The mechanisms of action of bacterial toxins have provided novel insights into the control of cellular regulatory processes, including protein synthesis, ion channel activity, and signal transduction (1). Indeed, cholera and pertussis toxins have been instrumental in the identification, respectively, of stimulatory and inhibitory G-proteins controlling adenyl cyclase activity (2, 3). To date, no intracellularly acting toxin has been described which leads to an activation of polyphosphoinositide-specific phospholipase C, another major transducer of transmembrane signaling (4). This phospholipase is involved in the action of many extracellular factors, including mitogenic neuropeptides and growth factors (5).

Recently, Pasteurella multocida toxin has been shown to be an extremely potent and effective mitogen for Swiss 3T3 cells. Other established cell lines, and early passage cultures (6). The toxin is a monomeric 146-kDa protein and has been purified (7–13), cloned (14–16), sequenced (17, 18), and expressed in Escherichia coli (14, 15). The deduced amino acid sequence of P. multocida toxin did not reveal any significant homologies with other toxins or proteins (17, 18). Both native and recombinant P. multocida toxin are mitogenic at picomolar concentrations (6). Several lines of evidence indicated that P. multocida toxin enters the cells and acts intracellularly to initiate and sustain DNA synthesis. Thus, a transient exposure of the cells to the toxin was sufficient to commit them to S phase and division. Furthermore, early but not late addition of either the lysosomotrophic agent methylamine or Pasteurella multocida toxin antiserum selectively blocked the mitogenic action of recombinant P. multocida toxin (6).

Prior to the stimulation of DNA synthesis, recombinant P. multocida toxin enhanced the formation of total inositol phosphates (6). The toxin also increased the cellular content of diacylglycerol, caused the translocation of protein kinase C, and stimulated the phosphorylation of 80 K (19), a major substrate of protein kinase C (20–22). Furthermore, the binding of epidermal growth factor to its receptor was decreased by recombinant P. multocida toxin, an action attributable in part to protein kinase C activation (19). The stimulation of protein kinase C by recombinant P. multocida toxin, like its mitogenic action (6), required cellular entry and, via a methylamine-sensitive process, activation of the toxin. The toxin did not increase the cellular content of cyclic AMP (6).

The isositol phosphate species present in recombinant P. multocida toxin-stimulated Swiss 3T3 cells have not been analyzed in detail. It is not known if P. multocida toxin causes PtdIns(4,5)P$_2$ breakdown, as do many extracellular stimuli, 1. The abbreviations used are: PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; inositol, myo-inositol; [Ca$^{2+}$], cytoplasmic-free Ca$^{2+}$ concentration; DMEM, Dulbecco’s modified Eagle’s medium; EGTA, ethylenebis(oxyethylentri-
the hydrolysis of other inositol-containing phospholipids, or even the formation of inositol phosphates from other sources. In the present report the P. multocida toxin-induced inositol phosphate response has been examined in detail, including an analysis of the formation of Ins(1,4,5)P3 and its metabolic products. The action of P. multocida toxin on the mobilization of intracellular Ca2+ stores has also been examined. We present evidence that P. multocida toxin, acting intracellularly, stimulates the phospholipase C-mediated breakdown of PtdIns(4,5)P2.

EXPERIMENTAL PROCEDURES

Materials—The Amersham Corp. supplied [2-3H]inositol (10-20 Ci/mmol), [3-3H]glycero-3-phospho-1-inositol (10 mM), and [3H]inositol phosphate standards. Bombesin and saponin were from Sigma. Dowex resin (AG 1-X8, 200-400 mesh) was from Bio-Rad. Fura-2/AM was from Molecular Probes, and GTP-S from Boehringer Mannheim. All other reagents were of the highest grade commercially available.

Preparation of Recombinant P. multocida Toxin—The gene encoding P. multocida toxin was cloned and expressed in E. coli (15). Transformed E. coli was grown on L agar, and recombinant P. multocida toxin was purified, after induction, by DEAE chromatography, preparative polyacrylamide gel electrophoresis, and electroelution (7). The toxin was stored at -20°C at 60 µg/ml in 50% glycerol (v/v). 25 mM Tris/HCl, pH 6.0.

Cell Culture—Stock cultures of Swiss 3T3 cells (23) were propagated as described previously (24). For experimental purposes, 106 cells were subcultured into 35-mm Nunc dishes in 2.5 ml of DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in a humidified atmosphere of 10% CO2, 90% air at 37°C for 6-8 days before use, in the same conditions, in the experiments. After this time the cells were washed 2-3 times with 37°C, and then loaded onto the column at 1 ml/min. After washing the column with the dilution buffer for a further 10 min to remove [3H]inositol the inositol phosphates were eluted by linear increasing the concentration of Na2SO4 in 30 min. Fractions (1 ml) were collected, mixed with 4 ml of Pic0 Fluor 15 (Packard), and then counted by liquid scintillation. The peak of radioactivity corresponding to Ins(1,4,5)P3 was used as the basis of coelution with standard [3H]Ins(1,4,5)P3.

Analysis of Total Inositol Phosphates—The cultures were labeled in 2 ml of medium as above but containing 10 µCi of [2-3H]inositol. Using one dish/data point, inositol phosphates were extracted by replacing the medium with 1 ml of ice-cold 3% HClO4. After centrifugation, the supernatants were neutralized on ice with 0.5 M KOH containing 25 mM HEPES, 5 mM EDTA, and 0.01% phenol red. Precipitated KClO4 was removed by centrifugation. FPLC Analysis of Inositol Phosphates—The cells were permeabilized by the method of Meek (34) using a Pharmacia LKB Biotechnology Inc. 5/5 Mono Q column. The entire extract was diluted to 10 ml in 10 mM HEPES/Na, 0.1 mM EDTA, pH 7.4, and then loaded onto the column at 1 ml/min. After washing the column with the dilution buffer for a further 10 min to remove [3H]inositol the inositol phosphates were eluted by linear increasing the concentration of Na2SO4 in 30 min. Fractions (1 ml) were collected, mixed with 4 ml of Pic0 Fluor 15 (Packard), and then counted by liquid scintillation. The peak of radioactivity corresponding to Ins(1,4,5)P3 was used as the basis of coelution with standard [3H]Ins(1,4,5)P3.

Analysis of Inositol Phosphate Release from Permeabilized Cells—Confluent and quiescent cultures of Swiss 3T3 cells were permeabilized with KCl. The gradient was followed as described for the HPLC analysis except that the cells were permeabilized for 4-6 days in DMEM containing 0.5% fetal bovine serum.

HPLC Analysis of Inositol Phosphates—The cells were washed twice with 2 ml of DMEM-Waymouth medium (1:1) and then incubated for 20 h in 1 ml of this medium containing 25 µCi of [2-3H]inositol. Additions were made to the cells as required, and LiCl was added to a final concentration of 20 mM for the last 20 min of the incubation. Inositol phosphates were extracted by rapidly replacing the medium with 0.25 ml of ice-cold 5% (w/v) CCl3COOH containing 1% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated in a humidified atmosphere of 10% CO2, 90% air at 37°C for 6-8 days before use, in the same conditions, in the experiments. After this time the cells were washed with 2 ml of medium as above but containing 10 pCi of [2-3H]inositol. Of pump A, containing deionized H2O, and pump B, 0.1 M triol[tetraacetic acid, GTP-S, guanosine 5'-O-(3-thiotriophosphate); PBl2, phosphor 12.13-diubutyrate; Ins, InsP, InsPn, InsP3, InsP4, and InsP5 (and equivalent abbreviations with added locants, e.g. Ins(1,4,5)P3, myo-inositol and the appropriate myo-inositol containing phosphates (numbered by reference to native-inositol 1-phosphate as InsP1 (see the recommendations of the Nomenclature Committee of the International Union of Biochemistry (1989) Biochem. J. 258, 1-2), in which when a single peak in the HPLC analysis is likely to contain more than one species, a hybrid abbreviation is used, e.g. Ins(1,4,5)P3 for a mixture of the enantiomers InsP1 and InsP3; GroPInsnP and GroPIns(4,5)Pn, glycerol-3-phospho-1-inositol 4-phosphate and 4,5-bisphosphate, respectively; InsnP, total inositol phosphates present in a cell extract, analyzed as an unresolved pool.

[NH4]HPO4 (pH 3.8 with H3PO4) 0 min, 0% B; 2 min, 0% B; 32 min, 30% B; 32 min, 100% B; 65 min, 100% B. The content of pump B was then changed to 1.0 M [NH4]HPO4 (pH 3.8 with H3PO4), and the gradient was continued as follows: 65:1, 100% B; 120 min, 100% B; 120 min, 100% B; 165 min, 100% B; 165 min, 0% B. Fractions were collected every 0.5 min, and a mixture of methanol/deionized H2O (5:5) was added: 0.8 ml, up to 0.1 M salt; 1.0 ml, up to 0.3 M salt; and then 1.4 ml, to the remaining vials. Finally, 4 ml of xylene/Triton (1:1) scintillant (see Ref. 27) was added and the samples counted on a Packard 2000CA scintillation counter with appropriate dual label protocols and quench correction.
FIG. 1. Recombinant (rPMT) \textit{P. multocida} toxin and bombesin cause similar increases in $[^3\text{H}]$inositol phosphates in Swiss 3T3 cells. HPLC analysis is shown of the $[^3\text{H}]$inositol phosphates present in control cells (A) and cells incubated with either 10 nM bombesin for 30 min (B) or 20 ng/ml recombinant \textit{P. multocida} toxin for 4 h (C). Confluent and quiescent Swiss 3T3 cells were labeled with $[^3\text{H}]$inositol for 20 h as described under "Experimental Procedures." Additions prior to extraction were as follows: recombinant \textit{P. multocida} toxin, 20 ng/ml for 4 h; bombesin, 10 nM for 30 min. LiCl, 20 mM, was present in all cases for 20 min prior to extraction of the inositol phosphates. Additions were made directly to the dishes. HPLC analysis was as described under "Experimental Procedures." The analysis of the \textit{Ins}P\textsubscript{1} and \textit{Ins}P\textsubscript{2} species employed 0.25 ml of extract for optimal resolution. This amount of extract was not sufficient to detect $[^3\text{H}]$\textit{Ins}(1,4,5)P\textsubscript{3} clearly. Therefore, to detect higher phosphorylated inositol species, 0.5 ml of extract was analyzed. The counts associated with the latter analysis were corrected for the 2-fold increase in volume analyzed and then multiplied by 20 in the interest of clarity. The \textit{continuous lines} indicate the elution of $[^3\text{H}]$-labeled material present in the cell extracts. The \textit{dashed lines} indicate...
Swiss 3T3 cells were labeled for 20 h with 5 μCi/ml [3H]inositol. Recombinant P. multocida toxin (rPMT) (20 ng/ml) was added for 4 h prior to extraction. Bombesin (10 nM) was present for 1 h. NaCl (control) or LiCl was added to final concentrations of 20 mM for 20 min prior to extraction. [3H]InsP₃ were determined as described under "Experimental Procedures." Values have been expressed relative to the control value (129 ± 11 cpm) and are the means ± S.D. of three incubations.

| Conditions | Control | LiCl  |
|------------|---------|-------|
| rPMT       | 100 ± 9 | 108 ± 15 |
| Bombesin   | 392 ± 126 | 1,756 ± 178 |

Relative InsP₃

RESULTS AND DISCUSSION

Chromatographic Analysis of Inositol Phosphates—To understand further the basis of P. multocida toxin action we have chromatographically analyzed the inositol phosphates present in recombinant P. multocida toxin-stimulated cells. Quiescent cultures of Swiss 3T3 cells were prelabeled with [2-H]inositol for 16 h. The cells were then treated for a further 4 h with 20 ng/ml of rPMT or recombinant P. multocida toxin known to result in a commitment to DNA synthesis (6) and protein kinase C activation (19). The inositol phosphate response was amplified by adding LiCl for 20 min prior to the termination of the incubation (4, 41, 42). Analysis by HPLC demonstrated that recombinant P. multocida toxin caused an increase in the cellular content of [3H]Ins(1,4,5)P₃ (Fig. 1), a product of PtdIns(4,5)P₂ hydrolysis. The toxin also caused a dramatic increase in the cellular content of [3H]Ins(1,3,4,5)P₄ and a lesser accumulation of [3H]Ins(1,3,4,5,6)P₅. Recombinant P. multocida toxin also caused the accumulation of distinct InsP₂ and InsP₃ species, Ins(1,4)P₃, Ins(1,4,5)P₂, and Ins(1,3,4)P₃. The accumulation of these products is clearly in accordance with known pathways of Ins(1,4,5)P₃ metabolism (4, 41, 42). The small accumulation in the acid extracts of Ins(2)P probably arises from the acid hydrolysis of Ins(1,2)cyclic P formed as a side product of phospholipase C action, either directly or via Ins(1,2cyc,4)P₃ and/or Ins(1,2cyc,4,5)P₃.

It is well established that in Swiss 3T3 cells the mitogenic neuropeptide bombesin (43), acting via a specific plasma membrane bombesin receptor (44) and an unidentified G-protein (45-48), activates phospholipase C, thereby leading to increased formation of inositol phosphates (39, 49, 50). The accumulation of inositol phosphates in recombinant P. multocida toxin-treated cells was compared with that occurring upon activation of the bombesin receptor. The cells were treated with toxin for 4 h and with bombesin for 30 min. Since the toxin only causes an accumulation of inositol phosphates after a lag period of several hours (6) and see below and bombesin acts within seconds (39), the incubation times were chosen such that the cells would have been activated for approximately the same period of time. Under these conditions, the profile of inositol phosphates in the recombinant P. multocida toxin-treated cells was very similar to that in cells treated in parallel with bombesin except for the presence of a small additional peak (coeluting with standard Ins(4,5)P₂) in the recombinant P. multocida toxin-treated cells and unidentified material eluting at 16 min (Fig. 1). It is clear that recombinant P. multocida toxin causes an activation of inositol phospholipid breakdown which is very similar to the receptor-mediated breakdown induced by bombesin.

For the HPLC analysis the cells were incubated with LiCl to facilitate the accumulation of inositol phosphate metabolites in the cells. The recombinant P. multocida toxin-induced...
pretreated for phate 1-phosphatase (4, 41, 42) and is consistent with the individual inositol phosphates revealed that the enhancement of the toxin, and after a further 30 min the cellular Ca$^{2+}$ was determined. Chromatographic analysis by FPLC of the toxin, and after a further 30 min the cellular Ca$^{2+}$ was determined. The values, expressed relative to the control values, are means for 3 determinations. The effects of 5 (●) or 20 ng/ml recombinant P. multocida toxin (●) on the [3H]InsP₃ and the [45Ca$^{2+}$] content of Swiss 3T3 cells with respect to control (C) are shown to the left. The effects of a 4-h incubation with 20 ng/ml recombinant P. multocida toxin (closed bars) on the [3H]InsP₃ and [45Ca$^{2+}$] content (mean ± S.D. of five determinations) of mouse embryo fibroblasts (MEF) are shown with respect to the control values, respectively, 51 ± 13 cpm and 4251 ± 252 cpm (open bars).

**TABLE II**

$^{45}$Ca$^{2+}$ mobilization by recombinant P. multocida toxin and bombesin: the lack of additivity

|          | Cellular $^{45}$Ca$^{2+}$ |
|----------|-----------------------------|
| rPMT     | 58.4 ± 4.3                  |
| Bombesin | 57.6 ± 6.0                  |
| rPMT + Bombesin | 51.8 ± 8.2               |

In response to growth factors acting via Ins(1,4,5)P₃, including bombesin, is subsequently released into the extracellular medium, leading to a decrease in the cellular Ca$^{2+}$ content (38, 39, 52). Recombinant P. multocida toxin mobilized Ca$^{2+}$ in Swiss 3T3 cells. Thus, the $^{45}$Ca$^{2+}$ content of $^{45}$Ca$^{2+}$-equilibrated cells was decreased by recombinant P. multocida toxin (20 ng/ml, 4-h incubation) to 62 ± 8% of the control value (mean ± S.D., 14 independent cell preparations). This decrease occurred after a lag period and paralleled, in a time- and dose-dependent manner, the increases in inositol phosphates (Fig. 3). The kinetics of Ca$^{2+}$ mobilization, InsP₃ accumulation, protein kinase C activation (19), and commitment to DNA synthesis (6) are very similar.

Recombinant P. multocida toxin also increased the accumulation of [3H]inositol phosphates and decreased the content of $^{45}$Ca$^{2+}$ in a time and dose-dependent manner, leading to a decrease in the cellular Ca$^{2+}$ content (38, 39, 52). Receptor activation by PtdIns(4,5)P₂ hydrolysis in Swiss 3T3 cells. Therefore, the effects of the toxin and the neuropeptide on Ca$^{2+}$ mobilization were compared. The magnitude of the decrease in the $^{45}$Ca$^{2+}$ content of cells pretreated with recombinant P. multocida toxin or bombesin is very similar (Table II). Furthermore, the
addition of 100 nM bombesin to recombinant *P. multocida* toxin-pretreated cultures only caused a small, but statistically insignificant, further decrease in the cellular "Ca\(^{2+}\)" content (Table I). These data are consistent with an identity of the intracellular pools of Ca\(^{2+}\) mobilized by the two agents.

Further evidence for a common source of mobilized Ca\(^{2+}\) comes from a study of the rapid and transient increase in [Ca\(^{2+}\)], elicited by bombesin (38, 39, 52). Pretreatment of the cells with recombinant *P. multocida* toxin under conditions that resulted in "Ca\(^{2+}\)" mobilization and enhanced inositol phosphate formation greatly reduced the increase in [Ca\(^{2+}\)], caused by bombesin (Fig. 4).

Protein kinase C activation is known to attenuate Ca\(^{2+}\) mobilization by low concentrations of bombesin (38), and recombinant *P. multocida* toxin activates protein kinase C (19). However, under identical conditions, it was confirmed that treatment of the cells with the protein kinase C activator PDBu did not decrease the Ca\(^{2+}\) mobilizing action of the higher concentrations of bombesin used in the experiments presented in Fig. 4 and Table II. Attenuation by recombinant *P. multocida* toxin pretreatment was observed in the presence or absence of extracellular Ca\(^{2+}\), indicating that the source of the Ca\(^{2+}\) mobilized by bombesin was intracellular. Furthermore, the decrease in the Ca\(^{2+}\) mobilizing action of bombesin caused by pretreatment with recombinant *P. multocida* toxin was dependent upon the time of exposure of the cells to the toxin, exhibiting a lag period (Fig. 4) similar to that observed for the enhancement of InsP, formation and "Ca\(^{2+}\)" mobilization (Fig. 3). The decrease of Ca\(^{2+}\) mobilization by bombesin in recombinant *P. multocida* toxin-treated cells is consistent with a common mechanism of Ca\(^{2+}\) mobilization by the two agents, i.e. both act via Ins(1,4,5)P\(_3\).

**Evidence for the Cellular Entry and Processing of Recombi-**
Fig. 7. Persistence of the recombinant *P. multocida* toxin-induced increase in inositol phosphates after removal of extracellular toxin: comparison with the action of bombesin. The determination of cellular \([^{3}H]In_{sP}\), has been described under "Experimental Procedures." The cells were labeled with 5 μCi/ml \([^{3}H]inositol*, Swiss 3T3 cells were incubated with 20 ng/ml recombinant *P. multocida* toxin for 4 h (●) or 10 nM bombesin for 1 h (■), and their \([^{3}H]In_{sP}\), content (means ± S.D., n = 3) was determined relative to the control value at 0 min, 267 ± 15 cpm (□). Other treated cells were either incubated for a further 50 min (dashed lines) or washed and then incubated for a further 50 min (continuous lines). Washing was achieved by rapidly replacing the medium twice with 2 ml of the labeling medium containing \([^{3}H]inositol* at the same activity as in the prelabeling medium. In all determinations, LiCl (20 mM) was added for 20 min prior to the extraction of the cells.

*nant P. multocida* Toxin—Many toxins require entry into cells and processing for biological activity (53, 54). The lag period in the action of recombinant *P. multocida* toxin may reflect its cellular entry and possible processing and activation. Indeed, the lysosomotropic agent methylamine selectively blocked the increase in InsP3, and Ca2+ mobilization caused by recombinant *P. multocida* toxin (Fig. 5). In contrast, this agent did not block the same responses to bombesin. An antiserum raised against native *P. multocida* toxin (Fig. 5), which recognizes recombinant *P. multocida* toxin by immuno blotting (6), blocked the actions of recombinant *P. multocida* toxin but did not prevent the responses to bombesin (Fig. 5). Addition of the antiserum 3 h after the toxin did not block the InsP3 response (results not shown), suggesting that the toxin had entered the cells and was therefore unavailable to the antiserum.

In addition to methylene, two other lysosomotropic agents, ammonium chloride and chloroquine, also inhibited the stimulation of InsP3 production by recombinant *P. multocida* toxin (Fig. 6). The selectivity of the inhibitory action of these agents is again demonstrated by the ability of bombesin to stimulate InsP3 production in their presence (although there was a slight inhibitory effect of chloroquine).

Inhibition of recombinant *P. multocida* toxin-stimulated InsP3 production by methylene was time dependent (Fig. 6). Addition of the lysosomotroph with the toxin, or 30 min after it, completely inhibited the InsP3 response. Addition at later times, up to 2 h, caused progressively less inhibition after which time there was essentially no effect (Fig. 6). Similarly, in 4-h incubations with 20 ng/ml recombinant *P. multocida* toxin, the addition of methylene 3 h after the addition of recombinant *P. multocida* toxin did not inhibit the decrease in Ca2+ (results not shown). The time dependence of the inhibitory action of methylene is consistent with a requirement for the cellular entry of recombinant *P. multocida* toxin via endocytosis and involving endosomal/lysosomal trafficking.

To explore further the role of endocytosis in the activation of recombinant *P. multocida* toxin, we investigated the effect of the toxin on inositol phosphate release in permeabilized cells. The direct addition of recombinant *P. multocida* toxin at 20 or 100 ng/ml to saponin-permeabilized 3T3 cells failed to stimulate the release of inositol phosphates (Table III). Under the same conditions we verified that either bombesin or GTPyS alone stimulated inositol phosphate release. Addition of both agents together resulted in synergistic stimulation. Thus, the permeabilized cell preparation was operationally defined to contain the molecular components necessary to couple the bombesin receptor via a G-protein to phospholipase C activation. The lack of effect of recombinant *P. multocida* toxin in this system lends further support to the notion that recombinant *P. multocida* toxin has to enter the cells via the physiological process of endosomal trafficking, resulting in release of active toxin into the cytosol to stimulate polyphosphoinositide breakdown.

In theory, the intracellular action of recombinant *P. multocida* toxin could be the result of the direct activation of intracellular processes controlling polyphosphoinositide breakdown. Alternatively, recombinant *P. multocida* toxin could act indirectly causing the synthesis and release of a factor into the culture medium which then stimulates inositol phosphate formation. The latter possibility is untenable since (a) cycloheximide (25 μM) did not prevent the recombinant *P. multocida* toxin-induced increases in \([^{3}H]In_{sP}\), accumulation and 45Ca mobilization (results not shown), indicating that *do novo* protein synthesis was not required for its actions; and (b) the incubation medium from cells treated for 4 h with 20 ng/ml recombinant *P. multocida* toxin failed to provoke a \([^{3}H]In_{sP}\) increase in untreated cells (Table IV). This result also argues against the possibility of extracellular processing of the toxin into an active form.

If recombinant *P. multocida* toxin acts intracellularly, the stimulation of InsP3 formation should persist after removal of extracellular toxin. As shown in Fig. 7, the InsP3 increase induced by recombinant *P. multocida* toxin persisted after removal of extracellular toxin. In marked contrast, the InsP3 response induced by bombesin was reversed by removal of extracellular ligand (Fig. 7).

Collectively, the dose-dependent lag period, the methylene block, the inactivity of the toxin in saponin-permeabilized cells, the persistence of toxin action after removal from activated cells, the lack of effect of toxin-conditioned medium argue that recombinant *P. multocida* toxin, in order to stimulate polyphosphoinositide breakdown, enters the cells by the action of endosomal/lysosomal traffic and thereby gains access to the cytosol in an activated state.

**CONCLUSIONS**

We have identified a toxin, *P. multocida* toxin, that enters cells both to enhance the formation of Ins(1,4,5)P3 and to mobilize intracellular Ca2+. The apparent requirement for cellular entry of the toxin to elicit InsP3 increases, and Ca2+ mobilization is also a feature of both its mitogenic action (6) and its ability to activate protein kinase C (19). Furthermore, the similarities in the inositol phosphate species formed in response to recombinant *P. multocida* toxin and bombesin suggest that *P. multocida* toxin modifies cellular regulatory processes physiologically involved in polyphosphoinositide hydrolysis. Thus, we propose that *P. multocida* toxin stimulates the phospholipase C-mediated hydrolysis of PtdIns(4,5)P2,hence causing the release of Ins(1,4,5)P3, Ca2+ mobilization, increase in diacylglycerol, translocation of protein kinase C, and the phosphorylation of the protein kinase C substrate 80 K. The molecular basis of the action of *P. multocida* toxin may provide new insights into the regulation of receptor-phospholipase C coupling. *P. multocida* toxin...
could also provide a novel tool with which to study cellular Ca\textsuperscript{2+} signaling under conditions free from constraints such as ligand-induced cellular desensitization.

REFERENCES
1. Middlebrook, J. C., and Dorland, R. B. (1984) *Microbiol. Rev.* **48**, 199–211
2. Moss, J., and Vaughan, M. (1988) *Adv. Enzymol.* **61**, 303–379
3. Pfeuffer, T., and Halmreich, E. J. M. (1988) *Curr. Top. Cell. Regul.* **29**, 129–208
4. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
5. Rozengurt, E. (1986) *Science* **234**, 161–166
6. Rozengurt, E., Higgins, T., Chanter, N., Lax, A. J., and Staddon, J. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 123–127
7. Chanter, N., Rutter, J. M., and Mackenzie, A. (1986) *J. Gen. Microbiol.* **132**, 1089–1097
8. Nakai, T., Sawata, A., Tsuji, M., Samejima, Y., and Kume, K. (1984) *Infect. Immun.* **46**, 429–434
9. Rimler, R. B., and Brogden, K. A. (1986) *Am. J. Vet. Res.* **47**, 730–737
10. Kamp, E. M., van der Heijden, P. J., and Tettenborn, B. J. (1987) *Vet. Microbiol.* **13**, 235–248
11. Foged, N. T. (1988) *Infect. Immun.* **56**, 1901–1906
12. Dominick, M. A., and Rimler, R. B. (1988) *Vet. Pathol.* **25**, 17–27
13. Foged, N. T., Pedersen, K. B., and Elling, F. (1987) *FEMS Microbiol. Lett.* **43**, 45–51
14. Peterson, S. K., and Foged, N. T. (1989) *Infect. Immun.* **57**, 3967–3973
15. Lax, A. J., and Chanter, N. (1990) *J. Gen. Microbiol.* **136**, 81–87
16. Kamps, A. M. E. K., Kamp, E. M., and Smits, M. A. (1990) *FEMS Microbiol. Lett.* **67**, 187–190
17. Lax, A. J., Chanter, N., Pullinger, G. D., Higgins, T., Staddon, J. M., and Rozengurt, E. (1990) *FEBS Lett.* **277**, 59–64
18. Petersen, S. K. (1990) *Mol. Microbiol.* **4**, 821–830
19. Staddon, J. M., Chanter, N., Lax, A. J., Higgins, T. E., and Rozengurt, E. (1990) *J. Biol. Chem.* **265**, 11841–11848
20. Rozengurt, E., Rodriguez-Pena, A., and Smith, K. A. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 7224–7228
21. Rodriguez-Pena, A., and Rozengurt, E. (1986) *EMBO J.* **5**, 77–83
22. Blackshear, P. J., Wen, L., Glynn, B. P., and Witters, L. A. (1986) *J. Biol. Chem.* **261**, 1459–1469
23. Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17**, 299–313
24. Dicker, P., and Rozengurt, E. (1980) *Nature* **287**, 607–612
25. Weggelt, K. A., Howe, L. R., Moore, J. R., and Irvine, R. F. (1987) *Biochem. J.* **245**, 933–934
26. Michell, R. H., Conroy, L. A., Finney, M., French, P. J., Brown, G., Creja, J. A., Bonce, C. M., and Lord, J. M. (1990) *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **327**, 193–207
27. Shears, S. B., Storey, D. J., Morris, A. J., Cubitt, A. B., Parry, J., B., Michell, R. H., and Kirk, C. J. (1987) *Biochem. J.* **242**, 393–402
28. Stephens, L. R., Hawkins, P. T., Morris, A. J., and Downes, C. P. (1988) *Biochem. J.* **249**, 283–292
29. Hawkins, P. T., Stephens, L., and Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
30. Grado, C., and Ballou, C. E. (1963) *J. Biol. Chem.* **236**, 54–60
31. Brown, D. M., and Stewart, J. C. (1966) *Biochem. Biophys. Acta* **125**, 413–421
32. Morris, A. J., Murray, K. J., England, P. J., Downes, C. P., and Michell, R. H. (1986) *Biochem. J.* **251**, 157–163
33. Graf, E. (1983) *Annu. Rev. Biochem.* **51**, 351–355
34. Meek, J. L. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 4162–4166
35. Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P., and Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
36. Lopez-Rivas, A., and Rozengurt, E. (1984) *Am. J. Physiol.* **247**, C156–C162
37. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
38. Lopez-Rivas, A., Mendoza, S. A., Nääbberg, E., Sinnett-Smith, J. W., and Rozengurt, E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 5768–5772
39. Nääbberg, E., and Rozengurt, E. (1988) *EMBO J.* **7**, 2741–2747
40. Tsien, R. Y., Pozzan, T., and Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334
41. Majerus, P. W., Connolly, T. M., Bansal, V. S., Inhorn, R. C., Ross, T. S., and Lips, D. L. (1989) *J. Biol. Chem.* **263**, 3051–3054
42. Shears, S. B. (1989) *Biochem. J.* **260**, 313–324
43. Rozengurt, E., and Sinnett-Smith, J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7616–7620
44. Zachary, I., and Rozengurt, E. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 7616–7620
45. Erusalimsky, J. D., Friedberg, L., and Rozengurt, E. (1988) *J. Biol. Chem.* **263**, 19188–19194
46. Hasegawa-Sasaki, H., Lutz, F., and Sasaki, T. (1988) *J. Biol. Chem.* **263**, 12970–12976
47. Sinnett-Smith, J., Lehmann, W., and Rozengurt, E. (1990) *Biochem. J.* **265**, 485–493
48. Coffer, A., Fabregat, I., Sinnett-Smith, J., and Rozengurt, E. (1990) *FEBS Lett.* **263**, 80–84
49. Heslop, J. P., Blakely, D. M., Brown, K. D., Irvine, R. F., and Berridge, M. J. (1986) *Cell* **47**, 703–709
50. Takaku, N., Takaku, Y., Bollag, W. E., and Rasmussen, H. (1987) *J. Biol. Chem.* **262**, 182–188
51. Irvine, R. F., Lefter, A. J., Heslop, J. P., and Berridge, M. J. (1986) *Nature* **320**, 631–634
52. Mendoza, S. A., Schneider, J. A., Lopez-Rivas, A., Sinnett-Smith, J. W., and Rozengurt, E. (1986) *J. Cell Biol.* **102**, 2223–2233
53. Neville, D. M., Jr., and Hudson, T. H. (1986) *Annu. Rev. Biochem.* **55**, 195–224
54. Ohnes, S., and Sandvig, K. (1986) in *Immunotoxins* (Frankel, A. E., ed) pp. 39–73, Kluwer Academic Publishers, Norwell, MA