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Action of Iron Nitrosyl Complexes, NO Donors, on the Activity of Sarcoplasmic Reticulum Ca^{2+}-ATPase and Cyclic Guanosine Monophosphate Phosphodiesterase

L. V. Tat'yanenko*, N. A. Sanina, O. V. Dobrokhotova, A. I. Kotelnikov, N. S. Goryachev, I. I. Pihteleva, G. I. Kozub, and T. A. Kondrateva

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Abstract—The effect of iron nitrosyl complexes, NO donors, of a general formula $[Fe_2(L)_2(NO)_4]$ with functional sulfur-containing ligands (L-3-nitro-phenol-2-yl, 4-nitro-phenol-2-yl, or 1-methyl-tetrazol-5-yl) on the activity of sarcoplasmic reticulum Ca^{2+}-ATPase and cyclic guanosine monophosphate phosphodiesterase (cGMP PDE) was studied. The test complexes uncoupled the hydrolytic and transport functions of Ca^{2+}-ATPase, thus disturbing the balance of Ca^{2+} ions in cells, which may affect the formation of thrombi and adhesion of metastatic cells to the endothelium of capillaries. They also inhibited the activity of cGMP PDE, thereby contributing to the accumulation of the second messenger cGMP. The studied iron nitrosyl complexes can be considered as potential drugs.

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Iron nitrosyl complexes, known as natural "stores" of nitric oxide (NO), a versatile compound functioning as a messenger in cell–cell interactions and an inhibitor of aggregation of platelets and their adhesion to the walls of blood vessels, etc., is formed in the reactions of endogenous NO with active sites of nonheme [2Fe–2S] proteins [1]. Their synthetic models are used in the development of new-generation drugs used in the NO-therapy of socially significant diseases [2].

The aim of this work was to investigate the effect of new NO donors of the family of neutral binuclear tetranitrosyl iron complexes **1–3** (Fig. 1) of two different structural types on the activity of sarcoplasmic reticulum (SR) Ca^{2+}-ATPase and cyclic guanosine monophosphate phosphodiesterase (cGMP PDE).

It is known that SR Ca^{2+}-ATPase catalyzes the active transport of calcium ions through biological membranes at the expense of the energy of hydrolysis of the enzyme substrate ATP. Inhibition of active transport of Ca^{2+} ions through SR membrane disturbs the ratio of calcium ion concentration inside and outside cells, which plays an important role in the processes of thrombus formation and adhesion of metastatic cells to capillary endothelium [3], thereby preventing the metastatic growth.

Inhibition of cGMP PDE function leads to the accumulation of cGMP, which is involved in important processes of life activity and determines the antiaggregant, vasodilator, and antihypertensive functions of the body [4].

Investigation of the effect of the new NO donors on the function of SR Ca^{2+}-ATPase and cGMP PDE contributes to the identification of the molecular mechanisms of their biological action, which make it possible to select compounds as potential drugs with antimetastatic, antiaggregant, antihypertensive, and vasodilator effects.

Polycrystalline powders **1–3** were synthesized and characterized by physicochemical methods as described in [5–7]. Complexes **1–3** have a binuclear structure (Fig. 1), in which each iron atom is coordinated with two NO groups and two functional sulfur-containing ligands. In compounds **1** and **2** (Fig. 1), iron atoms are connected into dimers by sulfur atoms (structural type μ-S); in compound **3**, by S-C-N bridges (structural type μ-N-C-S).

In this study, we used human albumin, imidazole, cGMP, cobra venom nucleotidase, ATP, and histidine from Sigma-Aldrich (United States). Dimethyl sulfoxide (DMSO), ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA), sucrose, and salts $MgCl_2$, NaCl, KCl, $CaCl_2$, sodium oxalate, and

Institute of Energy Problems of Chemical Physics, Chernogolovka Branch, Russian Academy of Sciences, Chernogolovka, Moscow oblast, 142432 Russia

** e-mail: kotel@icp.ac.ru; gor_ns@icp.ac.ru*

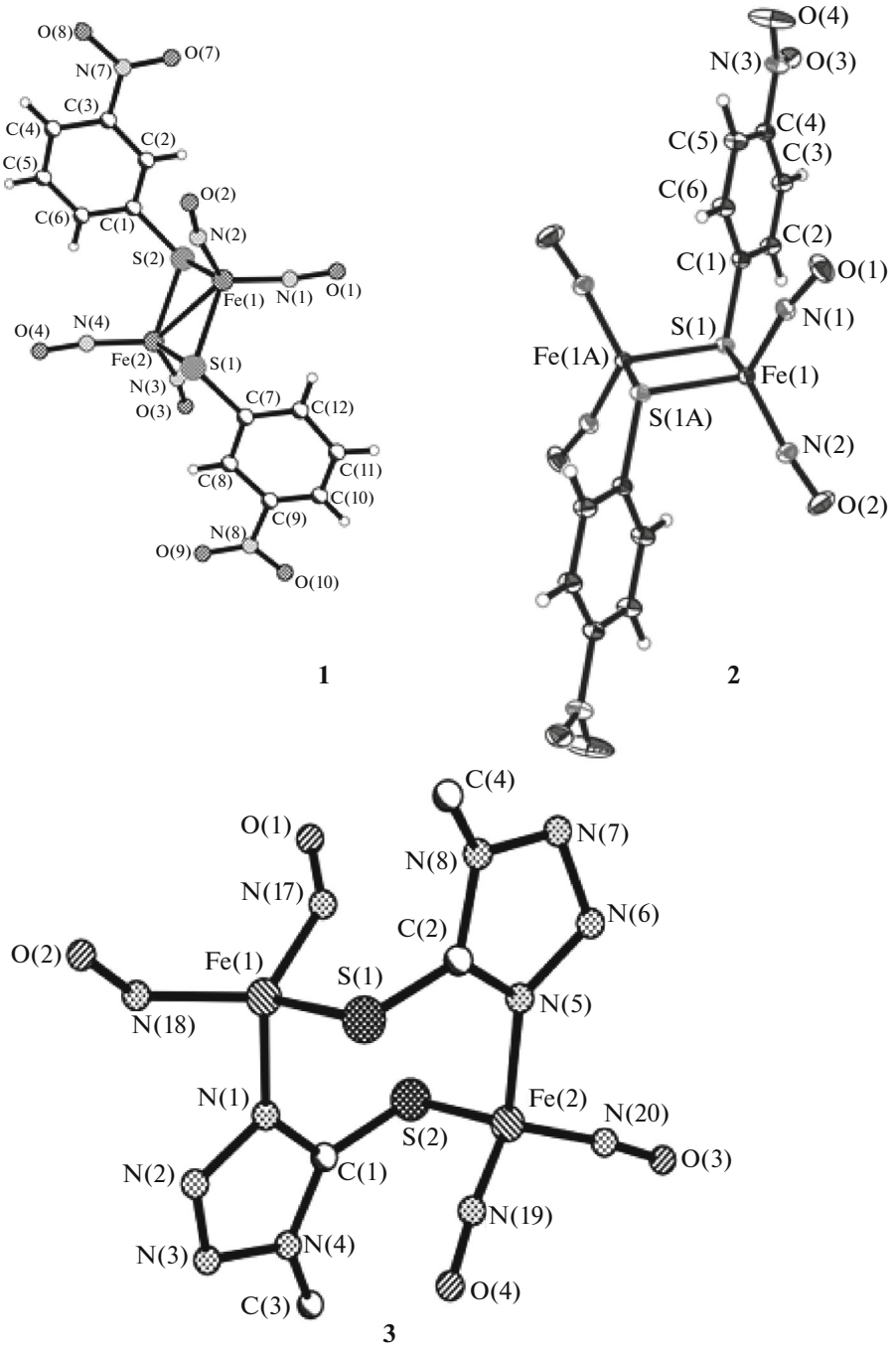

Fig. 1. Molecular structures of neutral binuclear tetranitrosyl iron complexes **1–3**.

ammonium molybdate from Reakhim (Russia) were subjected to appropriate additional purification.

All experiments on the study of the effect of complexes **1–3** on the function of enzymes were performed as described in [8]. Compounds **1–3** were dissolved in DMSO and added in a volume of 100 μL per 5.0 mL of the reaction medium. The hydrolytic and transport functions of SR Ca^{2+}-ATPase were investigated after 3 min preincubation of samples with the enzyme.

Sarcoplasmic reticulum Ca^{2+}-ATPase was isolated from rabbit hindlimb white muscles as described previously [8]. The specific activity of Ca^{2+}-ATPase activity was 15 μmol inorganic phosphate per milligram protein in 1 min. The hydrolytic activity of Ca^{2+}-ATPase activity was calculated from the slope of the kinetic curve of ATP hydrolysis. The rate of changes in the Ca^{2+} concentration was assessed by the kinetics of their uptake by SR vesicles.

Table 1. Effect of iron nitrosyl complexes on the activity of SR Ca^{2+}-ATPase

Compound no.	Inhibition of SR Ca^{2+}-ATPase activity (% of control)									
	Concentrations of compounds in samples, M									
	1×10^{-4}		1×10^{-5}		1×10^{-6}		1×10^{-7}		1×10^{-8}	
	active transport of Ca^{2+}	ATP hydrolysis	active transport of Ca^{2+}	ATP hydrolysis	active transport of Ca^{2+}	ATP hydrolysis	active transport of Ca^{2+}	ATP hydrolysis	active transport of Ca^{2+}	ATP hydrolysis
1	100 ± 10	100 ± 10	100 ± 10	100 ± 10	72 ± 7	18 ± 1*	54 ± 5	14 ± 0.4*	44 ± 4	0*
2	100 ± 10	100 ± 10	100 ± 10	100 ± 10	52 ± 5	22 ± 2*	37 ± 4	14 ± 1*	–	–
3	50 ± 5	39 ± 4*	41 ± 4	20 ± 2*	15 ± 0.5	10 ± 1	–	–	–	–

Data are represented as $M \pm m$, $n = 6–9$. * $p < 0.01$ when compared the parameters of ATP hydrolysis and active transport of Ca^{2+}. Dash means that determination was not performed.

The inhibition of the hydrolytic activity of the enzymes was calculated by the formula $I = 100(A_0 - A)/A$, where I is the relative activity, A is the specific content of inorganic phosphate in the control sample, and A_0 is the specific content of inorganic phosphate in the experimental sample (in the presence of complex). Protein concentration was determined by a modified Lowry method.

The kinetics of inhibition of SR Ca^{2+}-ATPase was determined from the dependence of the enzymatic reaction rate on the substrate (ATP) concentration in the presence and absence of test compounds **1**, **2**, and **3** at concentrations of 4×10^{-6}, 2×10^{-6}, and 1×10^{-4} M, respectively.

The reversibility of action of the compounds was determined by the dialysis of R Ca^{2+}-ATPase solution containing the test complexes at concentrations of 2×10^{-6} M (**1**, **2**) and 1×10^{-4} M (**3**). Dialysis against a 100-fold excess of the incubation medium was performed in the absence of complexes at 4–5°C for 24 h.

cGMP phosphodiesterase was isolated from the cerebral cortex of male Wistar rats. The activity of cGMP PDE in the presence and absence of test agents was determined by the amount of GMP formed in the enzymatic reaction, which was equal to the amount of inorganic phosphate formed from GMP after the addition of nucleotidase. Measurements were performed spectroscopically at the wavelength $\lambda = 735$ nm with a Specord M-40 spectrophotometer (Carl Zeiss Industrielle Messtechnik GmbH, Germany). The results were evaluated by parametric statistical methods. Differences were considered significant at $p \le 0.05$.

As can be seen from Table 1, compounds **1** and **2** completely inhibited active transport and ATP hydrolysis at concentrations of 1×10^{-4} and 1×10^{-5} M. At a concentration of 1×10^{-6} M, these compounds inhibited active transport of Ca^{2+} by 72 ± 7 and 52 ± 5%, respectively, and inhibited the hydrolysis of ATP by only 18 ± 1 and 22 ± 2%, respectively, thus uncoupling the hydrolytic and transport functions of the enzyme. A similar uncoupling effect of these compounds was observed at a concentration of 1×10^{-7} M. Complex **3** at a concentration of 1×10^{-4} M inhibited the transport function of the enzyme by 50 ± 5% and ATP hydrolysis by 39 ± 4%. Complex **1** at a concentration of 1×10^{-8} M inhibited Ca^{2+} transport by 44 ± 4% but had no effect on ATP hydrolysis.

Table 2 presents data on the effect of the test compounds on the activity of SR Ca^{2+}-ATPase. As can be seen from the table, as a result of hydrolysis of one ATP molecule, the amount of calcium ions that were transferred into the vesicles was smaller than in the control ($ATP/Ca^{2+} = 1 : 1.9$). As a result, the ratio of the extracellular and intracellular calcium ions changed, which certainly could disrupt the aggregation of platelet and their relationship with metastatic tumor cells and prevent the adhesion of the latter to the vessel walls [9–11].

At the next stage of the study, we investigated the mechanism of action of complexes **1–3** as modulators of SR Ca^{2+}-ATPase activity. As can be seen from Table 3, complexes **1** and **2** at a concentration of 1×10^{-6} M and compound **3** at a concentration of 1×10^{-4} M after dialysis almost completely stopped to inhibit the hydrolytic and transport functions of SR Ca^{2+}-ATPase. This indicates the noncovalent binding of the complexes to the enzyme, i.e., complexes **1–3** are reversible inhibitors of SR Ca^{2+}-ATPase. Complexes **1** and **2** exhibited the same inhibitory activity as their isostructural analogue complex **2** in our previous study [8], which contained the NH_2 group at position 4 of the phenyl ring. In addition, complex **1**, which was studied in this work, exhibited a pronounced inhibitory effect at the lowest concentrations used. Thus, the replacement of the substituent did not lead to a noticeable increase in the activity of SR Ca^{2+}-ATPase but make it possible to increase the activity of this enzyme at low concentrations of the complex.

Table 2. Effect of iron nitrosyl complexes at different concentrations on the $[Ca^{2+}]/[ATP]$ ratio

Compound no.	Concentration of compounds, M											
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
	ATP hydrolysis, units of specific activity				active transport of Ca^{2+}, units of specific activity				$[Ca^{2+}]/[ATP]$ ratio			
1	0	0	2.2	2.6	0	0	1.5	2.9	0	0	0.7*	1.1*
2	0	0	2.3	2.5	0	0	2.6	3.5	0	0	1.1*	1.4*
3	2.6	1.97	1.8	3.4	3.7	3.2	5.96	–	1.4*	1.6*	1.8	–

$[Ca^{2+}]/[ATP]$ ratio in the control was 1.9. * $p < 0.01$ compared to the control.

Table 3. Effect of iron nitrosyl complexes on the hydrolytic and transport functions of SR Ca^{2+}-ATPase before and after dialysis

Compound no.	Inhibition of SR Ca^{2+}-ATPase, % of control			
	before dialysis		after dialysis	
	active transport of Ca^{2+}	ATP hydrolysis	active transport of Ca^{2+}	ATP hydrolysis
1	75 ± 7	20 ± 2	23 ± 3*	0*
2	50 ± 5	25 ± 2	20 ± 2*	0*
3	50 ± 5	39 ± 4	15 ± 1*	0*

Test complexes were added to the dialyzed samples at concentrations of 1×10^{-6} M (compounds **1** and **2**) and 1×10^{-4} M (compound **3**). Data are represented as $M \pm m$, $n = 3$. * $p < 0.01$ compared to the data before dialysis.

A more complete understanding of the mechanism of action of the test compounds on the enzyme was obtained with the use of the kinetic method of investigation of enzymatic reactions, which provides information on the nature of the binding of the enzyme to the inhibitor. The nature of effect of a reversible inhibitor is determined by the dependence of inverse values of the enzymatic reaction rate ($1/V$) on the substrate concentration ($1/S$) [12]. Using the numerical values of maximum ATP hydrolysis rates, we calculated the inhibition constant (K_i) under the influence of compounds **1**, **2**, and **3** on SR Ca^{2+}-ATPase. As can be seen in Figs. 2–4, complexes **1–3** competitively inhibited the hydrolytic function of SR Ca^{2+}-ATPase with $K_i = 2 \times 10^{-6}$, 1×10^{-6}, and 3.3×10^{-4} M, respectively, which indicates that complexes **1–3** did not bind to the active site of the enzyme.

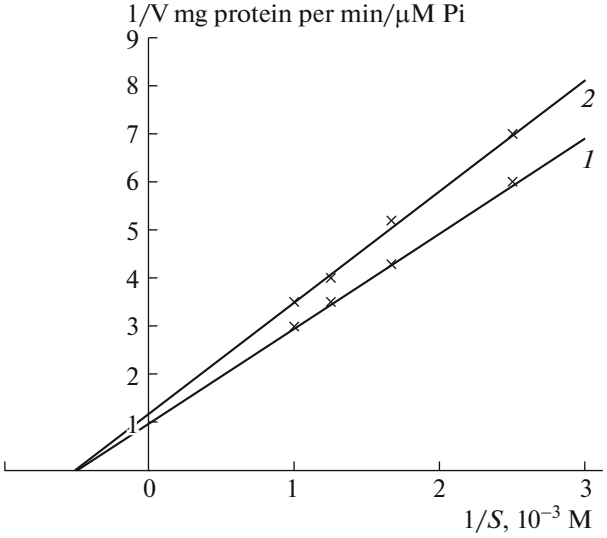

Fig. 2. Dependence of the rate of ATP hydrolysis by SR Ca^{2+}-ATPase of the substrate concentration (1×10^{-3}–4×10^{-4} M) in the Lineweaver–Burk coordinates (*1*) in the absence and (*2*) in the presence of complex **1** at a concentration of 4×10^{-6} M.

Complex **3** inhibited SR Ca^{2+}-ATPase two orders of magnitude more efficiently than its structural analogue, the complex with 1-phenyl-1H-5mercapotetrazolyl, which we investigated earlier (complex **5** in [8]): K_i of complex **3** was 3.3×10^{-4} M, and K_i of its structural analogue **5** (according to [8]) was 2×10^{-6} M.

As seen from Table 4, all the studied sulfur-containing–nitrosyl iron complexes inhibited cGMP PDE function at concentrations of 1×10^{-4} and 1×10^{-5} M. Compounds **1–3** reduced the activity of cGMP PDE by 88 ± 9, 96 ± 9, and 78 ± 9%, respectively, at a concentration of 1×10^{-4} M and by 61 ± 6, 40 ± 4, 30 ± 3%, respectively, at a concentration of 1×10^{-5} M. These results suggest that the complexes possess antiaggregant, antihypertensive, and vasodilator

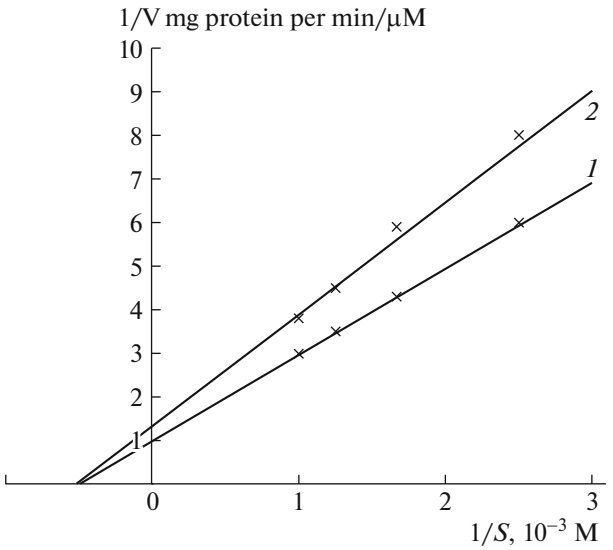

Fig. 3. Dependence of the rate of ATP hydrolysis by SR Ca^{2+}-ATPase of the substrate concentration (1×10^{-3}–4×10^{-4} M) in the Lineweaver–Burk coordinates (*1*) in the absence and (*2*) in the presence of complex **2** at a concentration of 2×10^{-6} M.

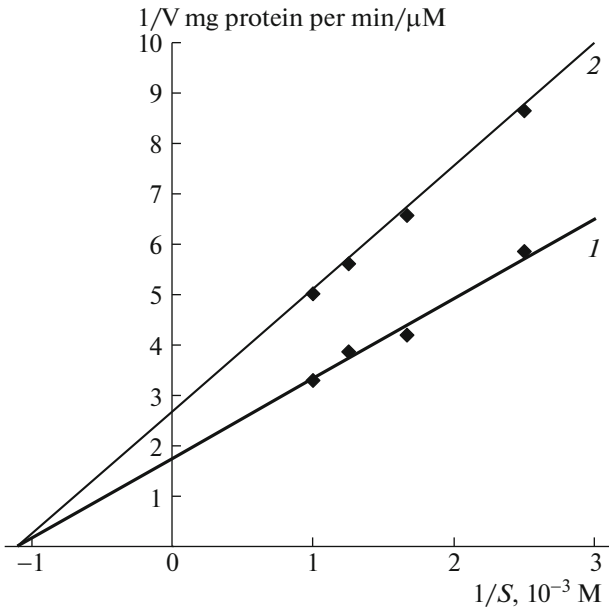

Fig. 4. Dependence of the rate of ATP hydrolysis by SR Ca^{2+}-ATPase of the substrate concentration (1×10^{-3}–4×10^{-4} M) in the Lineweaver–Burk coordinates (*1*) in the absence and (*2*) in the presence of complex **3** at a concentration of 1×10^{-4} M.

properties, which is associated with the accumulation of important second messenger cGMP. The inhibitory ability of complexes **1** and **2** was significantly higher than that of the isostructural complex containing the phenyl ligand (compound **2** in [8]) and, apparently, was determined by the presence of nitro groups in the phenyl ring of the ligand of complexes. In other words, the introduction of substituents into the phenyl ring (in this case, acceptor nitro groups) plays a positive role and opens new perspectives for designing nitrosyl complexes of the structural type μ-S—effective inhibitors of cGMP PDE. The necessity to use more bulky sulfur-containing ligands in the synthesis of tetranitrosyl binuclear iron complexes to enhance their inhibitory ability was confirmed by the data obtained for the complexes of the structural type μ-N-C-S, which were represented by complex **3** investigated in this work. The inhibitory activity of this complex was much lower at concentrations of 1×10^{-5} and 1×10^{-6} M than the inhibitory activity of its isostructural analogue containing the phenyl substituent instead of the methyl one in the tetrazole ring of the ligand (compound **5** in [8]).

Thus, the results obtained in the study of the effect of new sulfur-containing–nitrosyl iron complexes on the key enzyme systems of living organisms—SR Ca^{2+}-ATPase and cGMP PDE—allow us to recommend them for further study as potential drugs exerting antimetastatic, antiaggregant, antihypertensive, and vasodilator effects and to perform a directional design of mimetics of neutral [2Fe–2S] nitrosyls.

Table 4. Effect of iron nitrosyl complexes on the activity of cGMP PDE

cGMP PDE activity inhibition, % of control			
Compound no.	Concentration of compounds, M		
	1×10^{-4}	1×10^{-5}	1×10^{-6}
1	88 ± 9*	61 ± 6*	16 ± 2
2	96 ± 9*	40 ± 4*	12 ± 1
3	78 ± 8*	30 ± 3*	0

Data are represented as $M \pm m$, $n = 6$–8. * $p < 0.01$ compared to the control samples containing no compounds.

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