Lubiprostone Activation of Cl^- Currents Does Not Involve Ca^{2+}, cAMP, or PKA

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Abstract

Introduction

Lubiprostone stimulates electrogenic Cl^- transport in human intestinal T84 cells and Cl^- currents in HEK293 cells stably transfected with recombinant human ClC-2 (hClC-2) with an EC_{50} of approximately 20 nM [Cuppoletti et al AJP 287:C1173, 2004]. The purpose of this study was to determine whether lubiprostone (over the concentration range that activates electrogenic Cl^- transport) causes changes in $[Ca^{2+}]_i$, cAMP or PKA mediated phosphorylation.

Methods

T84 cells, HEK293 cells and HEK293 cells stably expressing hClC-2 or mutant hClC-2 were used. T84 cell $[Ca^{2+}]_i$ was measured using the calcium indicator dye indo-1/AM and intracellular [cAMP] was determined using a commercial ELISA kit. A commercial FLIPER assay was used to measure $[Ca^{2+}]_i$, and [cAMP] in HEK293 cells. Cl^- currents were measured by whole cell patch clamp.

Results

Lubiprostone at 1, 10 or 100 nM did not increase $[Ca^{2+}]_i$ in T84 cells and only slightly increased [cAMP] from 0.56 ± 0.02 to 4.0 ± 0.3 pmole/10^5 cells (n=4, P<0.001) at 100 nM (5 times the EC_{50} for lubiprostone activation of Cl^- currents). 1 µM PGE_1 significantly (P<0.0005) increased $[Ca^{2+}]_i$ from 80.8 ± 2.4 nM (control) to 1175.0 ± 18.7 nM (n=3) and greatly increased [cAMP] to 37.6 ± 0.6 pmole/10^5 cells (n=4) (P<0.0005). In contrast, 100 nM lubiprostone had no effect on $[Ca^{2+}]_i$ or cAMP in HEK293 cells. Nevertheless hClC-2 in HEK293 cells is activated by lubiprostone, thereby demonstrating that neither $[Ca^{2+}]_i$ or cAMP signaling are involved in lubiprostone activation of hClC-2. To rule out involvement of PKA phosphorylation of hClC-2 in lubiprostone activation of Cl^- currents, a mutant hClC-2 Cl^- channel lacking the two unique PKA consensus phosphorylation sites (RRAT and RGET) essential for PKA activation of ClC-2, was used [Cuppoletti, et al JBC 279:21849, 2004]. In wild-type hClC-2 expressing HEK293 cells, control Cl^- currents at -140 mV (n=6) of -27.2 ± 3.5 pA/pF, significantly increased (P<0.0005) to -100.9 ± 6.5 pA/pF with 20 nM lubiprostone and subsequently were reduced with 500 µM $CdCl_2$ to control levels, -29.9 ± 5.6 pA/pF (P<0.0005). Cl^- currents in mutant hClC-2 expressing HEK293 cells was also significantly (P<0.0005) increased with 20 nM lubiprostone and inhibited by $CdCl_2$ (n=6): control, -27.1 ± 6.9 pA/pF; with lubiprostone, -81.9 ± 5.1 (P<0.0005); after $CdCl_2$, -29.4 ± 3.7 pA/pF (P<0.0005).

Conclusions

Activation of Cl^- currents by lubiprostone is independent of $[Ca^{2+}]_i$ and cAMP signaling and PKA mediated phosphorylation of hClC-2. More direct action of lubiprostone on hClC-2 Cl^- channels may be involved.

Supported by Sucampo Pharmaceuticals, Inc.

Introduction

Lubiprostone stimulates electrogenic Cl^- transport in human intestinal T84 cells and Cl^- currents in HEK293 cells stably transfected with recombinant human ClC-2 (hClC-2) with an EC_{50} of approximately 20 nM[1]. The purpose of this study was to determine whether lubiprostone (over the concentration range that activates electrogenic Cl^- transport/Cl^- currents) causes changes in $[Ca^{2+}]_i$, cAMP or PKA mediated phosphorylation.

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Methods

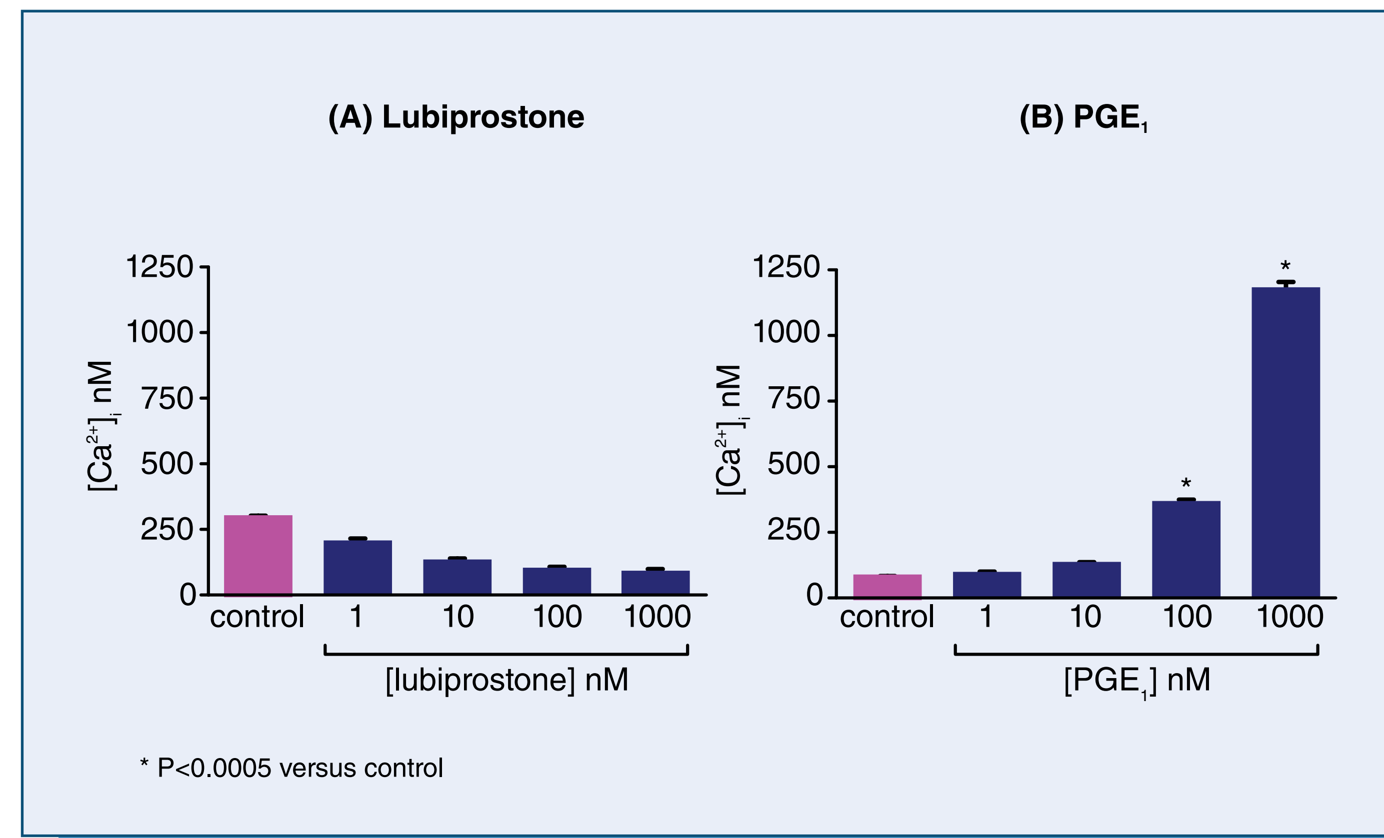
T84 cells, HEK293 cells and HEK293 cells stably expressing hClC-2 or mutant hClC-2 were used. $[Ca^{2+}]_i$ in T84 cells was measured using the calcium indicator dye indo-1/AM and intracellular [cAMP] was determined using a commercial ELISA kit. A commercial FLIPER assay was used to measure $[Ca^{2+}]_i$, and [cAMP] in HEK293 cells. Cl^- currents were measured by whole cell patch clamp. Mutant hClC-2 with RRAT(A) and RGET(A) was made using site-directed mutagenesis and stably expressed in HEK293 cells as previously described.[2]

Results

$[Ca^{2+}]_i$ was measured using indo-1/AM before and after addition of 1, 10, 100 or 1000 nM lubiprostone (A) or PGE_1 (B). Data are plotted as mean ± SEM (n=3).

1, 10, 100 or 1000 nM lubiprostone did not increase $[Ca^{2+}]_i$, in T84 cells. In contrast, 1 µM PGE_1 significantly (P<0.0005) increased $[Ca^{2+}]_i$ from 80.8 ± 2.4 nM (control) to 1175.0 ± 18.7 nM.

Figure 1. Effects of Lubiprostone (A) and PGE_1 (B) on $[Ca^{2+}]_i$ in T84 Cells



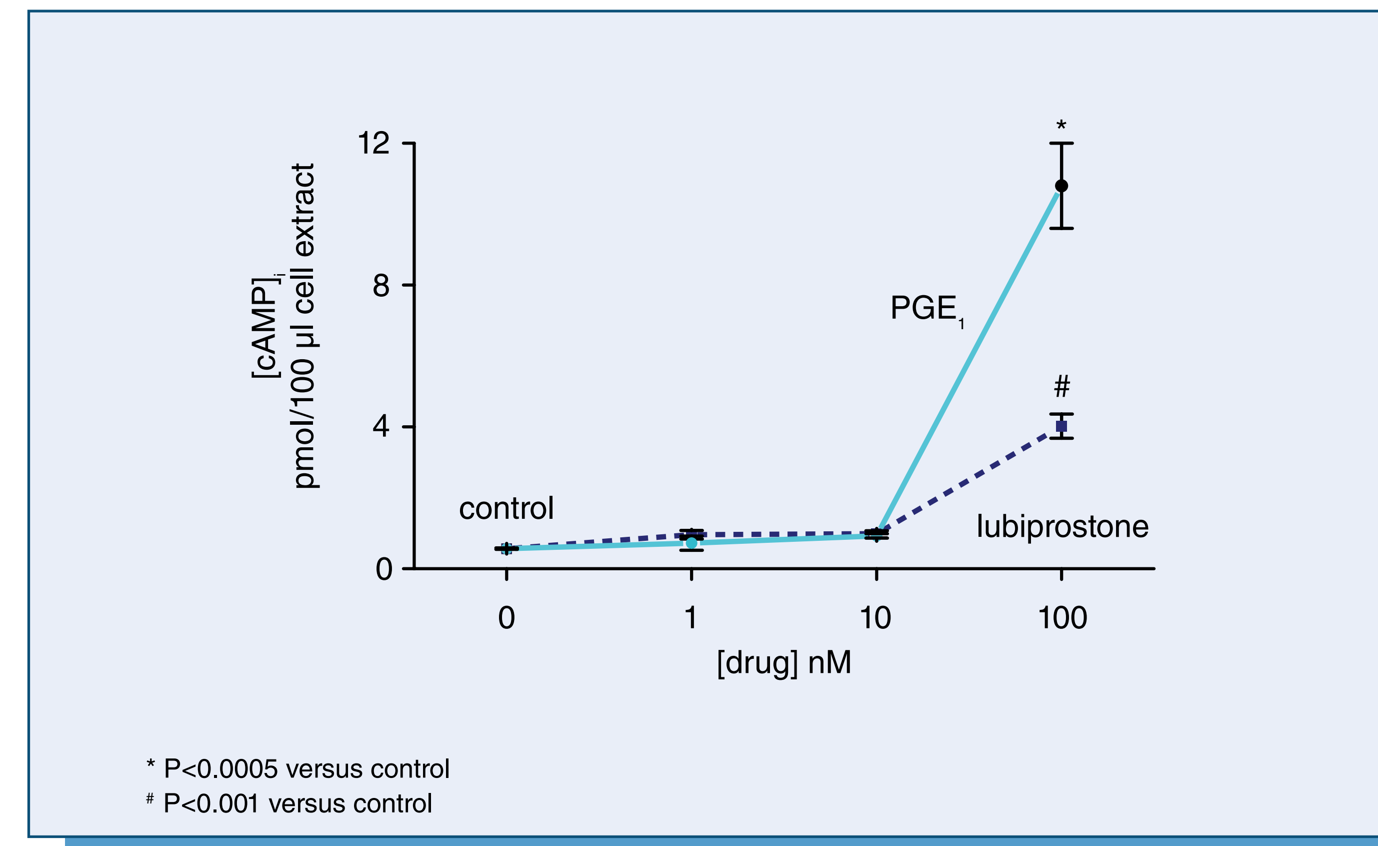
$[cAMP]_i$ was measured before and after addition of 1, 10, 100 nM lubiprostone (A) or PGE_1 (B). Data are plotted as mean ± SEM (n=3).

Lubiprostone at 1, 10 or 100 nM only slightly increased $[cAMP]_i$ from 0.56 ± 0.02 to 4.0 ± 0.3 pmole/100 µl cell extract (n=4, P<0.001) at 100 nM (5 times the EC_{50} for lubiprostone activation of Cl^- currents). In contrast, 100 nM PGE_1 significantly (P<0.0005) increased $[cAMP]_i$ to 10.8 ± 0.6 pmole/100 µl cell extract (n=4). 1 µM PGE_1 greatly increased $[cAMP]_i$ to 37.6 ± 0.7 pmole/100 µl cell extract (n=4). 150 µl cell extract ≈ 10^5 cells.

Similarly, 100 nM lubiprostone also had no effect on $[Ca^{2+}]_i$ or $[cAMP]_i$ in HEK293 cells. Nevertheless hClC-2 in HEK293 cells is activated by lubiprostone (Cuppoletti et al AJP 287:C1173, 2004), thereby demonstrating that neither $[Ca^{2+}]_i$ or cAMP signaling are involved in lubiprostone activation of hClC-2.

From data in Figs 1 & 2: in T84 cells and HEK293 cells lubiprostone had little or no effect on $[cAMP]_i$ and $[Ca^{2+}]_i$. In contrast, in T84 cells PGE_1 significantly increased $[cAMP]_i$ and $[Ca^{2+}]_i$.

Figure 2. Effects of Lubiprostone and PGE_1 on $[cAMP]_i$ in T84 Cells



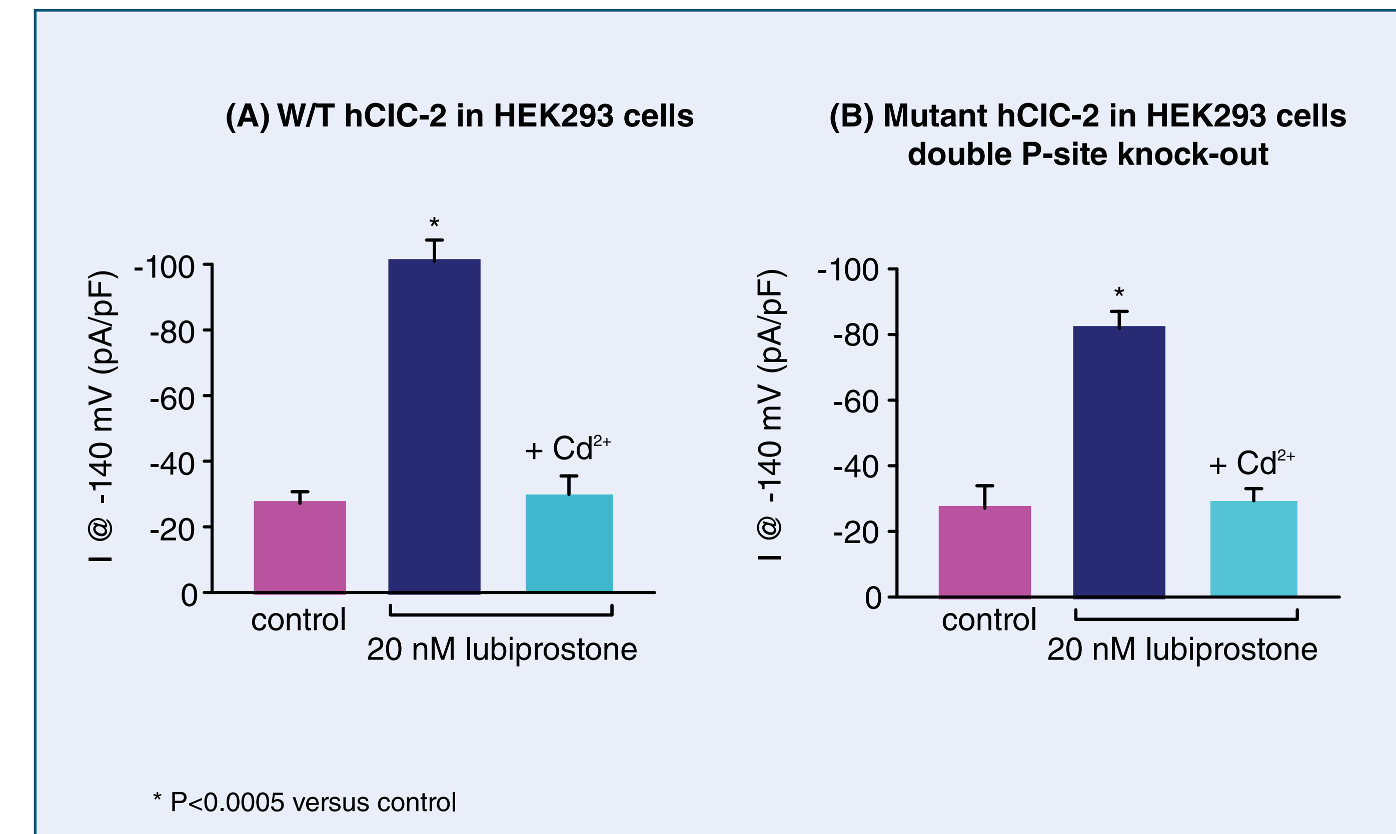
Effect of 20 nM lubiprostone on Cd^{2+}-inhibitable Cl^- currents in HEK293 cells expressing (A) wild-type recombinant human ClC-2 and (B) mutant human ClC-2 lacking the two unique PKA consensus phosphorylation sites, RRAT and RGET. To rule out involvement of PKA phosphorylation of hClC-2 in lubiprostone activation of Cl^- currents, a mutant hClC-2 Cl^- channel lacking the two unique PKA consensus phosphorylation sites (RRAT and RGET) essential for PKA activation of ClC-2, was used [Cuppoletti et al JBC 279:21849, 2004]. Cl^- currents were measured for 30 s in W/T and mutant hClC-2 stably transfected HEK293 cells by patch clamp before and after addition of 20 nM lubiprostone and then after addition of 500 µM $CdCl_2$. Currents normalized to cell capacitance at -140 mV are plotted as mean ± SEM.

(A) In wild-type hClC-2 expressing HEK293 cells, control Cl^- currents at -140 mV (n=6) of -27.2 ± 3.5 pA/pF, significantly increased (P<0.0005) to -100.9 ± 6.5 pA/pF with 20 nM lubiprostone and subsequently were reduced by 500 µM $CdCl_2$ to control levels, -29.9 ± 5.6 pA/pF (P<0.0005).

(B) Cl^- currents in mutant hClC-2 expressing HEK293 cells were also significantly (P<0.0005) increased with 20 nM lubiprostone and inhibited by $CdCl_2$ (n=6): control, -27.1 ± 6.9 pA/pF; with lubiprostone, -81.9 ± 5.1 (P<0.0005); after $CdCl_2$, -29.4 ± 3.7 pA/pF (P<0.0005).

Lubiprostone activation of hClC-2 mediated Cl^- currents is independent of PKA-mediated phosphorylation of hClC-2.

Figure 3. Effect of Lubiprostone Is Independent of PKA-Phosphorylation of hClC-2



Summary

- In T84 cells lubiprostone did not increase $[Ca^{2+}]_i$, or $[cAMP]_i$, while PGE_1 significantly increased both $[Ca^{2+}]_i$ and $[cAMP]_i$.
- In HEK293 cells 100 nM lubiprostone also had no effect on $[Ca^{2+}]_i$ or $[cAMP]_i$.
- Cd^{2+}- sensitive Cl^- currents were activated similarly by lubiprostone in HEK293 cells expressing wild-type or double PKA phosphorylation site knock-out [RRAT(A), RGET(A)] mutant human ClC-2.

Conclusions

Activation of Cl^- currents by lubiprostone is independent of $[Ca^{2+}]_i$ and cAMP signaling and PKA mediated phosphorylation of hClC-2.

More direct action of lubiprostone on hClC-2 Cl^- channels may be involved.

Acknowledgment

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References

1. Cuppoletti J, Malinowska DH, Tewari KP, Li QJ, Sherry AM, Patchen ML, Ueno R (2004) SPI-0211 activates T84 cell chloride transport and recombinant human ClC-2 chloride currents. Am J Physiol Cell Physiol 287: C1173-1183.
2. Cuppoletti J, Tewari KP, Sherry AM, Ferrante CJ, Malinowska DH (2004) Sites of protein kinase A activation of the human ClC-2 Cl^- channel. J Biol Chem 279: 21849-21856.