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CELL INTERACTIONS

Effect of Local Microapplication of Serotonergic Drugs on Membrane Currents of *Paracentrotus lividus* Early Embryos

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Abstract—It was shown that local application of agonists of the 3rd type receptors 5-HTQ and quipazine into the interblastomere cleft of *Paracentrotus lividus* embryos evoked specific membrane currents. At the same time, ligands of 5-HT_3- receptors specifically affected the cleavage patterns of half-embryos, i.e., imitated or avoided the interblastomere signal. In the view of the data obtained, we discuss a more precise concept of protosynapse, where the distribution of membrane serotonin receptors is restricted to the period of blastomere formation during cleavage and localized in the area of interblastomere contact.

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Neurotransmitters, such as serotonin, catecholamines, and acetylcholine, are involved in several key processes during the early (prenervous) embryogenesis. In sea urchins, they include, above all, the cell cycle triggering, control of the cytocortex state (Buznikov, 1987), completion of the cleavage furrow formation (Shmukler et al., 1999), adhesion of blastomeres after division (Buznikov and Shmukler, 1978), and, finally, direct blastomere interactions (Shmukler, 1981, 1992). The involvement of these substances in the early embryogenesis is characterized both by chemical specificity and different localization of the corresponding receptive structures (here and below: receptors): if the triggering of cleavage divisions and blastomere adhesion are mediated by intracellular receptors (Buznikov and Shmukler, 1978; Shmukler et al., 1986; Buznikov, 1987), interblastomere signalization appears to be mediated by the surface membrane receptors (Shmukler, 1992, 1993; Shmukler and Tosti, 2002).

The involvement of transmitters in blastomere interactions is substantiated by the capacity of serotonin antagonists to induce functional isolation of the blastomeres (Buznikov and Shmukler, 1978), as well as by specific effects of serotonergic substances on so-called micromere model, i.e., by their capacity to affect the pattern of cleavage in sea urchins developing form the blastomeres isolated during the 1st cleavage division (Shmukler, 1981). In the experiments on *Scaphechinus mirabilis* and *Paracentrotus lividus* embryos, the serotonergic substances, which poorly penetrate into the cell, influenced effectively the cleavage pattern, most likely, via the surface membrane receptors (Shmukler, 1992). In addition, the labeled ligand of serotonin receptors were specifically bound under the conditions, when its penetration in the cells was maximally limited (Shmukler, 1992).

At the first stage of these studies, the pharmacology of serotonin receptors was not sufficiently developed and it was not possible to determine their types. However, the efficiency of the 3rd type serotonin receptors was already demonstrated in the experiments on the influence of serotonergics on intracellular calcium ion levels (Shmukler et al., 1999). Under voltage patch-clump in the whole-cell configuration, the agonist of the 3rd type serotonin receptors (5-HT_3) 5-HTQ, which poorly penetrates in the cell, evoked specific inward currents timed to the formation of the 1st and 2nd cleavage furrows. On the contrary, the agonist of 5-HT_1 receptors did not evoke currents, while the agonist of 5-HT_2 receptors evoked them during the entire cell cycle (Shmukler and Tosti, 2002). This suggests, on the one hand, the presence of the above mentioned serotonin receptive structures in the surface membrane and, on the other, the presence of certain pharmacological specificity in these structures.

Here, we continue to study pharmacological sensitivity of the early embryos, in particular the specificity of their serotonin-receptive structures involved in blastomere interactions and undertake an attempt to investigate the distribution of these structures over the surface of blastomeres.

MATERIALS AND METHODS

Gametes. Female gametes of the sea urchin *P. lividus* (Bay of Naples, Mediterranean Sea, Italy) were obtained by injection of 0.05 ml 0.5 M KCl solution.

Male gametes were collected in total and the "dry sperm" was diluted by sea water immediately before artificial fertilization, which, as well as fertilization membrane and hyaline layer removal, was described earlier (Yazaki et al., 1995). Just before voltage clamp and current measurement, the embryos were transferred into normal sea water; measurements were started since the moment of appearance of the 1st cleavage furrow.

Voltage clamp in the whole-cell configuration was performed as described earlier (Yazaki et al., 1995); a micropipette with tip diameter ca. 1 μm resistance 5–10 MOhm filled with a solution close in composition to the intracellular medium, mM: 200 KCl, 20 NaCl, 250 sucrose, 10 EGTA, and 10 Hepes, pH 7.4, was used. After the **gigaOhmseal** reached 40–200 MOhm, the membrane was broken through; the negative membrane potential suggested the micropipette accessed the cytosol. The potential was fixed at the level -40 mV. Only those embryos were used in experiments, in which the holding current was below 200 pA; the values of ion currents in our work were obtained after subtraction of the holding current. Currents were recorded using a List EP7 amplifier and Clampfit software. The results were analyzed using Axoscope 10.0 and Microsoft Excel software and when application of chemical substances evoked output currents, they were averaged as experiments with zero results for more rigorous estimation of the obtained data.

Administration of chemical substances into chamber. 2-Methyl-serotonin, 5-HTQ, and nicotine (Sigma-RBI, USA) were administered to a final concentration of 100 μm by a micropipette into the most remote from the patch pipette part of a 2-ml experimental chamber, in order to exclude cell stalling from the pipette, avoid the effect of excessive concentration of the substance, and restrict mechanical noise during records.

Microapplication of chemical substances. The application micropipette filled with a solution of neurochemicals at a concentration of 1 mM and connected with a Picospitzer pneumatic microinjector (USA) was placed either at a distance of 5 to 10 μm from the first cleavage furrow, or at the same distance from the blastomere membrane out of the contact area (Fig. 1). The position of the applicating micropipette tip in the direct vicinity of the embryo surface was evaluated according to equal sharpness of its image and that of a voltage clamp micropipette. Application was performed by single pulses, 10 pl each. The agonists of 5-HT_3 receptors were used: 5-HTQ (trimethylserotonin iodide) and quipazine (2-(1-piperidiyl)quinoline maleate) (Sigma), as well as DMSO (dimethylsulfoxide) as a solvent.

Isolation of blastomeres. A suspension of embryos obtained like for the experiments on voltage clamp, were transferred from calcium-free sea water to the normal one directly before isolation. This is essential, since successful isolation of blastomeres in calcium-free water is impossible until a certain moment because

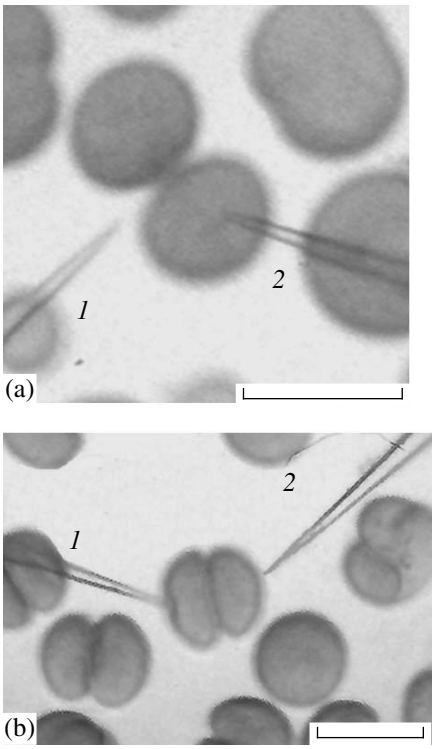

Fig. 1. Position of applicating micropipettes on an embryo after blastomere adhesion following cleavage division: (a) in the area of cleavage furrow before adhesion; (b) outside the contact area. Micropipettes: *1*, applicating; *2*, measuring. Scale: 100 μm.

of their destruction. The blastomeres were isolated during the 1st cleavage division before and after adhesion using a Γ-shaped glass needle with a tip diameter of about 5 μm, as described earlier (Shmukler et al., 1981b; Shmukler, 1993), in small Petri dishes in the presence of the studied chemical preparation. The change of calcium-free sea water for the normal one directly before isolation prevented the destruction of blastomere isolated prior to adhesion. Within 10 min, the isolated blastomeres were transferred in a Petri dish with normal sea water and then the moment of formation of the micromere in half-embryos was recorded. In addition, we studied the effects of quipazine and antagonist of 5-HT_3 receptors iodomethylate 3-tropanilindole-3-capboxylatan on the cleavage pattern in half-embryos.

RESULTS

Membrane currents evoked after introduction of chemical substances in experimental chamber. Specificity of the earlier demonstrated effect of the ligand of $5HT_3$ receptors 5-HTQ was tested in the experiments with another $5HT_3$ agonist 2-methylserotonin and nicotine, a ligand of n-cholinoreceptors. 2-Methylserotonin (100 μM) evoked an input current similar to that evoked by 5-HTQ (–335 ± 55 pA, $n = 7$). Nicotine at the same

Effects of microapplication of chemical substances onto blastomeres of *P. lividus*

Preparation	Site of microapplication	Number of experiments (with effect : without effect)	Current amplitude, pA	Latent period, s
5-HTQ	Area of contact, before adhesion	29 (25 : 4)	–119.4 ± 18.4	5.1 ± 0.7
	Other variants, including:	30 (13 : 17)	–47.0 ± 14.8	24.4 ± 2.3
	—area of contact, after adhesion	11 (5 : 6)	–61.0 ± 31.5	22.0 ± 2.4
	—outside the contact area	16 (8 : 8)	–43.4 ± 15.2	25.9 ± 3.7
Quipazine	Area of contact, before adhesion	12 (8 : 4)	–100.0 ± 42.3	6.1 ± 1.6
	Other variants, including:	12 (7 : 5)	–73.3 ± 32.6	29.6 ± 4.5
	—area of contact, after adhesion	6 (5 : 1)	–123.3 ± 58.9	25.3 ± 5.1
	—outside the contact area	6 (2 : 4)	–23.3 ± 14.8	45.5 ± 4.3
DMSO	Area of contact, before adhesion	17 (8 : 9)	–32.2 ± 9.8	30.0 ± 8.0
	Other sites	6 (2 : 4)	–21.2 ± 13.8	45.5 ± 14.5

concentration did not evoke significant currents (–52 ± 19 pA, $n = 16$) and inward currents were recorded only in six cases.

Membrane currents evoked after local application of chemical substances. When a single 5-HTQ pulse (100 μM) was applied into the area of interblastomere cleft, typical inward currents were evoked in the vast majority of experiments (25 out of 29). In the experiment shown in Fig. 2a, microapplication of 5-HTQ into the interblastomere cleft before adhesion evoked a maximum current: 103 pA with a latent period of about 0.5 s. The 5-HTQ application out of the contact area or after completion of blastomeres adhesion evoked inward currents in 13 out of 30 experiments and their amplitude was statistically lower than after application in the contact zone ($p < 0.01$, Fig. 2b). In many cases, when application outside the contact area did not evoke membrane currents, addition of 5-HTQ in the experimental chamber evoked pronounced currents, similar to those observed earlier (Shmukler and Tosti, 2002) and with higher amplitude than after microapplication.

It is even more important that the application into the contact area evoked currents with significantly shorter latent period than that onto the blastomere free surface (table, $p < 0.001$). Naturally, the latent period could be determined only in those cases, when application evoked a current of considerable amplitude. The results of 5-HTQ microapplication into the cleavage furrow area after completion of blastomeres adhesion and onto the blastomere free surface did not differ significantly (table).

Another agonist of 5-HT_3 receptors quipazine also evoked input currents when applied to cleavage furrow area before adhesion of the blastomeres (in 8 out of 12 experiments); the mean amplitude of current strength was 100 ± 42 pA with the latent period 6.1 ± 1.6 s. Note that if the effects of quipazine microapplication onto the free surface of blastomeres differed significantly and reliably from those of its microapplication into the interblastomere cleft in both amplitude and length (table), the quipazine microapplication into the contact area after adhesion completion evoked currents of similar amplitude but with longer latent period.

Application of DMSO, which was used as a solvent, into the contact area did not evoke pronounced currents. Nevertheless, the latent period of small changes in currents in these experiments was much longer than after 5-HTQ application into the interblastomere cleft (table, Fig. 3).

Application of serotonergic substances onto isolated blastomeres. Note that modified conditions of blastomere isolation allowed us to obtain different cleavage patterns as in *Scaphechinus mirabilis* and *Strongylocentrotus nudus* half-embryos isolated before and after adhesion of blastomeres in the course of the 1st cleavage division and, specifically, obtain a sustainable portion of half embryos that did not form micromeres during the 4th cleavage division (Fig. 4). Thus, we succeeded also to study the effect of the agonist, which, as was already shown (Shmukler, 1981), imitated the interblastomere signal and caused an increased proportion of embryos that formed micromeres during the 4th cleavage division.

When applied to the blastomeres isolated before adhesion during the 1st cleavage division, the agonist of 5-HT_3 receptors quipazine (100 μM) increased the proportion of half embryos that formed micromeres during the 4th cleavage division by 24.0 ± 0.5% ($p < 0.001$), as compared to the control in the absence of agonist (41.5 ± 0.5%). At the same time, in the experiments with the blastomeres isolated after adhesion during the 1st cleavage division, the antagonist of 5-HT_3 receptors 4-tropanylindole-3-carboxylate methiodide decreased the proportion of half-embryos that formed micromeres during the 4th cleavage division by 25.0 ± 0.8% ($p < 0.001$), as compared to the control in the absence of antagonist (85.5 ± 0.7%).

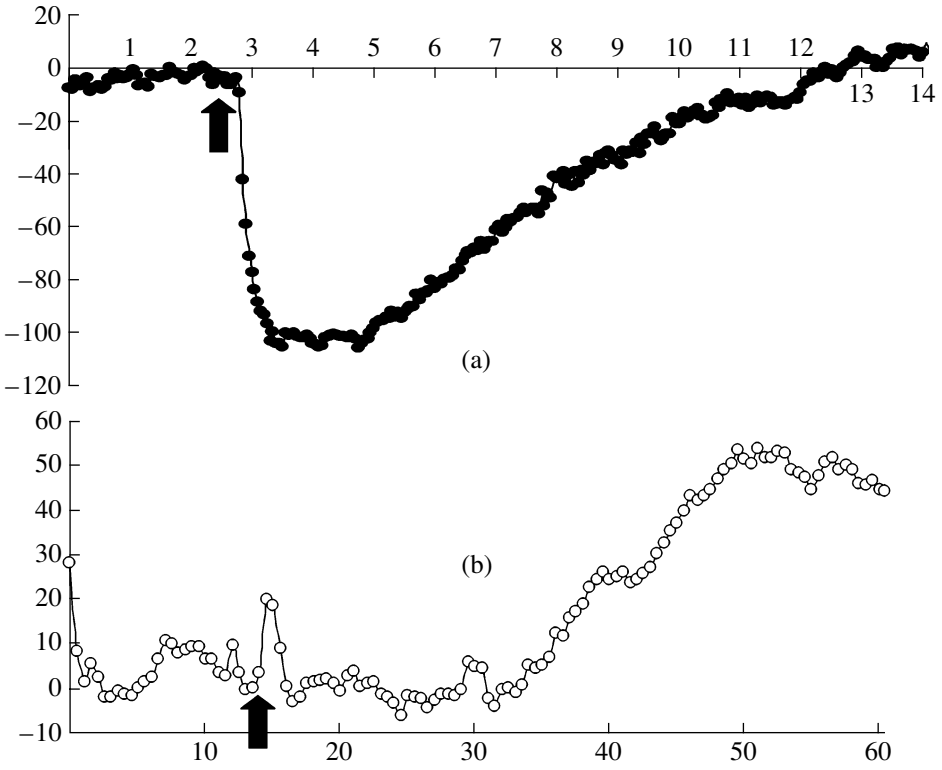

Fig. 2. Voltage clamp after 5-HTQ microapplication in the contact area (a) and onto the free surface of blastomeres (b) during the 1st cleavage division in *P. lividus* (single pulse). Here and in Fig. 3: abscissa: time, s; ordinate: current amplitude, pA; (➡) moment of pulse.

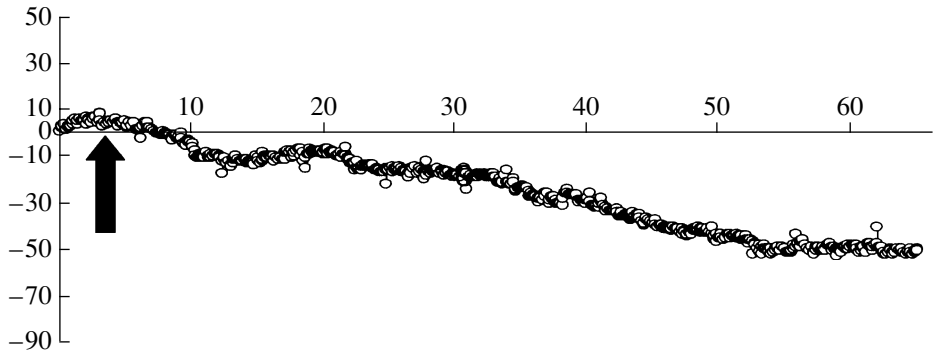

Fig. 3. Current amplitude after DMSO microapplication (single pulse) in the contact area of blastomeres during the 1st cleavage division in *P. lividus*.

DISCUSSION

The effect of the 5-HT_3 receptor agonist 5-HTQ had already been described (Shmukler and Tosti, 2002), while the 5-HT_2 receptor agonist DOI evoked currents during the entire cell cycle and the 5-HT_1 receptor agonist did not exert such an effect. Hence, it was proposed that embryonic receptor structures are similar to 5-HT_3 receptors of adults. The efficiency of the ligands of $5HT_3$ receptors is also confirmed in the present study.

In addition to the earlier described temporal organization of the processes related to 5-HT_3 receptors (Shmukler et al., 1999), we also demonstrated their spatial organization. The localization of receptor structures, similar to 5-HT_3 receptors, in the area of contact membrane of the blastomeres follows from both higher amplitude of the current strength upon local application of 5-HT_3 receptor agonists in the area of the 1st cleavage furrow, than upon application onto the free surface, and a markedly shorter latent period of such currents.

Significant variability of the latent period upon application in the contact area of 2-cell embryo can be accounted for by two factors: impossibility of full standardization of the application pipette tip according to

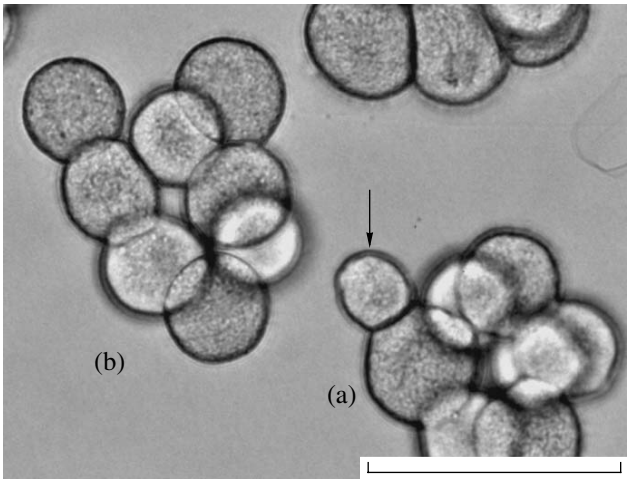

Fig. 4. Patterns of cleavage of half-embryos of *P. lividus* during the 4th cleavage division. Cleavage: (a) equal; (b) unequal. (⟶) Micromere.

the distance and angle to the contact surface and time from the return of the embryo into the normal sea water, when the formation of hyaline layer is resumed, which may affect the rate diffusion of the ligands to the receptors.

The appearance of currents upon application onto the free surface may be related to the leakage of a part of the applied ligand to the contact area. In these cases, the latent period is lengthened due to a longer distance from the micropipette tip, while the amplitude of current strength decreases due, apparently to the ligand dilution over a longer distance. Association of the sensitivity of embryos to the 5-HT_3 receptor agonists with the contact surface is indirectly confirmed by the appearance of currents upon the ligand addition in the experimental chamber in those experiments when the preceding application onto the free surface was ineffective. In addition, successive application in the contact area and onto the free surface was performed in arbitrary order and the results obtained coincided with the overall data. Association of the currents evoked by 5-HT_3 ligands with the contact area is indirectly confirmed by their earlier described timing to the cleavage furrow formation (Shmukler and Tosti, 2002).

The differences between the results of 5-HTQ and quipazine application in the contact area after adhesion of the blastomeres may be related to different lipophility of these agonists and, correspondingly, their capacity to penetrate to the receptors across two membranes. The former is characterized by a very low permeability across the membrane, which practically excludes its penetration to the receptors in the contact area after completion of the blastomeres adhesion after division. Quipazine is able of very fast penetration across the cell membrane and binding by intracellular structures (Buznikov, 1987; Shmukler, 1992), which decreases the probability of its interaction with the membrane receptors; however, the ligand, unlike 5-HTQ, may later pass across the contact membrane to the contact area.

Physiological activity of the 5-HT_3 receptor ligands is not limited by their ability to evoke membrane currents. In the experiments on the "micromere model" (Shmukler et al., 1981a, 1981b; Shmukler, 1981), the effects of 5-HT_3 receptor agonist and antagonist fully correspond to the previously described effects of sero-

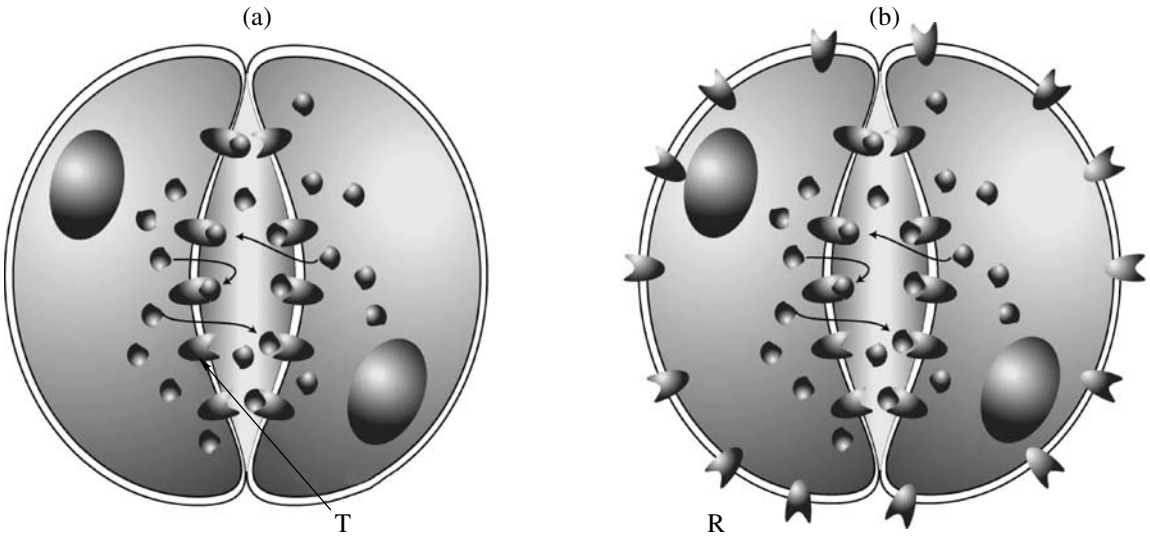

Fig. 5. Schematic diagram of protosynapse: (a) with uniform distribution of receptors over the blastomere surface; (b) with localization of receptors in the area of interblastomere contact. R, Receptor; T, transmitter.

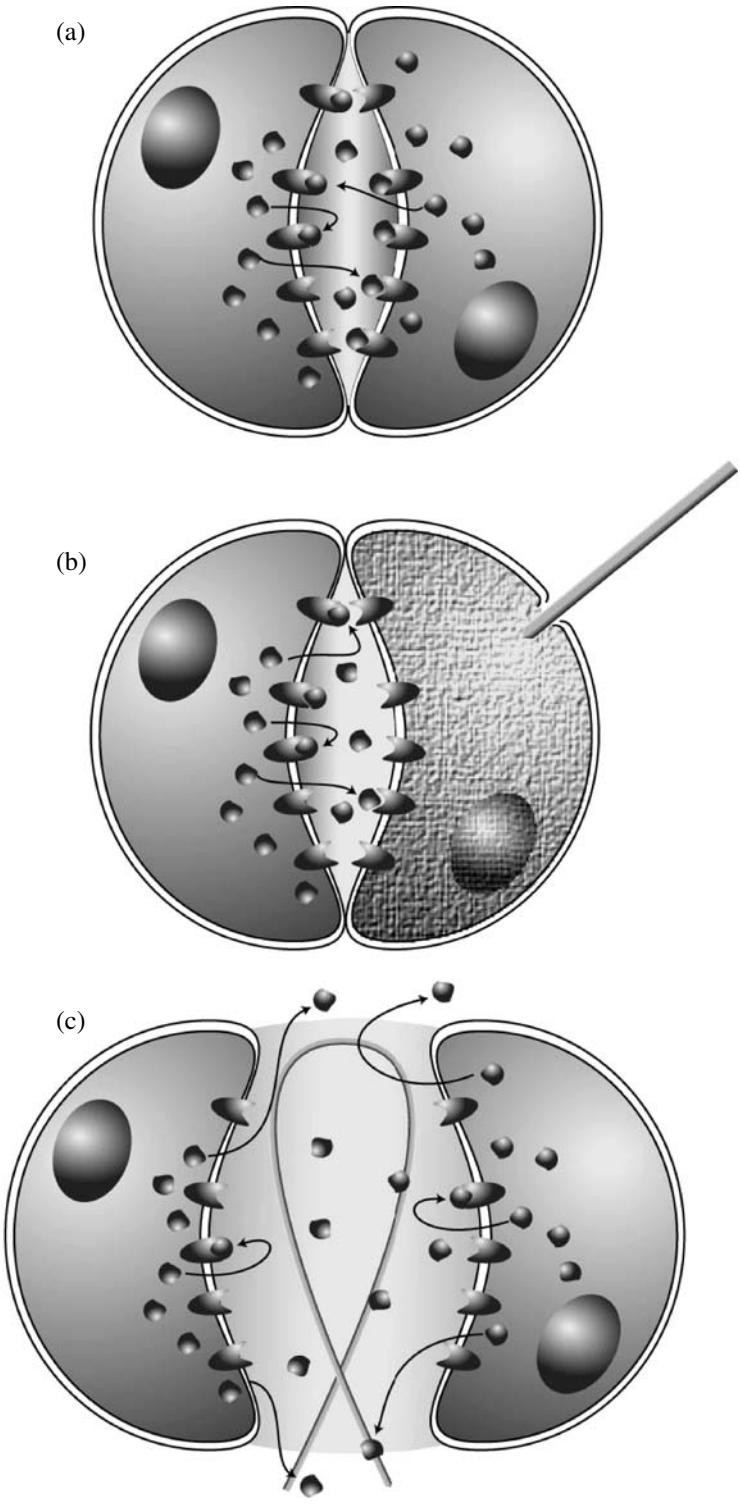

Fig. 6. Protosynapse (a) and schemes of Roux' (b) and McClendon (c) experiments.

tonergic substances (Shmukler, 1981, 1992); the cleavage pattern of half-embryos is changed correspondingly. The improved method of isolation allowed us to obtain for the first time a fraction of *P. lividus* blastomeres isolated before adhesion during the 1st cleavage division, which did not form micromeres during the 4th cleavage division, and to demonstrate the effect of the serotonin receptor agonist. It may well be that the effects of specially synthesized antagonists of the serotonin receptors (Shmukler, 1992), such as methiodides

of inmecarb and preparation KYuR-14, were due to their interaction with 5-HT_3 receptor-like structures. It can also be proposed that these effects of 5-HT_3 receptor ligands, like the membrane currents, are determined by their interaction with the receptors in the contact area during cleavage division. Thus, the state of blastomere cytocortex may be locally changed and predetermine the position of the next cleavage spindle and, correspondingly, the moment of micromeres formation.

These data correspond quite well to the concept of protosynapse, bilateral-symmetrical structure of blastomeres interaction, which implies that each sea urchin blastomere is both a source of the intercellular signal and its target and, in addition, provides for its accumulation in the interblastomere cleft representing a passive obstacle to the transmitter diffusion to the external medium (Shmukler, 1992). Thus, blastomeres can be polarized, which predetermines the position of the next cleavage division spindle and, hence, the cleavage pattern of a half-embryo. Finally, the totipotency of an isolated blastomere is limited.

The appearance and/or activity of these receptors are coupled in time with the 1st cleavage division and localized in the area of interblastomere contact. This means that the above concept should be made more precise and exclude the suggestion about uniform distribution of serotonin-receptive structures over the surface of blastomeres and restrict it by the time of cleavage divisions and area of interblastomere contact (Fig. 5).

Note that the concept of protosynapse, in addition to explanation of specific phenomena of early development and evolutionary foundations of subsequent formation of the synaptic mechanisms per se, can also explain the old contradiction of the classical experiments of Roux (1888) and McClendon (1910). In the Roux' experiments, coagulation of a blastomere in situ led to the formation of a half set of structures, while McClendon observed complete regulation of development after mechanical isolation of blastomeres. This contradiction may be explained by that in the case of coagulation of a blastomere, rather than its complete isolation, the intact blastomere is both source and target of transmitter and the coagulated blastomere serves as a passive obstacle for diffusion of the transmitter retaliated from the intact blastomere into the external medium (Fig. 6) and, hence, the asymmetrical effect of the transmitter is preserved. Thus, the prospective fate of the cell is determined and, correspondingly, the totipotency of cells of the regulative embryo is limited.

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