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Simultaneous Optical Measurements of Cytosolic Ca^{2+} and cAMP in Single Cells

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Abstract

Understanding the temporal and spatial integration of the Ca^{2+} and adenosine 3′,5′-monophosphate (cAMP) signaling pathways requires concurrent measurements of both second messengers. Here, we describe an optical technique to simultaneously image cAMP and Ca^{2+} concentration gradients in MIN6 mouse insulinoma cells using Epac1-camps, a Förster (or fluorescence) resonance energy transfer (FRET)-based cAMP biosensor, and Fura-2, a fluorescent indicator of Ca^{2+}. This real-time imaging method allows investigation of the dynamic organization and integration of multiple levels of signal processing in single living cells.

Introduction

Ca^{2+} and adenosine 3′,5′-monophosphate (cAMP) are key second messengers in cells. The intracellular signaling pathways mediated by these two second messengers are thought to be interconnected (*1–3*), and resolving the spatial and temporal interrelationships of Ca^{2+} and cAMP signaling requires simultaneous measurement of both molecules. FlCRhR, a bimolecular recombinant Förster or fluorescence resonance energy transfer (FRET)–based cAMP indicator, has been used together with the Ca^{2+} indicator Fura-2 or with patch-clamp recordings of Ca^{2+} current to concurrently measure cAMP and Ca^{2+} responses in single cells (*4*, *5*). Widespread use of FlCRhR, the first FRET-based real-time optical indicator of cAMP (*6*), is limited by the requirement for microinjection to load the recombinant FRET donor and acceptor molecules into cells. Recent development of several genetically targeted FRET-based unimolecular indicators has made measurements of cAMP in single cells much easier (*7*). Biosynthetic FRET indicators of cAMP can be expressed in cells by transient transfection with either plasmid or virus gene shuttle vectors.

The principles of FRET and the design of biosynthetic FRET sensors have been extensively reviewed elsewhere and will not be discussed here (*8*, *9*). Genetically targeted unimolecular biosensors employing FRET are powerful tools used to quantitatively visualize spatial and temporal changes in protein kinase activity, protein-protein interactions, and the concentration gradients of second messengers (such as Ca^{2+}, cyclic nucleotides, nitric oxide, and inositol 1,4,5-trisphosphate) (*10–12*). The most commonly employed FRET donor and acceptor pair are enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP), which are mutants of green fluorescent protein (GFP). Measuring FRET using ECFP and EYFP involves excitation of ECFP near its absorption maximum (436 nm) and monitoring ECFP and EYFP fluorescence emission at 485 nm and 535 nm, respectively. An increase in FRET results in a decrease in ECFP emission, due to radiationless transfer of energy to the chromophore of EYFP and an increase in EYFP emission intensity.

Fura-2, a high-affinity fluorescent polycarboxylate Ca^{2+} indicator, has been widely employed for 20 years to study cytoplasmic Ca^{2+} signaling, using digitized video dual-wavelength excitation microspectrophotometry (*13*). In most experimental settings, Fura-2 fluorescence is excited at 340 nm and 380 nm, the absorbance maxima of the Ca^{2+}-bound and Ca^{2+}-free forms of Fura-2, respectively, and emission is detected at a single wavelength, usually 520 nm.

The spectral properties of ECFP, EYFP, and Fura-2 are distinct. Because Fura-2 exhibits low absorbance at 430 to 440 nm, the range of excitation wavelengths typically used to excite ECFP, it is possible to simultaneously study two distinct signals in cells coloaded with Fura-2 and any FRET-based biosensor consisting of ECFP and EYFP. First to exploit the spectral differences between Fura-2 and ECFP were Roger Tsien and his co-workers, who used Fura-2 and D1ER, a FRET-based biosensor of endoplasmic reticulum (ER) Ca^{2+}, to simultaneously measure cytosolic and ER Ca^{2+} concentrations in HeLa and MCF-7 cells (*14*). We used a similar approach to simultaneously visualize changes in intracellular Ca^{2+} and cAMP in insulin-secreting MIN6 β-cells using Fura-2 and Epac1-camps, a FRET-based cAMP biosensor (*15*).

Here, we describe a method to concurrently visualize localized changes in cytoplasmic Ca^{2+} and cAMP concentrations. In addition, we provide detailed information about spectral interplay between Fura-2 and Epac1-camps that addresses concerns about possible interference of Fura-2 with FRET measurements. Although the protocol is focused on measuring cAMP and Ca^{2+} using Epac1-camps and Fura-2, Fura-2 can be used with any FRET biosensor composed of ECFP and EYFP to study the spatial and temporal interrelationships between changes in intracellular Ca^{2+} and other signals in single living cells.

Materials

2-mercaptoethanol, 55 mM in D-PBS [Invitrogen, #21985-023 (https://catalog.invitrogen.com/)]

200 mM L-glutamine

$CaCl_2 \cdot 2H_2O$

Cell culture pipettes and flasks

Coverslips, glass, sterile, 25 mm (Fisher No. 1)

D-glucose

Dimethyl sulfoxide (DMSO) [(Sigma-Aldrich, #D-2650 (http://www.sigmaaldrich.com)]

Dulbecco's modified Eagle medium containing 25 mM glucose (DMEM)

Fetal bovine serum

Forskolin (Sigma-Aldrich, #F-3917)

Fura-2, acetoxymethyl (AM) ester, special packaging (in 50 μg aliquots) [Molecular Probes, #F-1221 (http://probes.invitrogen.com/)]

Hepes-sodium salt

Ionomycin, Ca^{2+} salt (Molecular Probes, #I-24222)

KCl

KH_2PO_4

Lipofectamine-2000 (Invitrogen)

Mammalian expression vector for the biosynthetic FRET sensor Epac1-camps (can be obtained from Martin J. Lohse; see contact information above)

$MgCl_2 \cdot 7H_2O$

Microcentrifuge tubes

Micropipettors

MIN6 mouse insulinoma cell line

NaCl

$NaHCO_3$

Penicillin (10,000 units/ml)-streptomycin (10,000 μg/ml) (pen-strep)

Pluronic F-127, 20% solution in DMSO (Molecular Probes, http://probes.invitrogen.com/)

Tissue culture plates, 6-well

Equipment

Cell Culture

Laminar flow hood

Tissue culture incubator at 37°C, 5% CO_2

Microscopy

Charge-coupled device [16-bit Cascade 650 digital camera (Roper Scientific, http://www.roperscientific.com/)]

Computer-controlled high-speed excitation and emission filter wheels (Lambda 10-2 optical filter changer, Sutter Instruments, Novato, CA)

Coverslip dish, Teflon glass [Warner Instruments, #65-0051 (http://www.warneronline.com)]

Dichroic mirror for Fura-2 and Epac1-camps: 455-nm (455DCLP or 455DCXRU)

Note: Dichroic mirrors and filter sets are all from Chroma Technology (http://www.chroma.com/).

Epac1-camps FRET filter set: 436/10× excitation filter and 485/40 and 535/30 emission filters

Filter set for Fura-2: 340× and 380× excitation filters and 520/20 emission filter

Fluorescence objective (Nikon Super Fluor 40× oil immersion, NA 1.30 objective)

Inverted fluorescence microscope (Nikon TE-2000U)

Neutral density filters

Temperature-controlled perifusion microincubator [Model PDMI-2, Harvard Apparatus, (http://www.harvardapparatus.com)]

Temperature controller (Model TC-202A, Harvard Apparatus)

Data Acquisition and Analysis

Software for imaging data acquisition and analysis [MetaMorph/MetaFluor (Molecular Devices, http://www.moleculardevices.com)]

Perifusion (Optional)

Glass heating coil, 1.5 ml (Radnoti Glass Technology, #158823, www.radnoti.com)

Heated circulating water bath (Haake, Thermo Electron, http://www.thermo.com)

Note: For perifusion, we use a peristaltic pump to provide fluid flow into a temperature-controlled Teflon glass coverslip dish in combination with a Leiden aspirator connected to a vacuum pump. For experiments performed at 37°C, solutions are prewarmed by passing through a heating coil (connected to a heated circulating water bath).

Leiden aspirator (Harvard Apparatus, #65-0047)

Peristaltic pump [Minipuls3 (Gilson, http://www.gilson.com)]

Vacuum pump

Recipes

Recipe 1: Cell Culture Medium

Fetal bovine serum	50 ml
L-Glutamine, 200 mM	5 ml
Pen-strep	5 ml
2-mercaptoethanol, 55 mM	0.5 ml
DMEM	500 ml

Mix components and filter-sterilize. Store at 4°C; heat to 37°C before using.

Recipe 2: Krebs-Ringer Bicarbonate Solution (KRB)

Stock solution	***Volume for 1 liter***	***Final concentration***
NaCl, 1.19 M (69.54 g/liter)	100 ml	119 mM
KCl, 94 mM (7.01 g/liter)	50 ml	4.7 mM
$CaCl_2 \cdot 2H_2O$, 50 mM (7.35 g/liter)	50 ml	2.5 mM
$MgCl_2 \cdot 7H_2O$, 24 mM (5.92 g/liter)	50 ml	1.2 mM
KH_2PO_4, 24 mM (3.27 g/liter)	50 ml	1.2 mM
$NaHCO_3$, 250 mM (21 g/liter)	100 ml	25 mM
D-glucose, 1 M (180.2 g/liter)	2 ml	2 mM

Add stock solutions, then adjust volume to 1 liter. If not used immediately, store overnight at 4°C.

Bubble with 5% CO_2/95% O_2 before and during use to maintain a pH of 7.40.

Note: To avoid the possibility of microbial contamination in glucose-containing KRB and KRH buffers, we advise against preparing these buffers more than 24 hours prior to use, or reusing buffers that have been incubated at 37°C.

Recipe 3: 1 M Hepes-NaOH, pH 7.40

Dissolve 26.03 g of Hepes-sodium Salt in 50 ml of distilled H_2O. Adjust pH to approximately 8.0 with 10 N HCl, then to pH 7.40 with 1 N HCl, and then adjust volume to 100 ml.

Recipe 4: Krebs-Ringer Hepes Solution (KRH)

Stock solution	*Volume for 1 liter*	*Final concentration*
NaCl, 1.19 M (69.54 g/liter)	100 ml	119 mM
KCl, 94 mM (7.01 g/liter)	50 ml	4.7 mM
$CaCl_2 \cdot 2H_2O$, 50 mM (7.35 g/liter)	50 ml	2.5 mM
$MgCl_2 \cdot 7H_2O$, 24 mM (5.92 g/liter)	50 ml	1.2 mM
KH_2PO_4, 24 mM (3.27 g/liter)	50 ml	1.2 mM
Hepes, 1 M, pH 7.40 (260.3 g/liter)	10 ml	10 mM
D-glucose, 1 M (180.2 g/liter)	2 ml	2 mM

Add stock solutions, then adjust volume to 1 liter. Store at 4°C.

Note: KRH solution does not require bubbling with 5% CO_2/95% O_2 to maintain pH 7.40, and is therefore suitable for use when this gas mixture is not available, or when superfusion of the sample during imaging is not desired (e.g., when quantity of the drug being used is limiting, or when nonspecific binding to the walls of the perifusion tubing is a concern).

Recipe 5: 1 mM Fura-2, AM Stock Solution

Add 50 μl of DMSO to 50 μg of Fura-2, AM (the lipophilic, cell-permeable derivative of Fura-2) and vortex to dissolve. Store the stock solution desiccated at −20°C. Avoid exposure to light. Thaw thoroughly before use.

Recipe 6: Fura-2, AM Loading Solution

Add 0.5 μl of 1 mM Fura-2, AM Stock Solution (Recipe 5) and 0.625 μl of 20% Pluronic F-127 dissolved in DMSO to a 1.5-ml microcentrifuge tube containing 1 ml of KRB (Recipe 2) or KRH (Recipe 4). Vigorously pipette the solution with a 1-ml micropipettor several times to evenly disperse Fura-2, AM. Make up Loading Solution immediately before use.

Note: Pluronic F-127 is used at a concentration of 0.0125% (v/v) to aid in the dispersal of Fura-2, AM. Failure to completely disperse Fura-2, AM in the aqueous loading buffer will cause poor labeling of cells with Fura-2.

Recipe 7: 10 mM Forskolin Stock Solution

Add 2.43 ml of DMSO to 10 mg of Forskolin and vortex. Store desiccated at −20°C in 50 μl aliquots.

Recipe 8: 10 μM Forskolin Perifusion Solution

Add 20 μl of 10 mM Forskolin Stock Solution (Recipe 7) to 20 ml of KRB (Recipe 2) or KRH (Recipe 4).

Recipe 9: 100 μM Forskolin Nonperifusion Solution

Add 1 μl of 10 mM Forskolin Stock Solution (Recipe 7) to 100 μl of KRH (Recipe 4).

Recipe 10: 10 mM Ionomycin Stock Solution

Add 134 μl of DMSO to 1 mg of ionomycin and vortex. Store desiccated at −20°C in 10 μl aliquots.

Recipe 11: 10 μM Ionomycin Perifusion Solution

Add 10 μl of 10 mM ionomycin stock solution to 10 ml of KRB (Recipe 2) or KRH (Recipe 4).

Recipe 12: 100 μM Ionomycin Nonperifusion Solution

Add 1 μl of 10 mM ionomycin stock solution to 100 μl of KRH (Recipe 4).

Instructions

Generating Cells Expressing Biosynthetic FRET Sensors

The method described here is for production of MIN6 mouse insulinoma cells that transiently express Epac1-camps. MIN6 cells are more difficult to transfect by liposomal approaches than are other transformed cell lines, such as Chinese hamster ovary or HEK-293T cells. However, commercially available transfection reagents can be used to achieve suitably high transfection efficiencies, typically 10 to 20%. Transfection at this efficiency enables fluorescence microscopy to be performed simultaneously on multiple cells visible within a single field of view.

1. Place one uncoated, sterilized 25-mm circular glass coverslip in each well of 6-well tissue culture plates.
2. Plate MIN6 cells at a density of 8×10^5 cells per coverslip (approximately 50% confluence) in 3 ml of Cell Culture Medium (Recipe 1).

   Note: If using a cell line other than MIN6, the plating density may need to be adjusted so that the cells will not be overgrown 3 to 5 days after plating (48 to 96 hours after the transfection).
3. Incubate cells overnight in a humidified incubator at 37°C, 5% CO_2.
4. Transfect cells in each well with 1 μg of plasmid DNA encoding the FRET biosensor and 5 μl of Lipofectamine-2000 reagent, according to the manufacturer's instructions.

   Note: Transfection can be performed using any commercially available transfection reagent that works effectively with the cell line being transfected. The quantity of DNA and transfection reagent may need to be optimized for each cell line.
5. Replace medium daily with 3 ml of fresh Cell Culture Medium (Recipe 1).
6. Analyze the cells 48 to 96 hours after the transfection.

   Note: Although FRET biosensor expression can be detected after 24 hours, waiting 48 hours or longer results in a higher percentage of positive cells. We have found that cell viability after transfection is increased if the medium is changed daily.

Simultaneous Measurement of FRET and Fura-2 Emission: Data Acquisition and Analysis

For simultaneous FRET and Fura-2 measurements, cells that have previously been transfected with plasmid encoding the FRET sensor are loaded with Fura-2, AM. FRET and Fura-2 emission are visualized with an epifluorescence microscope equipped with a fluorescence objective and a digital video camera for image capture. A 455-nm dichroic filter is used for both FRET and Fura-2. The 340- and 380-nm excitation filters and a 520-nm emission filter are used for Fura-2 dual-wavelength excitation ratio imaging. Dual-emission wavelength ratio imaging of the FRET sensor is performed using a 436-nm excitation filter together with 485 nm (FRET donor, ECFP) and 535 nm (FRET acceptor, EYFP) emission filters. Optical filter sets are changed with a computer-controlled high-speed filter wheel system controlling both the excitation and emission filter sets.

1. Transfer a coverslip with the transfected cells to a 1-ml Teflon glass coverslip dish.
2. Add 1 ml of Fura-2, AM Loading Solution (Recipe 6) to the dish.

   Note: Esterified Fura-2 is brightly fluorescent at the excitation wavelengths used for both Fura-2 and FRET measurements, potentially interfering with FRET and Fura-2 analysis of the cells. For simultaneous imaging of both Fura-2 and FRET, we find that loading the cells with 500 nM Fura-2, rather than 2 to 5 μM as is often reported in the literature, results in brightly labeled cells, and it decreases the Ca^{2+}-chelating effect of overloading cells with Fura-2.
3. Incubate for 15 to 20 min at 37°C, 5% CO_2 in a humidified incubator.
4. Place the coverslip dish into the perifusion microincubator mounted on the specimen stage of an inverted microscope.
5. Begin superfusion with KRB (Recipe 2) or KRH (Recipe 4) at a rate of 2 to 5 ml/min, at 37°C.
6. Visualize cells on the epifluorescence microscope, using dual excitation (340 and 380 nm) and 520 nm emission for Fura-2, and 436-nm excitation and dual emission (485 and 535 nm) for Epac1-camps.
7. Record Fura-2 and Epac1-camps fluorescence in defined regions of interest, using MetaMorph/MetaFluor or other standard imaging acquisition and analysis software. We generally record Fura-2 and Epac1-camps fluorescence at 10-s intervals. Obtain baseline values for a 60-s period before addition of experimental reagents, and record images of two or more cell-free areas on the coverslip during each experiment for subsequent background subtraction and data analysis.

Note: Exposure of cells to excitation illumination, especially UV light, should be minimized. This can be accomplished by using a neutral density filter to block excitation light, and by decreasing the image exposure times. This decreases phototoxic effects on cells and reduces the rate of FRET sensor photobleaching. We use one or more neutral density filters to attenuate the excitation light intensity by 50% to 90%, and limit exposure time to ≤ 200 ms per image. Increasing the recording interval (e.g., from 10 to 60 s) can further minimize exposure to excitation light when conducting measurements over a prolonged period (e.g., ≥ 1 hour).

8. Verify Epac1-camps function in the transfected cells by treating the cells with forskolin, an activator of adenylate cyclase. Perfuse the cells with 10 μM Forskolin Perifusion Solution (Recipe 8) at a rate of 2 ml/min for 10 min while recording Epac1-camps fluorescence. Alternatively, to avoid perifusing the cells, carefully add 100 μl of 100 μM Forskolin Nonperifusion Solution (Recipe 9) to the cell dish containing 1 ml of KRH (Recipe 4), and record Epac1-camps fluorescence.

   Note: This step is optional. Activation of adenylate cyclase elevates intracellular cAMP concentrations, resulting in decreased 535 nm (FRET acceptor) emission and increased 485 nm (FRET donor) emission by Epac1-camps. To control for nonspecific effects of DMSO on the cells, a parallel experiment should be performed using 20 ml of KRB (Recipe 2) or KRH (Recipe 4) to which 10 μl of DMSO has been added.

9. Verify function of Fura-2 by treating the cells with ionomycin, a Ca^{2+} ionophore. Perifuse with 10 mM ionomycin perifusion solution at 2 ml/min for 5 min while recording Fura-2 fluorescence. Alternatively, to avoid perifusing the cells, carefully add 100 μl of 100 μM Ionomycin Nonperifusion Solution (Recipe 12) to the cell dish containing 1 ml of KRH (Recipe 4), and record Fura-2 fluorescence.

   Note: This step is optional. Addition of ionomycin to the cells increases intracellular Ca^{2+} concentrations, resulting in increased 520 nm emission at 340 nm excitation and decreased emission at 380 nm excitation.

10. Subtract average values from the cell-free background regions defined in step 7 from images obtained in regions of interest.

11. Express data as the ratio of FRET donor and acceptor emission (R485/535) and as the ratio of Fura-2 emission when excited at 340 and 380 nm (R340/380).

12. To facilitate comparisons of responses in different cells from independent experiments, normalize data to the average baseline values of R485/535 and R340/380 measured before addition of experimental reagents. Present data as fold-change or relative change in R485/535 and R340/380.

Troubleshooting

Little or No Expression of the FRET Sensor

Low expression of the FRET sensor can be the result of poor transfection efficiency. It may be necessary to optimize the amounts of plasmid DNA and transfection reagent used for transfecting a particular cell line. It is generally necessary to wait at least 48 hours after transfection before performing imaging experiments, although expression of the sensor can be detected after 24 hours. We have also found that transfection efficiency declines after using plasmid DNA preparations that have been subjected to repeated cycles of freezing and thawing. We suggest aliquoting maxipreps of plasmid DNA that will be used for biosensor transfection to avoid this problem. DNA sequences from all maxipreps should also be verified before use.

Appearance of Bright Fluorescent Clusters on Coverslips Exposed to Fura-2, AM

Fura-2, AM is poorly soluble in aqueous solutions. Vigorous trituration, use of detergents such as Pluronic F-127, or both are required to evenly disperse Fura-2, AM. Failure to disperse Fura-2, AM in solution reduces cell loading and can leave a fluorescent residue on the coverslip.

Weak Fura-2 Signal

A weak Fura-2 signal may be related to poor loading or incomplete de-esterification. It may also be caused by the choice of dichroic mirror. In our original experiments, we used the 455DCLP mirror, which allowed 20 to 30% of the excitation light (340 nm and 380 nm) to pass through the mirror rather than be reflected to the biological samples. More recently, we have employed a 455DCXRU dichroic, which reflects more excitation light to the sample and, compared to the 455DCLP, substantially improves Fura-2 fluorescence emission intensity.

No Change in FRET (R485/535) Observed in Epac1-Camps Transfected Cells Following Drug Treatment

Cellular autofluorescence from nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) in the wavelengths used to monitor FRET can be mistaken, especially in widefield imaging mode, for fluorescence from genetically encoded FRET-based biosensors composed of GFP mutants. The following information may help the discrimination of autofluorescence and FRET. Autofluorescence generally appears to be localized in subcellular puncta or fusiform structures (possibly mitochondria) and is present in all cells. In addition, autofluorescence is less intense than Epac1-camps emission, which is two- to three-fold above background. FRET emission by Epac1-camps exhibits a diffuse cytosolic distribution, is excluded from the nucleus, and should be evident in only a small fraction of cells (due to low transfection efficiency). It is important to note that genuine FRET is confirmed by observing an anti-parallel change in the 485- and 535-nm emission (for example, 485 nm emission increases while 535 nm emission decreases). This can be verified by plotting the background-corrected 485- and 535-nm emission intensities versus time. Any change in the R485/535 that occurs without this antiparallel change should be considered an artifact. We have observed small artifactual variations in the R485/535 during experiments resulting from movement of the cells or changes in the focal plane.

Notes and Remarks

Spectral Bleed-Through

The absorbance spectra of ECFP, EYFP, and Fura-2 are distinct. The difference in the spectral properties of Fura-2 and Epac1-camps allows simultaneous measurement of cytoplasmic Ca^{2+} and cAMP concentration ($[Ca^{2+}]_c$ and $[cAMP]_c$, respectively) in single cells. Ratiometric Ca^{2+} measurements with Fura-2 utilize dual wavelength excitation at 340 nm and 380 nm, the excitation maxima of the Ca^{2+}-bound and Ca^{2+}-free forms of Fura-2. In simultaneous imaging experiments with Fura-2 and Epac1-camps, we observed that the FRET donor ECFP exhibits absorbance at 340 nm and 380 nm (Figs. 1 and 2). In contrast, Fura-2 showed little absorbance at 436 nm.

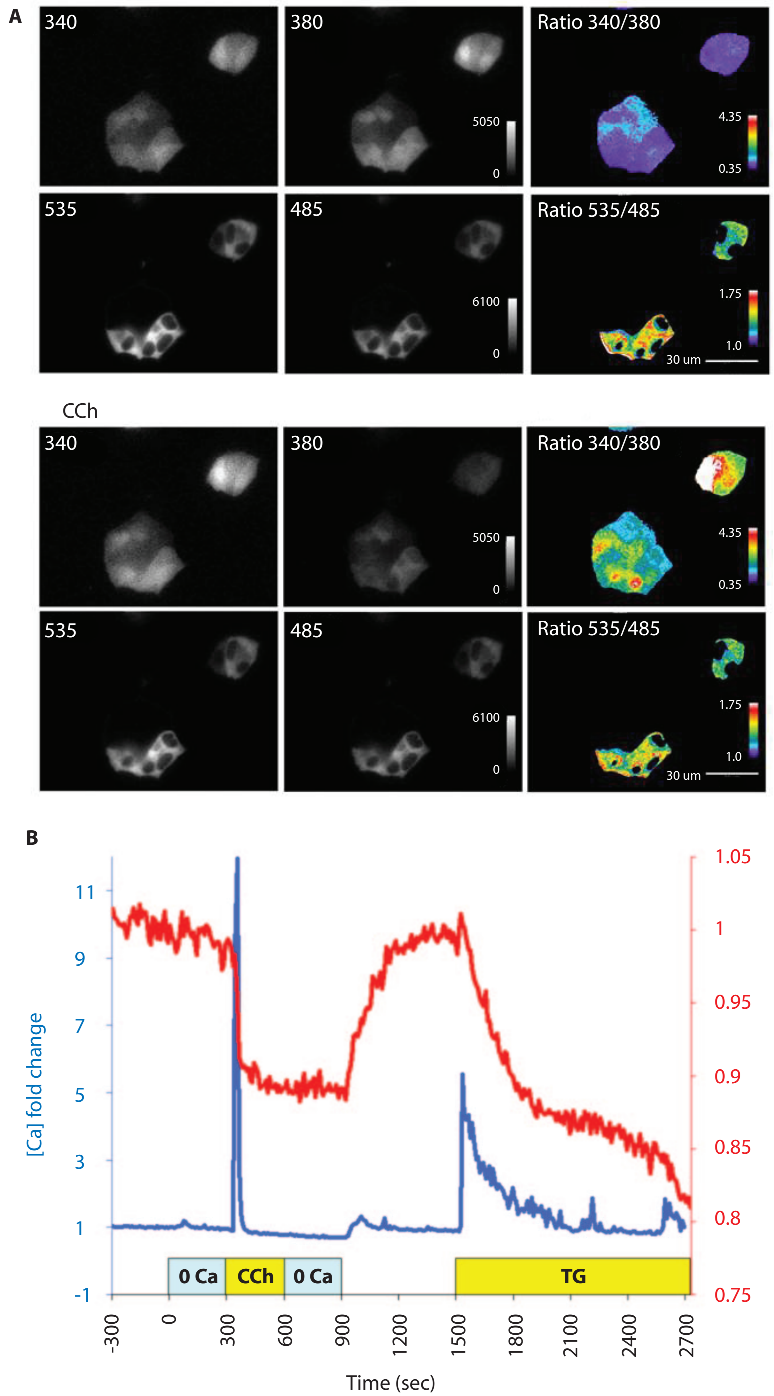

Fig. 1. Simultaneous measurements of cytoplasmic and ER Ca^{2+} in βTC6 cells. (**A**) 16-bit digital images of Fura-2-loaded βTC6 cells transiently expressing YC4.12er. The image in each panel was recorded from the same field of view (see "Instructions" for details). Fluorescence emission (in grayscale), and Ratio 340/380 and Ratio 535/485 (in pseudocolor) before (upper panel) and after (lower panel) stimulation with carbachol (250 μM; CCh) are shown. Note the differences between Fura-2 and YC4.12er distribution. Grayscale and color bars showing magnitude of signals and ratio, respectively, are depicted. Scale bar indicates 30 μm. (**B**) Simultaneous imaging with Fura-2 and YC4.12er reveals coordinated interplay of $[Ca^{2+}]_c$ (blue trace) and $[Ca^{2+}]_{ER}$ (red trace) in a single βTC6 cell following discharge of ER Ca^{2+} store with carbachol (CCh) in Ca^{2+}-free perifusion buffer solution (0 Ca). Refilling of ER Ca^{2+} store in response to increasing extracellular Ca^{2+} (at the 900-s time point) and the effect of SERCA inhibition with thapsigargin (TG) are shown. Data are expressed as change in $[Ca^{2+}]_c$ ([Ca] fold change) and FRET ratio (R535/485) relative to baseline.

To quantify the effect of ECFP and EYFP spectral interference or bleed-through on Fura-2 imaging measurements, we monitored cells transfected either with Epac1-camps or yellow cameleon-4.12er (YC4.12er), a FRET-based ER Ca^{2+} biosensor, at the Fura-2 excitation and emission wavelengths. In cells expressing Epac1-camps but not loaded with Fura-2, the application of forskolin and isobutyl methylxanthine (IBMX) decreased FRET, and did not affect absorbance at wavelengths used for Fura-2 imaging (Fig. 2A

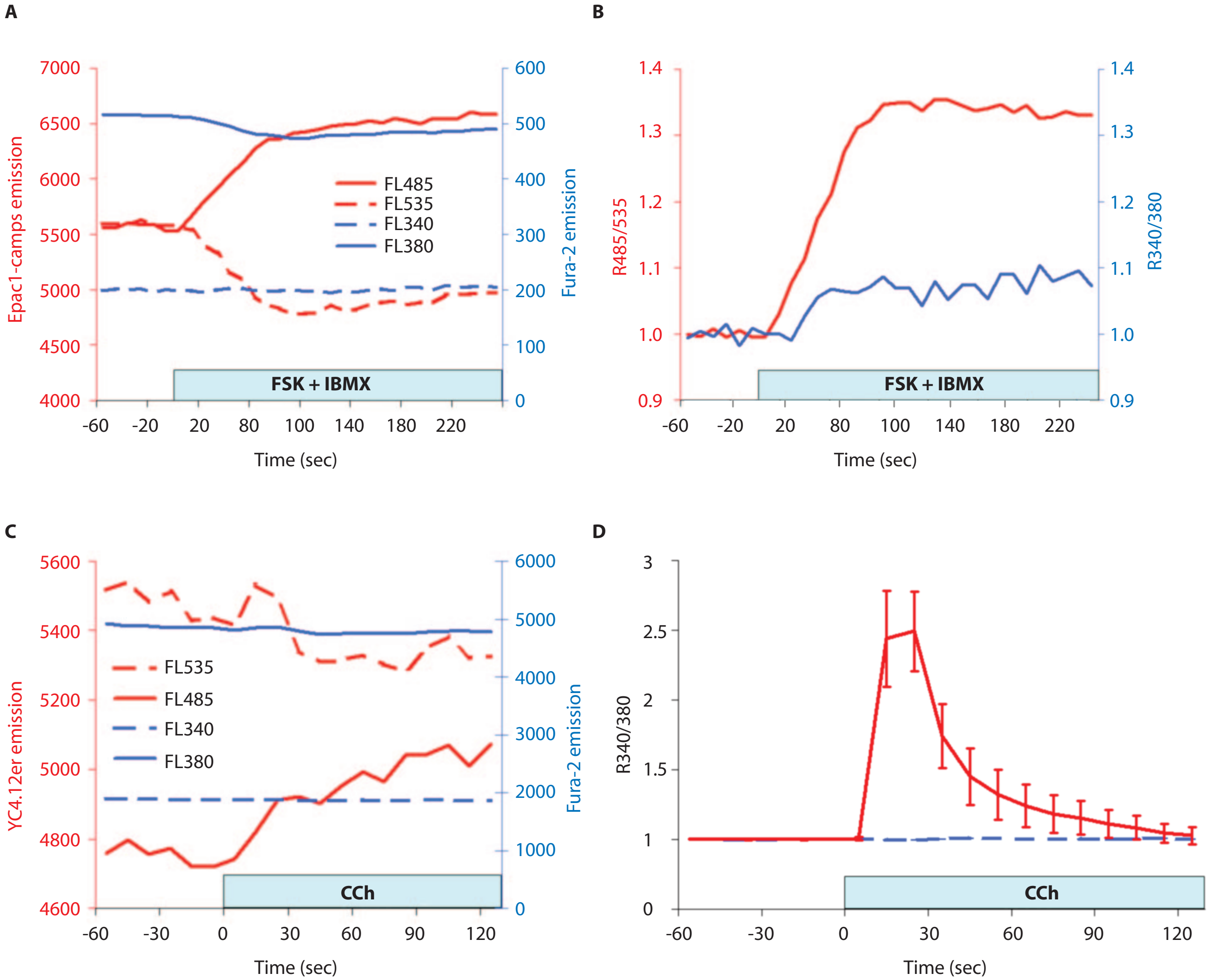

Fig. 2. Fluorescence emission of ECFP and EYFP does not interfere with Fura-2 measurements. Insulin-secreting βTC6 cells not loaded with Fura-2 but transiently expressing Epac1-camps or YC4.12er were imaged at the excitation and emission wavelengths used to measure Fura-2 (excitation: 340 nm and 380 nm; emission: 520 nm) and FRET (excitation: 436 nm; emission: 485 nm and 535 nm; see "Instructions" for details). (**A**) Time-dependent change in Epac1-camps FRET donor (ECFP; FL485) and acceptor (EYFP; FL535) fluorescence after application of 100 μM forskolin (FSK) and 100 μM IBMX. Traces illustrate the average fluorescence intensity of Epac1-camps FRET recorded at 485 nm (red solid) and 535 nm (red hashed), and at 520 nm following excitation at 340 nm (blue hashed) and 380 nm (blue solid) from a single βTC6 cell. Epac1-camps FRET decreases as $[cAMP]_c$ increases. As expected, following the application of forskolin and the phosphodiesterase inhibitor IBMX, ECFP emission increased and EYFP emission decreased. This indicates a decrease in FRET. The fluorescence resulting from 340 nm and 380 nm excitation remained relatively constant. (**B**) FRET donor/acceptor fluorescence emission ratio (R485/535) and Fura-2 emission ratio (R340/380) relative to baseline, measured in the cell from (A). The increase in the 485/535 ratio indicates an increase in $[cAMP]_c$. (**C**) Time-dependent change in YC4.12er fluorescence after application of 250 μM carbachol (CCh). Traces illustrate the average fluorescence intensity of YC4.12er FRET recorded at 485 nm (red solid) and 535 nm (red hashed), and at 520 nm following excitation at 340 nm (blue hashed) and 380 nm (blue solid) from a single βTC6 cell. As is the case for Epac1-camps, the fluorescence resulting from 340 and 380 nm excitation remained relatively constant while the YC4.12er donor/acceptor emissions ratio decreased, indicating a decrease in $[Ca^{2+}]_{err}$. (**D**) Change in Fura-2 emission ratio (R340/380) relative to base-line in βTC6 cells transiently expressing YC4.12er following stimulation with 250 μM carbachol. Traces indicate cells loaded with (red solid) or without (blue hashed) Fura-2, and are the means ± standard error for $n \geq 16$ cells. Note that the R340/380 ratio remains unchanged in the YC4.12er-expressing cells that have not been labeled with Fura-2, further demonstrating that FRET donor and acceptor emission at 520 nm does not alter the Fura-2 FL340/380 ratio.

and 3B). This lack of spectral bleed-through appears to be a general property of other unimolecular FRET biosensors based on ECFP and EYFP. For example, in cells expressing YC4.12er alone, carbachol (a muscarinic receptor agonist that generates inositol 1, 4 to 5-trisphosphate by a phospholipase C-dependent mechanism), decreased FRET (indicative of reduction of ER Ca^{2+} concentration) without affecting fluorescence measured at excitation wavelengths used for Fura-2 imaging (Fig. 2C). Carbachol treatment of Fura-2-loaded cells transiently expressing YC4.12er produced a 2.5-fold increase in R340/380 (Fig. 2D). No increase in

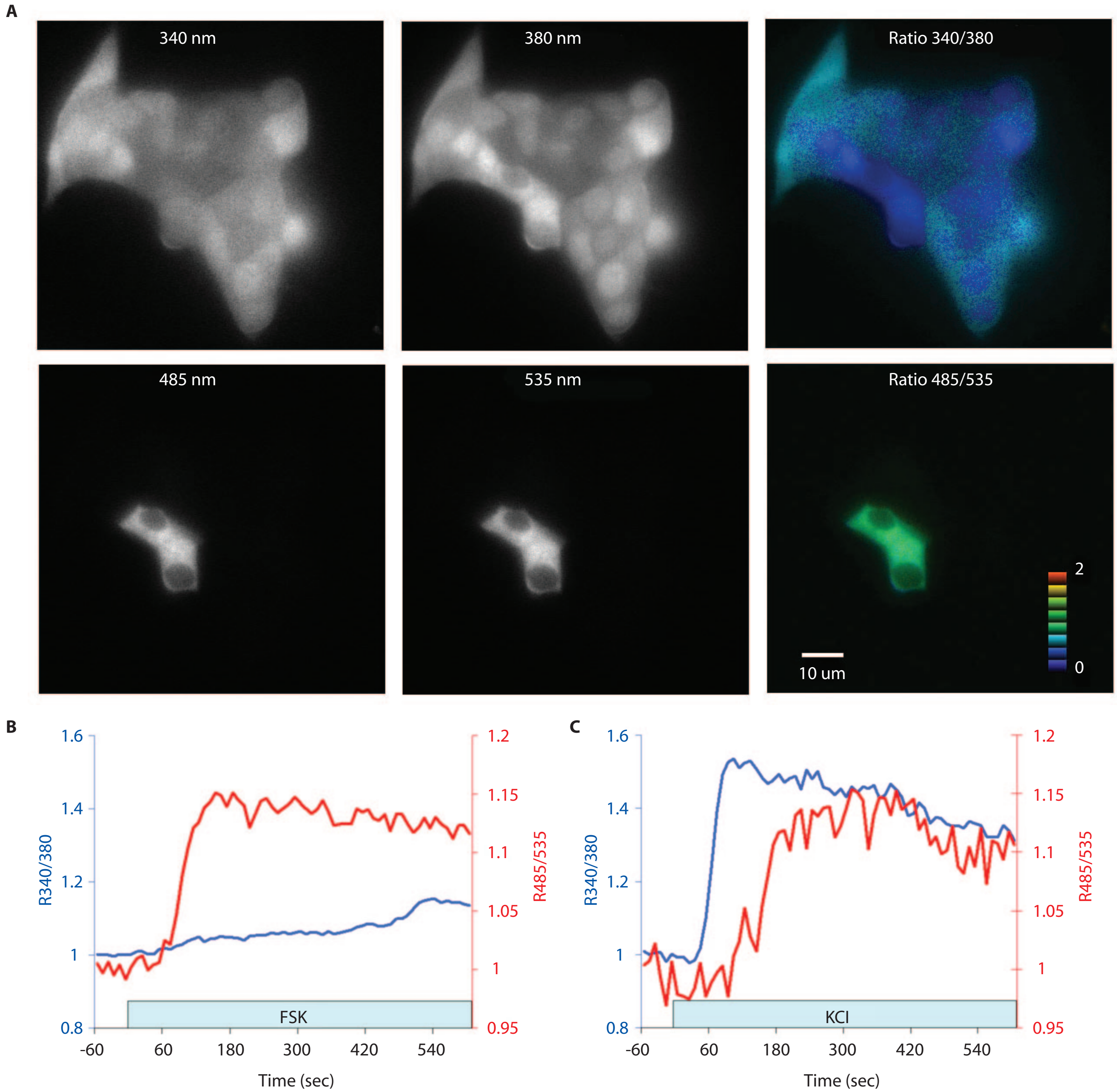

Fig. 3. Simultaneous imaging of cytoplasmic Ca^{2+} and cAMP in MIN6 cells. (**A**) 16-bit digital images of Fura-2-loaded MIN6 cells transiently expressing Epac1-camps. The image in each panel was recorded from the same field of view (see "Instructions" for details). The pseudocolored displays of $[Ca^{2+}]_c$ (Ratio 340/380 panel) and $[cAMP]_c$ (Ratio 485/535 panel) show diffuse labeling of all cells with Fura-2 and only two cells expressing Epac1-camps. Note the spectral separation between the fluorescent indicators; it is easy to distinguish Epac1-camps expression in cells loaded with Fura-2. The color bar indicates the range of Epac1-camps FRET 485/535 nm ratio values depicted in the pseudocolored display. Scale bar indicates 10 μm. (**B**) Effects of stimulating adenylyl cyclase with forskolin (FSK; 10 μM) on $[cAMP]_c$ (red line) and $[Ca^{2+}]_c$ (blue line) in a single MIN6 cell. The kinetics and amplitude of forskolin-induced cAMP production were not affected by Fura-2 loading. (**C**) 20 mM KCl-induced gradients of $[Ca^{2+}]_c$ and $[cAMP]_c$ in MIN6 cells. The traces are representative of cAMP and Ca^{2+} responses imaged simultaneously in individual cells ($n \geq 15$ cells for each treatment) [from (*15*) with permission].

R340/380, however, was observed in cells expressing YC4.12er, but without Fura-2 (Fig. 2D), further demonstrating that FRET biosensor absorbance at 340 nm and 380 nm does not cause substantial changes in the R340/380. The area under the R340/380 responses during a 2-min exposure to carbachol was 52.8 ± 16.4 for the Fura-2 loaded cells and 0.4 ± 0.2 for the cells without Fura-2 [expressed as means ± standard error ($n \geq 16$ cells)].

We also evaluated the effects of spectral overlap of Fura-2 fluorescence into the FRET signal. Using the optical filters described above, Fura-2 absorbance at 436 nm (the excitation for the FRET donor ECFP) was very low (Figs. 1 and 3). Excitation of Fura-2 at 436 nm produced fluorescence emission at 485 nm and 535 nm 1 to 4% above background levels. This extremely low emission of Fura-2 at the excitation wavelength used for the FRET-based biosensor imaging indicates little or no bleed-through from Fura-2 into the FRET wavelengths that could influence the FRET measurements. On the other hand, Fura-2, AM exhibits substantially greater absorbance at 436 nm than de-esterified Fura-2, and may cause spectral bleed-through and interfere with FRET imaging. We propose loading MIN6 cells with 500 nM Fura-2, AM, rather than 2 to 5 μM, to decrease the amount of residual esterified Fura-2 remaining in cells following the loading procedure, and reduce a potential source of spectral bleed-through between Fura-2 and the FRET biosensor.

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