Hypercholesterolemia inhibits L-type calcium current in coronary macro-, not microcirculation

D. K. Bowles,1,2 C. L. Heaps,1 J. R. Turk,1 K. K. Maddali,1 and E. M. Price1,2

1Department of Biomedical Sciences and 2Dalton Cardiovascular Research Center, University of Missouri, Columbia, Missouri 65211

Submitted 17 November 2003; accepted in final form 27 January 2004

Bowles, D. K., C. L. Heaps, J. R. Turk, K. K. Maddali, and E. M. Price. Hypercholesterolemia inhibits L-type calcium current in coronary macro-, not microcirculation. J Appl Physiol 96: 2240–2248, 2004. First published January 29, 2004; 10.1152/japplphysiol.01229.2003.—Hypercholesterolemia (HC) is a primary risk factor for the development of coronary heart disease. Coronary ion regulation, especially calcium, is thought to be important in coronary heart disease development; however, the influence of high dietary fat and cholesterol on coronary arterial smooth muscle (CASM) ion channels is unknown. The purpose of this study was to determine the effect of diet-induced HC on CASM voltage-gated calcium current (ICa). Male miniature swine were fed a high-fat, high-cholesterol diet (40% kcal fat, 2% wt cholesterol) for 20–24 wk, resulting in elevated serum total and low-density lipoprotein cholesterol. Histochemistry indicated early atherosclerosis in large coronary arteries. CASM were isolated from the right coronary artery (>1.0 mm ID), small arteries (~200 μm), and large arterioles (~100 μm). ICa was determined by whole cell voltage clamp. L-type ICa was reduced ~30% by HC compared with controls in the right coronary artery (~5.29 ± 0.42 vs. 7.59 ± 0.41 pA/pF) but not the microcirculation (small artery, ~8.39 ± 0.80 vs. 10.13 ± 0.60; arterioles, ~10.78 ± 0.93 vs. 11.31 ± 0.95 pA/pF). Voltage-dependent activation was unaffected by HC in both the macro- and microcirculation. L-type voltage-gated calcium channel (Ca v 1.2) mRNA and membrane protein levels were unaffected by HC. Inhibition of ICa by HC was reversed in vitro by the cholesterol scavenger methyl-β-cyclodextrin and mimicked in control CASM by incubation with the cholesterol donor cholesterol:methyl-β-cyclodextrin. These data indicate that CASM L-type ICa is decreased in large coronary arteries in early stages of atherosclerosis, whereas ICa in the microcirculation is unaffected. The inhibition of calcium channel activity in CASM of large coronary arteries is likely due to increases in membrane free cholesterol.

chondro; vascular smooth muscle; cycloxdextrin; voltage-gated calcium channels; porcine

HYPERCHOLESTEROLEMIA IS a primary causal factor in the development of coronary atherosclerosis. Population studies such as the Framingham Heart Study (39) and the Multiple Risk Factor Intervention Trial (31) have demonstrated a direct relationship between serum total and/or low-density lipoprotein (LDL) cholesterol and coronary heart disease. Accordingly, reductions in serum cholesterol, either pharmacologically or through dietary restrictions, reduce the incidence of coronary heart disease and produce beneficial remodeling of existing lesions (29, 36). The target cells and cellular mechanisms relating hypercholesterolemia and coronary atherosclerosis remain unclear. Alterations in ion regulation, particularly calcium homeostasis, in coronary vascular cells may contribute to the onset and progression of coronary heart disease by producing abnormal vasomotion (21) and/or alterations in gene expression (5). Diet-induced hypercholesterolemia has been reported to alter the activity of several smooth muscle membrane channels and pumps, including voltage-gated calcium channels (VGCC) and Na+–K+–ATPase (7, 9), an effect that has been attributed by some to changes in membrane free cholesterol content (34). Conclusions as to whether hypercholesterolemia inhibits or activates membrane channel and transporter activity are varied. Manipulation of membrane free cholesterol content, either through diet or through in vitro enrichment and depletion studies, has often shown an inverse relationship between membrane cholesterol content and channel activity (1, 6, 18, 22, 27). However, VGCC current (ICa) increases have been reported both in cholesterol-enriched cultured cells (28) and in rabbit portal vein smooth muscle after diet-induced hypercholesterolemia (9). Coronary arterial smooth muscle (CASM) cells play a substantial role in coronary lesion formation during coronary heart disease, and VGCCs have been implicated in enhanced coronary vasomotion in coronary disease (21), yet no information is available regarding the effects of hypercholesterolemia on CASM calcium channel function. Therefore, the purpose of this study was to determine the effect of diet-induced hypercholesterolemia on VGCC activity and expression. Furthermore, because coronary atherosclerotic lesion formation is primarily localized in the macro-, not microcirculation, we compared the effects of diet-induced hypercholesterolemia on ICa in CASM from both large conduit and resistance (~300 μm) coronary arteries.

MATERIALS AND METHODS

Animals. Adult male miniature swine weighing 25–40 kg were obtained from the breeder (Sinclair Farms, Columbia, MO) and housed in pens at the College of Veterinary Medicine. Animal protocols were approved by the University of Missouri Animal Care and Use Committee in accordance with the “Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.”

Diet. Animals were randomly divided into two dietary groups: a group fed a normal pig chow diet (control, n = 17) and a group fed a high-fat, high-cholesterol diet (n = 13). The pig chow fed to the control group was Mini-pig Diet Breeder chow (Purina Lab, St. Louis, MO) containing (by weight) 16.7% protein, 2.6% fat, and 53.2% total carbohydrate (22% kcal from protein, 8% from fat, and 70% from carbohydrate). The hypercholesterolemia diet consisted of Mini-pig Diet Breeder chow supplemented (by weight) with 20% cholesterol, 17.1% coconut oil, 2.3% corn oil, and 0.7% sodium cholate (13% kcal
from protein, 46% from fat, and 41% from carbohydrate). Pigs were fed an average of 15–20 g/kg once per day (20–24 wk). Water was given ad libitum.

**Blood sampling.** Blood samples were collected at baseline, after 4 wk and at 24 wk. Samples were collected from the jugular vein into a vacutainer containing EDTA and centrifuged at 2,000 g for 15 min at 4°C. Plasma was stored immediately at −70°C until analyzed as previously described (33).

**Preparation of coronary arteries.** At 20–24 wk, pigs were anesthetized with ketamine (30 mg/kg) and pentobarbital sodium (35 mg/kg). The hearts were removed and placed in ice-cold (4°C) Krebs bicarbonate solution during vessel isolation. Conduct (>1.0 mm ID) segments of right coronary artery (RCA) were trimmed of fat and connective tissue in sterile MEM containing 20 mM HEPES on ice. Small arteries (225–175 μm ID) and arterioles (75–125 μm ID) were dissected free from the subepicardial wall near the apex. All arteries were stored under sterile conditions in MEM at 4°C until dispersion (0–1 h).

**Cell dispersion.** Freshly dispersed CASM cells were obtained as described previously (2–4, 15). Resistance arteries were incubated in 200 μl of enzyme solution consisting of low-calcium (0.1 mM) physiological solution plus 294 μM collagenase (CLS II, Worthington), 6.5 μM elastase (Worthington), 2 mg/ml bovine serum albumin (fractions V, Sigma Chemical), 1 mg/ml soybean trypsin inhibitor (type 1-S, Sigma Chemical) and 0.4 mg/ml DNase I (type IV, Sigma Chemical). Cells were enzymatically dispersed by incubation for 60 min in a water bath at 37°C. Immediately after, the enzyme solution was replaced with enzyme-free low-calcium solution and isolated single cells were obtained with gentle trituration using a flame-polished Pasteur pipette. For conduit coronary arteries, arteries were opened longitudinally and pinned, lumen side up, onto a silicone rubber substrate in ~2 ml of low-calcium enzyme solution. Cells were enzymatically dispersed for 60 min in a water bath at 37°C. The enzyme solution was then replaced with enzyme-free low-calcium solution, and isolated single cells were obtained by repeatedly directing a stream of low-calcium solution over the artery via a fire-polished Pasteur pipette. All solutions used for conduit and resistance vessels were identical. Cell suspensions were stored in low-calcium (0.1 mM) buffer at 4°C until use (0–6 h).

**Whole-cell voltage clamp.** Whole-cell currents were determined by using a standard whole cell voltage-clamp technique (2–4, 15). Cells were initially superfused with physiological saline solution containing (in mM) 0.1 CaCl₂, 138 NaCl, 1 MgCl₂, 5 KCI, 10 HEPES, and 10 glucose, pH 7.4, during gigaseal formation. After whole cell configuration, the superfusate was switched to physiological saline solution with tetrathylammonium chloride substitution for NaCl and 10 mM Ba²⁺ as the charge carrier. The pipette solution contained (in mM), 120 CsCl, 10 tetrathylammonium chloride, 1 MgCl₂, 20 HEPES, 5 Na₂ATP, 0.5 Tris-GTP, and 10 EGTA, pH 7.1. Ionic currents were amplified by an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Whole cell currents were low-pass filtered with a cutoff frequency of 1,000 Hz, digitized at 2.5 kHz, and stored on computer. Current densities (pA/pF) were obtained for each cell by normalization of whole cell current to cell capacitance to account for differences in cell membrane surface area. Capacity currents were measured for each cell during 10-ms pulses from a holding potential of −80 mV to a test potential of −75 mV. Capacity currents were filtered at a low-pass cutoff frequency of 5 kHz. Leak subtraction was not performed. Data acquisition and analysis were accomplished using pCLAMP 7.0 software (Axon Instruments). All experiments were conducted at room temperature (22–25°C). Cells were continually superfused under gravity flow.

**Immunoblot.** RCA segments (5-mm axial length) were quick-frozen in liquid N₂ and stored at −80°C until processed. Samples were minced thoroughly in 200 μl of ice-cold buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1 mM PMSF, 2 mM leupeptin, 1 mM pepstatin A, 10% vol/vol glycerol) and subjected to 4 × 30 s homogenizations at a speed of 4.0–5.0 using a FASTPREP 120 system (QiBiogene) with samples kept on ice for 5 min between each pulse. After a low-speed centrifugation, supernatants were subjected to high-speed centrifugation (100,000 g for 1 h). Crude membrane pellets were dissolved by vortexing in homogenization buffer containing 0.5% Triton X-100 at room temperature. Membrane protein concentrations (3–5 μg/μl) were determined by using the Bio-Rad Bradford protein assay kit. Equal protein amounts (30 μg) of sample were electrophoresed on 5% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane at 30 V for 14–16 h at 4°C. After transfer, membranes were blocked for 1 h in Tris-buffered saline solution containing 5% dry milk plus 0.1% Tween 20 and probed with anti-Ca₂⁺,1.2 antibody (1:200; Alomone) for 2 h at room temperature. Immunoreactive bands were detected by chemiluminescence (ECL, Amersham) and quantified by use of Image J software (NIH).

**Quantitative RT-PCR.** Real-time PCR was performed to simultaneously amplify ("duplex") the Ca₂⁺,1.2 subunit of the VGCC and the constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RCA segments (10-mm axial length) were quick-frozen in liquid N₂ and stored in RNase-free microcentrifuge tubes at −80°C until processed. Arterial segments were pulverized under liquid N₂, and a crude lysate was prepared in lysis buffer containing (in mM) Tris-base, 10 EDTA, 100 Tris-HCl with 1% LIDs (pH 8.0) by vigorous agitation (vortex). Poly(A)+ RNA was isolated with paramagnetic oligo(dt) polystyrene beads [Dynabeads oligo(dt)₅₀, Dynal] and eluted for first-strand cDNA synthesis using reverse transcriptase (RT) and oligo(dt)₁₂₋₁₈ to prime the reaction (Superscript Preamplification System, Invitrogen). All PCR reactions were performed in a laminar flow benchtop hood. cDNA samples were used to perform real-time PCR in a 25-μl volume containing buffer [50 mM KCl, 20 mM Tris·HCl (pH 8.4), 5 mM MgCl₂], 0.15–0.20 μM of each primer or probe, 0.2 mM dNTP, and 2.5 U AmpliTaq Gold DNA polymerase (Roche) on a Cepheid Smart Cycler (model SC1000-1). The PCR reaction was initiated by activation of AmpliTaq Gold polymerase for 1 min at 95°C followed by 50 cycles of 95°C (15 s), 54°C (30 s) using conditions optimized for MgCl₂ concentration and annealing temperature.

Primers were designed on the basis of published sequences from human VGCC and domestic porcine (Sus scrofa) GAPDH. The primer sets were as follows: Ca₂⁺,1.2; sense, 5'-GAA GTG CCT CAC TGT CG-3'; antisense, 5'-CTG GTC CCA ACA GAA ATG AA-3'; and probe 5'-FAM-TCC ATT GAT GAC AAG CTT CC-TAMRA-3'; GAPDH; sense, 5'-ATG ATT CTA CCC ATG GCA AA-3'; antisense, 5'-TCC ATT GAT GAC AAG CTT CC-3'; and probe, 5'-ROX-CGC CAG ACT CAA GGC TGA GAA TG-3'. Nucleotide sequencing demonstrated complete homology between human and Yucatan porcine Ca₂⁺,1.2 PCR-amplified products. ROX- and FAM-conjugated fluorogenic probes (Biosearch Technologies) for GAPDH and Ca₂⁺,1.2, respectively, were designed for real-time PCR on the basis of the derived Yucatan nucleotide sequences. For quantification, the Taq polymerase-amplified PCR product from the Ca₂⁺,1.2 subunit was subcloned into a plasmid vector by use of the pCR2.1 TOPO TA cloning kit (Invitrogen) and transformed into competent E. coli, and a standard curve was generated plotting threshold cycle against known quantities of cDNA (Fig. 6). All standards and samples were evaluated in triplicate.

**Immunohistochemistry.** Samples of RCA were fixed in neutral buffered 10% formalin for a minimum of 24 h. Samples were split and one half was frozen, sectioned on a Leica CM 1850 cryostat, and stained with Oil Red O to assess lipid accumulation. The remaining sample was embedded routinely in paraffin and sectioned serially at 5-μm thickness. Sections were floated onto positively charged slides (Fisher), deparaffinized, and steamed in citric buffer at pH 6.0 (Dako S1699) for 30 min to achieve antigen retrieval and then cooled for 30 min. The slides were stained manually with Tris buffer or water wash steps performed after each step. Sections were incubated with

**References**
unesterified cholesterol and interacts with the hydroxy group of unesterified cholesterol (10). Cryosections of RCA were stained with fluorescent antibiotic lipin, which binds specifically to sterols and interacts with the 3β-hydroxy group of unesterified cholesterol (10). Cryosections of RCA were stained with lipin (5 mg dissolved in 1 ml dimethylformamide and diluted in 100 ml phosphate-buffered saline for 30 min as in Ref. 10). Filipin-stained sections were digitally photographed using a ×40 oil-immersion objective (N.A. 1.35) with 340-nm excitation and a 510-nm emission barrier filter. Images were acquired by use of Axon Imaging Workbench 2.2 with constant excitation intensity and exposure (4 s). Mean pixel intensity of three filipin-stained sections was determined from three control and three hypercholesterolemic animals (n = 18 total sections) by use of Image J software.

Statistics. Data are expressed as means ± SE. Repeated-measures analysis of variance was used for comparisons of current-voltage relationships between groups. Histochemistry was compared in control and hypercholesterolemic animals using a Mann-Whitney nonparametric analysis. All other comparisons were made by ANOVA with Bonferroni post hoc analyses. A P value < 0.05 was set as the criterion for significance in all comparisons. For electrophysiology experiments, data on three to five cells were obtained from each animal, and each cell was counted as one observation (n). For all other variables, each animal was counted as one observation.

RESULTS

Body weight and blood lipids. Body weight was significantly greater in animals after 20–24 wk on the hypercholesterolemic diet compared with controls (44.6 ± 2.2 kg vs. 36.56 ± 1.8 kg, respectively). As previously reported for this model (33), plasma total cholesterol increased about eightfold and LDL cholesterol increased about sixfold in pigs fed the high-fat, high-cholesterol diet compared with controls at 20–24 wk (Fig. 1). Plasma triglycerides were unaffected by diet. On the basis of this lipid profile, the high-fat, high-cholesterol diet-fed animals are referred to as hypercholesterolemic.

Diet and CASM cell size. Diet had no effect on whole cell membrane capacitance in cells from conduit arteries, resistance arteries, or large arterioles, indicating a similar cell surface area in CASM from control and hypercholesterolemic swine (Table 1). As previously reported (3), cell membrane surface area decreased as arterial diameter decreased, demonstrating a correlation between CASM membrane surface area and arterial size. Series resistance during voltage clamp was < 10 MΩ, unaffected by diet, and similar in all arterial sizes (data not shown).

Effect of hypercholesterolemia on coronary artery histology. Previous studies have demonstrated that our model of hypercholesterolemia is associated with the production of Starry type I–III lesions in the RCA and elevations in both serum and coronary C-reactive protein, macrophage accumulation, and monocyte chemoattractant protein 1 (35). The present study further demonstrated a 15-fold increase in coronary lipid deposition in hypercholesterolemia compared with controls (3.83 ± 0.50 vs. 0.25 ± 0.25, P < 0.05; Fig. 2A, left). In contrast, no detectable lipid accumulation occurred in microvessels from either group (Fig. 2A, right). Free cholesterol deposition, as indicated by filipin staining, was significantly greater in large coronary arteries from hypercholesterolemic animals compared with controls (P < 0.05; Fig. 2B). Cholesterol deposition was noted in both the intima and the neointima as well as the media. In addition, both endothelial and smooth muscle cells in RCA from hypercholesterolemic pigs demonstrated multifocal positive staining for Ki-67, a marker of proliferation (Fig. 2C).

VGCC in coronary smooth muscle. Figure 3 shows a representative family of currents obtained in conduit CASM from either control (A) or hypercholesterolemic (B) animals. Successive depolarization steps from −60 to +70 mV produced inward currents showing a peak near +20 mV and slow inward currents that were blocked by nifedipine (9). The voltage dependence of activation of these currents is shown in the upper panel of Figure 3. In control arteries, the voltage dependence of activation of 

| Table 1. Coronary smooth muscle cell capacitance is unaffected by diet |
|---------------|----------------|----------------|
|                | Conduit         | Small artery   | Large artery  |
| Control        | 21.0 ± 0.6      | 16.2 ± 0.7     | 13.6 ± 0.6    |
| (87, 17)       | (80, 16)        | (36, 8)        |               |
| HC             | 23.5 ± 1.9      | 14.6 ± 0.8     | 12.2 ± 0.7    |
| (58, 13)       | (38, 9)         | (46, 12)       |               |

Values are means ± SE; n values in parentheses refer to cells, animals, respectively; p < 0.05 vs. *control, **conduit, ***vessels small artery. HC, hypercholesterolemia.
inactivation (selected test potentials shown). We have previously shown that CASM from male swine are dominated by L-type current, with no discernable T-type current present in any arterial size or group (2). In the present study, hypercholesterolemia did not alter the dominant expression of L-type current as confirmed by the near complete block of inward current by nifedipine (Fig. 3C). However, the magnitude of both the peak and sustained inward current was decreased ~30% in cells from hypercholesterolemic animals compared with control. Current-voltage relationships demonstrated an overall reduction in inward VGCC current in conduit CASM from hypercholesterolemic animals (Fig. 4A). VGCC current density was unaffected in CASM from either small arteries (not shown) or arterioles (Fig. 4B). Both the membrane potential producing half-maximal activation ($V_{0.5}$) and voltage-sensitivity ($k$) were unaffected by diet in both conduit and resistance CASM (Fig. 4, C and D). The effect of hypercholesterolemia on peak $I_{Ca}$ was attenuated as arterial size decreased (Fig. 5), with only a trend for inhibition in small arteries (~200 μm) and no effect in arterioles (~100 μm). Thus the effect of hypercholesterolemia on VGCC current density is greatest in conduit CASM, with progressively diminishing effects in the microcirculation (Fig. 5).

Fig. 2. HC elicits early atherosclerosis. Histology of right coronary artery (RCA) from control and HC swine. A: photomicrographs of RCA in cross section stained for lipid using Oil Red O (ORO) in RCA of control (top left) and HC (bottom left) animals. Lipid deposition was increased in HC compared with controls ($P < 0.05$). ORO staining was undetectable in coronary microvessels in either control (not shown) or HC (right) animals. B: RCA sections from control (top) and HC (bottom) animals stained for free cholesterol using filipin. Brightfield images shown in left and pseudo-colored fluorescent images shown in right. Free cholesterol deposition was significantly increased in HC compared with control ($P < 0.05$). C: RCA sections identifying proliferating cells (Ki-67) from control (left) and HC (right). Proliferation (%Ki-67 positive cells) was significantly increased in HC. Scale bars in photomicrographs represent 100 μm; $n = 8$ control and $n = 7$ HC sections from individual animals in both A and C; $n = 3$ sections each from 3 control and 3 HC animals (total of 18 sections) in B.

Fig. 3. Effect of HC on voltage-gated calcium current in coronary smooth muscle. Representative current traces for voltage-gated calcium channel current in coronary artery smooth muscle (CASM) from an animal on the control diet (A) and an HC animal induced by the high-fat diet (B). Currents were elicited by 400-ms step-depolarizations from a holding potential (h.p.) of −80 mV to test potentials (t.p.) of −60 to +60 mV in 10-mV increments with 10 mM external Ba$^{2+}$ as the charge carrier (selected test potentials of −30, −10, and +20 mV shown). Depolarizations positive to −40 elicited a sustained, slowly inactivating inward current that was maximal near +20 mV. Inward currents were effectively abolished by nifedipine (nif; 1 μM) in both control (data not shown; see Ref. 2) and HC (C) animals, indicating a dominance of dihydropyridine-sensitive, L-type current in CASM that is unchanged by HC. Capacitance transients were eliminated for clarity.
Fig. 4. Effect of HC on the voltage-gated calcium channel (VGCC) current-voltage relationship. Current-voltage (I-V) relationships are shown for whole cell VGCC current in coronary myocytes from conduit arteries (A) and large arterioles (B) from control (○) and HC (□) animals. Current is plotted as the peak inward current measured during a 400-ms step depolarization to the membrane potential ($V_m$) indicated from a holding potential of $-80$ mV. Current is normalized to cell membrane capacitance (pA/pF). Data are means ± SE of 4–5 cells per artery per animal (n values as in Table 1). Repeated-measured ANOVA indicated a significant main effect of HC in conduit arteries but not arterioles. Voltage-dependent activation in CASM is shown from conduit arteries (C) and arterioles (D) from control (○) and HC (□) animals. Data are means ± SE, where relative conductance ($g/g_{max}$) = peak $I_{Ca}/g_{max} (V-E_{rev})$; $g_{max}$ is the maximal conductance obtained from the linear regression of the positive limb of the I-V relationship through the apparent reversal potential ($E_{rev}$), and $I_{Ca}$ was obtained from the I-V relationship. The $g/g_{max}$ was fit to a conventional Boltzmann distribution equation, $1/[1+exp((V_{0.5} - V)/k)]$, where $V_{0.5}$ is the membrane potential producing half-maximal activation and k is the slope factor. Voltage-dependent activation of currents ($V_{0.5}$, k) were unaffected by HC in any artery. Responses to HC in small arteries were similar to arterioles (data not shown).

Hypercholesterolemia and Ca	extsubscript{v}1.2 expression. The reduction in $I_{Ca}$ in conduit CASM with hypercholesterolemia must result from a reduction in the open probability, unitary conductance, and/or number of channels. To examine the possibility that hypercholesterolemia reduced $I_{Ca}$ by inhibiting the synthesis of VGCCs, we compared both Ca	extsubscript{v}1.2 mRNA and protein levels in RCA from both control and hypercholesterolemic animals. Ca	extsubscript{v}1.2 primers amplified a single product of predicted size (Fig. 6A), and subsequent sequence analysis confirmed complete homology between the amplified sequence of Yucatan porcine and human Ca	extsubscript{v}1.2. Quantitative PCR demonstrated that Ca	extsubscript{v}1.2 mRNA levels were unaffected by hypercholesterolemia, indicating no effect of hypercholesterolemia on Ca	extsubscript{v}1.2 gene transcription (Fig. 6C). Potential effects of hypercholesterolemia on Ca	extsubscript{v}1.2 translation were examined by immunoblots comparing equal amounts of membrane protein from RCA of control (n = 8) and hypercholesterolemic (n = 7) swine.

Fig. 5. HC inhibits peak voltage-gated calcium current ($I_{Ca}$) in macro-, not microvessel, CASM. Summary of peak $I_{Ca}$ obtained during I-V normalized to cell surface area (pA/pF) among arterial sizes. Current density was significantly inhibited only in CASM from conduit (>1.0 mm ID) coronary arteries and was unaffected in small (sm.) arteries (175–225 μm) and arterioles (75–125 μm). Data are means ± SE; *p < 0.05 vs. respective control.

Fig. 6. No effect of HC on Ca	extsubscript{v}1.2 mRNA expression in conduit CASM. Quantitative PCR of Ca	extsubscript{v}1.2 mRNA in RCA. A: Ca	extsubscript{v}1.2 PCR products with (+RT) or without (−RT) addition of reverse transcriptase. Ca	extsubscript{v}1.2 products were of predicted size (68 bp). Sequencing demonstrated complete homology between porcine and human Ca	extsubscript{v}1.2 in the amplified region. bp, 25 bp ladder. B: quantitative PCR standard curve of known amounts of cloned Ca	extsubscript{v}1.2 cDNA plotted against threshold cycle (Ct). Linear regression fit of the standard curve produced a slope (2.08), indicating a replication efficiency of 1.08. C: relative Ca	extsubscript{v}1.2 gene expression expressed as $2^{-ΔΔCt}$, where the threshold cycle difference ($ΔCt$) was determined as $Ct_{Ca\textsubscript{v}1.2} - Ct_{GAPDH}$ for each sample and normalized to average $ΔCt$ for control. Ca	extsubscript{v}1.2 expression was similar in control (n = 4) and HC (n = 4) swine.
animals probed with anti-Ca,1.2 antibody (Fig. 7). Similar to mRNA levels, membrane Ca,1.2 protein was unaffected by hypercholesterolemia, although low statistical power (<0.8) of this comparison may have allowed small changes in Ca,1.2 protein to go undetected. These data indicate that the reduction in I_Ca in conduit CASM in hypercholesterolemia is not due to a substantial reduction in channel synthesis or membrane targeting.

Reversal of hypercholesterolemic inhibition of I_Ca by β-cyclodextrin. Increased membrane free cholesterol content has been shown to inhibit the activity of a number of membrane ion channels and transporters (18, 22). To test the hypothesis that I_Ca in conduit CASM with hypercholesterolemia results from increased free cholesterol, I_Ca in isolated CASM from control and hypercholesterolemic swine were compared before and after incubation with the cholesterol scavenger methyl-β-cyclodextrin (mβCD) (Fig. 8). Before mβCD, I_Ca was reduced ~50% in hypercholesterolemic cells compared with control. Incubation with mβCD (5 mM, 60 min) completely reversed the inhibition of I_Ca by hypercholesterolemia. Furthermore, incubation of CASM from control animals with cholesterol-enriched mβCD inhibited I_Ca to a similar extent as that found in CASM from hypercholesterolemic animals. In cells from control animals, mβCD had no significant effect, although there was a trend for an increase in current with mβCD. These data indicate that reductions in CASM I_Ca in hypercholesterolemia result from an inhibition of channel activity due to increased plasma membrane free cholesterol.

Fig. 7. No effect of HC on Ca,1.2 protein in conduit CASM. A: representative immunoblot showing RCA membrane fractions from control and HC animals probed with anti-Ca,1.2 antibody. Ca,1.2-positive band appeared at ~220 kDa. B: densitometric analysis of Ca,1.2 protein levels in control (n = 8) and HC (n = 7) animals. Equal quantities of crude membrane protein (20 μg) were run in each lane. Densitometric values for each sample were quantified and normalized to the average control value obtained from the same blot (total of 2 blots). HC had no effect on membrane Ca,1.2 levels.

Fig. 8. Reversal of in vivo HC-induced calcium current inhibition by in vitro cholesterol removal. A: representative currents during step depolarization to +20 mV in CASM from a hypercholesterolemic animal before (○) and after (shaded circle) methyl-β-cyclodextrin (mβCD) (5 mM, 60 min). B: representative currents during step depolarization to +10 mV in CASM from a control animal before (●) and after (hatched circle) cholesterol-enriched mβCD (50 μg cholesterol/ml, 120 min). C: group data (means ± SE) for CASM I_Ca from control and HC animals before and after treatment with mβCD or cholesterol-enriched mβCD (cholesterol:mβCD). mβCD reversed the attenuation of calcium current by HC, whereas cholesterol:mβCD inhibited I_Ca in CASM of controls (n = 19, 20, 17, 9, and 14 cells for control, HC, HC+mβCD, control + mβCD, and control + cholesterol:mβCD, respectively). *P < 0.05 vs. all other groups.

DISCUSSION

The present study is the first to demonstrate a direct link between diet-induced hypercholesterolemia and alterations in coronary smooth muscle I_Ca. In large coronary arteries, smooth muscle I_Ca was attenuated in a large mammal model of total and LDL hypercholesterolemia, a model similar to hypercholesterolemia in humans (36) and one that mimics the early stages of coronary atherosclerosis (37). Furthermore, our data implicate increased membrane free cholesterol as a primary mechanism mediating this effect. These findings provide novel insights into coronary smooth muscle phenotypic changes in early atherosclerosis.

In humans, elevated serum total and LDL cholesterol greatly increase the risk for atherosclerosis. Increased dietary fat and cholesterol in our swine model produced a robust increase in both total and LDL cholesterol [see Thomas et al. (33) for a more detailed lipid profile of this model]. Compared with the rabbit model of hypercholesterolemia, this increase in both total and LDL cholesterol is more similar to the lipid profile found in hypercholesterolemic humans (19). Serum total and LDL cholesterol levels achieved in our porcine model (~350 and ~200 mg/dl, respectively) are in the range of humans at high risk for atherosclerosis and well above the recommended levels for reducing coronary risk in humans (<200 and <130 mg/dl, respectively).

The atherogenic nature of the cholesterol profile is evidenced by the finding that coronary arteries from hypercholesterolemic swine demonstrate elevated lipid and free cholesterol deposition and increased CASM proliferation (Fig. 2) in addition to previously reported production of Stary type I–III
lesions, elevations in both serum and coronary C-reactive protein, macrophage accumulation, and increased monocyte chemoattractant protein 1 (35). This pathological profile is indicative of the early stages of atherosclerosis (37). Thus the hypercholesterolemic swine appears to be a useful model for studying coronary arterial changes associated with early atherosclerosis.

The decreased VGCC activity found in CASM of hypercholesterolemic animals is consistent with studies showing an inverse relationship between membrane free cholesterol content and ion channel-pump activity (1, 6, 18, 22, 27). Jennings et al. (18) reported that increasing membrane free cholesterol 67% by cholesterol-enriched cyclodextrin in vitro almost completely abolished L-type VGCC in gallbladder smooth muscle. Interestingly, this attenuation was specific to VGCC as K+ currents were unaffected. Chen et al. (7) found a similar increase in membrane cholesterol reduced Na+ current de

The decreased VGCC activity found in CASM of hypercholesterolemic animals is consistent with studies showing an inverse relationship between membrane free cholesterol content and ion channel-pump activity (1, 6, 18, 22, 27). Jennings et al. (18) reported that increasing membrane free cholesterol 67% by cholesterol-enriched cyclodextrin in vitro almost completely abolished L-type VGCC in gallbladder smooth muscle. Interestingly, this attenuation was specific to VGCC as K+ currents were unaffected. Chen et al. (7) found a similar increase in membrane cholesterol reduced Na+ current de

Our findings are, however, in contrast to studies in portal vein smooth muscle from hypercholesterolemic rabbits (9). In this latter model of hypercholesterolemia, serum cholesterol increased 48-fold and membrane free cholesterol 2-fold, whereas VGCC current increased 33% and K+ current decreased 50%. A similar finding was reported in cultured cells (AT7/5) with liposme cholesterol enrichment (28). The discrepancy between our finding and these latter two reports is presently unresolved but may be due to the vascular location of the smooth muscle studied and/or species differences in cholesterol metabolism. The 48-fold increase in total serum cholesterol reported in the rabbit model of hypercholesterolemia (9) was much greater than the ~7-fold increase produced in our model or that seen in human hypercholesterolemia. Finally, modification and packaging of cholesterol in vivo may affect the response. Hypercholesterolemic swine, like humans, exhibit an increase in LDL cholesterol that is not seen in rabbits (36). It is possible that cholesterol transfer via LDL, particularly via oxidized LDL, may contribute to differences in response. However, our ability to manipulate ICa in CSM in vitro loses the potential of in vivo modification or packaging effects.

The inhibition of ICa with hypercholesterolemia does not appear to result from a downregulation of channel synthesis or net loss of functional channels. First, neither Ca,1.2 mRNA nor membrane protein levels were affected by hypercholesterolemia (Figs. 6 and 7). Second, acute treatment of cells from hypercholesterolemic animals in vitro with the cholesterol scavenger mβCD completely reversed the effect of hypercholesterolemia (Fig. 8), indicating an inhibition of channel function by excess cholesterol, rather than a loss of functional channels. Finally, the effect of hypercholesterolemia in vivo could be mimicked acutely in vitro by treating CASM from normal-fed animals with cholesterol-enriched mβCD (Fig. 8). The movement of cholesterol from the cell membrane and incorporation into extracellular acceptors, such as HDL, in reverse cholesterol transport, occurs in part through aqueous diffusion (8). mβCD is a cyclic oligosaccharide that acts as an artificial cholesterol shuttle. Exposing tissues or cells to “empty” mβCD results in a net efflux of free cholesterol from cell membranes, whereas exposure to cholesterol-enriched mβCD results in an increase in free cholesterol (22, 27). At the concentration of mβCD used in this study, reductions in membrane free cholesterol of 40–80% have been reported (8, 22), and our model of hypercholesterolemia increases free cholesterol in large coronary arteries approximately twofold (40). Thus the expected reduction in free cholesterol by mβCD should “normalize” free cholesterol in CASM from hypercholesterolemic animals. In CASM from control-fed swine, VGCC activity was relatively unaffected by mβCD, a finding similar to that reported by others (23). One possible interpretation is that a threshold of membrane cholesterol may exist that, when exceeded, inhibits VGCC activity. Lowering free cholesterol below normal levels may not dramatically affect channel activity. Together, these data indicate that hypercholesterolemia inhibits VGCC activity in coronary smooth muscle due to increases in membrane free cholesterol. Determining the precise relationship between membrane cholesterol and VGCC activity in coronary smooth muscle will require further study.

Although not directly addressed in this study, the pathophysiological consequences of hypercholesterolemia-induced inhibition of ICa on coronary vasoreactivity appear paradoxical. One might predict that a loss of VGCC activity would translate to an overall decrease in contractile response to vasoactive stimuli that activate VGCCs, such as vasosagpasm (21). Because abnormal vasomotion and vasospasm can provoke acute coronary events (37), an attenuation of vasoreactivity by loss of ICa could be interpreted as beneficial. However, the effects of hypercholesterolemia on coronary vasoreactivity are not only multifactorial but varied. Whereas abnormal vasomotion due to endothelial dysfunction is common, there is equivocal evidence that diet-induced hypercholesterolemia is linked to increased coronary smooth muscle reactivity (11, 14, 26, 30). Interestingly, the therapeutic effects of calcium channel blockers on atherosclerosis have been attributed to properties unrelated to the inhibition of channel activity and vasoreactivity, but their ability to counter effects of excess cholesterol on plasma membrane properties (24). Thus the contribution of hypercholesterolemia-induced reductions in VGCC activity on coronary vasomotion remains to be determined.

Inhibition of calcium channels by hypercholesterolemia may be more relevant as an early event marker in atherosclerosis. Interestingly, loss of VGCC activity is associated with smooth muscle dedifferentiation and modulation from a “contractile” to a “synthetic” phenotype (13). Phenotypic modulation of coronary smooth muscle plays a key role in the development of atherosclerosis (25) and contributes to the developing neointima of diseased vessels (37). This would be consistent with our model, which exhibits definitive hallmarks of early atherosclerosis, indicating that loss of CASM ICa may be an early event in atherosclerosis. Accordingly, it is interesting to note that lipid deposition and VGCC activity were unaffected in the coronary microcirculation in our model of early atherosclerosis. This is consistent with studies showing that macrovascular function is affected by hypercholesterolemia before changes in coronary microvascular function (32).

In conclusion, the present study provides novel, direct electrophysiological evidence that diet-induced hypercholesterol-
emia reduces L-type calcium channel current in coronary smooth muscle of the macro- but not microcirculation. Because this model mimics human hypercholesterolemia and early atherosclerosis, these findings implicate a role for VGCC specifically, and ion channels in general, in CASM phenotypic changes at the onset of coronary heart disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the significant contribution of Cathy Galle to this work and thank Darla Tharp for critical reading of the manuscript.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grant HL-52490.

REFERENCES


