Acetylcholine synthesis and possible functions during sea urchin development

C. Angelini, B. Baccetti,* P. Piomboni,* S. Trombino, M.G. Aluigi, S. Stringara, L. Gallus, C. Falugi
Department of Experimental, Environmental and Applied Biology (DIBISAA), University of Genoa;
*Center for the Study of Germinal Cells CNR, Institute of General Biology, University of Siena, Italy

Cholinergic neurotransmitter system molecules were found to play a role during fertilisation and early cell cycles of a large number of invertebrate and vertebrate organisms. In this study, we investigated the presence and possible function of choline acetyltransferase (ChAT, the biosynthetic enzyme of acetylcholine) in gametes of the sea urchin, *Paracentrotus lividus*, through localisation and functional studies. ChAT-like molecules were detected in oocytes, mature eggs and zygotes with indirect immunofluorescence methods. Positive immunoreactivity was found in the ovarian egg cytoplasm and surface as well as at the zygote surface. This suggests the eggs' capacity to autonomously synthesise acetylcholine (ACh), the signal molecule of the cholinergic system. Acetylcholinesterase (AChE, the lytic enzyme of acetylcholine) was also found in ovarian eggs, with a similar distribution; however, it disappeared after fertilisation. Ultrastructural ChAT localisation in sperms, which was carried out with the immuno-gold method, showed immunoreactivity in the acrosome of unreacted sperms and at the head surface of reacted sperms. In order to verify a functional role of ACh during fertilization and sea urchin development, *in vivo* experiments were performed. Exposure of the eggs before fertilisation to 1 mM ACh + 1 µM eserine caused an incomplete membrane depolarisation and consequently enhanced polyspermy, while lower concentrations of ACh caused developmental anomalies. The exposure of zygotes to 0,045 AChE Units/mL of sea water caused developmental anomalies as well, in 50% of the embryos. Altogether, these findings and other previously obtained results, suggest that the cholinergic system may subserve two different tasks during development, according to which particular type of ACh receptor is active during each temporal window. The first function, taking place in the course of fertilisation is a result of autonomously synthesised ACh in sperms, while the second function, taking place after fertilisation, is due to maternal ChAT molecules, assembled on the oolemma along with egg maturation and fertilisation processes.

Correspondence: Carla Falugi, University of Genoa, Department of Experimental, Environmental and Applied Biology (DIBISAA), viale Benedetto XV 5, 16132 Genoa, Italy.
Tel. international +39.010.3538055.
Fax. international +39.010.3538047.
E-mail: falugi@unige.it

In recent years, there has been an increasing awareness concerning the possible multiple-functional role of neurotransmitter molecules in cell-to-cell communication, or during cytoplasmic cytoskeleton arrangement mediated by intracellular ion changes (Falugi, 1993). These signalling systems might represent an ancestral mechanism of exchanging or transmitting cell messages, which evolved long before or along with structures as highly specialised and fast as synapses (Neumann and Nachmanson, 1975; Buznikov, 1980; Buznikov et al., 1996).

In male gametes of several invertebrate and vertebrate species, cholinergic neurotransmitter system molecules have been reported. For example, acetylcholinesterase (AChE, E.C. 3.1.1.7) activity was detected and quantified (Cariello et al., 1986) in flagellar structures of sea urchin sperms; at the same level, nicotinic receptors (nAChRs) were also detected (Nelson, 1976, 1978; Young and Laing, 1991). Such localisations were hypothesised to be related to sperm motility and function (Falugi et al., 1993). Bailey and Storey (1994) also reported muscarinic activation in mouse sperm. Recently in sperms of a great number of invertebrate and vertebrate species we found the concomitant presence of both nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors (Baccetti et al., 1995), and forwarded the hypothesis that the latter may be functional in the interaction with the egg. On the other hand, acetylcholine receptors have been found in eggs as well. In sea urchin eggs and in the following stages of early embryonic development, nicotinic receptors (Falugi and Prestipino, 1989), co-localised with AChE activity (Falugi et al., 1989) have been reported. Ivonnet and Chambers (1997) pharmacologically characterised such receptors as pertaining to the neuromuscular type, and showed that these nAChRs, upon excitation by specific ligands, are capable of gating Na+ entry, followed by membrane depolarisation. As described by Epel (1975), the first input of

egg membrane depolarisation by Na⁺ ions entry is caused by contact with the sperm. The result is a partial depolarisation which was shown to prepare the membrane for fusion with the sperm (McCulloh et al., 1987). This finding was confirmed by Jaffe (1980), who demonstrated that exposure to nicotine (cholinomimetic agonist of nAChR) can cause polyspermy.

As far as muscarinic acetylcholine receptors are concerned (mAChRs), we found that in the sea urchin, *Paracentrotus lividus*, molecules immunologically related to mAChRs are present in the cortical layer of unfertilised eggs, which are then exposed at the surface of the zygote membrane (Piomboni et al., 2001). After fertilisation, mAChRs are capable of responding to both acetylcholine (ACh) and carbachol, evoking an intracellular Ca^{2+} increase (Harrison et al., 2002). The presence of ACh had not been reported up to now, although several authors had hypothesised its role in vertebrate oocyte activation (Eusebi et al., 1979; 1984; Kusano, 1978; Kusano et al., 1977). This may be due to the fact that its secretion is quantal, and a small amount released is immediately hydrolyzed by the activity of AChE, which is present in both eggs of invertebrates (Piomboni et al., 2001) and vertebrates (Civinini et al., 1990), as well as in sperms (Falugi et al, 1991a).

In this study, an attempt was made to understand the possible localisation of ACh in early developmental events of the sea urchin, *Paracentrotus lividus*, by investigating the presence of molecules immunologically related to choline acetyltransferase (ChAT, E.C. 2.3.1.6), the biosynthetic enzyme of ACh. To be precise, the localisation in the cell membrane of such an enzyme indicates the cells ability to autonomously synthesise ACh. Moreover, by *in vivo* experiments of ACh exposure and ACh inhibition, a possible function of such an enzyme was studied, by analysing the effects on fertilisation and early development.

Materials and Methods

Unless otherwise specified, all materials were obtained from Sigma Chemical Company.

Gamete handling and fertilisation

Eggs from the sea urchin *Paracentrotus lividus* were obtained by intracoelomic injection of 0.5 M KCl and collected in ultra filtered seawater. Sperm was collected *dry* and stored at 4°C for no more than two days. For fertilisation, 5 μL of the dry sperm was added to 1 mL of sea water and 10 μL of this diluted sperm was added to the bowl containing the eggs in 10 mL of sea water. The extent of fertilisation was always checked 2-4 minutes after addition of sperm; experiments were carried out only when > 85% eggs raised the fertilisation layer (FL). Fertilisation and development were carried out al 19°C in a temperature-controlled room.

Electrophysiological recordings

The eggs were poured through a 100 μm mesh net, to remove the jelly coat, into a plastic Petri dish, coated with poly-l-lysine, and were penetrated by the microelectrode. Solution exchange was performed by introduction of clean sea water and then adding a new solution with a syringe. Eggs were impaled with a glass microelectrode filled with 3 M KCl. 100 μM ACh was added together with 10 μM eserine, an AChE inhibitor, in order to prevent degradation by AChE. The drugs, dissolved in sea water, were added to the eggs after the ΔV stabilisation at −70 mV. The sperms were added after 1 min. After addition of the drugs, the eggs were rinsed with clean sea water, to test the reversibility of the effect.

Cytochemical staining with aceto-lactic orcein

Slides carrying resin sections (obtained by use of the Kulzer 7100 resin, cut 3 μm with a Reichert microtome) were stained for 20 min in a solution of aceto-lactic orcein, then dried and mounted with DPX. Aceto-lactic orcein stains nucleic acids red-violet, including mRNA, so showing the transcription activity of nuclei.

Immunocytochemical reaction against molecules immunologically related to cholinergic molecules
A) Sperm-electron microscopy (ChAT).

Immunoreactivity against ChAT was obtained by pre-embedding, as follows: fixed cells were rinsed in cold PBS containing 0.5 M glycine, blocked with PBS containing 1% bovine serum albumin (BSA) and 5% normal goat serum (NGS). The incubation was carried out for 1h at room temperature or overnight at 4°C in the primary antibody diluted 1:200 in PBS-0.1% BSA -1% NGS. The primary antibody was a polyclonal antibody (AB 143) against choline acetyl transferase (ChAT, Biosys, France) raised in rabbit, diluted 1:500. After incu-

bation, samples were rinsed with PBS, and stained with a secondary anti-rabbit IgG conjugated to gold particles, diluted 1:100 in PBS containing 0.1% BSA and 1% NGS.

After dehydration, samples were embedded in Epon-Araldite, sectioned and observed at an electron microscope Philips CM10.

Specificity controls were performed by use of normal serum instead of the primary antibody, or by omitting the incubation in the primary antibody.

B) Eggs- ChAT immunofluorescent staining

Paraffin sections (5 μm thick) of oocytes and eggs were dewaxed and incubated for 4 h at room temperature with the primary antibody, diluted 1:250 in PBS containing NGS and BSA, as described above. After incubation, samples were rinsed in three changes of cold PBS and incubated for 2 h at room temperature in the dark in FITC-conjugated secondary antirabbit antibody (Cappel, Italy), diluted 1:200 in PBS. After thorough rinsing, sperms were mounted on a slide for microscopic analysis of immunoreactivity (IR) localisation by use of a Zeiss microscope, equipped with an UV apparatus and fluorescence filter set. Fading of fluorescence was prevented by mounting the samples with gelvatol (Lennet, 1978).

C) ACh- and AChE-like immunoreactivity

Some experiments were also performed by use of a polyclonal antibody anti-mammalian ACh conjugated with glutaraldehyde (Biosys, France), and anti-rabbit secondary antibody (Cappel, Italy). For AChE, a monoclonal antibody (BDA, Italy) and an anti-mouse FITC-conjugated secondary antibody (Cappel, Italy) were employed, as above described. The immunoreaction procedure and controls were performed as above described.

In vivo experiments:

A) Exposure of sperms to ACh

One mL of concentrated eggs were rinsed in sea water sterilised by ultra filtration (0.2 μm pores), and suspended in 25 mL of ultra filtered sea water. From this suspension, gently mixed by inversion, 1 ml was drawn and added to the exposure solution, containing ACh at concentrations of 10^{-3} M and 10^{-4} M. After 10 min, the eggs were rinsed twice in sea water, and placed in a fingerbowl containing 10 mL of sea water. The exposure lasted a short time, as ACh is promptly inactivated by sea water. These sperms were employed in the ratio 20/1 sperms/egg to fertilise the eggs, maintained in standard sea water. Embryos were collected after 15 min, 30 min and 70 min, to check the extent of fertilisation, the first cell cycle and their nuclear status by DNA staining. Other samples were collected after 20 h, to check their further development. Control samples were obtained by processing the eggs in the same way but without the addition of ACh.

B) Exposure to AChE

Inhibition of ACh was also performed by exposing the cells to AChE, the lythic enzyme of ACh. Exposure to AChE decreases the amount of ACh at the receptorial sites, mimicking an inhibition of ACh synthesis. Sperms and zygotes were exposed to different concentrations of AChE obtained from Electric eel by Sigma Chem. Co (I). AChE was used at 0.64 and 0.045 U/mL final concentrations in ultra filtered sea water (0.02 μm Millipore filter). Controls were performed by incubating the materials in normal ultra filtered sea water, using the same procedures.. Sperms were exposed for 30 min before being added to the eggs, in the previously mentioned proportions. Development took place in 30 mL finger bowls in a thermostated incubator at 18°C, supplied with a light/dark timer. Stages were then verified at 15, 30, 70 min, and 10 h (blastula stage). One fraction of the embryos was permitted to continue development as far as the pluteus stage.

Each of the exposure experiments were repeated 6 times with gametes from different specimens.

Analyses of exposed samples

Samples from exposed sperms were collected and fixed in 2% paraformaldehyde in sea water at 15 min, 30 min and 70 min after fertilisation. Samples from embryos exposed at the zygote stage were collected from the blastula stage (10 h development). From each finger bowl containing about 1000 embryos in 30 mL sea water, samples of about 100 embryos were drawn and fixed.

Morphological and histochemical analyses were then carried out on the samples.

Histochemical staining of DNA

Embryos stored in methanol were re-hydrated with 0.1 M citrate buffer, pH 7. The buffer was replaced twice, and then was substituted with a solution of 2,6-diamidine-4-phenylindole (DAPI; obtained from Sigma, I) 1:1000 in citrate buffer,
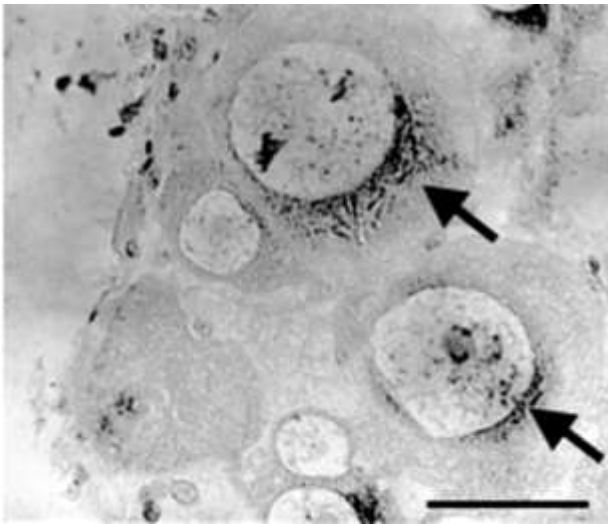
Figure 1. Ovarian oocytes at auxocytosis stage. Staining with aceto-lactic orcein shows active transcription of mRNA (arrows). 5 μm paraffin section. Bar equals 50 μm.

and incubated for 5 min in the dark. DAPI causes fluorescence of DNA, binding to A-T clusters in the minor groove of DNA. After rinsing with citrate buffer, samples were mounted on a slide supplied with antifading Gelvatol (Lennet, 1978) and observed on a Zeiss microscope, equipped with UV, at a λ = 360-400 nm wavelength.

Morphological analysis

Embryos collected from blastula up to pluteus stage were observed under a stereomicroscope Wild, at magnification X 50, and embryos presenting 3 different aspects were counted: 1: normal = embryos at the same stage as the controls; 2: anomalous = embryos differing from the controls; 3: uncleaved = zygotes in which development ceased after fertilisation.

Statistical analysis was performed by the ANOVA test, according to Sokal and Rolf (1981)

Results

Localisation studies

The histochemical and immunohistochemical analyses revealed a frame of cholinergic molecules starting from the initial events of gametogenesis and gamete interaction.

The first events that take place in the course of gametogenesis, seem to be related to the transcription and synthesis of molecules that will be active in
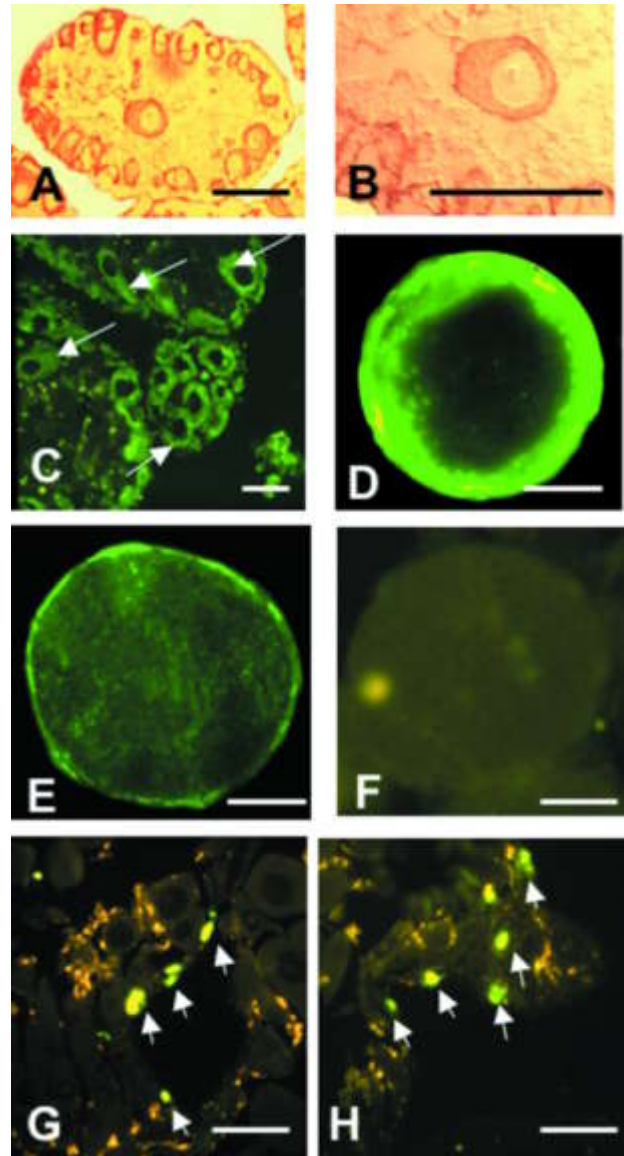

Figure 2. A, B: Ovary with oocytes at auxocytosis stage. ChAT immunoreaction marked by HRP reaction (brown precipitation). C-H: ChAT immunoreaction revealed by FITC (immunofluorescence = IR); C: ovarian oocytes at the beginning of auxocytosis, D: IR in mature unfertilised egg; E: IR in mature fertilised egg; F: control; G,H: IR for ACh in maturing ovaries: only somatic cells are stained by the fluorochrome. 5 μm paraffin section. Bars equal 50 μm for all the figures.

the following steps of cell interaction: aceto-lactic orcein staining of ovaries (Figure 1) showed the presence of nucleic acids in the cytoplasm, indicating the presence of maternal transcripts, with a polarised localisation and a gradient between the nucleus and the periphery. In the course of these early stages of oogenesis, molecules immunologically related to ChAT were found to be differently localised in relation to the egg stage. In immature
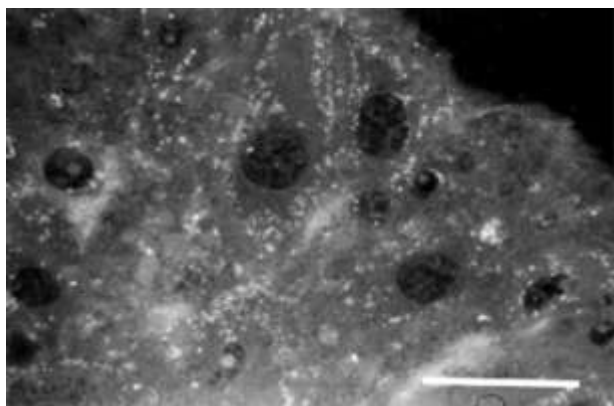
Figure 3. AChE immunofluorescence in oocytes during the aux-ocytosis stage. The enzyme is contained within intracytoplasmic vesicles, and not yet exposed at the egg surface. 5 μm paraffin section. Bar equals 50 μm.

Figure 4. ChAT immuno-gold reaction in *P. lividus* sperms. A: reacted sperm head; B: unreacted sperm head; C: control. (magnification X 20,000).

oocytes, recognised thanks to their large nucleus to cytoplasm ratio, ChAT IR was mainly found in per-inuclear locations. In the cytoplasm, positive zones showing the same polarity seen for aceto-lactic orcein, were observed (Figure 1). The ChAT-like immunoreactive molecules appeared as stained dots, in the cytoplasm, and, with a homogeneous distribution, in the cortical layer (Figure 2 A-C).

In mature unfertilised eggs, ChAT IR was found in a large cortical zone of cytoplasm, and in some regions of the egg membrane (Figure 2 D), while after fertilisation the staining was mainly associated with the membrane (Figure 2 E). No staining was present in control samples (Figure 2 F).

To verify whether the ChAT molecules revealed with the immunocytochemical reactivity were active at these stages, the presence of ACh was investigated by immunoreactive labelling. In ovaries, cells associated to the somatic portion presented ACh IR, but no staining was evident either in the oocytes (Figure 2 G-H), or in mature eggs (not shown). This suggests that ChAT-like molecules are present in the oocytes as far as fertilisation, but in an inactive form; the activity begins after fertilisation, when ChAT molecules are assembled at the zygote membrane to finalise their cholinergic function in ACh synthesis, as we previously reported (Piomboni et al., 2001).

AChE-like molecules were also synthesised early in the ovarian oocytes, where they appeared as small fluorescent vesicles under AChE immunoreactivity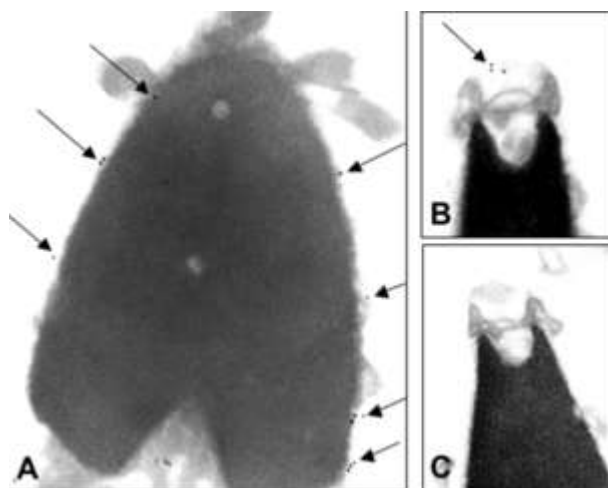. Positive vesicles were found in different positions in the course of egg maturation (Figure 3), and were assembled on the membrane in mature eggs (see Piomboni et al., 2001).

These observations reveal that a complete cholinergic set of molecules is ready to trigger signal transduction mechanisms related to fertilisation. Since ACh was not found at these stages, we hypothesised that the first signal might be carried by the sperm. To be precise, ChAT-like immunoreactivity, in the sperms, revealed by immunogold particles, was mainly localised in the head. In immotile sperm (*fixed-dry sperm*), gold particles were particularly concentrated in the acrosoma vesicle (Figure 4 A). However, when sperms began actively swimming as a result of their suspension in water containing eggs, the gold particles were observed in the membrane all along the head region (Figure 4 B). No staining was detected in controls (Figure 4 C). These results show that the sperm autonomously synthesises ACh, and that this function is active as far as the first sperm-egg interaction.

Functional studies

To verify this assumption, we performed *in vivo* experiments, by investigating the effects of variations of the amount of ACh on fertilisation and early developmental events.

The electrophysiological recordings of fertilisation potential in eggs exposed to 100 μM ACh and eserine showed that the degree of membrane depolarisation occurred to about -25 mV. However, subse-
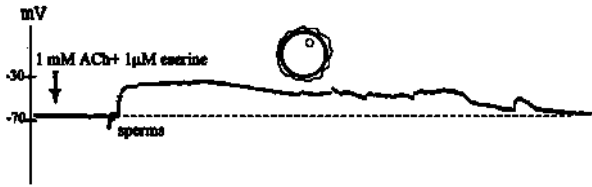
Figure 5. Electrophysiological recording of fertilisation membrane potential in eggs exposed to 1mM ACh + 1 μM eserine.

quent repolarisation was slow and irregular, lasting more than 30 min, and wasn't affected by the addition of neuroactive substances (Figure 5). Moreover, even though the cortical reaction took place normally, the fertilisation layer immediately collapsed on the egg membrane. This was probably caused by a loss of pressure throughout the perivitelline space, as a result of the perforation caused by the microelectrode.

In fertilisation experiments in presence of ACh, 15% zygotes showed polyspermy, as previously reported (Figure 6) (for the statistical validation of data, see Harrison et al., 2002).

Effects on development

In eggs fertilised with sperms exposed to 0.045 AChE Units/mL sea water, development proceeded normally as far as gastrulation. In zygotes exposed to 0.67 Units/mL after fertilisation, from the blastula stage onwards anomalies were evident, particularly in the development of the perioral arms of plutei (Figure 7 A,B: P<0.05). In zygotes exposed to 0.045 Units/mL, a strong effect was seen, as shown in Figure 7 B:

In Figure 7 E the histograms show the effect of both the exposures as compared to controls, when the morphology of embryos was observed at 10 h development, corresponding to blastula stage in controls. The exposure to 0.67 Units/mL sea water caused significant number of anomalous embryos, as compared with the lower concentration and with controls (p<0.05). In each exposure experiment, including controls, some uncleaved eggs were also found, but in this case the difference among the treatments was not significant (P=0.76).

Effects of exposure to ACh

Electrophysiological recordings of membrane potential showed that eggs, which had been exposed to ACh before fertilisation and fertilised in the presence of the signal molecule, maintained the ΔV of -70 mV, while the fertilisation potential was incomplete as the ΔV reached -30 mV, with a variation of only 40 mV (Figure 5). As a result, eggs exposed to 10^{-4} M ACh caused a 25% increase of polyspermic zygotes (Figure 6). Non-polispermic eggs followed a delayed/abnormal development (Figure 7 B,C).

Discussion

From the described results, and along with previous data obtained by our group (Baccetti et al., 1995; Falugi et al., 1993; Piomboni et al., 2001; Harrison et al., 2002), we can forward the hypothesis that cholinergic related molecules may play two different roles during the very early steps of sea urchin development, from fertilisation to the early cell cycles.

The cholinergic molecules present in the sperm acrosome might play a role in sperm-egg interaction, as hypothesised by Ibanez et al. (1991). ChAT molecules and their mRNAs were found by Ibanez et al. (1991) in the equatorial ring of the human sperm; at the same level we found the presence of mAChRs (Baccetti et al. 1995). This is the portion of the acrosome membrane that first comes into contact with the egg. In the sea urchin this contact takes place at the tip of the acrosome, where we have
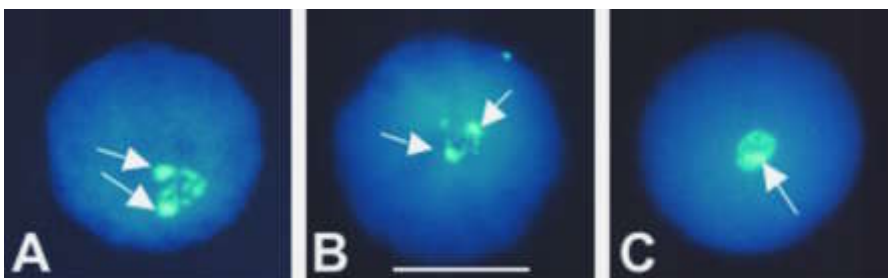

Figure 6: DAPI staining of zygotes fertilised after exposure to 0.1 mM ACh. A,B: two male pronuclei are seen as white spots close to the female pronucleus (arrows). C: Control. a single sperm chromatine is beginning to decondense within the female pronucleus (samples mounted in toto, bar equals 80 μm).
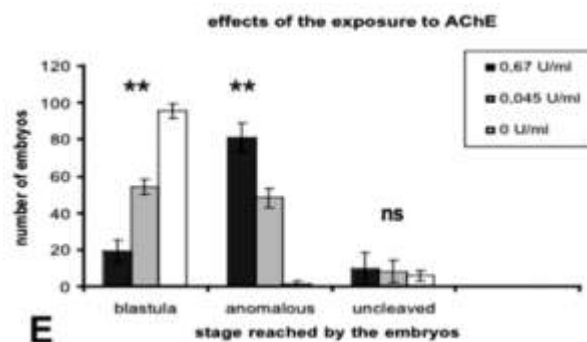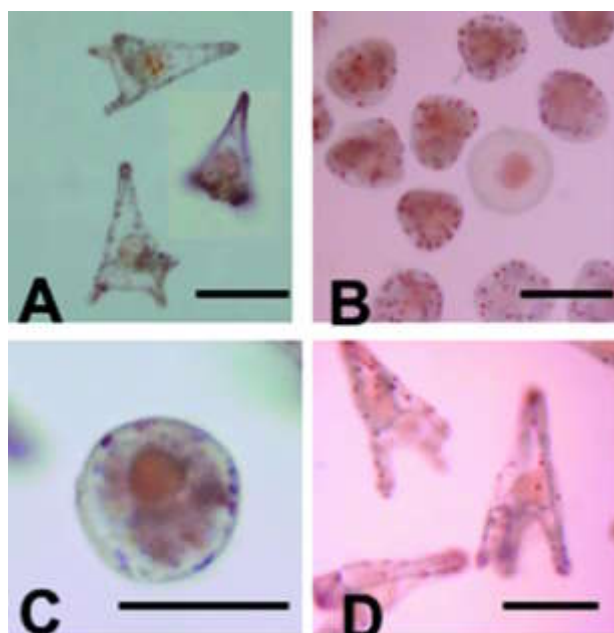
effects of the exposure to AChE

Figure 7. Morphological aspects of A: embryos obtained from eggs fertilised in the presence of 0.67 U/mL AChE by AChE-exposed sperms; B,C: embryos obtained from ACh-exposed eggs and fertilised in the presence of 0.1 mM ACh; D: control. Bars equal 100 µm for all the figures. E: histogram showing the frequency of anomalies in embryos exposed to AChE along development and observed at time corresponding to blastula stage in control embryos (0 U/ml). ** = p<0.05; ns: not significant.

found ChAT immunoreactivity (IR); at the same level mAChRs had been previously detected (Baccetti et al., 1995). Hence, the mAChRs present in the sperm acrosome may be activated at the first contact with the egg thanks to the ACh received from the mature egg membrane (where ChAT is partially exposed at the surface, as shown in Figure 2).

Thus, we forward the hypothesis that, prior to fertilisation, ACh autonomously synthesised in the egg membrane may be functional for sperm activation, through mAChRs present on the acrosome membrane (Baccetti et al., 1995) and through calcium intake by Ca^{2+} channels, while ACh synthesised by ChAT in the sperm membrane may be functional for the first depolarisation step as a result of nicotinic receptor activation; this enables sperms to fuse with the oolemma (the scheme of this hypothesis is shown in Figure 8).

This is supported by the fact that the sea urchin unfertilised egg has functional neural-type nicotinic AChRs in the membrane (as shown by Ivonnet and Chambers, 1997). Thus, the ChAT molecules present on the sperm head membranes appear to play a role in providing ACh to activate nicotinic receptors. Nicotinic receptors are channel molecules, that upon ACh activation cause a Na^+ influx (Changeux, 1994).Egg membrane depolarisation, due to Na^+ influx in the sea urchin, first described by Epel (1975), is very similar in amplitude to the one caused by ACh exposure in the experiment above

described. We now know that this step is crucial to the preparation of the membrane for fusion with the sperm (McCulloh et al., 1987). This may be confirmed by the fact that exposure to ACh causes a high percentage of polyspermic zygotes (Harrison et al., 2002), and that nicotine exposure also impairs the block to polyspermy (Jaffe, 1980; Falugi et al. 1993).

ChAT IR changes its localisation from inside the membrane at the tip of the acrosome in non-reacted sperms, to a scattered distribution over all the head membrane in reacted sperms: this is probably due to different functions at different moments of sperm-egg interaction; in fact, it is known that ChAT activity is regulated by its binding to the cholinergic membrane (Dobransky and Rylett, 2003) and by interaction with other cellular proteins related to this membrane (Gabrielle et al., 2003): in other words, the enzyme is not active when it is inside the cytoplasm, while it is active when it is exposed on the cholinergic membrane. This is a common feature of cholinergic molecules, including mAChRs, that are functional only when assembled on the cell surface, while inactive subunits are contained in ER-derived vesicles (Mei and Xiong, 2003). This supports our observations, indicating that no ChAT activity is present during oogenesis, although the enzyme is present. In this case, the antibody may bind to an epitope that is common to unassembled and assembled forms of the molecule, and this is not
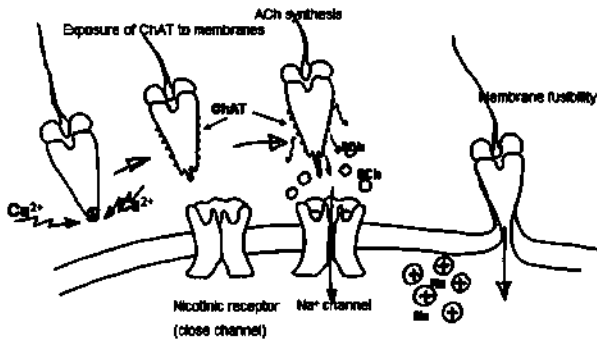
Figure 8. Scheme of the possible function of ChAT found on the sperm head during fertilisation.
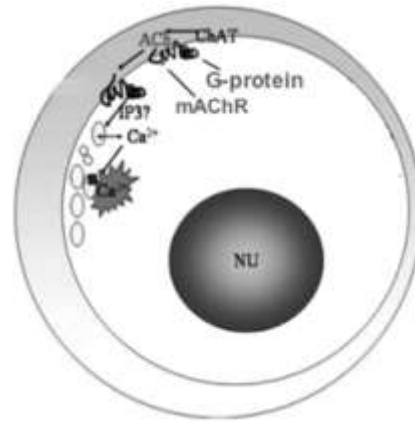

Figure 9. Possible autocrine mechanism in modulating Ca^{2+}-dependent development after fertilisation, via muscarinic receptors and subsequent G-protein activation. ACh = acetylcholine; ChAT = Choline-acetyltransferase; IP3 = inositol-triphosphate; mAChR = mature ACh receptor; NU = nucleus.

discriminated by specificity controls.

ChAT IR was observed at all stages of egg maturation, starting from the stage of auxocytosis: in these immature eggs, ChAT immunoreactive molecules were mainly retained in the sites of synthesis and elaboration, with a perinuclear to cytoplasmic localisation. Since in the course of maturation, no ACh was revealed by immunohistochemical methods in the eggs, this shows that ChAT IR at these stages is capable of binding inactive forms of the enzyme as well. Actually, in the ovary, ACh IR was only found in somatic cells, where it is probably associated to ovarian functions, such as the regulation of spawning, as described by Yokota (2000). In mature unfertilised eggs, the perinuclear localisation of ChAT IR is no longer detectable, and the enzyme is localised within the cytoplasm, towards the cortical layer, where it stains heavily: after fertilisation, the enzyme is present only at the membrane surface. In unfertilised eggs, we also found mAChRs in membrane vesicles in the cortical layer, and after fertilisation in the plasmalemma, (Piomboni et al., 2001), where they can be reached by ACh and cholinomimetic drugs.

The activation of $[Ca^{2+}]_i$ responses by muscarinic drugs was possible only after fertilisation, and no Ca^{2+} response was seen on preceding events, such as fertilisation (Harrison et al., 2002); thus, we advanced the hypothesis that mAChRs could be involved in the regulation of intracellular ionic dynamics related to the cleavage of the first cell cycles (Harrison et al., 2002). This seems to confirm the previous hypothesis, suggesting a model of autocrine regulation of these dynamics. To be precise, the ChAT molecules, that after fertilisation are exposed at the egg surface, may autonomously synthesise ACh. The egg cannot receive ACh from elsewhere, because after fertilisation the zygote is closed in the fertilisation membrane, completely surrounded by sea water, and transported by waves and currents. The ACh produced by the ChAT assembled on the egg membrane is released into the perivitelline space and reaches the mAChRs that at this stage are also exposed at the surface (Piomboni et al., 2001). As a result, it evokes a $[Ca^{2+}]$ response (Figure 9), that further enhances development.

These events are not involved in the calcium-induced cortical reaction (fast block to polyspermy), where other signal molecules play a relevant role: it has recently been discovered by Kuo et al. (2000) that egg activation and cortical reaction is caused by nitric oxide synthase (NOs), contained in the sperm head, whose activation to produce nitric oxide (NO) in the egg cytoplasmic domain causes both inositol-triphosphate (IP3) release and cyclic adenosyn diphosphate ribose (cADPR) synthesis.

Acknowledgments

References

Baccetti B, Burrini AG, Collodel G, Falugi C, Moretti E, Piomboni P. Localisation of two classes of acetylcholine receptor-like molecules in sperms of different animal species. Zygote 1995; 3:207-17.

Bailey JL, Storey L. Muscarinic activation of mouse sperm. Molec. Reprod. Devel 1994; 39:297-308.

Bonner TI. The molecular basis of muscarinic receptor diversity. Trends Neurosci 1989; 12:148-151.

Buznikov GA.. Biogenic monoamines and acetylcholine in protozoa and metazoan embryos. In: Salanki J, Turpaev M, eds. Neurotransmitters: Comparative Aspects. Akademial Kiado, Budapest,1980, pp. 7-28.

Buznikov GA, Koikov LN, Shmukler Yu B, Whitaker MJ. Nicotinic antagonist (piperidines and quinuclidines) reduce the susceptibility of early sea urchin embryos to agents evoking calcium shock. Gen Pharmac 1997; 29:49-3.

Buznikov GA, Shmukler YB, Lauder JM. From oocyte to neuron: do neurotransmitters function in the same way throughout development? Cell Molec Neurobiol 1996; 16:533-59.

Cariello L, Romano G, Nelson L. Acetylcholinesterase in sea urchin spermatozoa. Gamete Res 1986; 14:323-32.

Civinini A, Gallo VP, Mastrolia L. Fine localisation of the acetyl-cholinesterase activity in somatic and germ cells during the morphogenesis of chick ovarian cortex. Cell Molec Biol 1990; 36:497-508.

Dobransky T, Rylett RJ. Functional regulation of choline acetyltransferase by phosphorylation. Neurochem Res 2003; 28:537-542.

Epel D. The program and mechanism of fertilisation in the echinoderm egg. Am Zool 1975; 15:507-22.

Eusebi F, Mangia F, Alfei L. Acetylcholine-elicited responses in primary and secondary mammalian oocytes disappear after fertilisation. Nature 1979; 277:651-53.

Eusebi F, Pasetto N, Siracusa G. Acetylcholine receptors in human oocytes. J Physiol 1984; 346:321-30.

Falugi C. Localisation and possible role of molecules associated with the cholinergic system during "non nervous" developmental events. Eur J Histochem 1993; 37:287-94.

Falugi C, Borgiani L, Faraldi G, Tagliafierro G, Toso F, Drews U. Localisation and possible functions of neurotransmitter systems in sperms of different animal species. In: Baccetti B, ed. Comparative Spermatology, 20 years later, vol. 75. Raven Press, New York, 1991a, pp. 475-79.

Falugi C, Delgaudio R, Angelini C, Geraci G. Calcium signalling in sea urchin zygotes. Dev. Biol. Of SEa Urchin XIII, MBL, Woods Hole, Sept 29-Oct 2, 2000.

Falugi C, Pera C, Prestipino G. Localisation of α-bungarotoxin receptors and AChE activity in eggs and embryos of Paracentrotus lividus Lamk. Acta Embryol Morphol Exp n.s. 1989; 10:113-19.

Falugi C, Pieroni M, Moretti E. Cholinergic molecules and sperm functions. J Submicrosc Cytol Pathol 1993; 25:63-9.

Falugi C, Prestipino G. Localisation of putative nicotinic cholinoreceptors in the early development of Paracentrotus lividus. Cell Molec Biol 1989; 35:147-61.

Florman HM, Storey BT. Characterisation of cholinomimetic agents that inhibit in vitro fertilisation of the mouse. Evidence for a sperm-specific binding site. J Androl 1982; 3:157-64.

Gabrielle P, Jeana M, Lorenza EC. Cytosolic choline acetyltransferase binds specifically to cholinergic plasma membrane of rat brain synaptosomes to generate membrane-bound enzyme. Neurochem Res 2003; 28:543-9.

Harrison PK, Falugi C, Angelini C, Whitaker MJ. Muscarinic signalling affects intracellular calcium concentration during the first cell cycle of sea urchin embryos. Cell Calcium 2002; 31:289-97.

Ibanez CF, Pelto-Huikko M, Soder O, Ritzen EM, Hersh LB, Hokfelt T, Persson H. 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 1991; 88:3676-80.

Ivonnet PI, Chambers EL. Nicotinic acetylcholine receptors of the neuronal type occur in the plasma membrane of sea urchin eggs. Zygote 1997; 5:277-87.

Jaffe LA. Electrical polyspermy block in sea urchins: nicotine and low-sodium experiments. Dev Grow Diff 1980; 22:503-07.

Kuo RK, Baxter GI, Thompson SH, Stricker SA, Patton C, Bonaventura J, Epel D. NO is necessary and sufficient for egg activation at fertilisation. Nature 2000; 406:633-6.

Kusano K. Comparative electrophysiological study on the oocyte membrane neuro-receptors. Biol Bull 1978; 155: (Papers presented at Marine Biological Laboratory), p 450.

Kusano K, Miledi R, Stinnakre J. AChE receptors in the oocyte membrane. Nature 1977; 270:739-41.

Lennet EDA. An improved mounting medium for immunofluorescence microscopy. Am J Clin Pathol 1978; 69:647-8.

Marchi B, Trielli F, Pieroni M, Falugi C. A cholinergic system is involved in the regulation of early phases of fertilisation in the sea urchin, Paracentrotus lividus. In: Scalera Liaci L, C. Canicattì C eds. Echinoderm Research, Balkema Press, Rotterdam, 1991, pp. 165.

Mautner HG. Choline acetyltransferase. In: Boulton AA, Baker GB, Yu PH eds. Neuromethods. Neurotransmitter Enzymes, vol. 5, Humana Press, Clifton, New Jersey, 1986, pp. 273-304.

McCulloh DH, Lynn JW, Chambers EL. Membrane depolarisation facilitates sperm entry, large fertilisation cone formation, and prolonged current responses in sea urchin oocytes. Dev Biol 1987; 124:171-90.

Mei L., Xiong WC. Two birds with one stone: a novel motif for ACh receptor assembly quality control. Trends Neurosci 2003; 26:178-81.

Miki A, Mizoguti H. Acetylcholinesterase activity in the myotome of the early chick embryo. Cell Tiss Res 1982; 227:23-40.

Minganti A, Falugi C. An epithelial localisation of acetylcholinesterase in the ascidian Cyona intestinalis embryos and larvae. Acta Embryol. Morphol. Exp. n.s.1980; 1:143-55.

Nelson L. α-Bungarotoxin binding by cell membranes. Blockage of sperm cell motility. Exp Cell Res 1976; 101:221-4.

Nelson L. Chemistry and neurochemistry of sperm motility control. Fed Proc 1978; 37:2543-7.

Neumann E, Nachmanson D. Nerve excitability. Towards an integrated concept. In: Manson LA ed. Biomembranes, vol. 7. Plenum Press, New York, London, 1975, pp. 99-166.

Oettling G, Schmidt H, Drews U. An embryonic Ca^{++} mobilizing muscarinic system in the chick embryo heart. J Dev Physiol 1989; 12: 85-94.

Pieroni M, Falugi C. Effects of cholinergic drugs on cell interactions during fertilisation and early development of the sea urchin Paracentrotus lividus. Boll Soc It Biol Sper 1992; 68:113-19.

Piomboni P, Baccetti B, Moretti E, Gambera L, Angelini C, Falugi C. Localisation of molecules related to cholinergic signaling in eggs and zygotes of the sea urchin, Paracentrotus lividus. J Submicrosc Cytol Pathol 2001; 33:187-93.

Sastry BVR, Sadavongvivad C. Cholinergic systems in nonnervous tissues. Pharmacol Rev 1979; 30:65-132.

Sokal RR, Rohlf FJ. Biometry. 2nd ed. vol. 1. WH Freeman and Co, New York, 1981 pp. 859.

Trielli F, Politi H, Falugi C, Delmonte Corrado MU. 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 1997; 279:633-8.

Yokota Y. Introduction to the sea urchin biology. In: Yokota Y, Matranga V and Shmolenicka Z, eds. The Sea Urchin: from Basic Biology to Aquaculture. Balkema Publishers, Lisse, Abingdon, Exton (PA), Tokyo. 2000 pp 1-10.

Young MJ, Laing JC. The binding characteristics of cholinergic sites in rabbit spermatozoa. Molec Reprod Devel 1991; 28:55-61.