Isoform-specific Stimulation of Cardiac Na/K Pumps by Nanomolar Concentrations of Glycosides

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ABSTRACT It is well-known that micromolar to millimolar concentrations of cardiac glycosides inhibit Na/K pump activity, however, some early reports suggested nanomolar concentrations of these glycosides stimulate activity. These early reports were based on indirect measurements in multicellular preparations, hence, there was some uncertainty whether ion accumulation/depletion rather than pump stimulation caused the observations. Here, we utilize the whole-cell patch-clamp technique on isolated cardiac myocytes to directly measure Na/K pump current (I\textsubscript{p}) in conditions that minimize the possibility of ion accumulation/depletion causing the observed effects. In guinea pig ventricular myocytes, nanomolar concentrations of dihydro-ouabain (DHO) caused an outward current that appeared to be due to stimulation of I\textsubscript{p} because of the following: (1) it was absent in 0 mM [K\textsuperscript{+}], as was I\textsubscript{p}; (2) it was absent in 0 mM [Na\textsuperscript{+}], as was I\textsubscript{p}; (3) at reduced [Na\textsuperscript{+}], the outward current was reduced in proportion to the reduction in I\textsubscript{p}; (4) it was eliminated by intracellular vanadate, as was I\textsubscript{p}. Our previous work suggested guinea pig ventricular myocytes coexpress the \(\alpha_2\) and \(\alpha_3\)-isoforms of the Na/K pumps. The stimulation of I\textsubscript{p} appears to be through stimulation of the high glycoside affinity \(\alpha_2\)-isoform and not the \(\alpha_1\)-isoform because of the following: (1) regulatory signals that specifically increased activity of the \(\alpha_2\)-isoform increased the amplitude of the stimulation; (2) regulatory signals that specifically altered the activity of the \(\alpha_2\)-isoform did not affect the stimulation; (3) changes in [K\textsuperscript{+}], that affected activity of the \(\alpha_1\)-isoform, but not the \(\alpha_2\)-isoform, did not affect the stimulation; (4) myocytes from one group of guinea pigs expressed the \(\alpha_2\)-isoform but not the \(\alpha_3\)-isoform, and these myocytes did not show the stimulation. At 10 nM DHO, total I\textsubscript{p} increased by 35 \pm 10\% (mean \pm SD, n = 18). If one accepts the hypothesis that this increase is due to stimulation of just the \(\alpha_2\)-isoform, then activity of the \(\alpha_2\)-isoform increased by 107 \pm 30\%. In the guinea pig myocytes, nanomolar ouabain as well as DHO stimulated the \(\alpha_2\)-isoform, but both the stimulatory and inhibitory concentrations of ouabain were \sim 10-fold lower than those for DHO. Stimulation of I\textsubscript{p} by nanomolar DHO was observed in canine atrial and ventricular myocytes, which express the \(\alpha_2\)- and \(\alpha_3\)-isoforms of the Na/K pumps, suggesting the other high glycoside affinity isoform (the \(\alpha_2\)-isoform) also was stimulated by nanomolar concentrations of DHO. Human atrial and ventricular myocytes express all three isoforms, but isoform affinity for glycosides is too similar to separate their activity. Nevertheless, nanomolar DHO caused a stimulation of I\textsubscript{p} that was very similar to that seen in other species. Thus, in all species studied, nanomolar DHO caused stimulation of I\textsubscript{p} and where the contributions of the high glycoside affinity \(\alpha_2\) and \(\alpha_3\)-isoforms could be separated from that of the \(\alpha_1\)-isoform, it was only the high glycoside affinity isoform that was stimulated. These observations support early reports that nanomolar concentrations of glycosides stimulate Na/K pump activity, and suggest a novel mechanism of isoform-specific regulation of I\textsubscript{p} in heart by nanomolar concentrations of endogenous ouabain-like molecules.

KEY WORDS: cardiac electrophysiology • Na/K ATPase • cardiac glycosides

INTRODUCTION

The Na/K pump utilizes the energy stored in one ATP molecule to translocate three Na\textsuperscript{+} out of the cell and two K\textsuperscript{+} into the cell. Thus, the cycle generates a net outward current that tends to hyperpolarize the membrane voltage. Each Na/K pump comprises an \(\alpha\) and a \(\beta\) subunit, however, the \(\alpha\) subunit alone binds Na\textsuperscript{+} and K\textsuperscript{+} and possesses the ATPase activity. Three different isoforms (\(\alpha_1\), \(\alpha_2\), and \(\alpha_3\)) of the \(\alpha\) subunit are widely expressed in an organ-specific manner (Swadner, 1989), and recently Woo et al. (1999) identified a fourth isoform in testes.

We have reported previously guinea pig ventricular myocytes express two functionally distinct Na/K pumps: one with a high affinity for inhibition by dihydro-ouabain (DHO)* 1 \mu M DHO dissociation constant) and the other with a low affinity (100 \mu M DHO dissociation constant; Gao et al., 1995; for review see Mathias et al., 2000). This is consistent with the observations by others

\*Abbreviations used in this paper: DHO, dihydro-ouabain; ISO, isoproterenol; NE, norepinephrine; OUA, ouabain; PROP, propranolol.
(Mogul et al., 1989; Berrebi-Bertrand et al., 1991). We also have reported that mRNA for the α₁ and α₂ isoforms of the Na/K ATPase coexist in guinea pig ventricular myocytes (Gao et al., 1999a). Given the relative amounts of mRNA were consistent with the high and low DHO affinity currents, and since in rodent heart the α₂ isoform has a high affinity for ouabain (OUA) and the α₁ isoform has a low affinity (Sweadner, 1989), the α₂ and α₃ isoforms most likely represent the high and low DHO affinity pumps, respectively.

Our studies on regulation of these two isoforms in guinea pig ventricular myocytes (reviewed in Mathias et al., 2000) showed that transport by the α₂ isoform is increased by β-adrenergic activation but is unaffected by α-adrenergic activation. Conversely, transport by the α₁ isoform is modulated by β-adrenergic activation, but is not affected by α-adrenergic activation. Gao et al. (1995) showed that half-maximal activation of the α₂ isoform occurred at a K⁺ concentration of 0.4 mM, a concentration ~10-fold lower than that for the α₁ isoform (4 mM K⁺). In guinea pig myocytes, we have three markers that functionally separate the α₁ and α₂ isoforms: their affinity for DHO, their response to α- and β-adrenergic activation, and their response to changes in [K⁺].

Canine ventricular myocytes also have high and low DHO affinity Na/K pumps, but RNase protection assays indicate they express the α₁ isoform and α₂ isoform, which also has a high affinity for OUA (Maixent et al., 1996; Korth et al., 1997). Insofar as we have looked, the α₂ isoform in dog has the same functional properties as the α₂ isoform in guinea pig, however, our studies in dog are not as extensive as in guinea pig.

All of the three α isoforms (α₁, α₂, and α₃) of the Na/K pump are present in human heart (Shamraj et al., 1991; Zahler et al., 1993). However in the human, the affinities of these isoforms for OUA are nearly identical (Shamraj et al., 1993). The Na/K pumps of human atrial cells appear to share some of the functional properties we have determined for the α₂ isoform of the Na/K pump in guinea pig ventricle: they are stimulated by β-adrenergic activation, but are unaffected by α-adrenergic activation.

A number of studies have suggested that very low concentrations of cardiac glycosides can stimulate activity of the Na/K pumps in heart (for review see Noble, 1980). There are also reports of endogenous ouabain-like substances that are released at very low concentrations (Kolbel and Schreiber, 1996; Jortani and Valdes, 1997). This suggests the possibility of another isoform-specific regulatory input, coupled to endogenous ouabain-like substances. The purpose of the present study was to carefully characterize the effects of nanomolar [DHO] or [OUA] on the various isoforms of the Na/K pumps in heart cells from different species.

**MATERIALS AND METHODS**

Single cardiac myocytes were enzymatically isolated from adult male guinea pig hearts as previously described in Gao et al. (1992) or from mongrel dog hearts as described in Cohen et al. (1987). Guinea pigs, weighing 300–500 g, were killed with sodium pentobarbitone solution (1 ml of 390 mg ml⁻¹) by peritoneal injection, and adult mongrel dogs were killed with the same euthanasia solution but by intravenous injection. Human heart tissues were provided by the Department of Surgery and isolated into single cells following the procedures described in Cohen et al. (1987). The human tissue was a byproduct of surgery and obtained in accordance with an approved protocol in accordance with the National Institutes of Health guidelines. The isolated cells were stored in K⁺ solution (Isenberg and Klockner, 1982) containing the following (in mM): 83 KCl, 30 K₂HPO₄, 5 MgSO₄, 5 sodium pyruvic acid, 5 β-OH-butyric acid, 5 creatine, 20 taurine, 10 glucose, 0.5 EGTA, 2 KOH, and 5 Na₂ATP, pH 7.2.

An Axopatch 1A amplifier (Axon Instruments, Inc.) and the whole-cell patch-clamp technique were used to observe cell membrane current. Patch pipette resistances were 1–3 MΩ before sealing. The pipette solution contained the following (in mM): 70 sodium aspartic acid, 20 potassium aspartic acid, 30 CsOH, 20 TEACl, 5 HEPES, 1 CaCl₂, 10 glucose, 7 MgSO₄, and 5 Na₂ATP, pH 7.2. In the Na⁺-free pipette solution, sodium aspartic acid was replaced with the free acid of aspartic acid. In the experiments to examine the effects of α- and β-adrenergic agonists on Iₓ, 0.2 mM Na₂GTP was included in the pipette solution. The external Tyrode solution contained (mM) the following: 137.7 NaCl, 2.3 NaOH, 5.4 KCl, 1 MgCl₂, 10 glucose, 5 HEPES, 2 BaCl₂, and 1 CdCl₂, pH 7.4. In K⁺-free Tyrode solution, KC was deleted without ionic strength adjustment.

The heart cells were held at 0 mV after the formation of the whole-cell recording configuration. All experiments were conducted at 32 ± 0.5°C. External solutions containing various concentrations of DHO or ouabain (OUA) were superfused to observe changes in Na/K pump current (Iₓ). Based on our earlier work (for review see Mathias et al., 2000), current generated by the α₂ isoform is blocked with a dissociation constant of ~1 μM DHO, whereas that generated by the α₁ isoform is blocked with a dissociation constant of ~100 μM DHO. Thus, 5 μM DHO blocks most of the current generated by the α₂ isoform and almost none of the current generated by the α₁ isoform, so this concentration of DHO was used to separately assay activity of the α₂ isoform. Total Na/K pump activity due to the α₁ isoform plus the α₂ isoform was assayed as the current blocked by 1 mM DHO, 1 μM isoproterenol (ISO) plus 1 μM prazosin (PZ), or 10 μM noradrenaline (NE) plus 10 μM propranolol, were added to the external solution to study the effects of β- or α-adrenergic activation on Iₓ, respectively. All patch-clamp data were recorded on disc by the data acquisition program Axoscope 1.1 (Axon Instruments, Inc.), for later analysis. The sampling rate was 200 ms point, and the data were low pass filtered at 2 Hz. The paired t test was used to determine P values, with P < 0.05, indicating a significant difference between outcomes.

RNase protection assays were performed essentially as described previously (Gao et al., 1999a). For each experiment, 2 μg of total RNA was used. Cyclophilin probes were included in the hybridization reaction to confirm that the sample was not lost during the course of the experiment and to provide a standard for quantitative measurement of Na/K pump mRNA for each isoform. 5 μg of yeast tRNA was used as a negative control for probe self-protection bands. The RNase protection assay figures are all 4-d exposures. For the comparison of the α₁ and α₂ isoform mRNA levels in ventricle, the intensity of the specific protected signals were measured directly from RNase protection gels using
a Phosphor Imager (Molecular Dynamics). In this experiment, three independent samples of RNA were used.

RESULTS

As described in the INTRODUCTION, guinea pig ventricular myocytes coexpress the α2- and α2-isoforms of the Na/K pump (Gao et al., 1995). Since these two isoforms have an ∼100-fold difference in affinity for the cardiac glycosides, they can be studied separately by using 5 μM DHO to block the current generated by only the α2-isofrom (Ip), and using 1 mM DHO to block total current (IT) generated by the both isoforms. Total pump current is given by IT = Ip + Ip. With physiological pH and [K+]o, Ip is ∼60% of IT, but there is some cell to cell variation in this percent. There also is considerable cell to cell variation in myocyte size as well as in the density of total pumps per square centimeter of cell membrane. The original records displayed in this section reflect the variability in all of these parameters, so a wide range of current scales are used to optimally display each individual record. However, when we studied an effector of pump current, the standard protocol was to measure pump current in control, test conditions in the same cell, and then calculate the ratio of test to control current and average this ratio from at least five cells. This procedure uses each cell as its own control and removes the uncertainty due to cell to cell variation in size and pump density. In the data that follow, when we report an effect on the pump current, that effect was relative to control conditions in the same cell, and each effect was observed in 100% of the cells in which the protocol was completed. This procedure requires that each cell be held stably in the whole-cell patch configuration for time periods in excess of 10 min. The human heart cell data shown in Fig. 10 C are the only exception to the above protocol. After patch clamping these cells, they generally survived only a few minutes. Therefore, we made a quick measurement of cell capacitance at the beginning of each experiment, and this was used as a measure of cell size to normalize the subsequent measurements of IT.

Ip Is Stimulated by Nanomolar Concentrations of DHO in Guinea Pig Ventricular Myocytes

After whole-cell recording was initiated, a period of at least 5 min was required for the pipette and intracellular solutions to come to steady state (Gao et al., 1992). When steady state was achieved, different concentrations of DHO were superfused to observe the DHO-induced changes in holding current. In Fig. 1 A, when 5 μM DHO was applied rapidly, an inward shift in the holding current was observed, indicating inhibition of the current generated by the α2-isofrom of the Na/K pumps (Gao et al., 1999a; for review see Mathias et al., 2000). Upon washout of DHO, the holding current returned to its original level. Our first indication that nanomolar DHO might stimulate Ip was observed in the same cell, when 5 μM DHO was applied slowly. In this situation, the holding current experienced an initial outward transient (indicated by the arrow), which could not be attributed to any artifact, suggesting very low concentrations of DHO may stimulate the pumps. The same results were consistently observed in a total of eight cells, suggesting relatively low concentrations of DHO might have evoked the initial increase in holding current (i.e., as the solution containing 5 μM DHO was slowly perfused into the DHO-free bath, the initial [DHO] was much lower than 5 μM). Therefore, we used 10 nM DHO to investigate whether a steady state increase in outward current could be generated (Fig. 1 B). Upon washout of DHO, the holding current returned to its original level. However, when the external K+ was removed, 10 nM DHO did not induce any change in the holding current in the same cell. The same results were observed in a total of six cells, suggesting that the steady state increase in outward current by 10 nM DHO at 5.4 mM [K+]o could be due to stimulation of Ip.

The Na/K pump also requires intracellular Na+ for transport to take place. If the DHO effect is stimulation of Ip, then, if intracellular Na+ were removed, the 10 nM DHO-induced outward current should not occur. Therefore, we removed all Na+ from the pipette solution and observed the effect of 10 nM DHO on the holding current. In the top panel of Fig. 1 C, the patch pipette resistance was 1.5 MΩ, and the waiting period for the pipette and the intracellular solutions to come to steady state was 6 min. The application of 10 nM DHO did not induce any change in the holding current. A second exposure to a saturating concentration of 1 mM DHO (Gao et al., 1995) in the same cell also had no effect, indicating the Na/K pump current was eliminated by the removal of intracellular Na+. In a different cell, the pipette resistance was higher at 4 MΩ, and the waiting period before the application of 10 nM DHO was shorter at 5 min. Based on the data and analysis in Oliva et al. (1988), in this period of time, given this pipette resistance, some residual [Na+] should have been present. The same protocol as described above was applied. A relatively small outward shift in current by 10 nM DHO and a small inward shift by 1 mM DHO were observed, indicating a small amount of intracellular Na+ remained due to the higher resistance pipette and shorter waiting period. The remaining Na+ presumably activates a small fraction of total Na/K pump current. Therefore, the low [DHO] stimulation and the high [DHO] inhibition were present but smaller than normal (Fig. 1 C, middle). In another cell, the pipette resistance was 5 MΩ, and the waiting period was 4 min. A higher [Na+] should have remained, due to the further increase in pipette resistance and decrease...
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in waiting period. Therefore, a larger stimulation of $I_p$ and a larger $I_p$ blockade should be induced by low [DHO] and high [DHO], respectively, as shown in Fig. 1 C (bottom). In each experiment, the outward current shift (stimulation of $I_p$) by 10 nM DHO was about a third of the total $I_p$, supporting the hypothesis that the outward current evoked by low [DHO] is stimulation of $I_p$.

The application of vanadate inside of a cell has several effects (Akera et al., 1979; Takeda et al., 1980; Fox et al., 1983), including blockade of Na/K ATPase activity. Since we assay for $I_p$ by recording the change in current elicited by external DHO, a specific inhibitor of $I_p$, we can separate the effects of vanadate on $I_p$ from other effects. We included 1 mM sodium orthovanadate in the pipette solution to completely inhibit the Na/K pumps, and then performed a similar protocol to that described in Fig. 1 C. Fig. 1 D (left) shows that application of 10 nM DHO did not induce an increase in outward current, and 1 mM DHO did not inhibit any pump current. To ensure that this was not due to a

Thus, the outward shift in current at 10 nM DHO and blockade of $I_p$ at 1 mM DHO were highly correlated, providing further evidence that the outward current was due to stimulation of $I_p$. (D) Intracellular vanadate blocks both $I_p$ and the low DHO-dependent outward current. (left) When 1 mM sodium orthovanadate was included in the pipette solution, 10 nM DHO did not cause an outward shift in current and 1 mM DHO did not cause an inward shift in current, indicating inhibition of total $I_p$ also inhibited the low DHO stimulation of $I_p$. (right) Cells isolated from ventricles of the same heart show a typical stimulation and inhibition of $I_p$ by low and high [DHO], respectively, when vanadate was not in the pipette solution.

Figure 1. Stimulation of $I_p$ in guinea pig ventricular myocytes. (A) Slow superfusion of solution containing 5 pM DHO caused the bath concentration of DHO to slowly rise from 0 to 5 pM. This induced an initial outward transient in holding current (arrow) followed by the inward shift. The outward transient was not observed when 5 pM DHO was rapidly superfused. See Results for details. (B) The steady state increase in outward holding current induced by 10 nM DHO in the presence of 5.4 mM [K$^+$]o did not occur when the external K$^+$ was removed, suggesting the outward shift in current was due to stimulation of $I_p$. (C) Stimulation of $I_p$ was intracellular Na$^+$-dependent. (top) Intracellular Na$^+$ was completely removed after waiting 6 min in the whole-cell mode with a pipette resistance ($R_p$) of 1.5 MΩ. Neither the outward shift in current at 10 nM DHO nor inhibition of $I_p$ by 1 mM DHO was observed. (middle) In a second experiment, a small amount of Na$^+$ remained in the cell after waiting only 5 min with $R_p$ of 4 MΩ. A small shift in outward current at 10 nM DHO and a small inhibition of $I_p$ by 1 mM DHO were observed. (bottom) In a third experiment, a larger amount of Na$^+$ remained in the cell after waiting 4 min with $R_p$ of 5 MΩ. A larger outward shift in current and a larger blockade of $I_p$ were observed.
change in affinity for DHO, 2 mM DHO was applied, but $I_p$ was still not detectable. Similar results were observed in a total of five cells, suggesting that when $I_p$ was completely inhibited, low [DHO] could not induce the increase in outward current. However, in the absence of vanadate, the stimulation of $I_p$ by low [DHO] and the inhibition of $I_p$ by high [DHO] were observed in cells isolated from the same guinea pig hearts. Fig. 1 D (right) shows a holding current recording in the absence of vanadate. In this cell, the increase in outward current induced by 10 nM DHO was 24 pA, and $I_p$ inhibited by 1 mM DHO was 88 pA. Similar results were obtained in a total of five cells, thus, when $I_p$ was not blocked, the increase in outward current was observed. All of the results shown in Fig. 1 are consistent with the hypothesis that the low [DHO]-induced increase in outward current is due to stimulation of $I_p$. 

**Stimulation of $I_p$ by Nanomolar [DHO] Is Associated with the $\alpha_2$-Isoform and Not the $\alpha_1$-Isoform of the Na/K Pumps in Guinea Pig Ventricular Myocytes**

We have shown previously that $\beta$-adrenergic activation, through activation of PKA, specifically increases the current generated by the $\alpha_2$-isoform (for review see Mathias et al., 2000), either by increasing the number of pumps in the plasma membrane or by increasing the turnover rate of each pump. In either situation, if the stimulation of $I_p$ by nanomolar [DHO] is via the $\alpha_2$-isoform, the stimulation should increase in the presence of $\beta$-adrenergic activation. We examined the effects of $\beta$-adrenergic activation on the stimulation of $I_p$ by nanomolar [DHO].
gic activation with the specific β-agonist isoproterenol (ISO). Fig. 2 A shows the effect of ISO on the stimulation of I_p in the control solution (I_p(Con)) and that in the presence of ISO (I_p(ISO)) are 11 and 12 pA, respectively. The summary of the results from a total of five cells is shown in Fig. 2 B. The stimulation of I_p(Con) was normalized to 1. Then, the ratio I_p(ISO)/I_p(Con) in each cell was averaged to obtain the value 0.980.04 (SD), indicating ISO had no effect on the stimulation of I_p, suggesting the α_1-isoform is not involved.

We have also shown previously that α-adrenergic activation, through activation of PKC, specifically increases the current generated by the α_2-isoform (for review see Mathias et al., 2000), again either by increasing the number of pumps in the plasma membrane or by increasing the turn-over rate of each pump. In either situation, if the stimulation of I_p is via the α_2-isoform, α-adrenergic activation should increase it. We examined the effects of α-adrenergic activation with norepinephrine (NE) in the presence of the β-blocker propranolol (PROP). Fig. 2 C shows the effect of α-adrenergic activation on the stimulation of I_p. In this cell, the stimulation of I_p in control and that in the presence of NE + PROP are 7 and 11 pA, respectively. Fig. 2 D summarizes the results from a total of five cells. The stimulation of I_p in control was normalized to 1; in each cell, the ratio of the stimulation of I_p in the presence of NE + PROP to that in control was averaged to obtain the value 1.54 ± 0.11 (SD). Hence α-adrenergic activation increased the stimulation of I_p by nanomolar [DHO] but β-adrenergic activation had no effect. Based on our previous studies (Gao et al., 1999a; for review see Mathias et al., 2000), these results suggest that the stimulation of I_p by nanomolar [DHO] involves the α_2-isoform but not the α_1-isoform of the Na/K pump.

Gao et al. (1995) showed that the [K+]_o affinity of the α_2-isoform is much higher (K_{1/2} = 0.4 mM) than that of the α_1-isoform (K_{1/2} = 4 mM). Thus at 4 mM [K+]_o, α_2-isoform activity is at 0.92 of saturation, whereas α_1-iso-
form is at half-saturation. Hence, if $[K^+]_o$ is changed from 4 to 8 mM, $\alpha_1$-isoform activity will increase significantly, whereas $\alpha_T$-isoform activity will increase very little. If the stimulation of $I_P$ by nanomolar [DHO] is not via the $\alpha_1$-isoform, then changing $[K^+]_o$ from 4 to 8 mM will not change the stimulation of $I_P$ very much, but it will increase total $I_P$ by increasing $\alpha_T$-isoform activity. Therefore, two predictions emerge: (1) in each cell, the ratio of the stimulation of $I_P$ in 8 to 4 mM $[K^+]_o$ will be $\sim 1.04$, given the average numbers presented in Gao et al. (1995); (2) in each cell, the ratio of total $I_P$ in 8 to 4 mM $[K^+]_o$ will be $\sim 1.4$, again based on average numbers presented in Gao et al., 1995. Fig. 3 A shows an example of the protocol. In this cell, at 4 mM $[K^+]_o$, the stimulation of $I_P$ by 10 nM DHO was 26 pA, and the total $I_P$ indicated by 1 mM DHO was 71 pA. In the same cell when the external solution contained 8 mM $[K^+]_o$, the stimulation of $I_P$ was 27 pA and total $I_P$ indicated by 1 mM DHO was 96 pA. Thus, the ratio of the stimulation at 8 to 4 mM $[K^+]_o$ was 1.04, whereas the ratio of total $I_P$ in 8 to 4 mM $[K^+]_o$ was 1.35. Fig. 3 B summarizes the results from a total of five cells. The ratio of $I_P$ stimulation in 8 to 4 mM $[K^+]_o$ was $1.04 \pm 0.08$, and the ratio of total $I_P$ increased in all cells by an average value of $1.39 \pm 0.20$. These results are consistent with the predictions and further support the hypothesis that stimulation of $I_P$ is not associated with the $\alpha_1$-isoform of the Na/K ATPase.

Perhaps the best evidence that the $\alpha_T$-isoform is not involved in the stimulation of $I_P$ occurred serendipitously owing the adaptability of biological systems. During the summer, ventricular myocytes from guinea pigs ceased expressing the stimulation of $I_P$. Using the hearts from these animals, we conducted parallel studies on the stimulation of $I_P$ and RNase protection assays for mRNA levels of both the $\alpha_1$- and $\alpha_T$-isoforms. A piece of the left ventricle was removed and prepared for RNase protection assays, and then the rest of the heart was used to isolate single ventricular myocytes for measurement of $I_P$ by the patch-clamp technique.

Figure 4. RNase protection assays indicate the lack of $\alpha_2$-isoform in guinea pig ventricular myocytes that lacked the stimulation of $I_P$. mRNA for the $\alpha_1$, $\alpha_T$, and $\alpha_T$-isoforms of the Na/K pump are abundantly expressed in guinea pig brain. In contrast, the $\alpha_T$-isoform is the dominant transcript in guinea pig ventricle. The $\alpha_T$-isoform is present at very low levels, just $6 \pm 4\%$ (SD) of the total Na/K pump mRNA based on quantification from three different samples (compared with $18\%$ in normal samples). No $\alpha_T$-isoform mRNA was detected from any guinea pig heart sample.

Figure 5. PMA, an activator of the $\alpha_T$-isoform, had no effect on $I_P$ in guinea pig cardiac myocytes lacking mRNA for the $\alpha_T$-isoform. (A) An original holding current record showing the experimental protocol. $I_P$ was indicated by the holding current shift induced by 1 mM DHO. In this sample, $I_P$(Con) is 284 pA, and $I_P$(PMA) is 276 pA. (B) The averaged results from five cells. The pump currents measured in different conditions were normalized to $I_P$(Con). Thus, $I_P$(Con) = 1, and the normalized $I_P$(PMA) is $1.02 \pm 0.09$ (P = 0.46).
No α₂-isofrom mRNA was detected from any guinea pig heart sample. At 6% α₂-isofrom, our patch-clamp method is probably beyond its limit of resolution. This analysis assumes a linear relationship between [mRNA] and plasma membrane protein and equal maximum turnover rates for both pump types.

We previously reported that α-adrenergic stimulation of $I_p$ is only coupled to the α₂-isofrom of the Na/K pump through a PKC-mediated pathway (Wang et al., 1998; Gao et al., 1999a,b). If the α₂-isofrom is not present in these guinea pig ventricular myocytes, activation of PKC should not have any effect on $I_p$. We also reported PMA has a larger effect than norepinephrine on $I_p$ (Wang et al., 1998; Gao et al., 1999c). Therefore, we used PMA as the activator of PKC to examine its effect on $I_p$. Fig. 5 A shows a typical record of the effect of PMA on $I_p$ in cells lacking the α₂-isofrom of the Na/K pump. In these experiments, $I_p$ was measured by application of 1 mM DHO, which is essentially saturating (Gao et al., 1995). In this sample, $I_p$ in control ($I_p$(Con)) is 284 pA, and $I_p$ in the presence of PMA ($I_p$(PMA)) is 276 pA. Fig. 5 B shows the averaged results from a total of five cells. $I_p$(Con) was defined as 1. In each cell the ratio $I_p$(PMA)/$I_p$(Con) was calculated and averaged to obtain the value 1.02 ± 0.09 (SD), indicating PMA had no effect on $I_p$ in these heart cells. These results are consistent with our previous conclusion that PKC is coupled specifically to the α₂-isofrom of the Na/K pumps in guinea pig myocytes, and they reinforce the conclusions based on Fig. 2 (C and D).

**A Two-site DHO-binding Model for Stimulation and Inhibition of $I_p$**

To characterize the stimulation of $I_p$, we measured the DHO-sensitive currents as [DHO] ranged from $10^{-9}$ to $10^{-3}$ M. Then, a DHO dose–response curve was constructed. Fig. 6 A shows the protocol for measuring stimulation of $I_p$ by low [DHO] and the inhibition of $I_p$ by high [DHO]. In this cell, seven different concentrations of DHO from $10^{-11}$ to $10^{-3}$ M were applied, and then DHO was washed out. The DHO-sensitive currents ($\Delta I_p$, stimulation, or inhibition), were normalized to the max-

![Graph](image-url)

**Figure 6.** $\Delta I_p$-[DHO] relation in guinea pig ventricular myocytes. (A) An original record of holding current showing the protocol for observing stimulation of $I_p$ by low [DHO] and inhibition of $I_p$ by high [DHO]. In this cell, seven different concentrations of DHO from $10^{-11}$ to $10^{-3}$ M were applied. The DHO-sensitive currents indicated the change in $I_p$ ($\Delta I_p$). (B) The $\Delta I_p$-[DHO] curve. The normalized $\Delta I_p$ at each point was averaged from at least five cells, and the bars indicate SD (see results for normalization method). The points above the zero level (the dotted straight line) represent statistically significant increases in the holding current with $P$ values of $6 \times 10^{-3}$, $5 \times 10^{-5}$, $10^{-7}$, $5 \times 10^{-6}$, and $2 \times 10^{-5}$, respectively.
imal value of \( \Delta I_p \) obtained in the same cell by total pump inhibition on application of 1 mM DHO. The basal \( \Delta I_p \) before any application of DHO was defined as zero (Fig. 6 A, see dotted line at a holding current of 100 pA). When the DHO-induced current shifted above the basal level, due to stimulation of \( I_p \) by low [DHO], we assigned \( \Delta I_p \) a positive value. If the induced current shifted below the basal level, due to the inhibition of \( I_p \) by high [DHO], we assigned \( \Delta I_p \) a negative value. Since 1 mM DHO is a saturating concentration that completely blocks \( I_p \) (Gao et al., 1995), we defined the \( \Delta I_p \) induced by 1 mM DHO as \(-1\). In this cell, the maximum stimulation was \( \sim 20\% \) of total \( I_p \), whereas the average maximum stimulation shown in Fig. 6 B was \( 35 \pm 10\% \).

Fig. 6 B shows the \( \Delta I_p-[\text{DHO}] \) curve. The normalized \( \Delta I_p \) at each point is averaged from at least five cells, and error bars indicate SD. The points above the zero level indicate stimulation of \( I_p \), and the points below the zero level indicate inhibition of \( I_p \). The maximal stimulation occurred at \( \sim 10 \) nM DHO, and its percentage increase in total Na/K pump current (\( I_{pT} \)) is \( 35 \pm 10\% \) (SD, \( n = 8 \)).

A two-site binding model was developed to interpret our data (see APPENDIX). In guinea pig ventricular myocytes, \( I_{pT} \) is the sum of the high DHO affinity \( I_p \) contributed by the \( \alpha_2 \)-isoform (\( I_{p2} \)) and the low DHO affinity \( I_p \) contributed by the \( \alpha_1 \)-isoform (\( I_{p1} \)). Since only the \( \alpha_2 \)-isoform seems to be involved in the stimulation of \( I_p \) by low concentrations of DHO, the parallel model was described by the following equation (see APPENDIX for derivation),

\[
\Delta I_{pT} = f_2 \frac{kD(K^*_2 - D(D + K^*_2))}{(D + K^*_2)(D + K^*_1)} - f_1 \frac{D}{D + K^*_1},
\]

In this equation, \( k \) is the increase in \( I_{p2} \) when DHO is bound to the stimulatory site; \( K^*_2 \) and \( K^*_1 \) are the dissociation constants of the stimulatory and inhibitory DHO-binding sites on the \( \alpha_2 \)-isoform, respectively; and \( K_i \) is the dissociation constant for the inhibitory DHO-binding site on the \( \alpha_1 \)-isoform. The symbols \( f_2 \) and \( f_1 \) represent the fractions of \( I_{pT} \) due to \( I_{p2} \) and \( I_{p1} \).

Eq. 1 was used to fit our \( \Delta I_p-[\text{DHO}] \) data in Fig. 6 B. The values of \( K^*_2 \) and \( K^*_1 \) obtained by the curve fitting are 2.1 nM and 0.15 \( \mu \)M, respectively, and \( k = 1.32 \). Since \( I_{p1} \) is not involved in the stimulation of \( I_p \), the value of \( K_i \) is fixed at 72 \( \mu \)M, which was the number we previously reported (Gao et al., 1995). The values of \( f_2 \) and \( f_1 \) determined by the fitting are 0.35 and 0.65, respectively, which are the same as those reported in Gao et al. (1995). At 10 nM [DHO], the best-fit maximum stimulation of \( I_{p2} \) was 107%.

Fig. 7 compares the \( \Delta I_p-[\text{DHO}] \) curve in Fig. 6 with that recorded in cells isolated from the guinea pig hearts without the \( \alpha_2 \)-isoform. Data were collected as described in Fig. 6 A. Each point was averaged from at least five cells. No stimulation of \( I_p \) was observed even at 10 nM DHO in the guinea pig hearts lacking the \( \alpha_2 \)-isoform. The curve fitting indicates only \( I_{p1} \) (\( \alpha_1 \)-isoform) was present in these cells. The value of \( K_i \) given by the fitting was 74 \( \mu \)M, which is almost identical to the value (72 \( \mu \)M) we reported previously (Gao et al., 1995). These results suggest that when the \( \alpha_2 \)-isoform is absent, there is no stimulation of \( I_p \), and strengthen the suggestion that the stimulation of \( I_p \) involves only the \( \alpha_2 \)-isoform, and not the \( \alpha_1 \)-isoform.

![Figure 7. \( \Delta I_p-[\text{DHO}] \) curve in guinea pig ventricular myocytes lacking the \( \alpha_2 \)-isoform. The open circles and the dashed smooth curve are the same ones presented in Fig. 3 B, indicating the \( \Delta I_p-[\text{DHO}] \) curve with two isoforms (\( \alpha_1 \) and \( \alpha_2 \)). The closed circles and the solid smooth curve define the \( \Delta I_p-[\text{DHO}] \) curve lacking the \( \alpha_2 \)-isoform. Stimulation of \( I_p \) was not observed in the guinea pig hearts lacking the \( \alpha_2 \)-isoform. The curve fitting indicates only the \( \alpha_1 \)-isoform was present in these cells.](image)
Ouabain Stimulates the Na/K Pump at Lower Concentrations than DHO

Our studies with DHO, chosen for its rapid binding and unbinding, showed that low concentrations (10^-9 to 10^-7 M) resulted in an increase in outward current. However, they did not demonstrate whether this “pump stimulation” was a specific action of DHO or a more general property of a class of compounds. We therefore performed similar studies with ouabain (OUA). The results of these studies are provided in Fig. 8 (A and B). A sample of the protocol is provided in Fig. 8 A. An increase in outward current is observed in response to application of 10^-10 M OUA. The maximum outward current is observed at 1 nM (a concentration about an order of magnitude lower than the [DHO] required for its maximal stimulatory effect). A smaller outward current shift is observed at 10^-8 M OUA. On application of 5 x 10^-4 M, there is a large inward shift in holding current corresponding to total inhibition of the Na/K pump. When OUA is washed out, there is a slow return to the original holding current.

The results of all our experiments with OUA are summarized in Fig. 8 B. The figure demonstrates that application of OUA at low concentrations (10^-10 to 10^-8 M) results in an increase in outward current (pump stimulation), whereas higher concentrations result in an inward current shift (pump inhibition). Maximum inhibition is achieved at 10^-4 M. The smooth curve is the fit of the two-site model presented in the appendix to all our OUA data. The K_i’s for both the inhibitory and stimulatory sites of the high affinity pumps (I_P2), and for the low affinity pump (I_P1) are about an order of magnitude higher affinity than for DHO (parameters provided in Fig. 8 legend).

The α-Isiform of the Na/K Pump Also May Be Stimulated by Nanomolar [DHO]

It has been reported that canine cardiac myocytes contain two distinct molecular forms (α and α+).
Na/K ATPase catalytic subunit (Maixent et al., 1987). Of these two isoforms, [3H]ouabain-binding measurements and Na/K ATPase assays indicated $\alpha_2$ has a 150-fold higher affinity for ouabain than $\alpha$. Maixent and Berrebi-Bertrand (1993) and Zahler et al. (1996) reported that dog left ventricle expresses $\alpha_1$ and $\alpha_3$-isoforms, but no detectable $\alpha_2$-isoform of the Na/K pumps. RNase protection assays (unpublished data) also indicated that the $\alpha_1$- and $\alpha_3$-isoforms but not the $\alpha_2$-isoform of the Na/K pumps are present in canine ventricle. To examine if nanomolar [DHO] can stimulate the $\alpha_2$-isoform, we investigated the effects of low concentrations of DHO on $I_p$ in canine cardiac myocytes. Fig. 9 shows stimulation of $I_p$ by low concentrations of DHO and inhibition of $I_p$ by a high [DHO] in canine atrial (Fig. 9 A), ventricular epicardial (Fig. 9 B), and ventricular endocardial (Fig. 9 C) cells. Similar observations were obtained from at least five cells from each region.

Figure 9. Stimulation of $I_p$ in canine cardiac myocytes. Stimulation of $I_p$ by low concentrations of DHO ($10^{-9}$, $10^{-8}$, and $10^{-7}$ M) and the inhibition of $I_p$ by a high concentration of DHO ($10^{-4}$ M) were observed in canine atrial (A), ventricular epicardial (B), and ventricular endocardial (C) cells. The dotted lines indicate the holding current in the absence of DHO. Similar results were obtained from at least five cells from each region.

Stimulation of $I_p$ in Human Heart Cells

Three $\alpha$-isoforms ($\alpha_1$, $\alpha_2$, and $\alpha_3$) of the Na/K pump are reported to be present in human heart (Shamraj et al., 1991; Zahler et al., 1993). Thus, stimulation of $I_p$ by low concentrations of cardiac glycosides also may occur in human heart cells, but there have been no studies on this effect. Therefore, we examined the effect of low [DHO] on $I_p$ in human heart cells. Fig. 10 (A and B) shows original current recordings...
indicating stimulation of $I_p$ by low [DHO] and the inhibition of $I_p$ by high [DHO] are indeed present in human ventricular and atrial myocytes, respectively. Similar results were observed in eight cells in each cell type. The effects of $\alpha$- and $\beta$-adrenergic activation on $I_p$ in the human atrial cells were investigated using the same protocols described for Fig. 2. In Fig. 10 C, $I_p$ was normalized to the membrane capacitance. $I_p$ averaged from 12 cells in the control is $0.29 \pm 0.06 \text{ pA/pF (SD)}$, and $I_p$ averaged from 8 cells in the group of ISO treatment ($\beta$-adrenergic activation) was $0.29 \pm 0.04 \text{ pA/pF}$, suggesting no difference between control and ISO-treated cells. However, $I_p$ from 13 cells in the group of NE-treatment ($\alpha$-adrenergic activation) was $0.36 \pm 0.06 \text{ pA/pF}$, indicating a significant increase over control ($P < 0.01$). These results suggest that the Na/K pump current in human atrial cells has similar properties to the $\alpha_2$-isoform in guinea pig ventricle.

Fig. 10 D shows the $\Delta I_p$-DHO curve from human atrial cells. Data were collected and normalized as described in Fig. 6A. Each point was averaged from at least five cells. The points above the zero level indicate stimulation of $I_p$, and those below the zero level indicate inhibition of $I_p$. The fully blocking concentration of DHO for the human atrial cells was $10^{-10}$ M. As in guinea pig ventricular myocytes, the maximal stimulation of $I_p$ occurred at $\sim10$ nM DHO, and the percent increase in $I_p$ is $29 \pm 6\%$ (SD, $n = 8$). The equation to fit these data is the same as that used in guinea pig ventricular myocytes, but the term for $I_{p1}$ was omitted,
since all isoforms of the Na/K pumps in human heart have a relatively high affinity for DHO. This does not imply the α1-isoform of the Na/K pump is not present. It simply means the α1-isoform cannot be distinguished using DHO-binding assays. K+ and K− are the dissociation constants of the stimulatory and inhibitory binding sites, respectively. The values of K+ and K− given by the curve fitting are 0.84 nM and 1.7 μM, which are similar to those observed for the α2-isoform of guinea pig ventricular myocytes.

**Discussion**

We have presented evidence for stimulation of IP by low concentrations of glycosides in human (atrial and ventricular), canine (endocardial and epicardial), and guinea pig ventricular myocytes. An increase in outward current was observed at concentrations of DHO ranging from 1 to 100 nM. The increase did not occur when IP was blocked by removal of either external K+ or intracellular Na+, or addition of intracellular vanadate, suggesting the Na/K pumps generated the current. The maximum stimulation occurred at 10 nM DHO and ranged between 29 and 35% of total pump current, suggesting the Na/K pumps generated the current. The actual stimulation provided a strategy to identify which isoforms of the Na/K pump is stimulated by low concentrations of glycoside on IP. The pipette solution contained 80 mM Na+ to saturate the Na+ -binding sites of the Na/K pump, so changes in [Na+]o on the order of 10 mM would have little effect on Na/K pump current. To minimize K+ concentration changes in the T-system lumen, K+ conductance was blocked with 20 mM TEA+ and 30 mM Cs+ in the pipette solution, and 2 mM Ba2+ in the bath. We also added 1 mM Gd3+ in the external Tyrode solution to block the L-type Ca2+ channels and the Na/Ca exchanger. The heart cells were held at 0 mV, where the IP-voltage curve reaches its maximum, and where the cell membrane resistance is higher than at diastolic potentials, so the ratio of signal to noise was increased. In our experimental conditions, the increase in outward current induced by low concentrations of DHO was observed, and did not occur when IP was inhibited with removal of either extracellular K+ or intracellular Na+, or when vanadate was included in the pipette solution, suggesting low concentrations of glycoside indeed stimulate IP.

Studies using molecular biological techniques have demonstrated the Na/K pump is a multigene family of proteins (for reviews see Sweadner, 1989; Geering, 1990). To date, four α-isoforms of the Na/K pump have been found. Of the α-isoforms, α1 and α3 have a higher affinity for ouabain than α2. More recently, we reported that α1- and α2-isoforms coexist in guinea pig ventricle (Gao et al., 1999a), where they probably function as the low DHO affinity and the high DHO affinity pumps (Mogul et al., 1989; Gao et al., 1995). We also reported isoform-specific regulation of the Na/K pump by α- and β-adrenergic agonists. The α1-isoform could be stimulated or inhibited by the β-agonist ISO, depending on [Ca2+]o, but it is insensitive to α-adrenergic activation, whereas the α2-isoform is stimulated by α-adrenergic activation but is insensitive to β-adrenergic agonists (Gao et al., 1999a). This isoform-specific regulation provided a strategy to identify which α-isoform(s) of the Na/K pump is stimulated by low concentrations of glycoside. In guinea pig ventricular myocytes, stimulation of IP is increased by α-adrenergic activation but insensitive to β-adrenergic activation, the same as in IP2. In some guinea pig hearts, expression of the α2-isoform was dramatically decreased, and so was the stimulation of IP. These data suggest that only the α2-isoform is involved in the stimulation of IP in guinea pig heart. Moreover, the occasional loss of expression of the α2-isoform may explain why stimulation of IP was not observed in some studies (Kasturi et al., 1997). We observed stimulation of IP with OUA as well as DHO, suggesting that DHO is not unique in this action. The dissociation constants for stimulation and inhibition for OUA were about an order of magnitude higher affinity than those for DHO. We also observed stimula-
tion of $I_p$ in canine ventricular myocytes. Previous reports (Maixent et al., 1987; Maixent and Berrebi-Bertrand, 1993; Zahler et al., 1996) and our present study have found the $\alpha_1$- and the $\alpha_2$-isoforms of the Na/K pump coexist in canine heart cells. If the $\alpha_1$-isoform is insensitive to nanomolar DHO, as in guinea pig ventricle, the $\alpha_2$-isoform must be the source of the stimulation of $I_p$ in canine ventricle.

Our results from human heart cells have some ambiguities that make it uncertain which $\alpha$-isoform(s) are stimulated by nanomolar DHO. We do not know which isoforms were present in these cells or the regulatory paths for the different isoforms. Moreover, the dissociation constants for inhibition by DHO are similar for the three isoforms (Shamraj et al., 1993), so we could not functionally separate the responses of the high versus low DHO affinity pumps. Zahler et al., 1993, reported the $\alpha_1$-isoform mRNA in normal human left ventricle is 62.5% $\alpha_1$, 15% $\alpha_2$, and 22.5% $\alpha_3$. However, Allen et al. (1992) and Shamraj et al. (1993) reported proportions of the $\alpha_2$- and $\alpha_3$-isoforms that were double those reported by Zahler. The cells we used were from the atrial tab of diseased hearts, and Zahler et al. (1993) also reported there was an increase in $\alpha_3$-isoform in the failing heart. However, other studies in diseased hearts reported a decrease in [3H]ouabain binding (Shamraj et al., 1993; Ellingsen et al., 1994; Bundgaard and Kjeldsen, 1996; Larsen et al., 1997), suggesting a reduction in the expression of total Na/K pumps. Given these diverse results, it is not possible to make a reasonable guess on the isoform composition of the cells we used. And given the similar dissociation constants for inhibition by DHO, we could not even estimate the fraction of $\alpha_1$-isoform. The stimulation of $I_p$ observed in these heart cells might be due to the $\alpha_2$- and the $\alpha_3$-isoforms. The maximal stimulation of total $I_p$ recorded in Fig. 10 D was $\sim30\%$, which is a much smaller effect than the 107% increase in $I_{p2}$ in guinea pig and, therefore, consistent with the presence of a significant amount of $\alpha_3$-isoform that is insensitive to nanomolar DHO. The results in Fig. 10 C indicated a 24% increase of total $I_{p1}$ by $\beta$-adrenergic activation, which is a much smaller effect than the 38% increase in $I_{p2}$ observed in guinea pig ventricular myocytes (Gao et al., 1999a), which again is consistent with the presence of significant $\alpha_1$-isoform that is insensitive to $\alpha$-adrenergic activation. However, the results in Fig. 10 C also indicate that $\beta$-adrenergic activation had no effect on total $I_{p1}$, which is not consistent with the presence of $\alpha_1$-isoform, unless regulation in human atrium differs from that in guinea pig ventricle. More detailed information will only be available with heterologous expression as in the studies of Crambert et al. (2000).

The mechanism of the stimulation of $I_p$ by low concentrations of glycosides is not well understood. Hougen et al. (1981) observed an increase in $\text{Rb}^+$ uptake induced by nanomolar ouabain in guinea pig left atria. This stimulation of the Na/K pump was prevented by the $\beta$-adrenergic antagonist propranolol, by depletion of endogenous norepinephrine with either reserpine or 6-hydroxydopamine, or by pretreatment with $\beta$-adrenergic agonists. Other results indicated OUA promoted the release of endogenous norepinephrine from sympathetic nerve endings in intact tissue as well as inhibiting norepinephrine uptake (Seifen, 1974; Harvey, 1975). Therefore, Hougen et al. (1981) concluded that the stimulatory effect of low concentrations of OUA on the Na/K pump is mediated, at least in part, by $\beta$-adrenergic effects of endogenous catecholamines released from nerve terminals. However, we used isolated single heart cells, instead of heart tissue, and nerve terminals were absent. When the $\beta$-blocker propranolol was added to the bath, the stimulation was still present in our isolated cells. Therefore, the endogenous catecholamine release mechanism cannot explain stimulation of $I_p$ in the present study. Furthermore, in our experimental conditions, high $[\text{Na}^+]$, saturated the Na$^+$-binding sites of the Na/K pump. Moreover, K$^+$ conductance was significantly reduced by Cs$^+$, TEA$^+$, and Ba$^{2+}$. In these conditions, stimulation of $I_p$ is unlikely to be explained by a secondary effect of DHO. Our results suggest that the stimulation of $I_p$ is a direct action of low concentrations of glycosides on the cardiac Na/K pump, and is only associated with the $\alpha_2$- and the $\alpha_3$-isoforms.

In conclusion, we observed stimulation of $I_p$ in myocytes from guinea pig, canine, and human hearts. This stimulation appears to be a direct action of low concentrations of glycosides, and it is most likely coupled to only the $\alpha_2$- and $\alpha_3$-isoforms of the Na/K pump. Further studies are necessary to understand the molecular basis of the stimulation and its functional significance. It could represent another mechanism of isoform-specific regulation, in this instance by endogenous glycoside-like compounds (Kolbel and Schreiber, 1996; Jortani and Valdes, 1997). Finally, we do not know the relationship, if any, of this stimulation to the inotropic effect of cardiac glycosides.

APPENDIX

A Two Binding-site Model for DHO Stimulation and Inhibition of the Cardiac Na/K Pump

We do not have enough data to generate a unique model for our observations, however, it is useful to have a quantitative framework to store and easily recreate our observations. The simplest model that fits our data and is consistent with other observations assumes two binding sites for DHO: (1) a high affinity stimulatory site (R$^+$) and (2) a low affinity inhibitory site (R$^-$). Because the binding data suggest only 1 DHO per pump protein, we assume R$^+$ and R$^-$ are competitive for
DHO. Moreover, ATPase assays indicate total blockade by high concentrations of DHO, so we also assume that when R\(^+\) is filled, R\(^-\) is available, but once R\(^-\) is filled, R\(^+\) releases the bound DHO, if present, and becomes unavailable for binding.

\[
[DHO] + R^- \xrightleftharpoons{\alpha^-}{\beta^+} P^-
\]  

**(SCHEME I)**

\[ R^- = 1 - P^- , \]

where D is the DHO concentration, and P\(^-\) is the inhibited pump. Thus,

\[
P^- = \frac{[DHO]}{[DHO] + K} \times \frac{K^-}{[DHO] + K} \]

\[ K^- = \beta^- / \alpha^- . \]

When R\(^+\) is occupied by DHO, the pump is stimulated, i.e.,

\[
[DHO] + R^+ \xrightleftharpoons{\alpha^+}{\beta^-} P^+
\]  

**(SCHEME II)**

P\(^+\) is the stimulated pump. The R\(^+\) site is not available in those pumps with the R\(^-\) site filled, hence,

\[
R^+ = 1 - P^+ - P^- 
\]

\[
P^+ = \left( \frac{[DHO]}{[DHO] + K} \right) \times \left( \frac{K^-}{[DHO] + K} \right) \]

\[ K^+ = \beta^+ / \alpha^+ . \]

Then,

\[
I_p = I_{max}(kP^+ + P) ,
\]

where I\(_p\) is pump current, I\(_{max}\) is the pump current at zero [DHO], k is the increase in current by each pump when R\(^+\) is bound to DHO, and P is the fraction of unbound pumps, P = 1 - P\(^+\) - P\(^-\). The normalized change in pump current is therefore:

\[
\Delta I_p = \frac{I_p - I_{max}}{I_{max}}
\]


The above equation was used in Fig. 6 to fit the DHO effects on the \(\alpha_2\)-isoform.

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