test if the HOLO state had differences with the HOLO(-) in order to test what were the main differences induced by ATP binding. Fig. 17 shows that HOLO(-) had higher flexibility in the protein mostly in residues that interact closely with ATP, namely LYS 67, LYS 69, LYS313, while HOLO had a higher flexibility in residues ILE 218 and LYS298. This could mean that transition from closed to open state would happen by introducing spatial constrain to a few residues while others fluctuate randomly. Overall results indicate that HOLO(-) had a higher RMSF and this may be due to a stabilization of the open state once ATP molecules are bound (HOLO).

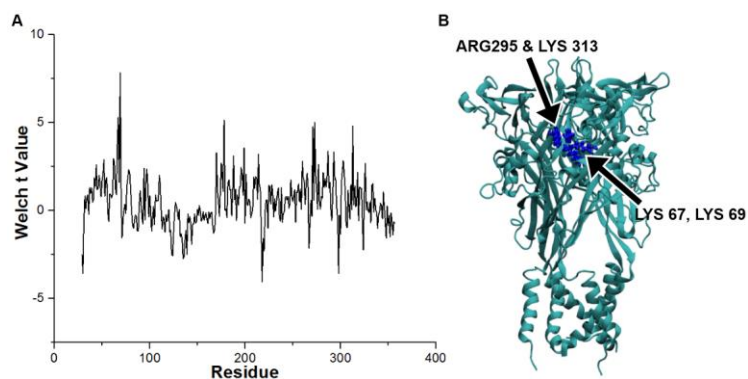


Figure 17. Flexibility differences between HOLO and HOLO(-) states in P2X4 receptor. (A) RMSF differences between HOLO(-) and HOLO states in the P2X4 receptor.(B) Among the residues with higher differences in flexibility, LYS 67, LYS 69 & LYS 313 are key along ARG 295 to stabilize

the four negative charges of ATP and therefore important in the opening of the receptor upon stimulation with this agonist.

With the idea that ATP has a role in P2X₄ receptor stabilization, the interaction energy between the receptor subunits and each ATP was calculated (Fig. 18, Fig. 19). The interaction energy that ATP makes with each subunit is of high importance since its value (~ -73.8 to -400 kcal/mol, Fig. 19) is comparable to subunit-subunit interaction energy whose value is -533.1 kcal/mol ($= -1599.4/3$ kcal/mol, Fig. 18). Therefore, it could restrain on how conformational changes occur by keeping residues located at the binding pocket of ATP (i.e. LYS 67, 69, 313 and ARG 295) tightly closed and acting like a hinge. This could also be an explanation of how the internal profile of the receptor in APO vs. HOLO state is (Fig. 20) in which it is observed that the bending of the internal profile follows as ATP acting as a hinge, producing a reduction of the pore size in the upper part (height > 75 Å) and a dilation at the level below it.

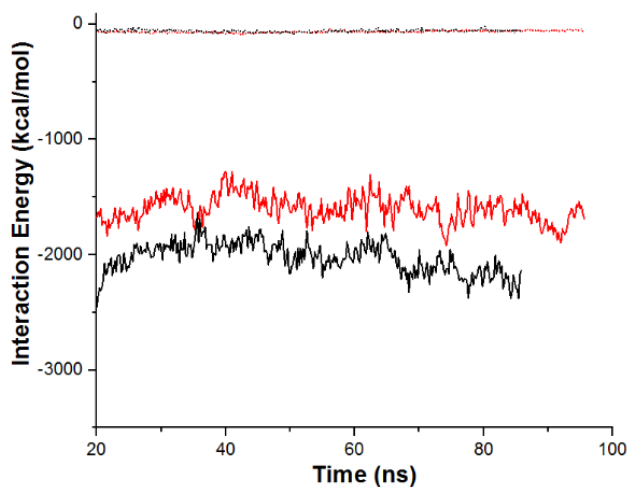


Figure 18. Total inter-subunit non-bonded interaction energy in the P2X₄ receptor. HOLO (red dotted line) and APO (black dotted line) inter-subunit TM non-bonded interaction energies has an average of -64.5 kcal/mol (SD 8.9 kcal/mol) and -57.6 kcal/mol (SD 10.9 kcal/mol) respectively, whereas HOLO (solid red line) and APO (solid black line) total inter-subunit non-bonded interaction energies has an average of -1599.4 kcal/mol (SD 114.7 kcal/mol) and -2027.4 kcal/mol (SD 136.3 kcal/mol) respectively.

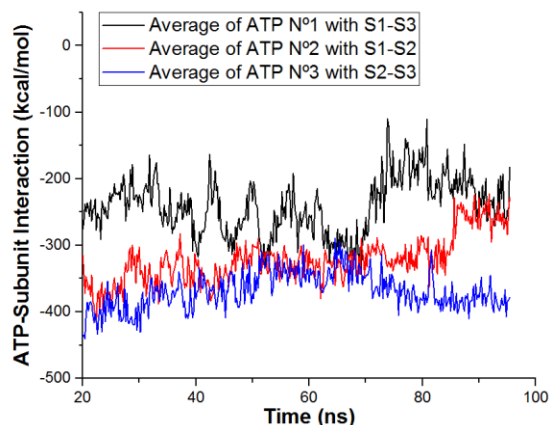


Figure 19. ATP-P2X4 receptor interaction energy. Interaction energy of an ATP molecule with the two subunits of the receptor is in contact with. (Black) ATP molecule N°1 has an averaged interaction energy with S1 and S3 of -241.1 kcal/mol, having an interaction S1-ATP1 and S3-ATP1 of -73.8 and -408 kcal/mol respectively. (Red) ATP molecule N°2 has an averaged interaction energy with S1 and S2 of -322.5 kcal/mol, having an interaction S1-ATP2 and S2-ATP2 of -215.5 and -429.4 kcal/mol respectively. (Blue) ATP molecule N°3 has an averaged interaction energy with S2 and S3 of -368.1 kcal/mol, having an interaction S2-ATP3 and S3-ATP3 of -255.3 and -480.8 kcal/mol respectively. S1, S2 and S3 represent the 3 subunits in the receptor.

Another key point to mention is that HOLO state has less energy interaction between subunits than APO state (Fig. 18). This could give a hint on why subunits are allowed to increase the distance between themselves. However, it is not clear if this lower interaction is because at some point the receptor dilated and/or if this lower interaction will lead to a higher dilation (Fig. 20).

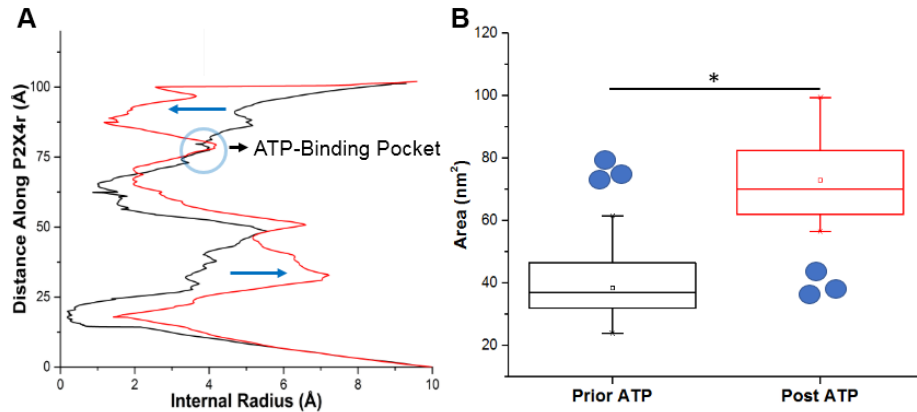


Figure 20. Internal profile & cross-sectional area of P2X4 receptor in the APO and HOLO states. (A) Internal profile of APO (black) and HOLO state (red), bottom indicates the start of the transmembrane segment -showing a narrow internal radius between ~ 12 and 25 Å for the APO state- as it goes to the extracellular segment (30 - 100 Å). At approximately 75 Å, ATP binding pocket is located. By contrasting APO and HOLO profile, it seems to resemble a hinge mechanism centered near the ATP-binding pocket, dilating beneath it and contracting above it (blue arrows). (B) Increase in cross-sectional area observed upon ATP stimulation as detected by AFM (data provided by Frederik Bergler, Prof. Edwardson's lab)

Taken together, MD simulations suggest that ATP has a number of effects on P2X4 receptor: it has a high interaction energy between the two subunits that is comparable to the energy of interaction that the two subunits have. This fact could have high relevance since P2X4 receptor, once bound to ATP, is subjected to a spatial restraint given by ATP due to its high interaction, giving the possibility that ATP acts as a hinge.

A third point to look at in the structural differences between APO and HOLO states of P2X4 receptor refers to protein conformational changes associated with the environmental interaction (solvent and lipid bilayer surrounding the membrane protein).

Protein-Water Interaction

In order to test possible differences between both APO and HOLO states at interacting with water, a distinction of first solvation layer was made. This approach consisted in calculating the interaction energy with water molecules that are within 3.5 Å of the protein (Laage, Elsaesser, & Hynes, 2017) versus the total interaction energy between the receptor and water, which was calculated applying a cutoff of 12Å for Van der Waals interactions. Fig. 21 shows the first solvation layer (dotted line) interaction energy for both states, being the APO state -1043.2 kcal/mol more negative than HOLO state and similarly the total interaction energy (bold line) presented a difference of -1213 kcal/mol between both states. It is important to highlight that water molecules present within the first layer of solvation can be separated in three groups: water present at interface subunit-subunit, ATP binding pocket (highly positive net charge) and at the outer and inner surface of the receptor. Interestingly, the interaction energy of water molecules and intersubunit residues (Fig. 22) provided a difference of -613.8 kcal/mol in favor of the APO state. Altogether these data suggest that inter-subunit residues interacting with water molecules account for approximately 60% of the difference between both states. The other difference may be due to solvation in the binding pocket since it has four positively charged residues, which are accesible to form hydrogen bonds with water in the APO but not in the HOLO state.

In addition, this difference at interface subunit-subunit can be explained by at least two factors: 1) surface that subunits in the APO state share is larger, so the number of water molecules is higher and therefore the net interaction is higher, 2) Charged or polar residues are more exposed in the inter-subunit space so they can interact stronger with water molecules (or conversely non-polar residues are less exposed in the APO state). In order to answer those two proposals, the number of water molecules was calculated for each state, yielding values of 325 (SD 18) and 299 (SD 17) for the APO and HOLO state respectively and had an average interaction of -1.5 (SD 0.13) and -1.43 (SD 0.23) kcal/mol for the APO and HOLO state respectively.

The analysis of protein-water interaction suggests that there is a larger interaction for the APO state which could be due to the number of molecules present between the P2X4 receptor subunits (protein-protein interface), having -on average- 26 additional water molecules in the APO state and a slightly larger interaction energy than in the HOLO state.

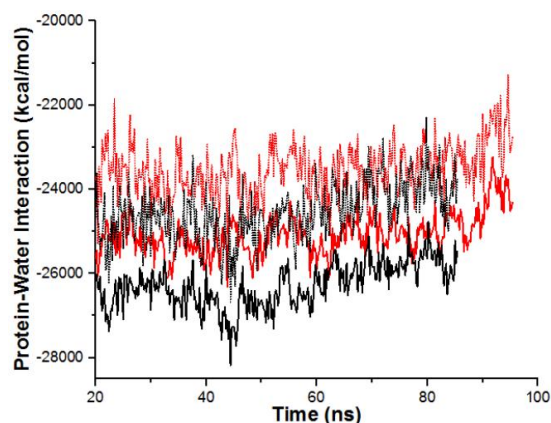


Figure 21. Interaction of P2X4 receptor with water molecules. (red dotted) Interaction energy between HOLO state and water molecules within 3.5 Å has a mean of -23512.8 kcal/mol (SD 620 kcal/mol) whereas for the (black dotted) APO state has a mean of -24556.4 kcal/mol (SD 699.7 kcal/mol). (Solid red) Total interaction energy (no cutoff) between HOLO state and water molecules has a mean -25071 kcal/mol (SD 489.6 kcal/mol) whereas for (solid black) APO state has a mean of -26284 kcal/mol (SD 533.5 kcal/mol). Time resolution = 0.2 ns.

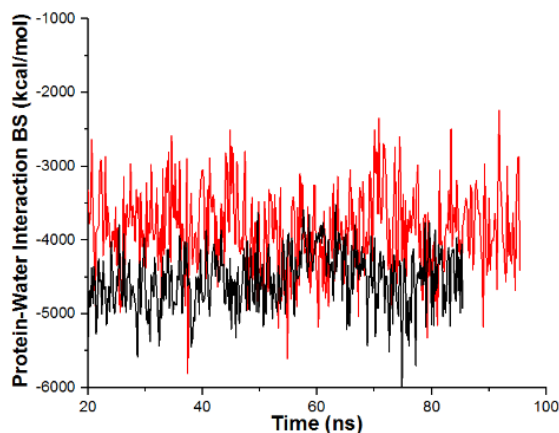


Figure 22. Interaction of P2X4 receptor with intersubunit water molecules. (Red line) Total interaction energy of HOLO state with intersubunit water molecules (cutoff of 3.5Å) has a mean of -3860 kcal/mol (SD 587 kcal/mol) whereas for the APO state (black line) has a mean of -4473.8 kcal/mol (SD 421.2 kcal/mol). Time resolution = 0.2 ns.

Protein-Lipid Interaction

In order to test the role of lipid bilayer on the structural behavior of APO and HOLO states of P2X4 receptors, we are going to focus the analysis on the interaction energy between receptor and lipid bilayer, the energy fluctuation between them, the interaction energy within lipids in the receptor surroundings and their energy fluctuation.

A protein that interacts stronger with lipids is more likely to have a larger effective mass since lipids are likely moving along with the protein. Before calculating protein-lipid interaction energy, it was worth asking what distance is necessary to capture a representative interaction (e.g. in water, 3.5 Å is a good cutoff to get the first solvation layer) between lipids and protein. Therefore, the interaction was calculated with different cutoffs: 3.5 Å, 4.5 Å, 5.5 Å and 7 Å (Fig. 23). Results show that interaction energy between POPC and HOLO state decreases rapidly and there is no major difference when using a cutoff 4.5 Å vs. 7 Å so the way of calculating the interaction energy would be similar to water, this is taking two cutoffs: 3.5 Å and 12 Å for the “first lipid layer” and for the total interacting lipids respectively. This would give us different information since “anchored” lipids are likely to be in the proximities, consequently fluctuations within this cutoff are more likely to be related to them. On the other hand, fluctuations with a higher cutoff are likely to give us information about all the lipids that have some degree of interaction with the protein while also capturing the intrinsic fluctuation of those lipids.

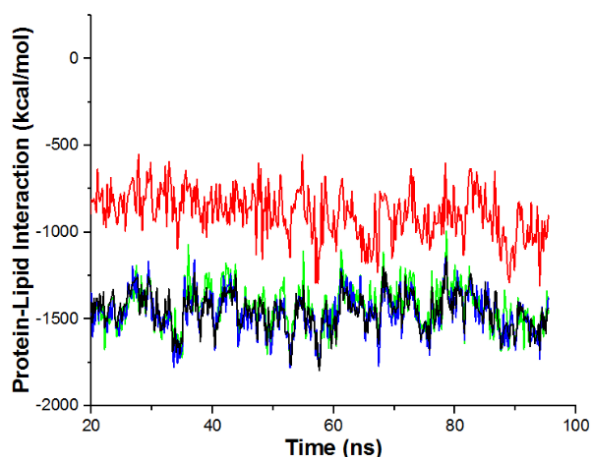


Figure 23. Interaction of the HOLO state in P2X4 receptor with POPC lipids at different cutoffs. (red line) Interaction energy of HOLO state with POPC lipids within 3.5Å, (green line), 5.5 Å (blue line) and 7 Å (black line). Time resolution=0.2 ns.

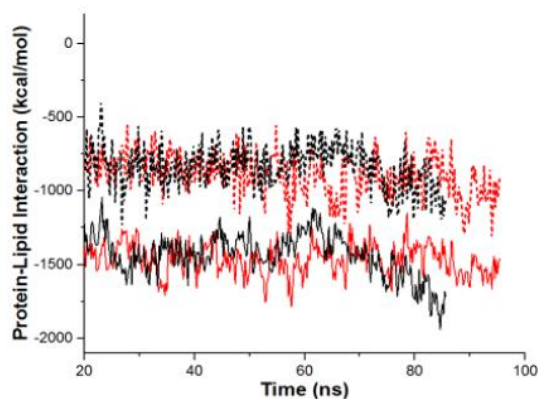


Figure 24. Interaction of the HOLO and APO states in P2X4 receptor with POPC lipids. (red dotted line) Interaction energy between HOLO state and lipids within 3.5Å has a mean of -890.5 kcal/mol (SD 140.9 kcal/mol) whereas for APO state (black dotted line) has a mean of -837.4 kcal/mol (SD 148.1 kcal/mol). (solid red line) Total interaction energy between HOLO state and lipids (no cutoff) has a mean -1467.3 kcal/mol (SD 103.7 kcal/mol) whereas for APO state (solid black line) has a mean of -1436.8 kcal/mol (SD 148 kcal/mol). Time resolution=0.2 ns.

Since proteins are surrounded by a highly heterogeneous lipid composition, some of them having a net charge (e.g., DOPS) and others not (e.g., L α -PC), it is worth to evaluate independently the contribution of both coulombic and van der Waals interactions (Fig. 25). Most of the fluctuations in APO and HOLO structures comes from electrostatic fluctuation. In the case of APO state, the SD of coulombic and vdW interactions are 135.2 and 28.8 kcal/mol respectively (being coulombic 369% higher than vdW) and for the case of HOLO state the SD of coulombic and vdW are 95.4 and 25.5 kcal/mol respectively (being coulombic fluctuations 274% higher than vdW). This fact is expected because vdW interaction decays as r^{-6} whereas Coulombic decrease as r^{-1} , and therefore the later captures fluctuation of non-tightly coupled lipids.

The importance of these findings comes from the fact that charged lipids (e.g., DOPS) may be closely interacting with charged residues, and by altering the fluctuation of coulombic interactions, the stability of the system will be modified largely. Moreover, P2X4 receptor has at least 10 charged residues per subunit that are accessible to lipid heads, namely: ARG 33, GLU 51, LYS 52, ASP 260, ASP 264, ARG 265, LYS 326, LYS 329, ASP 331 and ASP354. In a previous research (Shinozaki et al., 2009), the pore dilation of rat P2X4 receptor was measured yielding

more notorious values than our case (Fig. 20), however, their reconstituted lipid bilayer had two times the abundance of phosphatidylserine (L α -PC:PS=1:1) in comparison to our experiments (L α -PC:PS=3:1), which might suggest that the pore dilation mechanism is dependent on negatively charged lipids.

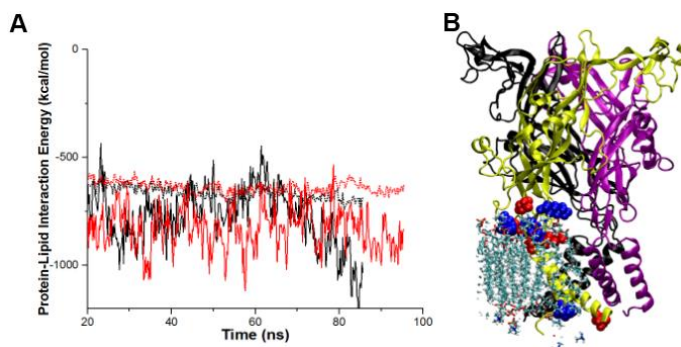


Figure 25. Contribution of electrostatic and van der Waals interactions energies between P2X4 receptor and lipids. (A) (black line) Electrostatic contribution in the total interaction energy between APO state and POPC has a mean of -774.5 kcal/mol (SD 135.2 kcal/mol) whereas Van der Waals (VdW) contribution in the total interaction energy (black dotted line) has a mean of -662.3 kcal/mol (SD 28.8 kcal/mol). (red line) Electrostatic contribution in the total interaction energy between HOLO state and POPC has a mean of -830.5 kcal/mol (SD 95.4 kcal/mol) whereas VdW contribution in the total interaction energy (red dotted line) has a mean of -636.8 kcal/mol (SD 25.5 kcal/mol). (B) P2X4 receptor showing charged residues (blue=positive, red=negative) that are in close contact with a segment of the lipid bilayer.

Finally, looking at the lipid-lipid interaction (Fig. 26), both the interaction energy and energy fluctuation differ just 1.8% and 4.6% respectively between the two states. Since both HOLO and APO states are surrounded by an homogeneous POPC bilayer, it is expected that in both cases the lipid-lipid interaction was similar. However, that may not be true when looking at the very close of the protein because different tilt of the α -helix domains could give rise to different lipid orientation, modification of bilayer thickness and their flexibility, and therefore other ways to interact between themselves (Casuso, Sens, Rico, & Scheuring, 2010).

The analysis of lipids yields three conclusions: total protein-lipid fluctuation was higher in the APO state, while most of the energy fluctuations in both states comes from coulombic interactions rather than vdW. In the case of lipid-lipid interaction no substantial differences were observed between APO and HOLO states.

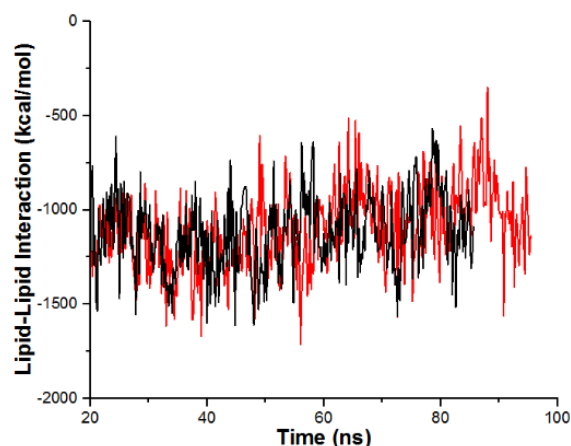


Figure 26. Lipid-lipid interaction within 7Å of the P2X4 receptor. (red line) For the HOLO state, lipid-lipid interaction has a mean of -1097.7 kcal/mol (SD 216.9 kcal/mol) whereas for the APO state (black line) this has a mean of -1117.9 kcal/mol (SD 207.2 kcal/mol). Time resolution= 0.2 ns.

Imaging porous alumina surface by AFM

When measuring movement of receptors embedded on supported lipid bilayer, protein diffusion is much less than expected mainly due to two factors: 1) high interaction between mica and lipids, which makes them be less mobile and 2) proteins with large intracellular or extracellular domains are more likely to have a strong interaction with the mica. In order to try overcoming this problem, porous alumina surface was used for supporting the lipid bilayer, however when “bilayer” was imaged by AFM, its quality was not good enough to distinguish P2X4 receptors (Fig. 27). It is possible to improve this result by using another functionalization method and instead of covering the surface with Poly-lysine (see Materials & Methods), a protocol using 1-octanethiol on gold-coated porous alumina could result in more stable bilayers, therefore imaging them would induce a lower perturbation of the system and chances to distinguish proteins should be higher.

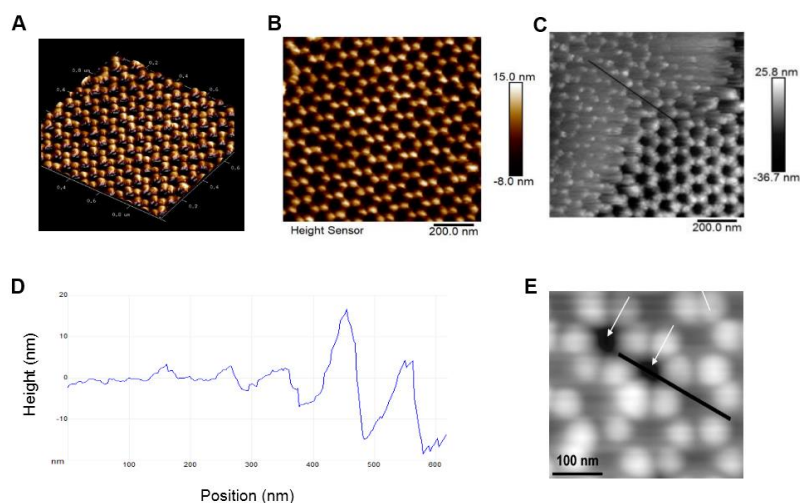


Figure 27. Imaging porous alumina surface by AFM. (A) 3D representation of an image of bare alumina with its 2D shown in (B). (C) Proteoliposome containing P2X4 receptors supported on porous alumina. (D) Height profile of line drawn in (C). (E) Image showing a liposome suspended on alumina indicating non-covered pores with white arrows, taken from (Steltenkamp et al., 2006).

Conclusion

Understanding of the structural mechanism associated with receptors activation is crucial to the development of new pharmaceutical drugs. Once P2X4 receptors are bound to ATP, our HS-AFM experiments indicate that they started diffusing less and broaden their outer domain.

The MD results tell us that energy fluctuation of protein-lipid interaction in HOLO state was lower and therefore more stable, and that a major component of this fluctuation in both APO and HOLO states comes from Coulombic interaction rather than van der Waals interaction. In the case of total protein-water interaction, the fluctuation was higher also in the APO state. Altogether, these structural features give support to a lower mobility of the HOLO state.

The decrease in diffusion might not be solely due to a change in the interaction of the protein with water/lipids, but also given a stronger interaction of the activated state with the mica. In order to address this possibility, proteoliposomes were suspended on porous alumina, but it was not possible to image single receptors mostly due the instability of the bilayer.

Regarding the broadening of the outer domain upon ATP activation, a previous study (Shinozaki et al., 2009) observed a larger dilation at the extracellular domain compared to our data, which

could be associated with a bigger DOPS concentration used in their experiments (50% versus 25% used herein). This fact could suggest that negatively charged lipids could modulate the activity and dilation of the receptor. In our computational study, it was measured a marked difference in the internal profile of the P2X4 receptor when comparing its APO and HOLO state, with an immobile region that could act as a hinge mechanism between both states located at the ATP binding pocket. However, it was not possible to measure a marked dilation in the upper external profile of the receptor through MD. This could be explained by current limitations on the MD time needed to explore long-term protein dynamics, the lack of negative lipids.

Future Work

In order to improve the results obtained on proteoliposomes deposited on porous alumina, we propose a methodology based on chemical functionalization by 1-octanethiol on gold-coated porous alumina. This method should give more stability to the lipid bilayer by forming a self-assembly monolayers on top of alumina and allowing to the proteoliposome to fuse with them (Prime & Whitesides, 1991), which would lead us to explore diffusion properties of individual P2X4 receptor not deposited over a flat mica.

In the case of our computational results, although they are based on single extended simulations for the APO and HOLO states, it is suggested to: 1) increase the number of replicas to verify reproducibility; 2) perform the simulation on bilayers containing DOPS/L α -PC as it was done in our experiments; and 3) use a MD protocol that boost conformational sampling (Bernardi, Melo, & Schulten, 2015).

In order to characterize the P2X4 receptor pore dilation mechanism and its dependence of negatively charged lipids (DOPS) two experiments could be performed: 1) analysis of the pore dilation by AFM in liposomes containing different DOPS/L α -PC ratios and 2) performing electrophysiological measurements of P2X4 receptor on the same modified liposomes. This could allow us to associate the structural/functional role of the charged lipids.

Appendix

Comparison of CSD and MSD

In order to review the features of both CSD and MSD methodologies, simulations with a system having two states were performed. Diffusion can be modeled as a succession of displacements given by a gaussian distribution (eq. 6) (i.e., a gaussian process), so for a system with two states it is necessary to: 1) define a transition probability from one state to the other and 2) give a gaussian distribution with standard deviation to each state:

$$f(v_i) dv_i = \left(\frac{m}{2\pi kT} \right)^{1/2} e^{-\frac{mv_i^2}{2kT}} dv_i \quad (eq. 6)$$

Where m represents the mass, k the Boltzmann constant, T the temperature, and v_i the velocity in the axis i .

By performing this simulation, it is possible to compare both MSD and CSD analysis. As seen in Fig. 28, it is observed that A and D show a corrallated trajectory that is effectively given into account by the MSD analysis by showing an initial linear regime and after decreasing; However, MSD it is not capable of showing that different states exist neither when transitions occur. In the case of CSD (C, F) it is seen a stair-like plot in which each break means a transition.

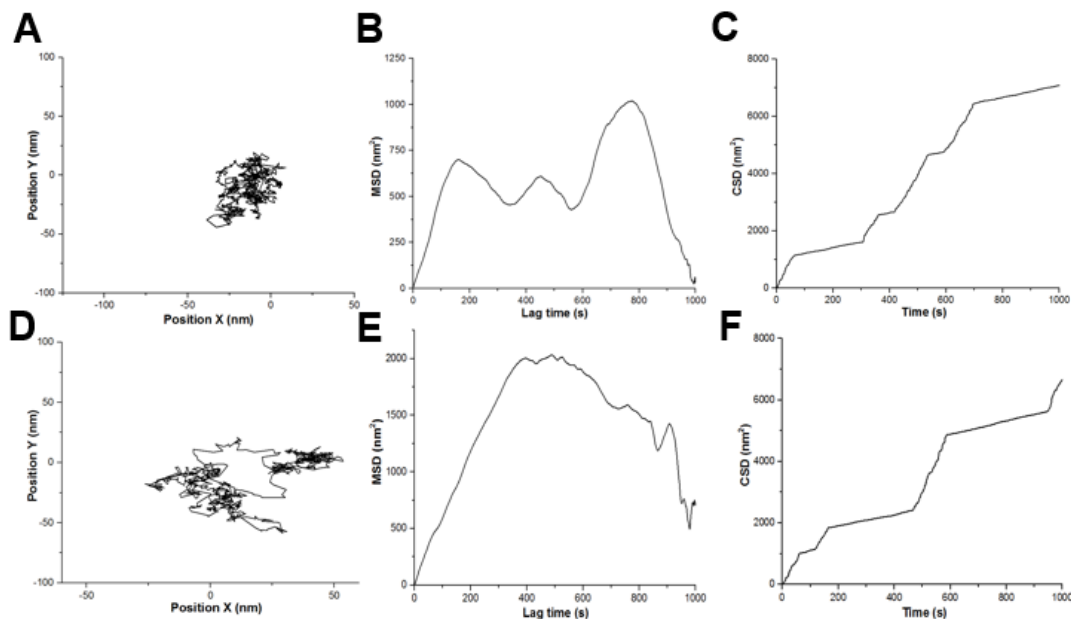


Figure 28. Simulation of a diffusion process with two states. (A,D) Trajectory XY of a particle. (B,E) MSD analysis and (C,F) CSD analysis.

In order to empirically study how many transitions did occur through the simulation via CSD analysis, a set of piecewise of linear function should be used and the Akaike information criterion (AIC) is calculated for different set of fittings in order to know the number of transitions. In the case of studying the number of distinguishable states, it is necessary to study how the slopes are distributed (Fig. 28 C,F). These transitions are visible in our results when comparing prior to post stimulation with ATP, showing different slopes; however, it was not attempted to find substates within the HOLO state. If they did exist, their lifetimes should be less than our temporal resolution, and this framework should work only when a “higher” number of points are available between two transitions. Moreover, if few points per state are available, AIC is most likely to see them as outliers of the previous state, therefore making not possible to tell if it was an actual transition.

Codes

Counting the number of water molecules between subunits (Implemented in Tool Command Language)

```
1. #set the cutoff
2. set cutoff 3.5
3.
4. #get the number of frames
5. set nf [molinfo top get numframes]
6.
7. #contacts will keep the number of atoms belonging to water molecules
8. set contacts [list]
9.
10. set filename "nwater_ang_${cutoff}.txt"
11.
12. #create and write in file
13. set fileId [open $filename "w"]
14.
15.   for {set i 0} {$i < $nf} {incr i} {
16.
17.     set sel1 [atomselect top "water and within $cutoff of segname P1 and within $cutoff of
       segname P2" frame $i]
18.     set contacto [$sel1 num]
19.
20.     puts $fileId "$i $contacto"
21.   }
22. #result must be divided by three
23.
24.
25. close $fileId
```

Average RMSF/RMSD per residue at “equilibrium” (Implemented in Python)

```
1. import csv
2. #load archive generated by timeline plugin, delete headers starting with #
3. reader=csv.reader(open("archive_name.dat"),delimiter=" ")
4.
5. #create arrays to store data
6. add = []
7. addsquare = []
8. count = []
9.
```

```

10. #number of residues
11. residues=303
12.
13. for i in range(1,residues):
14.     add.append(0)
15.     count.append(0)
16.     addsquare.append(0)
17.
18. #set frame number from where structure has a stable RMSD/RMSF
19. equilibrium = 100
20.
21. #read the file
22. for line in reader:
23.     for i in range(1,residues):
24.         if int(line[3])>equilibrium:
25.             if int(line[0]) == i:
26.                 add[i]=add[i]+float(line[4])
27.                 count[i] = count[i]+1
28.                 addsquare[i] = addsquare[i]+(float(line[4]))**2
29.
30. #create and write to the file that will store RMSD/RMSF per residue
31. file=open("RMSD_perResidue.txt","w")
32. for i in range(1,residues):
33.     if count[i] == 0:
34.         file.write(str(i))
35.         file.write(" ")
36.         file.write(str(0))
37.         file.write(" ")
38.         file.write(str(0))
39.         file.write(" ")
40.         file.write(str(0))
41.         file.write("\n")
42.     if count[i] > 1:
43.         file.write(str(i))
44.         file.write(" ")
45.         file.write(str(add[i]/count[i])) #average per residue
46.         file.write(" ")
47.         file.write(str(((addsquare[i]-(add[i]*add[i]/count[i]))/(count[i]-
1)**0.5)) # standard deviation
48.         file.write(" ")
49.         file.write(str(count[i]))
50.         file.write("\n")
51. file.close()

```

Measuring the center of mass in receptors (Implemented in ImageJ Macro software)

```

1. run("Text Window...", "name=Log width=120 height=16 menu");

```



```

2. x=1 //calculate the center of mass every 1 frames
3. num=165 //number of frames
4. for (i=0; i<num; i++)
5. {
6.   run("Adrian's FWHM", "single pinhole=15 pinhole=15 height=0");
7.   String.copyResults();
8.   selectWindow("Log");
9.   String.paste;
10.  selectWindow("name.avi");//name of .avi file
11.  for (j=0; j<x; j++)
12.  {
13.    run("Next Slice [>]");
14.  }
15.  close("Y Histo*");
16.  close("X Histo*");
17.  close("ROI*");
18. }

```

Simulating CSD and MSD in a two-state system (implemented in Wolfram Mathematica software)

```

1. lengthsim = 1000;
2. position = ConstantArray[0, {lengthsim, 2}];
3. speedx = ConstantArray[0, {lengthsim, 2}];
4. speedy = ConstantArray[0, {lengthsim, 2}];
5. displacement = ConstantArray[0, lengthsim - 1];
6. CSD = 0; (*Storage Cumulated Squared Displacement*)
7. Cumulant1 = ConstantArray[0, {lengthsim, 2}];
8. positionx = 0; (*initial position XY*)
9. positiony = 0;
10. MSD = ConstantArray[0, {lengthsim, 2}];(*Store MSD*)
11. Transitionprob = 0.5; (*if set 0.5, probability of staying in the \
12. same state is 0.5*)
13. i = 2;
14.
15. For[itr = 1, itr <= 1000, itr++,
16.
17.  x = RandomReal[];
18.  y = RandomInteger[
19.    BinomialDistribution[100, 0.7]]; (*length of state in steps,
20. modeled as a binomial distribution with mean 70*)
21. If[i >= lengthsim,
22.  Break[]; (*Stop the process if i surpass length simulation*)
23.
24.  If[x > Transitionprob && i <= lengthsim,
25.
26.   Print["State 1, at step ",
27.    i];(*Included to get track of the transition*)
28.   For[length = 0, length < y, length++,
29.
30.    positionx =

```

```

31. RandomReal[NormalDistribution[positionx, 1]];(*Gaussian process,
32. with mean X and standard dev 1 *)
33. positiony = RandomReal[NormalDistribution[positiony, 1]];
34. position[[i, 1]] = positionx;
35. position[[i, 2]] = positiony;
36. speedx[[i, 2]] = position[[i, 1]] - position[[i - 1, 1]];
37. speedx[[i, 1]] = i;
38. speedy[[i, 2]] = position[[i, 2]] - position[[i - 1, 2]];
39. speedy[[i, 1]] = i;
40. If[i > 1,
41. displacement[[i - 1]] =
42. Sqrt[(position[[i, 1]] -
43. position[[i - 1, 1]])^2 + (position[[i, 2]] -
44. position[[i - 1, 2]])^2];];
45. CSD =
46. CSD + (position[[i, 1]] -
47. position[[i - 1, 1]])^2 + (position[[i, 2]] -
48. position[[i - 1, 2]])^2;
49. Cumulant1[[i, 2]] = CSD;
50. Cumulant1[[i, 1]] = i;
51. i++;
52. If[i > lengthsim, Break[]];
53.
54. ];
55.
56. ];
57.
58. If[x <= Transitionprob && i <= lengthsim,
59.
60. Print["State 2, at step ", i];
61.
62. For[length = 0, length < y, length++,
63.
64. positionx =
65. RandomReal[NormalDistribution[positionx, 3]];(*Gaussian process,
66. with mean X and standard dev 3 *)
67. positiony = RandomReal[NormalDistribution[positiony, 3]];
68. position[[i, 1]] = positionx;
69. position[[i, 2]] = positiony;
70. speedx[[i, 2]] = position[[i, 1]] - position[[i - 1, 1]];
71. speedx[[i, 1]] = i;
72. speedy[[i, 2]] = position[[i, 2]] - position[[i - 1, 2]];
73. speedy[[i, 1]] = i;
74. If[i > 1,
75. displacement[[i - 1]] =
76. Sqrt[(position[[i, 1]] -
77. position[[i - 1, 1]])^2 + (position[[i, 2]] -
78. position[[i - 1, 2]])^2];];
79. CSD =
80. CSD + (position[[i, 1]] -
81. position[[i - 1, 1]])^2 + (position[[i, 2]] -

```

```

82.     position[[i - 1, 2]]^2;
83.   Cumulant1[[i, 2]] = CSD;
84.   Cumulant1[[i, 1]] = i;
85.   i++;
86.   If[i > lengthsim, Break[]];
87.
88. ];
89.
90. ];
91.
92. ];
93.
94. (*MSD calculation*)
95. maximumlag = lengthsim - 1;
96. MSD = ConstantArray[0, {lengthsim - 1, 2}];
97.
98. For[lag = 1, lag <= maximumlag, lag++,
99.
100.     numpoints = lengthsim - lag;
101.     squaredR = 0;
102.
103.     For[time = 1, time <= numpoints, time++,
104.
105.         squaredR += (position[[time + lag, 1]] -
106.             position[[time, 1]]^2 + (position[[time + lag, 2]] -
107.                 position[[time, 2]]^2;
108.
109.     ];
110.
111.     MSD[[lag, 1]] = lag;
112.     MSD[[lag, 2]] = squaredR/numpoints
113. ]
114.
115. (*Plot*)
116. ListPlot[position, Joined -> True]
117. ListPlot[Cumulant1, Joined -> True]
118. ListPlot[MSD, Joined -> True]
119. Histogram[displacement, ScalingFunctions -> {"Linear", "Linear"}]
120. Histogram[speedx[[1 ;; lengthsim, 2]]]
121. Histogram[speedy[[1 ;; lengthsim, 2]]]

```

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