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pH-Induced Changes in the Reactions Controlled by the Low- and High-Affinity Ca^{2+}-Binding Sites in Sarcoplasmic Reticulum†

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ABSTRACT: The effect of pH on the Ca^{2+}-binding sites of high and low affinity, located respectively on the outer and inner surfaces of the sarcoplasmic reticulum membrane, was investigated using intact and leaky sarcoplasmic reticulum vesicles. With the use of intact vesicles, different pH profiles of membrane phosphorylation and rates of nucleoside triphosphate hydrolysis were obtained depending on the assay temperature, on the Ca^{2+} concentration, and on whether ATP or ITP was used as substrate. The different pH profiles were related to the amount of Ca^{2+} accumulated by the vesicles, i.e., to different degrees of saturation of the inner, low-affinity Ca^{2+}-binding site. With the use of leaky vesicles, the saturation of the two Ca^{2+}-binding sites could be controlled more precisely since the Ca^{2+} concentration on both sides of the membrane was equal to the Ca^{2+} concentration of the assay medium. Using leaky vesicles and measuring the rates of nucleotide hydrolysis, nucleotide–phosphate exchange and membrane phosphorylation by nucleotide as an indication of the degree of saturation of the Ca^{2+}-binding sites, we observed that the affinity of both the high- and low-affinity sites increased three to four orders of magnitude when the pH of the assay medium was increased from 6.1 to 8.65.

The Ca^{2+} accumulation by sarcoplasmic reticulum vesicles isolated from skeletal muscle is mediated by a membrane-bound NTPase[1] (Hasselbach, 1974; MacLennan and Holland, 1975). The reversibility of the Ca^{2+} pump, involving NTP synthesis and Ca^{2+} efflux, has been demonstrated (Barlogie et al., 1971; Makinose and Hasselbach, 1971; Hasselbach, 1974). Two types of Ca^{2+}-binding sites having different affinities appear to be involved in the forward and reverse reaction. When only the high-affinity site, located on the outer surface of the membrane, is saturated, the transport enzyme (E) is phosphorylated by NTP ($E \sim P$) and the NTPase activity is maximal (Hasselbach, 1974; de Meis and Carvalho, 1974; Ikemoto, 1974; Souza and de Meis, 1976). Different steady-state levels of phosphoenzyme are obtained depending on whether ATP or ITP is used (Souza and de Meis, 1976). This has been interpreted to mean that ATP besides acting as substrate could also activate the rate of $E \sim P$ hydrolysis (de Meis and de Mello, 1973; Froehlich and Taylor, 1975; Carvalho et al., 1976; Souza and de Meis, 1976).

When only the low-affinity Ca^{2+}-binding site, located in the inner surface of the membrane, is saturated, the enzyme is phosphorylated by P_i, the phosphate is transferred to ADP, and ATP is synthesized (Makinose, 1972; Yamada et al., 1972; de Meis, 1976; de Meis and Carvalho, 1976). The simultaneous binding of calcium to both low- and high-affinity sites inhibits the NTPase activity and activates a steady exchange between P_i and the γ-phosphate of NTP (de Meis and Carvalho, 1974; Ikemoto, 1974; de Meis and Sorenson, 1975; Carvalho et al., 1976). This exchange is the result of the transport enzyme operating simultaneously forward (NTP hydrolysis) and backward (NTP synthesis from NDP and P_i), where part of the energy derived from the NTP hydrolysis seems to be conserved by the system for the synthesis of a new NTP molecule (de Meis and Carvalho, 1974; Carvalho et al., 1976).

In this paper, we attempted to modify the affinity of the two Ca^{2+}-binding sites by varying the pH of the assay medium (Meissner, 1973; Verjovski-Almeida and de Meis, 1975). The

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[1] Abbreviations used: NTPase, nucleoside 5′-triphosphatase; NTP, nucleoside 5′-triphosphate; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; P_i, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; SE, standard error.

aim was to study how such modifications would affect the different reactions catalyzed by the Ca^{2+} transport NTPase. In order to distinguish pH effects on the inner Ca^{2+}-binding sites from those on the external high-affinity sites, we have compared intact with leaky vesicles. In the former, the internal Ca^{2+} concentration increases as Ca^{2+} is accumulated; in the latter, both sides of the membrane can be exposed to the same Ca^{2+} concentrations over a wide range.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as previously described (de Meis and Hasselbach, 1971). When used with no further treatment, they are referred to as intact vesicles.

Leaky vesicles were prepared by incubating sarcoplasmic reticulum vesicles (15 to 20 mg of protein/ml) for 30 min at room temperature in a 20 mM Tris, pH 9.0 solution containing 1 mM EGTA (Duggan and Martonosi, 1970). Afterward the pH was adjusted to 7.0 by the addition of Tris-maleate buffer (pH 6.0) and kept at 0 °C. The leaky vesicles were then used within 30 min.

$^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy and further purified by the procedure described by Kanazawa and Boyer (1973).

NTP ⇌ P_i exchange was assayed by measuring the formation of [γ-^{32}P]NTP from NDP and $^{32}P_i$ at 30 °C, the excess of P_i being removed by extraction of the ammonium phosphomolybdate complex with a mixture of benzene and isobutyl alcohol (50% v/v) (de Meis and Carvalho, 1974).

Membrane Phosphorylation by [γ-^{32}P]NTP. The phosphorylation reaction was started by addition of vesicles and stopped after 10–20 s at 37 °C by adding 2.5 ml of an ice-cold solution of 1 M perchloric acid containing 2 mM orthophosphate. The suspension was centrifuged in the cold at 5000*g* for 15 min. The protein pellet was washed four times with 4-ml samples of ice-cold 125 mM perchloric acid solution containing 2 mM orthophosphate. After the washings, the pellet was resuspended in 1 ml of a solution containing 0.1 N NaOH, 2% Na_2CO_3, and 1 mM orthophosphate. The pellet was dissolved by heating the suspension in boiling water for 20 min. An aliquot of this solution was counted in a liquid scintillation counter and another aliquot was used for protein measurement by the method of Lowry et al. (1951). Controls for nonspecific [γ-^{32}P]NTP binding were performed as previously described (de Meis and de Mello, 1973).

NTPase activity was assayed by measuring the release of $^{32}P_i$ from [γ-^{32}P]NTP at 37 °C. The $^{32}P_i$ was extracted from the assay medium as previously described (de Meis and Carvalho, 1974). When the NTPase activity was measured simultaneously with the membrane phosphorylation by [γ-^{32}P]NTP, the extraction was carried out on an aliquot of 0.5 ml after stopping the reaction with perchloric acid.

The assay medium composition was as described in the figure legends. The reaction was started by the addition of intact or leaky vesicles and stopped after 20-s incubation by the addition of cold perchloric acid. The concentrations of vesicles and of ATP or ITP were chosen so that less than 30% of the substrate was hydrolyzed during the 20-s incubation. In the experiments of Figures 2, 4, and 5, in conditions of maximal NTPase activity, the percents of ATP or ITP hydrolyzed were respectively 5.6, 27.8, and 12.7.

Calculation of Free Calcium Concentration. In the experiments of Figures 2 and 3, 0.3 mM EGTA and different concentrations of $CaCl_2$ were included in the assay media in order to obtain the free Ca^{2+} concentrations shown in the figures.

TABLE I: Percent Inhibition of the Formation of Phosphorylated Intermediate (E ~ P) and of the ITPase Activity by CaEGTA Complex, in the Presence of 0.2 mM Free Calcium.[a]

	pH 7.0		pH 8.5	
CaEGTA (mM)	E ~ P Level	ITPase Act.	E ~ P Level	ITPase Act.
0 (control)	0	0	0	0
0.3	0	0	0	0
1.0	0	12 ± 5	27 ± 2	24 ± 6
3.0	24 ± 11	35 ± 5	62 ± 1	73 ± 6
10.0	27 ± 6	47 ± 6	69 ± 1	84 ± 6

[a] Conditions described under Materials and Methods. The figures represent the means and standard errors of three experiments.

The proportion of Ca^{2+} which was bound to EGTA at each pH was calculated from the pH of the medium, the pK_a values of EGTA, and the absolute association constants for the formation of CaEGTA complex from $EGTA^{4-}$ and $HEGTA^{3-}$ (10^{11} and 2.1×10^5 M^{-1}, respectively) (Schwarzenbach, 1957). A program was prepared for a Hewlett-Packard 9810A calculator to perform the calculations, which is a simplified version of the one used by Vianna (1975).

In the course of the experiments it was found that high concentrations of the CaEGTA complex inhibited both the ITPase activity and the membrane phosphorylation by ITP independently of the free Ca^{2+} concentration of the medium. Table I shows that the inhibitory effect of the CaEGTA complex was more pronounced at pH 8.5 than at pH 7.0. In this experiment, the assay medium composition of the control test was 0.1 mM [γ-^{32}P]ITP, 4 mM $MgCl_2$, 40 mM Tris-maleate buffer (pH 7.0 or 8.5), 0.1 mg/ml of leaky-vesicle protein, and 0.2 mM $CaCl_2$. Equimolar amounts of $CaCl_2$ and EGTA were included in the other tests, giving the CaEGTA concentrations shown in the table, without changing significantly the free Ca^{2+} concentration of the assay medium.

Because of this finding, the EGTA concentration used in the experiments of Figures 2 and 3 was 0.3 mM.

In all experiments, the pH of the assay media was checked before and after the incubation period and did not vary more than 0.15 unit.

Results

pH Effect on Membrane Phosphorylation by ATP and ITP in Intact Vesicles. The maximum amount of phosphoprotein formed from ITP is greater than that from ATP (Figure 1). This has been noted previously and a satisfactory explanation is not yet available (Friedman and Makinose, 1970; Pucell and Martonosi, 1971; de Meis and de Mello, 1973; Froehlich and Taylor, 1975). In this study we will focus on the differences in the profiles of membrane phosphorylation obtained at different pH values and Ca^{2+} concentrations.

The degree of membrane phosphorylation by ATP sharply increases in the pH range of 4.5 to 6.0 (Figure 1) and does not vary significantly when the pH is further raised to 8.5. A different pH profile is obtained when ITP is used as substrate. A significant degree of phosphorylation can only be measured at pH values above 5.5, and it increases abruptly when the pH is raised to 7.5. When the assay temperature is decreased from 37 to 0 °C, the pH profile of membrane phosphorylation by ATP remains essentially the same (data not shown), whereas

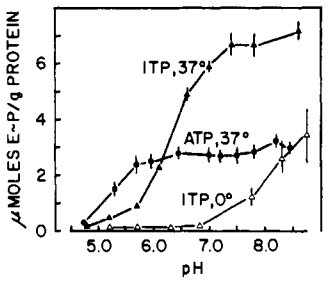

FIGURE 1: Different pH profiles of E~P from ITP and ATP in intact vesicles. The assay medium composition was 1 mM [γ-^{32}P]NTP, 4 mM $MgCl_2$, 40 mM Tris-maleate buffer adjusted to the desired pH with either NaOH or HCl and 0.1 mM $CaCl_2$. The reaction was started by the addition of the intact vesicles to a total of 0.067 mg of protein per ml and stopped after 20 s by the addition of cold perchloric acid (1 M). The assay was performed at 37 °C (▲, ●). In this experiment only, the temperature was also reduced to 0 °C (△). Membrane phosphorylation by ITP (▲, △) or ATP (●). The values represent the average ± SE of five experiments with ITP and six with ATP.

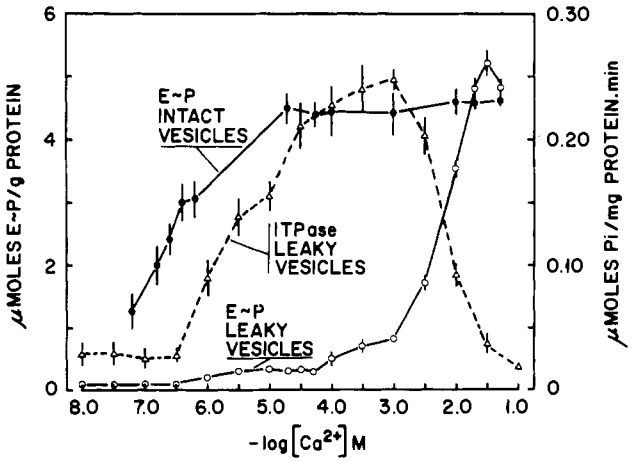

FIGURE 2: Ca^{2+} dependence of E ~ P level and ITPase activity in intact and leaky vesicles at pH 7.0. The assay medium composition and experimental conditions were as described for Figure 1, using 0.1 mM [γ-^{32}P]ITP and 0.067 mg/ml of leaky-vesicle protein (○, △) or 1 mM [γ-^{32}P]ITP and 0.5 mg/ml of intact-vesicle protein (●). The free Ca^{2+} concentration was controlled by means of CaEGTA buffer as described under Materials and Methods. Membrane phosphorylation (○, ●) and ITPase activity (△). The values represent the average ± SE of three experiments.

the pH profile of membrane phosphorylation by ITP is shifted to the alkaline side.

In the experiment of Figure 1, different amounts of Ca^{2+} had been accumulated by the vesicles at the time membrane phosphorylation was measured. In the pH range of 6.0 to 7.5 and at 37 °C, this was between 20 and 50 nmol of Ca^{2+}/mg of protein. The relatively low calcium accumulation observed is due to the absence of Ca^{2+}-precipitating agents such as oxalate or phosphate. No significant Ca^{2+} accumulation could be measured at pH values below 5.5 and above 8.0, nor at any pH when the temperature was decreased to 0 °C (data not shown).

In view of the different levels of internal Ca^{2+} the possibility arises that the pH profiles obtained in Figure 1 reflect different degrees of saturation of the internal low-affinity Ca^{2+}-binding site. Therefore in the following experiments the membrane phosphorylation and NTPase activity were compared in intact and leaky vesicles at different pH values. With the use of leaky vesicles, the Ca^{2+} concentration on both sides of the membrane is essentially the same as that of the assay medium.

Membrane Phosphorylation of ITP. Recently it was shown that the saturation of the Ca^{2+}-binding sites of high- and low-affinity could be inferred from measurements of the membrane phosphorylation by ITP in leaky vesicles (Souza and de Meis, 1976). This is shown in Figure 2, comparing leaky and intact vesicles. With the use of leaky vesicles at pH 7.0, an initial steady level of E~P was observed in the Ca^{2+}-concentration range of 3 to 50 μM (apparent K_m 1 to 3 μM). In this Ca^{2+}-concentration range, only the high-affinity Ca^{2+}-binding site is saturated (Figure 2) and the low steady-state level of E~P is due to the high rate of E~P hydrolysis (Carvalho et al., 1976; Souza and de Meis, 1976). A further increase of the Ca^{2+} concentration to the range of 20 to 60 mM promoted a simultaneous increase of the level of E~P and, at Ca^{2+} concentrations above 1 mM, inhibition of P_i release. The Ca^{2+} concentration required to promote 50% inhibition of ITPase activity and enhancement of E~P level to 4.5 to 5.0 nmol/mg of protein was in the range of 1-6 mM. This Ca^{2+} concentration is quite similar to the K_a reported for the low-affinity Ca^{2+}-binding site as measured by equilibrium dialysis (Carvalho, 1966; Carvalho and Leo, 1967; Chevallier and Butow, 1971; MacLennan et al., 1971; Hasselbach, 1972; Ikemoto, 1974). The saturation of this site inhibits the hydrolysis of the phosphoenzyme (Souza and de Meis, 1976). Thus the phosphorylated intermediate is accumulated and the P_i release decreases.

In intact vesicles (Figure 2), the rising phase of the membrane phosphorylation curve is difficult to interpret. It probably reflects both increasing saturation of the external, high-affinity Ca^{2+}-binding site and increased saturation of the internal Ca^{2+}binding site due to different degrees of filling.

Above the concentration of Ca^{2+} required to saturate the external, high-affinity site, the Ca^{2+} accumulated inside the vesicles due to active transport is high enough to saturate the Ca^{2+}-binding site of low affinity. Thus the steady-state level of E~P is maximal and does not change as the Ca^{2+} concentration of the assay medium is increased to 50 mM.

In Ca^{2+} concentrations above 20 μM, the ITPase activity in intact vesicles was three times lower than in leaky vesicles (data not shown).

The foregoing data indicate that, in Figure 1 with ITP at pH 7.0, both the internal and external Ca^{2+}-binding sites were fully saturated. However, as shown in Figure 3, at other pH values the concentrations of Ca^{2+} required to saturate these sites are quite different.

At pH 6.1 the apparent K_m for Ca^{2+} at the high-affinity site was in the range of 10 to 20 μM (Figure 3). For the additional late increase of E~P level associated with the low-affinity site, 100 mM did not show saturation. With alkalinization of the medium to pH 8.65, the high-affinity site was saturated in the nanomolar range (apparent K_m 2 to 4 nM), and the low-affinity site was saturated above 0.1 mM Ca^{2+} (apparent K_m 0.03 to 0.1 mM).

The effect of pH on the low-affinity site was also studied in a different experimental design, testing a wider pH range, as shown in the experiment of Figure 4.

With the use of leaky vesicles and 0.1 mM $CaCl_2$, the level of membrane phosphorylation was low in the pH range of 5.0 to 7.5, increasing abruptly when the pH was raised to 8.6 (Figure 4, middle). This pH profile was modified when the Ca^{2+} concentration of the medium was increased. The higher the Ca^{2+} concentration the lower was the pH in which the abrupt increase of the membrane phosphorylation occurred (Figure 4, middle). With the use of intact vesicles able to ac-

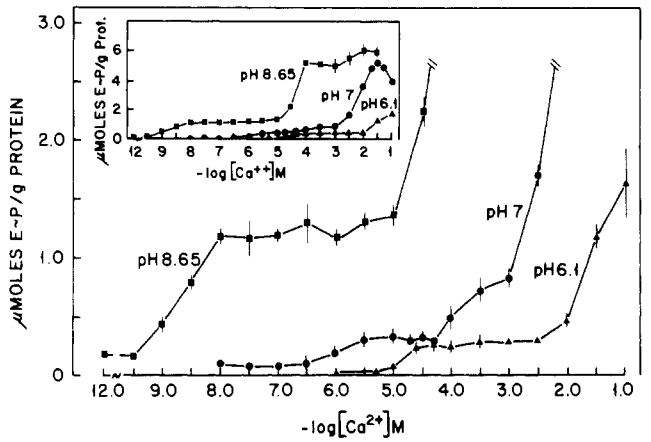

FIGURE 3: Ca^{2+} dependence of E~P from ITP at different pH values. The assay medium composition and experimental conditions were as described for Figure 1, using 0.1 mM [γ-^{32}P]ITP and 0.1 mg/ml of leaky-vesicle protein. The free Ca^{2+} concentration was controlled by means of CaEGTA buffer as described under Materials and Methods. In the inset, a reduced scale is used to show the upper parts of the curves, not included in the larger graph. The pH was 6.1 (▲), 7.0 (●), or 8.65 (■). The values represent the average ± SE of three experiments at each pH.

TABLE II: Effect of pH on the Ca^{2+} Requirement for Activation of ITP ⇌ P_i Exchange, the Late Increase in E ~ P Level, and for Inhibition of ITPase Activity.[a]

	Apparent K_m (mM)[b]		K_i (mM)[c]
pH	Exchange	E ~ P	ITPase Act.
6.5	2–3		5.8
7.0	1.8	1.5–1.7	2.5
7.5	0.6–0.7	0.6	1.2
8.0	0.3	0.3	0.3
8.3	0.2–0.3	0.1	0.25

[a] The reaction medium contained 5 mM ITP, 4 mM IDP, 10 mM $MgCl_2$, 6 mM $^{32}P_i$, and 0.6 mg/ml of leaky-vesicle protein at 30 °C for the exchange measurements. Other determinations were under the conditions described for leaky vesicles in Figure 4. [b] Ca^{2+} concentration required to promote 50% activation of the parameters, obtained from plots similar to those in Figures 3 and 6. [c] Ca^{2+} concentration required to promote 50% inhibition of the activity, obtained from plots of the activities at different pH values vs. calcium concentration.

cumulate Ca^{2+}, essentially the same pH profile was obtained when the Ca^{2+} concentration of the assay medium was either 0.1 or 10 mM (Figure 4, left). The pH profile obtained with leaky vesicles and 10 mM $CaCl_2$ was very similar to that obtained with intact vesicles (Figure 4, left).

In the presence of 0.1 mM $CaCl_2$, the ITPase activity of leaky vesicles reached a maximum at pH 7.8 (Figure 4, right). Higher Ca^{2+} concentrations both decreased the maximum ITPase activity and shifted the optimum pH to the acidic side. Comparison of Figure 4, middle, and Figure 4, right, shows that for each Ca^{2+} concentration and pH tested the abrupt increase of the membrane phosphorylation by ITP was coincident with a decrease of the ITPase activity (Table II).

Membrane Phosphorylation by ATP. The experiment of Figure 5 left shows that, when ATP was used as substrate in the pH range of 4.5 to 7.7, a similar profile of membrane phosphorylation was observed with the use of either intact or leaky vesicles in presence of 0.1 mM $CaCl_2$. With the use of leaky vesicles, an increment of the degree of membrane phosphorylation by ATP from 1.9 to 3.1 μmol/g protein was observed when the pH was raised from 7.7 to 8.5 (Figure 5, left). This increment was less pronounced than that observed with the use of ITP, where the alkalinization of the medium from 7.5 to 8.5 enhanced the degree of phosphorylation of the membrane of leaky vesicles from 1.15 to 6.9 μmol of E ~ P/g of protein (Figure 4).

At all pH values tested, the ATPase activity was higher in leaky vesicles than in intact vesicles (Figure 5, right). This difference was more pronounced in the pH range of 6.0 to 7.5, where the intact vesicles were able to accumulate larger amounts of Ca^{2+}.

Ca^{2+} Dependence of the NTP ⇌ P_i Exchange Reaction. In a previous paper, it was shown that leaky vesicles were able to catalyze an NTP ⇌ P_i exchange provided that the low-affinity Ca^{2+}-binding site was saturated (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Carvalho et al., 1976). Thus, the NTP ⇌ P_i exchange reaction provides another means of monitoring the effect of pH on the functional properties of the Ca^{2+}-binding site of low affinity.

At pH 7.0, little or no NTP ⇌ P_i exchange could be mea-

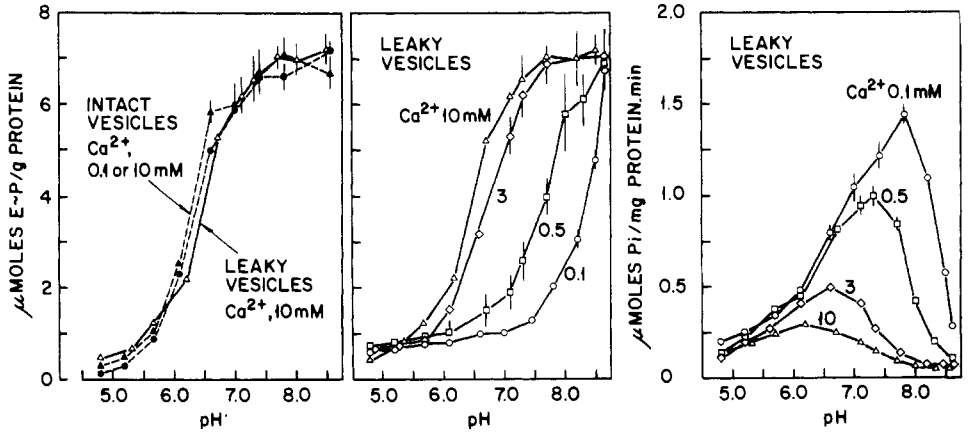

FIGURE 4: pH profiles of E~P level and of ITPase activity in intact and leaky vesicles. The assay medium composition and experimental conditions were as described for Figure 1, using 1 mM [γ-^{32}P]ITP and 0.6 mg/ml of either intact-vesicle (closed symbols, dotted lines) or leaky-vesicle protein (open symbols, solid lines). (Left) Phosphoenzyme level in either intact or leaky vesicles; (middle) phosphoenzyme level in leaky vesicles; (right) ITPase activity in leaky vesicles. The $CaCl_2$ concentrations were 0.1 (○, ●), 0.5 (□), 3 (◇), and 10 mM (△, ▲). The values represent the average ± SE of four to five experiments at each calcium concentration.

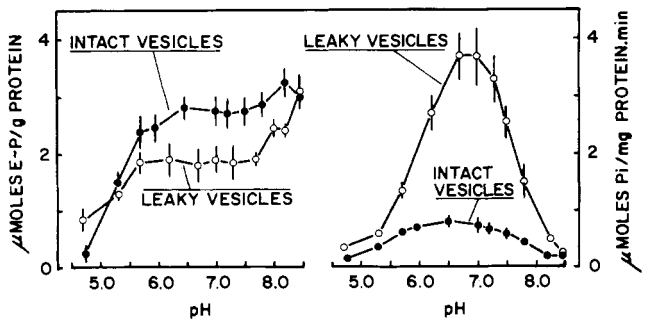

FIGURE 5: pH profiles of E~P level and of ATPase activity in intact and leaky vesicles. The assay medium composition and experimental conditions were as described for Figure 1, using 1 mM [γ-^{32}P]ATP and 0.1 mg/ml of either intact-vesicle (●) or leaky-vesicle protein (○). The $CaCl_2$ concentration was 0.1 mM. (Left) E~P level; (right) ATPase activity. The values represent the average ± SE of seven experiments.

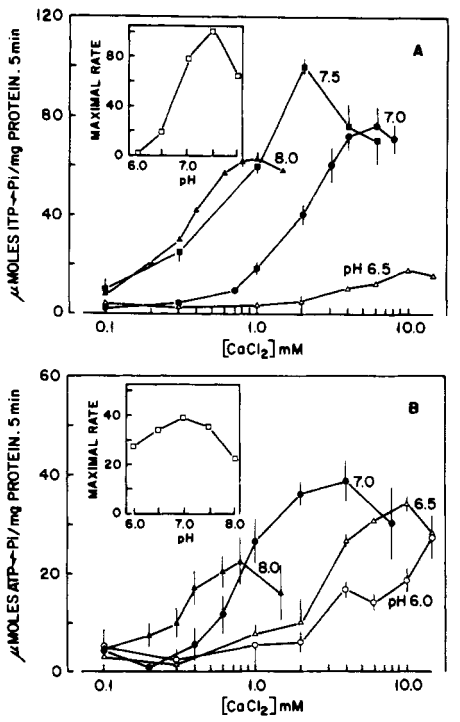

FIGURE 6: Ca^{2+} dependence of NTP ⇌ P_i exchange at different pH values. The assay medium composition was 5 mM ITP-4 mM IDP (A) or 10 mM ATP-1 mM ADP (B) and 10 mM $MgCl_2$, 6 mM $^{32}P_i$, 0.6 mg/ml of leaky-vesicle protein, and 40 mM Tris-maleate buffer adjusted to pH 6.0 (○), 6.5 (△), 7.0 (●), 7.5 (■), or 8.0 (▲). The assay was performed at 30 °C for 5 min. The insets show the rates of exchange (μmol per mg per 5 min) obtained at optimal Ca^{2+} concentration at each pH. The values represent the average ± SE of three experiments.

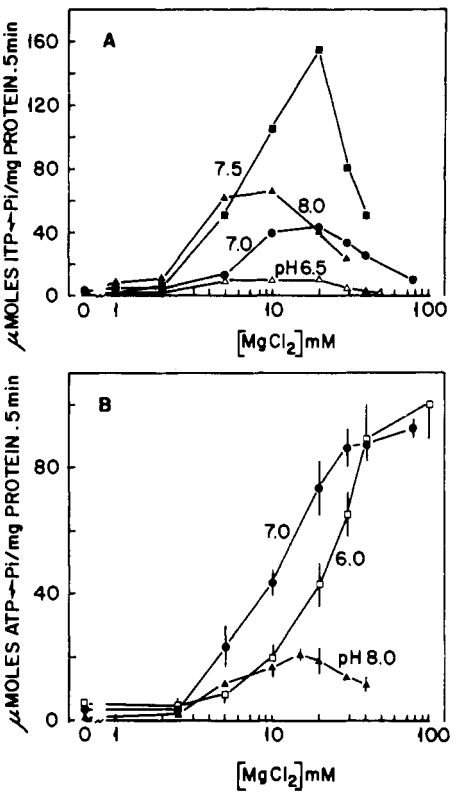

FIGURE 7: Mg^{2+} dependence of NTP ⇌ P_i exchange at different pH values. The assay medium composition was 5 mM ITP-4 mM IDP (A) or 5 mM ATP-0.5 mM ADP (B), 6 mM $^{32}P_i$, 0.2 mg/ml of leaky-vesicle protein, 40 mM Tris-maleate buffer adjusted to pH 6.0 (□), 6.5 (△), 7.0 (●), 7.5 (■), and 8.0 (▲), and the optimal calcium concentration at each pH (taken from Figure 6). The assay was performed at 30 °C for 5 min. The values represent the average ± SE of three experiments in B and a typical experiment in A. Identical results were obtained with three different preparations tested in A, although the maximal rates differed by 50-60%.

sured in the Ca^{2+} concentration range of 0.1 to 0.3 mM (Figure 6A and B). Both the rate of ATP ⇌ P_i exchange and the rate of ITP ⇌ P_i exchange were progressively activated by raising the Ca^{2+} concentration from 0.3 to 10 mM, half-maximal activation being observed in the Ca^{2+} concentration range of 1 to 2 mM. Figure 6 (A and B) and Table II show that the higher the pH, the lower the Ca^{2+} concentration required to activate the rate of NTP ⇌ P_i exchange. The inserts in Figure 6 (A and B) show that a modification of the pH of the assay medium also modifies the maximal rate of NTP ⇌ P_i exchange. This could in part be related to a modification of the Mg^{2+} requirement for the exchange reaction. Figure 7 (A and B) shows that the higher the pH of the medium, the lower the Mg^{2+} concentration required for maximal activation of the rate of NTP ⇌ P_i exchange. Therefore, in Figure 6, for some of the pH values tested, the concentration of Mg^{2+} used (10 mM) was suboptimal. Modification of the Mg^{2+} requirement with pH is currently being investigated in more detail.

Discussion

At pH 7, two main classes of Ca^{2+}-binding sites have been recognized by equilibrium dialysis experiments, one of high affinity with a K_a in the range of 1 to 3 μM, and another of low affinity with a K_a in the range of 1 to 3 mM (Carvalho, 1966; Carvalho and Leo, 1967; Chevallier and Butow, 1971; MacLennan et al., 1971; Hasselbach, 1972; Ikemoto et al., 1972; Ikemoto, 1974). Since these early experiments, the functional role of the high-affinity site has been correlated with NTP hydrolysis, membrane phosphorylation by NTP, and Ca^{2+} uptake. These parameters also exhibit half-maximal activation in the Ca^{2+}-concentration range of 1 to 3 μM (Hasselbach, 1972). The low-affinity Ca^{2+}-binding site has only recently been shown to have a functional role in control of the transport NTPase. Thus it has been shown that, in the Ca^{2+} concentration range in which this site is saturated, NTP hydrolysis is inhibited (Kielley and Meyerhof, 1948; Makinose and The, 1965; Weber et al., 1966; Ikemoto, 1974; de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Ikemoto, 1975; Carvalho et al., 1976), the NTP ⇌ P_i exchange is activated (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Car-

valho et al., 1976), and the steady-state level of membrane phosphorylation by ITP (and not by ATP) is enhanced (Souza and de Meis, 1976). Evidence has also been presented that saturation of this site is required to activate the reversal of the Ca^{2+} pump which leads to Ca^{2+} efflux and ATP synthesis (Knowles and Racker, 1975; De Meis, 1976). Thus, the saturation of the low-affinity site seems to block the forward reaction and, under the proper conditions, to activate the reversal of the Ca^{2+} pump (Carvalho et al., 1976).

Measuring Ca^{2+} binding and membrane phosphorylation by ATP, Meissner (1973) concluded that the affinity of the high-affinity site is increased by alkalinization of the assay medium. In this paper, his conclusion is confirmed (Figure 3) and evidence is presented that the Ca^{2+} affinity of the low-affinity site also increases with the pH of the assay medium. A significant variation of the Ca^{2+} affinity is measured in the pH range of 6.5 to 8 (Table II). This variation becomes much more pronounced when the pH is decreased below 6.5 or above 8.5. Thus, Figure 3 shows that the Ca^{2+} affinity increases three to four orders of magnitude when the pH is raised from 6.1 to 8.65. The experiments of Figures 2 and 4 (left) reinforce the earlier conclusion that the Ca^{2+}-binding site of low affinity is located on the inner surface of the membrane (de Meis and Carvalho, 1974; Ikemoto, 1975; de Meis, 1976; de Meis and Carvalho, 1976). Therefore, different pH profiles of membrane phosphorylation (Figure 1) and NTPase activity (Figure 4) may be expected when intact vesicles which are loaded with Ca^{2+} to different degrees are used (Figures 1 and 5).

In earlier papers it has been discussed how saturation of the low-affinity Ca^{2+}-binding site could control NTP $\rightleftharpoons$ P_i exchange (de Meis and Carvalho, 1974; de Meis and Sorenson, 1975; Carvalho et al., 1976) and, in conjunction with the high-affinity site, regulate the steady-state level of membrane phosphorylation by ATP or ITP (Souza and de Meis, 1976). At present we interpret most of the differences between ATP and ITP to indicate that these nucleotides activate to different extents the rate of hydrolysis of the phosphoenzyme (de Meis and de Mello, 1973; Froehlich and Taylor, 1975; Carvalho et al., 1976; Souza and de Meis, 1976).

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