The Rate of Activation by Calmodulin of Isoform 4 of the Plasma Membrane Ca\(^{2+}\) Pump Is Slow and Is Changed by Alternative Splicing

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A reconstitution system allowed us to measure the ATPase activity of specific isoforms of the plasma membrane Ca\(^{2+}\) pump continuously, and to measure the effects of adding or removing calmodulin. The rate of activation by calmodulin of isoform 4b was found to be very slow, with a half-time (at 235 mM calmodulin and 0.5 µM free Ca\(^{2+}\)) of about 1 min. The rate of inactivation of isoform 4b when calmodulin was removed was even slower, with a half-time of about 20 min. Isoform 4a has a lower apparent affinity for calmodulin than 4b, but its activation rate was surprisingly faster (half time about 20 s). This was coupled with a much faster inactivation rate, consistent with its low affinity. A truncated mutant of isoform 4b also had a more rapid activation rate, indicating that the downstream inhibitory region of full-length 4b contributed to its slow activation. The results indicate that the slow activation is due to occlusion of the calmodulin-binding domain of 4b, caused by its strong interaction with the catalytic core. Since the activation of 4b occurs on a time scale comparable to that of many Ca\(^{2+}\) spikes, this phenomenon is important to the function of the pump in living cells. The slow response of 4b indicates that this isoform may be the appropriate one for cells which respond slowly to Ca\(^{2+}\) signals.

Unlike other mechanisms for removing Ca\(^{2+}\) from the cytosol, the plasma membrane Ca\(^{2+}\) pump requires activation by another protein, calmodulin. The requirement for this extra step may have profound effects on the shape of Ca\(^{2+}\) spikes, particularly if the binding of calmodulin to the pump is slow. The activation by calmodulin of several calmodulin-regulated enzymes is fast, but it has been observed that the activation of the plasma membrane Ca\(^{2+}\) pump is slow in human erythrocytes (1). Since the binding of calmodulin to the plasma membrane Ca\(^{2+}\) pump is very tight, this slowness in the activation was surprising. Erythrocytes contain a mixture of isoforms 1 and 4 of the plasma membrane Ca\(^{2+}\) pump (2), so it was not clear which isoform was responsible for the slow activation. It was possible to study the rate of activation in erythrocytes because almost all of their Ca\(^{2+}\)-stimulated ATPase activity is due to the plasma membrane Ca\(^{2+}\) pump, but extension of such studies to other cell types has been difficult. The major difficulty is the presence in almost all kinds of cells of non-pump Ca\(^{2+}\) ATPases whose activity swamps that of the pump.

Some studies using Ca\(^{2+}\) indicators in whole cells other than erythrocytes have given results consistent with slow activation of the pump. In human neutrophils (3) it was concluded that a Ca\(^{2+}\) spike was caused by delayed activation of the plasma membrane Ca\(^{2+}\) pump. In this case the arguments were based in part on the use of a calmodulin antagonist, which is rather nonspecific. In vascular endothelial cells (4) a similar conclusion was based on the use of La\(^{3+}\), VO\(_4\)\(^{2-}\), and Hg\(^{2+}\) as inhibitors of the plasma membrane Ca\(^{2+}\) pump. Each of these reagents also inhibits other pumps and enzymes. In T cells (5), a similar conclusion was based on the behavior of the cells when the SERCA pump and the mitochondria were inhibited by specific inhibitors. Their conclusions were based on the assumption that the plasma membrane Ca\(^{2+}\) pump was the only remaining mechanism for Ca\(^{2+}\) efflux. It is evident from these studies that, since no highly specific inhibitor exists for the plasma membrane Ca\(^{2+}\) pump, complex experiments and reasoning are required to make a conclusion about the role of this pump in most kinds of whole cells.

In the present study, we have developed and applied a system to investigate the rate of activation by calmodulin of specific isoforms of the pump in a reconstituted system. This system overcomes the problem of non-pump ATPases, since their amount is drastically reduced by the reconstitution procedure. Using this system, we have measured the rate of activation of the pump on specific isoforms and constructs expressed in COS cells. The results show that isoform 4b of the pump has a very slow activation by calmodulin, which is partly due to the presence of the downstream inhibitory region of this pump. We show that isoform 4a is more rapidly activated than 4b; this result was surprising, since isoform 4a has a lower apparent affinity for calmodulin than 4b. We also show that the rate of dissociation of calmodulin from isoform 4b is even slower than the association rate, with a half-time of tens of minutes.

MATERIALS AND METHODS

Transfection—Transfection was carried out using LipofectAMINE (Life Technologies, Inc.) based on the protocol as described by the manufacturer and by Enyedi et al. (6). Briefly, transfection was initiated when the cells were 70 to 80% confluent in 150-cm\(^2\) flasks. The cells were incubated at 37 °C with the DNA-LipofectAMINE complex (formed by incubating 8 µg of DNA and 100 µl of LipofectAMINE in 3.6 ml of serum-free Opti-MEM medium) in 14.5 ml of serum-free Opti-MEM medium. After 5 h incubation, the cells were supplemented with serum and incubation continued for a total of 24 h. The medium con-

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In this equation, $Y_i$ is the initial optical density, $r_i$ is the rate of optical density increase for the enzyme alone, $r_c$ is the rate of optical density increase for the enzyme-calmodulin complex, and the integral is evaluated between zero and $t$. Substitution of Equation 3 in Equation 4 and integration results in the expression used to fit the data for activation of the enzyme, 

$$Y(t) = Y_i + \frac{r_c}{k} + \frac{r_c}{k_c} \left(1 - \exp(-k_c t)\right)$$  

(Eq. 6)

Following the same kind of derivation, we obtain an equation corresponding to Equation 5, but for the inactivation of the enzyme by calmodulin dissociation,

$$Y(t) = Y_i + v_c t + \left(\frac{r_c}{k} + \frac{r_c}{k_c}\right) \left(1 - \exp(-k_c t)\right)$$  

(Eq. 7)

Equations 5 and 7 were fitted to the experimental data using GraphPad Prism™ from GraphPad Software.

Calculation of First Derivative Curves—Direct numerical calculation of a first derivative from data leads to immense amplification of even small amounts of noise in the data. While there are complex procedures which make possible the reduction of noise in numerical differentiation, it is impractical to use them on this data, because these procedures involve transformation of the differentiation into an integral equation, which is then numerically solved with an appropriate regularization. Therefore, we chose a simpler, yet reasonable approach. The derivative of the theoretical curve (which closely fits the data) was used to represent the derivative of the original curves. The uncertainty in this derivative due to experimental errors was estimated by the Monte Carlo method.

To make the comparison of isoforms meaningful, we have considered the normalized form of the first derivative, i.e. 

$$Z(t) = \left(\frac{Y(t) - v_c t}{v_c}\right) = 1 - \exp(-k_c t)$$  

(Eq. 5)

The uncertainties were estimated from 50 simulated curves obtained from a random sample of $k$ values distributed according to a Gaussian distribution. The value of $k$ estimated by the original fitting procedure was taken as the mean value of the distribution. Two standard errors in the $k$ obtained by the fitting procedure (95% confidence limit) were chosen to represent the standard deviation of the distribution. In this way for each time point in the original data (every half-second) 50 values of $Z(t)$ were generated. The maximal and minimal values of these 50 were used to construct envelopes around the original curve ($Z(t)$ with the $k$ estimated by fitting). These envelopes indicate the region of uncertainty (the results are shown below, in Fig. 5).

RESULTS

The assay of the pump ATPase activity in microsomal membranes isolated from COS cells is almost impossible because of the high level of non-pump Mg$^{2+}$-ATPase in these membranes. One of the reasons for devising the reconstitution method was to reduce this interfering Mg$^{2+}$-ATPase. Experiments with the reconstituted preparation showed that its Mg$^{2+}$-ATPase activity was decreased to 10% of that seen in the original membranes, while its ATPase activity due to the pump was not affected. In the reconstituions with isoforms 4a and 4b, this resulted in a Mg$^{2+}$-ATPase activity of about 30% of the pump ATPase activity, a level which could easily be subtracted out.

To verify that the reconstructed enzyme behaves as the pump in the original membrane, we compared the affinity for Ca$^{2+}$ of the reconstituted preparation of PMCA4b with the enzyme present in erythrocyte membranes. The $K_{1/2}$ for Ca$^{2+}$ of the reconstituted preparation in the presence of 235 mM calmodulin was 0.32 μM Ca$^{2+}$ for the reconstituted preparation and 0.23 μM for the erythrocyte membranes. In the absence of calmodulin, the $K_{1/2}$ for Ca$^{2+}$ was over 10 μM in the reconstituted preparation, which is also like the corresponding result for erythrocyte membranes. These results show that the kinetic behavior of the reconstituted pump is like its behavior when it is in its natural

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1. The abbreviations used are: PMCA, plasma membrane Ca$^{2+}$ pump; TES, 2-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino)-ethanesulfonic acid.
environment (9).

To check the molecular integrity of isoforms 4a and 4b, and to estimate the amount of each present in our assays, we performed quantitative Western blots on reconstituted liposomes containing these forms. Quantitation was performed by scanning the blots directly, followed by computer analysis (10). The blots were stained with antibody 5F10, which recognizes all isoforms of PMCA (11), and purified human red cell PMCA (12) was used as standard. The sample of purified pump used contained a small amount of a proteolytic fragment. Such fragments are frequently found in human tissues, and their presence and size depends on the exact history of the tissue (11). Fig. 1 shows that the reconstituted preparation contained a band of the expected size for both isoforms 4a and 4b. A very faint band of slightly higher molecular weight was also visible, particularly when 4a was expressed. This band was due to the presence of a small amount of endogenous COS cell PMCA, which is isoform 1b. However, the addition of Ca\(^{2+}\) and/or calmodulin did not produce any noticeable change in the slope when ATPase assays were performed with membranes from cells transfected with empty plasmid. This shows that the contribution of this endogenous pump is negligible. From these Western blots, we found the pump concentration to be 3–7 nM loaded with calmodulin, and the calmodulin concentration was limited by the equipment and materials available at that time.

Our measurements use a method similar to that employed by Scharff and Foder in 1982 (1). Their pioneering work was limited by the equipment and materials available at that time. We were able to extend their work significantly by using ex-

**FIG. 1.** Western blot shows purity and amounts of isoform 4b and 4a. This figure shows a single, unedited blot. The molecular weights of the pertinent human isoforms are: 1b, 134,708; 4b, 133,929; 4a, 129,304. The tracks show hPMCA4b after incorporation into vesicles, hPMCA4a after incorporation into vesicles and 10, 20, and 40 ng of purified red cell plasma membrane Ca\(^{2+}\) pump.

**FIG. 2.** Slow activation of isoform 4b by 235 nM calmodulin. Calmodulin was added at zero time, and the rate of ATP splitting was measured. The experiment shown was one of six; the standard error of the mean of \(k\) for the six experiments was \(\pm 0.0016 \text{s}^{-1}\). The solid line is a line made up of the actual data points, and the dashed line is a least squares line fitted to the fitted curve linear portion of the curve (the data points from 120 s to the end of the experiment). Comparison of these two lines makes the curvature of the early data evident. The equation described under "Materials and Methods" was fitted to the data also, but it is not shown in the main panel of the figure, since it is nearly indistinguishable from the line made up of the data points. The inset shows the data points for the first 50 s, and a solid line representing the fit by Equation 5.

Some small deviations from the theoretical curve occurred in the first 30 s, as shown, but the fit was much better at all longer times. For this experiment, the value of \(k\) calculated from the fitted curve was 0.0151 \(\pm 0.0001 \text{s}^{-1}\), which gave a value of 3.9 \(\times\) 10\(^{-6}\) mmol mg\(^{-1}\) min\(^{-1}\) for \(k_{p}\), and a \(t_{1/2}\) of 46 s. The value of \(v_{0}\) was 62 nmol mg\(^{-1}\) min\(^{-1}\), and the sum of \(v_{0} + v_{a}\) was 147 nmol mg\(^{-1}\) min\(^{-1}\).

Since the rate at which calmodulin activates isoform 4b is slow, and calmodulin binds to the pump very tightly, the rate of inactivation of 4b when calmodulin dissociates is expected to be very slow. That this is the case was confirmed by the experiment shown in Fig. 6. In this experiment, the enzyme was loaded with calmodulin, and the calmodulin concentration was then lowered well below its \(K_{d}\) for the pump by adding an excess of a calmodulin-binding peptide. The slowing of the pump’s rate of reaction was then observed using the coupled enzyme reaction. The slowing of the pump's rate occurred over a long time, and was not complete when the reaction was terminated after an hour of incubation at 37 °C. The theory fitted the data quite well, and gave a half-time for inactivation of 23 min.

In the case of isoform 4a, the binding of calmodulin is less tight than for 4b, but the rate of activation was faster. In order to explain this, it was necessary to postulate a rate of inactivation of 4a which would be much faster than that of 4b. This was confirmed by the experiment shown in Fig. 7, done in the same way as Fig. 6. The half-time for inactivation was less than a minute, nearly 30 times faster than for 4b.

**DISCUSSION**

Our measurements use a method similar to that employed by Scharff and Foder in 1982 (1). Their pioneering work was limited by the equipment and materials available at that time. We were able to extend their work significantly by using ex-
pressed isoforms of the pump, rather than the mixture of isoforms available in natural membranes. In order to use expressed isoforms, it was necessary to develop a method for eliminating the large amount of non-pump Ca\(^{2+}\)-ATPase found on the surface of almost all cells. COS cell membranes have a large amount of such an ATPase, which makes the measurement of the pump ATPase inaccurate. The uncertainty of ATPase measurements on such membranes is made more acute by the fact that the true Ca\(^{2+}\)-pump ATPase consists of two components, the ATPase stimulated by Ca\(^{2+}\) and the ATPase stimulated by calmodulin. Since either Mg\(^{2+}\) or Ca\(^{2+}\) also stimulate the non-pump ATPases, only the calmodulin-stimulated component of the pump ATPase can be measured in normal cell membranes. The confusion is further compounded by the fact that the amount of calmodulin stimulation varies, depending on the membrane lipid composition. All of these factors make it impossible to measure the amount of Ca\(^{2+}\)-pump ATPase in normal cell membranes. Only human erythrocyte membranes are truly exempt from this difficulty, since they are nearly devoid of non-pump ATPases. Because of this situation, our prior studies of the pump using COS cell membranes have been restricted to measurements of Ca\(^{2+}\) uptake.

The reconstitution method described here solves this problem by preferentially solubilizing the pump from the membranes and reconstituting it into liposomes. The use of a non-ionic detergent for the extraction solubilizes the pump, while the non-pump ATPases remain insoluble. Although the pump is unstable after long exposures to such detergents, the prompt reconstitution of the pump into liposomes restores its stability. We accomplished the reconstitution by introducing Bio-Beads into the liposome suspension, which removed the detergent used for solubilization. Our experience shows that the amount of non-pump ATPase in these reconstituted liposomes is quite low, and the ATPase due to the pump can easily be measured. Since ATPase activity can be measured continuously by coupled enzyme systems, kinetics studies such as those described here are now possible.

By comparing the slopes in Figs. 2–4, 6, and 7 with the concentration of reconstituted pump present in the assay medium, we estimated that the activity of the enzyme was 4 to 8 \(\mu\)mol min\(^{-1}\) (mg of pump protein\(^{-1}\)). These numbers compared very well with the activities reported for the purified pump from human red cells (12).

Our data showed a difference between isoforms 4a and 4b in the rate of activation of the pump by calmodulin. This differ-
FIG. 6. Inactivation of isofom 4b after removal of calmodulin.
Free calmodulin was removed from the solution at zero time by addition of the calmodulin-binding peptide from myosin light chain kinase. Symbols are as described in the legend to Fig. 2, except that Equation 7 was used to fit the data. The experiment shown was one of three; the standard error of the mean of $k_1$ for the three experiments was $\pm 0.000278$ s$^{-1}$. Once again, the theory fits the data excellently, so that the fitted line was omitted in the main panel. The inset allows the data points and the fitted line to be distinguished, so that the nature of the fit can be seen better. This curve was representative of two separate experiments on vesicles reconstituted from different batches of COS cell membranes. For this experiment, the value of $k_1$, calculated from the fitted curve was $0.000493 \pm 0.000004$ s$^{-1}$ or $0.0296$ min$^{-1}$, which gave a $t_{1/2}$ of 23 min. The fitted curve had a correlation coefficient $r^2$ of 0.99999. The value of $v_0$, was 34 nmol mg$^{-1}$ min$^{-1}$, and the sum of $v_0 + v_1$ was 119 nmol mg$^{-1}$ min$^{-1}$.

ence was in a surprising direction. A previous study showed that isofom 4a required 125 nM calmodulin for half-maximal activation, while isofom 4b required only 18 nM (9). Since the affinity of calmodulin for isofom 4a was so much lower than for 4b, we expected the rate of activation of 4a by calmodulin to be even slower than 4b. The results here showed the opposite, with the rate of activation of 4a being about four times faster than that of 4b (Figs. 2 and 3). This result suggested that the rate of inactivation of 4a by loss of calmodulin must be very much faster than that of 4b, in order to give the observed relationship between the affinities of the two isoforms for calmodulin. The results shown in Figs. 6 and 7 showed that this was indeed the case, with the rate of inactivation of 4a being about 30 times faster than that of 4b!

The rates of calmodulin binding and dissociation have also been measured for three other calmodulin-activated enzymes: cyclic nucleotide phosphodiesterase, Ca$^{2+}$-calmodulin-dependent protein kinase, and myosin light chain kinase. In all cases the rates were much faster than were observed in this study. For cyclic nucleotide phosphodiesterase, $k_1$, was about 60 times faster than for isofom 4b at $2.4 \times 10^9$ M$^{-1}$ min$^{-1}$ and $k_1$ was 0.192 min$^{-1}$ (14). For unphosphorylated Ca$^{2+}$-calmodulin-dependent protein kinase, $k_1$ was about 600 times faster at $2.4 \times 10^9$ M$^{-1}$ min$^{-1}$ and $k_1$ was 130 min$^{-1}$ (15). For myosin light chain kinase, $k_1$ was about 1700 times faster at $6.6 \times 10^9$ M$^{-1}$ min$^{-1}$ and $k_1$ was 34 min$^{-1}$ (16). The same workers also measured the corresponding rates for a 17-residue peptide whose sequence was taken from the calmodulin-binding domain of myosin light chain kinase. For this peptide, $k_1$ was more than 13,000 times faster at $5.3 \times 10^{10}$ M$^{-1}$ min$^{-1}$ and $k_1$ was too slow to measure, being less than 0.6 min$^{-1}$ (16).

Comparison of the myosin light chain kinase peptide with the intact enzyme indicates that occlusion of the calmodulin-binding domain plays an important role in determining both the rate at which calmodulin binds and its affinity for the enzyme. The rate at which calmodulin bound to the same calmodulin-binding domain was about 80 times faster in the free peptide than in the intact enzyme, and the affinity of the peptide for calmodulin was about 30 times greater. Since the rate of activation of isofom 4b was so much slower than any of the other enzymes, it seems probable that the calmodulin-binding domain of 4b is more occluded than the others, making the penetration of calmodulin to its binding site more difficult.

The role of occlusion in determining the rate at which calmodulin binds is shown by the increased rate at which ct92 is activated by calmodulin. Two regions (the calmodulin-binding domain and the downstream inhibitory region) in the carboxyl terminus of isofom 4b inhibit the enzyme by interacting with the catalytic core (7, 13). In ct92, the downstream inhibitory region is removed and the degree of inhibition is less (13), indicating that the calmodulin-binding domain is less occluded. This causes the faster interaction of calmodulin with ct92.

The differences between isoforms 4a and 4b are difficult to

| Table I |
|---------|
| 4b      | 4a      | ct92    |
| $k_1$ (min$^{-1}$) | $3.9 \times 10^6$ | $15.2 \times 10^6$ | $7.6 \times 10^6$ |
| $k_2$ (min$^{-1}$) | 0.0296   | 0.81    | 7.6    |
| $K_c$ (nM)      | 26       | 53      |        |

$^a$ The values of $k_1$ and $k_2$, all had coefficients of variation of less than 3%.
analyze from a structural point of view. The calmodulin-binding domain of 4a is longer than that of 4b (17) and has a loop inserted into its middle which does not bind calmodulin (18). It is difficult to tell how these properties would affect the rates of calmodulin binding, but the generally faster rates observed for 4a may indicate that its calmodulin-binding domain is less occluded than that of 4b.

The slow binding and dissociation of calmodulin will certainly influence the role of this pump in living cells. The activation of isoform 4b, with $t_{1/2}$ of 46 s, occurs on the same time scale as do the Ca$^{2+}$ spikes in many kinds of cells. In cells for which 4b is the dominant mechanism of Ca$^{2+}$ efflux (such as the kinds of cells derived from hematopoietic stem cells), this slow response would allow a full development of the spike. The inactivation rate observed here for 4b is so slow ($23 \text{ min}$) as to imply that cells of this type would require a long period before a long quiet period had elapsed. While this may actually be the case, it is too early to make such a conclusion definitively. The experiments done here were necessarily different from the situation in a living cell. In these experiments, calmodulin was introduced or removed to measure the rates. In a living cell, the driving force for calmodulin binding and dissociation is the rise and fall of the Ca$^{2+}$ concentration. Experiments in which the Ca$^{2+}$ concentration is increased or decreased will be necessary to settle this problem.

Cells which respond more rapidly to calcium signals, such as nerve and muscle cells, will require a more rapidly responding isoform of the pump. 4a is one such isoform, and it is no accident that it is primarily found in such rapidly-responding cells. Studies of isoforms 1, 2, and 3 will be needed to determine where they fit relative to 4a and 4b.

By using a doubly integrated theoretical equation combined with computer handling of the data from the spectrophotometer, we were able to take data at the rate of two points per second, and fit all of the data to the theoretical curve. This gave a more accurate fit than the clever and laborious method available to Scharff and Foder in 1982 (1), which involved making measurements at longer intervals and plotting them on axes which linearized the resulting plot. Even though their measurements were made on erythrocyte ghost membranes and ours were made on reconstituted COS cell membranes, the two methods gave comparable values for $k_1$ and $k_1$. At 1.78 $\mu$M free Ca$^{2+}$, Scharff and Foder (1) found values of $1.5 \times 10^9$ or $2.6 \times 10^9$ M$^{-1}$ min$^{-1}$ for $k_1$ and 0.035 or 0.051 min$^{-1}$ for $k_1$. At 0.72 $\mu$M free Ca$^{2+}$, they found values of $0.27 \times 10^9$ or $0.24 \times 10^9$ M$^{-1}$ min$^{-1}$ for $k_1$ and 0.121 or 0.108 min$^{-1}$ for $k_1$. The two different values they obtained for each constant were gotten using different types of experiments (1). These values compare well with the values we obtained for isoform 4b at 0.5 $\mu$M free Ca$^{2+}$, which were $3.9 \times 10^9$ M$^{-1}$ min$^{-1}$ for $k_1$ and 0.0296 min$^{-1}$ for $k_1$. Their rate for binding of calmodulin at 0.72 $\mu$M free Ca$^{2+}$ was slower than the rate we obtained at 0.5 $\mu$M free Ca$^{2+}$ and their rate for dissociation of calmodulin was faster. Despite this difference, the agreement is remarkably good, considering that their results were obtained on a natural membrane which contained mixed isoforms, whereas ours were obtained on a single isoform reconstituted into liposomes, and that the conditions were different. This result shows that the erythrocyte ghost membranes they studied (which contain isoforms 1b and 4b) must be predominantly isoform 4b, a result which is consistent with other indications of the composition of these membranes.

The kinetic data for isoforms 4a and 4b will also allow the calculation of a true $K_d$ for the binding of calmodulin to the pump, based on the relationship $K_d = k_+ / k_1$. Table I shows the values calculated from this relationship (the values for ct92 are not included because the inactivation rate was not measured for that isoform). In the case of isoform 4b, the value obtained for $K_d$ can be compared with those previously measured on the pump from erythrocyte membranes. Measurements were made by observing the binding of iodinated calmodulin to erythrocyte membranes directly (19) and by kinetic titrations of erythrocyte membranes (20) and of purified pump (21). The direct binding measurement gave two kinds of results: six preparations of erythrocyte membranes gave curved Scatchard plots with a $K_d$ between 1 and 5 nM, while another three preparations gave linear Scatchard plots with a $K_d$ of 29.7 ± 3.4 nM. The kinetic titration of erythrocyte membranes gave a $K_d$ of 14.5 nM, and the kinetic titration of purified pump gave a $K_d$ of 4.5 nM. The value of 7.6 nM found here for $K_d$ is quite consistent with these previous values.

The $K_d$ for isoform 4a has not been measured before, and our new value can be compared only with the $K_d$ previously observed. For the pump expressed in COS cell membranes, the $K_d$ for isoform 4a was 126 nM and that for isoform 4b was 18 nM (9). The ratio of these two values was 7.0, exactly the same as the ratio of the $K_d$ values found here. The consistency of these measurements show that these new $K_d$ values can be taken as authoritative measurements.

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