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# ATP DEPENDENT CHARGE MOVEMENT IN ATP7B Cu+-ATPase IS DEMONSTRATED BY PRE-STEADY STATE ELECTRICAL MEASUREMENTS

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## Abstract

ATP7B is a copper dependent P-type ATPase, required for copper homeostasis. Taking advantage of high yield heterologous expression of recombinant protein, we investigated charge transfer in ATP7B. We detected charge displacement within a single catalytic cycle upon ATP addition and formation of phosphoenzyme intermediate. We attribute this charge displacement to movement of bound copper within ATP7B. Based on specific mutations, we demonstrate that enzyme activation by copper requires occupancy of a site in the N-terminus extension which is not present in other transport ATPases, as well as of a transmembrane site corresponding to the cation binding site of other ATPases.

### Keywords

charge transfer measurements; copper displacement; heterologous expression; phosphoenzyme intermediate; recombinant $Cu^+$-ATPase; solid supported membrane

## 1. Introduction

P-type ATPases are membrane-bound enzymes that utilize ATP for cation transport [1–3] using the catalytic mechanism of acid halogenases [4], which includes formation of a phosphoenzyme intermediate by covalent transfer of ATP γ-phosphate to an aspartyl residue within an invariant DKTG motif. In the P-type ATPases, ATP utilization is dependent on specific cation binding to an activating site, and phosphoenzyme formation is coupled to displacement/occlusion of the bound cation. Conformational isomerization of the phosphoenzyme intermediate is then followed by active transport of the occluded cation, and hydrolytic cleavage of Pi. The typical architecture of P-type ATPases, such as the well characterized sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase [5] or $Na^+$,$K^+$-ATPase [6], comprises a transmembrane region with ten transmembrane segments including cation binding sites involved in catalytic activation and active transport. The headpiece of these proteins protrudes from the membrane, and includes the ATP binding site in the N domain,

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the aspartyl residue undergoing phosphorylation in the P domain, and a conserved TGE sequence in the A domain required for catalytic assistance of the final hydrolytic reaction. The ATP7B copper ATPase, involved in the etiology of Wilson disease [7], is included in the $P_1$-type ATPase subfamily which is selective for soft and transition metals. Its function is to deliver copper to nascent metalloproteins and export excessive copper from the cell [8]. The ATP7B protein comprises a transmembrane region with eight membrane spanning segments, and a putative transmembrane metal binding site (TMBS) between the sixth and seventh transmembrane segments (Fig 1), corresponding to the specific cation binding/transport sites of other P-type ATPases. In addition to the A, N and P domains common to other P-type ATPases, the headpiece of ATP7B includes an N–metal binding domain (NMBD) with six additional copper binding sites (Fig 1). Furthermore, an important feature of ATP7B is the presence of serine residues that undergo phosphorylation attributed to protein kinase assistance, and may be involved in functionally relevant ATP7B interactions [9].

Much progress in characterization of ATP7B and its isoform ATP7A (Menkes disease protein) has been obtained by heterologous expression in cultured cells [10,11]. We reported very recently high yield heterologous expression of ATP7B in COS-1 cells infected with adenovirus vector, and functional characterization of membrane-bound ATPase obtained with the microsomal fraction of infected cells [12]. In the present manuscript we show that the membrane-bound recombinant $Cu^+$-ATPase obtained under the above-mentioned conditions is suited for adsorption on a solid supported membrane (SSM) and charge transfer measurements. The SSM method has been employed for detection of charge movements by primary and secondary active membrane transporters [13]. We demonstrate by pre-steady state charge measurements within a single catalytic cycle time frame, that the chemical potential of ATP is utilized for electrogenic movement of bound copper within the ATP7B protein. This charge movement is totally prevented by mutations of either a single NMBD site or the TMBS copper binding site. We also compare the time constants of cation displacement in the copper and in the calcium transport ATPases.

## 2. Materials and Methods

Recombinant adenovirus vector (rAdATP7Bmyc) containing CMV promoter driven WT human ATP7B cDNA, fused with 3′ c-myc tag, was constructed as explained previously [12]. Site directed mutations C983A and C985A (in the transmembrane copper binding site, TMBS) and C575A and C578A (in the 6$^{th}$ copper site of the N-metal binding domain, NMBD) were produced in pShuttleCMV-ATP7Bmyc plasmid using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) as per the manufacturer's instructions. The mutations were confirmed by sequencing, and related plasmids were used for construction of rAdATP7Bmyc mutants and infection of COS-1 cells. Microsomes were prepared from COS-1 cells and protein expression was evaluated by SDS gel electrophoresis or Western blotting as explained previously [12].

### 2.1. [$^{32}$P]Phosphoenzyme formation by incubation of microsomes with [γ-$^{32}$P]ATP

Microsomal protein (50 μg protein/ml) was incubated with 50 μM [γ-$^{32}$P]ATP at 30 °C, in a reaction mixture containing 50 mM MES triethanolamine, pH 6.0, 300 mM KCl, 10 mM DTT, 3 mM $MgCl_2$ and 5 μM $CuCl_2$. Samples were quenched at serial times with 5% trichloroacetic acid. The quenched samples were pelleted by centrifugation at 5000 rpm for 5 min, washed with 0.125 N TCA, and finally resuspended in pH 8.3 (half sample) or 6.3 loading buffer (remaining half sample), and separated by Laemmli [14] (alkaline buffer) or Weber and Osborn [15] (acid buffer) gel electrophoresis. The gels were dried and exposed to a phosphor screen followed by scanning on a Typhoon scanner (Amersham, Piscataway Township, NJ) for stoichiometric determination of phosphoprotein relative to three

[$\gamma$-$^{32}$P]ATP standards placed on the gels. Comparative experiments were performed with microsomes derived from COS-1 cells infected with rAdATP7Bmyc (WT and mutants) and cells infected with rAdGFP ("sham").

### 2.2. Measurement of charge movements

Charge movements were measured by adsorbing microsomes containing recombinant WT or mutant $Cu^+$-ATPase on a SSM (Fig 2). The SSM consists of an alkanethiol monolayer covalently bound to a gold electrode via the sulfur atom, and a phospholipid monolayer on top of it [16]. After adsorption, the protein was activated by a concentration jump of a suitable substrate, i.e. ATP (Fig 2). If at least one electrogenic step is involved in the relaxation process, a current transient can be recorded along the external circuit [13]. Numerical integration of each transient is related to a net charge movement, which depends upon the particular electrogenic event. In addition, kinetic information can be obtained by fitting a sum of exponentially decaying terms to the current versus time curves.

In ATP concentration-jump experiments, the non-activating solution contained 300mM KCl, 50 mM MES triethanolamine, pH 6.0, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM DTT, and $CuCl_2$ (5 μM) or BCS (1 and 5 mM); the activating solution contained, in addition, 100 μM ATP. The concentration jump experiments were carried out by employing the SURFE$^2$R$^{One}$ device (IonGate Biosciences, Frankfurt/M., Germany). The temperature was maintained at 22–23 °C for all the experiments.

To verify the reproducibility of the current transients generated within the same set of measurements on the same SSM, each single measurement of the set was repeated 6–8 times and then averaged to improve the signal to noise ratio. Standard deviations were usually found to be no >±5%. Moreover, each set of measurements was usually reproduced using three different SSM electrodes.

## 3. Results and Discussion

Taking advantage of high yield heterologous expression of ATP7B in COS-1 cells [12] and recovery of the expressed protein with the microsomal fraction of infected cells (Fig 3A), we clearly identified two distinct fractions of phosphoprotein formed by utilization of ATP (Fig 3B). An alkaline labile phosphoprotein fraction, corresponding to the catalytic ATPase intermediate (i.e., aspartyl phosphate), is formed rapidly and reaches steady state levels within one second at 30°C. Formation of the phosphoenzyme intermediate is prevented by mutation of the conserved Asp1027 or Cys residues within putative copper sites [12]. In addition, an alkaline stable fraction is formed within a more extended time scale (Fig 3B), due to phosphorylation of serine residues at least in part identified by mass spectrometry as Ser478, Ser481, Ser1121, and Ser1453 [12]. Phosphorylation of both aspartate and serine residues is copper dependent [12].

While detection of cation displacement can be demonstrated with radioactive isotopes for the SR $Ca^{2+}$-ATPase and the $Na^+$,$K^+$-ATPase, analogous measurements with the copper ATPase are difficult, due to the low native abundance of the protein and unfavorable features (very short half life and high gamma emission) of the $^{64}$Cu radioactive isotope. Even though $^{64}$Cu has been used in steady state copper transport assay [10], its use in single cycle experiments would be very difficult. We recently reported detection of pre-steady state calcium movements by charge measurements with native or recombinant SR $Ca^{2+}$-ATPase adsorbed on a SSM [17,18]. We have now applied this technique to studies of copper movements in the ATP7B protein. To this aim, we collected the microsomal fraction of COS-1 cells sustaining heterologous expression of recombinant ATP7B and allowed the microsomal membrane-bound protein to adsorb on the SSM electrode. We then activated

ATP7B with saturating (100 μM) ATP delivered through a concentration jump, and detected the related current transient by the SSM method.

It is shown in Fig 4A that, in the presence of 5 μM $CuCl_2$, the ATP concentration jump induces a current transient (Fig 4A, solid line), which is not observed in the presence of bathocuproine disulfonate (BCS), a specific copper chelator (Fig 4A, dotted line). To gain evidence for the functional relevance of the observed signal, we then repeated the measurements using specific mutants of ATP7B. To this aim, we produced mutations (see 2. Materials and Methods) at the TMBS or at the $6^{th}$ NMBD copper sites, i.e. C983A/C985A and C575A/C578A respectively, obtaining expression of mutant protein as high as that of WT ATP7B (Fig 4, inset). Both mutants were found to be catalytically inactive, inasmuch as no phosphoenzyme intermediate was formed upon addition of ATP. When we attempted measurements of charge movements with these ATPase mutants, we observed no current transient following addition of ATP in the presence of 5μM $CuCl_2$ (Figs 4B and 4C, solid lines) or in the presence of 1mM BCS (Figs 4B and 4C, dotted lines). Taken together, our electrical measurements indicate that the charge obtained by numerical integration of the ATP-induced current transient corresponds to an electrogenic event related to vectorial displacement of bound $Cu^+$ upon utilization of ATP.

It is noteworthy that the observed current transient is due to flow of electrons along the external circuit toward the electrode surface (Fig 2). Such current signal is required to compensate for the potential difference across the vesicular membrane produced by displacement of positive charge (i.e., bound cation) in the direction of the SSM electrode, if the applied voltage across the whole system is kept constant [18]. The current recorded by the SSM method is a measure of the rate of change of the transmembrane potential, and is not sensitive to stationary currents. Therefore, only electrogenic steps within the first catalytic cycle are detected, while steady-state events after the first cycle are not measured [18].

We have then compared (Fig 5) the different time frames of charge movements generated by addition of saturating ATP concentrations to the recombinant $Cu^+$-ATPase (in the presence of 5 μM $CuCl_2$, solid line), or to the recombinant SR $Ca^{2+}$-ATPase (in the presence of 10 μM free $Ca^{2+}$, dashed line). Analysis of the time frames then indicates that the charge transfer decay time constant for the recombinant WT $Ca^{2+}$-ATPase signal (25 ± 0.3 ms) is in good agreement with values obtained with native SR microsomes [19]. On the other hand the charge transfer decay time constant obtained with ATP7B (140 ± 4 ms) is about one order of magnitude longer. These time constants are derived from first order exponential decays, and are therefore independent of the protein concentrations. Finally, it is worth noting that the decay time constant of the $Cu^+$-ATPase current signal (140 ms) is within the time frame of aspartyl phosphate formation (Fig 3B), indicating its correspondence to completion of the first cycle of phosphorylation. This suggests a direct correlation between $Cu^+$ displacement and phosphoenzyme formation, preceding phosphoenzyme hydrolytic cleavage.

It should be pointed out that the observed decay time constants are pertinent to an initial partial reaction of the ATPase catalytic cycle. They are not equivalent to steady state turnover which is expected to be slower, due to rate limiting steps preceding the final hydrolytic cleavage of the phosphoenzyme intermediate.

In conclusion, we detected positive charge displacement within a single catalytic cycle of ATP7B upon addition of ATP and formation of phosphoenzyme intermediate. We attribute this positive charge displacement to movement of bound copper within the ATP7B protein, determining that a single cycle of the copper pump is electrogenic. We produced site

directed mutations demonstrating that ATP7B activation requires occupancy of a copper binding site in the N-terminus extension which is not present in other transport ATPases, as well as of a transmembrane site corresponding to the cation binding site of other ATPases. We also found that the catalytic time constant for ATP7B is one order of magnitude longer than for the $Ca^{2+}$-ATPase (SERCA).

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## Abbreviations used

| | |
|---|---|
| **BCS** | bathocuproine disulfonate |
| **NMBD** | N–metal binding domain |
| **SR** | sarcoplasmic reticulum |
| **SSM** | solid supported membrane |
| **TMBS** | transmembrane metal binding site |

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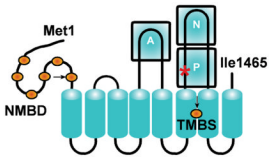


**Fig 1.**

Two-dimensional folding model of the ATP7B sequence. The diagram shows eight transmembrane segments including a copper (orange sphere) binding site (transmembrane copper binding site, TMBS). The extramembranous region includes: the nucleotide binding domain (N); the P domain, where D1027 (red asterisk) undergoes phosphorylation to form the catalytic phosphoenzyme intermediate; the actuator domain (A); the N-metal binding domain (NMBD) with additional six copper (orange spheres) binding sites; and a C-terminus chain. Mutations at the TMBS and NMBD copper sites are indicated by arrows.

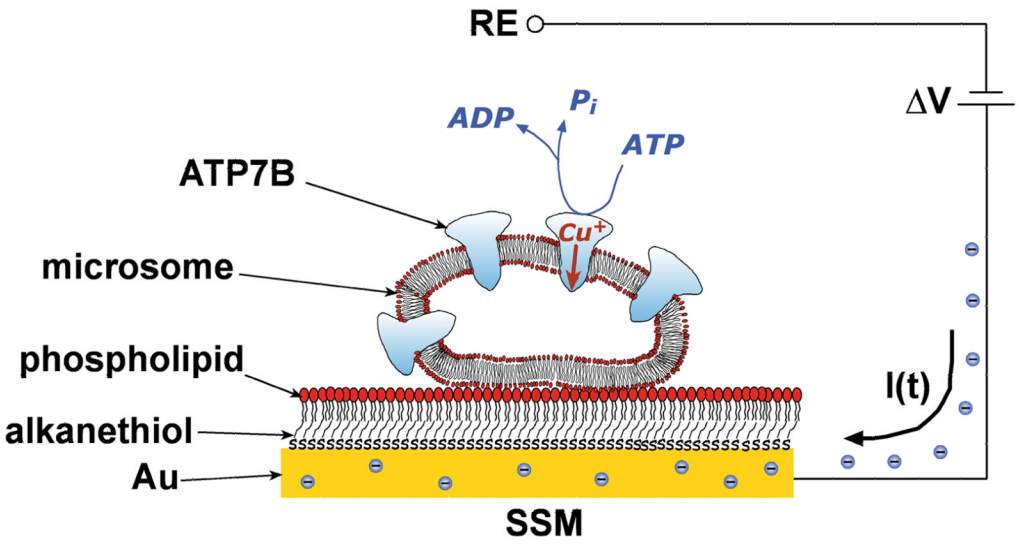


**Fig 2.**

Schematic diagram of a microsome containing ATP7B adsorbed on a SSM (not drawn to scale). When charge displacement does occur, a compensating current *I(t)* flows along the external circuit (the blue spheres are electrons) if the potential difference, *ΔV*, applied across the whole system is kept constant. RE is the reference electrode. For simplicity, only four $Cu^{+}$-ATPase molecules are shown within the microsome.

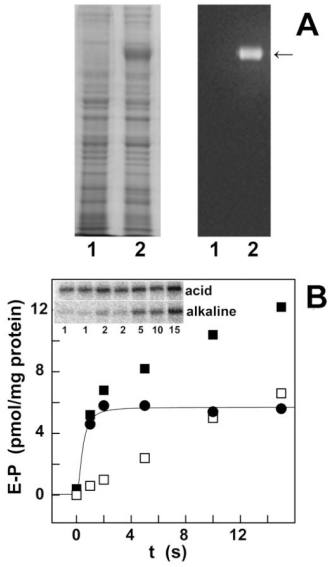


**Fig 3.**

Heterologous expression of ATP7B (A), and phosphorylation by ATP (B). (A): Electrophoretic analysis of the microsomal fraction of sham COS1 cells (left in each panel) and COS1 cells sustaining heterologous expression of ATP7B (right in each panel), followed by general staining of proteins with Coomassie Blue (left panel), and specific immunostaining of the expressed ATP7B protein using a monoclonal antibody for a c-myc tag (right panel). (B): Phosphorylation of ATP7B was obtained by addition of ($\gamma$-$^{32}$P)ATP, followed by acid quenching at times specified in the figure (inset). The quenched protein was then dissolved in detergent, and subjected to electrophoretic analysis in acid or in alkaline media to detect total phosphoprotein (■), and distinguish alkaline labile (●, aspartyl phosphate) and alkaline stable (□, phosphorylated serines) fractions.

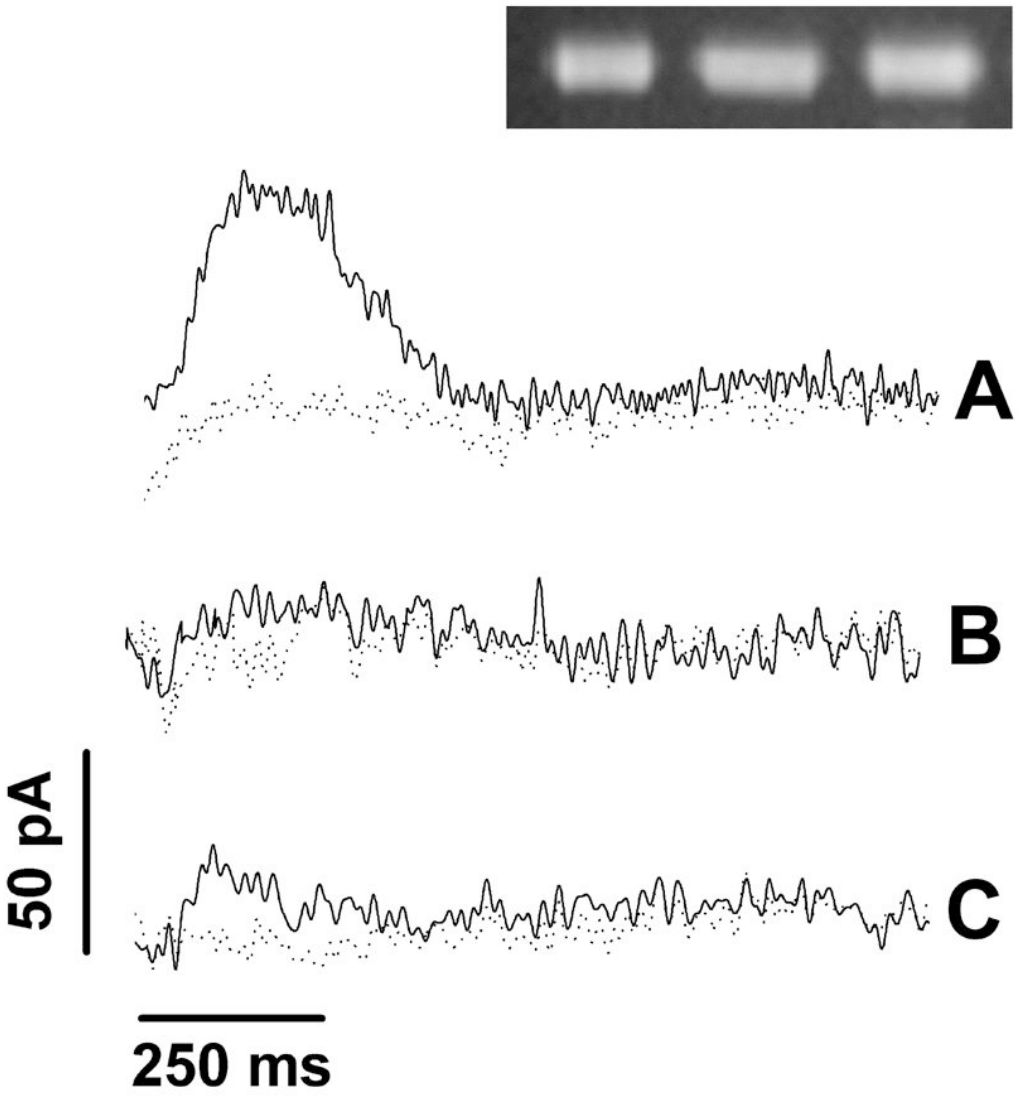


**Fig 4.**

Recombinant WT and mutant $Cu^+$-ATPase. Current transients induced by 100μM ATP concentration jumps in the presence of 5μM $CuCl_2$ (solid lines) or 1mM BCS (dotted lines) on WT and mutant $Cu^+$-ATPase: (A) WT $Cu^+$-ATPase, (B) C983A/C985A mutant, and (C) C575A/C578A mutant. The right upper corner inset shows Western blots of the WT and two mutant proteins demonstrating nearly identical expression levels. The Western blots were obtained with an antibody reacting with the same c-Myc tag present in the three constructs.

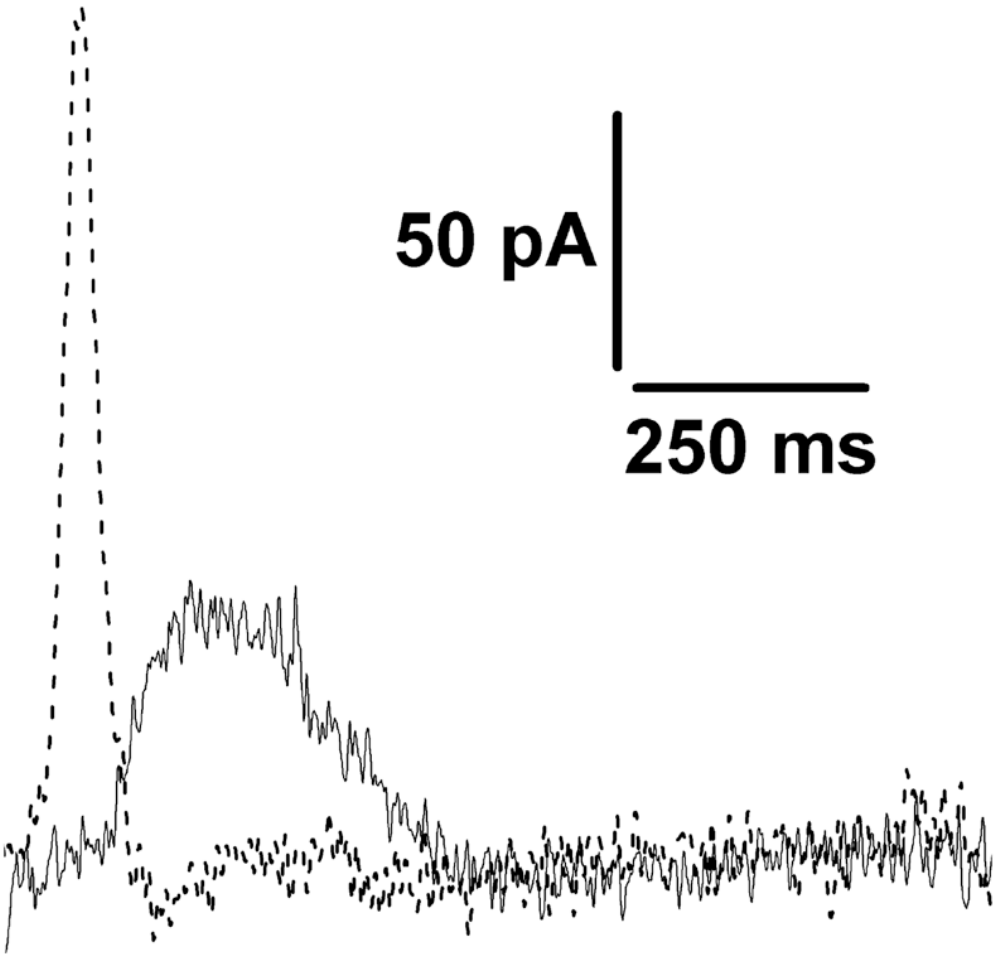


**Fig 5.**

Recombinant $Cu^{+}$-ATPase and SR $Ca^{2+}$-ATPase. Current transients obtained after 100μM ATP concentration jumps on $Cu^{+}$-ATPase (in the presence of 5μM $CuCl_2$, solid line) and on SR $Ca^{2+}$-ATPase (in the presence of 10μM free $Ca^{2+}$, dashed line).