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ORIGINAL PAPER

Cholesterol Reporter Molecules

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Abstract Cholesterol is a major constituent of the membranes in most eukaryotic cells where it fulfills multiple functions. Cholesterol regulates the physical state of the phospholipid bilayer, affects the activity of several membrane proteins, and is the precursor for steroid hormones and bile acids. Cholesterol plays a crucial role in the formation of membrane microdomains such as ''lipid rafts'' and caveolae. However, our current understanding on the membrane organization, intracellular distribution and trafficking of cholesterol is rather poor. This is mainly due to inherent difficulties to label and track this small lipid. In this review, we describe different approaches to detect cholesterol in vitro and in vivo. Cholesterol reporter molecules can be classified in two groups: cholesterol binding molecules and cholesterol analogues. The enzyme cholesterol oxidase is used for the determination of cholesterol in serum and food. Susceptibility to cholesterol oxidase can provide information about localization, transfer kinetics, or transbilayer distribution of cholesterol in membranes and cells. The polyene filipin forms a fluorescent complex with cholesterol and is commonly used to visualize the cellular distribution of free cholesterol. Perfringolysin O, a cholesterol binding cytolysin, selectively recognizes cholesterol-rich structures. Photoreactive cholesterol probes are appropriate tools to analyze or to identify cholesterol binding proteins. Among the fluorescent cholesterol analogues one can distinguish probes with intrinsic fluorescence (e.g., dehydroergosterol) from those possessing an attached fluorophore group. We summarize and critically discuss the features of the different cholesterol reporter molecules with a special focus on recent imaging approaches.

Keywords Cholesterol · Photoreactive sterols · Cyclodextrins · Fluorescent sterols · Cytolysins · Filipin · Cholesterol oxidase

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Abbreviations

ACAT	Acyl-coenzyme A:cholesterol acyltransferase
22-NBD Cholesterol	22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol
25-NBD Cholesterol	25-[*N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol
BCθ-toxin	A biotinylated and carlsberg protease-nicked derivative of perfringolysin O
Benzophenone-cholesterol	22-(*p*-benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MβCD	Methyl-β-cyclodextrin
NMR	Nuclear magnetic resonance
NPC	Niemann-Pick C

Introduction

Cholesterol is a major constituent of the plasma membrane in most eukaryotic cells. Actually, it is a multifunctional lipid. Cholesterol strongly influences the physical state of membranes. It has a condensing effect on phospholipids in monolayers or bilayers (i.e., decreasing the average area per lipid molecule). Cholesterol regulates the fluidity of the plasma membrane, increases the membrane thickness, and reduces the permeability of the membrane for small water-soluble compounds (Yeagle 1985). In addition, cholesterol is necessary for the functional activity of several membrane proteins and is the precursor for steroid hormones and bile acids (Pucadyil and Chattopadhyay 2006; Burger et al. 2000). Cholesterol is non-randomly distributed in cells and their membranes (Yeagle 1985). It plays an essential role in the formation of lateral membrane domains designated as ''lipid rafts'' (Simons and Ikonen 1997; Simons and Toomre 2000). Particularly, the study of ''lipid rafts'' has boosted a strong re-interest in cholesterol during the last years.

We report on different cholesterol reporter molecules that have been employed to study the localization and trafficking of cholesterol in vitro and in vivo. Principally, two different classes of cholesterol reporters can be distinguished: cholesterol binding molecules and cholesterol analogues. Among the cholesterol binding molecules, the enzyme cholesterol oxidase, polyenes (in particular filipin), and cytolysins play a dominant role as cholesterol reporters. In addition, cyclodextrins and their inclusion complexes with cholesterol have developed as versatile and popular compounds to analyze the trafficking of cholesterol and the integrity of lipid rafts. Susceptibility to cholesterol oxidase has been applied since more than 30 years as a versatile tool to study membrane organization and cholesterol localization. Filipin, a polyene antibiotic that forms a fluorescent complex with cholesterol, is commonly used to visualize the cellular distribution of free cholesterol. Perfringolysin O, a member of the large family of cholesterol-dependent cytolysins, has attracted attention due to its capacity to specifically bind to cholesterol-rich structures. As cytolysins are proteins, the full

repertoire of cytochemical labeling techniques can be exploited. Using cholesterol binding substances one normally visualizes the steady state distribution of cholesterol in the cells. Basic knowledge about the metabolism and trafficking of cholesterol has been derived from biochemical studies using radiolabeled cholesterol or its precursors. However, the biochemical approaches suffer from the fact that the isolation protocols for subcellular organelles are rather imperfect. To overcome these limitations, a variety of photoreactive, fluorescent, and spin-labeled cholesterol analogues have been developed. For many proteins a localization in cholesterol and sphingolipid enriched microdomains has been reported. This raises the question whether and how these proteins are in molecular contact with cholesterol. Photoreactive cholesterol probes provide appropriate tools to identify and analyze putative cholesterol binding proteins at the molecular level. To study the distribution and dynamics of cholesterol in membranes and cells, researchers can select among cholesterol analogues with different fluorescence properties. One can distinguish cholesterol analogues with intrinsic fluorescence (e.g., dehydroergosterol) from those possessing an attached fluorophore group (e.g., NBD-cholesterol). In this review, we summarize and critically discuss the features of the different cholesterol reporters, with a special focus on imaging approaches.

Cholesterol-Binding Molecules

Cholesterol Oxidase

Cholesterol oxidase is employed for the determination of the cholesterol concentration in serum and food, one of the most widely performed assays in biochemistry. The flavoenzyme converts cholesterol and oxygen into the products 4-cholesten-3-one and hydrogen peroxide that can be quantitated by spectrophotometry (or fluorometry) via an oxidative coupling reaction in the presence of peroxidase to form a chromogen (or fluorogen). Currently, cholesterol oxidase is immobilized onto different surfaces for the fabrication of cholesterol biosensors.

Cholesterol oxidase is produced by several microorganisms, e.g., *Nocardia erythropolis*, *Brevibacterium sterolicum*, *Streptomyces hygroscopicus*. The enzymes exhibit a broad range of steroid specificities dependent on the bacterial source. Commonly, they accept sterols with a 3β-hydroxy group including several of the intermediates in cholesterol biosynthesis after lanosterol. Structures of cholesterol oxidase from *Brevibacterium sterolicum* and *Streptomyces hygroscopicus* in its free and substrate-bound states have been determined at atomic resolution (Vrielink et al. 1991; Li et al. 1993; Yue et al. 1999). The water-soluble enzyme associates peripherally with the surface of the membrane. Most likely, it forms a complex with the lipid bilayer that allows cholesterol to move directly from the membrane into the active site (Bar et al. 1989; Ahn and Sampson 2004). In fact, it is known that the properties of the membrane strongly influence the accessibility of the enzyme to its substrate. Thus, cholesterol oxidase is a valuable probe for studying membrane organization and a sensor of the bilayer lipid phase, with a preferential binding to the solid phase (Patzer and Wagner 1978; Ahn and Sampson 2004). The properties and broad applications of cholesterol oxidase enzymes are reviewed elsewhere (Lange 1992; MacLachlan et al. 2000). Cellular cholesterol can be tracked by using its susceptibility to cholesterol oxidase (Lange 1992). In living intact cells, cholesterol is only a poor substrate

for cholesterol oxidase. However, this can dramatically change when certain substances or enzymes were added to the cells or membranes. Among the agents stimulating the enzymatic turnover are cholesterol, glutaraldehyde, low ionic strength buffer, phospholipase C, sphingomyelinase, detergents, and membrane intercalators such as decane or octanol (Lange et al. 1984; Slotte et al. 1989). Lysophosphatides are shown to inhibit the activity of cholesterol oxidase (Lange et al. 1984, 2005). It has been proposed that in the unperturbed plasma membrane, cholesterol is kept at a low chemical potential by its association with bilayer phospholipids (Radhakrishnan and McConnell 2000). Thus, stimulators of enzyme activity might act by increasing the chemical activity of cholesterol leading to a better accessibility of cholesterol to the enzyme, whereas inhibitors such as lysophosphatidylcholine might associate with the excess cholesterol and thereby lower its chemical activity (Lange et al. 2004). Variations of cholesterol oxidase accessibility have also been explained assuming the existence of regularly distributed sterol superlattices in membranes (Wang et al. 2004). According to this model, cholesterol within sterol superlattices is tightly packed and more accessible to the aqueous phase (i.e., to cholesterol oxidase) as compared with cholesterol localized in irregularly distributed lipid areas (Wang et al. 2004). Interestingly, the cholesterol oxidase-accessible plasma membrane pool may be the same pool of cholesterol removed by high density lipoproteins (Vaughan and Oram 2005).

The susceptibility to cholesterol oxidase has been exploited to gain information about the localization, transfer kinetics, and transbilayer distribution of cholesterol (Lange 1992). In human fibroblasts, not less than 90% of the cholesterol in fixed (e.g., glutaraldehyde treated) cells were rapidly oxidizied (~1 min) by the enzyme. The residual 10% of cholesterol resistant to cholesterol oxidase coincided with markers of endocytic membranes that are also to large parts derived from the plasma membrane (Lange et al. 1989; Lange 1991). This would indicate that in fibroblasts almost all of the cellular cholesterol is localized in the plasma membrane pool, whereas only minor cholesterol amounts (1% or less) are distributed to other organelles (e.g., the endoplasmic reticulum). Application of cholesterol oxidase in human erythrocytes suggested that cholesterol flips very rapidly across the plasma membrane (Lange et al. 1981). Given the reasonable assumptions that the enzyme has only access to the outer membrane leaflet and cholesterol is not exclusively distributed there, cholesterol moved with a half time of less than 3 s at 37°C across the membrane (Lange et al. 1981). In contrast, another group reported a half-time of 1–2 h for the transmembrane movement of cholesterol using susceptibility to cholesterol oxidase as reporter (Brasaemle et al. 1988). The transfer of newly synthesized cholesterol to the cell surface occurred with a half-time of 10 min to 1 h as measured by the cholesterol oxidase approach in fibroblasts (Lange et al. 1991; Lange and Matthies 1984).

In each case, the application of cholesterol oxidase as a cholesterol reporter requires the careful selection of reaction conditions and rigorous control experiments. When the enzyme is used on living cells, one has to consider marked alterations in protein localization or receptor signaling (Smart et al. 1994; Gimpl et al. 1997; Okamoto et al. 2000). Inherent difficulties in this approach are related with the fact that the enzyme does not only bind to but convert cholesterol to a steroid with substantially altered properties. For example, 4-cholesten-3-one does not condense a phospholipid monolayer to the same extent as cholesterol (Gronberg and Slotte 1990). In addition, 4-cholestene-3-one is a raft-dissolving steroid that, unlike cholesterol, favors the liquid disordered phase (Xu and London 2000). Its action promote a certain rate of leakage of the plasma membrane (Ghoshroy et al. 1997). Mutant enzymes with unimpaired

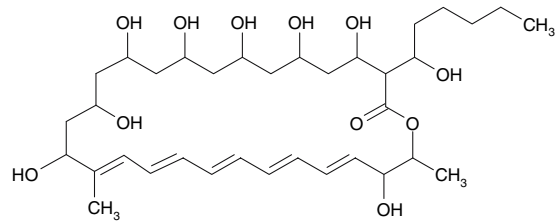

Fig. 1 Chemical structure of filipin III

membrane binding and low or lacking catalytic activity may overcome this limitation (Yin et al. 2002).

Polyenes

The family of polyenes are characterized by a macrolide structure with an amphipathic nature. Among the members of this family, filipin is certainly the most important tool to visualize the localization of free cholesterol in cells. Filipin is an antibiotic with antifungal properties and a mixture of four macrolides with minor differences in their structure, the fraction known as filipin III being the major component (Bolard 1986) (Fig. 1). Filipin performs its antibiotic action by inducing a structural disorder in sterol containing membranes. The disintegration of the membranes then leads to the leakage of cellular components. How does filipin interact with free cholesterol? For its action filipin requires a sterol partner with a free 3′-OH group. However, further details about the filipin–sterol interaction are rather speculative. Filipin may form large planar aggregates between the two layers of the membrane, it may be absorbed at the membrane surface or located at the upper layer of the membrane (Castanho et al. 1992). Different models have been generated to explain the organization of the filipin–sterol complexes within the membrane bilayer (de Kruijff and Demel 1974; Elias et al. 1979; Lopes et al. 2004).

Filipin has been used for decades to localize the distribution of free cholesterol in cells and tissues (Butler et al. 1987, 1992; Kinsky et al. 1967; Verkleij et al. 1973; Elias et al. 1979; Robinson and Karnovsky 1980; Orci et al. 1983). For example, filipin staining has been and still is a prominent diagnostic tool for the identification of cholesterol mislocalization in lysosomes of the Niemann-Pick C (NPC) phenotype (Butler et al. 1987, 1992). What are the disadvantages of using filipin as a reporter of cholesterol? First, the spectroscopic properties of filipin are not favorable. The excitation of the fluorophore is within the UV range where scattering effects and autofluorescence impair the quality of the images. In addition, the bleaching rate of the fluorophore is very high. Most notably, filipin is a cytotoxic compound. As mentioned above, it disrupts the integrity of sterol-containing membranes (Behnke et al. 1984). Thus, staining with filipin can only be employed in fixed cells or tissues. Moreover, the fluorescence of filipin reports on the steady-state level of cholesterol but does not gain information about the trafficking of free cholesterol. It has also been reported that some sterol-containing membranes are not labeled by filipin (Pelletier and Vitale 1994; Steer et al. 1984; Severs and Simons 1983).

Other polyene antibiotics such as nystatin and amphotericin B share the cholesterol binding property with filipin. Both substances form pores unlike filipin. Electrophysiologists use nystatin for the so called ‘‘perforated patch clamping.’’ Nystatin forms

complexes with cholesterol that lead to ''perforations'' in the bilayer inside the patch pipette. These holes are small but large enough to allow the permeabilization of ions such as sodium and potassium.

In conclusion, although filipin is frequently used as cholesterol reporter, it is not entirely clear whether its staining reflects the correct distribution of cholesterol, particularly at intracellular sites that are not easily accessible and/or prone to artifacts from fixation techniques.

Cholesterol-Dependent Cytolysins

Cholesterol-dependent cytolysins are produced by a variety of pathogenic Gram-positive bacteria including *Streptococcus pyogenes* (streptolysin O), *Streptococcus pneumoniae* (pneumolysin), *Listeria monocytogenes* (listeriolysin O), *Clostridium perfringens* (perfringolysin O), and *Bacillus anthracis* (anthrolysin) (Palmer 2001; Rossjohn et al. 1997; Tweten et al. 2001). The cytolysins form a large family of protein toxins that are active only in cholesterol-containing membranes. The monomeric forms of the cytolysins are water-soluble. When these proteins bind to cholesterol-containing membranes, they spontaneously self-associate to form large aqueous pores in the bilayer. These oligomeric complexes contain up to 45–50 individual monomers. Cytolysins interact with cholesterol also in the absence of any other lipids. Structural features of the cholesterol molecule required for interaction with the toxins include the 3β-OH group, the stereochemistry of the sterol ring system, and the isooctyl side chain (Watson and Kerr 1974; Prigent and Alouf 1976). Thus, a specific cholesterol binding site should be present although it has not been identified on the protein molecule. In addition to their potency as cholesterol reporters, cytolysins are useful tools in cell biology due to their pore-forming capacity. For example, application of a low concentration of streptolysin O allows to shuttle macromolecules into cells of interest (Lafont et al. 1995).

The crystal structure of a water-soluble cytolysin monomer was solved in case of perfringolysin O (Rossjohn et al. 1997) (Fig. 2). Accordingly, perfringolysin O is comprised of four domains. The C-terminal portion of perfringolysin O (designated D4 domain) folds into a separate β-sandwich domain composed of two four-stranded β-sheets at one end of the elongated molecule. This D4 domain is involved in cholesterol (and membrane) recognition and binding. However, only the short hydrophobic loops at the tip of the D4 β-sandwich (Fig. 2, arrowheads) are exposed to the bilayer interior while the remainder of the structure remains close to the membrane surface (Ramachandran et al. 2002). A protease-nicked and biotinylated derivative of perfringolysin O (designated as BCθ-toxin) was shown to retain specific binding to cholesterol without cytolytic activity. Fujimoto et al. (1997) introduced BCθ-toxin in combination with fluorophore-labeled avidin as a cholesterol reporter (Fujimoto et al. 1997). This probe was used for the localization of membrane cholesterol in various cells by fluorescence microscopy and by electron microscopy in cryosections (Iwamoto et al. 1997; Mobius et al. 2002; Sugii et al. 2003; Waheed et al. 2001; Reid et al. 2004; Tashiro et al. 2004). As expected from the structure of perfringolysin O, the D4 domain was sufficient to function as a cholesterol binding protein (Rossjohn et al. 1997). Visualization of the D4 probe has been achieved by two approaches: following conjugation of a fluorophore to D4 or by N-terminal fusion of EGFP to the protein (Shimada et al. 2002).

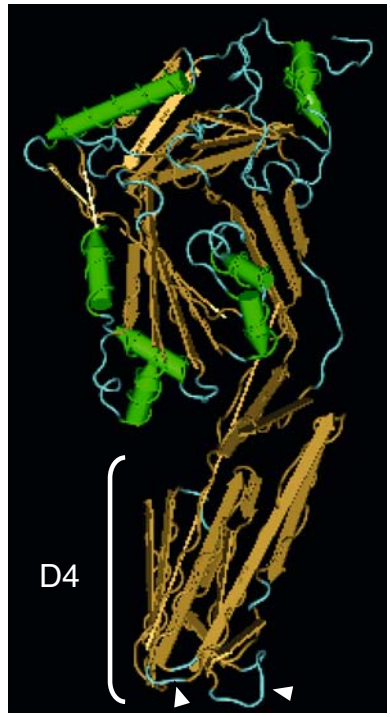

Fig. 2 Structure of the water-soluble monomeric form of perfringolysin O (Rossjohn et al. 1997). The D4 domain (C-terminal portion of perfringolysin with residues 301–500) is involved in the binding of the membrane and recognition of cholesterol. Only the short hydrophobic loops at the tip of the D4 β-sandwich (arrowheads) are exposed to the non-polar interior of the bilayer (Ramachandran et al. 2002). The picture was adapted from PubMed using the Cn3d program

It was commonly observed that perfringolysin O derivatives detect cholesterol primarily in cholesterol-rich membrane microdomains such as caveolae or ''lipid rafts'' (Waheed et al. 2001; Shimada et al. 2002; Sugii et al. 2003). Studying the pathophysiological cholesterol accumulation in the Niemann-Pick C mouse brain, Reid et al. (2004) found staining with BCθ-toxin superior to that achieved by filipin. Notably, in brain regions known to be affected by the neurodegenerative NPC disease, cholesterol accumulations were observed both at a better signal-to-noise ratio and at earlier time points with BCθ-toxin as compared with filipin (Reid et al. 2004). In contrast, in a hippocampal culture system, cholesterol was detectable by BCθ only at the cell surface of fully matured neurons, whereas filipin stained intracellular and cell surface cholesterol in neurons at all developmental stages. Additionally, the two cholesterol reporters showed different labeling patterns in cultured hippocampal neurons. While BCθ staining was observed mainly on axons, filipin labeled axons, dendrites and somata (Tashiro et al. 2004). Tashiro et al. (2004) also reported that neurons that were induced to the NPC phenotype by administration of certain reagents, lose cell surface BCθ staining on axons. Obviously, the distribution of cholesterol at the axonal surface is critical for recognition by BCθ. While the findings with NPC mice suggested that compared with BC staining, cells must accumulate a higher threshold level of cholesterol before they stain filipin-positive, the reverse appeared true for hippocampal neurons in culture.

What are the major considerations of using perfringolysin O derivatives as cholesterol reporters? Most importantly, perfringolysin O derivatives bind only to membranes or liposomes with high (>20–25 mol%) cholesterol (Ohno-Iwashita et al. 1992). The BCθ-toxin also labeled Triton-insoluble fractions, i.e., ''lipid rafts'' (Waheed et al. 2001). In addition, when membranes or cells were depleted of cholesterol by β-cyclodextrins, the binding of θ-toxin was completely abolished whereas significant filipin staining was retained (Waheed et al. 2001; Shimada et al. 2002). It was also suggested that the actual arrangement of cholesterol and other membrane components might influence the efficiency of cholesterol detection by BCθ-toxin (Mobius et al. 2002). As mentioned above, only the loops at the tip of the critical D4 β-sandwich are embedded in the phospholipid bilayer. Thus, it is likely that the toxin recognizes a certain arrangement of cholesterol at the outer leaflet of the bilayer.

Taken together, perfringolysin O derivatives might be good and selective reporters for cholesterol-rich domains such as caveolae or rafts, but are neither suitable to label cholesterol-poor organelles nor for quantitative in situ determination of membrane cholesterol.

Cyclodextrins

Cyclodextrins are torus-shaped cyclic oligosaccharides containing at least six glucose units attached by glycosidic bonds. They possess a hydrophilic outer surface and a hydrophobic inner cavity. Cyclodextrins enhance the solubility of non-polar substances (e.g., cholesterol) by incorporating them into their hydrophobic cavity and forming non-covalent water-soluble inclusion complexes. Cyclodextrins comprised of 6, 7, and 8 glucose units (α-, β- and γ-forms, respectively) were used to alter the lipid composition of cells (Ohtani et al. 1989). Among those, β-cycodextrins and derivatives therefrom such as methyl-β-cyclodextrin (MβCD) or 2-hydroxypropyl-β-cyclodextrin were found to selectively extract cholesterol from the plasma membrane, in preference to other membrane lipids (Irie et al. 1992; Klein et al. 1995; Gimpl et al. 1995, 1997; Kilsdonk et al. 1995). The kinetics of cyclodextrin-mediated cholesterol efflux provide information about the cholesterol pools in cells (Yancey et al. 1996). While ''empty'' β-cyclodextrins function as rather selective cholesterol acceptors, cholesterol-cyclodextrin complexes serve as very efficient sterol donors in vitro and in vivo (Klein et al. 1995; Gimpl et al. 1997; Kilsdonk et al. 1995). For example, up to 80% of the cholesterol can be extracted from fibroblasts via MβCD within 10–30 min. Vice versa, using cholesterol-MβCD as donor, cholesterol-depleted cells can be reloaded with cholesterol within the same time scale. Cholesterol can also be substituted by a variety of cholesterol analogues when the corresponding sterol–MβCD complexes are applied to the cholesterol-depleted membranes or cells (Gimpl et al. 1997). Performing reversible alterations of the cholesterol content in membranes and cells is now established as a standard methodology in the research of ''lipid rafts'' (Simons and Toomre 2000). As ''lipid rafts'' represent cholesterol enriched microdomains, the integrity of these structures is particularly sensitive to the treatment of β-cyclodextrins. Beyond their importance in cholesterol research, cyclodextrins have found a wide range of applications in food, pharmaceutical and textile industry, cosmetics, environmental engineering, and agrochemistry. For example, cyclodextrins are employed for the preparation of cholesterol-free products or for the delivery of drugs (Challa et al. 2005).

Cholesterol Analogues

Cholesterol is regarded as a molecule that has achieved evolutionary perfection to fulfill its different functions in membrane organization (Yeagle 1985). Features that have been found to be necessary for a biologically active cholesterol analogue are a free 3β-OH, a planar tetracyclic ring system with a $\Delta^{5(6)}$ double bond, angular methyl groups, and an isooctyl side chain at the 17β-position (Schroeder 1984) (Fig. 3A). While the 3′-OH group presumably interacts with the head group of phospholipids, the aliphatic side chain may be necessary to allow flip-flops and/or tail-to-tail transbilayer interaction of cholesterol molecules. Thus, the structural features of a biologically active substitute of cholesterol that supports ordered lipid domains (''lipid rafts'') are rather stringent (Schroeder et al. 1995; Yeagle 1985; Vainio et al. 2006; Megha et al. 2006). It is therefore a difficult task to design useful cholesterol probes. Certainly, none of the cholesterol analogues that are described below can claim to mimic all properties of the multifunctional cholesterol molecule.

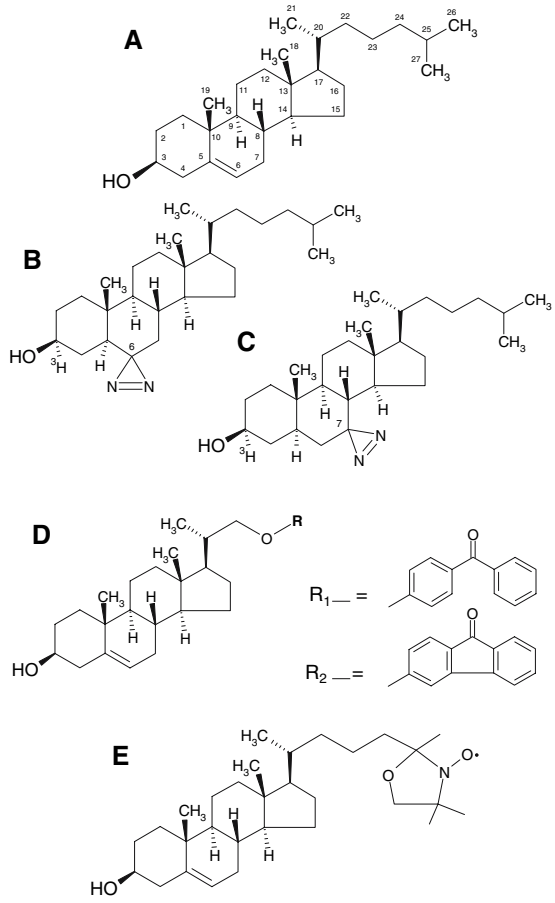

Fig. 3 Chemical structures of cholesterol (**A**), the photoreactive cholesterol analogues (**B**–**D**) [^{3}H]6-azi-5α-cholestanol (**B**), [^{3}H]7-azi-5α-cholestanol (**C**), 22-(*p*-benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol (**D**, R=R$_1$), the fluorenone moiety (**D**, R=R$_2$), and the spin-label probe 25-doxyl-cholestanol (**E**)

Photoreactive Cholesterol Analogues

The identification of specific cholesterol binding proteins can be achieved by the usage of photoreactive cholesterol analogues. In the first photoreactive cholesterol analogues that have been synthesized, the photoreactive groups were incorporated either at the C-3 position (cholesteryl diazoacetate, 3α-azido-5-cholestene, or 3α-(4-azido-3-iodosalicylic)-cholest-5-ene) (Corbin et al. 1998; Middlemas and Raftery 1987) or at the aliphatic side chain of cholesterol (25-azidonorcholesterol or sterols with diazoacetate, aryldiazirines or fluorodiazirine attached at C-22 or C-24) (Terasawa et al. 1986; Stoffel and Klotzbucher 1978). Unfortunately, not many applications have been described for most of these compounds. The nicotinic acetylcholine receptor binds cholesterol but reveals very low structure-activity requirements for cholesterol. Even analogues derivatized at the C-3 positions with a broad range of substituents or bile acid derivatives support receptor activity (Corbin et al. 1998; Fernandez et al. 1993). Using 3α-(4-azido-3-iodosalicylic)-cholest-5-ene or the bile acid *p*-azidophenacyl 3α-hydroxy-5*β*-cholan-24-ate as photoreactive probes, all subunits of the nicotinic acetylcholine receptor could be labeled in membranes or proteoliposomes (Corbin et al. 1998). Photoreactive cholesteryl diazoacetate also labeled the nicotinic acetylcholine receptor (Middlemas and Raftery 1987). Although this probe is modified at C-3, it immobilized in lipid bilayers like cholesterol and upon irradiation incorporated into the choline head group of phosphatidylcholine (Keilbaugh and Thornton 1983). However, cholesteryl diazoacetate behaved different from cholesterol concerning its exchange kinetics from unilamellar vesicles (Kan et al. 1992).

The acetylcholine receptor may certainly be an exception concerning its broad tolerance for cholesterol substitutes. To develop a probe with more general applicability, we synthesized a photoreactive cholesterol analogue in which both the C-3 and the isooctyl side chain left unattached. Based on the knowledge about photoreactive derivatives of bile salts (Kramer and Kurz 1983), we have developed the photoreactive cholesterol analogue [^{3}H]6-azi-5α-cholestanol (Fig. 3B). The azi-group has been introduced at position C-6 because modification at this position was functionally tolerated to a certain extent by the oxytocin receptor, a G protein coupled receptor that we studied in detail with respect to its specific requirement for cholesterol (Klein et al. 1995; Gimpl et al. 1995, 1997; Burger 2000). The first application of this compound (often designated simply as photocholesterol) has been published by Thiele et al. (2000). Up to now, several putative cholesterol binding proteins have been labeled with 6-azi-5α-cholestanol, among those are synaptophysin (Thiele et al. 2000), caveolin (Thiele et al. 2000), MLN64 (Alpy et al. 2005), vitellogenins (Matyash et al. 2001), proteolipid protein (Simons et al. 2000; Kramer-Albers et al. 2006), tetraspanins (Charrin et al. 2003), and cholesterol absorption proteins in small intestinal enterocytes (Kramer et al. 2003). Another related tritiated photoreactive cholesterol analogue, [^{3}H]7-azi-5α-cholestanol (Fig. 3C), has been synthesized by Cruz et al. (2002). A direct binding of this analogue with caveolin-1 and Niemann-Pick C1 (NPC1) protein has been demonstrated (Cruz et al. 2002; Ohgami et al. 2004).

Spencer et al. (2004) synthesized a series of eight benzophenone-containing photoreactive cholesterol analogues. Due to the larger size of these photophores compared with the diazirines, these sterol analogues have the disadvantage of being less cholesterol-like. On the other hand, benzophenone derivatives show a high crosslinking yield and a preferential reaction with C–H bonds which may be beneficial for the sterol

labeling of some proteins. In one group of benzophenone-containing cholesterol probes, the photophore moiety extended, or replaced most of, the cholesterol isooctyl side chain. In another group of analogues, the photophore was attached at C-3 via an amide linkage. Surprisingly, all of these analogues even those with modifications at C-3 were similarly effective as cholesterol when tested in an apolipoprotein A–I dependent sterol efflux assay. This indicates that at least related to certain transport pathways of cholesterol, biological membranes show an unexpected tolerance for cholesterol substitutes (Spencer et al. 2004). One of these analogues, tritiated 22-(*p*-benzoylphenoxy)-23,24-bisnorcholan-5-en-3β-ol (Fig. 3D, R=R_1), photolabeled caveolin effectively (Fielding et al. 2002). Fluorenone-containing cholesterol probes that are structurally similar to the corresponding benzophenone derivatives (Fig. 3D, R=R_2) represent a further interesting group of compounds since they are both photoreactive and fluorescent (Spencer et al. 2006). Two such analogues behaved similar as cholesterol in the above mentioned sterol efflux assay (Spencer et al. 2006). Their further potential has to be evaluated.

In experiments with photoreactive cholesterol analogues one has to consider that at least in the plasma membrane large amounts of cholesterol are always present. So, presumably each integral membrane protein faces cholesterol in its direct environment. The experimenter hopes or expects that only those membrane proteins are photolabeled that possess one ore more specific cholesterol docking site(s). Membrane proteins residing in cholesterol-enriched lateral lipid domains such as ''lipid rafts'' or caveolae are good candidates. On the other hand, even if these membrane proteins are functionally dependent on cholesterol, their affinity for cholesterol could be low (e.g., in the mM range) since embedded in such a cholesterol-rich environment they are always in contact with cholesterol. In contrast, cholesterol-dependent membrane proteins residing in cholesterol-poor organelles such as the mitochondrion or the endoplasmic reticulum, may be evolutionary selected towards higher affinity for cholesterol. In each case, the usage of exceeding high concentrations of photoreactive cholesterol could artifactually label proteins that do not possess a specific cholesterol binding site.

Spin-Labeled Cholesterol Analogues

Spin-labeled lipids provide information about the structure of biological membranes by using nuclear magnetic and electron spin resonance spectroscopy. Cholesterol analogues with a nitroxide spin-label (doxyl moiety) attached at the C-3 or C-25 position have been synthesized to analyze the orientation, distribution and transbilayer movements of the cholesterol probe in liposomes and biological membranes. Spin–spin interaction of 3β-doxyl-5α-cholestane in liposomes provided evidence for the formation of cholesterol-enriched domains (Tampe et al. 1991). The relative amount of the sterol in the liquid-ordered phase could increase when a protein (glycophorin) was incorporated into the liposomes (Tampe et al. 1991). It is yet unclear how cholesterol is distributed across the membrane bilayer. Using 3β-doxyl-5α-cholestane as a reporter it was observed that cholesterol undergoes a rapid transbilayer movement (<1 min) in liposomes and human erythrocytes (Muller and Herrmann 2002). Data obtained with a fluorescent cholesterol (dehydroergosterol) also suggested a spontaneous cholesterol diffusion from one leaflet to the other with a half-time of a few minutes even at 4°C (Schroeder et al. 1991). A flip-flop faster than 1 s was reported for cholesterol (Steck et al. 2002). Concerning its condensing effect on phospholipids the spin-labeled compound 25-doxyl-cholesterol (Fig. 3E) was found to be an excellent cholesterol analogue. This sterol probe revealed

a cholesterol-like orientation, with the doxyl group at C-25 facing the chain termini of the phospholipids (Scheidt et al. 2003). The localization of the doxyl group in the membrane interior was confirmed by the finding that the nitroxide label was inaccessible from the aqueous phase as it could not be reduced by ascorbate (Scheidt et al. 2003). Probes with the spin-label group accessible from the aqueous phase (e.g., at C-3 as in 3β-doxyl-5α-cholestane) allow to measure cholesterol flip-flop by chemical reduction of the nitroxide radical with ascorbate (Morrot et al. 1987). However, cholesterol analogues with modifications at C-3 are not regarded as faithful mimics of cholesterol. For example, 3β-doxyl-5α-cholestane was not able to exert a comparable condensing effect on phospholipids as cholesterol (Scheidt et al. 2003). Investigations on the transmembrane diffusion of lipids obtained with spin-labeled and fluorescent lipid probes have recently been summarized (Devaux et al. 2002).

Fluorescent Cholesterol Analogues

The applications of fluorescent sterols provide a powerful approach for studying cholesterol behavior in membranes and cells due to their sensitivity, time resolution, and multiplicity of measurable parameters. Two classes of probes can be distinguished: (i) intrinsically fluorescent sterols with dehydroergosterol as its most widely used representative, (ii) cholesterol probes with chemically linked fluorophores. Both classes of probes have their specific advantages and disadvantages. Sterols belonging to the first class may be regarded as being more cholesterol-like but possess unfavorable spectroscopic properties. Sterol analogues of the second class bear bulky reporter groups. However, their fluorescence properties are much better so that these probes can normally be applied at lower concentrations. Several fluorescent cholesterol analogues have been employed to address fundamental issues of distribution and trafficking of cholesterol. In particular, they enable the researcher to design pulse-chase experiments and/or to image the sterol in living cells.

Dehydroergosterol

Due to its conjugated triene system, dehydroergosterol (=ergosta-5,7,9(11),22-tetraene-3β-ol) is a cholesterol analogue with intrinsic fluorescence. Dehydroergosterol naturally occurs in yeasts and certain sponges (Schroeder 1984). Its structure differs from cholesterol only in possessing three additional double bonds and a methyl group at C-24 (Fig. 4A). Dehydroergosterol is doubtless one of the best studied cholesterol probes with respect to its physico-chemical properties (Schroeder 1984; Schroeder et al. 1995). In many respects, it faithfully mimics cholesterol. For example, it co-distributes with cholesterol in both model and biological membranes, exhibits the same exchange kinetics as cholesterol in membranes, is non-toxic to cultured cells or animals, and is a substrate for esterification (Schroeder et al. 1996; Schroeder 1984; Smutzer et al. 1986; Frolov et al. 1996). It can also replace up to 85% of L-cell fibroblast cholesterol without causing significant detrimental effects (Schroeder 1984). So, its employment as a cholesterol reporter should be a good choice. However, dehydroergosterol has unfavorable spectroscopic properties including a low quantum yield, excitation and emission in the UV region, and a rapid bleaching rate. Using sophisticated fluorescence instrumentation (e.g., UV optimized optics, multiphoton excitation), several reports on microscopic imaging with dehydroergosterol have recently been published (Hao et al.

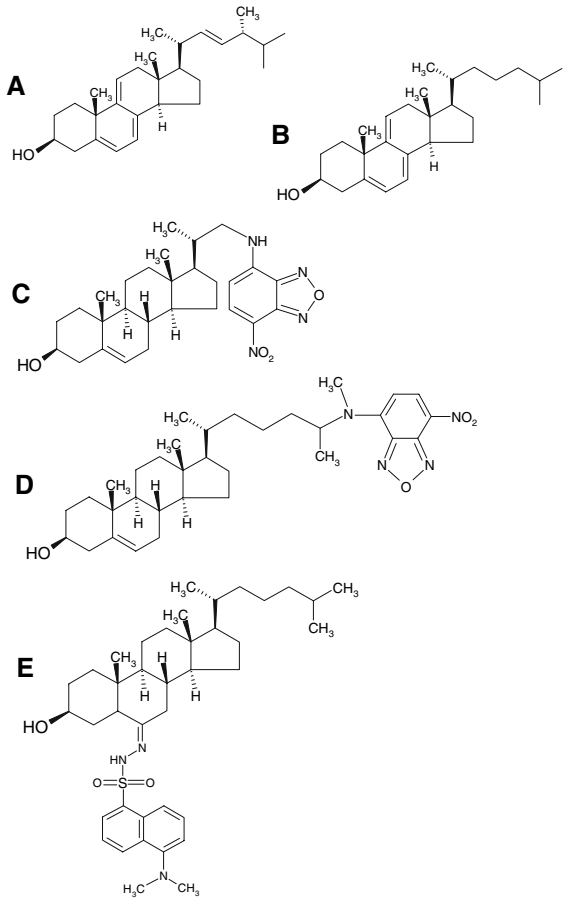

Fig. 4 Chemical structures of fluorescent cholesterol analogues: dehydroergosterol (**A**), cholestatrienol (=cholesta-5,7,9(11)-triene-3β-ol) (**B**), (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol) (=22-NBD-Cholesterol) (**C**), 25-[*N*-[(7-nitro-2-1,3-benzoxadiazol-4-yl)methyl]amino]-27-norcholesterol (=25-NBD-Cholesterol) (**D**), and 6-dansyl-cholestanol (**E**)

2002; Frolov et al. 2000; McIntosh et al. 2003; Wustner et al. 2004, 2005, 2002; Zhang et al. 2005; Pipalia et al. 2007; Wustner 2007; Mukherjee et al. 1998). Even short pulse-chase experiments were possible using dehydroergosterol incorporated in β-cyclodextrin as a water-soluble donor.

In two studies with mouse L-fibroblasts, dehydroergosterol was applied in form of large unilamellar vesicles as donor (Frolov et al. 2000; Zhang et al. 2005). Using multiphoton microscopy for imaging, the authors observed that dehydroergosterol was targeted rapidly from the plasma membrane to lipid droplets (Frolov et al. 2000; Zhang et al. 2005). In other studies, UV-microscopy was performed using dehydroergosterol complexed with methyl-β-cyclodextrin as donor (Hao et al. 2002; Wustner et al. 2002, 2005, 2004). Hao et al. (2002) found that in a CHO cell line, dehydroergosterol was preferentially incorporated into the endocytic recycling compartment within minutes and remained there for hours. Likewise, in polarized HepG2 hepatocytes and in J774 macrophages, the influx of dehydroergosterol was reported to occur via a vesicular

pathway and enrichment in recycling endosomes. In macrophages but not in hepatocytes, dehydroergosterol was also translocated to lipid droplets (Wustner et al. 2002, 2005, 2004). Since dehydroergosterol possesses a significant higher esterification rate (>7-fold) as compared with [^{3}H]cholesterol, one may expect that after hours the sterol will be stored as ester into lipid droplets (Frolov et al. 2000). The enzymes responsible for esterification are localized in the endoplasmic reticulum. So, one should assume that its substrate will also be localized there for some time. However, dehydroergosterol has not been observed to be translocated into the endoplasmic reticulum. It was speculated that technical limitations such as a low signal-to-noise ratio in dehydroergosterol imaging may be responsible for the failure to detect the sterol in other cellular compartments (Hao et al. 2002).

Dehydroergosterol has also been employed to address the question whether cholesterol-rich microdomains are present in vivo. The results of these studies provided some evidence in favor of the ''lipid rafts'' hypothesis (McIntosh et al. 2003; Zhang et al. 2005). However, the methodology selected for the isolation of microdomains (usage of detergents or not) was found to be critical (Gallegos et al. 2006). Additionally, cholesterol binding assays for various cholesterol-binding proteins such as fatty acid binding protein (Nemecz and Schroeder 1991), sterol carrier protein-2 (Schroeder et al. 1990), and Niemann-Pick C2 protein (Liou et al. 2006; Friedland et al. 2003) have been successfully established using the fluorescence properties of dehydroergosterol.

Cholestatrienol

Cholestatrienol (=cholesta-5,7,9(11)-triene-3β-ol) is a fluorescent cholesterol probe similar to deydroergosterol. It differs from dehydroergosterol in the absence of both the double bond Δ^{22} and the methyl group at C-24 (Fig. 4B). Thus, cholestatrienol possesses an isooctyl side chain like cholesterol and should therefore mimic cholesterol better than dehydroergosterol. Scheidt et al. (2003) demonstrated that this is indeed the case when comparing their effects on phospholipid condensation by NMR spectroscopy. Both fluorescent sterol analogues have been introduced at the same time as membrane and lipoprotein probes and can be used to measure the sterol exchange between membranes (Bergeron and Scott 1982; Nemecz et al. 1988). In each case, cholestatrienol is regarded as a cholesterol analogue that mimics the membrane behavior of cholesterol quite well (Fischer et al. 1984; Schroeder et al. 1988; Hyslop et al. 1990; Yeagle et al. 1990; Scheidt et al. 2003; Bjorkqvist et al. 2005; Smutzer et al. 1986). The reason why cholestatrienol has not been used as much as dehydroergosterol is probably ascribed to the fact that it is not commercially available up to now.

Cholestatrienol associates with liquid ordered domains and its quenching by nitroxide-labeled lipids can report on the formation or separation of lipid domains (Bjorkqvist et al. 2005; Heczkova and Slotte 2006). Cholestatrienol has also been evaluated as an appropriate reporter for sterol–protein interactions (Schroeder et al. 1985). A close interaction between cholesterol and rhodopsin has been demonstrated by fluorescence energy transfer from protein tryptophans to cholestatrienol in retinal rod outer segment disk membranes (Albert et al. 1996). Recently, even the imaging of cholestatrienol-specific fluorescence by confocal microscopy has been reported (Tserentsoodol et al. 2006). Low-density lipoproteins labeled with cholestatrienol crossed the blood–retina barrier and were taken up by the retina within 2 h of intravenous injection. The fluorescent sterol was observed to remain in photoreceptor outer segments for at least 24 h. Presumably, cholestatrienol became highly concentrated

in retinal tissues because otherwise imaging could not be expected under the described conditions (Tserentsoodol et al. 2006).

Clearly, imaging of dehydroergosterol and cholestatrienol has its limitations as it must somehow compensate for the low quantum yield and severe photobleaching of these fluorophores. Cells must be loaded with a relatively high sterol concentration and/or mainly those structures are visible in which the sterol probes became substantially enriched. It cannot be excluded that high concentrations required for the visualization of these sterols preferentially force the sterol into pathways which are untypical for cholesterol.

NBD-Cholesterol

The NBD (=7-nitrobenz-2-oxa-1,3-diazol-4-yl) fluorophore has been widely used as a reporter group for lipids (Chattopadhyay 1990). The term NBD-cholesterol causes considerable confusion in the literature, because there are actually two fluorescent cholesterol analogues available with this name. The NBD reporter group is the same, but it is attached to the cholesterol nucleus at different positions. To distinguish between both analogues, we will designate them herein 22-NBD-cholesterol and 25-NBD-cholesterol, respectively (Fig. 4C, D). In an older third variant termed NBD-cholesterol the fluorophore including a spacer has been attached at the C-3 OH via an ester linkage (Alecio et al. 1982). But this 3β-OH labeled analogue probably does not mimic cholesterol well due to the lack of the important free hydroxy group.

Both 22- and 25-NBD-cholesterol have been employed to study the distribution and dynamics of cholesterol in different systems. The behavior of lateral phases in cholesterol and phosphatidylcholine monolayers has been visualized by fluorescence microscopy using 22-NBD-cholesterol (Slotte and Mattjus 1995). Results with model membranes and 25-NBD-cholesterol as reporter indicated that cholesterol may form transbilayer, tail-to-tail dimers even at low sterol concentrations (Mukherjee and Chattopadhyay 1996; Rukmini et al. 2001). McIntyre and Sleight (1991) introduced the fluorescence quenching of NBD by dithionite as an approach to measure the membrane lipid asymmetry. If the NBD group is localized at the outer leaflet of the membrane and is accessible from the aqueous phase, it can be chemically reduced to a non-fluorescent state by water-soluble dithionite. In lipid vesicles, which were selectively labeled with 22-NBD-cholesterol at the outer leaflet, dithionite reduced 95% of the NBD fluorescence (McIntyre and Sleight 1991). Schroeder et al. (1991) studied the transbilayer cholesterol distribution of human erythrocytes using two approaches: photobleaching of 22-NBD-cholesterol and quenching of dehydroergosterol fluorescence. The results suggested an enrichment of cholesterol in the inner leaflet of the erythrocytes (Schroeder et al. 1991). In aggregates and micelles, taurocholic acid quenches the fluorescence of 22-NBD-cholesterol (Cai et al. 2002). Based on the dequenching of the NBD fluorescence, an in vitro assay has been developed to measure the exit of cholesterol from bile acid micelles (Cai et al. 2002). Concerning the quenching behavior of the NBD group, Martin et al. (1993) made an interesting observation in living cells. The photostability of a NBD-labeled ceramide was strongly dependent on the cholesterol status of the Golgi apparatus where the ceramide accumulates. Cholesterol deprivation of the cells accelerated the photobleaching of the NBD-labeled ceramide severalfold, suggesting that this lipid may be used to monitor cholesterol at the Golgi compartment (Martin et al. 1993).

In the last years, mainly 22-NBD-cholesterol has been used although the 25-NBD variant has some advantages as compared with 22-NBD-cholesterol. 25-NBD-cholesterol contains the full isooctyl side chain like cholesterol. A chain length of at least five carbons at the 17β-position was necessary for sterols to form visible sterol/phospholipid domains in lipid monolayers (Mattjus et al. 1995). The spectroscopic properties of 25-NBD-cholesterol have been characterized in detail (Chattopadhyay and London 1987, 1988). Loura et al. (2001) argued against the usage of 22-NBD-cholesterol due to its anomalous distribution behavior in phosphatidylcholine/cholesterol bilayers (Loura et al. 2001). A critical evaluation of both 22- and 25-cholesterol has been published by Scheidt et al. (2003) who observed that both sterols may adopt a reverse (up-side-down) orientation within a phospholipid bilayer. In contrast, Chattopadhyay and London (1987) found that the fluorophore of 25-NBD-cholesterol was deeply buried within the bilayer (Chattopadhyay and London 1987). Possibly, a high mobility of the sterol may explain these discrepant results. 22-NBD-cholesterol has successfully been employed to prove and characterize the cholesterol binding of the cholesterol-binding proteins ''steroidogenic acute regulatory protein'' and ''sterol carrier protein-2'' by spectroscopic techniques (Petrescu et al. 2001; Avdulov et al. 1999).

Mukherjee et al. (1998) applied dehydroergosterol, 22-NBD-cholesterol and 25-NBD-cholesterol to CHO cells and observed a mistargeting of both NBD-cholesterol analogues to mitochondria (Mukherjee et al. 1998). However, in L-cell fibroblasts, 22-NBD-cholesterol distributed similarly as dehydroergosterol from the plasma membrane via unidentified structures (endoplasmic reticulum?) into lipid droplets (Frolov et al. 2000; Atshaves et al. 2000). In hamster fed with a diet containing 22-NBD-cholesterol, the sterol was found to be absorbed (less efficiently than cholesterol) by intestinal epithelial cells and packaged into lipoproteins (Sparrow et al. 1999). Within the enterocytes most of the sterol was translocated into large apical droplets and was presumably stored there in esterified form. 22-NBD-cholesterol was verified as a good substrate for esterification in different cells (Sparrow et al. 1999; Lada et al. 2004; Frolov et al. 2000). HDL-associated 22-NBD-cholesterol was followed by microscopic imaging in 3T3-L1 fibroblasts differentiating to adipocytes (Dagher et al. 2003). At early stages of differentiation 22-NBD-cholesterol co-localized with scattered Golgi structures, while in developing adipocytes, the fluorescent sterol gradually concentrated in lipid droplets (Dagher et al. 2003).

Dansyl-Cholestanol

With the synthesis of 6-dansyl-cholestanol we have recently introduced a novel fluorescent cholesterol probe (Fig. 4E) (Wiegand et al. 2003). The introduction of a photoreactive azo-group at the same position (6-azi-5α-cholestanol, see above) has been proven to be a useful tool for cholesterol–protein interaction studies, as described above. Mintzer et al. (2002) showed that derivatization at position 6 did not change the biophysical parameters of the cholesterol analogue in model membranes by using two different assays: the condensing effect in phosphatidylcholine-containing monolayers, and the fusion of alphaviruses with liposomes (Mintzer et al. 2002). The ''dansyl''—group was chosen because it is one of the smallest fluorescent groups available. Dansyl-cholestanol can be incorporated into methyl-β-cyclodextrin similar good as cholesterol or dehydroergosterol (Gimpl et al. 1997). For in vivo experiments dansyl-cholestanol was excited at its red edge (390 nm) above its excitation maximum (~360 nm). This avoids potential UV-induced damage to the cells and reduces photobleaching.

Using CHO cells we compared the behavior of dansyl-cholestanol vs. [^{3}H]cholesterol with respect to esterification rate, efflux kinetics, and distribution in detergent-insoluble lipid domains (''rafts''). Dansyl-cholestanol showed the same kinetics of esterification by acyl-coenzyme A:cholesterol acyltransferase (ACAT) as compared with [^{3}H]cholesterol. This suggests that within the time scales studied (0–24 h), the translocation rates of dansyl-cholestanol and [^{3}H]cholesterol are similar if not identical. Also the efflux kinetics and subcellular distribution profile were found to be same for both sterols (Wiegand et al. 2003). In microscopic imaging, three further observations indicated the quality of dansyl-cholestanol as a reporter for cholesterol. First, the cellular influx of dansyl-cholestanol occurred rapidly by an energy-independent (probably non-vesicular) pathway via the endoplasmic reticulum. In previous biochemical studies with [^{3}H]cholesterol, it has been proposed that plasma membrane-derived cholesterol passes through the endoplasmic reticulum prior to its transfer to other intracellular sites (Liscum and Munn 1999; Lange et al. 1993). Second, following inhibition of ACAT the unesterified dansyl-cholestanol accumulated in the endoplasmic reticulum in accordance with earlier predictions for cholesterol. It has been observed that in cells in which ACAT was pharmacologically inhibited, LDL-derived cholesterol was not sequestered in lysosomes, but instead accumulated in the endoplasmic reticulum (Blanchette-Mackie 2000; Butler et al. 1992). Third, dansyl-cholestanol was finally translocated to lipid droplets. This agrees well with the trafficking behavior of 22-NBD-cholesterol and dehydroergosterol as described above. Under the pathophysiological conditions of the NPC phenotype, huge amounts of cholesterol are found in lysosomes. Thus, one might assume that lysosomes are part of the cholesterol trafficking pathways within the cells. Surprisingly, in all imaging studies previously performed with 22-NBD-cholesterol, dansyl-cholestanol, and dehydroergosterol, a distribution of the sterols to lysosomes has never been observed, irrespective of how the probe has been administered to the cells.

In conclusion, dansyl-cholestanol is a promising new cholesterol probe whose full potential has still not been exploited. One disadvantage of dansyl-cholestanol concerns its relatively high bleaching rate which shortens the imaging time. Additionally, the physicochemical properties of this cholesterol probe have to be characterized in more detail.

Concluding Remarks

A variety of cholesterol reporter molecules are currently available to study the cholesterol behavior in membranes and cells. Each of them has its specific application field. Cholesterol oxidase enzymes are not only applied to determine the cholesterol concentration in serum and food. Susceptibility to cholesterol oxidase provides a lot of information about the localization of cholesterol and the structure of cholesterol-containing membranes. The polyene filipin was and still is the standard reporter for the distribution of free cholesterol in fixed cells. However, one cannot always be sure whether its staining reflects the correct distribution of cholesterol. Perfringolysin O is one of the best probes to study the localization of cholesterol-rich microdomains, whereas it does not recognize structures with low cholesterol amounts. Possibly, other members of the cholesterol-dependent cytolysin family will be added as cholesterol probes in the future. Photoreactive cholesterol derivatives are the most appropriate tools to identify and characterize cholesterol binding proteins at the molecular level. Fluorescent sterols represent the most sensitive and versatile compounds to explore

several aspects of trafficking and distribution of cholesterol in vitro and in vivo. They also play an essential role to characterize cholesterol–protein interactions inasmuch as classical radioligand binding assays often provide unclear or non-reproducible results. With respect to its multiple functions as a membrane constituent in eukaryotes, cholesterol is an extraordinary molecule optimized during a long evolutionary process. Definitely no cholesterol analogue will be able to fully substitute the cholesterol molecule in all of its facets. Nevertheless, previous results with cholesterol analogues modified by various groups such as diazirine, benzophenone, NBD or dansyl have clearly demonstrated that specific aspects of the cholesterol behavior can well be studied with these probes. These are encouraging findings and should stimulate further work on the fascinating cholesterol molecule.

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