สกว
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รายงานวิจัยฉบับสมบูรณ์

โครงการ ฤทธิ์และกลไกการออกฤทธิ์ของสารบาราคอลในการแก้ไขและป้องกันความผิดปกติของทางเดินอาหาร

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สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ สำนักงานคณะกรรมการอุดมศึกษา (สกอ.) และ สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ที่ให้ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่ ประจำปี 2546 เพื่อสนับสนุนการทำวิจัย และเปิดโอกาสให้ผู้วิจัยได้ใช้ความรู้ความสามารถของตนเองในการทำวิจัยโดยเฉพาะอย่างยิ่งในสาขาวิชาที่ตนเองถนัดและมีความเชี่ยวชาญ เพื่อเป็นการเสริมสร้างประสบการณ์การทำวิจัย การพัฒนาทางด้านความคิดและวิชาการ และใช้ความรู้ทางวิชาการที่ได้เรียนมาให้เกิดประโยชน์มากที่สุด ผู้วิจัยขอขอบคุณเป็นอย่างยิ่งสำหรับ ศาสตราจารย์ นทีทิพย์ กฤษณามระ นักวิจัยพี่เลี้ยงในการให้คำปรึกษาและคำแนะนำ ตลอดจนความช่วยเหลือในทุก ๆ ด้านเป็นอย่างดียิ่งจนกระทั่งผลงานได้รับการตีพิมพ์ในวารสารระดับนานาชาติได้ อีกทั้งยังเกิดความร่วมมือในการทำวิจัยต่อไป และท้ายที่สุดขอขอบคุณภาควิชาสรีรวิทยา และคณะแพทยศาสตร์ ตลอดจนมหาวิทยาลัยศรีนครินทรวิโรฒ ที่ให้การสนับสนุนการทำวิจัย สนับสนุนด้านวัสดุครุภัณฑ์และเครื่องมือเครื่องใช้ในการทดลองที่จำเป็น ทั้งหมดนี้นับว่ามีส่วนช่วยให้การวิจัยครั้งนี้สำเร็จลุล่วงไปด้วยดี

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ABSTRACT

Project Code : MRG4680196

Project Title : An investigation on effects and mechanism of action of barakol on cure and prevention of gastrointestinal disorders

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Barakol is a purified extract of *Cassia siamea*, a plant that has been used as a laxative in traditional medicine. In this study the effects of barakol on anion transport and immunoglobulin A (IgA) secretion across the rat colon epithelium were investigated. Colonic epithelium was mounted in Ussing chambers and bathed with Ringer solution. Addition of 1 mM barakol to the basolateral solution produced a slow increase in Isc in proximal colon and distal colon by 24.5 ± 2.2 $\mu A/cm^2$ and 24.2 ± 1.4 $\mu A/cm^2$, respectively. Barakol increased Isc in a concentration dependent manner with an EC_{50} value of 0.4 mM. The barakol-stimulated increase in Isc was inhibited by subsequent treatment with 500 µM diphenylamine-2-carboxylic acid or 400 µM glibenclamide added to the apical solution, and 200 µM bumetanide added to the basolateral solution. Pretreatment of the tissues with 200 µM bumetanide, but not 10 µM amiloride, completely abolished the barakol-increased Isc. Ion substitution experiments showed an inhibition of barakol-stimulated Isc in choride-free solution, but not in bicarbonate-free solution. In addition, pretreatment of tissues with 10 µM tetrodotoxin or 10 µM indomethacin, but not 1 µM atropine or 10 µM hexamethonium, partially inhibited the Isc response by barakol. In the presence of both tetrodotoxin and indomethacin, the barakol-stimulated Isc response was nearly abolished. These results demonstrated the stimulatory effect of barakol on the bumetanide-sensitive chloride secretion in rat colon. The effect of barakol was partially mediated by stimulation of submucosal nerves and through the release of cyclooxygenase metabolites. In addition, barakol markedly stimulated the secretion of IgA both *in vivo* and *in vitro* studies in rat colon. All of these findings provide the effect and the mechanism of action of barakol as a secretagogue and its role on enhancement of non-specific mucosal immunity in mammalian colon.

Keywords : Barakol, *Cassia siamea*, Cl secretion, IgA, laxative, rat colon Ussing chamber

บทคัดย่อ

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ชื่อโครงการ : ฤทธิ์และกลไกการออกฤทธิ์ของสารบาราคอลในการแก้ไขและป้องกันความผิดปกติของทางเดินอาหาร

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บาราคอลเป็นสารสกัดบริสุทธิ์จากใบและดอกขี้เหล็กซึ่งเชื่อว่ามีสรรพคุณเป็นยาระบาย การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์และกลไกการออกฤทธิ์ของสารสกัดบาราคอลต่อการเปลี่ยนแปลงการขับหลั่งสารน้ำและอีเล็กโทรไลต์ในเยื่อบุทางเดินลำไส้ใหญ่ ซึ่งเป็นกลไกที่สำคัญในแง่ของการเป็นยาระบาย รวมถึงศึกษาผลของบาราคอลต่อการเปลี่ยนแปลงการขับหลั่งของ immunoglobulin A (IgA) ในลำไส้ใหญ่ของหนูขาว ทำการทดลองโดยนำเยื่อบุลำไส้ใหญ่มาติดตั้งเข้ากับ Ussing chambers ที่มีสารละลาย Ringer solution อยู่ทั้ง 2 ด้าน และเชื่อมต่อกับเครื่อง voltage clamp เพื่อทำการวัด short circuit current (Isc) และ transepithelial potential difference (PD) การทดลองให้บาราคอล (1mM) ทางด้าน basolateral solution พบว่ามีผลเพิ่ม Isc ที่ลำไส้ใหญ่ส่วนต้นและส่วนปลาย 24.5 ± 2.2 $\mu A/cm^2$ และ 24.2 ± 1.4 $\mu A/cm^2$ ตามลำดับ บาราคอลเพิ่ม Isc ตามความเข้มข้นที่เพิ่มขึ้นโดยมีค่า EC_{50} 0.4 mM การเพิ่ม Isc ของบาราคอลถูกยับยั้งโดยการใส่ diphenylamine-2-carboxylic acid (500 µM) หรือ glibenclamide (400 µM) ทางด้าน apical solution และ bumetanide (200 µM) ทางด้าน basolateral solution การให้ bumetanide (200 µM) ก่อนการให้บาราคอลสามารถยับยั้งผลของบาราคอลในการเพิ่ม Isc ได้อย่างสมบูรณ์ ในขณะที่ amiloride (10 µM) ก่อนไม่มีผลในการยับยั้งผลของบาราคอล ผลของบาราคอลยังถูกยับยั้งในสารละลายที่ปราศจากคลอไรด์แต่ไม่ถูกยับยั้งในสารละลายที่ปราศจากไบคาร์บอเนต นอกจากนี้การให้ tetrodotoxin (10 µM) หรือ indomethacin (10 µM) ก่อนสามารถยับยั้งผลของบาราคอลได้เป็นบางส่วน ในขณะที่การให้ atropine (1 µM) หรือ hexamethonium (10 µM) ก่อนไม่มีผลในการยับยั้งผลของบาราคอล จากการทดลองแสดงให้เห็นฤทธิ์ของบาราคอลต่อการกระตุ้นการหลั่งคลอไรด์ในลำไส้ใหญ่หนูโดยผ่านทาง bumetanide-sensitive pathway โดยมีกลไกการควบคุมผ่านระบบประสาททางเดินอาหารในชั้น submucosal และการหลั่ง cyclooxygenase metabolites นอกจากนี้บาราคอลยังสามารถกระตุ้นการหลั่ง IgA จากลำไส้ใหญ่ทั้งในหลอดทดลองและตัวสัตว์ทดลอง จากการทดลองทั้งหมดแสดงให้เห็นถึงประโยชน์และสรรพคุณของบาราคอลในการเป็นยาระบายอย่างอ่อนที่มีกลไกการออกฤทธิ์ที่แน่นอน และยังมีบทบาทสำคัญในการกระตุ้นระบบภูมิคุ้มกันของเยื่อเมือกในลำไส้ใหญ่ของสัตว์เลี้ยงลูกด้วยนมอีกด้วย

คำหลัก *Cassia siamea*, Immunoglobulin A, Ussing technique, การหลั่งคลอไรด์, บาราคอล, ยาระบาย, ลำไส้ใหญ่ของหนู

EXECUTIVE SUMMARY

ทุนพัฒนาศักยภาพในการทำงานวิจัยของอาจารย์รุ่นใหม่

1. ความสำคัญและที่มาของปัญหา

ในปัจจุบันความผิดปกติของร่างกายอันมีสาเหตุเบื้องต้นมาจากความผิดปกติของการทำงานของระบบทางเดินอาหาร เช่น ท้องเสียหรือท้องผูกเรื้อรัง การติดเชื้อฉวยโอกาสในคนปกติหรือผู้ป่วยที่มีภูมิคุ้มกันบกพร่อง (Human Immunodeficiency Syndrome) เป็นปัญหาที่สำคัญของประเทศซึ่งทำให้เกิดการสูญเสียทางเศรษฐกิจสำหรับการรักษา และสำหรับการสั่งซื้อยาแผนปัจจุบันที่ผลิตจากต่างประเทศเข้ามาเป็นจำนวนมาก ความผิดปกติดังกล่าวนั้นมีสาเหตุเกิดมาจากการทำงานที่มากหรือน้อยเกินไปของระบบทางเดินอาหารซึ่งประกอบด้วย การหดตัวของกล้ามเนื้อเรียบ การขับเคลื่อนตัวของลำไส้ การขับหลั่งสารน้ำและอีเล็กโทรไลด์ การดูดซึมสารอาหาร และระบบภูมิคุ้มกันของทางเดินอาหาร เป็นต้น โดยการทำงานดังกล่าวนี้อยู่ภายใต้การควบคุมของระบบประสาทอัตโนมัติเป็นส่วนใหญ่ และมักจะได้รับผลกระทบมาจากสิ่งแปลกปลอม เช่น จุลินทรีย์ที่ปนเปื้อนมากับอาหาร เป็นต้น

ในปัจจุบันได้มีการนำสารหลายชนิดในกลุ่ม inorganic acid หรือสารสมุนไพรที่มีฤทธิ์ทางชีวภาพ มาช่วยแก้ไข บรรเทา ป้องกันความผิดปกติและลดอาการไม่พึงประสงค์ที่มีสาเหตุมาจากการทำงานที่ผิดปกติของระบบทางเดินอาหารได้ จากรายงานการศึกษาที่ผ่านมาพบว่ามีสมุนไพรต่าง ๆ จำนวนมากโดยเฉพาะสมุนไพรจากต่างประเทศในตระกูล senna, sennosides A และ B, cascara sargrada และ danthron ได้ถูกสกัด (crude extract) มาใช้เป็นยาระบาย บรรเทา และแก้ไขอาการท้องผูก สมุนไพรเหล่านี้มีส่วนประกอบที่สำคัญคือ สารจำพวก anthraquinones ซึ่งออกฤทธิ์ส่วนใหญ่ที่ลำไส้ใหญ่โดยเพิ่มการขับเคลื่อนตัวของลำไส้ใหญ่ (peristalsis) และการขนส่งสารน้ำและอิเล็กโตรไลท์ ดังนั้นจึงมีการใช้สารสมุนไพรนี้กันอย่างแพร่หลายในประเทศไทยในรูปของสมุนไพรนำเข้าจากต่างประเทศซึ่งมีราคาแพง หากเราสามารถนำพืชสมุนไพรที่หาได้ง่ายและปลูกได้ทั่วไปในประเทศไทยมาทำการศึกษาอย่างจริงจัง และทำการสกัดสารบริสุทธิ์ (pure compound) จากพืชสมุนไพรเหล่านี้มาทำการศึกษาการออกฤทธิ์ทางชีวภาพ ความเป็นพิษต่ออวัยวะต่าง ๆ ของร่างกาย ตลอดจนกลไกการออกฤทธิ์แล้ว ก็สามารถที่จะพัฒนาต่อไปเป็นยาที่ใช้เสริมหรือทดแทนยาแผนปัจจุบัน เพื่อใช้กันในประเทศไทยตลอดจนส่งออกขายยังต่างประเทศ ก็จะเป็นการพึ่งพาตนเอง ช่วยส่งเสริมและพัฒนาเศรษฐกิจของประเทศให้ดียิ่งขึ้น

ตามความเป็นจริงแล้วต้นขี้เหล็ก (*Cassia siamea*) เป็นพืชสมุนไพรที่จัดอยู่ในตระกูลเดียวกันนี้สามารถปลูกได้ทั่วไปในแถบเอเซียตะวันออกเฉียงใต้ ราคาถูก และหาได้ง่ายในประเทศไทย ใบและดอกขี้เหล็กถูกนำมาปรุงอาหารเพื่อรับประทานกันอย่างกว้างขวางโดยเชื่อในสรรพคุณว่าสามารถใช้เป็นยาระบายได้ อย่างไรก็ตามยังไม่มีผลงานวิจัยทางวิทยาศาสตร์รองรับอย่างเด่นชัด ผลของการเป็นยาระบายของขี้เหล็กส่วนหนึ่งอาจเป็นผลมาจากสารจำพวก anthraquinones ที่พบ

อยู่ในใบและดอกของขี้เหล็กเช่นเดียวกัน รวมทั้งสารประกอบอื่น ๆ ที่ยังไม่ทราบแน่ชัด อย่างไรก็ตามมีรายงานว่าการรับประทานขี้เหล็กเข้าไปเป็นจำนวนมาก อาจก่อให้เกิดความเป็นพิษต่อตับได้ ในปี ค.ศ. 1969 Hassanali - walji และคณะได้สกัดสารออกฤทธิ์สำคัญตัวหนึ่งจากใบและดอกขี้เหล็ก สารสกัดนี้เรียกว่า บาราคอล (barakol) มีชื่อทางเคมี 3α, 4-dihydro-3α, 8-dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene ($C_{13}H_{12}O_4$) จากรายงานการศึกษาส่วนใหญ่ถึงผลของบาราคอลต่อการทำงานของระบบประสาทส่วนกลางพบว่า มีฤทธิ์ในการคลายความวิตกกังวล ต้านพฤติกรรมการสบัดหัวที่ถูกกระตุ้นโดยสารสื่อประสาทเซโรโตนิน ลดการหลั่งและลดระดับสารสื่อประสาทโดปามีนในสมอง เป็นต้น ส่วนผลของบาราคอลต่อการทำงานของระบบทางเดินอาหารยังมีการศึกษากันน้อยมาก การศึกษาในห้องปฏิบัติการของทีมผู้วิจัย พบว่าบาราคอลสามารถออกฤทธิ์เพิ่มการหดตัวของกล้ามเนื้อเรียบลำไส้เล็กที่ถูกกระตุ้นด้วยสนามไฟฟ้าได้ รวมทั้งสามารถต้านฤทธิ์ของนอร์อิพิเนฟฟรินที่ลดการหดตัวของกล้ามเนื้อ แสดงให้เห็นถึงฤทธิ์ของการเป็นยากลุ่ม prokinetic drug ของบาราคอล นอกจากนี้ยังพบว่าบาราคอลมีความเป็นพิษต่ำ มีค่า LD_{50} ของหนูขาวและหนูถีบจักร ~1000-1400 mg/kg และไม่ก่อให้เกิดความเป็นพิษต่อตับและไต จากการให้บาราคอลในความเข้มข้นสูงถึง 100 mg/kg แก่สัตว์ทดลองเป็นเวลา 30 วัน ดังนั้นบาราคอลจึงน่าจะเป็นสารที่ควรได้รับการศึกษาต่อไป และควรจะมีฤทธิ์ต่อการทำงานของระบบทางเดินอาหารในด้านอื่น ๆ ด้วย นอกเหนือจากฤทธิ์ของการเป็นยา prokinetic drug

จากการศึกษาเบื้องต้น (preliminary study) โดยทีมผู้วิจัยพบว่าบาราคอลมีแนวโน้มเพิ่มการขับหลั่งสารน้ำและอีเล็กโทรไลต์ในเยื่อบุทางเดินลำไส้ใหญ่ ซึ่งเป็นกลไกที่สำคัญในแง่ของการเป็นยาระบาย จึงมีความสนใจที่จะศึกษาถึงชนิดของไอออนรวมทั้งกลไกการออกฤทธิ์ของบาราคอลต่อไป และหากบาราคอลมีฤทธิ์เพิ่มการขนส่งสารน้ำและอิเล็กโทรไลต์แล้ว จะศึกษาต่อไปถึงผลต่อการขับหลั่งสารสำคัญที่มีบทบาทต่อระบบป้องกันของทางเดินอาหาร เช่น secretory Immunoglobulin A (sIgA) ซึ่งมักจะหลั่งออกมามากเมื่อมีการเพิ่มการหลั่งสารอีเล็กโทรไลต์ เพื่อช่วยทำลายจุลินทรีย์ที่ก่อให้เกิดโรคในทางเดินอาหาร นอกจากนี้การออกฤทธิ์เพิ่มการขับหลั่งอีเล็กโทรไลต์บางชนิด เช่น คลอไรด์ จะช่วยเพิ่มความเป็นกรดให้กับทางเดินอาหารส่วนลำไส้ใหญ่ ทำให้แบคทีเรียกลุ่ม Lactobacillus เจริญเติบโตส่งผลลดการเจริญของแบคทีเรียที่ก่อให้เกิดโรคที่จะลุกลามเข้าสู่ร่างกาย รวมทั้งสามารถลดอุบัติการของการเกิดมะเร็งในลำไส้ใหญ่ส่วนปลายได้อีกด้วย หากบาราคอลมีฤทธิ์เพิ่มการขับหลั่งสารน้ำและอีเล็กโทรไลต์ รวมทั้ง sIgA ด้วยกลไกการออกฤทธิ์ที่แน่นอน ถูกหลักการทางวิทยาศาสตร์ ไม่ก่อให้เกิดความเป็นพิษต่อร่างกาย ก็สามารถนำไปเป็นข้อมูลพื้นฐานทางการแพทย์ในการพัฒนายาที่ใช้เสริมหรือทดแทนยาแผนปัจจุบัน ในการที่จะช่วยรักษา แก้ไข บรรเทาอาการท้องผูก และเพื่อใช้เพิ่มการทำงานของระบบป้องกันของทางเดินอาหารและลดอัตราเสี่ยงต่อการติดเชื้อฉวยโอกาสที่ปนเปื้อนมาจากภายนอก โดยเฉพาะอย่างยิ่งผู้ป่วยที่มีความบกพร่องของระบบประสาททางเดินอาหาร เช่น ท้องผูกแบบเรื้อรัง หรือมีการขยายขนาดของลำไส้ใหญ่ (megacolon) ที่มักจะมีการขับเคลื่อนตัวของทางเดินอาหาร หรือการหลั่งสารน้ำและอีเล็กโทรไลต์ลดลงอย่างมาก รวมทั้งผู้ป่วยมีภาวะของภูมิคุ้มกันต่ำร่วมด้วยจากภาวะเครียดจากทาง

จิตใจ ภาวะเครียดจากการผ่าตัด หรือ ในภาวะของผู้ป่วย HIV ก็จะทำให้เชื้อสามารถเข้าสู่ร่างกายได้รวดเร็วยิ่งขึ้น

2. วัตถุประสงค์

2.1 ศึกษาฤทธิ์ของสารสกัดบาราคอลต่อการขนส่งสารน้ำและอิเล็กโทรไลต์ผ่านเซลล์เยื่อบุลำไส้ใหญ่ทั้งส่วนต้นและส่วนปลาย

2.2 ศึกษาถึงชนิดของไอออนที่ตอบสนองต่อการออกฤทธิ์ของบาราคอลที่ลำไส้ใหญ่

2.3 ศึกษากลไกการออกฤทธิ์ของสารสกัดบาราคอลต่อการขนส่งไอออนผ่านเซลล์เยื่อบุลำไส้ใหญ่

2.4 ศึกษาผลของสารสกัดบาราคอลต่อการเปลี่ยนแปลงปริมาณการขับหลั่งของ immunoglobulin A (IgA)

3. ระเบียบวิธีการวิจัย

การวิจัยครั้งนี้เป็นการศึกษา *in vitro* โดยใช้ลำไส้ใหญ่ของหนูขาว หลังจากเปิดช่องท้องเพื่อนำลำไส้ใหญ่ที่มีความยาวขนาด 10-12 ซม ออกมา ตัดให้เป็นชิ้นเล็ก ๆ แล้วนำมาติดตั้งเข้ากับ Ussing chamber ที่ต่อเข้ากับเครื่อง voltage clamp เพื่อทำการวัดค่าทางไฟฟ้าได้แก่ short circuit current, potential differences และ tissue conductance ที่แสดงถึงการขนส่งไอออนผ่านเซลล์เยื่อบุลำไส้อย่างต่อเนื่อง โดยทำการศึกษาผลและกลไกการออกฤทธิ์ของสารสกัดบาราคอลต่อการเคลื่อนผ่านไอออน ชนิดของไอออนที่ตอบสนองต่อการออกฤทธิ์ของบาราคอล ตลอดจนผลของบาราคอลต่อการขับหลั่ง Immunoglobulin A (IgA) โดยมีการทดลองต่าง ๆ ดังนี้

3.1 การศึกษาผลของบาราคอลต่อการขนส่งไอออนผ่านเซลล์เยื่อบุลำไส้ใหญ่ โดยศึกษาผลของบาราคอลที่ความเข้มข้นต่าง ๆ กันต่อการขนส่งไอออนในลำไส้ใหญ่ เพื่อหาค่าความเข้มข้นที่ทำให้เกิดการตอบสนอง 50% (EC_{50}) ทำการศึกษาทั้งลำไส้ใหญ่ส่วนต้น (proximal colon) และส่วนปลาย (distal colon) ตลอดจนการศึกษาเปรียบเทียบผลของบาราคอลกับผลของ acetylcholine หรือ cabachol และ norepinephrine ต่อการขนส่งไอออนในลำไส้ใหญ่ ตลอดจนดูผลในการต้านฤทธิ์และเสริมฤทธิ์โดยใช้ขนาดความเข้มข้นของสารต่าง ๆ ที่ให้การตอบสนองสูงสุด

3.2 การศึกษาชนิดของไอออนที่เกี่ยวข้องกับผลของบาราคอลในการดูดกลับและขับออกอีเล็กโทรไลต์ โดยใช้สารที่ยับยั้งการทำงานของ ion channel ร่วมกับการทดลองใช้ไอออนอื่นเพื่อทดแทน (ion substitution experiment) เพื่อยืนยันชนิดของไอออนที่คาดว่าจะเกี่ยวข้องกับการเปลี่ยนแปลงค่า Isc ของเนื้อเยื่อต่อบาราคอล

3.3 การศึกษากลไกการออกฤทธิ์ของบาราคอลต่อการขนส่งไอออน เพื่อดูว่าออกฤทธิ์โดยตรงหรือผ่านการทำงานของระบบประสาท submucosal plexus ในทางเดินอาหาร โดยการใช้สารที่ยับยั้งการเหนี่ยวนำของกระแสประสาท (tetrodotoxin) และสารที่ยับยั้งตัวรับระบบ

ประสาท cholinergic (atropine) นอกจากนี้ยังศึกษาถึงกลไกการออกฤทธิ์ของบาราคอลที่อาจเกี่ยวข้องกับการสร้าง prostaglandins โดยใช้สารที่ยับยั้งเอ็นไซม์ cyclooxygenase (indomethacin)

3.4 การศึกษาผลของบาราคอลต่อปริมาณการขับหลั่งของ IgA ที่สามารถตรวจวัดด้วยวิธี Enzyme – linked immunoabsorbent assay (ELISA)

ข้อมูลที่ได้จากการทดลอง จะถูกนำเสนอในรูปค่าเฉลี่ย ± SEM (standard error of mean) เปรียบเทียบผลการทดลองระหว่างกลุ่มควบคุมและกลุ่มที่ให้ยาในขนาดต่าง ๆ โดยใช้ Analysis of Variance (ANOVA) ค่า $P < 0.05$ ถือว่ามีนัยสำคัญทางสถิติ

4. แผนการดำเนินการวิจัยตลอดโครงการในแต่ละช่วง 6 เดือน

<u>ปีที่ 1</u> : ช่วง 6 เดือนแรก

- จัดเตรียมอุปกรณ์และเครื่องมือที่ใช้ในงานวิจัย
- ศึกษาผลของสารบาราคอลต่อการขนส่งไอออนผ่านเซลล์เยื่อบุลำไส้ใหญ่ส่วนต้นและส่วนปลาย
- ศึกษาชนิดของไอออนที่เกี่ยวข้องกับผลของบาราคอลในการดูดกลับและขับออกอีเล็กโทรไลต์

<u>ปีที่ 1</u> : ช่วง 6 เดือนหลัง

- ศึกษากลไกการออกฤทธิ์ของบาราคอลต่อการขนส่งไอออน
- รวบรวมและวิเคราะห์ข้อมูล
- สรุปผลงานวิจัยและเขียนรายงาน
- จัดทำ manuscript สำหรับตีพิมพ์

<u>ปีที่ 2</u> : ช่วง 6 เดือนแรก

- ศึกษาผลของบาราคอลต่อการขับหลั่งของ immunoglobulin A (IgA) ในลำไส้ใหญ่ส่วนต้นและส่วนปลาย

<u>ปีที่ 2</u> : ช่วง 6 เดือนหลัง

- รวบรวมและวิเคราะห์ข้อมูล
- สรุปผลงานวิจัยและเขียนรายงาน
- จัดทำ manuscript สำหรับตีพิมพ์

5. ผลงาน/หัวข้อเรื่องที่จะตีพิมพ์ในวารสารวิชาการระดับนานาชาติในแต่ละปี

<u>ปีที่ 1</u>: ชื่อเรื่องที่คาดว่าจะตีพิมพ์ : Barakol stimulates chloride secretion in rat colonic epithelium

ชื่อวารสารที่คาดว่าจะตีพิมพ์ : Journal of Pharmacology and Experimental Therapeutics (impact factor 3.555) หรือ European Journal of Pharmacology (impact factor 2.164)

<u>ปีที่ 2</u>: ชื่อเรื่องที่คาดว่าจะตีพิมพ์ : Effects of barakol on secretion of secretory immunoglobulin A (sIgA) in rat colon

ชื่อวารสารที่คาดว่าจะตีพิมพ์ : Journal of Pharmacology and Experimental Therapeutics (impact factor 3.555) หรือ European Journal of Pharmacology (impact factor 2.164)

เนื้อหางานวิจัย

INTRODUCTION

Barakol is a biologically active compound extracted from leaves and flowers of *Cassia siamea*, a plant that has been traditionally used for the treatment of fever, skin disease, constipation, diabetes, hypertension and insomnia (Kinghorn and Balandrin, 1992). An alcoholic extract of *C. siamea* was shown to inhibit central nervous system activity, increase tension in smooth muscle (Arunlakshana, 1949) and decrease blood pressure (Mokasmit, 1981). In 1969, Hassanali-Walji and coworkers originally extracted a biologically active constituent called barakol from *C. siamea.* The chemical structure of barakol was identified as 3α, 4-dihydro-3α, 8-dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene ($C_{13}H_{12}O_4$) or 2, 5-dimethyl-3αH-pyrano-[2, 3, 4-de]-1-benzopyran-3α, 8-diol and a proposed synthetic procedure was described in 1970 (Bycroft et al., 1970). Barakol is a chromone that is not related chemically to anthranoid laxatives derived from *Cassia* plants. In animal model studies, barakol has been shown to possess hypotensive activity (Suwan et al., 1992), serotonergic receptor antagonist activity (Tongroach et al., 1992) and appears to function as an anxiolytic in exploratory behavioral activities (Thongsaard et al., 1996). Further in vitro studies in the central nervous system have found that barakol inhibits K^+-stimulated dopamine release from striatal slices of rat brain (Thongsaard et al., 1997). Recent studies in our laboratory have shown that barakol increases smooth muscle contraction in the isolated rat ileum under basal conditions and during electrical field stimulation. In these studies, barakol was found to antagonize norepinephrine-suppressed smooth muscle contraction, suggesting a role for barakol as a prokinetic drug (Poonyachoti et al., 2002; Deachapunya et al., 2005).

The colonic epithelium plays an essential role in the absorption and secretion of water and electrolytes. Its transport function is regulated by a variety of neurotransmitters, hormones and inflammatory mediators. Under basal conditions, the colonic epithelium absorbs fluid from the lumen into the circulation and this process is driven by energy dependent Na^+ transport. In contrast, colonic secretion is activated by secretagogues that enhance Cl^- transport mechanisms, which in turn create electrochemical and osmotic driving forces for passive cation and water movement into the lumen (Kunzelmann and Mall, 2002). Decreased secretory function or increased fluid absorption by the colon is typically associated with constipation. Since *Cassia siamea* has been used as a laxative, we hypothesized that its active ingredient, barakol, may exert a laxative effect by stimulating chloride secretion and/or inhibiting NaCl absorption across the colonic epithelium.

Secretory immunoglobulin A (sIgA) plays an important role in mucosal immunity in many epithelia such as respiratory, gastrointestinal and genitourinary tracts. In intestine, it is produced from immunoglobulin producing plasma cells resided in laminar propria of small intestine and, to a lesser extent, in the large intestine. It is transported across epithelial cells in a receptor-mediated transcytosis which involve the binding of dimeric IgA to the polymeric immunoglobulin receptor (pIgR) on the basolateral membrane of epithelial cells. When translocated to the apical membrane, pIgR is proteolytically cleaved and the extracellular domain of the receptor, bound to IgA, is released into the mucosal secretions. This cleaved extracellular membrane of the receptor is known as the secretory component (SC). The secreted IgA, together with the SC, is known as secretory IgA which serves to prevent exogenous antigens, including microorganisms, from attaching to and penetrating the epithelial lining of mucous membranes and helps to promote their degradation and excretion. In addition, during transcytosis, pIgR-IgA complexes are important in intracellular virus neutralization and the clearing of antigens from the larmina propria (Rojas and Apodaca, 2002).

Secretion of IgA has been shown to be stimulated by several secretagogues such as cholecystokinin (CCK), substance P (SP) and neurokinin A (NKA) (McGee et al., 1995; Schmidt et al., 1999). The receptors for these neuropeptides have been found on plasma cells (Stanisz et al., 1987; Kimata et al., 1996). In addition, SP and VIP have been known to stimulate water and electrolyte secretion into the colonic lumen. This raises the possibility whether barakol may stimulate the secretion of IgA in the colon.

In the present study, we aimed to investigate the effect of barakol on ion transport across the rat colon epithelium and to determine the mechanisms involved in barakol action. If barakol exerts secretory activity, the effect of barakol on secretion of IgA in the colon will be also examined.

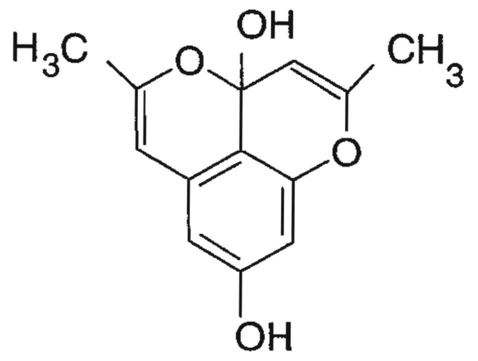

Figure 1 Chemical structure of barakol

MATERIAL AND METHODS

Plant extraction. Barakol was extracted and purified from *Cassia siamea* in our laboratory by a procedure as described in our previous report (Thongsaard et al., 2001). Briefly, fresh young leaves and flowers of *C. siamea* were obtained from a local market in Bangkok and the identification confirmed by comparison with the herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Co-operative, Thailand. They were cut into small pieces and boiled in 0.5% sulfuric acid for 30 min. The mixture was blended, filtered, and alkalinized with concentrated $NaHCO_3$ and then extracted with chloroform. The chloroform extract was further concentrated with 5% acetic acid and neutralized with 25% ammonium hydroxide. The crude barakol was obtained as greenish crystallized yellow needles with 0.3% yield. Concentrated hydrochloric acid was finally added to obtain barakol hydrochloride and the mixture was dried by vacuum filtration to form yellowish crystallized anhydrobarakol hydrochloride. The compound was shown to be a single chemical using thin layer chromatography on silica gel and the identification confirmed by nuclear magnetic resonance (NMR). Barakol was dissolved in distilled water immediately before testing its activity. When anhydrobarakol hydrochloride is dissolved in water, the reaction is reversed and the product used in all the biological experiments is a barakol solution with a pH of 3-4 at the stock concentration (50 mM) (Thongsaard et al., 2001). In the experiment, barakol was freshly dissolved in normal saline, wrapped with aluminum foil and kept on ice, and used within 3 hours after preparation.

Chemicals. Tetrodotoxin, atropine sulphate, hexamethonium, amiloride, glibenclamide, diphenylamine-2-carboxylic acid (DPC), bumetanide, indomethacin, acetazolamide, N-vanillylnonanamide (capsaicin synthetic) and high purity grade salts were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were made in aliquots and kept at -20 C before use. Some chemicals were dissolved in dimethyl sulfoxide (DMSO), the final DMSO concentration of which was less than 0.1% (vol/vol).

Animals and tissue preparation. Male Wistar rats (250-300 g) were obtained from National Animal Center, Mahidol University, Thailand. They were housed in stainless-steel cages in a room with a 12-12 hour light:dark cycle and allowed free access to food and water. All animals were taken care in accordance with the International Guiding Principles for Biomedical Research Involving Animals provided by the National Research Council of Thailand.

For experiment, rats were sacrificed with small animal decapitator (Harward, Kent, UK). After a laparotomy incision, the whole colon was removed, rinsed and placed in an ice-cold oxygenated Ringer solution (composition in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 11 D-Glucose; pH 7.4). The colon was longitudinally cut close to the mesentary and the serosal muscle layers were carefully stripped away by blunt dissection to obtain a mucosa-submucosal preparation. The appearance of palm-like foldings was used to distinguish between the distal and proximal colon.

Measurement of electrical parameters. The mucosa-submucosal preparation was mounted in Ussing chambers (0.62 cm^2) bathed on the apical and basolateral sides with identical Ringer's solutions at 37° C and gassed with 95% O_2 and 5% CO_2. Transepithelial potential difference (PD) and short circuit current (Isc) were measured with the use of voltage-clamp amplifier (EVC-4000, World Precision Instrument) using Ag/$AgCl_2$ electrodes connected to the bathing solution via agar bridges. Tissue conductance (G) was calculated using Ohm's law (G = Isc/PD). The tissues were continuously short-circuited, except for a brief interval of open-circuited readings before and after adding any chemicals. The data from the voltage clamp was connected to MacLab 4S A/D converter and recorded with a 400 MHz PowerPc Macintosh. After mounting, the tissues were equilibrated for at least 30 min to achieve a stable Isc before the addition of chemicals. Positive Isc corresponded to the movement of anions from the serosal to mucosal compartments or movement of cations from the mucosal to serosal compartments or a combination of both. In the anion replacement experiments, gluconate salts were substituted for chloride and HEPES buffer

(20 mM) was substituted for HCO_3^-. Experiments under HCO_3^--free conditions were performed in the presence of 100 μM acetazolamide and aerated with 100% O_2.

Measurements of IgA levels. The amount of total rat IgA was estimated in the mucosal (apical) and serosal (basolateral) solutions bathing the tissue preparations as well as in the caecal content. To obtain the barakol effect on secretion of IgA *in vitro*, the colonic epithelium was mounted in Ussing chamber and the mucosal and serosal solutions were collected at a 5 min period after serosal application of barakol (1 mM), which approximated the maximum barakol response. The solutions collected during a 5 min period prior to addition of barakol were used as control. To determine the effect of barakol on IgA secretion *in vivo*, rats were intraperitoneally injected with barakol (50 mg/kg in 1 ml normal saline) and 30 min later, the rats were sacrificed and the caecal contents were collected. All collected samples were immediately kept in -70°C until analysis. Before analysis, the ceacal contents were thawed out and diluted with 0.1% trypsin inhibitor cocktails (1 ml/mg of sample). The mixture was homogenized and solubilized by vortex spinning every 15 min for 60 min, after which the homogenate was centrifuged for 15 min at 8,000 rpm and supernatant was collected for measurement of IgA level.

The amount of total IgA was determined by a rat IgA quantitation kit (Bethyl Laboratories, Texas, USA). Briefly, microtitre plates were coated with 100 μl of affinity purified goat anti-rat IgA antibody diluted 1:100 in carbonate buffer pH 9.6 and kept at 4°C overnight. After washing and blocking steps following the standard protocol of enzyme linked immunoabsorbent assay (Bethyl Laboratories), 100 μl of 1:50 diluted sample were added to each well and incubated for 1 hr at room temperature. After a third washing step, each well was added 100 μl of capture antibody, the anti-rat IgA conjugated with horseradish peroxidase diluted 1:20,000 in PBS buffer pH 7.2 for 1 hr at room temperature. Color was then developed by 3,3',5,5' tetramethylbenzidine (TMB) substrate for 5 min and stopped by 2N sulfuric acid. Absorbance was read at 450 nm and 550 nm by ELISA plate reader (Tecan). The amount of IgA was determined from standard curve using a rat IgG reference and expressed as ng/ml/cm^2 or ng/mg of caecal content.

Data analyses. All values were expressed as means and standard error of mean (SEM), n was the number of different tissue preparations and N was the number of animals in each experiment. The increase in Isc was quantified by subtracting the peak of an Isc response from its respective baseline value before drug administration. The statistical differences between control and treatment means were analyzed using Student's paired or unpaired t-test when appropriate. The differences among groups were analyzed using a one way analysis of variance followed by Dunnett's multiple comparison. A p-value less than 0.05 was considered statistically significant.

RESULTS

Under basal conditions, after an equilibration period of 30-45 min, the distal colon showed average Isc, PD (lumen negative) and G values of 35.5 ± 2.4 $\mu A/cm^2$, -3.7 ± 0.3 mV and 11.0 ± 0.8 pS/cm^2 (n = 44 tissues, N = 17 rats), respectively. The proximal colon exhibited average Isc, PD and G values of 39.6 ± 5.1 $\mu A/cm^2$, -2.7 ± 0.3 mV and 15.3 ± 1.5 pS/cm^2 (n = 17 tissues, N = 12 rats), respectively.

Effect of barakol on Isc

Addition of 1 mM barakol to the basolateral solution produced a slow increase in Isc, which peaked in 5-10 min and was sustained for 30-45 min (Fig. 2A), before it gradually returned to the baseline level. The maximal increase in Isc produced by barakol was 24.4 ± 2.0 $\mu A/cm^2$ (n = 33 tissues, N = 18 rats) in distal colon and 24.0 ± 4.6 $\mu A/cm^2$ (n = 10 tissues, N = 7 rats) in proximal colon. The tissue conductance at the maximal barakol response was not different from its corresponding baseline value (control, 13.3 ± 1.0 pS/cm^2; after barakol, 12.8 ± 1.0 pS/cm^2; n = 35 tissues, N = 18 rats). Addition of 0.01 M hydrochloric acid in equal volume to barakol solution (200 μl) did not alter any baseline electrophysiological parameters. Since the tracings and maximal Isc responses to barakol in the distal and proximal colon were not different, the rest of the experiments were performed using distal colon unless otherwise stated. Barakol (10 μM-3 mM) induced a cumulative concentration-dependent increase in Isc with an apparent EC_{50} value of 0.4 mM (n = 9 tissues, N = 5 rats, Fig. 2B). The barakol-induced increase in Isc was completely inhibited by the Cl^- channel blocker, diphenylamine-2, 2′-dicarboxylic acid (DPC, 500 μM, n = 4 tissues, N = 3 rats, Fig. 3A) and partially inhibited by glibenclamide (400 μM) added to the apical solution. Glibenclamide produced a 52.6 ± 11.0% decrease in barakol-stimulated Isc (n =5 tissues, N = 4 rat, Fig. 3B). In contrast, the barakol-stimulated increase in Isc was not affected by a subsequent addition of 4, 4-diisothiocyanatostilbene-2, 2-disulfonic acid (DIDS) to the apical solution (n = 5 tissues, N = 4 rats, data not shown). Basolateral addition of bumetanide (200 μM), a Na^+-K^+-$2Cl^-$ cotransporter inhibitor, completely suppressed the barakol-stimulated Isc (n = 5 tissues, N = 4 rats, Fig. 3C). The Isc response to barakol did not significantly change in the presence of the Na^+ channel blocker amiloride (10 μM, Fig. 4A) in the apical solution (control, 33.5 ± 6.8 $\mu A/cm^2$, n = 6 tissues, N = 4 rats; after amiloride, 32.5 ± 10.1 $\mu A/cm^2$; n = 3 tissues, N = 3 rats). In contrast, pretreatment of tissues with bumetanide (200 μM, Fig. 4B) abolished the barakol-induced increase in Isc, from 33.5 ± 6.8 $\mu A/cm^2$ (n = 6 tissues, N = 4 rats) to 1.0 ± 3.6 $\mu A/cm^2$ (n = 3 tissues, N = 3 rats, p<0.01, Fig. 4C).

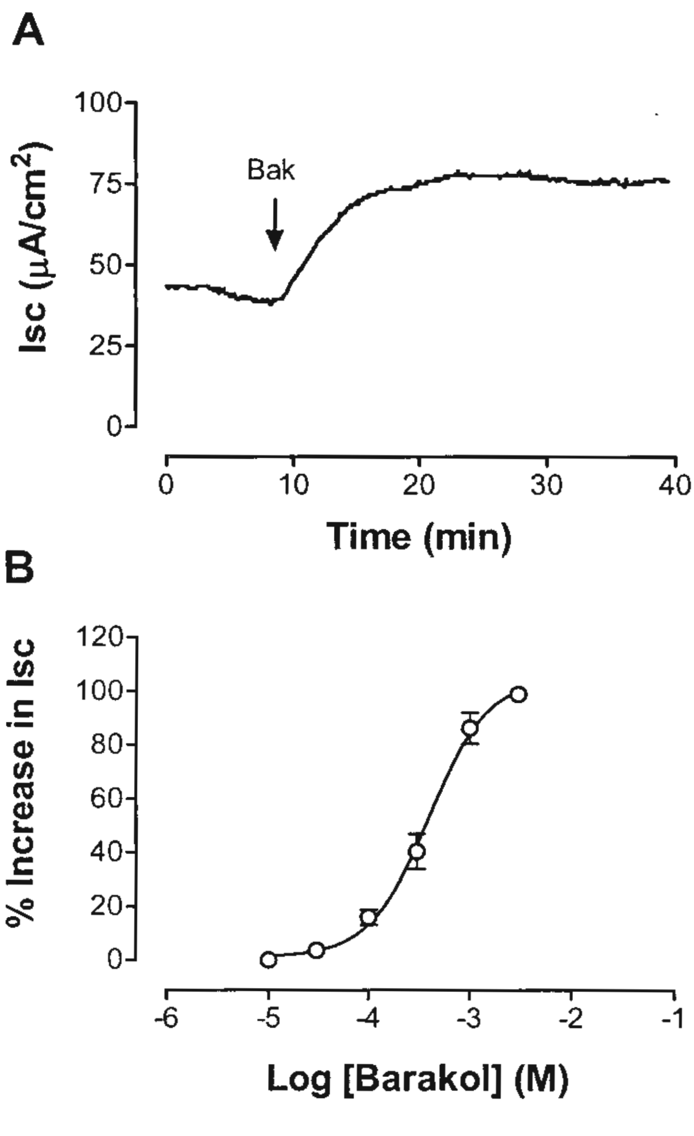

Figure 2 Effect of barakol on Isc in rat colon epithelium. (A) Representative recordings of the Isc response produced by addition of 1 mM barakol (Bak) to the basolateral solution. (B) Concentration-response relationship for barakol-stimulated Isc. The EC_{50} value was 0.4 mM. Each point represented mean ± SEM.

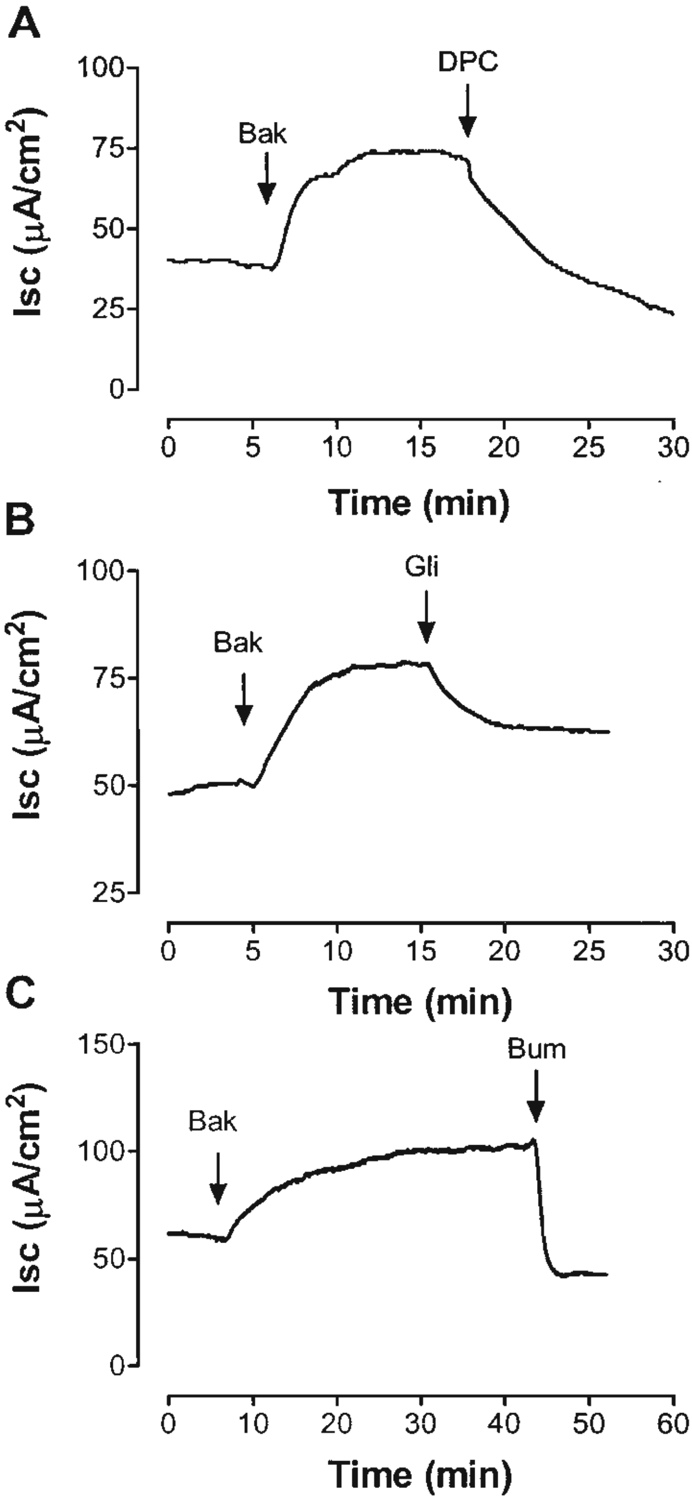

Figure 3 Effect of Cl^- channel blockers and a Na^+-K^+-$2Cl^-$ cotransport blocker on the barakol-stimulated Isc. Representative recordings of the Isc response produced by basolateral addition of 1 mM barakol (Bak) were inhibited by (A) apical addition of 500 μM DPC, (B) apical addition of 400 μM glibenclamide (Gli) or (C) basolateral addition of 200 μM bumetanide (Bum).

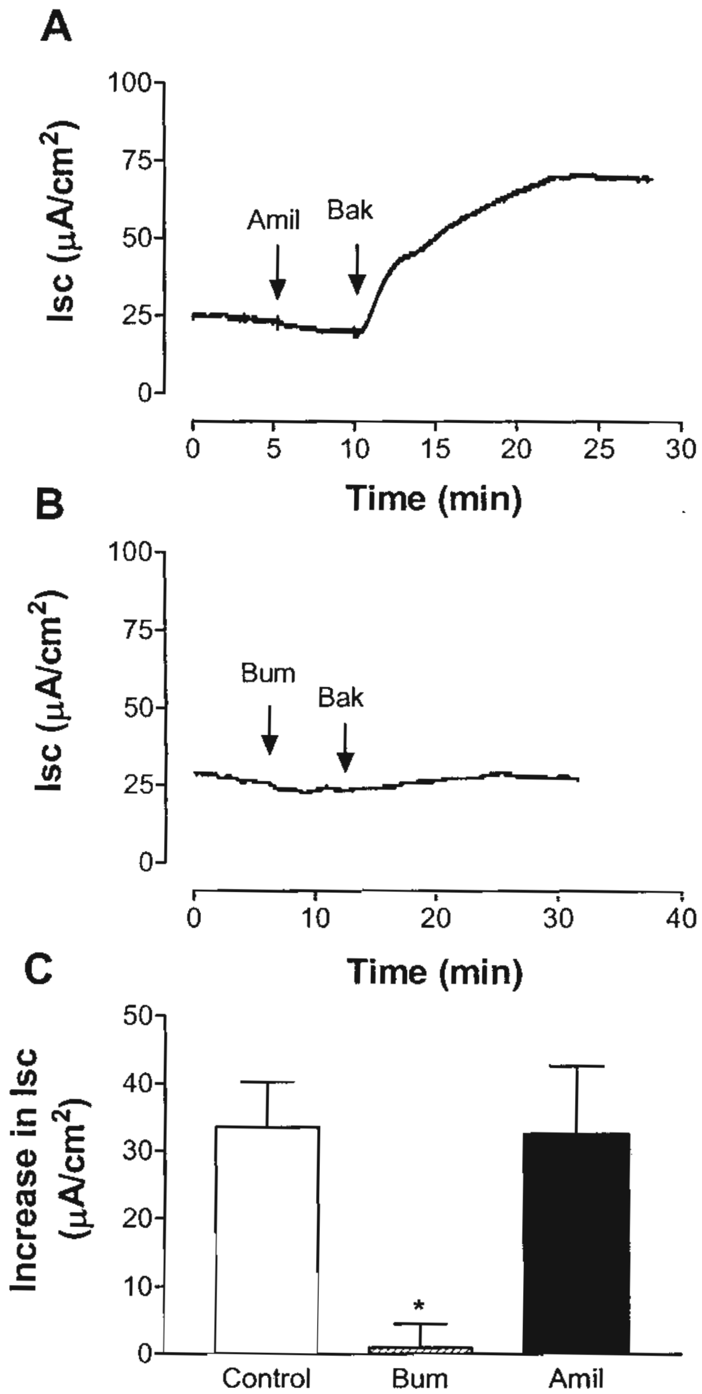

Figure 4 Effect of amiloride and bumetanide on the barakol-stimulated Isc. Representative tracings of the barakol (Bak)-stimulated Isc response after pretreatment with (A) apical addition of 10 μM amiloride (Amil) or (B) a basolateral addition of 200 μM bumetanide (Bum). (C) Bar graph illustrating the average maximal increases in Isc response produced by barakol alone (Control) and after pretreatment with bumetanide or amiloride. Values represent means ± SEM. *P<0.01 when compared to the control value.

Effect of ion substitution on barakol-increased Isc

Ion substitution experiments were performed to determine the ionic basis of the Isc response induced by barakol. In normal Ringer's solution, barakol at a concentration of 1 mM produced a mean increase in Isc of 31.1 ± 2.8 $\mu A/cm^2$ (n = 24 tissues, N = 13 rats). Replacement of Cl^- in both apical and basolateral solutions significantly inhibited the maximal Isc response to barakol (7.8 ± 0.6 $\mu A/cm^2$, n = 8 tissues, N = 6 rats, $p<0.05$), whereas replacement of HCO_3^- had no effect on the maximal barakol-induced Isc (29.4 ± 2.6 $\mu A/cm^2$, n = 5 tissues, N = 4 rats). Replacement of both Cl^- and HCO_3^- significantly inhibited the maximal Isc response to 2.6 ± 0.7 $\mu A/cm^2$ (n = 5 tissues, N = 4 rats, $p<0.05$, Fig. 5).

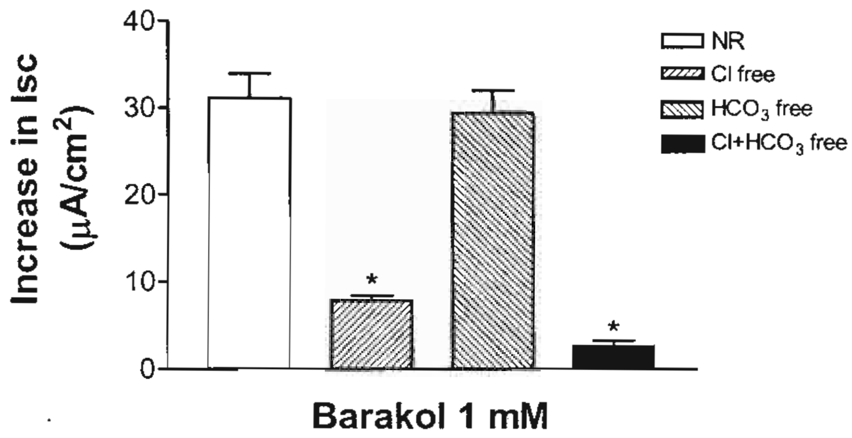

Figure 5 Effect of Cl^- and HCO_3^- substitution on the barakol-stimulated Isc. In standard Ringer's solution (NR), barakol at a concentration of 1 mM produced a mean increase in Isc of 31 $\mu A/cm^2$. Replacement of Cl^- (Cl free) or both Cl^- and HCO_3^- (Cl-HCO_3 free) inhibited the maximal Isc response to barakol by 74% and 91% respectively, whereas replacement of HCO_3^- (HCO_3 free) had no effect on barakol response. Values were means ± SEM. *P<0.01 when compared to the control value of NR.

Effects of submucosal neuronal blockers and indomethacin on barakol-increased Isc

To assess whether barakol activation of Cl^- secretion was mediated by submucosal neurons and prostaglandin synthesis, the tissues were pretreated with neuronal blockers or indomethacin for 5 minutes followed by basolateral addition of barakol (1 mM). The effect of barakol alone on control tissues is shown in figure 6 (30.4 ± 3.2 $\mu A/cm^2$, n = 22 tissues, N = 11 rats). Tetrodotoxin (TTX, 10 μM) significantly decreased the basal Isc from 51.9 ±

13.0 $\mu A/cm^2$ to 33.2 ± 9.9 $\mu A/cm^2$ and subsequent addition of barakol resulted in an Isc response that was 60% lower than that in control tissues (11.8 ± 4.2 $\mu A/cm^2$, n = 5 tissues, N = 4 rats, p<0.05). In contrast, pretreatment with the muscarinic receptor antagonist atropine (1 µM) in the basolateral solution did not significantly change the basal Isc or reduce the barakol-induced increase in Isc (24.7 ± 6.4 $\mu A/cm^2$, n = 4 tissues, N = 4 rats). In addition, hexamethonium pretreatment (10 µM) did not significantly alter the barakol-stimulated Isc (n = 5 rats, N = 4 rats, data not shown). Pretreatment of tissues with the cyclooxygenase inhibitor indomethacin (10 µM) in both apical and basolateral solutions significantly reduced the barakol-stimulated Isc response by 60% (12.3 ± 7.1 $\mu A/cm^2$, n = 7 tissues, N = 6 rats, p<0.05). The barakol-stimulated Isc response was decreased by 90% in the presence of indomethacin and TTX (4.95 ± 1.1 $\mu A/cm^2$, n = 4 tissues, N = 4 rats, p<0.05, Fig. 6). In addition, pretreatment with dihyphendamine (10 µM), a histamine (H_1) receptor blocker, did not change the barakol-stimulated Isc (n = 3 tissues, N = 3 rats, data not shown).

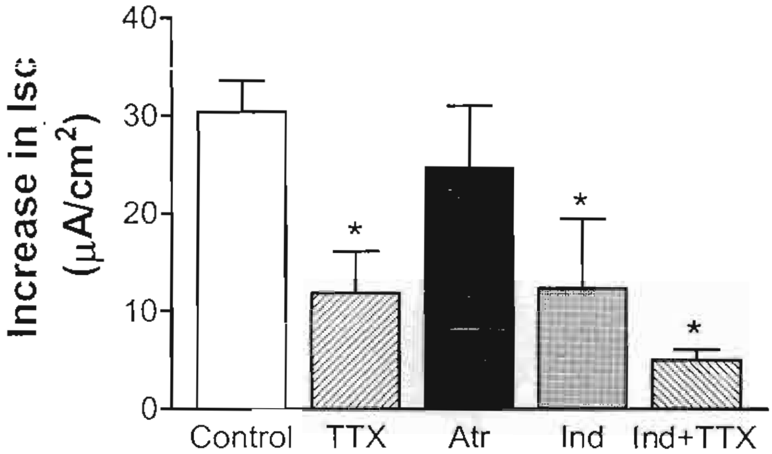

Figure 6 Effect of submucosal neuronal blockers and indomethacin on the barakol-stimulated Isc. Bar graph illustrating the maximal Isc response to barakol alone (Control) and after pretreatment with 10 µM tetrodotoxin (TTX), 1 µM atropine (Atr), 10 µM indomethacin (Ind) or a combination of Ind and TTX (Ind+TTX). Values were mean ± SEM. *P<0.05 when compared to the control value.

Effect of sensory neuron blocker on barakol-increase Isc

In our findings, barakol was shown to stimulate Cl^- secretory pathway partially due to stimulation of submucosal neurons. This stimulation was not dependent on motor cholinergic pathway. To further investigate whether barakol-stimulated Cl^- secretion may involve sensory neuron, the sensory neurons were chemically denervated following pretreatment of the tissues with capsaicin synthetic (3 μM and 10 μM) added to the basolateral solution. Capsaicin at the concentration of 3 μM increased the amplitude of Isc by 24.7 ± 6.4 $\mu A/cm^2$ (n = 4 tissues, N = 4 rats) when added to the basolateral solution. A subsequent addition of capsaicin (10 μM) had no further increase in Isc response, suggesting a successfully denervation of sensory nerve. In the presence of capsaicin, a small Isc response induced by barakol (1 mM) was observed (8.9 ± 2.7 $\mu A/cm^2$, n = 4 tissues, N = 4 rats, $p<0.05$, Fig. 7A and 7B), which accounted for an inhibition of 72% of barakol response alone (30.4 ± 3.2 $\mu A/cm^2$, n = 9 tissues, N = 22 rats). In addition, barakol pretreatment completely abolished the capsaicin-induced increase in Isc (Fig. 7C).

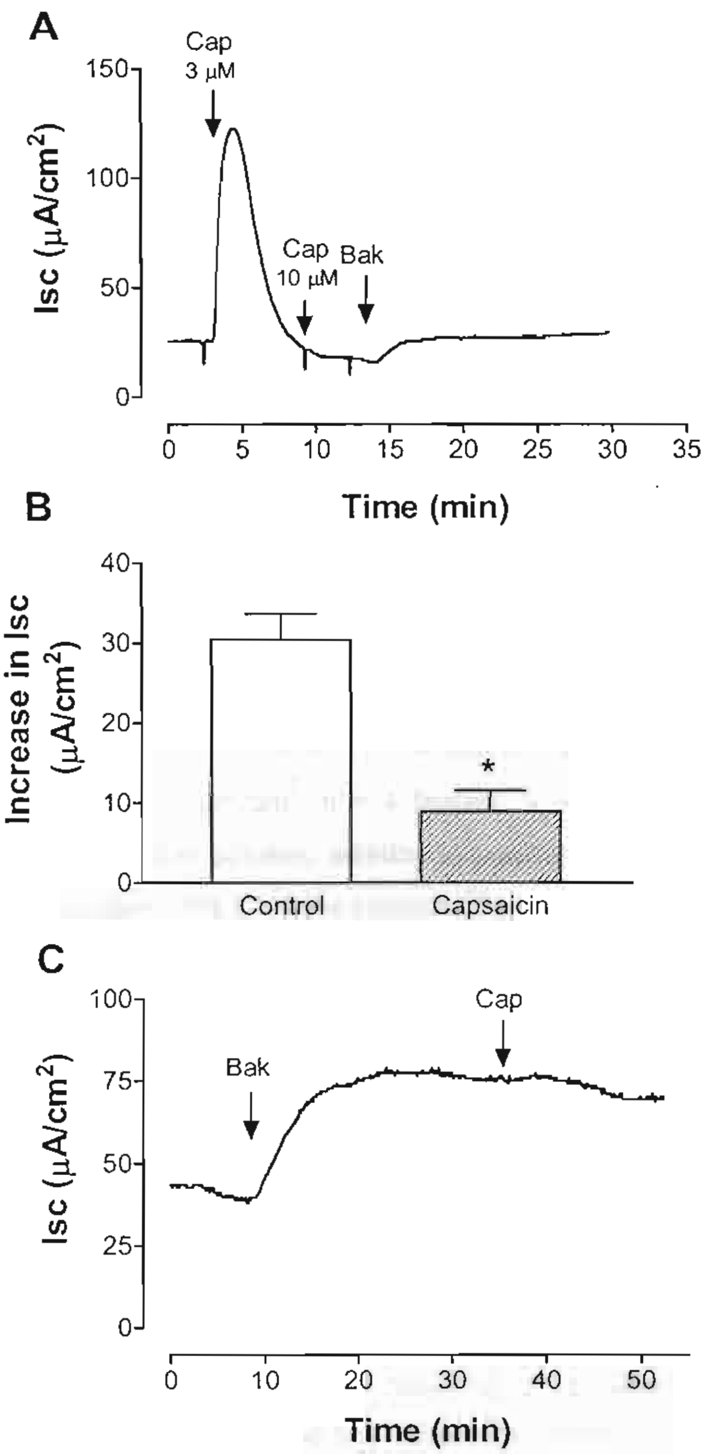

Figure 7 Effect of capsaicin on the barakol-stimulated Isc. (A) Representative tracing showing the Isc produced by basolateral addition of 3 and 10 µM capsaicin (Cap). Subsequent addition of 1 mM barakol (Bak) showed a small Isc response. (B) Bar graph illustrating the average maximal increases in Isc response produced by barakol alone (Control) and after pretreatment with capsaicin (3 µM). Values represent means ± SEM. *P<0.01 when compared to the control value. (C) Representative Isc showing that addition of 1 mM barakol abolished the increase in Isc produced by 3 µM capsaicin.

Effect of barakol on the immunoglobulin A (IgA) secretion

Secretagogues including substance P and cholecystokinin which stimulates electrolytes and water secretion have been shown to increase the secretion of IgA into the lumen of *porcine ileum (Schmidt et al., 1999). Since barakol had a potential effect on stimulation of Cl^-* secretion, we therefore investigated whether barakol could induce the secretion of IgA cross the colonic epithelium. By using the Ussing apparatus and voltage clamp techniques, the concentration of IgA in the mucosal (apical) and serosal (basolateral) solutions bathing the colonic epithelium could be determined before and after barakol treatment. Under normal Ringer solution, the level of IgA content in the mucosal and serosal solution bathing the colonic epithelium was 65.5 ± 16.5 ng/ml/cm^2 (n = 4 tissues, N = 4 rats) and 32.2 ± 7.2 ng/ml/cm^2 (n = 4 tissues, N = 4 rats), respectively. A serosal addition of barakol (1 mM) for 5 min produced a marked increase in the IgA concentration in the mucosal solution by 301.8 ± 15.8 ng/ml/cm^2 (n = 4 tissues, N = 4 rats, P<0.01) and in the serosal solution by 327.6 ± 11.1 ng/ml/cm^2 (n = 4 tissues, N = 4 rats, P<0.01). Replacement of Cl^- significantly increased the IgA content in the mucosal solution (205.9 ± 31.4 ng/ml/cm^2, n = 4 tissues, N = 4 rats, P<0.01), but not in the serosal solution (66.2 ± 8.5 ng/ml/cm^2, n = 4 tissues, N = 4 rats) compared to the normal Ringer solution. Under Cl^--free solution, addition of barakol (1 mM) had no effect on the IgA level in the mucosal solution (186.4 ± 27.4 ng/ml/cm^2, n = 4 tissues, N = 4 rats) but showed a small increase in the serosal IgA level (153.9 ± 36.1 ng/ml/cm^2, n = 4 tissues, N = 4 rats). However, the change of serosal IgA responses to barakol in Cl^--free solution significantly lowered than that in normal Ringer solution (Fig. 8). In both normal Ringer and Cl^--free solutions, application of barakol had no effect on tissue conductances (normal Ringer: before barakol 13.3 ± 1.0 pS/cm^2; after barakol 12.8 ± 1.0 pS/cm^2, n = 35 tissues, N = 18 rats; Cl^--free: before barakol 9.9 ± 1.7 pS/cm^2, after 10.8 ± 1.7 pS/cm^2, n = 12 tissues, N = 6 rats). In addition, the tissue conductances in Cl^--free solution before and after barakol addition was not different from its corresponding values in normal Ringer solution (Fig. 9).

To confirm the barakol effect on IgA secretion *in vivo* study, the amount of IgA was determined from caecal content collected from killed rats, 30 min after a peritoneal injection of barakol at the dose of 50 mg/kg. The dose of barakol used in this study was the dose that showed anxiolytic effect in our previous study (Thongsaard et al., 2001). Barakol treatment markedly increased the caecal IgA content to 172.2 ± 26.0 ng/mg (n = 4 tissues, N = 4 rats) from 36.3 ± 1.5 ng/mg (n = 5 tissues, N = 5 rats) in the normal saline control rats, Fig. 9). In addition, the tissue conductance of colonic epithelium taken from normal saline-treated control rats (22.7 ± 5.0 pS/cm^2, n = 4 tissues, N = 4 rats) and barakol-treated rats (24.6 ± 6.5 pS/cm^2, n = 4 tissues, N = 4 rats) did not show significant changes.

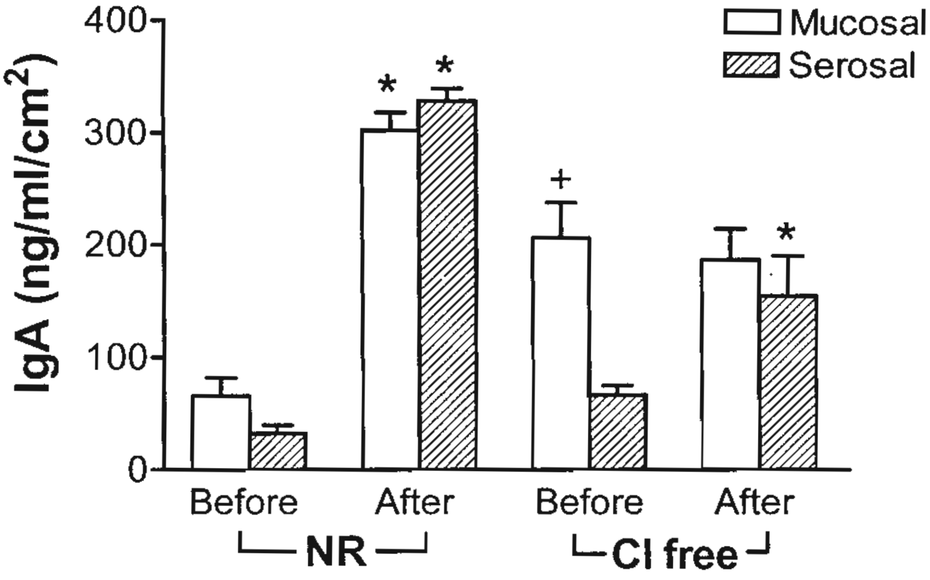

Figure 8 Effect of barakol on the secretion of IgA in isolated rat colon under standard normal Ringer solution and Cl^--free solution. The concentration of IgA was determined from mucosal and serosal solutions bathing the colonic epithelium before and after barakol treatment for 5 min. In normal Ringer's solution (NR), a basolateral addition of barakol (1 mM) produced a marked increase in the level of IgA in both musocal and serosal solutions. Replacement of Cl^- (Cl free) increased the IgA level in mucosal solution, but not in the serosal solution. Addition of barakol (1 mM) had no effect on the IgA level in mucosal solution but showed a small increase in serosal IgA. Values were means ± SEM. *P<0.01 when compared to the control value before barakol addition. $^+$P<0.01 when compared to the corresponding value under normal Ringer's solution.

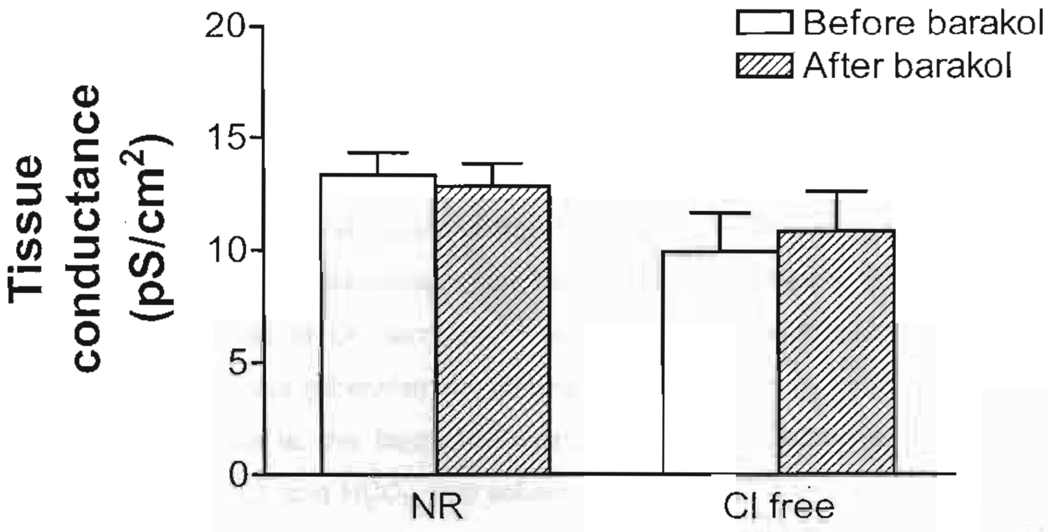

Figure 9 Transepithelial conductance of colonic tissues under normal Ringer' solution (NR) and Cl^--free solution (Cl free). The tissue conductance was determined immediately before and after addition of barakol into the basolateral solution of for 5 min. Values were mean ± SEM.

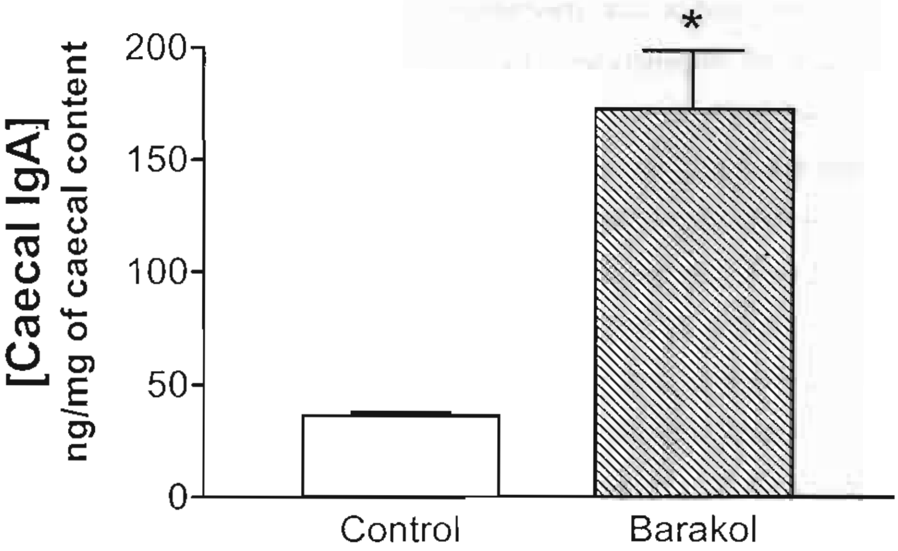

Figure 10 Effect of barakol on the level of IgA secretion isolated from caecal content. Bar graph illustrates the concentration of IgA isolated from caecal content using ELISA technique. The dramatically increase of the IgA secretion was seen 30 min after an intraperitoneal injection of barakol compared with normal saline-injected control rats. Values were mean ± SEM. *$P<0.01$ when compared to the control value.

DISCUSSION

Effect of barakol on chloride secretion

In the present study, the direct effect of barakol on the ion absorption and secretion was studied in vitro using serosal muscle-stripped colonic epithelium. Barakol was shown to increase Isc in both proximal and distal colon by the same magnitude. The increased Isc was due to an activation of Cl^- secretion. This was supported by the findings that Cl^- channel blockers, DPC and glibenclamide, inhibited the barakol-induced increase in Isc. In ion substitution experiments, the barakol-stimulated Isc was abolished in Cl^--free solution and further inhibited in Cl^- and HCO_3^--free solutions. In addition, pretreatment with the loop diuretic bumetanide completely inhibited the barakol-stimulated Isc. All of these findings were consistent with stimulation of transepithelial Cl^- secretion in human and rat colon (Dharmsathaphorn et al., 1985; Ko et al., 2002; Kunzelman and Mall., 2002). The finding that the increase in Isc by barakol was not affected by the presence of the Na^+ channel blocker amiloride, indicating that barakol did not stimulate electrogenic Na^+ absorption.

Cl^- secretion in mammalian colon involves Cl^- uptake across the basolateral membrane by a Na^+-K^+-$2Cl^-$ cotransport mechanism and subsequent efflux across the apical membrane through Cl^- channels. CFTR is the predominant Cl^- channel that plays a role in Cl^- .secretion in many epithelia including the colonic epithelium (Kunzelmann and Mall, 2002). CFTR is a cAMP-mediated Cl^- channel that has previously been shown to be inhibited by DPC and glibenclamide (Sheppard and Welsh, 1992). On the other hand, DIDS has been shown to block Ca^{2+}-activated Cl^- channel but had no effect on the activity and conductance of CFTR (Anderson et al., 1992; Schultz et al., 1999). Since the present study showed that the barakol-induced increase in Isc was completely inhibited by DPC and partly inhibited by glibenclamide, it is most likely that CFTR was the apical Cl^- exit pathway for barakol-stimulated Cl^- secretion. The insensitivity of the barakol response to DIDS further confirmed the involvement of CFTR-mediated Cl^- secretion, and suggested that the Ca^{2+}-activated Cl^- channel was not involved in the Isc response to barakol. The barakol-stimulated increase in Isc was also abolished by bumetanide, an inhibitor of Na^+-K^+-$2Cl^-$ cotransporter, suggesting that barakol-stimulated Cl^- uptake was mediated by this mechanism. Moreover, the alleviation of barakol-stimulated Cl^- secretion by CFTR Cl^- channel blockers and bumetanide indicated that the cellular mechanism of barakol may involve the cAMP dependent pathway. Our finding was consistent with Cl^- secretion induced by other plant-derived bioactive compounds, especially flavonoid baicalein.

Baicalein has been shown to stimulate Cl^- secretion across rat colonic epithelium by activation of the cAMP-dependent apical Cl^- channel and the basolateral K^+ channels (Ko et al., 2001). From studies in human colonic cancer cells (T84 cells), baicalein was found to potentiate the Ca^{2+}-mediated Cl^- secretion via an accumulation of cAMP and activation of protein kinase A activity (Yue et al. 2004). However, the signaling mechanisms involving either intracellular Ca^{2+} or cAMP and protein kinase A in the mediation of barakol-stimulated Cl^- secretion could not be ruled out and are subject to further investigation.

Most of the naturally occurring laxatives exert their effects on the colonic epithelium by stimulating Cl^- secretion and/or inhibiting Na^+ absorption, resulting in an accumulation of fluid and subsequent increased colonic motility. The increased Cl^- secretion by anthranoid laxatives, anthraquinone and sennosides, is due to disruption of epithelial tight junctions, leading to increased permeability of the epithelium (Wanitschke, 1980; Ewe, 1980). Their chemical structures related to junctional disruption have not been indicated. Although the direct effect of barakol on tight junction permeability was not examined in the present study, an increase in junctional permeability was unlikely to account for barakol-stimulated Cl^- secretion, since changes in tissue conductance were relatively small. The suppressive actions of anthranoid laxatives on Na^+ absorption could result from decreased ATP production or a direct inhibition of the Na^+, K^+-ATPase activity in the basolateral membrane (Wanitschke, 1980; Wanitschke and Karbach, 1988). Reduced Na^+, K^+-ATPase activity would, in turn, decrease net Na^+ absorption across the epithelium. In this study, the barakol-induced increases in Isc were not affected by amiloride, indicating that the barakol effect was not due to activation of epithelial Na^+ channels. This finding may be due to the fact that Na^+ channels normally play a relatively minor role in Na^+ absorption across the proximal and distal colon in rats (Kunzelmann and Mall, 2002). However, a question still remains as to whether barakol may have some effects on Na^+-H^+ or Cl^--HCO_3^- exchange, since the activities of these electroneutral transporters could not be detected by measurement of Isc.

To identify whether the barakol response involved neurotransmitter release from nerves within the submucosal plexus or was the result of a direct effect on the epithelium, the tissues were pretreated with tetrodotoxin to block the neuronal Na^+ channel activity and inhibit action potential propagation. A substantial portion of basal Isc (36%) was inhibited following addition of tetrodotoxin, suggesting that it was sustained by endogenous release of neurotransmitters from the submucosal plexus. Pretreatment with tetrodotoxin inhibited the barakol-induced increase in Isc by 48%, confirming that barakol-stimulated Cl^- secretion was partially mediated through the activation of submucosal nerves. In contrast, the lack of effect of the muscarinic cholinergic receptor blocker atropine or the nicotinic receptor blocker hexamethonium on the barakol-stimulated Isc response argued against involvement of the acetylcholine-containing submucosal neurons in barakol action on Cl^- secretion in the rat. The tetrodotoxin-insensitive barakol response suggested that barakol may act directly on the colonic epithelial cells to stimulate Cl^- secretion.

To further test whether the barakol activation of Cl^- secretion was due to prostaglandin release, tissues were pretreated with the cyclooxygenase inhibitor, indomethacin, to block the synthesis of prostaglandins. Prostaglandins are normally released in response to a variety of stimuli. They have been known to activate Cl^- secretion directly via prostanoid receptors located in rat colonic epithelial cells (Brown et al., 1992). The present findings demonstrated that indomethacin inhibited the effect of barakol on Isc by 62%, suggesting that its sustained stimulatory effect on Cl^- secretion was partially mediated through the release of prostaglandins. The combined presence of indomethacin and tetrodotoxin nearly abolished (90%) the stimulatory effect of barakol on Cl^- secretion, indicating that the direct interaction of barakol with epithelial cells may account in part for its response.

The synthesis and release of prostaglandins are known to be part of the mechanisms of the anthranoid laxatives. Damage to epithelial cells caused by anthranoids induces the release of histamine and serotonin from monocytes, mast cells and other intestinal monocytes, leading to increased biosynthesis of prostaglandin (Yagi et al., 1988; Nijs et al., 1992). Prostaglandin release, in turn, accelerates the large intestine transit and alters fluid absorption and secretion (Leng-Peschlow, 1986). In addition, the release of inflammatory mediators, especially histamine, is known to stimulate Cl^- secretion in colonic epithelium (Traynor et al., 1993; Yue et al., 2004). However, inflammatory mediators were unlikely to be responsible for the actions of barakol since the histamine antagonist diphenhydramine did not alter the Isc response to barakol.

From the present findings, it seemed that barakol response was substantially mediated by enteric nerves within the submucosal plexus. Being insensitive to atropine and hexamethonium, barakol actions may be mediated by other neurotransmitter substances of the noncholinergic pathways. Candidates including vasoactive intestinal peptide (VIP), substance P and calcitonin gene-related peptide (CGRP) that are among several neurotransmitters present in enteric nerves and sensory neurons of the small and large intestine (Cooke, 1994). These substances have been shown to be capable of inducing Cl^-

and neurotransmitters (McGee et al., 1995; Schmidt et al., 1999). In salivary gland, stimulation of autonomic nerve lining closely to plasma cells has been shown to stimulate IgA secretion (Carpenter et al., 2004). The IgA is uptaked by binding to dimeric or polymeric immunoglobulin receptors located on the basolateral membrane, the receptors are endocytosed and transported to the apical membrane where it is cleaved to release into the mucosal solution as secretory IgA (sIgA) containing secretory component (SC). Although the secretory component was not determined in the present study, the IgA detected in the mucosal solution was likely the sIgA. Basically, the increase of total IgA in the intestinal lumen was due to an increase of sIgA. In contrast, the IgA in the basolateral solution resulted from the locally produced IgA from plasma cells. In our studies, barakol (1 mM) was shown to cause fivefold increase of the total IgA level in the apical solution and tenfold increase in the basolateral solutions of colonic epithelium. The IgA response by barakol was observed within 5 min suggesting the acute effect of barakol on the secretion of pre-existing IgA in the colonic tissues.

In our previous studies, barakol was found to antagonize the inhibitory effect on norepinephrine-induced smooth muscle contraction (Deachapunya et al., 2005). It was also shown in the present study that barakol stimulated Cl^- secretion partially through noncholinergic motor and sensory neurons. Substance P, vasoactive intestinal peptide and calcitonin gene-related peptide (CGRP) are deserved to be candidates for mediating the barakol-stimulated IgA secretion because these substances have been shown to activate IgA and Cl^- secretion in large intestine (Cooke, 1994; Schmidt et al., 1999). Although the exact mechanism of barakol stimulation of IgA secretion is unknown, the increase in IgA by barakol may possibly due to an indirect activation of plasma cells through modulation of noncholinergic neurotransmission or direct plasma cell activation, resulting in the release of IgA from plasma cells. The increase in IgA secretion reflected the increased serosal IgA. Furthermore, the more produced IgA may be transported across epithelial cells and released as secretory IgA in the mucosal solution. In this study, the increase in junctional permeability was unlikely to account for the leakiness of IgA through paracellular pathway since the tissue conductance was unchanged upon barakol stimulation. However, the transportation of IgA through paracellular pathway could not be excluded in this study.

Since barakol and other secretagogues have been shown to stimulate Cl^- secretion as well as IgA secretion, we further investigate whether the secretion of IgA in the colon is dependent on Cl^- secretion. In this study, the changes of bathing solutions from standard Ringer solution to Cl^--free Ringer solution produced a markedly increase in the mucosal IgA and small increase in the serosal IgA. The mechanism responsible for increased IgA secretion in Cl^--free solution was still unknown. However, application of barakol had no effect on the mucosal IgA, suggesting that barakol-stimulated IgA secretion across the colonic epithelium was Cl^- dependent. About twofold increase in the serosal IgA was observed in Cl^--free solution, indicating the capability of barakol to activate plasma cells under this condition.

In vivo study, the dramatically increase of the IgA level in caecal content was observed after intraperitoneally barakol injection, but not in normal saline injection, confirming the effect of barakol on IgA secretion *in vitro*. Although the IgA in blood circulation was not measured, the locally release of IgA from plasma cells was suspected. Previous study has demonstrated that intraperitoneal injection of barakol inhibits the orally bacteria *Salmonella* enteritidis colonization and translocation to intestinal Peyer's patches and spleen (Poonyachoti et al., 2004). These findings reflect the protective role of local IgA on bacterial infection.

IgA is the major immunoglobulin in the mucosal colon and appears to be the first line for host defense mechanism against external pathogens. The increased IgA level in response to barakol stimulation both *in vitro* and *in vivo*, suggesting an important role of barakol in enhancement of non-specific mucosal immunity. These findings may be useful for therapeutic use of barakol in the protection of human and animals from gastrointestinal bacterial and viral infection.

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FUTURE DIRECTIONS

1. To study the modulatory effect of barakol on sensory neuron-mediated chloride secretion in rat colon
2. To study the intracellular signaling pathways of barakol-stimulated Cl^- secretion.
3. To study the regulation and mechanism of secretory IgA secretion in intestine

REFERENCES

Anderson MP, Sheppard DN, Berger HA and Welsh MJ (1992) Chloride channels in the apical membrane of normal and cystic fibrosis airway and intestinal epithelia. *Am J Physiol* 263:L1-L14.

Arunlakshana O (1949) Pharmacological study of the leaves of *Cassia siamea*. *Siriraj Hosp.Gaz.*,1: 434-444.

Brown DR, Parsons AM and O'Grady SM (1992) Substance P produces sodium and bicarbonate secretion in porcine jejunal mucosa through an action on enteric neurons. *J Pharmacol Exp Ther* 261:1206-1212.

Bycroft BW, Hassanali – walji A, Johnson AW and King TJ (1970) The structure and synthesis of barakol: a novel dioxaphenalene derivative from *Cassia siamea*. *J Chem Soc* 12:1686-1689.

Carpenter GH, Proctor GP, Ebersole LE and Garrett, JR (2004) Secretion of IgA by rat parotid and submandibular cells in response to autonomimetic stimulation in vitro. *Int Immunopharmacol* 4: 1005-1014.

Cooke HJ (1994) Neuroimmune signaling in regulation of intestinal ion transport. *Am J Physiol* 266:G167-G178.

Deachapunya C, Thongsaard W and Poonyachoti S (2005) Barakol suppresses the spontaneous smooth muscle contraction in rat ileum. *Journal of Ethnopharmacology* 101: 227-232.

Dharmsathaphorn K, Mandel KG, Masui H and McRoberts JA (1985) Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line. Direct participation of a basolaterally localized Na^+,K^+,Cl^- cotransport system. *J Clin Invest* 75(2):462-471.

Esfandyari T, Macnaughton WK, Quirion R, St Pierre S, Junien JL and Sharkey KA (2000) A novel receptor for calcitonin gene-related peptide (CGRP) mediates secretion in the rat colon: implications for secretory function in colitis. *FASEB J* 14:1439-1446.

Ewe K (1980) The physiological basis of laxative action. *Pharmacol* 20 (Suppl. 1):2-20.

Hassanali - Walji A, King TJ and Wallwork SC (1969) Barakol, a novel dioxzphenalene derivative from *Cassia siamea*. *J Chem Soc - Chem Commun* 12:678.

Kimata H, Yoshida A., Ishioka C et al, (1996) Vasoactive intestinal peptide enhances immunoglobulin production and growth in human plasma cells via mechanisms that may involve protein kinase C. J Clin Endocrinol Metab 81: 3024-3032.

Kinghorn AD and Balandrin MF (1992) Human traditional agents from plants. American Chemical Society, Washington DC.

Ko WH, Law VW, Yip WC, Yue GG, Lau CW, Chen ZY and Huang Y (2002) Stimulation of chloride secretion by baicalein in isolated rat distal colon. *Am J Physiol* 282 (3):G508-G518.

Kunzelmann K and Mall M (2002) Electrolyte transport in the mammalian colon: Mechanisms and implications for disease. *Physiol Rev* 82:245-289.

Leng-Peschlow E., 1986. Dual effect of orally administered sennosides on large intestine transit and fluid absorption in the rat. *J Pharm Pharmacol* 38:606-610.

McGee D, Eran M, McGhee JR and Freier S (1995) Substance P accelerates secretory component-mediated transcytosis of IgA in the rat intestine. Advances in Mucosal Immunology Edited by J Mestecky et al., 643-646.

Nijs G, De Witte P, Geboes K and Lemli J (1992) Influence of rhein anthrone and rhein on small intestine transit in rats: Evidence of prostaglandin mediation. *Eur J Pharmacol* 218:199-203.

Poonyachoti S, Kramomtong I, Niyomtham W and Deachapunya C (2004) Effect of barakol on the secretory immunoglobulin A secretion against *Salmonella* Enteritidis translocation to the systemic organs of Wistar rat. *Thai J Vet Med* 34(2): 133-134.

Poonyachoti S, Thongsaard W and Deachapunya C (2002) The prokinetic effects of barakol in rat intestine. *The Pharmacologist* 44(2):A35-36 (abstract).

Riegler M, Castagliuolo I, So PT, Lotz M, Wang C, Wlk M, Sogukoglu T, Cosentini E, Bischof G, Hamilton G, Teleky B, Wenzl E, Matthews JB and Pothoulakis C (1999) Effects of substance P on human colonic mucosa in vitro. *Am J Physiol* 276:G1473-G1483.

Rojas R and Apodaca G (2002) Immunoglobulin transport across polarized epithelial cells. Nature Reviews Molecular Cell Biology 3: 944-956.

Schmidt PT, Eriksen L, Loftager M, Rasmussen TN and Holst JJ (1999) Fast acting nervous regulation of immunoglobulin A secretion from isolated perfused porcine ileum. *Gut* 45: 679-685.

Schultz BD, Singh AK, Devor DC and Bridges RJ (1999) Pharmacology of CFTR chloride channel activity. *Physiol Rev* 70 (Suppl. 1):S109-S144.

Sheppard DN and Welsh MJ (1992) Effect of ATP-sensitive K^+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J Gen Physiol* 100:573-591.

Stanisz AM, Scicchitano R, Dazin P, et al, (1987) Distribution of substance P receptors on murine spleen and Peyer's patch T and B cells. *J Immunol* 139: 749-754.

Suwan G, Sudsuang R, Ghumma - Upakorn D and Werawong C (1992) Hypertensive effects of barakol estracted from leaves of *Cassia siamea* Lam. in rats and cats. *Thai J Physiol Sci* 5:53-65.

Thongsaard W, Chainakul S, Bennett GW and Marsden CA (2001) Determination of barakol extracted from *Cassia siamea* by HPLC with electrochemical detection. *J Pharmaceu Biomed Ana* 25:853-859.

Thongsaard W, Deachapunya C, Pongsakorn S, Boyd EA, Bennett GW and Marsden CA (1996) Barakol: A potential anxiolytic extracted from *Cassia siamea. Pharmacol Biochem Behav* 53:753-758.

Thongsaard W, Pongsakorn S, Sudsuang R, Bennett GW and Marsden CA (1997) Barakol, a natural anxiolytic, inhibits striatal dopamine release but not uptake in vitro. *Eur J Pharmacol* 319:157-164.

Tongroach P, Jantarayota P, Tantisira B, Kunluan P, Tongroach C and Chaichantipyuth C (1992) Barakol, a neuroactive compound from *Cassia siamea*. In *Proceeding of the First JSPS-NRCT Joint Seminar in Pharmaceutical Sciences: Advance in Research on Pharmacologically Active Substances from Natural Sources* pp 21, Chiangmai, Thailand.

Traynor TR, Brown DR and O'Grady SM (1991) Regulation of ion transport in porcine distal colon: effects of putative neurotransmitters. *Gastroenterology* 100:703-710.

Traynor TR, Brown DR and O'Grady SM (1993) Effects of inflammatory mediators on electrolyte transport across the porcine distal colon epithelium. *J Pharmacol Exp Ther* 264:61-66.

Wanitschke R (1980) Influence of rhein on electrolyte and water transfer in the isolated colonic mucosa. *Pharmacol* 20 (Suppl. 1):21-26.

Wanitschke R and Karbach U (1988) Influence of rhein on rat colonic Na^+,K^+-ATPase and permeability in vitro. *Pharmacol* 36 (Suppl. 1):98-103.

Yagi T, Miyawaki Y, Nishikawa T, Yamauchi K and Kuwano S (1988) Involvement of prostaglandin E-like material in the propulsive action of rhein anthrone, the intraluminal active metabolite of sennoside A and B in mice. *J Pharm Pharmacol* 40:27-30.

Yue GGL, Yip TWN, Huang, Y and Ko WH (2004) Cellular mechanism of Ca^{2+}-mediated Cl^- secretion by the flavonoid baicalein in intestinal epithelia. *J Biol Chem* 279:39310-39316.

OUTPUT ที่ได้จากโครงการ

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 2 ฉบับ

   - Deachapunya, C., Thongsaard, W. and Poonyachoti, S. Barakol suppresses norepinephrine-induced inhibition of spontaneous longitudinal smooth muscle contractions in isolated rat small intestine. *Journal of Ethnopharmacology*, 101(1-3): 227-232, 2005. (impact factor ปี 2004 เท่ากับ 1.420)
   - Deachapunya, C., Thongsaard, W., Poonyachoti, S. and Krishnamra, N. Barakol extracted from *Cassia siamea* stimulates chloride secretion. *Journal of Pharmacology, Experimental and Therapeutics* 314(2): 732-737, 2005. (impact factor ปี 2004 เท่ากับ 4.335)

2. ผลงานตีพิมพ์บทคัดย่อในวารสารวิชาการในประเทศ จำนวน 1 ฉบับ

   - Poonyachoti S, Kramomtong I, Niyomtham W and Deachapunya C (2004) Effect of barakol on the secretory immunoglobulin A secretion against *Salmonella* Enteritidis translocation to the systemic organs of Wistar rat. *Thai J Vet Med* 34(2): 133-134.

3. เสนอผลงานเรื่อง Barakol extracted from *Cassia siamea* stimulates chloride secretion in rat colon ในการประชุม นักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สำนักงานกองทุนสนับสนุนการวิจัย ณ โรงแรมเฟลิกซ์ จังหวัดกาญจนบุรี ระหว่างวันที่ 14 -16 มกราคม 2548

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Barakol suppresses norepinephrine-induced inhibition of spontaneous longitudinal smooth muscle contractions in isolated rat small intestine

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Abstract

The present study aimed to investigate the purgative effects of barakol, the purified extract of *Cassia siamea* Lam., on the longitudinal smooth muscle contractions of the rat ileum. The extract increased the force of spontaneous muscle contractions in a concentration-dependent manner (EC_{50} = 0.3 mM). Saxitoxin (0.3 μM) abolished the stimulatory effects of barakol, a result indicating a neural mechanism of action. In addition, atropine (10 μM) but not propanolol (10 μM) or phentolamine (10 μM), partially inhibited barakol-induced smooth muscle contractions suggesting that cholinergic nerves were involved. The motor effects of barakol were further examined in muscle strips treated with catecholamines to suppress spontaneous contractile activity and decrease muscle tone. Norepinephrine or dopamine (10 μM) decreased the amplitude of spontaneous contractions by 72% and 18%, respectively. Pretreatment of the tissues with barakol (1 mM) significantly decreased the inhibitory effect of norepinephrine by 60%, but not that of dopamine. Its ability to potentiate atropine- and saxitoxin-sensitive contractions and inhibit the antimotility actions of norepinephrine suggests that barakol may increase longitudinal smooth muscle contractions by decreasing the inhibitory effect of norepinephrine on excitatory cholinergic motor neurons. Barakol may produce a purgative action in small intestine which may be clinically important in patients with intestinal hypomotility disorders.

Keywords: *Cassia siamea*; Intestinal motility; Constipation

1. Introduction

Barakol is a biologically active constituent extracted from the leaves and flowers of *Cassia siamea* Lam. (or *Senna siamea* Lam. Irwin & Barneby) of the family *Caesalpiniaceae*. This plant is widely cultivated in southeast Asia including Thailand and traditionally used to treat insomnia, diabetes, fever, hypertension and constipation (Satyavati et al., 1979; Kinghorn and Balandrin, 1992). Alcoholic extracts of *Cassia siamea* have been shown to possess central nervous system (CNS) depressant activity, decrease spontaneous locomotor activity, increase smooth muscle tone (Arunlakshana, 1949) and decrease blood pressure (Mokasmit, 1981). Barakol was first isolated by Hassanali-Walji et al. (1969) and its chemical structure (3α, 4-dihydro-3α, 8-dihydroxy-2, 5-dimethyl-1, 4-dioxaphenalene or 2,5-dimethyl-3α*H*-pyrano-[2,3,4-de]-1-benzopyran-3α,8-diol) was identified by Bycroft et al. (1970). In animal models, barakol produces hypotension (Suwan et al., 1992), suppresses serotonergic activity as shown by decreasing 5-hydroxytryptophan-induced head shake behavior (Tongroach et al., 1992) and possesses anxiolytic activity on the elevated plus maze, a behavioral test for anxiolytic drugs (Thongsaard et al., 1996). Barakol also suppresses K^+-stimulated endogenous dopamine release from striatal slices of the rat brain (Thongsaard et al., 1997). These data suggest that barakol can alter CNS activity. However, little is known about its

Abbreviations: CNS, central nervous system; DA, dopamine; ENS, enteric nervous system; NE, norepinephrine

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ffects on the peripheral nervous system, another potential e of barakol action.

Gastrointestinal secretory and motor functions are regulated by the enteric nervous system (ENS; Guyton and ll, 1996). The myenteric ganglionated plexus (also termed erbach's plexus) within the ENS modulates propulsive tor activity along the intestinal tract. Stimulation of enteric olinergic neurons within the gut wall as well as those originating in the vagus increases intestinal motility by promoting ristalsis. On the other hand, noradrenergic neurotransmission in the gut is generally associated with decreased motility ange, 1996; Curry and Tatum-Butler, 1996). Decreases intestinal motility induced by psychological stress or opathic constipation may be mediated by norepinephrine E) and possibly the related catecholamine, dopamine (DA; ukada et al., 2002). Catecholamines may reduce motility rough direct actions on intestinal myocytes or by suppressing excitatory neurotransmission to smooth muscle (McEvoy, 00). As barakol modulates dopaminergic transmission in e CNS, it is possible that it may alter the inhibitory effects catecholamines on intestinal motor function. Therefore, e aim of the present study was to investigate the effect barakol on spontaneous longitudinal smooth muscle contractions in the isolated rat ileum. An additional objective s to determine the mechanism by which barakol alters e inhibitory actions of NE and DA on smooth muscle ntractility.

Materials and methods

. Drugs and chemicals

Barakol was extracted and purified from *Cassia siamea* in r laboratory as previously described (Thongsaard et al., 01). Briefly, a *Cassia siamea* plant was collected from e Ladkrabang area of Bangkok, Thailand in August. The rbarium specimen was authenticated by Wongpakam S., plant taxonomist, deposited, and given voucher specimen mber A011432 by the Department of Botany, Faculty of ience, Chulalongkorn University, Bangkok, Thailand. The ves and flowers were cut into small pieces and boiled twice).5% sulfuric acid for 30 min. The mixture was blended, filtered, and alkalinized with concentrated sodium bicarbonate d subsequently extracted with chloroform. The chloroform tract was further concentrated with 5% acetic acid and neutralized with 25% ammonium hydroxide. The crude barakol tract was obtained as greenish crystallized yellow needles th 0.3% yield. Concentrated hydrochloric acid was finally ded to obtain barakol hydrochloride and the mixture was ed by vacuum filtration to form yellowish crystals of anhydrous barakol hydrochloride. The purity of the compound s confirmed by thin layer chromatography on silica gels d nuclear magnetic resonance. When anhydrous barakol drochloride was dissolved in water, it converted to barakol a solution at pH 3–4 in the stock concentration of 50 mM. Prior to each experiment, barakol was freshly dissolved in normal saline solution, kept on ice and in the dark to prevent oxidation, and used within 3 h after preparation.

Arterenol bitartrate (norepinephrine), atropine sulfate, acetylcholine, dopamine hydrochloride, propanolol, phentolamine and saxitoxin were purchased from Sigma Chemical, St. Louis, MO, USA. Other chemicals and analytical grade salts were purchased from Fisher Scientific, Loughborough, UK. All drugs were dissolved in distilled water and maintained in concentrated stock solutions. Aliquots were diluted immediately before the start of each experimental session.

2.2. Animals

Male Wistar rats (250–300 g) were obtained from the National Laboratory Animal Center, Thailand. They were housed in stainless-steel cages in a room with a 12 h light:12 h dark cycle and allowed access to food (National Laboratory Animal Center, Thailand) and tap water ad libitum. All animals were sacrificed by decapitation. After a laparotomy incision, a portion of the ileum was removed and placed in an oxygenated physiological salt solution approximating the composition of rat extracellular fluid (composition in mM: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 0.5 $MgCl_2$, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 11 D-glucose; pH 7.4).

2.3. Measurement of smooth muscle contractility

Ileal segments were longitudinally cut along the antimesenteric border and placed in oxygenated ice-cold physiological salt solution. They were pinned out as a flap, with the mucosa uppermost and a 3 × 10 mm muscle strip was cut parallel to the longitudinal muscle layer of the ileum. The strip therefore contained the longitudinal smooth muscle, from which isometric recordings were made, as well as the circular smooth muscle and both the myenteric and submucosal plexuses.

Intestinal strips were oriented in the plane of the longitudinal muscle and mounted in 15 ml organ baths containing physiological salt solution that was gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C. Tissues were mounted under an initial tension of 9.8 millinewtons (mN). Strips were equilibrated for 45 min and the bathing media was changed every 15 min. Mechanical activity was recorded isometrically with a strain gauge force transducer (Maclab Model FT-100, AD Instruments, NSW, Australia) connected to a BRIDGE amplifier and a MacLab® 4S A/D converter (AD Instruments) and monitored with a 400 MHz PowerPC MacIntosh computer.

The average peak amplitude and frequency of contractions occurring after administration of each drug or drug concentration was determined and compared to average contraction amplitudes measured prior to drug administration. Due to the acidity of the barakol solution, the effect of 0.1 M hydrochloric acid was determined on spontaneous muscle contractions as a drug-free vehicle control. Hydrochloric acid, in a lume equivalent to that of the barakol solution (200 μl), did t produce changes in either the amplitude or frequency of ontaneous muscle contractions. In experiments with cate-olamines, 1 mM barakol was added to the bathing medium min before addition of 10 μM NE or DA. In experiments h pharmacological blocking drugs, 10 μM of propanolol, entolamine, or atropine or 0.3 μM of saxitoxin was admin-ered 10 min before addition of barakol or catecholamines.

. Data analysis

The results were expressed as mean ± S.E.M, with n noting the number of tissue preparations tested and N the al number of animals used in each experiment. Significant ferences between a single control and treatment means re analyzed by paired or unpaired two-tailed Student's t-ts. $P<0.05$ was considered statistically significant. Data re analyzed using the PRISM software program (Graph-d Software, Inc., San Diego, CA, USA).

Results

. Effects of barakol on spontaneous longitudinal ooth muscle contractions

Intestinal strips oriented in the plane of the longitudinal scle exhibited spontaneous, phasic contractions without nificant oscillations in tone. After a 45 min equilibration iod, the average amplitude of longitudinal contractions s 13.23 ± 0.10 mN relative to baseline tension in a repre-tative group of 78 tissues from 16 rats.

Barakol, at a bath concentration of 1 mM, increased the ce of muscle contractions which reached a maximal ten-n within 1–2 min without significant changes in contrac-n frequency. The maximal force of contraction was main-ed up to 5 min and then gradually returned to pre-drug eline values (Table 1, Fig. 1A). Barakol increased sponta-us contractions of ileal strips in a concentration dependent nner with an EC_{50} value of 0.3 mM (n = 4–22 tissues, 4–8 rats) (Fig. 2). It was 600-fold less potent in increas- contractions than acetylcholine, which had an EC_{50} e of 0.5 μM (n = 4–15 tissues, N = 4–6 rats). Barakol was

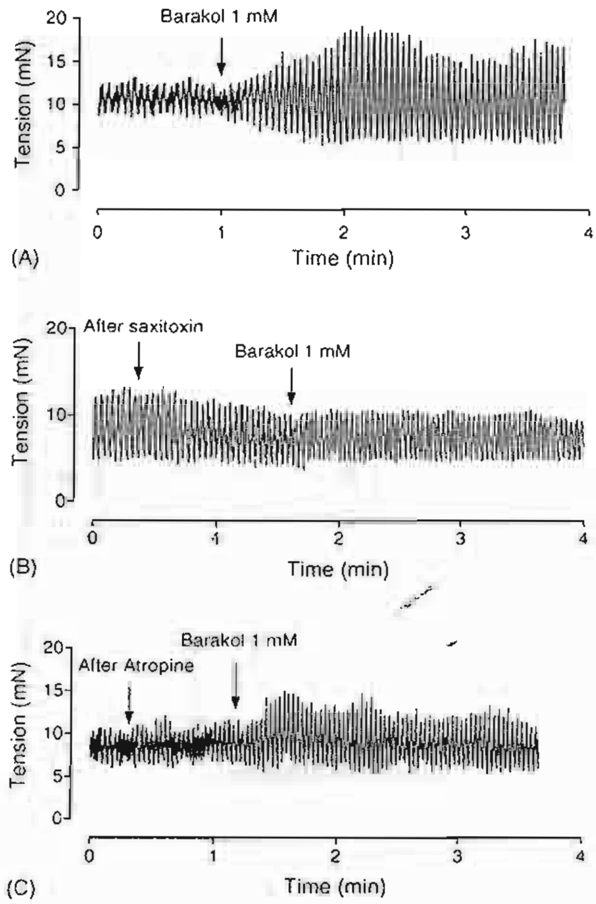


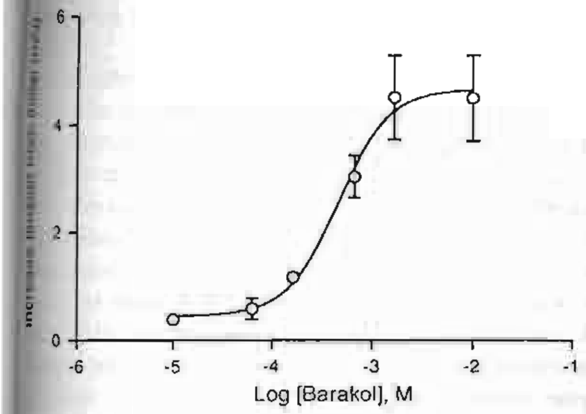
Fig. 1. Representative tracings of spontaneous ileal smooth muscle contractions in response to barakol treatment. (A) Effect of 1 mM barakol alone. (B) Lack of effect of barakol in a tissue pretreated with 0.3 μM saxitoxin, a neural Na^+ channel blocker. (C) Partial inhibition of barakol action in a tissue pretreated with the muscarinic cholinergic antagonist atropine (10 μM). Records are representative of responses in 4–28 tissues from 4–9 rats.

also less effective in increasing contraction amplitude than acetylcholine. At a concentration of 1 mM, it produced a 48.03 ± 8.76% increase in contraction amplitude relative to baseline values measured prior to barakol addition (n = 20 tissues, N = 5 rats). In comparison, 10 μM acetylcholine produced maximal contractions that were 180.90 ± 26.29% over baseline (n = 8 tissues, N = 4 rats). Barakol (1 mM) did not

le 1
Effect of various drugs on the amplitude of longitudinal smooth muscle contractions in rat ileal strips

nt	N rats	Before drug treatment[a]	After drug treatment[a]	Average change[a]
tylcholine (10 μM)	4	12.35 ± 1.08	34.69 ± 3.63*	22.34 ± 2.84
akol (1 mM)	5	9.41 ± 0.89	13.92 ± 0.78*	4.52 ± 0.78
epinephrine (10 μM)	6	11.66 ± 2.65	3.23 ± 1.08*	−8.33 ± 2.84
amine (10 μM)	6	10.29 ± 0.88	8.43 ± 0.98*	−1.86 ± 0.69
pine (10 μM)	9	12.45 ± 0.69	10.49 ± 0.59*	−1.96 ± 0.39
anolol (10 μM)	7	12.74 ± 0.78	12.54 ± 0.78	−0.20 ± 0.01
ntolamine (10 μM)	4	13.62 ± 1.18	13.97 ± 1.18	0.29 ± 0.20
itoxin (0.3 μM)	4	7.64 ± 0.88	7.55 ± 0.88	−0.01 ± 0.01

Values represent the mean ± S.E.M amplitude of spontaneous smooth muscle contractions, expressed in millinewtons.
* $P<0.05$ compared to contractile force before drug treatment (Student's t-test).



.1 Concentration–effect relationship of barakol in increasing the ampli- of isometric contractions in rat ileal strips. Each point represents the ± S.E.M change in isometric tension in response to each concentration akol relative to the amplitude of spontaneous contractions occurring drug administration. The data were obtained in 4–15 tissues from 4–9

the concentration–effect relationship of acetylcholine reasing intestinal contractions.

Effects of pharmacological blockers on ntaneous longitudinal smooth muscle contractions

To assess the mechanisms underlying the procontractile of barakol on intestinal strips, its action was exam- in tissues pretreated with different pharmacological ers. At 0.3 μM, the axonal conduction blocker saxi- n had no effect on the amplitude of basal contractions; ever, it completely inhibited the stimulatory effect of kol on muscle contractions by 98% ($n=4$ tissues, $N=4$). At a bath concentration of 10 μM, neither the α- noceptor blocker phentolamine nor the β-adrenoceptor ker propanolol altered the amplitude of smooth mus- contractions under baseline conditions or after treatment barakol. The muscarinic cholinergic receptor blocker ine (10 μM) decreased the amplitude of spontaneous actions by 14.60 ± 2.50% of initial values ($n=28$ tis- $N=9$ rats; Table 1) and abolished the contractile effect of lcholine. Barakol-induced spontaneous muscle contrac- were decreased by 41.80 ± 1.20% in tissues pretreated atropine ($n=28$ tissues, $N=9$ rats; Fig. 1C).

Effects of barakol on norepinephrine- or mine-suppressed smooth muscle contractions

The catecholamines NE and DA, at bath concentrations ging from 0.1–10 μM, decreased the force of sponta- us contractions in a concentration-dependent manner. 10 μM, NE and DA decreased contractile force by 57 ± 14.94% ($n=9$ tissues, $N=6$ rats) and 17.67 ± 3.93%, =14 tissues, $N=6$ rats) respectively (Table 1). Pretreatment tissues with 1 mM barakol decreased the inhibitory action

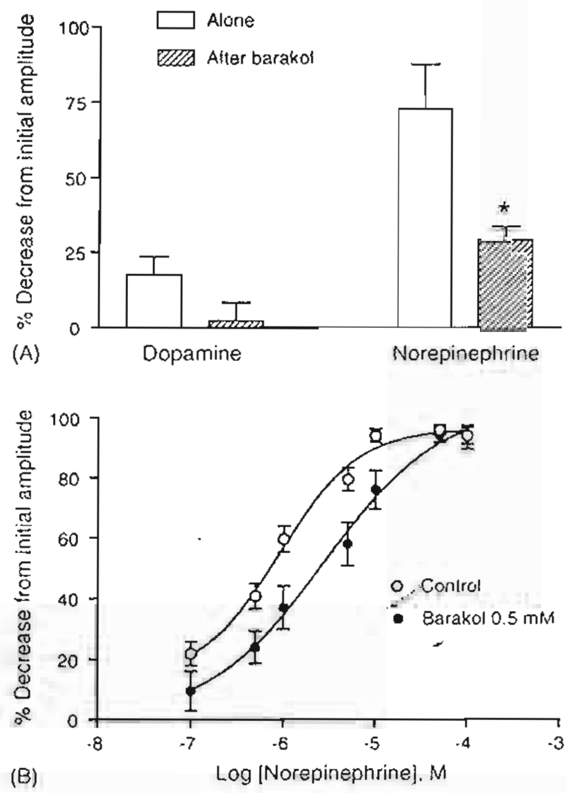


Fig. 3. (A) Effect of barakol on muscle contractions suppressed by catecholamines. (A) Dopamine (DA) or norepinephrine (NE) at a bath concentration of 10 μM inhibited spontaneous muscle contractions by 18% and 73%, respectively. In tissues pretreated with 1 mM barakol, DA or NE respectively inhibited the amplitude of spontaneous muscle contractions by 2% and 29% ($P<0.05$) relative to the contraction amplitude determined prior to catecholamine administration. (B) The concentration–response relationship of NE in depressing the amplitude of spontaneous contractions in tissues untreated (○) or pretreated (●) with 0.5 mM barakol. Barakol produced a 3-fold dextral shift in the NE concentration-effect curve without diminishing the maximum effect produced by NE. Values represent the mean ± S.E.M percentage of inhibition produced by NE based on the amplitude of the spontaneous contractions measured prior to NE addition.

of 10 μM NE from 72.57 ± 14.94% ($n=9$ tissues, $N=6$ rats) to 29.02 ± 4.32% ($n=7$ tissues, $N=4$ rats, $P<0.05$). In contrast, barakol pretreatment did not significantly decrease the inhibitory action of 10 μM DA (Fig. 3).

At a bath concentration of 0.5 mM, barakol decreased the potency of NE in inhibiting spontaneous contractions (EC_{50} for NE in absence and presence of barakol = 0.92 and 2.80 μM, respectively; $n=7$–11 tissues, $N=5$–7 rats, Fig. 3B). However, it did not alter the maximum inhibitory effect of NE on spontaneous muscle contractions (96.41 ± 3.76 and 104.2 ± 8.64% in absence and presence of barakol, respectively; $n=7$–11 tissues, $N=5$–7 rats). Barakol had no significant effect on either the potency and maximal inhibitory effect of DA on contraction amplitude (DA EC_{50} = 12.4 and 15.3 μM, maximal inhibition of 97.81 ± 12.21 and 102.41 ± 4.10%, respectively in absence and presence of barakol; $n=5$ tissues, $N=5$ rats).

Discussion

Anthranoids derived from herbal plants are widely used as atives. The most well-known anthranoid laxatives, the sen-des, are extracted from the leaves, roots, pods or even the ole plant of the species including *Cassia siamea*, *Cassia* a and *Cassia acutifolia*. Other bioactive anthranoids may m after metabolic processing by commensal bacteria in the e intestine, after extraction from different parts of *Cas-* plants, or as a result of the method chosen for extraction nz, 1993). Barakol, extracted from young leaves of the *ssia* spp. plant, is a chromone that is not related chemically nthranoids (Bycroft et al., 1970). It alters dopaminergic erotonergic transmission in the CNS (Thongsaard et al., 7; Grider et al., 1998). Given the overlapping complement eurotransmitters in the CNS and ENS (Hansen, 2003), hypothesized that it would potentially alter enteric neu-ansmission as well. Indeed, receptors for plant-derived chotropic drugs such as the cannabinoids or opioids are ent in the ENS and mediate gastrointestinal function twee, 2001; Kromer, 1990). Although *Cassia siamea* t preparations have been traditionally used as laxative s, the barakol extract has never been assessed for laxa-activity.

The use of intestinal muscle strips in this in vitro study per-ed us to examine the direct intestinal actions of barakol e absence of drug-biotransforming bacteria or metabolic ns. The observation that barakol affects intestinal motor tion in vitro indicates that it is biologically active per d does not require bioactivation by the intestinal flora tabolic processes. This stands in contrast to the anthra-s which must be converted to biologically active aglycon one metabolites by intestinal flora (De Witte and Lemli,). However, information pertaining to pharmacokinetics pharmacodynamics of barakol in vivo will be needed to port its potential therapeutic applications.

Using the isolated longitudinal smooth muscle contrac-assay, barakol was found to augment spontaneous muscle actions, but was considerably less potent and effective acetylcholine. Furthermore, it did not alter the potency aximum contractile effects of acetylcholine. This finding ests that barakol does not impair cholinergic neurotrans-ion in intestinal strips. Indeed, the contractile effects rakol were inhibited by both saxitoxin and atropine, ult indicating that barakol may increase the force of oth muscle contractions in part by facilitating cholinergic mission at myoneural junctions. Its action may be medi-by excitatory neurotransmitter substances in addition etylcholine. Substance P, serotonin, and histamine are ng several enteric neurotransmitters which stimulate con-ions in gastrointestinal smooth muscle (Hansen, 2003). involvement of excitatory, non-cholinergic transmitters rakol action is a subject worthy of future study.

Spontaneous muscle contractions were not affected by d β-adrenergic antagonists and it is therefore unlikely endogenous NE tonically modulates motor function, at least in the intestinal strip preparation. Nevertheless, adrenergic nerve fibers innervate both myenteric neurons and the intestinal smooth muscle and NE affects several gastrointestinal functions, including intestinal motility (Liu and Coupar, 1997). Indeed, NE has been implicated as a causal factor in some dysmotility syndromes, such as postoperative ileus or constipation. NE inhibited the amplitude of spontaneous contractions to a greater degree than DA. The antimotility effects of NE have been attributed to direct relaxant actions on smooth muscle cells and an indirect action to decrease the release of acetylcholine and other excitatory transmitters at myoneural synapses (Bolton, 1979). Barakol significantly decreased the inhibitory effect of NE but not DA on contraction amplitude. It slightly decreased the potency, but not the maximal effect produced by NE and did not alter the concentration–effect relationship for DA in depressing smooth muscle contractions. It is possible that barakol might act either as a weak adrenoceptor blocker or more likely, as a functional antagonist of NE action, but this hypothesis must be tested through further pharmacological analysis.

In general, the mechanisms of action of stimulant laxatives including the anthranoid derivatives encompass two independent mechanisms: increases in colonic motility, and alterations in colonic absorption and secretion (Leng-Peschlow, 1986). Barakol action was not investigated in a colonic smooth muscle preparation, but it is conceivable that barakol could possess antimotility activity in this gut segment as it does in the ileum. The effects of barakol on colonic absorption and secretion are presently under investigation. Furthermore, it remains to be determined whether the stimulatory effect of barakol on smooth muscle contractions in vitro can be extrapolated to an acceleration of intestinal transit in vivo. If it is proven to stimulate coordinated intestinal motor activity, barakol may represent an herbal laxative that is superior to the anthranoids. Indeed, the biologically-active anthranoid rhein anthrone appears to increase gut motility at least in part by damaging epithelial cells in the intestinal mucosa which in turn elicits the release of motility-enhancing cytokines or inflammatory mediators such as histamine and serotonin from monocytes, mast cells and other intestinal immunocytes (Nijs et al., 1992; Siegers et al., 1993). Epithelial cell damage may be involved in the increased incidence of colonic adenocarcinoma that has been associated with the chronic use of anthranoid laxatives (Siegers et al., 1993).

In summary, the results of this study clearly demonstrate that barakol increases the amplitude of spontaneous smooth muscle contractions in the isolated rat ileum. These results provide a pharmacological basis for the use of barakol, a biologically active compound extracted from *Cassia siamea*, as an herbal medicine for treatment of intestinal hypomotility. Although the precise mechanism(s) underlying the promotility actions of barakol require further characterization, it may be concluded from this study that barakol may act by promoting the release of stimulatory neurotransmitters such as acetylcholine or suppressing the effects of inhibitory transmitters such as norepinephrine on intestinal smooth

...contractility. Adrenergic receptor blockers adminis... in combination with cholinergic agonists have effec... been used to alleviate postperative ileus or constipation ...gston and Passaro, 1990). The promotility actions of ... may have clinical importance, particularly in dys... states involving elevated sympathetic outflow to the ...al tract, such as those secondary to stress and chronic ...thic constipation.

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...rences

...ana, O., 1949. Pharmacological study of the leaves of *Cassia* ... Siriraj Hospital Gazette 1, 434–444.

... T.B., 1979. Mechanisms of action of neurotransmitters and other ...nces on smooth muscle. Physiological Reviews 59, 606–616.

... B.W., Hassanali-walji, A., Johnson, A.W., King, T.J., 1970. The ... and synthesis of barakol: a novel dioxaphenalene derivative ... *Cassia siamea*. Journal of Chemical Society 12, 1686–1689.

..., C.E., Tatum-Butler, D., 1996. Laxative products. In: Handbook ...onprescription Drugs, vol. 225–244. 11th ed. American Pharma...al Association, Washington, DC, 83–97.

..., P., Lemli, L., 1990. The metabolism of anthranoid laxatives. ...togastroenterology 37, 601–605.

..., 1993. The senna drug and its chemistry. Pharmacology 47 ...pl. 1), 2–6.

...., Foxx-Orenstein, A.E., Jin, J.G., 1998. 5-Hydroxytryptamine ...ceptor agonists initiate the peristaltic reflex in human, rat, and ...a pig intestine. Gastroenterology 115, 370–380.

... A.C., Hall, J.E., 1996. The autonomic nervous system: the ...al medulla. In: Textbook of Medical Physiology, 9th ed. W.B. ...ders Company, Philadelphia, Pensylvania, pp. 769–781.

... M.B., 2003. The enteric nervous system III: a target for phar...ological treatment. Pharmacological Toxicology 93, 1–13.

...-Walji, A., King, T.J., Wallwork, S.C., 1969. Barakol, a novel ...phenalene derivative from *Cassia siamea*. Journal of Chemical ...ety and Chemistry Communication 12, 678.

..., A.D., Balandrin, M.E., 1992. Human Medicinal Agents from ... American Chemical Society, Washington, DC.

Kromer, W., 1990. Endogenous opioids, the enteric nervous system and gut motility. Digestive Diseases and Sciences 8, 361–373.

Lange, R.L., 1996. Antidiarrheal products. In: Handbook of Nonprescription Drugs, vol. 245–260, 11th ed. American Pharmaceutical Association, Washington, DC.

Leng-Peschlow, E., 1986. Dual effect of orally administered sennosides on large intesine transit and fluid absorption in the rat. Journal of Pharmacy and Pharmacology 38, 606–610.

Liu, L., Coupar, I.M., 1997. Characterization of pre- and post-synaptic α-adrenoreceptors in modulation of rat ileum longitudinal and circular muscle activities. Naunyn-Schmiedberg's Archrive of Pharmacology 356, 248–256.

Livingston, E.H., Passaro Jr., E.P., 1990. Postoperative ileus. Digestive Diseases and Sciences 35, 121–132.

McEvoy, G.K., 2000. AHFS Drug Information 2000. American Society of Health-System Pharmacists, Bethesda, MD.

Mokasmit, M., 1981. Hypertensive effects of Thai medicinal plants. Newsletter of National Research Council of Thailand 22, 3–4.

Nijs, G., de Witte, P., Geboe, K., Lemli, J., 1992. Influence of rhein anthrone and rhein on small intestine transit rate in rats: evidence of prostaglandin mediation. European Journal of Pharmacology 218, 199–203.

Pertwee, R.G., 2001. Cannabinoids and the gastrointestinal tract. Gut 48, 859–867.

Satyavati, G.V., Roana, M.K., Sharma, M., 1979. Medicinal Plants of India. Indian Council of Medical Research, New Delhi.

Siegers, C.P., von Hertzberg-Lottin, E., Otte, M., Schneider, B., 1993. Anthranoid laxative abuse-a risk for colorectal cancer? Gut 34, 1099–1101.

Suwan, G., Sudsuang, R., Ghumma-Upakorn, D., Werawong, C., 1992. Hypertensive effects of barakol extracted from leaves of *Cassia siamea* Lam. in rats and cats. Thai Journal of Physiological Science 5, 53–65.

Thongsaard, W., Chainakul, S., Bennett, G.W., Marsden, C.A., 2001. Determination of barakol extracted from *Cassia siamea* by HPLC with electrochemical detection. Journal of Pharmaceutical and Biomedical Analysis 25, 853–859.

Thongsaard, W., Deachapunya, C., Pongsakorn, S., Boyd, E.A., Bennett, G.W., Marsden, C.A., 1996. Barakol: A potential anxiolytic extracted from *Cassia siamea*. Pharmacology Biochemistry and Behavior 53, 753–758.

Thongsaard, W., Pongsakorn, S., Sudsuang, R., Bennett, G.W., Kendall, D.A., Marsden, C.A., 1997. Barakol, a natural anxiolytic, inhibits striatal dopamine release but not uptake in vitro. European Journal of Pharmacology 319, 157–164.

Tongroach, P., Jantarayota, P., Tantisira, B., Kunluan, P., Tongroach, C., Chaichantipyuth, C., 1992. Barakol, a neuroactive compound from *Cassia siamea*. In: Proceedings of the First JSPS-NRCT Joint Seminar in Pharmaceutical Sciences: Advances in Research on Pharmacologically Active Substances from Natural Sources, Chiangmai, Thailand. P21.

Tsukada, F., Sawamura, K., Kohno, H. Ohkubo, Y., 2002. Mechanism of inhibition of small intestinal motility by restraint stress differs from that with norepinephrine treatment in rats. Biology Pharmacology Bulletin 25, 122–124.

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arakol Extracted from *Cassia siamea* Stimulates Chloride ecretion in Rat Colon

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STRACT

akol is a purified extract of *Cassia siamea*, a plant that has en used as a laxative in traditional medicine. In this study, the ct of barakol on anion transport across the rat colon epi-lium was investigated. Colonic epithelium was mounted in sing chambers and bathed with Ringer's solution. Addition 1 mM barakol to the basolateral solution produced a slow ease in short-circuit current (Isc) in proximal colon and tal colon by 24.5 ± 2.2 and 24.2 ± 1.4 μA/cm^2, respectively. akol increased Isc in a concentration-dependent manner n an EC$_{50}$ value of 0.4 mM. The barakol-stimulated increase sc was inhibited by subsequent treatment with 500 μM henylamine-2-carboxylic acid or 400 μM glibenclamide ded to the apical solution and 200 μM bumetanide added to basolateral solution. Pretreatment of the tissues with 200 μM bumetanide, but not 10 μM amiloride, completely abolished the barakol-increased Isc. Ion substitution experiments showed an inhibition of barakol-stimulated Isc in chloride-free solution but not in bicarbonate-free solution. In addition, pretreatment of tissues with 10 μM tetrodotoxin or 10 μM indomethacin, but not 1 μM atropine or 10 μM hexamethonium, partially inhibited the Isc response by barakol. The present results demonstrated the stimulatory effect of barakol on the bumetanide-sensitive chloride secretion in rat colon. The effect of barakol was partly mediated by the stimulation of submucosal nerves and through the release of cyclooxygenase metabolites. These findings thus provide an explanation for the underlying mechanism of barakol as a secretagogue in mammalian colon.

Barakol is a biologically active compound extracted from ves and flowers of *Cassia siamea*, a plant that has been ditionally used for the treatment of fever, skin disease, nstipation, diabetes, hypertension, and insomnia (Kingrn and Balandrin, 1992). It was originally extracted by ssanali-Walji et al. in 1969. Its chemical structure was ntified as 3α,4-dihydro-3α,8-dihydroxy-2,5-dimethyl-1,4-oxaphenalene ($C_{13}H_{12}O_4$) or 2,5-dimethyl-3α*H*-pyrano-3,4-*de*]-1-benzopyran-3α,8-diol (Fig. 1), and a proposed nthetic procedure was described in 1970 (Bycroft et al., 970). In animal model studies, barakol has been shown to ssess hypotensive activity (Suwan et al., 1992) and serotonergic receptor antagonist activity (Tongroach et al., 1992), and it appears to function as an anxiolytic in exploratory behavioral activities (Thongsaard et al., 1996). Further in vitro studies in the central nervous system have found that barakol inhibits K$^+$-stimulated dopamine release from striatal slices of rat brain (Thongsaard et al., 1997). Recent studies in our laboratory have shown that barakol increases smooth muscle contraction in the isolated rat ileum under basal conditions and during electrical field stimulation. In these studies, barakol was found to inhibit norepinephrine-suppressed smooth muscle contraction, suggesting a role for barakol as a prokinetic drug (Poonyachoti et al., 2002).

The colonic epithelium plays an essential role in the absorption and secretion of water and electrolytes. Its transport function is regulated by a variety of neurotransmitters, hormones, and inflammatory mediators. Under basal conditions, the colonic epithelium absorbs fluid from the lumen into the circulation, and this process is driven by energy-dependent Na$^+$ transport. In contrast, colonic secretion is activated by secretagogues that enhance Cl$^-$ transport mechanisms,

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BREVIATIONS: DPC, diphenylamine-2-carboxylic acid; DIDS, 4,4-diisothiocyanatostilbene-2,2-disulfonic acid; PD, potential difference; Isc, ort-circuit current; G, tissue conductance; TTX, tetrodotoxin; CFTR, cystic fibrosis transmembrane conductance regulator.

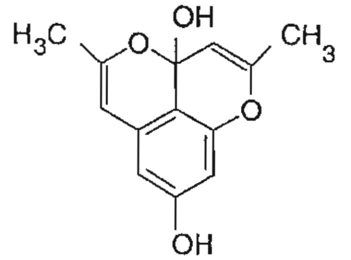


Fig. 1. Chemical structure of barakol.

ich in turn create electrochemical and osmotic driving es for passive cation and water movement into the lumen nzelmann and Mall, 2002). Decreased secretory function creased fluid absorption by the colon is typically associ- d with constipation. Since *Cassia siamea* has been used as xative, we hypothesized that its active ingredient, bara- may exert a laxative effect by stimulating chloride secre- and/or inhibiting NaCl absorption across the colonic thelium. Therefore, the aim of the present study was to estigate the effect of barakol on ion transport across the colon epithelium and to determine the mechanisms in- ved in barakol action.

Materials and Methods

Plant Extraction. Barakol was extracted and purified from *Cas- siamea* by a method modified in our laboratory (Thongsaard et 2001). Briefly, fresh young leaves and flowers of *Cassia siamea* e obtained from Ladkrabang, Bangkok, Thailand. The herbarium cimens were authenticated, deposited, and given the voucher cimen number A001432 by the Department of Botany, Faculty of ence, Chulalongkorn University, Bangkok, Thailand. They were into small pieces and boiled in 0.5% sulfuric acid for 30 min. The ture was blended, filtered, and alkalinized with concentrated HCO_3 and then extracted with chloroform. The chloroform extract further concentrated with 5% acetic acid and neutralized with ammonium hydroxide. The crude barakol was obtained as nish crystallized yellow needles with a 0.3% yield. Concentrated rochloric acid was finally added to obtain barakol hydrochloride, the mixture was dried by vacuum filtration to form yellowish tallized anhydrobarakol hydrochloride. The compound was wn to be a single chemical using thin-layer chromatography on ca gel, and the identification was confirmed by nuclear magnetic onance. Barakol was dissolved in distilled water immediately ore testing its activity. When anhydrobarakol hydrochloride is olved in water, the reaction is reversed, and the product used in the biological experiments is a barakol solution with a pH of 3 to the stock concentration (50 mM) (Thongsaard et al., 2001). In the eriment, barakol was freshly dissolved in normal saline, wrapped h aluminum foil, and kept on ice, and it was used within 3 h after paration.

Chemicals. Tetrodotoxin, atropine sulfate, hexamethonium, loride, glibenclamide, diphenylamine-2-carboxylic acid (DPC), -diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), bumetanide, yphendamine, indomethacin, acetazolamide, and high-purity ide salts were obtained from Sigma-Aldrich (St. Louis, MO). All micals were made in aliquots and kept at −20°C before use. Some micals were dissolved in dimethyl sulfoxide, the final dimethyl foxide concentration of which was less than 0.1% (v/v).

Animals and Tissue Preparation. Male Wistar rats (250–300 were obtained from National Animal Center, Mahidol University, ngkok, Thailand. They were housed in stainless steel cages in a m with a 12-h light/dark cycle and allowed free access to food and ter. All animals were taken care of in accordance with the International Guiding Principles for Biomedical Research Involving Animals provided by the National Research Council of Thailand.

For the experiment, rats were sacrificed with a small animal decapitator (Harward, Kent, UK). After a laparotomy incision, the whole colon was removed, rinsed, and placed in an ice-cold oxygenated Ringer's solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, and 11 mM D-glucose; pH 7.4). The colon was longitudinally cut close to the mesentery, and the serosal muscle layers were carefully stripped away by blunt dissection to obtain a mucosa-submucosal preparation. The appearance of palm-like foldings was used to distinguish between the distal and proximal colon.

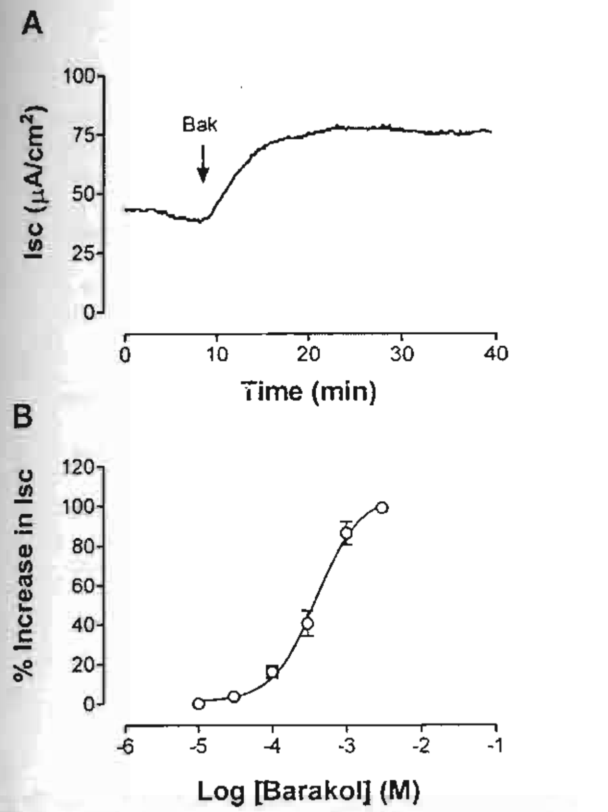
Measurement of Electrical Parameters. The mucosa-submucosal preparation was mounted in Ussing chambers (0.62 cm^2) bathed on the apical and basolateral sides with identical Ringer's solutions at 37°C and gassed with 95% O_2 and 5% CO_2. Transepithelial potential difference (PD) and short-circuit current (Isc) were measured with the use of an EVC-4000 voltage-clamp amplifier (World Precision Instruments, Inc., Sarasota, FL), using Ag/$AgCl_2$ electrodes connected to the bathing solution via agar bridges. Tissue conductance (G) was calculated using Ohm's law (G = Isc/PD). The tissues were continuously short-circuited, except for a brief interval of open-circuited readings before and after adding any chemicals. The data from the voltage clamp was connected to a MacLab 4S A/D converter and recorded with a 400-MHz PowerPC Macintosh. After mounting, the tissues were equilibrated for at least 30 min to achieve a stable Isc before the addition of chemicals. Positive Isc corresponded to the movement of anions from the serosal to mucosal compartments or movement of cations from the mucosal to serosal compartments or a combination of both. In the anion replacement experiments, gluconate salts were substituted for chloride, and 20 mM HEPES buffer was substituted for HCO_3^-. Experiments under HCO_3^--free and Cl^--HCO_3^--free conditions were performed in the presence of 100 μM acetazolamide and bubbled with 100% O_2.

Data Analyses. All values were expressed as means and standard error of mean (S.E.M.); n was the number of different tissue preparations, and N was the number of animals in each experiment. The increase in Isc was quantified by subtracting the peak of an Isc response from its respective baseline value before drug administration. The statistical differences between control and treatment means were analyzed using Student's paired or unpaired t test when appropriate. The differences among groups were analyzed using a one way analysis of variance followed by Dunnett's multiple comparison. A p value of less than 0.05 was considered statistically significant.

Results

Under basal conditions, after an equilibration period of 30 to 45 min, the distal colon showed average Isc, PD (lumen negative), and G values of 35.5 ± 2.4 $\mu A/cm^2$, −3.7 ± 0.3 mV, and 11.0 ± 0.8 pS/cm^2 (n = 44 tissues, N = 17 rats), respectively. The proximal colon exhibited average Isc, PD, and G values of 39.6 ± 5.1 $\mu A/cm^2$, −2.7 ± 0.3 mV, and 15.3 ± 1.5 pS/cm^2 (n = 17 tissues, N = 12 rats), respectively.

Effect of Barakol on Isc. Addition of 1 mM barakol to the basolateral solution produced a slow increase in Isc, which peaked in 5 to 10 min and was sustained for 30 to 45 min (Fig. 2A) before it gradually returned to the baseline level. The maximal increase in Isc produced by barakol was 24.4 ± 2.0 $\mu A/cm^2$ (n = 33 tissues, N = 18 rats) in distal colon and 24.0 ± 4.6 $\mu A/cm^2$ (n = 10 tissues, N = 7 rats) in proximal colon. The tissue conductance at the maximal barakol response was not different from its corresponding baseline value (control, 13.3 ± 1.0 pS/cm^2; after barakol, 12.8 ± 1.0 pS/cm^2, n = 35 tissues, N = 18 rats). Addition of 0.01 M



2. Effect of barakol on Isc in rat colon epithelium. A, representative rdings of the Isc response produced by addition of 1 mM barakol (Bak) he basolateral solution. B, concentration-response relationship for kol-stimulated Isc. The EC_{50} value was 0.4 mM. Each point repre-ted mean ± S.E.M.

lrochloric acid in equal volume to barakol solution (200 μl) not alter any baseline electrophysiological parameters. ce the tracings and maximal Isc responses to barakol in distal and proximal colon were not different, the rest of experiments were performed using distal colon unless erwise stated. Barakol (10 μM–3 mM) induced a cumula- concentration-dependent increase in Isc with an appar- EC_{50} value of 0.4 mM (n = 9 tissues, N = 5 rats; Fig. 2B). barakol-induced increase in Isc was completely inhibited the Cl^- channel blocker DPC (500 μM; n = 4 tissues, N = ts; Fig. 3A) and partially inhibited by glibenclamide (400) added to the apical solution. Glibenclamide produced a 5 ± 11.0% decrease in barakol-stimulated Isc (n = 5 ues, N = 4 rat; Fig. 3B). In contrast, the barakol-stimu- d increase in Isc was not affected by a subsequent addi- of DIDS to the apical solution (n = 5 tissues, N = 4 rats). olateral addition of bumetanide (200 μM), a Na^+-K^+- $^-$ cotransporter inhibitor, completely suppressed the akol-stimulated Isc (n = 5 tissues, N = 4 rats; Fig. 3C). Isc response to barakol did not significantly change in presence of the Na^+ channel blocker amiloride (10 μM; 4A) in the apical solution (control, 33.5 ± 6.8 μA/cm^2, 6 tissues, N = 4 rats; after amiloride, 32.5 ± 10.1 cm^2, n = 3 tissues, N = 3 rats). In contrast, pretreatment issues with bumetanide (200 μM; Fig. 4B) abolished the akol-induced increase in Isc, from 33.5 ± 6.8 μA/cm^2 (n = ssues, N = 4 rats) to 1.0 ± 3.6 μA/cm^2 (n = 3 tissues, N = ts, $p < 0.01$; Fig. 4C).

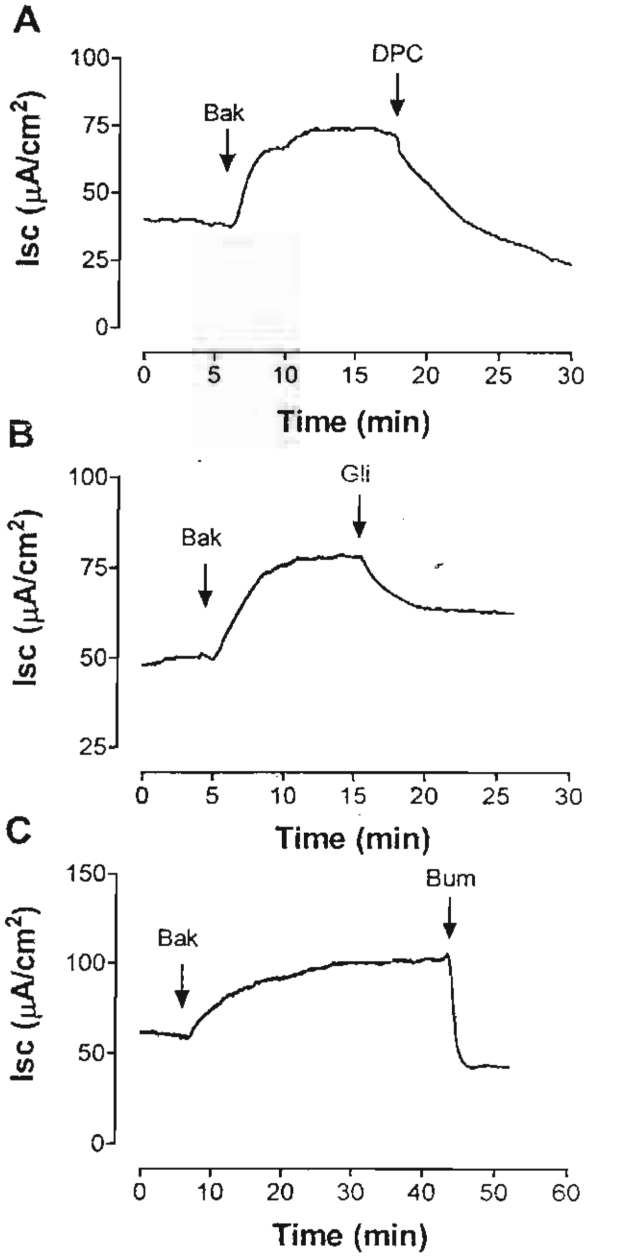
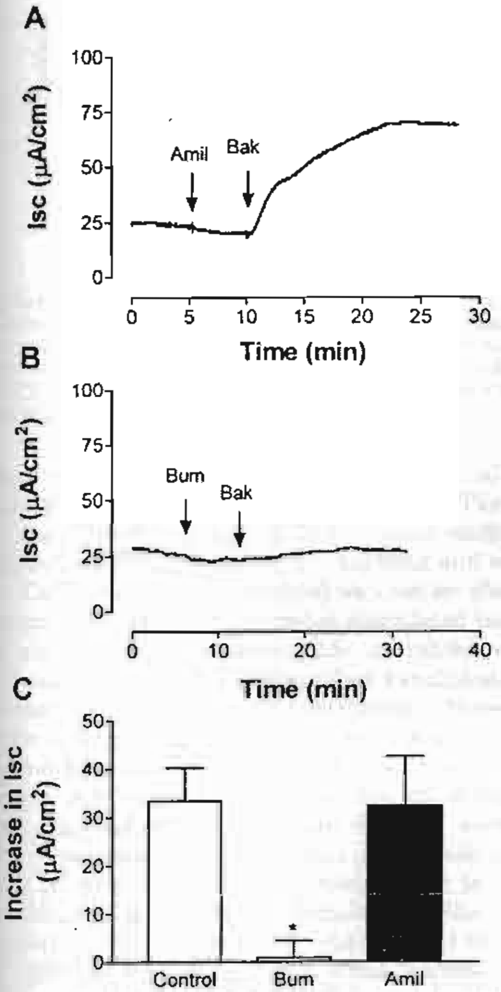


Fig. 3. Effect of Cl^- channel blockers and a Na^+-K^+-$2Cl^-$ cotransport blocker on the barakol-stimulated Isc. Representative recordings of the Isc response produced by basolateral addition of 1 mM barakol (Bak) were inhibited by (A) apical addition of 500 μM DPC, (B) apical addition of 400 μM glibenclamide (Gli), or (C) basolateral addition of 200 μM bumetanide (Bum).

Effect of Ion Substitution on Barakol-Increased Isc. Ion substitution experiments were performed to determine the ionic basis of the Isc response induced by barakol. In normal Ringer's solution, barakol at a concentration of 1 mM produced a mean increase in Isc of 31.1 ± 2.8 μA/cm^2 (n = 24 tissues, N = 13 rats). Replacement of Cl^- in both apical and basolateral solutions significantly inhibited the maximal Isc response to barakol (7.8 ± 0.6 μA/cm^2, n = 8 tissues, N = 6 rats, $p < 0.05$), whereas replacement of HCO_3^- had no effect on the maximal barakol-induced Isc (29.4 ± 2.6 μA/cm^2, n = 5 tissues, N = 4 rats). Replacement of both Cl^- and HCO_3^- significantly inhibited the maximal Isc response to 2.6 ± 0.7 μA/cm^2 (n = 5 tissues, N = 4 rats, $p < 0.05$; Fig. 5).



…4. Effect of amiloride and bumetanide on the barakol-stimulated Isc. …entative tracings of the barakol (Bak)-stimulated Isc response …pretreatment with (A) apical addition of 10 μM amiloride (Amil) or …basolateral addition of 200 μM bumetanide (Bum). C, bar graph …ating the average maximal increases in Isc response produced by … alone (Control) and after pretreatment with bumetanide or …ride. Values represent means ± S.E.M. *, $P < 0.01$ when compared …the control value.

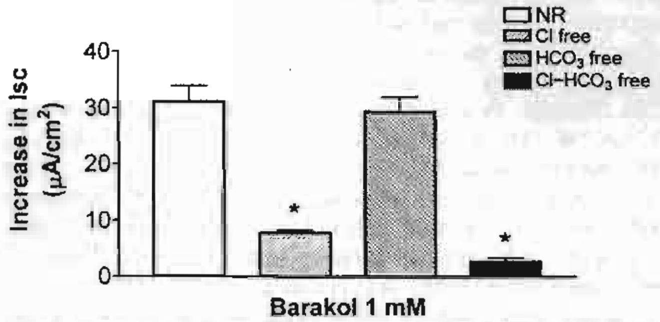


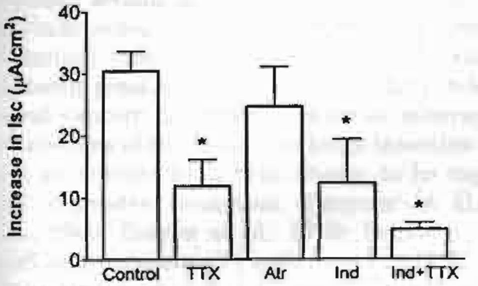
Fig. 5. Effect of Cl^- and HCO_3^- substitution on the barakol-stimulated Isc. In standard Ringer's solution (NR), barakol at a concentration of 1 mM produced a mean increase in Isc of 31 μA/cm^2. Replacement of Cl^- (Cl free) or both Cl^- and HCO_3^- (Cl-HCO$_3$ free) inhibited the maximal Isc response to barakol by 74 and 91%, respectively, whereas replacement of HCO_3^- (HCO_3 free) had no effect on barakol response. Values were means ± S.E.M. *, $P < 0.01$ when compared with the control value of NR.

…fects of Submucosal Neuronal Blockers and Indo…thacin on Barakol-Increased Isc. To assess whether …kol activation of Cl^- secretion was mediated by submu…l neurons and prostaglandin synthesis, the tissues were …eated with neuronal blockers or indomethacin for 5 min …wed by basolateral addition of barakol (1 mM). The effect …rakol alone on control tissues is shown in Fig. 6 (30.4 ± …μA/cm^2, n = 22 tissues, N = 11 rats). Tetrodotoxin (TTX; …μM) significantly decreased the basal Isc from 51.9 ± 13.0 …3.2 ± 9.9 μA/cm^2, and the subsequent addition of barakol …lted in an Isc response that was 60% lower than that in …trol tissues (11.8 ± 4.2 μA/cm^2, n = 5 tissues, N = 4 rats, $<$ 0.05). In contrast, pretreatment with the muscarinic …eptor antagonist atropine (1 μM) in the basolateral solu…n did not significantly change the basal Isc or reduce the …kol-induced increase in Isc (24.7 ± 6.4 μA/cm^2, n = 4 …es, N = 4 rats). In addition, hexamethonium pretreat…t (10 μM) did not significantly alter the barakol-stimu…d Isc (n = 5 tissues, N = 4 rats; data not shown). Pre…tment of tissues with the cyclooxygenase inhibitor indomethacin (10 μM) in both apical and basolateral solutions significantly reduced the barakol-stimulated Isc response by 60% (12.3 ± 7.1 μA/cm^2, n = 7 tissues, N = 6 rats, $p < 0.05$). The barakol-stimulated Isc response was decreased by 90% in the presence of indomethacin and TTX (4.95 ± 1.1 μA/cm^2, n = 4 tissues, N = 4 rats, $p < 0.05$; Fig. 6). In addition, pretreatment with 10 μM dihyphendamine, a histamine (H_1) receptor blocker, did not change the barakol-stimulated Isc (n = 3 tissues, N = 3 rats; data not shown).

Discussion

In the present study, the direct effect of barakol on the ion absorption and secretion was studied in vitro using serosal muscle-stripped colonic epithelium. Barakol was shown to increase Isc in both proximal and distal colon by the same magnitude. The increased Isc was due to an activation of Cl^- secretion. This was supported by the findings that Cl^- channel blockers, DPC and glibenclamide, inhibited the barakol-induced increase in Isc. In ion substitution experiments, the barakol-stimulated Isc was abolished in Cl^--free solution and further inhibited in Cl^-- and HCO_3^--free solutions. In addition, pretreatment with the loop diuretic bumetanide completely inhibited the barakol-stimulated Isc. All of these findings were consistent with stimulation of transepithelial Cl^- secretion in human and rat colon (Dharmsathaphorn et al., 1985; Ko et al., 2002; Kunzelman and Mall, 2002). The finding that the increase in Isc by barakol was not affected by the presence of the Na^+ channel blocker amiloride indicates that barakol did not stimulate electrogenic Na^+ absorption.

Cl^- secretion in mammalian colon involves Cl^- uptake across the basolateral membrane by a Na^+-K^+-$2Cl^-$ cotransport mechanism and subsequent efflux across the apical membrane through Cl^- channels. CFTR is the predominant Cl^- channel that plays a role in Cl^- secretion in many epithelia, including the colonic epithelium (Kunzelmann and Mall, 2002). CFTR is a cAMP-mediated Cl^- channel that has previously been shown to be inhibited by DPC and glibenclamide (Sheppard and Welsh, 1992). On the other hand, DIDS has been shown to block Ca^{2+}-activated Cl^- channel but had no effect on the activity and conductance of CFTR (Anderson et al., 1992; Schultz et al., 1999). Since the present study showed that the barakol-induced increase in Isc was completely inhibited by DPC and partly inhibited by gliben-



6. Effect of submucosal neuronal blockers and indomethacin on the kol-stimulated Isc. The bar graph illustrates the maximal Isc re- to barakol alone (Control) and after pretreatment with 10 μM 1 μM atropine (Atr), 10 μM indomethacin (Ind), or a combination of and TTX (Ind + TTX). Values were mean ± S.E.M. *, $P < 0.05$ when ared with the control value.

nide, it is most likely that CFTR was the apical Cl^- exit hway for barakol-stimulated Cl^- secretion. The insensi-ity of the barakol response to DIDS further confirmed the olvement of CFTR-mediated Cl^- secretion and suggested t the Ca^{2+}-activated Cl^- channel was not involved in the response to barakol. The barakol-stimulated increase in was also abolished by bumetanide, an inhibitor of Na^+-$2Cl^-$ cotransporter, suggesting that barakol-stimulated uptake was mediated by this mechanism. Moreover, the viation of barakol-stimulated Cl^- secretion by CFTR Cl^- nnel blockers and bumetanide indicated that the cellular hanism of barakol may involve the cAMP-dependent hway. Our finding was consistent with Cl^- secretion in-ed by other plant-derived bioactive compounds, especially onoid baicalein. Baicalein has been shown to stimulate secretion across rat colonic epithelium by the activation he cAMP-dependent apical Cl^- channel and the basolat-l K^+ channels (Ko et al., 2002). From studies in human nic cancer cells (T84 cells), baicalein was found to poten-te the Ca^{2+}-mediated Cl^- secretion via an accumulation of MP and activation of protein kinase A activity (Yue et al., 04). However, the signaling mechanisms involving either racellular Ca^{2+} or cAMP and protein kinase A in the ediation of barakol-stimulated Cl^- secretion could not be ied out and are subject to further investigation.

Most of the naturally occurring laxatives exert their effects the colonic epithelium by stimulating Cl^- secretion and/or ibiting Na^+ absorption, resulting in an accumulation of d and subsequent increased colonic motility. The in-ased Cl^- secretion by anthranoid laxatives anthraquinone l sennosides is due to disruption of epithelial tight junc-ns, leading to increased permeability of the epithelium we, 1980; Wanitschke, 1980). Their chemical structures lated to junctional disruption have not been indicated. Al-ough the direct effect of barakol on tight junction perme-ility was not examined in the present study, an increase in nctional permeability was unlikely to account for barakol-mulated Cl^- secretion since changes in tissue conductance re relatively small. The suppressive actions of anthranoid atives on Na^+ absorption could result from decreased ATP duction or a direct inhibition of the Na^+,K^+-ATPase ac-vity in the basolateral membrane (Wanitschke, 1980; Wan-schke and Karbach, 1988). Reduced Na^+,K^+-ATPase activ-y would, in turn, decrease net Na^+ absorption across the ithelium. In this study, the barakol-induced increases in were not affected by amiloride, indicating that the bara-kol effect was not due to activation of epithelial Na^+ channels. This finding may be due to the fact that Na^+ channels normally play a relatively minor role in Na^+ absorption across the proximal and distal colon in rats (Kunzelmann and Mall, 2002). However, a question still remains as to whether barakol may have some effects on Na^+-H^+ or Cl^--HCO_3^- exchange, since the activities of these electroneutral transporters could not be detected by measurement of Isc.

To identify whether the barakol response involved neurotransmitter release from nerves within the submucosal plexus or was the result of a direct effect on the epithelium, the tissues were pretreated with tetrodotoxin to block the neuronal Na^+ channel activity and inhibit action potential propagation. A substantial portion of basal Isc (36%) was inhibited following the addition of tetrodotoxin, suggesting that it was sustained by endogenous release of neurotransmitters from the submucosal plexus. Pretreatment with tetrodotoxin inhibited the barakol-induced increase in Isc by 48%, confirming that barakol-stimulated Cl^- secretion was partially mediated through the activation of submucosal nerves. In contrast, the lack of effect of the muscarinic cholinergic receptor blocker atropine or the nicotinic receptor blocker hexamethonium on the barakol-stimulated Isc response argued against involvement of the acetylcholine-containing submucosal neurons in barakol action on Cl^- secretion in the rat. The tetrodotoxin-insensitive barakol response suggested that barakol may act directly on the colonic epithelial cells to stimulate Cl^- secretion.

To further test whether the barakol activation of Cl^- secretion was due to prostaglandin release, tissues were pretreated with the cyclooxygenase inhibitor indomethacin to block the synthesis of prostaglandins. Prostaglandins are normally released in response to a variety of stimuli. They have been known to activate Cl^- secretion directly via prostanoid receptors located in rat colonic epithelial cells (Brown et al., 1992). The present findings demonstrated that indomethacin inhibited the effect of barakol on Isc by 62%, suggesting that its sustained stimulatory effect on Cl^- secretion was partially mediated through the release of prostaglandins. The combined presence of indomethacin and tetrodotoxin nearly abolished (90%) the stimulatory effect of barakol on Cl^- secretion, indicating that the direct interaction of barakol with epithelial cells may account in part for its response.

The synthesis and release of prostaglandins are known to be part of the mechanisms of the anthranoid laxatives. Damage to epithelial cells caused by anthranoids induces the release of histamine and serotonin from monocytes, mast cells, and other intestinal monocytes, leading to increased biosynthesis of prostaglandin (Yagi et al., 1988; Nijs et al., 1992). Prostaglandin release, in turn, accelerates the large intestine transit and alters fluid absorption and secretion (Leng-Peschlow, 1986). In addition, the release of inflammatory mediators, especially histamine, is known to stimulate Cl^- secretion in colonic epithelium (Traynor et al., 1993; Yue et al., 2004). However, inflammatory mediators were unlikely to be responsible for the actions of barakol since the histamine antagonist diphenhydramine did not alter the Isc response to barakol.

From the present findings, it seemed that barakol response was substantially mediated by enteric nerves within the submucosal plexus. Being insensitive to atropine and hexame-

...m, barakol actions may be mediated by other neuro-...itter substances of the noncholinergic pathways. ...dates include vasoactive intestinal peptide, substance ...d calcitonin gene-related peptide (CGRP), which are ...g several neurotransmitters present in enteric nerves ...sory neurons of the small and large intestine (Cooke, ... These substances have been shown to be capable of ...ing Cl^--dependent secretion (Traynor et al., 1991; ... et al., 1992; Riegler et al., 1999; Esfandyari et al., ...). The effect of substance P involved in barakol response ...ently under investigation. Possible involvement of ex-...ry, noncholinergic transmitters in barakol action is also ...ject worthy of future study.

...e fact that a decrease in Cl^- secretory activity in the ...n is one explanation for the increased incidence of con-...tion in the elderly; therefore drugs that increase fluid ...tion through modulating ion channel activity could be ...ficial for the treatment of constipation. Barakol, shown ...e present study to have a stimulatory effect on electro-...nd water secretion, could thus be therapeutically useful ...e management of age-related changes in colonic func-...or constipation associated with physiological or psycho-...al stress. By increasing the Cl^- secretion in both proxi-... and distal colon, barakol could work as a laxative by ...asing colonic volume and motility.

...conclusion, we have shown in rat colonic epithelium that ...kol stimulated chloride secretion without affecting elec-...nic sodium absorption. The transport mechanisms in-...d basolateral Na^+-K^+-$2Cl^-$ cotransporters and apical ... channels. Although not specifically addressed in this ...y, some increase in K^+ channel activity was also likely as ...eans to sustain the electrical driving force for Cl^- exit ...ss the apical membrane. The barakol-stimulated Isc was ...ially controlled by enteric nerves within the submucosal ...us and partially mediated by the release of cyclooxygen-... metabolites. Our findings provided a mechanistic expla-...n for the action of the active compound barakol and the ...tive effect of *Cassia siamea* plant extract.

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...rences

...son MP, Sheppard DN, Berger HA, and Welsh MJ (1992) Chloride channels in ... apical membrane of normal and cystic fibrosis airway and intestinal epithelia. ... *J Physiol* 263:L1–L14.

...a DR, Parsons AM, and O'Grady SM (1992) Substance P produces sodium and ...rbonate secretion in porcine jejunal mucosa through an action on enteric ...urons. *J Pharmacol Exp Ther* 261:1206–1212.

...& BW, Hassanali-Walji A, Johnson AW, and King TJ (1970) The structure and ...thesis of barakol: a novel dioxaphenalene derivative from *Cassia siamea*. ...*Chem Soc* 12:1686–1689.

...e HJ (1994) Neuroimmune signaling in regulation of intestinal ion transport. ...*n J Physiol* 266:G167–G178.

Dharmsathaphorn K, Mandel KG, Masui H, and McRoberts JA (1985) Vasoactive intestinal polypeptide-induced chloride secretion by a colonic epithelial cell line. Direct participation of a basolaterally localized Na^+,K^+,Cl^- cotransport system. *J Clin Investig* 75:462–471.

Esfandyari T, Macnaughton WK, Quirion R, St Pierre S, Junien JL, and Sharkey KA (2000) A novel receptor for calcitonin gene-related peptide (CGRP) mediates secretion in the rat colon: implications for secretory function in colitis. *FASEB J* 14:1439–1446.

Ewe K (1980) The physiological basis of laxative action. *Pharmacology* 20 **(Suppl 1)**:2–20.

Hassanali-Walji A, King TJ, and Wallwork SC (1969) Barakol, a novel dioxaphenalene derivative from *Cassia siamea*. *J Chem Soc Chem Commun* 12:678.

Kinghorn AD and Balandrin MF, editors (1992) *Human Traditional Agents from Plants*. American Chemical Society, Washington DC.

Ko WH, Law VW, Yip WC, Yue GG, Lau CW, Chen ZY, and Huang Y (2002) Stimulation of chloride secretion by baicalein in isolated rat distal colon. *Am J Physiol* 282:G508–G518.

Kunzelmann K and Mall M (2002) Electrolyte transport in the mammalian colon: mechanisms and implications for disease. *Physiol Rev* 82:245–289.

Leng-Peschlow E (1986) Dual effect of orally administered sennosides on large intestine transit and fluid absorption in the rat. *J Pharm Pharmacol* 38:606–610.

Nijs G, De Witte P, Geboes K, and Lemli J (1992) Influence of rhein anthrone and rhein on small intestine transit in rats: Evidence of prostaglandin mediation. *Eur J Pharmacol* 218:199–203.

Poonyachoti S, Thongsaard W, and Deachapunya C (2002) The prokinetic effects of barakol in rat intestine (Abstract). *The Pharmacologist* 44:A35–A36.

Riegler M, Castagliuolo I, So PT, Lotz M, Wang C, Wlk M, Sogukoglu T, Cosentini E, Bischof G, Hamilton G, et al. (1999) Effects of substance P on human colonic mucosa in vitro. *Am J Physiol* 276:G1473–G1483.

Schultz BD, Singh AK, Devor DC, and Bridges RJ (1999) Pharmacology of CFTR chloride channel activity. *Physiol Rev* 70 **(Suppl 1)**:S109–S144.

Sheppard DN and Welsh MJ (1992) Effect of ATP-sensitive K^+ channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. *J Gen Physiol* 100:573–591.

Suwan G, Sudsuang R, Ghumma-Upakorn D, and Werawong C (1992) Hypertensive effects of barakol extracted from leaves of *Cassia siamea* Lam. in rats and cats. *Thai J Physiol Sci* 5:53–65.

Thongsaard W, Chainakul S, Bennett GW, and Marsden CA (2001) Determination of barakol extracted from *Cassia siamea* by HPLC with electrochemical detection. *J Pharm Biomed Anal* 25:853–859.

Thongsaard W, Deachapunya C, Pongsakorn S, Boyd EA, Bennett GW, and Marsden CA (1996) Barakol: a potential anxiolytic extracted from *Cassia siamea*. *Pharmacol Biochem Behav* 53:753–758.

Thongsaard W, Pongsakorn S, Sudsuang R, Bennett GW, and Marsden CA (1997) Barakol, a natural anxiolytic, inhibits striatal dopamine release but not uptake in vitro. *Eur J Pharmacol* 319:157–164.

Tongroach P, Jantarayota P, Tantisira B, Kunluan P, Tongroach C, and Chaichantipyuth C (1992) Barakol, a neuroactive compound from *Cassia siamea*, in *Proceeding of the First JSPS-NRCT Joint Seminar in Pharmaceutical Sciences: Advance in Research on Pharmacologically Active Substances from Natural Sources*, 1992 Dec 3–5; Chiangmai, Thailand. pp 21, Faculty of Pharmacy, Chiangmai University, Chiangmai, Thailand.

Traynor TR, Brown DR, and O'Grady SM (1991) Regulation of ion transport in porcine distal colon: effects of putative neurotransmitters. *Gastroenterology* 100: 703–710.

Traynor TR, Brown DR, and O'Grady SM (1993) Effects of inflammatory mediators on electrolyte transport across the porcine distal colon epithelium. *J Pharmacol Exp Ther* 264:61–66.

Wanitschke R (1980) Influence of rhein on electrolyte and water transfer in the isolated colonic mucosa. *Pharmacology* 20 **(Suppl 1)**:21–26.

Wanitschke R and Karbach U (1988) Influence of rhein on rat colonic Na^+,K^+-ATPase and permeability in vitro. *Pharmacology* 36 **(Suppl 1)**:98–103.

Yagi T, Miyawaki Y, Nishikawa T, Yamauchi K, and Kuwano S (1988) Involvement of prostaglandin E-like material in the propulsive action of rhein anthrone, the intraluminal active metabolite of sennoside A and B in mice. *J Pharm Pharmacol* 40:27–30.

Yue GGL, Yip TWN, Huang Y, and Ko WH (2004) Cellular mechanism of Ca^{2+}-mediated Cl^- secretion by the flavonoid baicalein in intestinal epithelia. *J Biol Chem* 279:39310–39316.

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