Pseudomonas pyocyanine alters calcium signaling in human airway epithelial cells

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Denning, Gerene M., Michelle A. Railsback, George T. Rasmussen, Charles D. Cox, and Bradley E. Britigan. *Pseudomonas* pyocyanine alters calcium signaling in human airway epithelial cells. *Am. J. Physiol.* 274 (*Lung Cell. Mol. Physiol.* 18): L893–L900, 1998.—*Pseudomonas aeruginosa*, an opportunistic human pathogen, causes both acute and chronic lung disease. *P. aeruginosa* exerts many of its pathophysiological effects by secreting virulence factors, including pyocyanine, a redox-active compound that increases intracellular oxidant stress. Because oxidant stress has been shown to affect cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in other cell types, we studied the effect of pyocyanine on $[Ca^{2+}]_c$ in human airway epithelial cells (A549 and HBE). At lower concentrations, pyocyanine inhibits inositol 1,4,5-trisphosphate formation and $[Ca^{2+}]_c$ increases in response to G protein-coupled receptor agonists. Conversely, at higher concentrations, pyocyanine itself increases $[Ca^{2+}]_c$. The pyocyanine-dependent $[Ca^{2+}]_c$ increase appears to be oxidant dependent and to result from increased inositol trisphosphate and release of Ca^{2+} from intracellular stores. Ca^{2+} plays a central role in epithelial cell function, including regulation of ion transport, mucus secretion, and ciliary beat frequency. By disrupting Ca^{2+} homeostasis, pyocyanine could interfere with these critical functions and contribute to the pathophysiological effects observed in *Pseudomonas*-associated lung disease.

oxidants; inositol phosphates; A549 cells; HBE cells; G protein-coupled receptors

THE GRAM-NEGATIVE bacterium *Pseudomonas aeruginosa* causes acute lung disease with high mortality in patients with hospital-acquired pneumonias (23) and is commonly associated with the chronic lung disease observed in individuals with cystic fibrosis (9). Lung disease is currently the leading cause of morbidity and mortality in cystic fibrosis (26). During infection, *P. aeruginosa* secretes numerous virulence factors that contribute to its pathophysiological effects. Among these factors is the phenazine derivative pyocyanine (19), a redox-active compound that increases intracellular oxidant stress. Currently, the mechanisms by which these factors exert their effects are poorly understood.

Pyocyanine has been shown to have pathophysiological effects in numerous cell types. Most, if not all, of these effects appear to be due to increased intracellular oxidant formation and can be blocked by exogenous addition of antioxidants. In airway epithelial cells, pyocyanine inhibits ciliary beat frequency (16). This inhibition correlates with decreased cellular levels of ATP and cAMP.

Our studies were designed to identify other potentially pathophysiological effects by pyocyanine in human airway epithelial cells. Previous studies demonstrated that pyocyanine increases oxidant formation in these cells (3, 10). Because oxidant stress has been shown to affect Ca^{2+} homeostasis in other cell types (8, 20, 24), we examined the effect of pyocyanine on cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) using two human airway epithelial cell lines, A549 and HBE. We found that pyocyanine increases $[Ca^{2+}]_c$ under some conditions while inhibiting subsequent $[Ca^{2+}]_c$ increases in response to G protein-coupled receptor agonists such as the purinergic receptor agonist ATP. Additional studies were then performed to explore the mechanisms that underlie these effects.

MATERIALS AND METHODS

Materials. Buthionine sulfoximine (BSO), glutathione reductase, NADPH, human placental collagen, *N*-acetylcysteine (NAC), ATP, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and carbamylcholine chloride (carbachol) were purchased from Sigma (St. Louis, MO). Pyocyanine was generously provided by Dr. Cox.

Cell culture. The human alveolar type II cell line A549 (American Type Culture Collection CL-185) (18) was cultured in MEM with Earle's salts (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, 2 mM glutamine, and 500 U/ml each of penicillin and streptomycin. Passages from 85 to 120 were used in these studies. Stock but not experimental cultures of the human bronchial epithelial cell line HBE (14) were cultured in collagen-coated tissue cultureware (*passages 8–25*) in the same medium. Experiments were performed on cultures that were 80–90% confluent.

Spin trapping and electron paramagnetic resonance. Electron paramagnetic resonance (EPR) experiments were performed on airway epithelial cells cultured in 24-well tissue culture plates. Reaction mixture (0.5 ml/well) consisting of Hanks' balanced salt solution containing 100 mM 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO; OMRF Spin Trap Source, Oklahoma City, OK) and 100 μM diethylenetriamine pentaacetic acid (DTPA; Aldrich Chemical, Milwaukee, WI) with or without the indicated additions was placed on the cell monolayer. Samples were incubated for 15 min at room temperature, and then the reaction mixture was transferred to a quartz flat cell. All spectra are the result of seven signal-averaged scans and were obtained at room temperature using a Bruker model ESP300 EPR spectrometer (Karlsruhe, Germany). Instrument settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; receiver gain, 4×10^5; modulation amplitude, 0.501 G; time constant, 327.68 ms; sweep rate, 335.5 s.

Measurement of $[Ca^{2+}]_c$. Ca^{2+} measurements were performed at the Cell Fluorescence Core Facility (Veterans Affairs Medical Center, Iowa City, IA). Cells were cultured on collagen-coated 25-mm round glass coverslips. Cells were loaded in complete medium with fura 2 by direct addition of the cell-permeant form, fura 2-AM (Molecular Probes, Eugene, OR), to the culture dish containing the coverslip and

incubation for 30 min at 37°C. Cells were washed with HEPES-buffered saline [in mM: 135 NaCl, 5 KOH, 10 HEPES, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose (HBS-G)], and measurements of the apparent $[Ca^{2+}]_c$ were done in HBS-G using the Photoscan II spectrofluorometer (Photon Technologies International, New Brunswick, NJ) with a Nikon microscope (Nikon, Niles, IL). Final Ca^{2+} concentration ($[Ca^{2+}]$) values were determined using a PTI software package from the ratio of emission intensities [emission wavelength (λ_{em}) 510 nm] for excitation wavelengths (λ_{ex}) of 340 and 380 nm. Briefly, background fluorescence intensities for each λ_{ex} were obtained using unloaded cells and were subtracted from the raw data. The ratios of the corrected fluorescence intensities were then converted to $[Ca^{2+}]$ values using the formula $[Ca^{2+}] = K_d \cdot (R - R_{min})/(R_{max} - R)$, where the maximum and minimum ratios (R_{max} and R_{min}, respectively) as well as the apparent dissociation constant (K_d) were empirically derived from $[Ca^{2+}]$ curves generated using the instrument. In earlier work (6), we found that this method gives similar values for basal $[Ca^{2+}]$ as well as for Ca^{2+} increases in response to agonists as does the more laborious method that involves determining R_{max} and R_{min} using ionomycin followed by EGTA (7).

Fluorescence measurements of intracellular oxidant formation. To measure intracellular oxidant formation, we used an oxidant-sensitive dichlorodihydrofluorescein derivative (Iowa probe) generously provided by Dr. Stephen Hempel (Dept. of Internal Medicine, Veterans Affairs Medical Center and the Univ. of Iowa, Iowa City, IA). In cell-free studies (15), the Iowa probe behaved identically to commercially available dichlorodihydrofluorescein compounds (Molecular Probes). However, in cell culture studies, the Iowa probe was significantly more sensitive in detecting oxidant formation in response to pyocyanine as well as to other redox-active compounds (S. Hempel, unpublished observations). Although the exact mechanism by which these compounds detect oxidant formation is not fully understood, reaction of these probes with H_2O_2 appears to require peroxidase activity or iron (25).

For these studies, cells in 12-well tissue culture plates were washed twice with warm HBS-G and preincubated at 37°C for 30 min in HBS-G containing 5 μM Iowa probe. At the end of the preincubation period, the indicated concentration of pyocyanine was added, and the cells were incubated for 1 h at 37°C. To measure cell-associated fluorescence, the cells were washed twice with ice-cold PBS and incubated on ice with PBS containing 0.2% Triton X-100. The cell extract was removed from the cells, and the relative fluorescence intensity of the extract (λ_{ex}, 485 nm; λ_{em}, 512 nm) was determined using a Gilford Fluoro IV spectrofluorometer (Ciba-Corning Diagnostics, Park Ridge, IL).

Altering cellular antioxidant capacity. To reduce glutathione levels, cells were incubated with 100 μM BSO in complete medium for 48 h. To measure glutathione, cells were washed twice with ice-cold PBS, scraped into 0.01 N HCl, frozen overnight, and thawed. Samples were centrifuged at 12,000 rpm for 5 min to remove cellular debris, and total glutathione in the cell extract was determined by assaying the rate of DTNB reduction in the presence of NADPH and glutathione reductase (11). Oxidized glutathione was measured in cell extracts treated for 1 h with 2-vinylpyridine (Aldrich Chemical). Reduced glutathione was determined by subtracting oxidized glutathione from total. Values were generated by comparison with a reduced glutathione standard curve and were normalized to total cell protein, measured using the micro bicinchoninic acid assay (Pierce, Rockford, IL). For studies with NAC, cells were pretreated with the indicated concentration of NAC for 2–4 h before experiments were performed. NAC at these concentrations markedly acidifies the medium. Thus all NAC-containing solutions were adjusted to pH 7.3–7.5 before use.

Turnover of inositol phosphates. Cells were seeded into six-well tissue culture plates, allowed to attach overnight, and then cultured for 48 h in complete medium containing 1 μCi/ml of *myo*-[^{3}H]inositol (Amersham, Arlington Heights, IL). At the end of the labeling period, turnover of inositol phosphates (IPs) was measured as previously described (7). Briefly, cells were washed with HBS-G and incubated in HBS-G for 20 min at 37°C. Cultures were then incubated for 20 min with HBS-G containing 10 mM LiCl. Finally, cells were stimulated with the indicated agonist for 20 min or with pyocyanine for 10 min and then agonist for 10 min. IPs were extracted overnight at 4°C with 0.5 M perchloric acid. The acid extract was neutralized with 2.5 M KOH and 0.5 M HEPES (pH 7.4) and centrifuged to remove the precipitate. The IP species were then collected using anion-exchange column chromatography (Dowex AG, 1–8X, 100–200 mesh, formate form; Bio-Rad, Hercules, CA) as previously described (22).

RESULTS AND DISCUSSION

Pyocyanine increases oxidant formation in A549 and HBE cells. Previous work in our laboratories (3) and by Gardner (10) using two different assay techniques demonstrates that pyocyanine stimulates oxidant formation in A549 cells. To verify these results in our A549 cultures and to determine whether pyocyanine increases superoxide anion ($\cdot O_2^-$) formation in HBE cells, we used the spin-trapping agent DMPO and EPR spectroscopy. Figure 1 shows pyocyanine-dependent formation of the DMPO-$\cdot$OH adduct ($a^N = a^H = 14.9$ G, where a^N and a^H are the splitting constants for nitrogen and hydrogen, respectively) in HBE (Fig. 1*A*) and A549 (Fig. 1*B*) cells. Formation of this adduct could result from a reaction between DMPO and either $\cdot O_2^-$ or the

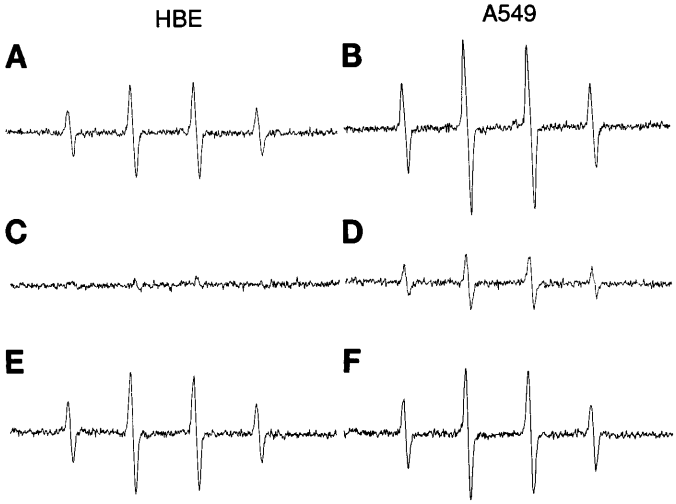

Fig. 1. Representative electron paramagnetic resonance (EPR) spectra from HBE and A549 cells exposed to pyocyanine. Cells were exposed for 15 min at room temperature to 80 μM pyocyanine alone (*A* and *B*), pyocyanine in presence of 3,000 U/ml of superoxide dismutase (*C* and *D*), or pyocyanine in presence of 5,000 U/ml of catalase (*E* and *F*). EPR spectra were subsequently obtained as described in MATERIALS AND METHODS using the 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin-trapping system. Spectra shown represent DMPO-$\cdot$OH spin adduct. Similar responses were observed in 2 other independent experiments.

hydroxyl radical (·OH). Addition of superoxide dismutase markedly attenuates or abolishes the signal (Fig. 1, *C* and *D*), demonstrating that $\cdot O_2^-$ is required for adduct formation. In addition, catalase has little or no effect (Fig. 1, *E* and *F*) on the signal. This result argues against ·OH formation, since its formation requires H_2O_2 and hence is inhibited by catalase. Consistent with this conclusion is our observation that the ·OH scavenger DMSO, which reacts with ·OH to form the methyl radical ($\cdot CH_3$) and subsequently DMPO-$\cdot CH_3$, did not alter the response to pyocyanine (data not shown). Together, these results indicate that the spectra shown in Fig. 1 reflect pyocyanine-induced $\cdot O_2^-$ formation.

Work by our laboratories (3) and others (13) suggests that pyocyanine induces $\cdot O_2^-$ formation by accepting an electron from cellular NAD(P)H to form the pyocyanine radical, which in turn reduces O_2 to form $\cdot O_2^-$. This mechanism suggests that pyocyanine will redox cycle, producing $\cdot O_2^-$ until these reducing sources are consumed or until the pyocyanine is metabolized.

Pyocyanine increases [Ca²⁺]c. Previous studies (8, 24) show that oxidants increase $[Ca^{2+}]_c$ in a variety of cell types. In early studies, we found that pyocyanine causes an acute, transient increase in $[Ca^{2+}]_c$. A representative example of this response in A549 cells is shown in Fig. 2*A*. In these studies, the apparent basal $[Ca^{2+}]_c$ values were ~100–150 nM for both A549 and HBE cells and were similar from experiment to experiment. In contrast, the magnitude of the maximal $[Ca^{2+}]_c$ increase in response to pyocyanine, although similar within an experiment, varied considerably from experiment to experiment, ranging from 50 to over 300 nM. Moreover, concentration-dependence studies indicate that a threshold concentration of pyocyanine is required to elicit the response. This threshold concentration also varied considerably from experiment to experiment (ranging from 80 to 250 μM). Responses to other Ca^{2+} agonists in these same studies did not exhibit the same degree of variability, suggesting that this is a specific characteristic of the pyocyanine response. The basis for this variability remains unclear but did not appear to depend on the preparation of pyocyanine used or on the confluency state of the cells. We speculate that the variability may reflect differences in uptake of pyocyanine, via a mechanism currently unknown, or differences in the level of reducing sources (NADPH, NADH). Further experiments will be necessary to test these and other possibilities.

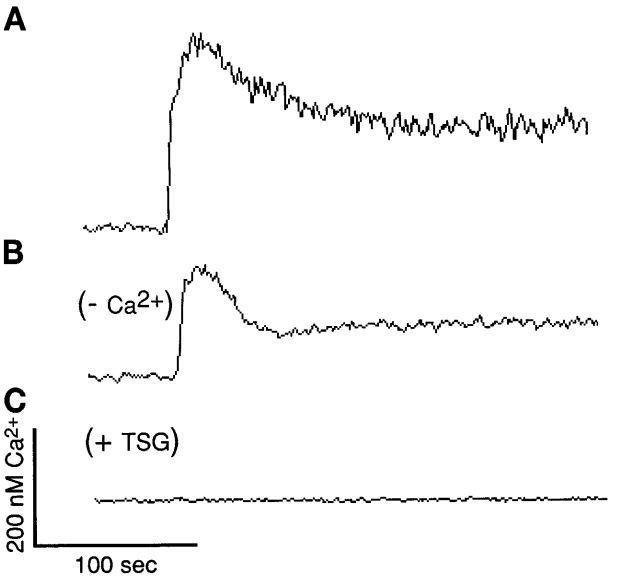

Fig. 2. Pyocyanine-dependent increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). A549 cells were loaded with fura 2 and stimulated with 300 μM pyocyanine in presence (+; *A*) or absence (−; *B*) of extracellular Ca^{2+}. *C*: cells were treated for 40 min with 2 μM thapsigargin (TSG) before loading with fura 2 and stimulation with pyocyanine (added at 60 s). Ca^{2+} measurements were performed as described in MATERIALS AND METHODS. Similar responses were observed in 2 other independent experiments.

As a first step in determining the mechanism by which pyocyanine increases $[Ca^{2+}]_c$, we designed experiments to identify the source of the $[Ca^{2+}]_c$ increase. As shown in Fig. 2*B*, the response to pyocyanine persists in the absence of extracellular Ca^{2+}. This suggests that pyocyanine stimulates release of Ca^{2+} from intracellular Ca^{2+} stores. Consistent with this conclusion, we found that depletion of these stores by treatment with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (TSG) abolishes the response to pyocyanine (Fig. 2*C*). Furthermore, the more transient nature of the $[Ca^{2+}]_c$ increase in the absence of extracellular Ca^{2+} (Fig. 2, compare *A* with *B*) suggests that influx also contributes to the pyocyanine-dependent increase in $[Ca^{2+}]_c$. However, the lack of a $[Ca^{2+}]_c$ increase after TSG treatment (see Fig. 3*C*) suggests that this influx is dependent on the stimulated release from stores. Similar results were obtained with HBE cells (data not shown). These data indicate that pyocyanine increases $[Ca^{2+}]_c$ by stimulating release of Ca^{2+} from TSG-sensitive stores and that this release triggers Ca^{2+} influx.

Pyocyanine stimulates IP turnover. Release of Ca^{2+} from intracellular Ca^{2+} stores can result from formation of the second messenger inositol 1,4,5-trisphosphate (IP_3). To determine whether pyocyanine increases $[Ca^{2+}]_c$ by increasing IP_3, we used an assay that measures turnover of IPs. This assay has been shown to be the most sensitive measure of increased IP_3. Moreover, in previous work (17), we found that results using this method accurately reflected increased IP_3 formation.

Using this approach, we found that pyocyanine increases IP turnover in a concentration-dependent manner in both A549 and HBE cells. In three independent experiments, the maximum increase observed in response to pyocyanine ranged from 116 ± 6 to 169 ± 11% of control value (means ± SD for triplicate samples). As a positive control in these studies, we used the purinergic receptor agonist ATP. We observed that 1 mM ATP stimulated IP turnover in the range of 141 ± 10 to 206 ± 18% of control value in these experiments. Moreover, as with our $[Ca^{2+}]_c$ measurements, we observed that the minimum pyocyanine concentration required to stimulate IP turnover varied markedly. As stated above, the basis for this variability remains unknown.

Cellular antioxidant capacity alters the response to pyocyanine. To determine whether oxidants contribute to the pyocyanine-dependent increase in $[Ca^{2+}]_c$, we decreased the antioxidant capacity of the cells by decreasing intracellular levels of the thiol antioxidant glutathione. To do this, cells were treated for 48 h with 100 μM BSO, an inhibitor of γ-glutamylcysteine synthetase (21). Total glutathione levels in control cells were 79 ± 7 and 42 ± 6 nmol/mg protein (means ± SE; combined data from 6 independent experiments with triplicate samples for each cell type) for A549 and HBE cells, respectively. In each case, the majority (84–90%) of the glutathione was in the reduced form. A 48-h treatment with BSO decreased total glutathione levels by 80–98% (A549, 2.2 ± 0.44 ng/mg protein; HBE, 9.5 ± 3.0 ng/mg protein; means ± SE; combined data from two independent experiments with triplicate samples for each cell type).

First, to determine whether BSO treatment affects pyocyanine-dependent oxidant formation, we used a cell-permeable, oxidant-sensitive fluorescent probe. For these experiments, cells were pretreated for 48 h with or without BSO, washed with buffer, preincubated for 30 min with 5 μM probe, and then stimulated for 1 h with the indicated concentration of pyocyanine. Cultures were then rapidly washed with ice-cold PBS and permeabilized with 0.2% Triton X-100. Finally, the relative fluorescence of the cell extract was determined. Figure 3 shows representative results from studies with A549 cells. Similar results were seen with HBE cells (data not shown). Under these conditions, pyocyanine increases cell-associated probe fluorescence in a concentration-dependent manner (Fig. 3*A*, solid line) and BSO enhances pyocyanine-dependent oxidation of the probe (Fig. 3*A*, dotted line).

We then measured pyocyanine-dependent $[Ca^{2+}]_c$ changes in control and BSO-treated cells (Fig. 3*B*). To do this, cells with or without BSO treatment were loaded with fura 2 and then individually placed in the spectrofluorometer. A baseline ratio was collected for 1–2 min. This ratio represents the apparent basal $[Ca^{2+}]_c$ for the sample. Pyocyanine at the indicated concentration was then added to the sample, and data were collected over the next 10 min. We found that this time period was sufficient to observe maximum changes. The peak ratio of the response was converted to $[Ca^{2+}]_c$ and used to determine the increase over baseline. Consistent with a role for oxidants in these changes, BSO treatment shifted the pyocyanine dose-response curve to the left. The kinetics of Ca^{2+} release in response to pyocyanine was not altered in BSO-treated cells. These data suggest that glutathione plays a role in removal of pyocyanine-generated oxidants and that oxidant formation contributes to the increase in $[Ca^{2+}]_c$ in response to pyocyanine.

To assess further whether pyocyanine increases $[Ca^{2+}]_c$ by increasing oxidant formation, we tested the effect of adding the thiol antioxidant NAC. In these experiments, cells were preincubated for 2 h with 30 mM NAC. When compared with controls (A549, 92 ± 10 ng/mg protein; HBE, 36 ± 8 ng/mg protein; means ± SE), NAC had no appreciable effect or slightly increased total glutathione levels (A549, 110 ± 37 nmol/mg protein; HBE, 26 ± 5 nmol/mg protein; means ± SE). These values represent combined data from two independent experiments with triplicate samples for each cell type. As illustrated in Fig. 4 for HBE cells, NAC inhibits the pyocyanine-dependent oxidation of the fluorescent probe (Fig. 4*A*, dotted line). Control studies indicate that this inhibition is not due to NAC-dependent quenching of probe fluorescence. NAC also inhibits the pyocyanine-dependent increase in $[Ca^{2+}]_c$ (Fig. 4*B*). Similar results were obtained with A549 cells (data not shown). These data provide further evidence that oxidants mediate the pyocyanine-dependent $[Ca^{2+}]_c$ increase.

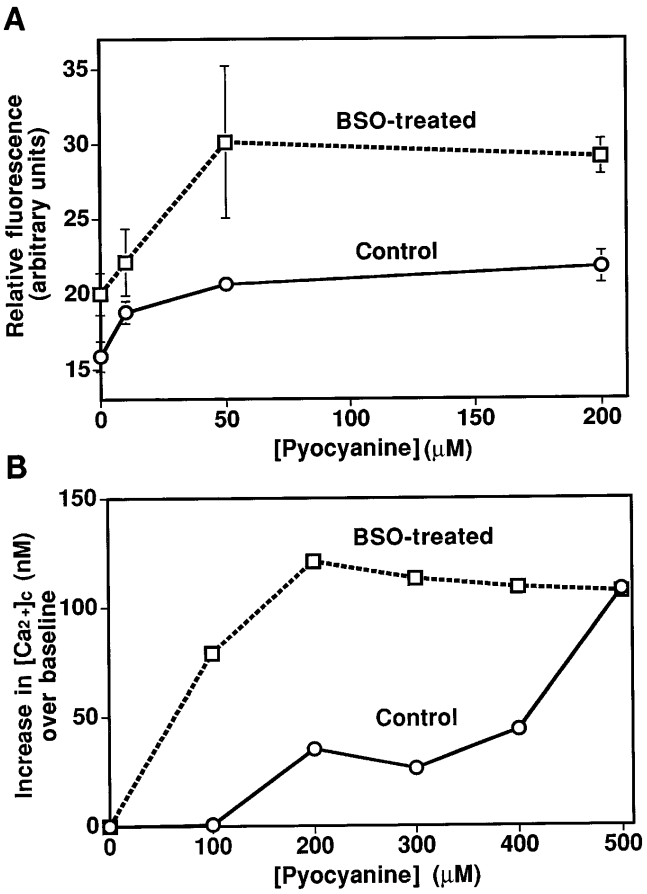

Fig. 3. Effect of reducing glutathione on response to pyocyanine. A549 cells were treated for 48 h with (dotted line) or without (solid line) 100 μM buthionine sulfoximine (BSO). *A*: pyocyanine-dependent oxidant formation was then measured using an oxidant-sensitive fluorescent probe as described in MATERIALS AND METHODS. Values are means ± SD of triplicate samples from a representative experiment. Similar results were obtained in 2 other independent experiments. *B*: pyocyanine-dependent changes in $[Ca^{2+}]_c$ were measured in fura 2-loaded cells. Values are means of duplicate samples from a representative experiment. A similar shift in sensitivity after BSO treatment was observed for selected pyocyanine concentrations in 2 other independent experiments. [Pyocyanine], pyocyanine concentration.

Pyocyanine inhibits the response to Ca^{2+} agonists. Because pyocyanine itself increases $[Ca^{2+}]_c$, we wondered whether pyocyanine would affect the subsequent response to Ca^{2+} agonists. The purinergic receptor

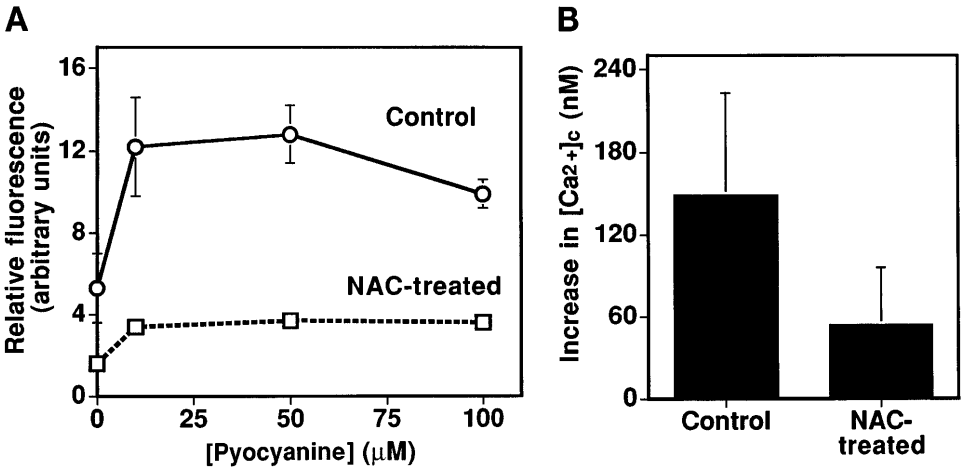

Fig. 4. Effect of adding thiol antioxidant *N*-acetylcysteine (NAC) on response to pyocyanine. HBE cells were treated for 2 h with or without 30 mM NAC. Measurements were then performed on control and NAC-treated cells to determine oxidant formation (*A*; fluorescent probe) and $[Ca^{2+}]_c$ changes (*B*; fura 2) in response to pyocyanine. Values are means ± SD of triplicate samples from a representative experiment. Similar results were seen in a separate independent experiment.

agonist ATP was used for these experiments, since it increases $[Ca^{2+}]_c$ in both A549 (Fig. 5*A*) and HBE (Fig. 5*B*) cells.

We found that pyocyanine inhibits the $[Ca^{2+}]_c$ increases in response to ATP in both A549 and HBE cells (Fig. 5, *E* and *F*, respectively; second arrow). Interestingly, this inhibition was often observed under conditions in which pyocyanine itself had little or no effect on $[Ca^{2+}]_c$ (Fig. 5, *E* and *F*; first arrow). This latter observation suggests that the inhibition is not simply due to depletion of Ca^{2+} from hormone-sensitive stores, a conclusion supported by our observation that pyocyanine does not appreciably inhibit a subsequent $[Ca^{2+}]_c$ increase in response to TSG or ionomycin (data not shown). In addition, these data suggest that the effect of pyocyanine on agonist-dependent increases in $[Ca^{2+}]_c$ is independent of the direct effect of pyocyanine on $[Ca^{2+}]_c$ levels. NAC pretreatment prevents pyocyanine from inhibiting the response to ATP (data not shown), suggesting that this pyocyanine effect is also mediated by oxidant formation.

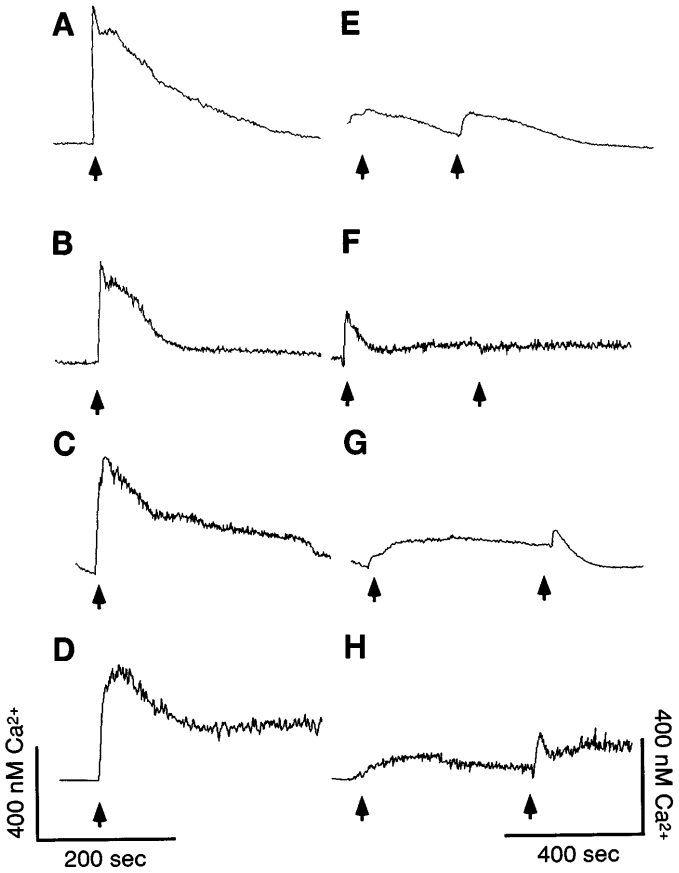

Fig. 5. Effect of pyocyanine on $[Ca^{2+}]_c$ increase in response to Ca^{2+} agonists. Representative tracings from 2 to 4 independent experiments for each cell type show an increase in $[Ca^{2+}]_c$ in response to 1 mM ATP in HBE (*A*), A549 (*B*), and HT-29 (*C*) cells. *D*: $[Ca^{2+}]_c$ increase in HT-29 cells in response to 100 μM carbachol. *E–H*: corresponding tracings of the same cell type and agonist first exposed to pyocyanine (100–300 μM; first arrow) and then to agonist (second arrow).

The inhibitory effect by pyocyanine is not restricted to ATP or to airway epithelial cells. Figure 5 also demonstrates that both ATP (Fig. 5*C*) and the muscarinic receptor agonist carbachol (Fig. 5*D*) increase $[Ca^{2+}]_c$ in the human intestinal epithelial cell line HT-29. Pyocyanine (Fig. 5, *G* and *H*; first arrow) inhibits the response to both agonists (Fig. 5, *G* and *H*; second arrow) in these cells. These results suggest that pyocyanine inhibits signaling in response to receptors that are coupled via G proteins to phosphatidylinositol 4,5-bisphosphate (PIP_2)-specific phospholipase C (PIP_2-PLC). Conversely, pyocyanine does not inhibit cytokine receptor-mediated signaling, since we found that pyocyanine has no effect on cytokine-dependent increases in polymeric IgA-receptor expression in HT-29 cells (Denning, unpublished data) and synergizes with cytokines in stimulating release of interleukin-8 by airway epithelial cells (Denning, unpublished observations).

Pyocyanine inhibits IP turnover in response to ATP. To determine whether pyocyanine inhibits the $[Ca^{2+}]_c$ increase in response to ATP by inhibiting agonist-dependent IP_3 formation, we measured the effect of pyocyanine on ATP-stimulated turnover of IPs. In these experiments, cells were labeled with *myo*-[^{3}H]inositol for 48 h, washed, and incubated for 20 min in buffer with 10 mM LiCl. The cells were then incubated for 10 min with or without pyocyanine and finally for 10 min with or without 1 mM ATP. We found that pyocyanine

inhibits IP turnover in response to ATP. Moreover, inhibition of the response to ATP can occur at pyocyanine concentrations that, within a given experiment, do not by themselves stimulate an increase in IP turnover. As an example of these results, we obtained values of 100 ± 11, 87 ± 2, 160 ± 27, and 86 ± 4% (means ± SD for triplicate samples from a representative experiment) for control, pyocyanine alone (200 μM), ATP alone, and pyocyanine followed by ATP, respectively. Similar results were seen in two other independent experiments. In studies in which we observed a pyocyanine-dependent increase in IP turnover, no additional increase was observed with subsequent exposure to ATP. These data further suggest that the effect of pyocyanine on IP_3 formation in response to ATP is independent of its effect on IP_3 formation itself.

Activation of protein kinase C (PKC) has been shown to inhibit signaling by G protein-coupled receptors linked to PIP_2-PLC (4). In addition, oxidant stress has been shown to activate PKC (27). Thus, to determine whether pyocyanine inhibits IP_3 formation in response to ATP by activating PKC, we tested the effect of the PKC inhibitors staurosporine and bisindolylmaleimide. Results from these experiments illustrate several points (Fig. 6). First, ATP stimulates IP turnover, and inhibitor treatment has little or no effect on this response. Second, activation of PKC by the phorbol ester phorbol 12-myristate 13-acetate (PMA) inhibits the ATP response, and this inhibition is prevented by pretreating the cell with either inhibitor. Finally, pyocyanine inhibits IP turnover in response to ATP, but, in contrast to PMA, PKC inhibitors do not block this effect. These data suggest that pyocyanine does not exert its inhibitory effect by activating PMA-sensitive isoforms of PKC. We cannot rule out the possibility, however, that the ATP response is inhibited by pyocyanine through activation of PMA-insensitive isoforms of PKC that are not inhibited by these concentrations of PKC inhibitors.

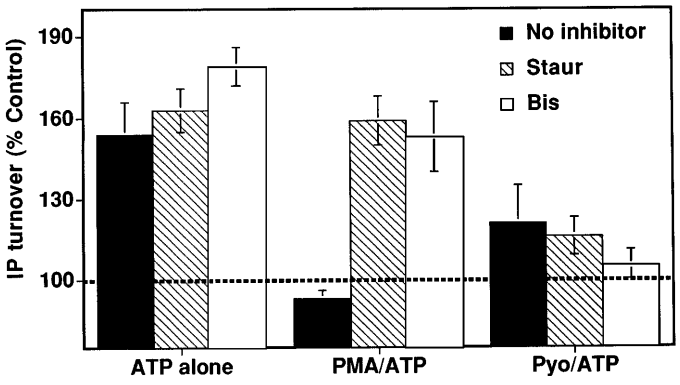

Fig. 6. Studies with protein kinase C (PKC) inhibitors. A549 cells were radiolabeled for 48 h with *myo*-[^{3}H]inositol and then pretreated for 1 h with or without 300 nM PKC inhibitors staurosporine (Staur) or bisindolylmaleimide (Bis). Inositol phosphate (IP) turnover was then measured in control cells (data not shown), in cells stimulated with 1 mM ATP, in cells pretreated for 10 min with 100 nM phorbol 12-myristate 13-acetate (PMA) before ATP stimulation, or in cells pretreated for 10 min with 200 μM pyocyanine (Pyo) and then stimulated with ATP. Values are means ± SD of triplicate samples. Similar results were obtained in a separate independent experiment.

Conclusions. These studies are the first to examine the effect of *Pseudomonas* pyocyanine on $[Ca^{2+}]_c$ in human airway epithelial cells. We found that pyocyanine has two such effects. Pyocyanine alone at higher concentrations increases $[Ca^{2+}]_c$, presumably by increasing IP_3, which stimulates Ca^{2+} release from intracellular Ca^{2+} stores and a subsequent influx of extracellular Ca^{2+}. Conversely, pyocyanine at lower concentrations inhibits IP_3 formation and the subsequent increase in $[Ca^{2+}]_c$ in response to Ca^{2+} agonists. Because of differences in the concentration dependence of these two effects and because they reflect opposite effects on IP metabolism, it follows that the two effects are independent and therefore must involve different molecular mechanisms. However, both effects appear to share the common feature of being mediated, at least in part, by pyocyanine-generated oxidants.

These effects occur at concentrations ranging from 80 to 250 μM. Pyocyanine has been detected at concentrations as high as 75–100 μM in sputum (28) and bronchoalveolar lavage fluid from patients with *Pseudomonas* infections. With consideration that dilution occurs as a result of lavage, it is reasonable to assume that higher concentrations of pyocyanine are present at the site of infection. Thus the concentrations used in our studies are likely to be physiologically relevant.

The molecular mechanisms by which pyocyanine increases IP_3 and $[Ca^{2+}]_c$ remain to be identified. Previous studies suggest several possibilities. One of these involves oxidant-dependent activation of protein tyrosine kinases (PTKs) with subsequent tyrosine phosphorylation and activation of PLC-γ. As an example, oxidants increase IP_3 and $[Ca^{2+}]_c$ in HL-60 cells by stimulating tyrosine phosphorylation and activation of PLC-γ2 (2). Currently, however, two pieces of data argue against this occurring in our system. First, the kinetics of the initial $[Ca^{2+}]_c$ rise is slower (minutes) when tyrosine phosphorylation is involved, whereas the response to pyocyanine is rapid (seconds). Second, preliminary results indicate that the pyocyanine-dependent increase in $[Ca^{2+}]_c$ is not inhibited by 300 μM PTK inhibitor genistein or by concentrations of staurosporine (300 nM) that inhibit PTKs (5) (data not shown). In parallel studies, these inhibitors prevented the pyocyanine-dependent increase in interleukin-8 release, demonstrating that pyocyanine does activate PTKs in these cells and that these inhibitors are effective under our experimental conditions. Although our data suggest that pyocyanine does not increase $[Ca^{2+}]_c$ by stimulating tyrosine phosphorylation and activation of PIP_2-PLC, further studies will be necessary to rigorously rule out this possibility.

Alternatively, pyocyanine could activate an isoform of PIP_2-PLC by a mechanism other than tyrosine phosphorylation, or it could have a direct effect on IP metabolism. Moreover, although it seems likely that the $[Ca^{2+}]_c$ increase results from increased IP_3, on the basis of its characteristic rapid onset, oxidants have been shown to have other effects that may be relevant

in this case. For example, oxidant stress has been shown to inhibit the Ca^{2+}-ATPases in both endothelial (12) and skeletal muscle (1) cells. If pyocyanine-induced oxidants inhibit the endoplasmic reticulum Ca^{2+}-ATPase, then this might contribute to the pyocyanine-dependent release of Ca^{2+} from intracellular stores.

The molecular mechanism by which pyocyanine inhibits its IP_3 formation and $[Ca^{2+}]_c$ increases in response to hormonal agonists is also currently unknown. We initially speculated that pyocyanine inhibits the response to ATP by activating PKC, as is often illustrated by pretreating cells with PMA. Our studies with PKC inhibitors suggest that if an isoform of PKC is involved, it is likely to be PMA insensitive as well as relatively insensitive to both staurosporine and bisindolylmaleimide. In addition to being inhibited by PKC, however, signaling by G protein-coupled receptors is inhibited by activation of receptor-associated kinases (4). Of note with respect to our studies, these kinases are not inhibited by inhibitors of PKC. It will be of interest to determine whether these kinases are activated by pyocyanine and whether activation of these kinases is responsible for the observed inhibition.

An alternative hypothesis is suggested by studies in human neuroblastoma SH-SY5Y cells exposed to H_2O_2 (17). In these cells, H_2O_2 inhibits IP_3 formation in response to carbachol. The authors provide evidence that PLC and phosphoinositide metabolism are not inhibited but rather that oxidant stress inhibits activation of the G proteins that couple to PIP_2-PLC. These results suggest that pyocyanine may inhibit G protein coupling to receptors through an oxidant-dependent mechanism. If this is the case, however, then pyocyanine-dependent increases in IPs must occur distal to or independent of G protein-mediated events. Further studies will be required to test these hypotheses.

Our work demonstrates that pyocyanine alters Ca^{2+} homeostasis in human airway epithelial cells, including inhibition of the response to Ca^{2+} agonists. These agonists regulate important epithelial cell functions including ion transport, mucus secretion, and ciliary beat frequency. These functions, in turn, influence mucociliary clearance. By altering Ca^{2+} homeostasis in these cells, pyocyanine could interfere with critical host defense mechanisms and thereby contribute to the pathophysiological effects observed in *Pseudomonas*-associated lung disease.

Antibiotic therapy has shown limited success in treating *Pseudomonas* infections. Understanding the mechanisms by which *Pseudomonas* virulence factors such as pyocyanine exert their effects may provide insight that will lead to more effective therapeutic approaches that bypass antibiotic resistance mechanisms and that specifically target this microorganism.

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