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D-*myo*-Inositol 1,4,5,6-tetrakisphosphate produced in human intestinal epithelial cells in response to *Salmonella* invasion inhibits phosphoinositide 3-kinase signaling pathways

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ABSTRACT **Several inositol-containing compounds play key roles in receptor-mediated cell signaling events. Here, we describe a function for a specific inositol polyphosphate, D-*myo*-inositol 1,4,5,6-tetrakisphosphate [Ins(1,4,5,6)P_4], that is produced acutely in response to a receptor-independent process. Thus, infection of intestinal epithelial cells with the enteric pathogen *Salmonella*, but not with other invasive bacteria, induced a multifold increase in Ins(1,4,5,6)P_4 levels. To define a specific function of Ins(1,4,5,6)P_4, a membrane-permeant, hydrolyzable ester was used to deliver it to the intracellular compartment, where it antagonized epidermal growth factor (EGF)-induced inhibition of calcium-mediated chloride (Cl^-) secretion (CaMCS) in intestinal epithelia. This EGF function is likely mediated through a phosphoinositide 3-kinase (PtdIns3K)-dependent mechanism because the EGF effects are abolished by wortmannin, and three different membrane-permeant esters of the PtdIns3K product phosphatidylinositol 3,4,5-trisphosphate mimicked the EGF effect on CaMCS. We further demonstrate that Ins(1,4,5,6)P_4 antagonized EGF signaling downstream of PtdIns3K because Ins(1,4,5,6)P_4 interfered with the PtdInsP_3 effect on CaMCS without affecting PtdIns3K activity. Thus, elevation of Ins(1,4,5,6)P_4 in *Salmonella*-infected epithelia may promote Cl^- flux by antagonizing EGF inhibition mediated through PtdIns3K and PtdInsP_3.**

Inositol-based compounds are central to many signal transduction pathways. For example, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is a precursor for the phospholipase C product inositol 1,4,5-trisphosphate, which is a key regulator of intracellular calcium levels (1). Moreover, PtdIns(4,5)P_2 is a substrate for phosphoinositide 3-kinase (PtdIns3K), which yields phosphatidylinositol 3,4,5-trisphosphate (PtdInsP_3). PtdIns3K is triggered by a wide range of growth factors and cytokines, and it and/or its products contribute to the regulation of many cell functions, including cell proliferation, differentiation, and apoptosis (2). However, although more than 30 inositol-containing phospholipids and inositol polyphosphates have been identified, relatively little is known about the roles of most of these compounds in cellular signaling.

Chloride (Cl^-) secretion by polarized epithelia at mucosal surfaces plays a key role in controlling salt and fluid secretion. In the intestine, for example, constitutive Cl^- flux is important in the maintenance of normal hydration and provides solvent for extracellular solutes. Furthermore, Cl^- and consequently water flux increase after infection with many enteric pathogens (e.g., *Salmonella*), and this host response contributes to the removal of the invading pathogen from the intestinal tract (3, 4). Several pathways have been identified that stimulate Cl^- secretion through the elevation of either cAMP or intracellular calcium levels, which act on separate apically located Cl^- channels (5). In addition, Cl^- secretion is controlled by negative regulatory mechanisms. For example, we have identified two separate receptor-activated signaling cascades that inhibit the calcium-mediated Cl^- secretory pathway [e.g., calcium-mediated Cl^- secretion (CaMCS)] in intestinal epithelia: (*i*) muscarinic stimulation of inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P_4] levels results in inhibition of CaMCS through a direct effect on Cl^- flux (6–8) and (*ii*) epidermal growth factor (EGF) inhibits CaMCS through a PtdIns3K pathway (9, 10). Thus, the overall level of Cl^- secretion is determined by a balance between stimulatory and inhibitory factors that act on the intestinal epithelium.

Invasive enteric bacteria enter and penetrate the intestinal epithelium to gain access to the underlying mucosa and initiate systemic infection. Bacterial entry into epithelial cells is an interactive process that requires bacterial and host cell signaling events (11, 12) and triggers epithelial cell responses that play a role in host protection against the invading bacteria (4, 13). A possible role for inositol polyphosphates in the epithelial response to bacterial invasion was suggested by earlier studies, in which *Salmonella* invasion elicited an overall increase in inositol polyphosphate turnover (14), but the nature and source of these changes remained undefined. Because inositol polyphosphates are potentially important in cell signaling, we examined the changes in the levels of specific cellular inositol polyphosphates in response to *Salmonella* invasion of intestinal epithelial cells. The data show that infection of intestinal epithelial cells with *Salmonella*, but not other invasive bacteria, induces Ins(1,4,5,6)P_4 production. Furthermore, this inositol isomer has a function in epithelial cells by antagonizing signaling through PtdIns3K pathways. As a consequence, the negative regulation of CaMCS by EGF is antagonized, and could thereby contribute to the bacterially induced diarrhea.

MATERIALS AND METHODS

Reagents. Inositol phosphates and their corresponding membrane-permeant acetoxymethyl esters were synthesized as described (6). Pluronic F-127 was a generous gift from BASF

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Abbreviations: PtdIns3K, phosphoinositide 3-kinase; PtdInsP_3, phosphatidylinositol 3,4,5-trisphosphate; CaMCS, calcium-mediated chloride secretion; EGF, epidermal growth factor; Ins(1,4,5,6)P_4, D-*myo*-inositol 1,4,5,6-tetrakisphosphate; ΔI_{sc}, short circuit current.
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Bioresearch (Leverkusen, Germany). Membrane-permeant analogues of $PtdInsP_3$ were prepared by total synthesis as will be described elsewhere (T.J., G. Sweeney, A. Klip, A.E.T.-K., and R.T., and C.S., M.T.R., H. H. Gillandt, and A.E.T.-K., unpublished work). The preparation of DiC_{16}-BtPtdInsP_3/AM was ≈5% DiC_{16}-BtPtdInsP_3/AM and 95% dipalmitoylglycerol whereas the preparations of DiC_8-PtdInsP_3/AM and DiC_{12}-PtdInsP_3/AM were >99% pure, as determined by proton nuclear magnetic resonance spectroscopy and positive ion mode electron spray mass spectroscopy. Radiolabeled inositol phosphates used as standards for HPLC were from NEN. Phosphatidylinositol for *in vitro* PtdIns3K assays was obtained from Avanti Polar Lipids. EGF was from Genzyme.

Cells. The T_{84} human colon epithelial cell line (15) was maintained in 50% DMEM and 50% Ham's F-12 medium, supplemented with 5% newborn calf serum and 2 mM glutamine. The LS174T human colon adenocarcinoma cell line (CL 188) was obtained from the American Type Culture Collection and maintained in DMEM supplemented with 10% fetal calf serum, 10 mM Hepes, and 2 mM glutamine.

Bacterial Strains. The following bacteria were used in these studies (13, 16): *Salmonella dublin*, *Salmonella typhi* BRD691 (an *aroA*/*aroC* mutant that invades epithelial cells normally but does not replicate inside the cells), *Yersinia enterocolitica*, enteroinvasive *Escherichia coli* (serotype O29:NM), enterohemorrhagic *E. coli* (serotype O157), *Shigella dysenteriae, Shigella flexneri,* and nonpathogenic *E. coli* DH5α (GIBCO/BRL). Bacteria were grown and prepared for infection as described before (13, 16).

[^{3}H]Inositol Labeling and Extraction of Cellular Inositol Polyphosphates. Confluent epithelial monolayers in 6-well plates were incubated for 4 days with 50 μCi/well [^{3}H]inositol (specific activity 80–120 Ci/mmol; Amersham) in inositol-free 50% DMEM, 50% Ham's F-12 medium, supplemented with 5% dialyzed newborn calf serum. Cultures were washed three times with prewarmed medium (50% DMEM/50% Ham's F-12 medium/1 mg/ml BSA) and infected with 5×10^8 bacteria/well using the same medium for various periods of time. Cultures were washed twice with ice-cold PBS and lysed for 5 min on ice in 0.5 ml/well 10% trichloroacetic acid/10 mM phytic acid, and extracts were neutralized using 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine. [^{3}H]inositol polyphosphates in the extracts were separated on an Adsorbosphere SAX column (Alltech Associates), and the radioactive peaks were quantitated as described before (17).

Enantiomeric Identification of Ins(1,4,5,6)P_4. Cell extracts were fractionated by HPLC using an Adsorbosphere SAX column. The [^{3}H]Ins(3,4,5,6)P_4/[^{3}H]Ins(1,4,5,6)P_4 peak was separated from all other [^{3}H]InsP_4 isomers, desalted, and then incubated with partially purified Ins(1,4,5,6)P_4 3-kinase with an internal standard of [^{33}P]Ins(1,4,5,6)P_4 (6). Rat hepatic Ins(1,4,5,6)P_4 3-kinase was purified 240-fold, with a yield of 3%, by Mono Q (Pharmacia) anion-exchange chromatography (18) followed by blue A affinity chromatography and Ins(1,4,5)P_3 affinity chromatography (A. Craxton, M. Hirata, and S.B.S., unpublished data). The enzyme preparation did not phosphorylate Ins(3,4,5,6)P_4. The [^{33}P]Ins(1,4,5,6)P_4 standard was prepared by dephosphorylation of [^{33}P]Ins(1,3,4,5,6)P_5 by using multiple inositol polyphosphate phosphatase (19). [^{33}P]Ins(1,3,4,5,6)P_5 was purified from turkey erythrocytes that were labeled radioactively by using methods described before except that [^{33}P]P_i was used instead of [^{32}P]P_i (20). Equal amounts of ^{3}H and ^{33}P radioactivity were added to the reaction, and the ratio of [^{3}H]Ins(3,4,5,6)P_4:[^{3}H]Ins(1,4,5,6)P_4 in the original peak was determined by comparing the relative amounts of ^{3}H- and ^{33}P-labeled Ins(1,3,4,5,6)P_5 formed. As an example, in a representative experiment, 2,263 dpm of the purified [^{3}H]Ins(3,4,5,6)P_4/[^{3}H]Ins(1,4,5,6)P_4 peak obtained from *S. dublin*-infected T_{84} cells was mixed with 1,675 dpm of [^{33}P]Ins(1,4,5,6)P_4 standard, and the appropriate buffer and Ins(1,4,5,6)P_4 3-kinase were added. After 30 min, the reaction was found to contain 1,842 dpm [^{3}H]Ins(1,3,4,5,6)P_5 and 1,458 dpm [^{33}P]Ins(1,3,4,5,6)P_5. Thus, phosphorylation efficiencies were 1,842 dpm/2,263 dpm × 100% = 81% for the [^{3}H]Ins(3,4,5,6)P_4/[^{3}H]Ins(1,4,5,6)P_4 fraction and 1,458 dpm/1,675 dpm × 100% = 87% for the internal standard. It follows that 81%/87% × 100% = 93% of the [^{3}H]Ins(3,4,5,6)P_4/[^{3}H]Ins(1,4,5,6)P_4 peak was [^{3}H]Ins(1,4,5,6)P_4, with the remainder being [^{3}H]Ins(3,4,5,6)P_4.

Ussing Chamber Experiments. T_{84} cells were seeded onto microporous filter inserts (Snapwells, Costar) and grown to confluence for 7–12 days. The inserts were mounted into modified Ussing chambers (Physiologic Instruments, San Diego), bathed with Ringer's solution warmed to 37°C and gassed continuously with 95% O_2/5% CO_2 at a rate of 30–35 ml/min. The spontaneous potential difference across the monolayer was short-circuited with a voltage clamp (model VCC MC6, Physiologic Instruments). Short circuit current (I_{sc}) and conductance were recorded at 4-s intervals by using ACQUIRE AND ANALYSE software 1.1 (Physiologic Instruments). Increased I_{sc} stimulated through cholinergic pathways (e.g., after carbachol addition) in T_{84} cells is wholly reflective of Cl^- secretion (21). $[Ca^{2+}]_i$ levels were elevated with carbachol (100 μM) applied to the medium bathing the basolateral side of the monolayers.

PtdIns3K Assays. PtdIns3K activity was measured in anti-PtdIns3K (rabbit polyclonal anti-85K subunit, Upstate Biotechnology, Lake Placid, NY) or mouse monoclonal anti-phosphotyrosine (clone 4G10, Upstate Biotechnology) immunoprecipitates exactly as described (10). For experiments to determine the effect of Ins(1,4,5,6)P_4 on PtdIns3K activity *in vitro*, T_{84} cells were stimulated with EGF (16.3 nM) for 1 and 5 min, and PtdIns3K was immunoprecipitated and resuspended in reaction buffer (10). Ins(1,4,5,6)P_4 (200 μM) was added 10 min before addition of [γ-^{32}P]ATP. Wortmannin (50 nM) was used as a control.

RESULTS

***Salmonella* Infection Induces Ins(1,4,5,6)P_4 Production in Intestinal Epithelial Cells.** To determine changes in inositol polyphosphate metabolism after *Salmonella* infection, monolayers of T_{84} human colonic epithelial cells were labeled with [^{3}H]inositol and infected for varying periods of time with *S. dublin*. Inositol polyphosphates were extracted and resolved by HPLC equipped with an on-line radioactivity detector. During the 60-min period immediately after infection, there were no significant changes in the levels of $InsP_6$ or inositol polyphosphates that typically accumulate when phospholipase C is activated, such as [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4,5)P_4, and [^{3}H]Ins(1, 4)P_2 (Fig. 1*a*). In contrast, levels of [^{3}H]Ins(3,4,5,6)P_4/[^{3}H]Ins(1,4,5,6)P_4 (enantiomers that cannot be resolved by HPLC) increased within 10 min after *S. dublin* infection, reaching a 14-fold maximum 30–40 min after infection (Fig. 1). After enantiomeric analysis, this peak was found to consist primarily of Ins(1,4,5,6)P_4 (Fig. 1). We estimate that the intracellular concentrations of Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 were 4.7 and 0.9 μM, respectively, 30 min after *S. dublin* infection, based on our previous determinations of the resting levels of Ins(1,4,5,6)P_4 and Ins(3,4,5,6)P_4 in T_{84} cells (6) and the relative increase of these enantiomers after infection. Levels of Ins(1,4,5,6)P_4 decreased slowly after 40 min and returned to near baseline by 3 h after infection (Table 1). The elevation in [^{3}H]Ins(1,4,5,6)P_4 levels was accompanied by a parallel decline in [^{3}H]InsP_5 levels, reaching a minimum of 50% of control levels 30–60 min after infection (Fig. 1*b*) and by smaller increases in an InsP_3 isomer (labeled Z in Fig. 1*a*) and two InsP_2 isomers (labeled X and Y in Fig. 1*a*). Compounds X, Y, and Z did not match elution patterns of available inositol phosphate standards. Similar observations

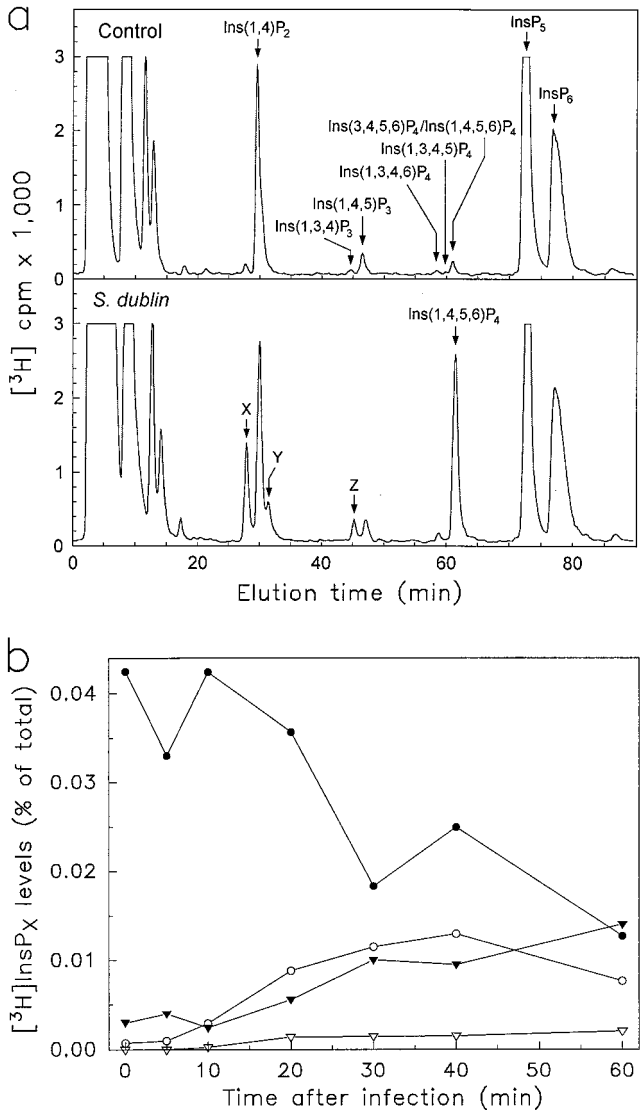

FIG. 1. HPLC analysis of inositol polyphosphate levels after *S. dublin* infection of T_{84} human colonic epithelial cells. [^{3}H]Inositol-labeled T_{84} monolayers were infected with *S. dublin*, and inositol polyphosphates were extracted and analyzed by an HPLC system that resolves three $InsP_4$ peaks, as demonstrated (17). (*a*) A representative radiochromatogram from control cells (top) and cells infected for 30 min with *S. dublin* (bottom). Arrows indicate elution times of known inositol phosphate standards. The $Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4$ peak was collected and analyzed as described in *Materials and Methods* and found to consist of 84 ± 5% (mean ± SEM, $n = 3$) $Ins(1,4,5,6)P_4$ after *S. dublin* infection, with the remainder being $Ins(3,4,5,6)P_4$. In unstimulated cells, the same peak contained approximately equal amounts of both isomers (data not shown) (6). (*b*) A time course of the levels of $InsP_5$ (●), $Ins(1,4,5,6)P_4$ (○), $InsP_3$-Z (▽), and $InsP_2$-X and Y (▼). Data are displayed as the fraction of radioactivity associated with the specific $InsP_x$ relative to the total radioactivity in all cellular $InsP_x$.

were made using another human intestinal epithelial cell line, LS174T, in which [^{3}H]$Ins(1,4,5,6)P_4$ levels increased 11.3-fold after *S. dublin* infection, indicating that the changes in cellular inositol polyphosphates after infection represent a general response of epithelial cells.

Infection of T_{84} cells with another invasive *Salmonella* strain, *S. typhi* BRD691 (16), also increased [^{3}H]$Ins(1,4,5,6)P_4$ levels (Table 1). In contrast, a mutant strain of *S. dublin*, SB133, which attaches normally to epithelial cells but does not invade them (22), increased [^{3}H]$Ins(1,4,5,6)P_4$ levels only minimally, indicating that invasion of host cells by *Salmonella* was required for this response. However, bacterial invasion alone was not sufficient to increase $Ins(1,4,5,6)P_4$ levels because infection of T_{84} cells with several other invasive Gram-negative bacteria, including *S. flexneri*, *S. dysenteriae*, *Y. enterocolitica,* and enteroinvasive *E. coli* (serotype O29:NM), caused only small increases in [^{3}H]$Ins(1,4,5,6)P_4$ levels (Table 1). Furthermore, addition to T_{84} monolayers of noninvasive Gram-negative bacteria such as enterohemorrhagic *E. coli* (serotype O157) or a nonpathogenic *E. coli* (strain DH5α) or addition of bacterial lipopolysaccharide, also had little to no effect on [^{3}H]$Ins(1,4,5,6)P_4$ levels (Table 1).

Table 1. Increase in $Ins(1,4,5,6)P_4$ levels after *Salmonella* infection of T_{84} intestinal epithelial cells

Bacteria added	Time after infection, min	[^{3}H]$Ins(1,4,5,6)P_4$ levels, ratio infected/control	*n*
S. dublin lane	30	13.9 ± 0.8	4
S. dublin lane	60	10.3 ± 1.3	5
S. dublin lane	120	3.6	2
S. dublin lane	180	1.9	2
S. typhi BRD691	30	10.5	2
S. dublin SB133 (*invA*)	30	1.9 ± 0.1	3
S. flexneri	60	1.9	2
S. dysenteriae	60	2.3	2
Y. enterocolitica	60	2.6 ± 0.1	3
E. coli O29:NM	60	2.0 ± 0.1	5
E. coli O157	60	2.3 ± 0.2	4
E. coli DH5α	60	2.1 ± 0.2	4
LPS	60	1.0	2

[^{3}H]Inositol-labeled T_{84} monolayers were infected with various bacteria for the indicated times. [^{3}H]$Ins(1,4,5,6)P_4$ levels were determined by HPLC and are expressed as ratio of the level in infected cells to that in control cells. Results are means ± SEM of the number of determinations indicated in the last column. LPS (lipopolysaccharide) from *E. coli* O111 was used at 10 μg/ml.

$Ins(1,4,5,6)P_4$ Inhibits EGF-Induced Inhibition of Calcium-Mediated Cl^- Secretion in Intestinal Epithelial Cells. We next investigated the functional relationship between the *Salmonella*-induced changes in $Ins(1,4,5,6)P_4$ levels and the secretory response to the infection. Such studies in bacteria-infected cells are hampered by the asynchronous nature of the infection with *Salmonella* and by the fact that a variable proportion of epithelial cells in tissue culture become infected. In addition, *Salmonella* infection is likely to have other effects on epithelial cells that would complicate the identification of a role for $Ins(1,4,5,6)P_4$. To circumvent these problems, we raised $Ins(1,4,5,6)P_4$ levels in resting T_{84} cells by using membrane-permeant D-2,3-di-*O*-butyryl-*myo*-inositol 1,4,5,6-tetrakisphosphate octakis(acetoxymethyl) ester ($Bt_2Ins(1,4,5,6)P_4$/AM), which diffuses into epithelial cells and subsequently is hydrolyzed by ubiquitous, nonspecific cellular esterases to release $Ins(1,4,5,6)P_4$ (6). As a functional assay for these experiments, we assessed the effect of $Ins(1,4,5,6)P_4$ on transepithelial Cl^- secretion across polarized T_{84} monolayers mounted in Ussing chambers.

We found previously that $Bt_2Ins(1,4,5,6)P_4$/AM had no effect on Cl^- secretion by itself, nor did it affect $[Ca^{2+}]_i$ levels (6). Furthermore, $Bt_2Ins(1,4,5,6)P_4$/AM neither potentiated nor inhibited the effects of known agonists of Cl^- secretion. For example, thapsigargin stimulates increased Cl^- secretion through an increase in $[Ca^{2+}]_i$ levels (i.e., CaMCS), and neither Cl^- secretion nor $[Ca^{2+}]_i$ levels are affected by 400 μM of $Bt_2Ins(1,4,5,6)P_4$/AM pretreatment (6). We therefore questioned whether $Ins(1,4,5,6)P_4$ might up-regulate Cl^- secretion by antagonizing a process that normally constrains transepithelial Cl^- flux. For example, $Ins(3,4,5,6)P_4$ inhibits CaMCS (6). However, $Ins(1,4,5,6)P_4$ did not affect this function of $Ins(3,4,5,6)P_4$ (6). We therefore turned our attention to EGF-mediated inhi-

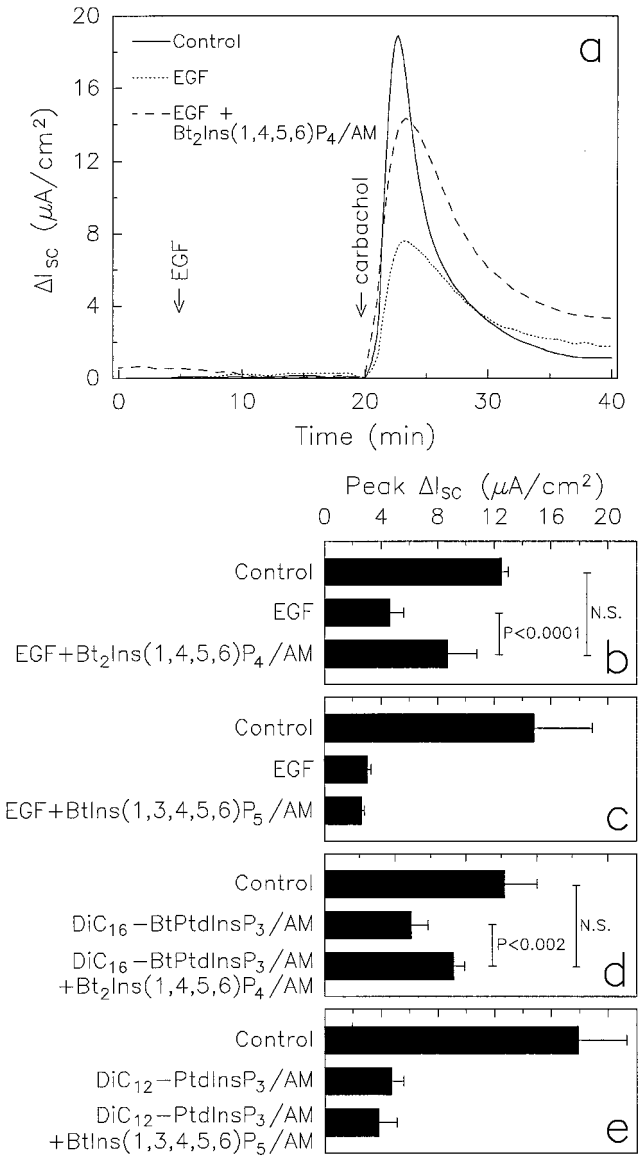

FIG. 2. Membrane-permeant $Ins(1,4,5,6)P_4$ reverses EGF- and $PtdInsP_3$-mediated inhibition of CaMCS. T_{84} monolayers were incubated for 30 min with the indicated membrane-permeant esters (200 μM) or vehicle (dimethyl sulfoxide/5% Pluronic) in Ringer's solution before mounting in Ussing chambers. Based on our previous findings that 1–2% of the membrane-permeant inositol polyphosphate esters enter T_{84} cells (6), we estimate that the intracellular concentration of $Ins(1,4,5,6)P_4$ was ≈2–4 μM after 30 min of incubation with its membrane-permeant ester. (*a*) Time course of ΔI_{sc}. EGF (16.3 nM) was added basolaterally, followed 15 min later by carbachol (100 μM) to acutely elevate $[Ca^{2+}]_i$ levels. Controls also were stimulated with carbachol but not pretreated with EGF. Data are means of duplicate measurements from one representative of three experiments. (*b-e*) Peak ΔI_{sc} after carbachol addition. Data are means ± SEM of 4–10 experiments. Statistical significance was determined by Student's *t* test (N.S., not significant). (*d*) DiC_{16}-$BtPtdInsP_3$/AM was used at 100 μM. Similar results were obtained with DiC_8-$PtdInsP_3$/AM (ΔI_{sc} in $\mu A/cm^2$; controls 14.0 ± 1.9; 200 μM DiC_8-$PtdInsP_3$/AM 7.3 ± 1.7; DiC_8-$PtdInsP_3$ plus $Bt_2Ins(1,4,5,6)P_4$/AM 9.9 ± 1.8; n = 7–8). Not shown, treatment of T_{84} cells with $Bt_2Ins(1,4,5,6)P_4$/AM, $BtIns(1,3,4,5,6)P_5$/AM, or up to 10 mM dipalmitoylglycerol (which was a contaminant in the DiC_{16}-$BtPtdInsP_3$/AM preparations) did not alter peak ΔI_{sc} after carbachol addition compared with control cells.

bition of CaMCS (9). Addition of $Bt_2Ins(1,4,5,6)P_4$/AM to T_{84} monolayers significantly reversed inhibition of CaMCS that was induced by EGF preincubation (Fig. 2 *a* and *b*). In contrast, this inhibitory EGF function was not attenuated by addition of membrane-permeant esters of $Ins(1,3,4,5,6)P_5$ (Fig. 2*c*) or $Ins(3,4,5,6)P_4$ (data not shown), the latter being the enantiomer of $Ins(1,4,5,6)P_4$. These findings demonstrate that $Ins(1,4,5,6)P_4$ can, in an enantiomerically specific manner, up-regulate CaMCS by reversing EGF-mediated inhibition of this process. Furthermore, the $Ins(1,4,5,6)P_4$ action was specific for CaMCS because addition of $Bt_2Ins(1,4,5,6)P_4$/AM to T_{84} monolayers did not reverse EGF inhibition of cAMP-mediated Cl^- secretion (data not shown).

$Ins(1,4,5,6)P_4$ Antagonizes Phosphoinositide 3-Kinase Signaling Pathways in Epithelial Cells. The $Ins(1,4,5,6)P_4$ effect on EGF-mediated inhibition of CaMCS is similar to that reported for the PtdIns3K inhibitor wortmannin (10). This raised the possibility that $Ins(1,4,5,6)P_4$ might interact with PtdIns3K signaling pathways. We first investigated whether PtdIns3K activity itself was affected by $Ins(1,4,5,6)P_4$. However, addition of up to 200 μM $Ins(1,4,5,6)P_4$ had no effect on the activity of immunoprecipitated PtdIns3K *in vitro* (data not shown). Furthermore, $Ins(1,4,5,6)P_4$ did not inhibit PtdIns3K activation after EGF stimulation of T_{84} cells because assays of PtdIns3K activity (see *Materials and Methods*) showed that EGF stimulated this activity in $Bt_2Ins(1,4,5,6)P_4$/AM-treated cells as much, or more, than it did in control cells [the ratio of PtdIns3K activity in EGF-stimulated relative to basal activity in unstimulated control cells was 3.3-fold in $Bt_2Ins(1,4,5,6)P_4$/AM-pretreated cells and 2.7-fold in untreated cells (n = 2); these values fall within the range of those reported (10)].

We next tested whether $Ins(1,4,5,6)P_4$ acted downstream of PtdIns3K. $PtdInsP_3$ is a major product of PtdIns3K in EGF-stimulated T_{84} cells, and our previous studies suggested that $PtdInsP_3$, or one of its metabolites, mediated the EGF effects on CaMCS (10). To test this hypothesis directly, three membrane-permeant analogues of $PtdInsP_3$ were synthesized: dipalmitoyl DL-6-*O*-butyryl-PtdIns(3, 4, 5)P_3/AM (DiC_{16}-$BtPtdInsP_3$/AM), dioctanoyl D-$PtdInsP_3$/AM (DiC_8-$PtdInsP_3$/AM), and dilauryl D-$PtdInsP_3$/AM (DiC_{12}-$PtdInsP_3$/AM). The three different analogues of $PtdInsP_3$ have different solubilities and were produced through separate synthetic pathways (T.J., G. Sweeney, A. Klip, A.E.T.-K., and R.T., and C.S., M.T.R., H. H. Gillandt, and A.E.T.-K., unpublished work). Preincubation of T_{84} monolayers with 100–200 μM of any of the three membrane-permeant forms of $PtdInsP_3$, but not with $PtdInsP_3$ itself (which is not expected to enter cells), inhibited CaMCS by up to 74% (Fig. 2 *d* and *e*) but had no effect on $[Ca^{2+}]_i$ levels after carbachol stimulation (T.J., G. Sweeney, A. Klip, A.E.T.-K., and R.T., unpublished work). Inhibition of CaMCS by DiC_{12}-$PtdInsP_3$/AM was dose-dependent, with 38.5 ± 3.8% inhibition (mean ± SEM, n = 4) after incubation with 20 μM of the ester and 17% inhibition (mean, n = 2) after incubation with 2 μM ester. The maximal inhibition of CaMCS by the membrane-permeant forms of $PtdInsP_3$ was comparable to that observed after EGF stimulation of T_{84} cells (9). Furthermore, addition of EGF to monolayers preincubated with DiC_{12}-$PtdInsP_3$/AM did not result in additional inhibition of CaMCS (data not shown), indicating that $PtdInsP_3$ and EGF work through the same mechanism. We then tested if co-incubation of monolayers with $Bt_2Ins(1,4,5,6)P_4$/AM and either DiC_{16}-$BtPtdInsP_3$/AM or DiC_8-$PtdInsP_3$/AM would reverse the effect of membrane-permeant $PtdInsP_3$ on CaMCS. As shown in Fig. 2*d*, preincubation of T_{84} monolayers with membrane-permeant forms of both $Ins(1,4,5,6)P_4$ and $PtdInsP_3$ led to significantly less inhibition of CaMCS than preincubation with membrane-permeant $PtdInsP_3$ alone. In contrast, preincubation with a membrane-permeant form of $Ins(1,3,4,5,6)P_5$ did not affect inhibition of CaMCS by $PtdInsP_3$ (Fig. 2*e*).

DISCUSSION

These findings represent the first evidence that a physiologically relevant stimulus, the entry of *Salmonella* into colonic epithelia, elicits the elevation of $Ins(1,4,5,6)P_4$ levels. The changes in

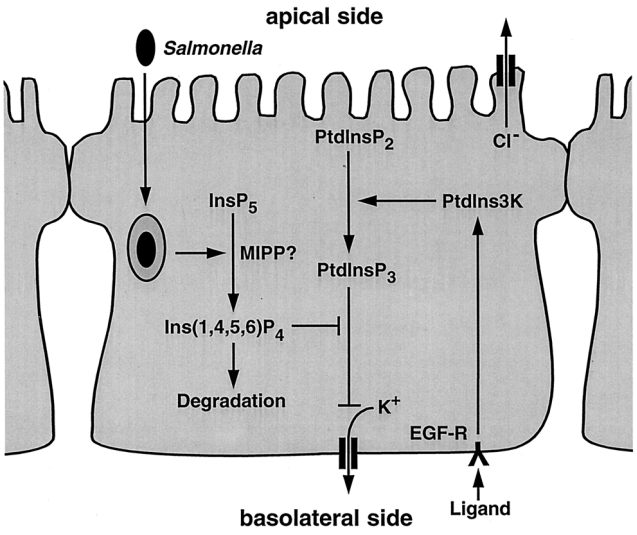

FIG. 3. Proposed model of the mechanism of the effect of *Salmonella*-induced increase in Ins(1,4,5,6)P4 levels on ion transport and PtdIns3K signaling. CaMCS is accompanied by basolateral K^+ efflux, which contributes to the driving force for Cl^- secretion and can be limiting for Cl^- secretion after elevation of $[Ca^{2+}]_i$ levels. We have found that DiC_{12}-$PtdInsP_3$/AM inhibited K^+ efflux (as measured by Rb^+ efflux) in carbachol-stimulated epithelial monolayers (T.J., G. Sweeney, A. Klip, A.E.T.-K., and R.T., unpublished work). Thus, $PtdInsP_3$-mediated inhibition of K^+ efflux can suppress CaMCS. MIPP, multiple inositol polyphosphate phosphatase; EGF-R, EGF receptor. Relevant ligands for the EGF receptor in this model include EGF and transforming growth factor α. Arrowheads depict stimulatory, and capped lines designate inhibitory effects.

inositol polyphosphates after *Salmonella* entry contrast markedly with those observed after receptor-mediated phospholipase C activation, such as increases in $Ins(1,4,5)P_3$, $Ins(1,3,4,5)P_4$, $Ins(1,3,4)P_3$, $Ins(1,3,4,6)P_4$, and $Ins(1,4)P_2$ (17), which suggests that the effects of *Salmonella* invasion on inositol phosphate levels are independent of phospholipase C. Furthermore, elevated $Ins(1,4,5,6)P_4$ levels have been attributed to constitutive src activation in transformed fibroblasts (23), but tyrosine kinases were not involved in the *Salmonella*-induced $Ins(1,4,5,6)P_4$ increase because specific tyrosine kinase inhibitors (genistein, herbimycin A, and tyrphostin A25) did not affect this cellular response (data not shown). Therefore, *Salmonella* appears to affect inositol phosphate metabolism through a unique mechanism. The fact that *S. dublin* did not alter $InsP_6$ levels and the demonstration of reciprocal changes in the levels of $InsP_5$ and $Ins(1,4,5,6)P_4$, together suggest that *S. dublin* infection might activate a phosphatase, such as multiple inositol polyphosphate phosphatase (previously inositol polyphosphate 3-phosphatase) (19), to dephosphorylate $InsP_5$ to $Ins(1,4,5,6)P_4$ (as schematically depicted in Fig. 3, left).

Our data suggest that $Ins(1,4,5,6)P_4$ prevents EGF from inhibiting CaMCS in polarized epithelial cells by antagonizing a $PtdInsP_3$ signaling pathway. Transforming growth factor α, which, like EGF, binds to and activates the EGF receptor, is constitutively expressed in intestinal epithelial cells (24) and inhibits Cl^- secretion through a PtdIns3K pathway (data not shown). Tonic inhibition through this pathway could be counteracted by the *Salmonella*-induced increase in $Ins(1,4,5,6)P_4$ (as schematically shown in Fig. 3, right). Such a mechanism would be expected to act early after infection and mostly in those epithelial cells that are infected with *Salmonella* and could result in diarrhea although other mechanisms also contribute to this outcome (3, 4, 25). Moreover, because $Ins(1,4,5,6)P_4$ disinhibits CaMCS, this inositol polyphosphate, or an analogue, may be useful in treating cystic fibrosis, in which mucosal Cl^- secretion is reduced and depends primarily on CaMCS (26). Because our data indicate that the effect of $Ins(1,4,5,6)P_4$ on Cl^- secretion was downstream of PtdIns3K, it is possible that $Ins(1,4,5,6)P_4$ competes with $PtdInsP_3$ for effector binding sites because $Ins(1,4,5,6)P_4$ is a partial structural analog of the $PtdInsP_3$ headgroup (27). As a result, $Ins(1,4,5,6)P_4$ could compete with phosphoinositide binding to pleckstrin homology domains of signaling proteins and inhibit the normal recruitment of signal transduction complexes to the plasma membrane (28, 29).

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