Deoxycholic acid inhibits smooth muscle contraction via protein kinase C-dependent modulation of L-type Ca^{2+} channels in rat proximal colon

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Abstract. The aim of this study was to investigate the effects of deoxycholic acid (DCA) on the contractions of rat proximal colonic smooth muscle (PCSM) *in vitro*. The contractile response of rat PCSM strips was tested using a polyphysio-graph. The whole cell patch-clamp technique was also used in rat colonic smooth muscle cells (SMCs) isolated by an enzymatic procedure to record the L-type calcium current (I_{Ca-L}) prior to and following the application of various concentrations of DCA. The application of DCA (10^{-6}-10^{-4} M) decreased the amplitude of spontaneous contractions of the PCSM strips in a dose-dependent manner. The administration of DCA (10^{-5} M) caused the relaxation of isolated smooth muscle strips pre-contracted by acetylcholine (Ach) or KCl (by 12.2±1.5 and 16.3±6.9%, respectively). The concentration-response curve of CaCl$_2$ was shifted to the right. Pre-treatment of the strips with the protein kinase C (PKC) inhibitor chelerythrine (1 μM) significantly attenuated the effects of DCA on the strips pre-contracted by Ach. DCA reduced the peak I_{Ca-L} by 6.02±0.87% at 10^{-6} M, 15.02±1.73% at 10^{-5} M and 47.14±3.79% at 10^{-4} M. DCA shifted the current-voltage (I-V) curve of I_{Ca-L} upward, but the contour of the I-V curve was unchanged, and the peak current-induced voltage remained at 0 mV. Pre-treatment with chelerythrine (1 μM) blocked the actions of DCA on the I_{Ca-L}. Taken together, the actions of DCA on I_{Ca-L} in rat colonic SMCs contributed to a negative inotropic effect. These actions appear to be mediated through protein kinase C. Furthermore, this study suggests another possible mechanism for the DCA-related modulation of gastrointestinal motility.

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Introduction

Bile acids are synthesized from cholesterol and are conjugated to either taurine or glycine. The primary bile acids are dehydroxylated by intestinal bacteria, which gives rise to secondary bile acids, including deoxycholic acid (DCA). Most bile acids are absorbed from the terminal ileum and are returned to the liver by enterohepatic circulation. Several bile acids, malabsorbed by the small intestine, are transported to the colon where they affect colonic motility. In certain pathological conditions, for example, irritable bowel syndrome and ileal surgical resection, bile acid reabsorption is compromised. Previous studies suggested that bile acids affect gastrointestinal motility (1-3). Bile acids have been reported to inhibit ileal motility in the isolated perfused rabbit terminal ileum and guinea pig gallbladder smooth muscle strips (1,2). Moreover, DCA has been shown to inhibit spontaneous mechanical contractions in canine colonic smooth muscle (3).

In non-muscle cells, bile acids act through a variety of signaling mechanisms, including the activation of protein kinase C (PKC) or cyclooxygenase, or the generation of reactive oxygen species (ROS) (4-6). DCA inhibits the pacemaker currents of the interstitial cells of Cajal (ICC) by activating K$_{ATP}$ channels through the production of PGE$_2$ (7). L-type Ca^{2+} channels in intestinal smooth muscle are thought to trigger contractile activity. Intestinal smooth muscle contractility is dependent on the activation of L-type Ca^{2+} channels, which result in Ca^{2+} entry and the initiation of contractions (8). The effects and mechanism of DCA activity on the contraction of colon smooth muscles are not completely understood. Our aim was to investigate the possible mechanism by which DCA affects colon motility *in vitro*.

Materials and methods

Animals. Male Wistar rats weighing 200-250 g were used in this study. Rats were given free access to food and water in a temperature- and relative humidity-controlled room (23-25°C, relative humidity 55-60%) with a 12-h light/dark cycle for 1 week. Animals were maintained according to animal care guidelines from the Institutional Animal Care and Use

Committee of Wuhan University. Approval was obtained from the ethics committee of Wuhan University.

Preparation of colonic strips. Rats were anesthetized with ethyl ether prior to cervical exsanguination. The proximal colon was cut along the mesenteric border and cleaned with Ca^{2+}-free physiological saline solution (PSS). Muscle strips (~3x10 mm) were removed and placed in Ca^{2+}-free PSS, which was oxygenated continuously. Following the careful removal of the mucosa and submucosa by dissection, smooth muscle strips were obtained.

Contraction recording of colonic smooth muscle strips. Following the method described in our previous study (9), the muscle strips were mounted in 7 ml Tyrode's solution and connected to an isometric force transducer (JZJOIH, Chengdu, China). The Tyrode's solution was kept at 37˚C and bubbled with carbogen (95% O$_2$ + 5% CO$_2$). A tension of 1.0 g was slowly applied to the muscle strips prior to drug treatment. The muscle strips were allowed to equilibrate for 30-60 min, with the solution being changed every 20 min. The strips were washed at least three times with Tyrode's solution between each experimental condition. The mean contractile amplitude and frequency were recorded as control values and the effects of drugs as response values. The results were presented as the percentage change, calculated as: percentage change = (response value - control value)/control value x 100%.

Cell isolation. Colon smooth muscle cells (SMCs) were dissociated by enzymatic digestion according to a previously described method (10). The colon tissue was pinned to the Sylgard surface of a Petri dish and the mucosa was carefully dissected away under an anatomical microscope. The smooth muscle strips were then cut into small strips (2x2 mm) and placed in 2 ml calcium-free PSS supplemented with 0.12% (w/v) collagenase (type II), 0.2% soybean trypsin inhibitor and 0.2% bovine serum albumin (BSA), and incubated for 20-30 min at 37˚C. Pieces of tissue were rinsed in Ca^{2+}-free PSS solution five times to remove the enzyme, and were maintained at 4˚C for 6 h until use. Single SMCs were isolated by several gentle triturations through the tip of a fire-polished Pasteur pipette.

Whole-cell patch clamp recordings. For patch-clamp recordings, colon SMCs were placed onto glass coverslips. After being allowed to adhere to the glass for 10 min, the cells were washed with bath solution at a rate of 3 ml/min. The ion channel currents were recorded using a conventional whole-cell patch clamp method with an EPC-10 amplifier (HEKA Instruments Inc., Bellmore, NY, USA). Typical pipette resistances were 3-5 MΩ. Data were filtered at 200 Hz and digitized at 10 KHz (filter 1) and 2.9 KHz (filter 2). The experiments were performed at 24±1˚C, and the temperature of the bath solution ranged from 20 to 22˚C. L-type Ca^{2+} currents were activated by step depolarisation every 10 sec from a holding potential of -80 or -60 mV, between -40 and +30 mV for 400 ms in 10-mV increments to obtain current-voltage (I-V) relationships.

Solutions and drugs. The cells were bathed in a solution (Tyrode's solution) containing (mM): NaCl, 147.0; KCl, 4.0; CaCl$_2$, 2.0; NaH$_2$PO$_4$, 0.42; Na$_2$HPO$_4$, 1.81; MgCl$_2$, 1.05; and

glucose, 5.5 (adjusted to pH 7.35 with NaOH). The pipette solution contained (mM): CsCl, 135; MgCl$_2$, 4; HEPES, 10; Na$_2$-ATP, 2; EGTA, 10; and tetraethylammonium (TEA), 20 (adjusted to pH 7.3 with Tris base). The Ca^{2+}-free PSS contained (mM): NaCl, 135; KCl, 5.0; MgCl$_2$, 1.2; glucose, 10.0; and HEPES, 10 (adjusted to pH 7.4 with NaOH). The drugs used were: acetylcholine (Ach), DCA, nifedipine, chelerythrine and phorbol 12,13-dibutyrate (PDBu), and were purchased from Sigma (St. Louis, MO, USA). Collagenase was purchased from Gibco (Invitrogen Life Technologies, Carlsbad, CA, USA). Nifedipine, chelerythrine and PDBu were prepared as stock solutions in dimethylsulfoxide (DMSO) and stored at -20˚C. The final concentration of DMSO used in the experiments was <0.03%, which had no effect on the strips and cells.

Statistical analysis. The drug-induced changes in contractile response relative to the basal levels were calculated as percentages, and all values were expressed as the mean ± standard error (SE). Differences in the data were evaluated by the Student's t-test. P<0.05 was considered to indicate a statistically significant result. The n values reported in the text refer to the number of cells used in patch-clamp experiments.

Results

Effects of DCA on contractions of proximal colon smooth muscle strips. DCA decreased the amplitude of spontaneous contractions of the proximal colonic smooth muscle (PCSM) strips in a concentration-dependent manner, but not the frequency of the spontaneous contractions. DCA affected the contraction of the strips when applied for 2-3 min. Fig. 1A shows representative traces which demonstrate the effect of DCA (10^{-5} M) on the resting tension of longitudinal and circular muscle strips.

The mean contractile amplitude of longitudinal muscle was reduced by 5.63±2.77, 10.95±2.76 and 19.89±3.33% at DCA concentrations of 10^{-6}, 10^{-5} and 10^{-4} M, respectively (all n=6; p<0.05). The contractile amplitude of circular muscle was reduced by 5.22±1.63, 14.84±2.09 and 21.15±3.28%, respectively (all n=6; p<0.05) (Fig. 1B).

When the contraction evoked by Ach or KCl had reached a plateau, DCA (10^{-5} M) was administered. The administration of DCA induced the relaxation of PCSM (followed by circular muscle) strips pre-contracted with Ach (10 μM) or KCl (80 mM) by 12.2±1.5%, p<0.05 and 16.3±6.9%, p<0.05, respectively. Data were expressed as the percentage changes in tension from the contraction induced by Ach or KCl, respectively.

Increasing the concentration of CaCl$_2$ resulted in a concentration-dependent contraction of the strips. Incubation with DCA (10^{-5} M) for 20 min shifted the concentration-response curves of CaCl$_2$ to the right and decreased the maximal response (Fig. 1C).

Pre-treatment of proximal colonic strips with the PKC inhibitor chelerythrine for 20 min significantly attenuated the effects of DCA (10^{-5} M) on the Ach-induced contractions (Fig. 1D).

Effects of DCA on L-type Ca^{2+} currents in colonic smooth muscle. L-type calcium current (I_{Ca-L}) was recorded with calcium as the current carrier when the pipette contained a

A longitudinal
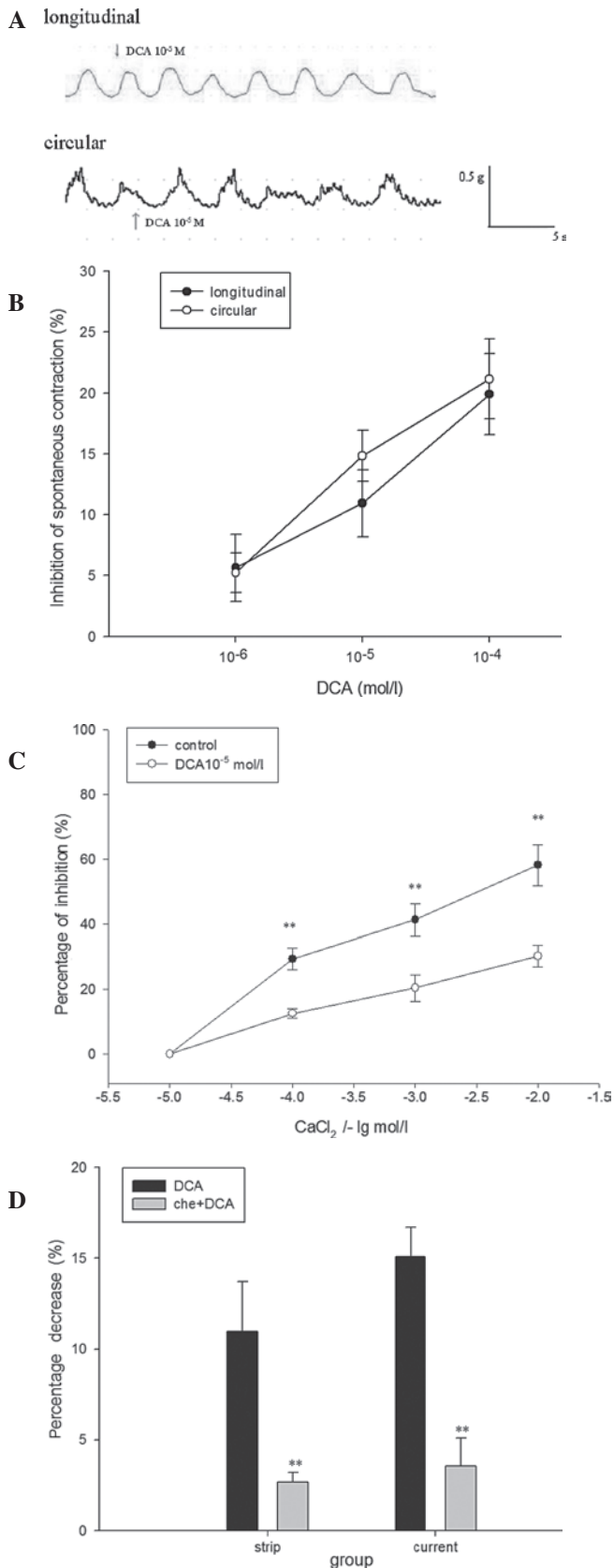

B

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Figure 1. Effects of deoxycholic acid (DCA) on the resting tension of rat proximal colonic smooth muscle strips. (A) Representative traces showing the effect of DCA (10^{-5} M) on the resting tension of longitudinal and circular muscle strips. (B) Curves showing the concentration-dependent effects of DCA on the amplitude of contraction. (C) Inhibitory effect of DCA (10^{-5} M) on the $CaCl_2$ concentration-response curve in isolated colon smooth muscle strips. (D) DCA (10^{-5} M) inhibited strip contraction and I_{Ca-L} in the absence and presence of chelerythrine (1 μM). **$P<0.05$ compared with the control. I_{Ca-L}, L-type Ca^{2+} current; che, chelerythrine.

high-Cs solution. The maximum current reached ~0 mV under these conditions, and was depressed by nifedipine (10 μM) by ~80% (n=6; p<0.05). The average cell capacitance (Cm) was 60.007±20.17 pF (n=60) and the access resistance was 5-10 MΩ. A series resistance compensation (lag of 60 us) of 70-75% was applied during each recording.

When DCA (10^{-5} M) was applied for 2-3 min, the peak I_{Ca-L} was reduced. As time increased, I_{Ca-L} reduced to a steady state level and was partially restored following a long-time washout. Fig. 2A shows the time-dependent effect of DCA in a representative cell. The currents were evoked by a step to 0 mV from a holding potential of -40 mV.

As shown in Fig. 2B, current traces were obtained under control conditions and during superfusion with DCA at concentrations from 10^{-6} to 10^{-4} M. Fig. 2C shows that DCA suppressed the I_{Ca-L} at 0 mV by 6.02±0.87, 15.02±1.73 and 47.14±3.79% at concentrations of 10^{-6}-, 10^{-5} and 10^{-4} M, respectively (all n=6; p<0.05).

Fig. 2D shows the average steady-state I-V relationships in the presence and absence of 10^{-5} M DCA, indicating that the drug did not change the shape of the I-V curve. When DCA was applied, the amplitude of the peak current was decreased from 213±4.6 to 181±4.9 pA compared with the control group (at 0 mV; n=6, p<0.05).

Effects of PKC on the action of DCA on I_{Ca-L} in colonic smooth muscle cells. To determine whether PKC was involved in the action of DCA, we used PDBu, a PKC activator, and chelerythrine, a potent PKC inhibitor.

Firstly, we tested the effects of PDBu (100 nM) and found that it promoted the I_{Ca-L} by 23.11±2.2% at 0 mV (n=6; p<0.05). The results demonstrated that PKC enhanced the L-type calcium currents in colonic SMCs. We then tested the effects of PDBu on the I_{Ca-L} in cells treated with DCA (10^{-5} M) for 3 min. We found that the promoting effect of PDBu on the I_{Ca-L} was reduced to 5.45±1.12% in the presence of DCA (Fig. 3A).

Secondly, pre-treatment of the colonic SMCs with the PKC inhibitor chelerythrine (1 μM) greatly reduced the decrease of I_{Ca-L} caused by DCA (10^{-5} M) (Fig. 3B). DCA decreased the I_{Ca-L} by only 3.57±0.35% in the presence of chelerythrine, compared with 15.09±1.65% in the absence of chelerythrine at 0 mV (n=6; p<0.05) (Fig. 1D). These results suggest that the inhibitory effects of DCA on the colonic SMC I_{Ca-L} are mediated by PKC.

Dissussion

In the present study, we investigated whether DCA inhibited the contraction of colonic smooth muscle strips *in vitro* and decreased I_{Ca-L}, possibly by the PKC pathway, in colonic SMCs.

It has been previously reported that concentrations of biliary DCA are increased in subjects with cholesterol gallstones, and that they alter gallbladder motor function (11). Therefore, it appears likely that DCA produced in the colon affects colonic motility. DCA has been shown to impair esophageal mucosal integrity via the induction of apoptosis (12). In our experiment, the effects of DCA were tested at concentrations of 10^{-6}-10^{-4} M. KCl- and Ach-induced contractions of colonic smooth muscle strips were relaxed following the administration of DCA. The concentration-response curve of $CaCl_2$ was shifted to the right
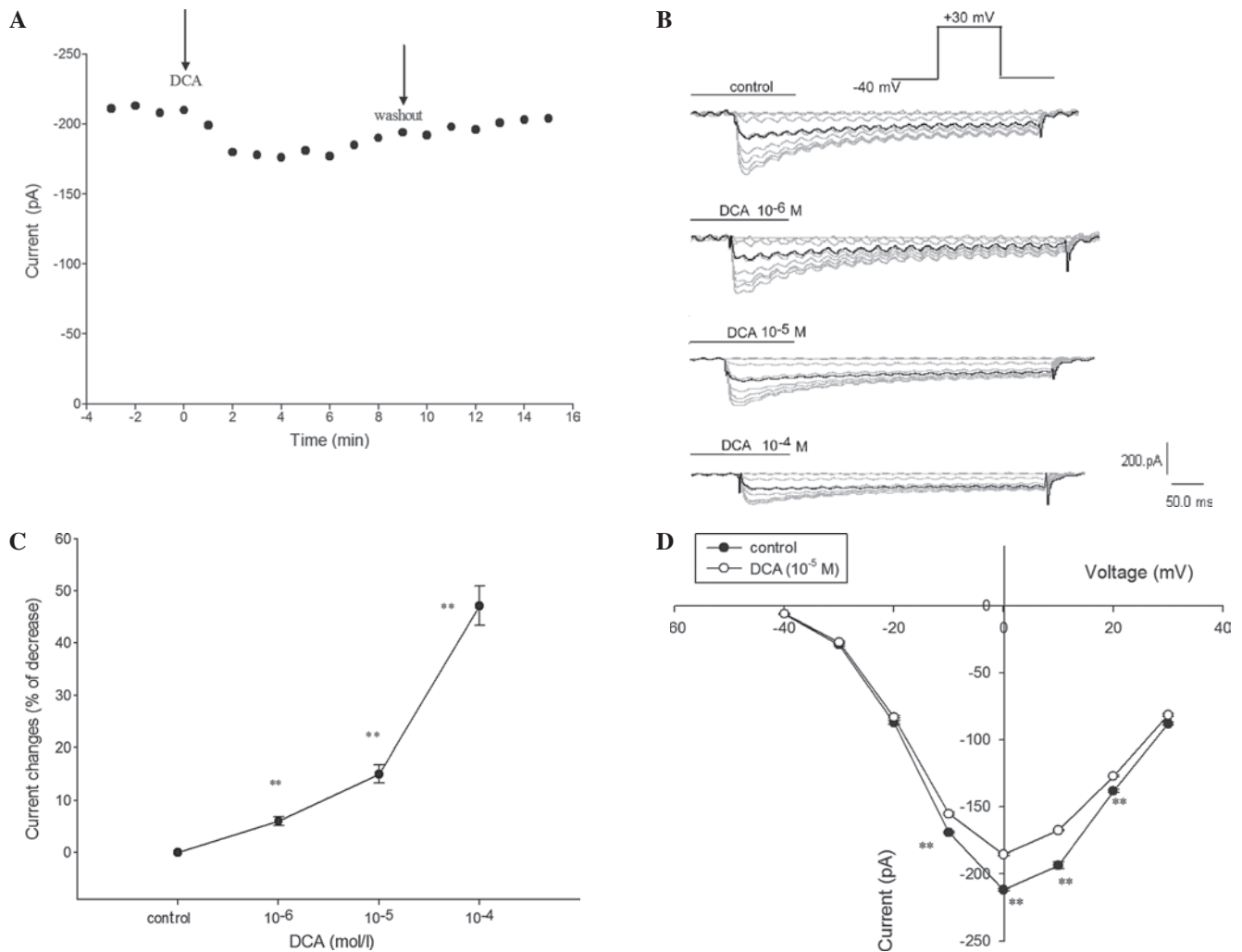
Figure 2. Effects of DCA on L-type Ca^{2+} currents in colonic SMCs. (A) Time-dependent course of peak Ca^{2+} currents prior to and following the application of DCA. (B) I_{Ca-L} traces were recorded following the superfusion of various concentrations with DCA. (C) Curve showing the concentration-dependent effects of DCA on I_{Ca-L}. (D) Current-voltage relationship for average peak Ca^{2+} currents in the presence and absence of DCA (10^{-5} M). [**]$P<0.05$ compared with the control (Tyrode's solution). DCA, deoxycholic acid; SMCs, smooth muscle cells; I_{Ca-L}, L-type Ca^{2+} current.
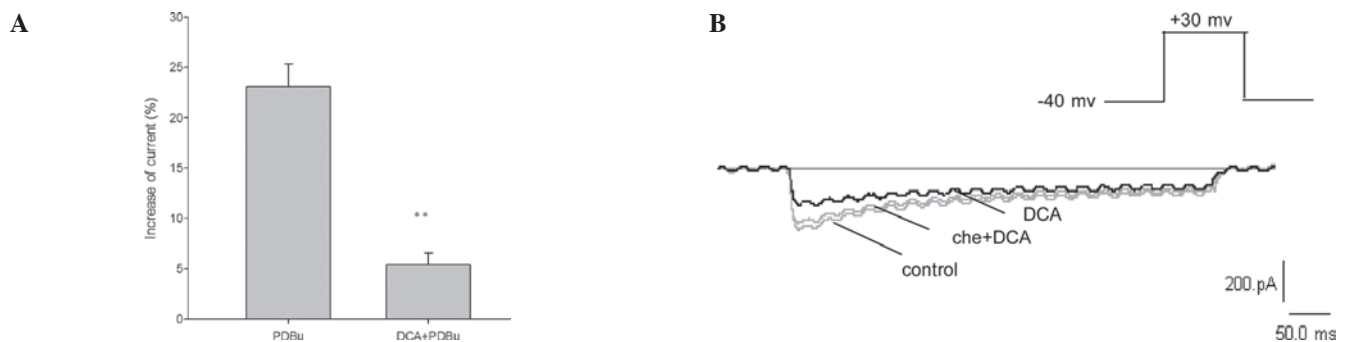

Figure 3. Chelerythrine inhibited the DCA-induced decrease of I_{Ca-L}. (A) PDBu induced an increase of I_{Ca-L} in the absence and presence of DCA (10^{-5} M); [**]$p<0.05$ compared with the PDBu group. (B) I_{Ca-L} traces were recorded in the presence and absence of chelerythrine (1 μM) while exposing to DCA (10^{-5} M) and a voltage step from -40 to 0 mV. DCA, deoxycholic acid; I_{Ca-L}, L-type Ca^{2+} current; PDBu, phorbol 12,13-dibutyrate; che, chelerythrine.

by incubation with DCA. These results indicate that DCA substantially inhibits extracelluar Ca^{2+} influx.

The activities of ion channels closely correlate with the movement of smooth muscle. In the present study, we found that the I_{Ca-L} in colonic SMC was decreased by DCA in a concentration-dependent manner. In addition, the char-

acteristics of the I-V curve were not significantly altered. The effects of DCA on calcium have been demonstrated in a previous study. For example, bile acids were shown to stimulate calcium mobilization through extracellular calcium influx and/or calcium release from intracellular organelles (as reviewed in ref. 13). Our patch clamp results directly

demonstrate that DCA inhibits the contraction of smooth muscles through the reduction of calcium entry through L-type calcium channels. The possible effect of DCA on the release of calcium from intracellular organelles needs to be further investigated. Previous studies have indicated that DCA inhibits the pacemaker currents generated by interstitial cells of Cajal (ICC); interaction with the activating K_{ATP} channels in the ICC resulted in the suppression of intestinal motility (7). Variations in the channel suggest that DCA may possess differing tissue and channel effects. The activity of DCA on ICC is consistent with its effects on smooth muscles. Thus, we presume that DCA activates K_{ATP} channels in colonic smooth muscles, but further investigation is required. Inhibition was observed when applying DCA for 2-4 min, and partly recovered following washout; thus, we suggest that the activity of DCA is non-genomic.

PKC, which is involved in cell responses to various stimuli, including neurotransmitters, growth factors and hormones, is essential for cell proliferation, differentiation and the regulation of ion channels. In smooth muscles, PKC is important for the regulation of movement. It is well known that activation of PKC enhances I_{Ca-L} in a variety of smooth muscles (14). For example, cholecystokinin (CCK) increases the I_{Ca-L} of gastric abtrum via the PKC pathway (15). Results showed that incubation with chelerythrine significantly affected the action of DCA on colonic smooth muscle strips pre-contracted by Ach, while the promoting effects of PDBu on the I_{Ca-L} were reduced in the presence of DCA. The I_{Ca-L} in the presence of chelerythrine was not greatly reduced by DCA. The results indicate that DCA regulates the PKC pathway. We conclude that DCA reduces PCSM contraction and inhibits L-type calcium channels in smooth muscle cells. This activity appears to be mediated through PKC. Furthermore, this study suggests another possible mechanism for the bile acid-related modulation of gastrointestinal motility.

Acknowledgements

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