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ABSTRACT

The aim of this study was to identify and characterize the receptor system involved in controlling encystment in Acanthamoeba using specific agonists and antagonists and to examine whether endogenous stores of catecholamines are produced by the organism. Acanthamoeba trophozoites suspended in axenic growth medium were exposed to adrenoceptor agonists and antagonists to determine which compounds promoted or prevented encystment. Second, trophozoites were cultured in medium containing a catecholamine synthesis inhibitor to investigate the effect this had on natural encystment. Nonspecific adrenoceptor agonists including epinephrine, isoprotenerol, and the selective β1 adrenoceptor agonist dobutamine were found to cause >90% encystment of Acanthamoeba trophozoites compared to <30% with the controls. The selective β1 antagonist metoprolol was able to inhibit epinephrine mediated encystment by >55%. Cultures of Acanthamoeba with the catecholamine synthesis inhibitor α-methyl-p-tyrosine significantly reduced the level of amoebic encystment compared to controls. In conclusion, Acanthamoeba appear to contain a functional adrenergic receptor system of unknown structure which is involved in initiating the encystment process that can be activated and blocked by β1 agonists and antagonists respectively. Furthermore, the presence of this receptor system in Acanthamoeba indicates that topical β adrenoceptor blockers may be effective adjunct therapy by reducing the transformation of trophozoites into the highly resistant cyst stage.

ACANTHAMOEBA is a common environmental free-living amoeba characterized by a life cycle of a feeding and replicating trophozoite which, in response to adversity, can transform into a highly resistant cyst stage (Byers et al. 1980; Cordingley et al. 1996). Acanthamoeba spp. are opportunistic pathogens of humans, notably causing keratitis in previously healthy persons. Contact lens wearers are most at risk from Acanthamoeba keratitis and account for some 90% of reported cases (Radford et al. 1998; Tu 2014).

Acanthamoeba keratitis is one of the most difficult ocular infections to manage successfully and can result in permanent blindness. Current medical therapy involves topical administration of a biguanide (either polyhexamethylene biguanide or chlorhexidine digluconate) in combination with a diamidine compound (propamidine isethionate or hexamidine diisethionate) (Lorenzo-Morales et al. 2015). Even with prompt diagnosis and aggressive medical therapy, the mean treatment period can be over 5 mo and in approximately 5% of cases the condition fails to respond to treatment (Dart et al. 2009). Many cases require a penetrating keratoplasty to either control the infection or resolve the damage caused by the organism and inflammatory response (Dart et al. 2009). The presence of dormant cysts in the corneal tissue can lead to recrudescence upon cessation of drug treatment causing infection of the corneal graft in up to 41% of patients after penetrating keratoplasty (Kitzmann et al. 2009). A variety of chemical mediators including antibiotics, ethidium bromide, cyclic adenosine monophosphate (cAMP), taurine, and epinephrine have previously been shown to induce encystment (Akins and Byers 1980;
Raizada and Krishna Murti 1972; Srivastava and Shukla 1983b). In many eukaryotic cells cAMP-dependent signalling pathways play a critical role in regulating cell growth, metabolism, and differentiation and the levels of cAMP have been observed to increase in encysting Acanthamoeba (Abel et al. 2001; Raizada and Krishna Murti 1972). In mammalian systems, one mechanism for elevating intracellular levels of cAMP is through the action of the endogenous catecholamine epinephrine. In the liver this results in increased glycogen phosphorylase activity and the subsequent breakdown of the liver’s glycogen stores (Riley and Haynes 1963). During encystment, glycogen metabolism is one of the key processes that has been observed in Acanthamoeba and it has been noted that the activation of adenylate cyclase, leading to polymer degradation, resembles the behaviour of the mammalian liver or muscle in its response to epinephrine (Verma and Murti 1976b; Weisman et al. 1970).

Furthermore, epinephrine and the synthetic catecholamine isoprotenerol have been shown to directly induce encystment in trophozoites of A. culbertsoni in the presence of magnesium (Srivastava and Shukla 1983b). Radio-labelled epinephrine has also been shown to be taken up by Acanthamoeba in a process blocked by the β adrenoceptor antagonist propranolol but not by the α antagonist phentolamine (Verma and Murti 1976a). Consequently, it has been suggested that the Acanthamoeba which interact with epinephrine, show some resemblance to the mammalian β adrenergic receptor in its sensitivity to the β adrenoceptor (Î²AR) antagonist propranolol (Verma and Murti 1976a).

Epinephrine has been reported to have been identified spectrophotometrically within A. culbertsoni in an unpublished observation (Verma et al. 1974) but conclusive evidence has not been published to date. Very little is known about exactly how Acanthamoeba senses and responds to its changing environment and the receptors and chemical pathways involved. However, previous studies with Entamoeba invades have indicated that this organism contains a functional adrenergic signalling system through studies demonstrating the encystment-promoting properties of catecholamines (Coppi et al. 2002) and through the identification of a G-protein-regulated adenylate cyclase that functions downstream of a adrenergic receptor (Frederick and Eichinger 2004).

Acanthamoeba keratitis is one of the most difficult ocular infections to treat and drugs including the diaminid compounds that are currently used in the treatment of Acanthamoeba keratitis have been reported to induce the encystment of Acanthamoeba at low concentrations by interfering with Acanthamoeba polyamine metabolism (Akins and Byers 1980; Ogbunude and Asiri 2001). Therefore, a compound which is able to prevent the transformation of trophozoites into the highly resistant cyst stage may prove to be useful tool for improving the treatment of this infection.

The aim of this study was to identify and characterize the receptor system involved in controlling encystment in Acanthamoeba using specific agonists and antagonists. This study will then determine if compounds related to epinephrine are synthesized and released by Acanthamoeba. To this end, this study has addressed the hypothesis that Acanthamoeba contains a functional adrenergic signalling system which enables them produce and store catecholamines that are released to initiate the encystment process via an interaction with adrenoceptors located on the surface of the trophozoite.

**MATERIALS AND METHODS**

**Acanthamoeba strains and culture methods**

Acanthamoeba castellanii (ATCC 30868) and A. culbertsoni (ATCC 30171) were obtained from LGC Standards (Ted-dington, UK). Acanthamoeba polyphaga (Ros) was isolated from a case of keratitis in 1991 (Kilvington et al. 1990). Acanthamoeba were cultured in 75 cm² tissue culture flasks (Nunc, Gloucester, UK) containing 30 ml of semi-defined axenic Acanthamoeba growth medium at 30 °C as previously described (Hughes et al. 2003). Acanthamoeba castellanii was selected for the encystment studies over A. culbertsoni and A. polyphaga because it has a more favourable encystment profile in growth medium supplemented with 50 mM MgCl₂ (Sigma, Gillingham, UK). Through personal observations, A. culbertsoni does not encyst in a nutrient encystment medium possibly through the presence of catabolite repressors that oppose encystment (Srivastava and Shukla 1983a). Acanthamoeba polyphaga too readily encysted making it difficult to tell what level of encystment was due to the exogenous catecholamines and what was due to natural encystment. Acanthamoeba castellanii was found to be in between these two extremes and gave intermediate levels of natural encystment in growth medium supplemented with 50 mM MgCl₂.

**Adrenoceptor agonist/antagonist studies**

Encystment studies were performed in 50 ml polypyrrole tubes containing 2.5 × 10⁸/ml trophozoites in 5 ml Acanthamoeba axenic growth medium. All adrenoceptor agonists and antagonists were obtained from Sigma (Gillingham, UK). The required concentrations of MgCl₂ and α adrenoceptor (αAR) or β adrenoceptor (βAR) agonists and antagonists were subsequently added and the tubes were placed in an orbital shaking incubator at 30 °C and 120 rpm. Each day over a 7 d period the percentage encystment was recorded using a modified Fuchs-Rosenthal haemocytometer (Hawksley, Lancing, UK).

**Inhibition of catecholamine synthesis**

Acanthamoeba polyphaga was selected for the inhibition of catecholamine synthesis study over the other two strains as it readily encysted in trophozoite growth medium supplemented with 50 mM MgCl₂ in the absence of exogenous catecholamines. Two cultures of the A. polyphaga strain were set up and passaged three times.
through either standard trophozoite growth medium or trophozoite growth medium supplemented with 10 mM of the catecholamine synthesis inhibitor α-methyl-p-tyrosine (AMPT) (Sigma). The two cultures were then transferred to trophozoite growth medium supplemented with 50 mM MgCl₂ to facilitate encystment.

**Data analysis**
Statistical analysis was performed using one-way analysis of variance (ANOVA) of data from triplicate experiments on the InStat statistical software package (GraphPad, La Jolla, CA).

**RESULTS**

Adrenoceptor agonists
The results for the adrenoceptor agonist studies with *A. castellanii* are shown in Table 1. A low level of encystment of *Acanthamoeba* occurred in standard growth medium (GM) in the absence of Mg²⁺ compared to 32% upon the addition of 50 mM MgCl₂ (p < 0.05). Encystment was further enhanced by the addition of epinephrine from 0.05, to 0.5, and up to 5 mM giving encystment of 56%, 72%, and 93% respectively (p < 0.001 for all relative to control). Isoproterenol induced approximately 90% encystment of *A. castellanii* (p < 0.001), whereas with norepinephrine only 45% encystment (p < 0.001). The selective βAR₁ agonist dobutamine gave encystment of 92% (p < 0.001) and the selective βAR₂ agonist salbutamol gave encystment of 39% at 10 μM (p < 0.01). Salbutamol could not be tested at higher concentrations as the compound caused extensive lysis of the *Acanthamoeba* trophozoites. The αAR agonist phenylephrine gave minimal encystment of approximately 10% at 100 μM (p > 0.05) and dibutyryl-cAMP, a lipophilic form of cyclic adenosine monophosphate which can cross intact membranes was also found to promote encystment of >70% (p < 0.001).

**Adrenoceptor antagonists**
The results for the adrenoceptor antagonist studies with *A. castellanii* are shown in Table 2. Epinephrine was added to the growth medium at a concentration of 500 μM along with 50 mM MgCl₂ and 500 μM of the antagonist to be tested. In the absence of antagonist, 0.5 mM epinephrine gave 72% encystment of A. castellanii. When the αAR antagonist phentolamine was added no decrease in the level of encystment was observed (p > 0.05). With the two nonselective βAR antagonists alprenolol and propranolol the level of encystment decreased to 39% and 44% respectively (p < 0.01 for both). A small decrease of <10% in encystment with the selective βAR₂ antagonist ICI-118,551 (p > 0.05) was observed in contrast with the >55% increase in the level of encystment seen with the βAR₁ antagonist metoprolol (p < 0.001).

**Inhibiting catecholamine synthesis using AMPT**
The results for the catecholamine synthesis inhibitor studies with *A. polyphaga* are shown in Table 3. *A. polyphaga* trophozoites grown in standard growth medium gave maximal encystment of 65% at 7 d compared to only 18% with the trophozoites grown in the presence of AMPT prior to encystment (p < 0.001).

**DISCUSSION**
The resistance of *Acanthamoeba* cysts to most antimicrobial agents at concentrations tolerated by the cornea makes Acanthamoeba keratitis one of the most difficult

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<th>Table 1. The effect of adrenoceptor agonists and dibutyryl cyclic adenosine monophosphate (cAMP) on the encystment of <em>Acanthamoeba castellanii</em></th>
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<td>Encystment conditions</td>
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<td>GM + Mg²⁺ + Norepinephrine (5 mM)</td>
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<td>GM + Mg²⁺ + Phenylephrine (100 μM)</td>
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<td>GM + Mg²⁺ + Dobutamine (100 μM)</td>
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<td>GM + Mg²⁺ + Salbutamol (10 μM)</td>
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<td>GM + Mg²⁺ + Dibutyryl-cAMP (100 μM)</td>
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*The values reported are ± the standard error of the mean from triplicate experiments.

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<th>Table 2. The effect of adrenoceptor antagonists on the epinephrine mediated encystment of <em>Acanthamoeba castellanii</em></th>
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<td>Encystment conditions</td>
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<td>GM + Mg²⁺ + Epinephrine (500 μM) + Alprenolol (500 μM)</td>
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<td>GM + Mg²⁺ + Epinephrine (500 μM) + Propranolol (500 μM)</td>
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<td>GM + Mg²⁺ + Epinephrine (500 μM) + Metoprolol (500 μM)</td>
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<td>GM + Mg²⁺ + Epinephrine (500 μM) + ICI 118,551 (500 μM)</td>
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the earlier observation that α-AR agonists did not enhance the encystment process. The βAR antagonists alprenolol and propranolol both caused a reduction in the level of encystment of around 30% with the specific βAR, antagonist metoprolol causing biggest decrease of > 55%. The βAR2 antagonist gave a < 10% reduction in the encystment levels which suggests that the βAR2 adrenoceptor is not involved in the encystment process. Combined, the results indicate that selective βAR1 agonists and antagonists can be used to promote and inhibit the encystment process. Furthermore, in an unpublished observation we have demonstrated that metoprolol can completely inhibit natural encystment triggered by Neff’s defined encystment medium (Neff et al. 1964) in all three strains of Acanthamoeba used in this study.

These findings mirror that of a previous study with E. invadens which also demonstrated that βAR1 agonists and antagonists can be used to promote and inhibit the encystment process. Using a radio-labelled nonselective βAR agonist [3H]-CGP-12177, the authors were also able to show that nonlabelled βAR antagonists including metoprolol could block the binding of [3H]-CGP-12177 and prevent encystment in a dose-dependent manner (Coppi et al. 2002). Other protozoa are believed to contain adrenergic signalling systems including the ciliate Tetrahymena through its response to αAR and βAR agonists and antagonists (Blum 1967).

Studies with dibutyl-cAMP a nonhydrolysable form of cAMP which readily crosses cell membrane demonstrated that it too promoted encystment. This combined with the agonist and antagonist studies initially lead us to believe that the receptor was similar to the mammalian βAR1 adrenoceptor. In the human body βAR1 is known to be primarily coupled to the Gs protein which activates adenylate cyclase leading to the intracellular accumulation of cAMP and the subsequent activation protein kinase A (Harmar et al. 2009). During normal amoebic growth intracellular cAMP levels are constant in the Acanthamoeba trophozoite but increase when the trophozoite encysts (Gessat and Jantzen 1974; Neff and Neff 1969). In mammalian systems cAMP is broken down to 5’ monophosphate by the enzyme phosphodiesterase (PDE) (Butcher and Sutherland 1962) and studies have shown that PDE inhibitors like theophylline can increase the level of encystment in A. culbertsoni (Verma et al. 1974).

However, although the results suggest the involvement of a βAR1 receptor a search of the recently sequenced Acanthamoeba genome indicates that there are no sequences that share homology with known mammalian adrenergic receptor systems present. Therefore, further studies will be required to determine the structure of the receptor that the βAR1 agonists and antagonists are interacting with. Interestingly the Acanthamoeba genome has revealed that they encode thirty-five G-protein-coupled receptors involved in cell signalling (Clarke et al. 2013) and so the authors believe that an as yet unidentified receptor is involved in adrenergic signalling in Acanthamoeba.

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<th>Table 3. The effect of the catecholamine synthesis inhibitor α-methyl-p-tyrosine (AMPT) on the encystment of Acanthamoeba polyphaga</th>
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<td>% Encystment (±SEM)</td>
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Magnesium Chloride (MgCl₂) was also present in the encystment medium and appears to play some role in mediating the encystment process, as when present there was a three fold increase in encystment compared to the control. One possibility for the encystment promoting activity of Mg²⁺ lies in its ability to increase the fluidity of the lipid bilayer by interacting with negatively charged phospholipids leading to the modification in activity of membrane bound enzymes (Fuyu et al. 1983). Previous authors have demonstrated that βAR stimulation in cardiac and liver cells results in an increase in levels of cAMP and a concomitant release of Mg²⁺ (Romani et al. 1991). Probably one of the most significant processes that has been shown to occur during the encystment of Acanthamoeba is the breakdown of intracellular glycogen stores (Weisman et al. 1970). In this process glycogen is degraded to glucose-1-phosphate by the enzyme glycogen phosphorylase (Heilmeyer 1991). The resulting glucose is then polymerized to form the cellulose cyst wall (Neff and Neff 1969). Divalent metal ions including Mg²⁺ have been demonstrated to increase the affinity of phosphorylase kinase for glycogen phosphorylase resulting in enzyme activation (Xu et al. 1996). Therefore, this may explain how the presence of Mg²⁺ in the medium promotes the encystment process by increasing the liberation of glucose from glycogen making more sugar available for cyst wall synthesis.

Synthesis of catecholamines like epinephrine occurs through a stepped synthesis pathway directly from the amino acid tyrosine or indirectly from phenylalanine. The rate limiting step in this pathway involves the conversion of tyrosine to DOPA by the enzyme tyrosine hydroxylase (Nagatsu et al. 1964). Assuming that Acanthamoeba have a catecholamine synthesis pathway, we incubated trophozoites with a catecholamine synthesis inhibitor to see if there was any effect on the encystment process. This study showed that 10 mM AMPT could competitively inhibit the enzyme tyrosine hydroxylase by competing with the free tyrosine in the medium (0.55 mM). This lead to a reduction in the encystment of A. polyphaga of approximately 40% compared to the control after 7 d in an encystment medium. Therefore, the results from this study indicate that Acanthamoeba spp. can synthesize and store catecholamines which are released to initiate encystment. This finding is supported by a previous study with the ciliate Encephalidium formosus and the flagellate Crithidia fasciculata which showed radiolabelled ¹⁴C-Tyrosine, ¹⁴C-Phenylalanine amino acids became incorporated into the catecholamines in these organisms (Janakidevi et al. 1996).

In conclusion, the findings of this study suggest that Acanthamoeba spp. contain a functional adrenergic receptor system of unknown structure that can be activated and blocked by exogenous βAR agonists and antagonists. In addition, the use of a catecholamine synthesis inhibitor this study demonstrates that Acanthamoeba synthesize and store catecholamines which are believed to be released to trigger the encystment process. Acanthamoeba have been shown to encyst in vivo during infection (Clarke et al. 2005), however, the exact reason for this unknown but it is possible they do this in response to catecholamines present in the patients circulatory system or contained within the lachrymal fluid.

The encysted form of Acanthamoeba is highly resistant to kill using topical antimirolbal agents and so it may be possible to use topical βAR antagonists as an adjunct therapy in Acanthamoeba keratitis alongside the current drug regime to reduce encystment of amoebae during treatment. Preventing encystment could potentially enhance treatment as the trophozoite is more susceptible to killing using topical antimicrobial agents. It is hoped that this approach could bring down the mean treatment time and prevent recrudescence of infection. Topical βAR antagonists are well tolerated in the eye and are currently used in the management of glaucoma (Ong et al. 2005).

ACKNOWLEDGMENTS

None.

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