Probabilistic Model Checking Analysis of Palytoxin Effects on Cell Energy Reactions of the Na^+/K^+-ATPase

Fernando A. F. Braz, Jader S. Cruz, Alessandra C. Faria-Campos, and Sérgio V. A. Campos

Abstract—Probabilistic Model Checking (PMC) is a technique used for the specification and analysis of complex systems. It can be applied directly to biological systems which present these characteristics, including cell transport systems. These systems are structures responsible for exchanging ions through the plasma membrane. Their correct behavior is essential for animal cells, since changes on those are responsible for diseases. In this work, PMC is used to model and analyze the effects of the palytoxin toxin (PTX) interactions with one of these systems. Our model suggests that ATP could inhibit PTX action. Therefore, individuals with ATP deficiencies, such as in brain disorders, may be more susceptible to the toxin. We have also used heat maps to enhance the kinetic model, which is used to describe the system reactions. The map reveals unexpected situations, such as a frequent reaction between unlikely pump states, and hot spots such as likely states and reactions. This type of analysis provides a better understanding on how transmembrane ionic transport systems behave and may lead to the discovery and development of new drugs to treat diseases associated to their incorrect behavior.

Index Terms— Probabilistic model checking, systems biology, sodium-potassium pump, palytoxin.

1 Introduction

Probabilistic Model Checking (PMC) is a formal technique to model and analyze probabilistic and complex systems. It explores a stochastic model exhaustively and automatically, verifying if it satisfies properties given in special types of logics. Properties can be expressed as, e.g., "What is the probability of a particular event happening?", offering valuable insight over the behavior of the model [6], [7], [8].

PMC can be applied to study biological systems which show probabilistic and non-deterministic behavior. It can obtain a better understanding than others methods, such as simulations, which present local minima problems that PMC avoids [9], [10], [15].

We present and check a PMC model of the sodium-potassium pump (or Na^+/K^+-ATPase), a transmembrane ionic transport system which is responsible for exchanging internal sodium ions for external potassium ions.

This pump exists in all animal cells and it is important to several biological processes, such as cell volume control and heart muscle contraction. Its irregular behavior can be related to several diseases and syndromes and it is one of the main targets of toxins and drugs [12].

- *F. Braz, A. Faria-Campos and S. Campos are with the Department of Computer Science, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 30123-970, Belo Horizonte, MG, Brazil. E-mail: {fbraz, alessa, scampos}@dcc.ufmg.br*
- *J. Cruz is with Department of Biochemistry and Immunology, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 30123-970, Belo Horizonte, MG, Brazil. E-mail: jcruz@icb.ufmg.br*

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In our model, the pump is exposed to a deadly toxin called palytoxin (PTX). This was done in order to better understand the disruptive effects of PTX interactions with the pump [13].

We have discovered that high doses of Adenosine Triphosphate (ATP), the cellular energy unit, could inhibit PTX action. When the concentration of ATP is increased from 10 mM to 100 mM, the probability of PTX related pump sub-states is reduced by 38.3520%. This suggests that individuals with ATP deficiency could be more susceptible to the toxin action. ATP deficiency appears in different forms, such as in brain disorders.

Since its production can not be directly stimulated, studying the ability of ATP to change our Na^+/K^+-ATPase model behavior is even more important. Experiments may overlook these extreme and difficult to explore conditions.

We have also used heat maps to enhance the kinetic model of the pump, which is used for describing system reactions. This map is a visual tool for model investigation, which is achieved by coloring each state and reaction in red (likely) or blue (unlikely), accordingly to its probability.

Heat maps reveal "hot spots" (active locations) and unexpected situations, such as a frequent reaction between unlikely pump states, which suggests that either these states are temporary; or there is an unknown state between those two. Either is worth being experimentally validated and could change the perception of researchers on the pump mechanisms. Our results have shown that PMC can be used successfully to explore the dynamics of cell transport systems.

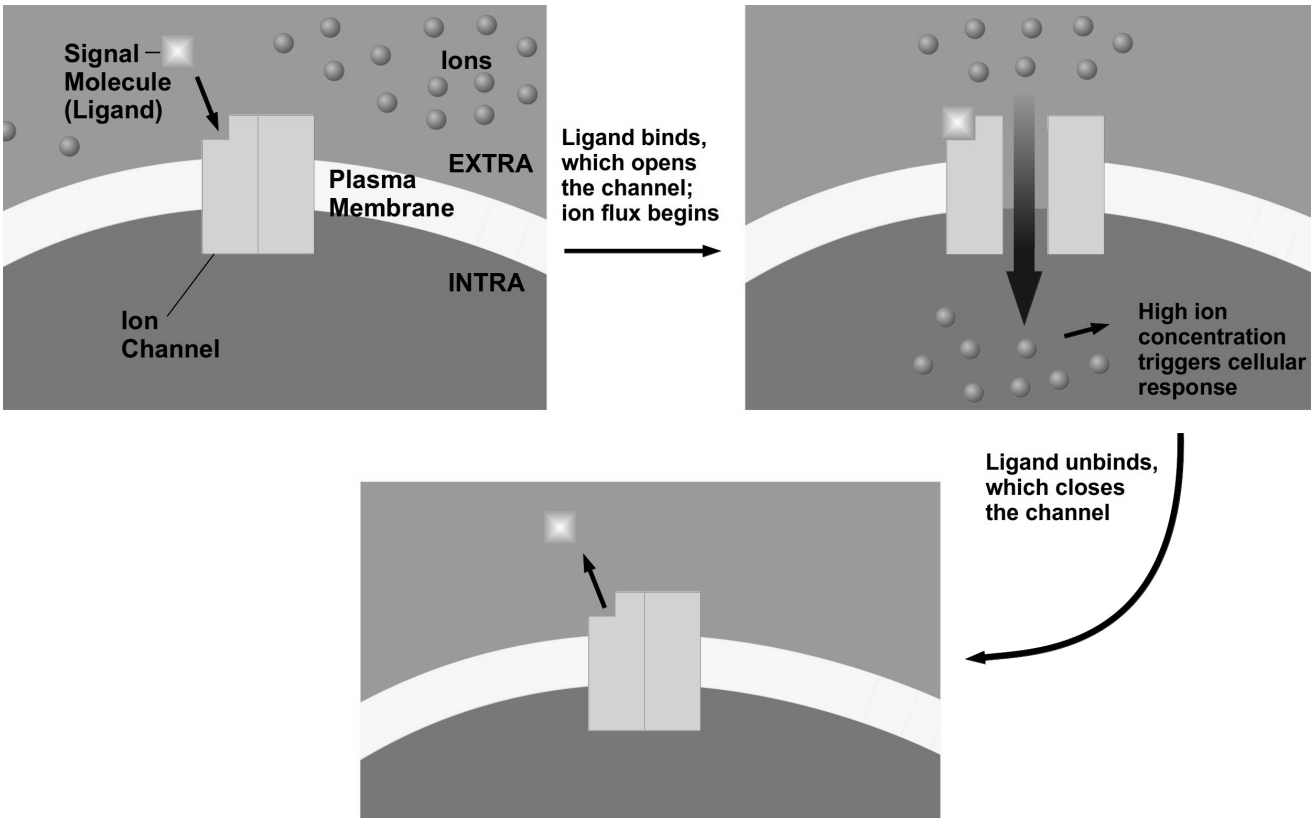

Fig. 1. Generic Ion Channel. An open ion channel transfers ions down their electrochemical gradient. For example, if the extracellular concentration is higher than the intracellular one, the ions would rapidly move into the cell. A high concentration of ions inside the cell could trigger a cellular response, which closes the channel.

This is part of an ongoing effort to better understand these systems. The PMC model of the pump was first described in [5]. PTX was included in the model in [3], where disturbances caused by the toxin in cell energy related reactions were studied.

A model which focused on sodium and potassium related reactions was described in [4]. It revealed that sodium enhances PTX action, while potassium inhibits it. Since the toxin is found in marine species, the sodium inhibitory effect is not a coincidence.

Outline. This paper describes cell transport systems in Section 2. Related works to the analysis of these systems and PMC are discussed in Section 3. Our model is covered in Sections 4 (the pump) and 5 (PTX interactions). Our experiments, properties and results are shown in Section 6. Finally, Section 7 presents our conclusions and future works.

2 Background

2.1 Transmembrane Ionic Transport Systems

Animal cells contain structures called transmembrane ionic transport systems, which are responsible for ion exchange between the inside and outside of the cell. The difference in charges and concentrations between ions in these sides creates an electrochemical gradient, which is essential for cells to perform their functions properly. Ionic transport systems are responsible for the maintenance of this gradient [16].

There are two types of transport systems: ion channels, a passive transport system which does not consumes energy to promote ion exchange; and ionic pumps, an active transport system that uses energy in the form of Adenosine Triphosphate (ATP) to perform ion exchange. Ion channels depend on the concentration gradient of the ions to be transported, moving them down their electrochemical gradient. Ionic pumps exchange ions against their concentration gradient [17], using ATP energy in the process. Once open, ion channels rapidly diffuse ions, allowing abrupt changes in ions concentrations. Ionic pumps, on the other hand, exchange ions very slowly, permitting only subtle changes in ions concentrations.

A generic ion channel is shown in Fig. 1. The ion channel is initially closed, and there is a high concentration of ions in the extracellular medium. A signaling molecule binds to the ion channel, which opens the ion channel. This allows the ions to diffuse rapidly from the extra to the intracellular side (low concentration of ions). The change in ion concentration inside the cell triggers a cellular response. The signaling molecule unbinds the ion channel, which closes the ion channel, therefore interrupting ion flux.

Ion channels and ionic pumps allow only the passage of specific ions such as sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}). For ionic pumps, the passage of ions can be viewed as two gates, one

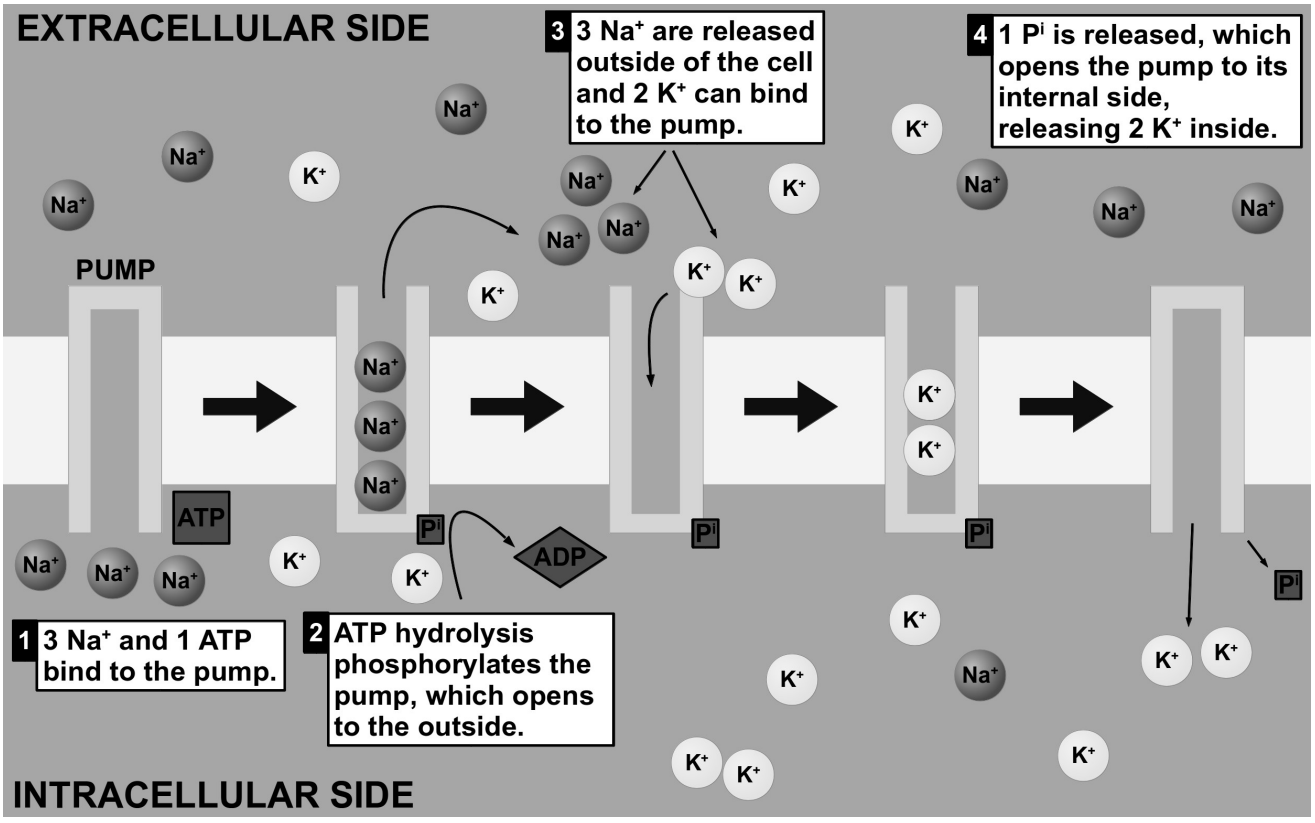

Fig. 2. The sodium-potassium pump. Na^+/K^+-ATPase exchanges three sodium ions from the intracellular side of the cell for two potassium ions from the extracellular side. This is an active transport and it hydrolyzes a molecule of ATP to phosphorylate the pump and change its shape.

internal and one external, which open or close based on different factors, such as chemical signals [16].

An example of a pump is the sodium-potassium pump or Na^+/K^+-ATPase (Fig. 2). This pump is responsible for exchanging three sodium ions from the intracellular medium (rich in potassium and poor in sodium) for two potassium ions from the extracellular medium (poor in potassium and rich in sodium).

This pump can be in two major states: open to the inside of the cell, or open to the outside. The pump cycle starts with three sodium ions binding to the pump when its open to the intracellular side. An ATP binds to the pump, which is followed by its hydrolysis. This breaks the ATP into two molecules, one of phosphate (P_i), which remains bound to the pump, and another of Adenosine Diphosphate (ADP), which is released inside the cell. This also causes the pump to release the sodium ions outside. Two potassium ions in the outside bind to the pump, which are released in the intracellular side, including the phosphate. The pump now can repeat the process [16].

Cell transport systems are involved in several biological processes such as cellular volume control, nerve impulse, coordination of heart muscle contraction and release of accumulated calcium in the sarcoplasmic reticulum for performance of muscle contraction.

These systems are one of the main targets in research for discovery and development of drugs, since its irregular behavior is associated with several diseases, such as hypertension and Parkinson's disease. Cardiac glycosides are one type of these drugs, for example digoxin (also known as digitalis) and ouabain, drugs that are used to improve heart performance by increasing its contraction force [16].

Due to their role in the nervous system, ion transport systems are affected by neurotoxins [16]. One of the toxins that affects these structures is the palytoxin (PTX, or $[PTX]^o$ for extracellular PTX concentration), a deadly toxin found in corals of the *Palythoa toxica* species. PTX disturbs the Na^+/K^+-ATPase, modifying its behavior to the one of an ion channel, which means that the pump transfers ions down their electrochemical gradient (from the ions high concentration side to their low concentration side), instead of against it [12].

Although cell transport systems have been discovered over 60 years ago, they still are an active research field [16]. Because of their different behaviors and transfer rates, ion channels and ionic pumps have been seen as different entities. However, discoveries such as the interaction between PTX and the Na^+/K^+-ATPase are forcing new studies about the mechanics of these structures and its perception by the scientific community [11], [12], [18], [19], [20], [21].

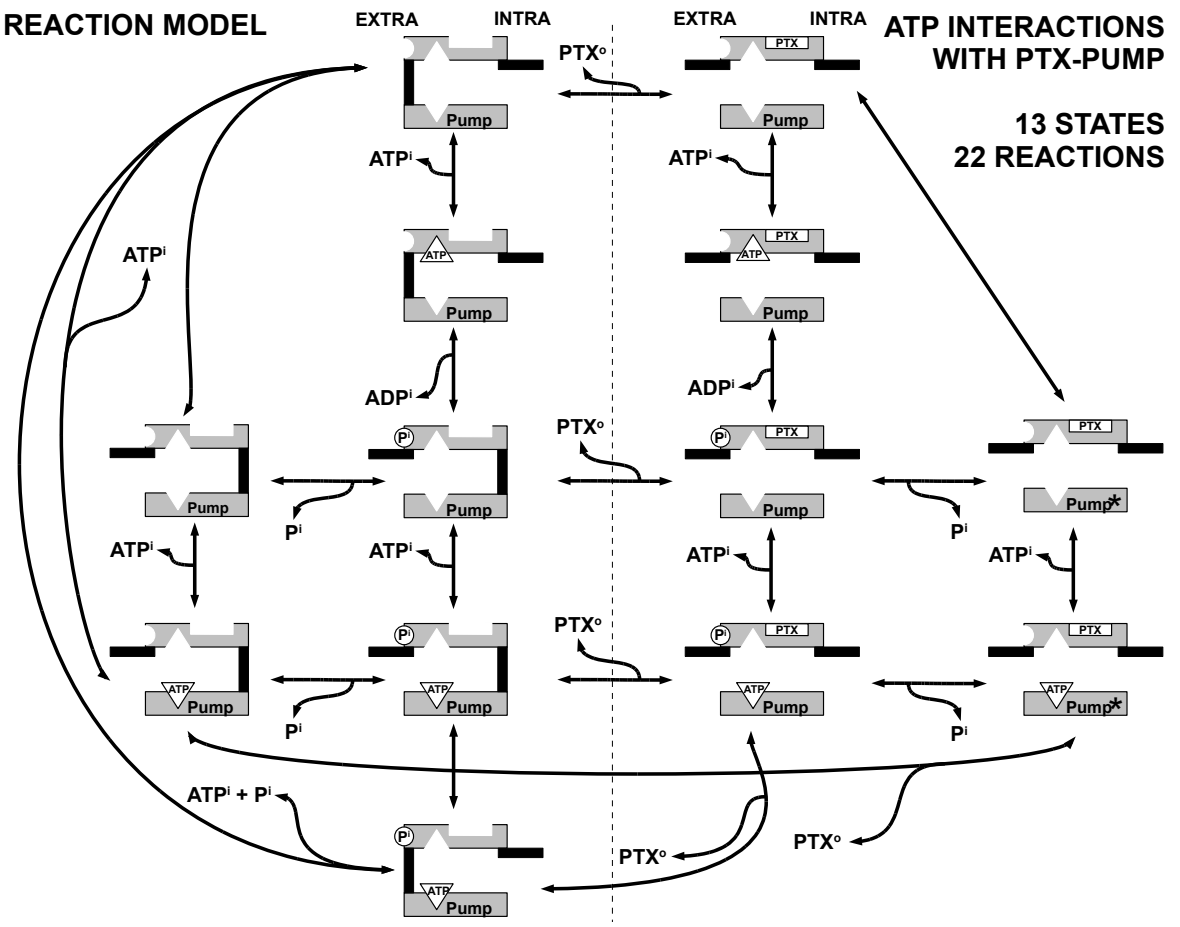

Fig. 3. The kinetic model for PTX interactions with the pump. It presents all the pump sub states (13) and reactions (22) of the model. The left side is the classical Albers-Post model [22], which represents the regular behavior of the pump, while the right side describes the PTX related pump states and reactions [13].

Channels and pumps usually are investigated using experimental results in laboratory benches, which are expensive for both financial and time resources. In order to avoid or minimize these costs, different types of simulations, mathematical and computational methods are employed, among these include sets of ordinary differential equations (ODEs) and Gillespie's algorithm for stochastic simulations [23].

Despite their ability to obtain valuable information, simulations do not cover every possible situation, and might never search certain regions of the state space, therefore possible overlooking some events, such as ion depletion, where all ions have been exchanged. These systems are also described as a kinetic model, which presents its states and possible reactions from one state to other states (for example, Figure 3).

2.2 Probabilistic Model Checking (PMC)

Probabilistic Model Checking is a formal, exhaustive and automatic technique for modeling and analyzing stochastic systems. PMC checks if the model satisfies a set of properties given in special types of logics.

A stochastic system M is usually a Markov chain. This means that the system satisfies the Markov property, i.e., its behavior depends only on its current state and not on the whole system history.

Given a property ϕ expressed as a formula in a probabilistic temporal logic, PMC attempts to check whether a model of a stochastic system M satisfies the property ϕ with a probability greater than or equal to a probability threshold $\theta \in [0, 1]$.

Tools called models checkers such as PRISM [8] attempt to solve this problem. It requires two inputs: a modeling description of the system, which defines its behavior (for example, through the PRISM language), and a probabilistic temporal logic specification of a set of desired properties (indicated as ϕ).

Properties can be expressed quantitatively as "What is the probability of ATP binding to the pump?" or qualitatively as "ATP eventually depletes.", offering valuable insight over the system behavior.

Let $\mathbb{R}_{\geq 0}$ be the set of positive reals and AP be a fixed, finite set of atomic propositions used to label states with properties of interest. A labeled Continuous-time Markov Chain (CTMC) $\mathcal{C}$ is a 4-tuple $\langle S, I, R, L\rangle$ where:

- S is a finite set of states;
- $I \in S$ is the initial state;

- $R : (S \times S) \to \mathbb{R}_{\geq 0}$ is the transition rate matrix, which assigns rates between each pair of states;
- $L : S \to 2^{\mathrm{AP}}$ is a labeling function which labels each state $s \in S$ the set $L(s)$ of atomic propositions that are true in the state.

The probability of a transition between states s and s' being triggered within t time-units is $1 - e^{-\mathbf{R}(s,s') \cdot t}$. The elapsed time in state s, before a transition occurs, is exponentially distributed with the *exit rate* given by $E(s) = \sum_{s' \in S} R(s, s')$. The probability of changing to state s' is given by $\frac{R(s,s')}{E(s)}$.

Further details on the formal definitions of PMC and CTMCs can be found in [24], [25].

Properties are specified using the Continuous Stochastic Logic (CSL) [26], which is based on the Computation Tree Logic (CTL) and the Probabilistic CTL (PCTL). The syntax of CSL formulas follows:

$$\Phi \ ::= \ true \mid a \mid \neg\Phi \mid \Phi \wedge \Phi \mid \mathcal{P}_{\trianglelefteq p}[\phi] \mid \mathcal{S}_{\trianglelefteq p}[\phi]$$

$$\phi \ ::= \ \mathbf{X}\ \Phi \mid \Phi\ \mathbf{U}^I\ \Phi$$

where a is an atomic proposition, $\trianglelefteq \in \{>, <, \geq, \leq\}$, $p \in [0, 1]$ and I is an interval of $\mathbb{R}_{\geq 0}$.

There are two types of CSL properties: transient ($\mathcal{P}_{\trianglelefteq p}$) and steady-state ($\mathcal{S}_{\trianglelefteq p}$). In this work we are interested in transient or time related properties. A formula $\mathcal{P}_{\trianglelefteq p}[\phi]$ states that the probability of the formula ϕ being satisfied from a state respects the bound $\trianglelefteq p$. Path formulas use the $\mathbf{X}$ (next) and the $\mathbf{U}^I$ (time-bounded until) operators. For example, formula $\mathbf{X}\,\Phi$ is true if Φ is satisfied in the next state.

This can be applied to check if one state leads to another with a probability p, for example, there is a chance of at least 10% that the state "open-in" is true until the state "open-out" becomes true: $\mathcal{P}_{\geq 0.1}[\text{“}open-in\text{''}\ \mathbf{U}\ \text{“}open-out\text{''}]$.

As described in [25], a CTMC $\mathcal{C}$ can be enriched with a reward structure $(\underline{\rho}, \boldsymbol{\iota})$, which is used to define two types of rewards: *state* rewards, which are associated with states through the reward function $\underline{\rho} : S \to \mathbb{R}_{\geq 0}$, and *transition* rewards, which are associated with transitions through the reward function $\boldsymbol{\iota} : S \times S \to \mathbb{R}_{\geq 0}$. The state reward $\underline{\rho}(s)$ is the reward accumulated in state s per time unit that is spent on that state, and the transition reward $\boldsymbol{\iota}(s, s')$ is incremented each time a transition between states s and s' occurs.

A reward structure can be used to quantify aspects of the system that the CTMC represents, for example the cell energy (ATP) consumption, the number of ions exchanged between the sides of the cell or the induced electric current by the ions.

3 Related Work

3.1 Experimental and Simulational Techniques

The authors of [12] investigated PTX and its interactions with the Na^+/K^+-ATPase. They have discovered that PTX drastically modifies the nature of the pump after binding to it, which changes the behavior of the pump to that of an ion channel. They suggest that PTX could be an useful tool in experiments to discover the control mechanisms for opening and closing the gates of ion pumps. This is later visited by the authors of [13] through mathematical simulations using non-linear ODEs and considering states and reactions related to the phosphorylation process (phosphate binding and unbinding to the pump).

Interactions of PTX with the complete model of the Na^+/K^+-ATPase are analyzed in [14]. This series of studies by Rodrigues and co-workers can be viewed as a simulational approach of the experimental results of Artigas and co-workers in [12]. This work can be seen as a probabilistic model checking approach to the same experiments.

3.2 Model Checking

The main tools used in the formal verification of biological systems that are related to this work are PRISM [8], BioLab [15], Ymer [27] and Bio-PEPA [28].

PRISM supports different types of models, properties and simulators [8]. It has been largely used in distinct fields, e.g. communication and media protocols, security and power management systems. We have used PRISM in this work for several reasons, which include: exact PMC in order to obtain accurate results; Continuous-time Markov Chain (CTMC) models, suited for our field of study; rich modeling language that allowed us to build our model; and finally property specification using Continuous Stochastic Logic (CSL), which is able to express qualitative and quantitative properties.

The authors of [15] introduce a new algorithm called BioLab. Instead of building all states of a model, the algorithm generates the minimum number of necessary simulations, given error bounds parameterized for acceptance of false positives and false negatives of the properties to be verified. This algorithm is based on the work of [27], author of the approximate model checker Ymer. We did not use these tools because our initial analysis demanded exact results. Future works include an approximate analysis.

In [29], the authors present a novel computational modeling approach using formal verification with the PRISM model checker. The authors treat their model – signaling pathways – as a distributed system, where its components can interact with each other similarly to computer processes. Rather than using an individual approach where each of the ligands are modeled individually, they treat these components as populations, in order to capture the behavior of a whole set of ligands.

The application of PMC to model and analyze different complex biological systems can be seen in [30], [9], for example the signaling pathway of Fibroblast

Growth Factor (FGF), a family of growth factors involved in healing and embryonic development. The analysis of other signaling pathways such as MAPK and Delta/Notch can be seen in [10].

The use of PMC is demonstrated also in [26], [31], where the authors examine and obtain a better understanding of mitogen-activated kinase cascades (MAPK cascades) dynamics, biological systems that respond to several extracellular stimuli, e.g. osmotic stress and heat shock, and regulate many cellular activities, such as mitosis and genetic expression.

4 The Na^+/K^+-ATPase Model

The model is written in the PRISM language (used by the PRISM model checker [8]) and consists of modules for each of the molecules (ATP, ADP and P_i), one main module for the pump, and one auxiliary module which defines the rate of each reaction.

This model does not include PTX because its interactions with the pump are an extension presented in Section 5. A fragment of the model is shown in Fig. 4 and its complete version can be seen in [1].

Na^+/K^+-ATPase PRISM Model

```
1  module p
2   // number of P inside cell
3   pIn : [0..(PI+ATPI+1)] init PI;
4   [r3] pIn<=(PI+ATPI) -> 1 : (pIn'=pIn+1);
5   [rr3] pIn>=1 -> pIn : (pIn'=pIn-1);
6  endmodule
7  module pump
8   // e2 state: pump open to the extracellular side
9   E2 : [0..1] init 1;
10  // e2 with a phosphate bound to it
11  PE2 : [0..1] init 0;
12  // reaction3: PE2 <-> Pi + E2
13  [r3] PE2=1 & E2=0 -> 1 : (PE2'=0) & (E2'=1);
14  [rr3] PE2=0 & E2=1 -> 1 : (PE2'=1) & (E2'=0);
15 endmodule
16 // base rates
17 const double r3rate = 1.90;
18 const double rr3rate = 6.00*pow(10,1)/(0.001*V*AV);
19 // module representing the base rates of reactions
20 module base_rates
21  [r3] true -> r3rate : true;
22  [rr3] true ->rr3rate : true;
23 endmodule
```

Fig. 4. The Na^+/K^+-ATPase model. The model is a stochastic system composed of modules for the molecules (ATP, ADP and P_i), which control their flow; the pump, which controls the pump sub-state and the base rates, which define the rate of the reactions.

Each molecule module contains a variable to store the current number of molecules, e.g. `pIn` for P_i (line 3 of the code fragment in Fig. 4). Each module is composed of PRISM commands (or transitions) that represent reactions, which are responsible for changing the number of molecules (lines 4 and 5).

A PRISM command uses the following structure: `[sync] conditions` $\rightarrow$ `rate : update`, where the `conditions` must be observed for the `update` to occur at a given `rate`. The `sync` is used to synchronize multiple commands. In our model, the `sync` is often used to synchronize a transition which represents a chemical reaction with another transition of the module `base_rates`, which is used to define the rate of that reaction through its transition rate. A transition rate is a variable `rXrate` for Albers-Post reactions and `rpXrate` for PTX related reactions, where `X` is the number of the reaction. The same applies to `sync` labels, `rX` and `rpX`.

Command conditions usually are lower and upper bounds, i.e. there must be at least one molecule for a binding reaction. The list of reactions can be found in [13] and in the comments of our model [1].

TABLE 1
Albers-Post states characteristics.

	Open		ATP Binding Site		
State name	Intra	Extra	High	Low	P_i
E1	✔				
ATPhighE1	✔		✔		
ATPlowPE1	✔			✔	✔
E2		✔			
PE2		✔			✔
ATPlowPE2		✔		✔	✔
ATPlowE2		✔		✔	

Albers-Post states of the pump cycle, which can be open to the intracellular side (2nd column) or to the extracellular side (3rd column). An ATP can bind to the pump in either its high or low affinity binding sites (4th and 5th columns). The pump can also be phosphorylated (6th column).

The main module controls the pump, keeping track of its current sub-state. The sub-states are a boolean vector (lines 9 and 11), where only one position can and must be true. There are also several constants which are used across the whole model, such as the ligands concentrations and pump volume. Fig. 5 is an overview of the model and its components.

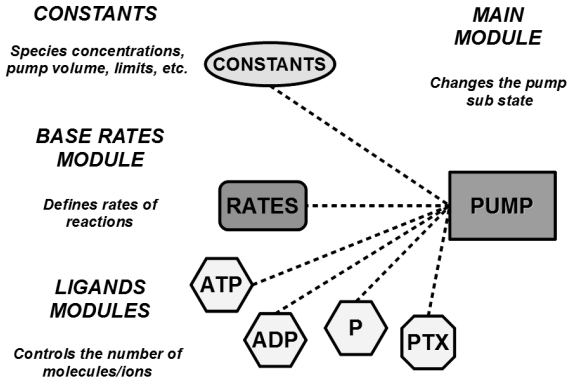

Fig. 5. Overview of the model. The model is composed of PRISM modules: one for each ligands (ATP, ADP, P_i and PTX), which control their flow; the pump, which controls its sub-state and the base rates, which controls the rate of each reaction. There are also several constants which are used across the whole model, such as ligands concentrations and pump volume.

The Albers-Post model [22] represents the Na^+/K^+-ATPase cycle and it can be seen on the left side of Fig. 3. According to it, the pump can be in different

sub-states, which change depending on different reactions involving ATP, ADP and P_i. Table 1 summarizes the states of the pump and their characteristics. The right side of Fig. 3 shows the Palytoxin extension model, which is discussed later.

The pump can be open or closed to either cell sides. An ATP can bind to the Na^+/K^+-ATPase in its high or low affinity binding sites. An ATP bound to the pump can be hydrolyzed, leaving one P_i bound to it and releasing one ADP. The reactions are bidirectional and their rates were obtained from [13].

4.1 Model Rewards

In PRISM, rewards (previously discussed in Section 2.2) are described using the following syntax:

rewards "$reward\ name$"
...
endrewards

Each reward is specified using multiple commands which follow the syntax below.

$$[sync]\, guard\ :\ reward\,;$$

These commands describe rewards for the states and transitions of our model, and also other aspects of the model, such as time. The predicate that must be observed is the *guard*. The *sync* is an optional label used to synchronize a set of commands into a single transition of the system. Finally, *reward* is an expression, which can contain variables and constants of the model, and when evaluated, it accumulates for the reward. If the *sync* is present, the reward is a transition one, otherwise it is a state one. A transition reward assigns its value to transitions where the *guard* is true, while a state reward assigns its value to states where the *guard* is observed.

One example of a reward is the ATP binding (reaction `r1`) to the pump open to the intracellular side of the cell (`E1`, in our model), described below. The *reward* value is one, since it is essentially counting the number of times when that particular reward was observed. The *guard* is the condition which must be true – `E1=1`, i.e. that state must be present. The *sync* `r1` is used to indicate that `r1`-labeled transitions are assigned the reward value. Therefore, the cumulative reward represents the expected number of times that the reaction `r1` happened while the pump was open to the intracellular side (state `E1`).

rewards "$E1$"
$[r1]\, E1 = 1\ :\ 1\,;$
endrewards

Reward properties can be applied to states and transitions. For example, "What is the expected reward for the phosphorylated pump open to the external side of the cell at time T?".

This reward can be instantaneous, obtaining its value at the given time through the property $\mathcal{R}_{=?}[\mathcal{I}^{=t}]$, or accumulated, calculating its value until the given time, using the property $\mathcal{R}_{=?}[\mathcal{C}^{<=t}]$. In this work, we have used cumulative rewards because they show the reward history, giving a better intuition on its role on the model, while instantaneous rewards yield only local information.

The probability of being in a state is obtained by dividing the time that the system spends in that state by the total time spent in all states. The time spent in a state is represented by the state reward, which increments each time unit spent on that state. The same procedure can be applied to transitions (or chemical reactions, in our model).

4.2 Discrete Chemistry

The main components of our model are molecules (ATP, ADP and P_i) and the Na^+/K^+-ATPase, which can interact with each other through several elementary reactions. There is one additional molecule (PTX) in the palytoxin extension for this model, covered in the next section.

The concentration of each of these components is a discrete variable, instead of a continuous function. Therefore, we have converted the amount of initial concentration of molecules from molarity (M) to number of molecules. The stochastic rates for forward and backward transitions are from [13]. The ligands concentrations ($[ATP]^i = 0.005$ M, $[P]^i = 0.00495$ M and $[ADP]^i = 0.00006$ M) are from [32]. The *cell volume* is from [33].

In order to convert the initial amount of molecules given in molarity ([X]) into quantities of molecules (#X), we have used the following biological definition:

$$\#X = [X] \times V \times N_{\mathrm{A}} \tag{1}$$

where V is the cell volume and N_{A} is the Avogadro constant.

5 The Palytoxin Model

The palytoxin model is an extension of the Na^+/K^+-ATPase model, described in the previous section. It corresponds to the right side of the Fig. 3, and it is based on the description of [13] and [12]. Once again, a fragment of the model extension is shown in Fig. 6, and its complete version can be seen in [1].

This extension consists of: one additional molecule module (PTX) which controls its flow; additional reactions in each of the already present modules; and additional sub-states and transitions for the pump module. Initial concentrations for $[PTX]^o$ and stochastic rates for reactions were obtained from [13].

The six additional sub-states correspond to the pump bound to PTX, when the pump is open to both sides behaving like an ion channel. Table 2 summarizes the PTX states and their characteristics.

Palytoxin PRISM Model

```
module ptx
 // number of PTX outside the cell
 ptxOut : [0..(PTXO)] init PTXO;
 //reaction p1: PTXo + E1 <-> PTXE
 [rp1] ptxOut>=1 -> ptxOut : (ptxOut'=ptxOut-1);
 [rrp1] ptxOut<=(PTXO-1) -> 1 : (ptxOut'=ptxOut+1);
endmodule
module pump
 PTXE : [0..1] init 0;
 //reaction p1: PTXo + E1 <-> PTXE
 [rp1] E1=1 & PTXE=0 -> 1 : (E1'=0) & (PTXE'=1);
 [rrp1] E1=0 & PTXE=1 -> 1 : (E1'=1) & (PTXE'=0);
endmodule
// base rates
const double rp1rate=2.73*pow(10,1)/(0.001*V*AV);
const double rrp1rate=6.0*0.0001;
// module representing the base rates of reactions
module base_rates
 [rp1] true -> rp1rate : true;
 [rrp1] true -> rrp1rate : true;
endmodule
```

Fig. 6. The palytoxin (PTX) extension of the model requires a new ligands module to control the number of PTX molecules and several new sub-states and transitions for the pump. Those are created to represent the coupling and uncoupling of PTX to the pump.

TABLE 2
PTX related states characteristics.

	ATP Binding Site			
State name	High	Low	P_i	After P_i
PTXE				
PTXATPhighE	✔			
PTXPE			✔	
PTXATPlowPE		✔	✔	
PTXATPlowE⋆		✔		✔
PTXE⋆				✔

PTX-pump complex states, when the pump is open to both sides. An ATP can bind to the pump in either its high or low affinity binding sites (2nd and 3rd columns). The pump can be phosphorylated (4th column). There is a distinction between states when the pump has been dephosphorylated (5th column).

6 Experimental Results

6.1 Parameters and Model Complexity

Our model can be explored in its three dimensions: $[PTX]^o$ (extracellular PTX concentration), $[ATP]^i$ (intracellular ATP concentration) and pump volume. Each dimension represents one aspect or parameter of the model, and can be changed to modify its behavior.

TABLE 3
Model complexity as function of pump volume.

Pump Volume	Model States	Model Transitions	Check Time
10^{-22}	376	1912	310.895 s
10^{-21}	1274	7140	321.506 s

These parameters directly influence the complexity of the model (number of states, transitions and topology), model build time and property verification time. For example, for $[PTX]^o = 0.001\ \mu M$, $[ATP]^i = 10\ mM$ and pump volume of 10^{-22} L, the model has 376 states and 1912 transitions, taking 0.004 s to build and 310.895 s to check a property discussed further below.

Table 3 shows how these values increase in function of pump volume, for $[PTX]^o = 0.001\ \mu M$, $[ATP]^i = 10\ mM$ and a reward property. This is similar to increasing the concentration of ATP. The machine used to perform experiments is an Intel(R) Xeon(R) CPU X3323, 2.50GHz and has 17 GB of RAM memory.

The volume of an animal cell is 10^{-12} L [33], which is prohibitive to represent using PMC since it would cause the classical problem of state space explosion for model checking. Our analysis is restricted to only one pump. As a consequence, it would also not be realistic to model a large volume because in the real cell this large volume is shared between several pumps and other cellular structures, not limited to pumps.

Our abstraction reduces the cell volume, focusing our analysis in one or few pumps and their surroundings. We achieve this by maintaining the proportions between all interacting components. Therefore, our dimension for cellular volume is called pump volume and is usually 10^{-22} L, which can be handled by PMC.

Although those values are orders of magnitude smaller from the real values, they still represent proper cell behavior, and can be interpreted as using a magnifying glass on a portion of the cell membrane.

On the other hand, for some dimensions we have used more values than intuition suggests, ranging from three orders of magnitude below and above their literature reference values, e.g. $5\ \mu M$ for $[PTX]^o$ and between 1 mM and 10 mM for $[ATP]^i$.

This is particularly interesting because we can model different situations for pump behavior, including abnormal concentrations levels for $[ATP]^i$ due to some disease or syndrome, and different degrees of exposure to $[PTX]^o$, from mild to fatal exposure.

We have created two scenarios – the Control scenario, where the pump is under normal physiological conditions (3 mM), and the High $[ATP]^i$ scenario, where the concentration of ATP is increased (100 mM).

Although the High $[ATP]^i$ concentration is significantly higher than the reference value, one has to consider that the usual concentration is an average, therefore it could be higher in certain pumps, and lower in others. Also, different diseases and syndromes could change the concentration of ATP. The literature has reported cases on the matter (although we were not able to find a quantitative study), for example, a case of Huntington's disease [34].

PMC allows to explore the model using values for its parameters which are difficult to be obtained in practice. It may be challenging to control the experimental conditions of a biological model to obtain, for example, an increased intracellular concentration of ATP. Exploring extreme values could also be used to reveal trends and latent behaviors of the model.

We have formulated many properties that can be

State Rewards PRISM Model

```
rewards "ptxe"
  (PTXE=1) : 1;
endrewards
```

Accumulated State Reward Property

$\mathcal{R}${"ptxe"}=? [$\mathcal{C}$ <=T]
What is the accumulated reward for the state `ptxe` at time T?

Fig. 7. Rewards are used to quantify aspects of the model, such as states (for example, `ptxe`) and reactions. The accumulated reward property is used to obtain the accumulated ($\mathcal{C}$ operator) value of a particular reward ($\mathcal{R}$ operator) at a given time (`T` variable).

seen in [1]. Due to space limitations we have chosen to present the most important ones: state and transition (rate) rewards; and ligands depletion events.

We have also enhanced the kinetic model with probabilities and turned it into a heat map, which reveals model dynamics.

6.2 PTX inhibition by high doses of ATP

In order to observe the probability of being in PTX and non-PTX related states over time, all states were labeled and quantified using rewards (explained previously in Section 2.2). The excerpt of the model in Fig. 7 shows the reward for the sub-state `PTXE`, where the pump is open to both sides of the cell and bound to PTX. Basically rewards are incremented each time its conditions are true.

A model with rewards for each state can be fully quantified, and allows, for example, counting the expected accumulated reward associated with each sub-state over time. For that, we use properties such as the one shown in Fig. 7. Using the operator $\mathcal{R}$ we are able to quantify the reward for some given event, for example the number of times the model was in sub-state `PTXE`. The operator $\mathcal{C}$ allows to quantify accumulated rewards for a given time T, therefore we are able to observe rewards over time.

Consider the following conditions: a single pump, a pump volume of 10^{-22} L, $[ATP]^i$ = 10 mM and $[PTX]^o$ = 10 μM, at instant T=100s. The expected accumulated rewards for the same sub-state `PTXE` and the sub-state `PE2`, where the pump is open to the external side and bound to a phosphate, are respectively 1.0100 and 33.3544. The state probability can be obtained by dividing the state reward by the sum of all state rewards. In other words, in 100 seconds, the pump is expected to be open to the extracellular side and phosphorylated approximately 34.1952% of the time, and the pump is expected to be bound exclusively to PTX only 1.0355% of the time.

Using a broad spectrum of different $[ATP]^i$ and $[PTX]^o$, for the pump volume of 10^{-22} L, we have found that there are only two sets of values for sub-state rewards. One set is associated with $[ATP]^i$ equals or below to 10 mM, while the other set is associated with $[ATP]^i$ above 10 mM.

For example, when $[ATP]^i$ = 100 mM, the expected rewards associated with the two sub-states `PE2` and `PTXE` change to respectively 37.3577 and 0.5904, or 39.2389% and 0.6201% of the time. Therefore, as we increased $[ATP]^i$, the likelihood of the pump being open to the extracellular side and phosphorylated increased 14.7496%, and for the pump to be bound exclusively to PTX decreased 40.1175%.

TABLE 4
PTX-pump state probability for different scenarios.

Pump State	Control	High $[ATP]^i$	Difference
`E1`	0.0072%	0.0045%	-37.7249%
`ATPhighE1`	0.0312%	0.0185%	-40.5495%
`ATPlowPE1`	0.0314%	0.0187%	-40.5245%
`E2`	34.1902%	39.2551%	+14.8137%
`PE2`	34.1952%	39.2389%	+14.7496%
`ATPlowPE2`	7.3272%	6.5347%	-10.8154%
`ATPlowE2`	0.0000%	0.0000%	+2.4527%
Non-PTX related	75.7824%	85.0704%	+12.2560%
`PTXE`	1.0355%	0.6201%	-40.1175%
`PTXATPhighE`	3.4466%	2.3806%	-30.9279%
`PTXPE`	8.3250%	5.8626%	-29.5785%
`PTXATPlowPE`	0.0308%	0.0177%	-42.4199%
`PTXATPlowE*`	0.7262%	0.1969%	-72.8886%
`PTXE*`	10.6535%	5.8517%	-45.0723%
PTX related	24.2176%	14.9296%	-38.3520%

Probabilities for states of the sodium-potassium pump interacting with palytoxin at time T=100s. Two states are more present than others: `E2`, where the pump is open to its external side; and `PE2`, that same state although the pump is phosphorylated. As $[ATP]^i$ increases, the probability of PTX related states decreases, which suggests that ATP inhibits PTX action.

The rewards can be divided in two groups: PTX related sub-states and Albers-Post (non-PTX) sub-states. Summing all the rewards of each group, and dividing each by the total, one can obtain the probability of the pump being inhibited by PTX. For $[ATP]^i$ = 10 mM and T=100s, PTX related states correspond to 24.2176%. As we increased $[ATP]^i$ to 100 mM, PTX related states correspond to only 14.9296%, suffering a 38.3520% reduction.

Therefore, this reduction indicates that as $[ATP]^i$ increases, the probability of being in PTX related sub-states decreases, suggesting that ATP is an inhibitor of PTX. As consequence, people with ATP depletion would be more vulnerable to this toxin. ATP deficiency appears in different forms, e.g. brain disorders, for example, stroke and encephalopathies [35]. Table 4 summarizes the state reward values and percentages for the Control and High $[ATP]^i$ scenarios, as well as the difference between them.

Similar reward structures to the ones of Fig. 7 were created for transitions (in our model, chemical

Ligands Depletion Events and Time Reward

```
label "ptxAllBounded" = ptxOut=0;

rewards "time"
  true: 1;
endrewards
```

Ligands Depletion Properties

$\mathcal{P}$>=1 [$\mathcal{F}$ "ptxAllBounded"]
The event "ptxAllBounded" eventually always happens.

$\mathcal{R}${"time"}=? [$\mathcal{F}$ "ptxAllBounded"]
How long it takes for the event "ptxAllBounded" to happen?

Fig. 8. The depletion of a ligand ATP and PTX) happens when the variable which stores its number reaches zero. These events can be observed using labels, one of the features of PRISM. One can check if a given event happens using reachability properties ($\mathcal{F}$ operator). A time reward allows quantifying when an event occurs.

reactions). For $[ATP]^i$ = 10 mM we found that during the first 100 seconds PTX related reactions correspond only to approximately 8.01%. Once we change $[ATP]^i$ to 100 mM the role of PTX related reactions decreases by approximately 42.97%, which reinforces our discovery that high doses of ATP inhibit PTX action. The most active reactions are dephosphorylation, changes in the pump conformational state, and coupling and releasing of ATP. Other pump volumes have one set of values for each $[ATP]^i$, even though the same behavior remains.

The experimental conditions used to study the major effects of various ligands including ATP on PTX-modified Na^+/K^+-ATPase [12] are rather different and this poses a problem in terms of comparison with our results. The inhibitory effect elicited by ATP as predicted by our model has been not verified experimentally and it was unexpected. This result raises an important point that may be worth being experimentally validated.

Our results suggest that in the presence of palytoxin, the extent of phosphorylation from ATP is greatly reduced probably by a PTX-promoted rapid dephosphorylation step that could, at high concentrations of ATP, lead to inhibition of ATP binding. This reinforces the notion that the phosphorylated intermediates formed from ATP are different and this may change PTX affinity and the overall behavior of the pump. There are some reports in the literature that could support this result [36]. These results have been obtained from a parametric study of the state and transitions rewards of our model.

6.3 Ligands Depletion

We have also investigated properties related to ligands (ions or molecules) depletion, i.e. when there are no ligands in one side of the cell. For example, the events "atpAllBounded" and "ptxAllBounded", where all ATP and palytoxin molecules are bound to the pump, respectively. These events can be created in PRISM using *labels*, one of its features (Fig. 8).

Ligands depletion properties state that these events eventually ($\mathcal{F}$ operator) will always happen ($\mathcal{P}$>=1 operator). For example, in every scenario the event "ptxAllBounded" always eventually happens.

One could check how long it takes for those events to happen. For that we have to use a time reward, and reward properties, such as the one shown in Fig. 8. The expected value of the occurrence time for the event "ptxAllBounded" is 30.4379 seconds in the $[ATP]^i$ = 10 mM scenario. This event is sensitive to the parameter $[ATP]^i$– in the 100 mM scenario, the value increases to 49.4342 seconds.

6.4 A Probabilistic and Quantified Kinetic Model

The Albers-Post model for the Na^+/K^+-ATPase was first proposed in [22]. It is a kinetic model (and also a directed graph) which describes the set of chemical reactions allowed from one state to another, consuming or producing ligands in the process.

We are able to quantify this kinetic model using PMC through state and transition (rate) rewards. We calculate a state probability dividing its reward by the sum of all state rewards. This is also applied to reactions and ligands (ATP, ADP, P_i and PTX).

We associate colors to states and reactions, in order to represent their probabilities. The kinetic model is colored using a jet palette, which is often associated with temperatures, where probabilities transit from red to blue, or from likely to unlikely. This modified kinetic model is called a heat map. Red states and reactions are more probable or hot while blue states and reactions are unlikely or cold. An example of the heat map can be seen in Fig. 9, where the states `PE2` and `ATPlowPE2` (phosphorylated pump open to the external side and that same state with ATP bound to its low affinity binding site, respectively) are more probable, and reactions between `E1` and `ATPlowE2` (pump open to the internal side and pump open to the external side with ATP bound to its low affinity binding site, respectively) occur more often.

For example, the reaction between the states `E1` and `ATPlowE2` is one of the most active reactions, while the states themselves are one of the most inactive states. This could suggest that either these states are temporary or there might be an intermediary and unknown state between these two states.

After contacting Rodrigues and co-workers, they have suggested that this intermediary state is the pump transitioning between open to the external side

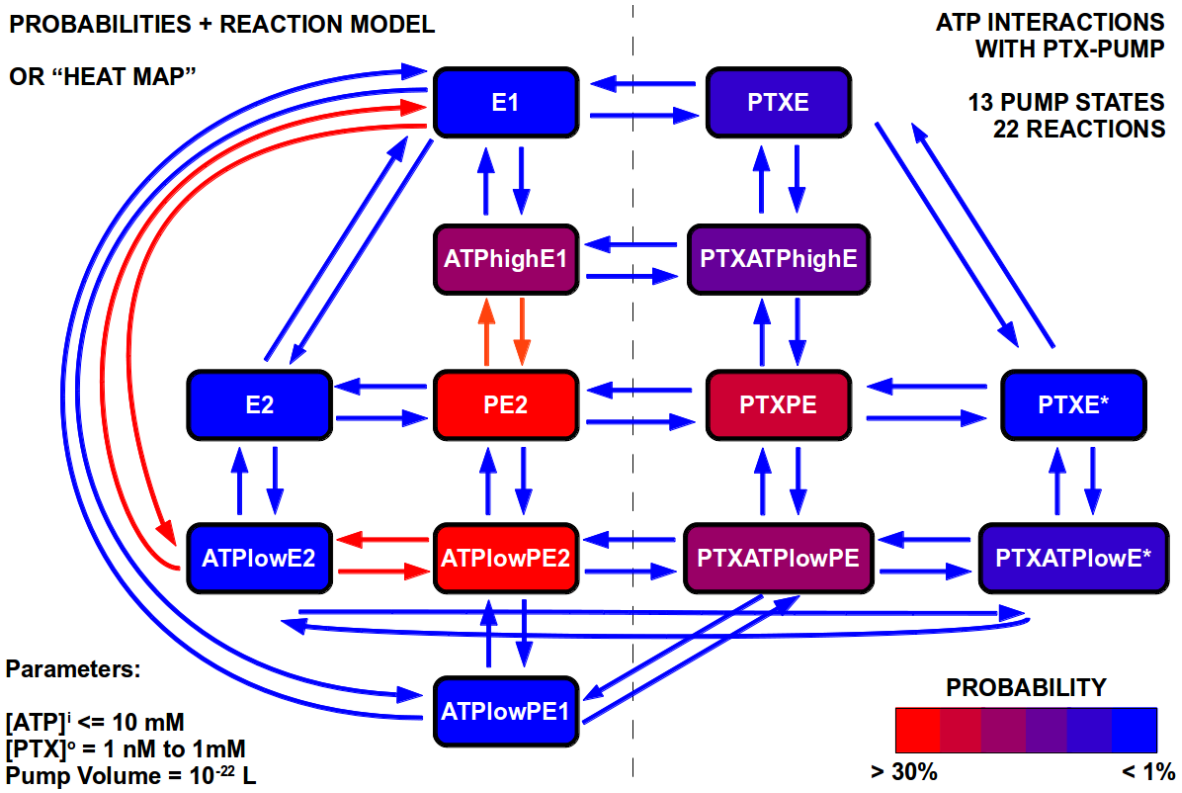

Fig. 9. Heat Map: kinetic model of the Na^+/K^+-ATPase with state and rate probabilities represented as colors. Each state and rate is colored based on its probability. Red states/rates are likely while blue states/rates are improbable. This could be a valuable visual tool for biologists as it shows model dynamics and suggests overlooked conditions.

to open to the internal side, which is exactly the effect of the reaction between those two states.

There are other hypotheses to explain this situation, such as a trap state where the system stays most of the time (for example, `ATPlowPE2` or `PE2`, the two most likely states).

Another possibility is that we have a fast occuring reversible reaction, which is the case of this particular reaction, as it is $5.00 \times 10^2 M^{-3} s^{-1}$ (although not the faster in the whole model).

This intermediary state might not explain this phenomena, as it could be another low populated state with a high reaction rate. Nonetheless, this low populated state might enrich the kinetic model.

This odd behavior could reflect an imprecision on the kinetic model description itself, which might not include all the existing states and reactions of the pump interacting with palytoxin. Novel experiments could be performed in order to validate this behavior and further improve the current description of PTX-pump interactions.

We have created a tool called `dot2heatmap` to enhance a graph description in DOT language with PMC results in order to automatically create a heat map. The tool is freely available in [2].

The heat map could be a valuable tool for biologists as it shows model dynamics and it could be used to suggest overlooked experiments. Since the kinetic model is an abstraction suggested by experimental data, it could be incomplete, which the heat map would assist towards its completion. It raises several questions, especially about likely reactions involved with improbable states.

7 Conclusion

The sodium-potassium pump (Na^+/K^+-ATPase) is a cellular structure which is responsible for exchanging ions through the plasma membrane at the expense of cell energy (ATP). Its correct behavior is necessary for all animal cells, otherwise the health of the individual could be in risk due to diseases.

A model of cell energy related reactions of a single pump interacting with the palytoxin toxin (PTX) has been built using a Probabilistic Model Checking approach, which has allowed formal, exhaustive and automatic exploration of the model. We have used the PRISM tool, a model checker which is used to model and analyze complex systems.

PTX essentially disrupts the Na^+/K^+-ATPase regular behavior, changing it to the one of an ion channel, i.e., ions freely move accordingly to their concentration gradient. PMC has allowed us to investigate the model, which shows unpredictable characteristics. Properties about biological events were expressed in probabilistic logics, e.g. "What is the probability of being in PTX related sub-states?".

We have discovered that as the concentration of $[ATP]^i$ increases, the probability of being in PTX related sub-states decreases. For example, when $[ATP]^i$ is increased from 10 mM (our Control scenario) to 100 mM (our High $[ATP]^i$ scenario), that probability is reduced by 38.3520%.

This suggests that high doses of $[ATP]^i$ could inhibit PTX action, which implies that individuals with $[ATP]^i$ depletion are more susceptible to PTX effects. This $[ATP]^i$ deficiency appears in different forms, such as in brain disorders, for example, stroke.

The study of the role and ability of $[ATP]^i$ to change our Na^+/K^+-ATPase model behavior is even more important, because the production of $[ATP]^i$ can not be stimulated directly.

Although $[ATP]^i$ is higher than normal physiological conditions in the High $[ATP]^i$ scenario, these concentrations are averages, therefore some pumps may have a higher $[ATP]^i$ than others. These extreme conditions can also be used to reveal trends or latent behaviors of the model, since it may be difficult to control the experimental conditions of the biological model to obtain these values.

We have also enhanced the kinetic model of the pump, which is used for describing the states and reactions of the system, with probabilities, creating a heat map. It reveals unexpected situations, such as a frequent reaction between unlikely states, which suggests that either these states are temporary; or there is an unknown state between those two.

This odd behavior could reflect an imprecision on the kinetic model description itself, which might not include all the existing states and reactions of the pump interacting with palytoxin. Novel experiments could be performed in order to validate this behavior and further improve the current description of PTX-pump interactions.

The impacts of our model to understand PTX interactions with the sodium-potassium pump include, for example, the suggestion that high doses of ATP might inhibit PTX action. We expect that our model can be used as a tool to improve the current kinetic model of the PTX interactions with the sodium-potassium pump, which might be incomplete.

We have shown in this work that PMC can be used to obtain valuable insight of transmembrane ionic transport systems in a simple and complete way. This type of analysis can provide a better understanding of how cell transport systems behave, give a better comprehension of these systems, and can lead to the discovery and development of drugs.

Future works: confront the results with wet lab experiments; expand the model to other Albers-Post sub-states (e.g. related to potassium and sodium); explore other dimensions such as the number of pumps; adapt our model to other toxins (for example, ouabain) or even drugs (e.g. digitalis); and use an approximate analysis to larger pump volumes.

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Fernando A. F. Braz received the master's degree in Computer Science at Universidade Federal de Minas Gerais (scholarship by CAPES) on the topics of Model Checking and Systems Biology. He has received the bachelor's degree in Computer Science from Pontifícia Universidade Católica de Minas Gerais (2010). He also has a technical course in Computer Management at Colégio Técnico de Minas Gerais (2004). He also worked in undergraduate research at the laboratory LICAP (Applied Computational Intelligence Laboratory) in Pontifícia Universidade Católica de Minas Gerais (2008-2010) developing a satisfiability solver for Stalmarck's proof procedure. He has been interested in software development, modeling and verification of formal models and bioinformatics.

Jader S. Cruz received the master's degree in Biology from the University of Brasilia and the PhD degree from the Federal University of Minas Gerais in Cell Biophysics. He was a visiting scientist at Kyushu University (Japan) working with Professors Akinori Noma and Hiroko Matsuda in cardiac electrophysiology. He spent 2 years at the Medical Biotechnology Center - University of Maryland (USA) doing his post-doctoral training under the supervision of Professor WJ Lederer working with confocal microscopy combined with cell electrophysiology to study calcium dynamics in cardiac cells. Now he is Associate Professor at the Biochemistry and Immunology Department, Federal University of Minas Gerais, Brazil.

Alessandra C. Faria-Campos works currently as a Postdoc at Federal University of Minas Gerais - UFMG (Brazil), on a project that addresses Laboratory Data Management and Integration at the Department of Computer Science. She is a Biology Major and earned her Master's and Undergraduate Degrees at UFMG also, where she has worked on protein analysis and molecular genetics. She has a Doctor's Degree from UFMG, earned working on Bioinformatics where she acquired extensive experience on database modeling and the SQL language.

Sérgio V. A. Campos has received the bachelors degree in computer science from Universidade Federal de Minas Gerais (Belo Horizonte – Brazil) in 1986, the masters degree in computer science from the same university in 1990 and the Ph.D. degree in Computer Science from Carnegie Mellon University in 1996 on the topic of formal verification of real-time systems. During his Ph.D. he has spent two summers working at Intel performing formal verification of hardware circuits. He has spent one year as a post-doctoral researcher at Carnegie Mellon and is currently an associate professor at the computer science department of Universidade Federal de Minas Gerais. While at UFMG Sergio has worked on various topics such as formal verification of hardware and software, the design and implementation of multimedia systems, scheduling problems and telecommunication network management. His interests include design, analysis and verification of real-time systems, hardware and software in general. More recently Sérgio has been the PI of the "Popular" PC project that developed a low cost computer for the use in remote and underdeveloped areas, which has received significant coverage from international press.