



Calmodulin Antagonist W-7 Enhances Intermediate Conductance Ca^{2+} - Sensitive Basolateral Potassium Channel (IK_{Ca}) Activity in Human Colonic Crypts

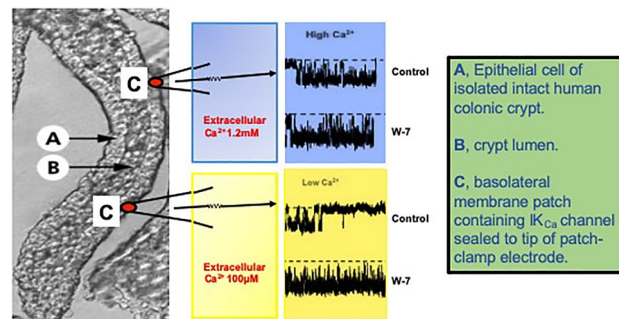
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Abstract

Intermediate conductance potassium (IK_{Ca}) channels are exquisitely Ca^{2+} sensitive, intracellular Ca^{2+} regulating channel activity by complexing with calmodulin (CaM), which is bound to the cytosolic carboxyl tail. Although CaM antagonists might be expected to decrease IK_{Ca} channel activity, the effect of W-7 in human T lymphocytes are conflicting. We therefore evaluated the effect of W-7 on basolateral IK_{Ca} channels in human colonic crypt cells. Intact crypts obtained from normal human colonic biopsies by Ca^{2+} chelation were used for patch clamp studies of basolateral IK_{Ca} channels in the cell-attached configuration. IK_{Ca} channel activity was studied when the bath Ca^{2+} concentration was changed from 1.2 mmol/L to 100 $\mu\text{mol/L}$ and back to 1.2 mmol/L, as well as from 100 $\mu\text{mol/L}$ to 1.2 mmol/L and back to 100 $\mu\text{mol/L}$, both in the absence and presence of 25 $\mu\text{mol/L}$ W-7. Decreasing bath Ca^{2+} from 1.2 mmol/L to 100 $\mu\text{mol/L}$ decreased IK_{Ca} channel activity reversibly in the absence of W-7, whereas there was a uniformly high level of channel activity at both bath Ca^{2+} concentrations in the presence of W-7. In separate experiments, increasing bath Ca^{2+} from 100 $\mu\text{mol/L}$ to 1.2 mmol/L increased IK_{Ca} channel activity reversibly in the absence of W-7, whereas there was again a uniformly high level of channel activity at both bath Ca^{2+} concentrations in the presence of W-7. We, therefore, propose that W-7 has a specific stimulatory effect on basolateral IK_{Ca} channel activity, despite its ability to inhibit Ca^{2+} /CaM-mediated, IK_{Ca} channel-dependent Cl^- secretion in human colonic epithelial cells.

Graphic Abstract



Keywords Calmodulin · Colon · Potassium channels · W-7

Introduction

Intermediate conductance Ca^{2+} -sensitive K^+ channels (IK_{Ca}) dominate the K^+ conductance of the basolateral membrane in human colonic crypts (Al-Hazza et al. 2012). These K^+ channels are activated by increases in intracellular Ca^{2+} and

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cAMP, and rapidly inhibited in a non-genomic manner by aldosterone (Sandle et al. 1994; Bowley et al. 2003). They are a major component of the electrogenic Cl^- secretory process in human colon and readily inhibited by somatostatin and its stable synthetic analogue octreotide, which is widely used as an anti-diarrhoeal agent (Sandle et al. 1999). Their inhibitory effects on human colonic IK_{Ca} channels are mediated by inhibitory G proteins and may reflect a decrease in the sensitivity of the channels to intracellular Ca^{2+} (Sandle et al. 1999). IK_{Ca} channels are exquisitely sensitive to Ca^{2+} , despite lacking Ca^{2+} -binding sites such as 'EF-hand' and 'Ca²⁺ bowl' motifs in their sequences (Joiner et al. 1997; Ishii et al. 1997; Logsdon et al. 1997). The high affinity of IK_{Ca} channels for Ca^{2+} reflects the constitutive Ca^{2+} -independent interaction of one calmodulin (CaM) molecule with the cytosolic COOH terminus of each channel subunit (Fanger et al. 1999; Khanna et al. 1999). Under these circumstances, Ca^{2+} binding to CaM induces a conformational change in CaM, which is transmitted to the channel subunits, leading to channel gating (Fanger et al. 1999).

CaM antagonists such as W-7, trifluoperazine and calmidazolium prevent interactions between CaM and target proteins by reversibly binding to the hydrophobic areas of CaM exposed after Ca^{2+} binding (Ikura 1996). Studies into the interaction between CaM and IK_{Ca} channel subunits have produced conflicting results, CaM antagonists either inhibiting or having no effect on IK_{Ca} channel activity (Fanger et al. 1999; Khanna et al. 1999; del Carlo et al. 2002). In this study, the initial aim was to investigate the role of CaM in Ca^{2+} -regulated gating of basolateral IK_{Ca} channels in human colonic crypt cells using the CaM antagonist W-7, which inhibits IK_{Ca} channel currents in human T lymphocytes (Fanger et al. 1999), but at the outset it became clear that W-7 itself had a marked stimulatory effect on IK_{Ca} channels in human colonocytes.

Methods

Isolation of Human Colonic Crypts

After obtaining written informed consent, 4–6 biopsies of normal-looking sigmoid colonic mucosa were taken during colonoscopy or flexible sigmoidoscopy in patients being investigated for altered bowel habit. None of the patients were receiving medication at the time of the procedure. Patients undergoing colonoscopy self-administered Klean-Prep® (Norgine) during the preceding 24 h, and those undergoing flexible sigmoidoscopy received a Fleet® ready-to-use enema (De Witt) before the procedure. Routine histology was normal in all cases. The study was approved by the Leeds Health Authority Ethics Committee. Intact crypts were isolated by a modification of a

previously described Ca^{2+} chelation technique (Bowley et al. 2003), suspended in a storage solution containing (mmol/L): 100 K^+ gluconate, 30 KCl, 20 NaCl, either 1.2 or 0.1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 5 glucose, 5 Na^+ pyruvate, 5 Na^+ butyrate, supplemented with 1 g/L bovine serum albumin, titrated to pH 7.4 with KOH, and kept on ice until required.

Patch Clamp Studies

Recordings were obtained from basolateral membrane patches of cells in the middle third of crypts in the cell-attached configuration. Experiments were performed at 20–22 °C rather than 37 °C to maintain cell viability (Sträter et al. 1996). The bath solution contained (mmol/L): 140 NaCl, 4.5 KCl, either 1.2 or 0.1 CaCl_2 , 1.2 MgCl_2 , 5 glucose, 10 HEPES, 5 Na^+ butyrate, and titrated to pH 7.4 with NaOH. Patch pipettes fabricated from borosilicate glass were filled with a solution containing (mmol/L): 145 KCl, 1.2 CaCl_2 , 1.2 MgCl_2 , 5 glucose, 10 HEPES, 5 Na^+ butyrate, and had resistances of 5–6 M Ω . Single channel currents were recorded with a patch clamp amplifier (List model EPC7, Darmstadt, Germany) at a holding voltage of –40 mV, referenced to the pipette interior. Currents were stored on videotape after pulse code modulation (PCM-701 ES, Sony, Japan). Stored currents were low pass filtered (750 Hz) and loaded into computer memory via a DigiData 1200 interface system using pClamp software (version 5.6, Axon Instruments Inc, USA) for off-line analysis. Single channel open probability (P_{O}) was calculated using an analysis programme written in Quick Basic 4.0 (Microsoft, USA), as previously described (Lomax et al. 1996).

All recordings were made in the cell-attached configuration, while crypts were superfused continuously with NaCl solution containing either 1.2 mmol/L or 100 $\mu\text{mol/L}$ unbuffered Ca^{2+} . The potential role of CaM in regulating basolateral IK_{Ca} channel activity was evaluated using the CaM antagonist W-7 at both bath Ca^{2+} concentrations. Crypts bathed initially in 100 $\mu\text{mol/L}$ Ca^{2+} were stored in a K^+ -rich solution containing 100 $\mu\text{mol/L}$ CaCl_2 rather than the usual 1.2 mM CaCl_2 . To determine the effect of W-7, crypts were pre-incubated with 25 $\mu\text{mol/L}$ W-7 for 20 min, and continuously exposed to this concentration throughout the experiment.

Statistical Analysis

Results are expressed as mean \pm standard error (SE). Statistical analyses were performed using the Student's *t* test, where $P < 0.05$ was considered significant.

Results

In preliminary experiments, 25 $\mu\text{mol/L}$ W-7 had no effect on channel activity at a bath Ca²⁺ concentration of 1.2 mmol/L. However, W-7 stimulated channel activity at a bath Ca²⁺ concentration of 100 $\mu\text{mol/L}$ (data not shown). Subsequent experiments were, therefore, done to test the effect of W-7 when the bath Ca²⁺ concentration was switched from 1.2 mmol/L to 100 $\mu\text{mol/L}$, and vice versa.

Effect of W-7 on IK_{Ca} Channel Activity in Response to Lowering Bath Ca²⁺

Representative recordings showing the effect of W-7 on the response of IK_{Ca} channel activity to lowering bath Ca²⁺ from 1.2 mmol/L to 100 $\mu\text{mol/L}$ are shown in Fig. 1. In the absence of W-7 (control), there was a high level of IK_{Ca} channel activity when the bath solution initially contained 1.2 mmol/L Ca²⁺. Within 2 min of lowering bath Ca²⁺ to 100 $\mu\text{mol/L}$, there was a sustained decrease in channel activity, consistent with a decrease in intracellular Ca²⁺ concentration. Channel activity was restored to its original high level by raising bath Ca²⁺ to 1.2 mmol/L. When this protocol was repeated in the presence of 25 $\mu\text{mol/L}$ W-7, the high level of channel activity at a bath concentration of 1.2 mmol/L was similar to that in the absence of W-7. However, a striking finding was that in the presence of W-7, lowering bath Ca²⁺ concentration to 100 $\mu\text{mol/L}$ failed to decrease channel activity, and the subsequent raising of bath Ca²⁺ to 1.2 mmol/L had no additional effect.

Data obtained from experiments when bath Ca²⁺ was changed from 1.2 mmol/L to 100 $\mu\text{mol/L}$ and then back to

1.2 mmol/L, either in the absence of W-7 (controls, $n=5$), or in the presence of W-7 ($n=6$), are summarized in Table 1. W-7 had no effect on IK_{Ca} channel activity at a bath Ca²⁺ concentration of 1.2 mmol/L. Whereas channel activity decreased by 49% ($P<0.005$) when bath Ca²⁺ was lowered from 1.2 mmol/L to 100 $\mu\text{mol/L}$ in the absence of W-7, there was no change in channel activity when bath Ca²⁺ was lowered in the presence of W-7. These data suggest that W-7 may have ‘clamped’ IK_{Ca} channel activity at a level (which was dependent on the initial intracellular Ca²⁺ concentration) when the bath solution contained 1.2 mmol/L Ca²⁺. To explore this possibility, additional experiments were done at an initial bath Ca²⁺ concentration of 100 $\mu\text{mol/L}$.

Effect of W-7 on IK_{Ca} Channel Activity in Response to Raising Bath Ca²⁺

Representative recordings showing the effect of W-7 on the response of IK_{Ca} channel activity to raising bath Ca²⁺ from 100 $\mu\text{mol/L}$ to 1.2 mmol/L are shown in Fig. 2. In the absence of W-7 (control) there was a relatively low level of IK_{Ca} channel activity when the bath solution initially contained 100 $\mu\text{mol/L}$. Within 2 min of raising bath Ca²⁺ to

Table 1 Effect of W-7 on single IK_{Ca} channel open probability (P_o) at high initial bath Ca²⁺ concentration

	Bath Ca ²⁺ concentration		
	1.2 mmol/L	100 $\mu\text{mol/L}$	1.2 mmol/L
Control ($n=5$)	0.612 \pm 0.064	0.321 \pm 0.087	0.500 \pm 0.095
W-7 ($n=6$)	0.645 \pm 0.073	0.700 \pm 0.042	0.646 \pm 0.084
<i>P</i>	N.S	<0.003	N.S

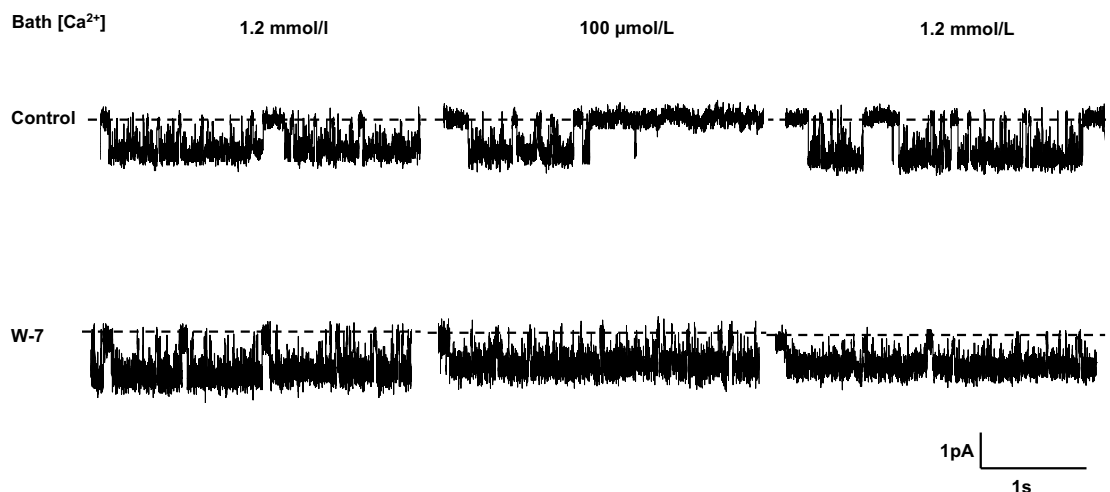


Fig. 1 IK_{Ca} channel activity when bath Ca²⁺ concentration changed from 1.2 mmol/L to 100 $\mu\text{mol/L}$, and back to 1.2 mmol/L in the absence (control) and presence of W-7

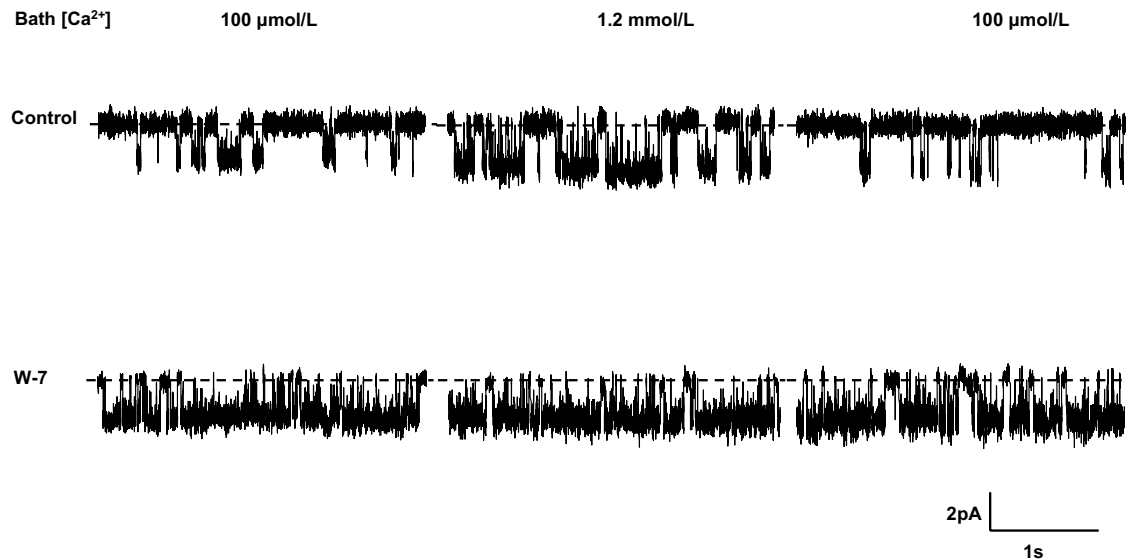


Fig. 2 IK_{Ca} channel activity when bath Ca^{2+} concentration changed from 100 $\mu\text{mol/L}$ to 1.2 mmol/L , and back to 100 $\mu\text{mol/L}$ in the absence (control) and presence of W-7

Table 2 Effect of W-7 on single IK_{Ca} channel open probability (P_O) at low initial bath Ca^{2+} concentration

Bath Ca^{2+} concentration	Bath Ca^{2+} concentration		
	100 $\mu\text{mol/L}$	1.2 mmol/L	100 $\mu\text{mol/L}$
Control ($n=6$)	0.271 ± 0.080	0.499 ± 0.090	0.128 ± 0.049
W-7 ($n=4$)	0.661 ± 0.073	0.703 ± 0.046	0.711 ± 0.053
<i>P</i>	<0.01	N.S	<0.0001

1.2 mmol/L , there was a marked increase in channel activity, consistent with an increase in intracellular Ca^{2+} concentration, and channel activity decreased to the basal level when bath Ca^{2+} was lowered to 100 $\mu\text{mol/L}$. Repeating this protocol in the presence of W-7, the high level of channel activity at an initial bath Ca^{2+} concentration of 100 $\mu\text{mol/L}$ was similar to that seen at an initial bath Ca^{2+} concentration of 1.2 mmol/L in the absence of W-7. Channel activity did not increase further when bath Ca^{2+} concentration was raised to 1.2 mmol/L , and was unchanged when bath Ca^{2+} was subsequently lowered to 100 $\mu\text{mol/L}$.

Data obtained from experiments when bath Ca^{2+} was changed from 100 $\mu\text{mol/L}$ to 1.2 mmol/L and then back to 100 $\mu\text{mol/L}$, either in the absence of W-7 (controls, $n=6$), or in the presence of W-7 ($n=4$), are summarized in Table 2. W-7 elicited a greater than twofold increase in IK_{Ca} channel activity at a bath Ca^{2+} concentration of 100 $\mu\text{mol/L}$. Whereas channel activity increased by 84% ($P < 0.001$) on raising bath Ca^{2+} from 100 $\mu\text{mol/L}$ to 1.2 mmol/L in the absence of W-7, channel activity was unaffected when the same change in bath Ca^{2+} was performed in the presence of

W-7. Thus, these observations suggest that W-7 stimulates basolateral IK_{Ca} channels in human colonic crypt cells by a mechanism that appears to be largely (if not entirely) independent of the intracellular Ca^{2+} concentration, rather than by ‘clamping’ channel activity at a high level commensurate with the intracellular Ca^{2+} concentration that prevailed at a bath Ca^{2+} concentration of 1.2 mmol/L .

Discussion

The initial aim of this study was to evaluate the effect of the CaM antagonist W-7 on the regulation of basolateral IK_{Ca} channels by Ca^{2+} -mediated agonists in human colonic crypt cells. However, in preliminary experiments, rather than producing little or no change in basal channel activity (that is, in the absence of a Ca^{2+} -mediated agonist), W-7 prevented the decrease in channel activity that occurred when bath Ca^{2+} concentration was lowered from 1.2 mmol/L to 100 $\mu\text{mol/L}$. We therefore focused on evaluating the effect of W-7 on human colonic crypt cells in the absence of Ca^{2+} -mediated agonists. The data in Table 1 and Table 2 show that in the absence of W-7, decreasing or increasing extracellular Ca^{2+} concentration elicited the expected changes in IK_{Ca} channel activity, which presumably reflected corresponding decreases or increases in intracellular Ca^{2+} concentration. In the absence of W-7, P_O was 0.5–0.6 at a bath Ca^{2+} concentration of 1.2 mmol/L , and decreased by ~50% when bath Ca^{2+} was lowered to 100 $\mu\text{mol/L}$. By contrast, P_O was invariably higher (~0.7) in the presence of W-7, irrespective of the bath Ca^{2+} concentration. This novel and entirely

unexpected observation suggested that amongst the CaM inhibitors, W-7 at least has the ability to stimulate IK_{Ca} channels, which raises questions about its suitability in studies designed to evaluate the role of CaM in IK_{Ca} channel regulation. Coincidentally, it may be that the level of basolateral IK_{Ca} channel activity seen in human colonic crypt cells in the presence of W-7 is the highest achievable, since previous studies have always indicated lower levels of activity, even after using thapsigargin to increase intracellular Ca^{2+} concentration (Sandle et al. 1999).

Unlike our results, previous studies have shown that CaM inhibitors such as W-7, trifluoperazine (TFP), and calmidazolium, either had no effect or inhibited the activity of native and cloned IK_{Ca} channels. Thus, W-7, TFP or calmidazolium had no effect on whole-cell IK_{Ca} currents at physiological membrane voltages in human T lymphocytes and KCNN4-transfected COS-7 cells (Fanger et al. 1999). Voltage-dependent inhibition of IK_{Ca} currents was seen in depolarized cells, probably reflecting a direct effect on the channel rather than via modulation of CaM activity (Fanger et al. 1999). The ineffectiveness of CaM antagonists at physiological voltages could reflect the constitutive nature of the binding between CaM and IK_{Ca} channels, which may prevent access of W-7 to its site of action during conformational changes in the N- and C-terminal domains of CaM triggered by Ca^{2+} binding to EF hands (Ikura 1996). CaM antagonists may therefore be unable to antagonize the interaction between the hydrophobic region of Ca^{2+} -bound CaM and the IK_{Ca} channel CaM-binding domain in the proximal C terminus (Fanger et al. 1999).

Our finding that W-7 altered the response of basolateral K_{Ca} channels in human colonocytes to changes in bath Ca^{2+} concentration differs from studies in human erythrocytes, which showed that Ca^{2+} -dependent IK_{Ca} channel activity in cell-attached and excised inside-out patches was unaffected by 10–100 $\mu\text{mol/L}$ W-7 (del Carlo et al. 2002). CaM binding to IK_{Ca} channel α -subunits is thought to be Ca^{2+} -independent, and the interaction between CaM and its binding domain on IK_{Ca} channel protein may occur early in channel biogenesis, before insertion into the cell membrane (Fanger et al. 1999). However, whole-cell IK_{Ca} currents in human T lymphocytes are inhibited by CaM antagonists and the CaM kinase antagonist KN-62, which suggests a role for CaM kinases in IK_{Ca} channel regulation (Khanna et al. 1999). In addition, cloned KCNN4 channels expressed in CHO cells were shown to be blocked by W-7 and TFP in a voltage-dependent manner, suggesting that these CaM antagonists may have inhibited channel activity by impeding the interaction of the hydrophobic region of CaM with IK_{Ca} channel protein (Fanger et al. 1999).

The results of the present study strongly suggest that W-7 has a direct stimulatory effect K_{Ca} channel activity in human colonocytes which is CaM-independent. Consistent with this

view, W-7 (20–70 $\mu\text{mol/L}$) has previously been shown to increase ^{86}Rb (K^+) influx and efflux in the human salivary epithelial cells line HSG-PA in a partially Ca^{2+} -dependent manner (Patton et al. 1991). The overall conclusion from those studies was that W-7 stimulated K^+ fluxes in HSG-PA cells via a mechanism involving direct or indirect interaction with K^+ channels in a way that differed from that seen during muscarinic (carbachol) stimulation or in response to the Ca^{2+} ionophore A23187 (Patton et al. 1991).

While the precise mechanism by which W-7 might interact with K^+ channels remains to be established, one attractive possibility has come from studies using large unilamellar vesicles (LUVs) (Sengupta et al. 2007). These studies were based on the fact that the naphthalene-sulfonamide derivatives W-7 and W-13 are amphipathic weak bases. At concentrations where they may bind to the Ca^{2+} /CaM complex by nonspecific hydrophobic and electrostatic interactions, they may also bind to the inner leaflet of the plasma membrane. This would have the effect of decreasing the inner leaflet's net negative charge, which mainly reflects the presence of the monovalent acidic lipid 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylserine (PS). Consistent with this view, the inclusion of PS produced a negative electrostatic potential in the aqueous phase close to the vesicle surface which decreased exponentially with distance, the zeta potential (ζ) being the electrostatic potential $\sim 2 \text{ \AA}$ from the membrane surface, and the addition of 30 $\mu\text{mol/L}$ W-7 to the LUVs decreased ζ by $\sim 23 \text{ mV}$ (Sengupta et al. 2007). We, therefore, speculate that if W-7 depolarized ζ at the inner leaflet of the colonocyte basolateral membrane, voltage-sensitive Ca^{2+} channels within the membrane may have been activated (Brice and Dolphin 1999), resulting in a rise in intracellular Ca^{2+} to a level sufficient to increase IK_{Ca} channel activity, even at a bath Ca^{2+} of 100 $\mu\text{mol/L}$. This offers a plausible explanation for our observation that IK_{Ca} channel activity at a bath Ca^{2+} concentration of 100 $\mu\text{mol/L}$ in the presence of W-7 was similar to that at a bath Ca^{2+} concentration of 1.2 mmol/L in the absence of W-7. We also considered two other explanations. The first is that W-7-induced depolarization of ζ might have resulted in a purely voltage-dependent increase in IK_{Ca} channel activity but this is unlikely since basolateral IK_{Ca} channels in human colon are inherently voltage-insensitive (Bowley et al. 2003). The second, which also seems unlikely, is that W-7 enhanced the already exquisite Ca^{2+} -sensitivity of IK_{Ca} channels to the extent that their activity at a bath Ca^{2+} of 100 $\mu\text{mol/L}$ increased to the level seen at a bath Ca^{2+} concentration of 1.2 mmol/L. Thus, in studies where W-7 is used to investigate CaM's role in Ca^{2+} -mediated Cl^- secretion (specifically in human colonic epithelium), where basolateral IK_{Ca} channels are a critical component (Tabcharani et al. 1994; Duan et al. 2019), W-7 may actually upregulate these channels independently of CaM while inhibiting overall Cl^- secretion.

Author Contributions Material preparation, data acquisition and analysis, KAB; data analysis and manuscript preparation, GIS; approval of final manuscript, GIS.

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Declarations

Ethical Approval The study was approved by the Leeds Health Authority Ethics Committee.

Informed Consent Included in Ethics Approval.

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