The Enteric Parasite Entamoeba Uses an Autocrine Catecholamine System during Differentiation into the Infectious Cyst Stage*

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Enteric amoebae of the genus Entamoeba travel from host to host in an encysted form. We previously showed that in vitro cyst development of Entamoeba invadens requires the addition of defined amounts of multivalent galactose-terminated molecules, such as mucin, to the cultures. The amoeba surface lectin that binds mucin is presumed to convey transmembrane signals when clustered by the ligand, but the signaling molecules that function downstream of the lectin are not known. We report here that Entamoeba encystation was induced in the absence of galactose ligand when catecholamines were added to the encystation medium. Micromolar amounts of both epinephrine and norepinephrine inhibited encystation, and did so even when exogenous catecholamines were not added, indicating that catecholamine binding is required for encystation and suggesting an endogenous source of the ligand. High performance liquid chromatography analysis of Entamoeba extracts showed that the amoebae themselves contain catecholamines and at least one of these is released when the cells are stimulated to encyst with galactose-terminated ligands. The presence of catecholamine binding sites on the surface of amoeba trophozoites was confirmed using radiolabeled catecholamine antagonist. Amoeba encystment was inhibited by addition of β₁-adrenergic receptor antagonist to cells that were stimulated to differentiate with either galactose ligand or catecholamines, but not with dibutyryl cAMP. This suggests that the amoeba catecholamine receptor functions downstream of the galactose lectin and upstream of adenyl cyclase. This enteric protozoan parasite, therefore, contains the components of an autocrine catecholamine ligand-receptor system that may act in conjunction with a galactose lectin to regulate differentiation into the infectious cyst stage.

Parasites of the genus Entamoeba have a simple life cycle that alternates between a feeding trophozoite stage, which multiplies within the colon of the host, and a dormant cyst stage that is passed into the external environment to infect another host. Morphological and biochemical changes associated with differentiation of the trophozoite into the cyst (encystation) have been described (1–3), but little is known about the stimuli that trigger this differentiation process.

No axenic in vitro system currently exists for studying encystation of the human-infecting parasite Entamoeba histolytica; however, such a system exists for Entamoeba invadens, a parasite of reptiles. These two organisms have the same two-stage life cycle and pathogenic potential toward their vertebrate hosts (4), and form quadrinucleated, chitinaceous cysts with osmotically resistant cyst walls (1). E. invadens will readily encyst in vitro in response to carbon source deprivation (5), hypoosmotic shock (6), or a combination of the two stimuli (7). One requirement for maximal encystation to occur in vitro is the presence of 5% adult bovine serum (ABS) in the encystation medium (8); dialyzed serum will also suffice, suggesting a role for a large molecular weight molecule (8). We have shown that specific concentrations of galactose-terminated glycoproteins, such as asialofetuin (ASF), or mucin, the major glycoprotein comprising the mucus layer of the colon, can substitute for the 5% serum and trigger the differentiation of the trophozoite to the cyst form (9). Mucin binds to Entamoeba trophozoites (10) via a surface galactose lectin (11, 12) that has possible transmembrane signaling capacity (13), but the downstream signaling systems that interact with the lectin are not defined.

During in vitro encystation, the motile E. invadens trophozoites form large multicellular aggregates within the first 2 h after they are transferred into encystation induction medium (5). Within these aggregates the trophozoites will eventually differentiate to the cyst form. Encystation appears to be dependent upon this aggregation; millimolar amounts of free galactose, which inhibit aggregation, also prevent differentiation of the trophozoites to cysts (14). Whether the parasite forms such aggregates in vivo is unknown. A common soil amoeba, Dictyostelium discoideum, forms large masses of 10⁶ or more cells when its food supply of bacteria is depleted (15). A number of diffusible factors are involved in signaling between D. discoideum cells during the aggregation phase that precedes differentiation (16–18). Additionally, a large number of Gram-positive and Gram-negative bacteria, as well as fungi, secrete signaling molecules (so-called autoinducers) that induce changes in gene expression, which can result in differentiation (19–21).

Catecholamines are biogenic amines that contain a catechol nucleus (a benzene ring with two adjacent hydroxyl substituents) and an amine group. In mammalian systems the cat-
A Catecholamine System in Entamoeba Differentiation

echolamines (dopamine, norepinephrine, and epinephrine) function as hormones and neurotransmitters that regulate a variety of physiologic functions, including heart rate, blood pressure, and levels of circulating glucose (22). In both plants and animals, these compounds are synthesized from tyrosine in the order: dopamine → norepinephrine → epinephrine. In mammalian systems, cell surface receptors that selectively bind catecholamines are divided into two major classes, dopaminergic and adrenergic. Adrenergic receptors can be divided into three types, α₁, α₂, and β, and each of these can be further divided into several subtypes, based on pharmacological differences and amino acid sequence (23, 24).

Hartmannella vermiformis, a nonpathogenic free-living amoeba that plays a major role in the continuous environmental presence and amplification of Legionella pneumophila, the causative agent of Legionnaire's disease (26), will encyst when one of a variety of bioactive amines, including catecholamines, are added to a non-nutrient medium (25). Uptake of epinephrine by these cells is inhibited by the β-adrenergic receptor (β-AR) antagonist propranolol, which also inhibits differentiation of the amoeba into the cyst form (27). A differentiation response similar to that obtained with these bioactive amines is also seen after the addition of dibutyryl cyclic AMP to the cultures (28), indicating the involvement of adenyl cyclase in the regulation of Hartmannella differentiation. These results imply the presence of a β-AR on the Hartmannella surface, but the characterization of such a receptor, and the source of its ligands, have not been reported.

Here we show that the enteric amoeba, E. invadens, also encysts in response to the binding of catecholamines. Trophozoite stages of both E. invadens and E. histolytica contain dopamine, norepinephrine, and epinephrine, and E. invadens trophozoites release epinephrine upon transfer to encystation medium that contains a ligand for the amoeba surface galactose lectin. E. invadens binds catecholamines via a β₁-adrenergic receptor on the trophozoite surface, and this catecholamine ligand-receptor system acts downstream of the galactose lectin and upstream of adenyl cyclase.

EXPERIMENTAL PROCEDURES

Materials—[3H]-CGP 12177 was obtained from PerkinElmer Life Sciences. Waters AccQ-Fluor reagent kit was obtained from Millipore (Milford, MA). Whatman GF/B glass fiber filters were obtained from Macherey-Nagel (Duren, Germany). Waters AccQ-Fluor reagent kit was obtained from Millipore (Milford, MA). Whatman GF/B glass fiber filters were obtained from Macherey-Nagel (Duren, Germany).

Preparation of Encystation Culture Lysates and Encystation Media for HPLC Analysis—For lysate preparations, 1 × 10⁵ Entamoeba trophozoites were harvested from 50-ml cultures in BYI-S-33, washed with ice-cold amoeba wash buffer (AWB, 65 mM NaCl, 75 mM Tris-HCl, pH 7.2) (30), and resuspended with 100 μl of borate buffer (0.2 M sodium borate, pH 8.5) containing 1 mM EDTA, pH 8.0. The tubes were held at 100 °C for 5 min and then centrifuged at 15,000 × g for 15 min at room temperature to remove protein. The supernatant was stored at −20 °C until analysis.

For analysis of medium from high and low cell density cultures, E. invadens trophozoites were harvested from 50-ml cultures in BYI-S-33 and washed with ice-cold AWB. The amoebae were resuspended in BYI-S-33 (without serum) to yield final concentrations of 3 × 10⁴ trophozoites/ml (low density) or 3 × 10⁵ trophozoites/ml (high density). After a 24-h incubation at 25 °C, the tubes were chilled in ice/water for 10 min, and the amoebae were pelleted by centrifugation. The medium was heated at 100 °C for 5 min, centrifuged at 15,000 × g for 15 min at room temperature to remove protein, and the supernatant was stored at −20 °C until analysis.

Forty-five microliters of trophozoite lysates or media were mixed with 75 μl of borate buffer by vortexing. Twenty microliter of the AccQ-Fluor reagent (N-hydroxy-2-aminobenzoyl-6-aminquinolyl carbamate) was then added to give a final volume of 100 μl, and the sample was vortexed vigorously. Samples were spiked with 10 μl of 1 mM aqueous solutions of (-)-epinephrine, (-)-norepinephrine, or (±)-dopamine to verify the presence of the three different catecholamine peaks. These samples were prepared as above with the exception of the addition of 65 μl of borate buffer. An internal standard, 2 μl of 1 mM 1,7-diminoheptane, was included in every injection. All derivatized samples were analyzed within 1 h of preparation, and each sample was analyzed in triplicate.

High Performance Liquid Chromatography Analysis—The Waters HPLC used for analysis of trophozoite lysates included a quaternary pump (model 625), a system controller (model 600E), an autosampler (model 715), and a fluorescence detector (model 470). Waters Millennium software was used to control the system, collect data, and calculate peak areas. A 5-μm silica particle C₈ Symmetry column (3.9 × 150 mm, inner diameter) with a 100-A pore size (Waters) was used for separation. The elution buffer consisted of 0.14 M sodium acetate, 0.017 M triethylamine-HCl, pH 5.05 (eluent A). Fluorescence excitation was at 250 nm, and emission was detected at 395 nm. Elution was at 1.0 ml/min. The mobile phase gradient was as previously described for polyamine analysis (31), except the last step was a linear gradient starting at 83% eluent A, 17% acetonitrile extending to 60% eluent A, 40% acetonitrile over 20 min.

The suitability of the analytical system was assayed by demonstrating that (+)-epinephrine, (-)-norepinephrine, and (-)-dopamine, and an internal standard were resolved from each other and from all other potentially interfering peaks in the biological sample. To demonstrate linearity, a series of standard aqueous solutions for each of the three catecholamines (±)-epinephrine, (−)-norepinephrine, and (±)-dopamine) ranging from 1.25 to 50 nmol were used to construct calibration curves. Linear curves with r values greater than 0.99 (r > 0.99) were obtained for each catecholamine standard. The internal standard, 2 μl of 1 mM 1,7-diminoheptane, was included in every injection. Each sample was analyzed in triplicate.

[3H]-CGP 12177 Binding to E. invadens and E. histolytica—E. invadens and E. histolytica trophozoites were harvested from 50-ml cultures in BYI-S-33 by centrifugation and washed with ice-cold AWB. Trophozoites were resuspended at 3 × 10⁶/ml in either BYI medium or 100% LG encystation medium containing either 5% ABS or 1 μg/ml ASF. After a 15-min incubation at room temperature, the tubes were placed on ice. [3H]-CGP 12177 was added to a final concentration of 40 μM in the presence or absence of 500 μM (±)-metoprolol and incubated on ice for 30 min. (Previous time-course experiments had indicated that equilibrium under these conditions is reached after 15 min.) The cells were bound to Whatman GF/B glass fiber filters, washed twice with 3 ml of ice-cold AWB, and dried overnight. Filters were subsequently counted in 3 ml of Betaplate Scint in an LKB model 1214 scintillation counter.
FIG. 1. Ability of catecholamines to support E. invadens encystation. Shown is the percentage of encystation obtained in encystation medium lacking 5% serum but containing the indicated amounts of (±)-epinephrine, (±)-norepinephrine, or (-)-norepinephrine. The solid and dotted arrows indicate the levels of encystation obtained with encystation medium containing and lacking 5% serum, respectively.

For saturation binding experiments, E. invadens and E. histolytica trophozoites were harvested from cultures grown in BYI-S-33 as described above and induced to encyst in 100% LG encystation medium containing 1 μg/ml ASF or 5% ABS for 15 min at room temperature. Trophozoites were chilled on ice, harvested, and resuspended in fresh medium containing various concentrations of [3H](-)-CGP 12177 and incubated on ice for 30 min. The cells were bound to glass fiber filters, washed, dried, and counted as described above. For competition binding experiments, E. invadens trophozoites were harvested from cultures grown in BYI-S-33 as described above, and induced to encyst in 47% LG medium containing 1 μg/ml ASF for 15 min at room temperature. Trophozoites were chilled on ice, harvested, and resuspended in fresh medium. [3H](-)-CGP 12177 was added to a final concentration of 8 pM in the presence or absence of 500 μM (±)-alprenolol, (±)-propranolol, (±)-metoprolol, or (±)-timolol, and incubation was continued on ice for 30 min. The cells were processed over glass fiber filters as above.

RESULTS
Exogenous Catecholamines Stimulate Encystation of Entamoeba—We had previously found that efficient (∼90%) in vitro encystment of E. invadens required the presence in the cyst induction medium of ligands for a cell surface galactose lectin (9), and that much lower levels (10–20%) of cyst formation occurred in their absence. To determine whether catecholamines were recognized by E. invadens and acted at a step subsequent to the required lectin–ligand interactions, catecholamines were added to cyst induction medium that was made without the bovine serum source of the galactose ligand(s). (+)-Epinephrine functionally substituted for serum and induced the same high levels of cyst formation normally obtained with complete encystation medium (Fig. 1). Titration of (+)-epinephrine in the serum-free encystation medium gave a biphasic curve with levels of maximum encystation at 5 μM and 5 mM. (-)-Epinephrine was effective at slightly lower concentrations (100–500 nM) (Fig. 1), reflecting the higher stimulatory activity of the purer (minus)-isomer of this compound. (-)-Norepinephrine supported maximal encystation at 1 μM and 1 mM (Fig. 1). Background levels of encystation were obtained with lower micromolar amounts of this compound, whereas higher millimolar amounts were amebicidal (data not shown). Addition of (+)-dopamine or 3-(3,4-dihydroxyphenyl)alanine (l-DOPA) to the serum-free induction medium was either toxic to the amoebae or yielded ~50% levels of encystation through a concentration range of 100 nM to 1 mM (data not shown).

To obtain maximal levels of encystment in vitro, the density of E. invadens trophozoites in encystation medium must be between 2 × 10⁵/ml and 5 × 10⁵/ml (8). To determine whether exogenous epinephrine could overcome this trophozoite density requirement, cultures at optimal (3 × 10⁵/ml) and suboptimal (3 × 10⁴/ml) cell densities of trophozoites containing and lacking 5% serum, respectively. The solid and dotted arrows indicate the levels of encystation obtained with optimal (3 × 10⁵/ml) and 5 mM (±)-epinephrine. A concentration of 5 μM (±)-epinephrine supported maximal levels of differentiation of only the high density culture, whereas 5 mM (±)-epinephrine induced maximal or near maximal encystment of both high and low density cultures (Fig. 2).

Encystation Is Selectively Induced with β-Adrenergic Receptor Agonists—Epinephrine and norepinephrine are ligands for both α- and β-ARs (32). To start to define the specificity of the presumed E. invadens AR, we tested adrenergic agonists that exhibit known α- and β-receptor specificities. At concentrations from 10 nM to 100 μM, the α-AR agonist l-phenylephrine did not stimulate levels of encystation much above background (Fig. 3), and higher amounts were amebicidal. In contrast, the β-AR agonist isoproterenol supported maximal levels of encystation at a concentration of 100 nM. The encystation levels obtained with this compound were similar to those obtained with induction medium containing either serum or 5 μM exogenous epinephrine. Approximately 50-fold less isoproterenol than epinephrine was required to elicit maximal encystation, consistent with the known increased potency of the synthetic compound in other adrenergic receptor systems (24). To demonstrate the presence in the ameba of a particular subtype of β-AR, we tested the β₁-specific agonist dobutamine. Dobutamine induced greater than 90% encystation at a concentration of 1 μM (Fig. 3).

Encystation Is Selectively Inhibited by β₂-Antagonists—The selectivity of the putative AR involved in ameba encystation was further addressed by testing the ability of AR subtype-
selective antagonists to block encystation that was induced by addition of either serum or exogenous catecholamines. The \(\alpha\)-AR antagonist phentolamine had little inhibitory effect on encystation in either type of medium even at 500 \(\mu\)M (Table I). In contrast, the \(\beta\)-AR antagonists alprenolol and propranolol substantially inhibited encystation in both types of medium.

Encystation levels were less than 50% of control when these antagonists were added at 25 to 100-fold molar excess over the levels of encystation that occurred in the presence of timolol or metoprolol, which provided only a carbon source deprivation stimulus, and resulted in the highest level of CGP 12177 binding, yielding a \(K_d\) of 2.35 \(\pm\) 0.59 pm, and a \(B_{max}\) value of 98.93 \(\pm\) 6.6 fmol/mg protein (Fig. 5).

\([\text{H}]\text{CGP}\) also bound in a saturable manner to live \(E.\ histolytica\) trophozoites transferred for 15 min to encystation medium, which provided only a carbon source deprivation stimulus (100% LG), bound a greater amount of \([\text{H}]\text{CGP}\) than did trophozoites that were transferred 15 min to encystation medium containing serum (BYI/ABS) bound less \([\text{H}]\text{CGP}\) than trophozoites in the same medium containing ASF in place of serum (BYI/ASF). Metoprolol (500 \(\mu\)M) inhibited \(\geq 70\%\) of the \([\text{H}]\text{CGP}\) binding to amebae in BYI-based media. Trophozoites transferred to encystation medium containing serum (100% LG/ABS) for 15 min bound \(\sim 30\%\) more \([\text{H}]\text{CGP}\) as cells in BYI/ABS, and an additional increase in binding was observed with cells transferred to 100% LG containing ASF. As before, \(\geq 75\%\) of the binding to the cells in the encystation medium was inhibited by 500 \(\mu\)M metoprolol. Saturation binding analysis of \([\text{H}]\text{CGP}\) to trophozoites exposed to the medium (100% LG/ASF) that most closely mimicked encystation induction conditions, and resulted in the highest level of CGP 12177 binding, yielded a \(K_d\) of 2.74 \(\pm\) 0.76 pm, and a \(B_{max}\) value of 82.48 \(\pm\) 6.4 fmol/mg protein (Fig. 5).

If regulation of encystation is indeed mediated by catecholamine ligands and receptors, then the relative effectiveness of \(\beta\)-antagonists to block binding of a defined ligand to the trophozoite surface (their \(K_v\) values) should correlate with their relative abilities to inhibit trophozoite encystation. Four antagonists, metoprolol, timolol, alprenolol, and propranolol, were tested for both their ability to inhibit \([\text{H}]\text{CGP}\) binding to trophozoites and to inhibit encystation. Both alprenolol and propranolol had similar \(K_v\) values (1.67 and 2.85 nm, respectively) and permitted encystation levels of 60–45% when present at 125–500 \(\mu\)M concentrations in encystation medium (Fig. 6). The \(K_v\) values of metoprolol and timolol were 10–18 times lower (0.096 and 0.137 nm), and these compounds permitted only 20–10% encystation when present at 125–500 \(\mu\)M. The two antagonists with the lower \(K_v\) values, then, were more effective at inhibiting encystation than the two compounds with the higher \(K_v\) values. However, concentrations of antagonists that were required to inhibit encystation were 100–1000 times greater than their \(K_v\) values toward \([\text{H}]\text{CGP}\). The same was

**Table I**

| Antagonist | Percentage of encystation |
|-----------|---------------------------|
| None      | 47% LG with serum          |
| Phenolamine (\(\alpha\)) | 8.2% ± 0.82 |
| Alprenolol (\(\beta\)) | 95.8 ± 0.30 |
| metoprolol (\(\beta\)) | 9.5 ± 2.70 |
| Metoprolol (\(\beta\)) | 12.2 ± 1.25 |
| ICI-118,551 (\(\beta_2\)) | 84.6 ± 1.45 |
| Propranolol (\(\beta\)) | 100% LG containing ASF |

**Fig. 3.** Effect of \(\alpha\)- and \(\beta\)-AR agonists on \(E.\ invadens\) encystation. Shown is the percentage of encystation obtained when the indicated amounts of agonists L-phenylephrine (\(\alpha\)-type), (-)-isoproterenol (\(\beta\)-type), or dobutamine (\(\beta_\text{AR}\)-type) were substituted for serum. The solid and dotted arrows indicate the levels of encystation obtained with encystation medium containing and lacking 5% serum, respectively.
true of CGP 12177 itself. Its $K_d$ toward *E. invadens* trophozoites, as mentioned above, was 2.35 pM, whereas its EC$_{50}$ in encystation medium was 2.45 mM. This is roughly 5 times the 0.5 mM level at which we could not detect catecholamines in medium containing 1 mg/ml of epinephrine, as compared with 5% ABS. We used these levels in further studies to determine the concentration of released epinephrine. The amount of released epinephrine was dependent on the concentration of ASF added to the medium. The greatest amount of epinephrine was detected in the medium containing 1 mg/ml of epinephrine, which was roughly 5 times the 0.5 mM amount of exogenously added (-)-epinephrine that was required to stimulate encystment of trophozoites in medium that did not contain a galactose ligand (Fig. 1).

**DISCUSSION**

*Entamoeba* parasites overcome the inefficiency of the oral-fecal transmission route by generating and releasing large numbers of cysts from infected carriers. Because each trophozoite gives rise to a single cyst, the release of large numbers of cysts requires that trophozoites themselves attain a high density in the colon. Perhaps reflecting this *in vivo* requirement, efficient encystation *in vitro* only occurs when a high cell density is exposed to encystation medium. This density require-
ment could be because of a need for a certain level of physical contact between the amebae and/or for the presence of critical levels of metabolites made by the trophozoites. When *E. invadens* was incubated with (H11006)-epinephrine, (H11002)-epinephrine, or (H11002)-norepinephrine, the trophozoites differentiated to the cyst forms. Two concentrations of (H11006)-epinephrine, 5\(\mu\)M and 5mM, yielded high levels of encystation when added in place of a ligand for the galactose lectin to cultures of optimal cell density (3 \(\times\) 10^7/ml). The cell density dependence of encystation appeared to be overcome by the addition of 5mM (H11006)-epinephrine, but not by 5 \(\mu\)M, suggesting that epinephrine levels may be limiting in the low-density cultures. However, it is questionable whether the response to the millimolar amounts of epinephrine or norepinephrine are physiologically relevant. The trophozoites do not appear capable of generating millimolar amounts of epinephrine, and the ameba \(\beta_1\)-AR appears to have a high affinity for catecholamines, suggesting that the differentiation response of low density cultures to high amounts of epinephrine reflects a receptor-independent mechanism. Interestingly, even when high levels of exogenous epinephrine were added, there still appeared to be a requirement for cells to be part of an aggregate to differentiate, as individual *E. invadens* trophozoites in close proximity to the aggregates did not encyst (data not shown). Consistent with this, those amebae that did

**FIG. 5.** Saturation binding of [3H]-(H11546)-CGP 12177 to live trophozoites at 4\(^\circ\)C. Trophozoites of *E. invadens* or *E. histolytica* were exposed for 15 min to 100% LG containing 1 \(\mu\)g/ml ASF and then examined for specific binding of radiolabeled \(\beta\)-antagonist CGP 12177.

**FIG. 6.** Competitive binding analysis of [3H]-(H11546)-CGP 12177 and four antagonists to *E. invadens* trophozoites. The indicated amounts of four antagonists, (H11001)-alprenolol, (H11001)-propranolol, (H11001)-metoprolol, and (H11002)-timolol, were used to compete with radiolabeled CGP 12177 for binding to live *E. invadens* trophozoites at 4\(^\circ\)C. Inset, the indicated amounts of the same four antagonists were used to inhibit *E. invadens* encystation, which was carried out at room temperature.
encyst in the low density cultures were invariably found in such aggregates. In contrast, Hartmannella trophozoites can encyst as individual cells. Why one ameba type needs to aggregate while the other does not remains unknown, but it is possible that the resulting unique mechanisms of triggering cAMP release or regulating the activation states of the cAMP-dependent protein kinases are different for the two cell types.

Encystation of *E. invadens* was stimulated by addition of the β-AR agonist isoproterenol but not the α-AR agonist phenylephrine, implying the involvement of a catecholamine receptor of the β-type (according to the vertebrate classification system). The putative *E. invadens* receptor was further characterized as a β1-subtype because of the ability of the β1-AR agonist dobutamine to stimulate encystation. This classification was consistent with results obtained using α- and β-AR antagonists, wherein the α-AR antagonist phentolamine did not compete with the stimulatory concentration of exogenously added epinephrine to prevent *E. invadens* encystation. β1-AR antagonists, however, were effective inhibitors when either normal (serum-containing) encystation medium was used or when exogenous epinephrine was used in place of serum. In these experiments, the *E. invadens* trophozoites still formed multicellular aggregates, but most cells within the aggregates did not differentiate. Significant also was the ability of the β1-AR antagonists to reduce *Entamoeba* differentiation to levels below the “background” level obtained when serum or galactose ligand was left out of the encystation medium, indicating that the background encystation was also occurring in a catecholamine-dependent manner and that binding of a ligand by a β1-type AR is therefore required for (in vitro) *Entamoeba* encystation.

The β1-AR antagonist metoprolol inhibited 74% of the encystation that was stimulated by mucin, suggesting that catecholamine binding by the trophozoite occurs after binding of galactose ligand by the lectin. Metoprolol had no inhibitory effect when encystation was induced with db-cAMP. β-ARs are coupled to adenylyl cyclase through G-proteins (34, 35), and adding db-cAMP would serve to artificially increase intracellular cAMP levels while bypassing the normal AR-dependent step of the process. It appears, then, that the presumptive ameba AR occupies a functional position downstream of the galactose lectin and upstream of adenylyl cyclase.

The ability of epinephrine and norepinephrine to trigger differentiation of both *E. invadens* and *H. vermiformis* implies the existence of ARs on the respective trophozoite surfaces. Similar evidence for such receptors in other protozoans, including *Dictyostelium discoideum* (36) and *Trypanosoma cruzi* (37, 38), have been reported, but the proteins have not been characterized. The radiolabeled antagonist [3H](-)-CGP 12177 bound to live trophozoites of both *E. invadens* and *E. histolytica*. Binding to both was inhibited by the β1-AR antagonists metoprolol and timolol, but not by the β2-type antagonist ICI 118,551 (data not shown). Binding of CGP 12177 was increased when the parasites were incubated in encystation induction medium (LG) instead of trophozoite culture medium (BYI). Whether this increased binding represented increased surface deposition of receptors from an internal pool, the functional unmasking of receptors already present on the trophozoite surface, or an effect of the medium composition on receptor

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**Table III**

Catecholamine content of Entamoeba trophozoites

| Catecholamine | *E. invadens* IP-1 | *E. histolytica* HM1 |
|---------------|-------------------|---------------------|
|               | nmol/10⁵ cells    | nmol/10⁵ cells       |
| Dopamine      | 5.17 ± 4.56       | 0.064 ± 0.002       |
| Norepinephrine| 10.79 ± 3.51      | 0.48 ± 0.015        |
| Epinephrine   | 1.13 ± 0.31       | 3.28 ± 0.12         |

**Table IV**

Epinephrine release during encystation of *E. invadens*

| ASF (in 47% LG) | Encystation | Epinephrine released into medium |
|-----------------|-------------|---------------------------------|
|                 | %           | nmol/10⁵ cells                   |
| 100 μg/ml       | 30          | 0.24 ± 0.07                      |
| 0.1 μg/ml       | 50          | 0.43 ± 0.05                      |
| 1 μg/ml         | 95          | 0.69 ± 0.12                      |

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**FIG. 7.** AccQ-Fluor/HPLC analysis of *E. histolytica* trophozoite lysate. The lower panel shows the unique mechanisms of triggering catecholamine release or regulating the activation states of the cAMP-dependent protein kinases are different for the two cell types.
function is not yet known. Preliminary experiments with cycloheximide-treated cells indicated that new protein synthesis is not required for the effect.² Why metoprolol, or other potent antagonists, did not inhibit all of the ameba differentiation that was induced by galactose ligands is not clear. One simple possibility is that increased aggregation of the amebae induced by the ligands served to exclude the antagonist from the inner regions of the aggregates. This same reason may partially account for the large difference in the amounts of antagonists needed to inhibit ³H]CGP 12177 binding to live cells versus the amounts of these antagonists that were required to inhibit encystation. The CGP 12177 binding experiments were performed with live cells that were maintained at 4 °C, which minimizes the formation of trophozoite aggregates, and which should also prevent release of the intracellular stores of catecholamines. Antagonists added to cultures encysting at room temperature, on the other hand, would need to gain access to receptors that are on the surface of cells within the multicellular aggregates. Approximately 61% of the epinephrine contained in trophozoites was released within the first 2 h following transfer of the cells to an optimal encystation induction medium, and the amount released was estimated to be more than sufficient to stimulate differentiation. The antagonist, then, would have to gain access to receptors while also competing with stimulatory levels of an endogenous agonist.

Epinephrine does not appear to be used by E. invadens as a constitutively operating quorum sensing molecule, as it was not released from vegetatively growing trophozoites, even when the cultures attained high cell densities. Epinephrine was, however, released from cells transferred to encystation medium that contained a galactose ligand. Entamoeba appears to use epinephrine, therefore, in a fashion similar to that used by chromaffin and nerve cells of higher eukaryotes, as an induced release signaling molecule. The amount of epinephrine that was released by trophozoites directly correlated with the levels of encystation that were induced by the different amounts of ASF added as galactose ligand. It remains to be shown, however, that engagement and/or clustering of the surface lectin by galactose ligands directly induces epinephrine release. Additionally, all the results described here were obtained using cultures grown in undefined medium, so at this point it is not known if the amebae synthesize the cat echolamines they contain de novo or take them up from the trophozoite growth medium either partially or completely pre-formed.

How the amebae use the binding of epinephrine to induce metabolic changes that lead to encystment can now be examined. Similar to epinephrine-responsive liver and muscle cells of higher organisms, Entamoeba stores large amounts of glucose as glycogen. These reserves in amebae are substantially decreased during encystation (2), presumably to provide metabolic energy for differentiation and to supply the amino sugar synthesis pathway with substrate for the synthesis of UDP-GlcNAc, which is ultimately polymerized into chitin found in the cyst wall. Whole E. invadens cells, as well as plasma membrane preparations, show increased adenylyl cyclase activity when exposed to epinephrine.³ If they are responsive to cAMP levels as in higher eukaryotes, the relative activities of glycogen synthase and glycogen phosphorylase, then, would be expected to change upon epinephrine binding by the amebae, which is readily testable.

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² A. Coppi, unpublished data.

³ J. Frederick and D. Eichinger, unpublished data.
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