Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle

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Jennings, Lee J., Qi-Wei Xu, Tracy A. Firth, Mark T. Nelson, and Gary M. Mawe. Cholesterol inhibits spontaneous action potentials and calcium currents in guinea pig gallbladder smooth muscle. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G1017–G1026, 1999.—Elevated cholesterol decreases agonist-induced contractility and enhances stone formation in the gallbladder. The current study was conducted to determine if and how the electrical properties and ionic conductances of gallbladder smooth muscle are altered by elevated cholesterol. Cholesterol was delivered as a complex with cyclodextrin, and effects were evaluated with intracellular recordings from intact gallbladder and whole cell patch-clamp recordings from isolated cells. Cholesterol significantly attenuated the spontaneous action potentials of intact tissue. Furthermore, calcium-dependent action potentials and calcium currents were reduced in the intact tissue and in isolated cells, respectively. However, neither membrane potential hyperpolarizations induced by the ATP-sensitive outward potassium channel opener, pinacidil, nor voltage-activated outward potassium currents were affected by cholesterol. Hyperpolarizations elicited by calcitonin gene-related peptide were reduced by cholesterol enrichment, indicating potential changes in receptor ligand binding and/or second messenger interactions. These data indicate that excess cholesterol can contribute to gallbladder stasis by affecting calcium channel activity, whereas potassium channels remained unaffected. In addition, cholesterol enrichment may also modulate receptor ligand behavior and/or second messenger interactions.

cholelithiasis; gallstone disease; biliary stasis; biliary tract motility

HUMAN BILIARY TRACT STUDIES have revealed a motility deficit in the gallbladders of patients with cholesterol gallstones. For example, diminished gallbladder emptying, in response to a meal and/or CCK infusion, occurs in a high proportion of patients with cholesterol gallstones (12, 14, 17, 26). Furthermore, patients with cholesterol stones have increased residual gallbladder volumes interprandially, as well as a decreased ejection fraction after a meal (12, 25). Finally, gallbladder smooth muscle strips from patients with cholesterol gallstones also have impaired contractile responses to a variety of stimuli in vitro, compared with gallbladder strips from patients with pigment stones (1, 6).

Several lines of data support the concept that decreased motility is a critical phase in the cascade of events that lead to cholesterol gallstone formation. When fed an elevated cholesterol diet, ground squirrels and prairie dogs undergo a progressive increase in cholesterol saturation with a subsequent decrease in gallbladder contractility, followed by cholesterol stone formation (9, 15). When gallbladder motility is increased with prokinetic agents, such as cisapride or erythromycin, the formation of cholesterol crystals is prevented in cholesterol-fed animals (29, 30). Conversely, when gallbladder stasis is pharmacologically induced by the administration of a CCK-A antagonist, gallstone formation is enhanced, especially in cholesterol-fed animals (28).

Together, these clinical and experimental observations indicate that gallstone formation is likely to result from an elevation in cholesterol levels within the bile and, in turn, a decrease in the contractility of the gallbladder. The link between cholesterol and gallbladder muscle contractility has been studied by examining muscle responses from prairie dog gallbladders that were incubated for 2 h in cholesterol-rich liposomes (33). These results were compared with data from the gallbladders of prairie dogs that were fed a cholesterol-rich diet. Treatment with the cholesterol-rich liposomes yielded results similar to those obtained from human gallbladders with cholesterol stones and from the gallbladders of animals that were fed a cholesterol-rich diet. CCK responsiveness was decreased in gallbladder muscle strips and from isolated myocytes that were exposed to cholesterol-rich liposomes. CCK-induced gallbladder myocyte shortening was reduced in these experimental paradigms as well. These results demonstrate that cholesterol can have a direct effect on the gallbladder muscle; however, the mechanisms by which cholesterol decreases gallbladder smooth muscle motility are not known.

Potential sites of the contractile defect caused by cholesterol could be at the smooth muscle membrane, relating to impaired ligand binding, signal transduction pathways, and/or ion channels, or cholesterol may disrupt the contractile machinery. The possibility that cholesterol disrupts the contractile machinery has been largely ruled out. No difference is detected between the contractile responses of gallbladder muscle strips from control and cholesterol-fed ground squirrels exposed to the calcium ionophore A-23187, which allows access of calcium to the contractile proteins and causes contractions (31). Therefore, it is likely that the primary smooth muscle defect in cholesterol gallstone disease does not reside in the contractile apparatus but rather involves the smooth muscle membrane.
In the studies reported here, we tested the hypothesis that the voltage-activated ionic currents, which underlie the spontaneous action potential in gallbladder smooth muscle, are affected by exposure to cholesterol. To accomplish this, intact and acutely dissociated gallbladder smooth muscles were exposed to cholesterol, which was complexed to cyclodextrin to make it soluble. Intracellular recordings were made from intact smooth muscle to determine the effects of cholesterol on the spontaneous action potential, responses to channel openers, and the neuroactive peptide, calcitonin gene-related peptide (CGRP). Whole cell patch-clamp recordings were made from isolated cells to test the effects of cholesterol on voltage-activated inward and outward currents that underlie the action potential in these cells.

METHODS

Adult guinea pigs of either sex, weighing 250–350 g, were used for this study. Animals were anesthetized with isoflurane and exsanguinated. This method has been reviewed and approved by the Institutional Animal Care and Use Committee of the University of Vermont.

Intracellular recordings. Gallbladders were cut open with a single incision from the end of the cystic duct to the base of the organ. They were pinned flat, mucosal side up, in a dish lined with Sylgard 184 elastomer (Dow Corning, Midland, MI). The mucosal layer and underlying connective tissue were gently removed with forceps under microscopic observation while bathed in ice-cold, recirculated modified Krebs solution that comprised (in mM) 121 NaCl, 5.9 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 25 NaHCO$_3$, 1.2 Na$_2$HPO$_4$, and 8 glucose. The preparations were then pinned out in a Sylgard-lined tissue chamber and placed on the stage of an inverted microscope (Nikon Diaphot). Smooth muscle bundles were visualized at ×200 with Hoffman Modulation Contrast optics (Modulation Optics, Greenvale, NY). The preparations were continuously perfused at a rate of 10–12 ml/min with the modified Krebs solution and aerated with 95% O$_2$–5% CO$_2$. Temperature was maintained between 36 and 37°C at the recording site. Wortmannin (500 nM), which inhibits myosin light-chain kinase activity without altering electrical properties or calcium transients in smooth muscle (4, 5), was added to the Krebs solution to inhibit tissue contractions and therefore to increase the durations of cell impalements. Glass microelectrodes used for intracellular recording were filled with 2.0 M potassium chloride and had resistances in the range of 50–110 MΩ. A negative-capacity compensation amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA) with bridge circuitry was used to record electrical activity.

Patch-clamp recordings. For patch-clamp studies, gallbladders were dissected into small strips (1 × 3 mm) in a calcium-free cell isolation solution that comprised (in mM) 55 NaCl, 80 sodium glutamate, 2 MgCl$_2$, 6 KCl, 10 glucose, and 10 HEPES (adjusted to pH 7.3). The resulting tissue was placed into isolation medium containing 1 mg/ml BSA, 1 mg/ml dithioerythritol, and 1 mg/ml papain ( Worthington, Lakewood, NJ) and incubated for 35 min at 37°C. The tissue was subsequently transferred to medium containing 1 mg/ml collagenase (Fluka, Milwaukee, WI), 1 mg/ml BSA, and 100 µM CaCl$_2$ and incubated for a further 10 min. After a wash with ice-cold medium, the tissue was triturated in cell isolation medium to yield single smooth muscle cells. Cells were stored until use at 4°C for up to 6 h.

Whole cell patch-clamp recordings were performed as previously described for isolated smooth muscle cells (35, 36) using an Axopatch 1D amplifier (Axon Instruments). Isolated cells (3–5 drops) were placed into a recording chamber on the stage of an inverted phase-contrast microscope (Nikon) under ×150 magnification. Currents were filtered with a low-pass eight-pole bessel filter at 1 kHz for calcium currents and 500 Hz for potassium currents. Calcium currents were sampled at 25 kHz and potassium currents at 1 kHz by a Digidata analog-to-digital board using pCLAMP software (Axon Instruments). Currents were corrected for leakage current, which is <20 pA at −80 mV and linear over the voltage range of −50 to −90 mV. This current was extrapolated to other potentials and electronically subtracted from all the recordings. All experiments were conducted at 22–26°C. Electrodes were pulled from borosilicate glass and had resistances in the range of 2–10 MΩ.

For whole cell calcium channel recordings, the pipette solution contained (in mM) 130 CsCl, 5 EGTA, 1 MgCl$_2$, 10 HEPES, 2 NaATP, 0.5 NaGTP, and 10 glucose (adjusted to pH 7.2 with CsOH). The bathing solution contained (in mM) 142 tetraethylammonium chloride, 10 HEPES, 1 MgCl$_2$, 2 CaCl$_2$, and 10 glucose (adjusted to pH 7.4 with NaOH). For outward potassium current recordings, the pipette solution contained (in mM) 107 KCl, 30 KOH, 1 NaCl, 2 MgCl$_2$, 10 HEPES, 1 CaCl$_2$, 1 NaATP, 1 NaGTP, and 10 EGTA (adjusted to pH 7.2 with KOH). The bath solution contained (in mM) 136 NaCl, 6 KCl, 1 MgCl$_2$, 10 HEPES, 10 glucose, and 2 CaCl$_2$ (adjusted to pH 7.4 with NaOH).

Cholesteryl ester tracer study. To visually examine the uptake of cholesteryl into isolated gallbladder smooth muscle cells and whole mount tissues, a BODIPY FL-tagged cholesteryl ester (cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate; Molecular Probes, Eugene, OR) was used as a marker. The BODIPY FL-tagged cholesteryl ester is marketed as a cholesterol analog with similar polarity properties to cholesterol and has been previously used as a tracer of cholesterol transport (21). The cholesteryl ester consists of a fatty acid esterified to the 3β-hydroxyl group of cholesterol.

For comparative purposes, BODIPY FL-tagged cholesteryl ester was delivered to the isolated cells and whole mount tissue as a complex with cyclodextrins. To do this, methyl-β-cyclodextrin was mixed with distilled water at a ratio of 45% wt/vol. After solubilization, either cholesterol, the BODIPY FL-tagged cholesteryl ester or the BODIPY FL fluorophore alone in the form of propionic acid (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid; Molecular Probes) was added to the solution at a ratio of 4% wt/wt to the cyclodextrin concentration. This material was shaken for 1 h at room temperature with care taken to protect the solution from light and then centrifuged (10,000 g) for 5 min. The supernatant was carefully drawn off and frozen before lyophillization, and the insoluble component was discarded. The supernatant was lyophilized overnight (FTS Systems, Stone Ridge, NY), and the resultant cyclodextrin complex powder was stored for later use at −20°C.

Gallbladder smooth muscle cell suspensions or whole mount tissues were exposed to either the cyclodextrin-cholesterol complex, the cyclodextrin-BODIPY-tagged cholesteryl ester complex, or the cyclodextrin-BODIPY FL fluorophore complex (10 mg/ml) and incubated at room temperature for 1 h while gently shaken. Aliquots of the cell suspension were then placed on coverslips and left at room temperature for 20 min to allow smooth muscle cells to adhere. Adherent cells were fixed with a 4% paraformaldehyde solution for 5 min at room temperature, washed several times with 0.1 M PBS, and...
mounted on slides with Citifluor (Citifluor, Canterbury, Kent, UK). After incubation, whole mount tissues were fixed in 4% paraformaldehyde for 1 h at 4°C, subsequently washed with 0.1 M PBS, and mounted on slides with Citifluor.

Isolated smooth muscle cells and whole mount preparations were initially visualized under light microscopy using a Nikon inverted microscope with a ×60 oil immersion objective. Imaging of both cells and tissue was undertaken with the use of a laser scanning confocal microscope (Nikon, Middleton, WI) together with a Silicon Graphics R5000 computer (Mountain View, CA) and Intervision software (Noran). The laser was used at only 5% of its maximum intensity (0.5 W beam) due to excessive photobleaching at higher intensities. Samples were excited at 488 nm, and observations were made at an emission wavelength of 515–550 nm. Images were obtained using a z-series configuration, taking 0.5-µm sections through the preparations before final reconstruction with Intervision software.

Cholesterol and phospholipid assay. Gallbladders were prepared as described above for intracellular recording. The tissues were exposed to either the cholesterol-cyclodextrin complex (50 µg/ml cholesterol), to methyl-β-cyclodextrin alone, or to physiological saline solution for 2 h. At the end of the 2-h period, the tissues were removed, washed in 0.1 M PBS, and then placed in 2 ml of 0.1 M PBS and frozen quickly on dry ice.

Gallbladder membrane preparation. Crude gallbladder membranes were prepared using a method modified from Shaw and colleagues (24a). Gallbladders were thawed (4 per condition), minced, and homogenized in 1.5 ml of a solution containing 0.25 M sucrose, 10 mM HEPES, 1 mM mercaptoethanol, 100 µM phenylmethylsulfonyl fluoride, and 0.01% BSA using a Polytron (Brinkmann Instruments, Westbury, NY). Homogenates were centrifuged at 25,000 g for 15 min; the supernatants were retained and subsequently spun at 150,000 g for a further 45 min. The pellets were resuspended in 1.1 ml of homogenization buffer (as above but excluding BSA). The protein content of each batch of membranes was determined with a protein assay kit (Bio-Rad Laboratories, Fullerville, WI) together with a Silicon Graphics R5000 computer (Mountain View, CA) and Intervision software.

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RESULTS

Delivery of cholesterol to the tissue. Cholesterol is an extremely hydrophobic molecule; therefore, a requisite to studying the effects of excess cholesterol on the membrane properties of gallbladder muscle was to devise a way to deliver cholesterol to the tissue in an aqueous solution. Our strategy was to add cholesterol to the bathing solution as a cholesterol-cyclodextrin complex, since cyclodextrins bind hydrophobic molecules at high concentrations and release them in dilute solutions. To verify that cyclodextrins could be delivered to gallbladder muscle for incorporation into the plasma membrane, two approaches were used. The first was to expose tissues to a complex composed of cyclodextrin and cholesterol tagged with the fluorophore BODIPY FL and then to examine the tissue to determine whether the fluorophore was incorporated into the muscle cells. The second was to expose gallbladder preparations to the cholesterol-cyclodextrin complex and then compare the membrane cholesterol levels with those of control membranes.

Attaching BODIPY FL to cholesterol involves the removal of a hydroxyl group from cholesterol and the formation of BODIPY FL cholesterol ester. When dissociated muscle cells (3 preparations) and whole mount muscle preparations (n = 3) were exposed to cyclodextrin complexed with BODIPY FL cholesterol, as they would be exposed to cholesterol-cyclodextrin in physiological studies, fluorescence was detected in isolated muscle cells and muscle bundles (Fig. 1). Examination of optical sections taken through the center of the cells using confocal microscopy also demonstrated that the fluorophore was present in association with intracellular organelles in addition to the cytoplasmic membrane, although resolution was insufficient to determine what intracellular membranes were enriched by
the BODIPY FL cholesteryl. Application of cholesterol alone or BODIPY FL alone to similar preparations did not result in any discernible fluorescence.

Application of cholesterol-cyclodextrin complex (50 µg cholesterol/ml) for 2 h resulted in an 83% increase in membrane cholesterol when compared with gallbladders exposed to cyclodextrins alone or normal Krebs solution ($P < 0.05$; Fig. 2).

Cholesterol disrupts the electrical activity of intact gallbladder smooth muscle. The results described above indicate that cholesterol can be delivered to gallbladder smooth muscle in aqueous solutions when complexed with cyclodextrin. To test for cholesterol-induced changes in the electrical activity of gallbladder muscle, intact smooth muscle cells were impaled with intracellular microelectrodes, and the properties of the spontaneous action potential were evaluated before and during cholesterol exposure. The spontaneous action potential of gallbladder smooth muscle consists of a spike and a plateau and occurs at a frequency of $0.3 \text{ Hz}$ (36). When bathed in normal Krebs solution, the intact muscle cells evaluated in the current studies had properties comparable to those described previously (36).

Treatment of gallbladder preparations with cholesterol-cyclodextrin resulted in a marked reduction in the frequency and amplitude of the spontaneous action potentials (Fig. 3) and membrane depolarization (control, $-54.4 \pm 2.0 \text{ mV}$; cholesterol, $-47.5 \pm 2.5 \text{ mV}$; $P < 0.05$; $n = 6$). Frequently, small transient membrane depolarizations occurred that were not accompanied by spike generation, especially after exposure to cholesterol for 1 h or more. Several features of action potentials were analyzed, including frequency, rise time,

![Image](https://example.com/image)

**Fig. 1.** Gallbladder smooth muscle was exposed to cholesterol that was tagged with the fluorophore BODIPY FL and complexed with cyclodextrin to determine whether cholesterol could be delivered to this tissue as a cyclodextrin complex. A and B: uptake of the lipid into isolated smooth muscle cells from 2 different preparations. C: uptake of the lipid into smooth muscle bundles in a gallbladder whole mount preparation. D: individual smooth muscle cell that was exposed to the BODIPY FL fluorophore alone, demonstrating specificity of lipid uptake.

![Image](https://example.com/image)

**Fig. 2.** Delivery of cholesterol (50 µg/ml) complexed with cyclodextrins to in vitro whole-mount gallbladder preparations resulted in a significant ($* P < 0.05$) increase in membrane cholesterol levels compared with controls. Superfusion of the cyclodextrin alone did not alter membrane cholesterol levels when compared with controls.

![Image](https://example.com/image)

**Fig. 3.** Intracellular recordings obtained from intact gallbladder smooth muscle cells in whole-mount preparations revealed a disruption in the frequency of spontaneous action potentials when exposed to cholesterol (50 µg/ml) complexed with cyclodextrins. In this example, recordings obtained from a cell while superfused with cholesterol demonstrate the dramatic disruption in action potential frequency over time. Exposure of cells to cyclodextrin alone under the same conditions did not result in any changes in frequency of the spontaneous action potentials (data not shown). Resting membrane potential for the cell in this example was $-52 \text{ mV}$. Scale bars (left) are in mV.
Effects of cholesterol on the properties of gallbladder smooth muscle action potential involving the activation of dihydropyridine-sensitive, voltage-dependent calcium channels (36). Because cholesterol decreased the amplitude of the spike as well as plateau duration in gallbladder muscle, experiments were conducted to determine whether cholesterol altered calcium channel function in these cells. In the intact preparation, responses to the dihydropyridine-sensitive calcium channel activator, BAY K 8644, were tested. Cholesterol exposure for ≥60 min resulted in a significant decrease in the depolarization that was elicited by 200 nM BAY K 8644 (Fig. 5; control, 3.6 ± 0.1 mV; cholesterol, 0.6 ± 0.3 mV; n = 6; P < 0.0001).

To further investigate the effect of cholesterol on the calcium conductance, gallbladder muscle cells were isolated and voltage-activated inward currents were evaluated, using calcium as a charge carrier. Cholesterol significantly reduced calcium current at all voltages (Fig. 6; P ≤ 0.005 at 0 mV; n = 5 cells for control and n = 10 cells for cholesterol group). Together, the data from intact and isolated cells indicate that cholesterol enrichment causes a marked reduction in the ability of calcium to enter gallbladder muscle through voltage-dependent calcium channels.

Effects of cholesterol on potassium conductances in gallbladder muscle. Because calcium channel function was disrupted by excess cholesterol, experiments were undertaken to test whether other ion channels were similarly affected by cholesterol. Voltage-activated potassium currents and ATP-sensitive potassium (K_{ATP}) currents have been previously described in gallbladder smooth muscle (11, 35, 36). Although large-conductance potassium (BK) channels have been detected in these cells, they contribute very little to the voltage-activated outward current, and blockade of these channels has no effect on the shape of the action potential (36). The effect of cholesterol on voltage-dependent potassium currents was examined. Cholesterol had no effect on voltage-dependent potassium channels (Fig. 7; n = 6 cells from 3 preparations).

Application of K_{ATP} channel openers (e.g., pinacidil) to gallbladder smooth muscle cells results in a membrane hyperpolarization with an accompanying decline in spontaneous action potential generation (35). Pinacidil (1–100 μM) was initially applied under control conditions and then reapplied following superfusion of 50 μg/ml cholesterol for at least 60 min. The K_{ATP} channel opener elicited a membrane potential hyperpolarization and a decrease in spontaneous action potentials. In the case of cholesterol-treated preparations, few action potentials were present before application of pinacidil. Cholesterol did not affect the membrane potential hyperpolarization to pinacidil (Fig. 8; 24.2 ± 1.3 mV for control and 24.6 ± 1.6 mV for cholesterol; n = 6).

Effects of cholesterol on CGRP-induced hyperpolarization in gallbladder muscle. Gallbladder excitability is modulated by a number of neurotransmitters and neuropeptides. Thus modifying the communication of receptors to ion channels could alter gallbladder smooth muscle excitability. The K_{ATP} channel can be indirectly activated by CGRP through the activation of the adenylyl cyclase-protein kinase A cascade (35). Because...
the results described in Effects of cholesterol on potassium conductances in gallbladder muscle indicate that the K\textsubscript{ATP} channel can function normally when cholesterol is elevated, we measured responses to CGRP (200 nM) in intact gallbladder smooth muscle before and during cholesterol treatment (Fig. 9). The CGRP-induced hyperpolarization was significantly reduced by cholesterol treatment (control, 10.9 ± 0.84 mV; cholesterol, 6.9 ± 0.78 mV; P < 0.005; n = 6). These data indicate that cholesterol enrichment of gallbladder smooth muscle membranes can alter the interaction of ligand-receptor binding and/or signal transduction in addition to decreasing the activity of calcium channels.

**DISCUSSION**

The aim of this study was to test the hypothesis that cholesterol alters the electrical properties of gallbladder smooth muscle. Biochemical and morphological analyses demonstrated that cholesterol can be delivered to intact and isolated gallbladder smooth muscle preparations when complexed with cyclodextrins and that this resulted in cholesterol enrichment of gallbladder smooth muscle membranes. Delivery of cholesterol resulted in a marked attenuation of spontaneous action potentials, depolarizations to BAY K 8644, and voltage-activated calcium currents. Voltage-activated potassium currents and pinacidil-induced K\textsubscript{ATP} channel hyperpolarizations were not altered by cholesterol enrichment. Finally, the effects of cholesterol on CGRP-induced K\textsubscript{ATP} channel hyperpolarizations were diminished in cholesterol-treated preparations. These data indicate that cholesterol enrichment results in selective deficits rather than a nondiscriminant disruption of membrane protein function.
In human (19) and animal models (8, 9), lithogenic bile has been related to impaired gallbladder contractility. Increasing the dietary cholesterol content in certain animal models results in increased biliary cholesterol levels and gallbladder hypomotility (31). This has led to the theory that excess cholesterol in gallbladder smooth muscle attenuates the ability of the muscle to contract, either through modulation of ion channel behavior, changes in membrane receptor-ligand interactions, alterations in contractile protein activity, and/or alterations in signal transduction efficacy.

The upstroke of the gallbladder smooth muscle action potential is caused by calcium influx through dihydropyridine-sensitive, voltage-dependent channels (36). These action potentials resemble those seen in the gut during slow waves but are shorter in duration and occur at higher frequency (2, 23, 24). In the gut, slow waves that are accompanied by spikes are directly coupled to mechanical gastrointestinal motility (27), and, in gallbladder, twitches of individual muscle bundles can be observed in coordination with spontaneous action potentials (36). It is likely then that alter-

Fig. 7. Voltage-dependent outward currents carried by potassium were examined using whole cell patch clamp in isolated gallbladder smooth muscle. Cells incubated with cholesterol (50 µg/ml; complexed with cyclodextrins) before recording were compared with cells not exposed to cholesterol, and little difference was found between the two groups. A: examples of typical currents from a control cell and from a cell that had been preincubated with cholesterol are shown together with the pulse protocol used (top). B: current-voltage relationships calculated from peak currents in control cells (●) and cells incubated with cholesterol (●) are shown for comparison.

Fig. 8. In gallbladder smooth muscle cells, pinacidil directly activates ATP-sensitive potassium channels, which hyperpolarize the cell membrane and disrupt production of spontaneous action potentials. Intracellular recordings obtained from cells before and during cholesterol (50 µg/ml; complexed with cyclodextrins) superfusion demonstrated that effects of pinacidil were not modulated by the presence of cholesterol. A: concentration dose response for pinacidil in cells before and during cholesterol superfusion is evident. B: example of gallbladder smooth muscle cell response to pinacidil application (60 s) before and during cholesterol superfusion. Break indicates 5 min. Resting membrane potential of this cell was −45 mV.
Resting membrane potential of this cell obtained in the presence of cholesterol.

ous action potentials in the recording experiment. Note absence of spontaneous action potentials in the recording obtained in the presence of cholesterol. Resting membrane potential of this cell was −48 mV.

ations in the gallbladder smooth muscle action potential and calcium channel activity would have significant impact on the ability of the gallbladder to contract in response to physiological stimuli. Attenuation of calcium influx through the dihydropyridine-sensitive calcium channels would have significant effects on the ability of the gallbladder to contract.

The data presented here demonstrate that cholesterol can alter the ability of gallbladder smooth muscle to fire action potentials by suppressing calcium flow through dihydropyridine-sensitive channels. In the presence of cholesterol, the action potential frequency, spike amplitude, plateau duration, and voltage-activated calcium currents were all significantly reduced. In the current study, it was noted that, whereas inward currents in the presence of cholesterol were almost totally inhibited, action potentials could still be observed in some intact preparations following prolonged exposure to cholesterol. The disparity may be related to temperature differences in the two techniques and/or to the concentration of cholesterol accessing the smooth muscle cells in the two conditions. Patch-clamp experiments were conducted at room temperature, whereas intracellular recordings were done at 37°C. Furthermore, isolated cells would be expected to have more immediate access to cholesterol than muscle cells in intact tissue because the cell membranes of isolated cells are directly exposed to the medium. Because action potentials and BAY K 8644-induced depolarizations were attenuated in cholesterol-enriched tissues, results between the two experimental conditions are consistent.

Recently, investigators have attempted to address the question of how cholesterol may modulate contractility in the gallbladder. It has been demonstrated in ground squirrels that cholesterol-induced gallbladder hypomotility was due to a defect in the plasma membrane and not in the second messenger system or the contractile apparatus (31). In agreement with these findings, others have also demonstrated that the primary defect in gallbladder contractility lies in the plasma membrane and that circumventing the sarclemma can restore contractility (7, 16, 33). We now present evidence that a primary defect in cholesterol-enriched gallbladder smooth muscle lies in the ability of calcium to traverse the sarcolemma. Remarkably, cholesterol appears to affect selectively voltage-dependent calcium channels and not voltage-dependent potassium channels or K_ATP channels. The mechanism by which cholesterol decreases voltage-dependent calcium channel activity in gallbladder smooth muscle remains to be determined.

In the current study, cholesterol enrichment caused a slight decrease in the voltage-activated outward current in gallbladder smooth muscle that was reminiscent of the slight change that is observed when calcium-activated BK channels are blocked by charybdotoxin (36). It is possible that decreased calcium entry through calcium channels, due to cholesterol enrichment, decreases BK channel activity in gallbladder muscle, and this may be responsible for the slight shift in the voltage-activated outward current and for the depolarization that was measured during prolonged exposure to cholesterol.

CGRP has a well-characterized effect on gallbladder smooth muscle (13, 35). This neurotransmitter is located in sensory nerve fibers and activates the CAMP-protein kinaseA cascade in gallbladder smooth muscle. Elevated protein kinaseA, in turn, activates the opening of K_ATP channels, which results in membrane potential hyperpolarization. In cholesterol-enriched gallbladders, the ability of CGRP to initiate hyperpolarizations via the K_ATP channel was significantly reduced, despite the finding that hyperpolarizations initiated by the direct actions of the K_ATP channel opener, pinacidil, are not affected by cholesterol. These data indicate that cholesterol interferes with ligand-receptor binding and/or the signal transduction pathway. Human gallbladders with cholesterol gallstones have impaired responsiveness to CCK, which appears to consist of a defect in receptor ligand binding (6, 34).

The data presented here validate the technique of delivering cholesterol to cells and tissue as a complex with cyclodextrins. Previous studies, primarily using cell cultures, have also demonstrated the efficacy of these cyclic oligosaccharides to deliver cholesterol in physiological solutions. Cholesterol enrichment of plasma membranes has been demonstrated in Fu5AH...
rat hepatoma cells (22), insect Sf9 cells (10), COS cells (20), and Hep G2 cells (18) using cholesterol complexed with cyclodextrins. Interestingly, some of these studies have also used cyclodextrins alone as a chelating agent to extract cholesterol from cell membranes. This chelating effect of cyclodextrins was not apparent when applied to our tissues, as measured by changes in electrical activity or cholesterol levels. Maximal time of cyclodextrin application to our tissues was 2 h in comparison to a daily application over several days in the cell culture systems (18). In a further study, it was demonstrated that cyclodextrins could chelate cholesterol from cells in <1 min (32). The concentration of cyclodextrin used to obtain these effects was in the millimolar range in comparison to a concentration of ~5 μM in our study. In addition, the same authors demonstrated little or no cholesterol chelation at the lower end of the millimolar range.

Cholesterol enrichment of gallbladder smooth muscle membranes clearly occurs in cholelithiasis, and experimental manipulation of cholesterol levels in gallbladder smooth muscle will certainly facilitate our understanding of the mechanisms of gallbladder stasis. Membranes from smooth muscle of human gallbladders with cholesterol disease have cholesterol levels that are 65% higher than those from gallbladders removed because of pigment stones (6). In prairie dogs fed a high-cholesterol diet, an examination of the cholesterol content of gallbladder smooth muscle membranes revealed that plasma membrane cholesterol levels were increased by 85%, with an associated decrease in contractile activity (33). It is now clear that cholesterol enrichment of smooth muscle membranes found in cholesterol disease and cholesterol-fed prairie dogs can be mimicked by application of cholesterol in vitro. In the current study, membrane cholesterol levels were increased by 83% following 2-h exposure to the cholesterol-cyclodextrin complex. Treatment of prairie dog gallbladder muscle cells with cholesterol-rich liposomes for 2 h results in a 56% increase in membrane cholesterol levels with an associated decrease in contractile activity (33).

In conclusion, the data presented here demonstrate that excess cholesterol is incorporated into the gallbladder smooth muscle and has an inhibitory effect on the production of action potentials. This inhibitory effect appears to be specific to the L-type calcium channels, which are responsible for the upstroke of the action potential. In addition, cholesterol enrichment appears to disrupt the ability of CGRP to open K<sub>ATP</sub> channels, although the channel itself appears to be unaffected by cholesterol, as measured by activation with pinacidil. Although the debate over which event happens first in gallbladder pathophysiology, cholesterol supersaturation, nucleation and stone formation, or gallbladder hypomotility, still continues, the data presented here suggest that excess cholesterol may contribute to gallbladder hypomotility by inhibiting calcium influx, leading to stasis and promoting the appropriate milieu for cholesterol nucleation.

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REFERENCES