Studies of Charge Translocation by *Bufo Marinus* Na\(^+\)/K\(^+\) ATPase in its Na\(^+\)/Na\(^+\) Exchange Mode

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This dissertation titled

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Exchange Mode

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ABSTRACT

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Studies of Charge Translocation by *Bufo Marinus* Na⁺/K⁺ ATPase in its Na⁺/Na⁺ Exchange Mode (97 pp.)

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The Na⁺/K⁺ ATPase (NKA) exports 3Na⁺ and imports 2K⁺ at the expense of the hydrolysis of 1 ATP. In the absence of K⁺, it carries on electroneutral Na⁺/Na⁺ exchange and produces a transient current containing faster and slower components in response to a sudden voltage step. Components with different speeds, representing sequential release of Na⁺ ions from three binding sites, were demonstrated by analysis using exponential fit of experimental data. Our data provides experimental support that the slow component can be present independently. Oligomycin is a NKA inhibitor favoring the 2Na⁺-occluded state without affecting the conformational change of the NKA. We studied the effects of oligomycin on both K⁺ activated currents and transient currents in wild-type *Bufo* NKA and a mutant form of *Bufo* NKA, NKA: G813A. Oligomycin almost completely blocked K⁺ activated current and the binding of oligomycin was voltage independent. Inhibition affected faster components without affecting the slow component carried by the first Na⁺ release/rebinding. The inhibited component shown using the P/4 leakage subtraction protocol had a rate coefficient and voltage dependence of charge similar to the medium component. Our study indicates that Na⁺ is released to the extracellular medium through at least two pathways.
In another set of studies, the effect of holding potential on slow charge movement was studied in the presence of different concentrations of ADP$_i$, Na$^+_i$, or Na$^+_o$. This was done to improve our understanding of Na$^+_i$ binding. However, the manipulation of [ADP$_i$] and [Na$^+_i$] did not cause as pronounced changes as predicted in magnitude of charge movement (Qtot), which indicated that our experimental conditions were not able to backwardly drive reaction across energy barrier to Na$^+_i$ release/rebinding steps. On the contrary, lowering [Na$^+_o$] caused evident dependence of Qtot on holding potential with characteristics suggesting pumps were escaping from E2P through the uncoupled Na$^+$ efflux activity.

Approved: _____________________________________________________________

Ralph A. DiCaprio

Professor of Biological Sciences
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GENERAL INTRODUCTION

The Na\(^+\)/K\(^+\) ATPase (NKA), a member of the P-type ATPase protein family, is an integral membrane protein that exports 3Na\(^+\) and imports 2K\(^+\) at the expense of the hydrolysis of 1 ATP. In most animal cells, this transport system is essential for the homeostasis of Na\(^+\) and K\(^+\). By establishing and maintaining the gradients for Na\(^+\) and K\(^+\) across the plasma membrane, the NKA plays key roles in both excitability and cell volume regulation. In addition, the energy stored in the Na\(^+\) gradient is extensively utilized in secondary active transport of ions and nutrients, such as Ca\(^{2+}\), glucose, amino acids, etc. (Kaplan 2002; Geering 2006)

A functional NKA is formed by the assembly of an α, a β, and in some instances, a γ subunit. The α subunit has ten transmembrane segments (TM) and it contains all components that are essential for NKA enzymatic activity and ion transport. The β subunit is a type II membrane protein with a single transmembrane domain and it functions, primarily, as a chaperone of the α subunit (Kaplan 2002; Geering 2006). Transport activity by the NKA is further regulated by a type I membrane protein, the γ subunit, which is thought to interact with the α subunit in a tissue- and isoform-specific way (Geering 2006).

A common characteristic of P-type ATPases is that they have a phosphorylated intermediate state that results from transferring the γ phosphate of ATP to a highly conserved aspartic residue in the large cytoplasmic loop between TM4 and TM5. This large loop is composed of a P (phosphorylation) domain and an N (nucleotide binding) domain, two of three important intracellular domains. The third domain is the A
When ATP binds to the N domain, rotation of the A domain and closing of the N domain bring ATP close to a highly conserved aspartic residue in the P domain and cause phosphorylation (Kaplan 2002).

The transport cycle by the $\text{Na}^+/\text{K}^+$ ATPase

Many experimental techniques have been used to study transport mechanisms of the NKA. Tracer flux studies were used to determine the stoichiometry of $3\text{Na}^+/2\text{K}^+$, ion occlusion of $\text{Na}^+$ and $\text{K}^+$ alternatively, and transmembrane movements of $\text{Na}^+$ outwardly and $\text{K}^+$ inwardly. But tracer flux studies could only be used on slow process. Electrophysiological methods are more widely used (Apell 2003). Comprehensive investigations were performed with the $\text{Na}^+/\text{K}^+$ ATPase because it is normally electronegative and exists in most animal cells. Due to the homology between NKA and other P-type ATPases, the transport mechanisms of other P-type ATPases can be extrapolated from research findings on the NKA (Apell 2003).

The reaction catalyzed by the NKA requires four substrates: intracellular $\text{Na}^+$ ($\text{Na}^+_i$), extracellular $\text{K}^+$ ($\text{K}^+_o$), ATP and $\text{H}_2\text{O}$, and produces four products: extracellular $\text{Na}^+$ ($\text{Na}^+_o$), intracellular $\text{K}^+$ ($\text{K}^+_i$), ADP, and orthophosphate. The NKA pumps ions in and out of the cell following a transport cycle usually referred to as the Albers-Post scheme (Fig. 1) (Albers 1967; Post, Hegyvary et al. 1972; Skou and Esmann 1992). In this model, the NKA can adopt two main conformations: E1 and E2. In E1, ion-binding sites are facing the cytoplasm and the apparent affinity for $\text{Na}^+$ is higher than for $\text{K}^+$. In E2, ion-binding sites are exposed to the extracellular solution and the apparent affinity for...
$K^+$ is higher than for $Na^+$. Phosphorylation of E1 by ATP promotes the occlusion (or trapping) of three intracellular $Na^+$, which are released to the external medium by a deocclusion transition associated with a conformational change to E2. Once two external $K^+$ bind to two of the three ion binding sites, they elicit dephosphorylation and $K^+$ occlusion. ATP binding favors transition back to E1, prompting $K^+$ release to the cytoplasm and binding of three $Na^+$ ions to complete the cycle.

**Figure 1.** Albers-Post scheme for the $Na^+/K^+$ ATPase.
Voltage dependence of Na\textsuperscript{+} transport and the access channel model

Under physiological conditions, the NKA cycles in the forward direction as indicated by the arrow in Fig 1. Therefore, in each cycle, the NKA produces a net movement of one positive charge in the outward direction. Consequently, the velocity at which the pump cycles (or turnover rate) must be sensitive to membrane potential (Vm). In 1985, Gadsby et al. determined the voltage dependence of the outward pump current (Ip) using whole cell patch clamp technique of isolated heart cells (Gadsby, Kimura et al. 1985). By minimizing the influence of potassium, calcium and sodium channels, Ip, defined as cardiotonic steroid-sensitive current with 144 mM Na\textsuperscript{+}{o} and 5.4 mM K\textsuperscript{+}{o} and, 34 mM Na\textsuperscript{+}{i}, was found to be rather small at -140 mV but increased steadily to a maximal level near 0 mV. Because the 3Na\textsuperscript{+}/2K\textsuperscript{+} stoichiometry of the NKA does not change with voltage (Rakowski, Gadsby et al. 1989), the decrease in Ip with negative potentials should represent a reduction in the turnover rate of the pump. Which step(s) of the pump cycle is/are voltage dependent? Most of the voltage dependence under normal ionic conditions comes from the transport of Na\textsuperscript{+}. An essentially voltage insensitive transport cycle can be achieved by replacing external Na\textsuperscript{+} with large cations that are incompatible with transport, such as NMG or TMA (Rakowski et al., 1993; Rakowski and Paxson, 1988; Nakao and Gadsby 1989; Sagar and Rakowski, 1994). The dramatic effect of Na\textsuperscript{+}{o} on voltage dependence of Ip indicates that the major voltage sensitive step occurs during Na\textsuperscript{+}{o} de-occlusion/occlusion and release/binding steps (Rakowski, Gadsby et al. 1989; Rakowski, Gadsby et al. 1997). This can be explained by postulating the existence of a narrow and deep access channel or “ion well” between the Na\textsuperscript{+} binding sites within the
protein and the extracellular aqueous solution. Because the electric field is expected to drop along an access channel, negative potentials will favor binding of external Na\(^+\) and consequently the forward pump turnover rate will be slowed down.

The access channel model predicts that changes in ion concentration and membrane voltage are equivalent. Supporting evidence for this model comes from studies in which the pump cycle has been greatly simplified by removing K\(^+\) from the experimental solutions, which restrict the pump to operate among the states encircled by dashed lines in Fig. 1. In this mode, usually referred to as electroneutral Na\(^+\)/Na\(^+\) exchange, the NKA exchanges Na\(^+\) from both sides of the membrane without net movement of ions or net hydrolysis of ATP. Under these conditions, Gadsby et al. (1993) showed that \(^{22}\)Na\(^+\) unidirectional efflux increased asymptotically at negative potentials and vanished at positive potentials. A simplified kinetic scheme of this transport mode is:

**Reaction scheme 1**

\[
E1 + 3Na_i \xrightleftharpoons[K_1^{-1}]^{K_1} E1(3Na) \xrightleftharpoons[K_2^{-2}]^{K_2} E2P + 3Na_o
\]

The sigmoid shape of the flux-voltage relationship can be modeled by an asymmetric voltage dependence of the rate constants where membrane potential only influences K\(_{-2}\). Any other voltage dependence of these rate constants will result in a bell-shaped flux-voltage curve, or in a sigmoid shaped curve but opposite to that found experimentally. Because K\(_{-2}\) represents rebinding of Na\(^+\)_o, voltage dependence of the apparent [Na\(^+\)]_o can be explaining if external Na\(^+\), as it approaches its binding site, senses part of the membrane field. In other words, Na\(^+\) traverses part of the membrane field through a narrow high-field access channel before reaching the binding site. This can be formulated
by transforming $K_{-2}$ into a pseudo-first order rate coefficient influenced by $[Na^+]_o$ and a Boltzmann factor $\{exp(-\lambda FV/RT)\}$, where $\lambda$ is the dielectric coefficient representing the electric field dropped between external medium and the binding site, $V$ is voltage, $F$ is Faraday’s constant, $R$ is gas constant and $T$ is temperature. Therefore, $K_{-2}$ can be written as $K_{-2}^0[Na^+]_oexp(-n\lambda FV/RT)$, where $n$ is the Hill coefficient representing the apparent molecularity of the charge moving process and $n\lambda$ can be expressed as the apparent valence $z$. As predicted for an access channel model, previous results showed that when $[Na^+]_o$ was doubled from 100 mM to 200 mM, and then to 400 mM, the Na$^+$ efflux vs voltage curve was parallel shifted to the right with equal intervals: 26mV for each doubling, equivalent to a value for $\lambda$ of $\sim$0.7 (Gadsby, Rakowski et al. 1993).

Transient currents carried by the NKA in its Na$^+$/Na$^+$ exchange mode

Na$^+$/Na$^+$ exchange transport kinetics have been further examined through transient currents elicited by sudden voltage steps. These currents originate from the influence of voltage on the rate constant of the system. At any given potential, the state occupancy is determined by the values of the rate constants. A sudden voltage step will instantly change any voltage dependent rate constant, and the system will relax to the newly imposed steady state following a time course established by the new values of the rate constants. Transient currents mediated by the NKA were first recorded on ventricular myocytes in 1986 as current sensitive to strophathidin, a specific inhibitor of NKA (Nakao and Gadsby 1986). Depolarizing and hyperpolarizing steps elicited outward and inward transient currents, respectively. These currents decayed exponentially with time.
constants that were faster at hyperpolarizing voltages and reached a minimum at positive potentials, which can also be explained by a solitary voltage dependence on binding. The voltage dependence of the charge moved \( (Q) \) followed a sigmoid shape with saturations at large hyperpolarizing and depolarizing potential. These data could be well fitted by a Boltzmann distribution \( \{Q(V) - Q_{\text{min}}\}/Q_{\text{tot}} = 1/\left(1 + \exp\left((V_{\text{mid}} - V)/A\right)\right) \), where \( Q_{\text{tot}} \) is the total amount of charge, \( Q_{\text{min}} \) is the minimum value of charge reached at extreme negative voltages, \( V_{\text{mid}} \) (~20 mV) is the mid point voltage, and \( A \) (~26 mV) equals \( RT/zF \) representing the steepness of the curve. The equation can be rewritten as

\[
\frac{Q(V) - Q_{\text{min}}}{Q_{\text{tot}}} = \frac{1}{1 + \exp\left[\frac{zF(V_{\text{mid}} - V)}{RT}\right]}
\]  

Equation 1

From these results, the authors proposed that these transient currents originate from the movement of a single positive charge between two states.

Later transient currents were intensively studied in cardiac myocytes, squid giant axons, and \textit{Xenopus} oocytes. Holmgren & Rakowski first investigated transient currents mediated by the NKA in its Na\(^+\)/Na\(^+\) exchange mode in \textit{Xenopus} oocytes using the cut-open technique and obtained a \( z \) value of 1.10\( \pm \)0.07 for dihydrooubain-sensitive transient current by fitting the \( Q \) vs \( V \) curve with equation 1 (Holmgren and Rakowski 1994). Their later publication showed that \( V_{\text{mid}} \) right-shifted 25.3\( \pm \)0.4 mV when \([Na^+]_o\) was doubled but other conditions were kept constant. And the \( z \) value was 0.99\( \pm \)0.03 obtained from the best fit to the \( Q \) vs \( V \) curve (Holmgren and Rakowski 2006). Given \( z=n\lambda \), \( \lambda \) was subsequently calculated as 0.63\( \pm \)0.05, which was consistent with the value estimated by other methods such as charge pulse experiments using the NKA accumulated on planar
lipid bilayer (Wuddel and Apell 1995; Holmgren and Rakowski 2006). The consistency of $\lambda$ strongly supports the access channel model, through which $Na^+$ crosses 70% of the electric field from the external medium to its binding sites.

The studies on transient currents cited above were done with a time resolution that was unable to resolve the very fast component that occurred right after the application of voltage steps. Hilgemann (1994) successfully separated the fast component from the slow component of transient currents recorded from cardiac myocytes using a “giant” patch clamp that allowed fast voltage clamp within 4 $\mu$s. Pump-mediated transient charge movement was assayed as ATP-sensitive transient currents in the absence of $K^+$ and intracellular ADP (ADP$_i$). Slow components with a single exponential time course dominated the records when voltage steps ranged from 2 to 4 ms. When the voltage dependence of the slow charge movement ($Q_{slow}$) was fitted with a Boltzmann function, it gave a $z$-value of 0.68. Fast components appeared as a charge jump within the first 100 $\mu$s during voltage steps and $Q_{fast}$ vs $V$ curve had a shallow slope with $z$ value of 0.26. Fast charge movement was interpreted as the result of $Na^+$ binding to or unbinding from E2P. The magnitude of $Q_{fast}$ ($Q_{free}$) was affected by holding potential ($V_h$) and the $Q_{free}$ vs $V_h$ curve had a sigmoid shape with a similar Boltzmann slope (1.1) as compared to the Boltzmann slope (0.68) of $Q_{slow}$ vs $V$ curve. This was hypothesized as being due to the slow charge transfer having the most voltage dependence so that it affected the availability of extracellular $Na^+$ binding site (Hilgemann 1994). Fast components of transient currents were further investigated by Holmgren and colleagues in squid giant axons with high speed voltage jumps in the absence of $K^+$ and ADP$_i$ (Holmgren, Wagg et
Pump-mediated transient currents were assayed as currents sensitive to dihydrodigitoxigenin, another NKA specific inhibitor. When recordings were obtained with a sampling rate of 20 kHz and low-pass filtered at 5 kHz, slow (reflecting Na⁺ occlusion caused by conformational change) and fast (reflecting Na⁺ binding through the access channel) components were separated with similar characteristics as described by Hilgemann (1994). In addition, Holmgren and colleagues (2000) demonstrated that the two components occurred in sequence, supported by the discrepancy between the “on” and the “off” transient currents elicited by stepping from 0 mV to -110 mV, and then back to 0 mV. The “on” transient current had a large fast component followed by a slow component. However, after the pump completely relaxed at -110 mV for enough time (20 ms), stepping back to 0 mV elicited a much smaller fast component followed by a slow component with comparable amount of charge as in the “on” transient. This indicated the sequential occurrence of Na⁺ binding and occlusion. Jumping from 0 mV to -110 mV caused Na⁺ to bind to E2P, which was manifested as large fast charge movement. Prolonged -110 mV voltage steps drove most pumps to the occluded state E1P(Na3) through a slow relaxation process so that fewer pumps were able to release Na⁺ instantly to create the fast component when the voltage was stepped back to 0 mV. In contrast, when a voltage step from 0 mV to 50 mV and then back to 0 mV was applied, there was a slightly larger fast component observed in the “off” transient as compared to the “on” transient. The explanation is that jumping to 50 mV drove more pumps from E1P(Na3) to E2P state, thus providing more sites for instant Na⁺ binding during the “off” transient. Because the conformational change E1P(Na3) ↔ E2P·Na3 was shown to be nearly
electroneutral (Apell and Karlish 2001), the slow component caused by ion deocclusion was presumed to have a rather small size, and not a dominant part of the overall current. To explain this discrepancy, the access channel is postulated as a high-energy state with short open time. Even though all ions must go through the access channel for binding and release, only some ions interact with pumps occupied in this high energy state and constitute the fast charge movement at the beginning of the transient currents. Other ions into or out of pumps have to go through a slow process before the access channel becomes available, so the charge carried by them is embedded in the slow component. In other words, the slow component reflects not only conformational change but also Na\(^+\) binding and rebinding through access channels following slow conformational changes. To resolve the detailed events, Holmgren and colleague increased sampling the rate to 2 MHz (0.5 \(\mu\)s sample interval) and low-pass filtered to 100 kHz. Three components were the able to be separated reflecting the de-occlusion/release of three Na\(^+\), which occur in sequence with increasing rate constants from the first (1000 s\(^{-1}\)), the second (about 6000 to 20000 s\(^{-1}\)) to the third Na\(^+\) ion (10\(^6\) s\(^{-1}\)) (Holmgren, Wagg et al. 2000). The release or rebinding of the first Na\(^+\) from E2P (Na3) or to E2P (Na2) is the dominant charge-carrying step with dielectric coefficient 0.65-0.7. (Wuddel and Apell 1995; Holmgren, Wagg et al. 2000; Holmgren and Rakowski 2006).

High resolution structures and ion pathways

High resolution crystal structures of SERCA, one of P-type ATPases, have been available since 2002 and homology models of the NKA were built based on the
homology between SERCA and the NKA (Toyoshima, Nakasako et al. 2000; Ogawa and Toyoshima 2002; Toyoshima and Nomura 2002; Toyoshima and Mizutani 2004). The predicted location of three Na\(^+\) binding sites was verified by mutagenesis studies (Li, Capendeguy et al. 2005). In 2007, crystal structures of a NKA were determined in Rb\(^+\) (K\(^+\) congener) bound state (Morth, Pedersen et al. 2007). All these structures are snapshots of the whole transport cycle when ions are occluded inside. To elucidate the entire dynamic process including the ion release pathway, many more high resolution structures are needed. Studies utilizing cysteine-scanning mutagenesis predicted that the ion pathway is located between transmembrane domain 4, 5, and 6 (Guennoun and Horisberger 2002; Horisberger, Kharoubi-Hess et al. 2004). Studies with palytoxin have shown the existence of an ion channel-like structure opened by palytoxin (Artigas and Gadsby 2002; Artigas and Gadsby 2003; Artigas and Gadsby 2004). But it is still not known where there exists more than one ion releasing pathway, which was investigated in the first part of my dissertation.

Intracellular Na\(^+\) binding and the subsequent enzyme conformational change

Forward Na\(^+\) transport (E1\(\cdot\)ATP\(+3\) Na\(^+\)\(i\)\(\rightarrow\)\(\rightarrow\)E2-P \(+\) 3Na\(^+\)\(o\)) requires energy from ATP hydrolysis, and ATP hydrolysis occurs only with 3 Na\(^+\) bound to the protein. Other congener cations (K\(^+\), Rb\(^+\), Cs\(^+\), NH4\(^+\), Tl\(^+\)) antagonize Na\(^+\) binding and cause a conformational change into the occluded E2 state after two ions have bound. The suggested reason for this phenomenon is that the third Na\(^+\) binding site becomes available after two Na\(^+\) ions have already bound and binding of the third Na\(^+\) ion occurs with a higher affinity than binding of the second (Schneeberger and Apell 2001). Binding of the
first two intracellular Na\(^+\) ions was found to be electroneutral since it is accompanied by exchange for bound H\(^+\). It has been shown that E1 has a higher pK (7.9) than E2 so that 83% of the sites bind H\(^+\) ions at physiological pH (7.2). The release of H\(^+\) ions abolishes the charge movement caused by Na\(^+\) binding. Binding of the third Na\(^+\) ion is electrogenic with a dielectric coefficient of 0.25 (Domaszewicz and Apell 1999). The time resolution of the techniques available to study cytoplasmic Na\(^+\) binding has not been able to determine the rate constant of these reaction steps; only equilibrium dissociation constants have been obtained.

Occupation of the third highly selective Na\(^+\) binding site in transmembrane parts of the Na\(^+\)/K\(^+\) ATPase causes a structural transition in the nucleotide binding site, which can be detected by the fluorescent FITC-labeled enzyme. This transition results in a movement of bound ATP into a position where its \(\gamma\) phosphate is able to coordinate with Asp-371 at the phosphorylation site. Thus, the sequence of enzyme phosphorylation following binding of the third Na\(^+\) ion ensures that no ATP is hydrolyzed unless three Na\(^+\) ions are bound to the pump (Apell 2003).

The phosphorylation of the enzyme occludes the three Na\(^+\) ions inside the transmembrane domains of the pump (Na3E1·ATP \(\rightarrow\) (Na3)E1-P + ADP). This process is electroneutral because no net charge movement within the membrane domain could be detected (Arato-Oshima, Matsui et al. 1996; Apell 2003). The phosphorylation-induced occluded state, (Na3)E1-P, is transient. Conformational change from E1P to E2P follows and three Na\(^+\) are subsequently released to extracellular side, as discussed previously.
The kinetics of internal Na\(^+\)/Na\(^+\) exchange is the least well studied part of the transport cycle. In the second part of my study, efforts were made to investigate it. Since this step has a rate that is not readily to be detected by current techniques, we were trying to observe it indirectly through including this step in the reaction scheme and recording its effect on extracellular Na\(^+\) release.
PART I: THE INHIBITING EFFECTS OF OLIGOMYCIN ON TRANSIENT CURRENT CARRIED BY RELEASE OF NA$^+$ IONS AND ENZYME CONFORMATIONAL CHANGE OF *BUFO* NA$^+$/K$^+$ ATPASE
Part I.I. Introduction

The release of three Na\(^+\) ions into extracellular media has been shown to follow three distinct and sequential steps (Holmgren, Wagg et al. 2000). However, it is not clear whether three Na\(^+\) are released through one single pathway to the extracellular medium or not (Gadsby 2007). To investigate whether Na\(^+\) ions are released through one single pathway, oligomycin was employed due to its unique inhibiting properties on the Na\(^+\)/K\(^+\) ATPase.

Oligomycin is an inhibitor of ATP synthase. The studies on the working mechanism underlying its effect on Na\(^+\)/K\(^+\) ATPase started in 1966 when Fahn, Covel and Albers were seeking means to study the effect of intracellular Na\(^+\) on enzyme phosphorylation. Ouabain was initially used for this purpose (Fahn, Koval et al. 1966). However, ouabain reduced the enzyme phosphorylation while at the same time reducing enzyme activity. 10 mM ouabain inhibited 100% of the enzyme activity and 96% of the enzyme phosphorylation. In contrast, with application of 20 \(\mu\)g/ml oligomycin, 90% of the enzyme activity was inhibited while enzyme phosphorylation was slightly enhanced (Fahn, Koval et al. 1966). Later oligomycin’s effects on the Na\(^+\)/K\(^+\) exchange and Na\(^+\)/Na\(^+\) exchange modes were examined using sealed ghost red blood cell (Garrahan and Glynn 1967). The efflux of radioactive Na\(^+\) was partially inhibited by 1-10 \(\mu\)g/ml oligomycin, both in the presence and absence of K\(^+\). 1\(\mu\)g/ml oligomycin inhibited 47% and 48% of Na\(^+\) efflux in the presence and the absence of K\(^+\), respectively and 10 \(\mu\)g/ml oligomycin inhibited 78% and 91% of Na\(^+\) efflux in the presence and the absence of K\(^+\), respectively. In their study, the formation of the phosphorylated intermediate was also
evaluated in the absence of K⁺. Interestingly, oligomycin had no effect on the formation of this intermediate even though it partially inhibited Na⁺ efflux, which is in contrast with the inhibiting effect of ouabain on both the formation of the phosphorylated intermediate and Na⁺ efflux. It was postulated that Na⁺/Na⁺ exchange requires both the phosphorylation of the enzyme and some “other process” and it is the “other process” that oligomycin affected (Garrahan and Glynn 1967). It was postulated that oligomycin would prevent the K⁺ activated dephosphorylation of NKA and that was demonstrated in later studies (Fahn, Koval et al. 1968; Fortes and Lee 1984). In the absence of K⁺, purified NKA was labeled with P³² after incubation with Na⁺ and P³² labeled ATP. The addition of K⁺ drove more pumps to the E2(K) conformation, thus decreasing the level of P³² labeled enzyme. Oligomycin appeared to block the reaction from going further, as supported by the observation that addition of K⁺ maintained high levels of ³²P labeled enzyme.

Yoda and Yoda (1986) showed that there were three phosphorylated intermediate states in the absence of K⁺ and oligomycin. They were the K⁺ sensitive E2P state, the ADP sensitive E1P state, and the K⁺ and ADP sensitive E*P state. Oligomycin favors the K⁺ insensitive states (including E1P and E*P) without affecting the occupancy of the K⁺ sensitive E2P state (Yoda and Yoda 1986; Glynn and Karlish 1990). Yoda and Yoda also showed that the occupation of E*P was favored by a high percentage of cholesterol in the lipid membrane, which causes the ratio of Na⁺ efflux : Na⁺ influx : ATP hydrolysis to be reduced from about 3:2:1 to 1.6:0.6:1 (Yoda and Yoda 1987). Therefore, E*P was able to transport one net Na⁺ out at the expense of one molecule of ATP. Given the results from
Garrahan and Glynn’s study (1967) showing that oligomycin only partially inhibited Na\(^+\) efflux, it is highly possible that E\(^*\)P is the state favored by the binding of oligomycin. Based on our current understanding of NKA transport, E\(^*\)P could be either one or two Na\(^+\) bound E2P state, i.e., E2P·Na or E2P·2Na.

Oligomycin was later utilized in electrophysiology studies on currents carried by the NKA. Transient current studies on endogenous *Xenopus* NKA done by Holmgren and Rakowski (1994) found that oligomycin inhibited about 80% of the charge translocation carried by electroneutral Na\(^+\)/Na\(^+\) exchange. Arato-Oshima and colleagues extensively investigated the working mechanism of oligomycin on Na\(^+\)/K\(^+\) ATPase obtained from canine (ouabian-sensitive) or rat (ouabian-resistant) kidney outer medulla (Arato-Oshima, Matsui et al. 1996). The ouabin sensitivity of binding of Na\(^+\), K\(^+\), or Rb\(^+\) binding to the NKA was calculated by comparing the difference between the presence and absence of oligomycin. The binding of Na\(^+\) was greatly increased by the presence of oligomycin but the binding of K\(^+\) was not affected by oligomycin. When trypsin was used to digest the NKA in non-denatured state, the digestion pattern of the NKA was not affected by the presence of oligomycin under different conditions, which indicated oligomycin did not induce a unique conformation. H\(^3\) labeled ATP binding on the NKA was not increased by more than 1.5 fold, and the presence of ATP did not change the effect of oligomycin in promoting Na\(^+\) binding. Since ATP binds to the E1 conformation, the lack of effect of ATP on the enhancement of Na\(^+\) binding by oligomycin indicated that it was not necessary for Na\(^+\) to bind to the E1 conformation in the presence of oligomycin. Na\(^+\)/Na\(^+\), Na\(^+\)/K\(^+\), or Rb\(^+\)/Rb\(^+\) exchange was studied on human ghost red blood cells (Arato-
Oshima, Matsui et al. 1996). The Na\(^+\)/Na\(^+\) and the Na\(^+\)/K\(^+\) exchange were both reduced by inclusion of oligomycin but the Rb\(^+\)/Rb\(^+\) exchange was not affected.

All these studies using either biochemical means or radioactive exchange methods showed that oligomycin has its effect on NKA activity by partially blocking Na\(^+\) release to the extracellular medium and in turn stopping pumps from going into the de-phosphorylation half cycle. However, the direct effect of oligomycin on steady state current mediated by the NKA has not yet been investigated. Since oligomycin blocks the pump from going through Na\(^+\) de-occlusion step to complete the full cycle, we propose that oligomycin in saturating concentrations is able to totally inhibit the steady state pump current.

One intriguing characteristic of oligomycin’s action is that the blockage by oligomycin seems able to leave the pump working in a narrow part of Na\(^+\)/Na\(^+\) electroneutral exchange half cycle. Studies have proposed that there are at least two components in the charge movement mediated by this half cycle, representing three Na\(^+\) ions are released sequentially. The employment of oligomycin in the working solution would be able to block release of some Na\(^+\) ions, thereby excluding some components and allowing more direct view of these components. So the study of transient currents before and after application of oligomycin would firmly verify present theory on mechanism of Na\(^+\) transportation by the NKA.
Part I.II. Materials and Methods

Oocytes preparation and maintenance

The charge movement \((Q)\) mediated by \(\text{Na}^+\) release and re-binding was measured using the cut-open oocyte voltage clamp technique in *Xenopus laevis* oocytes expressing *Bufo marinus* NKA \(\alpha 1\) and \(\beta 1\) subunits. Oocytes at stages V-VI were collected and treated with 0.3% collagenase in \(\text{Ca}^{2+}\) free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 2.5 mM Na pyruvate, 5 mM Tris HEPES, pH = 7.3, osmolality = 200±5) for 40-60 minutes to remove the follicular layer. Oocytes remained viable for up to 6 days at 15°C in ND96 solution (\(\text{Ca}^{2+}\) free ND96 plus 1.8 mM CaCl\(_2\)).

RNA preparation and injection

Wild type *Bufo marinus* NKA \(\alpha 1\) and \(\beta 1\) cRNAs were transcribed from their corresponding cDNAs, which were generous gifts from Jean-Daniel Horisberger (Institute of Pharmacology and Toxicology, University of Lausanne, Lausanne, Switzerland). *Bufo marinus* NKA \(\alpha 1\) and \(\beta 1\) cDNAs in plasmid pSD5 were transformed into competent bacteria DH5\(\alpha\), single colonies were grown overnight and plasmids were prepared using QIAprep Spin Miniprep Kit #27104 (QIAGEN Valencia, California). We sequenced our *Bufo* \(\alpha 1\) and \(\beta 1\) cDNAs and compared them to the published sequences, NCBI Z11798 and Z11097 (Jaisser, Canessa et al. 1992). We found two nucleotide differences in \(\alpha 1\) compared with the originally published sequences, G1860C and C1861G. However, our sequence corresponds to the updated sequence in Horisberger (1994). The \(\beta 1\) sequence showed changes T322C C323T T325C, which would cause one
amino acid difference Leu37Phe. However, the original sequence was likely an error. Jingping Hao in our laboratory has found that 37Ph is functional and 37Leu is not functional (personal communication).

The extracted α1 and β1 cDNAs were linearized by restriction enzymes SpeI and BglII (New England Biolab, Ipswich, Massachusetts), respectively. Linerized cDNAs were treated with proteinase K and extracted using phenol/chloroform. cRNAs were transcribed from linearized cDNAs using MAXIscript® SP6 Kit (#AM1308, Ambion, Austin, Texas). 10ng α1 cRNA and 1ng β1 cRNA were coinjected into oocytes 1 day after oocytes were collected. Injected oocytes were incubated in ND96 solution for 3 days to obtain good membrane expression. Oocytes were incubated overnight in 0.2 μM ouabain containing ND96 solution to inhibit the endogenous *Xenopus laevis* NKA current (Horisberger and Kharoubi-Hess 2002). *Bufo marinus* α1 is more ouabain resistant than *Xenopus laevis* α1 so that pretreatment in 0.2μM ouabain completely inhibits the endogenous pump current, but has little effect on the introduced *Bufo* pump current, while the 1 mM ouabain applied during electrophysiological measurement inhibits both kinds of currents.

*TWO ELECTRODE OOCYTE VOLTAGE CLAMP SETUP*

The steady state current mediated by *Bufo* NKA was studied using a two electrode oocyte voltage clamp preparation. Voltage pulses of 100 ms in duration were made from a holding potential -20mV to command potentials over the range −120 to +60 mV in increments of 20 mV. Pulses were applied every 300 ms. Data were acquired using an
analog to digital converter system and software (TL-1 DMA interface, 100 KHz, PCLAMP version 9; Molecular Devices, Sunnyvale, CA) running on an Dell compatible computer system (Dell Computer Corp., Austin, TX). The analog signal was sampled every 100 μs (10kHz).

Cut-open setup

Saponin-permeabilized Xenopus oocytes mounted with guarded seals between the intracellular and extracellular compartments (Taglialatela, Toro et al. 1992) were used to study transient currents mediated by Bufo NKA (Figure 2). Barriers A and B form Vaseline seals with the oocyte, establishing three electrically separated chambers. A lower chamber (I) is used to permeabilize the oocyte membrane with 0.5% saponin and to internally perfuse the oocyte, a middle compartment acts as a guard shield (GS) to prevent current flow between the lower and upper chambers, and an upper compartment (P) allows the exposed surface of the oocytes to be voltage clamped (Taglialatela, Toro et al. 1992). Compared to a regular two microelectrode voltage clamp recording of whole-oocyte currents, this setup has several advantages including (1) high frequency response and low noise recording; (2) access to cell interior; and (3) stable recording conditions lasting for several hours.
The oocyte is mounted with its animal (dark) pole oriented towards the bottom, where it was permeabilized by adding 0.5% saponin for approximately 1 minute. Permeabilization resulted in a decrease in access resistance and a large increase in total capacitance, which resulted in an increase in the time constant of transient currents induced by small amplitude (20 mV) test voltage clamp pulses. Then the saponin solution was replaced by the experimental intracellular solution and allowed to equilibrate until there was no change in transient current magnitude. Oocytes were stable with small leakage for more than 2 hours. The voltage clamp system was obtained from DAGAN Corporation (Minneapolis, MN; Model CA-1 High Performance Oocyte Clamp).

To observe the inhibiting effect of oligomycin on the fast component, three protocols were utilized. A). Voltage pulses of 40 ms in duration were made from \( V_h \) to command potentials over the range \(-160 \) to \(+60 \) mV in increments of 20 mV. Pulses were applied every 300ms. The current records were obtained by averaging 4 repetitions of the pulse protocol. Data were acquired using an analog to digital converter system and

**Figure 2.** Schematic drawing of the experimental chamber.
software (TL-1 DMA interface, 100 KHz, PCLAMP version 9; Molecular Devices, Sunnyvale, CA) running on a Dell compatible computer system (Dell Computer Corp., Austin, TX). The analog signal was low-pass filtered at 5 kHz before being digitized, and was sampled every 20 μs. B) two 20 ms voltage steps (+60 and -100 mV) from holding potential -20 mV are utilized to minimize the affect of voltage steps on membrane capacitance. The analog signal was low-pass filtered at 10 kHz before being digitized, and was sampled every 10 μs. C) P/4 leakage subtraction protocol was utilized on a mutant *Bufo* NKA: G813A to remove linear capacitance. Four small voltage steps with ¼ magnitude from -180 mV were applied before a voltage step from holding potential -20 mV was applied in the same direction. The slope of the nonlinear capacitance mediated by the pump at extreme negative potentials was assumed to be zero so only linear capacitance is present in these small pre-steps. The subsequent subtraction of these small steps from the following voltage step will result in the residual non-linear capacitance mediated by the NKA.

**Solutions**

For the oocyte cut-open setup, two basic K⁺-free solutions were used. One is 100Na⁺ external solution, and the other is 10Na⁺ internal solution. 100Na⁺ external solution had the following composition: 100 mM Na glutamate, 20 mM tetraethylammonium (TEA) glutamate, 3 mM Mg glutamate, 5 mM Ba(NO₃)₂, 2 mM Ni(NO₃)₂, 0.01 mM Gd(NO₃)₃, 0.3 mM niflumic acid and 10 mM Tris HEPES, pH=7.6. The composition of 10Na internal solution was 10 mM Na glutamate, 20 mM TEA
glutamate, 10 mM MgSO₄, 5 mM MgATP, 5 mM TrisADP, 5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 70 mM N-methyl D-glucamine (NMDG) glutamate, 10 mM MOPS (pH=7.3). Note that both ATP and ADP were present in the intracellular solution to promote electroneutral Na⁺/Na⁺ exchange (De Weer, 1970; Glynn, 1971). One millimolar AP5A was included in all ADP containing internal solutions to prevent phosphate exchange between ATP and ADP. The solutions were designed to minimize non-pump mediated current. Extracellular TEA⁺ and Ba²⁺ were present to block passive K⁺ conductance (Holmgren and Rakowski 1994). NMDG was used as an internal Na⁺ substitute because it would be less likely to compete for Na⁺ binding sites than smaller cations. The solutions were nominally chloride-free and are also Ca²⁺-free to prevent activation of Ca²⁺ dependent anionic current. Niflumic acid was present to block Cl⁻ channel. Ni²⁺ and Gd³⁺ were present to block Na⁺/Ca²⁺ exchange (Kimura, Miyamae et al. 1987) and stretch-activated cation channels (Yang and Sachs 1989), respectively.

For two voltage oocyte clamp setup, the perfusing solutions have the same composition as 100Na⁺ external solution except that they contain either an extra 5 mM TMA (0K solution) or an extra 5 mM K⁺ (5K solution).

Two forms of oligomycin were used in our NKA studies. One is an oligomycin mixture of 65% oligomycin A, and B and C in undetermined percentage; the other is pure oligomycin B. They both have effective and specific effects on the Na⁺/K⁺ ATPase. The oligomycin mixture was dissolved in DMSO (SIGMA St. Louis, MO) with a concentration of 25 mg/ml. Rakowski and his colleagues have established that DMSO
has no effect on the steady state current mediated by NKA in the presence of potassium (Rakowski, Gadsby et al. 1989). An appropriate amount of this stock solution was then added to the 100Na external solution to get solutions with final desired concentrations of oligomycin. Oligomycin B (USBIOLOGICAL, Swampscott, MA) was dissolved in DMSO to 30 mM. Similarly, different amounts of the oligomycin B stock solution in DMSO was added to the 100Na external solution to get final desired concentrations. The concentration of DMSO in all final solutions was kept constant throughout experiments on one single oocyte.

Steady state current analysis

Steady state currents (I) mediated by Bufo NKA after adding 1mM ouabain or oligomycin in different concentrations were subtracted from the currents in the presence of 5 mM potassium using software clampfit 9.2. The dependence of I on inhibiting factors was further analyzed, plotted and fitted with least-squares fitting procedure with SIGMAPLOT 8.0

Transient current analysis

Transient Na+/Na+ exchange current was calculated by subtraction of the current records obtained after arresting Na+/Na+ exchange by the addition of 1 mM ouabain from records acquired just prior to the addition of ouabain (Figure 3). To obtain its relaxation rate, the transient current was fitted with either a single exponential function or a two component exponential function. The charge translocated was determined by direct
numerical integration of the subtracted current records or by multiplying the two parameters $A$ and $\tau$ obtained by the single exponential fit. All of this analysis was done with Clampfit 9.2 software. Further analysis, least-squares curve fitting, and preparation of figures were done with SIGMAPLOT 8.0. Curve fit parameters were obtained from the least-squares fitting procedure. Experiments were performed at room temperature ($\sim 22 \, ^\circ C$).
Figure 3. Ouabain-sensitive transient currents from a permeabilized oocyte. A) Voltage pulse protocol. The voltage pulses were done in increments of 20mV from -160 to +60 mV with duration 40ms. For clarity, voltage pulses are shown every 40 mV. Holding potential was -20. B) Current time course in 100Na 0K external solution. C) Currents in 1mM ouabain 100Na 0K external solution. D) Ouabain sensitive currents determined by subtraction of currents in the presence of 1mM ouabain (C) from those in its absence (B).

The voltage dependence of the charge movement (QV curve) is given by equation 1, which has a sigmoid shape. To simplify the meaning of the curve, $Q_{\text{max}}$ represent the maximum occupation of pumps at $Na^+$ nonbinding form E2P and $Q_{\text{min}}$ represents the maximum occupation of pumps at $Na^+$ binding form E1P(Na3) (Fig 4).
Figure 4. An example of voltage ($V_m$) dependence of charge distribution ($Q$) ($Q/V$ curve). The solid line represents the best fit line of the data points using equation 1. Qmax at extreme positive $V_m$ represents when 100% of the measured pumps are in state E2P (no Na$^+$ bound) and Qmin at extreme negative $V_m$ represents 100% are in state E1P(Na3) (3 Na$^+$ bound). Vmid represents when half of the pumps are in state E2P and Half in E1P(Na3).
Part I. III. Results

First control experiments were done to make sure the observed currents are from the *Bufo* cRNAs that were injected into oocytes. Oocytes were incubated in 0.2μM ouabain overnight before measurements. Figure 5 shows that the endogenous currents were totally inhibited when *Bufo* cRNAs were not injected. But large currents remained when *Bufo* cRNAs were injected into oocytes.

**Figure 5.** Steady state currents in oocyte with or without injection of *Bufo* NKA α1 and β1 subunit. A) the steady currents of Xenopus oocyte without injection of *Bufo* cRNAs after incubation in 0.2μM ouabain overnight. B) the steady state current carried by *Bufo* NKA expressed in Xenopus oocytes after incubation in 0.2μM ouabain overnight.

We studied the inhibiting effect of oligomycin on both steady state current and transient current mediated by *Bufo marinus* Na⁺/K⁺ ATPase injected into Xenopus oocytes. The study was initially done on wild type *Bufo* Na⁺/K⁺ ATPase with a mixture of oligomycin species.
Inhibition of oligomycin on steady state current mediated by Bufo NKA

The study of inhibition by oligomycin mix of the K\(^+\) activated steady state current was done in 100 mM Na\(^+\) and 5 mM K\(^+\) solutions. One example is shown in Figure 6. The addition of 7.5\(\mu\)g/ml oligomycin first slightly enhanced pump current and then inhibited pump current. With the increase in [oligo], more and more pump current was inhibited (Fig 6 A). The difference currents between 5 mM K\(^+\) and oligomycin at different concentrations are the oligomycin sensitive currents (I\(_{\text{oligo}}\)). The difference current between 5 mM K\(^+\) and 1 mM ouabain is the ouabain sensitive current (I\(_{\text{ouab}}\)), which is treated as total available pump current. The ratio I\(_{\text{oligo}}\)/I\(_{\text{ouab}}\) is plotted against [oligo] to show the dose dependence curve for the inhibition of I by oligomycin (Fig 6 B). The apparent affinity of the sodium pump for oligomycin is obtained through fitting to equation 2.

\[
\frac{I_{\text{oligo}}}{I_{\text{ouab}}} = \frac{a \cdot [\text{Oligo}]^n}{(K_i^n + [\text{Oligo}]^n)} \quad \text{Equation 2}
\]

Where a is maximum inhibition by oligomycin, n is Hill coefficient and Ki is the apparent affinity for oligomycin.
Figure 6: The inhibiting effect of oligomycin on K⁺ activated current mediated by wild type *Bufo* NKA. A) Chart recording of oligomycin inhibition on 5mM K⁺ activated current in 100mM Na⁺ solution. (B) Dose dependence curve for the inhibition of K⁺ activated current by oligomycin. The solid line represents best fit to data points using equation 2, the best fit parameters: Ki = 12.3 μg/ml, n = 2.26, and a= 80%
The inhibition of oligomycin on $Q_{\text{whole}}$ of transient currents

The apparent affinity of oligomycin was obtained by studying its inhibiting effect on charge movement of the whole “on” trace ($Q_{\text{whole}}$). Charge movement was measured in the absence of oligomycin and ouabain, in the presence of oligomycin at different concentrations, and then in the presence of 1 mM ouabain. The amount of charge inhibited by oligomycin at different concentrations ($Q_{\text{oligo}}$) relative to the all movable charge ($Q_{\text{ouab}}$), $Q_{\text{oligo}}/Q_{\text{ouab}}$, is plotted to show its apparent affinity and its maximum inhibiting effect (Fig 6). The data is fitted using equation 3.

$$Q_{\text{oligo}}/Q_{\text{ouab}} = b*[\text{oligo}]/(K_i+[\text{oligo}]) \quad \text{Equation 3}$$

where $b$ is the maximum inhibition by oligomycin and $K_i$ is the apparent affinity of oligomycin.

The inhibiting effect of oligomycin was analyzed at different membrane potentials to see if the binding of oligomycin shows any voltage dependence (Fig 7 A). The $K_i$ value of 24±4 μg/ml at -160 mV is not significantly different from 28.9 ±4.7 μg/ml at +60 mV. A two-tailed t test was performed and $p=0.35$. At a significance level of 0.05, the comparable means between -160 mV and +60 mV are not statistically significantly different. The maximum inhibition of oligomycin is 0.70 and 0.75 at -160 mV and +60 mV, respectively. When individual oligo-sensitive QV curves were fitted to equation 1, the fitted maximum inhibition on whole charge ($Q_{\text{total,oligo,whole}}$) is obtained. The plot of
$Q_{\text{total, oligo, whole}}$ vs [oligo] shows similar results with $K_i$ equal to 24.8 μg/ml and maximum inhibition equal to 71% (Fig 7 B).
Figure 7. Dose response curve of oligomycin inhibition of *Bufo marinus* NKA. (A) Oligomycin inhibition curves obtained at two different voltages, -160 mV and +60 mV. Data points (mean ± SE) represent inhibited charge by a certain [oligo] at -160 mV (filled circles) or +60 mV (open circles). The solid lines represent the best fit using equation 3, the best fit parameters: $K_i = 24.0 \pm 4 \mu g/ml$ and $b = 0.70 \pm 0.03$ at -160 mV; $K_i = 28.9 \pm 4.7 \mu g/ml$ and $b = 0.75 \pm 0.03$ at +60 mV. (B) The inhibition of oligomycin on the $Q_{tot}$ of $Q_{whole}$. The data points ($Q_{total\_oligo\_whole}/Q_{total\_ouab\_whole}$) were obtained from fitting the individual $Q_{oligo\_whole}$ vs V curves at different [oligo] with equation 1. The solid line represents the best fit using equation 3, the best fit parameters: $K_i = 24.8 \pm 4.1 \mu g/ml$ and $b = 71\%$. Data are from 6 oocytes.
Inhibition of oligomycin on faster components

Preliminary data showed that 120 μg/ml oligomycin inhibited most of the faster component, as indicated by the disappearance of the peak of ouabain-sensitive transient currents after application of oligomycin (Fig 8) and the change in slope of the $Q_{\text{whole}}$ vs $V$ curve. The slope was obtained by integrating the entire “on” trace of transient current elicited by voltage steps (Fig 9), which is introduced as protocol (a) used in the oocyte cut-open setup.
A) Graph showing membrane potential (mV) over time (ms) for 120ug/ml Oligomycin and 1mM ouab.
Figure 8. The average 120μg/ml oligomycin sensitive and insensitive transient current measured in oocytes internally incubated with 10 Na⁺ 0 K⁺ and externally perfused with 100Na⁺ 0K⁺ solution. (A) The chart view of an entire experiment with IV steps labeled as a, b, c, d, e, f, and g applied under different conditions. Difference currents generated by IV steps under different conditions are shown in (B), (C), and (D). (B) Transient currents inhibited by 120μg/ml oligomycin (a-c). (C) Transient currents that were oligomycin insensitive but inhibited by 1mM ouabain (d-f). (D) Control obtained by subtracting two ouabain inhibited transients that had similar time intervals as compared to files used to obtain oligo-sensitive and oligo-non sensitive transient currents. Data are from 9 oocytes.
Without addition of oligomycin, there are at least two components, a fast and a slow component, in the whole “on” transient currents. The fast component has a shallower slope while the slow component has a steeper slope (Hilgemann 1994). When the integration is done over the whole trace, the voltage dependence of $Q_{\text{whole}}$ reflects the combined characteristics of slower and faster charge movement, that is, it is shallower than the voltage dependence of the slow component. The application of oligomycin made the steepness of $Q_{\text{whole}}$ less shallow, i.e., closer to the steepness of $Q_{\text{slow}}$, which is shown as the increase of $z$ from 0.4 to 0.57 (Fig 9). In addition, the application of oligomycin shifted $Q_{\text{whole}}$ vs $V$ curve to the left, which indicates that oligomycin favors the E2P conformation over E1 conformation.
Figure 9. Comparison of ouabain sensitive $Q_{\text{whole}}$ in the presence and absence of 120μg/ml oligomycin. Dots represent data points (mean±SE), and solid lines represent the best fit by equation 1. Circles are data points of ouabain inhibited $Q_{\text{whole}}$ obtained in the absence of oligomycin, the best fit parameters, $V_{\text{mid}} = -67.0\pm 3.5$ and $z = 0.40\pm 0.02$; triangles are data point of ouabain inhibited $Q_{\text{total}}$ obtained in the presence of 120μg/ml oligomycin, the best fit parameters, $V_{\text{mid}} = -91.2\pm 2.5$ mV and $z = -0.56\pm 0.04$. Both sets of data are from 7 oocytes.

**The effect of oligomycin on mutant Bufo NKA: G813A**

G813A is a mutated form of *Bufo* Na$^+$/K$^+$ ATPase α1 subunit (Li, Capendeguy et al. 2005). Glycine813 is thought to contribute to the third Na$^+$ binding site. The replacement of glycine by alanine increased both intracellular and extracellular Na$^+$ binding affinity, which indicates that the mutation affected the third Na$^+$ binding site rather than simply disturbing the E1-E2 equilibrium. Otherwise, the affinity decrease on
one side will compensate for the increase on the other side. (Li, Capendeguy et al. 2005)

There are two advantages in utilizing G813A to study transient currents, 1) Higher affinity to Na$^+$ at both intracellular and extracellular sides lower the possibility of a proton leak found by several laboratories (Vasilyev, Khater et al. 2004). In our lab, G813A is also found to produce steady state and transient currents with less leakage (data not shown). 2) Due to its promoting effect on Na$^+$ binding thus favoring E1, the QV curve is significantly shifted to the right (Li, Capendeguy et al. 2005), which makes it possible to use the P/4 protocol to determine the non-linear capacitance mediated by the sodium pump. Compared to the method using subtraction of the ouabain sensitive current, the nonlinear capacitance determined from the P/4 protocol has no time dependent leak current involved and no current introduced through slight differences in initiation of voltage steps before and after ouabain addition.

The P/4 protocol was utilized to obtain the nonlinear capacitance mediated by *Bufo* NKA G813A (Fig 10). A saturating concentration of oligomycin, 120 $\mu$g/ml was applied to see the evident effect on transient current.
**Figure 10.** The inhibition of 120 μg/ml oligomycin on transient current mediated by G813A. The experiment was done in 100Na 0K external solution and transient currents were obtained through p/4 protocol. A) Time control before addition of oligomycin. B) Time control after addition of ouabain. C) Transient currents before oligomycin addition. D) Close view of transient current induced by voltage step from -20 mV to -160 mV, one of the traces in C. The blue line represents the best fit by a two-component exponential function. The two green lines represent the two fit components. The best fit parameters, A1= -140±6.8 nA, τ1= 1.17±0.05 ms, A2= -322±7 nA, τ2= 0.27±0.01 ms. E) 120μg/ml oligomycin sensitive transient currents, the difference between transients in the absence of 120 μg/ml oligomycin and transients in its presence. F) Close view of transient current induced by voltage step from -20 mV to -160 mV, one of traces in E. the blue line represents the best fit by a one-component exponential function. The best fit parameters, A= -308±2.4 nA, τ= 0.31±0.003 ms. G) 120 μg/ml oligomycin insensitive transients. H) Close view of transient current induced by voltage step from -20 mV to -160 mV, one of traces in G. The blue line represents the best fit by a one component exponential function. The best fit parameters, A= -141±0.6 nA, τ= 0.99±0.01 ms. I) The voltage dependence of charge obtained from direct integrating 120 μg/ml oligomycin sensitive currents shown in E or insensitive currents shown in G. Dots represent data point and Solid lines represent best fit lines by equation 1. Filled circles represent oligomycin sensitive charge, the best fit parameters: Qmax=988, z=0.65 and Vmid=32 mV. Empty circles represent oligomycin insensitive charge, the best fit parameters, Qmax=837 z=0.74 and Vmid=27 mV.

Before the addition of oligomycin in saturating concentration, voltage jumps initiate transient currents with peak magnitude ranging from 200 nA to -450 nA and one of the voltage jumps, -20 mV to -160 mV, produces a transient containing two components with A1= -140 nA and τ1= 1.17 ms for slower component and A2= -322 nA
and $\tau_2=0.27\text{ms}$ for faster component (Fig 10 C & D). The magnitude of 120$\mu$g/ml oligomycin sensitive transient currents ranges from 150 nA to -350 nA and the fit of the transient at the voltage step -20mV to -160mV requires only 1-exponential function with $\tau \approx 0.31$ ms and $A=308$ nA (Fig 10 E & F). Not all recordings of transient currents were sensitive to 120$\mu$g/ml oligomycin. The oligomycin insensitive currents range from 50 nA to -150 nA in peak magnitude. The fit of transient currents at the voltage jump from -20 mV to -160 mV also only requires 1-exponential function with $\tau \approx 1$ ms and $A=140$ nA (Fig 10 G & H). The addition of 120$\mu$g/ml oligomycin therefore successfully separates the two components of the transient currents obtained using the P/4 protocol. The slope of the QV curve of oligomycin sensitive current is close to the steepness of medium component found in squid giant axon (Fig 10 I) (Holmgren, Wagg et al. 2000). The slope of the QV curve of oligomycin insensitive transient currents is consistent with values obtained on slow component (Holmgren and Rakowski 2006).

**The effect of oligomycin B on mutant Bufo NKA: G813A**

In additional experiments, more efforts were made to show the inhibiting effect of oligomycin on the fast component. In order to minimize amount of DMSO and lipophilic chemical oligomycin, pure oligomycin B was utilized. Oligomycin B was referred as the effective component at inhibiting NKA in some literatures (Nakao and Gadsby 1986; Holmgren and Rakowski 1994). The chart view and the dose dependence curve of inhibition by oligomycin B on steady state current mediated by G813A are shown in Figure 11 A and B. The application of oligomycin B in low concentrations first also
shows enhancement on pump current before current gradually decreases as [oligo B] increases (Fig 11 A). Ki of oligomycin B to G813A is determined to be 3.9 uM (3μg/ml) (Fig 11 B). Even though the addition of 3.2 uM oligomycin B inhibited almost half of steady state current mediated by pump, the voltage dependence of remaining I is fitted as well by the Boltzmann equation with a slope of 1 in the presence 3.2 uM oligomycin B as in the absence of oligomycin B (Fig 11 C). The consistency of voltage dependence of I indicates that a net single positive charge across membrane was carried by functional NKA in the presence of 3.2 uM oligomycin B. In the presence of 12.8 uM oligomycin B, current is close to 0 and does not show any change with a change in membrane potential.
Figure 11. The inhibiting effect of oligomycin B on 5mM K⁺ activated current mediated by Mutant *Bufo* NKA: G813A (A) Chart view of oligomycin B inhibition on K⁺ activated current mediated by G813A. Experiment was done in 100Na solution. (B) Inhibition of K⁺ activated current by oligomycin B. Black circle represents data points and solid line represents best fit to data point by equation 2, the best fit parameters, Ki = 3.9uM, a = 73%, and n = 2.9. (C) voltage dependence of current in the presence of 5mM K⁺ and different concentrations of oligomycin B. Dots represent data points (Black circle: 0 mM oligo B, red circles: 3.2uM oligo B, and green triangles: 12.8uM oligo B). Solid lines represent best fit curves by Boltzmann equation with slope of 1. Data were from 3 oocytes of at least two frogs.
The subtracted ouabain sensitive current is beneficial on keeping all components, especially very fast one with shallow slope, even though it requires oocyte to hold strictly tight through a period of time to avoid contamination with linear capacitance. Two different concentrations of oligomycin B were utilized in this method to show the direct inhibiting effect of oligomycin B on fast component.

The study of steady state current has shown that 3.2 uM is far from saturating (Ki =3.9 uM for steady state current mediated by G813A). This low concentration oligomycin B only inhibited a part of fast component with Tau close to 0.06ms (Fig 12 C). The remaining super fast component in 3.2uM oligomycin B insensitive current (Fig 12 D) has a similar time constant (Tau=0.05).
Figure 12. The inhibition by 3.2uM oligomycin B on transient currents mediated by mutated *Bufo* NKA: G813A. A) Voltage steps used to induce transient currents shown in B), C), D). B) Time control before addition of oligomycin and ouabain. C) 3.2 uM oligomycin B sensitive transient current. D) 3.2 uM oligomycin B insensitive but 1mM ouabain sensitive current.

As [oligomycin B] was increased to 12.8 uM, almost all fast component was inhibited and only slow component was left (Fig 13). The inhibited fast component has $\tau$ close to 0.07 ms at both -160 mV and +60 mV (Fig 13 C). The remaining slow component in oligomycin B insensitive current has time constant in the millisecond range with 0.3 ms at -160 mV and 0.4 ms at 40 mV(Fig 13 D).
Figure 13. The inhibition by 12.8 μM oligomycin B on transient current mediated by mutated *Bufo* NKA: G813A. **A)** Voltage steps are used to induce current shown in **B), C),** and **D).** **(B)** Time control before addition of oligomycin and ouabain. **(C)** 12.8 μM oligomycin sensitive transient currents. The best fit tau values are 0.07 ms and 0.07 ms for -160 mV and 40 mV voltage steps, respectively. **D)** 12.8μM oligomycin B insensitive but 1 mM ouabain sensitive currents. The best fit tau values are 0.3 and 0.4 ms for -160 mV and 40 mV voltage steps, respectively.

The inhibition by oligomycin B on the transient currents obtained by using P/4 protocol has similar characteristics to the inhibition by oligomycin mixture. The oligomycin B sensitive currents quickly relax to steady state and the charge movement has steepness of 0.85 (**Fig 14 C & E**). The 12.8 μM oligomycin B insensitive currents have slower relaxation rate and the charge carried by it moves across a field with dielectric coefficient of 0.83 (**Fig 14 D & F**).
Figure 14. The inhibition of 12.8 μM oligomycin B on transient current mediated by *Bufo* NKA G813A. The experiment was done in 100Na 0K external solution and transient current is obtained by using p/4 protocol. **A)** Voltage steps used to induce transient currents shown in B), C), D). **B)** Transient currents before oligomycin addition. **C)** 12.8 μM oligomycin B sensitive transient current. **D)** 12.8 μM oligomycin B insensitive transient. **E)** The voltage dependence of charge obtained from direct integrating 12.8 μM oligomycin B sensitive current. Dots represent data points (Mean ± SE). Solid line represents the best fit line by equation 1, the best fit parameters, \( z=0.85\pm0.22 \) and \( V_{mid}=-4\pm10 \) mV. **F)** The voltage dependence of charge obtained from direct integration of 12.8 μM oligomycin B insensitive current. Dots represent data points (mean ± SE). Solid line represents best fit line by equation 1, the best fit parameters, \( z=0.83\pm0.31 \) and \( V_{mid}=-43\pm11 \) mV. Data are from 3 oocytes of at least two different frogs.
Part I. IV. Discussion

The enhancing effect of oligomycin in low concentration on steady state current

In the presence of 100mM Na⁺, the addition of oligomycin in low concentrations first enhanced pump current and then inhibited pump current (Fig 6 A & 11 A). The concentration of K⁺ was kept constant at 5 mM after it was added to activate pump current. The enhancement of pump current by small amount of oligomycin can be viewed as an increase in apparent K⁺ affinity. This can be explained by two known facts. One is that potassium binds to E2P conformation (Kaplan 2002). The other is that oligomycin increases the population of pumps in the phosphorylated conformation (Fahn, Koval et al. 1966; Yoda and Yoda 1986). So, the addition of small amounts of oligomycin first provided more pumps in a conformation ready for K⁺ binding and enhanced I activated by 5 mM K⁺. The fact that oligomycin subsequently inhibited pump current may be explained as further incubation with oligomycin blocked the release of sodium to the extracellular medium, thus stopping the pumps from working through their full cycle (Arato-Oshima, Matsui et al. 1996).

The residual I mediated by wild type *Bufo* NKA in the presence of 120μg/ml oligomycin (Fig 6) and mediated by *Bufo* NKA G813A in the presence of 12.8um oligomycin B (Fig 11) indicates that the inhibited pumps were still able to carry net charge across the membrane. But the lack of voltage dependence of the residual current in the presence of 12.8 uM oligomycin B indicates that pumps are not going through their normal working cycle (Fig 11 C). The same slope of the IV curves in the presence of 3.2
μm oligomycin B and in the absence of oligomycin indicates that the binding of oligomycin is voltage independent.

*The partial inhibiting effect of oligomycin on transient current mediated by wild type Bufo α1 and β1*

The study on transient current in the presence of oligomycin done by Holmgren and Rakowski (Holmgren and Rakowski 2006) showed that oligomycin was not able to inhibit transient current completely. Our data showed a similar result (Fig 7). The total moveable charges inhibited by oligomycin and ouabain were calculated and the maximum inhibition by oligomycin is 71±3%. The apparent affinity obtained by this method is 24.8±4.1 μg/ml and does not show any voltage dependence (Fig 7). The exclusion of K⁺ in the solution lowered the apparent affinity for oligomycin.

A detailed analysis of the transient currents before and after addition of oligomycin suggests that the inhibition by oligomycin is dominant on the faster component. The peak of the transient currents obtained after addition of oligomycin was a much smaller magnitude and 100 μs delayed as compared to the transient currents before addition of oligomycin (Fig 8). The ouabain sensitive \( Q_{\text{whole}} \) vs V curve in the presence of 120 μg/ml oligomycin has a much steeper slope (\( z=0.56 \)) than the curve in the absence of oligomycin (\( z=0.4 \)) (Fig 9). In the absence of oligomycin, \( Q_{\text{whole}} \) contains fast component and slow components. The faster component has lower slope (about 0.26) and the slower component has steeper slope (Hilgemann 1994; Holmgren, Wagg et al. 2000). In this study, we have found that the slow component has a \( z \) value close to 0.65.
and the z value obtained in the presence of 120 μg/ml oligomycin is also close to 0.65. This once again suggests the application of oligomycin mainly abolishes the faster component with a shallower slope. Another effect of oligomycin on Q_total vs V curves is the leftward shift of the midpoint voltage from -67.0±3.5 mV to -91.2±2.5 mV, which indicates that fewer pumps are in the E1P(Na3) state.

The inhibiting effect of oligomycin on G813A

It is not clear how the three sodium ions are released into extracellular medium. Do three Na ions go through the same ion pathway? Further investigation done on G813A provides some insight to this question.

The rightward shift of QV curve in this mutation enables the P/4 protocol preserve more currents, even though super fast component with shallow slope could not be reserved. Before the addition of oligomycin at a saturating concentration, the transient current initiated by a voltage jump from -20 mV to -160 mV required a 2-exponential fit (Fig 10 D). The slow component, $\tau \approx 1.17$ ms, is in the normal range of the slow component in ouabain subtracted transient current. Another component, $\tau \approx 0.27$ms, has similar speed to the medium component described by Holmgren et al. (Holmgren, Wagg et al. 2000). The fit to the oligomycin sensitive transient requires only 1-exponential function with $\tau \approx 0.31$ ms and A=308, which closely resembles the medium component in the current transient obtained before the addition of oligomycin (Fig 10 D&F). The oligomycin insensitive current also needs only 1-exponential function for a good fit with $\tau \approx 1$ ms and A=140 nA, which closely resembles the slow component in the transient
obtained before addition of oligomycin (Fig 10 D&H). The P/4 protocol preserves only medium and slow components are preserved, and the medium component is sensitive to 120 μg/ml oligomycin (Fig 10 I and Fig 14 C & E).

Because the slow and the medium component represent two Na⁺ ions releasing from two binding sites through an ion pathway, it is legitimate to speculate that the phenomena resulted from the fact that separate releasing pathways exist for these two Na⁺ ions. The release of the first Na⁺ (forming the slow component in transient currents) is not blocked by oligomycin, but the release of the second Na⁺ (forming the medium component in transient currents) is blocked by oligomycin.

The study done on steady state current shows that G813A has the same affinity for oligomycin B (Ki=3.9 μM, i.e., 3μg/ml) as wild type Bufo NKA for oligomycin (Ki =12.4 μg/ml. If 20% of the mixture is oligo B (Kramar, Hohenegger et al. 1984), it is therefore about 2.5 μg oligo B/ml). To observe the fast component of pump-mediated transient current, a short protocol was applied to give the membrane as little stress as possible so that the minimal time dependent leakage contaminated the subtracted currents. In the presence of 3.2 μM oligomycin B, only a portion of very fast component with time constant 0.05 ms was inhibited, which can be due to the direct blockage of Na⁺ release involved in very fast component (Fig 12). As [oligomycin B] was increased to a more saturating concentration 12.8 μM, the fast component was totally inhibited and only slower component was left (Fig 13). So the release of the third Na⁺ (forming the fast component in transient currents) was also blocked by oligomycin, which suggests that the release of the third Na⁺ might share the same pathway with the second Na⁺.
The results on transient currents strongly suggest that oligomycin blocks release/rebinding of two Na\(^+\) ions involved in the medium and fast components of charge translocation mediated by electroneutral Na\(^+\)/Na\(^+\) exchange. The reaction in the presence of oligomycin can be expressed as reaction scheme 2.

**Reaction scheme 2**

\[
E_1 + 3Na_i \leftrightarrow E_1P(Na_3) \leftrightarrow E_2P\cdot3Na \leftrightarrow E_2P\cdot2Na + Na_o
\]

The fact that the slow component remained intact suggests that the release of three Na\(^+\) ions goes through at least two separate pathways. The application of oligomycin did not affect the release of the first Na\(^+\) into the extracellular medium through its own releasing pathway. But the release of other two Na\(^+\) ions through their common or separate pathways was blocked by oligomycin.

Horisberger and colleagues did cysteine-scanning mutagenesis study to evaluate the accessibility of some amino acids of transmembrane domain 4, 5, and 6. They found that there existed an ion pathway between transmembrane domain 4, 5 and 6 (Guennoun and Horisberger 2002; Horisberger, Kharoubi-Hess et al. 2004). Future studies can be done to evaluate the effect of oligomycin to the accessibility of those amino acids that were proposed to contribute to the ion pathway.
PART II: THE EFFECT OF HOLDING POTENTIAL ON CHARGE
TRANSLOCATION BY THE NA⁺/K⁺ ATPASE IN THE ABSENCE OF POTASSIUM
Part II. Introduction

In general introduction, we have described the extracellular Na\(^+\) release and its electrogenicity in details. However, it is not the only electrogenic step in the transport cycle of the Na\(^+\)/K\(^+\) ATPase. But there is less understanding of other steps including the intracellular Na\(^+\) binding step, which is the concentration of my work in part II.

Normally, the Na\(^+\)/K\(^+\) ATPase transports 3Na\(^+\) out of the cell followed by the import of 2K\(^+\), at the expense of 1ATP. At negative resting membrane potentials, the velocity of the pump is inhibited by \(~50\%\) of its maximal capacity. This is because the negative membrane potential drives external Na\(^+\) back towards their binding sites deep inside the protein. Most of the voltage dependence of the pump cycling comes from these transitions. However, there are three other electrogenic partial reactions in the Post-Albers cycle: 1) the 3\textsuperscript{rd} intracellular Na\(^+\) binding, Na\(2\cdot E1\cdot ATP + Na^+_{i} \rightarrow Na3\cdot E1\cdot ATP\) (Heyse, Wuddel et al. 1994; Wuddel and Apell 1995); 2) the conformational change, E1P(Na3)\(\rightarrow\)E2P(Na3) (Heyse, Wuddel et al. 1994; Wuddel and Apell 1995); and 3) the extracellular K\(^+\) binding, E2P+2K\(^+\)\(\rightarrow\)E2\cdot K2 (Heyse, Wuddel et al. 1994; Sagar and Rakowski 1994; Wuddel and Apell 1995). Because the pump moves one net charge per cycle, the sum of all dielectric coefficients from each electrogenic partial reaction has to be one (Apell 2003). The values presently determined for the dielectric coefficients are: the 3\textsuperscript{rd} Na\(^+\) binding to the intracellular binding site, 0.25 (Domaszewicz and Apell 1999); the conformational change, 0.1-0.2 (Heyse, Wuddel et al. 1994); the release of the 1\textsuperscript{st} Na\(^+\) to the extracellular medium, 0.65 (Hilgemann 1994; Holmgren, Wagg et al. 2000); the release of other two Na\(^+\) to the extracellular medium, each 0.1 (Heyse, Wuddel et al.
the binding of two K⁺ to their binding sites, each -0.1 to -0.2 (Rakowski, Vasilets et al. 1991; Heyse, Wuddel et al. 1994). The sum of these values is approximately 1.

In this part of my work, I have also been studying the major voltage dependent transition in the absence of K⁺, but have focus on the effect of the holding potential (Vₜₜ) on the kinetics of the Na⁺/Na⁺ exchange. As discussed previously, the slow charge translocation (Qslow) that we measure comes from transitions involving extracellular Na⁺ binding/unbinding and occlusion/de-occlusion, which we could isolate fairly well by specifying the composition of the internal and external solutions. If all pumps are confined to these states, we would expect no difference in the total amount of charge at different Vₜₜ. We hypothesize that by manipulating [ADPᵢ], [Na⁺ᵢ] or [Na⁺ₒ], we will allow pumps to exit these states through a Vₜₜ dependent route, and therefore infer the nature of the voltage dependence of those steps.

At a constant Vₜₜ, changes in [Na⁺ₒ] and [ADPᵢ] did not affect the magnitude of total mobile charge (Holmgren and Rakowski 2006), but Vₘᵢᵩ was shifted toward more positive voltage by increasing [Na⁺ₒ] or [ADPᵢ]. A change of [ADPᵢ] from 0 to 4.3 mM shifted Vₘᵢᵩ 16±6 mV (Peluffo 2004), and every doubling of [Na⁺ₒ] similarly shifted Vₘᵢᵩ by ~20-25 mV (Holmgren and Rakowski 2006).

At a constant Vₜₜ, changes in [Na⁺ᵢ] did change the total amount of charge following a simple dose response curve with an apparent affinity of 3.2 mM (Holmgren and Rakowski 2006). Because these data involve intracellular Na⁺ binding and occlusion, the authors based their results using the following scheme:
Reaction scheme 3:

\[ 3\text{Na}_i + \text{E}_1\text{ATP} \xrightleftharpoons[K_m(V_h)]{K_m(V_h)} (\text{Na}_3)\text{E}_1\text{P} \rightleftharpoons \text{ADP} \xrightarrow[k_1]{k_1} (\text{Na}_3)\text{E}_1\text{P} \xrightarrow[k_2]{k_2} \text{E}_2\text{P} + 3\text{Na}_o \]

And therefore charge \( Q \) becomes a function of voltage and \([\text{Na}_i^+]\) by:

\[
Q(V) = \left[ \frac{Q_{tot}}{1 + \exp\left(\frac{zF(V_{mid} - V)}{RT}\right)} + Q_{min} \right] \left[ \frac{[\text{Na}]_i}{[\text{Na}]_i + K_m(V_h)} \right]
\]

Equation 4

Where \( K_m(V_h) \) is a voltage dependent dissociation coefficient for binding of the third intracellular \( \text{Na}^+ \). Therefore, estimating \( Q_{tot} \) at different \( V_h \) but constant \([\text{Na}_i^+]\), we expect to learn about the intrinsic voltage dependence of intracellular \( \text{Na}^+ \) binding.
Part II. II. Materials and Methods

Oocytes preparation and maintenance and RNA preparation and injection are the same as in Part I.

Cut-open setup and Recording protocols

The cut-open setup is as described in Part I. The effects of three different \( V_h \), -20 mV, -60 mV, and -100 mV, were studied in permeabilized oocytes as \([Na^+]_o\), [ADP]_o or \([Na^+]_o\) were varied. Voltage pulses of 40 ms in duration were made from \( V_h \) to command potentials over the range −160 to +60 mV in increments of 20 mV. Pulses were applied every 300 ms and the current records were obtained by averaging 4 repetitions of the pulse protocol. Data were acquired using an analog to digital converter system and software (TL-1 DMA interface, 100 KHz, PCLAMP version 9; Molecular Devices, Sunnyvale, CA) running on an Dell compatible computer system (Dell Computer Corp., Austin, TX). The analog signal was filtered at 5 KHz before being digitized, and was sampled every 20 \( \mu \)s.

Solutions

100Na0K external solution had the following composition: 100 mM Na glutamate, 20 mM tetraethylammonium (TEA) glutamate, 3 mM Mg glutamate, 5 mM Ba(NO\(_3\))\(_2\), 2 mM Ni(NO\(_3\))\(_2\), 0.01 mM Gd(NO\(_3\))\(_3\), 0.3 mM niflumic acid and 10 mM Tris HEPES (pH=7.6). 25Na 0K external solution was obtained by equimolar substitution of tetramethylammonium (TMA) for Na\(^+\). The composition of 50Na0K internal solution
was 50 mM Na glutamate, 20 mM TEA glutamate, 10 mM MgSO₄, 5 mM MgATP, 5 mM TrisADP, 5 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 30 mM N-methyl D-glucamine (NMDG) glutamate, 10 mM MOPS (pH=7.3). Na⁺ free internal solution was prepared by equimolar substitution of NMDG for Na⁺. Intermediate intracellular Na⁺ concentrations were obtained by mixing internal solutions containing 50 and 0 mM Na⁺. Note that both ATP and ADP were present in the intracellular solution to promote electroneutral Na⁺/Na⁺ exchange (De Weer, 1970; Glynn, 1971). Compositions of all external solutions and internal solutions are listed in Table 1. One milimolar AP5A was included in all ADP containing internal solutions to prevent phosphate exchange between ATP and ADP. To study the ADP sensitivity of Qslow, 5mM phosphocreatine substituted 5mM ADP to make sure no ADP was formed from ATP hydrolysis. As described in part I, the solutions were designed to minimize non-pump mediated current.
Table 1. Composition of external and internal solutions used in the cut-open setup.

### External solutions

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<th>Na glutamate</th>
<th>TMA glutamate</th>
<th>others</th>
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<tbody>
<tr>
<td>100Na 0K</td>
<td>100 mM</td>
<td>20 mM TEA glutamate, 3 mM Mg glutamate, 5 mM Ba(NO₃)₂, 2 mM Ni(NO₃)₂, 0.01 mM Gd(NO₃)₃, 0.3 mM niflumic acid and 10 mM Tris HEPES, pH=7.6</td>
</tr>
<tr>
<td>25Na 0K</td>
<td>25 mM</td>
<td>0.01 mM Gd(NO₃)₃, 0.3 mM niflumic acid and 10 mM Tris HEPES, pH=7.6</td>
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</tbody>
</table>

### Internal solutions

<table>
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<th>NMDG glutamate</th>
<th>ADP</th>
<th>others</th>
</tr>
</thead>
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<td>30 mM</td>
<td>0</td>
</tr>
<tr>
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<td>50 mM</td>
<td>30 mM</td>
<td>5 mM</td>
</tr>
<tr>
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<td>10 mM</td>
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<tr>
<td>1Na 5ADP</td>
<td>1 mM</td>
<td>79 mM</td>
<td>5 mM</td>
</tr>
</tbody>
</table>
Transient current analysis

Transient Na⁺/Na⁺ exchange current was calculated as shown in Part I (Figure 3). The slow component of the transient current was measured using the CLAMPFIT software in PCLAMP 9.0. Previous studies have demonstrated that the slow ouabain sensitive transient current is preceded by at least one fast component with a time course close to that of the voltage step (relaxation rate > 2000 s⁻¹) (Hilgemann 1994; Holmgren, Wagg et al. 2000; Holmgren and Rakowski 2006). Therefore, to obtain the relaxation rate of slow components, transient currents were fitted with a single exponential function starting 2-3 ms after the initiation of voltage steps over a 30 ms period and the fit was extended to about 0.5 ms after the start of voltage steps to include the complete slow component without contamination by faster components. Qslow was then determined by multiplying the two parameters, A (the fitted initial value of transient currents) and tau (time constant of exponential decay of transient currents) obtained by the single exponential fit. Further analysis, least-squares curve fitting, and preparation of figures were done with SIGMAPLOT 8.0. Curve fit parameters were obtained from the least-squares fitting procedure. Experiments were performed at room temperature (~22 ºC).
Part II. III. Results

As in Part I, equation 1 was used to fit QV curves obtained under different conditions. The value of $V_{\text{mid}}$ depends on the ratio between the occupancies of E1P(Na3) and E2P states (Fig 5; part I). The $z$ value is the apparent valence of the charge moved between those states, which based on our data using Bufo marinus NKA $\alpha_1 \beta_1$ has been determined to be 0.65.

The effect of $V_h$ on the magnitude of $Q_{\text{slow}}$ in 100mM Na$^+$ o and 50mM Na$^+$ i with or without 5 mM ADP

In the absence of ADP, scheme 2 is reduced to:

\[
\text{Reaction scheme 4}
\]

\[
\text{E1P(Na3) } \leftrightarrow \text{ E2P+3Na}^+
\]

which includes the transitions eliciting the charge translocation that we measure. Therefore, we might expect that adding intracellular ADP should result in a reduction of the amount of charge.

Figure 15 shows how charge distributes (QV curve) at three different values of $V_h$, -20 (black), -60 (red) and -100 mV (green), and in the absence of ADP. All data was normalized to the $Q_{\text{tot}}$ obtained at $V_h$ of -20 mV. As expected, $Q_{\text{tot}}$ did not show significant dependence on $V_h$. $Q_{\text{tot}}$ was $0.88\pm0.02$ at $V_h$ of -60 mV and $0.82\pm0.03$ at $V_h$ of -100 mV.
Figure 15. The $V_h$ dependence of QV curve of ouabain sensitive transient currents in oocytes in internal solution 50mM Na$^+$, 5mM ATP, 0 ADP and 0 K$^+$ and externally perfused with 100mM Na$^+$ 0K$^+$. Dots are data points (Mean± SE) at $V_h$ of -20mV (black dots), -60mV (red dots), and -100mV (green dots). Solid lines represent best fit to equation 1 with z constrained to 0.65. The best fit parameter values are $Q_{tot}=1.00\pm 0.03$ $V_{mid}=-59.1\pm 5.9$ mV for $V_h=-20$mV; $Q_{tot}=0.89\pm 0.02$ $V_{mid}=-64.1\pm 5.3$ mV for $V_h=-60$mV; $Q_{tot}=0.82\pm 0.03$ $V_{mid}=-63.6\pm 7.1$ mV for $V_h=-100$mV. Data were from 5 oocytes of at least two different frogs.

Figure 16 shows the charge distribution from a similar set of experiments, but in the presence of 5 mM intracellular ADP. Clearly, under the present experimental conditions, ADP has no effect on the amount of charge moved at different $V_h$, suggesting that ADP could not displace pumps towards the intracellular Na$^+$ binding/release transitions. This failure could be because there was not enough difference between intracellular and extracellular [Na$^+$] to drive the pumps backwards.
Figure 16. The $V_h$ dependence of $QV$ curve of ouabain sensitive transient currents in oocytes in internal solution 50mM Na$^+$, 5mM ATP, 5mM ADP and 0 K$^+$ and externally perfused with 100mM Na$^+$ 0K$^+$. Dots are data points (Mean$\pm$ SE) at $V_h$ of -20mV (black dots), -60mV (red dots), and -100mV (green dots). Solid lines represent best fit to equation1 with $z$ constrained to 0.65. The best fit parameter values are $Q_{tot}=1.00\pm0.03$ $V_{mid}=-62.2\pm5.0$ mV for $V_h=-20mV$; $Q_{tot}=0.93\pm0.03$ $V_{mid}=-59.7\pm5.6$ mV for $V_h=-60mV$; $Q_{tot}=0.88\pm0.03$ $V_{mid}=-61.5\pm7.0$ mV for $V_h=-100mV$. Data were from 5 oocytes from at least two different frogs.

The effect of $V_h$ on the magnitude of $Q_{slow}$ in 100mM Na$^+$o and 10mM Na$^+$i or 1mM Na$^+$i

In an attempt to increase the transmembrane difference in [Na$^+$], we performed similar experiments in the presence of 5 mM ADP and 100 mM [Na$^+$o], but decreased the intracellular [Na$^+$] from 50 to 10 or 1 mM. Previously, it has been shown that $K_m(0)$ for intracellular Na$^+$ binding is 3.2 mM for endogenous *Xenopus* NKA (Holmgren and
Rakowski 2006). Assuming that *Bufo* NKA α1β1 has a similar apparent affinity, then the changes of $[\text{Na}^+]$ in our study would cover a large range of the dose response curve.

**Figure 17** shows that in the presence of 10 mM (A) or 1 mM $\text{Na}^+$ (B), $Q_{\text{tot}}$ does not change with different holding potentials, suggesting that not even a 100 fold difference between intracellular and extracellular $[\text{Na}^+]$ was enough to drive the pumps toward the inward-facing $\text{Na}^+$ binding sites.
Figure 17. The Vh dependence of QV curve of ouabain sensitive transient currents in 10 and 1 mM Na+ i. Oocytes were incubated in internal solution (in mM) 5 ADP, 5 ATP, and 0 K+ and externally perfused with 100Na+ 0K+ . (A) The voltage dependence of normalized charge (Qslow) in 10 mM at Vh of -20mV (black dots), -60mV (red dots), and -100mV (green dots). Dots are data points (Mean± SE). Solid lines represent best fit to equation1 with z constrained to 0.65. The best fit parameters are $Q_{tot}=1.00\pm 0.02$ and $V_{mid}=67.6\pm 3.5$ for Vh=-20mV; $Q_{tot}=0.93\pm 0.01$ and $V_{mid}=62.1\pm 2.8$ for Vh=-60mV; $Q_{tot}=0.92\pm 0.02$ and $V_{mid}=64.4\pm 3.6$ for Vh=-100mV. (B) The voltage dependence Qslow in 1 mM. Symbols are the same as in (A). The best fit parameter values are $Q_{tot}=1.00\pm 0.02$ and $V_{mid}=59.3\pm 3.7$ for Vh=-20mV; $Q_{tot}=0.99\pm 0.02$ and $V_{mid}=55.2\pm 3.9$ for Vh=-60mV; $Q_{tot}=0.97\pm 0.02$ and $V_{mid}=60.5\pm 4.1$ for Vh=-100mV. Data were from 5 or 6 oocytes of at least 2 frogs.
The effect of $V_h$ on the magnitude of $Q_{slow}$ at low $[\text{Na}^+_{o}]$

Figure 18 A shows QV curves in the presence of 25 mM Na$^+_{o}$ obtained at different $V_h$. Interestingly, the more negative $V_h$ was, the less $Q_{tot}$ we could estimate, as if holding at negative potential effectively removed pumps from the states shown in scheme 3. In fact, $Q_{tot}$ appeared to follow a sigmoid distribution with $V_h$ (Fig 18 B). If we assume that our system is losing pumps through a single voltage dependent transition, then it would be rather strong voltage dependence with an apparent valence of $\sim$4.
Figure 18. The Effect of holding potential on ouabain sensitive transient current in oocytes in internal solution 10mM Na⁺, 5mM ADP, 5mM ATP, and 0 K⁺ and externally perfused with 25mM Na⁺. (A) The voltage dependence of Qslow at Vh of -20mV (black dots), -60mV (red dots), and -100mV (green dots). Dots are data points (Mean± SE). Solid lines represent best fit to equation1 with z=0.65. The best fit parameter values are Qtot=1.00±0.03 Vmid=-105.3±3.8 for Vh=-20mV; Qtot=0.79±0.04 Vmid=-98.9±6.0 for Vh=-60mV; Qtot=0.63±0.02 Vmid=-76.8±4.8 for Vh=-100mV. (B) The Vh dependence of Qtot. Data were from 5 oocytes of at least two frogs.
Part II. IV. Discussion

_E1P(Na3)/E2P occupancy under different conditions_

To interpret the results, we have to once again review the rationale behind transient currents. There exists a dynamic equilibrium between the intermediate states in electroneutral Na⁺/Na⁺ exchange mode, which can be expressed in a more detailed form of scheme 3 (Wuddel and Apell 1995).

**Reaction scheme 5**

\[
E1\cdot\text{ATP}+3\text{Na} \leftrightarrow E1\cdot\text{ATP} \cdot 3\text{Na} \leftrightarrow E1P(\text{Na3}) \leftrightarrow E1P(\text{Na3}) \cdot \text{ADP} \leftrightarrow E2P \cdot 3\text{Na} \leftrightarrow E2P \cdot 2\text{Na} \leftrightarrow E2P \cdot \text{Na} \leftrightarrow E2P
\]

The distribution of pumps between these states depends on the concentration of substrates and products, and on the membrane potential. In this study, we varied the concentrations of [ADP], [Na⁺]ᵢ, and [Na⁺]ₒ, and the holding potential, Vₜ.

The charge translocation that we measure derives from a subset of transitions of scheme 4, which relate to extracellular Na⁺ binding/unbinding and occlusion/de-occlusion \((E1P(\text{Na3}) \leftrightarrow E2P \cdot 3\text{Na} \leftrightarrow E2P \cdot 2\text{Na} \leftrightarrow E2P \cdot \text{Na} \leftrightarrow E2P)\). Within these transitions, negative potentials will displace the system towards the E1P(3Na), whereas positive potentials will shift the pumps toward the E2P state. If any experimental condition allows pumps to exit from this subset of charge translocation transitions, then we would expect a change in Qₜₜ. If the escape route is a voltage dependent process, then the changes in Qₜₜ will be different depending on Vₜ, and these differences will provide information on the voltage dependent steps that do not relate to the extracellular Na⁺ binding/release transitions.
The effect of \( V_h \) on the magnitude of \( Q_{\text{slow}} \) with 100mM \( \text{Na}^+ \) in the presence and absence of ADP

In the presence of ADP, it is expected that pumps in the E1P(Na3) could, in principle, transit to E1P(Na3):ADP, and then de-occlude and release three \( \text{Na}^+ \) to the intracellular medium with the production of ATP. However, our results in Fig 15 show that the presence of 5 mM ADP in the intracellular solution did not elicit significant change of \( Q_{\text{tot}} \) as \( V_h \) became more negative, similarly to the results obtained in the absence of ADP (Fig 15). Therefore, under both conditions, 50 mM \( \text{Na}^+ \) and/or 5 mM ATP were able to keep pumps from exiting the subset of charge translocation transitions.

\[
\text{The effect of } V_h \text{ on the magnitude of } Q_{\text{slow}} \text{ with 5mM ADP and varying } \text{Na}^+ \text{ present intracellularly}
\]

According to equation 2, we were expecting that changing \( V_h \) would affect the \( Q(V) \) by a factor of \([\text{Na}]/(\text{Na}^+ + K_m(V_h))\), where \( K_m \) and \( V_h \) are related by:

\[
K_m(V_h) = K_m(0)\exp\left(-\lambda_i F V_h / RT\right)
\]

Equation 5

Where \( K_m(0) \) is the apparent affinity for intracellular \( \text{Na}^+ \), \( \lambda_i \) is the dielectric coefficient, and \( F, R \) and \( T \) have their usual meaning and values. We studied the effect of \( V_h \) on the pump-mediated charge translocation at three different \([\text{Na}^+]) (1, 10 \text{ and } 50 \text{ mM}). In each \([\text{Na}^+]), V_h \) has only minor changes in \( Q_{\text{tot}} \) (Fig 16, and 17), indicating that we were not able to displace pumps towards the intracellular \( \text{Na}^+ \) binding steps. The most feasible explanation is that the magnitude of the forward rate is difficult to overcome by changes in occupancy of the E1(3Na)-P state with voltage, once the intracellular ATP is 5
mM. This idea is supported by our results using a mutant pump, *Bufo* α1:G813A, in which the QV distribution is substantially biased towards the E1P(3Na), and still we did not observe a change in $Q_{tot}$ with $V_h$ (Fig. 19).

**Figure 19.** The $V_h$ dependence of QV curve of ouabain sensitive transient current in oocytes expressing mutant *Bufo* NKA: G813A. The oocytes were externally perfused with 100Na$^+$ 0K$^+$ and incubated in internal solution 50Na$^+$, 5 ADP, 5 ATP, and 0 K$^+$. Dots are data points (Mean±SE) at three different $V_h$, -20mV (black dots), -60mV (red dots), and -100mV (green dots). Solid lines represent best fit to equation1 with $z=0.65$. The best fit parameter values are $Q_{tot}=1.00\pm0.01$ and $V_{mid}=11.9\pm2.0$ for $V_h=-20$ mV; $Q_{tot}=1.02\pm0.02$ and $V_{mid}=9.8\pm2.3$ for $V_h=-60$ mV; $Q_{tot}=1.12\pm0.02$ and $V_{mid}=10.7\pm2.9$ for $V_h=-100$ mV. Data are from 6 oocytes of at least two frogs.

*The effect of $V_h$ on the magnitude of $Q_{slow}$ in 25 mM Na$^+$*

By reducing extracellular Nao to 25 mM, we observed an unexpected result. At low Nao, $Q_{tot}$ follows a sigmoidal shape with $V_h$, suggesting that negative $V_h$ somehow
remove pumps from the subset of charge translocation transitions. Furthermore, the route by which these pumps are escaping is a strong voltage dependent transition.

What route is being followed by the pumps? In the absence of K⁺, it has been reported since the 1960s (Garrahan and Glynn 1967) that pumps could operate in several modes. The most favorable is the traditional Na⁺/Na⁺ electroneutral exchange, but under certain conditions the pump could either transport 3Na⁺/2Na⁺, sometime called Na⁺ ATPase activity (Fig 20 A), or it could transport 3Na⁺ outwardly by an uncoupled ATPase activity (Fig 20 B) (Glynn and Karlish 1976; Heyse, Wuddel et al. 1994; Wuddel and Apell 1995; Peluffo, Arguello et al. 2000).
Figure 20. Two possible operating modes of the NKA other than electroneutral Na⁺/Na⁺ exchange in the absence of K⁺. A) the 3Na⁺/2Na⁺ transport mode; B) uncoupled ATPase activity with 3Na⁺ efflux and no cation influx. The numbers besides lines indicate the dielectric coefficients that the particular partial reactions go through. Question mark indicates that the value was calculated and has not been verified by experiments.
Both transports are expected to be very slow processes. Therefore, experimental estimations of their reaction rate have been difficult. However, they have been estimated by numerical simulation utilizing other available values of the reaction cycle and they are in $10^{-1} \text{s}^{-1}$ range. (Heyse, Wuddel et al 1994). In principle, both of these non canonical transport processes could provide escaping routes for E2P. However, there are two reasons that suggest that uncoupled ATPase activity is the main source for escape. First, it has been previously documented that uncoupled ATPase activity is favored in low $[\text{Na}^+]_o$, contrary to the $3\text{Na}^+//2\text{Na}^+$ ATPase activity which is enhanced by high $[\text{Na}^+]_o$ (Glynn and Karlish 1976; Heyse, Wuddel et al. 1994). And second, our observation that the escaping route is strongly voltage dependent is consistent with previous theoretical estimations (Heyse, Wuddel et al 1994).
SUMMARY

To elucidate whether there exists more than one ion pathway, we studied the effects of oligomycin on *Bufo* Na\(^+\)/K\(^+\) ATPase expressed in *Xenopus* oocytes in Na\(^+\)/K\(^+\) and Na\(^+\)/Na\(^+\) exchange mode. The apparent affinity of the oligomycin mixture is 12.3 μg/ml in the presence of K\(^+\) and 24.8 μg/ml in the absence of K\(^+\). The binding of oligomycin was shown to be voltage independent.

Oligomycin could only inhibit about 70% charge movement mediated by the NKA in its Na\(^+\)/Na\(^+\) exchange mode. Preliminary data showed that oligomycin-sensitive currents occurred about 100 μs earlier and relaxed to steady state faster than oligomycin-insensitive currents. Further studies with other two protocols were done to investigate the effect of oligomycin on three components of transient currents in Na\(^+\)/Na\(^+\) exchange mode. First, to avoid contamination of pump-mediated transient current by time-dependent linear capacitance, a P/4 protocol was utilized with a mutated *Bufo* NKA: G813A, whose rightward-shifted QV curve allows maximum preservation of pump-related nonlinear capacitance. Results from this set of studies showed that the oligomycin-sensitive transient current had characteristics, including a steep QV relationship and a time constant of around 0.3 ms, similar to the medium component. The oligomycin-insensitive transient current had characteristics, including a steep QV relationship and a time constant of around 1 ms, that are similar to the slow component. Secondly, a short protocol was applied to introduce the minimal linear capacitance to the subtracted currents. Oligomycin B was utilized in this set of studies to reduce the amount of lipophilic substance added and to keep the inhibitor as a pure chemical. Oligomycin B
at unsaturating concentrations only inhibited part of transient currents with a time constant (about 0.05 ms) similar to the fast component. When the concentration was increased to a saturating concentration, most of the fast component was inhibited. So the release of the second and the third Na\(^+\) (forming the medium and the fast components in transient currents) was blocked by oligomycin, which suggests that the release of these two Na\(^+\) might share the same pathway.

In the second part of my study, attempted to elucidate the nature of the intracellular Na\(^+\) binding/release step, the holding potential dependence of slow charge movement in Na\(^+\)/Na\(^+\) exchange mode was studied in different [ADP\(_i\)], [Na\(^+\),\(_i\)] or [Na\(^+\),\(_o\)]. However, the manipulation of [ADP\(_i\)] and [Na\(^+\),\(_i\)] did not change the magnitude of slow charge movement, which indicates that pumps were not displaced from extracellular Na\(^+\) release subset. Lowering [Na\(^+\),\(_o\)] brought out a big change in the magnitude of slow charge movement. At negative holding potentials there was less charge movement and the magnitude of Q\(_\text{slow}\) was strongly dependent on V\(_h\) with a z value close to 4. In low [Na\(^+\),\(_o\)], the reaction is favored to the forward direction instead of displacing pumps to intracellular Na\(^+\) binding steps, so a voltage dependent step other than the intracellular Na\(^+\) binding step was influencing the availability of extracellular Na\(^+\) release. It is possible that pumps were escaping though the uncoupled ATPase activity, which is favored in low [Na\(^+\),\(_o\)] and causes 3 net Na\(^+\) to be released to extracellular solution.
REFERENCES


Glynn, I. M. and S. J. D. Karlish (1976). "ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: evidence for allosteric effects of


APPENDIX A: ABBREVIATIONS AND SYMBOLS

- **Vm**: Membrane potential
- **Vh**: Holding potential
- **Vmid**: Midpoint voltage
- **Q**: Charge
- **Qtot**: Magnitude of charge movement
- **Qslow**: Charge carried by slow charge movement
- **Qwhole**: Charge carried by the whole "on" trace
- **Qoligo**: Oligomycin sensitive charge
- **Qouab**: Ouabain sensitive charge
- **I**: Current
- **Ioligo**: Oligomycin sensitive current
- **Iouab**: Ouabain sensitive current
- **K**: Apparent rate coefficient
- **F**: Faraday’s constant
- **R**: Gas constant
- **T**: Temperature
- **λ**: Dielectric coefficient
- **λi**: Dielectric coefficient for intracellular Na binding
- **z**: Apparent valence
- **Na⁺**: Extracellular Na⁺
- **Na⁺**: Intracellular Na⁺
\[ \text{[Na}^+\text{o]} \quad \text{Concentration of extracellular Na}^+ \]

\[ \text{[Na}^+\text{i}] \quad \text{Concentration of intracellular Na}^+ \]

\[ \text{[Oligo]} \quad \text{Concentration of oligomycin} \]

Km  \quad \text{Apparent affinity}

Ki  \quad \text{Apparent affinity of inhibitor}

IV  \quad \text{Current-Voltage relationship}

QV  \quad \text{Charge-Voltage relationship}
APPENDIX B: EQUATIONS AND REACTION SCHEMES

Equation 1: \[
\frac{Q(V) - Q_{\text{min}}}{Q_{\text{tot}}} = \frac{1}{1 + \exp\left(\frac{zF(V_{\text{mid}} - V)}{RT}\right)}
\]

Equation 2: \[
\text{I}_{\text{oligo}}/\text{I}_{\text{ouab}} = a \cdot \text{[Oligo]}^n / (K_i^n + [\text{Oligo}]^n)
\]

Equation 3: \[
\text{Q}_{\text{oligo}}/\text{Q}_{\text{ouab}} = b \cdot \text{[oligo]} / (K_i + \text{[oligo]})
\]

Equation 4: \[
Q(V) = \frac{Q_{\text{tot}}}{1 + \exp\left(\frac{zF(V_{\text{mid}} - V)}{RT}\right)} + Q_{\text{min}} \left[\frac{[\text{Na}]_i}{[\text{Na}]_i + K_m(V_h)}\right]
\]

Equation 5: \[
K_m(V_h) = K_m(0) \exp(-\lambda_i F V_h / RT)
\]

Reaction scheme 1:

\[
E1 + 3Na_i \xrightarrow{K_1} E1(3Na) \xrightarrow{K_2} E2P + 3Na_o
\]

Reaction scheme 2:

\[
E1 + 3Na_i \leftrightarrow E1P(\text{Na}_3) \leftrightarrow E2P \cdot 3Na \leftrightarrow E2P \cdot 2Na + Na_o
\]

Reaction scheme 3:

\[
3Na_i + E_1ATP \xleftarrow{K_{n(V_h)}} (\text{Na}_3)E_1P \cdot ADP \xrightarrow{k_1} (\text{Na}_3)E_1P \xrightarrow{k_2} E_2P + 3Na_o
\]

Reaction scheme 4: \[
E1P(\text{Na}_3) \leftrightarrow E2P + 3Na^+
\]

Reaction scheme 5:

\[
E1 \cdot \text{ATP} + 3Na \leftrightarrow E1 \cdot \text{ATP} \cdot 3Na \leftrightarrow E1P(\text{Na}_3) \cdot \text{ADP} \leftrightarrow E1P(\text{Na}_3) \leftrightarrow E2P \cdot 3Na \leftrightarrow E2P \cdot 2Na
\]

\[
\leftrightarrow E2P \cdot Na \leftrightarrow E2P
\]