Inhibition of the purified human red-cell Ca^{2+} pump by a monoclonal antibody

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1. A monoclonal antibody (1G4) was raised against the red-cell Ca^{2+} pump, and it reacted with the pump, as verified by Western blot analysis and by the e.l.i.s.a. method. 2. At 1 mM-ATP and 10 μM - Ca^{2+} , 1G4 inhibited the activity of the purified Ca^{2+} pump by 40 %. 3. Ca^{2+} pump inhibition by the antibody was non-competitive with regard to Ca^{2+} , calmodulin and the high-affinity portion of the ATP curve. Thus its mechanism was quite different from that of the antibody previously reported [Verbist, Wuytack, Raemaekers, VanLeuven, Cassiman & Casteels (1986) *Biochem. J.* **240**, 633–640], which partially caused inhibition by competition at the ATP site. 4. Antibody 1G4 reduced the steady-state level of phosphorylated intermediate and increased by 50 % the calmodulin-activated p-nitrophenyl phosphatase activity of the pump. 5. The experimental results are consistent with the hypothesis that 1G4 inhibits the Ca^{2+} pump by decreasing the rate of the transition from the E_2 form to the E_1 form, causing a higher concentration of E_2 . 6. Analysis by Western blot of the pattern of cross-reaction of 1G4 after tryptic digestion of the pump showed that this antibody reacts with bands of M_r 90 000, 85 000, 50 000 and 33 000. After chymotryptic digestion, the antibody reacts almost exclusively with a fragment of M_r 105 000 that is fully active but is not responsive to calmodulin. Altogether, the results indicate that 1G4 binds to an epitope involved in the functional properties of the enzyme but which is not related to the calmodulin-binding domain.

INTRODUCTION

Following its purification from human red-cell membranes [1,2], considerable information has been obtained about the structure and function of the plasma membrane Ca^{2+} pump. Its amino acid composition was determined [3], important functional sites (including the fluorescein isothiocyanate site [4], the phosphorylation site [5] and the calmodulin-binding site [6]) have been sequenced, and recently the complete sequences of some isoenzymes of the plasma membrane Ca^{2+} pump have been elucidated [7,8].

One difficulty that has been encountered in the study of the plasma membrane Ca^{2+} pump is the lack of specific inhibitors. Vanadate and calmodulin antagonists such as trifluoperazine also inhibit other cation-transporting ATPases [9–11]. An approach that can be used to study the function of the Ca^{2+} pump is to raise monoclonal antibodies and to study their effect on the activity of the system. This approach has given valuable information with other cation-transporting ATPases [12,13].

Previously this laboratory has reported inhibition of the plasma membrane Ca^{2+} pump by a polyclonal antibody [14]. Also, we have produced several monoclonal antibodies that were very useful for the localization of the Ca^{2+} pump in different tissues [15,16]. None of those monoclonal antibodies had an effect on the activity, however. Now we report a monoclonal antibody that has inhibitory properties on the activity of the red-cell Ca^{2+} pump. A kinetic mechanism for the effect of the antibody is proposed.

MATERIALS AND METHODS

RPM-1640 medium was from M.A. Bioproducts, Walkerville, MD, U.S.A. Fetal calf serum was from HyClone, Logan, VT, U.S.A. Glutamine, hypoxanthine/aminopterin/thymidine concentrated supplement and NCTC-135 were from Gibco, Grand Island, NY, U.S.A. Balb-c mice were from Jackson Laboratory, Bar Harbor, ME, U.S.A. Poly(ethylene glycol) 1450 was from J. T. Baker, Phillipsburg, NJ, U.S.A. E.l.i.s.a. plates (96-well) were from Costar, Cambridge, MA, U.S.A. Sterile culture plates (96-, 24- and 6-well) were from Becton-Dickinson, Oxnard, CA, U.S.A. Sterile culture flasks were from Corning, Corning, NY, U.S.A. Alkaline phosphatase-conjugated goat anti-(mouse IgG) was purchased from Pel-Freez, Brown Deer, WI, U.S.A. Peroxidase-conjugated goat anti-(mouse IgG) and peroxidase substrate were from Bio-Rad, Richmond, CA, U.S.A. Tos-Phe- CH_2Cl -treated trypsin was from Cooper Biomedicals, Malvern, PA, U.S.A., and chymotrypsin was from Worthington Enzymes (Freehold, NJ, U.S.A.). Chymostatin was from Boehringer-Mannheim, Indianapolis, IN, U.S.A. Gentamycin, ATP, p-nitrophenyl phosphate (p-NPP) and bovine serum albumin were from Sigma Chemical Co., St. Louis, MO, U.S.A. CaCl_2 was prepared from Ca_2CO_3 from J. T. Baker, Phillipsburg, NJ, U.S.A. $^{45}\text{CaCl}_2$, and [γ - ^{32}P]ATP were from New England Nuclear, Boston, MA, U.S.A. H.p.l.c.-grade methanol was purchased from Fisher Scientific, Fair Lawn, NJ, U.S.A. All the other reagents were analytical grade. Free Ca^{2+} was calculated using

Abbreviations used: p-NPP, p-nitrophenyl phosphate; TCA, trichloroacetic acid; Tos-Phe- CH_2Cl , 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK).

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computer programs as previously described [17], with association constants corrected for H^+ activity.

Ca^{2+} -ATPase was purified from human red-cell ghosts using calmodulin-affinity chromatography as previously described [3].

Inside-out vesicles from human red cells were prepared by the procedure of Sarkadi *et al.* [18].

Immunization, cell culture, cell fusion between mouse spleen cells and the FO myeloma cells and the subcloning procedure was done as described [16]. Also, ascites fluid production and antibody purification were done using previously published procedures [19].

Binding of antibodies to purified Ca^{2+} -ATPase was performed by the e.l.i.s.a. method [20] modified as described [19].

SDS/polyacrylamide-gel electrophoresis was performed as described by Laemmli [21]. Pyronin Y was used as tracking dye. Electrophoretic transfer of the protein from the gel to nitrocellulose paper was done by the procedure of Towbin *et al.* [22] modified as described [19], and the nitrocellulose sheet was treated for antibody binding using the procedure of Burnette [23] with the described modifications [19].

Silver staining of the gels was performed as described [24].

ATPase activity was estimated by measuring the release of ^{32}P from $[^{32}P]ATP$ by the procedure of Richards *et al.* [25]. ATPase activity was measured at 37 °C in 0.5 ml of media containing 20 mM-Na-Hepes (pH 7.4 at 37 °C), 130 mM-NaCl, 1 mM-EGTA, 0.08 mM-EDTA, 1 μ g of purified Ca^{2+} pump, 2.5 mM- $MgCl_2$ and the concentrations of $[^{32}P]ATP$, Ca^{2+} , calmodulin and antibody indicated in the Results section. Ca^{2+} -dependent ATPase activity is the activity measured in the media described above minus the activity measured in a similar medium but without added Ca^{2+} .

The level of phosphorylated intermediate was measured after 20 s of incubation at 0 °C in 0.5 ml of media containing 25 mM-Tes/triethanolamine (pH 7.4 at 0 °C), 150 mM-KCl, 1 mM-EGTA, 0.5 mM-EDTA, 7.5 mM-NaCl, 0.025 % Triton-X-100, 0.05 % phosphatidylcholine, 150 mM-sucrose, 1.5 mM- $MgCl_2$ (0.85 mM free Mg^{2+}), 10 μ M- $[^{32}P]ATP$, 300 nM-calmodulin, 10 μ M free Ca^{2+} , 10–15 μ g of purified red-cell Ca^{2+} pump and 50 μ g of purified monoclonal antibody or mouse IgG/ml. Because of the increased amount of enzyme in this experiment, the concentration of antibody was also increased to keep the ratio of antibody/enzyme constant. The reaction was stopped by the addition of 1 ml of 20 % (v/v) trichloroacetic acid (TCA)/5 mM-ATP/250 mM- H_3PO_4 . Then, 1 mg of red-cell ghost protein was added to each tube. The tubes were centrifuged at 3000 rev./min during 5 min (at 4 °C). The pellets were washed three times with 20 % TCA/250 mM- H_3PO_4 . The final pellet was dissolved in 5 % SDS and the radioactivity measured by liquid scintillation counting. The amount of phosphoenzyme was measured with and without Ca^{2+} and the difference was taken to be the amount of pump phosphoenzyme.

p-NPPase activity was estimated by measuring the release of *p*-nitrophenol from *p*-NPP [26]. Calmodulin-activated *p*-NPPase activity was measured at 37 °C in media containing 165 mM-KCl, 20 mM-Hepes/Na-Hepes (pH 7.4 at 37 °C), 10 mM- $MgCl_2$, 10 mM-*p*-NPP, 1 mM-EGTA, 0.05 mM-EDTA, 1 μ g of purified red-cell Ca^{2+} pump, 7.5 mM-NaCl, 300 nM-calmodulin and enough

$CaCl_2$ to give 0.8 μ M free Ca^{2+} . The activity measured in similar media but in the absence of calmodulin was subtracted. ATP-dependent *p*-NPPase activity was measured at 37 °C in medium containing 165 mM-KCl, 10 mM- $MgCl_2$, 10 mM-*p*-NPP, 1 mM-EGTA, 0.05 mM-EDTA, 7.5 mM-NaCl, 20 mM-Hepes/Na-Hepes (pH 7.4 at 37 °C), 1 mM-ATP and enough $CaCl_2$ to keep the free Ca^{2+} concentration at 100 μ M. The activity measured in similar media but without ATP was subtracted. After 30–60 min of incubation, the reaction was stopped by the addition of 1 ml of 1 M-NaOH and the absorbance at 410 nm was measured. Tryptic digestion of the purified Ca^{2+} pump was done at 37 °C during 2 min in media containing 130 mM-NaCl, 20 mM-Hepes/Na-Hepes (pH 7.4 at 37 °C), 0.05 % Triton X-100, 0.05 % phosphatidylcholine, 1 mM- $MgCl_2$, 2 mM-EDTA, 75 μ g of purified Ca^{2+} pump/ml and 3 μ g of trypsin/ml. Chymotryptic digestion was done during 30 min at 0 °C, in media containing 130 mM-NaCl, 20 mM-Hepes/Na-Hepes (pH 7.4), 0.05 % Triton X-100, 0.05 % phosphatidylcholine, 1 mM- $MgCl_2$, 2 mM-EDTA, 75 μ g of purified Ca^{2+} pump/ml and 93 μ g of chymotrypsin/ml. Digestions were stopped by the addition of electrophoresis sample buffer followed by immediate boiling of the samples for 2 min. When, after chymotryptic digestion, the ATPase activity was measured, the digestion was stopped by the addition of chymostatin (final concentration 100 μ g/ml).

RESULTS

Reaction of monoclonal antibody 1G4 with the Ca^{2+} pump

The antibody was isotypized as IgG2b. Fig. 1 shows an SDS/gel electrophoresis of the purified Ca^{2+} pump stained (after transfer to a nitrocellulose sheet by the Western blot procedure) with antibody 1G4. The antibody recognized a band at M_r approx. 140 000, characteristic of the plasma membrane Ca^{2+} pump, a heavy band at M_r 160 000–200 000 and a band at M_r approx. 95 000. As discussed previously [16], the bands at M_r 160 000–200 000 and M_r 95 000 are formed by aggregation and proteolysis products of the pump. Therefore, antibody 1G4 is directed against the Ca^{2+} pump molecule and all its effects are attributable to direct binding to the pump. Fig. 2 shows a plot of the binding of 1G4 to the Ca^{2+} pump as a function of monoclonal antibody concentration as measured by the e.l.i.s.a. method.

Effect of 1G4 on the activity of the pump

Fig. 3 shows the effect of 1G4 on the Ca^{2+} -dependent ATPase activity of the purified Ca^{2+} pump in the presence of calmodulin. It can be seen that 5 μ g of antibody/ml produced 40 % inhibition of the activity, and that much higher concentrations of the antibody did not induce further inhibition. In the same Figure, it is shown that mouse IgG did not produce any effect on the Ca^{2+} -ATPase activity of the pump. Similar results were obtained in the absence of calmodulin.

In Fig. 4, a plot of the Ca^{2+} -ATPase activity is shown as a function of Ca^{2+} concentration from 0–50 μ M in the absence of any addition, and in the presence of either 50 μ g of IgG/ml or 5 μ g of 1G4/ml. The incubation medium contained 60 nM-calmodulin. It can be seen that the effect of the antibody was the same at all the Ca^{2+} concentrations tested. From a Hill plot

Fig. 1. Western Blot of the purified erythrocyte Ca^{2+} pump stained with monoclonal antibody 1G4

SDS/gel electrophoresis in a 7.5% polyacrylamide gel, electrophoretic transfer to nitrocellulose and incubation with antibodies were performed as described in Materials and methods section.

Fig. 2. Binding of antibody 1G4 to purified Ca^{2+} pump as a function of antibody concentration

Binding of antibody was estimated by the e.l.i.s.a. method.

Fig. 3. ATPase activity of the Ca^{2+} pump as a function of antibody concentration

Incubation media contained $10 \mu\text{M}$ free Ca^{2+} , 1 mM -ATP and 60 nM -calmodulin. $\circ$, Antibody 1G4; $\bullet$, non-immune IgG. The concentration of antibody required for inhibition cannot be compared directly with those required for binding in Fig. 2, because of the much higher amount of ATPase used in this experiment.

Fig. 4. ATPase activity of the Ca^{2+} pump as function of Ca^{2+} concentration

Incubation media contained 1 mM -ATP and 60 nM -calmodulin. $\circ$, No addition; $\bullet$, $50 \mu\text{g}$ of non-immune IgG/ml; $\triangle$, $5 \mu\text{g}$ of 1G4/ml. Inset: same data represented as a percentage of the maximal activity of each curve.

(not shown), the value of V_{max} was estimated to be $1310 \pm 19 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the absence of antibody and $882 \pm 22 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the presence of 1G4; the K_{Ca} was $0.20 \pm 0.03 \mu\text{M}$ in the absence of antibody and $0.23 \pm 0.05 \mu\text{M}$ in the presence of 1G4 and the values of the Hill coefficient (h) were 1.25 ± 0.12 and 1.47 ± 0.17 respectively. In the inset of Fig. 4, the data are represented as a percentage of the V_{max} obtained in either case. It can be seen that all the data can be represented by the same curve, indicating again that 1G4 decreased V_{max} , but it did not change significantly either K_{Ca} or h . Similar results were obtained in the absence of calmodulin.

Fig. 5 shows a plot of the ATPase activity of the Ca^{2+}

Fig. 5. ATPase activity of the Ca^{2+} pump as a function of ATP concentration

Incubation media contained 10 mM free Ca^{2+} and 60 nM-calmodulin. $\circ$, No addition; $\bullet$, 50 μg of non-immune IgG/ml; $\triangle$, 5 μg of 1G4/ml. Inset: Eadie-Scatchard plot of the same data.

pump as a function of ATP concentration, without any addition, and in the presence of either 50 μg of IgG/ml or 5 μg of 1G4/ml. The curves show a biphasic response to ATP, characteristic of the Ca^{2+} pump [25,27,28]. This curve was treated as a sum of two Michaelis-Menten equations, and the values for the parameters of the curve in Fig. 5 were estimated by non-linear regression. Thus, $V_1 = 604 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $K_{m1} = 12 \mu\text{M}$, $V_2 = 1201 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_{m2} = 392 \mu\text{M}$ in the absence of antibody or in the presence of non-immune IgG, and $V_1 = 229 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, $K_{m1} = 9.8 \mu\text{M}$, $V_2 = 1155 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and $K_{m2} = 421 \mu\text{M}$ in the presence of 5 μg of 1G4/ml. Therefore monoclonal antibody 1G4 decreased the value of V_1 by about 60% without any significant effect on the value of V_2 or on the apparent affinity for ATP of both components of the curve.

The concentration of calmodulin required for half-maximal activation was 3.7 nM in the absence of antibody and 4.3 nM in the presence of 5 μg of 1G4/ml, indicating that the apparent affinity of the pump for calmodulin was not affected by the antibody (results not shown).

Effects of 1G4 on partial reactions of the Ca^{2+} pump

Table 1 shows that 50 μg of monoclonal antibody 1G4/ml decreased the steady-state level of phospho-

Fig. 6. Reaction of 1G4 with different fragments of the Ca^{2+} pump after tryptic and chymotryptic digestion

Lane 1, electrophoresed tryptic digest of the Ca^{2+} pump stained by the silver impregnation method; lane 2, the same tryptic digest stained with antibody 1G4; lane 3, electrophoresed chymotryptic digest of the Ca^{2+} pump stained by the silver impregnation method; lane 4, same chymotryptic digest stained with antibody 1G4.

enzyme to about 50% of the control. Table 1 also shows the effect of 1G4 on the p -NPPase activity of the Ca^{2+} pump. It can be seen that this antibody stimulated the calmodulin-activated p -NPPase activity by about 50%, whereas it had no effect on the ATP-activated p -NPPase activity.

Reaction of 1G4 with proteolytic fragments of the pump

In Fig. 6, proteolytic patterns of the Ca^{2+} pump, obtained by tryptic or chymotryptic digestion, are shown. Silver staining of the electrophoresed digest (lane 1) showed bands at M_r 90000, 85000, 50000, 42000 and 33000. Lane 2 shows the cross-reaction of 1G4 with bands of M_r 90000, 85000, 50000 and 33000, indicating good correlation between the bands seen by antibody staining and by protein staining.

In lane 3 (Fig. 6), the silver-stained fragments of the

Table 1. Effect of monoclonal antibody 1G4 on partial reactions of the plasma membrane Ca^{2+} pump

Experiments were done in duplicate, and the errors shown are the standard deviations for two tubes. Other results suggest that the S.D. for the population of all identical experiments may be larger. Because of this probably larger S.D., we do not interpret the increase in the ATP-activated p -NPPase caused by 50 μg of 1G4/ml as being significant.

Addition	EP level (% of control)	Calmodulin-activated p -NPPase		ATP-activated p -NPPase	
		(nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	(%)	(nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	(%)
IgG (50 μg /ml)	100	107.7 $\pm$ 12.1	100	256 $\pm$ 6.1	100
1G4 (5 μg /ml)	Not done	158.5 $\pm$ 5.4	147	261.4 $\pm$ 2.0	102
1G4 (50 μg /ml)	48.5 $\pm$ 1.8	154.9 $\pm$ 1.2	144	288.0 $\pm$ 3.6	112

Table 2. Activity of the M_r -105000 product of chymotryptic digestion of the Ca^{2+} pump

Abbreviation: CaM, calmodulin.

	Ca^{2+} -ATPase activity (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	
	-CaM	+CaM
Control	420	3500
Digested	3080	3460

Ca^{2+} pump produced by 10 min of chymotryptic digestion can be seen. The digestion produced almost exclusively a product of M_r 105000. The line that appeared at M_r 68000 is an artefact due to the presence of mercapto-ethanol [29,30], and it was seen even when there was no protein in the lane. Lane 4 shows the same electrophoresed digest stained with 1G4. It can be seen that the product of M_r 105000 contains the epitope for the antibody.

Table 2 shows that the activity of the Ca^{2+} pump digested by chymotrypsin is the same as that of the undigested control in the presence of calmodulin. In the absence of calmodulin, the digested enzyme is as active as in its presence. Therefore, the M_r 105000 product that contains the epitope for 1G4 is fully active but is no longer regulated by calmodulin.

DISCUSSION

This paper reports the inhibition of the red-cell Ca^{2+} pump by a monoclonal antibody and suggests a mechanism for this inhibition.

The reaction of 1G4 with the pump was verified by e.l.i.s.a., which showed that the antibody binds with high affinity to the pump, and by the Western blot procedure. This antibody reacts with the intracellular components of the pump, as was verified by its lack of reactivity with intact red cells [19]. A monoclonal antibody against the pig stomach plasma membrane Ca^{2+} pump that inhibited the activity has been reported, but that antibody had no reactivity by Western blot analysis [31]. The maximal inhibition of ATPase activity observed was about 40% of the total activity. Antibody 1G4 showed non-competitive kinetics with respect to Ca^{2+} and calmodulin.

The activation by ATP of the Ca^{2+} pump can be described as a sum of a high-affinity component with low maximal velocity and a low-affinity component with high maximal velocity [32]. 1G4 decreased by about 50–60% the maximal velocity of the high-affinity component without affecting significantly any of the other parameters describing ATP activation of the pump. This differs from the actions of the inhibitory monoclonal antibody reported by Verbist *et al.* [31], since that antibody was reported to compete with ATP.

Antibody 1G4 decreased the level of phosphorylated intermediate by 50%, in good agreement with the observed inhibition of the ATPase activity.

The red-cell Ca^{2+} pump displays p -NPPase activity. This activity can be activated by calmodulin and low Ca^{2+} concentration [17] or by ATP and higher Ca^{2+} concentrations [33]. It has been proposed that the p -

Scheme 1. Simplified kinetic scheme of Ca^{2+} pump function

NPPase activity is a property of the E_2 conformation of the pump [34]. Using this interpretation, the increase of calmodulin-activated p -NPPase activity produced by 1G4 suggests that the antibody shifts the $E_1 \rightleftharpoons E_2$ equilibrium towards E_2 .

We have made a simple interpretation of the effects of 1G4 on the Ca^{2+} pump on the basis of Scheme 1. This Scheme is not intended to be a complete model for the functioning of the Ca^{2+} pump but is to show the effect of a decrease in k_1 on the various activities measured. Since the antibody did not modify the low-affinity activation by ATP, and since, at present, there is not a universally accepted interpretation of this phenomenon, low-affinity activation was not considered in this model. The discussion in the Appendix shows that a decrease in k_1 would account for the observed decrease in V_m of the ATPase, the decrease in EP and the increase in calmodulin-stimulated p -NPPase. The K_m of the ATPase and the ATP-stimulated p -NPPase would remain unchanged, as was also observed.

The reactivity of our antibody by the Western blot procedure allowed us to determine which fragments of the pump react with it after tryptic and chymotryptic digestion. Results of the tryptic digestion indicated that the epitope for 1G4 is located in fragments of M_r 90000 and around 80000–85000. In our hands, chymotryptic digestion of the Ca^{2+} pump produced almost exclusively a fragment of M_r 105000, which is fully activated so that calmodulin has no additional effect. All these facts suggest that 1G4 reacts with a functional domain of the Ca^{2+} pump but that this domain is not related to the calmodulin-binding site. When inside-out vesicles from red cells are digested by chymotrypsin, a larger fragment (M_r 125000) is produced [35]. In a slightly different preparation of purified pump, James *et al.* [6] reported that both fragments (M_r 125000 and 105000) were present after chymotryptic digestion. This result suggests that in the purified Ca^{2+} pump, chymotrypsin digestion produced a slightly smaller fragment than when the pump is in the native membrane. Nevertheless, both fragments seem to have the same functional properties.

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APPENDIX

A simple kinetic model of the Ca²⁺ pump

In the absence of ADP and P_i, the equation for the ATPase activity predicted by Scheme 1 is:

$$v = \frac{V_m \cdot [\text{ATP}]}{K_m + [\text{ATP}]} \quad (1)$$

where

$$V_m = \frac{k_1 \cdot k_3 \cdot k_4}{k_1 \cdot k_4 + k_1 \cdot k_3 + k_3 \cdot k_4} \quad (2)$$

A decrease in the value for k_1 would lead to a decrease in the value of V_m , as was observed.

Also,

$$K_m = \frac{ak_1 + b}{ck_1 + d} \quad (3)$$

where $a = k_4 \cdot (k_3 + k_{-2})$; $b = k_{-1} \cdot k_4 \cdot (k_3 + k_{-2})$; $c = k_2 \cdot (k_4 + k_3)$ and $d = k_2 \cdot k_3 \cdot k_4$. K_m would not change (the experimentally observed result) for any value of k_1 between 0 and an infinite value if k_{-1} is as shown in eqn. (4):

$$k_{-1} = \frac{k_4 \cdot k_3}{k_4 + k_3} \quad (4)$$

On the other hand, the equation for the steady-state level of phosphorylated intermediate predicted by this model is:

$$\frac{[\text{EP}]}{[\text{E}_{\text{tot}}]} = \frac{\left(\frac{k_1 \cdot k_3}{k_1 \cdot k_4 + k_1 \cdot k_3 + k_3 \cdot k_4} \right)}{K_m + [\text{ATP}]} \quad (5)$$

From eqn. (5) it can be clearly seen that a decrease in k_1 will result in a decrease in the EP level.

The relative concentration of the E₂ form in the absence of ATP is shown by eqn. (6), and in the presence of saturating ATP by eqn. (7):

$$\frac{[\text{E}_2]}{[\text{E}_{\text{tot}}]} = \frac{k_{-1}}{k_1 + k_{-1}} \quad (6)$$

$$\frac{[\text{E}_2]}{[\text{E}_{\text{tot}}]} = \frac{k_3 \cdot k_4}{k_1 \cdot k_4 + k_1 \cdot k_3 + k_3 \cdot k_4} \quad (7)$$

If k_1 is much smaller than k_3 and k_4 , then a decrease in k_1 will increase E₂ in the absence of ATP and not change it in the presence of ATP. If E₂ is responsible for p-NPPase, this is also consistent with the experimental observations.