Changes in membrane cholesterol affect caveolin-1 localization and ICC-pacing in mouse jejunum

E. E. Daniel, Gregory Bodie, Marco Mannarino, Geoffrey Boddy, and Woo-Jung Cho

Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Submitted 20 August 2003; accepted in final form 7 February 2004

Daniel, E. E., Gregory Bodie, Marco Mannarino, Geoffrey Boddy, and Woo-Jung Cho. Changes in membrane cholesterol affect caveolin-1 localization and ICC-pacing in mouse jejunum. Am J Physiol Gastrointest Liver Physiol 287: G202–G210, 2004. First published February 19, 2004; 10.1152/ajpgi.00356.2003.—Pacing of mouse is dependent on the spontaneous activity of interstitial cells of Cajal in the myenteric plexus (ICC-MP). These ICC, as well as intestinal smooth muscle, contain small membrane invaginations called caveolae. Caveolae are signaling centers formed by insertions of caveolin proteins in the inner aspect of the plasma membrane. Caveolins bind signaling proteins and thereby negatively modulate their signaling. We disrupted caveolae by treating intestinal segments with methyl β-cyclodextrin (CD) to remove cholesterol or with water-soluble cholesterol (WSC) to load cholesterol. Both of these treatments reduced pacing frequencies, and these effects were reversed by the other agent. These treatments also inhibited paced contractions, but complete reversal was not observed. To evaluate the specificity of the effects of CD and WSC, additional studies were made of their effects on responses to carbamoyl choline and to stimulation of cholinergic nerves. Neither of these treatments affected these sets of responses compared with their respective time controls. Immunohistochemical and ultrastructural studies showed that caveolin 1 was present in smooth muscle membranes and ICC-MP. CD depleted both caveolin 1 and caveolae, whereas WSC increased the amount of caveolin 1 immunoreactivity and altered its distribution but failed to increase the number of caveolae. The effects of each agent were reversed in major part by the other. We conclude that signaling through caveolae may play a role in pacing by ICC but does not affect responses to acetylcholine from nerves or when added exogenously.

Caveolin 1 knockout mice have been shown to lack caveolae in vascular smooth muscle and endothelial cells and to have deficiencies in signaling through nitric oxide (NO) synthase (NOS) (8, 10, 23, 28, 29). Caveolae are closely associated with lipid rafts containing high concentrations of cholesterol and are involved in cholesterol transport into the membrane (7, 13, 25, 39, 40, 42). Removal of membrane cholesterol from the membrane using molecules such as methyl β-cyclodextrin (CD) causes loss of caveolin 1 and caveolae and dysfunction of caveolin-associated proteins in various cell types (7, 9, 11, 13, 15, 19, 21, 22, 24, 26, 30, 37, 42).

Previously, we showed that small isolated segments of longitudinal (LM) or circular muscle (CM) from mouse intestine contracted regularly, and the contractions behaved as if they were controlled by ICC pacing: i.e., contractions were independent of nerve function, were reduced but not abolished by block of VDCC, were faster in jejunum than ileum, had high temperature dependence, and were reduced in frequency by block of the sarco(endo)plasmic reticulum Ca2+-ATPase pump.

Our objectives were to determine whether caveolin 1 is present in ICC-MP and other ICC of mouse intestine, to evaluate the effects of removal of cholesterol with CD on ICC-MP pacing on responses to cholinergic stimulation and on the distribution of caveolin 1 and caveolae, and to determine whether any such effects could be reversed by restoration of cholesterol as water-soluble cholesterol (WSC). We also evaluated the effects on pacing of adding WSC first followed by CD and its reversal by treatment with CD.

MATERIALS AND METHODS

Tissue preparation. BALB/c mice were euthanized by cervical dislocation according to a protocol approved by the Health Sciences Laboratory Animal Services at the University of Alberta. Through a midline incision, the gastrointestinal tissue from the lower esophagus to the rectum was excised and immediately placed into an oxygenated Krebs solution at room temperature, and the mesentery was removed. Tissues were maintained at room temperature in Krebs solution that was constantly bubbled with 95% O2-5% CO2. Segments of the jejunum measuring ~1.5 cm for LM and 1 cm for CM were prepared for study as previously described (34).

In brief, the tissues were suspended in a 10-ml organ bath. To study CM contractions, the tissue was placed in the bath horizontally with an L-shaped holder through the lumen and stimulator electrodes on either side of the tissue. An aluminum wire triangle was inserted through the lumen and attached to a force transducer (Grass FT03; Grass Instruments, Quincy, MA) using silk thread. To study LM contractions, the tissue was tied at both ends and suspended vertically.

Address for reprint requests and other correspondence: E. E. Daniel, Rm. 9–10, Medical Sciences Bldg., Dept. of Pharmacology, Univ. of Alberta, Edmonton, AB, T6G 2H7, Canada (E-mail: edaniel@ualberta.ca).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
abolished. If needed, an additional 100 μM L-NNA to each bath to ensure that inhibitory neural activity was abolished after L-NNA were abolished by 0.1 M CD. The effects of the removal of the WS vs. the addition of CD were compared 30 min after the wash. Third, both test and control tissues were incubated for at least 60 min with WS. After the wash plus the addition of fresh Krebs and L-NNA, test tissues were given CD as above, and other tissues had WSC restored for 30 min.

In some experiments, if a fall in contraction frequency was not observed after 60 min incubation with WSC or CD, the tissues were exposed for an additional 15 min to the respective drug before each bath was washed out.

Additional experiments were carried out in which carbamoyl choline (CCH) was applied at 1 or 10 μM cumulatively before and after CD or WSC to LM segments of ileum and jejunum. Also, the effects of EFS (0.5, 2, 4, and 16 pulses/s) were applied to segments before and after CD and WSC. Changes were evaluated by comparison with responses of time controls. Only LM segments were used after block of neuronal NOS by 10−4 M L-NNA because they produced more reliable cholinergic responses.

Drugs and solutions. Krebs-Ringer solution was prepared with (in mM) 115.5 NaCl, 21.9 NaHCO3, 11.1 dextrose, 4.6 KCl, 1.16 MgSO4·H2O, 1.16 NaHPO4·H2O, and 2.5 CaCl2·H2O. The 10−2 M WS, and CD solutions were prepared by dissolving 0.131 g of drug in 1 ml of Krebs solution. A stock solution of 10−2 M L-NNA or CCH was prepared by dissolving the appropriate amount of drug in distilled water. All drugs used were obtained from Sigma-Aldrich (Oakville, ON).

Tissue manipulation. Before drugs were added, enteric nerves of the tissue preparations were stimulated using electrical field stimulation (EFS) from a Grass S88 stimulator set at 50 V/cm, 5 pulses/s, and 0.5-ms pulse duration to observe whether any inhibition of contractile activity took place. CM contractions were consistently inhibited by nerve stimulation, whereas LM contractions were stimulated. To prevent NO-mediated neural inhibition of muscle contraction, 100 μl of a 10−2 M solution of Nω-nitro-L-arginine (L-NNA), an inhibitor of NOS, was added to each bath, giving a final concentration of 10−3 M. EFS was subsequently performed 5 min after the addition of L-NNA to each bath to ensure that inhibitory neural activity was abolished. If needed, an additional 100 μl of L-NNA were added, giving a final concentration of 2 × 10−3 M. The contractions present after L-NNA were abolished by 0.1 μM atropine.

In each experiment either 1 ml of a 10−1 M solution of CD or WSC was added to the test tissues, giving a drug concentration of 10−2 M in the muscle bath. Warmed oxygenated Krebs solution (1 ml) was added to time control tissues in place of WSC or CD. Two experimental protocols are reported involving both LM and CM. First, after at least 60 min or more incubation with CD, the bath was washed out and fresh Krebs and L-NNA were added with WSC to restore membrane cholesterol. The effects of WSC recovery were measured 30 min later. Time control tissues were also washed out and given fresh Krebs and L-NNA. Second, both test and control tissues were incubated for at least 60 min with WS. After the wash plus the addition of fresh Krebs and L-NNA, control tissue was given 1 ml of warmed oxygenated Krebs solution. Test tissues were given 1 ml of 10−1 M CD. The effects of the removal of the WS vs. the addition of CD were compared 30 min after the wash. Third, both test and control tissues were incubated for at least 60 min with WS. After the wash plus the addition of fresh Krebs and L-NNA, test tissues were given CD as above, and other tissues had WSC restored for 30 min.

For the duration of each experiment, the muscle baths were continuously bubbled with 95% O2-5% CO2 at a constant rate and maintained at 37°C. Before experimental manipulations were performed, the tissues stabilized under slight tension for 5–10 min.

Fig. 2. A: figure organized as in Fig. 1 (time controls are shown in filled bars and experimental tissues in open bars), but it shows data of the normalized amplitudes of contractions and changes after CD for 60 min in LM and the lack of significant recovery in WSC for 30 min. These data are from the same tissues as in Fig. 1. Note that compared with time controls, there was no significant difference after recovery. B: the same data for CM with similar results, *P < 0.05; **P < 0.01.
sections of 10-μm thickness. The sections were attached on slide glasses coated with 2% (3-aminopropyl)triethoxysilane (cat. no. A3648; Sigma) in acetone and were dried for at least 1 h or overnight at room temperature. The sections were washed in 0.4% Triton X-100 (TX) in PBS for 15 min × 3. The sections were blocked with 10% normal sera that were raised in the host of secondary antibody for 1 or 1.2 h at room temperature. For immunohistochemistry, mouse anti-caveolin mAb (cat. no. 610406; BD Biosciences) was incubated for 18 to 19 h at 4°C. The sections were washed in 0.4% TX in PBS for 15 min × 3. For immunofluorescent-labeling secondary antibodies, immunoglobulin conjugated with Cy3 was used for 1 h at room temperature. The sections were washed in 0.4% TX in PBS for 15 min × 2 and were then washed in PBS for 15 min × 1. The sections mounted with aquamount medium were observed by confocal laser scanning microscope (CLSM; LSM 1500; Zeiss).

**Double immunofluorescent labeling using whole mount preparations.** CM and LM layers were separated from the mucosa and the submucosa layer under the dissection microscope. The muscle layers were washed in 0.5% TX in PBS for 15 min × 4 and were then blocked with 10% normal sera that were raised in the host of secondary antibody for 1.5 h at room temperature. For double immunohistochemistry, mouse anti-caveolin mAb and rat anti-cd117 (c-kit; Cedarlane) examined for whole mount were incubated for 48 h at 4°C and were then washed in 0.5% TX in PBS for 15 min × 4. For immunofluorescent-labeling secondary antibodies, immunoglobulin conjugated with Cy3 or FITC were incubated for 2 h at room temperature. The muscle layers were washed in 0.5% TX in PBS for 15 min × 3 and were then washed in PBS for 15 min × 1. The muscle layers mounted with aquamount medium were observed by CLSM.

**Preparation of sections for ultrastructural study.** Either fresh or after-organ bath experiment segments of jejunum were opened and pinned in ice-cold oxygenated Krebs solution as described in Immuno.

Fig. 3. This figure is organized similar to Figs. 1 and 2 except that both sets of tissues (filled and open bars) received 10 mM WSC after initial control recordings. The effects of 10 mM WSC for 60 min on frequencies of interstitial cells of Cajal (ICC)-driven contractions in longitudinal muscle (LM; A) and CM (B) in the second pair of bars and the subsequent effects of either 10 mM CD (open bars) or Ringer solution without WSC (filled bars) in the third pair of bars. Note that WSC exposure decreased frequencies, and these effects were reversed by CD but not by washing out WSC. ***P < 0.01; **P < 0.01.

**Data analysis.** At each time point (before WSC or CD, 60 min after WSC or CD, and 30 min after wash), contraction frequencies were measured over at least a 20-s interval. Contraction amplitudes were measured and converted to a fraction of the amplitude at time 0 (before WSC or CD), which was set as 1. For contraction frequencies, the statistical program Instat 3 was used to perform mean comparison tests using a one-way ANOVA. Prism 3 software was used to construct graphs using mean frequency values with error bars representing the SE. For the amplitude data, Prism 3 was used to construct graphs comparing the amplitude at each time point and conduct paired t-tests to compare the amplitudes at pairs of time points. Analysis of LM and CM data was done separately.

**Immunohistochemistry preparations.** Segments of jejunum, either fresh or after organ bath experiments, were placed in Krebs-Ringer solution, opened along the mesenteric border, and pinned on a petri dish of Sylgard silicon rubber (mucosa side down). For immunofluorescent labeling after cryosection, the segments were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight at 4°C. The fixed tissues were washed in PBS for 30 min × 8 and were cryoprotected in graded sucrose solutions (10, 20% sucrose in PBS) for 2 h each. They were then placed in 30% sucrose in PBS for 24 h at 4°C and stored at −80°C until used. For whole mount preparation, the jejunum was fixed at 4°C overnight in a fixative the same as mentioned earlier. The fixed tissue was washed in PB for 30 min × 8 and was dehydrated and cleared in DMSO for 10 min × 3 and was then rehydrated in PB for 15 min × 4 at room temperature.

**Immunofluorescent labeling using cryosections.** Frozen tissues were sectioned by a cryostat (Leitz 1720 digital cryostat) to make...
Signalizing through Caveolae Involved in ICC Pacing

RESULTS

Effects of CD and WSC on frequencies and amplitudes of paced contractions. Segments of both LM and CM exposed to 10 mM CD for 60 min decreased their pacing frequencies. Pacing frequencies were restored by the addition of 10 mM WSC for 30 min (Fig. 1). In time controls, there were no significant changes in frequencies during these time periods. Amplitudes of contractions also significantly decreased in CD compared with initial values as shown but not significantly compared with highly variable time controls. They were not restored by WSC (Fig. 2).

In some experiments, all segments were treated with CD for 60 min, and half had CD replaced with CD-free Ringer solution and the other half had WSC. There was no recovery of frequency when Ringer solution without WSC was used (not shown).

A possible explanation of our data showing that WSC restored frequencies after reduction by CD was that WSC had some physiological antagonism to CD. However, when 10 mM WSC was added first for 60 min, frequencies in LM or CM did not increase, but they decreased and were restored only in segments to which 10 mM CD was added for 30 min (Fig. 3). Moreover, washout of WSC in half of the tissues after 60 min was followed by the addition of CD or a replacement of WSC with Ringer solution. Only the presence of CD restored con-

Ultrastructural morphometric study: number of caveolae per unit length of ICC-MP and CM membrane. For the measuring of lengths of ICC-MP and CM membrane, sections (control, CD, CD followed by WSC, WSC alone, and WSC followed by CD) for ultrastructural study were examined. The lengths of each cell membrane were manually drawn and automatically measured by an image analytic program (Laser Scanning Systems LSM510 Image Examiner v.2.80.1123, Carl Zeiss). For counting of caveolae on ICC-MP and CM membranes, open and/or close caveolae on cell membranes, which had membrane length measured, were carefully counted and accepted after agreement by two persons. Uncertain caveolae, which looked similar to sarcoplasmic reticulum or damaged mitochondria and which were located in cytoplasm, were not counted. The number of caveolae per unit length of ICC-MP and CM membrane was shown as means ± SE and was analyzed by one-way ANOVA followed by Tukey-Kramer posttest using the statistical program Instat3.

Fig. 5. First panel shows a typical example of the effects of CD (60 min) and of subsequent WSC (WS; 30 min) on CM segments, contraction frequency, and amplitude. Note the decreases in frequency and amplitude of contractions, as well as tone and recovery of frequency after WSC. The last section was amplified 2.5 × other sections and the calibrations at the bottom. Second panel shows similar results to top panel in an LM segment. Note the decrease in frequency of contractions, with a decrease in their amplitudes after CD with recovery of frequency after WSC. The last segment was amplified 5 × other sections and the calibrations at bottom. Third panel shows a typical example of the effect of WSC (60 min) followed by CD (30 min) in a CM segment. Note the decrease in frequency or amplitude of contractions in CM after WSC and recovery of frequency after CD. All sections were amplified 5 × compared with the initial sections of other panels and the calibrations at bottom. Fourth panel shows a similar result to the third panel in LM. Note the decrease in frequency and amplitude after WSC and recovery after CD.
traction frequencies. WSC exposure for 60 min also decreased amplitudes of contractions in LM but not in CM in which it increased. There was no recovery in CD or Ringer solution after 30 min in LM (Fig. 4). Figure 5 shows an example of these findings. In other additional protocols (not shown), WSC reduced frequencies of contractions, and these changes were reversed after washout and replacement of L-NNA Ringer solution with CD but not when the replaced Ringer solution had more WSC.

Effects of CD and WSC on amplitudes of contractions of LM segments to CCH and EFS. Compared with time controls, contractile responses to CCH were unchanged after either 1 or 10 mM CD or WSC (Fig. 6, A and B). Although, responses were reduced after both CD and WSC, the responses of time controls were similarly reduced. Similarly, responses to cholinergic nerve stimulation were unchanged compared with those of time controls after CD or WSC (not shown). These, similar to the responses to CCH, decreased after CD and WSC but no more than time controls.

Fig. 7. The distributions of caveolin 1 immunoreactivity (IR) in control tissue (a), tissue treated with 10 mM CD for 60 min (b), and tissue treated with CD and subsequently with WSC for 30 min (c). All tissues were sectioned with the CM on cross section. In controls, caveolin 1 IR was located primarily at the muscle cell peripheries, often seen to be punctate in both LM and CM. Asterisks label what appear to be ICC cells labeled diffusely with caveolin 1 IR. Other studies shows that caveolin 1 IR was colocalized with c-kit IR in ICC in both the myenteric plexus and deep muscular plexus. After CD, caveolin 1 IR was much diminished and no longer clearly localized to cell peripheries or located in ICC. After CD and WSC, caveolin 1 IR was restored in all layers and ICC. Scale bars = 20 μm.

Fig. 8. The colocalization of c-kit (shown in red) and caveolin 1 (shown in green) in whole mounts of the myenteric plexus (Figs. a-c) and the deep muscular plexus (d-f). Note the excellent colocalization resulting in yellow staining.

Fig. 9. The effect of treatment with 10 mM WSC for 60 min on caveolin 1 IR (a) and the effect of subsequent treatment with 10 mM CD for 30 min (b). Note the heavy labeling of cell peripheries after WSC and diffusely in ICC (*). Subsequent treatment with CD reduced labeling in smooth muscle and ICC. Scale bars = 20 μm.
Locations of caveolin 1 in mouse intestine muscularis externa and effects of CD. Figure 7a shows that caveolin 1 immunoreactivity is located in punctate sites at the periphery of smooth muscle of both LM and CM. This figure also shows that it is also located in what appear to be ICC-MP. Caveolin 1 did not appear to be punctate in distribution in ICC. Figure 7b shows that caveolin 1 immunoreactivity is markedly decreased after CD, and Fig. 7c shows recovery of caveolin 1 immunoreactivity after WSC. One location of caveolin 1 was established to be in ICC membranes, as demonstrated by the colocalization of caveolin 1 and ckit (Fig. 8).

Effects of WSC on distribution of caveolae. Caveolae are present in ICC-MP, CM, and ICC-deep MP (DMP) in mouse intestine in control tissues after exposure to muscle bath conditions (Fig. 10). Although the several hours of exposure in the muscle bath affected mitochondrial status and caused other changes, caveolae were present in normal arrays. After exposure to CD, very few caveolae were present in ICC-MP, CM, or ICC-DMP, and those present were often opened in character (Fig. 11). After exposure to WSC, following CD, caveolae were present in normal-appearing arrays in ICC-MP, CM, and ICC-DMP (Fig. 12). After exposure to WSC, caveolae appeared abundant in ICC-MP, CM, and ICC-DMP (Fig. 13) and were restored toward the normal distribution after CD (not shown).

Quantitative evaluation of caveolae distributions. The numbers of caveolae in ICC and CM were examined quantitatively in ultrastructural profiles from tissues used in these experiments. Figure 14 shows the results of these analyses. CD treatment reduced the number of caveolae per unit membrane length of ICC-MP, and muscle cells of CM and subsequent WSC increased these numbers, which were still reduced but were no longer significantly different from controls. WSC treatment did not increase the number of caveolae per unit membrane length in either ICC-MP or CM cells.

DISCUSSION

Our main functional findings were that CD, which reduces cholesterol and also caveolin 1 and caveolae in membranes, reduced pacing frequencies in mouse LM and CM in intestine, and this effect was reversed by restoration of cholesterol by WSC, i.e., CD preloaded with cholesterol. Contractile amplitudes were reduced after CD compared with controls but not...
compared with the variable changes in time controls. To our surprise, WSC given first also inhibited pacing frequencies, and this effect was reversed by CD. This suggests that the ability of WSC to reverse the effects of CD on frequency were not some sort of physiological antagonism, because when given first, it had an opposite effect from when it was given after CD. In the case of WSC given first, there was also a decrease in contraction amplitude in LM, but CD did not reverse this significantly in LM. In CM, there was no decrease in contraction amplitude, and CD actually increased contractions compared with controls as well as compared with after WSC.

CD clearly altered the distribution of caveolin 1 and caveolae, which we demonstrated in LM, CM, and ICC-MP. It reduced the level of immunoreactivity in all of these cell types and nearly abolished the presence of caveolae. These effects were mostly reversed by subsequent exposure to WSC. Smooth muscle and ICC after these manipulations had abnormal mitochondria and other changes, suggesting that damages had occurred that might account for the failure of contraction amplitudes to recover.

Fig. 12. The recovery of caveolar structures after CD exposure when 10 mM WSC was applied for 30 min. a: The MP area. b: The CM. Note the increased number of caveolae in ICC-MP (some labeled with arrows) and CM cells. Scale bars = 2 μm.

Fig. 13. The effect of 60-min exposure to WSC on caveolar distribution. a: MP area. b: CM region. Note the very dense occurrence of caveolae in ICC-MP (a few labeled with arrows), CM, and BV endothelium. ICCp, ICC process. Scale bars = 2 μm.

Fig. 14. Summary of the distribution of caveolae in the membranes of ICC (A) and CM (B). Note that CD significantly reduced the numbers of caveolae in ICC and in CM, whereas subsequent WSC cholesterol partially restored these numbers. The values for ICC were not significantly different from control (CTR) values. WSC given first had no significant effect on the numbers of caveolae in ICC, but the numbers in CM were actually decreased. Subsequent CD still reduced these numbers in ICC, *P < 0.05; **P < 0.01; ***P < 0.001.
Given first, exposure to WSC increased the level of immuno-reactive caveolin 1 and altered its distribution. It was now distributed widely in the cytosol of smooth muscle as well as in the plasma membrane. There were high levels of caveolin present in ICC and smooth muscle, but these were not significantly increased compared with controls. CD exposure after WSC reduced the number of caveolae, but the distribution of immuno-reactive caveolin 1 was not fully restored. The actions of WSC on ICC and frequency of pacing may be related to overloading of cholesterol into inappropriate sites or to altered caveolin 1 function with excess cholesterol.

We interpret these changes to mean that caveolae, dependent on caveolin 1 in ICC and affected by cholesterol depletion, participate in the mechanisms controlling pacing. At this point, it is unclear whether ion channels or receptors in ICC are affected. The facts that WSC cholesterol also decreases pacing frequency and this effect is reversed by CD indicate that these interactions are not a kind of physiological antagonism but that there is an optimum level of cholesterol and caveolin 1 in ICC, with either too much or too little leading to decreased pacing frequencies.

Our findings also suggest that the levels of cholesterol in ICC control the level of caveolin 1 as well as its distribution. The literature (7, 13, 25, 39, 40, 42) suggests that caveolin 1 and caveolae contribute to the entrance and exit of cholesterol from smooth muscle cells. So far there has been no study of pacing by ICC in caveolin 1 knockout mouse intestine. Comparison of results from such a study with our results would be of interest. However, studies with cholesterol depletion and restoration or vice versa have some advantages over studies in knockout animals. First, because the effects of cholesterol removal and restoration on structure and function can be studied acutely, there is no possibility that the results reflect a developmental anomaly rather than a change in caveolar function. Second, further analysis is possible to determine what component of caveolar signaling has been affected. Third, comparisons can be made with intestinal segments from the same animals without caveolar disruption, and time controls can be included.

In conclusion, pacing by ICC in mouse intestine may be affected by cholesterol depletion, participate in the mechanisms controlling pacing. At this point, it is unclear whether ion channels or receptors in ICC are affected. The facts that WSC cholesterol also decreases pacing frequency and this effect is reversed by CD indicate that these interactions are not a kind of physiological antagonism but that there is an optimum level of cholesterol and caveolin 1 in ICC, with either too much or too little leading to decreased pacing frequencies.

ACKNOWLEDGMENTS

The authors acknowledge the expert assistance of summer research students Nipunue Rajapakse and Karen Ma in studies of CD and WSC on cholinergic responses.

GRANTS

This research was supported by the Canadian Institutes for Health Research.

REFERENCES


