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Steady-state kinetic analysis of the Na^+/K^+-ATPase. The effects of adenosine 5′-[β,γ-methylene]triphosphate on substrate kinetics

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We studied the substrate kinetics of the Na^+/K^+-ATPase in media with adenosine 5′-[β,γ-methylene]triphosphate ([β,γ-CH_2]ATP), an analog of ATP that is resistant to enzymatic hydrolysis. The aim was to analyze from the point of view of steady-state kinetics the mechanism that generates the biphasic response of the Na^+/K^+-ATPase to ATP. In the absence of K^+, the analog acted as a dead-end inhibitor, K_i for this effect was 43.4 μM. In media with K^+ and non-saturating concentrations of ATP, [β,γ-CH_2]ATP stimulated ATPase. With high concentrations of [β,γ-CH_2]ATP, the response of activity to the concentration of ATP changed from biphasic to hyperbolic. Comparison of these effects with the predictions of reaction mechanisms that display biphasic responses to the substrate showed that: (i) when this response is caused by two independent and non-interacting active sites, an analog of the substrate will not activate but may change the substrate curve from biphasic into hyperbolic, (ii) if there were negative interactions in affinity and positive interactions in reactivity between two active sites, an analog may activate and change the substrate curve from biphasic into hyperbolic, (iii) in models, such as that proposed for the Na^+/K^+-ATPase by Plesner et al. in 1981 (Biochim. Biophys. Acta 643, 483–494) in which biphasic kinetics is caused by the existence of two reaction cycles for a single active site, the analog will not activate, and (iv) the observed effects of the analog are compatible with models such as that of Albers-Post model and the more recent versions of the Plesner et al. model in which ATP apart from being the substrate is required to accelerate the rate-limiting step of the reaction.

Introduction

The response of the Na^+/K^+-ATPase activity to the concentration of ATP is biphasic and can be described by the sum of two Michaelis-Menten equations, one with much lower V_m and K_m than the other (for references see Ref. 1). The usually accepted interpretation of this behaviour is that apart from being the substrate ATP accelerates the rate-limiting step of the reaction acting with low affinity through a mechanism that does not involve its hydrolysis (for references see Ref. 2). This view has been recently contested by Plesner et al. [3,4] who proposed for the Na^+/K^+-ATPase a reaction mechanism which eliminates the non-catalytic role of ATP and postulates that hydrolysis takes place through two different reaction cycles depending on the presence or absence of K^+.

Practically all the experimental evidence for a non-catalytic role of ATP comes from studies on partial reactions. Steady-state kinetics is of little avail for exploring this because, as we have previously shown [1], many reaction mechanisms display biphasic substrate curves which can be written as the sum of two Michaelis-Menten equations. The studies reported in this paper were based on the hypothesis that the limitations of steady-state substrate kinetics would be substantially reduced when examined together with the effects of a non-hydrolyzable analog of ATP, thus allowing to obtain information on the mechanism of the biphasic effect of ATP on Na^+/K^+-ATPase activity based on the overall functioning of the enzyme.

Non-hydrolyzable analogs of ATP are obtainable replacing the oxygen the β,γ phosphate bond of ATP by a methylene or an imino group. The analogs bind to the Na^+/K^+-ATPase [7], are resistant to enzymatic attack and replace some of the effects of ATP on cation fluxes [8] and on partial reactions [9]. In a preliminary communication [10] we reported that the ATP analog adenosine 5′-[β,γ-methylene]triphosphate ([β,γ-CH_2]-ATP) modified the substrate kinetics of the Na^+/K^+-

Abbreviation: [β,γ-CH_2]ATP, adenosine 5′-[β,γ-methylene]triphosphate.

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ATPase and activated the enzyme. The activating effect was confirmed recently by Suzuki et al. [11]. Similar effects were reported for the Ca^{2+}-ATPase of sarcoplasmic reticulum by Taylor and Hattan [12].

This paper contains a rather brief experimental section and what intends to be a sufficiently generic study of the expected responses of the enzyme to an unreactive analog of its substrate as to be useful for the kinetic analysis of other enzyme displaying non-hyperbolic substrate kinetics.

Materials and Methods

Na^+/K^+-ATPase was a partially purified preparation obtained from the outer medulla of dog kidney by the simpler of the two procedures described by Jörgensen [13]. ATPase activity was estimated measuring the release of $[^{32}P]P_i$ from [γ-^{32}P]ATP following the procedure already described [14]. Incubations were performed at 37 °C in media containing 0.33 mg/ml of bovine serum albumin and the salt composition given in the legends of the figures. pH was kept at 7.4 with 20 mM imidazole-HCl. In all cases ouabain-insensitive activity (measured with 1 mM ouabain) was less than 1% of the total activity. Labelled ATP was prepared according to the method of Glynn and Chapeil [15] except that no unlabeled orthophosphate was added.

The concentration of free Mg^{2+} was estimated from the total concentrations of ATP, [β,γ-CH_2]ATP and magnesium and the equilibrium constants for the dissociation of MgATP (87 μM) and of Mg[β,γ-CH_2]ATP (159 μM). The values of the dissociation constants were obtained measuring with a Ca^{2+}-sensitive membrane electrode (W. Möller, Zurich) the Ca^{2+} displaced by Mg^{2+} from CaATP or from Ca[β,γ-CH_2]ATP. The measurements were performed in solutions of the same composition and temperature as those in the experiments.

ATP ('Vanadate-free'), bovine serum albumin and enzymes and cofactors for the synthesis of [γ-^{32}P]ATP were from Sigma (U.S.A.). Adenosine 5'-[β,γ-methylene]triphosphate ([β,γ-CH_2]ATP) was from Boeringher AG (F.R.G.). $[^{32}P]$Orthophosphate was from the Comisión Nacional de Energía Atómica (Argentina). Salts and reagents were of analytical reagent grade.

Treatment of the data. The equations were fitted to the experimental data by a non-linear regression procedure based on the Gauss-Newton algorithm. The velocity variable was assumed to be homoscedastic (constant variance) and the concentration variable to have negligible error. When necessary, the concentration of ATP was adjusted for consumption, following the procedure described by Segel [16]. Convergence to the least squares solution was insured by the inclusion of a 'damping factor' [17,18].

The 'best fitting equation' was considered as that which gave the minimal standard deviation of the regression and the least biased fit. Bias was estimated plotting the difference between theoretical and experimental values vs. the independent variable. The universe of alternatives tested was necessarily restricted by practical considerations. Depending on the case it was composed either of equations derived from plausible mechanistic assumptions or of empirical expressions based on rational expressions. Examples of both are given under Results in this and in the following papers of this series.

Simulations were performed plotting solutions of steady-state rate equations. Except when otherwise indicated, values of rate and equilibrium constants were taken from the literature. In most cases these have not been obtained at 37 °C. Since no information on the temperature dependence of the constants is available, no correction for temperature was possible (for a more detailed comment on this see Ref. 1) and the simulations were used only for looking at general types of responses and not to perform quantitative predictions.

Results and Discussion

The effects of [β,γ-CH_2]ATP on the activity in the absence of potassium (Na^+-ATPase)

In the experiment shown in Fig. 1, Na^+-ATPase activity was measured as a function of the concentration of ATP from 0.2 to 4.8 μM, in media with 130 mM Na^+, non-limiting concentrations of free Mg^{2+} and either 0, 0.1, 0.2, 0.3 or 0.4 mM [β,γ-CH_2]ATP. It can be seen that the response of the activity to the concentration of ATP was hyperbolic and that as the concentration of [β,γ-CH_2]ATP increased the curves were shifted to the right. The whole set of data could be

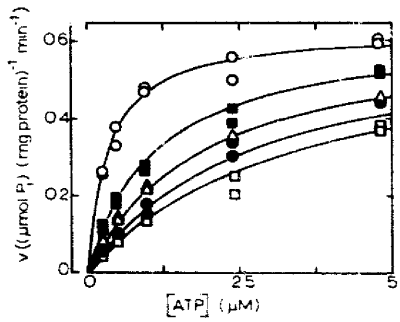

Fig. 1. A plot of Na^+-ATPase activity as a function of the concentration of ATP in media containing 0 (○), 0.1 (■), 0.2 (△), 0.3 (●) or 0.4 (□) mM [β,γ-CH_2]ATP, 130 mM NaCl and enough $MgCl_2$ as to keep constant at 0.3 mM the concentration of free Mg^{2+}. Continuous lines are the graphical representation of Eqn. 1 where each parameter was replaced by its best-fitting value. The values for each parameter (±S.E.) were: $V_m = 0.627 \pm 0.012$ μmol $P_i \cdot mg^{-1} \cdot min^{-1}$, $K_m = 0.331 \pm 0.028$ μM, $K_i = 43.4 \pm 3.6$ μM.

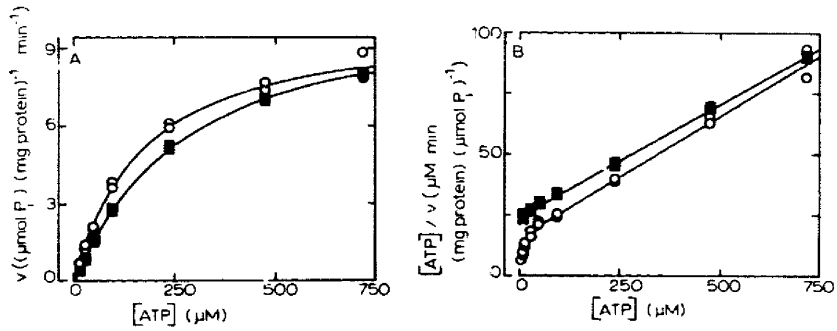

Fig. 2. (A) A plot of Na^+/K^+-ATPase activity as a function of the concentration of ATP in media containing either 0 (○) or 3 (■) mM [β,γ-CH_2]ATP, 130 mM NaCl, 5 mM KCl and enough $MgCl_2$ as to keep at 0.3 mM the concentration of free Mg^{2+}. The continuous line that fits the data at zero [β,γ-CH_2]ATP concentration is the graphical representation of:

$$v = V_{m1} + V_{m2}[ATP]/([ATP] + K_{m2}) \quad (2)$$

where the parameters were replaced by their best-fitting values. These (±S.E.) were: $V_{m1} = 0.175 \pm 0.099$ μmol $P_i \cdot mg^{-1} \cdot min^{-1}$, $V_{m2} = 10.06 \pm 0.28$ μmol $P_i \cdot mg^{-1} \cdot min^{-1}$, $K_{m2} = 176 \pm 17$ μM. Eqn. 2 corresponds to the sum of two Michaelis-Menten equations with its high-affinity component saturated, as it is to be expected since the lowest [ATP] used (2 μM) is about 10-fold higher than K_{m1} [1]. The continuous line that fits the data at 3 mM [β,γ-CH_2]ATP is the graphical representation of a Michaelis-Menten equation whose parameters were replaced by their best-fitting values. These (±S.E.) were: $V_m = 10.99 \pm 0.11$ μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ and $K_m = 273.1 \pm 6.7$ μM. (B) Hanes-Woolf plots of data in Fig. 2 (A). The continuous lines were calculated from the equation that fitted the data in Fig. 2 (A) using the best-fitting values of the parameters obtained by non-linear regression.

adequately described assuming simple dead-end (competitive) inhibition by [β,γ-CH_2]ATP, i.e.:

$$v = V_m[ATP]/([ATP] + K_m(1 + [AN]/K_i)) \quad (1)$$

where AN is [β,γ-CH_2]ATP.

Since it is generally accepted that in the absence of K^+ ATP behaves only as a substrate, it is likely that the dead-end effect is direct competition between [β,γ-CH_2]ATP and ATP at the active site. This and the fact that after binding [β,γ-CH_2]ATP does not undergo transformations [7], allowed us to consider K_i (Eqn. 1) as valid estimate of the equilibrium constant for the dissociation of the analog from the catalytic site of the ATPase. In the experiment in Fig. 1 the best-fitting value of K_i was 43.4 ± 3.6 μM which is not very different from that reported for the equilibrium dissociation constant of [β,γ-CH_2]ATP at 10°C (10 μM, [7]). Comparison of these values with K_m for ATP in the experiment of Fig. 1 (0.331 ± 0.028 μM) and with the reported values of the equilibrium constant for the dissociation of ATP from the active site of the ATPase (0.1–0.2 μM [7,19]) would indicate that the affinity of the catalytic site of the Na^+/K^+-ATPase for [β,γ-CH_2]ATP is almost two hundred times lower than for ATP.

The effects of [β,γ-CH_2]ATP on the substrate kinetics of the Na^+/K^+-ATPase

To see if the response of Na^+/K^+-ATPase activity to [β,γ-CH_2]ATP differed from that of the Na^+-ATPase activity (Fig. 1), Na^+/K^+-ATPase activity was measured as a function of the concentration of ATP from 2 to 750 μM, in media with non-limiting concentrations of the other ligands and either 0 or 3 mM [β,γ-CH_2]ATP (Fig. 2 (A and B)). Available experimental evidence indicates that 3 mM [β,γ-CH_2]ATP is considerably higher than the concentration at which the analog replaces some of the effects of ATP on the Na^+/K^+-ATPase. For example, [β,γ-CH_2]ATP acting with $K_{0.5} \approx 180$ μM is as effective as ATP in reversing the increase in the intrinsic fluorescence of the enzyme caused by K^+ [9]. Since the lowest concentration of ATP tested was 10-fold higher than the K_m of the high-affinity component of the ATPase [1], the function that gave best fit to the control data was the sum of a constant term, representing the V_m of this component plus a Michaelis-Menten equation. In contrast with this, in the presence of [β,γ-CH_2]ATP best fit was attained with a single Michaelis-Menten equation (Fig. 2A). The difference in substrate kinetics is appreciated more clearly in the Hanes-Woolf plots in Fig. 2B, which show that in the control experiment at low [ATP] the points curved downwards deviating from a straight line, while in the experiment with [β,γ-CH_2]ATP all points could be adjusted by a single straight line.

It would seem therefore that the main action of high concentrations of [β,γ-CH_2]ATP on the substrate curve of the Na^+/K^+-ATPase is to transform it into a single Michaelis-Menten curve. The best-fitting values of the parameters of the equations that were adjusted to the data (legend to Fig. 2) show that with [β,γ-CH_2]ATP, K_m was somewhat higher than K_{m2} of the control curve, and that V_m was not significantly different from V_{m2} of the control curve, indicating that the effects of [β,γ-CH_2]ATP are surmountable by ATP.

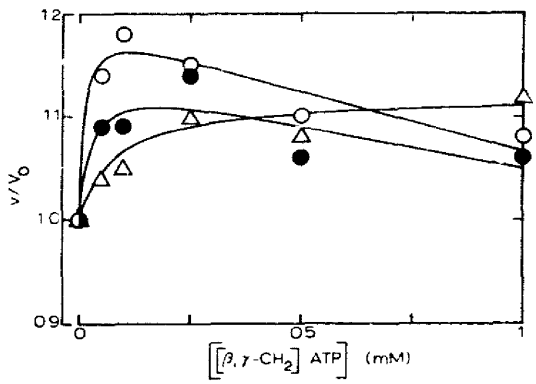

Fig. 3. A plot of Na^+/K^+-ATPase activity as a function of the concentration of $[\beta,\gamma\text{-}CH_2]$ATP in media containing 0.05 (○), 0.2 (●) or 1 (△) mM ATP, 130 mM NaCl, 5 mM KCl and enough $MgCl_2$ as to keep constant at 0.3 mM the concentration of free Mg^{2+}. The empirical equation:

$$v/V_0 = (K_a K_i + (V_m/V_0) K_i [AN])/(K_a K_i + K_i [AN] + [AN]^2) \quad (3)$$

was fitted to the experimental results at each concentration of ATP. For this reason [ATP] is not a variable in Eqn. 3. The continuous lines are the graphical representation of this equation where each parameter was replaced by its best-fitting value.

To analyze the effects of lower $[\beta,\gamma\text{-}CH_2]$ATP concentrations, we measured Na^+/K^+-ATPase activity as a function of the concentration of the analog in a 0 to 1 mM range in media containing non-limiting concentrations of Na^+ and K^+, enough $MgCl_2$ as to keep free $[Mg^{2+}]$ constant at 0.3 mM and either 0.05, 0.20 or 1.00 mM ATP. The concentrations of ATP were selected trying to insure that the high-affinity component of the substrate curve would remain near saturation at all the levels of the analog tested.

Results in Fig. 3 show that for the whole range of ATP concentrations assayed, $[\beta,\gamma\text{-}CH_2]$ATP increased Na^+/K^+-ATPase activity over the control values. This contrasts sharply with the hyperbolic decrease in velocity that would have taken place if $[\beta,\gamma\text{-}CH_2]$ATP acted only as a simple competitive inhibitor as it is the case with Na^+-ATPase activity. In media with 0.05 or 0.20 mM ATP as the concentration of $[\beta,\gamma\text{-}CH_2]$ATP increased, activity first raised and then decreased after passing through a maximum but remained above the control value up to the highest analog concentration tested. In the media with 1.00 mM ATP activity tended to saturation. Maximal relative activation of about 20% was attained with 0.05 mM ATP. In this condition activation was followed by a quick decline in activity. As the concentration of ATP increased relative activation diminished, its maximum was shifted towards higher $[\beta,\gamma\text{-}CH_2]$ATP concentrations and the apparent affinity for activation decreased.

The response to $[\beta,\gamma\text{-}CH_2]$ATP at each concentration of ATP could be fitted by the ratio between a linear and a parabolic function of the concentration of the analog (Eqn. 3 in the legend to Fig. 3). This equation is an empirical expression which does not intend to depict a particular mechanism but just to describe hyperbolic activation and hyperbolic inhibition by the same ligand. Eqn. 3 predicts that: (i) as [AN] goes from zero to infinity the activity will go from V_0 to zero, (ii) if there were no inhibition (K_i tending to infinity), activity would reach V_m and be half-maximal at $[AN] = K_a$, and (iii) if the apparent affinity for activation were very high (K_a tending to zero) the only observable effect would be inhibition which would be half-maximal at $[AN] = K_i$. Regression analysis of the data in Fig. 3 showed that as [ATP] raised K_i increased ($K_i \approx 8.5$ and 11.4 mM for 0.05 and 0.20 [ATP] and unmeasurable for 1.00 mM [ATP]) indicating that competition between ATP and $[\beta,\gamma\text{-}CH_2]$ATP persisted during Na^+/K^+-ATPase activity.

The mechanism of the effects of $[\beta,\gamma\text{-}CH_2]$ATP

The rate equations of reaction mechanisms that display biphasic substrate kinetics are rational expressions of degree equal or larger than two [1]. We have shown that, if certain relations hold among their coefficients, expressions of degree two can be written as the sum of two Michaelis-Menten equations [1] in which the physical meaning of the K_m values and the V_m values depends on the mechanism involved. Hence the observation that the response of Na^+/K^+-ATPase activity to ATP can be described as the sum of two Michaelis-Menten equations gives little information about the underlying mechanism. In what follows we will show how this indetermination is lessened when the study of substrate kinetics is accompanied by the analysis of its modification by inert analogs of the substrate.

(i) Two non-interacting active sites for the same substrate. This is the only mechanism in which each term of the sum of the Michaelis-Menten equations will measure the contribution of one of the sites to the overall activity and in which the K_m values and V_m values have their usual meaning. Substrate analogs will cause a linear increase in apparent K_m values with the consequent decrease in activity at non-saturating [ATP]. Hence in this model $[\beta,\gamma\text{-}CH_2]$ATP will be unable to activate. However, in systems of this kind substrate analogs are capable of eliciting Michaelis-Menten kinetics, i.e., if the K_m having the lower value raised with the concentration of the analog more steeply than the other, at a given concentration both K_m values will become equal and the substrate curve will become a single hyperbola. Alternatively, if the ratio: K_i/K_m were the same for both sites, the apparent K_m values would raise along parallel lines and Michaelis-Menten behaviour

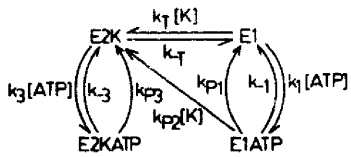

Fig. 4. A simplified version of the kinetic model for the Na^+/K^+-ATPase proposed by Plesner et al. [3].

would be approached at high concentrations of the analog.

(ii) Two interacting active sites for the same substrate. If the occupation of one of the sites decreased the affinity and increased the catalytic activity of the other, the substrate curve will be still describable by the sum of two Michaelis-Menten equations but, the K_m values and V_m values will loose their usual meaning. Taylor and Hattan [12] discarded this mechanism as an explanation of their findings on the effects of [β,γ-CH_2]ATP on the Ca^{2+}-ATPase of sarcoplasmic reticulum. This, however, is wrong: If the drop in affinity and the blockage of the sites were more than offset by the increase in the catalytic activity of the sites that remain available, the analog will activate, showing that activation by inert analogs of the substrate does not necessarily require, as we might intuitively think, the existence of a non-catalytic effect of the substrate. Furthermore in the presence of enough analog as to saturate the sites in the absence of the substrate, the interaction in affinity will be fully expressed by the action of the analog and Michaelis-Menten kinetics will ensue.

Hence, this class of systems are capable of responding to the analog in a way that is compatible with the experiments reported in this paper.

(iii) A single active site and two reaction cycles: the 1981 Plesner et al. model. None of the current models of the Na^+/K^+-ATPase proposes the coexistence of two interacting active sites as in the case just discussed. The scheme postulated by Plesner et al. in 1981 [3,4] approaches it in the sense that it only assigns catalytic effects to ATP. Its main postulate is "the existence of two distinct hydrolysis cycles for Na^+/K^+-ATPase with only one active substrate site" [4], one cycle representing Na^+-ATPase and the other Na^+/K^+-ATPase. In this model biphasic substrate kinetics, describable as the sum of two Michaelis-Menten equations, requires the simultaneous operation of both cycles. Hence biphasic response to ATP at high K^+ concentrations will only be possible if the affinity for K^+ as promoter of the transition between cycles is much less than that for activation of the K^+-dependent cycle.

Since the complexity of the model makes it impractical to derive its rate equation, we analyzed its predictions on the effects of substrate analogs by means of the simplified scheme of Fig. 4. We assumed that the analog is a dead-end inhibitor at E_1 and E_2K (Fig. 4). This allowed us to include its effects multiplying k_1 and k_T by $(1+([AN]/K_{AN1}))^{-1}$ and k_{-T} and k_3 by $(1+([AN]/K_{AN2}))^{-1}$ were AN is [β,γ-CH_2]ATP and K_{AN1} and K_{AN2} are the dissociation constants of AN from E_1 and E_2K, respectively. This yielded the following equation for the rate as a function of the concentrations of ATP and of the analog:

$$v = \frac{i[ATP] + j[ATP]^2}{k_0 + \kappa_1[AN] + (l_0 + l_1[AN])[ATP] + m[ATP]^2} \quad (4)$$

where i, j, k_0, k_1, l_0, l_1 and m are functions of the rate constants.

Eqn. 4 shows that at constant ATP, the activity will decrease continuously with the concentration of [β,γ-CH_2]ATP strongly suggesting that models like those shown in Fig. 4 are unable to account for activation by inert analogs of the substrate.

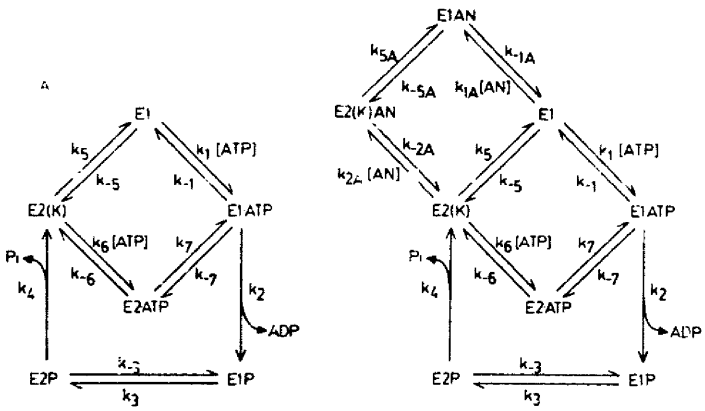

Fig. 5. (A) The version of Moczydlowski and Fortes [23] of the Albers-Post model for the Na^+/K^+-ATPase. The version assumes the existence of a single site for the addition of ATP. For the sake of clarity the actions of cations were omitted. (B) A modification of the same model including the assumptions given in the text for the effects of a non-hydrolyzable analog of ATP.

(iv) The substrate as activator: the Albers-Post model

More recent versions of the model of Plesner et al. [5,6] uphold the bicyclic hypothesis but include the additional postulate that in the $(Na^+ + K^+)$ cycle the binding of ATP is necessary for the release of K^+. In what regards substrate kinetics, this effect is analogous to that previously proposed for the Albers-Post model. The model explains biphasic substrate kinetics postulating that ATP not only binds at its catalytic site in E_1, but also with much lower affinity to E_2K accelerating the $E_2K \rightarrow E_1 + K^+$ transition without undergoing hydrolysis [2]. Kinetic studies cannot discriminate if the two effects of ATP are exerted at different sites [20,21] or at two states of the same site. Evidence in favour of the latter view has been provided by Moczydlowski and Fortes [22,23] who developed the reaction scheme shown in Fig. 5A. We incorporated the effects of analogs as [β,γ-CH_2]ATP into this scheme assuming that they bind to the same sites as ATP, being effective in accelerating the $E_2K \rightarrow E_1$ transition but still acting as competitive inhibitors of the nucleotide at the catalytic site in E_1 (Fig. 5B).

Inspection of Fig. 5B makes it likely that in the presence of an excess of analog the substrate curve will approach a single hyperbola. To envision this, let us imagine that ATP is added to an incubation medium containing sufficient [AN] as to occupy all the sites for ATP in the enzyme. Since our premise is that the displacement of the analog by ATP from E_2 will have little effect, the ATPase activity elicited by ATP will result mainly from the replacement of the analog by ATP in E_1. Under these conditions the curve of Na^+/K^+-ATPase vs. [ATP] will approach a single hyperbola since it will chiefly reflect the occupation of the catalytic site by the nucleotide.

Likewise, the scheme in Fig. 5B allows for activation by the analog. For this to be so, the rate through the new pathway for the $E_2 \rightarrow E_1$ transition opened by the analog must be sufficiently high as to outweigh, for a given range of concentrations of ATP and of AN, the negative effects of competition between the analog and ATP at E_1.

To see if these predictions can be quantitatively accounted for, we derived the rate equation for the scheme in Fig. 5B assuming steady-state for the binding of ATP and rapid-equilibrium for the binding of the analog. The following expression was obtained:

$$v = \frac{(a + a_1[AN])[ATP] + b[ATP]^2}{c[ATP]^2 + (d + d_1[AN])[ATP] + e + e_1[AN] + e_2[AN]^2} \qquad (5)$$

where the meanings a, a_1, b, c, d, d_1, e, e_1 and e_2 are given in Table I.

Notice that if all the terms of the right-hand side were divided by e_2 then at constant [ATP] Eqn. 5 would become formally identical to the empirical Eqn. 3 we employed to fit the results in Fig. 3. This gives indirect suport to the consistency between the model in Fig. 5B and the experimental results.

TABLE I

The meaning of coefficients in Equation 5

The values calculated replacing the coefficients into Eqn. 5 will be given in s^{-1}. To express them as specific activities they have to be multiplied by μmol enzyme per mg protein.

$$a = k_5\left(\frac{k_{-6}+k_7}{k_6}\right) + k_{-5}\left(\frac{k_7}{k_1}\right)$$

$$a_1 = \frac{k_{5A}}{K_{AN2}}\left(\frac{k_{-6}+k_7}{k_6}\right) + \frac{k_{-5A}}{K_{AN1}}\left(\frac{k_7}{k_1}\right);\ b = k_7;\ c = 1 + k_7F + \frac{k_{-7}}{k_2}$$

$$d = \frac{k_{-6}+k_7}{k_6}(1+k_5F) + \frac{k_{-5}}{k_1}(1+k_7F) + \frac{k_{-1}}{k_1}\left(\frac{k_7+k_{-5}}{k_2}\right) + \frac{k_{-7}}{k_2}\left(\frac{k_{-6}+k_5}{k_6} + \frac{k_{-5}}{k_1}\right)$$

$$d_1 = \frac{k_{-6}+k_7}{k_6}\left(\frac{1+k_{5A}F}{K_{AN2}}\right) + \frac{k_{-5A}}{k_1}\left(\frac{1+k_7F}{K_{AN1}}\right) + \frac{k_{-1}}{k_1}\left(\frac{k_7+k_{-5A}}{k_2K_{AN1}}\right) + \frac{k_{-7}}{k_2}\left(\frac{k_{-6}+k_{5A}}{k_6K_{AN2}} + \frac{k_{-5A}}{k_1K_{AN1}}\right)$$

$$e = E(k_5 + k_{-5})$$

$$e_1 = E((k_5 + k_{-5A})/K_{AN1} + (k_{5A} + k_{-5})/K_{AN2})$$

$$e_2 = E\left(\frac{k_{5A}+k_{-5A}}{K_{AN1}K_{AN2}}\right)$$

$$E = \left(\frac{k_{-6}+k_7}{k_1k_6}\left(1+\frac{k_{-1}}{k_2}\right) + \frac{k_{-6}k_{-7}}{k_1k_2k_6}\right)$$

$$F = \frac{1}{k_2} + \frac{1}{k_3} + \frac{1}{k_4}\left(1+\frac{k_{-3}}{k_3}\right)$$

$$K_{AN1} = \frac{k_{-1A}}{k_{1A}};\ K_{AN2} = \frac{k_{-2A}}{k_{2A}}$$

The use of rational expressions like Eqn. 5 in enzyme kinetics has been studied in detail by Ferdinand [24] who has given the conditions under which such expressions become hyperbolic. Applying these to Eqn. 5 the response to ATP would follow Michaelis-Menten kinetics only if:

$$(a + a_1[AN])^2c + b^2(e + e_1[AN] + e_2[AN]^2) - (a + a_1[AN])b(d + d_1[AN]) = 0 \qquad (6)$$

Eqn. 6 makes clear that in the absence of AN the only general way to obtain Michaelis-Menten kinetics is to consider that $a = e = 0$. This demands k_5 and k_{-5} to be zero (Table I). Hence in the Albers-Post model the

TABLE II

Values of rate (k_i) and equilibrium (K_{ANi}) constants in the scheme of Fig. 5

Except for K_{AN1}, all values are given for room temperature (20–25 °C). The subscripts within parenthesis are the cations present in the media.

Constant	Value	Units	Source	Ref.
k_1	10	$s^{-1} \cdot \mu M^{-1}$	Measured	25
k_{-1}	2	s^{-1}	Estimated as $k_1 K_{s1}$	19
k_2	180	s^{-1}	Measured	26
k_3	75	s^{-}	Measured	27
k_{-3}	23	s^{-1}	Estimated	27
$k_{4(Na)}$	2.5	s^{-1}	Measured	28
$k_{4(Na,K)}$	233	s^{-1}	Measured	26
$k_{5(Na)}$	54	s^{-1}	Assumed equal to k_7	1
$k_{5(Na,K)}$	0.26	s^{-1}	Measured	29
$k_{-5(Na,K)}$	0.0026	s^{-1}	Assuming that with 130 mM Na^+ and 10 mM K^+ $[E_1]/[E_2] = 100$	
k_6	1.6	$s^{-1} \cdot \mu M^{-1}$	Estimated	29
k_{-6}	833	s^{-1}	Estimated	29
k_7	54	s^{-1}	Measured	29
k_{-7}	0.00023	s^{-1}	Estimated from k_5, k_{-5}, k_6, k_{-6}, k_1, k_{-1} and k_7 using the constraints of the cycle in Fig. 5A	
k_{5A}	54	s^{-1}	Assumed to be equal to k_7	
k_{-5A}	0.0038	s^{-1}	Estimated from k_{5A}, k_5, k_{-5}, K_{AN1} and K_{AN2} using the constraints of the cycle in Fig. 5B	
K_{AN1}	43.4	μM	Equal to K_i for Na^+-ATPase (legend to Fig. 1)	
K_{AN2}	6250	μM	Taken as: $(K_{0.5AN}/K_{0.5ATP})/(k_{-6}/k_6)$, where $K_{0.5}$ values were from Ref. 8	

ATP-independent $E_2 \rightarrow E_1$ transition is indispensable for biphasic substrate kinetics.

When [AN] tends to infinity, Eqn. 6 will tend to:

$$\frac{k_{5A} - k_7}{K_{AN2}^2} \frac{k_{-6} + k_7}{k_6} + \frac{k_7(k_{-5A} + k_7)}{k_1 K_{AN1} K_{AN2}} = 0 \qquad (7)$$

where K_{AN1} and K_{AN2} are the equilibrium constants for the dissociation of the analog from E_1 and E_2, respectively.

Eqn. 7 shows that when [AN] tends to infinity the system will display Michaelis-Menten kinetics if k_{5A} were equal to k_7 and if the second term of Eqn. 7 were zero. For the latter condition the affinity of E_1 for AN must tend to zero that is K_{AN1} must tend to infinity (the mathematically equivalent case of K_{AN2} tending to infinity is irrelevant because k_{5A} would not exist). Notice these conditions are self-evident from the intuitive reasoning we made at the beginning of this section since if [AN] were as effective as ATP at E_2 and were not recognized by E_1, the kinetic effects of ATP added to an enzyme fully saturated with AN would result only from the occupation of the catalytic site in E_1.

Eqns. 6 and 7 demonstrate that in the scheme of Fig. 5 the substrate curve will deviate from Michaelis-Menten kinetics at zero and non-limiting [AN]. This feature is independent of the particular values of the constants, it only requires $k_5 > 0$ (Eqn. 6) and it is not submitted to the uncertainties mentioned in Materials and Methods.

Using the values in Table II we can calculate that the left-hand side of Eqn. 6 will be $-3\,763\,620$ at zero and 32.18 at non-limiting [AN]. Not very different values for the upper limit are obtained when $k_{5A} < k_7$ but still large enough as to give activation. Taking due account of the limitations of these calculations, these values indicate that: (i) the deviation from Michaelis-Menten-like behaviour will be much larger at zero [AN] than when [AN] tends to infinity and, (ii) as the sign of the extremes is different, Eqn. 6 will be fulfilled at an intermediate value of [AN] for which the substrate curve will become hyperbolic.

We checked the predictions of Eqn. 6 by computer simulations of Hanes-Woolf plots of the activity vs. [ATP] at various concentrations of [β,γ-CH_2]ATP (results not shown). At low [AN] the plots were noticeably concave downward. As [AN] increased the plots first become linear, that is hyperbolic, and then slightly concave upwards. Hyperbolic behaviour was observed aroun 1 4 mM [AN]. This is close to the concentration of [β,γ-CH_2]ATP used in the experiment in Fig. 2. The deviation from hyperbolic behaviour a high [AN] was sufficiently small as to make it likely that it would be difficult to detect experimentally due to the expected error of the measurements.

Activation by AN would take place within the ranges of [ATP] and of [AN] within which Eqn. 5 yielded a higher value than the same equation but with [AN] = 0. Subtracting from Eqn. 5 the same equation with [AN] = 0 this can be formally expressed as:

$$A[ATP]^2 + B[ATP] + C > 0 \qquad (8)$$

where:

$$A = a_1c - d_1b \quad (9)$$

$$B = a_1d - ad_1 - be_1 - be_2\,[AN] \quad (10)$$

$$C = a_1e - ae_1 - ae_2[AN] \quad (11)$$

and the coefficients in lower case are those of Eqn. 7 and Table I.

The concentration of the nucleotide at which, for a given value of [AN], activity would be equal as that in the absence of AN ($[ATP]_0$) will be given by the roots of the left-hand side of expression (8) i.e.:

$$[ATP]_0 = -B \pm (B^2 - 4AC)^{1/2}/2A \quad (12)$$

Eqn. 12 allows us to define three conditions: (i) if there are no real-positive roots there will be no activation, (ii) if there is only one real-positive root activation will take place at all finite concentrations of ATP larger than $[ATP]_0$ and (iii) if there are two real-positive roots activation will occur within the range defined by the two values of $[ATP]_0$. Since as we will show in the Appendix, C and A are negative, two real-positive values of $[ATP]_0$ will exist only if B is positive and large enough for $B^2 > 4AC$.

As B and C are linear decreasing functions of [AN] and C is always negative, activation will be not possible at sufficiently high [AN] or at sufficiently low [ATP]. This is intuitively obvious, since in both conditions competition between AN and ATP will predominate over activating effects.

The predictions of Eqn. 8 are independent of the actual values of the constants and are not submitted to the uncertainties mentioned in Materials and Methods. They only require the fulfillment of the assumptions of the scheme in Fig. 5B. Notice that though in Table II we assumed that $k_{5A} = k_7$, for a certain range of values of k_{5A} that are less than k_7 but larger than k_5, B will remain positive, A will remain negative and activation will occur because B^2 will stay larger than 4 AC.

The considerations of the preceding paragraphs are illustrated by the simulations in Fig. 6 in which the ratio between the calculated activity in the presence and in the absence of [β,γ-CH_2]ATP is plotted as function of the concentration [β,γ-CH_2]ATP for several concentrations of ATP. It can be seen that: (i) AN activates, (ii) activation requires a minimal ATP concentration and disappears at higher concentrations of the nucleotide, (iii) relative activation is maximal at very low ATP concentrations, (iv) in all cases activation is followed by a decline in activity, (v) as ATP concentration increases the activating and inhibitory phases of the curve and the position of the maximum are shifted to the right so that for a given range of [AN] its effect may appear as saturable as in the curve with 1 mM ATP in Fig. 3.

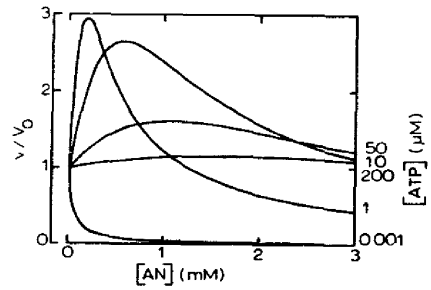

Fig. 6. Simulations of the effect of [β,γ-CH_2]ATP on Na^+/K^+-ATPase activity. The values were calculated using Eqn. 5 and represented as the ratio between the calculated activity in the presence and in the absence of [β,γ-CH_2]ATP as a function of the concentration of [β,γ-CH_2]ATP at the indicated concentrations of ATP given at the right of the graph. The calculations were performed using the values in Table II assuming that $k_{-5} = k_{-7} = k_{-5A} = 0$.

We have already mentioned that in our activation experiments we selected the concentrations of ATP with the idea of insuring that ATP would not be displaced by [β,γ-CH_2]ATP from E_1. The chosen concentrations were much higher than the surprisingly low ones required for maximal activation in the simulations of Fig. 6. A likely reason for this is that we did not take into account that, as judged by the values of the ratios: $K_{AN2}/(k_{-6}/k_6)$ and $K_{AN1}/(k_{-1}/k_1)$ (Table II), the nucleotide site in E_1 appears to be 20-times more selective for ATP relative to [β,γ-CH_2]ATP than that in E_2. This would insure effects of the analog at E_2 at low concentrations of ATP without significant displacement of the nucleotide from E_1.

The predicted requirements for ATP during activation by [β,γ-CH_2]ATP (Fig. 6) agree with the observations by Suzuki et al. [11] who obtained a substantially larger activation than that reported in this paper using the same preparation of the Na^+/K^+-ATPase but only 2.7 μM ATP. This may indicate that the selectivity is maintained at 37 °C, a view which is also supported by the studies reported in this paper on the affinity for [β,γ-CH_2]ATP as dead-end inhibitor of the Na^+-ATPase.

In spite of the qualitative agreement between the experimental and the simulated results, the predicted value for the apparent affinity for [β,γ-CH_2]ATP as activator is smaller and the predicted value of the maximal relative effect is larger than the experimentally observed ones both for the results in this paper (cf. Figs. 3 and 6) and for those of Suzuki et al. [11]. As mentioned under Materials and Methods the simulations were run using values for rate and equilibrium constants measured at temperatures lower than 37 °C. It cannot be discarded that this is the cause of the discrepancies with the experimental results. In fact by arbitrary modifications of the values in Table II it is possible achieve a much better quantitative agreement without loosing

the predictions of the model in what regards the effects of [β,γ-CH_2] ATP on the shape of the substrate curve.

The theoretical predictions in what regards the effects of [β,γ-CH_2]ATP on the shape of the substrate curve and on activation make it reasonable to conclude that among the current schemes of the Na^+/K^+-ATPase, those which propose an activating effect of ATP are able to predict the observed interactions between ATP and [β,γ-CH_2]ATP during Na^+/K^+-ATPase activity. It must be stressed, however, that neither the experimental results nor the theoretical considerations in this paper allow to discard models in which the biphasic response to ATP and the effect of [β,γ-CH_2]ATP are a consequence of the presence of two interacting active sites for the nucleotide.

Appendix

Activation by [β,γ-CH_2]ATP in the Albers-Post model

The coefficients of Eqn. 12: Using Table I and Eqns. 9–11 these can be expressed as:

$$A=\frac{k_{-6}/k_6}{K_{AN2}}\left(1+\frac{k_7}{k_{-6}}\right)(k_{5A}-k_7)-\frac{k_{-1}/k_1}{k_{AN1}}\left(\frac{k_7^2}{k_2}\right)\left(1+\frac{k_{-5A}}{k_{5A}}\right) \tag{13}$$

which shows that A will be negative if $k_{5A} \leq k_7$.

$$B=\frac{k_{-6}+k_7}{k_6}\left[\frac{k_{-6}+k_7}{k_6}\frac{k_{5A}-k_5}{K_{AN2}}-\frac{k_7}{k_1}\left(\frac{k_{5A}}{K_{AN2}}-\frac{k_5}{K_{AN1}}\right)\right.$$
$$\left.-\frac{k_7}{k_2}\left[\frac{k_{-1}+k_2}{k_1}+\frac{k_{-7}}{k_1(1+k_7/k_{-6})}\right]\right]\left[2\left(\frac{k_5}{K_{AN1}}+\frac{k_{-5}}{K_{AN2}}\right)\right.$$
$$\left.+\frac{k_{5A}+k_{-5A}}{K_{AN1}K_{AN2}}[AN]\right]\Bigg] \tag{14}$$

The positive factor $(k_7k_5)/(k_1K_{AN1})$ in the second term is canceled out by the same factor in the third term. Hence last two terms of Eqn. 14 will be negative and for B to be positive k_{5A} must be larger than k_5 and the first term of the equation must be greater than the sum of the absolute values of the others. However, since the third term of Eqn. 14 is a linear function of [AN], this can only hold below a given value of [AN].

The value of C at zero [AN] (C_0) will be:

$$C_0=-\left(\frac{k_5}{K_{AN1}}+\frac{k_{-5}}{K_{AN2}}\right)\left(\frac{k_{-6}+k_7}{k_6}k_5+\frac{k_{-5}k_7}{k_1}\right),$$
$$\times\left[\frac{k_{-6}+k_7}{k_6k_1}\left(1+\frac{k_{-1}}{k_2}\right)+\frac{k_{-6}k_{-7}}{k_6k_1k_2}\right] \tag{15}$$

Since C is a decreasing function of [AN] (Eqn. 11), its maximal value will be C_0 so that C will be always negative.

As A and C are negative their product will be positive, this can be written for the case $k_{5A}=k_7$ as:

$$AC=\frac{k_{-1}/k_1}{K_{AN1}}\left(\frac{k_7^2}{k_2}\right)\left(1+\frac{k_{-5A}}{k_{5A}}\right)\left[\frac{k_{-6}+k_7}{k_6k_1}\left(1+\frac{k_{-1}}{k_2}\right)+\frac{k_{-6}k_{-7}}{k_6k_1k_2}\right]$$
$$\times\left(\frac{k_{-6}+k_7}{k_6}k_5+\frac{k_{-5}k_7}{k_1}\right)$$
$$\times\left(\frac{k_5}{K_{AN1}}+\frac{k_{-5}}{K_{AN2}}+\frac{k_{5A}+k_{-5A}}{K_{AN1}K_{AN2}}[AN]\right) \tag{16}$$

It is convenient to consider Eqn. 16 as a linear function of [AN] with positive intercept and slope multiplied by a factor which includes A.

Conditions that favour activation. We have established that A and C are negative. This justifies the statement in Discussion that activation by an analog requires B to be positive and large enough for $B^2 > 4AC$. Except for the requirement about the relative values of k_5, k_{5A} and k_7, this is independent of the values of rate and equilibrium constants and therefore free of the restrictions given in Material and Methods.

It is evident that activation will be favoured by large values of B and small absolute values of AC. In what follows we will show that the conditions that help this are those which assist the displacement of ATP by the analog in E_2, were it is effective, and/or the displacement of the analog by ATP from E_1 were it is ineffective, i.e.:

(a) A large affinity for ATP compared to that for the analog in E_1. If this were so $(k_{-1}/k_1)/K_{AN1}$ would be small. A decrease in this ratio will increase B and decrease AC (eqns. 14 and 16). The high selectivity for ATP of E_1 at 37 °C is supported by the results in this paper on the affinity of [β,γ-CH_2]ATP as dead-end inhibitor of the Na^+-ATPase.

(b) A large value of k_2 with respect to k_7. This will drive the steady-state level of E_1ATP below its equilibrium value providing a kinetic mechanism for promoting the release of analog and the binding of ATP at E_1. When the ratio: k_7/k_2 decreases B raises and the product AC decreases (Eqns. 14 and 16).

(c) A large value of the ratio $(k_{-6}/k_6)/K_{AN2}$. This measures the relative affinities for ATP and for the analog of E_2. The larg : the ratio the easier the displacement of ATP from E_2. An increase in its value will increase B (Eqn. 14).

(d) A large value of the term $1+(k_7/k_{-6})$. This term measures the increase in the apparent dissociation constant for ATP caused by the non-equilibrium binding of the nucleotide to E_2. An increase in its value will increase B (Eqn. 14).

(e) Small values of k_{-5A}. This implies that the equilibrium concentration of E_1AN is high respect to that of E_2AN favouring the accumulation of the species from

which the analog must be replaced by ATP. Low values of k_{-5A} increase B and decrease AC (Eqn. 14 and Eqn. 16).

Notice that conditions (a) to (e) are independent of the uncertainties about the actual values of rate and equilibrium constants. In addition is is interesting to point out that if all these conditions were fulfilled to the limit then AN would be as effective as ATP at E_2 and ineffective at E_1. Hence the condition for optimal activation is analogous as that required to obtain Michaelis-Menten behavior at [AN] tending to infinite (see comments to Eqn. 7).

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