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# Properties of Potassium Activated *p*-Nitrophenyl Phosphatase of Plasma Membranes Isolated from Rat Stomach Muscle

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## Abstract

The plasma membrane-enriched fraction isolated from smooth muscle of rat gastric fundus was found to contain a substantial level of potassium-stimulated *p*-nitrophenylphosphatase activity (K-*p*NPPase), and its subcellular distribution closely resembled that of other plasma membrane enzyme markers. The kinetic profile of K-*p*NPPase and its sensitivity toward ouabain and vanadate confirmed the identification of this activity with the partial reaction of the sodium pump. The specific activity of K-*p*NPPase and its sensitivity to ouabain was significantly increased in the presence of saponin, indicating that part of this activity is latent when assayed on native membrane preparation. K-*p*NNPase was sensitive to the presence of calcium ions in the assay medium. The $Ca^{2+}$-inhibition of K-*p*NNPase was accompanied by increased sensitivity of the enzyme to ouabain. On the other hand, calmodulin and Ca antagonists had no effect on K-*p*NPPase activity nor its sensitivity to calcium.

**Key Words:** Smooth muscle; sodium pump; *p*-nitrophenyl phosphatase; plasma membranes; calcium.

## Introduction

Na, K-ATPase, an enzymatic manifestation of the sodium pump, is considered to be ubiquitous in all eukaryotic cell membranes. Na, K-ATPase has been well characterized in a variety of tissues; however, substantially less progress has been achieved in studies of this enzyme in smooth muscles (Allen and Navran, 1984). The specific activity of Na, K-ATPase in smooth muscles is generally low, which is primarily due to the lower density of sodium pump

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sites per cell membrane area in these tissues (Wallick *et al.*, 1982). Further, reliable measurements of Na, K-ATPase are hampered by high levels of basal Mg-ATPase encountered in plasma membrane-enriched preparations from many smooth muscles and by the complex pattern of interactions of sodium and potassium with basal Mg-ATPase and Mg-ADPase. In addition, ouabain was found to have a small activating effect on the Mg-ATPase of some smooth muscle plasma membranes (Kwan and Kostka, 1983).

One alternative to circumvent the difficulties in obtaining reliable measurement of the activity of Na, K-ATPase in smooth muscles is to study potassium-activated, ouabain-sensitive *p*NPPase. Identification of this activity with terminal hydrolysis of phosphoenzyme complex formed by interaction of Na, K-ATPase with macroergic phosphate donor is well established (Glynn and Karlish, 1975, and references therein). In smooth muscles, although K-*p*NPPase activity has been detected in a variety of vascular and nonvascular smooth muscle tissues (Kwan *et al.*, 1984a; Grover *et al.*, 1981), its properties have not been systematically studied.

The present study examines the properties of K-*p*NPPase in a plasma membrane-enriched fraction isolated from rat gastric fundus smooth muscle with emphasis on its kinetics, sensitivity to model inhibitors, interaction with $Ca^{2+}$, calmodulin, and $Ca^{2+}$ antagonists, and activation by saponin. The findings are discussed with respect to the enzymatic properties of K-*p*NPPase in smooth muscles and their relevance to the biochemical characteristics of Na, K-ATPase in these tissues.

## Methods

A plasma membrane-enriched fraction from rat gastric fundus smooth muscle was isolated by differential and sucrose density gradient centrifugation of tissue homogenate as described previously in detail (Kwan *et al.*, 1982). The light-density fraction at the interphase of 15–30% sucrose layers is the plasma membrane-enriched fraction (F2) which was about 14- to 18-fold enriched over the postnuclear supernatant (PNS) in several plasma membrane marker enzymes including 5′-nucleotidase, phosphodiesterase I, and Mg-ATPase, but poorly or not enriched in mitochondrial and endoplasmic reticulum membrane markers, such as cytochrome *c* oxidase and NADPH cytochrome *c* reductase activities, respectively (Kwan *et al.*, 1982).

K-*p*NPPase activity was assayed by incubation of membrane fraction (20–40 μg of protein) in a medium containing 50 mM imidazole-HCl, pH 7.8, 5 mM $MgCl_2$, 3 mM *p*-nitrophenyl phosphate, and millimolar concentrations of KCl as indicated in the text. Basal ($K^+$-independent) phosphatase activity was assayed in the medium of the same composition, but in the absence of

potassium. $K^+$-activated *p*NPPase activity was defined as the difference between activities observed in the presence and absence of potassium. Ouabain-sensitive activity refers to the differences in activities observed in the absence and presence of ouabain. The final volume of the incubation mixture was 1 ml. After 30 min incubation at 37°C the reaction was terminated by the addition of 1 ml ice-cold 1 M NaOH. Samples were subsequently centrifuged to remove precipitated proteins, and the amount of liberated *p*-nitrophenol was determined spectrophotometrically at 400 nm (molar absorption coefficient = 18,000 $M^{-1} cm^{-1}$). The value of the molar absorption coefficient for *p*-nitrophenol at 400 nm was not affected by the presence of Mg and Ca ions up to 10 mM concentrations.

Assays of plasma membrane markers 5′AMPase and phosphidiesterase I were performed according to previously described techniques (Kwan *et al.*, 1982).

The specific activity of K-*p*NPPase near saturation (10 mM $K^+$) varied from 1.0 to 2.5 μmol/mgh. These variations may reflect differences in the stability of the enzyme in different membrane preparations as we have observed a slight decline in specific activity after storage of membranes at −20°C. Such storage, however, did not alter the kinetic profile of enzyme nor the response to the inhibitors and activators we have studied. However, a sharp decline in the specific activity of K-*p*NPPase was observed when the membrane fractions were subjected to an additional freezing–thawing step. For that reason, all the assays were carried out on freshly prepared fractions or fractions subjected to only one freezing–thawing step.

In order to eliminate variations from preparation to preparation and the effect of aging on relative magnitude of K-*p*NPPase activity, the data sets presented further were assayed using the same membrane preparation unless indicated otherwise. The findings were subsequently confirmed by independent measurement on at least two additional membrane preparations.

*p*-Nitrophenyl phosphate (Tris salt) was purchased from Sigma, and *p*-nitrophenol was from Calbiochem. Calmodulin prepared from pig brain was a gift from Dr. S. Law, and was biologically active in the stimulation of smooth muscle plasma membrane $Ca^{2+}$-pump (Kwan *et al.*, 1986).

## Results

### *Subcellular Distribution of K-pNPPase Activity*

Analysis of various subcellular fractions isolated from smooth muscle of rat gastric fundus has revealed that K-*p*NPPase activity is confined in plasma membranes. The specific activity of this enzyme in purified plasma membranes was increased approximately 16-fold in comparison with the activity

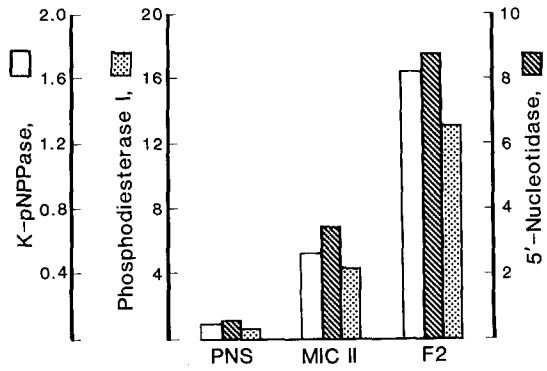


**Fig. 1.** The distribution of K-*p*NPPase, phosphodiesterase I, and 5′-nucleotidase activities in postnuclear supernatant (PNS), microsomal (MIC II) and plasma membrane (F2) fractions. See Kwan *et al.* (1982) for details of membrane isolation and characterization using other marker enzyme activities. Units for enzyme activities: K-*p*NPPase, μmol/mg h; phosphodiesterase I, $\Delta A_{400\,nm}$, mg min; 5′-nucleotidase, μmol/mg h.

**Table I.** Distribution of K-*p*NPPase Activity in Subcellular Fractions Isolated from Dog Aorta, Dog Mesenteric Artery, and Rat Mesenteric Arteries[a]

| Tissue | Postnuclear supernatant | Microsomal fractions | Plasma membranes |
|---|---|---|---|
| Dog aorta | 0.062 ± 0.020 | 0.357 ± 0.063 (5.8) | 1.130 ± 0.105 (18.2) |
| Dog mesenteric artery | 0.050 ± 0.012 | 0.364 ± 0.053 (7.1) | 0.834 ± 0.068 (16.7) |
| Rat mesenteric artery | 0.145 ± 0.083 | 0.752 ± 0.061 (5.2) | 2.030 ± 0.205 (14.0) |

[a]Subcellular fractions were prepared according to previously published fractionation techniques (dog aorta: Kwan *et al.*, 1984b; dog mesenteric arteries: Kwan *et al.*, 1983; rat mesenteric arteries: Kwan *et al.*, 1979). In fractionation of dog aorta the pretreatment of tissue with 0.6 m KCl was omitted in order to allow the measurement of K-*p*NPPase activity in postnuclear supernatant. Assay of K-*p*NPPase activity was performed in the standard medium as described in Methods. Numbers in parentheses stand for the enrichment of K-*p*NPPase over the postnuclear supernatant. Results are expressed as means ± S.D. from six replicates from two separate preparations. Activities are expressed as μmol/mg h.

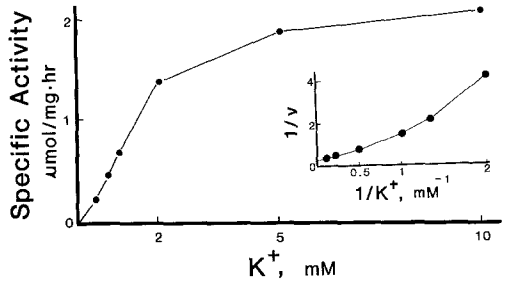


**Fig. 2.** Kinetics of K-*p*NPPase activation by potassium ions. Inset: Double-reciprocal plot of K-*p*NPPase activity at different potassium concentrations. Points represent means of three replicates.

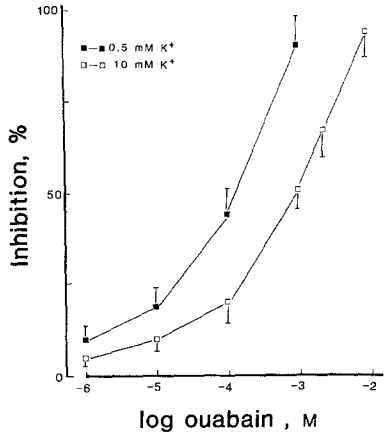


**Fig. 3.** Inhibition of K-*p*NPPase activity by ouabain at low (0.5 mM) and high (10 mM) potassium concentration. Results are mean ± S.D. from measurements on three different plasma membrane preparations.

in postnuclear supernatant, and this enrichment ratio closely paralleled the enrichments of other plasma membrane markers such as 5′AMPase and phosphodiesterase I (Fig. 1). For comparative purposes, Table I shows that a similar pattern of subcellular distribution of K-*p*NPPase was also observed in several other smooth muscle preparations, indicating the general applicability of using K-*p*NPPase activity as a plasma membrane marker.

### *Kinetics of Activation of K-pNPPase by Potassium Ions*

The kinetic profile of activation of K-*p*NPPase by potassium ions is shown in Fig. 2. The potassium concentration-dependent curves followed a sigmoidal pattern, resulting in a nonlinear double-reciprocal plot (Fig. 2 inset). The concentration of potassium ions required for half-maximal activation was 1.5–2 mM. A logarithmic plot of fractional saturation vs. potassium concentration from five different preparations yielded Hill coefficients between 1.5–1.8 (not shown).

### *Sensitivity of K-pNPPase to Inhibitors*

Both well-known inhibitors of sodium pump, ouabain and vanadate, inhibited K-*p*NPPase in a concentration-dependent manner. The concentration of ouabain required for 50% inhibition at a potassium concentration near saturation (10 mM) was 1 mM, while an approximately 10-fold lower concentration of ouabain produced the same degree of inhibition at low potassium concentration (0.5 mM; Fig. 3).

Micromolar concentrations of vanadate strongly inhibited K-*p*NPPase ($ID_{50}$ = 1 $\mu$M). The decline in the activity of K-*p*NPPase with increasing concentrations of vanadate was accompanied by a proportional decrease in ouabain-sensitive activity, suggesting that vanadate did not interfere with the response of K-*p*NPPase to ouabain (not shown).

## Effect of Sodium

Sodium ions in the absence of potassium slightly activated the hydrolysis of *p*NPP (basal phosphatase activity), and this activating effect of sodium was inhibited by ouabain. In the presence of potassium, sodium ions at 20 mM concentration has a biphasic effect on K-*p*NPPase activity depending on the concentration of potassium. At low potassium concentration (0.5 mM) the presence of 20 mM sodium caused approximately 50% activation of K-*p*NPPase activity. This stimulatory effect was reversed by increasing concentration of potassium, resulting in approximately 30% inhibition of K-*p*NPPase by 20 mM sodium at a potassium concentration near saturation (10 mM; Table IIB). These findings are compatible with the kinetic model of interaction of Na ions with K-*p*NPPase previously described by Robinson *et al.* (1983).

**Table II.** Effect of Sodium Ions on Basal Phosphatase and $K^+$-*p*NPPase Activities[a]

| A. Phosphatase activity ($\mu$mol/mg h) | | |
|---|---|---|
| [$Na^+$] concentration (mM) | No ouabain | 1 mM ouabain |
| 0 | 0.732 ± 0.012 | 0.690 ± 0.020 |
| 10 | 0.821 ± 0.018 | 0.702 ± 0.019 |
| 20 | 0.836 ± 0.017 | 0.705 ± 0.025 |
| **B. K-*p*NPPase activity ($\mu$mol/mg h)** | | |
| [$K^+$] concentration (mM) | No $Na^+$ | 20 mM $Na^+$ |
| 0.5 | 0.082 ± 0.015 | 0.122 ± 0.018 (+50%) |
| 1.0 | 0.213 ± 0.021 | 0.264 ± 0.023 (+24%) |
| 5.0 | 0.964 ± 0.022 | 0.648 ± 0.026 (−33%) |
| 10.0 | 1.245 ± 0.028 | 0.853 ± 0.025 (−31%) |

[a]Phosphatase activity was assayed in standard medium in the absence of potassium ions as described in Methods. K-*p*NPPase was measured in the same medium, but in the presence of potassium ions at the concentrations indicated. Specific activities of K-*p*NPPase were corrected for corresponding basal activities. Numbers in parentheses stand for the percentage activation (+) or inhibition (−) by $Na^+$. Results are expressed as means ± S.D. from three replicates.

### *Effect of Detergents*

Several nonionic detergents have been tested for their potency to modulate K-*p*NPPase activity and its sensitivity to ouabain. Table III shows that K-*p*NPPase was only marginally affected in the presence of digitonin and Na-deoxycholate in the assay medium at concentrations of 100 μg/ml. The enzyme activity was completely diminished by 0.01% Triton X. However, a substantial increase of K-*p*NPPase was observed in the presence of saponin (100 μg/ml).

Both digitonin and saponin at the applied concentrations significantly increased the sensitivity of K-*p*NPPase to ouabain, while the opposite effect, i.e., decrease in ouabain sensitivity, was observed in the presence of Na-deoxycholate. Among the detergents tested, saponin had the most profound effect on K-*p*NPPase as it activated both ouabain-sensitive and ouabain-insensitive components. Maximal activation of K-*p*NPPase (1.5- to 2.5-fold increase in comparison with control values) was observed at a saponin concentration of 50 μg/ml, and this activation was not further enhanced by increased saponin concentration up to 500 μg/ml (Fig. 4). Saponin did not alter the nonlinear profile of the double-reciprocal plot of stimulation of enzyme by $K^+$ ions nor the Hill slope for these ions (not shown).

The relative proportion of ouabain-sensitive activity also increased with increasing saponin concentration (Fig. 4). Interestingly, a higher concentration of saponin (200 μg/ml) was required for maximal increase of ouabain sensitivity in comparison with the concentration of detergent required for maximal enhancement of $K^+$-stimulated activity. Investigation of the

**Table III.** Effect of Detergents on $K^+$-*p*NPPase Activity and Its Sensitivity to Ouabain[a]

| Detergent | Phosphatase | $K^+$-*p*NPPase | | |
|---|---|---|---|---|
| | | No ouabain | 1 mM ouabain | % of ouabain inhibition |
| None (control) | 0.656 ± 0.020 | 2.221 ± 0.027 | 1.103 ± 0.022 | 50% |
| Saponin, 100 μg/ml | 0.720 ± 0.023 | 4.051 ± 0.032[a] | 1.320 ± 0.026 | 67%[b] |
| Digitonin, 100 μg/ml | 1.133 ± 0.029 | 1.932 ± 0.019[a] | 0.462 ± 0.018 | 76%[b] |
| Sodium deoxycholate, 100 μg/ml | 0.924 ± 0.019 | 2.028 ± 0.025[a] | 1.195 ± 0.031 | 41%[b] |
| Triton X, 0.01% | 0.550 ± 0.054 | Not detected | — | — |

[a] Phosphatase and K-*p*NPPase activities (μmol/mg h) were measured in standard assay media as described in Methods. $K^+$-*p*NPPase activity was assayed at 10 mM $K^+$. Results are expressed as means ± S.D. of three replicates.

[b] Significantly different ($P < 0.05$) from control value.

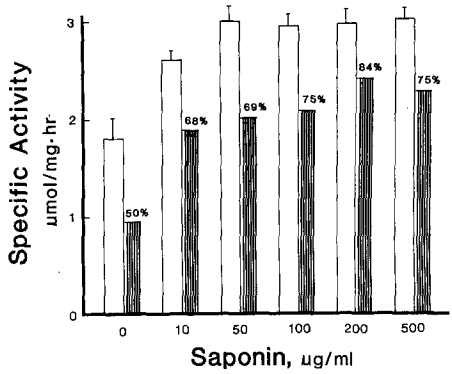


**Fig. 4.** Activation of K-*p*NPPase by different concentrations of saponin at potassium concentration near saturation (10 mM). Numbers at the top of hatched bars refer to the percent of ouabain inhibition of the control (open bars) at concentration of ouabain 1 mM. Values are mean ± S.D. of three replicates.

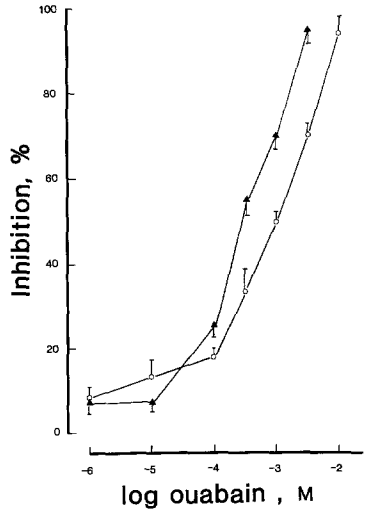


**Fig. 5.** Concentration-dependent profile of ouabain inhibition in the presence (▲) and absence (○) of saponin (100 μg/ml). K-*p*NPPase was assayed at 10 mM $K^+$. Results represent mean ± S.D. of three replicates.

saponin effect at different ouabain concentrations has shown that the relative increase in ouabain sensitivity due to saponin was present only at higher ouabain concentrations (> 0.5 mM; Fig. 5).

### *Inhibition by Calcium*

K-*p*NPPase was inhibited by $Ca^{2+}$ in the submillimolar concentration range (Fig. 6). The $Ca^{2+}$ concentration required for 50% inhibition at 10 mM

$K^+$ was approximately 0.5 mM. The calcium-induced decline in specific activity of K-*p*NPPase was accompanied by increased sensitivity of enyme to ouabain (Fig. 6, top). The inhibition of K-*p*NPPase by $Ca^{2+}$ and the concomitant increase in ouabain sensitivity was not affected by the presence of saponin (Fig. 6, bottom).

Inhibition of K-*p*NPPase activity by $Ca^{2+}$ was found to be invariant to the substrate concentration as 0.5 mM $Ca^{2+}$ inhibited the enzyme to a comparable degree at 1.5, 3, and 10 mM *p*NPP at a fixed concentration of magnesium and potassium ions (5 and 10 mM, respectively). Similarly, increasing Mg concentration from 5 to 10 mM at a constant level of *p*NPP (3 mM) and potassium (10 mM) caused no significant changes in the degree of inhibition by calcium nor was the inhibition affected by the presence of calmodulin (5 $\mu$g/ml) or several Ca antagonists (verapamil, nifedipine, and nitrendipine at 10 $\mu$M).

## Discussion

K-*p*NPPase activity in plasma membrane-enriched fraction isolated from the smooth muscle of rat gastric fundus resembles in several ways the properties of this enzyme observed in isolated membrane fractions or in purified form from other tissues. For example, the enzyme is activated by $K^+$ ions in millimolar concentrations ($K_{0.5}$ 1.5–2 mM) and reaches saturation at 10 mM $K^+$.

K-*p*NPPase activity was inhibited by ouabain, although relatively higher concentrations of glycoside were required to exert this effect. This seems to be due to the generally lower sensitivity of rat tissues to this inhibitor (Schwartz *et al.*, 1975). The inhibitory effect of ouabain was more prominent at lower potassium concentrations, which is consistent with the well-documented antagonism between glycoside and potassium ions.

The sigmoidal profile of the $K^+$ concentration dependence, with a Hill coefficient significantly higher than 1, is consistent with the kinetic model of K-*p*NPPase drawn from studies on other sources of enzyme (Robinson *et al.*, 1983). According to this model, potassium ions activate *p*NPPase activity by interaction with the low-affinity sites located on the cytoplasmic surface of plasma membrane. The sigmoidal profile of $K^+$ activation reflects the potassium-induced shift in the conformational equilibrium of Na, K-ATPase from the sodium-dependent ($E_1$) form to the potassium-dependent ($E_2$) form of the enzyme, with the reaction velocity of K-*p*NPPase being proportional to the $E_2$ form. In addition, Drapeau and Blostein (1980) have shown that in the presence of sodium, low concentrations of potassium ($\leqslant 1$ mM) can activate *p*NPPase activity as well by interaction with the high-affinity site

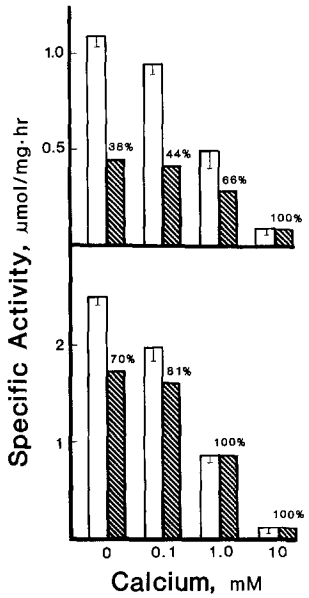


**Fig. 6.** Inhibition of K-*p*NPPase by calcium ions in the absence (top) and presence (bottom) of 100 μg/ml saponin. Open bars: $K^+$-stimulated activity (10 mM $K^+$). Hatched bars: Ouabain (1 mM)-sensitive activity. Numbers at the top of bars refer to the percent of ouabain inhibition. Values are mean ± S.D. of three replicates.

located on the external surface of plasma membrane. Such an additional interaction may lead to the biphasic effect of sodium; i.e., inhibition at high $K^+$ concentrations and activation at low $K^+$ concentrations, or introduction of a second inflection point in the double-reciprocal plot at low $K^+$ concentration (Robinson *et al.*, 1983). In our study, the presence of such additional reaction pathway was observed in the presence of 20 mM sodium as these ions caused substantial stimulation of K-*p*NPPase activity at 0.5 mM $K^+$ in contrast to the inhibition of enzyme at potassium concentrations near saturation.

The K-*p*NPPase activity and its sensitivity to ouabain was differentially affected by inclusion of nonionic detergents into the assay medium. In contrast to Na-deoxycholate, digitonin, and Triton X, only saponin induced a substantial increase in K-*p*NPPase activity. This increase was not accompanied by changes in $K_{0.5}$ for potassium. Such noncompetitive nature of the saponin effect indicates that the increase in K-*p*NPPase activity may be related to detergent-induced changes in membrane lipids. Two mechanisms are to be considered: Firstly, since saponin is known to interact with membrane-bound cholesterol and thus alter the fluidity of membrane, the

observed increase in K-*p*NPPase activity would suggest that this enzyme is in part controlled by membrane lipids and their physicochemical properties. Secondly, as we have shown (Kwan and Lee, 1984), saponin at high concentrations ($> 100\,\mu g/ml$) damages the membrane vesicles and thus eliminates the permeability barrier for large polar molecules. Thus, the saponin-induced increase in K-*p*NPPase activity may also be related to increased accessibility of the substrate. Since a 1.5- to 2.0-fold increase of the K-*p*NPPase activities was readily observed both in the presence and absence of ouabain at saponin concentrations ($10–50\,\mu g/ml$) low enough to permeate the cell membrane without breakdown of the membrane integrity (Kwan and Lee, 1984; Endo and Iino, 1980), this may be interpreted to indicate that approximately 30-50% of the enzyme activity remained latent and was probably due to inside-out oriented vesicles. However, with increasing saponin concentrations ($100–500\,\mu g/ml$), the plasma membrane vesicles became fragmented and potentially solubilized (Kwan and Lee, 1984). The enhancement of K-*p*NPPase activity remained unchanged whereas the inhibition by ouabain became potentiated. This is apparently due to the detergent effect of saponin at high concentrations on the ouabain sensitivity of the enzyme.

The inhibitory effect of calcium on sodium pump is well documented by numerous previous studies (Akera and Brody, 1982, and references therein), although the physiological relevance of this inhibition remains disputable. In our experiments $Ca^{2+}$ inhibited K-*p*NPPase activity in submillimolar concentrations; 50% inhibition was observed at 0.5–0.7 mM $Ca^{2+}$. Lack of changes in inhibitory response of K-*p*NPPase to $Ca^{2+}$ ions by manipulations in concentrations of *p*NPP and Mg eliminates the possibility that the observed effect of calcium could result from physicochemical interactions of calcium with *p*NPP or Mg-*p*NPP complex. These observations also suggest that $Ca^{2+}$ inhibits K-*p*NPPase activity by interaction with sites distinct from binding sites for the substrate and $Mg^{2+}$. Interestingly, $Ca^{2+}$ substantially increased the sensitivity of K-*p*NPPase to ouabain. Although we did not pursue this effect of $Ca^{2+}$ in more detail, it is quite possible that extracellular $Ca^{2+}$ might be involved in the modulation of the sensitivity of fundus smooth muscle sodium pump to ouabain.

Recently, several authors have proposed that calcium can also interact with sodium pump indirectly, particularly via Ca-sensitive intracellular proteins including calmodulin (Lelievre *et al.*, 1983; Cirillo *et al.*, 1984). However, in our study we failed to detect any changes in interaction of Ca ions with K-*p*NPPase in the presence of calmodulin. It seems unlikely that such lack of changes is due to the inaccessibility of calmodulin binding sites in our membrane preparation as our recent findings have shown the stimulation of ATP-dependent Ca-transport of smooth muscle plasma membranes by the same batches of calmodulin (Kwan *et al.*, 1986).

In conclusion, this study provides several lines of evidence that the properties of K-*p*NPPase activity encountered in smooth muscle are comparable to those established by studies carried out on plasma membrane-enriched fractions or purified Na, K-ATPase preparations from other tissues. This suggests that the sodium pump in smooth muscles may have enzymatic properties similar to those observed in other plasma membranes. These features of smooth muscle K-*p*NPPase justify the use of K-*p*NPPase as a marker for plasma membrane in the fractionation of smooth muscles, where in many cases the direct determination of Na, K-ATPase is complicated by the substantially low specific activity of Na, K-ATPase in comparison with the basal Mg-ATPase activity.

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