Effects of Inhibitors on Luminal Opening of Ca\textsuperscript{2+} Binding Sites in an E2P-like Complex of Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase with Be\textsuperscript{2+}-fluoride\textsuperscript{*S}

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We document here the intrinsic fluorescence and \textsuperscript{45}Ca\textsuperscript{2+} binding properties of putative “E2P-related” complexes of Ca\textsuperscript{2+}-free ATPase with fluoride, formed in the presence of magnesium, aluminum, or beryllium. Intrinsic fluorescence measurements suggest that in the absence of inhibitors, the ATPase complex with beryllium fluoride (but not those with magnesium or aluminum fluoride) does constitute an appropriate analog of the “ADP-insensitive” phosphorylated form of Ca\textsuperscript{2+}-ATPase, the so-called “E2P” state. \textsuperscript{45}Ca\textsuperscript{2+} binding measurements, performed in the presence of 100 mM KCl, 5 mM Mg\textsuperscript{2+}, and 20% Me\textsubscript{2}SO at pH 8, demonstrate that this ATPase complex with beryllium fluoride (but again not those with magnesium or aluminum fluoride) has its \textsuperscript{45}Ca\textsuperscript{2+} binding sites accessible for rapid, low affinity (submillimolar) binding of \textsuperscript{45}Ca\textsuperscript{2+} from the luminal side of SR. In addition, we specifically demonstrate that in this E2P-like form of ATPase, the presence of thapsigargin, 2,5-di-

dihydroxybenzene, or cyclopiazonic acid prevents \textsuperscript{45}Ca\textsuperscript{2+} binding (i.e. presumably prevents opening of the \textsuperscript{45}Ca\textsuperscript{2+} binding sites on the SR luminal side). Since crystals of E2P-related forms of ATPase have up to now been described in the presence of thapsigargin only, these results suggest that crystallizing an inhibitor-free E2P-like form of ATPase (like its complex with beryllium fluoride) would be highly desirable, to unambiguously confirm previous predictions about the exit pathway from the ATPase transmembrane Ca\textsuperscript{2+} binding sites to the SR luminal medium.

Sarcoplasmic reticulum (SR)\textsuperscript{2} Ca\textsuperscript{2+}-ATPase (SERCA1a) is an ion pump, belonging to the family of P-type ATPases and responsible in muscle cells for active transport of Ca\textsuperscript{2+} from the cytosol into the sarcoplasmic reticulum lumen. It takes up Ca\textsuperscript{2+} ions from an aqueous compartment (the cytosol) where the concentration of these ions is low and releases them on the other side of the membrane into a compartment where the concentration of Ca\textsuperscript{2+} is already high. This requires that at some step during the catalytic cycle of ATP hydrolysis (which provides the required energy), the pump’s affinity for Ca\textsuperscript{2+} changes from high affinity to low affinity, coupled with topological reorientation of the binding sites from one side of the membrane to the other. SR Ca\textsuperscript{2+}-ATPase is generally described as having high affinity for cytosolic Ca\textsuperscript{2+} in its so-called “E” or “E1” conformation and low affinity for luminal Ca\textsuperscript{2+} in its phosphorylated so-called “EP” or “E2P” conformation (e.g. see Refs. 1–6 for a review).

Although high affinity binding of Ca\textsuperscript{2+} to the E1 state of Ca\textsuperscript{2+}-ATPase has been extensively documented, low affinity binding of Ca\textsuperscript{2+} to the ATPase E2P state has in most cases been deduced from indirect experiments only, because of the difficulties inherent in directly measuring low affinity \textsuperscript{45}Ca\textsuperscript{2+} binding to a species that, in addition, is not necessarily stable; only one attempt for such a direct measurement was described recently (7). Nevertheless, for complete description of the complete transport process, availability of a robust assay for characterizing the sites of Ca\textsuperscript{2+} release from phosphorylated ATPase toward the SR lumen would be highly desirable.

Meanwhile, thanks to the recent crystallization of several forms of SR Ca\textsuperscript{2+}-ATPase, elucidation of the structural basis for ATP-driven ion pumping has made significant steps forward. Detergent-solubilized SR Ca\textsuperscript{2+}-ATPase has been crystallized under various forms, with the hope of understanding the structural changes occurring sequentially during its catalytic cycle (8–14). However, in none of these forms, including the Ca\textsuperscript{2+}-free fluoride forms once thought to be related to E2P, was the exit pathway from the transport sites toward the luminal medium found fully open (13, 14), and only hints concerning this exit pathway could be proposed (13).

Moreover, it should be recalled that detergent-solubilized Ca\textsuperscript{2+}-ATPase is highly unstable in the absence of Ca\textsuperscript{2+}. Thus, to avoid Ca\textsuperscript{2+}-ATPase irreversible denaturation on the time-scale of three-dimensional crystallization experiments, the crystals grown in the absence of Ca\textsuperscript{2+}, including those for Ca\textsuperscript{2+}-free fluoride forms (9, 13–14), have up to now been prepared in the presence of thapsigargin (TG). Thapsigargin is a strong inhibitor of the protein activity (15, 16), which binds with very high affinity to one of the functionally relevant conformations of Ca\textsuperscript{2+}-ATPase, the “E2” conformation that the ATPase specifically adopts in the absence of Ca\textsuperscript{2+} (e.g. see Refs. 17 and 18). In this conformation, it binds in a cleft located between transmembrane segments 3, 5, and 7 (9, 19, 20), and it probably glues these segments together, resulting in ATPase protection against denaturation (21). Very recently, it was found that the presence, in addition to TG, of 2,5-di-tert-butyl-1,4-
dihydroxybenzene (BHQ; an inhibitor of Ca\textsuperscript{2+}-ATPase also thought to be specific for E2P) improved the quality of “E2P” crystals (22). It is fair to ask whether the presence of TG or other inhibitors could have biased
the conclusions derived from crystals that failed to reveal transport sites open toward the lumen.

Previous experimental results do not clearly suggest one answer or another. On the one hand, proteolysis experiments previously suggested that the presence of TG affects only little the overall ATPase conformation in its cytosolic region; for instance, the almost complete protection against proteolytic cleavage afforded by binding of vanadate or metal-fluoride complexes to the ATPase was observed to occur both in the presence and absence of TG (23). Vanadate and fluoride are both thought to allow formation of complexes that are more or less closely related analogs of the ADP-insensitive phosphorylated state of the enzyme or of the transition state for dephosphorylation; they are here dubbed “E2P-related” states, in a loose sense. TG also did not greatly affect the superfluorescent state of TNP-AMP bound to the ATPase complex with beryllium fluoride (24). On the other hand, it was previously shown that TG altered the affinity of Ca\(^{2+}\)-free ATPase for ATP (16), and it was suggested that the interaction of ATPase with thapsigargin (or BHQ) might result in formation of modified E2 species (25–27). More specifically, TG was also shown to induce pretty large structural changes in two-dimensional crystals of the E2P-related E2-V0\(_{4}\) species (28), and TG was suspected from Trp fluorescence experiments (but with no direct evidence) to close, in E2P-related states, the postulated Ca\(^{2+}\) release pathway from the ATPase sites toward the SR lumen (24).

Relevant to these issues, it should also be mentioned that the so-called “E2P-related” complexes of Ca\(^{2+}\)-free ATPase with fluoride described so far did not all appear to be equally close to the “true” E2P form; all fluoride forms tested were inactive, but reactivation by exposure to a high luminal Ca\(^{2+}\) concentration was faster for the ATPase complex with beryllium fluoride than for those with aluminum fluoride or magnesium fluoride. Thus, the ATPase complex with beryllium fluoride was suggested to have its transport sites most widely open to luminal Ca\(^{2+}\); for those with aluminum fluoride or magnesium fluoride, respectively. Consequently, the ATPase complex with beryllium fluoride was less affected by the slower reactivation was due to reduced stability of the ATPase-fluoride complex in the presence of beryllium, compared with the two other metal ions.

Here, to address these questions, critical ones indeed for the sensible interpretation of presently available or future x-ray structures of Ca\(^{2+}\)-free ATPase forms, we establish a robust assay of 45Ca\(^{2+}\) binding to the luminal sites of Ca\(^{2+}\)-ATPase forms where these sites appear, which gives results somewhat different from those previously reported by Vieyra et al. (7). Simultaneously, we study the effect of TG and other inhibitors on this binding. The properties of Ca\(^{2+}\)-free ATPase-fluoride complexes formed in the presence of magnesium, aluminum, or beryllium and in the absence or presence of TG or other inhibitors (namely BHQ or cyclopiazonic acid (CPA)) are documented here. Intrinsic fluorescence measurements and 45Ca\(^{2+}\) binding measurements both demonstrate that in the absence of inhibitors, the ATPase complex with beryllium fluoride (but not that with magnesium or aluminum fluoride) constitutes an appropriate analog of the E2P state of Ca\(^{2+}\)-ATPase, with its Ca\(^{2+}\) binding sites readily accessible from the SR lumen. In addition, we specifically demonstrate that in this E2P-like form of ATPase, the presence of TG, BHQ, or CPA prevents binding of 45Ca\(^{2+}\) (i.e. presumably prevents opening of the luminally oriented 45Ca\(^{2+}\) binding sites).

As an unfortunate result of our study, there is therefore little hope that crystals grown in the presence of any of these inhibitors could reveal in an open form the pathway of Ca\(^{2+}\) ions from their binding sites (within the bundle of ATPase transmembrane segments) toward the SR lumen (where these ions are released as a result of their transport). On the more positive side, our results establish a new tool for monitoring low affinity binding of Ca\(^{2+}\) to the luminally oriented low affinity sites of E2P-like forms of Ca\(^{2+}\)-ATPase. They simultaneously suggest that crystallizing an inhibitor-free E2-BPF\(_4\) complex would indeed be of utmost interest, to confirm previous predictions about the Ca\(^{2+}\) internalization pathway from transmembrane binding sites in Ca\(^{2+}\)-ATPase toward the SR luminal medium (13).

**EXPERIMENTAL PROCEDURES**

**Membrane Preparation and Stock Solutions for Chemicals**—SR membrane vesicles were prepared from rabbits as previously described (29), after 2-day fasting of the animals and inclusion of 1 \(\mu\)g/ml a-amylose in the initial homogenization buffer (to avoid SR contamination with muscle phosphorylase; see, for example, Ref. 30). Potassium fluoride (stock solution was 1 \(\mu\)l in water) was from ICN (catalog no. 156339), TG (stock solution was 1 mg/ml in Me$_2$SO; i.e. about 1.5 mM) was from Calbiochem (catalog no. 586005), CPA (stock solution was 10 mg/ml in Me$_2$SO; i.e. about 30 mM) was from Sigma (catalog no. C1530), BHQ (stock solution was 22 mg/ml in Me$_2$SO; i.e. 100 mM) was from Aldrich (catalog no. 11,297-6), BeCl$_2$ (stock solution was 991 \(\mu\)g/ml in 1% HCl; i.e. about 110 mM in water) was Aldrich (catalog no. 20,498-9), and AlCl$_3$ (stock solution was 100 mM) was from Sigma (catalog no. A-3017). Proteinase K (stock suspension was 1.5 mg/ml) was from Roche Applied Science (catalog no. 745723).

**Formation of ATPase-Fluoride Complexes**—Reaction of ATPase with fluoride in the presence of various metals was performed in buffer A (100 mM KCl, 5 mM Mg\(^{2+}\), and 50 mM Mops-Tris at pH 7 and 20 °C) supplemented with 250 \(\mu\)M EGTA, in the absence of Me$_2$SO for Trp fluorescence measurements or in the presence of 20% Me$_2$SO (v/v) for 45Ca\(^{2+}\) binding measurements; 1 mM KF was added, either alone (i.e. simply with Mg\(^{2+}\)) or together with 50 \(\mu\)M BeCl$_2$ or 50 \(\mu\)M AlCl$_3$ (and Mg\(^{2+}\)), present from the start. The ATPase complex was then analyzed, in the absence of fluoride, SR was present at 0.1 mg of protein/ml from the start; when 45Ca\(^{2+}\) binding was to be measured after preincubation, SR was generally added to the complete fluoride-containing reaction medium, at either 0.3 or 0.4 mg of protein/ml. To measure residual ATPase activity, aliquots were taken after preincubation for various periods and diluted into a standard ATPase assay medium containing 0.5 mg/ml octaethylene glycol monododecyl ether.

**Intrinsic Fluorescence Measurements**—SR membrane vesicles were suspended at 0.1 mg/ml in the thermostatted and continuously stirred cuvette of a SPEX Fluorolog fluorometer (e.g. see Ref. 31), and Trp fluorescence was monitored using an excitation wavelength of 295 nm and an emission wavelength of either 315, 330, or 355 nm. Bandwidths were 2 and 10 nm, respectively.

**45Ca\(^{2+}\) Binding Measurements**—45Ca\(^{2+}\) binding to Ca\(^{2+}\)-ATPase was measured as previously described (32), using nitrocellulose Millipore filters to adsorb the ATPase membranes and including [H]glucose in the 45Ca\(^{2+}\)-containing buffer to serve as a marker of the amount of fluid trapped in the filter (and therefore of the amount of nonbound 45Ca\(^{2+}\), trapped in the filter together with bound 45Ca\(^{2+}\)). The buffer for 45Ca\(^{2+}\) binding generally contained 100 mM KCl, 5 mM MgCl$_2$, 100 mM Tes-Tris (at pH 8 and 20 °C), 20% (v/v) Me$_2$SO, [H]glucose, and 45Ca\(^{2+}\), as well as, in some cases, a small concentration of residual EGTA from the preincubation medium (for instance, in Fig. 4 experiments, 225 \(\mu\)M total 45Ca\(^{2+}\) and 25 \(\mu\)M residual EGTA; i.e. a free Ca\(^{2+}\) concentration of 200 \(\mu\)M). Tricine was avoided as a buffer, because Tricine binds Ca\(^{2+}\) with a significant affinity under our conditions (data not shown; see also Ref. 33). Note that “nonspecific” sites (with respect to Ca\(^{2+}\)-ATPase), including those inside the SR compartment if the
vesicles are made leaky (e.g. calsequestrin), also contribute to the measured amount of bound $^{45}\text{Ca}^{2+}$, especially at pH 8; this was minimized by working at 5 mM MgCl$_2$ and in the presence of 100 mM KCl. Experiments in which 0.3 or 0.6 mg of SR protein was to be retained by the filter were performed with Millipore HA or GS filters, respectively. In the latter case, the smaller pore diameter (0.22 μm instead of 0.45 μm) results in a larger surface available for adsorption of SR membranes. Filters were not rinsed, and they were counted for both $^3\text{H}$ and $^{45}\text{Ca}^{2+}$ radioactivity.

RESULTS

Background—As already mentioned in the Introduction, one of the ATPase intermediate forms during its catalytic cycle must have its $\text{Ca}^{2+}$ binding sites opened toward the luminal side and simultaneously endowed with poor affinity for $\text{Ca}^{2+}$, to permit $\text{Ca}^{2+}$ release into an SR lumen already containing a high concentration of free $\text{Ca}^{2+}$ (and therefore active transport of $\text{Ca}^{2+}$). This ATPase form is usually described as the ATPase “E2P” form, and a major aim of the present work was to measure binding of $^{45}\text{Ca}^{2+}$ to such a form.

A first prerequisite for such measurement is availability of a membrane preparation in which the SR luminal side is made accessible to $^{45}\text{Ca}^{2+}$ added to the outer medium. For this purpose, we used a $\text{Ca}^{2+}$ ionophore (calcymicin, A23187) in most of our experiments (but not in all of them, to make it possible to discriminate between binding sites accessible from the cytosolic side and binding sites accessible from the luminal side).

A second prerequisite is the availability of a tool allowing discrimination between binding of $^{45}\text{Ca}^{2+}$ to specific sites on the ATPase and “nonspecific” binding to $^{45}\text{Ca}^{2+}$ to other sites in the SR preparation. We chose to perform experiments in the absence or presence of TG, as TG is known, at least as concerns $\text{Ca}^{2+}$, to block or dramatically slow down $\text{Ca}^{2+}$ binding to the ATPase high affinity cytosolic sites (15–18, 25).

Third, demonstrating $\text{Ca}^{2+}$ binding to E2P is made difficult both by the transient nature of the E2P form and by its above-mentioned poor affinity for $\text{Ca}^{2+}$. Thus, we chose to increase the stability of the E2P form by including Me$_2$SO in the solution (34) and to increase its affinity for $\text{Ca}^{2+}$ by making the pH in the solution more alkaline (35). A recent report already made use of such conditions to try and measure the binding of $\text{Ca}^{2+}$ to E2P (7). In our hands, however, even such conditions were not sufficient to make the experiments reliable, as judged from dephosphorylation experiments (see below).

Thus, in addition to the above general conditions, but instead of using a genuine E2P form, we chose to use more stable ATPase forms, namely ATPase complexes with fluoride, which are thought to be potential E2P analogs and which can be formed in the absence of $\text{Ca}^{2+}$ but in the presence of various other metals: either magnesium alone or, in addition, aluminum or beryllium (these complexes will be referred to below as E2-MgF$_4$, E2-AlF$_4$, and E2-BeF$_4$).

A few properties of these complexes have already been described, and two of them (E2-MgF$_4$, E2-AlF$_4$) have in fact already been crystallized (13–14, 24, 36–40). According to previous indirect evidence, it is in the fluoride complex formed in the presence of beryllium, E2-BeF$_4$, that the ATPase conformation is most similar to that of the E2P form and that its $\text{Ca}^{2+}$ sites are most accessible from the luminal medium, as judged, in particular, from the highest efficiency of luminal $\text{Ca}^{2+}$ for reversing the inhibition of this particular complex (24).

Trp Fluorescence Properties of Complexes of $\text{Ca}^{2+}$-free ATPase with Fluoride (Especially E2-BeF$_4$) in the Absence or Presence of TG or BHQ—To start with, the conformational properties of the various ATPase complexes with fluoride were examined with intrinsic fluorescence. It had been shown previously that the drop in intrinsic fluorescence typically observed upon binding of TG to $\text{Ca}^{2+}$-free ATPase (17) was significantly larger for E2P or the E2-BeF$_4$ complexes than for either E2 or the other ATPase-fluoride forms (24). Here, this point was investigated further.

Fig. 1A first confirms the finding in Ref. 38 that the addition of fluorooraluminate to $\text{Ca}^{2+}$-free ATPase, leading to formation of an E2-AlF$_4$ species, only induces a slow decrease in the overall fluorescence intensity of the ATPase and the finding in Ref. 24 that the subsequent addition of TG quenches the fluorescence of this species only moderately, as initially described for E2 (17). Fig. 1B also confirms that the same addition of TG to E2-BeF$_4$ induces a much larger fluorescence decrease, as previously described (24).

But in addition, Fig. 1B reveals that formation of E2-BeF$_4$ itself results in significant Trp fluorescence enhancement, at a rate consistent with the rate of inhibition of ATPase under identical conditions (half-time of about 2 min in the absence of Me$_2$SO; data not shown). After the formation of this E2-BeF$_4$ complex, ATPase fluorescence was no longer sensitive to the addition of cytosolic $\text{Ca}^{2+}$, as expected (see supplemental Fig. S1B). The fact that beryllium fluoride enhances ATPase fluorescence in the absence of $\text{Ca}^{2+}$ contrasts with the fact that in the presence of $\text{Ca}^{2+}$ and ADP, no such rise is detectable (see supplemental Fig. S1D), again despite the fact that aluminum fluoride, as previously described (31), now triggers formation of an E1-ADP-AlF$_4$ form of high fluorescence (supplemental Fig. S1C). The rise in fluorescence observed upon the formation of E2-BeF$_4$ (Fig. 1B) was only observed in the presence of...
Mg\(^{2+}\) (data not shown), as expected from the Mg\(^{2+}\)-dependence of ATPase inhibition in the presence of beryllium and fluoride (37) (data not shown). Note that a rise in ATPase intrinsic fluorescence is also observed upon formation of the E2P (29, 41).

Remarkably enough, this rise in Trp fluorescence was converted into a fluorescence drop in the presence of A23187 (calcimycin), as used as an hydrophobic fluorescence quencher (Fig. 1D), whereas the large drop observed upon the addition of TG was wiped off. The drop in Trp fluorescence observed in the presence of A23187 upon formation of the E2-BeF\(_x\) complex is reminiscent of the fact that a drop in the presence of A23187 or other hydrophobic quenchers was also observed upon formation of the genuine E2P phosphoenzyme (42, 43). Thus, both in the absence and presence of quencher, these fluorescence results support the previous claim by Danko et al. (24) that the E2-BeF\(_x\) complex (but not the E2-AlF\(_x\) complex) is a fair analog of genuine E2P.

We also tried to add beryllium fluoride to Ca\(^{2+}\)-free ATPase previously cleaved at Thr\(^{242}\)–Glu\(^{243}\) by proteinase K, under conditions resulting in a fairly homogeneous \({ }^\text{p28N-p83C}\) complex, which is no longer phosphorylatable by P\(_i\), although it remains phosphorylatable by ATP (44). Fluorescence experiments with such a \({ }^\text{p28N-p83C}\) complex showed that the cleaved ATPase was not able to form a high fluorescence E2-BeF\(_x\) complex, although it did form the Ca\(_{2+}\)E1-ADP-AlF\(_x\) complex (data not shown). This again supports a close resemblance between E2-BeF\(_x\) and genuine E2P.

Interestingly, a second inhibitor, BHQ, also thought to react specifically with E2 forms of Ca\(^{2+}\)-ATPase, behaves like TG as regards its effect on ATPase intrinsic fluorescence (Fig. 2). BHQ has already been shown to quench the fluorescence of the E2 form of ATPase (27). We found that, as for TG, this quenching is larger for BHQ binding to E2-BeFx, than for its binding to E2-AlFx (with binding to E2 being intermediate) and only minimal after the addition of BHQ to E1 forms (supplemental Fig. S2). Moreover, both for BHQ and for TG, this quenching depends on the wavelength used for observing fluorescence, being largest at the shortest emission wavelengths (Fig. 2).

It was already known that going to the blue edge of Trp fluorescence (315 nm) increases the amplitude of the fluorescence rise induced by Mg\(^{2+}\) addition, whereas going to the red edge (355 nm) results in a signal of opposite sign for Mg\(^{2+}\) (45), and this was confirmed here. As a corollary, we found that the E2-BeF\(_x\) signal itself is even more prominent at 355 nm (Fig. 2, C and F). These data therefore provide spectroscopic characterization of those Trp residues that respond to E2-BeF\(_x\) formation or TG binding. In contrast, parallel experiments did not reveal any clear dependence on observation wavelength of the signal associated with formation of the Ca\(_{2+}\)E1-ADP-AlF\(_x\) complex (data not shown).

**Measurement of Rapid Binding of \(^{45}\)Ca\(^{2+}\) to E2-BeF\(_x\), an E2P-like Form of Ca\(^{2+}\)-ATPase**—We then performed \(^{45}\)Ca\(^{2+}\) binding experiments (Fig. 3A). For these experiments, Ca\(^{2+}\)-free SR vesicles made leaky by ionophore A23187 were preincubated with fluoride and beryllium (here in the presence of 20% Me\(_2\)SO), leading to formation of the E2-BeF\(_x\) species (and inhibition of ATPase activity) (e.g. see supplemental Fig. S3). Control vesicles were prepared similarly but in the absence of beryllium fluoride (i.e. in the E2 form). Then, preincubated samples were loaded on a nitrocellulose filter and immediately perfused for various periods manually, with a \(^{45}\)Ca\(^{2+}\)-containing binding buffer at pH 8 and in the continued presence of 20% Me\(_2\)SO. The final free \(^{45}\)Ca\(^{2+}\) concentration in the perfusion buffer was 200 nM, to render possible detection of \(^{45}\)Ca\(^{2+}\) binding with relatively poor affinity. Filters were counted without washing. In some cases, preincubated samples had been supplemented with TG immediately before loading onto the filter and \(^{45}\)Ca\(^{2+}\) perfusion.

Starting with measurements performed with ATPase initially in the E2 form (circles), Fig. 3A shows that the presence of TG together with E2 reduced the amount of \(^{45}\)Ca\(^{2+}\) bound on the filter by about 10 nmoI of \(^{45}\)Ca\(^{2+}\)/mg of protein, from 18–19 nmoI/mg in the absence of TG (open circles) to 8–9 nmoI/mg in its presence (closed circles). Since TG prevents rapid \(^{45}\)Ca\(^{2+}\) binding to ATPase in the E2 state (17), this difference (i.e. the TG-sensitive fraction of \(^{45}\)Ca\(^{2+}\) binding, 10 nmoI/mg) is to be attributed to \(^{45}\)Ca\(^{2+}\) binding to the ATPase, and this is indeed the level expected for binding with a stoichiometry of two binding sites per ATPase monomer (e.g. Refs. 32 and 46). The open circles were labeled E2 in A because the ATPase initially was in its E2 form, but in this case the ATPase of course adopts its Ca\(_{2+}\)E1 state after Ca\(^{2+}\) binding, with Ca\(^{2+}\) bound to the classical high affinity sites on the ATPase cytosolic side.

The residual 8–9 nmoI/mg \(^{45}\)Ca\(^{2+}\) bound in the presence of TG (closed circles in Fig. 1A) must be attributed to binding to other sites, "nonspecific" with respect to Ca\(^{2+}\)-ATPase, probably mostly (see below) Ca\(^{2+}\) binding sites inside the SR lumen, like calsequestrin, made...
This fast binding also fits with what can be expected for binding to sites confirming that the added ionophore allows fast access to the SR lumen. Various preincubation media, at 0.3 mg/ml protein and here in the additional presence of Ca\(^{2+}\) (ATPase in its E2 form); inverted triangles correspond to experiments where this medium contained 1 mM Kf and 50 \(\mu\)M BeCl\(_2\) (ATPase was converted to its E2BeF\(_2\), form). In B, squares correspond to experiments where this medium contained 1 mM (open squares) or 3 mM (dotted squares) F\(_2\) (ATPase in its E2 form); inverted triangles correspond to experiments where this medium contained 1 mM vanadate, either orthovanadate (open inverted triangles) or decavanadate (dotted open inverted triangles). At the end of this preincubation period (which, when fluoride was present, was more than sufficient to lead to complete inactivation; see supplemental Fig. S3), 2-ml aliquots of the preincubated samples (i.e. 0.6 mg of protein) were loaded onto Millipore GS filters and filtered for various periods manually (i.e. with variable volumes of perfusion medium), with a perfusion (and \(45\)Ca\(^{2+}\) binding) medium containing 200 \(\mu\)M \(45\)Ca\(^{2+}\), 200 \(\mu\)M \(^{3}H\)glucose, 100 mM KCl, 5 mM Mg\(^{2+}\), 200 \(\mu\)M Mg\(^{2+}\), and 100 mM Tes-Tris pH 8 and 20 °C. The filter was counted after washing, and bound \(45\)Ca\(^{2+}\) was obtained (thanks to the TH counts) by subtracting \(45\)Ca\(^{2+}\) trapped together with the washing fluid from the total \(45\)Ca\(^{2+}\) on the filter. For closed symbols in A, TG (at 0.03 mg/ml) was added to the preincubated samples immediately before loading them onto the filter and perfusing them with \(45\)Ca\(^{2+}\)-sp. (specific) and non-sp. (nonspecific) refer to binding of Ca\(^{2+}\) to Ca\(^{2+}\)-ATPase and to other binding sites, respectively.

accessible by the presence of ionophore (note that under these conditions, binding to ionophore itself is hardly detectable), or perhaps sites on the external surface of the vesicles, which can bind Ca\(^{2+}\) at the alkaline pH used here (pH 8). This residual binding of \(45\)Ca\(^{2+}\) to permeabilized SR vesicles with ATPase in its E2-TG state was relatively fast, confirming that the added ionophore allows fast access to the SR lumen. This fast binding also fits with what can be expected for binding to sites of poor affinity. Since it has been suggested that TG does not fully block Ca\(^{2+}\) binding to Ca\(^{2+}\)-ATPase but only reduces the ATPase affinity for Ca\(^{2+}\) (18, 25), it might be argued that part of the bound \(45\)Ca\(^{2+}\) measured in the presence of TG might reside on the Ca\(^{2+}\)-ATPase. However, the dramatic slowing down, over minutes, of the kinetics of \(45\)Ca\(^{2+}\) binding in the presence of TG simultaneously reported by the same authors (18, 25) makes that alternative interpretation of our results unlikely, since in our experiments \(45\)Ca\(^{2+}\) binding in the presence of TG was already complete after 2–3 s (more also below).

In the absence of TG, binding of \(45\)Ca\(^{2+}\) to ATPase initially in its E2-BeF\(_2\) state (Fig. 3A, open triangles) was lower than binding to E2 (open circles) but quite significant. This reduced binding is consistent with low affinity binding to E2-BeF\(_2\) (see below). Per se, it also proves that binding of \(45\)Ca\(^{2+}\) did not kick beryllium and fluoride out of the ATPase catalytic site within the time period (60 s) of these relatively rapid binding measurements, despite the fact that perfusion washed away fluoride or beryllium from the filter. This binding of \(45\)Ca\(^{2+}\) to E2-BeF\(_2\) was completed within a few seconds too, which again fits with what can be expected for binding to sites of poor affinity but disagrees with the putative very slow binding of \(45\)Ca\(^{2+}\) to E2 recently suggested in Ref. 7.

Importantly, in the presence of TG, binding of \(45\)Ca\(^{2+}\) to ATPase initially in its (E2-BeF\(_2\) + TG) state (Fig. 3A, closed triangles) dropped to the same low level as when ATPase was in its E2-TG state. This shows that the low affinity Ca\(^{2+}\) binding sites present in E2-BeF\(_2\) are no longer available in the presence of TG (see also subsequent figures below).

Fig. 3B shows an experiment similar to that in Fig. 3A, except that SR vesicles had now been preincubated under the same conditions either with 1 or 3 mM inorganic phosphate (open or dotted squares, respectively), leading to very significant ATPase phosphorylation to E2P (as checked in standard phosphorylation assays with \(^{32}\)P-iP, data not shown), or with 1 mM vanadate, leading to formation of E2\(\Delta\)VO\(_{4}\) and E2dVO\(_{4}\) states (ortho- or decavanadate, open or dotted inverted triangles, respectively). Binding of \(45\)Ca\(^{2+}\) to the initially E2-VO\(_{4}\) forms was...
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time-dependent over this 60-s period of time, which we interpret as revealing Ca\(^{2+}\)-induced destabilization of E2-VO\(_4\) sites within this period; this was confirmed by measuring the rate of Ca\(^{2+}\)-induced recovery from ATPase inhibition under similar conditions (supplementary Fig. 5A).

Binding of \(^{45}\)Ca\(^{2+}\) to ATPase initially in its E2P form (Fig. 3B, squares) almost immediately reached the same level as the one found after binding of \(^{45}\)Ca\(^{2+}\) to E2 (Fig. 3A, circles); however, classical dephosphorylation assays (not shown) showed that this was mainly due to the fact that 20% Me\(_2\)SO (in the presence of 100 mM KCl) does not slow down E2P dephosphorylation sufficiently. Thus, under our experimental conditions, only the E2-BeFx form (Fig. 3A) was stable enough to make analysis of the low affinity \(^{45}\)Ca\(^{2+}\) binding curves significant.

**Luminal Binding of \(^{45}\)Ca\(^{2+}\) to E2-BeFx, and Lack of Binding to Other E2-Fluoride Forms**—To discriminate between binding sites accessible from the cytosolic side and binding sites accessible from the luminal side, we repeated similar measurements in the absence or presence of the Ca\(^{2+}\) ionophore A23187 (Fig. 4). These experiments were performed over a moderately longer time scale (up to 4 min) and with a slightly different protocol, to see how robust the previous results were. Here, intact SR vesicles, therefore mostly tight (however, see below), were first incubated (at a protein concentration 10-fold larger than previously) either in the absence of fluoride (A and D), or in its presence together with magnesium and beryllium (B and E) or together with magnesium alone (C and F); they were subsequently diluted 10-fold into a \(^{1}\)H][sucrose- and \(^{45}\)Ca\(^{2+}\)-containing medium, and \(^{45}\)Ca\(^{2+}\) binding was allowed to occur during various periods of incubation. Some of these samples had been supplemented with ionophore and/or TG immediately before dilution into the \(^{45}\)Ca\(^{2+}\) binding medium. Only after dilution and \(^{45}\)Ca\(^{2+}\) binding were the samples loaded onto nitrocellulose filters, for subsequent double radioactivity counting of the filter without washing. The final free \(^{45}\)Ca\(^{2+}\) concentration during \(^{45}\)Ca\(^{2+}\) binding (after dilution) was kept at 200 \(\mu\)M, taking into account the residual EGTA from the preincubation medium.

Fig. 4, A–C, first shows results obtained with this new protocol when vesicles had been made leaky with ionophore, as previously; results identical to the previous ones were obtained for ATPase initially in its E2 state (Fig. 4D) or in its E2-BeFx, state (Fig. 4B). In the absence of TG, a lower amount of bound \(^{45}\)Ca\(^{2+}\) was again found for ATPase initially in the E2-BeFx state (Fig. 4B, open triangles) than for ATPase initially in the E2 state (Fig. 4A, open circles), whereas in the presence of TG, the amount found was again similar in both cases (Fig. 4, A and B, closed symbols). The TG-sensitive fraction of bound \(^{45}\)Ca\(^{2+}\), although not very large, was definitely significant, considering the range of possible errors in the various subtractions (data corresponding to three independent experiments all fall on the same line, as will be shown in Fig. 6). The very fact that \(^{45}\)Ca\(^{2+}\) binding to E2-BeFx remained fairly stable again ensures that CA\(^{2+}\)-dependent destabilization of this fluoride form was not too much of a concern in these experiments over a few minutes at 200 \(\mu\)M free Ca\(^{2+}\) (see below for further discussion of the effect of Ca\(^{2+}\) concentration on this destabilization).

When similar experiments in the presence of ionophore were repeated with ATPase initially in the E2-MgF\(_4\) state (Fig. 6C), the measured amount of bound \(^{45}\)Ca\(^{2+}\) was found to be hardly larger in the absence of TG than in its presence (Fig. 6C, open and closed diamonds, respectively). TG-resistant \(^{45}\)Ca\(^{2+}\) binding was identical to that in A and B, considered to represent binding to non-ATPase sites. These results are nicely consistent with the previous suggestion that luminal Ca\(^{2+}\) sites are much less accessible in E2-MgF\(_4\) than in E2-BeFx, (24), and in fact they prove that the much slower recovery from inhibition observed in that paper for E2-MgF\(_4\) (compared with E2-BeFx) was not due to greater stability of this complex with fluoride but was indeed due to poorer exposure of its Ca\(^{2+}\) binding sites.

Fig. 4, D–F, then shows results obtained in the absence of ionophore. In the presence of TG (closed symbols), \(^{45}\)Ca\(^{2+}\) binding was in all cases much lower than in the presence of ionophore, supporting the previous interpretation that TG-resistant binding to SR vesicles represents \(^{45}\)Ca\(^{2+}\) binding to sites different from those on Ca\(^{2+}\)-ATPase and residing mainly inside the SR lumen. In the absence of TG, \(^{45}\)Ca\(^{2+}\) binding to ATPase initially in its E2 state was again about 10 nmol/mg higher than in the presence of TG (compare open and closed circles in Fig. 4D), and this difference, the TG-sensitive fraction of bound \(^{45}\)Ca\(^{2+}\), again represents the amount of \(^{45}\)Ca\(^{2+}\) rapidly and specifically bound to the cytosolically accessible high affinity Ca\(^{2+}\) binding sites on the ATPase (32). \(^{45}\)Ca\(^{2+}\) binding to ATPase initially in its E2-MgF\(_4\) state was again hardly different from that in the presence of TG (Fig. 4F), and the TG-sensitive fraction of \(^{45}\)Ca\(^{2+}\) bound to E2-BeFx, (i.e. the difference between open and closed triangles) was definitely smaller in the presence of ionophore (Fig. 4E) than in its presence (Fig. 4B), demonstrating that Ca\(^{2+}\) binds to sites on E2-BeFx, mostly from the luminal side, as previously suggested.

It is fair to recognize that this TG-sensitive fraction of \(^{45}\)Ca\(^{2+}\) bound to E2-BeFx, although smaller in the absence of ionophore than in its presence, was not exactly zero in the absence of ionophore (Fig. 4E). Similarly, Ca\(^{2+}\) was previously found to partially stimulate ATPase recovery in the absence of ionophore (24). These somewhat puzzling observations do not ruin the above claim of a luminal orientation for the Ca\(^{2+}\) binding sites in E2-BeFx. We think they simply reflect the presence, in most SR vesicle preparations (including ours), of a fraction of vesicles that are not completely tight, for instance because of imperfect SR rescaling during muscle homogenization (or after freezing and thawing), or because of partial denaturation of a few ATPases (opening leaks in the membrane (see, for example, Ref. 47). In fact, in the old days, selecting the subpopulation of vesicles that were completely tight and therefore could be actively loaded with Ca\(^{2+}\) has been the purpose of a number of attempts (e.g. see Ref. 48).

Note that the amount of \(^{45}\)Ca\(^{2+}\) bound in the absence of ionophore to SR vesicles with ATPase initially in its E2 state slowly rose with time (over minutes), and this was the case both in the absence or presence of TG (open and closed circles in D) and also to some extent for fluoride complexes (Fig. 4, E and F). This slow rise most probably represents slow passive \(^{45}\)Ca\(^{2+}\) entry into the lumen of the tight vesicles (32). Conversely, the presence of a subpopulation of SR vesicles that are not tight accounts for part of the extrapolation to zero time of the amount of bound \(^{45}\)Ca\(^{2+}\) in the presence of TG and absence of ionophore (closed circles in Fig. 4D). This excludes further the hypothesis, alluded to above, that TG-resistant binding could represent binding to TG-inhibited ATPase. The similar levels found after preincubation with or without fluoride also exclude it (closed symbols in Fig. 4, A–C or D–F). Related measurements with E2-AlF\(_4\) will be shown below.

**Submillimolar Affinity for \(^{45}\)Ca\(^{2+}\) Binding to E2-BeFx, and Ca\(^{2+}\)-induced Destabilization**—The reduced binding of \(^{45}\)Ca\(^{2+}\) to E2-BeFx, compared with E2 (in Figs. 3 and 4) was interpreted above as implying poor affinity for Ca\(^{2+}\) of the luminal binding sites in E2-BeFx, This is because of the result of \(^{45}\)Ca\(^{2+}\) binding experiments similar to those shown in Fig. 4 but performed at a lower free Ca\(^{2+}\) concentration, 100 \(\mu\)M instead of 200 \(\mu\)M (Fig. 5); in those experiments, whereas TG-resistant \(^{45}\)Ca\(^{2+}\) binding to leaky SR vesicles was reduced to about 5 nmol/mg (closed symbols in Fig. 5, A–C), the TG-sensitive fraction of (cytosolic) binding to E2 remained 9–10 nmol/mg (difference between open and closed circles in Fig. 5A), but the TG-sensitive fraction of (luminal) bind-
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FIGURE 5. 45Ca2+ (at 100 μM) binds to E2BeFx, from the luminal side and not to E2AIF4. This experiment is similar to that illustrated in Fig. 4, except that in this case the third sample (squares) was prepared as E2AIF4, instead of E2-MgF4, i.e. it was preincubated with 1 mM KF and 50 μM AICl3, for 15 min, and the final 45Ca2+ binding medium was at pCa 4 instead of pCa 3.7 (final total concentrations were 125 μM for 45Ca2+ and 25 μM for EGTA); Tes-Tris was at 45 mM in this experiment instead of 90 mM. 0.3-mg protein filters were used instead of GS filters.

Conversely, measuring binding of 45Ca2+ to the luminal sites of E2-BeFx, at free Ca2+ concentrations higher than 200 μM, would be desirable to confirm the stoichiometry of 45Ca2+ binding; however, this would be made very difficult by signal/noise problems, resulting from both the increased amount of 45Ca2+ trapped in the wetting volume of the filter (which has to be subtracted) and the increased nonspecific binding of 45Ca2+ to SR internal sites (corresponding to the closed symbols in Fig. 4, D–F). Thus, the free Ca2+ concentration of 200 μM chosen for the experiments illustrated in Figs. 3 and 4 was a fair compromise. In addition, 45Ca2+ binding experiments performed at higher free Ca2+ concentrations would probably suffer from significant Ca2+-induced destabilization of the E2-BeFx state, as now discussed.

The latter prediction was confirmed by ATPase activity recovery measurements, similar to those in Ref. 24 and designed to more precisely evaluate under our own exact conditions the rate of this destabilization. Interpolation of measurements, performed at two different Ca2+ concentrations (100 μM and 1 mM), of the Ca2+-induced recovery of E2-BeFx from activity inhibition (supplemental Fig. S4) showed that under our experimental conditions over a few minutes, as in Fig. 4B, destabilization by 200 μM Ca2+ probably affects less than 10–20% of the ATPase E2-BeFx complexes, whereas going to 1 mM Ca2+ would result in marked (possibly cooperative) destabilization over the same period. Supplemental Fig. S4, D–F, also shows that the presence of 20% Me2SO in our 45Ca2+ binding buffer was essential for making E2-BeFx sufficiently stable in these activity recovery experiments and therefore also in our 45Ca2+ binding experiments at 100 or 200 μM free Ca2+.

Even at 100 or 200 μM, there is, in fact, a slight upward drift, over minutes, in the curves for 45Ca2+ binding to E2-BeFx. This can be made even more apparent in longer term 45Ca2+ binding experiments. For instance, at 200 μM Ca2+, experiments similar to those in Fig. 4 were conducted over up to 30 min and did show a significant time-dependent rise at the end of this period. This is shown in supplemental Fig. S5.

Note that the latter experiment was performed with spontaneously leaky membranes of deoxycholate-purified ATPase (kindly provided by Prof. J. V. Moller) instead of SR vesicles made leaky with ionophore; the smaller amount of calsequestrin in these purified ATPase membranes results in a lower level of TG-resistant 45Ca2+ binding but a similar level of TG-sensitive 45Ca2+ binding to E2-BeFx, over the first minutes, compared with calsequestrin-containing SR made leaky with ionophore (supplemental Fig. S5), also consistent with the above interpretation of our data.

BHQ and CPA, Too. Inhibit the Luminal Binding of 45Ca2+ to E2-BeFx — Additionally, experiments were performed to test for the effect, on luminal 45Ca2+ binding to E2-BeFx, of additional inhibitors of Ca2+-ATPase previously also described to interact with the E2 form: BHQ and CPA. E2 and E2-BeFx forms were again prepared, ionophore was again added, and aliquots were then supplemented with either TG as above, CPA, or BHQ. Fig. 6 makes clear that, just as with TG, ATPase prein-
cubation with CPA (inverted open triangles) inhibited both the (cytosolic) binding of $^{45}\text{Ca}^{2+}$ to the E2 form (Fig. 6A), as well known, and the (luminal) binding of $^{45}\text{Ca}^{2+}$ to the E2-BeFx form (Fig. 6B). BHQ (squares) also did so; however, under our conditions, BHQ appeared to bind with poorer affinity than TG or CPA, since a larger molar excess of BHQ over ATPase was required for almost complete inhibition (closed versus open squares). The poor apparent affinity with which BHQ binds to the ATPase under our conditions probably is an unfavorable consequence of the presence of Me$_2$SO in our buffers.  

**DISCUSSION**

Opening of the ATPase Ca$^{2+}$ Transport Sites toward the Luminal Side of SR—in crystals of Ca$^{2+}$-ATPase (SERCA1a) containing bound Ca$^{2+}$ ions, the binding sites for Ca$^{2+}$ located in the transmembrane section of SERCA1a are found shielded from the aqueous medium (8, 11, 12). Based on functional measurements, Ca$^{2+}$ ions had previously also been described as being "occluded" in some conformations of the Ca$^{2+}$ pump, for instance in the so-called E1P state of ATPase (e.g. see Ref. 49 for a review). However, to make active transport of Ca$^{2+}$ possible, these binding sites for Ca$^{2+}$—must of course transiently open, first toward the cytosolic medium, to take up Ca$^{2+}$ with high affinity (a binding generally described to be associated with the so-called E1 form of ATPase) and then toward the luminal side of the membrane, after experiencing simultaneously transition to a state where they have lost most of their affinity for Ca$^{2+}$, so that Ca$^{2+}$ can be released into the SR lumen. Previous studies have dubbed "E2P" this putative state in which the Ca$^{2+}$-ATPase, after phosphorylation, has its transport sites accessible from the cytosolic medium, to take up Ca$^{2+}$ with high affinity (a binding generally described to be associated with the so-called E1 form of ATPase) and then toward the luminal side of the membrane, after experiencing simultaneously transition to a state where they have lost most of their affinity for Ca$^{2+}$, so that Ca$^{2+}$ can be released into the SR lumen. Previous studies have dubbed "E2P" this putative state in which the Ca$^{2+}$-ATPase, after phosphorylation, has its transport sites accessible from the cytosolic medium, to take up Ca$^{2+}$ with high affinity (a binding generally described to be associated with the so-called E1 form of ATPase) and then toward the luminal side of the membrane, after experiencing simultaneously transition to a state where they have lost most of their affinity for Ca$^{2+}$, so that Ca$^{2+}$ can be released into the SR lumen.

$^{45}\text{Ca}^{2+}$ Binds from the Luminal Side to SR Ca$^{2+}$-ATPase in Its E2-BeFx Form—In the present work, we were able to reveal these luminally oriented sites in a stable ATPase complex with fluoride formed in the presence of beryllium, the "E2-BeFx$_x$" form. On the basis of indirect measurements, this form had previously been suggested to be the ATPase-fluoride form in which Ca$^{2+}$ binding sites were the most accessible to luminal Ca$^{2+}$. Our Trp fluorescence results confirm its resemblance to the genuine E2P form. They also provide spectroscopic characterizations for it (although alternative explanations for some of the observed changes in fluorescence are conceivable, in line with previous results; see further discussion in supplemental material and Refs 50 and 52). In addition, we were able to demonstrate the remarkable previous suggestion that Ca$^{2+}$ sites in the E2-BeFx$_x$ form are readily accessible to luminal Ca$^{2+}$ by directly measuring rapid $^{45}\text{Ca}^{2+}$ binding to these sites. Conversely, we found that $^{45}\text{Ca}^{2+}$ does not bind well to other ATPase-fluoride complexes, either E2-AlF$_x$ or E2-MgF$_x$, and these results again are the proof of the previous suggestion that opening of the Ca$^{2+}$ sites in these forms is restricted (24). As concerns other ATPase forms, like E2P-like intermediates, formed in the absence of Ca$^{2+}$ ions, the binding sites for Ca$^{2+}$ will take place because fast binding of luminal Ca$^{2+}$ will affect the transmembrane helices harboring the Ca$^{2+}$-liganding residues, this strain will be transmitted to the catalytic site (through a transition inverse of the so-called E1P to E2P transition, whereby phosphorylation at the catalytic site somehow triggers rotation of the A domain and reorganization of the Ca$^{2+}$ binding transmembrane segments), and the beryllium-fluoride complex bound at the catalytic site will now have a chance to dissociate, on a much slower time scale, however.

**Effects of TG, BHQ, and CPA on SERCA1a** Compared with the previously reported attempt to measure $^{45}\text{Ca}^{2+}$ binding to a genuine E2P form (7), a remarkable feature of our results is that luminal binding of $^{45}\text{Ca}^{2+}$ to E2BeFx$_x$ was fast on a time scale of seconds (Fig. 3), which contrasts with the slow (tens of minutes) rate of $^{45}\text{Ca}^{2+}$ binding to E2P suggested by that previous report (see further discussion in supplemental material). For binding to a low affinity site, however, our fast rate of binding sounds perfectly reasonable. Nevertheless, deciding whether the luminal sites in E2BeFx$_x$ remain open permanently or whether they flicker from closed states to open states but spend a significant fraction of their time open cannot be deduced from the present data (even if the latter view sounds the most likely, based on general principles of protein dynamics). $^{45}\text{Ca}^{2+}$ Binding to Its Luminal Sites Induces Time-delayed Destabilization of the E2-Fluoride Complex—The fact that binding of $^{45}\text{Ca}^{2+}$ to these luminal sites was completed within a few seconds (Fig. 3), whereas Ca$^{2+}$-dependent recovery from inhibition occurred on a much longer time scale (supplemental Fig. 54), has mechanistic implications. First, they bear on the long debate (e.g. see Ref. 53) about whether the species previously dubbed "E2P-Ca$_{cyt}$" may exist. We show that at least as concerns the fluoride complex E2-BeFx$_x$, a complex of Ca$^{2+}$ with an E2P-like form does exist, resulting from fast, low affinity binding of Ca$^{2+}$ from the luminal side. Second, this fast binding of Ca$^{2+}$ to E2-BeFx$_x$ reveals that for Ca$^{2+}$-induced destabilization of the fluoride complex, Ca$^{2+}$ must of course bind, but binding per se is not rate-limiting for Ca$^{2+}$-dependent recovery from inhibition. The rate-limiting step for recovery from inhibition of fluoride is much slower than Ca$^{2+}$ binding. Incidentally, this is why, in contrast with direct Ca$^{2+}$-binding measurements, the observation of a slow rate of recovery for E2-MgF$_x$ or E2-AlF$_x$ as in Ref. 24, may suggest but does not strictly prove accessibility or nonaccessibility of the binding sites (we had a similar difficulty in interpreting the experiments with E2-Vo$_x$ forms).

Presumably, Ca$^{2+}$-induced destabilization of the E2-fluoride complex will take place because fast binding of luminal Ca$^{2+}$ will affect the transmembrane helices harboring the Ca$^{2+}$-liganding residues, this strain will be transmitted to the catalytic site (through a transition inverse of the so-called E1P to E2P transition, whereby phosphorylation at the catalytic site somehow triggers rotation of the A domain and reorganization of the Ca$^{2+}$ binding transmembrane segments), and the beryllium-fluoride complex bound at the catalytic site will now have a chance to dissociate, on a much slower time scale, however.

**TG and Other Inhibitors Block Accessibility of the Binding Sites to the Luminal Medium**—Back to the E2-BeFx$_x$ form, in addition to making it possible to clearly reveal binding of $^{45}\text{Ca}^{2+}$ to the luminal side of ATPase, our use of this very stable form as a mimic of the normal E2P ATPase form provided an additional advantage; it allowed us to demonstrate the effect of TG and other inhibitors on accessibility of the binding sites to the luminal medium. In this respect, measuring $^{45}\text{Ca}^{2+}$ binding to E2P in the presence of TG would not be as easy, because the addition of TG has been shown to reduce significantly the amount of phosphoenzyme present (21, 54), and this may occur relatively rapidly, as judged from dephosphorylation measurements under our conditions (data not shown). In contrast, the stability of the ATPase-fluoride forms is sufficient to allow TG binding to occur without promoting a concomitant change of the fluoride ligand. This is demonstrated, for instance, by the fact that although the ATPase in its E2 form is susceptible to mild proteinolysis by either proteinase K (at Lys$^{120}$) or trypsin (at the T2 site) in the absence or presence of TG, the ATPase susceptibility is reduced to nearly zero in E2-fluoride forms both in the absence and presence of TG; this implies that TG does not release fluoride (see Table 2 in Ref. 23 and Table 1 in Ref. 24). Thus, the results in Fig. 1 also prove that TG binding

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3 D. McIntosh, personal communication.
to E2-BeF₄, blocks the opening of the transport sites toward the luminal side of the ATPase nearly completely. The same is of course probably true for the other fluoride forms, which already in the absence of TG do not open easily.

The fact that TG not only prevents binding of cytosolic Ca²⁺ to E₂, as already found long ago (15–18, 25), but also binding of luminal Ca²⁺ to E₂-BeF₄ (as found in the present report), is probably due to TG gluing transmembrane segments together (as many people in the field think, based on the structure of the E₂-TG forms in Ref. 9 and simply on its protecting effect). In fact, accessibility of the transport sites to one side of the membrane and accessibility to the other side probably share a common prerequisite, namely the possibility for the protein to “breathe” and thereby open cytosolic or luminal “gates” between the aqueous medium and the transmembrane binding sites. These breathing movements are probably prevented by TG (21). The fact that in E₂P-related forms TG stabilizes a closed conformation of Ca²⁺ binding sites that would otherwise open toward the luminal side might be the reason why, in previous electron microscopy experiments, the luminal region of ATPase proved to be significantly different in the presence or absence of TG in two-dimensional crystals of ATPase grown in the absence of Ca²⁺ but the presence of vanadate (28).

Implications for the ATPase Three-dimensional Structures to be Derived from Crystals—Crystallization of an enzyme is a difficult task, for which the presence of strong inhibitors (among which are transition state analogs and other inhibitors) has generally been found to be favorable, presumably because the formation of dead end complexes of the enzyme with such inhibitors slows down protein dynamics and/or selects well defined conformations. In many cases, these “frozen” conformations are a great help for understanding the catalytic cycle of the normal enzyme; in less favorable cases, certain features of the inhibitor-selected conformations might be more indicative of the enzyme-inhibitor complex than of the active enzyme itself. In the case of SR Ca²⁺-ATPase, the small number of crystalline forms available has provided immensely valuable insight into the functioning of the catalytic cycle, by revealing details we would not have ever known without crystallography. Conditions of crystallization might have, however, prevented in certain cases the acquisition of all desirable information.

From the present results, it appears that such was the case for the two crystals of Ca²⁺-free ATPase-fluoride forms that have already been described, E₂-MgF₄ and E₂-AlF₃ (13, 14), in which the Ca²⁺ release pathway from the ATPase transport sites toward the SR lumen was found closed. This can now most likely be ascribed to the fact that, those ATPase-fluoride forms were prepared in the presence of either aluminum fluoride or magnesium fluoride, and second, they were prepared in the presence of TG. In relation to these crystals, it would have been desirable for us to be able to repeat our ⁴⁵Ca²⁺ binding experiments under less alkaline conditions, since the presently available Ca²⁺-free crystals of ATPase were all prepared at slightly acidic pH. Unfortunately, the low affinity with which, a priori, Ca²⁺ binds to E₂P luminal sites at acidic pH (34, 35) makes the experiment hopeless.

A likely (and unhappy) consequence of our findings is that future crystals of E₂P-like forms, if they are grown in the presence of TG (as would seem reasonable to compensate for the instability (e.g. see Ref. 55) of these Ca²⁺-free forms in detergent), will have only very little chance of ever being able to reveal in an open state the Ca²⁺ release pathway from the ATPase occlusion sites toward the SR lumen. Unfortunately again, this conclusion can probably be extended to crystals that might be grown in the presence of BHQ or CPA, despite the fact that these two other inhibitors of the ATPase probably bind to sites different from the one to which TG binds (22, 27, 56). Nevertheless, to be more positive, our present results show that the E₂-BeF₄ form, if it can be stabilized in the absence of such a gluing agent, will hopefully provide the possibility of revealing the open state of this Ca²⁺ release pathway. Our Trp fluorescence measurements further substantiate the similarity between E₂-BeF₄ and genuine E₂P. Based on the E₂-MgF₄ structure (13) and judging from the Trp fluorescence results, we may anticipate that in genuine E₂P and E₂-BeF₄, the luminal half of the M₄ helix (which harbors Trpᵟₓ) and M₁ helix (which harbors Trpᵟₓ) could be more inclined (i.e. horizontal) than what was seen in E₂-MgF₄, bringing those Trp residues into a hydrophobic environment and forming a larger space for Ca²⁺ to access from the luminal side.

Note, finally, that we did not obtain any evidence (see final discussion in supplemental material) for the existence of more (7, 57, 58) than the two classical Ca²⁺ binding sites per ATPase monomer (although it is fair to recognize that our data cannot exclude the existence of those additional sites). These two binding sites for Ca²⁺, endowed with relatively poor affinity in E₂P or E₂-BeF₄ states, can most probably be formed by reorganization of the residues that are responsible, in other Ca²⁺-ATPase states, for the high affinity binding of cytosolic Ca²⁺. Such sites are likely to be similar to those predicted for the K⁺-binding sites in Na⁺,K⁺-ATPase (39), because the only critical difference is that Asnᵠ in Ca²⁺-ATPase is replaced with Asp in Na⁺,K⁺-ATPase.

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