Functional Comparison between Secretory Pathway Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase (SPCA) 1 and Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase (SERCA) 1 Isoforms by Steady-state and Transient Kinetic Analyses*

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Steady-state and transient kinetic studies were performed to functionally analyze the overall and partial reactions of the Ca\(^{2+}\) transport cycle of the human secretory pathway Ca\(^{2+}\)/Mn\(^{2+}\)-ATPase 1 (SPCA1) isoforms: SPCA1a, SPCA1b, SPCA1c, and SPCA1d (encoded by ATP2C1, the gene defective in Hailey-Hailey disease) upon heterologous expression in mammalian cells. The expression levels of SPCA1 isoforms were 200–350-fold higher than in control cells except for SPCA1c, whose low expression level appears to be the effect of rapid degradation because of protein misfolding. Relative to SERCA1a, the active SPCA1a, SPCA1b, and SPCA1d enzymes displayed extremely high apparent affinities for cytosolic Ca\(^{2+}\) in activation of the overall ATPase and phosphorylation activities. The maximal turnover rates of the ATPase activity for SPCA1 isoforms were 4.7–6.4-fold lower than that of SERCA1a (lowest for the shortest SPCA1a isoform). The kinetic analysis traced these differences to a decreased rate of the \(E_2\sim P(Ca)\) to \(E_2\)-P transition. The apparent affinity for inorganic phosphate was reduced in the SPCA1 enzymes. This could be accounted for by an enhanced rate of the \(E_2\)-P hydrolysis, which showed constitutive activation, lacking the SERCA1a-specific dependence on pH and K\(^+\).

Secretory pathway Ca\(^{2+}\)/Mn\(^{2+}\)-ATPases (SPCAs)‡ represent a new group of ion-motive ATPases consisting of single subunit integral membrane enzymes specifically mediating the ATP-powered uphill transport of either Ca\(^{2+}\) or Mn\(^{2+}\) from cytosol into the Golgi lumen (1–4).

SPCAs (encoded by ATP2C1–2 genes) as well as sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs; encoded by ATP2A1–3 genes) and plasma membrane Ca\(^{2+}\)-ATPases (PMCs; encoded by ATP2B1–4 genes) are characterized by the obligatory autophosphorylation during their reaction cycle (5, 6). Analysis of the deduced amino acid sequences of human (7), rat (8), worm (9), insect (9), and yeast (2) SERCA proteins indicates that despite the relatively low amino acid sequence homology (30–35%) with SERCAs, the SPCAs retain the key architectural features revealed in the recently elucidated high resolution crystal structures of the rabbit SERCA1a (994 amino acids) in Ca\(^{2+}\)-free thapsigargin-bound \(E_1\) and Ca\(^{2+}\)-bound \(E_1\) forms either with or without bound nucleotide (10–15). Relative to SERCA1a, SPCAs appear to be similarly composed of 10 hydrophobic helices (M1–M10) displaying strong membrane-spanning propensity. Other SERCA1a-specific structural determinants conserved in SPCAs are the phosphorylation (P, containing the phosphorylatable Asp\(^536\) in human SPCA1 and Asp\(^535\) in SERCA1a), nucleotide-binding (N) and actuator (A) domains present in the large “headpiece” emerging into the cytosol. The direct structural information obtained from SERCA1a crystals (10) has confirmed that two Ca\(^{2+}\) ions are bound at two high affinity binding sites (site I and site II) located in the transmembrane region, as previously proposed on the basis of extensive mutational studies (16–18). The sequence alignment of SPCAs from different species (9) together with functional studies involving mutants derived from yeast (19) and human (20) SERCA proteins have shown that only the amino acid residues contributing to site II (corresponding to Glu\(^306\) in M4 and Asn\(^738\) and Asp\(^742\) of M6 in human SPCA1) are conserved in SPCAs. Hence, SPCAs are most likely able to couple the chemical energy derived from the hydrolysis of one ATP molecule to the vectorial transport of only one divalent ion (Ca\(^{2+}\) or Mn\(^{2+}\)). SPCAs owe their selectivity for Mn\(^{2+}\) binding and transport to the critical packing interaction between Gln\(^783\) in M6 and Val\(^835\) in M4 in yeast SPCA (21); Gln\(^747\) and Val\(^314\) are the corresponding residues in human SPCA1. Our recent functional study on human SPCA1 mutants (20) has revealed that Gly\(^309\) in M4 (Gly\(^309\) in yeast SPCA) may also play an important role in conferring human SPCA1’s selectivity for Mn\(^{2+}\) transport. Similar to SERCA1a (except for the number of transported Ca\(^{2+}\) per cycle), it may be suggested that the binding of Ca\(^{2+}\) (or Mn\(^{2+}\)) in the transmembrane region of SPCA1 (\(E_2\) to \(E_1\) Ca transition) activates the phosphorylation of Asp\(^530\) by ATP, thus forming the \(E_2\sim P(Ca)\) (ADP-sensitive high energy) phosphoenzyme in which Ca\(^{2+}\) is occluded (Scheme 1). The next reaction step involves the transformation of this intermediate to the \(E_1\)-P (ADP-insensitive low energy) phosphoenzyme with the concomitant release of Ca\(^{2+}\) into the lumen and represents a known crucial rate-limiting step in the Ca\(^{2+}\) transport cycle.
transient expression in the mammalian expression vectors pMT2 or pCA

**Kinetics of Human SPCA1 Isoforms**

**Transfection, Microsome Preparation, and Protein Characterization—**

Transfection of HEK-293 and COS-1 cells with each of the cDNAs in the mammalian expression vectors pMT2 or pCAβ was performed using the transfection reagent FuGENE™ 6 (Roche Applied Science). The microsomal fraction containing the transiently overexpressed Ca2+-ATPase was isolated from transfected cells by differential centrifugation (27) and used for immunochemical and enzymatic assays. Protein concentration determination, denaturing gel electrophoresis, semi-dry blotting, and blot immunostaining were performed as reported earlier (28, 29). The specific polyclonal antibody able to recognize human SPCA1 proteins was prepared as we previously described (20, 30).

**Functional Assays—**

45Ca2+ fluxes were carried out on saponin-permeabilized COS-1 cells as described earlier (9, 20). The methods used for the analysis of the catalytic cycle in steady-state and transient-kinetic conditions have been previously established from studies with SPCA1a mutants (31, 32) and used very recently to characterize SPCA2 (33) and SPCA3 isoforms (34, 35) as well as Darier disease (SCA) patients. These methods were directly applicable to expressed human SPCA1 enzymes. The rapid quench-flow methodol-
ogy, which allows kinetic measurements to be performed on a millisecond scale, was employed as described (31, 33). Additional details of the functional assays are given in the figure legends. The background phosphorylation levels built up in reactions with $\gamma^{32}$P]ATP or $^{32}$P, were determined by adding, respectively, excess EGTA or Ca$^{2+}$ prior to phosphorylation. The constant level of phosphorylation remaining after the exponential decay of phosphoenzyme was used as background in some of the dephosphorylation experiments.

Data Analysis—All data presented are average values corresponding to two or more experiments. Standard errors (S.E.) larger than the symbols are shown as error bars in the figures. Experimental data were fitted by linear and non-linear regression analysis using the SigmaPlot program (SPSS Inc.) or by means of the kinetic simulation software SimZyme (31). The values extracted for $V_{\text{max}}$, $K_m$, $K_{\text{cat}}$, Hill coefficient, and different rate constants are listed in TABLES ONE TO THREE.

RESULTS

Expression of Human SPCA1 Isoforms—The expression levels of human SPCA1a, SPCA1b, SPCA1c, and SPCA1d isoforms in HEK-293 cells transfected with the corresponding cDNA were qualitatively examined and compared by Western blotting and immunocytochemistry analyses. The human SPCA1-specific polyclonal antibody used in these studies was raised against the large cytosolic loop connecting membrane-spanning helices M4 and M5 in human SPCA1 isoforms as described (20, 30). Fig. 1A shows a typical Western blot analysis performed on microsomal proteins isolated from transfected HEK-293 cells. The immunoblot analysis demonstrates that, with the exception of SPCA1c, all SPCA1 isoforms were clearly expressed in HEK-293 cells at levels much higher than that of the endogenous SPCA1 in control cells transfected with empty vector. SPCA1c appeared to be expressed at a level just slightly higher than that found in control microsomes. Human SPCA1 proteins were well resolved according to their sizes by denaturing gel electrophoresis: SPCA1a (919 amino acids), SPCA1b (939 amino acids), and SPCA1d (949 amino acids). The faint band corresponding to SPCA1c (888 amino acids) showed a slower electrophoretic mobility than expected (Fig. 1A). Additionally, the upper bands in Fig. 1A detected just below the 188-kDa molecular mass standard most likely represent SPCA1a, SPCA1b, and SPCA1d-derived dimers. Finally, the immunocytochemical analysis showed that the expression of SPCA1a, SPCA1b, and SPCA1d appeared to be largely confined to a juxtanuclear Golgi-like compartment, whereas SPCA1c was expressed in only a few cells and showed a more ER-like distribution (data not shown). Furthermore, our immunoblot analysis using a SERCA2b-specific antibody showed that the expression level of the endogenous SERCA2b did not change following the transient overexpression of the human SPCA1 isoforms in HEK-293 cells (data not shown).

The expression levels of SPCA1 isoforms in HEK-293 cells were further quantified by measuring their maximum capacity for phosphorylation with $\gamma^{32}$P]ATP (“active site concentration”) in the presence of activating Ca$^{2+}$ (forward reaction 3 in Scheme 1) and at 0 °C and neutral pH, i.e. under conditions known to preserve the stability of the phosphorylated intermediate $E_1-P(Ca)$. The phosphorylation reactions were performed in the presence of thapsigargin (0.1 μM), which inhibits the endogenous SERCA2b activity in both transfected and control HEK-293 cells. Fig. 1B illustrates the results of this functional approach. SPCA1-specific phosphorylation levels ranging from 100 to 200 pmol of active enzyme/mg microsomal protein, i.e. 1–2% of the total amount of microsomal protein, were obtained for the human SPCA1a, SPCA1b, and SPCA1d isoforms. These phosphorylation levels are comparable to those documented for SERCA pumps (33, 34). For direct comparison the expression level reached for SERCA1a in the present series of experiments was ~200 pmol/mg (data not shown). Importantly, the expression levels of the human SPCA1 isoforms were 200–350-fold higher than that of the endogenous SPCA1 pump in control HEK-293 cells. Therefore, the contribution of the endogenous SERCA1 enzyme to the enzymatic measurements described below is negligible. Even in the absence of thapsigargin, the background phosphorylation level generated by the combined action of SERCA2b and SPCA1 in control HEK-293 cells does not rise above more than 0.5–1.0 pmol of Ca$^{2+}$-ATPase/mg microsomal protein (33). Hence, the overexpression of exogenous SPCA1a, SPCA1b, and SPCA1d and the low background level allowed the reliable use of the heterologous mammalian cell expression system for the functional characterization of these SPCA enzymes. On the other hand, the phosphorylation level of SPCA1c (Fig. 1B) is as low as that obtained with control microsomes.
**Kinetics of Human SPCA1 Isoforms**

**45Ca2+ Transport Activity**—The thapsigargin-insensitive ability of each of the transiently overexpressed SPCA1 isoforms to actively transport Ca2+ into a membrane-delineated Ca2+ store was assessed following expression in COS-1 cells as previously described (9, 20). Using this whole cell approach the competence of the overexpressed enzyme is evaluated within a well preserved cellular environment. The filling state of the internal Ca2+ stores was studied in saponin-permeabilized cells, following a 90-min loading with a 45Ca2+-containing solution in the presence or absence of oxalate (Fig. 1C). Oxalate precipitates Ca2+ intracellular compartments, thus reducing the luminal free Ca2+ concentration and, thereby, relieving the back inhibition exerted by Ca2+ at the luminaly facing low affinity Ca2+ binding site of Ca2+-ATPase. COS-1 cells were preferred to HEK-293 cells in these experiments, because of their increased adherence to the gelatin-coated wells, thus being less prone to detaching during permeabilization, loading, and washing steps (9). At the end of the loading period (corresponding to the zero time point in each panel of Fig. 1C), the level of 45Ca2+ accumulated in the presence of oxalate by SPCA1a, SPCA1b, and SPCA1d, respectively, was 2.8-, 2.9-, and 4.0-fold increased relative to that of control cells. Furthermore, when the calcium ionophore A23187 was included in the efflux medium, the rate of 45Ca2+ release increased, thus demonstrating that during the loading period the overexpressed SPCA1 isoforms had indeed accumulated Ca2+ within intracellular membranous Ca2+ stores. No 45Ca2+ transport activity specific for the human SPCA1c isoform could be measured (Fig. 1C). Because of the low level of expression (Fig. 1A) and lack of phosphorylation (Fig. 1B), no other functional measurements were further performed on SPCA1c. These results suggest altogether that SPCA1c represents a rapidly degradable non-functional SPCA1 protein.

**ATPase Activity**—Steady-state ATPase activity at 5 mM MgATP was determined for each of the well expressed SPCA1a, SPCA1b, and SPCA1d isoforms by monitoring the release of inorganic phosphate. For each SPCA1 isoform, Fig. 2 shows the Ca2+ dependence of the ATPase turnover rate determined at pH 7.0 and 37 °C, in the absence and presence of the calcium ionophore A23187. The maximum turnover rate for ATP hydrolysis, the effect of the calcium ionophore on the turnover rate, and the Ca2+ titration data obtained for SPCA1 and SERCA1 enzymes are summarized in **TABLE ONE**. For SERCA1a, the E0.5 (P(Ca0)) to E1-P transition (step 4 in Scheme 1) is inhibited by the binding of accumulated Ca2+ at luminal low affinity sites. When the Ca2+ concentration of the medium is within micromolar range, the addition of calcium ionophore relieves the inhibition by allowing rapid equilibration of Ca2+ between the two sides of the microsomal membrane through passive Ca2+ efflux. Similar effects of ionophore addition were observed for the SPCA1 enzymes. Surprisingly, the maximum ATPase turnover rates determined for SPCA1a, SPCA1b, and SPCA1d, respectively, were 64.5-, 57.4-, and 47-fold lower than that of SERCA1a. Nevertheless, the apparent affinities for Ca2+ activation of the ATPase activity displayed by SPCA1a, SPCA1b, and SPCA1d were much higher (K0.5 7.5–18.2-fold lower in **TABLE ONE**) than that of SERCA1a. Finally, the Hill coefficient values (listed in **TABLE ONE**) of ~1.0 for SPCA1 isoforms indicate that the cooperativity seen for SERCA1a is absent for SPCA1 isoforms. The latter finding is consistent with the proposal that the active transport of Ca2+ performed by each human SPCA1 isoform takes place via a single high affinity Ca2+ binding site (i.e., each Ca2+ is transported per each ATP-driven catalytic cycle) instead of the two cooperatively interacting Ca2+ binding sites of SERCA pumps.

**Phosphorylation by [γ-32P]ATP**—The Ca2+ dependence of phosphorylation with [γ-32P]ATP was studied at pH 7.0 and 25 °C (Fig. 3). This analysis allows a very accurate determination of the apparent affinity (K0.5) for Ca2+ and Hill coefficient. Relative to SERCA1a, the K0.5 values

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**TABLE ONE**

| Type of Ca2+-ATPase | ATPase turnover ratea | Ionophore effect at pH 7.0b | K0.5 and Hill coefficient (nH) for Ca2+ activation of: | Ca2+ binding transitionc | Rate of Ca2+ dissociationd |
|---------------------|-----------------------|-----------------------------|------------------------------------------------------|--------------------------|---------------------------|
|                     | s⁻¹                   |                             | ATPase activitye | Phosphorylationd | ATPase activity | Phosphorylation | Initial offset | Initial offset |
| SERCA1a             | 130                   | 3.20                        | 0.310 ± 0.212  | 1.65          | 0.284 ± 0.004  | 1.81          | 11.44          | 42            | 27.0         |
| SPCA1a              | 20.27                 | 2.67                        | 0.041 ± 0.003  | 1.10          | 0.010 ± 0.001  | 0.67          | 8.64           | 52            | 43.5         |
| SPCA1b              | 22.50                 | 3.81                        | 0.017 ± 0.003  | 0.90          | 0.009 ± 0.001  | 0.75          | 24.85          | 50            | 34.4         |
| SPCA1d              | 27.44                 | 2.60                        | 0.037 ± 0.004  | 0.98          | 0.010 ± 0.001  | 0.76          | 22.42          | 45            | 36.7         |

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a Maximum turnover rate for ATP hydrolysis at 37 °C in the presence of calcium ionophore A23187 at pH 7.0 (Fig. 2).

b The ratio between the maximal ATPase activities with and without calcium ionophore A23187 at pH 7.0 (Fig. 2).

c The Ca2+ concentration giving half-maximal activation of ATPase activity at 37 °C in the presence of calcium ionophore A23187 (Fig. 2).

d The Ca2+ concentration giving half-maximal activation of phosphorylation by [γ-32P]ATP at 25 °C (Fig. 3).

e Rate constants corresponding to appearance of ability to phosphorylate with [γ-32P]ATP at 25 °C upon addition of 100 μM CaCl2 to the Ca2+-deprived enzyme and the initial phosphorylation levels at time 0 (Fig. 4).

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order monoexponential function with an initial offset to the data. The best fits are shown phosphorylation is shown relative to the maximum level calculated from fitting a first-order approach to the notion of a single cytoplasmically facing high affinity Ca\(^{2+}\) see TABLE ONE) are also in agreement with the data from Fig. 2 superimposed.

Obtained for SPCA1 enzymes (TABLE ONE) were ~30-fold decreased, consistent with the data from the Ca\(^{2+}\)-titrations of the ATPase activities presented above. Furthermore, the Hill coefficient values for the human SPCA1 isoforms (<1 in contrast to the value of 1.8 for SERCA1a, see TABLE ONE) are also in agreement with the data from Fig. 2 supporting the notion of a single cytoplasmically facing high affinity Ca\(^{2+}\) binding site per human SPCA1 molecule.

Rapid Kinetic Analyses of Ca\(^{2+}\) Binding and Phosphorylation—To obtain further information on the Ca\(^{2+}\) binding transition (forward reactions 1 and 2 in Scheme 1), Ca\(^{2+}\) dissociation (reverse reaction 2 in Scheme 1), and phosphorylation (reaction 3 in Scheme 1) properties of the human SPCA1 enzymes at 25°C, rapid kinetic measurements of the phosphorylation with \([\gamma-\text{32P}]\text{ATP}\) were conducted using the quench-flow methodology previously described and validated for SERCA1, SERCA2, and SERCA3 isoforms (31–34).

The rate constant of the Ca\(^{2+}\) binding transition was determined according to the double mixing protocol shown as a diagram at the top of Fig. 4. This procedure takes advantage of the fact that only Ca\(^{2+}\)-bound enzyme is able to be phosphorylated from ATP. Accordingly, the Ca\(^{2+}\)-deprived enzyme is incubated with saturating Ca\(^{2+}\) (100 \(\mu\text{M}\) final free Ca\(^{2+}\) concentration) for a variable period of time (\(t\) in Fig. 4) followed by a 50-ms incubation with 5 \(\mu\text{M}\) \([\gamma-\text{32P}]\text{ATP}\) before acid quenching. The results obtained for SPCA1 and SERCA1a isoforms are shown in Fig. 4. The rate constants corresponding to the Ca\(^{2+}\) binding transition (determined by fitting a monoexponential function with an initial offset) are presented in TABLE ONE together with the initial offset, which depends on the initial amount of enzyme present as \(E_1\) and the amount of \(E_1\) formed from \(E_2\) during the 50-ms incubation with Ca\(^{2+}\) and \([\gamma-\text{32P}]\text{ATP}\). SPCA1a and SERCA1a displayed rather similar rate constants (8.64 s\(^{-1}\) versus 11.44 s\(^{-1}\)). By contrast, the rate constants determined for human SPCA1b and SPCA1d were ~2-fold enhanced. The initial offset values for the SPCA1a isoforms were only slightly higher than that of SERCA1a (TABLE ONE).

The Ca\(^{2+}\) interaction of the SPCA1 pumps in the \(E_1\) form was further investigated by determining the rate of Ca\(^{2+}\) dissociation (\(k_{\text{off}}\)) from the Ca\(^{2+}\)-bound \(E_1\) form, again taking advantage of the fact that only Ca\(^{2+}\)-bound enzyme is able to be phosphorylated from ATP. In this procedure (31–34), the level of phosphoenzyme (\(E_{\text{PATP}}\)) is measured 34 ms after the simultaneous addition of excess EGTA (to initiate Ca\(^{2+}\) dissociation) and \([\gamma-\text{32P}]\text{ATP}\) to the enzyme pre-equilibrated with saturating Ca\(^{2+}\), and compared with the phosphoenzyme level (\(E_{\text{PATP}}\)) measured after 34 ms incubation with \([\gamma-\text{32P}]\text{ATP}\) in the presence of Ca\(^{2+}\), i.e., without allowing Ca\(^{2+}\) to dissociate. The data obtained at 25°C and pH 7.0 with the SPCA1 and SERCA1a enzymes are shown in Fig. 5, and TABLE ONE displays the rate constants for Ca\(^{2+}\) dissociation derived as previously explained (31), taking into consideration the difference in phosphorylation rate constant (see below). Because both Ca\(^{2+}\) sites must be occupied for activation of phosphorylation in...
SERCA1a, the Ca\(^{2+}\) dissociation assay monitors only the first Ca\(^{2+}\) dissociation step in this enzyme, corresponding to the Ca\(^{2+}\) ion bound last, at site II (31). Because this is the site equivalent to that present in SPCA1, it is reasonable to compare directly the Ca\(^{2+}\) activities does not reflect an increase of the intrinsic (true) affinity of SPCA1, it is reasonable to compare directly the Ca\(^{2+}\) dissociation step in this enzyme, corresponding to the Ca\(^{2+}\) ion bound last, at site II (31). Because this is the site equivalent to that present in SPCA1a, SPCA1b, and SPCA1d were, respectively, 1.61-, 1.27-, and 1.36-fold enhanced relative to SERCA1a. These results clearly demonstrate that the high apparent affinity of any functional SPCA1 isoform for cytosolic Ca\(^{2+}\) (low K\(_{50}\)) value manifested during the Ca\(^{2+}\) titration of ATPase (cf. Fig. 2) and phosphorylation (cf. Fig. 3) activities does not reflect an increase of the intrinsic (true) affinity of the E\(_1\) form for Ca\(^{2+}\).

The time course of phosphorylation was monitored with the enzyme initially present in the Ca\(^{2+}\)-saturated form (Fig. 6). As previously described (31, 33), the phosphorylation profile for SERCA1a displayed a slight overshoot that could be accurately reproduced by computation (shown as a line in Fig. 6). As indicated in Fig. 6, no phosphorylation overshoot was present for the SPCA1 enzymes, as the data could be fitted by monoexponential functions (lines in Fig. 6), and the rate constants were 3.4-, 2.7-, and 2.8-fold lower for SPCA1a, SPCA1b, and SPCA1d, respectively, relative to the phosphorylation rate constant of SERCA1a, see TABLE TWO. The lack of phosphorylation overshoot can be accounted for by the reduced phosphorylation rate. In addition, the reduced phosphoenzyme turnover rate (see below, Fig. 8) may also be instrumental in removing the overshoot.

**ATP Dependence of Phosphorylation**—To further examine the reduced rate of phosphorylation of the SPCA isoforms, the ATP concentration dependence of phosphorylation with [\(\gamma^{-32}\)P]ATP was studied, both at steady-state (Fig. 7A) and by determination of initial rates (Fig. 7B). As shown in Fig. 7A, SPCA1 isoforms displayed 2–4.1-fold lower apparent affinities for ATP (increased K\(_{0.5}\) values in TABLE TWO) than SERCA1a, thus in good agreement with their reduced phosphorylation rates (cf. Fig. 6). The K\(_{0.5}\) values obtained at steady-state depend on the affinity for ATP, the maximal phosphorylation rate corresponding to saturating ATP concentration (V\(_{\text{max}}\)), as well as the phosphoenzyme turnover rate (K\(_{\text{m}}\) increases with increasing phosphoenzyme turnover rate). Because the phosphoenzyme turnover rate of SPCA1 isoforms is markedly reduced relative to SERCA1a (see below, Fig. 8), only a reduced affinity for ATP or a reduced V\(_{\text{max}}\) for phosphorylation can account for the data in Fig. 7A. For SPCA1b and SPCA1d the V\(_{\text{max}}\) for phosphorylation was determined by examining the ATP concentration dependence of the initial phosphorylation rate at 25°C under the same conditions as those described for Fig. 6. The double reciprocal (Lineweaver-Burk) plots of the initial phosphorylation rate per Ca\(^{2+}\)-ATPase molecule versus the ATP concentration shown in Fig. 7B and the values extracted for K\(_{\text{m}}\) and V\(_{\text{max}}\) listed in TABLE TWO showed that: (i) the V\(_{\text{max}}\) for SPCA1b and SPCA1d was, respectively, 7- and 4.9-fold lower than that of SERCA1a, and (ii) the respective K\(_{\text{m}}\) values were 1.8- and 1.3-fold reduced relative to SERCA1a. Both effects can be explained by a reduced k\(_{3}\) in the simplified Michaelis-Menten reaction Scheme 2,

\[
E_1 + \text{ATP} \xrightleftharpoons{k_1} E_1\cdot\text{ATP} \xrightarrow{k_2} E_1\sim\text{P} + \text{ADP}
\]

**TABLE TWO**

| Type of Ca\(^{2+}\)-ATPase | Rate of phosphorylation* \(s^{-1}\) | \(K_{0.5}\) for ATP activation of phosphorylation \(\mu M \pm S.E.\) | ATP activation of phosphorylation at pre-steady-state | \(K_{0.5}\) for \([\gamma^{-32}\text{P}]\) titration of phosphorylation with \([\gamma^{-32}\text{P}]\) ATP | \(K_{0.5}\) for vanadate inhibition of phosphorylation \(\mu M \pm S.E.\) |
|--------------------------|-----------------|-----------------|--------------------------|-----------------|-----------------|
|                          |                 |                 | \(V_{\text{max}}\) \(s^{-1}\)| \(K_{\text{m}}\) \(\mu M\) | \(K_{0.5}\) | \(K_{0.5}\) |
| SERCA1a                  | 35.00           | 0.065 ± 0.003   | 192.4                   | 10.3            | 11.02 ± 0.610  | 0.140 ± 0.001  |
| SPCA1a                   | 10.44           | 0.130 ± 0.009   | ND                      | ND              | 67.06 ± 13.43  | 0.322 ± 0.006  |
| SPCA1b                   | 12.97           | 0.270 ± 0.004   | 27.4                    | 5.8             | 64.06 ± 12.12  | 0.635 ± 0.136  |
| SPCA1d                   | 12.36           | 0.162 ± 0.019   | 39.5                    | 8.1             | 61.43 ± 13.05  | 0.692 ± 0.188  |

*Rate constants corresponding to phosphorylation with \([\gamma^{-32}\text{P}]\)ATP of enzyme preincubated in a medium containing 100 \(\mu M\) CaCl\(_2\) at 25°C (Fig. 6). The phosphorylation reaction denotes the transition from \(E_1\) to \(E_1\sim\text{P}\) (Ca\(_2\)) for SPCA1 enzymes and \(E_1\cdot\text{Ca}_2\) to \(E_1\sim\text{P}\) (Ca\(_2\)) for SERCA1a.

**The ATP concentration giving half-maximal activation of phosphorylation by \([\gamma^{-32}\text{P}]\)ATP at 0°C when \(n_1\) is set to 1 (Fig. 7A).**

**The maximal phosphorylation rate (\(V_{\text{max}}\)) and the Michaelis-Menten constant (\(K_{\text{m}}\)) were calculated by linear regression from the double reciprocal plots of the initial phosphorylation rate versus ATP concentration (Fig. 7B).**

**The \([\gamma^{-32}\text{P}]\) concentration giving half-maximal activation of phosphorylation by \([\gamma^{-32}\text{P}]\)ATP at 0°C (Fig. 7A).**

**The vanadate concentration giving half-maximal inhibition of phosphorylation by \([\gamma^{-32}\text{P}]\)ATP at 0°C (Fig. 7B).**

**ND, not determined.**
where \( K_{\text{m}} = (k_{-1} + k_2)/k_1 \) and \( k_2 = V_{\text{max}} \) per \( Ca^{2+} \)-ATPase molecule. Because the observed \( K_{\text{m}} \) values for SPCA1b and SPCA1d are reduced (as expected because of the reduced \( k_2 \)) rather than increased, relative to SERCA1a, there is no evidence for a reduction in SPCA1b and SPCA1d of the true affinity for ATP (increase of the dissociation constant for the enzyme-ATP complex, \( K_{\text{D}} = k_{-1}/k_1 \)).

Dephosphorylation of the Phosphoenzyme Intermediate Formed from ATP—As illustrated in Fig. 8, the decomposition of phosphoenzyme intermediate was studied after phosphorylation with \( \gamma^{32}\text{P}\)ATP. Phosphorylation was carried out at neutral pH, 0 °C, and presence of K\(^+\), i.e. under conditions known for SERCA1a, SERCA2, and SERCA3 isoforms to lead to the accumulation of the high energy ADP-sensitive \( E_1\sim\text{P}(\text{Ca}) \) intermediate (33, 34). For SPCA1, it is expected that the phosphoenzyme intermediate \( E_1\sim\text{P}(\text{Ca}) \) contains only a single occluded \( Ca^{2+} \). These intermediates were chased either with excess ATP and EGTA (Fig. 8A) leading to dephosphorylation in the forward direction of the reaction cycle (via reactions 4 and 5 in Scheme 1) or with excess ADP and EGTA (Fig. 8B) leading to dephosphorylation in the backward direction with formation of ATP via the transfer of the phosphophoryl group to ADP (reverse reaction 3 in Scheme 1). The rate constants are shown in TABLE THREE. Following the ATP/EGTA chase, SPCA1a, SPCA1b, and SPCA1d isoforms displayed dephosphorylation rates that were, respectively, 14.2-, 12.0-, and 9.0-fold slower than that obtained for SERCA1a. Such pronounced reductions of the rate of dephosphorylation can explain the observed differences in the maximal ATPase turnover rate (cf. Fig. 2 and TABLE ONE) and the high apparent affinity for \( Ca^{2+} \) (cf. Figs. 2 and 3 and TABLE ONE) described above. It is furthermore interesting that the slightly different dephosphorylation rates of the SPCA1 isoforms appeared to correlate quite well with the length of their alternatively spliced C-terminal part: the isoform with the shortest C terminus (SPCA1a) dephosphorylated slower than the other SPCA1 isoforms and SPCA1d (the longest) was the fastest. It is also noteworthy that the dephosphorylation rates obtained following the ADP/EGTA chase of the SPCA1 \( E_1\sim\text{P}(\text{Ca}) \) intermediates were 2.3–4.1-fold reduced relative to SERCA1a (Fig. 8B). This represents significant reductions in the rate of reverse partial reaction 3 (Scheme 1), thus matching the decreased rates observed for the phosphorylation step (forward reaction 3 in Scheme 1, cf. Fig. 6).

Phosphoenzyme Formation from \( ^{32}\text{P} \) and Its Dephosphorylation—Backdoor phosphorylation with \( ^{32}\text{P} \) (reverse reaction 5 in Scheme 1) was analyzed for SPCA1 isoforms under conditions (acid pH, absence of \( Ca^{2+} \) and alkali metal ions, but presence of dimethyl sulfoxide) known for SERCA1a (36) to increase its affinity for P\(^+\) and to result in the formation of a rather stable ADP-insensitive \( E_2\sim\text{P} \) phosphoenzyme product. As seen in Fig. 9A, SPCA1 isoforms displayed rather similar apparent affinities for \( ^{32}\text{P} \), which were 5.5–6.0-fold reduced (larger \( K_{\text{m}} \) values in TABLE THREE) relative to SERCA1a. The decay of the \( E_2\sim\text{P} \) intermediate formed by phosphorylation with \( ^{32}\text{P} \) under conditions described in relation to Fig. 9A was monitored (Fig. 9, B–E and TABLE THREE) following its dilution in dephosphorylation media of varying pH at 0 °C in the presence or absence of K\(^+\). For SERCA1a, the \( E_2\sim\text{P} \) dephosphorylation becomes rate limiting for the overall activity at acidic or alkaline reaction conditions, but it is very
TABLE THREE

| Type of Ca\(^{2+}\)-ATPase | Dephosphorylation of ADP-sensitive phosphoenzyme\(^{a}\) | Dephosphorylation of ADP-insensitive phosphoenzyme\(^{b}\) |
|--------------------------|-------------------------------------------------|-------------------------------------------------|
|                          | ATP/EGTA chase                                  | pH 6.0 (no K\(^{+}\))                           | pH 7.0 (no K\(^{+}\))                           |
|                          | s\(^{-1}\) ± S.E.                               | s\(^{-1}\) ± S.E.                               | s\(^{-1}\) ± S.E.                               |
| SERCA1a                  | 0.299 ± 0.033                                  | 0.010 ± 0.001                                  | 0.081 ± 0.006                                  |
|                          | 1.081 ± 0.027                                  | 0.110 ± 0.009                                  | 0.004 ± 0.000                                  |
| SPCA1a                   | 0.021 ± 0.002                                  | 0.370 ± 0.051                                  | 0.323 ± 0.026                                  |
|                          | 0.390 ± 0.051                                  | 0.578 ± 0.036                                  | 0.952 ± 0.086                                  |
| SPCA1b                   | 0.025 ± 0.003                                  | 0.457 ± 0.049                                  | 0.413 ± 0.024                                  |
|                          | 0.475 ± 0.049                                  | 0.383 ± 0.054                                  | 0.270 ± 0.034                                  |
| SPCA1d                   | 0.033 ± 0.003                                  | 0.260 ± 0.019                                  | 0.340 ± 0.018                                  |
|                          | 0.752 ± 0.013                                  | 1.100 ± 0.025                                  | 0.682 ± 0.056                                  |

\(^{a}\) Rate constants corresponding to dephosphorylation of phosphoenzyme formed in the presence of [\(\gamma\)-\(^{32}\)P]ATP and 100 mM Ca\(^{2+}\); the phosphoenzyme was chased with either 0.6 mM ATP and 6.7 mM EGTA (ATP/EGTA chase; Fig. 8A) or 1.0 mM ADP and 10 mM EGTA (ADP/EGTA chase; Fig. 8D).

\(^{b}\) Rate constants corresponding to dephosphorylation at 0 °C and the indicated pH values (in the presence or absence of potassium ions) of phosphoenzyme formed in the presence of [\(\gamma\)-\(^{32}\)P]Pi (panels B–E in Fig. 9).

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**Kinetics of Human SPCA1 Isoforms**

**FIGURE 9.** P\(_i\) concentration dependence (A) and dephosphorylation of the phosphoenzyme formed from [\(\gamma\)-\(^{32}\)P]ATP (B–E). A, phosphorylation was carried out for 10 min at 25 °C in the presence of 100 mM MES/Tris, pH 6.0, 10 mM MgCl\(_2\), 2 mM EGTA, 30% (v/v) dimethyl sulfoxide, 0.1 \(\mu\)M thapsigargin and the indicated concentrations of \(^{32}\)P\(_i\), followed by acid quenching. Phosphorylation of SERCA1a was performed without thapsigargin. The data were normalized separately by taking as 100% the maximum phosphorylation level reached, and the lines for the indicated proteins show the best fits of the Hill equation with \(n_H = 1\). The \(K_0\) values (μM ± S.E.) are listed in TABLE TWO. B, phosphorylation with 0.5 μM \(^{32}\)P\(_i\), was performed as in A. Following cooling of the sample to 0 °C, the phosphoenzyme was chased by a 10-fold dilution of an aliquot into a medium (kept at 0 °C) containing 100 mM MES/Tris, pH 6.0, 10 mM MgCl\(_2\), 2 mM EGTA, and 0.5 mM non-radioactive \(P_i\) and acid quenching was performed at the indicated serial time intervals. C, the same as in B, but in addition the phosphorylation medium contained 80 mM KCl. D–E, the same as in B, but the dephosphorylation buffer contained 100 mM MOPS/Tris, pH 7.0 (D) or 100 mM Tris/HisCl, pH 8.5 (E) instead of 100 mM MES/Tris, pH 6.0. The phosphorolysis levels in B–E are shown relative to the level determined by acid quenching after the 10-min phosphorylation at 25 °C. The lines represent the best fits of a mono-exponential decay function, and the obtained rate constants (s\(^{-1}\) ± S.E.) are shown in TABLE THREE.

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rapid at neutral pH (34, 37, 38). Moreover, the dephosphorylation rate depends on the presence of monovalent alkali metal ions, being accelerated more than 10-fold by addition of K\(^{+}\) (38, 39). Surprisingly, the results illustrated in Fig. 9, B–E, with the rate constants being shown in TABLE THREE, demonstrated that the \(E_2\)-P dephosphorylation in SPCA1 enzymes was markedly accelerated relative to SERCA1a under all conditions examined. In the absence of K\(^{+}\), three pH conditions were tested, and the acceleration relative to SERCA1a was 73–93-fold at pH 6.0, 4–14-fold at pH 7.0, and 68–238-fold at pH 8.5. There was little influence of pH on the dephosphorylation of SPCA1 isoforms, and only SPCA1d resembled SERCA1a in displaying a higher dephosphorylation rate at pH 7.0 compared with pH 6.0 and pH 8.5. The addition of K\(^{+}\) at pH 6.0 resulted in an 11-fold increase in the rate of \(E_2\)-P dephosphorylation for SERCA1a (cf. Fig. 9, B and C), but K\(^{+}\) did not accelerate the \(E_2\)-P dephosphorylation in the SPCA1 isoforms (cf. Fig. 9, B and C). In fact, when compared with the decay rates obtained in the absence of K\(^{+}\) (TABLE THREE), it seemed that K\(^{+}\) actually exerted a slightly inhibitory effect on the dephosphorylation of \(E_2\)-P in SPCA1 isoforms, which was more pronounced in SPCA1b and SPCA1d isoforms than in SERCA1a. However, the rates with which SPCA1 enzymes dephosphorylated from \(E_2\)-P at pH 6.0 in the presence of 80 mM K\(^{+}\) were still 3–5-fold faster than that of SERCA1a (Fig. 9C). It can be concluded that irrespective of pH and K\(^{+}\) concentration, the \(E_2\)-P dephosphorylation in SPCA1 isoforms takes place faster relative to SERCA1a, and that the dependence of this step on pH and K\(^{+}\) is too weak to be of importance in SPCA1 isoforms.

**Inhibition by Vanadate**—Vanadate, an analog of phosphate, binds to the \(E_2\) dephosphoenzyme leading to a dead end complex (stable in ice-cold conditions) believed to resemble the transition state between \(E_2\)-P and \(E_2\)-P\(_{2}\), occurring during enzyme turnover. Therefore, any change brought to this transition state or to the \(E_2\) state reacting with vanadate would affect the apparent affinity of the enzyme for vanadate. Vanadate binding was studied under equilibrium conditions (i.e. in the absence of phosphoenzyme turnover) in which vanadate was allowed to bind \(E_2\) in the absence of Ca\(^{2+}\) and ATP (1 h at 25 °C). After the incubation, the fraction of phosphorylatable vanadate-free enzyme (\(E_2\)) was measured by phosphorylation (at 0 °C) following the addition of excess Ca\(^{2+}\) and [\(\gamma\)-\(^{32}\)P]ATP as previously described (33). The results shown in Fig. 10 demonstrate that SPCA1a, SPCA1b, and SPCA1d isoforms displayed, respectively, 2.3-, 4.5-, and 4.9-fold lower apparent affinities for vanadate than SERCA1a (higher \(K_0\) values in TABLE TWO), thus consistent with the data obtained from the P\(_i\) titration of P\(_i\) phosphorylation (cf. Fig. 9A).
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In the present study, we have performed for the first time a detailed characterization of the reaction cycle mediated by the human SPCA1 isoforms heterologously expressed in mammalian HEK-293 and COS-1 cells. The validity of the mammalian expression systems employed in this study was clearly demonstrated, as SPCA1a, SPCA1b, and SPCA1d were well expressed to levels comparable to SERCA pumps and functionally active (cf. Fig. 1, B and C). The low expression level, the anomalous electrophoretic mobility (slower than expected) and the lack of phosphorylation from alous electrophoretic mobility (slower than expected) and the lack of phosphorylation from SPCA1d have been summarized in TABLES ONE to THREE. In comparison with SERCA1a, the human SPCA1 isoforms display essential differences with respect to the overall Ca\(^{2+}\)-ATPase reaction (cf. Fig. 2): (i) much higher apparent affinities for Ca\(^{2+}\) activation at the cytosolic site and (ii) lower catalytic turnover rates. The Hill coefficient values extracted from the data describing the Ca\(^{2+}\) titration of ATP hydrolysis and phosphorylation (Figs. 2 and 3) moreover support the presence of only a single cytoplastically oriented high affinity ion-binding site per SPCA1 molecule. With regard to the partial reaction steps of the Ca\(^{2+}\) transport cycle illustrated in Scheme 1, the human SPCA1 isoforms also display kinetic properties distinct from SERCA1a as follows: (i) a markedly reduced maximum rate (V\(_{\text{max}}\)) of phosphorylation (E\(_{1}\)Ca to E\(_{2}\)~P(Ca) transition, cf. Figs. 6 and 7B); (ii) a markedly reduced rate of the E\(_{1}\)~P(Ca) to E\(_{2}\) transition (decreasing with the length of the alternatively spliced C terminus, cf. Fig. 8A); (iii) a markedly increased rate of E\(_{2}\)-P dephosphorylation, which furthermore does not show the dependence on pH and K\(^+\) characteristic of SERCA1a (cf. Fig. 9, B–E); (iv) a 2-fold increase of the rate of the Ca\(^{2+}\)-binding transition in SPCA1a and SPCA1d (cf. Fig. 4); and (v) a slight increase in the rate of Ca\(^{2+}\) dissociation from E\(_{2}\)Ca (cf. Fig. 5). As will be discussed below, the characteristics of the partial reaction steps of SPCA1 isoforms can explain the distinct features of their overall reaction described here (cf. Figs. 2, 3, 7A, 9A, and 10).

Increased Apparent Affinity for Cytosolic Ca\(^{2+}\)—Relative to SERCA1a, a 7.5–18.2-fold increase in the apparent affinity for Ca\(^{2+}\) was observed for SPCA1 isoforms by Ca\(^{2+}\) titration of ATPase activity at steady-state (Fig. 2). An even larger difference between SPCA1 isoforms and SERCA1a (~30-fold) was observed for the apparent Ca\(^{2+}\) affinity determined in studies of the Ca\(^{2+}\) dependence of phosphorylation from [γ-32P]ATP (Fig. 3). In principle, the increased apparent Ca\(^{2+}\) affinities of SPCA1 isoforms could be caused by a shift of the equilibrium between E\(_{2}\) and E\(_{1}\) in favor of the E\(_{1}\) form. As shown in Fig. 4, our rapid kinetic analysis instead demonstrated a 2-fold increase of the rate of the Ca\(^{2+}\) binding transition, thus in apparent agreement with the E\(_{2}\) to E\(_{1}\) equilibrium being shifted toward E\(_{1}\). However, it can be generally demonstrated by computer simulation of the Ca\(^{2+}\) dependence of phosphorylation with ATP (32) that the 2-fold increase in the E\(_{2}\) to E\(_{1}\) transition rate seen here for both SPCA1b and SPCA1d is too small a change to account for the observed ~30-fold decrease of the K\(_{\text{0.5}}\) values for Ca\(^{2+}\) activation of phosphorylation (Fig. 3). Moreover, SPCA1a did not show the increase in the E\(_{2}\) to E\(_{1}\) transition rate seen for the other SPCA1 isoforms (TABLE ONE). Therefore, other effects on partial reaction steps must be involved as well. For SPCA2b and SPCA3 enzymes, we recently demonstrated significant changes of the Ca\(^{2+}\) dissociation (E\(_{1}\)Ca to E\(_{2}\)Ca transition) rate, which could explain the observed differences from SERCA1a with respect to the apparent affinity of the E\(_{1}\) form for cytosolic Ca\(^{2+}\) (33, 34). Therefore, we expect to find a reduced Ca\(^{2+}\) dissociation rate for SPCA1 isoforms correlating with the increased apparent Ca\(^{2+}\) affinity. Surprisingly, the rates of Ca\(^{2+}\) dissociation (Fig. 5 and TABLE ONE) from the Ca\(^{2+}\)-bound E\(_{2}\) form (E\(_{2}\)Ca) found in the SPCA1 isoforms were instead slightly enhanced relative to SERCA1a and, thus, did not explain the enhanced apparent Ca\(^{2+}\) affinity of SPCA1 enzymes.

In SERCA1a, the transition from E\(_{1}\)~P(Ca\(_{2}\)) to E\(_{2}\)-P constitutes the most rate-limiting step of the cycle at physiological pH and substrate concentrations. The ensuing hydrolysis reaction E\(_{2}\)-P → E\(_{1}\)P is much faster than the E\(_{1}\)~P(Ca\(_{2}\)) to E\(_{2}\)-P transition at neutral pH and presence of K\(^+\). Computation based on the reaction cycle of SERCA1a demonstrated that acceleration or slowing of the rate-limiting E\(_{1}\)~P(Ca\(_{2}\)) to E\(_{2}\)-P transition causes, respectively, a decreased or increased apparent affinity for Ca\(^{2+}\) ions (32). Hence, the observed slow processing of the ADP-sensitive E\(_{1}\)~P(Ca) phosphoenzyme (cf. Fig. 8A and TABLE THREE) seems to be a major determinant of the high apparent affinity for Ca\(^{2+}\) displayed by all SPCA1 isoforms (when phosphoenzyme turnover is slow, more phosphoenzyme is accumulated at low Ca\(^{2+}\) concentrations, thus shifting the Ca\(^{2+}\) activation curve toward lower K\(_{\text{0.5}}\) values). Because the dephosphorylation of E\(_{2}\)-P is rapid in SPCA1 isoforms, the E\(_{1}\)~P(Ca) to E\(_{2}\)-P transition must be the slow step in the E\(_{1}\)~P(Ca) processing.

The finding that the high apparent affinity for Ca\(^{2+}\) displayed by SPCA1 isoforms owes to a "kinetic effect" rather than a true increase of affinity relative to SERCA1a has implications for understanding the structure of the Ca\(^{2+}\) binding site of the SPCA1 isoforms. Our data are consistent with the hypothesis that the Ca\(^{2+}\) binding site of SPCA1 is similar to site II in SERCA1a, i.e. the site composed of side chains from Glu\(^{100}\), Asn\(^{396}\), and Asp\(^{100}\) which have counterparts, Glu\(^{308}\), Asn\(^{738}\), and Asp\(^{742}\), in SPCA1. Because the rate of Ca\(^{2+}\) dissociation in SPCA1 isoforms is comparable to that observed for SERCA1a, it seems likely that residue Asp\(^{742}\) in SPCA1 contributes with only one of its two oxygen ligands to the site, exactly like its counterpart (Asp\(^{100}\)) in SERCA1a.

Lower Turnover Rate—We showed here for the first time that the SPCA1 isoforms display much lower catalytic ATPase turnover rates relative to SERCA1a (cf. Fig. 2 and TABLE ONE). The increase in the catalytic turnover rate upon addition of the calcium ionophore A23187 demonstrated that the E\(_{1}\)~P(Ca) to E\(_{2}\)-P transition plays indeed a rate-

**DISCUSSION**

**FIGURE 10. Vanadate inhibition of phosphorylation.** Equilibration with vanadate was performed for 1 h at 25 °C in medium containing 40 mM MOPS/Tris pH 7.0, 80 mM KCl, 5 mM MgCl\(_{2}\), 2 mM EGTA, 0.1 mM thapsigargin, and the indicated concentrations of vanadate. Phosphorylation of SERCA1a was performed without thapsigargin. Following cooling for 15 min at 0 °C, phosphorylation was initiated by sequential addition of 2.5 mM CaCl\(_{2}\) and 5 mM [γ-32P]ATP, and acid quenching was performed 15 s later. The lines show the best fits of the Hill equation, giving the K\(_{0.5}\) values (μM ± S.E.) listed in TABLE TWO.
limiting role in SPCA1 enzymes (TABLE ONE), because this transition should be sensitive to the back inhibition exerted by Ca\(^{2+}\) accumulated inside the microosomal vesicles (in the millimolar concentration range). Even in the presence of the calcium ionophore, the maximal turnover rates of the ATPase activity for SPCA1 isoforms were 4.7–6.4-fold lower than that of SERCA1a (TABLE ONE). The lower overall rate of ATP hydrolysis in SPCA1 can be explained by the slow transition of \(E_1\sim(P(Ca))\) to \(E_2\)-P. It is interesting that both the rate of ATP hydrolysis and the rate of phosphoenzyme processing increased with the length of the alternatively spliced C terminus, *i.e.* in the order SPCA1a, SPCA1b, and SPCA1d.

Our rapid kinetic measurements indicated that the SPCA1 enzymes are characterized by decreased phosphorylation rates (TABLE TWO). However, the rate limitation of the overall ATPase reaction imposed by the latter effect is insignificant in comparison with that imposed by slowing of the subsequent \(E_1\sim(P(Ca))\) to \(E_2\)-P transition, because phosphorylation basically is one of the fastest steps of the cycle. Our examination of the ATP concentration dependence of the initial phosphorylation rate data showed that the reduced phosphorylation rate is a \(V_{\text{max}}\) effect rather than a consequence of reduced affinity for the substrate. Interestingly, the reverse transfer of the phosphoryl group from \(E_2\sim(P(Ca))\) to ADP was also slower in the SPCA1 isoforms compared with SERCA1a (Fig. 8B and TABLE THREE), indicating a difference with respect to catalytic ability in both directions.

Enhanced (pH- and K\(^{+}\)-independent) \(E_2\)-P Dephosphorylation—The functional properties displayed by SPCA1 enzymes when present in \(E_2\)-P conformation were also distinct from those of SERCA1a. Hence, the apparent affinities for reaction of \(E_2\) with inorganic phosphate and the phosphate analog vanadate were both found reduced (*K_d* increased) relative to SERCA1a (Figs. 9A and 10, and TABLE TWO). The kinetic studies showed that the rate of \(E_2\)-P dephosphorylation was markedly enhanced in SPCA1 isoforms, relative to SERCA1a, thus explaining the reduced apparent affinity for \(P_i\). It is conceivable that the same factors that are responsible for destabilization of \(E_2\)-P also destabilize the vanadate-bound \(E_2\) form. The acceleration of dephosphorylation was observed irrespective of the presence of K\(^{+}\) and pH, and, unlike SERCA1a, the SPCA1 isoforms showed little dependence of the \(E_2\)-P dephosphorylation on K\(^{+}\) and pH. The inhibition of \(E_2\)-P dephosphorylation seen for SERCA1a at alkaline pH may in part be ascribed to lack of binding of protons (to be countertransported) at the transport sites in their luminal facing configuration (38). Hence, it seems that the SPCA1 isoforms either bind the protons with an unusually high affinity compatible with activation at pH values as high as 8.5, or have no need for such proton binding for activation of \(E_2\)-P dephosphorylation. Such an effect might be a consequence of the lack in SPCA1 of residues equivalent to Glu\(^{771}\) and Glu\(^{908}\) at Ca\(^{2+}\) site I in SERCA1a, as these residues might be involved in the proton countertransport and/or its regulation in SERCA1a.

It is well known for SERCA1a that monovalent alkali metal ions, in particular K\(^{+}\), accelerate the \(E_2\)-P dephosphorylation rate (38, 39). X-ray crystallography of SERCA1a recently demonstrated a binding site for a K\(^{+}\) ion located in domain P (12, 40, 41). Mutational analysis showed that Glu\(^{732}\) (contributing to the binding site) is of major importance for the stimulatory effect of K\(^{+}\) (40). For SPCA1 isoforms, the experiments illustrated in Fig. 9, B and C clearly demonstrated that addition of K\(^{+}\) to the dephosphorylation medium did not increase the rate of \(E_2\)-P dephosphorylation. Interestingly, the SPCA1 residue at the position equivalent to Glu\(^{732}\) in SERCA1a is Asp\(^{674}\). Mutagenesis experiments with SERCA1a\(^{4}\) indicate that this subtle difference (only the length of the side chain differs between glutamate and aspartate) is sufficient to attenuate the K\(^{+}\) regulatory effect. It is furthermore interesting that Pro\(^{1099}\) of SERCA1a is substituted by Val\(^{1100}\) in SPCA1 isoforms. In SERCA1a, this proline residue appears critical for the formation of a loop (residues 710–715), which together with Glu\(^{1102}\) contributes to coordination of K\(^{+}\) via backbone carbonyls (40). Thus, it is possible that in SPCA1 isoforms K\(^{+}\) is not bound, or is bound only weakly relative to SERCA1a.

The constitutive high activation of \(E_2\)-P dephosphorylation in SPCA1 isoforms could be a necessary mechanistic consequence associated with the slowness of the \(E_1\sim(P(Ca))\) to \(E_2\)-P step, because the involved destabilization of the \(E_2\)-P conformation inevitably leads to an enhanced \(E_2\)-P dephosphorylation, thus precluding any regulation. This would be similar to certain SERCA1 mutants with alterations to residues in the linker regions connecting the cytoplasmic domains with the membrane, where a reduced rate of the \(E_1\sim(P(Ca))\) to \(E_2\)-P transition was found associated with enhanced \(E_2\)-P dephosphorylation (42, 43).

**Physiological Relevance**—The biochemical properties of the SPCA1 pumps revealed in the present study appear to be compatible with its function in the secretory pathway. A high Ca\(^{2+}\) transport rate is not actually required in the SPCA1-containing Golgi compartment, which appears to be insensitive to inositol trisphosphate-generating agonists and is depleted of Ca\(^{2+}\) only by the probably slower process of downstream trafficking of Ca\(^{2+}\)-rich vesicles. In these non-releasable Ca\(^{2+}\) stores, a high affinity and low turnover rate pump such as SPCA1 can be expected to render the adjustment of the luminal Ca\(^{2+}\) level less dependent on the cytosolic Ca\(^{2+}\) concentration, mainly by limiting the fluctuation of pumping activity during cytosolic Ca\(^{2+}\) transients. In contrast, the function of SERCA2b in the endoplasmic reticulum and SERCA1a in the sarcoplasmic reticulum appears to be rather well adapted to the faster Ca\(^{2+}\) replenishment of releasable Ca\(^{2+}\) stores and the decline of cytosolic Ca\(^{2+}\) transients after cell stimulation.

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