Catecholamines in *Entamoebae*: recent (re)discoveries

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Free-living and enteric amoebae have similar two-stage life cycles, and both organisms depend on being able to monitor environmental conditions to determine whether to continue multiplying as trophozoites, or to differentiate into dormant or transmissible cysts. Conditions that support high trophozoite densities might also be expected to select for mechanisms of information exchange between these cells. We recently determined that trophozoites of at least one species of *Entamoeba* release and respond to catecholamine compounds during differentiation from the trophozoite stage into the cyst stage. It turns out that this is not an entirely novel finding, as a number of previous studies have demonstrated parts of this story in free-living or enteric amoebae. We briefly review here major points of the previous studies and describe some of our recent results that have extended them.

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1. Introduction

Three amino acids serve as precursors to a number of compounds that mediate a large variety of functions in multicellular organisms. Tryptophan, histidine, and tyrosine are converted into serotonin, histamine, and several catecholamines, respectively, by short biochemical pathways present in many types of mammalian tissues and cells. These compounds are typically referred to as neurotransmitters because of their essential functions in signalling between nerve cells or between nerve and muscle cells, and in thereby regulating brain function, sympathetic and parasympathetic muscle tonicity, circulating glucose levels, etc. Not long after the chemical structures of the active forms of the compounds found in mammals were determined, they were also shown to be present in single-celled protozoans, revealing the ancient origin of both the synthetic pathways that produce them as well as their roles as intercellular (and in the case of protozoans, interorganismal) signalling molecules.

The catecholamines are derived from tyrosine in the sequence: tyrosine → DOPA → dopamine → norepinephrine → epinephrine. To allow for controlled synthesis, to protect the synthesizing cell from reactive side products, and to permit their selective and rapid release, the compounds are in several steps made, and in all cases stored, within cytoplasmic vesicles. Uptake of these compounds into the vesicles can be inhibited by several compounds (e.g. reserpine) that interfere with the vesicular monoamine transporters that concentrate the compounds out of the cytoplasm. Catecholamines released from the synthesizing cell bind to a subclass of seven-transmembrane domain, G-protein-coupled receptors (GPCRs). These receptors convey the extracellular binding of ligand to intracellular secondary effector molecules such as adenylyl cyclase and phospholipase C. The most evolutionarily ancient of the biogenic amine receptors are those recognizing serotonin, with subsequent branches off of this primordial GPCR lineage acquiring specificity for catecholamines and histamine ligands. It should not

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Abbreviations used: GPCRs, G-protein-coupled receptors; PPO, polyphenol oxidase.

be surprising, therefore, that protozoans shown to contain and/or respond to catecholamines would also contain and have receptors for histamine or serotonin.

In addition to signalling molecules, catecholamines also serve other functions. DOPA, dopamine, and modified versions of the two, are used by fungi, insects, and crustaceans as substrates for several oxidases that convert the dihydroxyphenolic catecholamines into reactive quinones. These latter compounds are then used to cross-link and modify molecules in the chitin-containing layers in each organism type, yielding rigid, and in some cases, water-impermeable layers in the cell walls of fungi and the exoskeletons of insects and crustaceans (Brunet 1980).

2. Prevalence of biogenic amines in, and their effects on, various protozoa

Several model protozoan organisms under study in the 1960s were found to respond to exogenous biogenic amines. Blum (1967) described an intracellular adrenergic metabolic control system in *Tetrahymena* that regulated stores of glycogen. He subsequently found that growth of *Crithidia* and *Tetrahymena* were each sensitive to addition of serotonergic and adrenergic drugs. *Crithidia* and *Tetrahymena* had previously been suggested to possess and synthesize norepinephrine and epinephrine, and *Crithidia* was proposed to have a biopterin-dependent tyrosine hydroxylase involved in the first step of the catecholamine synthetic pathway (Janakidevi *et al* 1966). A suggestion that *Tetrahymena* itself contained catecholamine compounds was revealed when cell growth and glycogen levels were shown to be sensitive to the vesicular monoamine transporter inhibitor reserpine (Blum 1970). Both *Crithidia* and *Tetrahymena* were demonstrated to contain norepinephrine, epinephrine, and serotonin (Blum 1969). For a more recent review of serotonin in *Tetrahymena* see Csaba (1993). The disease-causing kinetoplastid *Trypanosoma cruzi* was also reported to be responsive to catecholamines. DeCastro and Oliviera (1987) showed that the adrenergic agonist isoproterenol caused an increased level of intracellular cAMP in *T. cruzi* epimastigotes. These authors also used ligand saturation analysis, together with the stereospecificity and reversibility of ligand binding, to demonstrate the presence of *b*-type catecholamine cell surface receptors. *T. cruzi* epimastigotes could be induced to differentiate into metacyclic trypomastigotes by increased cAMP levels that resulted from addition of epinephrine or cholera toxin (Gonzalez-Perdomo *et al* 1988). Functional desensitization of both *a*- and *b*-type cell surface catecholamine receptors occurred upon prolonged exposure of *T. cruzi* to catecholamines (Ayala and Kierszenbaum 1990).

In a series of reports by Murti *et al* during the 1970s the free-living amoeba *Hartmannella* was shown to respond to catecholamines. During encystment, *Hartmannella* trophozoites decrease internal glycogen stores, increase RNA and protein content, and synthesize cyst coat walls containing cellulose and mucopolysaccharides (Raizada and Murti 1972a). All these changes, and complete differentiation itself, could be induced by increasing internal (cAMP) through addition of exogenous cAMP or by inhibition of phosphodiesterase activity with theophyline (Raizada and Murti 1972b). The differentiation process could be inhibited by addition of free glucose, suggesting a catabolic repression mechanism used by the amoeba to monitor growth conditions that was also sensitive to cAMP levels (Verma and Raizada 1975). A review of the understanding of the molecular aspects of *Hartmannella* encystment was published by Murti in 1975, in which he described changes in cellulose synthetase activity and glycogen content when *Hartmannella* trophozoites were transferred to taurine, epinephrine, or db-cAMP-containing media. Also mentioned in the review was the lack of a response of axenically grown *Entamoeba histolytica* trophozoites to taurine or epinephrine-containing media, and the apparent inability of *E. histolytica* trophozoites to bind biogenic amines. *Hartmannella* was then shown to bind epinephrine during encystment via *b*-type receptors (Verma and Murti 1976). Further attempts to translate the *Hartmannella* differentiation system to *E. histolytica* were reported two years later (Mitra and Murti 1978). Now, axenic *E. histolytica* trophozoites, if starch-fed and treated with either *Escherichia coli* or *Vibrio cholera* toxins, or with epinephrine, were found to round up, deposit microscopically visible walls around the cells, increase amylase activity, and decrease internal glycogen content. These apparently differentiating cells did not, however, yield tetranucleate, acid-resistant cells resembling mature infectious cysts. The *Hartmannella* encystation system, in particular the use of epinephrine or increased cAMP to induce encystment, did effectively transfer to another free-living amoeba, *Acanthamoeba* (Srivastava and Shukla 1983). A hint that this organism may use exogenous catecholamines not just as ligands for surface receptors but also as substrates for secreted enzymes was revealed when Sykes and Band (1985) purified and characterized a polyphenol oxidase (PPO) activity made and secreted exclusively by encysting trophozoites. The function of the polyphenol oxidase and the source of its substrates were not established, but inclusion of PPO inhibitors in the encystment medium did lower the yield of cysts, suggesting the need for reaction products of the *Acanthamoeba* laccase-type oxidase for assembly of normal cysts.

3. Current understanding and hypotheses of encystation in *Entamoeba*

Most of the recent information about encystation of *Entamoeba* parasites is derived from studies using the reptile parasite *E. invadens* (Eichinger 2001a,b), which produces cysts with similar features, including a chitin-containing wall, to those of *E. histolytica*. *In vitro* encystment of *E. invadens* is triggered by removal of the glucose carbon source (Vazquezdelara-Cisneros and Arroyo-Begovich 1984), dilution of growth medium (Avron *et al* 1986), or a combination of both stimuli (Sanchez *et al* 1994). Efficient encystment is also cell-density dependent, and takes place only within aggregates of trophozoites that form when high-density cultures are transferred to encystment media. Trophozoites that remain apart from the aggregates, even though exposed to the same encystment medium, do not differentiate, implying a need for cell : cell contact or close apposition of trophozoite plasma membranes to start the differentiation process. The cell : cell interactions that mediate aggregate formation involve interactions between galactose binding lectins on the trophozoite surface and galactose-terminated ligands in the fluid phase, the latter being provided in the standard *in vitro* system by the serum component of the medium (Coppi and Eichinger 1999). This requirement for galactose-terminated ligands can be met using defined gal-terminated molecules, such as mucin, which is the most likely ligand for the surface lectin in the amoeba-infected colon. There is a concentration-dependent effect of the galactose-terminated ligand, indicating that cross-linking or clustering of the lectin on the trophozoite surface serves to transmit a signal intracellularly. These findings suggested a very simple model of regulation of encystation in which changes in the integrity of the surrounding mucus layer were detected by trophozoites via the surface galactose lectin. High local trophozoite density, together with a decreased concentration of mucus containing colonic bacteria – a major nutrient source for the trophozoites – would induce aggregation of trophozoites, clustering of surface lectin, transmission of an intracellular signal, and entry into the encystation pathway.

To determine which downstream transmembrane signalling effector systems may be tied to the lectin, the galactose ligand was omitted and replaced with a variety of known effector molecules, some of which had been used previously by investigators studying *Hartmannella* and *Acanthamoeba* encystment. Forskolin, pertussis toxin (J Frederick, unpublished) and db-cAMP (Coppi *et al* 2002) all bypassed the lectin : ligand requirement and triggered maximal levels of encystment of *E. invadens*, indicating that increased intracellular levels of cAMP were involved. The catecholamines norepinephrine and epinephrine were also found to be capable of substituting for galactose ligand, and when titrated downward, amounts one thousand-fold lower that those used by previous investigators were found to be effective for both *Entamoeba* and *Hartmannella* (Coppi *et al* 2002; A Coppi, unpublished). Importantly, these effective low micromolar concentrations were in the range attainable under expected physiological conditions, and were also suggestive of the presence on trophozoites of adrenergic receptors with strong ligand affinities similar to those of defined receptors on mammalian cells.

Using a series of synthetic adrenergic agonists and antagonists as stimuli, the resulting *Entamoeba* encystation response was found to be consistent with the presence of a receptor with ligand specificities of b_1-type mammalian receptors, whereas a-type ligands were without effect. Classic saturation binding studies using a hydrophilic, non-membrane permeable b-antagonist established the presence of catecholamine binding sites on the surface of both *E. invadens* and *E. histolytica* trophozoites. These presumptive receptors had K_d's for synthetic antagonists in the low picomolar range, and the number of such sites increased when trophozoite stage cells were exposed to encystation medium (Coppi *et al* 2002). Affinity purification of solubilized trophozoite membranes has yielded a protein with b-antagonist binding properties that migrates at approximately 63 kDa on SDS-PAGE, which falls within the 55–70 kDa size range of mammalian receptors (A Coppi, unpublished).

A direct correlation was found between the ability of four antagonists to inhibit *E. invadens* encystation and their K_i's toward the hydrophilic, surface-binding antagonist used in the saturation binding studies, indicating that the same surface receptor was being assessed in each condition. The three separate stimuli that were previously shown to trigger *E. invadens* encystment, gal ligand, cAMP, and catecholamines, were then used either alone or together with a b-antagonist to determine the relative order of functioning of the lectin, adenylyl cyclase, and the catecholamine receptor during initiation of encystation. This experiment showed that the catecholamine receptor in *E. invadens* functioned downstream of the galactose lectin and upstream of adenylyl cyclase (Coppi *et al* 2002). We have subsequently found that membrane preparations from *E. invadens* trophozoites express increased adenylyl cyclase activity when incubated with epinephrine and GTP (J Frederick, unpublished).

The source of the ligands for the *Entamoeba* catecholamine receptors was then examined. Since trophozoite cultures that are to be encysted are normally washed before placement in encystation medium, and since serum could be completely left out of encystation medium, the medium itself was not likely to have contributed catecholamines used during the initiation of encystment. The previous descriptions of catecholamines in other protozoa

suggested that the *Entamoeba* trophozoites themselves might be a source of the compounds, and HLPC analysis of trophozoite lysates showed this to be true. Both *E. histolytica* and *E. invadens* trophozoites contain dopamine, norepinephrine and epinephrine (the only three catecholamines that have been looked for). *E. invadens* trophozoites do not release any of these compounds during vegetative growth, but do release epinephrine, and only epinephrine, following transfer to encystation medium. The amount of epinephrine released during the first two hours in encystment medium correlates with both the amount of galactose ligand added to stimulate encystment and the eventual percent encystation, suggesting that clustering of the galactose lectin could regulate epinephrine release (Coppi *et al* 2002).

For reasons previously mentioned, the possibility existed that catecholamines were not the only bioactive amine that might be present in *Entamoeba* trophozoites and capable of triggering differentiation. In fact, McGowan *et al* (1983) reported that *E. histolytica* contained serotonin, and Nayeem *et al* (1993, 1994) reported that xenic cultures of *E. histolytica* would encyst in response to changes in carbon source (starch) concentrations and exogenously added histamine. We have found that both serotonin and histamine will individually substitute for galactose ligand and induce encystment of *E. invadens*. The differentiation induced by serotonin and histamine is not inhibited by adrenergic antagonists, but is blocked by serotonergic or histaminergic antagonists, suggesting the presence of two other types of bioactive amine ligand/receptor systems in *Entamoeba*. Both *E. invadens* and *E. histolytica* trophozoites contain serotonin and histamine, and both species contain trophozoite surface receptors for the compounds. *E. invadens* encystment can be regulated by serotonin agonists and antagonists with specificity for 5-HT_1-type receptors, and by histamine agonists and antagonists with specificity for H_2-type receptors (A Coppi, S Merali and D Eichinger, unpublished).

While replicating the encystment conditions for *Hartmannella*, we also readily detected release of PPO from encysting trophozoites (as a colour change of the medium that contained epinephrine), the activity of which could be decreased by addition of the phenoloxidase inhibitor tropolone. When added to encysting *E. invadens* cultures, tropolone did not appear to alter the normal timing or efficiency of trophozoite aggregation and encystment. The cysts that were produced, however, were sensitive to water and levels of detergent to which normally produced cysts are highly resistant (A Coppi, unpublished).

4. Model of *Entamoeba* encystation with regard to biogenic amines

These results allow for consideration of a more complex model of regulation of cyst formation, now involving both the galactose lectin as well as GPCRs that are functionally linked to adenylyl cyclase. Trophozoites would still monitor the concentration of surrounding galactose-terminated ligands, predominantly mucin, and form cellular aggregates when levels of ligand drop through a particular concentration range. The resulting clustering of the lectin molecules on the surface of the aggregated trophozoites would serve to stimulate release of epinephrine, which in turn would bind *b*-type catecholamine receptors on adjacent or the same epinephrine-releasing cells and thereby trigger internal increases in cAMP.

This model, however, is based on results obtained with an axenic *in vitro* system, and leaves several questions unanswered, the most obvious of which are: Is this response to catecholamines also seen when *E. invadens* encysts *in vivo*? Or is what we are monitoring an artifact of the culture conditions which allows for any stimuli (including serotonin and histamine), that cause an increased cAMP level to tip the balance and stimulate differentiation of the IP-1 strain of *E. invadens*, which may be aberrantly close to tipping as a result of the axenic *in vitro* culture conditions? In support of a normal role for catecholamines during encystation is the finding that epinephrine is not released constitutively during trophozoite growth but rather only during the encystment process, as well as the presence of surface receptors tied to regulation of cAMP levels that bind catecholamines with high affinity. Also consistent with this model are preliminary results of encystation experiments using a modified axenic culture medium. In these ongoing studies, vegetative growth medium made with short chain fatty acids instead of glucose allows for normal rates of *E. invadens* trophozoite multiplication until the cell density is high enough to support formation of cellular aggregates. Cyst formation then occurs "spontaneously" within the aggregates. The encystment, but not the aggregation, is inhibited by inclusion of the *b*-antagonist metoprolol in the medium (A Coppi and D Eichinger, unpublished). Encystment (of at least *E. invadens*) therefore does not require the transfer of trophozoites to a hypo-osmotic medium or the acute removal of a carbon source, which are supplied by the normal encystment media, but does require binding of catecholamine agonists. Intriguing also is the possibility that catecholamine products made further up in that synthetic pathway, such as DOPA or dopamine, may be used to modify the chitinaceous layer of the cyst wall and contribute to the hardiness of cysts traveling between hosts. Further studies on their release and receptors are also needed to determine how serotonin and histamine may contribute to the model of regulation of the encystment process, and how divergent the evolutionarily "ancient" amoebic versions of these GPCRs are from their mammalian counterparts. It might be interesting also to consider whether the use of these bioactive compounds

by *Entamoeba* trophozoites may account for any of the symptoms the host displays during *Entamoeba* infection, or whether receptors on the trophozoite allows the parasite to monitor and respond to ligands that are made by the host or the colonic bacteria.

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