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Pharmacological Evidence Suggests that the Lysozyme/PACAP Receptor of *Tetrahymena thermophila* is a Polycation Receptor

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Summary. Pituitary adenylate cyclase activating polypeptide (PACAP) is a peptide hormone that exists in two biologically active forms: PACAP-38 and PACAP-27. Several types of PACAP receptors have been characterized, and these have been classified into three families: the $VPAC_1$, the $VPAC_2$, and the PAC_1 receptors. In this study, we used *in vivo* behavioral assays along with pharmacological inhibitors to investigate the behavior of the lysozyme/PACAP receptor in *Tetrahymena.* This receptor behaves like a PAC_1 receptor in some respects; however, PACAP 6-38 serves as an agonist, rather than an antagonist, for this receptor. These results are consistent with the existence of a generalized polycation receptor rather than a PACAP-specific receptor.

Key words: chemorepellent, G-protein, PACAP-38, PKA, PKC, polycation receptor, *Tetrahymena*.

Abbreviations: BIS IV - bisindolylmaleimide IV; cAMP - adenosine 3'5'monophosphate; cGMP - guanosine 3'5' monophosphate; EC_{50} - concentration of repellent which causes 50% avoidance; EIA - enzyme-linked immunoassay; GDP-β-S - guanosine 5'-O-(2-thiodiphosphate); IP_3 - inositol 1,4,5 trisphosphate; PACAP - pituitary adenylate cyclase activating polypeptide; PKA - cAMP-dependent protein kinase; PKC - protein kinase C; Rp-cAMPs - Rp-adenosine-3', 5' cyclic monophosphorothioate.

INTRODUCTION

Pituitary adenylate cyclase activating polypeptide (PACAP) is a peptide hormone derived from a larger polypeptide precursor. This peptide exists in two biologically active forms: a 38-amino acid peptide, PACAP-38 (Miyata *et al*. 1989), and an N-terminal amidated 27-amino acid peptide, PACAP-27 (Miyata *et al*. 1990). Both forms of this peptide are polycations at physiological pH. Receptors for this peptide have been found in nearly every organ in the body (Vaudry *et al*. 2000). The nearly ubiquitous nature of this peptide in mammalian systems makes it an interesting ligand to study in other cell systems. PACAP-38 has previously been shown to act as a chemorepellent in *Tetrahymena thermophila* (Mace *et al.* 2000, Hassenzahl *et al.* 2001).

Since the initial discovery of PACAP, several types of receptors have been identified and classified based on their affinities for the following ligands: PACAP-38,

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PACAP-27, vasoactive intestinal peptide (VIP), and other members of the same peptide hormone superfamily. Three receptor classes have been identified so far: the $VPAC_1$ receptor, the $VPAC_2$ receptor, and the PAC_1 receptor (Harmar *et al*. 1998). Of these three receptor types, previously obtained pharmacological data (Hassenzahl *et al*. 2001) suggest that the *Tetrahymena* receptor may share some characteristics with the PAC_1 class of receptors in terms of the second messenger pathways utilized. The pathways through which previously identified PAC_1 receptors signal include cyclic AMP (cAMP) as well as inositol trisphosphate (IP_3) and diacylglycerol (DAG)-mediated activation of protein kinase C (PKC) (Delporte *et al*. 1995, Hahm *et al*. 1998).

Two forms of the PAC_1 receptor have been identified. Type IA receptors have an equal affinity for PACAP-38 and PACAP-27, while type IB receptors have a higher affinity for PACAP-38 than for PACAP-27 (Harmar *et al*. 1998, Robberecht *et al*. 1991). Both types receptors have an affinity for VIP that is about 1000 times less than the affinity for PACAP-38. In addition, PAC_1 receptors are selectively inhibited by the antagonist PACAP 6-38, a fragment of PACAP-38 that binds to the receptor but does not activate the second messenger pathway (Harmar *et al*. 1998). These pharmacological profiles have not previously been evaluated in *Tetrahymena*.

Previous work in *Tetrahymena* has demonstrated that the lysozyme response is mediated through a receptor that shows specific, saturable binding (Kuruvilla *et al*. 1997). The receptor has also been isolated and characterized electrophoretically as a 42 kD protein (Kuruvilla and Hennessey 1998). Adaptation studies with PACAP-38 have shown that cells exposed to lysozyme for at least 10 min no longer show avoidance to PACAP- 38 and *vice versa* (Mace *et al*. 2000). The fact that both lysozyme and PACAP are polycations, combined with the previously mentioned adaptation data, suggests that these two ligands may utilize a common receptor and second messenger pathways.

Recent work in our laboratory indicates that signaling through the lysozyme/PACAP receptor of *Tetrahymena thermophila* involves both cAMP production and the activation of PKC (Hassenzahl *et al*. 2001). While this suggests the possibility of PAC_1 receptor involvement, the potencies of PACAP-27, VIP, and the PAC_1 receptor antagonist PACAP 6-38 have not been previously tested in *Tetrahymena*. In our current study, we have used *in vivo* behavioral assays as well as pharmacological inhibitors to suggest the presence of a receptor that shares some characteristics with the PAC_1 receptor family, specifically, ligand potency and the use of common signaling pathways. However, there is one notable difference: namely, the action of PACAP 6-38, an antagonist to PAC_1 receptors (Vaudry *et al*. 2000), which acts as an agonist (chemorepellent) in our system. These data suggest that the lysozyme/PACAP receptor is probably a generalized, G-protein linked, polycation receptor which signals through several second messenger pathways.

MATERIALS AND METHODS

Cell cultures

Tetrahymena thermophila B, strain SB715, a generous gift from T. M. Hennessey (SUNY at Buffalo) was used throughout the study. Cells were incubated in the axenic medium Dentler (1988) at 25°C for 48 h after inoculation without shaking and without addition of antibiotics.

Chemicals and solutions

As in earlier studies with lysozyme and PACAP (Kuruvilla *et al*. 1997, Mace *et al*. 2000, Hassenzahl *et al*. 2001), behavioral bioassays were carried out in a buffer containing 10 mM Trizma base, 0.5 mM MOPS, 50 μM $CaCl_2$, pH 7.0. PACAP-38 was obtained from Calbiochem, La Jolla, CA. PACAP-27 was obtained from Peninsula Laboratories, San Carlos, CA. PACAP 6-38 and VIP were obtained from the American Peptide Co, Sunnyvale, CA. The G-protein inhibitor guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) was obtained from Alexis Biochemicals, San Diego, CA. The PKC inhibitor Calphostin C and the PKA inhibitor Rp-cAMPs were obtained from BIOMOL Research Laboratories, Plymouth Meeting, PA. Another PKC inhibitor, BIS IV, was obtained from LC Laboratories, Woburn, MA. The cAMP EIA kit was obtained from Cayman Chemical, Grand Rapids, MI. All other chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Behavioral bioassays

In vivo behavioral assays were carried out as previously described (Mace *et al*. 2000, Hassenzahl *et al*. 2001). Briefly, cells were first washed twice in the assay buffer and then transferred to the first well of a 3-well spot microtiter plate. Individual cells were then transferred, using a micropipette, to a well containing either buffer or the inhibitor being tested. If an inhibitor was being tested, cells were adapted to the inhibitor for a minimum of 10 to 15 min before individual cells were transferred to a third well containing a combination of the chemorepellent and the test concentration of inhibitor and scored for avoidance (+ or -) for each trial. Cells were scored as (+) for avoidance if they showed backward swimming, characterized by jerky, backward motions, backward motions in a straight line, or backward "tumbling", with the cell rotating anterior-over-posterior or spinning rapidly, like a fan blade. If no inhibitor was being tested, cells were simply transferred from the buffer to a well containing the

chemorepellent of interest and scored for avoidance (+ or -) for each trial. The mean ± SD was calculated for a minimum of three trials and was expressed as " Cells Showing Avoidance, [%]".

EIA assays

Enzyme-linked immunoassays (EIAs) for cAMP were performed using an EIA kit from Cayman Chemical, Grand Rapids, MI, Catalog Number 581001. Briefly, two-day old cell cultures were washed twice in behavioral buffer and then placed into the same behavioral buffer containing 1 mM theophylline. Cells were then exposed to 0.1 µM PACAP-38, PACAP-27, or PACAP 6-38, or simply left in buffer. Aliquots of 1 ml, containing approximately 7.66E06 cells, were immediately frozen in liquid nitrogen. Upon thawing, the cell lysate was centrifuged at 12,500 g for 10 min to remove particulate matter. The supernatant was acetylated according to the EIA kit manufacturer's instructions (diluted 1:100 for maximum performance on the standard curve) and used for the EIA assay.

RESULTS

In vivo behavioral assays indicate that PACAP-38, PACAP-27, and PACAP 6-38 all act with similar potencies as chemorepellents in *Tetrahymena* (Fig. 1). The EC_{50} (concentration of repellent which causes 50% of cells to show avoidance) of all three compounds was approximately 10 nM. VIP, which was also a chemorepellent, was much less potent, with an EC_{50} of approximately 10 µM, 1000 times higher than that of the other three ligands (Fig. 1). The concentrations of repellent necessary to elicit 100% avoidance was 0.1 µM for PACAP-38, PACAP-27, and PACAP 6-38, and 100 µM for VIP (Fig. 1). These concentrations of repellent were used in the adaptation studies (Fig. 2), cross-adaptation studies (Table 1), inhibitor studies (Table 2), and cAMP assays which follow.

Chemorepellent responses to 0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, and 100 µM VIP all declined with time (Fig. 2). By four min, approximately 50% of the cells had adapted to the repellent, while a baseline (≤ 20%) avoidance response was seen at 10 min. Based on the behavior of *Tetrahymena* when placed in buffer under our assay conditions (10-20%; Kuruvilla *et al*. 1997, Mace *et al*. 2000, Hassenzahl *et al*. 2001), it appears that cells are fully adapted after 10 min of exposure to the repellents. All of the ligands showed a similar time course of adaptation (Fig. 2). As seen in previous studies with other repellents in *Tetrahymena*, adaptation to all of the repellents could be reversed by placing cells in buffer for about 10 min before reintroducing them to the repellent (unpublished observations; Kuruvilla *et al.* 1997).

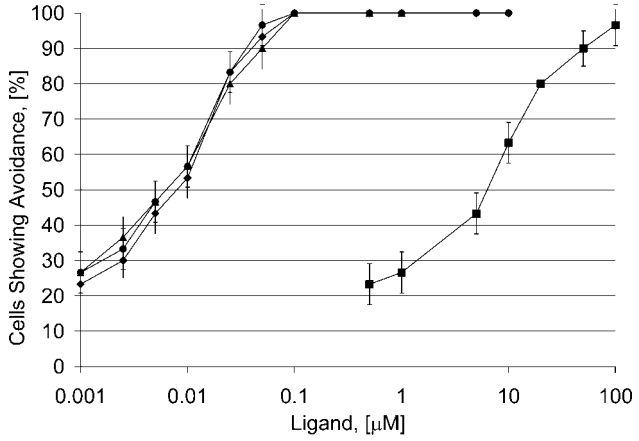

Fig. 1. PACAP-38, PACAP-27, PACAP 6-38, and VIP are effective chemorepellents in *Tetrahymena.* Behavioral bioassays (see Materials and Methods) were used to show the concentration dependencies for avoidance reactions to PACAP-38 (closed diamonds), PACAP-27 (closed triangles), PACAP 6-38 (closed circles), and VIP (closed squares). The percentage of cells showing avoidance was determined by observation of a single cell after transfer to the test solution. Each trial consisted of ten cells, which were individually scored as to whether or not avoidance occurred. Each point represents the mean ± SD of ≥ 3 trials. Error bars, representing the standard deviation, are shown for each point. The EC_{50} values for these repellents were approximately 10 nM for PACAP-38, PACAP-27, and PACAP 6-38 and 10 µM for VIP.

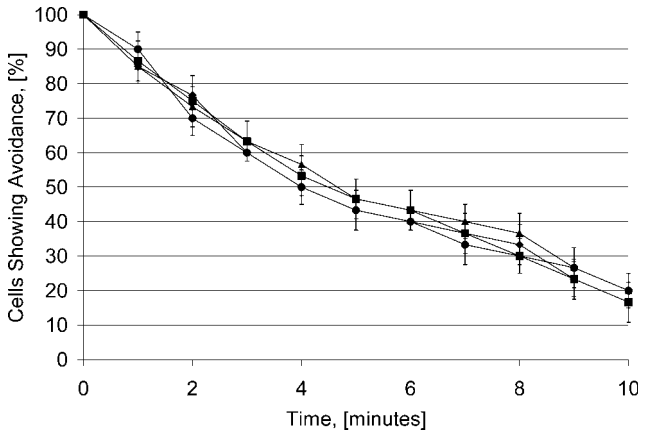

Fig. 2. Adaptation to PACAP-38 (closed diamonds), PACAP-27 (closed triangles), PACAP 6-38 (closed circles), and VIP (closed squares) all occurred over a similar time period (approximately 10 min), consistent with a common signaling mechanism. Each point represents the mean ± SD of ≥ 3 trials. Error bars, representing the standard deviation, are shown for each point.

Table 1. Cross-adaptation between members of the VIP/PACAP family.

	PACAP-38	PACAP-27	PACAP-6-38	VIP
PACAP-38	16.6 ± 5.8	20.0 ± 5.0	13.3 ± 5.8	20.0 ± 5.0
PACAP-27	16.6 ± 5.8	16.6 ± 5.8	20.0 ± 5.0	20.0 ± 5.0
PACAP 6-38	20.0 ± 5.0	20.0 ± 5.0	13.3 ± 5.8	16.6 ± 5.8
VIP	20.0 ± 5.0	20.0 ± 5.0	16.6 ± 5.8	20.0 ± 5.0

Means ± SD for ≥ 3 trials of 10 cells are listed. Each cell was adapted to the chemorepellent for 10 min before testing for cross-adaptation in a different repellent. In all experiments, adaptation to one chemorepellent in the VIP/PACAP family effectively eliminated avoidance to the other repellents in the family (avoidance in buffer alone under our assay conditions ranges from 10-20% (Kuruvilla *et al*. 1997, Mace *et al*. 2000).

Table 2 Effect of various pharmacological inhibitors upon avoidance behavior in various members of the VIP/PACAP family.

	No inhibitor	GDP-β-S 1mM	Rp-cAMPs 50 μM	Calphostin C 10 μM	BIS IV 50 μM	Neomycin 5 μM
PACAP-38, 0.1 μM	100 ± 0.0	16.6 ± 5.8	10 ± 5.0	16.6 ± 5.8	20.0 ± 5.0	16.6 ± 5.8
PACAP-27, 0.1 μM	100 ± 0.0	13.3 ± 5.8	10 ± 0.0	16.6 ± 5.8	16.6 ± 5.8	16.6 ± 5.8
PACAP 6-38, 0.1 μM	100 ± 0.0	6.6 ± 5.8	16.6 ± 5.8	20.0 ± 5.0	16.6 ± 5.8	20.0 ± 5.0
VIP, 100 μM	96.6 ± 5.8	20.0 ± 5.0	20.0 ± 5.0	20.0 ± 5.0	20.0 ± 5.0	20.0 ± 5.0

Means ± SD for ≥ 3 trials of 10 cells are listed. Each cell was incubated with the inhibitor for 10 min before testing for avoidance in the chemorepellent. In all experiments, exposure to the pharmacological inhibitor nearly eliminated avoidance to the chemorepellent (avoidance in buffer alone ranges from 10-20% under these assay conditions; Kuruvilla *et al*. 1997, Mace *et al*. 2000).

Cross-adaptation studies (Table 1) were also conducted to determine whether a cell adapted to one chemorepellent in the VIP/PACAP family would also be adapted to the other peptides in the family. Cells were first adapted to either 0.1 μM PACAP-38, 0.1 μM PACAP-27, 0.1 μM PACAP 6-38, or 100 μM VIP for 10 min and then exposed to one of the other three repellents. In all cases, adaptation to one repellent reduced avoidance to the other three repellents to baseline levels of 10-20% (Table 1).

Several potent and specific pharmacological inhibitors of second messenger pathways have been effective in eliminating avoidance to PACAP-38 in *Tetrahymena* (Mace *et al*. 2000, Hassenzahl *et al*. 2001). These compounds were tested using *in vivo* behavioral assays in order to determine whether the same second messenger pathways are involved in the signaling of the other ligands in the VIP/PACAP family (Fig. 2). The concentrations of inhibitors used in the current assay were based on previously published data (Mace *et al*. 2000, Hassenzahl *et al*. 2001).

The G-protein inhibitor, 1 mM GDP-β-S (Hassenzahl *et al*. 2001), effectively eliminated avoidance to 0.1μM PACAP-38, 0.1μM PACAP-27, 0.1μM PACAP 6-38, and 100 μM VIP (Table 2), lowering avoidance in all cases to the baseline avoidance of 10-20%. A cAMP analogue and competitive inhibitor of PKA, 50 μM Rp-cAMPs (Hassenzahl *et al*. 2001), also eliminated avoidance to all four chemorepellents, as did two PKC inhibitors, 10 μM Calphostin C (Hassenzahl *et al*. 2001), and 50 μM BIS IV (Hassenzahl *et al*. 2001), (Table 2). A competitive inhibitor of the lysozyme receptor (Kuruvilla *et al*. 1997) and an inhibitor of the PACAP-38 response (Mace *et al*. 2000), 5 μM neomycin sulfate, effectively eliminated the avoidance response to 0.1μM PACAP-38, 0.1μM PACAP-27, 0.1μM PACAP 6-38, and 100 μM VIP (Table 2).

Indirect determination of cytosolic cAMP levels was performed by EIA. These preliminary data (N=2) suggest that detectable levels of cAMP are present even in control cells (7.73 ± 0.02E-06 picomoles/cell). Exposure to PACAP-38 raised the cAMP level to 255% of the

control level (1.96 ± 0.11E-05 picomoles/cell), and PACAP-27 caused an increase in cAMP levels to 239% of the control level (1.85 ± 0.12E-05 picomoles/cell). Exposure to PACAP 6-38 caused a lesser increase, raising the level of cAMP to 131% of control levels (1.01 ± 0.08E-05 picomoles/cell). Although these data are preliminary, they are consistent with the Rp-cAMPs data mentioned above.

DISCUSSION

PAC_1 receptors are characterized by a number of common features, including a low affinity for VIP, signaling through cAMP and PKC, and inhibition by the PACAP fragment PACAP 6-38. Data from *in vivo* behavioral assays in *Tetrahymena* (Fig. 1) indicates that the potency of PACAP-38 and PACAP-27 is nearly identical, and the potency of VIP is nearly 1000 times lower than that of PACAP. These results are consistent with signaling through a PAC IA receptor. However, PACAP 6-38 acted as a repellent in our assay (Fig. 1) rather than an antagonist of receptor activity. This is in clear contrast to other systems where PACAP 6-38 serves as an antagonist to the PAC_1 receptor, binding to the receptor but not activating the second messenger pathway (Harmar *et al*. 1998). However, PACAP 6-27, also considered a competitive inhibitor of the PACAP receptor, has been shown to stimulate serotonin release from rat peritoneal mast cells (Seebeck *et al*. 1998). In this case, a receptor-independent mechanism of G-protein activation has been proposed. We have found that PACAP 6-27 is also an effective chemorepellent in our system (unpublished data), with potency nearly indistinguishable from PACAP 6-38. While a receptor-independent mechanism of signaling such as the one proposed for rat peritoneal mast cells is also possible in our system, the high potency of PACAP 6-38, which is nearly equivalent to that of PACAP-38 and PACAP-27, along with the shape of the activation curve, is more consistent with the existence of a receptor, linked to a G-protein. Use of common signaling pathways for all the ligands being studied (PACAP-38, PACAP-27, PACAP 6-38, and VIP) also supports the hypothesis that a common receptor is being used.

Time course studies of *in vivo* behavioral adaptation show a similar kinetic for all four repellents being tested. This result is consistent with the hypothesis that PACAP-38, PACAP-27, PACAP-6-38, and VIP are all signaling through a common receptor. Adaptation occurs over a fairly short time period (about 10 min) and is reversible. These data are consistent with an adaptation mechanism such as modification of the receptor or second-messenger pathway rather than a mechanism such as receptor-mediated endocytosis, which would require a longer period of time for adaptation and recovery or de-adaptation. In the case of lysozyme, which appears to work through the same receptor as PACAP-38 (Mace *et al*. 2000), receptor-mediated endocytosis is not the mechanism of adaptation (Cantor *et al*. 1999). Our current data support these earlier findings.

Cross-adaptation studies (Table 1) are also consistent with the hypothesis that all four ligands being tested (0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, and 100 µM VIP) share a common receptor and/or second messenger pathway. Adaptation to any one of the ligands in the VIP/PACAP family results in a lack of responsiveness to any of the other ligands, resulting in a baseline avoidance of 10-20%, similar to that seen in buffer alone (Kuruvilla *et al*. 1997, Mace *et al*. 2000).

The polycation, lysozyme, is a chemorepellent in *Tetrahymena* (Kuruvilla *et al*. 1997, Kuruvilla and Hennessey 1998). The lysozyme receptor has been purified by affinity chromatography, and a polyclonal antibody has been raised against the purified protein (Kuruvilla and Hennessey 1998). This antibody, along with a competitive inhibitor of the lysozyme receptor neomycin sulfate (Kuruvilla *et al*. 1997, Kuruvilla and Hennessey 1998) has been used to show that PACAP-38 and lysozyme share a common receptor in *Tetrahymena* (Mace *et al*. 2000). Avoidance to PACAP-38 may also be inhibited by the G-protein inhibitor GDP-β-S, the cAMP analogue Rp-cAMPs, and the PKC inhibitors Calphostin C and BIS IV (Hassenzahl *et al*. 2001). These potent, specific inhibitors can be effectively used to determine involvement of specific enzymes/receptors in the *in vivo* behavioral response of *Tetrahymena*.

In vivo behavioral assays done with 0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, or 100 µM VIP gave approximately 100% avoidance in all cases (Table 2). The G-protein inhibitor 1 mM GDP-β-S reduced avoidance to near the baseline response of ~10-20% in all cases (Table 2), implicating G-protein involvement in the signaling pathway. This is consistent with previous results for PACAP-38 and

lysozyme, which appear to share a receptor and signaling pathways (Mace *et al*. 2000, Hassenzahl *et al*. 2001).

The cAMP analogue, 50 µM Rp-cAMPs, also inhibited avoidance to 0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, and 100 µM VIP (Table 2). These data are also consistent with what has been seen for PACAP-38 and lysozyme (Hassenzahl *et al*. 2001). This indicates that cAMP is involved in signaling, possibly as an activator of PKA, or perhaps in another capacity, such as opening a plasma membrane calcium channel. A calcium influx has previously been implicated in the response of *Tetrahymena* to lysozyme (Kuruvilla and Hennessey 1998). These data also support the hypothesis of a common receptor/signaling pathway used by all four ligands.

Preliminary data from EIA experiments measuring the concentration of cAMP in the cytosol after PACAP stimulation showed that all PACAP isoforms tested stimulate cAMP production above control levels. This is consistent with our current Rp-cAMPs data, which suggests cAMP involvement in avoidance, as well as data from previous studies (Hassenzahl *et al*. 2001). Interestingly, PACAP 6-38, a PACAP receptor antagonist in many systems, caused less of an increase in cAMP production than the native PACAP forms (PACAP-38 and PACAP-27). Since the data are sparse, repeated studies will be necessary to determine whether this pattern is consistent. PACAP 6-38 was as potent an agonist in our system as were the native forms of PACAP, and future experiments continue to show lower cAMP levels elicited by PACAP 6-38, this would suggest that the high cAMP levels caused by the native forms of PACAP may not be necessary or sufficient to cause avoidance. The data discussed below implicate the PKC pathway as an integral part of avoidance, and recent data from our laboratory suggest that PKG may be important as well (unpublished observations). Clearly, cAMP is not the only essential component of this repellent pathway.

As seen in Table 2, the PKC inhibitors 10 µM Calphostin C and 50 µM BIS IV effectively reduced avoidance to 0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, and 100 µM VIP to near the baseline avoidance of 10-20%. This is consistent with previous results seen for PACAP-38 and lysozyme (Hassenzahl *et al*. 2001) and implicates PKC in the avoidance of chemorepellents in the PACAP family. These data also support the hypothesis of a shared second messenger pathway used by all four ligands.

Neomycin sulfate has been shown to be a competitive inhibitor of lysozyme binding to its receptor in *Tetrahymena* (Kuruvilla *et al*. 1997, Kuruvilla and Hennessey 1998) as well as an inhibitor of PACAP-38 binding (Mace *et al*. 2000). In this assay, 5 µM neomycin sulfate reduced avoidance to 0.1 µM PACAP-38, 0.1 µM PACAP-27, 0.1 µM PACAP 6-38, and 100 µM VIP to near the baseline of 10-20%. This, too, is consistent with previous results and supports the hypothesis that all four of these ligands are working through the previously identified lysozyme receptor. This low (5 µM) concentration of neomycin is 60 times lower than the concentration required to block calcium channels on the plasma membrane of *Paramecium* (~300 µM; Gustin and Hennessey 1988) and is consistent with previous inhibition data (Kuruvilla *et al*. 1997, Kuruvilla and Hennessey 1998, Mace *et al*. 2000).

In the current study, we have explored adaptation kinetics, cross-adaptation of cells adapted to one ligand and exposed to another, and the involvement of G-proteins, cAMP, and PKC in signaling. While some of the inhibitors used in our study may have pleiotropic effects on the cell, when taken collectively, our data suggest that PACAP avoidance in *Tetrahymena* occurs through activation of a G-protein linked receptor. The *Tetrahymena* receptor shows some similarities to previously described PAC_1 receptors (Miyata *et al*. 1989, Delporte *et al*. 1995, Hahm *et al*. 1998) in that it signals through cAMP and PKC. However, the N-terminal amino acids of PACAP do not appear to be required for receptor activation, since PACAP 6-38 is as potent a chemorepellent as PACAP-38 or PACAP-27. All of the PACAP isoforms used in the assay, as well as VIP, lysozyme (tested previously), and the lysozyme fragment CB_2 (Kuruvilla and Hennessey 1999) are positively charged polypeptides. Interestingly, the more highly charged molecules, such as PACAP (+ 12 at physiological pH), whose EC_{50} is 10 nM, are more potent than the less highly charged molecules, such as CB_2 (+ 4 at physiological pH) whose EC_{50} is 100 nm (Kuruvilla and Hennessey 1999, Mace *et al*. 2000). All of these data are consistent with signaling of these repellents through a polycation receptor, rather than through a specialized PACAP receptor. This polycation receptor is likely the previously isolated lysozyme receptor, based on the cross-adaptation studies, similar inhibition profiles, and the fact that the antibody raised against the lysozyme receptor blocks PACAP avoidance (Kuruvilla and Hennessey 1998, Mace *et al*. 2000, Hassenzahl *et al*. 2001). Molecular cloning of the receptor, perhaps using

the amino acid sequence previously isolated from the purified receptor preparation (Kuruvilla and Hennessey, 1998), will provide an important step toward identifying sequences responsible for ligand binding and allow for molecular characterization of this polycation receptor.

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