The Journal of Experimental Biology 204, 1901–1907 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
JEB3376



# SYNTHESIS OF THE SIGNAL MOLECULE ACETYLCHOLINE DURING THE DEVELOPMENTAL CYCLE OF *PARAMECIUM PRIMAURELIA* (PROTISTA, CILIOPHORA) AND ITS POSSIBLE FUNCTION IN CONJUGATION

MARIA UMBERTA DELMONTE CORRADO[1,*], HUGUETTE POLITI[1], MARZIA OGNIBENE[1], CRISTIANO ANGELINI[2], FRANCESCA TRIELLI[1], PATRIZIA BALLARINI[3] AND CARLA FALUGI[2]

[1]*Department for the Study of the Territory and its Resources, University of Genoa, Corso Europa 26, I-16132 Genoa, Italy,* [2]*Department of Experimental, Environmental, and Applied Biology, University of Genoa, Viale Benedetto XV 5, I-16132 Genoa, Italy* and [3]*Department of Molecular, Cellular, and Animal Biology, University of Camerino, Via Camerini 2, I-62032 Camerino, Italy*

*e-mail: corrado@dipteris.unige.it

*Accepted 9 March 2001*

**Summary**

**We recently discovered, in mating-competent *Paramecium primaurelia*, the presence of functionally related molecules of the cholinergic system: the neurotransmitter acetylcholine (ACh), both its nicotinic and muscarinic receptors and its lytic enzyme acetylcholinesterase (AChE). Our results on the inhibition of mating-cell pairing *in vivo* in mating-competent cells treated with cholinomimetic drugs support the hypothesis that the cholinergic system plays a role in cell-to-cell adhesion. To investigate the possible function of the signal molecule ACh in conjugation in *P. primaurelia*, we attempted to detect the intracellular sites of ACh synthesis by localizing the ACh biosynthetic enzyme choline acetyltransferase (ChAT). Using immunocytochemical and histochemical methods, we have demonstrated the presence and activity of ChAT principally on the surface membrane of mating-competent cells and of mature but non-mating-competent cells. No evidence for ChAT activity was found in immature cells. Immunoblot analysis revealed the presence of immunoreactive bands, ranging in molecular mass from 42 to 133 kDa, as reported for ChAT isolated from higher organisms. *In vivo* experiments showed that inhibition of ChAT activity by Congo Red, known to be a potent competitive inhibitor of acetyl coenzyme A, did not affect mating-cell pairing. Conversely, inhibition of AChE with BW 284c51 or eserine, which block enzyme activity by reacting with a specific serine within the catalytic centre, significantly inhibited mating-cell pairing. Our results suggest that ACh has a negative modulating effect on conjugation in *P. primaurelia*.**

Key words: acetylcholine, choline acetyltransferase, mating type, cell-to-cell adhesion, conjugation, *Paramecium primaurelia*, Protista.

## Introduction

Conjugation, a sexual process of ciliates, includes two early events: cell recognition between mating-competent cells and cell-to-cell adhesion leading to mating-cell pairing (Hiwatashi, 1951; Hiwatashi, 1969). Whereas some of the molecules involved in cell recognition have been extensively characterized in a few ciliates (Luporini and Miceli, 1986; Luporini et al., 1996), the mechanisms involved in cell-to-cell adhesion and their possible homology to adhesion systems present in higher organisms are still unknown. *Paramecium primaurelia* appears to be an excellent biological model in which to study this process. In this species, cell-to-cell adhesion followed by cell pairing occurs soon after mixing of mature and appropriately starved cell populations belonging to the two physiologically discrete cell types able to pair in conjugation, referred to as mating type (mt) I and mt II.

Previous studies have demonstrated the involvement of the surface glycosidic residues sialic acid (NeuAc) and *N*-acetylglucosamine (GlcNAc) in the cell-to-cell adhesion process in *P. primaurelia* (Delmonte Corrado et al., 1997). However, the treatment of mt I and mt II cells with cholinomimetic drugs, either agonists or antagonists of acetylcholine (ACh) receptors, dramatically inhibited mating-cell pairing. This finding led to studies investigating both the presence of a cholinergic signalling system and its possible role in regulating cell interactions leading to cell-to-cell adhesion. It is known that the cholinergic system consists of a set of functionally related molecules, including the neurotransmitter ACh, its lytic enzyme acetylcholinesterase (AChE) and both its nicotinic (gating $Na^+$ channels) and muscarinic (associated with the transduction cascade) receptors (Laasberg et al., 1987; Bonner, 1989; Stroud et al., 1990; Le Novere and Changeux, 1995). Signal molecules such as ACh have been shown to be

involved in regulating cell interactions during cell-to-cell communication (Buznikov, 1990; Falugi, 1993), such as between gametes during fertilization (Baccetti et al., 1995). Using histochemical and immunocytochemical approaches, we showed for the first time that a ciliate protozoan possesses a complete set of molecules belonging to the cholinergic system, localized at the cell surface of mating-competent mt I and mt II cells (Trielli et al., 1997). True AChE activity, also detected in the cytoplasm of immature cells, was due to a 260 kDa molecule similar to the membrane-bound tetrameric form found in human erythrocytes (Delmonte Corrado et al., 1999), indicating that this enzyme belongs to a cell signalling system acquired early in evolutionary history.

In this paper, we investigated the possible function of the signal molecule ACh in the conjugation of *P. primaurelia*. As reported previously, receptor-bound ACh is quantitatively and rapidly hydrolyzed by AChE (Dale, 1914), so its intracellular localization is difficult to demonstrate, especially in a non-nervous system such as *Paramecium*. Consequently, we attempted to detect the intracellular sites of ACh synthesis in *P. primaurelia* by localizing the ACh biosynthetic enzyme choline acetyltransferase (ChAT). ChAT catalyses the acetylation of choline by acetyl coenzyme A (acetyl-CoA) (Nachmansohn and Machado, 1943); the presence of ChAT has been reported in nervous tissues and in a wide variety of non-neuronal tissues (Mautner, 1986; Wessler et al., 1999). We assessed ChAT activity histochemically and the presence of ChAT-like molecules both immunocytochemically and using immunoblotting procedures.

Mature and suitably starved cells able to conjugate, referred to as mating-competent mt I and mt II cells, were examined together with mature cells unable to conjugate because of their well-fed conditions, referred to as non-mating-competent cells, and immature cells unable to conjugate even under appropriate conditions of starvation. Furthermore, to investigate the modulating activity of ACh in cell-to-cell adhesion, experiments were performed in which mating-competent cells, before mixing, were exposed to either AChE- or ChAT -specific inhibitors. The inhibition of AChE exerted by BW 284c51 and eserine, which block enzyme activity by reacting with a specific serine residue within the catalytic centre (Kennedy, 1991), prevents AChE from cleaving ACh and leads to overproduction of ACh at its receptor sites (for a review, see Massoulié et al., 1993). Conversely, blockade of ChAT activity with Congo Red, known to be a potent competitive inhibitor of acetyl-CoA (Mautner et al., 1981), inhibits the synthesis of ACh, inducing a decrease in ACh levels.

## Materials and methods

### *Cells and culturing methods*

*Paramecium primaurelia* was cultured at 25 °C in lettuce medium (pH 6.8) inoculated with *Enterobacter aerogenes* (Sonneborn, 1970). Two mating-type stock 90, mt I and mt II cells, was used as experimental material, and one mating-type stock P, mt I cells, was employed as a tester stock.

Cell lines re-isolated daily entered autogamy in response to depletion of their food supply. The products of the first post-autogamous fission were allowed to multiply separately and maintained in logarithmic growth phase under well-fed conditions. Autogamy is normally followed by the maturity phase; therefore, cells growing logarithmically were mature but unable to conjugate because of their well-fed conditions (non-mating-competent cells). At the transition from the logarithmic to the stationary growth phase, resulting from depletion of the food supply and leading to starving conditions, the cells became able to conjugate (mating-competent cells) and expressed their mating type. The mt, II or I, was revealed by the capacity of the cells to pair with stock P mt I or stock 90 mt II cells, respectively. Cells unable to conjugate under starving conditions (immature cells) were obtained by allowing the vegetative progeny originating from 90×90 crosses to multiply and to reach the stationary growth phase within the immaturity period that usually follows conjugation.

### *Immunocytochemical staining*

To detect the presence of molecules immunologically related to ChAT (E.C.2.3.1.6.), mating-competent mt I and mt II cells, non-mating-competent cells and immature cells were fixed for 30 min in 2% paraformaldehyde in 0.1 mol $l^{-1}$ phosphate-buffered saline (PBS) (pH 7.4) and transferred onto microscope slides. Incubation was performed using anti-human placenta ChAT polyclonal antibody AB143, raised in rabbit (Chemicon International Inc., Temecula, CA, USA) and diluted 1:250 in PBS, and was carried out for 2 h at 37 °C in a moist chamber. The samples were then rinsed in PBS and incubated with anti-rabbit secondary fluorescein isothiocyanate (FITC)-labelled antibody (Sigma, Milan, Italy), diluted 1:250 in PBS, for 3 h at 37 °C in a moist chamber in the dark. Controls were incubated with the secondary antibody only.

### *Confocal microscopy*

We acquired optical sections using an optical confocal laser scanning microscope workstation (Bio-Rad M600) equipped with a krypton/argon laser and based on a Nikon Diaphot-TMD inverted microscope. Images were acquired by using a ×60 apoplan objective (numerical aperture 1.4). A BHS filter (488 nm) was used. The photomultiplier tube output and aperture were standardized to make the images as bright as possible against a minimal background on a control sample. These standard conditions were maintained for all measurements. We acquired collections of optical slices in confocal mode in 1 μm axial steps. Analysis was performed using the Comos software package MPL program provided by Bio-Rad (Melville, NY, USA).

### *Histochemical procedure*

For histochemical detection of ChAT activity, mating-competent mt I and mt II cells, non-mating-competent cells and immature cells were fixed as described above and incubated in

the medium suggested by Burt (Burt, 1970) for at least 4 h at 37 °C in a moist chamber. To this medium (4 mmol $l^{-1}$ choline, 0.2 mmol $l^{-1}$ acetyl-CoA, 1.8 mmol $l^{-1}$ lead nitrate and 0.2 mol $l^{-1}$ maleate buffer), 10 µl of BW 284c51 (anti-AChE) (Sigma) was added to prevent AChE already present in the cells from destroying any newly formed ACh, so inducing salt precipitation. Controls were incubated in the same mixture without acetyl-CoA. After staining, the cells were examined in a Zeiss microscope.

### *Immunoblot analysis*

Mating-competent mt I and mt II cells, non-mating-competent cells and immature cells were centrifuged at 600 ***g*** to a density of $40\times10^3$ cells $ml^{-1}$. The samples were treated with the protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (Sigma) and leupeptin (Sigma) at concentrations of 2 mmol $l^{-1}$ and 5 µg $ml^{-1}$, respectively, maintained at –80 °C for 10 min and centrifuged at 13 000 ***g*** for 30 min. The pellet, containing the membrane fraction, and the supernatant, containing the cytoplasmic fraction, were treated with 2 % sodium dodecyl sulphate (SDS) (Sigma) and kept at –20 °C for 48 h. After centrifugation at 16 000 ***g***, 2 % sample buffer (10 % SDS, 10 % glycerol, 0.25 mmol $l^{-1}$ Tris-HCl and 10 % β-mercaptoethanol, pH 6.8) was added to the supernatant, and the mixture was boiled for 5 min with commercial ChAT from human placenta (Sigma; 2.1 µg $\mu l^{-1}$), which was used as a control. The total protein content of the experimental samples was evaluated spectroscopically using the method of Millon-Nasse (Oser, 1965). Bromophenol Blue (Sigma) was added to all the samples, and protein (40 µg $\mu l^{-1}$) from each sample was layered onto the gel. ChAT-like molecules were separated on a 4 % stacking gel and an 8 % polyacrylamide gel with 10 % SDS (according to the method of Ornstein and Davis, 1962). Electrophoresis was performed at room temperature (25 °C) for 2 h at 150 V. The gels were then transferred to nitrocellulose according to standard procedures. The blots were incubated with anti-human placenta ChAT polyclonal antibody AB143, raised in rabbit (Chemicon International Inc., Temecula, CA, USA) and diluted 1:1000 in 5 % bovine serum albumin (BSA) in Tris-buffered saline/Tween-20 (TBS-T) and with secondary antibody anti-rabbit immunoglobulins, diluted 1:1000 in BSA in TBS-T, and conjugated with alkaline phosphatase (Sigma). The blots were stained using a NBT/BCIP kit (Boehringer, Mannheim, Germany). The resulting bands were compared with bands stained in the lane containing commercial ChAT, and molecular masses were estimated by comparison with a commercial molecular mass standard marker (Celbio, Milan, Italy).

### In vivo *AChE and ChAT activity inhibition assays*

BW 284c51 (anti-AChE) or eserine (anti-cholinesterases) (Sigma) solution ($10^{-3}$ mol $l^{-1}$), stored at –20 °C, was added to mating-competent mt I and mt II cell populations at a final concentration of $10^{-4}$ mol $l^{-1}$. After a 30 min incubation, 30 treated mt I and 30 treated mt II cells were mixed either with a treated or with an untreated cell population of the opposite mating type. Congo Red (anti-ChAT) (Sigma) solution ($10^{-2}$ mol $l^{-1}$), stored at 4 °C, was added to mating-competent mt I and mt II cell populations for 30 min at a final concentration of $10^{-3}$ mol $l^{-1}$. Then, 30 treated mt I and 30 treated mt II cells were mixed with untreated mt II and mt I cell populations, respectively. Other crosses, referred to as control crosses, were carried out by mixing 30 untreated mt I and 30 untreated mt II cells with untreated mt II and mt I cell populations, respectively. The number of pairs formed in experimental and control crosses was recorded for 60 min after mixing the cells. All experiments were performed at least in duplicate. Student's *t*-test was used to compare the mean number of pairs formed in the experimental crosses with the control mean.

## Results

### *Immunocytochemical staining*

Immunocytochemical staining, analyzed by confocal imaging, demonstrates the presence of molecules immunologically related to ChAT. In mating-competent cells, ChAT-like molecules are found mostly at the cell surface. In mt I cells, strong fluorescence is detected mainly at the anterior apex (Fig. 1Ai,ii), the site where mating-cell pairing starts. In mt II cells, the fluorescence is weaker and distributed within the cytoplasm, apparently in the food vacuoles (Fig. 1Bi,ii). Non-mating-competent cells exhibit a strong immunofluorescence over the whole cell surface (Fig. 1Ci,ii), whereas the labelling in immature cells is much fainter (Fig. 1D–I).

### *Histochemical detection of ChAT activity*

The activity of ChAT was revealed using the method of Burt (Burt, 1970), which produces a dark precipitate at the active sites. Such activity was detected at the cell surface in mt I cells (Fig. 2Ai,ii). The staining in mt II cells was weaker and was localized in the same areas (Fig. 2Bi,ii), whereas in immature cells no reaction was evident (Fig. 2Ci,ii). Non-mating-competent cells show strong ChAT activity localized at the cell surface (Fig. 2D).

### *Immunoblot analysis of ChAT-like molecules*

The immunoreactive bands obtained by electrophoresis of *P. primaurelia* proteins were compared with both a molecular mass standard (Fig. 3, lane A) and commercial ChAT from human placenta (Fig. 3, lane B); placental ChAT is detected as eight immunoreactive bands corresponding to different molecular forms. The other lanes in Fig. 3 (lanes C–L), containing membrane and cytoplasmic lysates from different stages of the developmental cycle of *P. primaurelia*, also contain multiple molecular forms, which vary according to the developmental stage. Four immunoreactive proteins (42, 46, 56 and 133 kDa) are found in the membrane fraction of non-mating-competent cells (Fig. 3, lane E). One of these proteins, 46 kDa, is absent from membrane lysates from mt I cells (Fig. 3, lane C) and another, 133 kDa, from membrane lysates

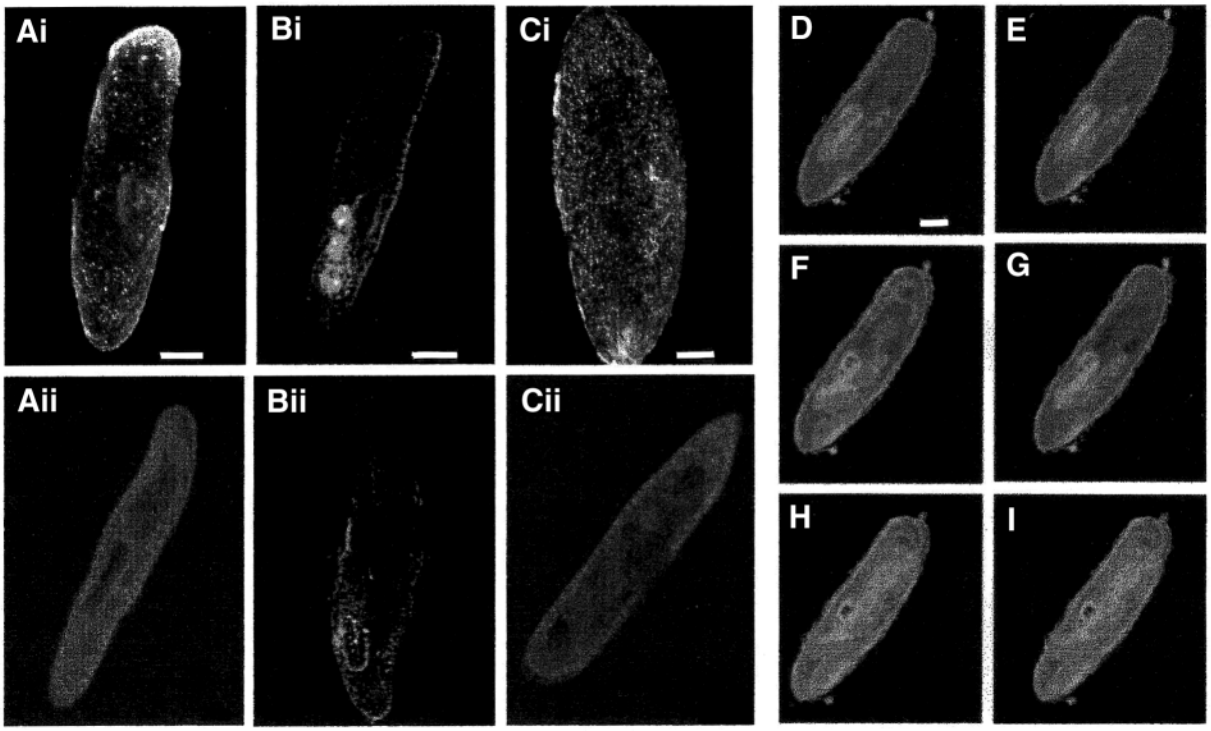


Fig. 1. Indirect immunofluorescence of choline acetyltransferase-related molecules analyzed by confocal laser scanning microscopy. Mating-competent mt I cell (Ai) and control (Aii). Mating-competent mt II cell (Bi) and control (Bii). Non-mating-competent cell (Ci) and control (Cii). Sections of an immature cell from the inside (D) to the surface (I). Scale bars, 20 μm.

from mt II cells (Fig. 3, lane D); in immature cells, only the two lightest proteins are present in membrane lysates (Fig. 3, lane F). The heaviest protein, 133 kDa, is absent from all cytoplasmic fractions. In cytoplasmic fractions from mating-competent mt I (Fig. 3, lane G), non-mating-competent (Fig. 3, lane I) and immature cells (Fig. 3, lane L), a further protein (62 kDa) is present; moreover, in cytoplasmic fractions from immature cells, the lightest protein (42 kDa) is absent. Only the two lightest proteins are present in cytoplasmic fractions from mating-competent mt II cells (Fig. 3, lane H).

### *Effects of inhibition of AChE and ChAT activity on conjugation*

Incubation of mating-competent mt I and mt II cell populations with inhibitors of AChE activity, BW 284c51 or eserine at a final concentration of $10^{-4}$ mol $l^{-1}$, prior to mixing treated cells with untreated cells of the opposite mating type inhibited mating-cell pairing in both cases. The mean number of cell pairs formed 60 min after mixing the BW-284c51-treated cells with untreated cells was significantly lower ($P$<0.001) than that in control crosses between untreated mt I and mt II cells (Table 1). A similar effect to that of BW 284c51 occurs in response to exposure to eserine: the percentage of pairs formed in the experimental crosses is less than 40 % compared with control crosses (Table 2). BW 284c51 treatment of both mt I and mt II cells before mixing results in the formation of a similar number of pairs to that recorded in crosses between BW-284c51-treated cells and untreated cells (Table 1). In crosses in which both mt I and mt II cells were exposed to eserine, the percentage of pairs formed is only approximately 20 % of the value for control crosses (Table 2). Exposure of mating-competent mt I and mt II cell populations to an inhibitor of ChAT activity, Congo Red at a final concentration of $10^{-3}$ mol $l^{-1}$, prior to mixing the treated cells with an untreated cell population of the opposite mating type does not affect mating-cell pairing. As shown in Table 3, the mean number of pairs formed in the experimental crosses does not differ significantly ($P$>0.05) from the number in the control crosses.

Table 1. *Effects on mating-cell pairing of exposure to BW 284c51, an inhibitor of acetylcholinesterase*

| Cross | Number of pairs formed | $t$ | $P$ |
|---|---|---|---|
| *mt I × mt II | 13±2.4 | 28.09 | <0.001 |
| mt I × *mt II | 15±1.4 | 34.40 | <0.001 |
| *mt I × *mt II | 13±1.6 | 37.10 | <0.001 |
| mt I × mt II | 27±1.3 | | |

The number of pairs formed was recorded 60 min after mixing mt I and mt II cells.

Values are means of at least two experiments ± S.D.

Student's $t$-test was used to compare the mean of the experimental crosses with the control mean (mt I × mt II).

Variances were homoscedastic.

*Exposed to $10^{-4}$ mol $l^{-1}$ BW 284c51.

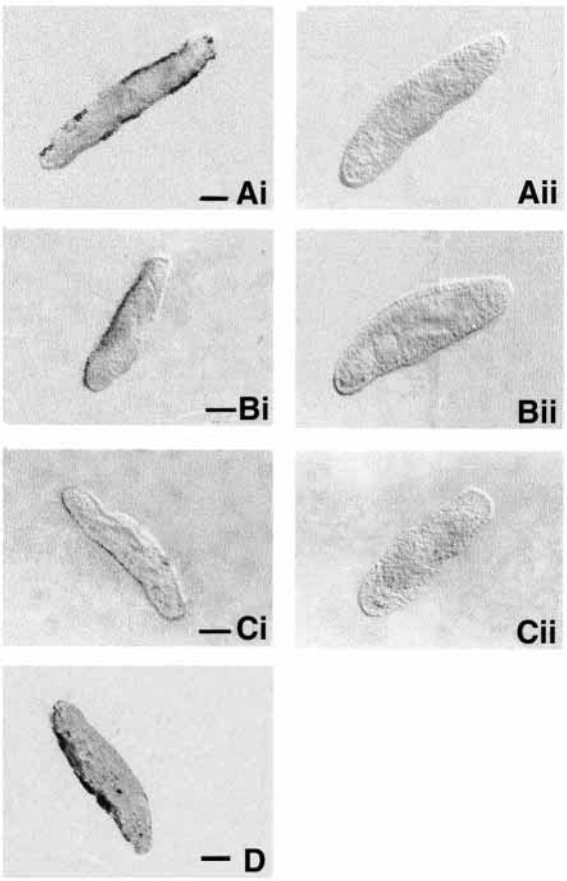


Fig. 2. Choline acetyltransferase activity, revealed as a dark precipitate, produced using the method of Burt (Burt, 1970). Mating-competent mt I cell (Ai) and control (Aii). Mating-competent mt II cell (Bi) and control (Bii). Immature cell (Ci) and control (Cii). Non-mating-competent cell (D). The control for the non-mating-competent cell is not shown because the complete absence of staining made it impossible to photograph. Scale bars, 20 μm.

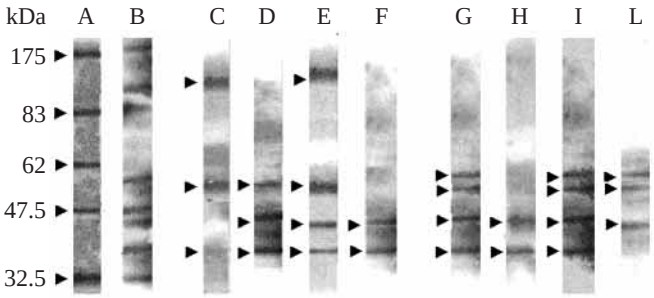


Fig. 3. Choline acetyltransferase (ChAT)-like molecules examined by immunoblot. Lane A, molecular mass standard marker; lane B, ChAT from human placenta; lanes C and D, membrane lysates from mating-competent mt I and mt II cells, respectively; lanes E and F, membrane lysates from non-mating-competent and immature cells, respectively; lanes G and H, cytoplasmic fractions from mating-competent mt I and mt II cells, respectively; lanes I and L, cytoplasmic fractions from non-mating-competent and immature cells, respectively.

Table 2. *Effects on mating-cell pairing of exposure to eserine, an inhibitor of acetylcholinesterase*

| Cross | Number of pairs formed | *t* | *P* |
|---|---|---|---|
| *mt I × mt II | 9±1.4 | 52.56 | <0.001 |
| mt I × *mt II | 10±2.3 | 36.61 | <0.001 |
| *mt I × *mt II | 5±2.6 | 42.66 | <0.001 |
| mt I × mt II | 28±1.4 | | |

The number of pairs formed was recorded 60 min after mixing mt I and mt II cells.

Values are means of at least two experiments ± S.D.

Student's *t*-test was used to compare the mean of the experimental crosses with the control mean (mt I × mt II).

Variances were homoscedastic.

*Exposed to $10^{-4}$ mol $l^{-1}$ eserine.

## Discussion

Our results demonstrate the presence and activity of ChAT at different stages of the developmental cycle of *P. primaurelia*. Immunoreactivity and enzyme activity were found in mating-competent mt I and mt II cells and in non-mating-competent cells, and they were localized mainly at the cell surface. However, by comparing the whole-cell ChAT immunofluorescence and histochemical localization of ChAT activity in mt I cells (Fig. 1A and Fig. 2A, respectively), it appears that not all the ChAT-like molecules detected using immunofluorescence are likely to be active at the same time. There is no evidence for the presence of ChAT activity in immature cells. Therefore, the synthesis of ACh appears to occur under conditions of maturity and at the cortical level of both mating-competent and non-mating-competent cells. This finding suggests a function for the cholinergic system in cell-to-cell, or at least in autocrine, signalling during the differentiation of mating type leading to the ability of cells to pair in conjugation.

The immunoblot analysis of mating-competent mt I and mt II cells, non-mating-competent cells and immature cells revealed the presence of several immunoreactive bands,

Table 3. *Effects on mating-cell pairing of Congo Red, an inhibitor of choline acetyltransferase*

| Cross | Number of pairs formed | *t* | *P* |
|---|---|---|---|
| *mt I × mt II | 24±3.5 | 1.5 | >0.05 |
| mt I × *mt II | 25±1.9 | 1.95 | >0.05 |
| mt I × mt II | 28±1.4 | | |

The number of pairs formed was recorded 60 min after mixing mt I and mt II cells.

Values are means of at least two experiments ± S.D.

Student's *t*-test was used to compare the mean of the experimental crosses with the control mean (mt I × mt II).

Variances were homoscedastic.

*Exposed to $10^{-3}$ mol $l^{-1}$ Congo Red.

ranging between 42 and 133 kDa, as reported for ChAT isolated from higher organisms (Brandon and Wu, 1978; Driskell et al., 1978; Malthe-Sørenssen et al., 1978; Ryan and McClure, 1979; Slemmon et al., 1982), distributed differently according to the stage of the developmental cycle. This finding is consistent with the pattern for commercial ChAT from human placenta, used as a control, which shows eight ChAT antibody-immunoreactive proteins in the range between 32.5 and 175 kDa. Four immunoreactive bands are found in *P. primaurelia* membrane lysates. Not all these proteins are enzymatically active because no histochemical reaction for ChAT was detected in immature cells displaying the two lightest bands, 42 and 46 kDa. These proteins may be produced by proteolytic degradation of ChAT, as reported by Badamchian and Carroll (Badamchian and Carroll, 1985) for mammalian brain low-molecular-mass forms. However, according to Grosman et al. (Grosman et al., 1995), the observation of Cozzari and Hartman (Cozzari and Hartman, 1983), that smaller ChAT-associated proteins can regulate ChAT activity, raises the possibility that these smaller molecules may play a role in the synthesis of ACh. The membrane lysates from both mating-competent and non-mating-competent cells contain, in addition, proteins with a molecular mass of 56 and 133 kDa. It is worth noting that the 133 kDa protein is absent from mt II cells, suggesting that the absence of this heaviest molecular form is related to mating type. Furthermore, the stronger histochemical reaction for ChAT in both mt I and non-mating-competent cells compared with mt II cells suggests that the 133 kDa protein may possess ChAT activity, as reported by Chao and Wolfgram (Chao and Wolfgram, 1973) for the 120 kDa ChAT molecular form found in bovine brain. The four immunoreactive bands present in the cytoplasmic lysates do not appear to possess catalytic activity because no histochemical reaction for ChAT was detected in the cytoplasm of the intact cells. We propose that the ChAT antibody-immunoreactive proteins found in this cell domain correspond to precursor proteins not yet possessing enzyme activity.

Taken together, these results suggest that cell-to-cell adhesion may be regulated by a signal, due to ChAT-synthesized ACh, originating principally in the mt I cells and received by the ACh receptors localized at the surface membrane in both mt I and mt II cells. Moreover, all these findings are in line with the localization of AChE activity, which is present within the cytoplasm of immature cells and at the surface membrane of mating-competent cells (Delmonte Corrado et al., 1999). Therefore, the activity of AChE in conjugation seems to be initiated in cells unable to conjugate and to be fully developed in mating-competent cells, which display AChE activity at their surface membrane, the site in the cell where ChAT synthesizes the target molecule for AChE.

Our results from *in vivo* experiments also demonstrate the role of the cholinergic system in conjugation. The effects of inhibition of ChAT and AChE activity suggest a negative modulating effect of ACh on mating-cell pairing, i.e. ACh would not promote cell pairing, which may be enhanced by other signal molecules, but would be able to decrease the ability of cells to conjugate or to prevent conjugation. The blockade of ACh synthesis seems not to affect cell-to-cell adhesion, while the blockade of the function of AChE in cleaving ACh appears to inhibit cell-to-cell adhesion. An excess of ACh at its receptor sites can have either an overexcitatory or an inhibitory effect on ACh receptor function, resulting in the blockage or impairment of cell function, as demonstrated in sea urchin spermatozoa (Falugi et al., 1993).

On the basis of the considerations mentioned above and our previous results, a possible functional scheme for the cholinergic system in *P. primaurelia* conjugation can be outlined. ACh synthesized by ChAT could be the signal molecule negatively modulating cell-to-cell adhesion performed by the surface glycoconjugates bearing NeuAc and GlcNAc as terminal glycosidic residues (Delmonte Corrado et al., 1997). ACh may interact with these surface-exposed glycosidic molecules so that their pattern of distribution would be modified according to the behaviour of the cell (the ability or inability to pair in conjugation) during the developmental cycle. In this light, the surface pattern of distribution of these glycosidic residues could be a result of a transduction cascade triggered by ACh-mediated excitation of muscarinic receptors located at the cell surface and associated with an intracellular transduction system activated by G-proteins (Laasberg et al., 1987; Bonner, 1989). We have shown previously (Trielli et al., 1997) that exposure to cholinomimetic drugs caused both an alteration in the surface pattern of distribution of glycosidic residues and an inhibition of mating-cell pairing. These results pave the way to further investigations focused on identifying other signal molecules involved in regulating mating-cell pairing and on detecting the surface glycoconjugates responsible for mating-cell pairing.

In conclusion, the cell-to-cell interactions during conjugation in *P. primaurelia* can provide a powerful model with which to investigate the first event during metazoan development, namely fertilization. Cell-to-cell interactions between gametes have been extensively studied in numerous animal groups on the basis of two different conceptual approaches related to the role of signal molecules, in particular neurotransmitter signalling (Eusebi et al., 1984; Falugi et al., 1990; Ibanez et al., 1991; Young and Laing, 1991; Baccetti et al., 1995; Kuo et al., 2000) and the role of the carbohydrate moieties of surface glycoproteins (for a review, see Rosati, 1995). The molecules involved in regulating and performing cell-to-cell adhesion in *P. primaurelia* make this biological system suitable for exploitation to elucidate how different mechanisms may interact at both the organism and cellular levels.

This work was supported by a 1999 grant from Ministero dell'Università e della Ricerca Scientifica e Tecnologica and by a 2000 grant from the University of Genoa (both to M.U.D.C.).

## References


**Baccetti, B., Burrini, A. G., Collodel, G., Falugi, C., Moretti, E. and Piomboni, P.** (1995). Localization of two classes of acetylcholine receptor-like molecules in sperms of different animal species. *Zygote* **3**, 207–217.

**Badamchian, M. and Carroll, P. T.** (1985). Molecular weight determinations of soluble and membrane-bound fractions of choline acetyltransferase in rat brain. *J. Neurosci.* **5**, 1955–1964.

**Bonner, T. I.** (1989). The molecular basis of muscarinic receptor diversity. *Trends Neurosci.* **12**, 148–151.

**Brandon, C. and Wu, J. Y.** (1978). Purification and properties of choline acetyltransferase from *Torpedo californica*. *J. Neurochem.* **30**, 791–797.

**Burt, A. M.** (1970). A histochemical procedure for the localization of choline acetyltransferase activity. *J. Histochem. Cytochem.* **18**, 408–415.

**Buznikov, G. A.** (1990). *Neurotransmitters in Embryogenesis*. Chur: Harwood Academic Publishers.

**Chao, L. P. and Wolfgram, F.** (1973). Purification and some properties of choline acetyltransferase (EC 2.3.1.6.) from bovine brain. *J. Neurochem.* **20**, 1075–1081.

**Cozzari, C. and Hartman, B. K.** (1983). An endogenous inhibitory factor for choline acetyltransferase. *Brain Res.* **276**, 109–118.

**Dale, H. H.** (1914). The action of certain esters of choline and their relation to muscarine. *J. Pharmac. Exp. Ther.* **6**, 147–190.

**Delmonte Corrado, M. U., Locatelli, D., Paleari, L. and Bottiroli, G.** (1997). Lectin-binding sites involved in *Paramecium primaurelia* mating pair formation. *J. Euk. Microbiol*. **44**, 303–308.

**Delmonte Corrado, M. U., Politi, H., Trielli, F., Angelini, C. and Falugi, C.** (1999). Evidence for the presence of a mammalian-like cholinesterase in *Paramecium primaurelia* (Protista, Ciliophora) developmental cycle. *J. Exp. Zool*. **283**, 102–105.

**Driskell, W. J., Weber, B. H. and Roberts, E.** (1978). Purification of choline acetyltransferase from *Drosophila melanogaster*. *J. Neurochem.* **30**, 1135–1141.

**Eusebi, F., Pasetto, N. and Siracusa, G.** (1984). Acetylcholine receptors in human oocytes. *J. Physiol., Lond.* **346**, 321–330.

**Falugi, C.** (1993). Localization and possible role of molecules associated with the cholinergic system during 'non nervous' developmental events. *Eur. J. Histochem.* **37**, 287–294.

**Falugi, C., Faraldi, G. and Tagliafierro, G.** (1990). Cholinesterase activity and neurotransmitter systems in amphibian gametogenesis. In *Cholinesterases*, vol. I (ed. J. Massoulié, F. Bacou, E. Barnard, A. Chatonnet, P. B. Doctor and D. M. Quinn), pp. 194–195. Washington DC: American Chemical Society.

**Falugi, C., Pieroni, M. and Moretti, E.** (1993). Cholinergic molecules and sperm functions. *J. Submicrosc. Cytol. Pathol.* **23**, 63–69.

**Grosman, D. D., Lorenzi, M. V., Trinidad, A. C. and Strauss, W. L.** (1995). The human choline acetyltransferase gene encodes two proteins. *J. Neurochem.* **65**, 484–491.

**Hiwatashi, K.** (1951). Studies on the conjugation of *Paramecium caudatum*. IV. Conjugation behavior of individuals of two mating types marked by a vital staining method. *Sci. Rep. Tohoku Univ. Ser. 4*, **19**, 95–99.

**Hiwatashi, K.** (1969). *Paramecium*. In *Fertilization*, vol. 2 (ed. C. B. Metz and A. Monroy), pp. 255–293. London, New York: Academic Press.

**Ibanez, C. F., Pelto-Huikko, M., Soder, O., Ritzen, E.,M., Hersh, L. B., Hokfelt, T. and Persson, H.** (1991). Expression of choline acetyltransferase mRNA in spermatogenic cells results in an accumulation of the enzyme in the postacrosomal region of mature spermatozoa. *Proc. Natl. Acad. Sci. USA* **88**, 3676–3680.

**Kennedy, S. W.** (1991). The mechanisms of organophosphate inhibition of cholinesterase. Proposal for a new approach to measuring inhibition. In *Chemical in Agriculture. Cholinesterase Inhibiting Insecticides. Their Impact on Wildlife and the Environment*, vol. 2 (ed. P. Mineau), pp. 74–87. Amsterdam, London, New York: Elsevier.

**Kuo, R. K., Baxter, G. I., Thompson, S. H., Stricker, S. A., Patton, C., Bonaventura, J. and Epel, D.** (2000). NO is necessary and sufficient for egg activation at fertilization. *Nature* **406**, 633–636.

**Laasberg, T., Pedak, A. and Neuman, T.** (1987). The muscarinic receptor-mediated action of acetylcholine in the gastrulating chick embryo. *Comp. Biochem. Physiol.* **86**C, 313–316.

**Le Novere, N. and Changeux, J. P.** (1995). Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. *J. Mol. Evol.* **40**, 155–172.

**Luporini, P. and Miceli, C.** (1986). Mating pheromones. In *The Molecular Biology of Ciliated Protozoa* (ed. J. G. Gall), pp. 263–299. Orlando, London: Academic Press.

**Luporini, P., Miceli, C., Ortenzi, C. and Vallesi, A.** (1996). Ciliate pheromones. In *Signaling Mechanisms in Protozoa and Invertebrates* (ed. G. Csaba and W. E. G. Muller), pp. 80–104. Berlin, Heidelberg: Springer Verlag.

**Malthe-Sørenssen, D., Lea, T., Fonnum, F. and Eskeland, T.** (1978). Molecular characterization of choline acetyltransferase from bovine brain caudate nucleus and some immunological properties of the highly purified enzymes. *J. Neurochem.* **30**, 35–46.

**Massoulié, J., Pezzementi, L., Bon, S., Krejici, E. and Vallette, F. M.** (1993). Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* **41**, 41–91.

**Mautner, H. G.** (1986). Choline acetyltransferase. In *Neuromethods*, series 1, *Neurochemistry Neurotransmitters Enzymes* (ed. A. A. Boutton, G. B. Baker and P. H. Yu), pp. 273–317. Clifton, NJ: Humana Press.

**Mautner, H. G., Merrill, R. E., Currier, S. F. and Harvey, G.** (1981). Interaction of aromatic dyes with the coenzyme A binding site of choline acetyltransferase. *Proc. Natl. Acad. Sci. USA* **78**, 7449–7452.

**Nachmansohn, D. and Machado, A. L.** (1943). The formation of acetylcholine, a new enzyme 'choline acetylase'. *J. Neurophysiol.* **6**, 397–403.

**Ornstein, L. and Davis, B.** (1962). *Disk Electrophoresis*, parts I and II. New York: Distillation Products Industries.

**Oser, B. L.** (1965). *Hawk's Physiological Chemistry*. London: McGraw-Hill Book Co.

**Rosati, F.** (1995). Sperm–egg interactions during fertilization in invertebrates. *Boll. Zool.* **62**, 323–334.

**Ryan, R. L. and McClure, W. O.** (1979). Purification of choline acetyltransferase from rat and cow brain. *Biochemistry* **24**, 5357–5365.

**Slemmon, J. R., Salvaterra, P. M., Crawford, G. D. and Roberts, E.** (1982). Purification of choline acetyltransferase from *Drosophila melanogaster*. *J. Biol. Chem.* **257**, 3847–3852.

**Sonneborn, T. M.** (1970). Methods in *Paramecium* research. In *Methods in Cell Physiology*, vol. 4 (ed. D. M. Prescott), pp. 241–399. New York, London: Academic Press.

**Stroud, R. M., McCarthy, M. P. and Shuster, M.** (1990). Nicotinic acetylcholine receptor superfamily of ligand-gated ion channels. *Biochemistry* **29**, 11009–11023.

**Trielli, F., Politi, H., Falugi, C. and Delmonte Corrado, M. U.** (1997). Presence of molecules related to the cholinergic system in *Paramecium primaurelia* (Protista, Ciliophora) and possible role in mating pair formation: an experimental study. *J. Exp. Zool.* **279**, 633–638.

**Wessler, I., Kirkpatrick, C. J. and Racke, K.** (1999). The cholinergic 'pitfall': acetylcholine, a universal cell molecule in biological systems, including humans. *Clin. Exp. Pharmac. Physiol*. **26**, 198–205.

**Young, M. J. and Laing, J. C.** (1991). The binding characteristics of cholinergic sites in rabbit spermatozoa. *Mol. Reprod. Devl*. **28**, 55–61.