Long-wavelength iodide-sensitive fluorescent indicators for measurement of functional CFTR expression in cells

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Am. J. Physiol. 277 (Cell Physiol. 46): C1008–C1018, 1999.—Limitations of available indicators [such as 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ)] for measurement of intracellular Cl– are their relatively dim fluorescence and need for ultraviolet excitation. A series of long-wavelength polar fluorophores was screened to identify compounds with Cl– and/or I– sensitivity, bright fluorescence, low toxicity, uniform loading of cytoplasm with minimal leakage, and chemical stability in cells. The best compound found was 7-[(β-D-ribofuranosylamino)-pyrido[2,1-h]-pteridin-11-ium-5-olate (LZQ). LZQ is brightly fluorescent with excitation and emission maxima at 400–470 and 490–560 nm, molar extinction 11,100 M–1·cm–1 (424 nm), and quantum yield 0.53. LZQ fluorescence is quenched by I– by a collisional mechanism (Stern-Volmer constant 60 M–1) and is not affected by other halides, nitrate, cations, or pH changes (pH 5–8). After LZQ loading into cytoplasm by hypotonic shock or overnight incubation, LZQ remained trapped in cells (leakage <3%/h). LZQ stained cytoplasm uniformly, remained chemically inert, did not bind to cytoplasmic components, and was photobleached by <1% during 1 h of continuous illumination. Cytoplasmic LZQ fluorescence was quenched selectively by I– (50% quenching at 38 mM I–). LZQ was used to measure forskolin-stimulated I–/Cl– and I–/NO3– exchange in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing cell lines by fluorescence microscopy and microparticle reader instrumentation using 96-well plates. The substantially improved optical and cellular properties of LZQ over existing indicators should permit the quantitative analysis of CFTR function in gene delivery trials and high-throughput screening of compounds for correction of the cystic fibrosis phenotype.

Cystic fibrosis transmembrane conductance regulator; cystic fibrosis; 6-methoxy-N-(3-sulfopropyl)quinolinium; chloride transport; fluorescence; high-throughput screening

SeveraL haloID-senSITIve fluorescent indicators have been introduced to study the functional properties of Cl– transporters in biomembrane vesicles, proteoliposomes, living cells, and epithelia (for review, see Refs. 28 and 30). The archetype indicator 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ) (11) is a polar quinolinium compound whose fluorescence is quenched by Cl– and I– by a collisional mechanism. SPQ and related quinolinium indicators have been useful in studying the cystic fibrosis transmembrane conductance regulator (CFTR) Cl– channel expressed in native and transfected cell models (4, 6, 7, 21, 22) and, recently, in assaying functional CFTR delivery in human gene therapy trials (10, 20). Various SPQ derivatives have been synthesized for specific applications including cell-permeable/trappable compounds (3), dual-wavelength Cl– indicators for ratio imaging (12), dextran-linked conjugates (1), and fiberoptic halide sensors (15).

Although used in numerous studies of CFTR function in cell culture models, the existing quinolinium-based halide indicators have imperfect optical and physical properties that limit their utility in more challenging applications, including the analysis of CFTR function in gene therapy trials and high-throughput drug screening. SPQ and related indicators have relatively dim fluorescence in cells (molar extinction <6,000 M–1·cm–1; quantum yield <0.1) and require ultraviolet excitation (excitation 320–365 nm; emission 420–460 nm). Significant technical limitations include the need for sensitive detection instrumentation with high numerical aperture optics, and photodynamic cell injury and background autofluorescence resulting from ultraviolet excitation. In addition, the quinolinium halide indicators are quenched by intracellular proteins and organic anions, resulting in decreased indicator sensitivity to cytoplasmic Cl– and undesired indicator sensitivity to cell volume changes (5, 26). We previously synthesized long-wavelength Cl– indicators containing the acridinium chromophore (2); although these indicators are useful to measure Cl– transport in liposomes and biomembrane vesicles, they are chemically unstable in cytoplasm, a problem that could not be overcome by derivatization.

The purpose of this project was to identify/synthesize bright, long-wavelength fluorescent halide indicators for assay of CFTR-mediated anion conductance in living cells. A specific goal was to develop a sensitive, robust anion transport assay suitable for high-throughput drug screening. The desired specifications of the
indicator(s) included: high Cl\textsuperscript{-} and/or I\textsuperscript{-} sensitivity, bright fluorescence with excitation wavelength > 400 nm and emission wavelength > 500 nm, minimal photobleaching, no cellular toxicity, rapid loading into cytoplasm, uniform distribution and chemical stability in cytoplasm, and slow leakage out of cells. A series of candidate fluorescent compounds were screened for these strict criteria. The compound luminarosine (LZQ), a pyrido[2,1-h]-pteridin, satisfied all of the above requirements. LZQ was characterized in vitro and in CFTR-expressing cells, and a microplate reader assay of functional CFTR was established.

METHODS

Materials. Forskolin, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt (CPT-cAMP), IBMX, nigericin, valinomycin, and carbonyl cyanide m-chlorophenylhydrazone were purchased from Sigma (St. Louis, MO). Rhodamine 110 and rhodamine green were purchased from Molecular Probes (Eugene, OR). Acridine yellow, acridine orange, 9-aminoacridine, 6-aminoquinoline, benzo[c]cinoline, phenazine, resorufin, and phenosafranine were obtained from Aldrich (Milwaukee, WI). 2,7-Diphenylpyrido[3,2-g]quinoline was provided by Dr. Helmut Quast, University of Wuerzburg, Germany.

Organic synthesis. Benzo[c]cinoline and phenazine were quaternized with methyl iodide to give compounds V and VI, respectively (see Fig. 1 and Table 2). Compound VII was synthesized by reaction of 2,7-diphenylpyrido[3,2-g]quinoline with trimethyloxonium tetrafluoroborate. Acridine yellow and acridine orange were quaternized with dimethyl sulfate to give compounds IX and X, respectively. 6-Aminoquinoline and 9-aminoacridine were quaternized with propane sulfone to give compounds XI and XII, respectively.

Compounds I (LZQ) and II (LMQ) were synthesized by phototransformation of N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride in aqueous solutions (24, 25). 2',3',5'-Tri-O-acetyl-inosine (0.5 mmol) was dissolved in 2.5 ml of dry pyridine and reacted with 0.75 mmol 4-chlorophenyl dichlorophosphate in the dark for 24 h at room temperature. Pyridine was then removed by evaporation, and the product N-[9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride was dissolved in water, neu-
formamido-6-(2N
irradiated at
solution of this compound (at pH 5.8–6.2) was deaerated and
confirmed by TLC (yield 70%). Two liters of a 0.3 mM aqueous
tralized, washed with chloroform, and lyophilized. Purity was
Table 1. Buffer compositions and protocols for perfusion experiments

<table>
<thead>
<tr>
<th>Protocol</th>
<th>1st Buffer Added</th>
<th>2nd Buffer Added</th>
<th>3rd Buffer Added</th>
<th>4th Buffer Added</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS + forskolin (20 µM)</td>
<td>Buffer A (100 mM I⁻) + forskolin (20 µM)</td>
<td>KSCN (150 mM)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Buffer A (100 mM I⁻)</td>
<td>PBS</td>
<td>PBS + cAMP stimulation</td>
<td>KSCN (150 mM)</td>
</tr>
<tr>
<td>3</td>
<td>PBS</td>
<td>Buffer B (50 mM I⁻)</td>
<td>PBS</td>
<td>Buffer B (50 mM I⁻)</td>
</tr>
<tr>
<td>4</td>
<td>Buffer C (20 mM I⁻)</td>
<td>Buffer D (NO₃⁻)</td>
<td>Buffer D (NO₃⁻) + cAMP stimulation</td>
<td>KSCN (150 mM)</td>
</tr>
<tr>
<td>5</td>
<td>Buffer D (NO₃⁻) + forskolin (20 µM)</td>
<td>Buffer C (20 mM I⁻) + forskolin (20 µM)</td>
<td>KSCN (150 mM)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>PBS + forskolin (20 µM)</td>
<td>Buffer D (NO₃⁻) + forskolin (20 µM)</td>
<td>KSCN (150 mM)</td>
<td>Buffer D (150 mM NaNO₃)</td>
</tr>
<tr>
<td>7</td>
<td>Buffer D (NO₃⁻)</td>
<td>Buffer D + water (3:1)</td>
<td>Buffer D (NO₃⁻)</td>
<td>Buffer D + 150 mM NaNO₃</td>
</tr>
</tbody>
</table>

Buffer compositions (in mM) are as follows. PBS: 137 NaCl, 2.7 KCl, 0.9 CaCl₂, 0.5 MgCl₂, 8 NaH₂PO₄, 1.5 KH₂PO₄. Buffer A: 100 NaI, 37 NaCl, 2.7 KCl, 0.9 CaCl₂, 0.5 MgCl₂, 8 NaH₂PO₄, 1.5 KH₂PO₄. Buffer B: 50 NaI, 87 NaCl, 2.7 KCl, 0.9 CaCl₂, 0.5 MgCl₂, 8 NaH₂PO₄, 1.5 KH₂PO₄. Buffer C: 20 NaI, 117 NaNO₃, 2.7 KNO₃, 0.9 Ca(NO₃)₂, 0.5 Mg(NO₃)₂, 8 NaH₂PO₄, 1.5 KH₂PO₄. Buffer D: 137 NaNO₃, 2.7 KNO₃, 0.9 Ca(NO₃)₂, 0.5 Mg(NO₃)₂, 8 NaH₂PO₄, 1.5 KH₂PO₄.

tralized, washed with chloroform, and lyophilized. Purity was
cirmed by TLC (yield 70%). Two liters of a 0.3 mM aqueous
olution of this compound (at pH 5.8–6.2) was deaerated and
irradiated at >300 nm wavelength in a Pyrex, air-cooled
reactor. After conversion to the intermediate N-[5-
formamido-6-(2',3',5',6'-trioctyl-1N-2H-pyridin-6-y)
pyridinium chloride was complete as determined by TLC, a
concentrated aqueous solution of the sensitizer (9-methylpu
rin-6-yl) pyridinium perchloride (0.15 M) was added and
irradiation was continued after adjusting the pH to 7.5.
Photoconversion to the bright yellow 2',3',5',6'-trioctyl-
5
larosine was checked by absorption spectrophotometry.

Cell culture. For fluorescence microscopy experiments, 3T3
fibroblasts (control and CFTR expressing), T84 cells, and
Calu-3 cells were cultured on 18-mm round coverslips in
DMEM/H21 medium supplemented with 5% FCS (3T3 fibro-
blasts) or 10% FCS (Calu-3 cells), penicillin (100 U/ml), and
streptomycin (100 µg/ml). T84 cells were grown in DMEM/ F-
12, Ham’s nutrient mix (1:1) containing 5% FCS, penicillin
(streptomycin (100 µg/ml), and streptomycin (100 µg/ml). Cells were grown at 37°C in 95% air-5% CO2 and used when 90% confluent. For microplate reader assays cells were cultured on Costar 96-
well black plates with a clear flat bottom and used when
nearly confluent.

Fluorescence microscopy. Fluorescence microscopy measure-
ments were performed as described previously (5). Briefly,
coverglasses were mounted in a 0.5-ml perfusion chamber in
which the cell-free glass surface made contact with an oil
immersion objective (Nikon ×40 magnification, numerical
aperture (NA) 1.3). Cell fluorescence was excited at 365 ± 10
nm (SPQ) or 420 ± 10 nm (LZQ and LMQ). Emitt
e fluorescence was detected by a photomultiplier in a Nikon inverted
epi-epifluorescence microscope using a 410-nm dichroic mirror
and 420-nm barrier filter (SPQ) or a 455-nm dichroic
and 500-nm barrier filter (LZQ). For cells labeled with SPQ
and LZQ, SPQ fluorescence was excited at 365 ± 10 nm and
was detected using a 410-nm dichroic mirror and 455 ± 30
nm interference filter. Confocal fluorescence micrographs
were obtained using a Nipkow wheel confocal microscope and
cooled charge-coupled device camera detector using a ×60 oil

Table 2. Optical and cellular properties of some analyzed compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>λ_ex (nm)</th>
<th>λ_em (nm)</th>
<th>Ksv (I⁻) (M⁻¹)</th>
<th>Brightness</th>
<th>Cellular Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Luminarosine</td>
<td>424</td>
<td>528</td>
<td>60</td>
<td>+++</td>
<td>Staining uniform; leakage rate very low; slow photobleaching</td>
</tr>
<tr>
<td>II</td>
<td>Luminarine</td>
<td>424</td>
<td>530</td>
<td>70</td>
<td>+++</td>
<td>Staining uniform; leakage rate moderate; slow photobleaching</td>
</tr>
<tr>
<td>III</td>
<td>Rhodamine-110</td>
<td>496</td>
<td>520</td>
<td>40</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>IV</td>
<td>Rhodamine green</td>
<td>505</td>
<td>530</td>
<td>30</td>
<td>+++</td>
<td>Accumulates in mitochondrial compartments</td>
</tr>
<tr>
<td>V</td>
<td>1-Methyl-ber-teenolinium</td>
<td>416</td>
<td>505</td>
<td>130</td>
<td>++</td>
<td>Uniform cytoplasmic staining but stains nucleus also; photobleached rapidly</td>
</tr>
<tr>
<td>VI</td>
<td>1-Methyl-phenazinium</td>
<td>440</td>
<td>520</td>
<td>90</td>
<td>++</td>
<td>Uniform cytoplasmic staining but also stains nucleus</td>
</tr>
<tr>
<td>VII</td>
<td>1-Methyl-2,7-diphenylpyrido[3,2,1-g] quinolinium</td>
<td>405</td>
<td>515</td>
<td>46</td>
<td>+++</td>
<td></td>
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<tr>
<td>VIII</td>
<td>Resorufin</td>
<td>571</td>
<td>585</td>
<td>76</td>
<td>+++</td>
<td>Accumulates in intracellular vesicles</td>
</tr>
<tr>
<td>IX</td>
<td>10-Methyl acridinium yellow</td>
<td>440</td>
<td>520</td>
<td>90</td>
<td>+++</td>
<td>Strong nuclear staining; very rapid photobleaching</td>
</tr>
<tr>
<td>X</td>
<td>10-Methyl acridinium orange</td>
<td>440</td>
<td>550</td>
<td>30</td>
<td>+++</td>
<td>Strong nuclear staining; very rapid photobleaching</td>
</tr>
<tr>
<td>XI</td>
<td>6-Amino-1-(3-sulfopropyl)quinolinium</td>
<td>396</td>
<td>545</td>
<td>240</td>
<td>+</td>
<td>Uniform cytoplasmic staining; rapid photobleaching</td>
</tr>
<tr>
<td>XII</td>
<td>9-Amino-10-(3-sulfopropyl)acridinium</td>
<td>422</td>
<td>457</td>
<td>253</td>
<td>+++</td>
<td>Nuclear staining; rapid photobleaching</td>
</tr>
<tr>
<td>XIII</td>
<td>Phenosafranine</td>
<td>520</td>
<td>586</td>
<td>33</td>
<td>++</td>
<td>Accumulates in intracellular vesicles</td>
</tr>
</tbody>
</table>

λ_ex, Excitation wavelength; λ_em, emission wavelength; Ksv, Stern-Volmer quenching constant; ++++, very bright; ++++, bright; ++, moderate brightness; +, weak.
immersion objective (Nikon, NA 1.4). LZQ-labeled cells were viewed using a fluorescein filter set (excitation 480 ± 15 nm, emission 520 ± 20 nm).

Fluorescence microplate reader measurements. Microplate reader measurements were performed in a BMG Fluostar microplate reader (BMG LabTechnologies, Durham, NC) equipped with two syringe pumps for automated solution additions. After cell loading with LZQ, extracellular LZQ was washed using a Labsystems Cellwash-4 (Franklin, MD). LZQ fluorescence was excited using a liquid fiberoptic and 425 ± 15 nm interference filter (HQ, Chroma Optical), and emitted fluorescence was collected using a liquid fiberoptic and 530 ± 15 nm filter. The fiberoptic was positioned just below the plate. Fluorescence was recorded continuously in 2-s intervals, each representing the average of 100 20-µs pulses of the xenon illumination lamp source.

Transport measurements. Table 1 lists the solution compositions and protocols for the transport measurements. Cells were labeled with LZQ or SPQ by hypotonic shock (loading buffer/water 1:1 containing 2 mM LZQ or 7.5 mM SPQ) for 5 min at room temperature or by overnight incubation with indicators in the cell culture medium. Extracellular indicator was removed by washing just before measurements. For fluorescence microscopy, glass coverslips were then mounted in a perfusion chamber (flow 2 ml/min) in which solutions were exchanged using a four-way valve. For microplate reader measurements, cells in 96-well plates were bathed in 20 µl of the first buffer (see Table 1), and 160 µl of the Cl−-containing, I−-free solution was injected into the well to drive Cl−/I− exchange (protocol 1). For stimulation of CFTD using protocol 2, 60 µl of a 1:8 mixture of the first buffer and the second buffer containing 80 µM forskolin, 2 mM CPT-cAMP, and 400 mM IBMX were added.

Time-resolved fluorescence measurements. Fluorescence lifetime and anisotropy decay measurements were carried out in the frequency domain by cuvette fluorometry using a Fö rier transform fluorometer (48000 MHF; SLM Instruments, Urbana, IL) or by fluorescence microscopy using epi fluorescence microscope optics in place of the cuvette compartment (29). For microscopy measurements, impulse-modulated vertically polarized light (442 nm, He-Cd laser 35 mW) was reflected onto the sample by a 510-nm dichroic mirror and objective lens; emitted fluorescence was filtered by a 515-nm barrier filter and passed through a rotatable analyzing calcite polarizer. In some experiments, a biconvex lens was introduced just in front of the dichroic mirror to diverge beam diameter in the focal plane to ~40 µm.

Analysis of lifetime and time-resolved anisotropy was performed by a comparative approach. Generally six pairs of measurements (each acquisition 8 s) were obtained, comparing sample and reference (fluorescein in 0.1 N NaOH, lifetime 4.0 ns) for measurement of lifetime, and parallel and perpendicular orientations of the emission polarizer for measurement of anisotropy decay. The phase-modulation data consisted of phase angles and modulation ratios at 40 discrete, equally spaced modulation frequencies (5–200 MHz). The analyzing polarizer was positioned at the magic angle for lifetime measurements. Additional details of the data acquisition and analysis routines were described previously (29). Median phase and modulation values for paired data were analyzed by nonlinear least squares for determination of lifetimes and rotational correlation times.

Computations. In vitro fluorescence quenching studies were carried out at peak excitation and emission wavelengths. Microliter aliquots of the sodium salt of the quenching anion (1 M) were added to indicator solution in 5 mM sodium phosphate (pH 7.2, unless indicated otherwise). Fluorescence intensities in the absence (F₀) and presence (F) of quencher anion ([Q]) were measured to give the Stern-Volmer equation:

\[
\frac{F_o}{F} = \frac{1}{K_q} + \frac{1}{K_q}[Q]
\]

where Fo is fluorescence intensity in the absence of I− or Cl−, F is fluorescence intensity in the presence of I− or Cl−, and Kq is the intracellular quenching constant for I− or Cl−.

RESULTS

Long-wavelength fluorophores were screened for sensitivity to Cl− and I− and for intracellular properties (loading, intracellular distribution, leakage, stability). Table 2 summarizes the optical and cellular properties of some of the fluorophores tested. The available Cl−...
indicators are based on fluorescence quenching of the quinolinium chromophore and similar positively charged heterocycles. Various classes of compounds were tested with extended conjugation, one or more heteroatoms, and linear or angular geometry. Several rhodamines were tested based on reports indicating rhodamine sensitivity to I\(^-\) (31). Although several of the compounds tested showed good sensitivity to Cl\(^-\) (compounds V, VI) and I\(^-\) in vitro, they were not useful as intracellular indicators because of instability, nonuniform intracellular distribution, and/or rapid leakage out of cells as given in Table 2. Of the compounds tested, the pyrido[2,1-h]-pteridins LZQ and LMQ had potentially useful optical and cellular properties and were characterized further.

Figure 2A shows the fluorescence spectra of LZQ. LZQ (and LMQ, not shown) have broad excitation and emission peaks with maxima at 424 and 528 nm, respectively. The molar extinction coefficient of LZQ was 11,100 M\(^-1\)cm\(^-1\) at 424 nm, and the quantum yield in the absence of I\(^-\) was 0.53. Figure 2B is a Stern-Volmer plot for quenching of LZQ and LMQ by I\(^-\) and Cl\(^-\), with data for SPQ shown for comparison. In water (or saline), LZQ fluorescence was quenched by I\(^-\) with a Stern-Volmer constant of 60 M\(^-1\). LZQ fluorescence was not quenched (Stern-Volmer constant, <1 M\(^-1\)) by Cl\(^-\), NO\(_3\), phosphate, acetate, Na\(^+\), and K\(^+\). For comparison, Stern-Volmer constants for SPQ quenching are 118 M\(^-1\) (Cl\(^-\)) and 282 M\(^-1\) (I\(^-\), not shown). LZQ fluorescence and I\(^-\) sensitivity were not affected by pH changes in the range 4.5–8. Fluorescence lifetime analysis indicated a 7.1-ns lifetime for LZQ in the absence of I\(^-\), which decreased in proportion to fluorescence intensity with increasing [I\(^-\)], indicating a collisional mechanism. Stopped-flow measurements showed that LZQ fluorescence responds in 1 ms to rapid addition and removal of I\(^-\) (not shown), as expected for a collisional quenching mechanism.

LZQ and LMQ were loaded into cells efficiently by hypotonic shock or passive incubation. The confocal fluorescence micrograph in Fig. 3B shows LZQ fluorescence labeling of the aqueous compartments in cytoplasm and nucleus. The green/yellow LZQ fluorescence was quite uniform and remarkably more intense than the blue fluorescence of SPQ (Fig. 3A), even though a substantially higher SPQ concentration was used. All cell types tested, including epithelial cells (T84, Calu-3, JME, LLC-PK\(_1\), Madin-Darby canine kidney) and non-epithelial cells (3T3 fibroblasts, Chinese hamster ovary cells, HeLa cells), could be labeled with LZQ and showed uniform labeling of the cytoplasm and nucleus. As was found for SPQ, LZQ and LMQ were nontoxic to cells in assays of cell growth (2 mM in culture medium for 72 h). TLC of lysates from LZQ-loaded cells showed that LZQ remained chemically stable in cells.

It was found that LZQ had better cytoplasmic retention properties than LMQ, probably because of its sugar moiety. Figure 4A, top, shows the time course of LZQ fluorescence during perfusion with physiological saline at 23°C. A small group of 20–30 cells was illuminated continuously, and fluorescence was detected by a photomultiplier using a ×40, 1.3 NA objective. Cellular fluorescence decreased slowly at a rate of 2.5%/h. To determine the relative contributions of indicator leakage vs. photobleaching, the perfused cells were illuminated intermittently (Fig. 4A, bottom). The decline in fluorescence during the illumination periods was almost negligible, indicating that photobleaching was the dominant cause of fluorescence loss.

**Fig. 3.** Confocal fluorescence micrographs of Swiss 3T3 fibroblasts labeled with SPQ (A) and LZQ (B). Cells were loaded by hypotonic shock in PBS-water (1:1) containing 2 mM LZQ or 7.5 mM SPQ. Images were acquired and printed under identical conditions to compare brightness. Scale bar, 10 µm.
cence was not different from that during continuous illumination, indicating the absence of photobleaching under the low-light-level conditions employed here. Similar LZQ loading and leakage properties were found for multiple different epithelial and nonepithelial cells, as well as for cells grown on plastic and on porous Transwell filters. Figure 4B shows the time course of cellular LZQ fluorescence in response to repeated addition and removal of 50 mM I\(^{-}\). There were large, reversible changes in fluorescence with signal-to-noise ratios generally \(\gtrsim 500:1\). Background fluorescence (in nonlabeled cells) was generally \(\lesssim 5\%\) of the fluorescence of LZQ-labeled cells.

A calibration study was done to determine the intracellular sensitivity of LZQ to I\(^{-}\). 3T3 fibroblasts expressing CFTR were perfused with solutions containing high K\(^{+}\) concentration and ionophores to equalize solution and intracellular I\(^{-}\). LZQ fluorescence changed reversibly in response to changes in solution I\(^{-}\) (Fig. 4C). Figure 4D shows the Stern-Volmer analysis indicating a Stern-Volmer constant of 26 M\(^{-1}\) for quenching of intracellular LZQ by I\(^{-}\), which is better than that of 12–18 M\(^{-1}\) for quenching of intracellular SPQ by Cl\(^{-}\) (5). Fluorescence lifetime analysis was done to determine whether LZQ fluorescence is quenched in cells by substances other than I\(^{-}\). Nanosecond lifetimes were measured by frequency-domain microfluorometry. Figure 5A shows a phase-modulation plot indicating a single-component LZQ lifetime in cells of 6.5 ± 0.3 ns (\(n = 4\)) in the absence of I\(^{-}\), close to that of 7.1 ns for LZQ in water. This finding contrasts with results for SPQ, where the SPQ fluorescence lifetime was eightfold decreased in cells (in the absence of Cl\(^{-}\)) vs. water (5). It is concluded that LZQ fluorescence is quenched little by intracellular components, a significant advantage over SPQ.

The similar LZQ lifetime in cells and water predicts that LZQ fluorescence should be relatively insensitive to cell volume in the absence of I\(^{-}\). Figure 5B compares the time course of intracellular SPQ and LZQ fluorescence in response to osmotically induced changes in cell volume in the absence of Cl\(^{-}\). Although SPQ fluorescence decreased by 30 ± 2% (SE; \(n = 3\)) on exposure to 600 mosmol/kg\(_2\)O, LZQ fluorescence changed by only 5 ± 1% (in the absence of I\(^{-}\)). When cells were exposed to hyposmotic medium (225 mosmol/kg\(_2\)O), SPQ fluo-
and nucleoplasm and binds little to intracellular proteins and lipids. Time-resolved anisotropy was measured to quantify intracellular LZQ binding. In water, LZQ rotated freely with a single-component rotational correlation time of 121 ± 3 ps (n = 3; not shown). For LZQ in cells, a plot of differential phase and modulation amplitude ratio (Fig. 5C) indicated a two-component anisotropy decay model with a major component (fractional amplitude 0.90–0.94) of 144 ± 10 ps, similar to that in water. Together these results indicate little binding of LZQ to intracellular components.

The utility of LZQ for functional measurement of CFTR expression was tested. Figure 6A compares the time course of LZQ and SPQ fluorescence in forskolin-stimulated CFTR-expressing cells using identical I⁻/NO₃⁻ exchange protocols. Although the amplitudes and curve shapes of the data differed for SPQ vs. LZQ because of unequal indicator Stern-Volmer constants, the computed I⁻ influx rates [SPQ: 0.26 ± 0.03 mM/s; LZQ: 0.25 ± 0.01 mM/s (SE, n = 3)] and efflux rates (SPQ: 0.08 ± 0.01 mM/s; LZQ: 0.09 ± 0.03 mM/s) were not different. Figure 6B shows a comparison of data obtained for cells loaded with LZQ and SPQ initially loaded with I⁻, followed by replacement of I⁻ with NO₃⁻ in the absence of forskolin, followed by addition of forskolin. The initial rates of forskolin-stimulated I⁻ efflux (SPQ: 0.09 ± 0.02 mM/s; LZQ: 0.08 ± 0.01 mM/s) were similar. Measurements of Cl⁻/NO₃⁻ exchange were also carried out by measuring SPQ fluorescence using cells labeled with SPQ alone vs. cells colabeled with SPQ and LZQ (Fig. 6C). The presence of LZQ (which is quenched by I⁻ but not by Cl⁻) did not affect the time course of SPQ fluorescence, indicating that LZQ did not itself affect CFTR-mediated Cl⁻ transport.

Because LZQ fluorescence is sensitive to I⁻ but not to Cl⁻, it was possible to measure Cl⁻/I⁻ exchange without the need to introduce NO₃⁻. Figure 7A shows the kinetics of forskolin-stimulated Cl⁻ efflux on replacement of solution Cl⁻ by I⁻ measured by LZQ fluorescence. A rapid decrease in LZQ fluorescence was seen in the CFTR-expressing fibroblasts and T84 cells but not in control fibroblasts. Figure 7B shows cAMP-stimulated I⁻ efflux in response to replacement of solution I⁻ by Cl⁻ and addition of cAMP agonists. There was a slow increase in fluorescence on solution exchange, representing basal anion transport, followed by a prompt increase in LZQ fluorescence on addition of cAMP agonists to the CFTR-expressing cell types.

The low rate of LZQ leakage out of cells and its bright long-wavelength fluorescence permitted measurements of CFTR-mediated Cl⁻/I⁻ or NO₃⁻/I⁻ exchange in a fluorescence microscope reader with cells cultured directly on 96-well plastic dishes. To establish a protocol for the microscope reader assay, the background fluorescence of clear flat-bottom, 96-well plates obtained from different vendors was measured at LZQ excitation and emission wavelengths. Fluorescence signals [in instrument units ± SD (to assess interwell variability) at gain typically used in transport assays] were 12,258 ± 386 (Falcon clear); 9,749 ± 568 (Nunc clear); 23,167 ± 755 (Greiner white); 39,782 ± 555 (Falcon white); 8,955 ± 116 (Packard Polyfibronics black); 9,582 ± 211.
(Falcon black); 6,989 ± 192 (Nunc black); 6,420 ± 562 (Greiner black); and 5,068 ± 268 (Costar black). Costar black plates were used for subsequent studies because of their relatively low background signal and interwell variability. Background fluorescence was generally 5- to 10-fold lower than cellular LZQ fluorescence, with very little (<2%) instrument background (with 96-well plate removed) or cellular autofluorescence (measured in unlabeled cells). Another technical consideration was selecting the flow rate and fluid volume added by the syringe pumps to adapt the microscopy protocol in Fig. 7 to the microplate reader. Exchange of I\(^-\) for Cl\(^-\) was effected in the microplate reader by diluting the I\(^-\)-containing buffer with a Cl\(^-\) buffer. To minimize rapid signal changes due to a rise in meniscus level and the associated light scattering, cells were initially bathed in a minimum 20-µl vol of the first buffer (Table 1) and diluted with 160 µl of the second buffer. The rates of fluid addition by the automated syringe pump was maintained at 150 µl/s to avoid bubbles (causing light scattering) and displacement of the cells from the flat-bottomed plastic well.

Figure 8 shows microplate reader data. Cells were loaded with 2 mM LZQ by a 5-min hypotonic shock, and extracellular LZQ was removed by rinsing the wells. Protocols 1 and 2 (Table 1) were compared for measurement of CFTR-stimulated I\(^-\) transport. Figure 8A shows rapid I\(^-\) influx in the CFTR-expressing fibroblasts and T84 cells but not in control fibroblasts. Figure 8B shows cAMP-stimulated I\(^-\) efflux in T84 cells but not in a cell line (JME) expressing the ΔF508 CFTR. Although the fluorescence signal-to-noise ratio of the microplate studies was inferior to the microscopy data in Fig. 7, cAMP-stimulated CFTR-mediated transport was clearly detected. As described in the discussion, technical improvements should further increase signal-to-noise ratio and thus assay sensitivity.

**DISCUSSION**

The goal of this study was to identify bright, long-wavelength halide indicators suitable for analysis of CFTR function in gene delivery trials and high-throughput drug discovery. With quinolinium-type Cl\(^-\) indicators as a starting point, the strict optical and cellular requirements of the indicator as outlined in the Introduction limited the types of potentially useful compounds. The best compound identified, LZQ, was I\(^-\) sensitive, brightly green fluorescent, stable and uniformly distributed in cells, and well retained in cells. The substantially improved optical properties and cell retention of LZQ compared with existing halide indicators (like SPQ, 6-methoxy-N-ethylquinolinium, MQAE) permitted measurements of CFTR-mediated Cl\(^-\)-I\(^-\) exchange with excellent signal-to-noise ratio without the need for signal detection by microscopy.
LZQ fluorescence was quenched by I\(^{-}\) by a collisional mechanism in which its nanosecond fluorescence lifetime decreased in proportion to quantum yield. A collisional quenching mechanism implies a rapid, reversible response of LZQ fluorescence to changes in [I\(^{-}\)] without static LZQ-I\(^{-}\) binding. Recently, analysis of the quenching mechanism of quinolinium-type indicators by anions indicated a charge-transfer mechanism in which electron transfer occurs during the formation of a transient indicator-anion complex (13). Because the efficiency of a charge-transfer quenching mechanism depends on anion redox potential (19, 23), quinolinium indicators are quenched significantly by a wide variety of anions, including intracellular organic anions and proteins. In contrast, the selective quenching of LZQ fluorescence by I\(^{-}\) suggests that fluorescence quenching is dominated by a heavy-atom quenching mechanism rather than a charge-transfer mechanism (17). The specificity of LZQ fluorescence quenching by I\(^{-}\) was supported by the similar nanosecond fluorescence lifetimes of LZQ in cells vs. water and by the relative insensitivity of intracellular LZQ fluorescence to changes in cell volume. The specificity of LZQ fluorescence quenching constitutes a distinct advantage over SPQ.

LZQ was readily loaded into the aqueous compartment of cytoplasm by a brief hypotonic shock or by overnight incubation. Loading by either method produced brightly stained cells with excellent spatial uniformity, as judged by confocal microscopy for epithelial cells. LZQ has the most uniform intracellular distribution of the many dyes we have studied. Time-resolved anisotropy measurements indicated that the majority of LZQ molecules rotated freely and thus were not bound to cytoplasmic components. Therefore, apart from its use as an intracellular halide indicator, LZQ appears to be an excellent aqueous-phase probe for analysis of cytoplasmic rheology (9) and comparative dye distribution. The calibration of intracellular LZQ fluorescence vs. I\(^{-}\) concentration gave a Stern-Volmer constant of 26 M\(^{-1}\), indicating that LZQ fluorescence is 50% quenched by 38 mM I\(^{-}\). The lower apparent...
Stern-Volmer constant for LZQ quenching by I– in cells vs. water may be related to the crowded/mildly viscous intracellular environment and/or to imperfect equalization of cytoplasmic and external I– in the calibration procedures. The similar fluorescence lifetimes of LZQ in cells vs. water indicates that LZQ fluorescence quenching by components other than I– is not responsible for the decreased apparent Stern-Volmer constant. In any case, the strong sensitivity of LZQ fluorescence to intracellular I– permits measurements of Cl–/I– exchange using <10 mM I–. The high intracellular fluorescence of LZQ in combination with its red-shifted fluorescence spectra and I– selectivity make it superior to quinolinium-based Cl–-indicators for measurements of CFTR function.

Although we believe that LZQ is the best available halide indicator for measurement of CFTR function, a few potential concerns are noted. This report established the application of LZQ in a limited number of cell types. LZQ loading, leakage, and I– sensitivity should be established in each cell type and tissue studied. Although the measurement of CFTR-mediated I– transport (rather than Cl– transport) is an advantage because the non-CFTR cotransporters are generally Cl– selective, possible inhibitory effects of I– on CFTR function have been noted (27). Despite this potential concern, the majority of reported CFTR functional measurements utilizing SPQ fluorescence or tracer efflux use I– transport protocols (reviewed in Ref. 18). In addition, because LZQ is a single-wavelength indicator, it is not suitable for ratio imaging as would be required for measurements of I– transport by cell cytometry. As accomplished recently for the quinolinium-based Cl– indicators (12), chemical modification and/or chromophore conjugation will be needed to develop dual-wavelength I– indicators based on the LZQ chromophore.

Notwithstanding these concerns, LZQ should find applications in studies of CFTR gene delivery and drug screening. The use of SPQ has been problematic for CFTR transport in freshly isolated nasal or tracheal epithelial cells in gene therapy trials (reviewed in Ref. 18). The efficient incorporation of LZQ and its bright fluorescence in cells should make attractive the use of a fluorescent indicator assay as a surrogate marker for functional CFTR gene delivery. The application of LZQ for measurement of CFTR-mediated halide transport using automated microplate reader technology establishes the basis for high-throughput drug screening. The correction of ΔF508 CFTR mistrafficking by low temperature (8), chemical chaperones (3), and other agents (14) raises the exciting possibility that high-potency modifiers of the cystic fibrosis phenotype might be identified. The data here were obtained on a standard commercial fluorescence plate reader without modification. Substantial improvements in signal-to-noise ratio and assay sensitivity are anticipated on optimization of light source and detector stability, light collection optics, and fluid addition hardware.

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