

## Membrane Cholesterol Regulates Smooth Muscle Phasic Contraction

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**Abstract.** The regulation of contractile activity in smooth muscle cells involves rapid discrimination and processing of a multitude of simultaneous signals impinging on the membrane before an integrated functional response can be generated. The sarcolemma of smooth muscle cells is segregated into caveolar regions—largely identical with cholesterol-rich membrane rafts—and actin-attachment sites, localized in non-raft, glycerophospholipid regions. Here we demonstrate that selective extraction of cholesterol abolishes membrane segregation and disassembles caveolae. Simultaneous measurements of force and  $[Ca^{2+}]_i$  in rat ureters demonstrated that extraction of cholesterol resulted in inhibition of both force and intracellular  $Ca^{2+}$  signals. Considering the major structural reorganization of cholesterol-depleted sarcolemma, it is intriguing to note that decreased levels of membrane cholesterol are accompanied by a highly specific inhibition of phasic, but not tonic contractions. This implies that signalling cascades that ultimately lead to either phasic or tonic response may be spatially segregated in the plane of the sarcolemma. Replenishment of cholesterol restores normal contractile behavior. In addition, the tissue function is re-established by inhibiting the large-conductance  $K^+$ -channel. Sucrose gradient ultracentrifugation in combination with Western blotting analysis demonstrates that its  $\alpha$ -subunit is associated with detergent-resistant membranes, suggesting that the channel might be localized within the membrane rafts in vivo. These findings are important in understanding the complex signalling pathways in smooth muscle and conditions such as premature labor and hypertension.

**Key words:** Membrane segregation — Caveolae — Raft microdomain —  $Ca^{2+}$  — Sarcolemma — Ion channels

### Introduction

Smooth muscle cell contraction is regulated by a complex interplay of periodically changing transmembrane currents that lead to fluctuations in the intracellular concentration of free  $Ca^{2+}$   $[Ca^{2+}]_i$ . Over the last decade significant progress has been achieved in understanding the mechanisms regulating  $[Ca^{2+}]_i$  and the impact of  $[Ca^{2+}]_i$  on the contractile machinery (Horowitz et al., 1996; Somlyo & Somlyo, 2000). It is widely recognized that the control of membrane conductance and membrane potential is the major determinant of  $[Ca^{2+}]_i$  and, thus, of the contractile response (Bolton et al., 1999). A variety of ion channels and receptors localized on the plasma membrane of smooth muscle cells contribute to the modulation of transmembrane currents and are subject to a complex regulation by agonists, membrane potential,  $Ca^{2+}$  and protein-protein interactions (Bolton et al., 1999; Sanders, 2001).

However, it has only recently become clear that the elaborate spatial architecture of the plasma membrane of smooth muscle cells might also contribute to the regulation of membrane-associated proteins (de Weerd, 1997; Babiychuk & Draeger, 2000; Kone, 2000; Dreja et al., 2002). The plasma membrane is subdivided into microdomains of sphingomyelin- and cholesterol-rich rafts and glycerolipid-containing non-raft regions. Rafts are planar associations of membrane lipids that act as phase-separating platforms for receptors and components of signalling cascades (Simons & Ikonen, 1997; Harder & Simons, 1997; Brown & London, 1998; Babiychuk & Draeger, 2000; Simons & Toomre,

2000). A sub-type of lipid rafts, caveolae, are abundant in the smooth muscle plasma membrane (Parton & Simons, 1995). They have been proposed to play an important role in signal transduction and contain high concentrations of receptors and ion channels (de Weerd et al., 1997; Ishizaka et al., 1998; Anderson, 1998). Yet, despite their potential importance for smooth muscle cell pathology, membrane segregation has hardly been investigated in this cell type (Babychuk & Draeger, 2000; Dreja et al., 2002).

Being a major component of membrane rafts, cholesterol is essential for their stability (Brown & London, 2000; Parton, 2001). The efflux of cholesterol from biological membranes can be significantly accelerated by a cyclic oligosaccharide, methyl- $\beta$ -cyclodextrin [(MCD), (Kilsdonk et al., 1995; Yancey et al., 1996)]. As a cholesterol-extracting agent, MCD is widely used in evaluating the role of rafts in various biological systems (Watson et al., 2001; Gimpl & Fahrenholz, 2000) and its effects on smooth muscle contraction have recently been demonstrated for vascular smooth muscle (Dreja et al., 2002).

This study investigates the effects of cholesterol depletion on sarcolemmal architecture at the molecular level, and reveals the putative role of membrane segregation in the regulation of smooth muscle contraction. We demonstrate that in smooth muscle, extraction of cholesterol by MCD abolishes segregation of membrane lipids and flattens caveolae. These structural rearrangements lead to a specific modulation of the muscle's contractile response, a modulation that likely is associated with changes in activity of raft-associated large-conductance potassium channel.

## Materials and Methods

Thin-layer chromatography (TLC), Western Blotting, 5'-nucleotidase activity measurements, electron microscopy, purification of porcine stomach smooth muscle microsomes and their extraction with non-ionic detergent were performed as described previously (Babychuk et al., 1999; Babychuk & Draeger, 2000). Polyclonal antibodies against caveolin and the  $\alpha$ -subunit of the large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^{+}$  channel were purchased from Transduction Laboratories (Lexington, KS) and Alomone Labs (Jerusalem, Israel), respectively.

### EXTRACTION/REPLENISHMENT OF CHOLESTEROL

Cholesterol was extracted (30 min, 30°C, if not stated otherwise) or replenished after extraction (30 min, 30°C, if not stated otherwise) by incubation in Krebs' solution containing either 2% methyl- $\beta$ -cyclodextrin (MCD; Sigma) alone or 5 mg/ml (total weight) of cholesterol balanced with methyl- $\beta$ -cyclodextrin (water-soluble cholesterol, Sigma). After extraction/replenishment the tissue was extensively washed with Krebs' solution before proceeding with the experiment.

### $\text{Ca}^{2+}$ AND FORCE MEASUREMENTS

Ureters and portal veins were excised from rats or guinea-pigs humanely killed by cervical dislocation after  $\text{CO}_2$  anaesthesia.

Small longitudinal strips about 2–3 mm in length and 1 mm in width were dissected and placed on the stage of an inverted microscope for tension recording. In some experiments, strips were loaded with the  $\text{Ca}^{2+}$ -sensitive indicator indo-1 (10  $\mu\text{M}$  for three hours) at room temperature, as described previously (Burdyga & Wray, 1998). Changes in  $[\text{Ca}^{2+}]_i$  were represented by the ratio of the 400 nm and 500 nm emissions. Contractions (30°C) were evoked electrically (1 Hz, 6 V, 600 ms) or by application of 40 mM KCl or 100  $\mu\text{M}$  carbachol (Cch). Tetraethyl ammonium (TEA, 5 mM), Bay K8644 (1  $\mu\text{M}$ ) and cyclopiazonic acid (CPA, 20  $\mu\text{M}$ ) were added to the superfusion solution as required.

### SUCROSE GRADIENT ULTRACENTRIFUGATION

Smooth muscle microsomes (1 ml; 16 mg/ml of total protein) were diluted twofold in buffer containing 80% sucrose, 2% Triton X-100 and 0.2 mM  $\text{CaCl}_2$  and overlaid by discontinuous gradient of 30% (10 ml)/5% (5 ml) sucrose containing 1% Triton X-100 and 0.2 mM  $\text{CaCl}_2$ . The gradients were subjected to ultracentrifugation at 4°C (16 hours 100,000  $\times$  g) in a swing-out rotor. Fractions of 1 ml were collected starting from the top of a centrifugation tube and were analyzed by Western blotting for protein- and by TLC for lipid-composition.

## Results

### MANIPULATION OF CHOLESTEROL CONTENTS IN THE PLASMA MEMBRANE OF SMOOTH MUSCLE CELLS

In a variety of smooth muscle tissues, MCD preferentially solubilizes cholesterol, leaving other major lipid constituents of the plasma membrane unaffected (Fig. 1*a, b*;  $n = 3$ ). Neither are membrane-associated proteins extracted by MCD, be they peripheral (caveolin), GPI-anchored (5'-nucleotidase) or transmembrane (large conductance  $\text{K}^{+}$ -channel, Fig. 1*c*;  $n = 3$ ). The cholesterol contents in the extracted preparations can be replenished by their incubation in the presence of "water-soluble cholesterol" (Fig. 1*d*;  $n = 3$ ). Thus, MCD-extraction in combination with "water-soluble cholesterol"-replenishment provides a selective tool for effective manipulation of cholesterol contents in the plasma membrane of smooth muscle cells.

### EXTRACTION OF CHOLESTEROL DESTABILIZES MEMBRANE RAFTS

Stabilized by cholesterol, lipid rafts are resistant to solubilization by non-ionic detergents and can therefore be isolated as detergent-insoluble glycosphingolipid-enriched membrane domains [(DIGs), (Harder & Simons, 1997; Brown & London, 1998, 2000; Babychuk & Draeger, 2000)]. In smooth muscle, DIGs are highly enriched in cholesterol and sphingomyelin (Fig. 2*a*;  $n = 5$ ). Extraction of cholesterol leads to raft destabilization; as a result, sphingomyelin as well as raft-associated proteins (*not shown*) lose their resistance to non-ionic detergents

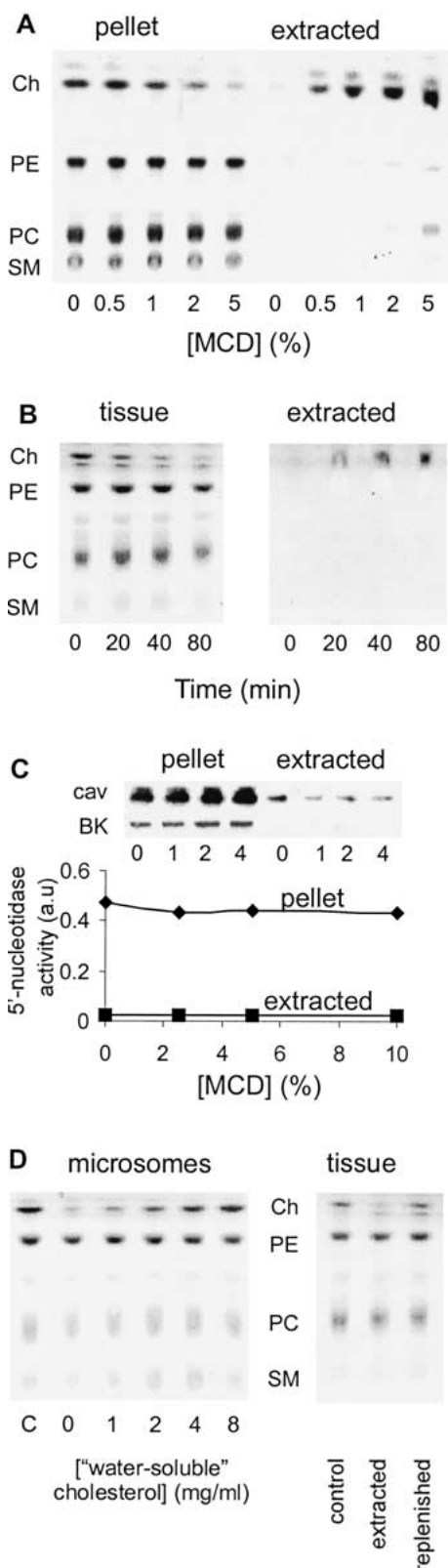
and show an extraction pattern similar to that of non-raft glycerophospholipids (Fig. 2*b*;  $n = 3$ ).

#### EXTRACTION OF CHOLESTEROL DISASSEMBLES CAVEOLAE

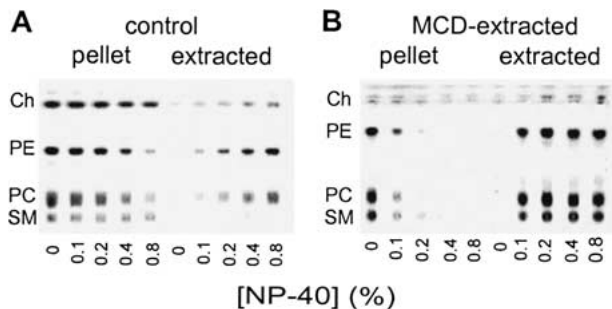
Extraction of cholesterol leads to a structural reorganization of the smooth muscle plasma membrane, marked by disassembly of caveolae (Fig. 3;  $n = 5$ ), which can be reversed by cholesterol replenishment (Dreja et al., 2002). Changes caused by the extraction of cholesterol remain restricted to the sarcolemma, inducing no obvious alterations in general morphology of the sarcoplasmic reticulum or mitochondria, two other key players in the regulation of  $\text{Ca}^{2+}$ -homeostasis (Fig. 3).

#### EXTRACTION OF CHOLESTEROL INHIBITS ELECTRICALLY-EVOKED CONTRACTIONS OF RAT URETER

Simultaneous measurements of force and  $[\text{Ca}^{2+}]_i$  in rat ureters perfused by MCD-containing solution demonstrated transient increases in both parameters (Fig. 4;  $n = 10$ ). As these are too brief to be a consequence of actual extraction of cholesterol and are likely to occur due to MCD-binding and subsequent initial rearrangement of the sarcolemma, we do not discuss those effects further. The initial transient increase was followed by a sustained inhibition of mechanical activity (control  $0.53 \pm 0.1$  mN, extracted  $0.12 \pm 0.04$  mN) and  $\text{Ca}^{2+}$  transients (21.5% amplitude of Indo-1 ratio, compared to 100% control) in response to electrical stimulation (Fig. 4;  $n = 10$ ). That the inhibition remained, even after removal of MCD, strongly argues for its reason being the cholesterol extraction per se. Correspondingly, super-



**Fig. 1.** MCD selectively extracts cholesterol from smooth muscle plasma membrane. (A–C). Smooth muscle microsomes (A,C) or strips of rat ureter (B) were extracted at 30°C with indicated concentrations of MCD for 30 min or with 2% MCD for indicated time periods. Extract was separated from insoluble residue either by centrifugation (30 min, 13,000 ×  $g$ ; microsomes) or decantation (strips). MCD-extraction of membrane lipids [cholesterol (*Ch*); phosphatidylethanolamine (*PE*), phosphatidylcholine (*PC*); sphingomyelin (*SM*)] was analyzed by thin-layer chromatography, TLC (A,B). The extraction of membrane-associated proteins caveolin (*cav*); large-conductance  $\text{K}^+$  channel (*BK*) and 5'-nucleotidase were analyzed by Western blotting analysis and enzymatic activity measurements (C). (D) Membrane cholesterol in MCD-extracted smooth muscle microsomes was replenished by microsome incubation in the presence of the indicated concentrations of “water-soluble cholesterol” (Sigma) at 30°C for 30 min. Likewise, membrane cholesterol of MCD-extracted strips of rat ureter was replenished by 2 mg/ml of “water-soluble cholesterol” (Sigma). After centrifugation (30 min, 13,000 ×  $g$ ; microsomes) or decantation (strips), pellets and tissue strips were analyzed by TLC. Line “C” represents control, unextracted microsomes.



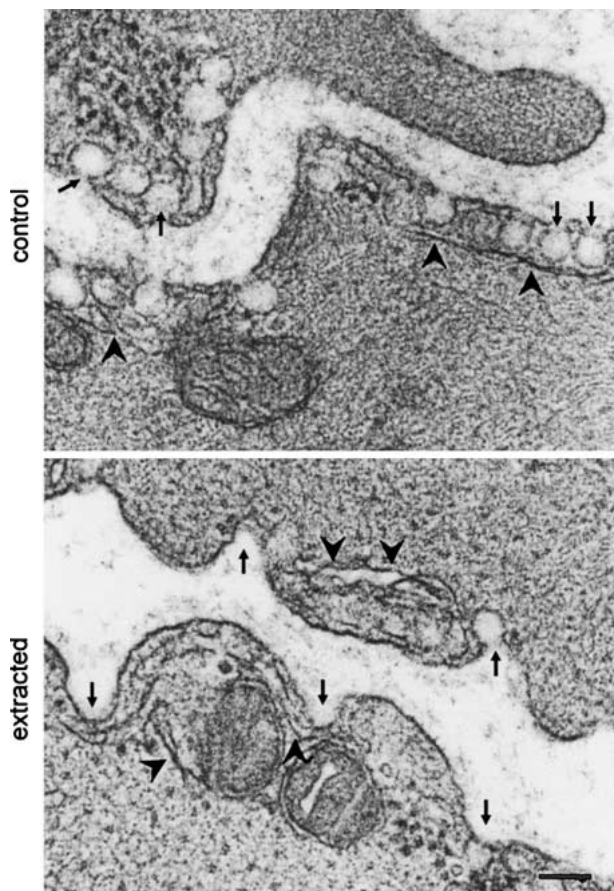
**Fig. 2.** Extraction of cholesterol results in destabilization of membrane rafts. Control (A) or MCD-pre-extracted (B) smooth muscle microsomes were incubated with the concentrations of non-ionic detergent (NP-40) for 30 min on ice, as indicated. After low-speed centrifugation (30 min,  $13,000 \times g$ ) the resulting pellets and supernatants were analyzed by TLC. In control preparations, non-raft glycerophospholipids (PE and PC) were preferentially extracted rather than raft-localized cholesterol and sphingomyelin. However, after selective removal of cholesterol by MCD, the remaining sphingomyelin was extracted by NP-40 as readily as the glycerophospholipids.

fusion with cholesterol reversed both the decrease in force and the  $Ca^{2+}$  transients (Fig. 4).

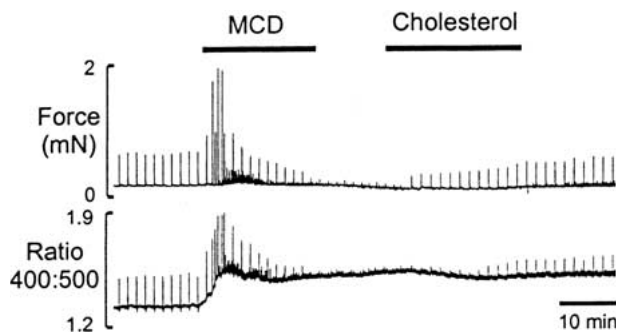
#### CHOLESTEROL-DEPENDENT INHIBITION OF CONTRACTILITY IS A CONSEQUENCE OF DECREASED $Ca^{2+}$ ENTRY THROUGH THE SARCOLEMMA

The inhibitory effect on force was preceded by a similar change in the amplitude of  $Ca^{2+}$  transients (Fig. 4), which suggests that it was not due to impairment or modulation of the  $Ca^{2+}$  sensitivity of the contractile machinery but to changes in the levels of  $[Ca^{2+}]_i$ .

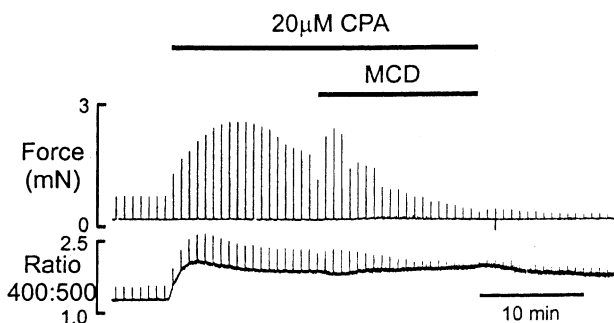
A sustained rise in basal  $Ca^{2+}$  occurred in MCD-treated tissue (Fig. 4), as also reported by Dreja et al. (2002). To determine if the rise in basal  $Ca^{2+}$  per se was responsible for the MCD-induced inhibition, the ureters were pre-incubated with an inhibitor of the sarcoplasmic reticulum (SR) Ca-ATPase, cyclopiazonic acid (CPA), to disable the SR. Application of CPA increased the basal level of  $Ca^{2+}$  but, in contrast to MCD, markedly potentiated contractility (Fig. 5;  $n = 3$ ), in agreement with our earlier findings (Burdyga & Wray, 1998). Application of MCD in the presence of CPA produced a transient increase in electrically-evoked force, followed by inhibition (Fig. 5), as seen in the absence of CPA (Fig. 4). These results suggest that the inhibitory effects of MCD on electrically-evoked contractions and  $Ca^{2+}$  transients of rat ureter are not produced via the SR or solely by the rise in the basal  $Ca^{2+}$ . The observed rise in the basal  $Ca^{2+}$  level also suggests that the inhibition is not a consequence of increased  $Ca^{2+}$  removal due to the activation of  $Ca^{2+}$ -extrusion mechanisms.



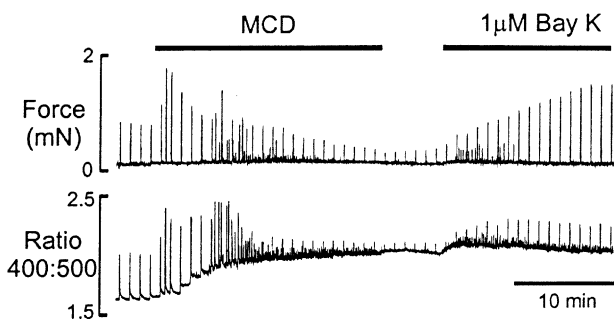
**Fig. 3.** Extraction of cholesterol leads to structural disassembly of caveolae. Electron micrograph of control rat ureter smooth muscle cell (upper panel) showing numerous caveolae (arrows). Sarcoplasmic reticulum (arrowheads) and mitochondria are seen in close proximity to the caveolar membrane domain. Cholesterol depletion (lower panel), does not affect the structure of the underlying sarcoplasmic reticulum or mitochondria but selectively targets caveolae, which gradually flatten until their complete disappearance. Bar = 0.2  $\mu m$ .



**Fig. 4.** Effects of cholesterol-extraction/replenishment on force and  $Ca^{2+}$  measured simultaneously in electrically stimulated rat ureter. 2% MCD added to the superfusion solution for a 20 min period resulted in inhibition of electrically-evoked contractions. After a 10–15 min washout, 5 mg/ml water-soluble cholesterol resulted in a recovery of force production.



**Fig. 5.** Effects of cholesterol extraction are not modulated by inhibition of SR. The tissue was pre-incubated with 20  $\mu\text{M}$  CPA for 20 min to inhibit any effects of the SR on electrically-evoked contractions. 2% MCD, in the continued presence of CPA, still resulted in inhibition of force production, suggesting that the MCD-induced effects were not mediated by the SR.



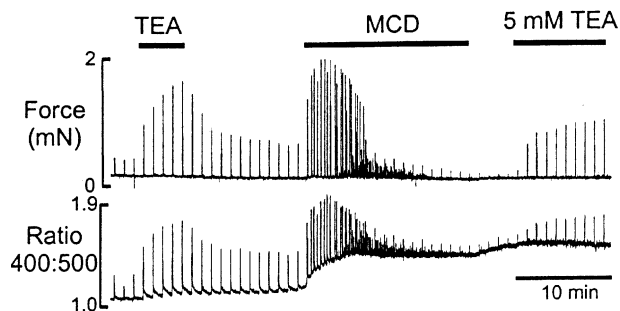
**Fig. 6.** Effects of cholesterol extraction: modulation by Bay K8644. Application of 2% MCD for 20 min inhibited electrically evoked contractions, an effect which was reversed by the  $\text{Ca}^{2+}$ -channel agonist Bay K8644 (1  $\mu\text{M}$ ).

#### EXTRACTION OF CHOLESTEROL DECREASES THE EXCITABILITY OF THE SARCOLEMMA

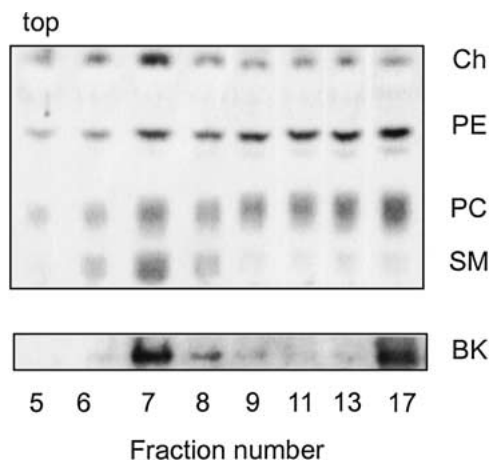
The major source of  $\text{Ca}^{2+}$  for contraction in ureter is the extracellular space, its entry being effected via L-type voltage-operated  $\text{Ca}^{2+}$  channels. We therefore investigated whether the  $\text{Ca}^{2+}$ -channel agonist Bay K8644 was able to restore mechanical activity in cholesterol-depleted tissues. Application of Bay K8644 reversed the inhibitory effects of MCD on both the  $\text{Ca}^{2+}$  transient and force (control,  $0.43 \pm 0.09$  mN; MCD,  $0.14 \pm 0.04$  mN; MCD plus Bay K8644,  $1.14 \pm 0.22$  mN; Fig. 6;  $n = 5$ ). Therefore, the MCD-induced inhibition was due to a decrease in  $\text{Ca}^{2+}$  influx, most likely via reduced membrane excitability.

#### INHIBITION OF THE LARGE-CONDUCTANCE $\text{K}^{+}$ CHANNEL ABOLISHES THE EFFECTS OF CHOLESTEROL EXTRACTION

Since membrane  $\text{K}^{+}$  conductance is largely responsible for determining excitability, we investigated whether the  $\text{K}^{+}$ -channel inhibitor tetraethyl-ammo-



**Fig. 7.** Effects of cholesterol extraction: modulation by TEA. 5 mM TEA alone produced potentiation of electrically-evoked contractions. TEA was also able to restore contractions after they had been inhibited by application of 2% MCD for 20 min.

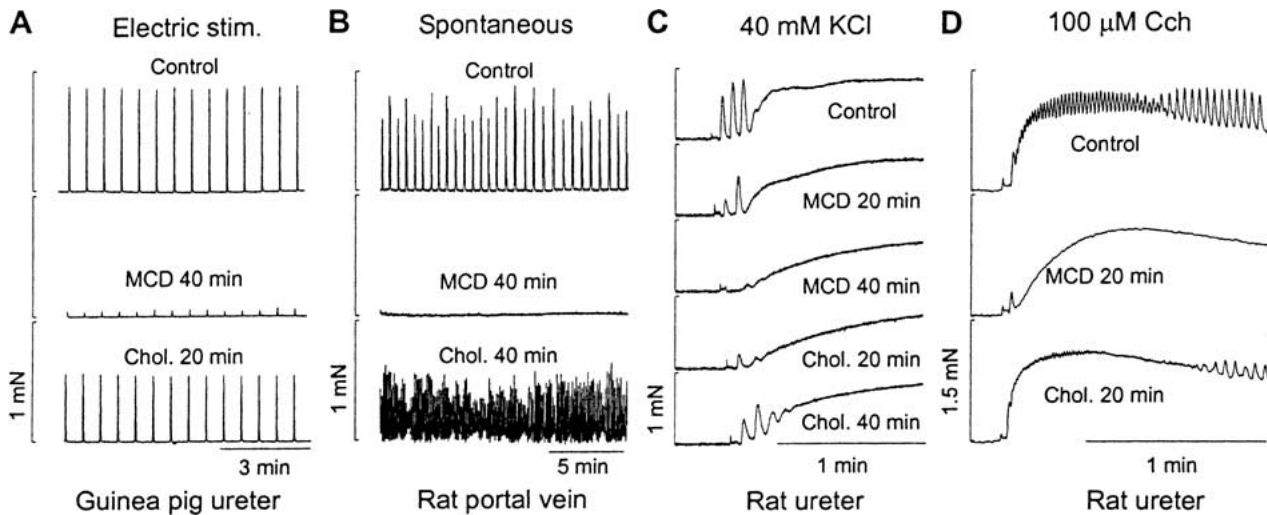


**Fig. 8.** The  $\alpha$ -subunit of large-conductance  $\text{K}^{+}$  channel localizes to membrane rafts. Smooth muscle microsomes were subjected to ultracentrifugation within a discontinuous gradient of sucrose in the presence of 1% Triton X-100. Fractions were analyzed by TLC for lipid composition (*upper panel*; cholesterol (*Ch*); phosphatidylethanolamine (*PE*), phosphatidylcholine (*PC*); sphingomyelin (*SM*)) and by Western blotting (*lower panel*) for the localization of the  $\alpha$ -subunit of the large-conductance  $\text{K}^{+}$ -channel (*BK*). Fraction 5 contains 5% sucrose, fractions 6–13, 30% sucrose; fraction 17, “soluble” 40% sucrose.

nium (TEA) could restore phasic activity in cholesterol-extracted preparations. Application of TEA was indeed able to restore force in cholesterol-extracted ureters (control,  $0.31 \pm 0.09$ ; MCD,  $0.10 \pm 0.03$  mN; MCD plus TEA,  $0.76 \pm 0.19$  mN; Fig. 7;  $n = 9$ ). The return of contractions was accompanied by a restoration of the  $\text{Ca}^{2+}$  transients, strongly suggesting that inhibition of large-conductance  $\text{K}^{+}$  channels counters the effects of cholesterol extraction.

#### $\alpha$ -SUBUNIT OF LARGE-CONDUCTANCE $\text{K}^{+}$ CHANNEL IS ASSOCIATED WITH DETERGENT-RESISTANT MEMBRANES

To assess the large-conductance  $\text{K}^{+}$  channel's association with membrane rafts, we investigated its microdomain localization. Ultracentrifugation of



**Fig. 9.** Extraction of cholesterol abolishes phasic but not tonic contractions of smooth muscles derived from various organs and species. The mechanical activity recorded from control electrically stimulated guinea pig ureter (*A*), spontaneously active rat portal vein (*B*) is exclusively phasic, while rat ureter stimulated with 40 mM KCl (*C*) or 100  $\mu$ M carbachol (*Cch*, *D*) shows rapid phasic and slow tonic contractions. After extraction of muscle strips by MCD, for indicated time periods, phasic but not tonic contractions are almost completely inhibited. This inhibition was reversed by incubation of muscle strips in the presence of “water-soluble” cholesterol (*Chol.*).

smooth muscle microsomes within a discontinuous sucrose gradient containing 1% Triton X-100, results in the separation of cholesterol- and sphingomyelin-enriched rafts from the glycerophospholipid milieu of the bulk membrane (Fig. 8, upper panel). The  $\alpha$ -subunit of the large-conductance  $K^+$  channel partially colocalizes with the highly buoyant cholesterol- and sphingomyelin-containing raft membranes (Fig. 8).

#### EXTRACTION OF CHOLESTEROL SELECTIVELY INHIBITS PHASIC SMOOTH MUSCLE CONTRACTIONS

The observed structural rearrangements of the sarcolemma result in alterations in the contractile activity of smooth muscle cells derived from a number of different organs and species. Similar to rat ureter (Fig. 4), the electrically evoked phasic mechanical activity of guinea pig ureter (Fig. 9*a*,  $n = 5$ ) and the spontaneous contractions of rat portal vein (Fig. 9*b*,  $n = 5$ ) were severely decreased after 40 min of cholesterol extraction. Rapid phasic contractions of rat ureter, triggered by the application of either 40 mM KCl (Fig. 9*c*,  $n = 10$ ) or 100  $\mu$ M carbachol (Fig. 9*d*,  $n = 10$ ), were likewise completely inhibited. In contrast, the sustained tonic component of these contractile responses remained unchanged (Fig. 9*c, d*). The inhibited phasic activity of the extracted tissues was “rescued” by cholesterol replenishment (Fig. 9*a-d*).

#### Discussion

Numerous studies have emphasized a significant role for cholesterol in the regulation of smooth

muscle contraction (reviewed in Bastiaanse et al., 1997). In smooth muscle, changes in the bulk amount of membrane cholesterol can alter the function of L-type  $Ca^{2+}$  channels (Tulenکو et al., 1990; Gleason et al., 1991; Sen et al., 1992), large-conductance  $K^+$  channels (Bolotina et al., 1989) and ATP-dependent  $K^+$  channels (Pongo et al., 2001). However, Lohn et al. (2000) reported L-type  $Ca^{2+}$  channel activation by membrane depolarization to be normal in cholesterol-depleted preparations.

Simultaneous measurements of force and  $[Ca^{2+}]_i$  in rat ureters perfused by MCD-containing solution demonstrated that the extraction of cholesterol resulted in inhibition of both force and  $Ca^{2+}$  signals during phasic, but not tonic contractions. This implies that signalling cascades that ultimately lead to either phasic or tonic response might be spatially segregated in the plane of the sarcolemma, in addition to the suggestion that different agonist signalling mechanisms may also be separate (Dreja et al., 2002). The inhibition of phasic contractions in cholesterol-extracted tissue, observed in the present study, may be accounted for by hyperpolarization of the sarcolemma due to an increased activity of the large-conductance  $K^+$  channels.

Changes in the cholesterol contents of the plasma membrane affect a number of important membrane properties, such as ion transport and the activity of membrane-associated proteins. These effects are generally attributed either to the specific alteration of cholesterol-protein interaction or to bulk changes in membrane fluidity. However, it has become increasingly evident that cholesterol is not randomly dis-

tributed in the plasma membrane, but concentrated in spatially confined microdomains or rafts. Consequently, the physico-chemical properties of a particular microdomain rather than that of the bulk membrane may selectively affect the function of membrane-associated proteins. While significant progress has been made in the biochemical and biophysical characterization of raft structure, molecular composition and dynamics, their physiological importance is poorly understood.

Here we show that in smooth muscle, the highly selective extraction of cholesterol by MCD results in the destabilization of membrane rafts and, as a consequence, in the loss of membrane microdomain segregation, disassembly of caveolae and decreased contractility. It is suggested, however, that MCD may have effects that are unrelated to rafts (Bastiaanse et al., 1997). This study demonstrates that in smooth muscle the level of membrane cholesterol and the stability of membrane rafts are intimately linked.

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