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Characterization of a BODIPY-labeled fluorescent fatty acid analogue. Binding to fatty acid-binding proteins, intracellular localization, and metabolism

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Abstract

The BODIPY-labeled fatty acid analogues are a useful addition to the tools employed to study the cellular uptake and metabolism of lipids. In this study, we show that BODIPY FL C_{16} binds to purified liver and intestinal fatty acid-binding proteins with high affinity at a site similar to that for the physiological fatty acid oleic acid. Further, in human intestinal Caco-2 cells BODIPY FL C_{16} co-localizes extensively with mitochondria, endoplasmic reticulum/Golgi, and L-FABP. Virtually no esterification of BODIPY FL C_{16} was observed under the experimental conditions employed. We conclude that BODIPY FL C_{16} may be a useful tool for studying the distribution and function of FABPs in a cellular environment. (Mol Cell Biochem **299:** 67–73, 2007)

Key words: BODIPY, fatty acid-binding protein, FABP, fluorescence, microscopy

Abbreviations: BODIPY FL C_{16}: 4,4-difluoro-5, 7-dimethyl-4-bora-3a, 4adiaza-s-indacene-3-hexadecanoic acid; FABP: fatty acid-binding protein

Introduction

Long-chain fatty acids (LCFA) are ligands for selected nuclear receptors, essential components of phospholipids, triacylglycerols and cholesterol esters, and a major substrate for ATP production via β-oxidation [1]. The mechanisms of cellular LCFA uptake and transport remain controversial [2, 3] and the sub-cellular site(s) of LCFA localization under different physiological conditions have not been directly identified. In the study of cellular LCFA uptake radiolabeled ligands are often used, but for cellular localization studies, a separate approach is to use fluorescent lipid analogues; NBD-labeled lipids have been a method of choice in the past [4, 5]. However, BODIPY-labeled fluorophores are an appealing alternative to NBD-labeled lipids for a number of reasons: (1) BODIPY fluorophores are non-polar and therefore anchored better in lipid bilayers than NBD-labeled fluorophores (2), they are highly photo stable (3), have a strong absorption band in the blue-green region of the visible spectrum (4), display a high fluorescence quantum yield, and (5) BODIPY fluorophores are well suited to confocal fluorescence microscopy [6–9]. In this study we have investigated the fluorescence characteristics of a fluorescent long-chain fatty acid analogue (BODIPY FL C_{16}) and studied its binding to liver and intestinal fatty acid-binding proteins (L-FABP and I-FABP, respectively). Additionally, we have used the human enterocyte-like

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Caco-2 cell line to begin to investigate the cellular uptake, localization and metabolism of the BODIPY-labeled fatty acid probe.

Materials and methods

Materials

BODIPY FL C_{16}, fluorescein, fluorescent stains for subcellular organelles, i.e. Mitotracker, Lysotracker and BODIPY TR ceramide, and all other fluorescent compounds were obtained from Molecular Probes (OR, USA). The Caco-2 cell line was obtained from American Type Culture Collection (VA, USA). Polyclonal antibodies against human L-FABP were kindly provided by Dr. J. Veerkamp (University of Nijmegen, The Netherlands). PBS-CGM buffer contains 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM $NaHPO_4$, 10 μM $CaCl_2$, 200 μM $MgCl_2$ and 25 mM glucose, pH 7.5.

Purification of FABP

Recombinant rat FABPs were purified from bacterial cultures by hydrophobic interaction and gel filtration chromatography and delipidated by passage over a Lipidex column, as previously described [10].

Determination of dissociation constants (K_D) and binding stoichiometry

BODIPY FL C_{16} was titrated with FABP and the K_D determined by fitting the data to a hyperbolic equation ($F_{OBS} = F_{MAX} \times [FABP]/(K_D + [FABP])$), where F_{OBS} is the observed (measured) fluorescence intensity at each concentration of FABP and F_{MAX} is the fluorescence intensity at saturating concentrations of FABP.

Fluorescence quantum yields

The quantum yield for BODIPY FL C_{16} in several solvents, and when bound to FABPs, was determined. Fluorescence emission was measured at an excitation wavelength of 488 nm and emission wavelength of 500–550 nm and corrected for inner filter effects ($F_{CORR} = F_{OBS} \times$ antilog $((OD_{ex} + OD_{em})/2)$), where F_{CORR} and F_{OBS} are the corrected and observed fluorescence values, respectively. OD_{ex} and OD_{em} are the optical densities at the excitation and emission wavelengths, respectively. Fluorescence quantum yield was determined by using fluorescein, with a quantum yield of 0.85 in 0.1 M NaOH, as a reference standard [7, 11–13].

Cell culture

Stock cultures of Caco-2 cells were grown as previously described [14, 15]. For experiments the cells were plated on poly-lysine-treated coverslips at a density of 10^5 cells per 20 cm^2 well, in six-well tissue culture plates, and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1% nonessential amino acids at 37°C in air and 5% CO_2. The cells were generally confluent by day 8 and experiments were performed on day 24, at which time the cells are fully differentiated [15].

Microscopy

Fluorescence microscopy was performed on a Zeiss LSM 410 inverted microscope and BODIPY FL C_{16} was viewed using a 488 nm argon/krypton laser, with a 515–540 nm band pass filter used for detection. Mitotracker, Lysotracker and BODIPY TR ceramide were viewed with a 565 nm helium/neon laser and a 590 nm band-pass emission filter. The cells were viewed through a 63× oil immersion objective (NA 1.3) and digitized data collected [12, 16, 17].

Sub-cellular localization of BODIPY FL C_{16}

Cells grown on coverslips were incubated with 5 μM BODIPY FL C_{16} for 15 min (37°C), then mitochondria, lysosomes or golgi/endoplasmic reticulum were labelled for 15 min with 500 nM Mitotracker, 75 nM Lysotracker or 5 μM BODIPY TR ceramide, respectively.

Immuno-fluorescence

Cells were plated on coverslips and incubated in standard media, as described above. Before the start of labelling experiments the cells were washed with PBS-CGM (37°C), incubated for 15 min with 5 μM BODIPY C_{16} in PBS-CGM (37°C) and then washed with PBS-CGM for 15 min (37°C). Cells were then placed on ice and fixed with 4% paraformaldehyde (60 min, room temperature, with shaking), followed by incubation with a rinse solution containing saponin (0.1 mg/ml), goat IgG (2.5 mg/ml) and 0.2 M glycine for 45 min. The permeabilized cells were subsequently incubated with primary anti L-FABP antibody (diluted 1:50 in blocking solution) for 1 h at room temperature and then overnight at 4°C. The cells were then washed

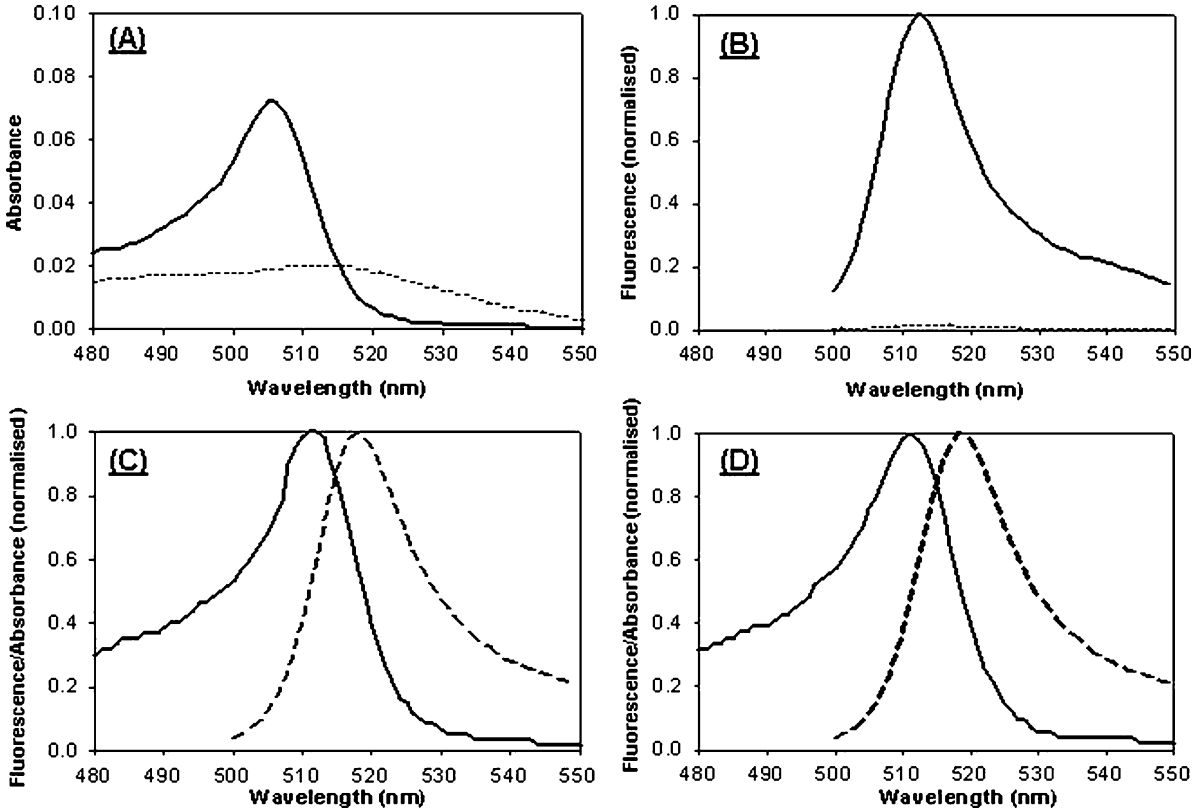

Fig. 1. Spectral properties of BODIPY FL C_{16} in buffer, methanol and bound to FABP. Absorbance (A) and fluorescence emission (B) scans of 0.25 μM BODIPY FL C_{16} in buffer (dotted line) or methanol (solid line). Absorbance (solid line) and fluorescence emission (dashed line) scans of 0.25 μM BODIPY FL C_{16} in the presence of 2.5 μM I-FABP (C) or L-FABP (D).

three times with rinse solution (room temperature) before incubation with a rhodamine-labelled secondary antibody (diluted 1:50 in blocking solution without glycine) for 1 h at room temperature (protected from light). Before viewing, the cells were washed once with rinse solution and twice with phosphate-buffered saline, pH 7.4 (PBS), before mounting in PBS buffer containing 70% glycerol and 2% DABCO (1,4-diazabicyclo[2.2.2]octane). The coverslips were sealed with nail polish, protected from light and stored at 4°C until viewing (described above) [18].

Lipid extraction and analysis

Total cellular lipids were extracted from Caco-2 cells incubated with BODIPY FL C_{16} by the method of Bligh and Dyer [19], and lipids in the organic phase separated by thin-layer chromatography, using a two-phase solvent system [20]. To detect BODIPY-labelled esterification products BODIPY-labeled lipids (Molecular Probes, OR, USA) were used as standards.

Results

Fluorescence properties of BODIPY FL C_{16}

The fluorescence properties of BODIPY FL C_{16} are environment dependent, as indicated by the differences in fluorescence yield and spectral shifts observed in aqueous buffer (PBS-CGM) and methanol. In the presence of both L-FABP and I-FABP there is a large increase in fluorescence quantum yield relative to the quantum yield in buffer (Fig. 1; Table 1),

Table 1. Fluorescence quantum yields of BODIPY FL C_{16} in the presence of L-FABP and I-FABP

	Methanol[1]	Buffer[2]	L-FABP[2]	I-FABP[2]
Quantum yield	0.94, 0.93	0.03, 0.03	0.88, 0.86	0.74, 0.70

Samples contained 0.5 μM BODIPY FL C_{16} in either methanol[1] or PBS-CGM buffer[2]. FABP was added at a concentration of 5 μM. Quantum yields were calculated as described under *Materials and Methods*. The results from two different experiments are shown.

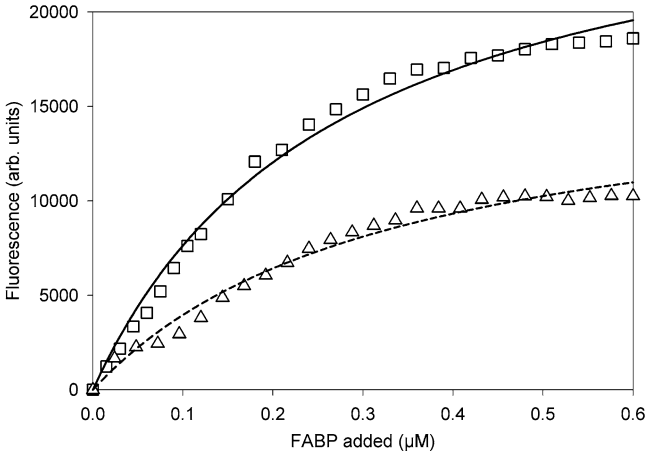

Fig. 2. Determination of BODIPY FL C_{16} binding affinity for L-FABP and I-FABP. Samples contained 0.5 μM BODIPY FL C_{16} to which aliquots of L-FABP (□, solid line) or I-FABP (△, dashed line) were added. Fluorescence was measured after equilibration of the samples for approximately 1 min (excitation 488 nm; emission 515 nm). The curves were generated by fitting the data to the following equation: $F_{OBS} = (F_{MAX} \times [FABP])/(K_D + [FABP])$.

an indication of BODIPY FL C_{16} binding to these proteins. In methanol, BODIPY FL C_{16} displayed an absorbance maximum at approximately 502.5 nm, with fluorescence emission at a maximum at approximately 514 nm. For binding to L-FABP and I-FABP the absorbance and fluorescence emission maxima were at approximately 510 and 518 nm, respectively (Fig. 1). In PBS-CGM buffer virtually no fluorescence emission was observed and at 514 nm the fluorescence in the PBS-CGM buffer was only 1.2% of the value that was observed in methanol (Fig. 1). The Stoke's shift for BODIPY FL C_{16} in methanol, and when bound to L-FABP or I-FABP, was in the range of 8–11 nm (Fig. 1) [7].

BODIPY FL C_{16} is known to be a fluorophore with a high fluorescence quantum yield, which makes it attractive for utilization in fluorescence microscopy applications. As shown here the fluorescence quantum yield of BODIPY FL C_{16} in methanol is very high (∼0.93) (Table 1), which compares well with the previously published value of 0.92 [7]. However, this probe is apparently very sensitive to either self-quenching characteristics or solvent effects since the quantum yield decreases dramatically in the presence of buffer (Table 1). In the presence of saturating concentrations of both L-FABP and I-FABP the quantum yield of BODIPY FL C_{16} is significantly increased relative to its value in buffer alone (Table 1), although the quantum yields are slightly lower than those obtained in methanol, suggesting that the BODIPY FL C_{16} binding site(s) may be less hydrophobic than methanol.

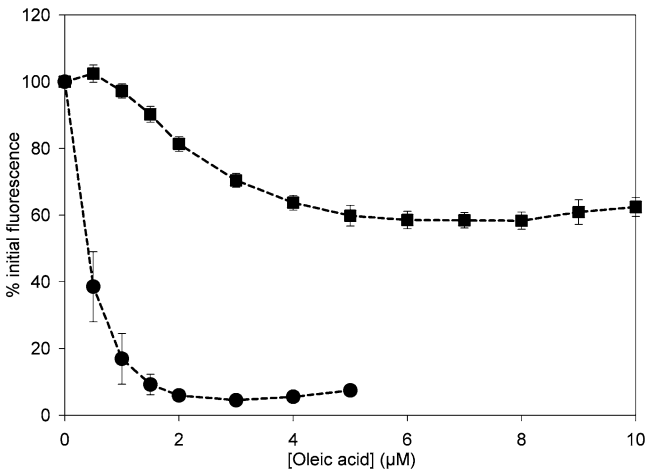

Fig. 3. Displacement of BODIPY FL C_{16} from FABP by oleic acid. Samples contained 1 μM L-FABP (■) or I-FABP (●) and 1 μM BODIPY FL C_{16} in buffer, to which oleic acid aliquots were sequentially added to obtain the total oleic acid concentration indicated.

BODIPY binding to FABP

Both L-FABP and I-FABP bound BODIPY FL C_{16} with high affinity, displaying apparent dissociation (K_D) values of 270 and 330 nM, respectively (Fig. 2). The binding of BODIPY FL C_{16} to L-FABP and I-FABP is also demonstrated by fluorescence displacement studies with unlabelled oleic acid as the second, competing ligand (Fig. 3). The latter data also suggests that BODIPY FL C_{16} binds to both FABPs in a configuration that at least partially overlaps with the binding site(s) for oleic acid.

Tissue culture studies: Co-localisation of BODIPY FL C_{16} and sub-cellular organelles

Using organelle-specific fluorescent probes it could be demonstrated that BODIPY FL C_{16} co-localises with BODIPY TR ceramide, a probe for the endoplasmic reticulum and Golgi apparatus (Fig. 4), and Mitotracker, a probe for mitochondria (Fig. 4), thus showing that BODIPY FL C_{16} is targeted to these organelles in Caco-2 cells. By contrast, little co-localisation was observed for BODIPY FL C_{16} and Lysotracker, the latter a probe with relative specificity for lysosomes (Fig. 4). This observation suggests that BODIPY FL C_{16}, as an assumed fatty acid analogue, is targeted to and potentially utilized by cellular degradation (e.g. β-oxidation) and lipid synthesis (esterification) pathways. However, within the time-scale of the experiments shown here, essentially none of the BODIPY FL C_{16} was metabolized in Caco-2 cells (data not shown).

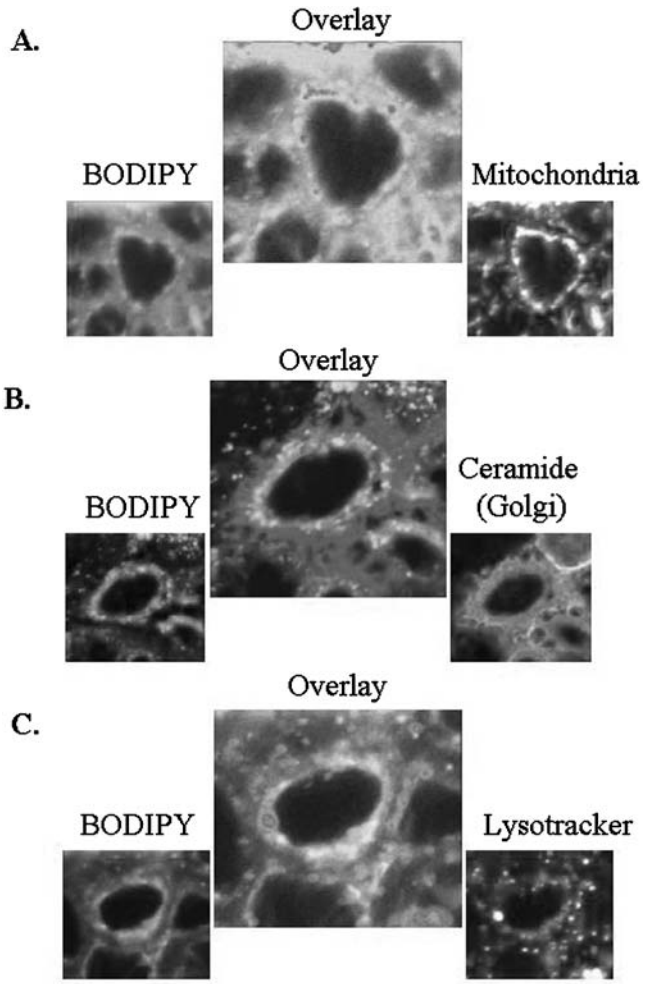

Fig. 4. Co-localization of BODIPY FL C_{16} with sub-cellular organelles in Caco-2 cells. Cellular BODIPY FL C_{16} fluorescence is shown as green and fluorescent organelle probes as red. Co-localization of the probes is shown in the overlay images as yellow/purple. Experimental details are provided under *Materials and methods*.

Tissue culture studies: Co-localisation of BODIPY FL C_{16} and FABP

Immuno-fluorescence techniques, together with the incubation of Caco-2 cells with BODIPY FL C_{16}, were used to investigate the co-localization of FABP and BODIPY FL C_{16}. As shown, L-FABP co-localized extensively with BODIPY FL C_{16} (Fig. 5), indicating that BODIPY FL C_{16} binds to FABP in the Caco-2 cells.

BODIPY FL C_{16} metabolism in Caco-2 cells

To determine whether the BODIPY FL C_{16} remained unesterified or was incorporated into higher lipids, cells labelled

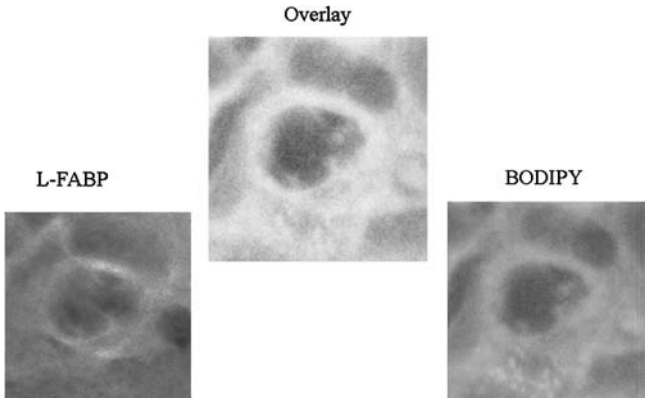

Fig. 5. Co-localization of BODIPY FL C_{16} and L-FABP. Cellular BODIPY FL C_{16} fluorescence is shown as green and L-FABP-specific immunofluorescence as red. Co-localization of the probes is shown in the overlay images as yellow. Experimental details are provided under *Materials and methods*.

under identical conditions as those used for the microscopy localization experiments were harvested, and the lipids extracted and separated by thin-layer chromatography. Detection of BODIPY-labelled lipids was done using a hand-held long-range UV lamp. Under the time and temperature conditions used the BODIPY FL C_{16} remained as the unesterified fatty acid; virtually none of the BODIPY FL C_{16} was incorporated into cholesteryl ester, phospholipid or acylglycerols (data not shown).

Discussion

The BODIPY probes have proven particularly beneficial for confocal microscopy studies as their fluorescence can be detected using a 488 nm argon/krypton laser. In this study we have confirmed previous reports that BODIPY FL C_{16} has spectral absorbance characteristics showing a maximum absorbance at approximately 510 nm, apparently irrespective of the probe environment, with a relatively small Stoke's shift of 5–10 nm (Fig. 1) [7]. However, although the wavelengths of maximum absorbance and fluorescence emission show very little dependence on the probe environment, the fluorescence quantum yield is markedly affected. We found that BODIPY FL C_{16} displays high fluorescence emission intensity in methanol and low fluorescence in the aqueous PBS-CGM buffer (Fig. 1). This finding was confirmed in a more detailed analysis of fluorescence quantum yields that demonstrated an increase in quantum yield from 0.03 in buffer to approximately 0.93 in methanol.

In the presence of L-FABP and I-FABP the quantum yield of BODIPY FL C_{16} increases to approximately 0.87 and 0.72, respectively, as compared to 0.03 in buffer alone (Table 1). The quantum yields obtained with L-FABP and I-FABP are

relatively high and sufficiently different from the values obtained in buffer to suggest that BODIPY FL C_{16} is a ligand for both L-FABP and I-FABP. This was further demonstrated by titration of BODIPY FL C_{16} with FABP, and in displacement studies with oleic acid. The titration curves showed hyperbolic binding characteristics that indicated relatively high affinity of both L-FABP and I-FABP for BODIPY FL C_{16}, with dissociation constants (K_D) in the sub-micromolar range (Fig. 2). The binding of BODIPY FL C_{16} to both FABPs was also supported by competitive displacement studies, which showed that oleic acid is an effective competitor of BODIPY FL C_{16} binding to both FABPs (Fig. 3). Interestingly, even though L-FABP and I-FABP show similar apparent affinity for BODIPY FL C_{16}, oleic acid more readily displaces the probe from I-FABP (Fig. 3). This observation can be explained by the different oleic acid binding characteristics of L-FABP and I-FABP. These FABPs display somewhat different affinities for oleic acid and, of likely importance here, I-FABP binds oleic acid at an equimolar ratio, whereas L-FABP displays two binding sites for oleic acid [21–24]. The displacement data for I-FABP appear to indicate that this protein binds both BODIPY FL C_{16} and oleic acid in an equimolar fashion. In contrast, BODIPY FL C_{16} seemingly binds to only one of the two oleic acid sites located on L-FABP, as indicated by the partial displacement of the fluorescent ligand by oleic acid (Fig. 3). A similar pattern of oleic acid displacement was observed for the fluorescent probe PA-DPH binding to L-FABP [25].

The fluorescence and binding characteristics of BODIPY FL C_{16} with L-FABP and I-FABP also suggested that this fluorophore is a potentially useful probe for studying FABP function in a cellular environment. This hypothesis was assessed by investigating the uptake and metabolism of BODIPY FL C_{16} in the Caco-2 cell line, a useful model system for the small intestine that expresses L-FABP and possibly I-FABP [26–29]. Utilizing both organelle-specific fluorescent probes and immuno-fluorescence techniques it could be demonstrated that BODIPY FL C_{16} is effectively taken up by Caco-2 cells and co-localises with L-FABP and markers for the mitochondria and endoplasmic reticulum/golgi apparatus (Figs. 4 and 5). The co-localisation of BODIPY FL C_{16} with fluorescent probes for mitochondria and endoplasmic reticulum/golgi apparatus appears to demonstrate that BODIPY FL C_{16} is targeted to these organelles in Caco-2 cells. Little or no localization of BODIPY FL C_{16} was found in lysosomes. These observations would suggest that BODIPY FL C_{16}, as a LCFA analogue, is targeted for subsequent use as an energy substrate via β-oxidation, for esterification to phospholipids and triacylglycerols, and for export in lipoproteins. BODIPY FL C_{16} was not metabolized within the time period investigated here (15–30 min) and the metabolic fate of the probe at longer time points was not further investigated. Though attempts to measure the kinetics of BODIPY FL C_{16} uptake into Caco-2 cells have thus far been complicated by photo-bleaching effects (data not shown), it is assumed that such studies would be feasible with improved technology, e.g. two-photon microscopy.

In summary, BODIPY FL C_{16} is a ligand for both L-FABP and I-FABP, binding to both proteins with an apparent dissociation constant (K_D) of 0.3 μM. Competitive displacement by oleic acid indicates that BODIPY FL C_{16} binds at the fatty acid binding site on I-FABP, and at one of the two fatty acid binding sites on L-FABP. In Caco-2 intestinal cells the probe appears to co-localize extensively with mitochondrial and endoplasmic reticulum/Golgi markers, but little with a lysosomal marker. Co-localization of BODIPY FL C_{16} and L-FABP was also observed. Therefore, we envisage that BODIPY FL C_{16} may be a useful tool for studying the distribution and physiological function of FABPs in a cellular environment.

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