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Original

(2*R*,3*S*,2''*R*,3''*R*)-manniflavanone, a new gastrointestinal smooth muscle L-type calcium channel inhibitor, which underlies the spasmolytic properties of *Garcinia buchananii* stem bark extract

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Abstract

Garcinia buchananii Baker stem bark extract (GBB) is a traditional medication of diarrhea and dysentery in sub-Saharan Africa. It is believed that GBB causes gastrointestinal smooth muscle relaxation. The aim of this study was to determine whether GBB has spasmolytic actions and identify compounds underlying these actions. Calcium (Ca^{2+}) imaging was used to analyze the effect of GBB on Ca^{2+} flashes and Ca^{2+} waves in guinea pig gallbladder and distal colon smooth muscle. Intracellular microelectrode recording was used to determine the effect of GBB, six fractions of GBB, M1–5 and M7, and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone, a compound isolated from M3 on action potentials in gallbladder smooth muscle. The technique was also used to analyze the effect of GBB, M3, and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone on action potentials in the circular muscle of mouse and guinea pig distal colons, and the effect of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone on slow waves in porcine ileum. GBB inhibited Ca^{2+} flashes and Ca^{2+} waves. GBB, M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited action potentials. L-type Ca^{2+} channel activator Bay K 8644 increased the discharge of action potentials in mouse colon but did not trigger or increase action potentials in the presence of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited action potentials in the presence of Bay K 8644. GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone reduced the amplitude but did not alter the frequency of slow waves in the porcine ileum. In conclusion, GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone relax smooth muscle by inhibiting L-type Ca^{2+} channels, thus have potential for use as therapies of gastrointestinal smooth muscle spasms, and arrhythmias.

Key words: muscle relaxant, flavonoid, biflavanoid, intestine, calcium transient

Abbreviations used: GBB, *Garcinia buchananii* Baker stem bark extract; GBSM, Gallbladder smooth muscle; M1–M8, GBB fractions obtained by medium pressure liquid chromatography; MNF, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone; Bay K 8644, 1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluoromethyl)phenyl]-3-pyridinecarboxylic Acid Methyl Ester); VDCC, Voltage-dependent calcium channels.

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Introduction

Garcinia buchananii Baker stem bark extract (GBB) is a traditional medication for acute and chronic diarrhea in sub-Saharan Africa (1–4). It is believed that GBB has spasmolytic effects in gastrointestinal smooth muscle. This idea is supported by findings showing that extract from seeds of a plant species from the same genus, *Garcinia kola* Heckle has anti-diarrheal effects and it inhibits rat intestinal motility through spasmolytic effects (5). Additional support comes from findings showing that Kolaviron, which is a mixture of biflavanoids (GB1, GB2 and kolaflavanone) isolated from the extract of seeds of *Garcinia kola* Heckle causes smooth muscle relaxation by inhibiting Ca^{2+} influx , intracellular Ca^{2+} release, and activation of potassium channels (5–7).

GBB is a flavanoid-rich preparation that inhibits intestinal motility by inhibition of synaptic transmission in the myenteric ganglia (4) and 5-hydroxytryptamine receptor subtype 3 and subtype 4 (8). The major bioactive components of GBB, and its antimotility fractions, are flavonoids (8–10) especially 3,8″-linked biflavanones and flavanone-C-glycosides (9, 10). If GBB has spasmolytic effects, the bioactive compounds and mechanisms of action are not yet known.

Flavonoids are the primary antidiarrheal agents of various natural products. Their antidiarrheal properties involve anti-secretory (11) and anti-motility actions (5, 12–14). Flavonoid-induced antimotility effects involve causing relaxation by direct actions on smooth muscle cells. Typically, this is considered to be due to inhibition of Ca^{2+} mobilization and Ca^{2+} antagonistic activity in smooth muscle cells (5, 12–14).

In Ca^{2+} imaging, Ca^{2+} influx into smooth muscle cells via voltage-dependent Ca^{2+} channels, which manifests as fast propagating, global Ca^{2+} transients called Ca^{2+} flashes (15–17). Calcium flashes reflect Ca^{2+} entry into smooth muscle cells in association with action potentials or slow waves. Calcium flashes couple to intracellular sarcoplasmic reticulum-mitochondrial Ca^{2+} handling, which is visualized as the slow, intracellular propagating transients called Ca^{2+} waves (16–20). Ca^{2+} flashes, Ca^{2+} waves, and localized sarcoplasmic reticulum Ca^{2+} release via ryanodine channels called Ca^{2+} sparks regulate the excitability of gastrointestinal smooth muscle (15–17, 19–24). Given that flavanoids are abundant in GBB (8–10), we hypothesized that GBB has spasmolytic flavanoids and that these flavanoids inhibit Ca^{2+} flashes and Ca^{2+} waves, spontaneous action potentials in gallbladder and gastrointestinal smooth muscle cells. Furthermore, we hypothesized that these flavonoids inhibit action potentials and slow waves in intestinal smooth muscle cells. To test these hypotheses, we used Ca^{2+} imaging to identify whether GBB inhibits Ca^{2+} flashes and Ca^{2+} waves in gallbladder and colon smooth muscle cells. Intracellular microelectrode recording was used to conduct bioactivity-guided screening of GBB fractions collected by medium pressure liquid chromatography (9, 10, 25) to identify the fraction and then the compound, which inhibit action potentials and slow waves.

Materials and Methods

Animals

Three animal species including guinea pig, mouse and porcine were used in the study. Different animal species were used due to difficulties of obtaining specimens from a single species and to test the effect of GBB and spasmolytic compounds on both action potentials and slow wave type action potentials (slow waves). Porcine was chosen because it is considered the best animal model for human gastrointestinal physiology and motility (26).

Calcium imaging

Ca^{2+} imaging studies were performed at the University of Vermont School of Medicine using guinea pig samples. Animals were exsanguinated under deep halothane anesthesia, according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Vermont. Gallbladders and segments of distal colon samples were immediately collected into aerated (95% O_2-5% CO_2), ice-chilled Krebs solution (mM: 121 NaCl, 5.9 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$, 1.2 NaH_2PO_4 and 8 glucose; pH 7.38) following a midline laparotomy. These samples were dissected into flat muscularis wholemount preparations. In addition, full thickness gallbladder preparations were used to analyze the difference of the effect of GBB on tissues with and without intact mucosa (15, 20). Ca^{2+} imaging was performed after loading these preparations with 10 µM fluo-4 acetoxymethyl ester (fluo-4 AM) in Hepes buffer (composed of (mM): 134 NaCl, 6 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, 10 HEPES; pH adjusted to 7.4 with NaOH) containing 2.5 µg mL^{-1} pluronic acid at room temperature using previously described procedures (15).

Calcium data acquisition and analysis

Tissues were equilibrated to 36.5°C by continuous superfusion with constantly aerated (95% O_2-5% CO_2), re-circulating (at a rate of 3 ml/min) physiological saline solution (in mM) 119 NaCl, 7.5 KCl, 1.6 $CaCl_2$, 1.2 $MgCl_2$, 23.8 $NaHCO_3$, 1.2 NaH_2PO_4, 0.023 EDTA, and 11 glucose; pH 7.3) for 25 min. GBB was delivered onto tissues by superfusion via physiological saline solution after collecting baseline data for 25 min. GBB was freshly made by suspending 0.5 g stem bark powder into 100 mL physiological saline solution under constant stirring for 30 min at room temperature and then the mixture filtered to remove stem bark particles. Movies of Ca^{2+} flashes and Ca^{2+} waves (six hundred images each, acquired at a rate of thirty images per second) were captured every 5th min for 25 min as described previously (15, 20).

Imaging to capture Ca^{2+} flashes and Ca^{2+} waves was performed using an inverted Nikon TMD Microscope, Noran Oz laser confocal system (Noran Instruments, Middleton, WI). For each assay, movies of either Ca^{2+} flashes or Ca^{2+} waves or both were taken in selected fields of view using Intervision software (Noran Instruments, WI) on an Indy work station (Silicon Graphics, Mountain View, CA). Previous criteria were used to distinguish Ca^{2+} flashes and Ca^{2+} waves (15, 16, 20). Recorded videos were used to generate baseline Ca^{2+} flashes and Ca^{2+} waves data and to measure the effect of the GBB on the frequency (Hz), amplitude and duration of Ca^{2+} flashes and Ca^{2+} waves off-line using a custom software written by Dr. Adrian D. Bonev as previously described (15).

Action potentials and slow wave action potential (slow waves) analysis

Studies of spontaneous rhythmic action potentials and slow waves were performed at the University of Idaho. Action potentials were studied in guinea pig gallbladder smooth muscle and in the circular muscle of distal colons from C57BL/6 mice and guinea pig. The effect GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone on slow waves was studied in the circular muscle of porcine ileum.

Mice and guinea pigs were exsanguinated under deep isoflurane anesthesia, according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Idaho. Guinea pig gallbladders, and guinea pig and mouse colon samples were collect using aerated (95% O_2-5% CO_2), ice-chilled Krebs solution and then dissected into muscularis externa as described above. Porcine ileum samples were obtained from C& L Locker Co. butcher in the outskirts (5 min drive) of the city of Moscow, 10–15 min after animals were killed by captive bolt and exsanguination methods. Sample collection was performed according to the Institutional Animal Care and Use Committee of the University of Idaho regulations. Samples were transported

in ice-chilled Hepes buffer (composed of (mM): 134 NaCl, 6 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, 10 HEPES; pH adjusted to 7.4 with NaOH) to the laboratory. Pieces of about 2 × 4 cm were pinned stretched mucosal surface up in Sylgaard-lined Petri dishes. Mucosal and submucosal layers were teased off using sharp forceps under a stereo microscope. Hepes buffer was used to collect porcine intestinal samples to optimize preservation of samples because in some cases, it took 2–3 hours of waiting for porcine scheduled for slaughter to be brought from farms to the butcher. After dissections, *muscularis externa* wholemounts (~1 × 1.5 cm) were individually pinned stretched mucosal surface up in Sylgaard-lined 3.5 mL recording chambers, mounted on an inverted Nikon Ti-S microscope and visualized using ×20 objective. Tissues were equilibrated at 35.5–36.0°C by continuous perfusion with an oxygenated (95% O_2-5% CO_2) Krebs solution (~10 ml/min) for two and a half hours before taking measurements.

Intracellular microelectrode recordings to measure transmembrane potentials were performed using glass microelectrodes (tip resistance 90–120 MΩ), an MLB870B71 intracellular recording system amplifier (ADInstruments, Colorado Springs, CO, USA), and a PowerLab 8/30 with LabChart Pro (ADInstruments, Colorado Springs, CO, USA) software to save and analyze the electrical signals on a computer. In all intracellular microelectrode-recording experiments, the penetration of circular smooth muscle cells was done from mucosal surface. The measurements included the resting membrane potential, the frequency and amplitudes of action potentials and slow waves.

Statistical analysis

This was done using either unpaired Student t-test or ONEWAY ANOVA and GraphPad Prism 5 (La Jolla, CA, USA). Data show means + SE. n, represent the number of animals used for specific experiments. *P<0.05 indicates statistically significant differences.

GBB fractions and isolated pure compounds

Medium pressure liquid chromatography fractions (M1–M5 and M7) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were isolated using procedures published by Stark et al. (9, 10, 25). M1, M2 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were dissolved directly into Krebs (30 min constant stirring under protection from light). M4, M5, and M7 were solubilized in DMSO to prepare stock solutions prior to being dissolved into Krebs.

Reagents and commercial drugs

These include fluo-4 AM and pluronic acid (F-127; Molecular Probes); and EDTA, $MgCl_2$ $6H_2O_2$, sucrose, glucose, KCl, $NaHCO_3$, sodium phosphate monobasic, potassium phosphate monobasic, HEPES, DMSO, NaCl (Sigma-Aldrich). Others are $CaCl_2$ (Acros Organic) and Bay K 8664 (1,4-Dihydro-2,6-dimethyl-5-nitro-4-[2′-(trifluoromethyl)phenyl]-3-pyridinecarboxylic Acid Methyl Ester) (R&D Systems, Inc., Minneapolis, MN, USA).

Results

GBB inhibits Ca^{2+} flashes and Ca^{2+} waves in GBSM and colon smooth muscle

We investigated the effect of GBB on the discharge, frequency, and rhythmic pattern of Ca^{2+} flashes and Ca^{2+} waves in guinea pig GBSM and distal colon smooth muscle preparations. GBB (0.5 g/100 mL PSS) inhibited Ca^{2+} flashes and Ca^{2+} waves in gallbladder and colon smooth muscle preparations within 3–5 min (Fig. 1A–E). Typically, GBB inhibited Ca^{2+} flashes prior to the inhibition of Ca^{2+} waves (Fig. 1A–C). The actions of

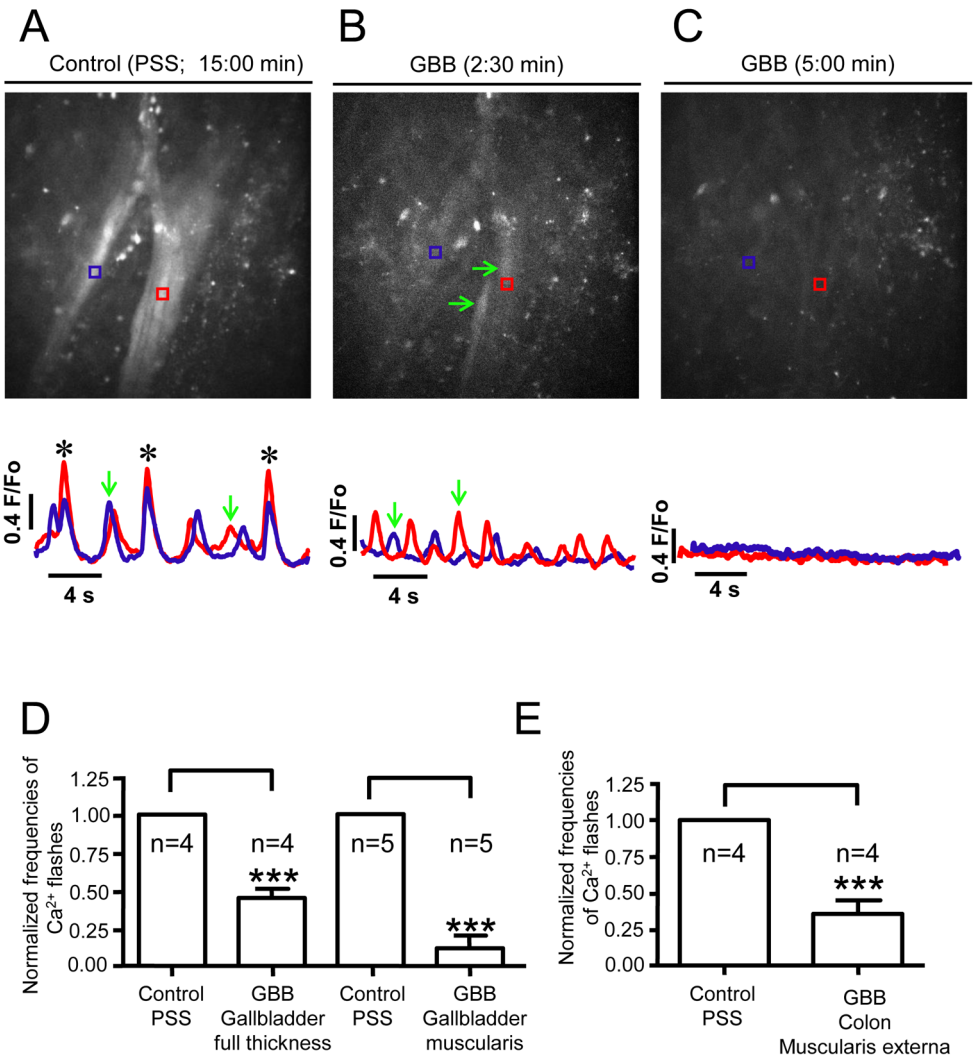

Fig. 1. GBB inhibits Ca^{2+} mobilization in guinea pig GBSM (A–D) and intestinal (E) smooth muscle cells. A–C. Pictures of guinea pig GBSM and traces of Ca^{2+} flashes (A; synchronized peaks, asterisks) and Ca^{2+} waves (A–B; asynchronous peaks) showing that the GBB (0.5 g/100 ml physiological saline solution) inhibited Ca^{2+} flashes before inhibiting Ca^{2+} waves in GBSM cells (B). Arrows in Fig. 1B show smooth muscle cells with Ca^{2+} waves at the time the captured. D. Summary data showing that mucosal layer reduced the dramatic effects of GBB to inhibit Ca^{2+} flashes in GBSM (D; ***$P < 0.001$), and GBB reduced Ca^{2+} flashes in colon smooth muscle cells (E; ***$P < 0.001$).

GBB were significantly greater in gallbladder preparations without mucosal layer (muscularis) compared with full thickness preparations (Fig. 1D). Collectively, the results suggested that GBB contains compounds that inhibit Ca^{2+} flashes and Ca^{2+} waves in GBSM and colon smooth muscle cells.

GBB inhibits action potentials and sub-threshold membrane depolarizations in GBSM

Spontaneous, rhythmic Ca^{2+} flashes correspond to action potentials. Ca^{2+} flashes and action potentials are essential for smooth muscle contractions that maintain gallbladder tone and cause emptying (15, 24). We analyzed the effect of GBB (0.5 g/100 ml Krebs) on action potentials in intact gallbladder muscularis preparations and observed that application of GBB inhibited the discharge of action potentials (Fig. 2A–C). GBB rapidly inhibited the discharge of action potential spikes and reduced the frequency of action potentials after 2–5 min

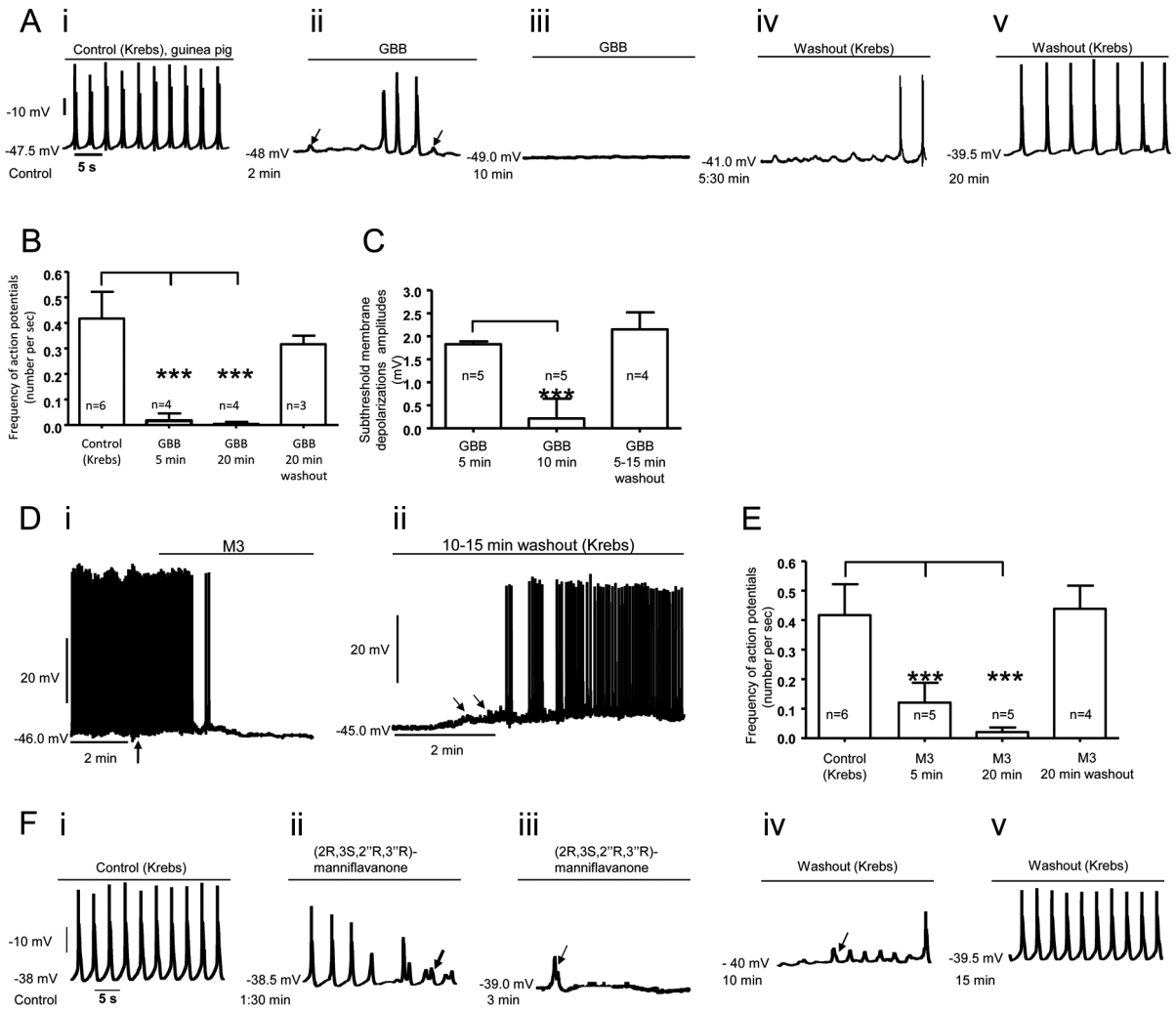

Fig. 2. GBB (0.5 g/100 ml Krebs), M3 (41.0 mg/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (41.0 mg/100 ml Krebs, which is 694.3 μM) inhibit action potentials in guinea pig gallbladder smooth muscle cells (GBSM). A. Traces obtained by intracellular recording of action potentials in guinea pig GBSM cells. Compared with control (Krebs), GBB inhibited the spikes elicited by the rapid upstroke membrane depolarizations during action potentials prior to blocking sub-threshold membrane depolarizations (arrows) (2Ai–iii). Krebs washout restored sub-threshold membrane depolarizations first (2A.iv), followed by action potentials (sub-threshold membrane depolarizations with superimposed spikes) (2A.v). B. Summary data showing that GBB significantly inhibited the discharge of action potentials after 5 min (***$P < 0.001$). This effect was maintained for 20 min. Washouts for 20 min restored the discharge of action potentials to normal frequency. C. Summary data of sub-threshold membrane depolarization amplitudes, showing that GBB inhibited these events (***$P < 0.001$), while Krebs washout restored sub-threshold membrane depolarizations to normal amplitudes after 5–15 min. D. M3 (41 mg/100 ml Krebs) inhibited action potentials and sub-threshold membrane depolarizations (not resolved in 2D.i), and washout restored these sub-threshold membrane depolarizations (arrows; 2D.ii) and action potentials in similar fashion as GBB. The arrow in Fig. Di depicts application of M3 during an experiment. E. Summary data showing that M3 significantly inhibited the discharge of action potentials after 5 min. This effect was maintained for 20 min (***$P < 0.001$). Washouts for 20 min restored the discharge of action potentials to normal frequency. F. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited spikes of action potentials prior to blocking sub-threshold membrane depolarizations (arrows). Washout restored sub-threshold membrane depolarizations and then action potentials to normal rhythmic pattern and amplitudes after 10–15 min.

(Fig. 2A, B). This effect gave rise to the observation of the spontaneous sub-threshold membrane depolarizations, which are normally overlaid by action potentials (Fig. 2A–C). GBB inhibited sub-threshold membrane depolarizations completely after 10–15 min. Interestingly, the exposure of gallbladder muscularis preparations to GBB for 25–30 min inhibited action potentials and spontaneous sub-threshold membrane depolarizations. However, subsequent washouts restored the discharge of sub-threshold membrane depolarizations and action potentials. The discharge of sub-threshold membrane depolarizations occurred after about 5 min. This was followed by the discharge of a mix of sub-threshold membrane depolarizations and action potentials. The discharge of action potentials without sub-threshold membrane depolarizations occurred after about 10–15 min washout. The discharge of action potentials was restored to the original rhythmic discharge pattern, frequency, and amplitude after 15–20 min (Fig. 2A–C).

The spasmolytic compound in GBB is contained in one individual fraction collected by medium pressure liquid chromatography

Bioactivity analysis of the effect of M1–M5 and M7 on GBSM action potentials showed that M3 (41.0 mg/100 ml Krebs) inhibited the discharge of action potentials and sub-threshold membrane depolarizations in a manner similar to that of GBB. In addition, like GBB, the inhibitory effects of M3 were dramatic and readily reversed by washout (Fig. 2D, E). Having discovered that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone is the principal compound of M3 (9, 10), we tested the effect of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (41.0 mg/100 ml Krebs) on GBSM action potentials. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited action potentials and sub-threshold membrane depolarizations in GBSM in a similar manner as M3 and GBB (Fig. 2F). To summarize, GBB and M3 inhibited the discharge of action potentials and sub-threshold membrane depolarizations in GBSM. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone was found to be the bioactive compound exerting these effects. M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were studied at a concentration of (41.0 mg/100 ml Krebs, which is 694.34 µM of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone) because for each 100 mg of M1–M8 combined, M3 constitutes 41.0 mg (9, 10, 25). Therefore, concentrations used here represent the natural fraction of M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone in the whole GBB.

GBB inhibits action potentials in mouse and guinea pig colon smooth muscle

In mouse and guinea pig colon, intestinal motility depends on rhythmic myogenic electrical activity that underlies smooth muscle contraction called action potentials (27, 28). In order to test whether GBB affects action potentials in intestinal smooth muscle cells GBB (0.5 g/100 ml Krebs) was applied on muscularis externa preparations from both guinea pig and mouse distal colon. We analyzed the effects of GBB on the discharge and frequency of action potentials in circular smooth muscle cells. GBB caused time-dependent inhibition of action potentials in both mouse and guinea pig distal colon. The initial effect involved inhibiting the discharge of the spikes and reducing the frequency of action potentials 3–5 min after application. This lead to the observation of sub-threshold membrane depolarization (20) that underlie action potentials (Fig. 3A–C). GBB inhibited spontaneous sub-threshold membrane depolarizations after another 3–5 min duration. Importantly, GBB did not completely block the ability of smooth muscle cells to discharge action potentials. Instead, it caused prolonged quiescent intervals between periodic discharges of action potentials. The longer the incubation of tissues with GBB, the greater was the interval between action potentials. Washout restored action potentials starting with the spontaneous sub-threshold membrane depolarizations. The original frequency and rhythmic pattern were restored after 15–25 min of washout. In summary, GBB inhibited action potentials in smooth muscle cells in the circular muscle of guinea pig and mouse colon and washout reversed this effect.

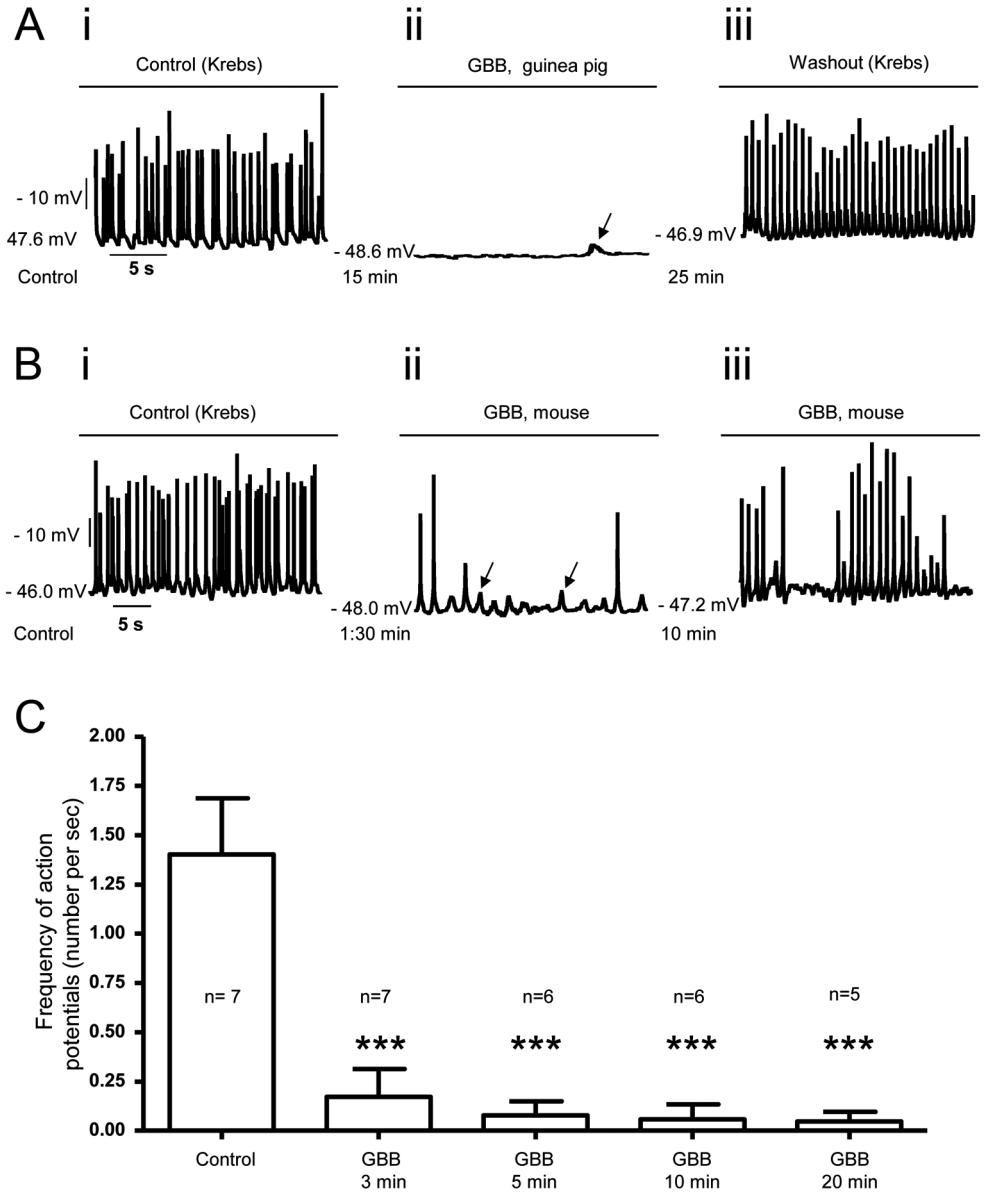

Fig. 3. A–C. Qualitative and quantitative data demonstrating the effects of GBB (0.5 g/100 ml Krebs) on the discharge and frequency of action potentials and sub-threshold membrane depolarizations in the circular muscle of guinea pig and mouse colons. A–B. Traces of action potentials recorded from smooth muscle cells of the inner circular layer of distal colon from guinea pig (A) and mouse (B). Compared with controls, GBB inhibited the spikes of the action potentials prior to inhibiting sub-threshold membrane depolarizations (A. ii, arrow). Washouts restored action potentials to the original rhythmic pattern (A. iii) after 10–25 min. GBB did not eliminate action potentials rather caused long quiescent intervals between discharges of action potentials (B. iii). C. Summary data showing that GBB significantly reduced the frequency of action potentials in the circular muscle of mouse distal colon after 3-min and this effect was maintained the entire 20–25 min exposure time (***$P < 0.001$).

(2R,3S,2''R,3''R)-manniflavanone is the bioactive compound in GBB, which inhibits action potentials in intestinal smooth muscle cells

The varying concentrations of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were applied to muscularis externa preparations from mouse colon for 30 min to measure its effect on action potentials in the circular muscle.

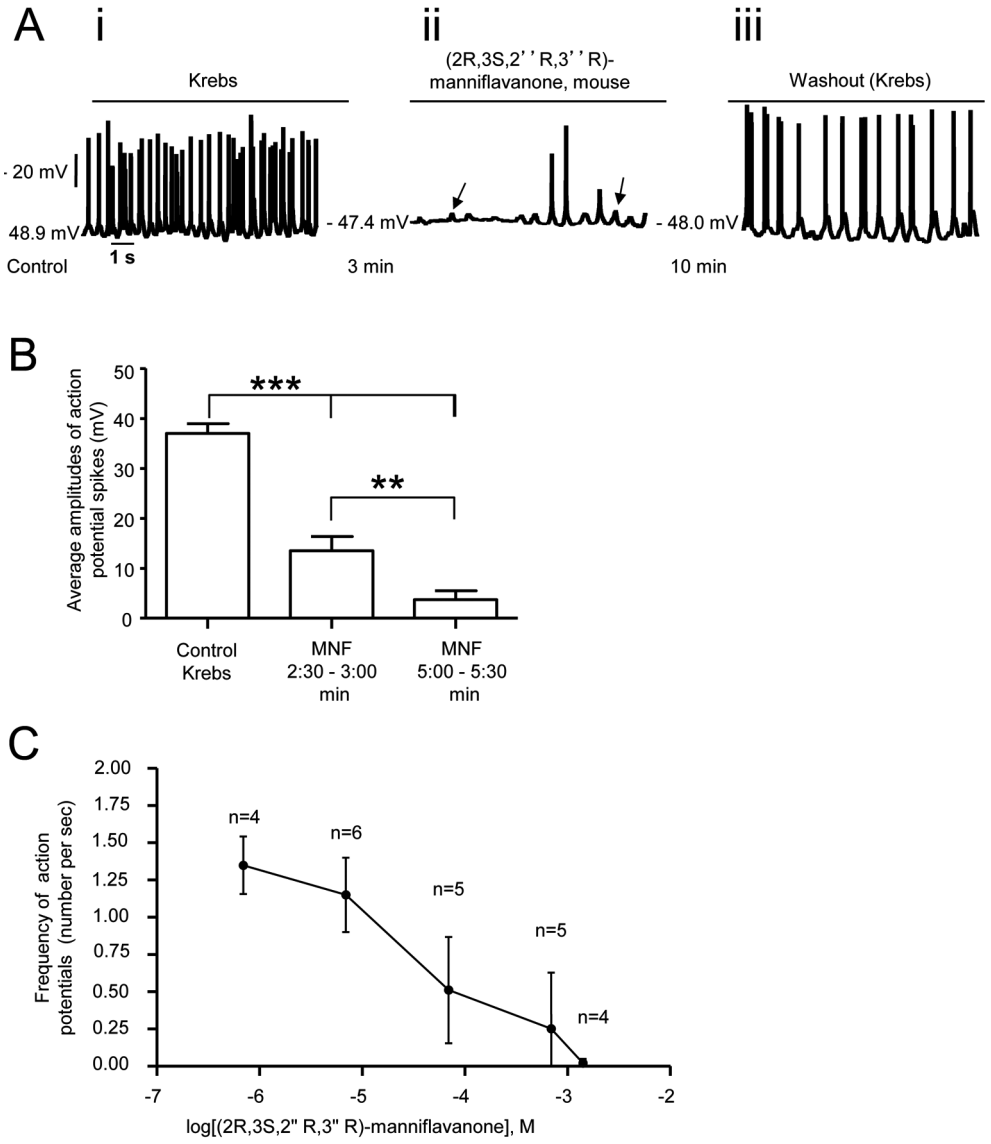

Fig. 4. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (694.3 μM) inhibits action potentials in intestinal smooth muscle cells of mouse. A. Traces of action potentials recorded from circular smooth muscle cells of mouse distal colon. Compared with control, GBB inhibited action potential spikes prior to inhibiting sub-threshold membrane depolarizations (arrows). Washouts restored action potentials to the original rhythmic pattern. B. Summary data showing that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone significantly reduced the average amplitudes of action potentials in the circular muscle of mouse distal colon after 2:30–5:30 min (***$P <$ 0.001). The difference between its effect at 3 min and 5 min was significant (*$P < 0.01$). C. The log plot showing that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibits action potentials in a concentration-dependent manner.

(2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (69.4 μM–1.4 mM) reduced the frequency of action potentials after 2–5 min. As with GBSM preparations, 41.0 mg/100 ml Krebs (694.34 μM) was the optimal concentration of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. At this concentration, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone initially caused the rapid inhibition of the spikes of action potentials before inhibiting sub-threshold membrane depolarizations, 10–15 min after application. 15–25 min of washout restored the discharge of action potentials to the original frequency and rhythmic pattern (Fig. 4A, B).

The effect of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone was concentration-dependent. At 1.4 mM, 694.3 μM

and 69.4 μM, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone significantly reduced the frequency of action potentials (Fig. 4C). 6.9 μM (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited the discharge of spikes without significantly reducing the frequency of action potentials. However, prolonged applications (15–30 min) were associated with inhibition of the discharge of action potentials. At the lowest concentration tested (0.69 μM), (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone did not affect action potentials (Fig. 4C). Taken together, these observations suggest that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone, the most abundant constituent of GBB (9, 10) has spasmolytic actions in intestinal smooth muscle cells.

GBB and (2R,3S,2''R,3''R)-manniflavanone inhibit L-type Ca^{2+} channels in intestinal smooth muscle cells

To determine whether GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibit the influx of Ca^{2+} into intestinal smooth muscle cells via L-type voltage-dependent calcium channels (VDCC), muscularis externa preparations from mouse distal colon were pre-treated with GBB (0.5 g/100 ml Krebs) for 5 min or 694.3 μM (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone for 15 min to inhibit action potentials. This was followed by the application of L-type Ca^{2+} channel activator, Bay K 8644 (1.0 μM) in the presence of GBB or (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (Fig. 5A, B). In control experiments, Bay K 8644 alone increased the frequency of action potentials after 2–3 min. However, Bay K 8644 did not trigger or affect the frequency action potentials in the presence of GBB or (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. Conversely, GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited action potentials in preparations pre-treated with Bay K 8644 for 3–5 min (Fig. 5C, D). Collectively, the findings of these experiments suggest that GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited L-type Ca^{2+} channels in intestinal smooth muscle cells. BAY K-8644 does not have competitive or cooperative effects with GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone.

GBB and (2R,3S,2''R,3''R)-manniflavanone inhibit spikes of slow waves in the circular muscle of porcine ileum

In the small intestine of mice, guinea pig and large animals, myogenic electrical activities are called slow waves. Each slow wave consists of a plateau phase and superimposed spike(s) (19, 22, 23, 29–31). The discharge of slow waves is regulated by spontaneous, rhythmic electrotonic depolarizations and repolarizations (pacemaker potentials) generated by the pacemaker interstitial cells of Cajal (22, 29–33). The effect GBB (0.5 g GBB/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (694.34 μM ml Krebs) on slow waves was tested in the circular muscle of porcine ileum. GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited spikes and reduced the amplitudes of slow waves without affecting the discharge and frequency of slow waves (Fig. 6A, B). Interestingly, GBB (0.5 g GBB/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (694.3 μM) did not affect slow waves in tissues treated with 2 μM nifedipine and 1 μM atropine to block smooth muscle contractions (Fig. 7A–C). These results suggest that GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited calcium influx into circular smooth muscle cells of porcine ileum but did not affect the discharge of slow waves.

Finally, GBB, M3, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone and Bay K 8644 did not affect the resting membrane potential (Fig. 8).

Discussion

The goal of this study was to identify whether GBB has spasmolytic effects in gallbladder and intestinal smooth muscle cells, and identify the bioactive compounds. We report for the first time that GBB inhibits

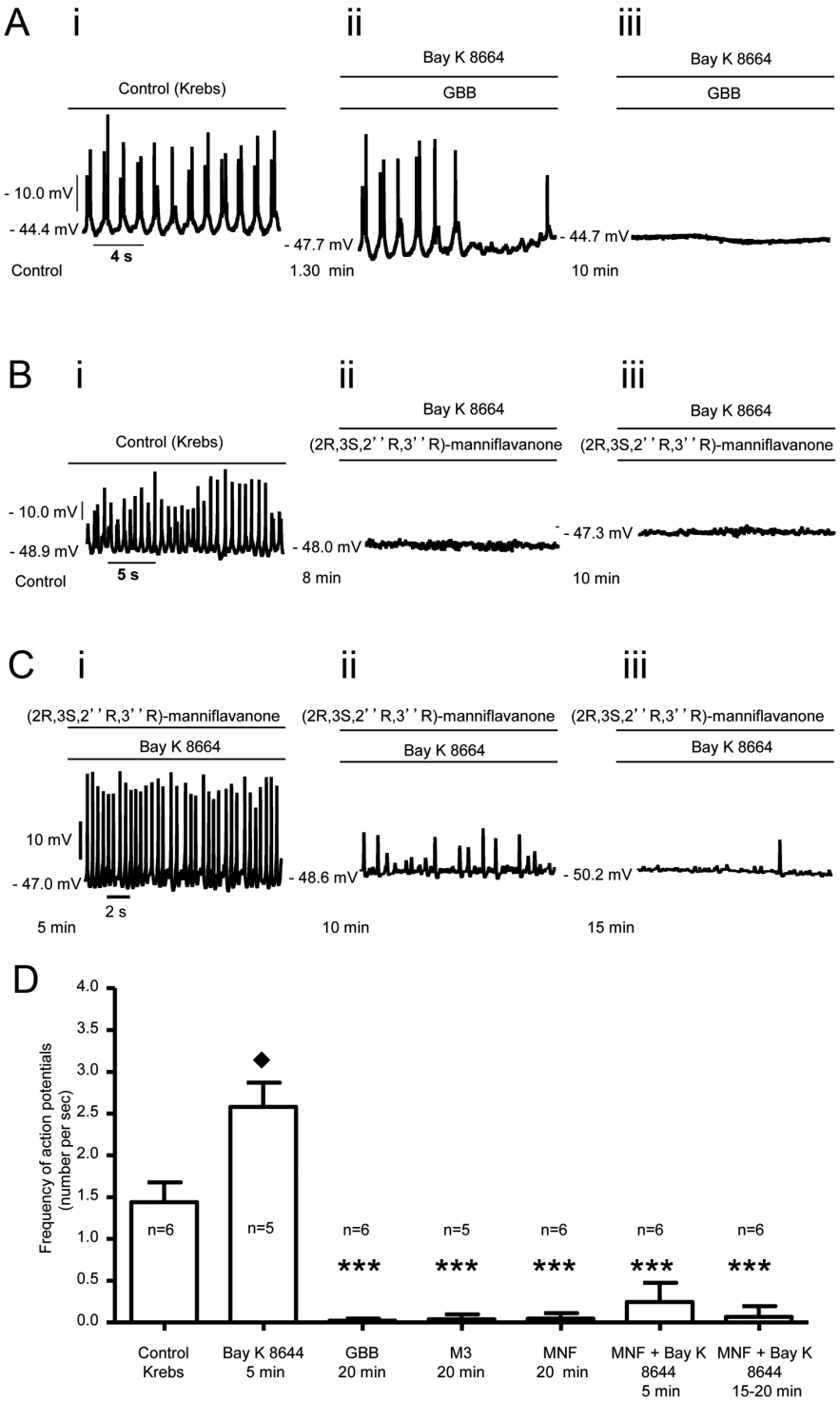

Fig. 5. GBB (0.5 g/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (694.3 μM) inhibit rhythmic electrical activity in gastrointestinal smooth muscle by blocking L-type VDCC. A. Traces showing that in the presence of GBB, Bay K 8644 (1.0 μM), the L-type VDCC activator did not increase the discharge of action potentials in the inner circular muscle of mouse distal colon. B. Likewise, Bay K 8644, did not trigger the discharge of action potentials in the presence of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone in similar preparations. C. Bay K 8644 did not block (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone from inhibiting action potentials. D. Quantitative data demonstrating that Bay K 8644 significantly increased the frequency of action potentials in the circular muscle of mouse distal colon (rectangle; ***$P < 0.001$). GBB, M3 (41.0 mg/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (MNF) significantly inhibited the discharge of action potentials with the same magnitude after 20 min (***$P < 0.001$). The actions of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were not altered by Bay K 8644 (MNF + Bay K 8664; ***$P < 0.001$).

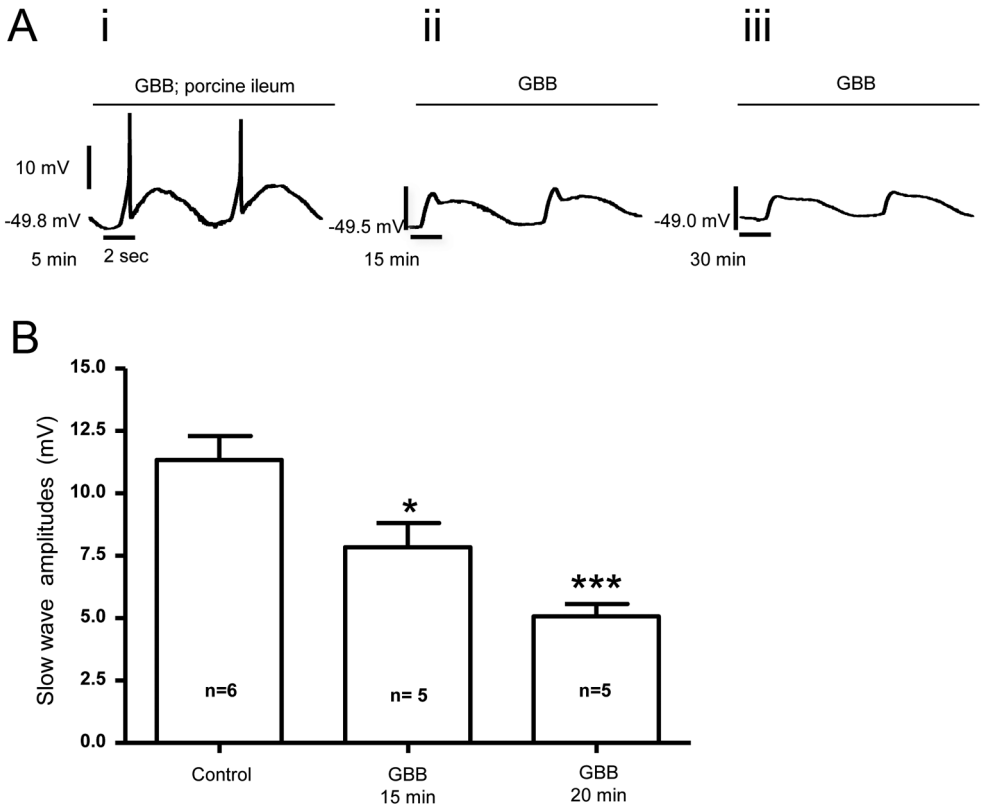

Fig. 6. GBB (0.5 g/100 ml Krebs) did not inhibit slow waves in the porcine ileum. A. Traces showing that GBB blocked the discharge of action potentials (spikes) normally superimposed on slow waves after 10–15 min. It reduced the amplitudes but not the frequency of slow waves. B. Quantitative data showing reduction of slow wave amplitudes following the application of GBB for 15–20 min (**P* < 0.05; ****P* < 0.001).

the discharge of rhythmic Ca^{2+} flashes and the corresponding rhythmic membrane depolarizations: action potentials in GBSM and in the inner circular muscle of mouse and guinea pig distal colons. Furthermore, it inhibited spikes and reduced the amplitudes but did not affect the discharge of slow waves in porcine ileum. Bioactivity analysis of GBB fractions identified a single fraction (M3) and a single bioactive compound isolated from M3, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone as the bioactive ingredient underlying these actions of GBB. The spasmolytic effects of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone were due to the inhibition of Ca^{2+} influx via L-type VDCC. GBB inhibited intracellular Ca^{2+} mobilization in the form of Ca^{2+} waves, and spontaneous sub-threshold membrane depolarizations. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited sub-threshold membrane depolarizations.

In this study, GBB inhibited Ca^{2+} flashes and Ca^{2+} waves. GBB, M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited spontaneous sub-threshold membrane depolarizations and action potentials while GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited spikes that are normally superimposed on slow waves. Ca^{2+} flashes indicate Ca^{2+} influx into smooth muscle cells via VDCC during an action potential and a slow wave (15, 17, 20, 23, 24, 34). Spontaneous sub-threshold membrane depolarizations are the rhythmic electrical activity that underlie the discharge of action potentials in gallbladder smooth muscle cells (20, 24) as well as action potentials and slow waves in gastrointestinal smooth muscle cells (16–18, 23, 28). These events are also called unitary potentials or spontaneous transient depolarizations (23, 31). Sub-threshold membrane depolarizations are thought to correspond to the summation of asynchronous intracellular Ca^{2+} waves (17, 20). Therefore, taken

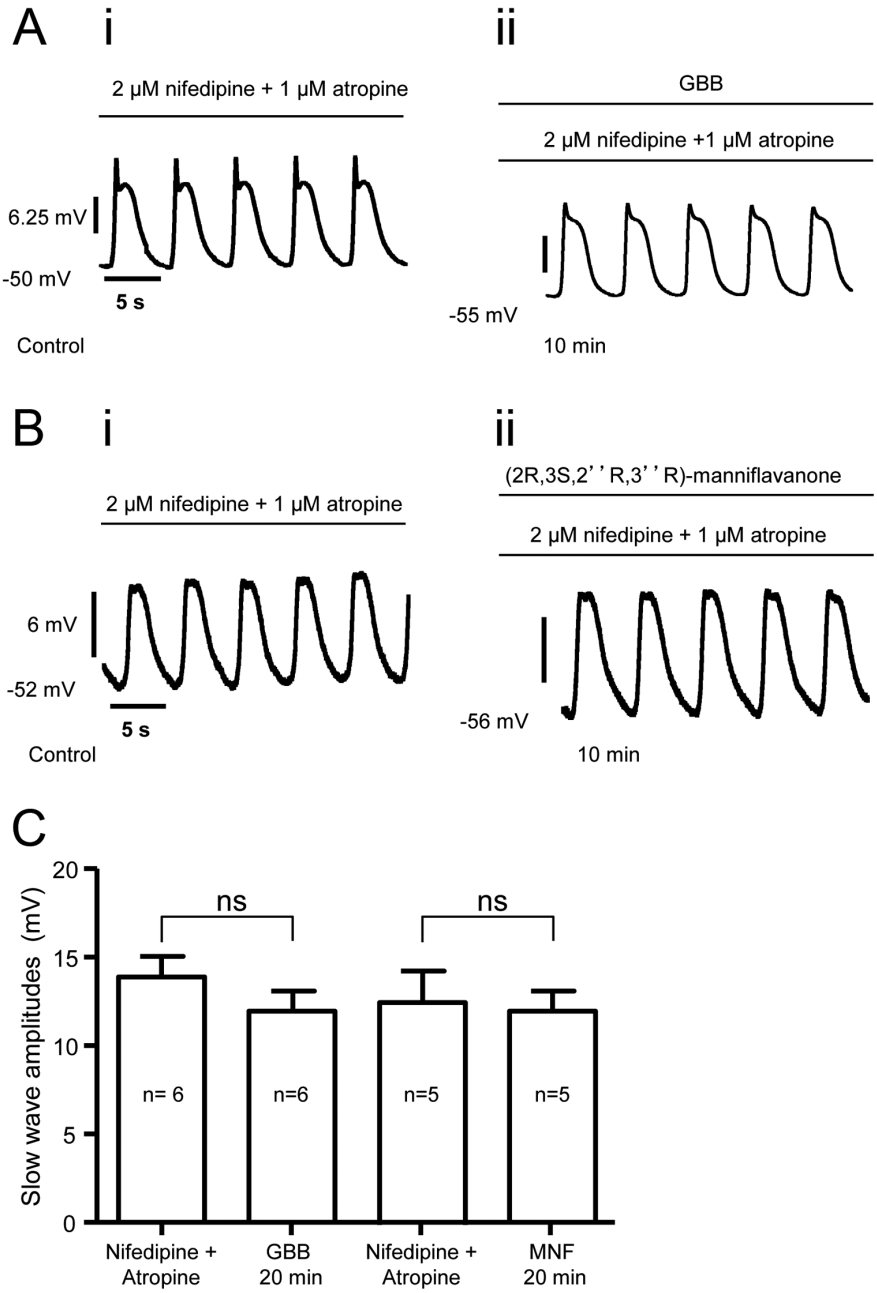

Fig. 7. GBB (0.5 g/100 ml Krebs) and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (41.0 mg/100 ml Krebs), did not inhibit slow waves in the presence of 2.0 μM nifedipine and 1 μM atropine. Traces of slow wave recorded from the inner circular muscle layer of porcine ileum. GBB (A) and (2*R*,3*S*,2''*R*,3''*R*)- manniflavanone (B) did not affect slow waves in the presence of nifedipine and atropine after 10 min. Notice inhibition of spikes by GBB in A. ii. This effect was seen as early as 3 min after the application of GBB. C. Summary data showing that and (2*R*,3*S*,2''*R*,3''*R*)- manniflavanone did not affect slow wave amplitudes after 20 min in tissues treated with nifedipine (2.0 μM) and atropine (1.0 μM) ($P > 0.05$). In these experiments, nifedipine (2.0 μM) and atropine (1.0 μM) were used to block muscle contractions in order to measure inhibitory junction potentials. The results will be reported in the future publications.

together, our results suggest that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone is likely the bioactive compound in GBB that inhibits Ca^{2+} transients and the corresponding spontaneous sub-threshold membrane depolarizations, action potentials, and spikes of slow waves. This is likely to cause a relaxation of GBSM and intestinal smooth muscle.

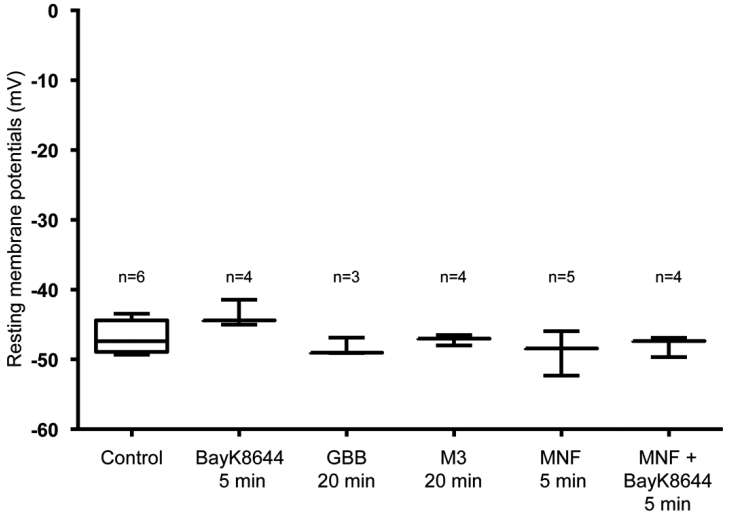

Fig. 8. Summary data showing that GBB (0.5 g/100 ml Krebs), M3 (41 mg/100 ml Krebs), (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone (41 mg/100 ml Krebs) and Bay K 8664 (1 μM) did not affect the resting membrane potential of smooth muscle cells in circular muscle of muscularis externa of mouse distal colon ($P > 0.05$).

The phytochemical composition of GBB is complex due to numerous compounds in it. The predominant compounds are biflavanones and these compounds are structurally related (9, 10). A group of structurally related polymethoxylated flavonoids all contribute to the spasmolytic effect of *Casimiroa tetrameria* leaf extract (13). Our findings strongly suggest that the spasmolytic component of GBB is a single compound, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. The main structurally related biflavanones isolated from GBB are the 3,8″-linked biflavanones (9, 10), which are (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone in M3, (2*R*,3*S*,2''*R*,3''*R*)-GB-2 (in M4), and (2*R*,3*S*,2''*S*)-buchananiflavanone (in M5) (9, 10). In this study, M4 and M5 did not affect action potentials in GBSM cells suggesting that (2*R*,3*S*,2''*R*,3''*R*)-GB-2, and (2*R*,3*S*,2''*S*)-buchananiflavanone do not contribute to the spasmolytic actions of GBB. The differences between these compounds can be explained by the 3D structure and stereochemistry, which are key determinants for the bioactivity of an individual molecule (35).

Gallbladder and gastrointestinal motility is modified by neurohormonal modulation of spontaneous rhythmic action potentials and slow waves (15, 19, 21, 33, 34, 36). We previously reported that GBB inhibits colon motility by inhibiting synaptic transmission (4). The current study revealed that GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone cause myorelaxation, thus highlights an additional mechanism, which is likely utilized by GBB to reduce biliary and gastrointestinal motility. Our findings correspond with antispasmodic and spasmolytic effects caused by kolaviron, a mixture of flavanoid compounds including GB-1, GB-2 and kolaflavanone from seeds of *Garcinia kola* in intestinal smooth muscle of rat (5).

The rhythmic electrical activity underlying gallbladder and gastrointestinal motility depends on Ca^{2+} entry into smooth muscle cells via L-type VDCCs, and Ca^{2+} release from the sarcoplasmic reticulum via inositol 1,4,5-tris-phosphate [Ins (1,4,5)P3] receptors and subsequent mitochondria Ca^{2+} handling, which causes Ca^{2+} waves (16, 19, 23, 24, 37). Rhythmic electrical activity also depends on localized sarcoplasmic reticulum Ca^{2+} release via ryanodine channels (Ca^{2+} sparks) and sarcoplasmic reticulum Ca^{2+} uptake via SERCA pumps (19, 21–24). The inhibition of Ca^{2+} flashes, Ca^{2+} waves, action potentials and spikes superimposed on the plateau of slow waves suggest that multiple mechanisms are utilized by GBB, and likely M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone to exert spasmolytic actions.

It is possible that GBB inhibits Ca^{2+} waves and sub-threshold membrane depolarization at least in part by inhibiting Ca^{2+} influx. This idea is supported by the finding that GBB inhibited Ca^{2+} flashes and action potentials prior to the inhibiting Ca^{2+} waves and sub-threshold membrane depolarization. An additional support is the fact that Ca^{2+} waves and sub-threshold membrane depolarization depend on intracellular calcium handling and Ca^{2+} influx is essential for refilling intracellular Ca^{2+} stores (15, 17, 21, 23, 28). It is also possible that GBB, M3, and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibit sub-threshold membrane depolarization by blocking sarcoplasmic reticulum-mitochondria Ca^{2+} handling (15, 20–24, 37). In support of the ideas highlighted above, GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone have outstanding antioxidative actions (9, 10) and mitochondrial-targeting antioxidants inhibit intracellular Ca^{2+} oscillations because mitochondrial production of oxidants is necessary for physiological Ca^{2+} oscillations (38). In addition, flavones 3,3'-di-O-methylquercetin (39) and hispidulin (40) relax gastrointestinal smooth muscle by inhibiting Ca^{2+} influx, Ca^{2+} release from intracellular stores, and Ca^{2+} binding to intracellular Ca^{2+}-receptor proteins. Kolaviron causes vasorelaxation using similar mechanisms (7). Therefore, mechanistic analyses of the effects of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone on L-type VDCC and intracellular Ca^{2+} signaling are needed to ascertain if GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibit intracellular Ca^{2+} mobilization to cause relaxation of smooth muscle cells.

GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibit L-type VDCC in gallbladder and intestinal smooth muscle cells. L-type VDCC agonist, Bay K 8644 failed to elicit the discharge of action potentials in colon smooth muscle in the presence of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. This finding indicates that GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibit L-type VDCC in intestinal smooth muscle cells. These results support observations showing that flavonoids such as quercetin and genistein inhibit L-type DVCC and block Bay K 8644 from causing contractions in vascular smooth muscle (7, 41). Bay K 8644 reverses the inhibitory action of dihydropyridine L-type VDCC antagonists (42) but it did not reverse the effects of GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. Furthermore, (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone inhibited action potentials in the presence of Bay K 8644. Overall, our results suggest GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone are L-type VDCC antagonists. BAY K-8644 does not have competitive or cooperative effects with GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone. It is likely that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone alters the conformation structure of L- type VDCC and through this action, it blocks Ca^{2+} entry while inhibiting Bay K 8644 from binding its receptor site quite effectively. A similar mechanism was proposed for a flavonoid monomer quercetin (41). However, these claims need to be confirmed because (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone could utilize other mechanisms such as inhibiting protein tyrosine kinase (43) to block Ca^{2+} influx into smooth muscle cells.

GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone did not affect the discharge of slow waves in the porcine ileum. This suggests that these preparations do not affect conductances and intracellular pathways responsible for the discharge of pacemaker potentials in interstitial cells of Cajal and slow waves in smooth muscle cells (22, 30–33, 37). These findings support the notion that in gastrointestinal smooth muscle, slow waves are insensitive to L-type Ca^{2+} channel inhibitors (23, 29, 31–33). Further studies are needed to confirm whether GBB and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone do not affect the discharge of slow waves in interstitial cells of Cajal.

Reversing the effect of GBB, M3 and (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone to the original rhythmic pattern by washout suggest that (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone interacts with L-type calcium channels with low binding affinity and its toxicity can be reversed.

In conclusion, GBB has spasmolytic actions in gallbladder and gastrointestinal smooth muscle. (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone is constituent of GBB that underlies these actions. It acts by inhibiting L-type calcium channels. Additional studies are needed to identify how (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone affect intracellular Ca^{2+} signaling and other cellular mechanisms, which cause relaxation of smooth muscle. The

results of this study suggest the need to test the potential of (2*R*,3*S*,2''*R*,3''*R*)-manniflavanone for treating fecal incontinence (44), gastrointestinal spasms (12) and arrhythmias (45) especially in conditions requiring both VDCC blockers and antioxidative supplements.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions

Participated in research design: Balemba OB, Mawe GM, and Stark TD.

Conducted experiments: Balemba OB, Patterson S, and McMillan JS.

Isolation of fractions and (2*R*,3*S*,2''*R*,3''*R*)- manniflavanone: Lösch S, and Stark TD.

Wrote or contributed to the writing of the manuscript: Balemba OB, Stark TD, Hofmann T, and Mawe GM.

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