Activation of cardiac ryanodine receptors by cardiac glycosides

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duration and with no discernible voltage dependence to this effect (24). Similar results were obtained with ouabain and digitoxin. These observations offered an additional explanation for glycoside actions in the heart by suggesting that glycosides might also have a direct, high-affinity action to promote SR Ca\(^{2+}\) release in addition to increasing the amount of Ca\(^{2+}\) stores in the SR via Na\(^{+}\) pump inhibition.

The current study was intended to examine the interaction of cardiac glycosides with the RyR2 channel, with an emphasis on the factors that might modulate glycoside actions on single-channel activity. We selected the glycosides digoxin and actodigin because the former is used clinically and is therefore of considerable interest whereas the latter is a semisynthetic agent that possesses very distinct effects on electrophysiological, mechanical, and toxic behavior of cardiac preparations that differ markedly from conventional glycosides (9, 32).

**METHODS**

**Preparation of crude and purified RyR2 protein.** Crude cardiac SR vesicles were prepared from left ventricles of 16 dogs according to a modification of Tsushima et al.'s (31) method. Skeletal SR vesicles were prepared from three dogs with the use of the muscle from the hindlimb. Dogs were anesthetized with pentobarbital sodium (35 mg/kg iv) and the heart was quickly removed. Skeletal muscle was removed immediately thereafter. The hearts of eight rats were obtained after removal of the heart under pentobarbital anesthesia (35 mg/kg ip) and combined in a single preparation of crude SR vesicles. Each muscle preparation was homogenized and the heavy SR vesicles were separated from the crude homogenate by ultracentrifugation. Vesicles were stored in 200 mM NaCl solution and snap-frozen in liquid nitrogen where they were stored for subsequent use in single-channel experiments.

Purification of RyR2 channel protein was accomplished by a modification of the method of Anderson et al. (3), as described in an earlier publication (31). Briefly, heavy SR vesicles were solubilized in 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution. The homogenate was separated with the use of sucrose gradient centrifugation and the purified protein was dialyzed to remove the detergent. Purified protein was suspended in proteoliposomes and stored in liquid nitrogen.

**Single-channel recordings.** Single-channel events were recorded with the use of the Muller-Rudin technique of artificial lipid bilayers. Crude vesicular RyR2 channels were recorded with the use of a mixture of 40 µg of phosphatidylethanolamine and 40 µg of phosphatidylserine (bovine brain, Avanti Polar Lipids) suspended in 20 µl of n-decane. The cis (cytoplasmic) side of the bilayer apparatus contained 250 mM Cs-methanesulfonate and the trans (luminal) side contained 20 mM Cs-methanesulfonate (both sides buffered to pH 7.2 with 20 mM HEPES). Unless stated otherwise, the trans chamber also contained 1 mM CaCl\(_2\) to simulate the normally high concentration of luminal [Ca\(^{2+}\)]. The artificial lipid mixture was then painted over the 200-µm hole separating the two sides, forming a bilayer. Three microliters of vesicle suspension was then added to the cis chamber, and the solution was stirred until vesicle fusion with the bilayer occurred. Each type of experiment was performed in at least three separate SR preparations, each representing a different animal. The Cs\(^{+}\) gradient was then abolished by addition of the appropriate amount of Cs-methanesulfonate from a 3 M stock. In most experiments, the holding potential was +40 to +50 mV to measure drug effects when mixed Cs\(^{+}\) and Ca\(^{2+}\) movements are in the physiological direction (lumen to cytoplasm). In some instances, −40 mV was chosen to show that drug effects were not dependent on either transbilayer voltage or the direction of current flow.

Data files were recorded using an Axoclamp-200 or −200A amplifier and a DMA interface from Axon Instruments. Data were filtered at 2 KHz with the use of an eight-pole Bessel filter (model 902; Analog Devices) and digitized at 5 kHz for 30-s recording intervals. Single-channel analysis was performed with the use of pCLAMP software to obtain channel open and closed times and to calculate single-channel open probability (P\(_o\)). Open and closed events were measured using an algorithm based on half-amplitude threshold detection.

Single-channel recordings of purified protein were performed with the use of KCl instead of Cs-methanesulfonate because no K\(^{+}\) or Cl\(^{−}\) channels were present. Data files were handled in an identical manner as described above.

Drugs were added from dilute ethanol stocks directly to the solutions on either side of the bilayer. Equivalent ethanol concentrations (up to 1%) showed no effect on single-channel activity in separate control experiments (n = 4).

**Statistics.** Statistical analyses were performed using SigmaStat software (Jandel). Data are presented as means ± SE. Comparisons between sample means were made using Student's t-tests or a one-way analysis of variance, with secondary comparisons made using a Newman-Keuls test. Differences between means were considered significant if P values were <0.05, unless indicated otherwise.

**RESULTS**

The effects of digoxin on single-channel activity recorded from crude vesicular RyR2 channels are shown in Fig. 1. The control recording shows typical single-
channel activity under our experimental conditions (holding potential +50 mV). With a free [Ca\(^{2+}\)] previously measured at 5 μM (by Ca\(^{2+}\) electrode), brief channel openings during the course of the recording period gave a \(P_0\) of 0.010. After the addition of 0.3 nM digoxin to the cis chamber, single-channel activity increased, giving a \(P_0\) of 0.020. The increase of digoxin concentrations in a cumulative manner caused a progressive increase in \(P_0\) up to 1 nM (\(P_0 = 0.057\)).

The concentration dependence of the effects of digoxin on single-channel activity are summarized in Fig. 2A. Activation of \(P_0\) occurred beginning above 0.1 nM digoxin and reached a maximum at ~1 nM. The fitted line shows the best sigmoidal fit to the data (Hill coefficient of 1.2), and a concentration at which 50% of maximal effect (EC\(_{50}\)) was ~0.2 nM. When the data are presented as percentage of control \(P_0\) (Fig. 2B), the results indicate that digoxin produces an increase in single-channel activity of about fivefold at maximal concentrations.

The effects of the semisynthetic agent actodigin on single-channel activity recorded under identical conditions are shown in Fig. 3. Increasing drug concentration cumulatively on the cis side of the bilayer from 0 to 0.6 nM resulted in progressive increase in \(P_0\) from 0.002 in control to 0.022 at the highest concentration of actodigin. These results are similar to those obtained with digoxin in Fig. 1.

Figure 4A summarizes the concentration dependence of the effects of actodigin on \(P_0\). The concentration dependence of channel activation is similar to that of digoxin, with an EC\(_{50}\) of ~0.3 nM (Hill coefficient of 1.7). The maximal effect of actodigin produces about a sixfold increase in \(P_0\) (Fig. 4B). These results demonstrate that the effect of glycosides to activate the RyR2 channel occurs at low concentrations that are quite close to the nanomolar concentrations of digoxin found in plasma of patients undergoing glycoside therapy (14).

Cardiac glycoside effects and MgATP. One of the most important physiological regulators of the RyR2 channel is Mg\(^{2+}\)-ATP on the cytoplasmic face of the channel. We examined the effect of glycosides in the presence of MgATP (2 mM) to determine whether or not the activation of RyR2 channels by digoxin was affected by this important regulator. In this series of experiments, MgATP was present under control conditions. In seven experiments (5 with 10 nM digoxin and 2 with 10 nM actodigin, initial \(P_0 = 0.02 \pm 0.03\); not significant (NS) compared with experiments in the absence of MgATP) single channels exposed to glycoside caused a 196 ± 18.0% increase in \(P_0\) in the presence of 2 mM MgATP (\(P < 0.05\)). These results demonstrate that glycoside activation of the channel occurs independently from cis MgATP, although the activating effect appears to be somewhat diminished.

Species differences in cardiac RyR2 channel activation by digoxin. To determine if a high sensitivity of RyR2 to digoxin is shared by other glycoside-sensitive species, we also performed a series of experiments in human cardiac RyR2 channels isolated from three nor-
mal hearts. As shown in Fig. 5, single-channel $P_o$ increased from 0.03 to 0.10 during exposure to 1 nM digoxin. In a total of four experiments, $P_o$ increased from 0.059 $\pm$ 0.018 to 0.111 $\pm$ 0.039 ($P < 0.02$), indicating that human cardiac RyR2 channels are activated by low concentrations of digoxin just as in dog heart. These observations are particularly important because both species are known to be sensitive to the inotropic and toxic effects of glycosides. Therefore, we would expect high sensitivity of the RyR2 channel to the effects of glycosides if this effect contributes to a change in cardiac cell contraction and/or development of toxicity.

In contrast to the high sensitivity of human heart to glycosides, rat heart is known to be less sensitive to the effects of cardiac glycosides than many other mammalian species, including the dog, cat, guinea pig, and human (2, 18). We tested the effects of digoxin on rat RyR2 channel to determine whether or not the purification process might alter sensitivity to glycosides. Figure 7A shows that $P_o$ in control was 0.195; after addition of digoxin (1 nM) to the cis side,

![Fig. 4. Summary of concentration-dependence of actodigin effects on $P_o$. A: average $P_o$ values at different concentrations of actodigin. B: $P_o$ values as a percentage of $P_o$ under control conditions. Each point represents the mean $\pm$ SE of 4–5 experiments.](http://ajpheart.physiology.org/)

Fig. 4. Summary of concentration-dependence of actodigin effects on $P_o$. A: average $P_o$ values at different concentrations of actodigin. B: $P_o$ values as a percentage of $P_o$ under control conditions. Each point represents the mean $\pm$ SE of 4–5 experiments.

...more apparent at 1 $\mu$M (0.066). Figure 6B summarizes the effects of these concentrations of digoxin on RyR2 channel activity. There was a significant increase in $P_o$ only when concentration was increased to 1 $\mu$M. These results demonstrate that, unlike in sensitive species, RyR2 channel activity in rat heart demonstrates a much lower sensitivity to digoxin, requiring concentrations 1,000-fold higher to produce significant activation observed in the sensitive species. These data suggest that activation of RyR2 channel activity at low concentrations might contribute to the cellular effects of digoxin in glycoside-sensitive species but are less likely to occur in rat.

**Addition of digoxin to luminal side of bilayer.** To determine the location of the binding site for digoxin on the dog cardiac RyR2 channel, we performed several experiments, in which glycoside (10 nM) was added to the trans side of the bilayer. In six experiments, $P_o$ was 0.021 $\pm$ 0.005 in control and 0.020 $\pm$ 0.004 (NS) during exposure to digoxin. These results indicate that the site of digoxin activation resides on the cytoplasmic face of the channel and that drug application to the lumen does not allow access to that site. Thus it is more likely that the binding site is present in the cytoplasmic domain of the channel protein than either the transmembrane or luminal portions of the protein sequence.

**Effects of glycosides on purified cardiac RyR2 and crude skeletal RyR1.** We next measured the effects of digoxin on single-channel activity in purified RyR2 channels from the dog heart to determine whether or not the purification process might alter sensitivity to glycosides. Figure 7A shows that $P_o$ in control was 0.195; after addition of digoxin (1 nM) to the cis side,
there was little change in $P_o$ (0.230). In six experiments, $P_o$ was $0.04 \pm 0.03$ in control and $0.05 \pm 0.04$ after addition of digoxin (NS). These data indicate that, in contrast to the crude vesicular membrane-bound form of the channel, the purified RyR2 channel bound to proteoliposomes is not activated by digoxin. One possible explanation is that the binding site of digoxin to the channel is removed by the purification process and thus may reside on an associated protein. Alternatively, the occupied receptor on the RyR2 channels may be unable to cause channel activation, possibly because the internal (luminal) Ca$^{2+}$-sensitive binding sites are no longer available to induce activation. Other possible explanations exist but must await further examination.

We also investigated the effects of digoxin on dog skeletal RyR (RyR1) activity to determine if RyR channel activation is a general action of glycosides in striated muscle. Figure 7B shows the results of an experiment in which single-channel activity was recorded before and during exposure to digoxin. Single-channel $P_o$ was 0.005 in control and 0.004 after addition of digoxin (1 nM) to the cis side of the bilayer and unchanged, even at 100 nM digoxin, indicating little effect of the glycoside. In seven channels, $P_o$ was $0.003 \pm 0.002$ in control and $0.001 \pm 0.002$ after addition of 100 nM (2 with digoxin, 5 with actodigin, NS). These results demonstrate that the glycoside effect occurs in the crude cardiac isoform of the channel and not in the skeletal isoform. Thus the effect of glycoside is not the result of a nonspecific drug action on the RyR2 in general but rather a specific action on the cardiac isoform of the channel.

**Luminal Ca$^{2+}$ and glycoside activation of RyR2.** All of the experiments to this point included 1 mM Ca$^{2+}$ on the luminal side of the bilayer. We examined the possibility that this condition might be required for glycoside action.

The effect of digoxin on single-channel activity at different concentrations of luminal (trans) Ca$^{2+}$ is...
shown in Fig. 8. Under control conditions (holding potential +40 mV), single-channel $P_o$ doubled at 30 μM and then declined slightly as [Ca$^{2+}$] was increased over the concentration range of 300 μM to 10 mM, giving a slight bell shape to the [Ca$^{2+}$]-$P_o$ relationship. These results differ somewhat from those reported by several other investigators (21, 30) who found a greater reliance of $P_o$ on luminal [Ca$^{2+}$] but under quite different experimental conditions. In a separate series of experiments, the same protocol was repeated with 10 nM digoxin. After control experiments were obtained ([Ca$^{2+}$] = 5 μM), addition of digoxin (10 nM) had little effect on single-channel activity. Subsequent increases in trans [Ca$^{2+}$] to 100 μM caused identical changes in $P_o$ to those found in the absence of digoxin. However, at [Ca$^{2+}$] ≥300 μM and up to 10 mM, there was a significant increase in $P_o$. Thus, in the presence of digoxin, there is little effect at [Ca$^{2+}$] <300 μM but a significant increase in $P_o$ at 0.3–10 mM. These data suggest that the ability of digoxin to activate the RyR2 channel is related to SR Ca$^{2+}$ load. In control, there may be a slight increase in RyR2 channel activity in the physiological range of luminal [Ca$^{2+}$]. However, when Na$^+$ pump inhibition causes an increased SR Ca$^{2+}$ load, the ability of the RyR2 to release that stored Ca$^{2+}$ is also enhanced.

**DISCUSSION**

It is well known that cardiac glycosides inhibit the sarcolemmal NKA and that this action is thought to underlie both the positive inotropic (therapeutic) and arrhythmogenic (toxic) actions of these agents. However, a single cellular mechanism of action for all such agents is unlikely to explain the numerous reports over the past 40 years detailing the variety of actions of different cardiac glycosides in whole heart (1) and at the cellular level. Most notably, Isenberg (10) reported that intracellular application of nanomolar concentrations of digoxin or ouabain produced a positive inotropic action in bovine cardiac myocytes, despite the absence of Na$^+$ or the presence of saturating extracellular concentrations of digoxin antibody. Subsequently, it was reported that hydrophilic glycosides (e.g., ouabain) failed to produce a positive inotropic action in guinea pig atria when membrane transport inhibitors were present whereas the effects of lipophilic agents were unaffected (26). This result was interpreted as suggesting that hydrophilic agents required active transmembrane transport to an intracellular site of action whereas lipophilic agents were capable of passive diffusion to that site. These interesting results recalled earlier suggestions that glycosides might have an intracellular site of action, possibly involving the sarcoplasmic reticulum (5, 8).

With this potential target in mind, we investigated the effects of low concentrations of glycosides on RyR2 channels and found that cardiac glycosides activated single-channel activity of dog RyR2 channels in artificial lipid bilayers (27). Subsequently, McGarry and Williams (24) found that digoxin increased single-channel $P_o$ in sheep cardiac RyR2 channels with an estimated EC$_{50}$ of ~650 pM. In addition, they found that digoxin acted to increase the sensitivity of RyR2 channels to cytoplasmic Ca$^{2+}$ in the range of 1–10 μM, thus suggesting a mechanism by which glycosides increase RyR2 channel activity over the physiological range of cytosolic [Ca$^{2+}$]. These investigators also reported specific binding of [3H]digoxin to heavy SR vesicles, demonstrating both high- and low-affinity sites on the cardiac RyR (23).

The present results demonstrate that as in another glycoside-sensitive species (sheep), low concentrations of digoxin and actodigin activate the RyR2 channel in the dog and human heart. Glycoside activation takes the form of increased number of openings with little change in either open or closed time durations. We have also measured the EC$_{50}$ to be ~200–300 pM, values quite close to that estimated at 650 pM by McGarry and Williams (24). In addition, this effect does not require the presence of Mg$^{2+}$ and/or ATP on the cytoplasmic side of the channel, suggesting that glycoside actions occur independent of the interactions of these two ligands with their receptors. Just as with sheep heart (24), we also found that digoxin activated only the cardiac isoform of the channel and had no effect on the skeletal channel in dog.

Interestingly, both digoxin and actodigin had very similar effects on concentration dependency and maximal extent of stimulation of RyR2 activity. This is in contrast to the pronounced differences in cellular actions that have been reported between these agents (9, 32). Because both agents produce similar effects to stimulate RyR2 activity, the differences at the cellular level must therefore not be due to the quality of interaction between these drugs and the RyR2, which appears to be similar for each. The difference between agents may be related to differences in lipid solubility and therefore access of each drug to this intracellular site (26) rather than simply to efficacy of sarcolemmal

**Fig. 8. Summary of the effect of digoxin on the relationship between single-channel $P_o$ and luminal (trans) [Ca$^{2+}$]. Data are presented as a percentage of control $P_o$ ([Ca$^{2+}$]$_{trans}$ = 5 μM). Each experiment was then conducted with cumulative additions of CaCl$_2$ to the trans side of the bilayer, either in the absence (Control) or presence (+ Digoxin) of the glycoside, respectively. After each addition of CaCl$_2$, the chambers were stirred for 90 s before data were acquired. Each point represents the means ± SE of 4–11 experiments. *$P < 0.05$. Holding potential = +40 mV.**
Na"\(^+\) pump inhibition, with the balance of these two actions causing the apparent distinctions in drug effects on cardiac preparations in vitro.

Species sensitivities of glycoside actions. One of the most striking effects of cardiac glycosides is the species difference in sensitivity to the inotropic and toxic actions of these agents. It is well known that rat heart is generally insensitive to glycoside actions, requiring at least 10-fold and often far greater drug concentrations, depending on experimental conditions (2, 18). This was thought to be the result of a selective expression of a glycoside-insensitive isoform of the NKA (\(\alpha_1\)) in the adult rat rather than the more common glycoside-sensitive isoform (\(\alpha_3\)) predominantly present in sensitive species (28). However, it is also possible that different amino acid sequences may exist in a single isoform, which may also impart varying degrees of glycoside sensitivity to that isoform (22).

The current results suggest an alternative, or perhaps parallel, explanation. Independently of sarcolemmal actions, the rat RyR2 channel is activated only by extremely high concentrations of glycoside. In contrast, the dog and human isoform of RyR2 channel is sensitive to activation by low concentrations of glycoside. The fact that RyR2 channel activation in rat occurs at a 1,000-fold higher concentration than in sensitive species suggests that the rat channel is regulated differently (binding site, regulatory factors, etc.) from sensitive species. Most importantly, the fact that rat RyR2 channel is activated at such high concentrations makes it less likely to contribute to its cellular actions in this species. Conversely, the results also suggest that part of the sensitivity of human and dog heart to glycoside inotropy might reside in an intracellular action on the SR because a high-affinity action on rat RyR2 channel would have been unlikely to account for a low-affinity inotropic effect in this insensitive species.

The fact that digoxin was unable to activate rat RyR2 except at 1,000-fold higher concentration than in the other species suggests that the effect of digoxin on RyR2 is not the result of a nonspecific interaction with either the channel or the lipid. Such nonspecific effects would also be expected to result in activation of RyR1, which did not occur. In addition, a nonspecific effect would be likely to occur from either side of the bilayer, which was not the case. The current results, as well as those of McGarry and co-workers (23, 24), suggest that the interaction between cardiac glycosides and RyR2 is a specific, high-affinity interaction that occurs only in glycoside-sensitive species.

Role of luminal \([\text{Ca}^{2+}]\) on glycoside-induced activation of RyR2. It is well known that the functional unit regulating SR \([\text{Ca}^{2+}]\) release in both skeletal and cardiac SR includes several \([\text{Ca}^{2+}]\) binding proteins aside from the RyR protein. The high-affinity \([\text{Ca}^{2+}]\) binding protein calsequestrin is located in the SR lumen and has long been thought to serve as a major mechanism of concentrating \([\text{Ca}^{2+}]\) in the vicinity of the RyR thus ensuring that sufficient \([\text{Ca}^{2+}]\) is available for effective release (7, 25). Junctin and triadin are thought to serve as anchoring proteins to stabilize calsequestrin at the inner face of the RyR (4, 12, 15, 16, 25).

This functional unit is responsible for ensuring that sufficient concentrations of \([\text{Ca}^{2+}]\) are available at the mouth of the RyR to promote contraction, for coupling the luminal \([\text{Ca}^{2+}]\) source (calsequestrin) to the RyR and for allowing fine regulation of RyR activity and, there-
fore, of SR Ca$^{2+}$ release. Our results also suggest a pharmacological role for this arrangement. The effect of digoxis requires that luminal Ca$^{2+}$ be in the physiological range to regulate RyR2 channel operation. This result suggests that the functional release unit must be intact for digitalis to be effective. Alternatively, it is possible that at low luminal [Ca$^{2+}$], the glycoside may no longer bind to a cytoplasmic receptor.

This observation might also suggest the mechanism by which to explain the lack of effectiveness of digoxin on the purified cardiac RyR2 channel; in the absence of associated proteins, the glycoside cannot activate the channel. Even though the same concentration of Ca$^{2+}$ (1 mM) is present on the luminal side of the channel, digoxin can no longer modulate activity. Several possibilities might explain this effect: 1) purification might remove the glycoside binding site from the RyR2 channel protein itself or from one of the associated proteins with access to the cytoplasm (junctin or triadin); 2) removal of the ancillary Ca$^{2+}$-binding proteins might prevent glycoside binding to its receptor; or 3) receptor occupation may no longer be capable of translation to the effector system responsible for increased RyR2 channel activity. It is not possible to discriminate between these possibilities at this time because all we know is that purification of the cardiac isofrom of the RyR2 channel removes these proteins, with the exception of the integral protein FK-506 binding protein (FKBP12.6) which remains associated with the channel during purification (17).

Possible mechanism for cardiac glycoside action in heart. The requirement of digoxin activation of RyR2 channels on physiological concentrations of Ca$^{2+}$ suggests a mechanism for glycoside actions on RyR2 channel activity (Fig. 9). There appears to be a range of luminal [Ca$^{2+}$] in which the RyR2 channel is most sensitive to glycoside activation. In the top panel (at normal SR Ca$^{2+}$ load), there is little effect of glycoside on SR Ca$^{2+}$ release even though its receptor on the RyR2 channel is occupied, as might occur at drug concentrations below the level of significant inhibition of NKA. When the NKA is inhibited (Fig. 9, bottom), not only does the increased SR Ca$^{2+}$ load make more Ca$^{2+}$ available for release but it also promotes the ability of the glycosides to activate the RyR2 channel directly, amplifying SR release beyond that expected simply from the increase in SR load secondary to NKA inhibition alone. Thus the ability of glycoside to activate RyR2 and stimulate SR Ca$^{2+}$ release is a result of both direct and indirect actions on the RyR2; the action is direct because it occurs as the result of a high-affinity binding interaction to stimulate single-channel activity and indirect in that it also relies on the ability of NKA inhibition to increase SR Ca$^{2+}$ load. This mechanism, combining increased SR load (via NKA inhibition) with direct increase in SR release, could explain how the action of glycosides to increase release could contribute to a sustained positive inotropic action without depleting the SR (6).

Interestingly, the range of SR [Ca$^{2+}$] causing maximal direct activation of RyR2 channel activity by digoxin coincides with that thought to be present under normal physiological conditions and during Na$^{+}$ pump inhibition. The extent of SR Ca$^{2+}$ loading, achieved through inhibition of the NKA, might therefore serve as a fine control system for the secondary action of glycoside-induced regulation of RyR2 channel activity. This explanation could provide a mechanism by which glycosides might produce their inotropic regulation of the heart, as well as of toxicity. In addition, the existence of these separate but interrelated actions could also explain how different glycosides produce different toxic:therapeutic ratios in vivo as well as in vitro (1, 13, 29, 32). It is also important to note that it is not clear if this effect on the RyR2 channel contributes to either or both the toxic and inotropic effects. However, we would expect that lipophilic agents (including digoxin) would gain access to the intracellular site faster than hydrophilic agents, thus explaining some of the differences between agents described in previous studies (9, 11, 26). Despite similar potencies of actodigin and digoxin for this intracellular action and a shared ability to inhibit the Na$^{+}$ pump, it may be the differences in physiochemical properties and access to these different mechanisms that may ultimately underlie the apparent difference in cellular actions between agents.

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