Beneficent and Maleficent Effects of Cations on Bufadienolide Binding to Na⁺,K⁺-ATPase

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ABSTRACT: Kinetic properties and crystal structures of the Na⁺,K⁺-ATPase in complex with cardiotonic steroids (CTS) revealed significant differences between CTS subfamilies (Laursen et al.). Thus, we found beneficial effects of K⁺ on bufadienolide binding, which strongly contrasted with the well-known antagonism between K⁺ and cardenolides. In order to understand this peculiarity of bufalin interactions, we used docking and molecular dynamics simulations of the complexes involving Na⁺,K⁺-ATPase, bufadienolides (bufalin, cinobufagin), and ions (K⁺, Na⁺, Mg²⁺). The results revealed that bufadienolide binding is affected by (i) electrostatic attraction of the lactone ring by a cation and (ii) the ability of a cation to stabilize and “shape” the site constituted by transmembrane helices of the α-subunit (αM1−6). The latter effect was due to varying coordination patterns involving amino acid residues from helix bundles αM1−4 and αM5−10. Substituents on the steroid core of a bufadienolide add to and modify the cation effects. The above rationale is fully consistent with the ion effects on the kinetics of Na⁺,K⁺-ATPase/bufadienolide interactions.

INTRODUCTION

The Na⁺,K⁺-ATPase, or the Na⁺ pump, is crucial for cell homeostasis and therefore represents an obvious pharmacological target. The enzyme’s abundance, however, imposes limitations on the use of inhibitors and modulators of its activity, since they inevitably cause a number of dose-dependent adverse effects and are fated to have a narrow therapeutic window. That is certainly true for cardenolides and bufadienolides were even more pronounced. The same authors also show that in the case of a K⁺-dependent adverse effects of an active compound may be diminished by inferring selectivity toward particular isoforms of a target protein. Thus, individual Na⁺,K⁺-ATPase isoforms revealed preferences to certain CTSs, and these tendencies might be amplified by derivatization of the already known compounds. Another approach would be to design a new synthetic compound complementary to a site on the target protein. In this case, the CTS binding site is well suited as a potential point of interaction for the future drug. It has two advantages: (i) it is accessible from the extracellular side, whereby negating membrane permeability as a requirement for a potential bioactive compound; (ii) the site has certain plasticity since it accommodates CTSs of highly variable structures. The implementation of either strategy would gain from the availability of detailed information about the spatial organization of the binding site and modes of ligand binding, as well as ways of interfering with their interactions.

Available crystallographic data describe high affinity complexes of the Na⁺,K⁺-ATPase with CTSs varying in the structure of the steroid core (cardenolides vs bufadienolides) as well as in the degree of glycosylation (aglycones vs mono/triglycosylated). These complexes revealed that CTSs bind to the α-subunit at the entrance to the extracellular cation transport sites. The nature of the ion bound within the site had a tremendous effect on CTS binding. Complexes with cardenolides (e.g., ouabain, digoxin) contained Mg²⁺, and substitution of Mg²⁺ with K⁺ or its congener Rb⁺ induced a rearrangement of transmembrane helix 4 (αM4) whereby changing the position of the cardenolide in the site. Functionally, it is manifested as a K⁺-induced decrease in affinity in vitro and described as an increased toxicity of digitals under hypokalemic conditions in vivo.

Bufadienolide binding is less affected by K⁺ as shown by relatively small variations in the IC₅₀ values for the Na⁺,K⁺-ATPase reaction at suboptimal and optimal K⁺ concentrations. The same authors also show that in the case of a K⁺-pNPase reaction, the differences in inhibiting potencies of cardenolides and bufadienolides were even more pronounced. Analysis of the crystal structure of the bufalin–enzyme complex suggests that bufalin binding, in contrast to ouabain
and digoxin, is facilitated by K⁺ in the binding sites. The six-membered unsaturated lactone directly coordinated K⁺ bound in cation transport site II. Electrostatic pull from K⁺ and a lack of hydroxyl substituents on the steroid core allowed bufalin to slide ~1.5 Å deeper into the site. Kinetic analysis revealed that, in the presence of K⁺, bufalin had higher affinity, and its complex with Na⁺,K⁺-ATPase composed a homogeneous pool which allowed for crystallization. Na⁺,K⁺-ATPase/bufalin complexes formed in the absence of K⁺ had different stabilities and were clearly divided into fast- and slow-dissociating pools.

Thus, the above data put forward two questions: (i) is K⁺-potentiation characteristic for all bufadienolides? And, (ii) what is the structural basis for the observed heterogeneity of Na⁺,K⁺-ATPase/bufalin complexes and how does K⁺ affect it? To find an answer, we applied an in silico approach (docking and molecular dynamics (MD) simulations) in conjunction with biochemical experiments. The results characterize the CTS binding cavity and ligand binding process. This information in the future will allow synthesis of compounds with predetermined properties, e.g., with improved isoform selectivity.

## RESULTS AND DISCUSSION

The crystallized high affinity CTS complexes of the Na⁺,K⁺-ATPase are based on the E2Pᵢ conformation of the enzyme. Therefore, all experiments in the present report describe bufadienolide’s interactions with that particular enzyme conformation exclusively, and reported values characterize equilibrium binding of CTS to the Na⁺,K⁺-ATPase in the presence of inorganic phosphate, 3 mM MgCl₂ and cations of choice.

We set out by comparing the effect of K⁺ on the kinetics of Na⁺,K⁺-ATPase interactions with cinobufagin and bufalin. Cinobufagin is a bufadienolide with polar substituents on ring D of the steroid core, which might interfere with the electrostatic interactions between the lactone ring and K⁺ in the cation binding site described for bufalin.

![Figure 1](https://dx.doi.org/10.1021/acs.jcim.0c01396)

Figure 1 compares the residual activity of Na⁺,K⁺-ATPase after enzyme preincubation with varying concentrations of bufalin and cinobufagin in the presence or absence of K⁺. The degree of inhibition corresponds to an amount of the enzyme-inhibitor complex, and kinetic analysis of these curves allows for the extraction of apparent affinity values for each inhibitor. Both bufadienolides exhibit high affinities toward Na⁺,K⁺-ATPase in the absence of any other ions but Mg²⁺. As shown previously, bufalin affinity was high (Kᵦ = 0.01 μM), yet incomplete inactivation revealed formation of both fast- and slow-dissociating complexes. Presence of K⁺ converted all fast-dissociating complexes into the tight one, increasing the percent of inhibition from 83.6% ± 0.36% in the absence of K⁺ (weighted mean, five curves) to 100% ± 0.44% with 10–200 mM K⁺ (three curves). The statistical significance of such a difference is equal to p = 10⁻¹⁰. In addition, K⁺ seemingly improved bufalin affinity for the enzyme (Figures 1 and S1, Table S1). Monitoring of bufalin substitution with anthroylouabain estimates the dissociation rate constant in the absence of K⁺ as 5.7 × 10⁻⁴ s⁻¹. It becomes 300 times slower in the presence of K⁺ (2.2 × 10⁻⁶ s⁻¹, Figure 1B). Note that the kₒ from the fast-dissociating complex could not be determined in our experimental setup. This clear K⁺-stabilizing effect on the Na⁺,K⁺-ATPase/bufalin complex was only partially observed for cinobufagin. Cinobufagin binding in the absence of K⁺ revealed the same pattern as that of bufalin: high affinity (Kᵦ = 0.05 μM) but incomplete inhibition (Table S1). The proportion between fast- and slow-dissociating complexes was essentially identical to that of bufalin. Addition of K⁺ promoted inhibition, i.e., converted the fast-dissociating complex into a tight one (p = 1 × 10⁻⁴), but the affinity for cinobufagin decreased 2-fold (p = 0.03; Figure 1A, Table S1). The latter resembles the K⁺ effect on cardenolide binding to Na⁺,K⁺-ATPase. Thus, the consequences of K⁺’s presence for bufalin and cinobufagin binding are not alike. While K⁺ ensures homogeneity of both bufadienolide/enzyme complexes, it has opposite effects on their apparent affinities.
In order to understand the mechanisms underlying the multiple and diverse K+ effects on bufadienolide binding to Na+,K+-ATPase, we turned to computational experiments. Note, that it is not feasible to obtain crystallographic evidence of the complexes of interest due to their transient nature. First, we performed docking calculations to probe the possible binding modes of each bufadienolide in the CTS binding site. The docking calculations consider both ligand and protein flexibility, which allows for more variation in the binding modes. They were performed for bufalin and cinobufagin in Na+,K+-ATPase with and without two K+ ions bound to ion sites I and II. The resulting poses of each bufadienolide were clustered according to their conformational and positional similarity, and the output of the calculations is summarized in Table S2.

Bufalin always bound with the lactone ring facing down; an upside down orientation of that compound (as previously suggested for an ouabain derivative\(^1\)) was never observed. Its docking into Na\(^+\),K\(^-\)-ATPase with and without two K+ ions bound to ion sites I and II. The resulting poses of each bufadienolide were clustered according to their conformational and positional similarity, and the output of the calculations is summarized in Table S2.

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therefore set out to test the effect of Na\(^+\) and Mg\(^{2+}\) on bufalin binding. Figure S1 and Table S1 summarize the effects of Mg\(^{2+}\), K\(^+\), and Na\(^+\) in various concentrations on the apparent affinities of bufalin. It is clear that only K\(^+\) prevents formation of the fast-dissociating complexes and improves affinity. The effect is effectuated at very low K\(^+\) concentrations as expected, as the affinity of the extracellular binding sites for K\(^+\) is high (Figure S1A). An increase in Mg\(^{2+}\) concentration (above 3 mM Mg\(^{2+}\), i.e., standard condition) has no further effect on bufalin binding. The sites are therefore either saturated with Mg\(^{2+}\) under standard conditions or Mg\(^{2+}\) does not affect bufalin binding (Figure S1B). Na\(^+\) affinity for the extracellular sites, on the other hand, is very low since the change in bufalin binding kinetics demands a high Na\(^+\) concentration (Figure S1C). Importantly, the effects of Na\(^+\) and K\(^+\) are ion-specific since 200 mM NMG\(^+\) (N-methyl-D-glucamine), used as a control for the effect of ionic strength alone, had no influence on bufalin binding (Figure S1B).

Thus, the effects of K\(^+\) on bufadienolides’ interactions with the Na\(^+\),K\(^+\)-ATPase are distinct from that of other ions bound to the extracellular sites. This finding triggered MD simulations of the Na\(^+\),K\(^+\)-ATPase/bufadienolide complexes in the presence of different ions. Analyses of the dynamical aspects of bufadienolide binding aim to reveal the structural consequences of the ions present in the binding site.

MD simulations were performed with the crystallized Na\(^+\),K\(^+\)-ATPase/bufalin complex\(^4\) as a starting structure in the presence of the ions tested above as well as an apo conformation. Simulations with K\(^+\) considered full occupation of the sites (i.e., two K\(^+\) ions). Since Mg\(^{2+}\) is mandatory for phosphorylation and always present in the biochemical experiments, the series included Mg\(^{2+}\)-bound enzymes. The Na\(^+\)-bound system was simulated with both one and two Na\(^+\) ions since affinity for Na\(^+\) was low. Singular ions were always located in site II. The Na\(^+\),K\(^+\)-ATPase/cinobufagin complex obtained from the docking calculation (C\(_{3K}\)) was simulated with either two K\(^+\) ions or one Mg\(^{2+}\) ion bound. Each system was simulated for 500 ns in three repeats, except for the

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**Figure 3.** Bufalin and cinobufagin stability in the binding site in the presence of Na\(^+\), K\(^+\), and Mg\(^{2+}\). The stability is evaluated from the rotational state of the bufadienolide (BM\(_0\)−3), and for each rotational state from the fluctuations up and down the binding site reflected in the z coordinate of the bufadienolide center relative to the starting structure. The rotational state was obtained from the conformational clustering of the data. All trajectories were aligned prior to analysis using nMDS−10 of the enzyme. Simulation repeats are named MD1−5 and colored separately. The combined distributions are shown as histograms (gray) on the right-hand side of each plot. The resulting probability density function (PDF, black dashed lines) was obtained by fitting a Gaussian mixture model. The number of Gaussian components was determined by the Bayesian information criterion.
binding modes (BM0) state of the bufadienolide. Across all systems, four major interactions, and the primary di- 

Figure 4. Direct coordination of the ion in cation site II by bufalin and cinobufagin. The distance between the ion in site II and carbonyl oxygen in the bufadienolide lactone ring as a function of time is reported for each molecular system. The running average is shown in opaque hues, while the raw data are shown in transparent hues based on the color legend below the plot. The combined distance distribution is shown as a histogram (gray) on the right-hand side of each plot. The resulting probability density function (PDF, black dashed lines) was obtained by fitting a Gaussian mixture model. The number of Gaussian components was determined by the Bayesian information criterion.

Na⁺,K⁺-ATPase/bufalin/2×K⁺ system, which was simulated in five repeats (MD1–5). Each molecular system was evaluated for (i) ligand stability within the binding site, (ii) ligand/ion coordination (site II), (iii) ion/protein coordination (site II), and (iv) protein/ligand interaction strength. Combined, these four metrics characterize both the direct ion effect through coordination of the ligand and the indirect ion effect due to stabilizing the binding site around the bufadienolide.

A caveat of this study is the physical description of ions by the force field. In the force field applied in this work, as well as in the majority of biologically relevant force fields, each ion is represented by a point charge and two parameters describing the van der Waal forces. By construction, the description is symmetric, and the ions are distinguishable by the coordination distance alone and not by the coordination geometry. As details are lost, the ions, especially the divalent ions, behave too similarly in MD simulations. Nevertheless, focusing on the reproducible aspects, such as the coordination distance, we obtain reliable data.

A clustering calculation provided an initial overview of the behavior of the bufadienolides within the binding site. In all cases, the steroid core was mainly stabilized by hydrophobic interactions, and the primary difference was in the rotational state of the bufadienolide. Across all systems, four major binding modes (BM0–3) were determined (Figure 2). Thus, in BM0 the β-surface of the bufadienolide points toward αM2 in agreement with the crystal structure of the Na⁺,K⁺-ATPase/bufalin complex. In BM1, BM2, and BM3, the β-surface points toward αM1, αM4, and αM6, respectively. The core hydroxyl group (C14β) of bufalin forms a hydrogen bond with Thr797 only in BM0 and BM1. An equivalent hydrogen bond was observed in crystal structures for all CTS-enzyme complexes. The equivalent epoxy group on cinobufagin is not able to form a hydrogen bond to Thr797 due to the altered local conformation near the oxygen atom leading to a suboptimal hydrogen bonding angle. In addition to the rotational state, the depth of binding, i.e., the z coordinate, showed some degree of variation. Thus, in order to assess the stability of the bufadienolides within the site, it is necessary to track their rotational state as well as the depth of their location. Figure 3 presents an overview of ligand stability for all systems. Bufalin bound to Na⁺,K⁺-ATPase in the presence of K⁺ is stable and in BM1 (β-surface facing αM1) despite being initiated from BM0. Bufalin changes from BM0 to BM1 within the first nanosecond. Minor fluctuations in binding depth were detected in all repeats except MD2 and the last 10 ns of MD4 where it moves even further down the binding site. In the presence of one or two Na⁺ ions, the behavior of bufalin is highly similar. In simulations with Mg²⁺, bufalin is less stable and retains BM1 only in one repeat simulation. Instead, bufalin binds in all four major binding modes and in additional minor binding modes in varying depths. The movements up and down the site have larger amplitudes than otherwise observed except for the apo conformation, where bufalin retains BM1 exclusively, but moves up and down in the binding site even more. In fact, in two simulations (MD1 and MD2), bufalin almost exits to the extracellular milieu (Figure S4). Intriguingly, the tilted binding mode observed in the docking calculations reappeared in MD1 as a semistable binding mode during bufalin’s exit path (Figure S4B).

Overall, BM1 appears to be a preferable binding mode of bufalin as it appeared in the majority of the simulation repeats regardless of ions bound, with the exception of Mg²⁺. The crystal structure of the Na⁺,K⁺-ATPase/bufalin complex, however, represents BM0 with the steroid core rotated almost 45° compared to BM1. This rotation alters the position of the hydrophobic β-surface in such a way that it faces the apolar αM1 (BM1) instead of the considerably more polar αM2 (BM0). The difference between the MD simulation results and the crystallized form with a less favorable orientation of bufalin
might be caused by the crystal packing. Note that all CTS crystallized in complexes with Na\(^+\), K\(^+\)-ATPase have the same binding mode (BM0) irrespective of the hydrophobicity of their steroid core.\(^{3,11,14}\)

The Na\(^+\), K\(^+\)-ATPase/cinobufagin complex was simulated in the presence of either K\(^+\) or Mg\(^{2+}\) ions. With K\(^+\) in the site, cinobufagin was overall stable in BM0 (\(\beta\)-surface facing \(\alpha\)M2) albeit positioned slightly higher up in the binding site compared to bufalin (Figure 3). Close-up inspection of the simulations revealed that this mode was best at accommodating the larger acetyl group of the core above the shorter \(\alpha\)M6 helix. With Mg\(^{2+}\) in the site, BM0 was observed in two repeats, while BM2 was observed in one (MD1). Cinobufagin in BM2 moves a few Ångström further out of the site (toward the extracellular milieu). Thus, cinobufagin is most stable in the presence of K\(^+\) ions, although the large acetyl group prevents optimal coordination with K\(^+\) in site II. The preferable binding mode of cinobufagin, BM0, is consistent with crystal structures of other CTSs.\(^{3,11,14}\)

Monitoring of the coordination distances for the ion in site II is a mode of evaluation of its direct interaction with the lactone oxygen of the bound bufadienolide. The first-shell coordination distance of Mg\(^{2+}\), Na\(^+\), and K\(^+\) is approximately 2.1 \(\text{Å}\), 2.4 \(\text{Å}\), and 2.8 \(\text{Å}\), respectively.\(^{15}\) In the calculations, direct coordination was defined as distances shorter than the optimal coordination distance plus 0.1 \(\text{Å}\).

Simulations of the Na\(^+\), K\(^+\)-ATPase/bufalin/2\(\times\)K\(^+\) system revealed that the distance distribution peak at 2.8 \(\text{Å}\) was most populated (Figure 4). They indicate direct coordination and were maintained in 63% of the total simulation time of MD2–MD5. MD1 was an exception: the direct coordination occurred only in <2% of the time. Instead, we registered a peak at 4.5 \(\text{Å}\). Thus, the distance distributions suggest that K\(^+\) does not always interact with bufalin, but direct coordination is common.

Simulations of Na\(^+\), K\(^+\)-ATPase/bufalin/2\(\times\)Na\(^+\) revealed two peaks at 2.4 and 4.2 \(\text{Å}\). In all three repeats, the second (distant) peak was most populated, while direct coordination of the ion was only detected in \(\sim\)7% of the total simulation time. The same trend, with even fewer occurrences of direct coordination (<2%), was observed with a single Na\(^+\) present.

Analysis of the Mg\(^{2+}\)-simulations revealed two peaks, but the populations were not similar across repeats. In MD1 and MD2, direct coordination was observed in approximately half of the simulation time (62 and 43%, respectively), while in MD3, direct coordination was never observed. Simulations revealed “irreversibility” of the breakage of direct coordination: when the direct coordination was broken, the interaction distance remained long for the rest of the simulation time. This feature is in contrast with the other simulations, where direct coordination broke and restored multiple times. Visual inspection revealed that coordination broke as Mg\(^{2+}\) tended to move deeper into site II, while bufalin was unable to follow Mg\(^{2+}\) due to steric hindrance by the enzyme.

Simulations of Na\(^+\), K\(^+\)-ATPase/cinobufagin/2\(\times\)K\(^+\) also displayed two distance distributions, but direct coordination to cinobufagin was only observed in 19% of the combined simulation time, in sharp contrast to bufalin. Direct coordination broke and reformed multiple times. In simulations of Na\(^+\), K\(^+\)-ATPase/cinobufagin/Mg\(^{2+}\), direct Mg\(^{2+}\)-coordination was observed initially, but was lost rapidly in all three repeats. All repeats were similar to the Na\(^+\), K\(^+\)-ATPase/bufalin/Mg\(^{2+}\) simulations where direct coordination was never restored after the initial loss.

In summary, bufalin in the preferable BM1 binding mode maintained direct coordination of K\(^+\) in site II most of the simulation time. In contrast, the BM1 mode of bufalin is not reconcilable with Na\(^+\) and Mg\(^{2+}\) binding, and direct Na\(^+\) and Mg\(^{2+}\) coordination by bufalin was rare. In both cases, bufalin can either move deeper into the site and achieve direct coordination or move higher up the site and occupy the second coordination shell of the ion.\(^{16}\) In practice, the latter was detected for bufalin, which would result in decreased bufalin affinity. Cinobufagin favored the BM0 binding mode, likely due to steric hindrances from its large acetyl moiety, and direct coordination by both K\(^+\) or Mg\(^{2+}\) was rare.

The paragraph above describes direct interactions of the ion in site II with the bufadienolide of choice. The ion, however, also coordinates the protein and may thus alter the geometry of the CTS binding site wedged between the flexible \(\alpha\)M1–4 helices and the rigid \(\alpha\)M5–M10 bundle. In the simulations, the ions in site II are coordinated by the backbone carboxyl of Val325 (\(\alpha\)M4) and one or two of the side chain oxygen atoms of Glu327 (\(\alpha\)M4), Glu779 (\(\alpha\)M5), and/or Asp804 (\(\alpha\)M6; Figure 5A). Coordination to Ala323 was not detected.\(^{16}\) Since it is feasible that coordination of an ion by both helix bundles simultaneously stabilizes the composite CTS site, the duration of coordination in conjunction with the helical location of a given coordinating residue may serve to quantify the indirect influence of an ion on CTS binding. It is particularly relevant.
for bufalin and cinobufagin as their β-surface has fewer polar substituents than most CTSs.

In the Na⁺,K⁺-ATPase/bufalin/2XK⁺ simulations, we observed coordination of K⁺ by Glu327 and Asp804 (single and double coordination, respectively) in approximately 50–65% of the simulation time (Figure S5B). Coordination to Val325 varied more and ranged from 6 to 84% depending on the repeat simulation. Some coordination to Glu779 in up to half of the simulation time was also present. The overall coordination pattern observed in double Na⁺ occupation was very similar to that in double K⁺: Na⁺ was consistently coordinated by Val325, Glu327, and Asp804. In contrast to K⁺, Na⁺ did not coordinate to Glu779, and double coordination by Asp804 was rare. However, since Na⁺ affinity to E2P is low, the existence of the E2P(2Na⁺)/bufalin form might be irrelevant under physiological conditions. Coordination of a single Na⁺ ion was different: it was very strong to Glu779, and coordination by Val325 and double coordination by Asp804 were rare. This altered behavior could be a consequence of a loss of stabilizing effect of the ion in site I on the ion in site II. The RMSD of the ion in site II was sometimes higher when only one site was occupied (Figure S5). The single Mg²⁺ ion in site II was strongly coordinated to Glu779 and doubly coordinated to Asp804, while coordination to Val325 and Glu327 was rarer. The Na⁺,K⁺-ATPase/cinobufagin simulations highlight the same observations as described for the bufalin systems (Figure S5B).

In short, in the MD simulations K⁺ was always surrounded by five complexing agents belonging to both αM1−4 and αM5−10 bundles. Complexing agents of a single Na⁺ included one residue from the αM1−4 bundle and two residues from the αM5−10 bundle, while Mg²⁺ exclusively coordinated to αM5−10 residues. Thus, K⁺ optimally spanned the separate bundles. Na⁺ also spanned the bundles but via fewer residues, while Mg²⁺ associated only with one helix bundle. Thus, the αM1−4/αM5−10 interface, and the contact surface for CTS binding, may vary depending on the ion. As the bufadienolides establish primarily hydrophobic interactions with the residues in the site, an increase in contact area is a main contributor for improving affinity. Therefore, we considered the calculation of the enzyme/bufadienolide surface overlap (i.e., contact area) as an indication of the indirect influence exerted by cations.

It appeared, that the overall contact surface for bufalin in Na⁺,K⁺-ATPase was increased in the presence of any cation compared to the system without ions. The calculations suggested that bufalin’s interactions with the binding site are weaker in the case of the least constrained site (Figure S6), but the calculations were not sensitive enough to reveal differences between the ions. In an attempt to locate hotspots for the ion-induced changes in local conformations of the binding site, the extent of bufalin contacts with selected amino acids (based on Qiu et al.¹) as well as that with individual helices (αM1−6) were calculated. Unfortunately, the detected fluctuations were not sufficiently system-dependent, possibly due to the limited time scale of the simulations. The importance of an ion for cross-linking the site remains hypothetical and awaits further investigation. However, certain observations from the MD simulations speak in favor of the ion-mediated confinement of the binding site. For example, bufalin unbinding from the apo enzyme occurred via a semistable tilted binding mode in which the lactone was above the short αM6 helix and the β-surface was facing the extracellular solvent (Figure S4B). This tilting was associated with an outward motion of αM1 and αM2 away from the αM5−10 bundle. With K⁺ in the ion sites, the helices would be restrained from such an outward movement, and bufalin unbinding will be hindered.

The results of the MD simulations allow assignment of structural characteristics to the complexes with certain kinetic behaviors. It seems that the tilted binding mode represents the fast-dissociating complex in biochemical experiments, and the coordination of both helix bundles by K⁺ is responsible for the disappearance of the fast-dissociating complex. K⁺-binding induces a different enzyme conformation which interacts with bufalin with only slightly better affinity but results in a very different dissociation rate constant (Table S1 and Figure 1B). Thus, K⁺ in the site affects bufalin association to almost the same degree as its dissociation. K⁺ alone, but not Na⁺, Mg²⁺, or NMG⁺, was able to ensure homogeneity of the bufadienolide complexes in binding-dissociation studies, and Na⁺ and Mg²⁺ were not able to stabilize the helix bundles to the same extent as K⁺ in the MD simulations.

Thus, the in silico study of bufadienolide interactions with Na⁺,K⁺-ATPase performed in parallel with their biochemical characterization with Na⁺,K⁺-ATPase provided an explanation for the unexpected and specific effects of K⁺ in these reactions. As a specific ligand for the enzyme, K⁺ has high affinity for the extracellular cation transport sites accessible in the E2P form. The same conformation has high affinity for CTSs. While the presence of K⁺ decreases the affinity for cardenolide binding, it has a complex effect on bufadienolides. The crystal structure of the enzyme/bufadienolide complex revealed electrostatic interactions between K⁺ and the bufadienolide lactone ring. The slight K⁺-induced improvement in affinity, however, turned out to be valid for bufalin (and possibly for its derivatives) but not for all bufadienolides. The in silico calculations allowed us to look at two aspects of K⁺ presence in the extracellular sites: the direct effect on bufadienolide binding due to lactone/K⁺ coordination and the indirect effect mediated by changes in protein structure. K⁺ turned out to be the only cation able to optimally span the two helix bundles and stabilize a well-defined local conformation of the CTS binding site. Therefore, K⁺ promotes formation of a homogeneous pool of bufadienolide complexes. For bufalin, this preformed site has an additional advantage: an optimal arrangement for electrostatic interactions between the ion and the enzyme/bufalin complex. The structural fluctuations within the site are also reflected in the heterogeneity of Na⁺,K⁺-ATPase complexes with aglycones from the cardenolide family. Cations occupying the extracellular sites seemed to influence the process as well. The cause of K⁺/cardenolide antagonism has been described on the basis of crystal structures, and the cause of K⁺/bufadienolide agonism is described herein. The question of how glycosylation affects CTS binding is a subject for our ongoing in silico study.

### MATERIALS AND METHODS

**Biochemical Characterization of E2P⁺-Bufadienolide Complex of Na⁺,K⁺-ATPase. Enzyme Preparation.** Purified pig kidney Na⁺,K⁺-ATPase was prepared as previously
Table 1. Overview of Simulation Protocol

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“NVT and NPT abbreviate constant number of atoms, volume/pressure, and temperature ensemble, respectively.

The Na+,K+-ATPase activity of the preparation was about 1800 μmol P_i/hour per mg protein at 37 °C.

**Binding of Bufadienolides to the E2P_i Conformational State of Na+,K+-ATPase.** The effect of cations on bufadienolides interactions with E2P, state was estimated from the residual Na+,K+-ATPase activity after preincubation of bufadienolides with the enzyme essentially as described by Yatime et al. for ouabain interactions. The specific Na+,K+-ATPase activity was determined from the difference in the amount of phosphate released in the absence and presence of 1 mM ouabain in a solution containing 130 mM NaCl, 20 mM KCl, 4 mM MgCl_2, and 3 mM ATP at 37 °C. The reaction was stopped after 2 min. The data were analyzed as in Yatime et al. using the commercial program KyPlot 5 (Kyence Inc.).

**Rate Constants for Bufalin Dissociation from the E2P_i**. Kinetics of bufalin dissociation from its complex with E2P_i was recorded on a SPEX Fluorolog-3 spectrophotometer equipped with a thermostated cell compartment, and a magnetic stirrer used in a kinetic mode: excitation wavelength, 370 nm (bandpass 5 nm); emission wavelength, 485 nm (bandpass 5 nm). The E2P_i-bufalin complex was formed by incubation of the 33 μg/mL enzyme with 0.13 μM bufalin in 20 mM histidine at pH 7.0, 4 mM H_3PO_4 (adjusted with NaOH), and 4 mM MgCl_2 in the absence or presence of 10 mM KCl at room temperature overnight. Then, the 100 μL aliquots were diluted in 3 mL cuvettes with the same media but containing either 1.5 μM anthroylouabain or 1.5 μM anthroylouabain and 1 mM ouabain. The dissociation of bufalin is considered irreversible due to dilution and the presence of anthroylouabain (the concentration exceeds that of bufalin in the cuvette by a factor of 37°). One millimolar ouabain prevents binding of both ligands, and this sample thus serves as a control for nonspecific anthroylouabain binding and stability of its fluorescence in time.

**Computational Protein and Ligand Preparation.** The protein model was prepared from PDB entry 4RES (α1β1γ subunits from pig) using the Protein Preparation Wizard in Maestro (Schrödinger Suite 2019, Schrödinger LLC, New York, NY). This entailed capping of termini; the addition of hydrogen atoms; potential flipping of His, Gln, and Asn; and a restrained minimization of the protein (max. 0.3 Å RMSD for heavy atoms). The protonation states of titratable residues were assessed by PROPKA 3 however, the protonation states of residues in the ion binding site were manually adjusted to be in accord with experiments by the Roux lab. The resulting model included disulfide bridges between CysB126-CysB149, CysB159-CysB175, and CysB213-CysB276; neutral AspA808, AspA926, GluA244, GluA327, GluA779, and GluA954. Histidines HisB212, HisA286, HisA517, HisA550, HisA613, HisA659, HisA678, HisA875, and HisA912 were modeled as the ε-tautomeric; and AspA369 was phosphorylated, while all other residues were modeled in the default state.

The chemical structure of bufalin was extracted from PDB entry 4RES, while cinobufagin was extended manually from the bufalin structure using the build panel available in Maestro (Schrödinger Suite 2019). Both compounds were minimized using a conjugate gradient algorithm in 5000 steps and submitted to a conformational search by using a mixed torsional/low mode sampling algorithm as implemented in MacroModel (Schrödinger Suite 2019). The lowest energy conformation of each compound was used in the docking calculation and for force field parameter generation.

**Docking Calculations.** All docking calculations were performed using the induced fit docking protocol, employing Glide and Prime (Schrödinger Suite 2019). In the initial docking all vdW interactions were scaled to 50%, the SP level was applied, and a maximum of 200 poses were allowed. The centroid of the binding site was defined based on the cocrystallized bufalin (PDB ID: 4RES). In the optimization step, residues within 5 Å of the ligand were subjected to side chain optimization. The final docking step was performed in XP, and a maximum of 100 poses with associated energies within 30 kcal/mol of the lowest energy pose were reported in the results. The resulting poses were clustered based on their in-plane conformation using the conformer cluster script available in Maestro (Schrödinger Suite 2019).

**System Building for MD Simulations.** Each simulated system contains one Na+,K+-ATPase, one ligand, one or two structural cations, a POPC membrane patch, solvent, and 0.2 M KCl. The system was built from scratch using a combined structural cations, a POPC membrane patch, solvent, and 0.2 M KCl. The system was solvated and neutralized with 0.2 M KCl before being minimized and equilibrated according to steps 1 and 2 in Table 1. The system was then
Molecular Dynamics Simulations. All simulations were performed in Gromacs 2019.2 using periodic boundary conditions. For the CG simulations, the MARTINI 2.2 force field was used. The vdW interactions were treated using cutoffs at 11 Å and the potential-shift-Verlet modifier, while electrostatic interactions were treated using the reaction-field method cutoff at 11 Å and a dielectric constant of 0 (= infinite) beyond the cutoff. The neighbor list was maintained using Verlet buffer lists. Temperature was kept at 310 K and pressure at 1 bar. The AA resolution simulations were performed using the CHARMM36m force field, and the TIPS3P water model, and CHARMM-compatible ligand parameters as described below. The vdW interactions were treated by cut-offs at 12 Å and a force-switch modifier after 10 Å, while electrostatic interactions were treated using PME. The neighbor list was maintained using Verlet buffer lists, and bonds linking hydrogen atoms to heavy atoms were restrained using LINCS. The temperature was maintained at 310 K using a coupling constant of 1, and pressure was maintained at 1 bar using a coupling constant of 4, a compressibility factor of 4.5 × 10⁻⁵, and semi-isotropic coupling to x, y, and z dimensions separately.

Force Field Parameters for Ligands and Phosphorylated Aspartate. Force field parameters for bufalin and cinobufagin were obtained by analogy using the ParamChem Web server and the CHARMM generalized force field. The associated penalties were assessed and indicated the parameters were a good fit. The applied parameters can be found in Supporting Data Files S1–S4. The CHARMM compatible parameters for the phosphorylated aspartate were taken from Damjanovic et al.

Calculation of Enzyme/Bufadienolidine Contacts. The interatomic contact surface between bufadienolides and Na⁺,K⁺-ATPase was calculated using the dr_sasa software in mode 4. Mode 4 calculates the contact surface (i.e., overlapping surface area between ligand and protein) based on close atom—atom interactions and thus not by using a spherical water probe as done commonly. This ensures a more thorough calculation of the intramolecular contacts. The calculation was performed for every 100th frame of each trajectory, i.e., a frame per every 40 ps.

Computational Analyses. All analyses other than the contact calculations were performed in in-house scripts. All figures of molecular systems were made using VMD 1.9.3 or ChemDraw.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jcim.0c01396. (Table S1) Effect of cations on bufalin and cinobufagin binding; (Table S2) induced fit docking scores; (Figure S1) cation effect on bufadienolidine interactions with Na⁺,K⁺-ATPase as reflected by enzyme inhibition; (Figure S2) major bufalin binding clusters from docking calculation; (Figure S3) major cinobufagin binding clusters from docking calculation; (Figure S4) bufalin unbinding; (Figure S5) RMSD of the ion in site II; (Figure S6) enzyme/bufadienolidine contacts; (Figure S7) enzyme RMSD; (Supporting Data File S1) bufalin itp file; (Supporting Data File S2) bufalin itp file; (Supporting Data File S3) cinobufagin itp file; (Supporting Data File S4) cinobufagin itp file (PDF)
methyl-d-glucamine; NPT, constant number of atoms, pressure, and temperature ensemble; NVT, constant number of atoms, volume, and temperature ensemble; OPM, orientation of proteins in membranes; POPC, 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine; RMSD, root-mean-square deviation

REFERENCES


(38) Ribeiro, J.; Rios-Vera, C.; Melo, F.; Schuller, A. Calculation of accurate interatomic contact surface areas for the quantitative analysis of non-bonded molecular interactions. Bioinformatics 2019, 35 (18), 3499–3501.