Control of Ion Transport in Mouse Proximal and Distal Colon by Prolactin

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Key Words

Prolactin • BK channels • CFTR • EnaC • Ca²⁺-activated Cl⁻ channels • Epithelial transport • Secretion • Colon • Ion channels

Abstract

The lactogenic hormone prolactin (PRL) has been known to affect Ca²⁺ and electrolyte transport in the intestinal epithelium. In the present study we analyzed ion transport in mouse proximal and distal colon, and acute changes induced by PRL. In the proximal colon, carbachol activated a Ca²⁺ dependent Cl⁻ secretion that was sensitive to DIDS and NFA. In the distal colon, both ATP and carbachol activated K⁺ secretion. Ca²⁺-activated KCl transport in proximal and distal colon was inhibited by PRL (200ng/ml), while amiloride sensitive Na⁺ absorption and cAMP induced Cl⁻ secretion remained unaffected. Luminal large conductance Ca²⁺-activated K⁺ (BK) channels were largely responsible for Ca²⁺-activated K⁺ secretion in the distal colon, and basolateral BK channels supported Ca²⁺-activated Cl⁻ secretion in the proximal colon. Ca²⁺ chelating by BAPTA-AM attenuated effects of carbachol and abolished effects of PRL. Both

inhibition of PI3 kinase with wortmannin and blockage of MAP kinases with SB 203580 or U 0126, interfered with the acute inhibitory effect of PRL on ion transport, while blocking of Jak/Stat kinases with AG 490 was without effects. PRL attenuated the increase in intracellular Ca²⁺ that was caused by stimulation of isolated colonic crypts with carbachol. Thus PRL inhibits Ca²⁺ dependent Cl⁻ and K⁺ secretion by interfering with intracellular Ca²⁺ signaling and probably by activating PI3 kinase and MAP kinase pathways.

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Introduction

Regulation of salt and water balance is essential to maintain homeostasis in most organisms. The lactogenic hormone, prolactin (PRL) is involved in water and electrolyte balance in most vertebrates. PRL receptors (PRL-R) are present in kidney and other epithelial tissues involved in salt balance. Thus PRL has been shown to control salt and water balance in teleosts and amphibians

[1, 2]. Although its effects are less clear in mammals, PRL has been shown to increase fluid and NaCl absorption in the rat proximal colon [3]. Increases in endogenous PRL levels after pituitary transplants cause enhanced intestinal absorption of fluid and Na⁺/Cl⁻ in the rat jejunum, and PRL increased fetomaternal fluid transport across the placental membrane [4, 5]. In contrast, a decrease in water absorption by PRL in the rat small intestine and colonic epithelium was reported in other studies [6, 7]. In these studies, the effects of PRL on water absorption in jejunum [4] and colon [7] were largely dependent on the luminal ion composition and the transport of electrolytes. These results prompted us to study the potential effects of PRL on electrogenic ion transport in mouse colon.

As electrogenic transport differs between proximal and distal colon, ion transport in both segments was analyzed separately in Ussing chamber recordings. In principle, ion transport in the colon occurs in both absorptive and in secretory direction [8]. Electrogenic Na⁺ absorption is due to amiloride sensitive epithelial Na⁺ channels (ENaC), while Cl⁻ secretion is due to cAMP activated cystic fibrosis transmembrane conductance regulator (CFTR) Cl⁻ channels [8]. We report in the present study that mouse proximal colon expresses another, CFTR independent Cl⁻ channel that is activated through increase in intracellular Ca²⁺. In contrast, the distal colonic epithelium does not show Ca²⁺ dependent Cl⁻ secretion but secretes K⁺ in response to stimulation with carbachol. While PRL has no effects on amiloride sensitive Na⁺ absorption and CFTR-mediated Cl⁻ secretion, it clearly interferes with Ca²⁺ dependent Cl⁻ and K⁺ secretion, which is activated through stimulation with the secretagogues carbachol and ATP.

Materials and Methods

Ussing chamber recordings

Proximal and distal colon were removed from mice (C57BL/6N, Charles River, Germany) after sacrificing the animal by anesthesia and cervical dislocation. Tissues were put immediately into an ice cold buffer solution of the following composition (mmol/l): NaCl 145, KCl 3.8, D-glucose 5, MgCl₂ 1, HEPES 5, Ca-gluconate 1.3. The tissues were mounted into a perfused micro Ussing chamber with a circular aperture of 0.95 mm². The apical and basolateral surfaces of the epithelium were perfused continuously at a rate of up to 10 ml/min (chamber volume 2 ml). The bath solution contained (mmol/l): NaCl, 145; KH₂PO₄, 0.4; K₂HPO₄, 1.6; D-glucose, 5; MgCl₂, 1; HEPES 5; and Ca-gluconate, 1.3. The pH was adjusted to 7.4 and all experiments were carried out at 37°C. Experiments were

performed under open circuit conditions. Transepithelial resistance (R_{te}) was determined by applying short (1s) current pulses ($\Delta I=0.5\mu A$) and the corresponding changes in V_{te} (ΔV_{te}) and basal V_{te} were recorded continuously. Values for the transepithelial voltage (V_{te}) were referred to the serosal side of the epithelium. The equivalent short circuit current (I_{sc}) was calculated according to Ohm's law from V_{te} and R_{te} ($I_{sc}=V_{te}/R_{te}$).

Isolation of colonic crypt

After removal the colon was rinsed with cold Ringer solution, inverted, and tied at both ends. The sac was filled with Ca²⁺-free solution (mM: NaCl 127, KCl 5, Glucose 5, MgCl₂ 1, Na-pyruvate 5, HEPES 10, EDTA 5, 1% BSA, pH 7.4), then incubated in the same solution for 10min at 37°C. Isolated crypt cells were obtained by mechanical vibration and gentle centrifugation (1,000rpm, 2min). Crypts were stored in a high K⁺ solution (mM: K⁺ gluconate 100, KCl 30, NaCl 20, HEPES 10, CaCl₂ 1.25, MgCl₂ 1, Glucose 12.2, Na-pyruvate 5, 1% BSA, pH 7.4) at 4°C and used for RNA- and protein isolation and for measurement of [Ca²⁺]_i within 5h after isolation. Viability was tested by trypan blue exclusion.

Immunohistochemistry

5μm cryosections of mouse colon were fixed by perfusion with 3% paraformaldehyde and frozen in liquid nitrogen. Cryosections were incubated in 0.1% SDS for 5min, washed and incubated with the affinity-purified primary antibody rabbit anti mouse bestrophin diluted (1:2000) in PBS with 2% bovine serum albumin and 0.04% Triton X-100 overnight at 4°C. A secondary antibody Alexa Fluor 546 labelled goat anti-rabbit IgG (1:400, Invitrogen, Karlsruhe, Germany) and Alexa Fluor 647 phalloidin (1:400, Invitrogen, Karlsruhe, Germany) were used to visualize bestrophin and actin using an inverted microscope (Axiovert 200M, Zeiss, Jena, Germany) with standard filter systems.

Detection of BK channel protein by Western blot

Protein was isolated from isolated mouse colonic crypts using a buffer containing 150mmol NaCl, 50mmol Tris, 100mmol DTT, 1% NP-40, and 1% protease inhibitor cocktail (Sigma, Taufkirchen, Germany). Equal amounts of total protein (150μg) were separated on a 7% SDS-PAGE and transferred to a PVDF membrane (Amersham Bioscience, Freiburg, Germany), which was blocked with 3% nonfat dried milk in PBS-Tween 20 (0.1%) buffer for 1h at RT. The membrane was incubated with anti-mouse BK antibodies (1:2000) overnight at 4°C. Proteins were visualized using a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (1:30.000, Acris Antibodies, Hiddenhausen, Germany) and ECL Advance Western Blotting Detection Kit (Amersham Bioscience, Freiburg, Germany). Signals were detected by a Fluor-STM MultiImager system (Bio-Rad Laboratories, Hercules, USA).

RNA isolation and RT-PCR

Total RNA was isolated from isolated colonic crypts, according to the manufacture's description (NucleoSpin RNAII, Machery and Nagel, Duren, Germany). After reverse transcription (Promega, Heidelberg, Germany), cells were

Primer Name	Primer Sequence	PCR product	Annealing	GenBank Accession No.
Bestrophin 1				
sense	ACA CAA ACA TTC TGG GTG C	246bp	58°C, 30s	AY450427
antisense	TTC AGA AAC TGC TTC CCG ATC			
BK $_{\alpha}$				
sense	GGT CCA CGA GCC CAA GAT G	505bp	58°C, 30s	NM010610
antisense	GTA GAG GAG GAA GAA CAC GTT G			
BK $_{\beta 1}$				
sense	GGG GAA GAA GCT GGT GAT G	220bp	58°C, 30s	NM031169
antisense	GTA CTG GGG CAC CTT CTT G			
BK $_{\beta 2}$				
sense	CGG ACC TCT TCA TCT TAC AG	305bp	58°C, 30s	BC058957
antisense	GAG AGC TTC CAA CAG TCG G			
BK $_{\beta 4}$				
sense	GAA CTC GGA GAG CGT CAT G	532bp	58°C, 30s	NM021452
antisense	CCG ACT TCT TTG AGG GTT TC			

Table 1. Primers used for RT-PCR experiments

Fig. 1. PRL does not affect ion transport by ENaC and CFTR in mouse proximal and distal colon. Original Ussing chamber recording of the transepithelial voltage V_{te} in mouse colon and summaries of the short circuit currents. PRL (200ng/ml) had no effects on amiloride (A, 20 μ) sensitive Na^+ transport in distal colon (A, B). PRL had no effects on IBMX (1,100 μ M) and forskolin (F, 2 μ M) induced Cl^- secretion in proximal (C, D) and distal (E, F) colon. (number of experiments).

incubated at 70°C for 5min, 20°C for 10min, 42°C for 1h, and then at 70°C for 15min. cDNAs encoding the putative Ca^{2+} -activated Cl^- channel bestrophin 1, the large Ca^{2+} -activated K^+ channel BK $_{\alpha}$ (Slo 1), and the three known mouse β -isoforms BK $_{\beta 1}$, BK $_{\beta 2}$ and BK $_{\beta 4}$ were amplified by PCR (94°C for 2min; 40 cycles of 94°C for 30s, 60°C for 30s and 72°C for 60s; 72°C for 10min) using sense and antisense primers as shown in Table 1.

Measurement of the intracellular calcium concentration

For fluorescence measurements cells were perfused with Ringer solution (mmol/l: NaCl 145; KH $_2$ PO $_4$ 0.4; K $_2$ HPO $_4$ 1.6; Glucose 5; MgCl $_2$ 1; Ca $^{2+}$ -Gluconat 1.3) at 37°C. Cell fluorescence was measured continuously using an inverted microscope IMT-2 (Olympus, Germany) and a high speed polychromator system (VisiChrome, Visitron Systems, Germany). Cells were loaded with 5 μ M Fura-2 AM (Molecular Probes) in OptiMEM (Invitrogen) with 0.02% pluronic (Molecular Probes) for 30min at RT. Fura-2 was excited at 340/380nm, and emission was recorded between 470 and 550nm using a CCD-camera (CoolSnap HQ, Visitron Systems, Germany). Experiments were controlled and analyzed using the software package Meta-Fluor (Universal imaging, USA). All optical filters and dichroic mirrors were from AHF (Tübingen, Germany).

Fig. 2. PRL effects on carbachol and ATP induced transport. A, B) Carbachol (C, 100 μ M, basolateral) induced Cl^- secretion in the proximal colon is reduced by pre-stimulation with PRL. C, D) Carbachol induced K^+ secretion in the distal colon is reduced in the presence of PRL. E, F) ATP (100 μ M, luminal) induced K^+ secretion in the distal colon is reduced in the presence of PRL. *indicate significant difference (paired *t*-test). (number of experiments).

Materials

The anti-BK α antibody was provided by Dr. M. Sausbier (Pharmazeutisches Institut der Universität Tübingen, Germany). All other compounds were of highest purity and were all from Sigma, Merck, or Calbiochem. *n* indicates the number of tissue samples. Paired student's *t*-test was used for statistical analysis. *P*-values < 0.05 were accepted to indicate statistical significance.

Results

PRL does not affect ion transport by ENaC and CFTR in mouse proximal and distal colon

In order to examine potential effects on basal transport, we applied 200 ng/ml PRL to the basolateral side of the non-stimulated mouse proximal and distal colon. This PRL concentration is equivalent to

physiological plasma levels occurring during lactation. It did not cause any changes in baseline transport, when applied for 10 or even 40 min (data not shown). Because PRL has been shown previously to increase fluid absorption and to affect fluid secretion [3, 7, 9], we examined the acute effects of PRL exposure on amiloride sensitive Na^+ absorption and Cl^- secretion by the cystic fibrosis transmembrane conductance regulator (CFTR). Amiloride sensitive Na^+ absorption was only present in the distal colon, but was not detectable in the proximal colon (Fig. 1A,B). Cl^- secretion in proximal and distal colon was activated through stimulation of CFTR with IBMX (100 μ M) and forskolin (2 μ M) (Fig. 1C-F). Neither Na^+ absorption nor Cl^- secretion was affected by pre-stimulation with PRL (200 ng/ml for 10 min).

Fig. 3. Carbachol activates a Ca^{2+} dependent Cl^- secretion in the proximal colon. A, C) Original recordings of V_{te} and effects of carbachol (C, $100\mu\text{M}$, basolateral) in the absence or presence of the Cl^- channel blockers NFA ($10\mu\text{M}$) or DIDS ($10\mu\text{M}$). B, D) Summary of the effects of carbachol (CCh) on I_{sc} in the absence or presence of NFA and DIDS. *indicate significant difference (paired t -test). (number of experiments).

Inhibition of Ca^{2+} dependent Cl^- and K^+ secretion in proximal and distal colon by PRL

In order to examine Ca^{2+} -activated transport, all experiments were performed in the presence of the cyclooxygenase inhibitor indomethacin ($10\mu\text{M}$), which suppressed formation of endogenous cAMP [8]. Intracellular Ca^{2+} was increased by basolateral application of carbachol ($100\mu\text{M}$), which caused a transient lumen negative V_{te} response in the proximal colon and a steady lumen positive V_{te} in the distal colon (Fig. 2A-D). Stimulation of luminal P2Y receptors by ATP ($100\mu\text{M}$) in the distal colon also induced a lumen positive response, albeit with a much faster time course (Fig. 2E-F). PRL reduced carbachol and ATP activated electrolyte transport by about 40%.

In the proximal colon, the lumen negative response was due to activation of Ca^{2+} dependent Cl^- channels (CaCC). Cl^- secretion via CaCC could be activated in the absence CFTR, pointing out to a separate molecular entity (data not shown). Inhibition of the basolateral $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ cotransporter NKCC1 by bumetanide ($50\mu\text{M}$) reduced carbachol induced Cl^- secretion only after stimulation with IBMX/forskolin, indicating that NKCC1 is only active in the presence of intracellular cAMP. Similar results were obtained with the azosemide ($10\mu\text{M}$), another inhibitor of NKCC1 (data not shown). It was possible to repetitively activate Cl^- secretion by carbachol under control conditions. Thus we could demonstrate that

Cl^- secretion was inhibited by the CaCC inhibitors niflumic acid (NFA, $100\mu\text{M}$) and DIDS ($100\mu\text{M}$) (Fig. 3A-D), respectively. A small but significant K^+ secretion was activated by carbachol in parallel with Cl^- secretion. Thus the lumen negative response induced by carbachol was larger after blocking luminal K^+ channels with Ba^{2+} (Fig. 4A,B). The K^+ channel inhibitors paxillin ($1\mu\text{M}$) and TRAM-34 ($1\mu\text{M}$) had no clear effects on luminal K^+ conductance (data not shown). Moreover, basolateral K^+ channels are activated during Ca^{2+} dependent stimulation, since carbachol induced Cl^- secretion was significantly reduced from 115.7 ± 20.1 to $76.7 \pm 8.1 \mu\text{A}/\text{cm}^2$ ($n=8$) in the presence of basolateral BaCl_2 (5mM). In order to determine the type of Ca^{2+} dependent K^+ channel present in basolateral membranes of proximal epithelial cells, we examined inhibitors of large (BK), intermediate (IK) and small (SK) conductance K^+ channels. Basolateral application of the BK inhibitor paxillin ($1\mu\text{M}$) and the IK inhibitor TRAM-34 ($1\mu\text{M}$) reduced carbachol induced Cl^- secretion significantly (Fig. 4C-F), suggesting activation of BK and IK channels during stimulation with carbachol. The SK inhibitor apamin (30nM) was without effects on CCh induced transport (116.2 ± 14.1 (-apamin) vs. 117.7 ± 12.3 (+apamin) $\mu\text{A}/\text{cm}^2$; $n=4$). Moreover, secretion was supported by basolateral KCNQ1 K^+ channels, since Cl^- secretion was reduced from 125 ± 13.1 to $75.7 \pm 8.3 \mu\text{A}/\text{cm}^2$ ($n=9$) in the presence of the KCNQ1 inhibitor 293B ($10\mu\text{M}$). In the distal colon, K^+ secretion could be

Fig. 4. Effect of K⁺ channel inhibitors on carbachol induced Cl⁻ secretion in mouse proximal colon. A, B) Luminal application of BaCl₂ (5mM) increased negative voltage deflection and I_{sc} induced by carbachol (C, 100μ, basolateral). C, D) Basolateral application of TRAM-34 (1μM) reduced negative voltage deflection and I_{sc} induced by carbachol. E, F) Basolateral application of paxillin (1μM) reduced negative voltage deflection and I_{sc} induced by carbachol. *indicate significant difference (paired *t*-test). (number of

repetitively activated by carbachol. Activation of luminal K⁺ conductance was largely reduced in the presence of luminal BaCl₂ (5mM) or paxillin (1μM) (Fig. 5A-D). Thus K⁺ secretion in mouse distal colon by carbachol is due to activation of luminal BK channels. Both, carbachol activated Cl⁻ secretion in the proximal colon and carbachol activated K⁺ secretion in the distal colon were inhibited by carbachol.

Expression of BK channels was further confirmed by RT-PCR analysis in isolated colonic crypts. In mouse, large conductance (BK) Ca²⁺-dependent K⁺ channels are composed of α (BK_α) and β1 (BK_{β1}), β2 (BK_{β2}), or β4 (BK_{β4}) subunits. Expression of BK_α and all three β-

subunits was clearly detectable in proximal and distal colonic epithelial cells (Fig. 6A). Expression of BK_α was confirmed by Western blot analysis which detected expression in the distal colon, but only very weak signals for the proximal colon (Fig. 6B). Accordingly, immunolabeling of BK was stronger in distal when compared to proximal colon (Fig. 6D). In contrast, bestrophin 1, a member of the family of Ca²⁺-activated Cl⁻ channels [10] was clearly detected in proximal epithelial cells, but was only weakly expressed in the distal colon (Fig. 6C). Transcripts for bestrophin 1 could be detected by RT-PCR in both distal and proximal colon (Fig. 6A).

Fig. 5. Effect of K^+ channel inhibitors on carbachol induced K^+ secretion in mouse distal colon. A, B) Carbachol induced K^+ secretion in the absence but not presence of luminal $BaCl_2$ (5mM). E, F) Carbachol induced K^+ secretion in the absence but not presence of luminal paxillin (1 μ M). *indicate significant difference (paired t -test). (number of experiments).

Signal transduction of the PRL effects

PRL inhibited Ca^{2+} -activated Cl^- and K^+ secretion in proximal and distal colon, respectively. In the presence of the Ca^{2+} chelator BAPTA-AM (10 μ M), activation of ion secretion by carbachol was reduced and under these conditions not other effects of PRL could be recognized (Fig. 7A,F). In the proximal colon, inhibition of the PI3 kinase by wortmannin (30nM) or blocking of the MAP kinase pathway with the compounds U 0126 (30 μ M) and SB 203580 (25 μ M) [11-13] did not affect carbachol induced Cl^- secretion, but abolished the inhibitory effects of PRL (Fig. 7B-D). In the distal colon, wortmannin, U 0126 and SB 203580, all markedly reduced CCh activated K^+ secretion, and PRL no longer attenuated the transport (Fig. 7G-I). In contrast, an inhibitor of Jak/Stat kinases, AG 490 (50 μ M) [14] did not show any effects in either proximal or distal colon (Fig. 7E,J).

We further examined if Ca^{2+} signaling was directly affected by PRL. To that end colonic crypts were isolated and loaded with the Ca^{2+} sensitive fluorescence dye Fura-2. The isolated crypts were stimulated with carbachol, which induced a typical transient (peak) and steady state (plateau) Ca^{2+} increase (Fig. 8A). Exposure to PRL did

not change intracellular baseline Ca^{2+} concentrations (Fig. 8C). However, CCh induced Ca^{2+} increase was attenuated by pre-stimulation with PRL (Fig. 8B,D). Thus PRL has a direct inhibitory effect on carbachol induced Ca^{2+} signaling. Taken together, PRL attenuates Ca^{2+} dependent Cl^- and K^+ secretion in the proximal (Fig. 9A) and distal (Fig. 9B) colonic epithelium, by affecting intracellular signaling through Ca^{2+} as well as MAP kinase and PI3 kinase pathways.

Discussion

Spatial distribution of Ca^{2+} dependent Cl^- and K^+ secretion in mouse colon

An essential finding of the present study is that increase in intracellular Ca^{2+} activates Cl^- secretion and K^+ secretion in proximal and distal colon, respectively. A recent study identified luminal large conductance BK channels as the K^+ secretory pathway in mouse distal colon, using BK $^{-/-}$ knockout animals [15]. Luminal Ca^{2+} dependent BK channels are activated through stimulation of luminal purinergic P2Y or basolateral muscarinic M3

Fig. 6. Expression of large conductance potassium (BK) channels in mouse colon. A) mRNA analysis of proximal and distal colon indicates expression of the alpha-subunit of large conductance K⁺ channels (BK α). Analysis of beta-subunits suggested weak expression of BK β 1 and BK β 4 but strong expression of BK β 2 in the proximal and distal colon. Transcripts for bestrophin 1 were found in proximal and distal colon. B) Western blot analysis demonstrates expression of BK α in distal colon, but weak signals for proximal colon. C) Immunohistochemistry of bestrophin 1 demonstrates expression in the proximal colon but only weak staining in the distal mouse colon. D) Immunohistochemistry of BK demonstrates stronger expression in the distal proximal when compared to proximal colon.

receptors. However, the time courses for activation via the two mechanisms are remarkably different. While activation through luminal P2Y receptors is almost instantaneous and transient, activation through basolateral M3 receptors is delayed and long lasting. This may be explained by the unequal contribution of intracellular second messenger pathways for luminal P2Y and

basolateral M3 receptors. Although both receptors couple to intracellular PI3 kinase and MAP kinase pathways [16], we propose that stimulation of luminal P2Y receptors leads to a rise in intracellular Ca²⁺ in close proximity to the BK channel, thereby rapidly activating K⁺ secretion. In contrast, Ca²⁺ signaling by basolateral M3 receptors may be restricted to the basolateral compartment and

Fig. 7. Signal transduction of carbachol and PRL in proximal (left panels) and distal (right panels) colon. A, F) The Ca^{2+} chelator BAPTA ($10\mu\text{M}$) reduced CCh induced transport in proximal and distal colon. PRL no longer inhibited ion transport in the presence of BAPTA. B, G) In the proximal colon, the inhibitor of the PI3 kinase, wortmannin (30nM), had no effects on CCh induced transport but abolished the effects of PRL. In distal colon, Wortmannin abolished CCh induced transport completely. C, H) In the proximal colon, the MEK1,2 inhibitor U 0126 ($30\mu\text{M}$) had no effects on CCh induced transport, but abolished the effects of PRL. In distal colon, U 0126 largely reduced CCh induced transport and abolished the effects of PRL. D, I) In the proximal colon, the MAP kinase inhibitor SB 203580 ($25\mu\text{M}$), had no effects on CCh induced transport but abolished the effects of PRL. In distal colon, SB 203580 abolished CCh induced transport completely. E, J) The inhibitor of Jak/Stat signaling, AG 490 ($50\mu\text{M}$), had no effects on CCh induced I_{sc} and did not suppress the inhibitory effects of PRL on CCh induced transport. *indicate significant difference (paired t -test). (number of experiments).

activation of the intracellular PI3 kinase/MAP kinase pathway may be essential to activate luminal BK channels [17-19]. BK channels have been shown to be activated by stimulation of both PI3 kinase and MAP kinase pathways [20, 21], which is in line with the present findings. Carbachol stimulates PI3 and MAP kinases probably by a mechanism involving intracellular Ca^{2+} . Similar observations have been described for T_{84} colonic

carcinoma cells and HEK cells overexpressing M3 receptors [18, 19]. Moreover, BK channels have been detected not only in the luminal but also in the basolateral membrane of rabbit distal colon [22, 23], and in the basolateral membrane of mouse colon as shown in the present study (Fig. 4). Thus BK channels play an important role in the overall regulation of salt and water transport in the colon.

Fig. 8. PRL affects intracellular Ca^{2+} signaling in isolated colonic crypts. A, B) Carbachol induced increase in intracellular Ca^{2+} in the absence and presence of PRL (200ng/ml). C) Summary of the effects of PRL on intracellular baseline Ca^{2+} levels. D) Summary of the effects of PRL on carbachol (CCh, 100 μM) induced Ca^{2+} increase. Both peak and plateau response were reduced in the presence of PRL. *indicate significant difference (paired *t*-test). (number of experiments).

Fig. 9. Simplified transport model of proximal (A) and distal (B) colonic epithelial cells and effects of prolactin (PRL). Activation of the prolactin receptor involves ligand-induced sequential receptor dimerization driven by the prolactin molecule containing two binding sites. As a result of receptor activation, Ca^{2+} dependent Cl^- secretion in the proximal colon and Ca^{2+} dependent K^+ secretion in the distal colon is inhibited.

In contrast to the distal colon where CCh activates BK channels, a Cl^- conductance is activated in mouse proximal colon when stimulated with muscarinic agonists. The molecular identity of this luminal Ca^{2+} -activated Cl^- conductance is still unclear, however, it is clearly different from CFTR since it is activated in the presence of indomethacin and in the absence of cAMP dependent stimulation. This conductance was inhibited by typical blockers of Ca^{2+} -activated Cl^- channels, such as NFA and DIDS. Currently, two families of proteins may serve as molecular candidates for the Ca^{2+} -activated Cl^- channel in the colonic epithelium, CLCA and Bestrophin. In fact in a previous RT-PCR analysis, we detected expression of the isoforms CLCA1 and CLCA2 in colon and T₈₄ colonic carcinoma cells (data not shown). Moreover, CLCA1 has been proposed as the Ca^{2+} -activated Cl^- channel stimulated by carbachol in the rat colonic epithelium [24]. The CLCA gene locus has even been

proposed as a modulator of the gastrointestinal basic defect in cystic fibrosis [25]. Despite these findings, there has

been considerable reluctance to accept the CLCA family as a candidate for Ca^{2+} -activated Cl^- channels, as summarized in previous reviews [10, 26]. Moreover, in a previous study, we identified bestrophin as the likely candidate for CaCC in epithelia [27]. Bestrophins comprise a family of 4 homologous proteins, which have been identified as Ca^{2+} -activated Cl^- channels [10]. The present data supply additional evidence for expression of bestrophin 1, particularly in the proximal colonic epithelium (Fig. 6).

Prolactin inhibits Ca^{2+} -activated conductances in mouse colon

Regulation of electrolyte transport in the mammalian intestine by PRL has been reported in previous studies [28, 29]. While these studies examined both acute and chronic effects of PRL, we focused in the present report on acute effects of PRL on electrolyte transport. Major electrogenic conductances in the colonic epithelium comprise the absorptive ENaC channel and the secretory CFTR [8]. Absorption or secretion via these pathways is supported by the $\text{Na}^+/\text{2Cl}^-/\text{K}^+$ cotransporter, the Na^+/K^+ -ATPase and basolateral K^+ channels, such as intermediate conductance Ca^{2+} -activated and cAMP activated KCNQ1 K^+ channels. While no evidence exists that PRL regulated these conductances, it acutely reduces KCl secretion stimulated by cholinergic agonist, carbachol. PRL has been largely considered to be the adapting hormone in fresh-water fish and teleosts [20, 30] that functions synergistically with cortisol to decrease ion efflux rates in cultured bronchial epithelia from fresh-water rainbow trout [31]. In mammals, PRL is a hormone that has important roles in a wide range of adaptations in the mother [32]. Stress during late pregnancy and lactation was attenuated markedly by PRL [33]. Hyperprolactinemic condition was also reported to decrease the acute stress response in lactating rats [34]. Increased epithelial ionic secretions in small and large intestine have been reported to occur in response to acute stress [35]. From our results, therefore, we speculate that the suppressive action of PRL in colon inhibits a stress induced KCl secretion. During lactation, PRL might be

able to adjust intestinal transport to maintain ionic homeostasis.

The acute inhibitory effects of PRL appear to be limited to Ca^{2+} -activated KCl transport. As demonstrated here, an increase of intracellular Ca^{2+} through muscarinic stimulation is attenuated by PRL. Although PRL exerts its biological actions mainly through the activation of Jak/Stat signaling molecules [36], this pathway does not seem to be relevant for the acute effects on KCl secretion. Apart from Jak/Stat, PRL-R is also coupled to PI3 [36] and MAP kinase pathways, and it appears that these pathways are relevant for the regulation of KCl secretion in the large intestine [19, 37]. However, the present results do not rule out additional effects of PRL on electroneutral Na^+ absorption, via parallel Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchanger [8].

Abbreviations

PRL (prolactin); IBMX (3-isobutyl-1-methylxanthine); ATP (adenosine 5'-triphosphate); CCh (carbachol); NFA (niflumic acid); DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid); cAMP (cyclic adenosine monophosphate); BAPTA-AM (1,2-bis (2 aminophenoxy) ethan- $\text{N},\text{N},\text{N}',\text{N}'$ -tetraacetic acid acetoxymethyl ester); CFTR (cystic fibrosis transmembrane conductance regulator); ENaC (epithelial Na^+ channels).

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References

- Hasegawa S, Hirano T, Kawauchi H: Sodium-retaining activity of chum salmon prolactin in some euryhaline teleosts. *Gen Comp Endocrinol* 1986;63:309-317.
- Moriya T: Prolactin induces increase in the specific gravity of salamander, *Hynobius retardatus*, that raises adaptability to water. *J Exp Zool* 1982;223:83-88.
- Mainoya JR: Effect of prolactin on fluid and NaCl absorption by the rat proximal and distal colon. *Experientia* 1979;35:1060-1061.

- 4 Mainoya JR: Analysis of the role of endogenous prolactin on fluid and sodium chloride absorption by the rat jejunum. *J Endocrinol* 1975;67:343-349.
- 5 Bakker-Teunissen OJ, Arts NF, Mulder GH: Fluid transport across human fetal membranes affected by human amniotic fluid prolactin: an in vitro study. *Placenta* 1988;9:533-545.
- 6 Krishnamra N, Wirunrattanakij Y, Limlomwongse L: Acute effects of prolactin on passive calcium absorption in the small intestine by in vivo perfusion technique. *Can J Physiol Pharmacol* 1998;76:161-168.
- 7 Krishnamra N, Ousingsawat J, Limlomwongse L: Study of acute pharmacologic effects of prolactin on calcium and water transport in the rat colon by an in vivo perfusion technique. *Can J Physiol Pharmacol* 2001;79:415-421.
- 8 Kunzelmann K, Mall M: Electrolyte transport in the colon: Mechanisms and implications for disease. *Physiol Rev* 2002;82:245-289.
- 9 Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA: Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr Rev* 1998;19:225-268.
- 10 Hartzell HC, Putzier I, Arreola J: Calcium-Activated Chloride Channels. *Annu.Rev.Physiol* 2005;67:719-758.
- 11 Dickson JL, Conner TD, Ecay TW: Inhibition of phosphatidylinositol 3-kinase does not alter forskolin-stimulated Cl(-) secretion by T84 cells. *Am J Physiol Cell Physiol* 2000;278:C865-C872.
- 12 Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC: SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* 1995;364:229-233.
- 13 Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM: Identification of a novel inhibitor of mitogen-activated protein kinase. *J Biol Chem.* 1998;273:18623-18632.
- 14 Nielsen M, Kaltoft K, Nordahl M, Ropke C, Geisler C, Mustelin T, Dobson P, Svejgaard A, Odum N: Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. *Proc Natl Acad Sci USA* 1997;94:6764-6769.
- 15 Sausbier M, Matos JE, Sausbier U, Beranek G, Arntz C, Neuhuber W, Ruth P, Leipziger J: Distal Colonic K⁺ Secretion Occurs via BK Channels. *J Am Soc Nephrol* 2006;17:1275-1282.
- 16 Elia MG, Muscella A, Romano S, Greco S, Di Jeso B, Verri T, Storelli C, Marsigliante S: Effects of extracellular nucleotides in the thyroid: P2Y2 receptor-mediated ERK1/2 activation and c-Fos induction in PC Cl3 cells. *Cell Signal* 2005;17:739-749.
- 17 Lavie Y, Agranoff BW: Carbachol inhibits insulin-stimulated phosphatidylinositol 3-kinase activity in SH-SY5Y neuroblastoma cells. *J Neurochem* 1996;67:1245-1251.
- 18 Slack BE: The m3 muscarinic acetylcholine receptor is coupled to mitogen-activated protein kinase via protein kinase C and epidermal growth factor receptor kinase. *Biochem J* 2000;348 Pt 2:381-387.
- 19 Keely SJ, Barrett KE: p38 mitogen-activated protein kinase inhibits calcium-dependent chloride secretion in T84 colonic epithelial cells. *Am J Physiol Cell Physiol* 2003;284:C339-C348.
- 20 Shanley LJ, O'Malley D, Irving AJ, Ashford ML, Harvey J: Leptin inhibits epileptiform-like activity in rat hippocampal neurones via PI 3-kinase-driven activation of BK channels. *J Physiol* 2002;545:933-944.
- 21 O'Malley D, Harvey J: Insulin activates native and recombinant large conductance Ca(2+)-activated potassium channels via a mitogen-activated protein kinase-dependent process. *Mol Pharmacol* 2004;65:1352-1363.
- 22 Hay-Schmidt A, Grunnet M, Abrahamse SL, Knaus HG, Klaerke DA: Localization of Ca(2+)-activated big-conductance K(+) channels in rabbit distal colon. *Pflugers Arch* 2003;446:61-68.
- 23 Wachter C, Turnheim K: Inhibition of high-conductance, calcium-activated potassium channels of rabbit colon epithelium by magnesium. *J Membr Biol* 1996;150:275-282.
- 24 Schultheiss G, Siefjediers A, Diener M: Muscarinic receptor stimulation activates a Ca(2+)-dependent Cl(-) conductance in rat distal colon. *J Membr Biol* 2005;204:117-127.
- 25 Ritzka M, Stanke F, Jansen S, Gruber AD, Pusch L, Woelfl S, Veeze HJ, Halley DJ, Tummler B: The CLCA gene locus as a modulator of the gastrointestinal basic defect in cystic fibrosis. *Hum Genet* 2004;115:483-491.
- 26 Eggermont J: Calcium-activated chloride channels: (un)known, (un)loved? *Proc Am Thorac Soc* 2004;1:22-27.
- 27 Barro Soria R, Spitzner M, Schreiber R, Kunzelmann K: Bestrophin 1 enables Ca²⁺ activated Cl⁻ conductance in epithelia. *J Biol Chem (in press)* 2006.
- 28 Mainoya JR, Bern HA, Regan JW: Influence of ovine prolactin on transport of fluid and sodium chloride by the mammalian intestine and gall bladder. *J Endocrinol* 1974;63:311-317.
- 29 Charoenphandhu N, Limlomwongse L, Krishnamra N: Prolactin directly stimulates transcellular active calcium transport in the duodenum of female rats. *Can J Physiol Pharmacol* 2001;79:430-438.
- 30 Pickford GE, Phillips JG: Prolactin, a factor in promoting survival of hypophysectomized killifish in fresh water. *Science* 1959;130:454-455.
- 31 Zhou B, Kelly SP, Ianowski JP, Wood CM: Effects of cortisol and prolactin on Na⁺ and Cl⁻ transport in cultured branchial epithelia from FW rainbow trout. *Am J Physiol Regul Integr Comp Physiol* 2003;285:R1305-R1316.
- 32 Grattan DR: Behavioural significance of prolactin signalling in the central nervous system during pregnancy and lactation. *Reproduction* 2002;123:497-506.
- 33 Lightman SL: Alterations in hypothalamic-pituitary responsiveness during lactation. *Ann NY Acad Sci* 1992;652:340-346.
- 34 Tilly BC, Kansen M, Gageldonk PG, van den Berghe N, Galjaard H, Bijman J, de Jonge HR: G-proteins mediate intestinal chloride channel activation. *J Biol Chem* 1991;266:2036-2040.
- 35 Soderholm JD, Perdue MH: Stress and gastrointestinal tract. II. Stress and intestinal barrier function. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G7-G13.
- 36 Freeman ME, Kanyicska B, Lerant A, Nagy G: Prolactin: structure, function, and regulation of secretion. *Physiol Rev* 2000;80:1523-1631.
- 37 McCole DF, Keely SJ, Coffey RJ, Barrett KE: Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-alpha. *J Biol Chem* 2002;277:42603-42612.