Biochemical and Biophysical Research Communications 426 (2012) 158–164

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

New fluorescent reagents specific for Ca^{2+}-binding proteins

Danya Ben-Hail [a,1], Daniela Lemelson [a,1], Adrian Israelson [b], Varda Shoshan-Barmatz [a,*]

[a] Department of Life Sciences and the NIBN, Ben-Gurion University, Beer-Sheva 84105, Israel
[b] Department of Physiology, Ben-Gurion University, Beer-Sheva 84105, Israel

ARTICLE INFO

Article history:
Received 10 August 2012
Available online 19 August 2012

Keywords:
Ca^{2+}-binding proteins
Fluorescent reagent
Ca^{2+} transport
Ca^{2+} transport inhibitor

ABSTRACT

Ca^{2+} carries information pivotal to cell life and death via its interactions with specific binding sites in a protein. We previously developed a novel photoreactive reagent, azido ruthenium (AzRu), which strongly inhibits Ca^{2+}-dependent activities. Here, we synthesized new fluorescent ruthenium-based reagents containing FITC or EITC, FITC-Ru and EITC-Ru. These reagents were purified, characterized and found to specifically interact with and markedly inhibit Ca^{2+}-dependent activities but not the activity of Ca^{2+}-independent reactions. In contrast to many reagents that serve as probes for Ca^{2+}, FITC-Ru and EITC-Ru are the first fluorescent divalent cation analogs to be synthesized and characterized that specifically bind to Ca^{2+}-binding proteins and inhibit their activity. Such reagents will assist in characterizing Ca^{2+}-binding proteins, thereby facilitating better understanding of the function of Ca^{2+} as a key bioregulator.

1. Introduction

Ca^{2+} carries information pivotal to cell life and death via its interactions with specific Ca^{2+}-binding sites in proteins. As a ligand, Ca^{2+} has special flexibility and is able to bind to sites with irregular geometry, which makes its binding to proteins particularly easy [1,2].

Several reagents which interact specifically with Ca^{2+}-binding proteins and inhibit their activity have previously been reported. Ruthenium red, $[(NH_3)_5Ru–O–Ru(NH_3)_4–O–Ru(NH_3)_5]Cl_6{\cdot}4H_2O$ (RuR), a water-soluble hexavalent cation [3–5], competes for the Ca^{2+}-binding site [5]. Ruthenium amine binuclear complex (Ru360), a derivative of RuR and inhibitor of the mitochondrial Ca^{2+} uniporter [6] and some other Ca^{2+}-binding proteins [4]. However, these compounds are not very specific and cannot be used for localizing Ca^{2+}-binding sites.

Recently, we developed a novel photoreactive reagent, Azido Ruthenium (AzRu), which specifically and irreversibly binds to calcium-binding proteins upon UV irradiation, and strongly inhibits their Ca^{2+}-dependent activities [4,7]. AzRu was used as a photoreactive reagent to characterize the Ca^{2+}-binding sites of the mitochondrial voltage-dependent anion channel (VDAC) [8].

Several fluorescent and bioluminescent Ca^{2+} indicators have been developed that enable the monitoring of changes in Ca^{2+} concentration in defined cell compartments (e.g. organelles and cytosolic sub-regions) [9,10]. In this study, we report on the development of fluorescent indicators which are specific for Ca^{2+}-binding proteins, rather than the cation, Ca^{2+}. These compounds constitute a new class of fluorescent reagents which can be used to specifically inhibit the activity of Ca^{2+}-binding proteins, and to monitor their binding to Ca^{2+}-binding proteins thus serving as a novel tool in the study of Ca^{2+}-dependent processes.

2. Materials and methods

2.1. Materials

Calmodulin from bovine testes, Fluorescein isothiocyanate (FITC), glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, Hepes, lactate dehydrogenase, leupeptin, myosin from porcine heart, NAD^+, NaN_3, phenylmethylsulfonyl fluoride (PMSF), sucrose, Tris and yeast hexokinase were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Human recombinant calbindin-D_{28K} [11] was produced by PPS, (Rehovot, Israel). [^{45}Ca] and [^{103}Ru] were purchased from NEN Life Science Products (Boston, MA). Sephadex LH-20 was purchased from Amersham Biosciences. Thin layer chromatography (TLC) plates were purchased from Merck (Darmstadt, Germany). Eosin 5-isothiocyanate (EITC) and dimethylformamide (DMF) were purchased from Fluka. Ruthenium chloride was purchased from Aldrich.

2.2. Synthesis, purification and characterization of FITC-Ru and EITC-Ru

AzRu was synthesized, purified and characterized as previously described [4,7]. FITC-Ru was synthesized by incubating AzRu and

* Corresponding author. Tel.: +972 8 6461336; fax: +972 8 6479207.
E-mail address: vardasb@bgu.ac.il (V. Shoshan-Barmatz).
[1] These authors contributed equally to this study.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.08.060

FITC, each at a final concentration of 10 mM, in a total volume of 2 ml containing 50% DMF, at 25 °C overnight. The reaction solution was loaded onto a Sephadex LH-20 column (2.5 × 50 cm) previously equilibrated with 10 mM Tricine, pH 8. The loaded column was then washed with 10 mM Tricine, pH 8. 1.5 ml fractions were collected and those with maximal absorbance at 495 nm were combined and lyophilized. The product was analyzed on silica gel F_{254} plates by TLC using 80% 1 M ammonium formate, pH 8.5, and 20% methanol as developer. The product migrated as a single spot with $R_f = 0.6$, while FITC and AzRu migrated with R_f values of 0.8 and 0, respectively. All steps were carried out in the dark.

The purified product FITC-Ru had a maximal absorbance at 495 nm. A molar absorption coefficient of ~92,000 $cm^{-1}\,M^{-1}$ was estimated. The exact mass of FITC-Ru (854.74 Da) and the mass fragmentation pattern of FITC-Ru were determined by liquid chromatography-mass spectrometry (LC/MS) and used to reveal the reagent structure (performed by Dr. Dhananjaya Nauduri).

Radiolabeled FITC-Ru was synthesized as described above for the non-radioactive reagent, using $Az^{103}Ru$ as starting material. EITC-Ru was synthesized as described for FITC-Ru, except that the reaction was carried out in 60% DMF. The reaction solution was loaded onto a Sephadex LH-20 column (1.5 × 75 cm) previously equilibrated with 30% DMF in double distilled water (DDW). The column was washed with 30% DMF in DDW and the fractions with maximal absorbance at 528 nm were collected and lyophilized. The product was then analyzed on silica gel F_{254} plates by TLC using 60% ethyl acetate, 30% methanol and 10% DDW as developer. The product migrated as a single spot with $R_f = 0.7$ but was determined to not be completely free of EITC. Thus, further purification was carried out using a Phenyl-Sepharose column (1 × 34 cm) previously equilibrated with 20% DMF in 10 mM Tricine, pH 8. The reagent was eluted from the column with 20% DMF in 10 mM Tricine, pH 8, and the fractions with maximal absorbance at 528 nm were collected and lyophilized. The product was then analyzed on silica gel F_{254} plates by TLC as above. All steps were carried out in the dark. The purified product EITC-Ru had a maximal absorbance at 528 nm and was deemed to be about 95% pure.

2.3. Membrane preparations and protein purification

Sarcoplasmic reticulum (SR) membranes were prepared from rabbit fast twitch skeletal muscle as described [12]. Mitochondria were isolated from rat liver as described previously [3].

2.4. Transport and enzymatic activities

2.4.1. Ca^{2+} accumulation by SR membranes

SR membranes (0.44 mg/ml) were incubated for 10 min with FITC-Ru or EITC-Ru (5–80 μM and 2–30 μM, respectively) in the dark in a solution containing; 20 mM MOPS, 0.1 M KCl, pH 6.8. Following incubation with the reagents, $^{45}Ca^{2+}$ uptake was assayed for 2 min in a solution containing 20 mM MOPS, 0.1 M KCl, 1.5 mM $MgCl_2$, 1.5 mM ATP, 0.5 mM EGTA, 0.5 mM $CaCl_2$ (containing 8×10^3 cpm/nmol $^{45}Ca^{2+}$) and 50 mM Pi, pH 6.8, as described previously [12]. $^{45}Ca^{2+}$ uptake was terminated by rapid Millipore filtration followed by a wash with 5 ml of 150 mM KCl.

2.4.2. Mitochondrial Ca^{2+} uptake

Freshly isolated rat liver mitochondria (1 mg/ml) were incubated for 5 min with EITC-Ru (2–30 μM) in the dark in a solution containing 220 mM mannitol, 70 mM sucrose, 5 mM $MgCl_2$, 5 mM ATP, 20 mM Tris–HCl, pH 7.4. Following incubation, $^{45}Ca^{2+}$ uptake by mitochondria (1 mg/ml) was initiated by addition of 120 μM $CaCl_2$ (containing $^{45}Ca^{2+}$ 8×10^3 cpm/nmol). After 2 min of incubation at 30 °C, uptake was terminated by rapid Millipore filtration followed by a wash with 5 ml of 150 mM KCl.

2.4.3. Enzymatic activities

Lactate dehydrogenase, pyruvate kinase [13], yeast hexokinase, glucose-6-phosphate (G-6-P) dehydrogenase, glutamate dehydrogenase and Ca^{2+}-ATPase activities were assessed at room temperature by monitoring the reduction of NAD^+ or the oxidation of NADH at 340 nm using an Ultraspec 2100 spectrophotometer, accordingly (Amersham Pharmacia Biotech). Lactate dehydrogenase and pyruvate kinase were used in coupled reactions.

2.5. Fluorescence measurements

All excitation and emission spectra and fluorescence intensity were measured using a Perkin- Elmer LS55 fluorometer at room temperature (25–26 °C), using 5 nm slit widths for all measurements. Changes in fluorescence intensity upon FITC-Ru binding to a protein were measured in a cuvette (0.2 ml) containing 150 mM NaCl and 40 mM Tris–HCl, pH, 7.4, and aliquots of FITC-Ru or protein solution were added successively. The difference between the fluorescence intensity (ΔF) in the presence and absence of the protein was taken as resulting from FITC-Ru interaction with the protein. An excitation wavelength of 491 nm and an emission wavelength of 515 nm were used. Curve fitting to the data was done using Sigma Plot 2000 software.

2.6. Reconstitution of purified intact VDAC into a planar lipid bilayer (PLB), single channel current recording and data analysis

VDAC purified from sheep liver mitochondria was solubilized with LDAO and purified using hydroxyapetite resin, as described previously [14]. Purified VDAC was used for channel reconstitution into a planar lipid bilayer (PLB) prepared from soybean asolectin dissolved in *n*-decane (50 mg/ml). Purified VDAC was added to the *cis* chamber containing 1 M NaCl and 10 mM Hepes, pH, 7.4. After one or more channels were inserted into the PLB, currents were recorded by voltage-clamping using a Bilayer Clamp BC-525B amplifier (Warner Instruments, Hamden, CT). Current was measured with respect to the *trans* side of the membrane (ground). The current was digitized on-line using a Digidata 1200 interface board and pCLAMP 6 software (Axon Instruments, Union City, CA).

3. Results

3.1. Synthesis, purification and characterization of FITC-Ru and EITC-Ru

The aim of this study was to develop new fluorescent reagents for characterizing the Ca^{2+}-binding properties of proteins. Since AzRu interacts with Ca^{2+}-binding proteins [4], we hypothesized that it may interact with the fluorescent Fluorescein isothiocyanate (FITC) or Eosin 5-isothiocyanate (EITC) to yield Ca^{2+} analog fluorescent reagents. Accordingly, we synthesized two new reagents, FITC-Ru and EITC-Ru, from AzRu (or $Az^{103}Ru$) and FITC or EITC, respectively. FITC-Ru was purified on a Sephadex LH-20 column, with AzRu(5+) eluted first, followed by the charged FITC-Ru molecule, while FITC remained bound to the matrix (Fig. 1). A similar method was used to synthesize and purify EITC-Ru. The obtained IR spectrum indicated that the synthesized reagents contained no azide groups (data not shown).

Radioactive [^{103}Ru]FITC-Ru was also synthesized and purified (Fig. 1) and its specific activity was used to estimate its concentra-

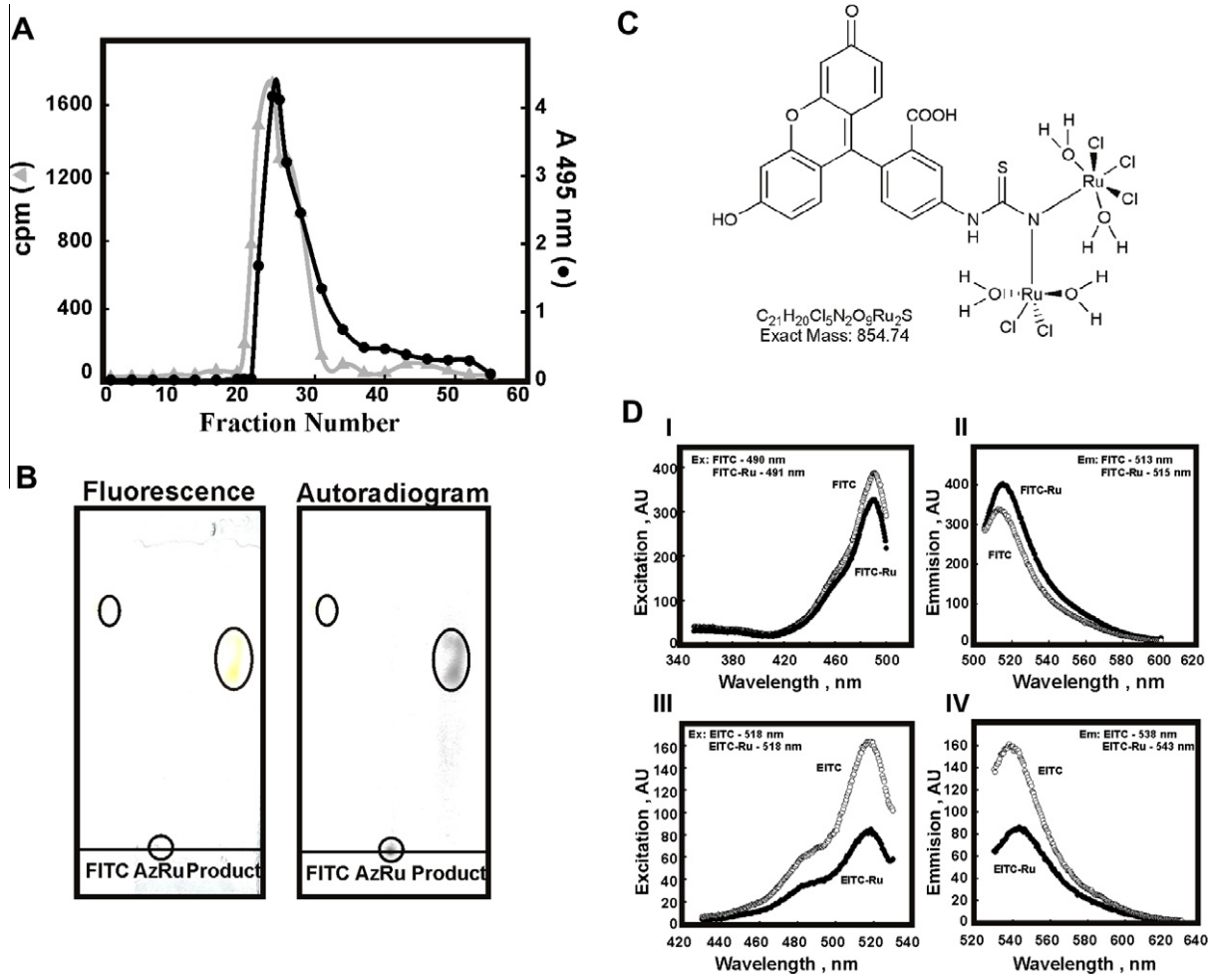

Fig. 1. Purification of FITC-Ru and [^{103}Ru]FITC-Ru and their characterization (A) [^{103}Ru]FITC-Ru and FITC-Ru, synthesized as described in Section 2, show a similar elution profile either upon radioactive counting (▲) or absorbance at 495 nm (●). (B) TLC analysis demonstrated that FITC-Ru is free of its precursors, AzRu and FITC, used in its synthesis, as visualized by fluorescence and autoradiography. (C) The structural formula of FITC-Ru was determined by LC/MS. (D) Excitation (I) and emission (II) spectra of FITC (0.5 μM) and FITC-Ru (2 μM), diluted in 20 mM MOPS, pH 7. Excitation (III) and emission (IV) spectra of EITC (1 μM) and EITC-Ru (5 μM), diluted in 20 mM MOPS, pH 7. The spectra were recorded using a Perkin Elmer LS55 fluorometer.

tion. Absorbance at 495 nm was used to determine a molar absorption coefficient of ~92,000 cm^{-1} M^{-1} for non-radioactive FITC-Ru.

Structural analysis of FITC-Ru by liquid chromatography-mass spectrometry (LC/MS), providing the exact mass of FITC-Ru (854.74 Da) and its mass fragmentation pattern. Based on these results, we propose a structural formula for FITC-Ru (Fig. 1C).

FITC-Ru and EITC-Ru show maximal excitation at 491 nm and 518 nm, respectively (Fig. 1D I and D III) and maximal fluorescence at 515 nm and 543 nm, respectively (Fig. 1D II and D IV). It is important to note that the fluorescence intensities of FITC-Ru and EITC-Ru were weaker than those of FITC and EITC, respectively.

3.2. FITC-Ru and EITC-Ru inhibit Ca^{2+} accumulation in isolated membranes as well as VDAC channel activity

FITC-Ru and EITC-Ru inhibited the Ca^{2+}/Mg^{2+}-ATPase activity and the coupled Ca^{2+} accumulation in SR membranes isolated from rabbit skeletal muscle. FITC-Ru and EITC-Ru inhibited Ca^{2+} accumulation with half-maximal inhibition (IC50) at 37 μM and 8 μM, respectively and the Ca^{2+}/Mg^{2+}-ATPase activity, in a concentration-dependent manner, with IC50 values of 40 μM and 14 μM, respectively (Fig. 2A and B).

Mitochondria contain several recently identified systems for transporting Ca^{2+} [15] that were identified recently. The mitochondrial Ca^{2+} uniporter (MCU) [16,17], its regulatory protein, the MICU1 (mitochondrial calcium uptake 1) [18], the high-affinity mitochondrial Ca^{2+}/H^+ exchanger, Letm1 [19], as well as the Na^+/Ca^{2+} exchanger NCLX [20].

EITC-Ru inhibited Ca^{2+} accumulation in mitochondria freshly isolated from rat liver with an IC_{50} of 14 μM (Fig. 3A).

VDAC is a channel protein which possesses Ca^{2+}-binding sites and transports Ca^{2+} across the mitochondrial outer membrane [3]. Channel activity of purified VDAC reconstituted into a planar lipid bilayer (PLB) was measured in terms of ions passing current across the bilayer in response to voltage gradient. In the absence of FITC-Ru, at −10 mV, VDAC was stable in a long-lived, fully open state. However, addition of FITC-Ru to the same channel induced VDAC closure, in a time-dependent manner (Fig. 3B I). At −40 mV, the channel showed different sub-conducting states, addition of FITC-Ru induced channel closure to a stable sub-state (Fig. 3B II). In multichannel measurements, the protein exhibits typical voltage-dependence conductance, with the highest conductance obtained at membrane potentials between −20 and +20 mV and with decreasing conductance at both high negative and positive potentials. In the presence of FITC-Ru, the channel showed almost no voltage-dependent conductance, and exhibited low conductance values at all voltages tested (Fig. 3C).

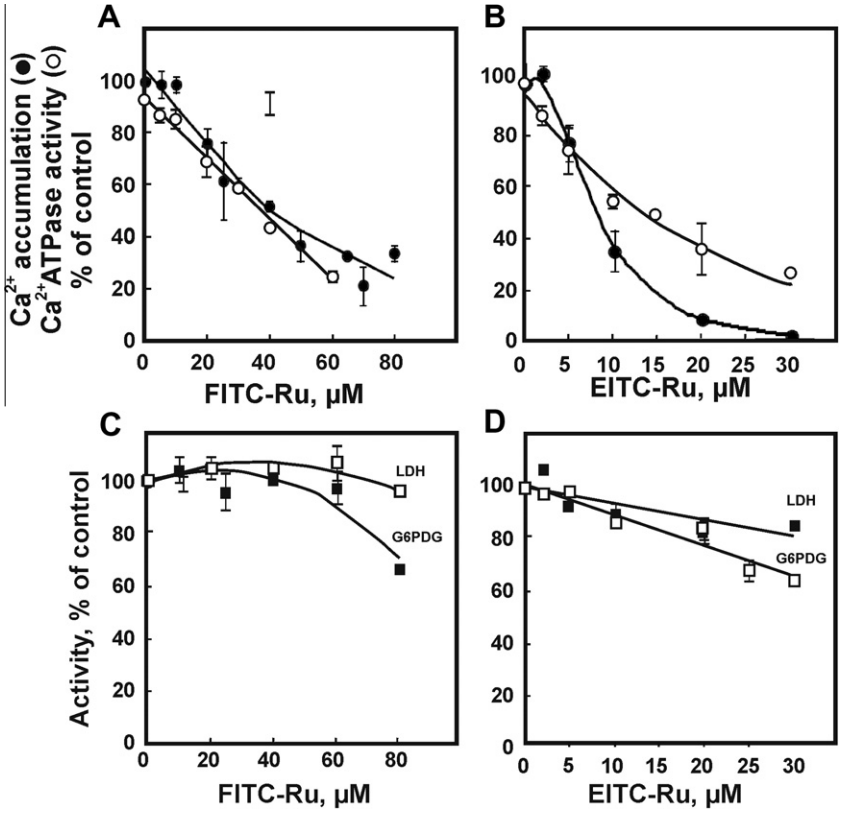

Fig. 2. FITC-Ru and EITC-Ru inhibit Ca^{2+}-dependent but not Ca^{2+}-independent reactions. SR was incubated for 10 min at 24 °C with the indicated concentrations of FITC-Ru (A) or EITC-Ru (B), and assayed for Ca^{2+} uptake (●) and ATPase activity (○). Lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) were incubated for 3 min at 24 °C with the indicated concentrations of FITC-Ru (C) or EITC-Ru (D). The enzymatic activities were assayed as described in Section 2.

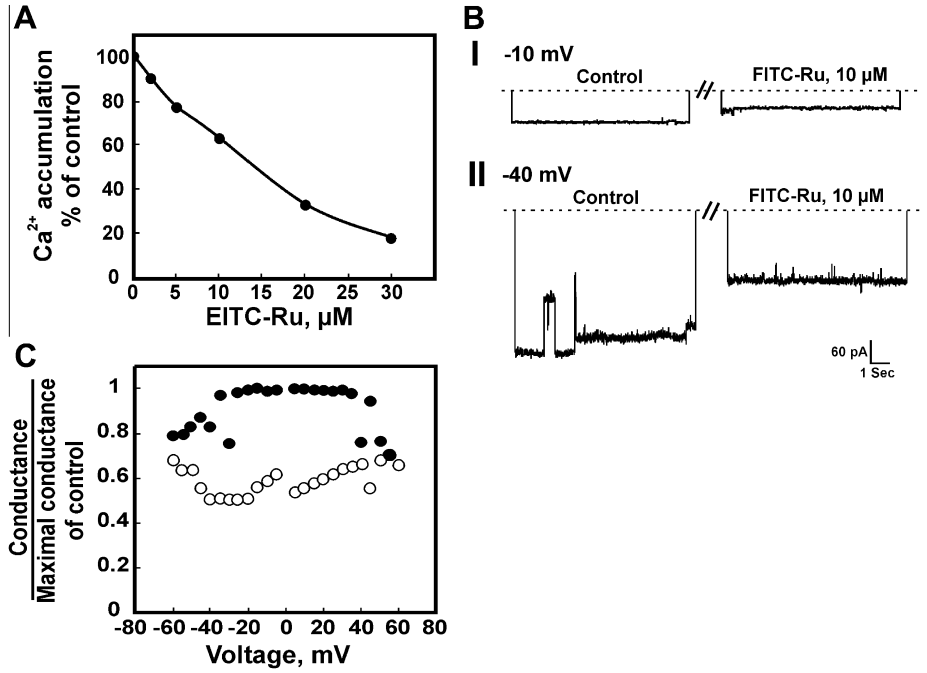

Fig. 3. EITC-Ru inhibits Ca^{2+} uptake in isolated mitochondria and FITC-Ru reduced channel conductance of VDAC. (A) Isolated mitochondria were incubated for 10 min at 24 °C with the indicated concentrations of EITC-Ru and assayed for Ca^{2+} uptake as described in Section 2. Control activity (100%) was 64 nmol/mg protein. Shown is a representative of three similar experiments. (B) FITC-Ru reduced VDAC channel conductance. VDAC was reconstituted into a PLB, and channel currents through VDAC, in response to voltage steps from 0 to −10 mV (I) or −40 mV (II), were recorded before and 30 min after the addition of FITC-Ru (10 μM). A representative of three independent experiments is shown. The dashed lines indicate zero-current. C. The effect of FITC-Ru on VDAC conductance as a function of voltage. The average steady-state conductance at a given voltage was normalized to the conductance at 10 mV (conductance/maximal conductance of the control). The recordings were taken before (●) and after (○) the addition of FITC-Ru.

Table 1
FITC-Ru and EITC-Ru specifically inhibit Ca^{2+}-dependent activities Summary of the effects of FITC-Ru and EITC-Ru on the activities of Ca^{2+}-(1–3) or Mg^{2+}-dependent (4) or -independent proteins (5–8). The degrees of inhibition by 60 μM of FITC-Ru or 20 μM of EITC-Ru is presented. The results represent means ± SEM of three experiments.

Activity assayed	Activity (% of control)			
	FITC-Ru		EITC-Ru	
	$K_{0.5}$ (μM)	60 μM	$K_{0.5}$ (μM)	20 μM
1. Ca^{2+} uptake by SR	37	35	8	8 ± 1
2. Ca^{2+} uptake by mitochondria	–	–	14	33
3. Ca^{2+}-ATPase activity	40	32 ± 2	14	36 ± 10
4. Yeast hexokinase	–	68 ± 3	–	57 ± 4
5. Glutamate dehydrogenase	–	71.5 ± 0.5	–	74 ± 8
6. G-6-P dehydrogenase	–	97 ± 3	–	85 ± 4
7. Pyruvate kinase	–	99 ± 3	–	75.5 ± 0.5
8. Lactate dehydrogenase	–	107 ± 7	–	84 ± 5

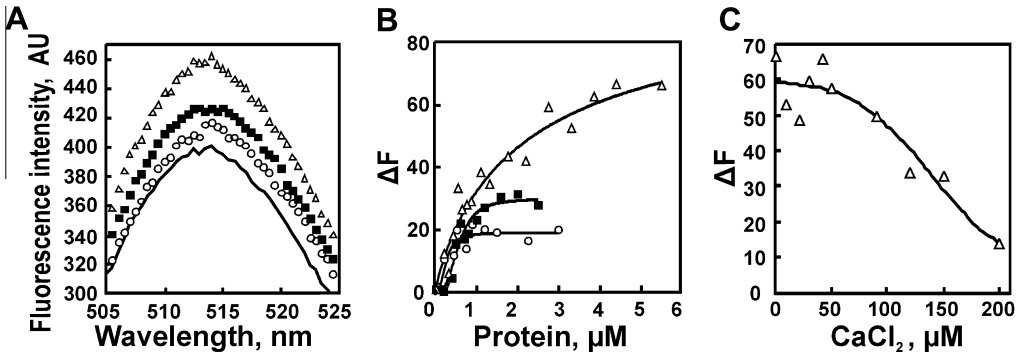

Fig. 4. FITC-Ru fluorescence is enhanced upon binding to Ca^{2+}-binding proteins (A) Relative fluorescence emission spectra of FITC-Ru (0.3 μM) in the absence (–) and presence of 2 μM calmodulin (Δ), myosin (○) or calbindin-D_{28K} (■) measured in a solution containing 150 mM NaCl and 40 mM Tris–HCl, pH 7.4, as described in Section 2. Emission spectra were collected upon excitation at 491 nm. (B) Change in FITC-Ru (0.3 μM) fluorescence as a function of calmodulin (Δ), calbindin-D_{28K} (■) or myosin (○) concentration. The difference between the fluorescence intensity of FITC-Ru in the presence and the absence of the protein (ΔF) is plotted as a function of protein concentration. Samples were excited at 491 nm and emission was measured, 1 min after each addition, at 515 nm. Solid lines represent best fits of the data, using Sigma Plot 2000 software. Results are shown in AU (arbitrary units). (C) Competitive displacement of FITC-Ru (0.3 μM) from calmodulin (5.5 μM) by $CaCl_2$. ΔF is plotted as a function of calcium concentration. Results are shown in AU (arbitrary units).

3.3. FITC-Ru and EITC-Ru interact specifically with Ca^{2+}-binding proteins

The specificity of the interactions of FITC-Ru and EITC-Ru with Ca^{2+}-dependent proteins was established by testing their effects on known Ca^{2+}-dependent and Ca^{2+}-independent proteins (Table 1). Both FITC-Ru and EITC-Ru were found to inhibit the activity of Ca^{2+}-dependent proteins tested, in a concentration-dependent manner. In contrast, neither FITC-Ru nor EITC-Ru had any effect on the activity of four different Ca^{2+}-independent enzymes, including glucose-6-phosphate dehydrogenase and lactate dehydrogenase, at the concentrations tested (Fig. 2C and D). Furthermore, FITC-Ru and EITC-Ru only weakly inhibited the activity of the Mg^{2+}-dependent protein, yeast hexokinase (Table 1). These results strongly support the specific interaction of these reagents with Ca^{2+}-binding proteins.

3.4. Characterization of the interaction of FITC-Ru with Ca^{2+}-binding proteins

In order to investigate whether these fluorescent Ca^{2+} analogs could be used as probes for monitoring reagent interaction with Ca^{2+}-binding proteins, we analyzed changes in FITC-Ru fluorescence in the presence of Ca^{2+}-binding and non-binding proteins. Fig. 4 shows the fluorescence spectra of FITC-Ru alone and in the presence of calmodulin, calbindin-D_{28K} or myosin. FITC-Ru binding to calmodulin [21] or calbindin-D_{28K} [22], Ca^{2+}-binding proteins of the EF-hand family, resulted in enhanced FITC-Ru fluorescence, with no significant shift in emission or excitation wavelengths (Fig. 4A). Calmodulin enhanced fluorescence two times higher than calbindin and four times higher than myosin (Fig. 4A). Myosin has been shown to possess Ca^{2+}-dependent ATPase activity [23]. No FITC-Ru fluorescence enhancement was obtained with hexokinase, a protein containing Mg^{2+}-binding sites (data not shown).

The difference in the fluorescence intensity (ΔF) between free and protein-bound FITC-Ru, was used to follow FITC-Ru binding. A typical plot of ΔF (at 515 nm) versus protein concentration generates a fluorescence titration curve for the Ca^{2+}-binding proteins, calmodulin, calbindin-D_{28K} and myosin (Fig. 4B), with no changes in fluorescence observed with hexokinase (data not shown). The change in the FITC-Ru fluorescence is saturable, protein concentration-dependent and allows for determination of the dissociation constants (Fig. 4B). To calculate binding affinity, we used the following equation, obtained from Sigma Plot 2000 software:

$$\Delta F = \frac{\Delta F_{\max}[P]^h}{[P]_{50}^h + [P]^h}$$

where $[P]$ represents the concentration of the protein, $[P]_{50}$ represents the concentration of protein needed for half-maximal binding of the ligand, and the h represents the Hill constant, i.e. the degree of cooperativity in the binding process.

Formation of the protein-FITC-Ru complex increased with increasing protein concentration, with half-maximal binding of FITC-Ru to the protein achieved at 2.26, 0.56 and 0.26 μM for calmodulin, calbindin-D_{28K} and myosin, respectively. The calculated cooperativity constants are 0.877, 3 and 2.6 for calmodulin, calbindin-D_{28K} and myosin, respectively (Fig. 4B). Calmodulin contains four Ca^{2+}-binding sites, of which 2 are high-affinity and are

considered to be occupied with Ca^{2+} in the native state of the protein [21]. Calbindin-D_{28K} presents six EF-hand domains and is known to bind four calcium ions [24], while myosin includes two Ca^{2+}-binding sites with distinct affinities [25]. Since FITC-Ru contains two Ru molecules, it is difficult to correlate the calculated cooperativity constant, i.e. the Hill coefficient, with the calculated cooperativity constant of the complex with those reported for calmodulin, calbindin and myosin [21–25].

Enhanced fluorescence of FITC-Ru binding to calmodulin was also analyzed as a function of FITC-Ru concentration. At low FITC concentrations (up to 0.6 μM), fluorescence was enhanced in the presence of calmodulin (data not shown). Beyond 0.6 μM, however, a decrease in ΔF was observed. This may result from quenching due to FITC-Ru binding to the second binding site, as such a decrease was not obtained with myosin or without protein.

Ca^{2+} addition to the FITC-Ru-calmodulin complex decreased the enhanced fluorescence of bound FITC-Ru, suggesting that Ca^{2+} displaces FITC-Ru from its binding site in calmodulin (Fig. 4C).

It should be noted that when monitoring binding of FITC-Ru to membrane proteins, such as purified SR Ca^{2+}-ATPase, we found that FITC-Ru was associated also with hydrophobic sites in the protein and/or the detergent surrounding the protein (data not shown).

4. Discussion

A variety of fluorescent Ca^{2+} indicators that allow monitoring changes in intracellular free Ca^{2+} concentrations are commercially available [26]. These probes, however, do not allow for the monitoring and characterization of Ca^{2+}-binding proteins and their Ca^{2+}-binding sites. In this study, we report the synthesis and characterization of the first fluorescent Ca^{2+}-analog reagents, FITC-Ru and EITC-Ru, which bind specifically to Ca^{2+}-binding proteins. The specificities of FITC-Ru and EITC-Ru for Ca^{2+}-binding proteins, together with their fluorescence properties, and their relative simple synthesis opens the opportunity for characterizing the Ca^{2+}-binding sites of proteins, including the determination of binding kinetics.

The specific interaction of FITC-Ru and EITC-Ru with Ca^{2+}-binding proteins was reflected in the ability of these reagents to inhibit Ca^{2+}-dependent activities. Both the SR Ca^{2+}-pump that transports Ca^{2+} using the energy of ATP hydrolysis (Fig. 2) and the mitochondrial uniporter, which is situated in the inner membrane and is driven by the membrane potential to transport Ca^{2+} to the matrix (Fig. 3A), were strongly inhibited by the reagents. Moreover, FITC-Ru induced channel closure of VDAC, which is located in the outer membrane of the mitochondria, possesses Ca^{2+} binding sites and transports Ca^{2+} (Fig. 3B and C). In contrast, the reagents had no effect on Ca^{2+}-independent reactions and only slightly affected Mg^{2+}-dependent activities, indicating the specificity of FITC-Ru and EITC-Ru for Ca^{2+}-binding proteins (Fig. 2 and Table 1).

FITC-Ru and EITC-Ru allowed the Ca^{2+}-binding properties of proteins to be characterized by monitoring the change in their fluorescence. Our results demonstrate that FITC-Ru displayed enhanced fluorescence upon binding to Ca^{2+}-binding proteins, calmodulin and calbindin-D_{28K}, but not with non-Ca^{2+}-binding or Mg^{2+}-binding proteins, such as hexokinase (Fig. 4). The enhanced FITC-Ru fluorescence upon binding to a Ca^{2+}-binding site suggests that the FITC moiety is found in an environment different than that presented in aqueous solution.

Ca^{2+} was able to decrease the fluorescence enhancement obtained upon FITC-Ru binding to calmodulin, suggesting displacement of FITC-Ru from its binding site(s) by Ca^{2+}. This finding, together with the specific inhibition of Ca^{2+}-dependent activities by FITC-Ru (Figs. 2 and 3 and Table 1), suggests that FITC-Ru binds to the Ca^{2+}-binding sites in the protein and proves the reagents as potentially useful as a Ca^{2+} analog.

In conclusion, these novel fluorescent probes, FITC-Ru and EITC-Ru, are the first fluorescent divalent cation analogs to be synthesized and characterized that specifically bind to Ca^{2+}-binding proteins and inhibit their activity. Such reagents with their specificity for Ca^{2+}-binding proteins and their fluorescence properties are attractive for the study of Ca^{2+}-mediated activities, and will assist in characterizing Ca^{2+}-binding proteins, thereby facilitating better understanding of the function of Ca^{2+} as a key bio-regulator and likely prove useful for many other applications.

Funding resource

The support from Phil and Sima Needleman to VSB is highly acknowledged.

Acknowledgments

We thank Prof. Itzhak Fishov for helpful discussions, as well as Prof. Galila Agam for providing calbindin.

References

[1] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell. Biol. 4 (2003) 517–529.
[2] E. Carafoli, Calcium - a universal carrier of biological signals. Delivered on 3 July 2003 at the Special FEBS Meeting in Brussels, FEBS J. 272 (2005) (July 2003) 1073–1089.
[3] D. Gincel, H. Zaid, V. Shoshan-Barmatz, Calcium binding and translocation by the voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial function, Biochem. J. 358 (2001) 147–155.
[4] A. Israelson, L. Arzoine, S. Abu-hamad, V. Khodorkovsky, V. Shoshan-Barmatz, A photoactivable probe for calcium binding proteins, Chem. Biol. 12 (2005) 1169–1178.
[5] T. Sasaki, M. Naka, F. Nakamura, T. Tanaka, Ruthenium red inhibits the binding of calcium to calmodulin required for enzyme activation, J. Biol. Chem. 267 (1992) 21518–21523.
[6] W.L. Ying, J. Emerson, M.J. Clarke, D.R. Sanadi, Inhibition of mitochondrial calcium ion transport by an oxo-bridged dinuclear ruthenium ammine complex, Biochemistry 30 (1991) 4949–4952.
[7] A. Israelson, N. Zilberberg, V. Shoshan-Barmatz, Azido ruthenium: a new photoreactive probe for calcium-binding proteins, Nat. Protoc. 1 (2006) 111–117.
[8] A. Israelson, S. Abu-Hamad, H. Zaid, E. Nahon, V. Shoshan-Barmatz, Localization of the voltage-dependent anion channel-1 Ca^{2+}-binding sites, Cell Calcium 41 (2007) 235–244.
[9] A. Miyawaki, J. Llopis, R. Heim, J.M. McCaffery, J.A. Adams, M. Ikura, R.Y. Tsien, Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin, Nature 388 (1997) 882–887.
[10] V.A. Romoser, P.M. Hinkle, A. Persechini, Detection in living cells of Ca^{2+}-dependent changes in the fluorescence emission of an indicator composed of two green fluorescent protein variants linked by a calmodulin-binding sequence. A new class of fluorescent indicators, J. Biol. Chem. 272 (1997) 13270–13274.
[11] E. Thulin, S. Linse, Expression and purification of human calbindin D28k, Protein Expression Purif. 15 (1999) 265–270.
[12] I. Orr, V. Shoshan-Barmatz, Modulation of the skeletal muscle ryanodine receptor by endogenous phosphorylation of 160/150-kDa proteins of the sarcoplasmic reticulum, Biochim. Biophys. Acta 1283 (1996) 80–88.
[13] P. Singh, M. Salih, J.J. Leddy, B.S. Tuana, The muscle-specific calmodulin-dependent protein kinase assembles with the glycolytic enzyme complex at the sarcoplasmic reticulum and modulates the activity of glyceraldehyde-3-phosphate dehydrogenase in a Ca^{2+}/calmodulin-dependent manner, J. Biol. Chem. 279 (2004) 35176–35182.
[14] V. Shoshan-Barmatz, D. Gincel, The voltage-dependent anion channel: characterization, modulation, and role in mitochondrial function in cell life and death, Cell Biochem. Biophys. 39 (2003) 279–292.
[15] T.E. Gunter, L. Buntinas, G.C. Sparagna, K.K. Gunter, The Ca^{2+} transport mechanisms of mitochondria and Ca^{2+} uptake from physiological-type Ca^{2+} transients, Biochim. Biophys. Acta 1366 (1998) 5–15.
[16] J.M. Baughman, F. Perocchi, H.S. Girgis, M. Plovanich, C.A. Belcher-Timme, Y. Sancak, X.R. Bao, L. Strittmatter, O. Goldberger, R.L. Bogorad, V. Koteliansky, V.K. Mootha, Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter, Nature 476 (2011) 341–345.

[17] D. De Stefani, A. Raffaello, E. Teardo, I. Szabo, R. Rizzuto, A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter, Nature 476 (2011) 336–340.
[18] J. Santo-Domingo, N. Demaurex, Calcium uptake mechanisms of mitochondria, Biochim. Biophys. Acta 1797 (2010) 907–912.
[19] D. Jiang, L. Zhao, D.E. Clapham, Genome-wide RNAi screen identifies Letm1 as a mitochondrial Ca^{2+}/H^{+} antiporter, Science 326 (2009) 144–147.
[20] R. Palty, W.F. Silverman, M. Hershfinkel, T. Caporale, S.L. Sensi, J. Parnis, C. Nolte, D. Fishman, V. Shoshan-Barmatz, S. Herrmann, D. Khananshvili, I. Sekler, NCLX is an essential component of mitochondrial Na^{+}/Ca^{2+} exchange, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 436–441.
[21] S. Linse, A. Helmersson, S. Forsen, Calcium binding to calmodulin and its globular domains, J. Biol. Chem. 266 (1991) 8050–8054.
[22] W.T. Cheung, D.E. Richards, J.H. Rogers, Calcium binding by chick calretinin and rat calbindin D28k synthesised in bacteria, Eur. J. Biochem. 215 (1993) 401–410.
[23] M.J. Holroyde, J.D. Potter, R.J. Solaro, The calcium binding properties of phosphorylated and unphosphorylated cardiac and skeletal myosins, J. Biol. Chem. 254 (1979) 6478–6482.
[24] C.A. Hobbs, L.J. Deterding, L. Perera, B.G. Bobay, R.J. Thompson, T.A. Darden, J. Cavanagh, K.B. Tomer, Structural characterization of the conformational change in calbindin-D28k upon calcium binding using differential surface modification analyzed by mass spectrometry, Biochemistry 48 (2009) 8603–8614.
[25] J.G. Watterson, L. Kohler, M.C. Schaub, Evidence for two distinct affinities in the binding of divalent metal ions to myosin, J. Biol. Chem. 254 (1979) 6470–6477.
[26] R.M. Paredes, J.C. Etzler, L.T. Watts, W. Zheng, J.D. Lechleiter, Chemical calcium indicators, Methods 46 (2008) 143–151.