Review

The effect of membrane cholesterol content on ion transport processes in plasma membranes

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Abstract

Cholesterol is a prominent component of mammalian plasma membranes and is one of the factors that determine membrane function. In this review the effects of cholesterol content on transport processes in biological membranes are summarized. Membrane cholesterol affects a variety of membrane proteins, including ion channels, transporters, and receptors. Present concepts concerning the mechanistic basis of lipid-induced modulation of transport protein function range between two extremes: modulation by bulk properties, or by specific interactions. Interest in bulk properties has been focussed mainly on membrane fluidity. The fluidity of biomembranes is diminished particularly by enrichment with cholesterol. As a change in membrane composition alters the environment in which the proteins are dissolved, any process which depends on membrane protein function may be affected by alterations in membrane composition, such as a change in cholesterol content. This review emphasizes the inhibitory effect of cholesterol enrichment on all membrane ATPases studied, and the stimulating effect of cholesterol enrichment on most other membrane transport proteins. Together with the intriguing feature that the cholesterol content of plasma membranes is considerably higher than that of subcellular membranes, there is ample evidence for a significant role of plasma membrane cholesterol in transmembrane protein function.

Keywords: Cholesterol; Sarcolemma; Ion transport; Ion channels

1. Introduction

Epidemiological studies have indicated that elevated plasma levels of cholesterol are associated with an increased risk of coronary heart disease [1,2]. However, cholesterol is an essential component of biological membranes and a precursor in the synthesis of steroid hormones and bile acids [3–5]. This review focuses on the multiple roles of cholesterol with regard to membrane function.

All biological membranes, including the plasma membrane, have a common basic structure [3,6] consisting of assemblies of lipid and protein molecules held together by non-covalent interactions. The most abundant lipids in plasma membranes are phospholipids and cholesterol. The cholesterol molecule is mostly hydrophobic, whereas the 3β-hydroxyl group is polar. This amphipathic character causes cholesterol to be surface-active, allowing interaction with phospholipid molecules by cholesterol’s polar hydroxyl group to the polar head of the phospholipid, while the hydrophobic steroid ring is oriented parallel to, and buried in, the hydrocarbon chains of the phospholipid bilayer [3,6]. This lipid bilayer provides the basic structure of the membrane and serves as a relatively impermeable barrier to most water-soluble molecules.

Cells maintain their cholesterol homeostasis (see Fig. 1) through a regulated balance between endogenous biosynthesis, receptor-mediated uptake of cholesterol from circulating lipoproteins, such as low-density lipoprotein (LDL), and cellular release of cholesterol to circulating lipoproteins, such as high-density lipoprotein (HDL) [3,4,7]. Cholesterol is synthesized from small carbon units via a series of reactions that take place in the cytosol and in the membranes of the endoplasmic reticulum [8]. Generally, the activity of the β-hydroxy-β-methylglutaryl-coenzyme A (HMG-CoA) reductase reaction is the rate-limiting step in de novo cholesterol synthesis. The activity of HMG-CoA

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reductase is regulated at several points including transcription and translation, phosphorylation, activation of sulfhydryl groups, and protein degradation. Cellular cholesterol content is the major factor in the regulation of HMG-CoA reductase activity. By negative feedback control, HMG-CoA reductase activity is repressed when cellular cholesterol content exceeds the demand [8,9].

It has become increasingly evident that cholesterol is not randomly distributed in either artificial or biological membranes, but is organized into structural and kinetic domains or pools [5,10]. The physiological importance of these domains is poorly understood. It is suggested that several membrane proteins are selectively localized in cholesterol-rich domains (e.g., the acetylcholine receptor) or in cholesterol-poor domains (e.g., the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase). Consequently the structure and properties of the different domains rather than of the bulk lipid may selectively affect the function of membrane proteins located in the different domains.

Another intriguing observation is that the cholesterol content of plasma membranes is much higher than that of subcellular membranes. The way in which this difference is maintained still presents an enigma [5,11], but it could be indicative of a distinctive role of cholesterol in plasma membranes, necessary to communicate between intracellular and extracellular spaces.

An important characteristic of the membrane is its fluid character. Since the introduction of the fluid mosaic model [12], attempts have been made to quantify membrane lipid fluidity and to relate membrane fluidity to other membrane characteristics. The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) [13] has been used most frequently, but nowadays a wealth of other probes and methods, including electron spin resonance (ESR) and nuclear magnetic resonance (NMR) [6], is available to sense the fluidity of membranes. For an ordinary liquid such as water or oil, fluidity is defined as the reciprocal of viscosity [14]. However, the lipid bilayer differs from a macroscopic fluid in several aspects. It is a fluid with stringent boundary conditions, essentially two-dimensional in nature, and anisotropic. Moreover, the viscoelastic properties of the membrane cannot easily be separated from those of the whole cell and its microscopic environment [13].

As applied to membranes, the term ‘fluidity’ is used to describe the motional freedom of lipid-soluble molecular probes within a lipid bilayer [13,14]. Besides temperature, fatty acid composition, and protein content, the fluidity of a membrane is determined by its cholesterol content [15]. It has been demonstrated that the higher the cholesterol content in membranes, the lower its fluidity, and vice versa [16–19].

Membranes do not only confine compartments, they also determine the nature of all communication between the interior and exterior of the cell. This communication includes transport of ions or molecules across the membrane, and is accomplished by the action of specific transport proteins. In addition, receptors and enzymes are localized in or on membranes, and are involved in mediating the communication between the interior and exterior of the cell.

Changes in the molar ratio of cholesterol/phospholipid of cell membranes induced by a change in cholesterol content affect a number of important membrane properties, including permeability, transport functions, membrane enzyme activities, the availability of membrane components as substrates, conformation of membrane proteins, and exposure of proteins [5,20,21]. These alterations are mediated either by the change in membrane cholesterol content itself or by a concomitant change in membrane fluidity.

This review deals with the influence of cholesterol on plasma membrane transporters. We have confined ourselves mainly, but not exclusively, to ion transporters located in the plasma membrane of myocytes. Proposed mechanisms by which cholesterol may affect the different transporters are presented.

2. Calcium

The calcium ion acts both as an electrical and chemical signal. This dual role is facilitated by its high transmembrane gradient ([Ca\(^{2+}\)]\(_i\)/[Ca\(^{2+}\)]\(_o\) \approx 10000). Alterations in the cytosolic free calcium concentration triggered by chemical or electrical signals regulate many intracellular processes such as muscle contraction, activation of enzymes, and membrane ionic conductance [22–24].
Intracellular free calcium concentration can be increased by: (i) increased \(Ca^{2+}\) influx through transmembrane channels; (ii) decreased \(Na^+/Ca^{2+}\) exchange activity in the direct mode (i.e., \(Ca^{2+}\) efflux); (iii) decreased sarcolemmal \((Ca^{2+},Mg^{2+})\)-ATPase activity; (iv) release of \(Ca^{2+}\) from internal stores, and/or diminished calcium uptake capacity of these stores; or (v) any combination of the previous items (i to iv). In cardiac and smooth muscles the main subcellular structures capable of accumulating calcium are the sarcoplasmic reticulum (SR) and the mitochondria. Calcium efflux is accomplished by sarcolemmal \((Ca^{2+},Mg^{2+})\)-ATPase and by \(Na^+/Ca^{2+}\) exchange. Like the sarcolemma, the SR membrane contains a \((Ca^{2+},Mg^{2+})\)-ATPase which is responsible for calcium uptake in the SR.

2.1. \(Ca^{2+}\) channel

Several studies have demonstrated that an increase in cholesterol content of plasma membranes leads to increased \(Ca^{2+}\) flux through the \(Ca^{2+}\) channel in plasma membranes [25–28].

Enrichment of arterial smooth muscle cell membranes with cholesterol was accompanied by a selective increase in sensitivity to the vasoconstrictor action of norepinephrine, but not to vasoconstriction mediated by histamine, serotonin or KCl [25, 26]. Since this increase in sensitivity was reversed by verapamil, Tulenko et al. [27] concluded that excess membrane cholesterol in arterial smooth muscle cells increased \(Ca^{2+}\) influx through the \(Ca^{2+}\) channel under norepinephrine stimulation. Gleason et al. [28] and Bialecki et al. [29] investigated the relation between membrane cholesterol content, \(^{34}Ca^{2+}\) movements across the plasma membrane, intracellular \(Ca^{2+}\) concentration ([\(Ca^{2+}\)]) and membrane fluidity in smooth muscle cells and isolated plasma membranes. Enrichment of vascular smooth muscle cells with cholesterol (leading to an increase in free cholesterol content of 52%) is associated with an increase in \([Ca^{2+}]\), of about 34% [28]. If the procedure to enrich vascular smooth muscle cells with cholesterol was performed in the presence of a calcium antagonist (either nifedipine, verapamil, or diltiazem), the rise in \([Ca^{2+}]\), was attenuated by 25–50%, whereas the calcium antagonist alone slightly reduced \([Ca^{2+}]\), by 6–13%. Thus, Gleason et al. [28] hypothesized that "cholesterol enrichment alters lipid dynamics in the arterial smooth muscle plasma membrane and results in the appearance of a new, or an unmasking of an otherwise silent, dihydropyridine-sensitive calcium channel." This is consistent with the findings of Strickberger et al. [30] who reported that in cholesterol-fed rabbits the intracellular calcium content of aortic segments is increased 4–8-fold compared to that of aortic segments of rabbits fed a normal chow.

Similar results (i.e., an increased cholesterol content of the plasma membrane is associated with an increased \([Ca^{2+}]\), and vice versa) were also found in smooth muscle cells of bovine arteries [31], rat heart myocytes [32], platelets [33], monocytes [34], and erythrocytes [35]. The study performed with erythrocytes showed that \(Ca^{2+}\) influx through the \(Ca^{2+}\) channel was increased by 100% if the membrane cholesterol content was increased by 80%, and was reduced by 47% if the membrane cholesterol content was reduced by 45% [35]. If cultured neonatal rat cardiomyocytes were incubated for 5 h with cholesterol-rich or cholesterol-free liposomes having a molar free cholesterol/phospholipid ratio of 2 and 0, respectively, the molar free cholesterol/phospholipid ratio of the cells changed from a baseline value of 0.328 ± 0.017 to 0.538 ± 0.081 (with cholesterol-rich liposomes) and to 0.257 ± 0.040 (with cholesterol-free liposomes) [32]. Subsequent determination of the fluorescence steady-state anisotropy with the probe TMA-DPH (1-(4-trimethylammonium-phenyl)-6,13,5-hexatriene) in cholesterol-loaded and in cholesterol-depleted cardiomyocytes gave values 8.0% higher and 4.2% lower (corresponding to diminished and augmented membrane fluidity, respectively) than in untreated cardiomyocytes, respectively. Enrichment of the sarcolemma of cultured cardiomyocytes with cholesterol was associated with a raised \([Ca^{2+}]\), and cholesterol depletion of the sarcolemma was associated with diminished \([Ca^{2+}]\) (Fig. 2). Since the rise in \([Ca^{2+}]\), induced by cholesterol loading of the cells was abolished by the presence of verapamil throughout and after the incubation of the cells with cholesterol-rich liposomes, Bastiaanse et al. argued that cholesterol enrichment of cardiomyocytes causes activation of \(Ca^{2+}\) channels either by opening more channels per myocyte or by inducing a higher conductance per channel [32]. Renaud et al. [36] demonstrated that if the cholesterol content of chicken embryonic ventricular cells in culture was reduced by mevinolin, a HMG-CoA reductase in-
hibitor, spontaneous electrical activity and contractions were suppressed. The number of tetrodotoxin binding sites (representative of the number of Na⁺ channels) was unchanged as was the number of nitrendipine binding sites (representative of the number of L-type Ca²⁺ channels). However, treatment of the cells with mevinolin did not alter the activation characteristics of the Na⁺ channels, but completely abolished the activation of the Ca²⁺ channels normally induced by high K⁺, isoproterenol or Bay K8644.

2.2. (Ca²⁺,Mg²⁺)-ATPase in sarcolemma and sarcoplasmic reticulum

Ortega and Mas-Oliva [37] measured the (Ca²⁺,Mg²⁺)-ATPase activity of a sarcolemmal preparation isolated from hearts of New Zealand white rabbits. Cholesterol incorporation into the sarcolemma was achieved by in vivo and in vitro procedures. Cholesterol-depleted membranes were obtained in vitro after incubation of the sarcolemma preparation with inactivated plasma. The (Ca²⁺,Mg²⁺)-ATPase was inhibited by an increase of the sarcolemmal cholesterol content, whereas the withdrawal of small amounts of cholesterol from the membranes had a considerable stimulatory effect. Changes in (Ca²⁺,Mg²⁺)-ATPase activity due to an altered membrane cholesterol content were fully reversible [37].

Ortega and Mas-Oliva [38] and Mas-Oliva and Santiago-Garcia [39] suggested that cholesterol interacts with the sarcosomal calcium pump in a direct manner, thereby inhibiting its enzymatic activity. They demonstrated that this inhibition could be, at least in part, explained by a complete loss of the pump’s sensitivity to calmodulin [38]. It is possible that by interaction with the boundary lipid of ATPase, cholesterol might alter the amount of water associated with the enzyme in such a way that the intermolecular hydrogen bonds of the protein are altered, leading to a modification of the protein structure [39]. Since the Km of sarcolemal (Ca²⁺,Mg²⁺)-ATPase for ATP in different membrane preparations is independent of the sarcolemmal cholesterol content, it is likely that specific regions of the enzyme, such as the low-affinity site for ATP, are not affected by the inclusion of cholesterol in the membrane [38,39]. The reversible modification of the enzyme caused by cholesterol impairs the catalytic activity of the ATPase (i.e., reduces the Vmax), but—at the same time—renders the ATPase resistant to thermal inactivation [39]. The effects might be mediated by a more general mechanism probably related to the overall change in the tertiary structure of the ATPase. Apparently, a restriction in the freedom of movement of the ATPase interferes with conformational changes occurring during the catalytic cycle, and moreover renders the enzyme less susceptible to thermal inactivation. Likewise, the lower [Ca²⁺]i in cholesterol-depleted cardiomyocytes described by Bastiaanse et al. [32] was ascribed by them to the activation of sarcolemmal Ca²⁺-ATPase.

The sarcoplasmic reticulum (SR) is a useful membrane system to investigate the influence of cholesterol on intrinsic membrane proteins. (Ca²⁺,Mg²⁺)-ATPase is the major intrinsic protein of the membrane and is responsible for the active transport of calcium ions into the SR. Two calcium ions are taken up into purified SR vesicles for each ATP molecule hydrolyzed [40].

Warren et al. [41] have proposed a general theory concerning the interaction of membrane lipids with the intrinsic membrane protein (Ca²⁺,Mg²⁺)-ATPase of the SR and the biochemical consequences of this interaction. The theory postulates that about 30 phospholipid molecules interact directly with the hydrophobic surface of the ATPase spanning the bilayer, and form the minimal lipid environment required to maintain full ATPase activity of the protein. Cholesterol is considered to be excluded from the phospholipid annulus (i.e., the direct environment of the protein in the SR membrane), which is relatively poor in cholesterol. This would suggest that cholesterol is unable to modulate the enzyme activity in the SR. Moreover, it has been suggested that the annulus protects the enzyme from changes in bulk membrane fluidity caused by the incorporation of cholesterol into the membrane. To force cholesterol to interact directly with the ATPase, it was necessary to use high concentrations of the ionic detergent, cholate, which strips phospholipid from the annulus. The activity of the (Ca²⁺,Mg²⁺)-ATPase is unaffected by the presence of cholesterol if 30 or more phospholipid molecules per ATPase molecule are preserved in the annulus. Below this value, cholesterol inhibits ATPase activity by replacing phospholipid in the annulus, and equimolar amounts of cholesterol and phospholipid in the annulus completely and reversibly inhibit ATPase activity [41]. In vivo, however, the phospholipid annulus of SR Ca²⁺-ATPase normally excludes cholesterol, rendering the enzyme relatively insensitive to changes in SR cholesterol content.

Madden et al. [40] examined the biochemical evidence which supports the annulus hypothesis. They found that increasing amounts of cholesterol caused a proportional decrease in enzyme activity. This result is in contrast with the observation reported by Warren et al. [41]. Apart from the method of reconstituting the enzyme system with cholesterol, another major difference between the studies of Warren et al. [41] and Madden et al. [40] concerns the method of membrane preparation. Madden et al. [40] showed that in vesicles of SR with or without supplemented cholesterol prepared in the presence of a protease inhibitor (phenylmethyl-sulphonylfluoride) and dithiothreitol, the ATPase is in a tightly coupled state in that enzyme activity is geared to the transport of calcium into the vesicles. The effect of membrane cholesterol on enzyme activity in these preparations is obscured by the fact that the rate-limiting step of the process is likely to be the efflux of Ca²⁺ from the vesicles. Collapsing the Ca²⁺ gradient in coupled preparations with a calcium ionophore allows an expression of maximal catalytic action and
unmasks the inhibitory effect of cholesterol on the (Ca\(^{2+}\).Mg\(^{2+}\))-ATPase. The results of Johansson et al. [42] confirm the findings of Warren et al. [41]. Johansson et al. [42] showed that under conditions in which the ATPase activity in SR vesicles is fully uncoupled from Ca\(^{2+}\) movements or in which the ATPase activity is coupled to calcium accumulation, cholesterol had no effect on ATPase activity.

Simmonds et al. [43] used a fluorescence quenching method to measure relative binding constants of hydrophobic compounds such as cholesterol and phospholipids to the phospholipid/protein interface. Cholesterol is capable of displacing only negligible quantities of phospholipid from the annulus around the ATPase, as the relative binding constant of the ATPase for cholesterol compared to that for phosphatidylcholine is 0.1 or less. These authors supported the concept that phospholipid molecules interact with this transmembrane protein more effectively than does cholesterol. Thus, while the sarcolemmal (Ca\(^{2+}\).Mg\(^{2+}\))-ATPase activity is inhibited by sarcolemmal cholesterol enrichment (and vice versa) [37–39], the (Ca\(^{2+}\).Mg\(^{2+}\))-ATPase of sarcoplasmic reticulum appears to be insensitive to changes in membrane cholesterol content [41–43], likely due to the inability of cholesterol molecules to become incorporated into the annulus around the ATPase molecule.

2.3. Na\(^{+}\)/Ca\(^{2+}\) exchange

By the process of Na\(^{+}\)/Ca\(^{2+}\) exchange calcium ions run in either direction across the membrane depending on the transmembrane Na\(^{+}\) and Ca\(^{2+}\) gradients and on the membrane potential. Na\(^{+}\)/Ca\(^{2+}\) exchange is electrogenic; the stoichiometry of the sarcolemmal exchange is three Na\(^{+}\) ions per Ca\(^{2+}\) ion [44].

Kutryk and Pierce [45] demonstrated that incorporation of cholesterol into isolated cardiac sarcolemmal vesicles resulted in a significant stimulation of Na\(^{+}\)/Ca\(^{2+}\) exchange by up to 48% over control values. This stimulation was specific to Na\(^{+}\)/Ca\(^{2+}\) exchange as the activities of (Ca\(^{2+}\).Mg\(^{2+}\))-ATPase and (Na\(^{+}\),K\(^{+}\))-ATPase were depressed by cholesterol enrichment [45]. The observation that oxidation of membrane cholesterol by treatment of cardiac sarcolemmal vesicles with cholesterol oxidase completely eliminated Na\(^{+}\)/Ca\(^{2+}\) exchange activity suggests that cholesterol is intimately associated with the Na\(^{+}\)/Ca\(^{2+}\) exchange protein [45,46].

These results indicate that the Na\(^{+}\)/Ca\(^{2+}\) exchange protein is surrounded by a cholesterol-rich annulus which modulates its activity.

3. Potassium and sodium

In this section the (Na\(^{+}\),K\(^{+}\))-ATPase and the Ca\(^{2+}\)-dependent K\(^{+}\) channel, both involved in the maintenance of transmembrane Na\(^{+}\) and K\(^{+}\) gradients, are discussed.

3.1. (Na\(^{+}\),K\(^{+}\))-ATPase

The (Na\(^{+}\),K\(^{+}\))-ATPase is responsible for the energy-dependent transmembrane transport of Na\(^{+}\) and K\(^{+}\) ions. When energized by intracellular ATP, the pump transports two K\(^{+}\) ions inwardly and three Na\(^{+}\) ions outwardly [47], leading to hyperpolarization.

Claret et al. [48] have shown that a 20–25% depletion in cholesterol of erythrocyte membranes caused substantial modifications of the kinetic parameters of the (Na\(^{+}\),K\(^{+}\))-ATPase: the apparent affinity for internal Na\(^{+}\) was decreased and the maximal translocation rate was increased, whereas the apparent affinity for external K\(^{+}\) remained unchanged. It is not known whether these effects are a reflection of a direct interaction of cholesterol molecules or are mediated indirectly by a change in the packing of phospholipids (i.e., a change in membrane fluidity). The observation by Giraud et al. [47] that the Na\(^{+}\) and K\(^{+}\) sites of the (Na\(^{+}\),K\(^{+}\))-ATPase of erythrocyte membranes were affected differently by the lipid order of the leaflet in which the cation sites were located, suggests that the Na\(^{+}\) and K\(^{+}\) sites are located in different lipid environments.

In membranes of human erythrocytes [49], guinea pig erythrocytes [50], rat liver cells [51], rabbit kidney outer medulla cells [52] and bovine kidney basolateral cells [53], a supranormal cholesterol content (by 50–100% higher than normal values) inhibits the (Na\(^{+}\),K\(^{+}\))-ATPase activity by 30–70%. This inhibitory effect of cholesterol is likely caused by a diminished molecular motion or conformational freedom of the enzyme in the bilayer, limited by the increase in molecular order (‘freezing’) of the phospholipid acyl chains in the membrane.

3.2. Ca\(^{2+}\)-dependent K\(^{+}\)-channel

The Ca\(^{2+}\)-dependent K\(^{+}\) channel opens in response to a raised [Ca\(^{2+}\)]. Patch-clamp techniques and fluorescence polarization analysis with DPH were used to study whether Ca\(^{2+}\)-dependent K\(^{+}\) channel kinetics and membrane fluidity depended on the cholesterol content of the plasma membranes of cultured rabbit aortic smooth muscle cells [54]. Mevinolin, a potent inhibitor of endogenous cholesterol biosynthesis by inhibition of HMG-CoA reductase, was used to deplete the cholesterol content of the plasma membrane. Treatment of smooth muscle cells with mevinolin led to an increase in membrane fluidity (corresponding to a decrease in fluorescence anisotropy of 31%) and to a ninefold increase in the probability of the Ca\(^{2+}\)-dependent K\(^{+}\) channels being open (\(P_o\)), suggesting a modulatory role of membrane composition and lipid dynamics on the function of this channel. Direct evidence for the role of cholesterol was obtained in experiments with cholesterol enrichment of cell membranes and isolated membrane patches [54]. After the membranes had been enriched with cholesterol, membrane fluidity was decreased (corresponding to an increase in fluorescence anisotropy of 36%) and
$P_r$ was decreased by 50%. Thus, the conductance of the $Ca^{2+}$-dependent $K^+$ channel is inversely related to the membrane cholesterol content.

A direct interaction of cholesterol with the ion channel is unlikely since cholesterol was shown to be absent in the annulus and located mainly in the bulk [5,15]. Several interventions designed to vary membrane fluidity were reported to affect ion channel function in general and $Ca^{2+}$-dependent $K^+$ channel function in particular. Bregestovski et al. [55] described elevated activity of the $Ca^{2+}$-dependent $K^+$ channel from human aorta by increasing the lateral motion of membrane lipids using 2-decenoic acid. Thus, the conductance of the $Ca^{2+}$-dependent $K^+$ channel appears to be sensitive to membrane fluidity.

4. Miscellaneous

4.1. Gap junctions

A gap junction is a special kind of solute transporter which mediates intercellular communications. The gap junction channels permit the exchange of ions and of small hydrophilic molecules of up to 1000 Da. By providing a low-resistance pathway for conduction of the action potential, gap junctions serve to synchronize electrical, and thereby contractile, events in the heart [56,57].

Gap junctions are localized in plasma membrane domains rich in cholesterol (i.e., the lipid environment of the gap junctions has a higher cholesterol content than the extrajunctional plasma membrane). This phenomenon may indicate that cholesterol plays a role in gap junction assembly, structure and function [58].

The effect of cholesterol on gap junction assembly and function was studied in Novikoff hepatoma cells by incubating the cells in medium containing serum supplemented with cholesterol [58–60]. Depending on the cholesterol concentration, gap junction assembly and permeability were either stimulated or inhibited. If cellular cholesterol content was increased by about 50% of control levels, maximal stimulation was observed: a seven-fold increase in the number of aggregated gap junction particles, and a three-fold increase in gap junction plaque area. The rate of Lucifer yellow transfer between cells was increased. At higher cholesterol content, gap junctional formation was negatively affected, or was even lower than in control cells [59].

In human smooth muscle cells, cholesterol supplementation resulted in an increase in gap junctional communication to levels of 130% compared to control values [61].

In a study on gap junctional conductance of neonatal rat cardiomyocytes, different fluorescent probes were used to assess the fluidity of the various domains: TMA-DPH for bulk fluidity and DHE (dehydroergosterol) to assess the fluidity of cholesterol-rich domains [62]. These probes allow differentiation between effects of lipophilic substances on bulk fluidity and on the fluidity of cholesterol-rich domains. The uncoupling action of 1-heptanol (2 mM) (i.e., the decrease of gap junctional current to zero) was associated with increased bulk membrane fluidity and decreased fluidity of the cholesterol-rich domains in which the gap junctions reside. Thus, gap junctional conductance of neonatal rat cardiomyocytes is affected by the fluidity of cholesterol-rich domains, rather than by fluidity of the bulk lipids [62].

4.2. Receptors

Several receptors can be recognized as transporters of ions (i.e., upon stimulation they transport ions across the membrane). Barnett et al. [63] used chick atrial myocytes cultured in medium supplemented with either 6% fetal calf serum (FCS) which represents control conditions, 6% lipoprotein-deficient serum (LPDS) to stimulate cellular cholesterol synthesis, or 6% LPDS + 30 μM mevinolin to inhibit cellular cholesterol synthesis. Under control conditions (in medium + FCS) isoproterenol stimulated adenylate cyclase activity by 128% over basal. The number of β-adrenergic receptors assayed by $[^{3}H]/$ipindol binding was 24 fmol/mg protein. If incubated in medium with LPDS, isoproterenol-stimulated adenylate cyclase activity was only 35% over basal, and the number of β-adrenergic receptors was reduced by 50% to 12 fmol/mg protein. Blockade of cholesterol synthesis by addition of mevinolin restored the isoproterenol-stimulated adenylate cyclase activity to a value of 118% over basal, in combination with a number of β-adrenergic receptors of 28 fmol/mg protein. Moreover, atrial myocytes incubated in medium with LPDS had lower (by 70%) levels of $\alpha_x$-subunits of the stimulatory guanine nucleotide binding protein (G_s), which couples the receptor–agonist interaction to adenylate cyclase stimulation, compared to cells incubated in medium with FCS. Blockade of cellular cholesterol synthesis partially reversed the effects of LPDS on the levels of $\alpha_x$-subunits, and increased the relative amounts of $\alpha_x$-subunits to 73% of that in FCS. Thus, stimulation of cellular cholesterol synthesis blunted the responsiveness of atrial myocytes to β-adrenergic stimulation. However, the responsiveness of muscarinic receptors was increased in these cells under conditions of stimulated cellular cholesterol synthesis [64]. Increased cholesterol synthesis of the cells was associated with a two-fold increase in the number of muscarinic receptors which bind agonist with high affinity, and a two-fold increase in the levels of the $\alpha_x$-subunits of G_s and G_i. Addition of mevinolin was associated with a reduction in the number of muscarinic receptors to a value equal to that observed under control conditions (medium with FCS; 180 fmol/mg protein).

In view of these results, the findings of Dalziel et al. [65] and Criado et al. [66] are worth mentioning here. Dalziel et al. [65] demonstrated that in reconstituted vesicles of electroplax tissue from Torpedo californica chole-
terol was necessary to maintain Na\(^+\) flux through the acetylcholine receptor channel. In these experiments cholesterol appeared to have no effect on the ligand binding properties of the acetylcholine receptor, nor on its ability to undergo affinity transitions (i.e., a shift from a low-affinity to a high-affinity binding state for the agonist). They suggested that cholesterol may be involved in modulating the ion channel protein itself without affecting the agonist-binding properties of the receptor. However, in a study on the acetylcholine receptor isolated from the electroplax tissue of *Torpedo marmorata* and incorporated into vesicles of pure synthetic lipids, Criado et al. [66] found that exogenous cholesterol is essential for maintenance of agonist-induced state transitions of the acetylcholine receptor in lipid vesicles.

### 5. Integrative view

Present concepts concerning the mechanisms of lipid-induced modulation of transport protein function range between two extremes: modulation by bulk properties, or by specific interactions [14]. Interest in bulk properties has been focused mainly on membrane fluidity. As mentioned in Section 1, the fluidity of biomembranes is diminished particularly by cholesterol enrichment. Little is known about the nature and specificity of interactions of the cholesterol molecule with specific protein sites.

Table 1 presents an overview of the effects of cholesterol enrichment and cholesterol depletion on several transporters in the membrane. A striking point is that cholesterol enrichment has an inhibitory effect on all membrane ATPases studied, whereas most other membrane transport proteins are stimulated by an increased membrane cholesterol content.

The ATPases listed in Table 1 are so-called E1/E2-type cation transporters, and are related by mechanistic similarities as well as amino acid homologies [67]. One of their characteristics is the conformational change that takes place during the transport process of the ions [67,68]. The fluidity of the lipid environment may affect the conformational change during the transport cycle, which is a key element of the function of these transporters, since a large free energy barrier requires a change in protein conformation to enable transport. It is likely that an increase in the rigidity of the membrane, as a result of an increased cholesterol content, hinders the conformational change of the protein. This is consistent with the proposal of Mas- Oliva and Santiago-Garcia [39] who stated that the restriction in freedom of movement interferes with the conformational changes of the sarcolemmal Ca\(^{2+}\)-ATPase during the catalytic cycle.

Besides interference in protein conformational changes by membrane rigidity, other mechanisms cannot be excluded. In fact, the similar catalytic units of the ATPases studied could also be a common target for direct interactions of cholesterol with the enzyme, causing diminished activity.

The acetylcholine receptors are activated by an elevated cholesterol content of the membrane (Table 1). A generally accepted theory to explain this phenomenon is not yet available. In this respect, the theory of Trudell about the way anesthetics affect ion channel conductance is worth mentioning [69]. This theory implies that there is a lateral phase separation in the region of a protein–lipid interface, and this phase separation is destroyed by anesthetics. For the function of Na\(^+\) channels, for instance, the conversion of high-volume fluid-phase lipids in the annulus into the low-volume solid phase of the membrane lipids surrounding the annulus allows the Na\(^+\) channel to expand. Anesthetic molecules fluidize the entire bilayer, including the annulus region, thereby preventing, partly or completely, the conformation change (expansion) of the protein to allow Na\(^+\) ions to pass [69,70]. Cholesterol may have an effect opposite to that of anesthetics (i.e., a stabilisation of the phase separation by an anti-fluidizing action).

To elucidate the effect of the annulus on the function of the transport protein, the physical characteristics of lipid domains must be examined. This is achieved by using structurally distinct spin probes and fluorescent probes. Probes that closely approximate intrinsic membrane components would be expected to partition differentially among the various lipid domains, thereby revealing the heterogeneous structure of biological membranes [71]. If this approach is combined with enrichment (or depletion) of cholesterol, more detailed knowledge of the effect of fluidity changes (as a result of changes in cholesterol content) on membrane function is likely to emerge.

#### 5.1. Cell dysfunction

Maintenance of ion homeostasis by the sarcolemma is necessary for proper cell function. In heart cells many processes are controlled or affected by particular ion species, including the control of membrane potential, cell contraction processes, synthesis reactions, and signalling of receptor stimulation. Thus, any process which depends on membrane protein function may be affected by alterations in membrane composition (e.g., due to a change in cholesterol content). Two pathophysiologic processes—hypercholesterolemia and ischemia/anoxia—will be discussed.

In hypercholesterolemia, membrane cholesterol content of vascular cells, such as endothelial cells, macrophages, and smooth muscle cells, is increased. There are several reasons for considering an increased membrane cholesterol content of vascular cells as essential in atherosclerosis. An increased sarcolemmal cholesterol content of vascular smooth muscle cells, as will occur in atherosclerotic arteries, was proposed to increase the number of active Ca\(^{2+}\) channels, leading to increased basal and norepinephrine-stimulated calcium uptake [25,26,28]. In atherosclerotic
Table 1
The effect of membrane cholesterol content on transport proteins, ATPases and receptors

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<th>Function is increased (+) or decreased (-)</th>
<th>Measurement</th>
<th>Reference</th>
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<td><strong>If membrane cholesterol content is increased</strong></td>
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<td>Ca(^{2+}) channel in SL</td>
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<td>(^{45})Ca(^{2+}) uptake</td>
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<td>(Ca(^{2+}), Mg(^{2+})) ATPase in SL</td>
<td>–</td>
<td>Enzyme assay</td>
</tr>
<tr>
<td>(Ca(^{2+}), Mg(^{2+})) ATPase in SR</td>
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<td>Enzyme assay</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Enzyme assay (^1)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Enzyme assay (^2)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Enzyme assay (^3)</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Enzyme assay (^4)</td>
</tr>
<tr>
<td>Na(^{+}/Ca(^{2+}) exchange in SL</td>
<td>+</td>
<td>Na(^{+})-dependent (^{45})Ca(^{2+}) uptake</td>
</tr>
<tr>
<td>(Na(^{+}, K^{+}))-ATPase</td>
<td>–</td>
<td>Enzyme assay</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Oaahain-sensitive Na(^{+}) efflux</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Oaahain-sensitive (^{36})Rb(^{+}) uptake</td>
</tr>
<tr>
<td>Ca(^{2+})-dependent K(^+) channel</td>
<td>–</td>
<td>Single channel conductance</td>
</tr>
<tr>
<td>Gap junction</td>
<td>+</td>
<td>Aggregated gap junction particles</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Dye transfer</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Aggregated gap junction particles</td>
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<tr>
<td>(\beta)-Adrenergic receptor</td>
<td>–</td>
<td>([^{125}])Pindolol binding sites</td>
</tr>
<tr>
<td>Muscarinic receptor</td>
<td>+</td>
<td>([^{3}H])QNB binding sites</td>
</tr>
<tr>
<td>Na(^{+}) flux AchR</td>
<td>+</td>
<td>Agonist-stimulated (^{36})Rb(^{+}) influx</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>Agonist-stimulated (^{36})Rb(^{+}) influx</td>
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<table>
<thead>
<tr>
<th>Function is increased (+) or decreased (-)</th>
<th>Measurement</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>If membrane cholesterol content is decreased</strong></td>
<td></td>
<td></td>
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<tr>
<td>Ca(^{2+}) channel in SL</td>
<td>–</td>
<td>(^{45})Ca(^{2+}) uptake</td>
</tr>
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<td></td>
<td>+</td>
<td>Intracellular Ca(^{2+}) ion concentration</td>
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<td>(Ca(^{2+}), Mg(^{2+})) ATPase in SL</td>
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<td>Enzyme assay</td>
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<tr>
<td>(Ca(^{2+}), Mg(^{2+})) ATPase in SR</td>
<td>+</td>
<td>Enzyme assay</td>
</tr>
</tbody>
</table>

1 If cholesterol if forced to replace phospholipids in the annulus of the ATPase.
2 If the phospholipid annulus is complete.
3 If SR was prepared in the presence of a thiol-reducing agent.
4 In fully-uncoupled ATPase.

SL = sarcolemma; SR = sarcoplasmic reticulum; AchR = acetylcholine receptor; QNB = quinuclidinyl benzilate.
arteries of rabbits fed a rabbit chow supplemented with 1% cholesterol, smooth muscle cell calcium levels were reported to be elevated. Thus, in hypercholesterolemia the increased membrane cholesterol content and increased calcium entry into vascular smooth muscle cells will promote calcium-dependent atherogenic cell processes, such as migration, proliferation, and stimulus-secretion coupling. Several findings indicate that the cholesterol-induced increase in vascular smooth muscle cell calcium content is a crucial, if not imperative, event in atherogenesis: (i) calcium antagonists inhibit serum-stimulated proliferation and DNA synthesis in cultured vascular smooth muscle cells; (ii) calcium antagonists inhibit atherosclerotic plaque formation in animals and retard the progression of minimal atherosclerotic lesions in human coronary arteries, defined as less than 20% in stenosis diameter on the angiogram made before randomization to nicardipine; (iii) a characteristic feature of advanced atherosclerosis is the presence of calcium deposits in the vascular wall. These extracellularly localized calcium deposits are considered to be the remains of calcium-overloaded vascular smooth muscle cells following their migration, proliferation, lipid accumulation and necrosis/apoptosis.

Normothermic no-flow ischemia of isolated rat hearts for 60 min resulted in a decline in sarcolemmal cholesterol content of 43% and a reduction in cellular cholesterol content of 34% in cultured neonatal rat cardiomyocytes. In this study, the decrease in cellular cholesterol content was significant after 15 min of metabolic inhibition induced by a combination of 2-deoxyglucose and NaCN, which caused a decrease in cellular free cholesterol content of 22% in the latter study. A decrease in the free cholesterol concentrations was significant after 15 min of metabolic inhibition already (Fig. 3). Fig. 4, taken from the same study, shows that the decrease in fluorescence steady-state anisotropy using TMA-DPH observed in the first 90 min of metabolic inhibition is correlated with the decrease in cellular free cholesterol content at corresponding time points, suggesting that the increase in sarcolemmal fluidity which occurs early after the onset of metabolic inhibition, may be due to loss of cholesterol from the sarcolemma. The decrease of sarcolemmal cholesterol preceded the onset of irreversible cell damage. The occurrence of irreversible cell death is primarily caused by ATP depletion and its consequences, such as loss of K⁺ ions and accumulation of Na⁺, Ca²⁺ and H⁺ ions. The subsequent destruction of the cellular architecture includes the degradation of cytoskeletal and membrane components. Cellular calcium overload to resting [Ca²⁺]i levels of 2 µM or higher will lead to severe mitochondrial calcium overload causing dissipation of mitochondrial inner membrane potential, impairment of oxidative phosphorylation, and the inability to regenerate a sufficiently high cytoplasmic phosphorylation potential to drive active calcium transport systems of the plasma membrane and SR. The mitochondrial calcium overload is considered to be the consequence of the formation of a non-specific 'channel' regulated by Ca²⁺ [79] or a Ca²⁺-activated pore which is opened by complete oxidation of the mitochondrial glutathione redox couple [80]. Thus sarcolemmal cholesterol content determine the duration of the reversible phase of cell injury during ischemia/anoxia? The time-dependent decrease of membrane cholesterol may indicate a degradative action operating at the membrane level. In our laboratory, it has been shown that the tolerance to anoxia of cultured cardiomyocytes is dependent on the cholesterol content of the sarcolemma. The higher the initial cholesterol content of the sarcolemma, the higher the tolerance to anoxia, and vice versa (Fig. 5). Where does the cholesterol go during ischemia/anoxia after it has left the sarcolemma? Roussin et al. demonstrated that although whole tissue cholesterol in canine myocardial tissue made...
ischemic by 60 min of coronary artery occlusion decreased by 24%, mitochondrial cholesterol content rose by 67% in that time [82]. The extent to which mitochondrial membrane cholesterol increased closely paralleled the concomitant impairment of mitochondrial state-3 respiration, suggesting that increased mitochondrial cholesterol may be regarded as a marker of mitochondrial membrane injury. Thus, the autolytic processes which are set in motion by severe energy depletion cause (i) redistribution of cholesterol from cholesterol-rich membranes (sarcolemma) to cholesterol-poor membranes (mitochondria), and (ii) cholesterol degradation. The question whether an increased mitochondrial cholesterol concentration (or a decreased mitochondrial membrane fluidity) is responsible for the impairment of mitochondrial function during ischemia is still unresolved.

6. Conclusions

Several transport proteins are affected by the cholesterol content of the membranes in which the proteins are located. The effect of cholesterol may be mediated by membrane fluidity or by the cholesterol molecules themselves, or both. For most of the transporters studied, the mechanism by which their activity depends on cholesterol content has not been elucidated yet. Interestingly, an increase in membrane cholesterol content inhibits ATPases and activates most other transport proteins.

As transporters are affected by the cholesterol content, a change in cholesterol content should influence cell function. To date, the concept of cholesterol’s influence on cell function has not attracted much attention. It is also of interest to know what the effect of a change in cholesterol content is on several other transport proteins, for instance transporters located in mitochondrial membranes.

References


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