2004

PACAP-38 Signaling in *Tetrahymena thermophila* Involves NO and cGMP

John Lucas

John Riddle

Janine Bartholomew

Brendan Thomas

Jason Forni

See next page for additional authors

Authors
John Lucas, John Riddle, Janine Bartholomew, Brendan Thomas, Jason Forni, L. Emery Nickerson, Bradley Van Heukelum, Joshua Paulick, and Heather G. Kuruvilla

Acta Protozool. (2004) 43: 15 - 20

ACTA
PROTOZOOLOGICA

PACAP-38 Signaling in *Tetrahymena thermophila* Involves NO and cGMP

John LUCAS, Mark RIDDLE, Janine BARTHOLOMEW, Brendan THOMAS, Jason FORNI, L. Emery NICKERSON, Bradley Van HEUKELUM, Joshua PAULICK and Heather KURUVILLA

Cedarville University, Department of Science and Mathematics, Cedarville, OH, USA

Summary. Chemorepellents are signaling molecules, which have been shown to be important for mammalian neuronal development, and are presumed to have a role in protozoan defense. *Tetrahymena thermophila* represent a good model system in which to study repellents because of their ease of use in biochemical, behavioral, electrophysiological, and genetic analyses. In this study, we have used *Tetrahymena* as a model in which to study the chemorepellent, PACAP. Using behavioral and biochemical (EIA) assays, we have found that the NO/cGMP pathway plays an important role in PACAP signaling. An increase in intracellular calcium is also critical for PACAP avoidance, which appears to be mediated through a pertussis toxin-sensitive G-protein.

Key words: chemorepellent, G-protein, nitric oxide, PACAP-38.

Abbreviations: cGMP - guanosine 3'5' monophosphate, IMP - 2-imino-4-methylpiperidine, NO - nitric oxide, NOS - nitric oxide synthase, PACAP - pituitary adenylate cyclase activating polypeptide.

INTRODUCTION

Chemorepellents are compounds that cause a cell to exhibit avoidance. Some of these compounds have been shown to be important in human neuronal development (Zhu *et al.* 1999). Still, relatively little is known about repellents and how they work. Ciliate model systems, such as *Tetrahymena thermophila*, are a useful tool in which to study repellents because they exhibit a distinctive "avoidance behavior", easily seen under a dissection microscope. They have been widely used in genetics studies (Orias 1998), and can even be used in electrophysiological experiments (Kuruvilla and Hennessey 1998, 1999; Kim *et al.* 1999).

Tetrahymena respond to a number of compounds as repellents, including ATP (Kim *et al.* 1999, Rosner *et al.* 2003), GTP (Kuruvilla *et al.* 1997, Iwamoto and Nakaoka 2002), and PACAP (Mace *et al.* 2000, Hassenzahl *et al.* 2001, Keedy *et al.* 2003), all of which appear to use different receptors. Although avoidance behavior to each of these compounds appears identical when observed under a dissection microscope, the intracellular pathways involved are not. Previous studies in our laboratory have implicated a G-protein linked receptor in PACAP signaling (Hassenzahl *et al.* 2001), which is similar to signaling pathways utilized by vertebrate PACAP receptors. ATP also appears to signal through a G-protein linked receptor (Rosner *et al.* 2003); however,

Address for correspondence: Heather Kuruvilla, Cedarville University, Department of Science and Mathematics, 251 North Main St. Cedarville, OH 45314, USA

GTP signaling appears to involve a tyrosine kinase pathway (Bartholomew *et al.*, submitted).

PACAP has myriad actions in vertebrates. Although originally isolated from brain, target cells include smooth muscle cells along with a number of different neuronal cell types (Forssmann and Said 1998). Vertebrate second messenger pathways activated by PACAP include protein kinases A and C, and pathways inhibited include the JNK/SAPK pathway (Forssmann and Said 1998).

In parallel with mammalian systems, PACAP-38 avoidance in *Tetrahymena* has previously been shown to involve an intricate pathway, which includes adenylyl cyclase and phospholipase C (Hassenzahl *et al.* 2001). Recent studies show that these pathways are also involved in ATP avoidance, along with an NO pathway (Rosner *et al.* 2003). In this study, we use pharmacological and EIA assays as evidence that the NO/cGMP pathway is also involved in PACAP-38 signaling in *Tetrahymena*. The PACAP-38 response is also shown to be calcium dependent and pertussis toxin sensitive. Finally, a model of PACAP-38 signaling attempts to pull together all of the pathways involved and postulates how these may contribute to avoidance.

MATERIALS AND METHODS

Cell cultures. *Tetrahymena thermophila* B, strain SB2086, a generous gift from T. M. Hennessey (SUNY at Buffalo) was used throughout the study. Cells were incubated under sterile conditions at 25°C for 48 h after inoculation, without shaking, in the axenic medium of Dentler (1988). No antibiotics were added to the media.

Chemicals and solutions. Behavioral bioassays were carried out in a buffer containing 10 mM Trizma base, 0.5 mM MOPS, 50 µM $CaCl_2$, pH 7.0. All repellents and inhibitors were dissolved in this buffer. Compounds which were insoluble in aqueous solution were first dissolved into a small quantity of DMSO, then immediately diluted into behavioral buffer at a dilution of 1:10,000 or higher.

PACAP-38 was obtained from the American Peptide Co. Sunnyvale, CA. The G-protein inhibitor, pertussis toxin, was obtained from Alexis Biochemicals, San Diego, CA. The calcium chelator, BAPTA-AM, the guanylyl cyclase inhibitor, NS-2028, and the Quantizyme Assay System© NO Kit were all obtained from BIOMOL Research Laboratories, Plymouth Meeting, PA. The NOS inhibitors, 1400W and 2-Imino-4-Methylpiperidine (IMP), the guanylyl cyclase inhibitor, LY-83583, and the cGMP EIA kit, were obtained from Cayman Chemical, Ann Arbor, MI. All other chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Behavioral bioassays. *In vivo* behavioral bioassays were carried out as previously described (Kuruvilla *et al.* 1997, Kim *et al.* 1999, Mace *et al.* 2000). Cells were first washed in the assay buffer and transferred to the first well of a 3-well spot microtiter plate. Cells were then transferred to the second well of the microtiter plate, which contained the inhibitor being tested, and were incubated for 10-15 min, to allow time for cellular uptake of the inhibitor. If no inhibitor was being tested, cells were simply incubated in fresh buffer for 10-15 min. Individual cells were then transferred to the third well of the microtiter plate, which contained a combination of the chemorepellent (0.1 µM PACAP 38) and the test concentration of inhibitor. If no inhibitors were being tested, cells were simply transferred to the repellent. Each cell was then scored for avoidance (+ or -) for each trial. Each trial consisted of ten cells. The mean ± SD was calculated for at least three trials, and was expressed as "cells showing avoidance, (%)".

EIA assays. NO assays were carried out using two-day old cell cultures. Cells were washed twice in buffer and diluted to a final concentration of approximately 720 cells/ml. Cells were exposed to 9 µM PACAP 38 for approximately 30 seconds, then spun down in a benchtop microcentrifuge for 30 s. Supernatant was analyzed for NO using a kit from BIOMOL, according to the manufacturer's instructions.

cGMP assays were carried out using two day old cell cultures. Cells were concentrated to approximately 7.66×10^6 cells/ml. Cells were exposed to 1 µM PACAP 38 for approximately 30 seconds, then immediately lysed by freezing in liquid nitrogen and thawing. Theophylline (1 µM) was added to the lysate to inhibit phosphodiesterases. Lysate was spun at 16,000 g for 30 min. Supernatant was assayed for cGMP using a kit from Cayman Chemicals according to the manufacturer's instructions.

RESULTS

As seen previously (Mace *et al*. 2000, Hassenzahl *et al*. 2001, Keedy *et al*. 2003), *in vivo* behavioral bioassays indicate that PACAP-38 is an effective chemorepellent in *Tetrahymena* (Fig. 1). The potency of PACAP-38 was concentration dependent, and a maximum potency, causing 100% of cells to show avoidance, was seen at 0.1 µM. This concentration was used in the inhibitor studies which follow. The EC_{50} of this compound was approximately 0.01 µM.

In order to determine whether NO was involved in PACAP-38 avoidance, two NO inhibitors, 1400W and IMP were used. Both inhibitors (Figs 2, 3) blocked avoidance in a concentration-dependent manner. An avoidance response near baseline (10-20%; Mace *et al.* 2000) was achieved at 10 µM 1400W (23.3 ± 5.8%), and 500 nM IMP (20 ± 5%). The EC_{50} for 1400W was near 1 nM, and for IMP was near 0.1 nM. These data implicate the NO pathway in PACAP signaling. In order to confirm these results biochemically, EIA assays were performed on intact cells. Using 9 µM PACAP, our EIA assays showed a 148 ± 4.8% increase in NO levels compared to control cells, confirming NO involvement in the *Tetrahymena* response.

Since NO often activates guanylyl cyclase within cells, and since the presence of a membrane-bound

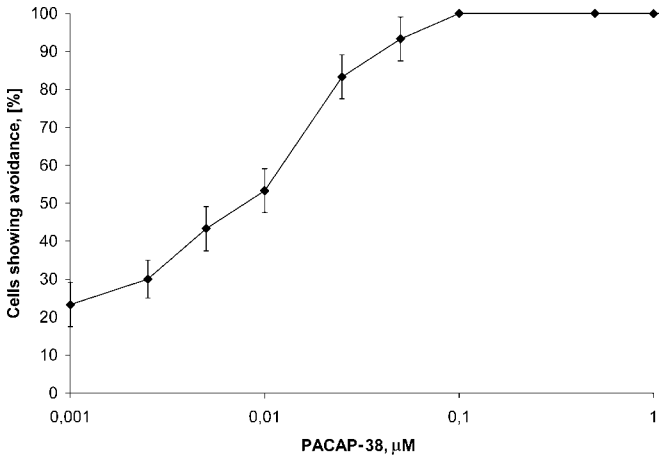

Fig. 1. PACAP-38 is an effective chemorepellent in *Tetrahymena*. *In vivo* behavioral bioassays (see Materials and Methods) were used to show the concentration dependencies for avoidance reactions to PACAP. The percentage of cells showing avoidance was determined by observation of a single cell after 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 three trials. Error bars, representing the standard deviation, are shown for each point. Minimum concentration required to give 100% avoidance was 0.1 μM for PACAP-38. This concentration was used in the inhibition studies that follow. The EC_{50} of this compound was approximately 0.01 μM.

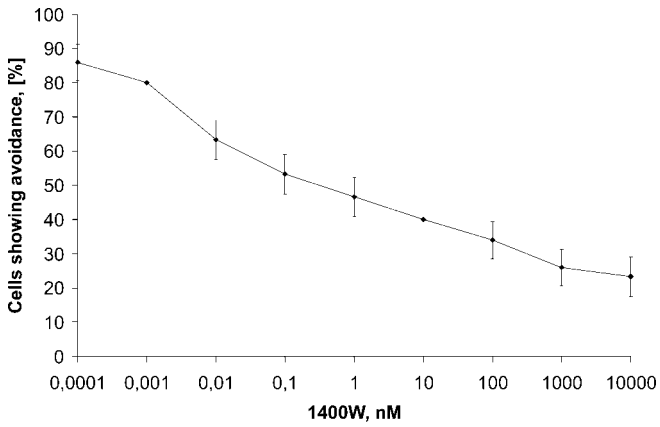

Fig. 2. *In vivo* behavioral assays (see Materials and Methods) show that the iNOS inhibitor, 1400W, reduced avoidance behavior to 0.1 μM PACAP to near baseline levels at a concentration of 10 μM 1400W. The IC_{50} of 1400W was approximately 1 nM.

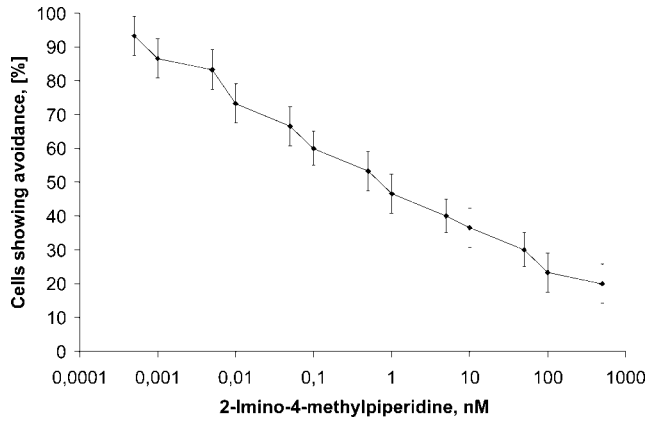

Fig. 3. *In vivo* behavioral assays (see Materials and Methods) show that the NOS inhibitor, IMP, reduced avoidance to 0.1 mM PACAP to baseline levels at a concentration of 500 nM IMP. The IC_{50} of IMP was approximately 1 nM.

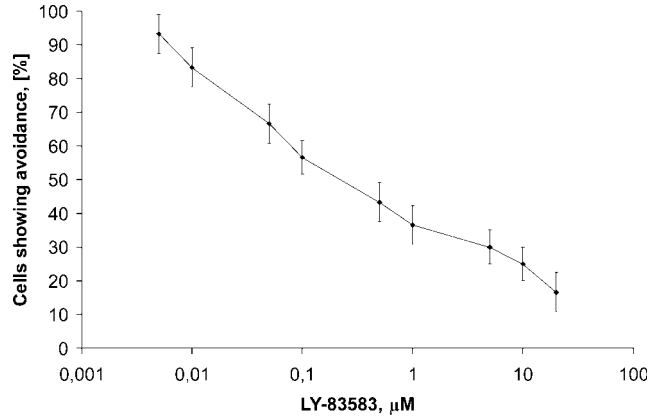

Fig. 4. *In vivo* behavioral assays (see Materials and Methods) show that the guanylyl cyclase inhibitor, LY-83583, reduced avoidance behavior to 0.1 mM PACAP to near baseline levels at a concentration of 20 mM LY-83583. The IC_{50} of LY-83583 was approximately 0.1 μM.

guanylyl cyclase in *Tetrahymena* has been documented (Linder and Schultz 2002), we used two guanylyl cyclase inhibitors, LY-83583, and NS-2028, in an attempt to inhibit PACAP-38 avoidance. Both inhibitors (Figs 4, 5) blocked avoidance in a concentration-dependent manner. An avoidance response near baseline was achieved using 20 μM LY-83583 (16.6 ± 5.8%), and 1 nM NS-2028 (13.3 ± 5.8%). The EC_{50} for LY-83583 was near 0.1 nM, and for NS-2028 was near 0.05 nM. These data implicate guanyl cyclase in PACAP-38 avoidance. In

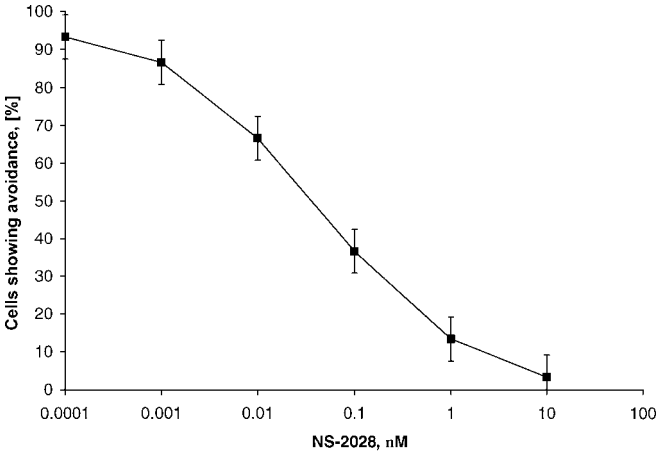

Fig. 5. *In vivo* behavioral assays (see Materials and Methods) show that the guanylyl cyclase inhibitor, NS-2028, effectively eliminated avoidance behavior to 0.1 mM PACAP at a concentration of 10 nM NS-2028. The IC_{50} of NS-2028 was approximately 0.05 nM.

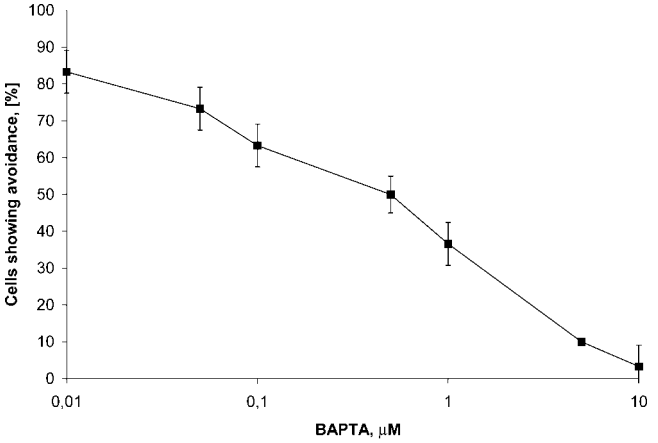

Fig. 6. *In vivo* behavioral assays (see Materials and Methods) show that the membrane-permeable calcium chelator, BAPTA-AM, effectively eliminated avoidance behavior to 0.1 mM PACAP at a concentration of 10 mM BAPTA-AM. The IC_{50} of BAPTA-AM compounds was 0.5 μM.

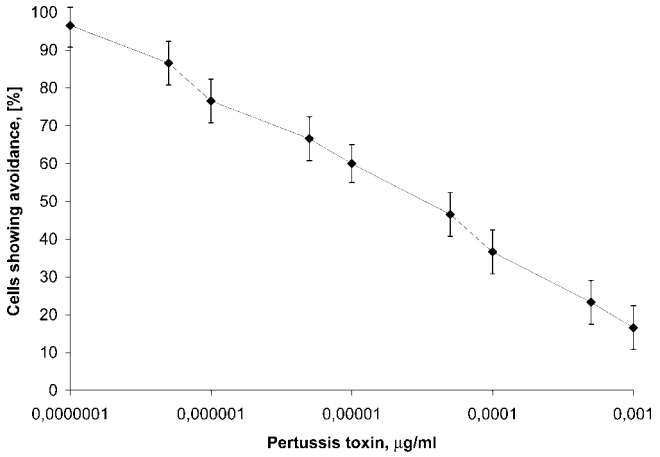

Fig. 7. *In vivo* behavioral assays (see Materials and Methods) show that pertussis toxin, an inhibitor of $G_{i/o}$ proteins, reduced avoidance behavior to 0.1 mM PACAP to baseline levels at a concentration of 0.001 mg/ml pertussis toxin. The IC_{50} of pertussis toxin was near 0.00001 mg/ml.

order to confirm these results biochemically, we used EIA assays performed on *Tetrahymena* lysate. Exposure to 1 μM PACAP-38 increased cGMP levels to 351 ± 4.6% of control levels.

Since many NO synthases are calcium-dependent, the membrane-bound guanylyl cyclase of *Tetrahymena* has been shown to be calcium dependent (Linder and Schultz 2002), and since *Tetrahymena* exhibit a calcium-based depolarization when stimulated with lysozyme (Kuruvilla and Hennessey 1998), which presumably acts through the same receptor as PACAP (Mace *et al.* 2000, Keedy *et al.* 2003), we used the membrane-permeable calcium chelator, BAPTA-AM, to see whether avoidance would be blocked. As seen in Fig. 6, exposure to 5 μM BAPTA-AM was sufficient to achieve baseline avoidance. The EC_{50} of this compound was 0.5 μM.

Previous studies (Hassenzahl *et al.* 2001, Keedy *et al.* 2003) indicate that the PACAP receptor is a G-protein linked receptor. However, the pertussis toxin sensitivity of this receptor has not previously been tested. We found that pertussis toxin was an effective inhibitor of PACAP avoidance, requiring a concentration of just 0.01 μg/ml to achieve baseline avoidance (Fig. 7). The EC_{50} of this compound was approximately 0.0001 μg/ml.

A proposed model of PACAP avoidance, based on these and previous studies, is shown in Fig. 8. Arrows broken with a question mark represent hypothetical steps in the model, while unbroken arrows represent confirmed steps in the pathway.

DISCUSSION

PACAP-38 is a convenient ligand to use for *Tetrahymena* avoidance assays because low concentrations are

required in order to achieve avoidance (Fig. 1; Mace *et al.* 2000, Hassenzahl *et al.* 2001, Keedy *et al.* 2003) and because the ligand has been well studied in other systems. In *Tetrahymena*, however, the pharmacological profile of the receptor is different from known PACAP receptors in other systems. For example, the antagonists, PACAP 6-27 and 6-38, which competitively inhibit many PACAP receptors actually serve as agonists for *Tetrahymena* PACAP receptors (Keedy *et al.* 2003). Previous studies (Hassenzahl *et al.* 2001) have shown that the PACAP-38 pathway in *Tetrahymena* is linked to a G-protein pathway which involves adenylyl cyclase and phospholipase C, presumably resulting in the formation of cAMP as well as the release of calcium from internal stores. Earlier studies (Kuruvilla and Hennessey 1998) link the lysozyme response, which presumably occurs through the same receptor as PACAP-38 (Mace *et al.* 2000) to a calcium-linked depolarization. However, the mechanism of avoidance has not yet been established. In this study, we present evidence that NO and cGMP are also critical for PACAP avoidance (Figs. 2-5). Intracellular calcium (Fig. 6) is also essential in order for avoidance to occur. A pertussis toxin-sensitive G protein is presumably involved (Fig. 7). How do all these data, together with that from other laboratories, come together to form a coherent model of PACAP avoidance? Questions remain, yet when the available data are compiled, a complex picture begins to emerge.

One of the first points made clear by the data is that calcium is critical for avoidance. When the membrane-permeable calcium chelator, BAPTA-AM, is introduced into the cells, avoidance ceases (Fig. 6). Interestingly, we have seen this same behavior with the repellents ATP and GTP (Rosner *et al*. 2003; Bartholomew *et al.*, submitted). This is consistent with past electrophysiological data (Kuruvilla and Hennessey 1998, Kim *et al.* 1999) showing that lysozyme, GTP, and ATP all exhibit calcium-based depolarizations. In addition, PACAP (Hassenzahl *et al.* 2001) and ATP (Rosner *et al.* 2003) also utilize the phospholipase C pathway, presumably releasing calcium from internal stores.

The roles for calcium in avoidance are also beginning to emerge. For example, many nitric oxide synthase isoforms are calcium dependent. Our pharmacological data with 1400W (Fig. 2) and IMP (Fig. 3) suggest a role for NO in PACAP avoidance, and our EIA detection assay confirms this result. We have seen similar results with ATP (Rosner *et al.* 2003) and GTP (Bartholomew *et al.*, submitted, personal comn.). Interestingly, 1400W is selective for iNOS isoforms, which are calcium independent, and the low concentrations of IMP needed for inhibition are also characteristic of an iNOS. NO has previously been implicated in *Tetrahymena* survival (Christensen *et al.* 1996); however, the type of NOS involved is unknown. It is possible that the NOS of *Tetrahymena* is calcium-dependent. Our data seem to support this, since the rise in NO is instantaneous, correlating with the calcium depolarization and presumably the phospholipase C pathway as well. However, inducible nitric oxide synthases are turned on at the transcriptional level, requiring hours to take effect (for example, see Arimoto and Bing 2003). Future experiments are required to determine whether the NOS of *Tetrahymena* is calcium-dependent.

Another possible role of calcium in avoidance is the triggering of guanylyl cyclase. In *Tetrahymena*, a membrane bound isoform of this enzyme has been shown to be calcium-dependent (Linder and Schultz 2002). Our pharmacological studies indicate that guanylyl cyclase is involved in PACAP-38 avoidance, as the guanylyl cyclase inhibitors, LY-83583 and NS-2028, block avoidance to PACAP-38 (Figs 4, 5). Both of these inhibitors have been previously shown to block soluble forms of guanylyl cyclase. If the guanylyl cyclase activated by PACAP-38 is indeed a soluble isoform, its calcium dependence is unknown. If, however, the drugs were blocking the membrane-bound cyclase mentioned previously, then calcium would have a role in cGMP formation.

As detected by EIA, cGMP is also produced at higher levels in cells, which have been exposed to PACAP-38. We have seen similar EIA and pharmacological results for ATP and GTP (Rosner *et al.* 2003; Bartholomew *et al.*, submitted), suggesting that the cGMP pathway, like intracellular calcium, may be critical for ciliary reversal.

Another potential role of calcium in avoidance has emerged from recent data. Hennessey *et al.* (2003), have shown that calcium-dependent ciliary reversal in *Tetrahymena* depends on properly functioning inner arm dynein 1 (I1). Their hypothesis is that a "calcium sensor" protein binds to and activates I1. The calcium sensor protein may be regulated by the NO/cGMP pathway or another of the pathways involved in avoidance.

The G-protein involved in avoidance is also interesting to consider. Our data suggest that the G-protein is pertussis toxin-sensitive (Fig. 7), a characteristic of $G_{i/o}$ proteins. This same pattern is seen in the ATP chemoresponse of *Tetrahymena* (Rosner *et al.* 2003). In many mammalian cells, PACAP acts through a G_s protein; however, in smooth muscle, PACAP acti-

vates a calcium-dependent, membrane-bound NO synthase *via* a G_{i1-2} protein (Murthy and Makhlouf 1996). Perhaps something similar is occurring during PACAP avoidance in *Tetrahymena*.

The diagram shown in Fig. 8 represents the pathways involved in PACAP-38 avoidance. As seen in the figure, the pathway is rather complex, and many questions still remain unanswered. This model represents a work of continuing research and will continue to develop as more is discovered regarding this specific signaling pathway and ciliary function in general.

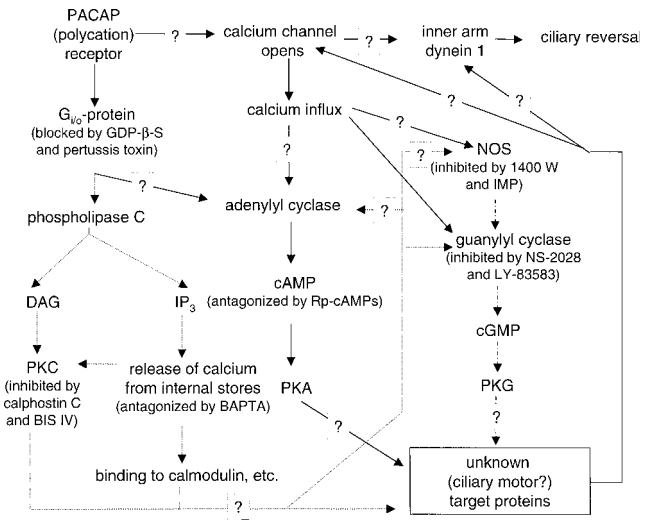

Fig. 8. Proposed mechanism for PACAP-38 avoidance in *Tetrahymena thermophila* based on current data. Dotted lines indicate components of the phospholipase C pathway. Dashed lines indicate components of the NO pathway. Arrows broken with a question mark indicate hypothetical components.

Further studies will be necessary to fully understand *Tetrahymena* avoidance behavior. The data we have obtained highlight the elegance and complexity of this model system for behavioral studies. Its adaptability for genetic experiments will make it an excellent candidate for future studies that may help us to understand protists better, as well as to gain a more complete knowledge of chemorepellent signaling in higher organisms.

REFERENCES

Arimoto T., Bing G. (2003) Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. *Neurobiol. Disease* **12:** 35-45

Christensen S. T., Kemp K., Quie H, Rasmussen L. (1996) Cell death, survival, and proliferation in *Tetrahymena thermophila*: effects of insulin, sodium nitroprusside, 8-bromo cyclic GMP, N^G-methyl-L-arginine, and methylene blue. *Cell Biol. International* **20:** 653-666

Dentler W. L. (1988) Fractionation of *Tetrahymena* ciliary membranes with Triton X-114 and the identification of a ciliary membrane ATPase. *J. Cell Biol.* **107**: 2679-2688

Forssmann W-G., Said S. I.; Eds. (1998) VIP, PACAP, and related peptides: Third International Symposium. *Ann. NY Acad. Sci.* 865

Hassenzahl D. L., Yorgey N. K., Keedy M. D., Price A. R., Hall J. A., Myzcka C. C., Kuruvilla H. G. (2001) Chemorepellent signaling through the PACAP/lysozyme receptor occurs through both cAMP and PKC in *Tetrahymena thermophila*. *J. Comp. Physiol. A* **187:**171-176

Hennessey T. M., Kim D.Y., Oberski D. J., Hard R., Rankin S. A., Pennock D.G. (2002) Inner arm dynein 1 is essential for Ca^{++}-dependent ciliary reversals in *Tetrahymena thermophila*. *Cell. Motil. Cytoskel.* **53:** 281-288

Iwamoto M., Nakaoka Y. (2002) External GTP binding and induction of cell division in starved *Tetrahymena thermophila. Eur. J. Cell Biol.* **81:** 517-524

Keedy M., Yorgey N., Hilty J., Price A., Hassenzahl D., Kuruvilla H. (2003) Pharmacological evidence suggests that the lysozyme/PACAP receptor of *Tetrahymena thermophila* is a polycation receptor. *Acta Protozool.* **42:** 11-17

Kim M. Y., Kuruvilla H. G., Raghu S., Hennessey T. M. (1999) ATP reception and chemosensory adaptation in *Tetrahymena thermophila*. *J Exp. Biol.* **202:** 407-416

Kuruvilla H. G., Hennessey T. M. (1998) Purification and characterization of a novel chemorepellent receptor from *Tetrahymena thermophila*. *J. Memb. Biol.* **162:** 51-57

Kuruvilla H. G., Hennessey T. M. (1999) Chemosensory responses of *Tetrahymena thermophila* to CB_2, a 24-amino acid fragment of lysozyme. *J. Comp. Physiol. A.* **184:** 529-534

Kuruvilla H. G., Kim M. Y., Hennessey T. M. (1997) Chemosensory adaptation to lysozyme and GTP involves independently regulated receptors in *Tetrahymena thermophila*. *J. Euk. Microbiol.* **44:** 263-268

Linder J. U., Schultz J. E. (2002) Guanylyl cyclases in unicellular organisms. *Mol. Cell. Biochem.* **230:** 149-158

Mace S. R., Dean J. G., Murphy J. R., Rhodes J. L., Kuruvilla H. G. (2000) PACAP-38 is a chemorepellent and agonist for the lysozyme receptor in *Tetrahymena thermophila*. *J. Comp. Physiol. A* **186:** 39-43

Murthy K. S., Makhlouf G. M. (1994) Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide-dependent activation of membrane-bound NO synthase in smooth muscle mediated by pertussis toxin-sensitive G_{i1-2}. *J. Biol. Chem.* **279:** 15977-15980

Orias E. (1998) Mapping the germ-line and somatic genomes of a ciliated protozoan, *Tetrahymena thermophila*. *Genomic Research* **8:** 91-99

Rosner B. N., Bartholomew J. N., Gaines C. D., Riddle M. L., Everett H. A., Rulapaugh K. G., Nickerson L. E., Marshall M. R., Kuruvilla H. G. (2003) Biochemical evidence for a P2Y-like receptor in *Tetrahymena thermophila. J. Comp. Physiol. A* **189:** 781-789

Zhu Y., Li H., Zhou L., Wu J.Y., Rao Y. (1999) Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron* **23:** 473-85

Received on 28th May, 2003; revised version on 22nd September, 2003; accepted on 27th October, 2003