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Phorbol Myristate Acetate Inhibits HeLa 229 Invasion by *Bordetella pertussis* and Other Invasive Bacterial Pathogens

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The microfilament inhibitors cytochalasins B and D have been traditionally used to indirectly evaluate the requirement for actin in the uptake of invasive bacterial pathogens by nonprofessional phagocytes. Through their effects on microfilaments, both cytochalasins also impart profound alterations in cellular morphology and surface topology, which likely interfere with adherence. Alterations affecting adherence would complicate interpretation of the effect of cytochalasins on entry alone. As an alternative to cytochalasins, the effect of the tumor promoter phorbol myristate acetate (PMA) was examined for its effects on uptake of several invasive bacterial pathogens by HeLa 229 cells. In this communication, PMA was shown to induce a similar change in HeLa cell actin distribution, but, in contrast to cytochalasins B and D, PMA had no significant effect on gross cell morphology. The modified actin distribution was shown to reduce internalization of *Bordetella pertussis*, *Yersinia pseudotuberculosis*, *Shigella flexneri*, and *Salmonella hadar* in a dose-dependent manner at concentrations ranging from 1 to 1,000 ng/ml. The magnitude of reduction at a PMA concentration of 1,000 ng/ml was greater than the reduction elicited by cytochalasin B at 2.5 μg/ml but was less than that elicited by cytochalasin D at 2.5 μg/ml. Mezerein, a functional analog of PMA, caused a similar dose-dependent reduction in uptake of *B. pertussis*, whereas an inactive analog of PMA, α-4-phorbol-12,13-didecanoate was without effect on invasion. Binding studies further reveal that pretreatment of HeLa cells with PMA or mezerein did not significantly impair the ability of *B. pertussis* to adhere, in contrast to cytochalasins B and D, which caused a marked reduction in adherence.

The uptake of several known invasive bacterial pathogens by nonprofessional phagocytes is known to involve cytoskeletal actin microfilaments (1, 7, 15, 17). The invading bacterium initially adheres to the cell membrane, and then transient actin polymerization at sites proximal to the site of entry is thought to trigger ingestion by the formation of a phagocytic vesicle enclosing the bacterium. F-actin is required for this process, because bacterial uptake by a variety of tissue culture cell types is markedly reduced in the presence of cytochalasins, a group of fungal metabolites which bind to actin and modify its polymerization. For example, eucaryotic cell invasion by *Shigella*, *Salmonella*, *Yersinia*, and *Bordetella* species is cytochalasin sensitive (4, 11, 12, 14). Cytochalasins B and D have been used for this purpose; however, cytochalasin D is much more potent than cytochalasin B in terms of its effects on actin assembly. This is likely due to the fact that cytochalasin D binds directly to the faster-growing (barbed) ends of actin filaments, whereas cytochalasin B binds to filament promoters distinct from those within the barbed ends (29). The disruption of actin architecture produced by cytochalasin treatment of cultured cells also disrupts their normal external morphology. For example, cytochalasins induce the formation of large numbers of bleblike knobby protuberances at the cell membrane as a result of endoplasmic herniation (16), enucleation (6), and cellular rounding due to microfilament contracture and loss of substrate attachment sites (20). Although such effects are of interest from a physiological point of view, they may complicate interpretation of the effects of cytochalasins on bacterial invasion if they interfere with the abilities of the bacteria to initially adhere to cultured cells.

The tumor promoter phorbol myristate acetate (PMA) causes numerous cellular changes through the activation of protein kinase C, among which is a profound and rapid alteration in cytoskeletal protein distribution. PMA-treated cells rapidly lose their ordered actin stress fiber arrangement as filaments are displaced toward the periphery and appear in large ribbonlike networks at the cell margins (10, 22, 24–26). Despite the alteration in actin distribution, however, the change in external appearance of cells treated with PMA for relatively short periods (i.e., <12 h) is limited to mild disruption of cell-cell contacts and focal contacts, partial rounding, and acquisition of an irregular outline (19, 23–25), all of which are minor in comparison with the changes produced by cytochalasins B and D. Therefore, because PMA rapidly alters actin distribution without markedly altering external morphology, it is a potentially useful reagent for investigating the postadherence, microfilament-dependent phase of bacterial invasion of eucaryotic cells. In this communication, we compare the effects of cytochalasins B and D, PMA, mezerein (a functional analog of PMA), and α-4-phorbol-12,13-didecanoate (an inactive PMA analog) on actin distribution and bacterial adherence and invasion of HeLa 229 monolayers.

MATERIALS AND METHODS

Bacterial strains. The nalidixic acid-resistant *Bordetella pertussis* Tohama 1 (BP338) was kindly provided by Alison A. Weiss, Virginia Commonwealth University, Richmond. The *Shigella flexneri*, *Salmonella hadar*, and *Yersinia pseudotuberculosis* strains have been described previously

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(12). Strain BP338 was cultivated on Bordet-Gengou agar with 13% sheep blood for 2 days prior to use in invasion assays. *Yersinia pseudotuberculosis* type A1, cultivated on brain heart infusion agar, was grown overnight in brain heart infusion broth at 28°C and recovered by centrifugation at 8,000 × *g* prior to use in invasion assays.

Fluorescence labeling of F-actin and vinculin. To visualize F-actin, HeLa monolayers grown in four-well slides (Nunc, Naperville, Ill.) and treated with drugs at the specified concentrations for 1 h at 37°C were fixed with 3.7% formaldehyde for 10 min at room temperature, washed with phosphate-buffered saline (54 mM Na_2HPO_4, 13 mM KH_2PO_4, 73 mM NaCl), extracted with acetone at 4°C for 5 min, dried, then stained with a 5 U/ml solution of rhodamine-phalloidin (Molecular Probes, Inc., Junction City, Oreg.) in phosphate-buffered saline for 30 min at room temperature. After being washed, slides were mounted with a 90% glycerol–10% phosphate-buffered saline solution and viewed under a Leitz photomicroscope by using epifluorescence with a 545 nm excitation filter and a 720 nm emission filter. For vinculin visualization, a similar fixation-extraction procedure was followed. After acetone extraction, cells were stained with mouse anti-chicken vinculin monoclonal antibody, clone VIN-11-5 (Sigma Chemical Co., St. Louis, Mo.) (cross-reacts with human vinculin) for 30 min followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma) for an additional 30 min. Slides were viewed by using fluorescence filters for fluorescein (excitation at 490 nm and emission at 525 nm).

Treatment of HeLa cells and invasion assay. PMA, cytochalasins B and D, mezerein, and α-4-phorbol-12,13-didecanoate, all obtained from Sigma, were dissolved in 100% ethanol (PMA) or dimethyl sulfoxide (all others) and stored at −70°C. Reagents were added to HeLa monolayers at the indicated concentrations and incubated at 37°C for 1 h prior to use in invasion assays. Control monolayers received dimethyl sulfoxide or ethanol at concentrations equivalent to those found in monolayers incubated with the highest concentrations of drugs tested. Invasion assays were performed as previously described (11). The concentrations of each of the drugs tested were maintained throughout the duration of each assay.

Adherence assays. Adherence assays were performed as described for the invasion assay, except that monolayers were washed extensively and harvested for total counts after coincubation with the bacteria.

RESULTS

Effects of PMA and cytochalasin B and D on actin distribution. PMA and cytochalasins B and D promoted rapid changes in the distribution of F-actin in HeLa cells. Within untreated control HeLa cells (Fig. 1A), multiple stress fibers composed of F-actin were visualized spanning the cell. Vinculin associated with the ends of actin at focal contacts was distributed evenly throughout the cells (data not shown). Within 1 h of treatment with PMA at 40 ng/ml (Fig. 1B), stress fibers disappeared with the concurrent appearance of large actin bundles arranged as ribbons at the cell periphery. The normal polygonal outline of the cells was replaced by a more irregular one, marked by numerous lamellar protuberances. This effect was dose dependent and noted with concentrations of PMA as low as 1 ng/ml, whereas the effect of PMA at 0.1 ng/ml was much less pronounced. Vinculin was similarly concentrated at the cell periphery in conjunction with actin (data not shown). Pretreatment of HeLa cells with mezerein, a non-phorbol tumor promoter with protein kinase C activation ability similar to that of PMA (21), produced effects indistinguishable from those of PMA (Fig. 1D). In contrast, the inactive PMA isomer α-4-phorbol-12,13-didecanoate showed none of these effects (Fig. 1C). The effects of treatment with cytochalasins B and D were more dramatic. The most pronounced effects of cytochalasin B treatment of HeLa cells were the overall rounding of the cells and formation of spherical protrusions which stained avidly with phalloidin (Fig. 1E). Stress fibers were also present, but in reduced numbers compared with untreated controls. Cytochalasin D produced the most dramatic alterations in actin architecture; it transformed the ordered filar arrangement of actin into a disorganized, stellarlike punctate distribution (Fig. 1F), which mirrored the marked cellular rounding and peripheral arborization seen under the inverted microscope.

Effect of PMA on bacterial invasion of HeLa monolayers. To determine the effect of PMA on invasion of *Shigella flexneri*, *Salmonella hadar*, *Y. pseudotuberculosis*, and *B. pertussis*, semiconfluent monolayers were pretreated with PMA at concentrations ranging from 0.1 to 1,000 ng/ml for 1 h prior to addition of bacteria (Fig. 2A through D). In each case, pretreatment of HeLa cells with PMA resulted in a dose-dependent reduction in invasion. At 1,000 ng of PMA per ml, the highest concentration tested, invasion of *B. pertussis*, *Salmonella hadar*, and *Y. pseudotuberculosis* was reduced to a level of <15% of untreated controls. In contrast, the invasion of *Shigella flexneri* was more refractory to the inhibitory effect of PMA, since invasion was reduced to a level of 30% of the control at a similar concentration of PMA. Figure 3 illustrates a similar dose-dependent reduction in uptake of strain BP338 in the presence of mezerein at concentrations ranging from 10 to 1,000 nM, whereas the inactive PMA analog α-4-phorbol-12,13-didecanoate was without effect on invasion at 1,000 nM.

In separate experiments, neither PMA at 1,000 ng/ml nor mezerein at 1,000 nM reduced the viability of strain BP338 or those of the other bacterial species tested following an 8-h coincubation period. Similarly, viability of the HeLa cells as assayed by trypan blue exclusion was not compromised after 8 h of coincubation with either PMA or mezerein at the concentrations indicated above.

Comparison of effects of cytochalasins B and D and PMA on bacterial invasion. Table 1 presents a direct comparison of the abilities of cytochalasins B and D and PMA to inhibit bacterial uptake by HeLa 229 monolayers. For each of the strains tested, cytochalasin D at 2.5 μg/ml had the most pronounced inhibitory effects on invasion, reducing uptake to approximately 1% of untreated controls. In separate experiments with *Shigella flexneri* and *B. pertussis*, we attempted to titrate cytochalasin D down to a concentration which elicited a significant reduction in invasion yet preserved the cell morphology. We were not able to do so in either case, indicating that the effects of cytochalasin D on actin are inseparable from morphological changes produced as a result of its actions. Cytochalasin B, which is significantly less potent than cytochalasin D in terms of its effects on actin, had varied effects depending on the species tested (Table 1). For example, cytochalasin B markedly reduced the uptake of *B. pertussis* to a level comparable to that observed in the presence of an equivalent concentration of cytochalasin D (approximately 2% of the control value). In contrast, *Shigella flexneri* was significantly more refractory to the inhibitory effect of cytochalasin B at 2.5 μg/ml than *B. pertussis*, *Salmonella hadar*, and *Y. pseudotuberculosis*,

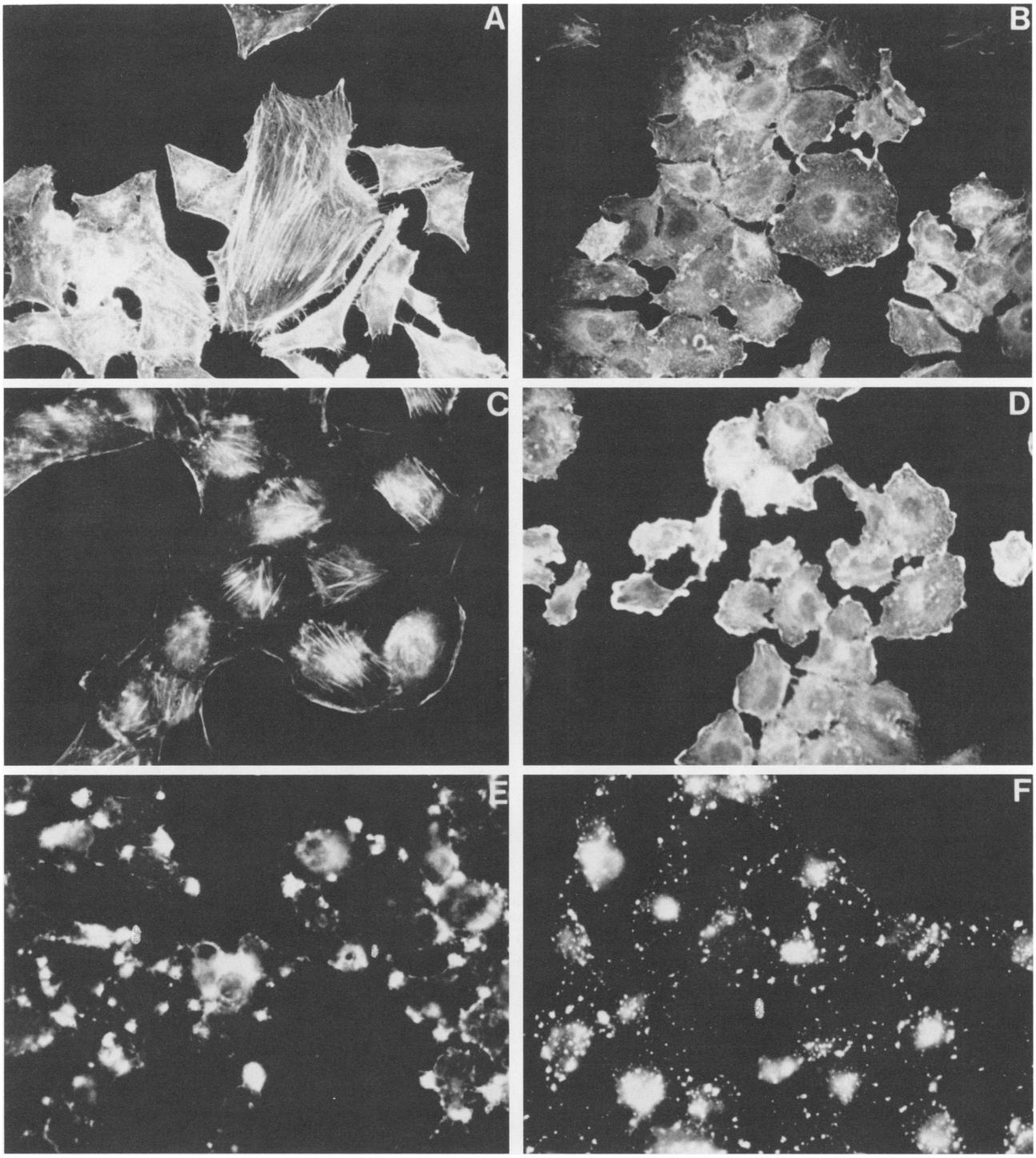

FIG. 1. Rhodamine-phalloidin fluorescence labeling of F-actin in HeLa 229 monolayers. Monolayers were incubated with no additions (as a control) (A), PMA at 40 ng/ml (B), α-4-phorbol-12,13-didecanoate at 1,000 nM (C), mezerein at 100 nM (D), cytochalasin B at 2.5 μg/ml (E), or cytochalasin D at 2.5 μg/ml (F) for 1 h at 37°C prior to processing as described in Materials and Methods. Magnification, ×3,800.

which also corresponded to the relatively lesser effect of PMA on *Shigella* species uptake. The magnitude of the effects of PMA at 1,000 ng/ml was intermediate between cytochalasins B and D, reducing uptake to approximately 15% of untreated controls for *B. pertussis*, *Salmonella hadar*, and *Y. pseudotuberculosis* and 30% of untreated controls for *Shigella flexneri*.

Effects of PMA and cytochalasins B and D on adherence of BP338 to HeLa monolayers. Pretreatment of HeLa monolayers with cytochalasin B or D resulted in pronounced alterations in actin distribution and overall cellular morphology, especially in the case of cytochalasin D. We therefore found it necessary to determine whether these alterations reduced the numbers of bacteria which initially bound to the cells.

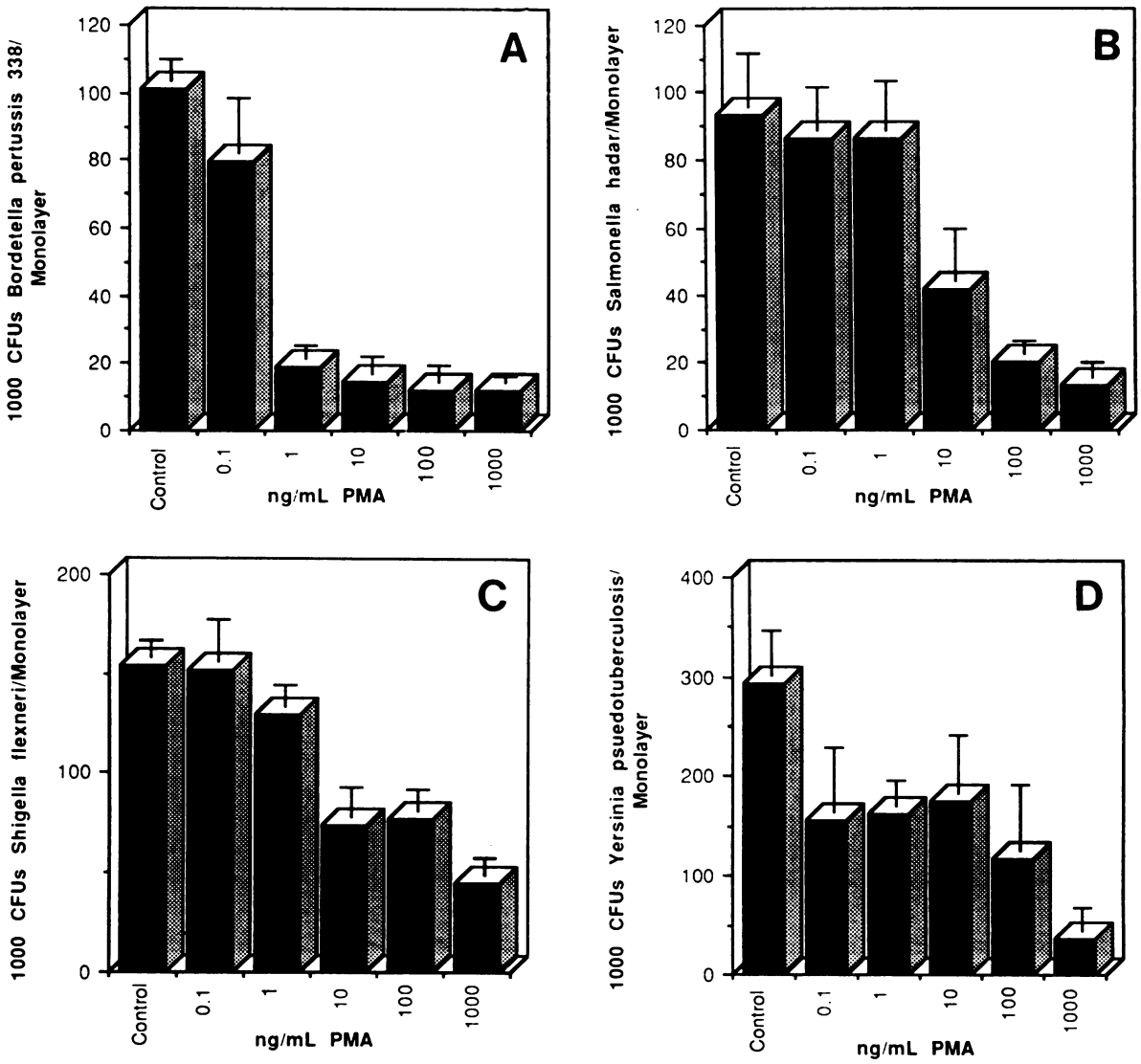

FIG. 2. Dose-dependent inhibition of invasion of HeLa 229 monolayers by various invasive bacteria mediated by PMA. (A) *B. pertussis* BP338; (B) *Salmonella hadar*; (C) *Shigella flexneri* serotype 0:3 E2549; (D) *Y. pseudotuberculosis* type A1. Values represent the mean ± standard deviation CFU (in thousands) recovered from gentamicin-treated monolayers from three independent determinations. HeLa monolayers were incubated with PMA at the concentrations indicated above for 1 h prior to addition of bacteria.

Initially, adherence assays were performed at 4°C to abrogate uptake by the cells so that the numbers of bacteria harvested after 5 h would reflect extracellular adherent bacteria only and not a combination of extra- and intracellular bacteria. However, we found that *B. pertussis* does not form stable associations with the cells at 4°C, precluding quantitation of adherence in the absence of internalization at lower temperatures. Consequently, adherence assays were performed at 37°C, and the data presented in Fig. 4 represents total CFU per monolayer (i.e., intracellular + extracellular). For *B. pertussis*, the ratio of intracellular to extracellular CFU was low (i.e., approximately ≤1:10), so that the values for untreated control, PMA, mezerein, and α-4-phorbol-12,13-didecanoate, which reflect numbers of intracellular and extracellular adherent organisms, did not change significantly. Thus, Fig. 4 illustrates that adherence of *B. pertussis* was unaltered in the presence of the highest concentrations of PMA, mezerein, and α-4-phorbol-12,13-didecanoate tested. In contrast, adherence in the presence of cytochalasins B and D at 2.5 μg/ml was reduced to approximately 50% of the control. This finding was expected, because of the dramatic morphological alterations of HeLa cells elicited by both cytochalasins B and D, as described above. Similar data were obtained by enumerating fluorescent bacteria adherent to HeLa monolayers directly by fluorescence microscopy (data not shown). In separate experiments, cytochalasin B or D at 2.5 μg/ml did not affect the viability of strain BP338 after an 8-h coincubation period.

DISCUSSION

A characteristic common to several invasive bacterial pathogens is the ability to induce a transient rearrangement of the F-actin architecture at sites proximal to entry in nonprofessional phagocytic cell types (7, 15). The stimulus that induces this rearrangement is unknown, although it may involve differential phosphorylation of a ubiquitous class of transmembrane receptors concentrated in focal contacts

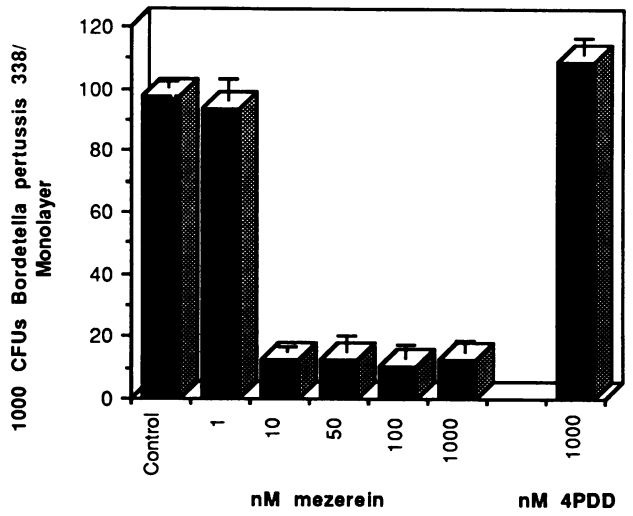

FIG. 3. Effects of mezerein and α-4-phorbol-12,13-didecanoate on uptake of *B. pertussis* BP338 by HeLa 229 monolayers. Each point represents mean ± standard deviation of gentamicin-resistant CFU (in thousands) from three independent determinations.

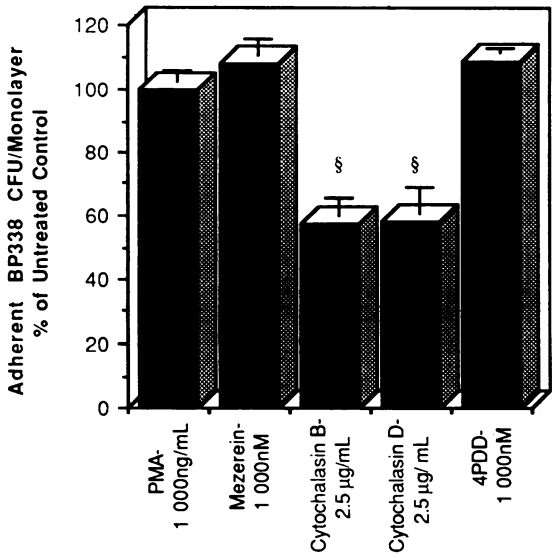

FIG. 4. Effects of PMA, mezerein, cytochalasins B and D, and α-4-phorbol-12,13-didecanoate on adherence of *B. pertussis* BP338 at the highest concentrations tested. Data are expressed as a percentage of untreated controls and represent the mean ± standard deviations from three independent assays. §, Statistically significant at $P < 0.0005$ compared with untreated controls (t test value).

known as integrins, which appear to transduce signals directly from the cell membrane to the actin network via intermediate proteins such as talin, vinculin, and α-actinin (5, 8, 9, 13, 18, 27, 28).

The requirement for F-actin in bacterial uptake by nonprofessional phagocytes has been demonstrated by several different techniques. In 1987, Clerc and Sansonetti (7) demonstrated this requirement by using two novel methods. One involved the use of phallacidin, a phallotoxin which specifically binds F-actin, linked to a fluorescent dye. By using this reagent, Clerc and Sansonetti observed fluorescent aggregates of filamentous actin beneath the membranes of HeLa cells whose appearance coincided with the penetration of invasive *Shigella flexneri*. Such a technique was later used by Finlay et al. (15) to demonstrate the transient appearance of F-actin on penetration of Madin-Darby canine kidney cells by invasive salmonella. In addition, Clerc and Sansonetti directly quantitated a decrease in the monomeric (G)-to-total (F+G)-actin ratio on penetration of *Shigella flexneri*, which reflects de novo polymerization of monomeric actin induced by the invading bacteria (2, 3, 7).

TABLE 1. Comparative efficacies of agents used to inhibit bacterial invasion of HeLa 229 monolayers

Organism	CFU[a]		
	Cytochalasin B	Cytochalasin D	PMA
Bordetella pertussis BP338	1.7 ± 2.4	1.4 ± 0.5	11.8 ± 2.1
Shigella flexneri	50.9 ± 16.3	1.1 ± 1.8	28.9 ± 6.7
Salmonella hadar	26.8 ± 21.6	0.3 ± 0.2	14.7 ± 6.3
Yersinia pseudotuberculosis	28.3 ± 17.7	0.1 ± 0.1	11.6 ± 6.8

[a] Results represent the average number of CFU per monolayer expressed as a percentage of the control ± standard deviation; $n = 3$ independent determinations for each. Monolayers were preincubated with cytochalasin B or D (2.5 μg/ml) or PMA (1.0 μg/ml) for 1 h prior to addition of bacteria. Data corresponding to 100% CFU control are shown in Fig. 2. Numbers of intracellular bacteria were determined by using the gentamicin assay as described previously (11).

To date, however, the majority of studies which demonstrate a requirement for filamentous actin in bacterial uptake have utilized cytochalasins B and D. Although these drugs bind specifically to actin, their actions result in corresponding changes in cell morphology which, as shown in this study, interfere with the abilities of invading bacteria to adhere to the plasma membrane. Consequently, the reduced numbers of intracellular CFU obtained following cytochalasin treatment do not accurately reflect the reduction due to microfilament dysfunction alone.

The tumor promoter PMA causes a rapid and dramatic alteration in actin distribution in numerous cell lines (10, 22–26), including HeLa cells, as demonstrated in this study. Unlike cytochalasins, however, treatment of HeLa cells with PMA up to a concentration of 1,000 ng/ml resulted in only minor changes in cell morphology compared with the cytochalasins. Therefore, we decided to determine whether PMA would reduce invasion of several different bacterial pathogens, as a possible alternative to the use of cytochalasin B or D for delineating the microfilament requirement for uptake.

The data presented here demonstrate a dose-dependent reduction in uptake of virulent *B. pertussis*, *Shigella flexneri*, *Salmonella hadar*, and *Y. pseudotuberculosis* by HeLa cells following 1 h of incubation with PMA at concentrations ranging from 0.1 to 1,000 ng/ml. A similar reduction in uptake of *B. pertussis* was demonstrated with a functional analog of PMA, mezerein (21), whereas an inactive isomer, α-4-phorbol-12,13-didecanoate, was without effects on invasion. Compared with cytochalasin B, cytochalasin D was the most potent drug in terms of its effects on actin and invasion. However, cytochalasin D also caused the most dramatic disruption of cellular morphology. The reduction in invasion elicited by an equivalent concentration of cytochalasin B varied according to the species tested. The invasion of *B. pertussis*, at one extreme, was reduced to approximately 2%

of untreated controls, whereas *Shigella flexneri* was more resistant to its effects and was reduced to only 50% of control. PMA at 1,000 ng/ml elicited a more uniform reduction in uptake among the species tested which was intermediate in magnitude between cytochalasins B and D. Similar to the result obtained with cytochalasin B, *Shigella flexneri* was also more refractory to the reductive effects of PMA. Perhaps the differences in susceptibilities to cytochalasin B and PMA demonstrated by the species tested reflect differences in relative requirements for actin in uptake by nonprofessional phagocytes.

An important finding was that the profound alterations in cellular morphology and the actin network elicited by both cytochalasins B and D caused not only a reduction in invasion but also a significant reduction in adherence. In contrast, we demonstrated that PMA caused a significant reduction in invasion without affecting adherence. This is important, because it permits study of the invasion process independently of the initial stage of adherence. One potential disadvantage to the use of PMA is its lack of specificity, since it may, if present for an extended period, interfere with other cellular processes required for subsequent intracellular survival, receptor synthesis or recycling, and overall cellular homeostasis. In addition, the possibility that PMA alters the ability of the molecule(s) responsible for invasion to bind to its corresponding receptor, thereby causing a reduction in invasion, cannot be excluded by this study.

To summarize, this study introduces a novel reagent for use in examining the bacterial requirement for microfilaments in uptake by cultured cells. PMA is well known to cell biologists for its ability to mimic the effects of tumor viruses on the actin cytoarchitecture of cultured cells. It is similarly capable of inhibiting the uptake of several different species of invasive bacteria through its effects on actin distribution. Importantly, it does so without causing deterioration of cellular morphology as do cytochalasins B and D, thereby allowing bacterial adherence to proceed unhindered.

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