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Short Contribution

Action of serotonin antagonists on cytoplasmic calcium levels in early embryos of sea urchin *Lytechinus pictus*

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ABSTRACT Possible interaction of the serotonergic system with intracellular calcium mechanisms was investigated using techniques of ratio imaging measurement of intracellular Ca^{2+} and confocal microscopy in cleaving embryos of sea urchin *Lytechinus pictus.* Some serotonin antagonists specifically increase free intracellular Ca^{2+} and evoke transient regression of the first cleavage furrow, suggesting possible linkage of serotonergic and calcium mechanisms in the regulation of cellular events during cleavage divisions. These effects were more pronounced in the experiments with hydrophilic 5-HT-antagonists, quarternary ammonium salts that do not penetrate the cell membrane. Thus, it appears that 5-HT-receptors which mediate these effects are localised on the cell membrane, whereas previously studied receptors mediating the cytostatic action of lipophilic 5-HT-antagonists are localised intracellularly.

KEY WORDS: *serotonin, Ca^{2+}, cleavage divisions, cytoskeleton, receptor*

It is well known now that neurotransmitters are multifunctional substances playing, in particular, an important role in regulatory events of embryogenesis, including the pre-nervous stages of development (Buznikov, 1967, 1990; Buznikov *et al.*, 1996). One of the most interesting peculiarities of pre-nervous neurotransmitter systems, is the co-existence of intracellular and plasma membrane neurotransmitter receptors (Buznikov, 1990; Shmukler, 1993; Buznikov *et al.*, 1996). Both types of receptors are functionally coupled to various second messengers, including cyclic nucleotides (Shmukler and Grigoriev, 1984; Shmukler *et al.*, 1986; Capasso *et al.*, 1988), phosphoinositides (Buznikov *et al.*, 1993), and Ca^{2+} (Shmukler *et al.*, 1986; Buznikov *et al.*, 1993, 1996, 1997). The present work is devoted to the study of the direct effects of drugs related to one pre-nervous neurotransmitter, 5-HT, on cytoplasmic Ca^{2+} levels in early sea urchin embryos.

Effects of 5-HT antagonists and agonists during the first cleavage division

TIC methiodide (PPM antagonist of $5HT_3$-receptors) applied during the first cleavage division (when cleavage furrow formation has already started) in Fura-2-dextran-ratio imaging experiments, evoked a Ca^{2+}-rise in a dose-dependent manner (total of 41 experiments, Table 1, Fig. 1). DMSO (0.5%), used as a solvent of neurochemicals, had no significant effect (Table 1).

Using the confocal microscope, the increase of free intracellular Ca^{2+} caused by IM methiodide (100 μM) was observed in all of 6 experiments (intensity comparing to resting level increased by 45±6.4% with a latent period of 30 sec) (Fig. 2a,b). By comparison with the data from ratio imaging experiments, the rise in Ca^{2+}-level corresponds to approximately 0.27±0.04 μM. The duration of intracellular free Ca2+ elevation was from 1.5 to 7 min. Similar results were obtained in experiments with KYuR-14 methiodide (PPM 5-HT-antagonist, 75 μM, 3 experiments.

Specificity of the effects of 5-HT-antagonists

5-HTQ (PPM $5HT_3$-agonist, 100 μM) administered 10-40 sec before TIC methiodide (100 μM) significantly decreased the effect of the latter in Fura-2-Dextran ratio imaging experiments (Table 2, Fig. 3), but only areas under the peaks differed significantly. Preliminary data show that the protective action of 5-HT (100 μM) was weaker than 5-HTQ.

In confocal microscope experiments, 5-HTQ (100 μM) administered 1 min before IM methiodide (100 μM) completely prevented Ca^{2+} increase in 2 out of 5 experiments, and left it in more or less unchanged form in another 3 experiments. No significant effects of 5-HTQ (100 μM) itself were observed.

Abbreviations used in this paper: 5-HT, 5-hydroxytryptamine, serotonin; EDTA, ethylenediaminetetraacetic acid disodium salt; ASW, artificial sea water; BAPTA/AM, 1,2-*bis* (2-Aminophenoxy) ethane-N,N,N,N-tetraacetic acid acetoxymethyl ester; $Ca^{2+}{}_{(i)}$, intracellular level of free calcium ions; Fura-2/DX, Fura-2 dextran; CG-1/DX, Calcium Green-1 dextran; PIPES, piperazine; N,N', tetraacetic acid; D-600, methoxyverapamil; 5-HTQ, trimethylserotonin methiodide; TIC, 3-tropanyl-indole-3-carboxylate hydrochloride (tropisetron); TIC methiodide, 3-tropanyl-indole-3-carboxylate methiodide; IM, inmecarb hydrochloride; IM methiodide, inmecarb methiodide; DMSO, dimethylsulfoxide; EPM, substance, easily penetrating the cell membrane; PPM, substance, poorly penetrating the cell membrane.

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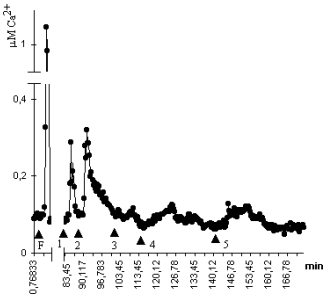

Fig. 1. Effect of TIC methiodide on intracellular free Ca^{2+} during first cleavage division. *Graph from the "dose-effect" experiment. Abscissa, time (min); ordinate, Ca^{2+} (µM). Arrows: F, fertilisation; 1, TIC methiodide (100 µM); 2, TIC methiodide (150 µM); 3, TIC methiodide (20 µM); 4, TIC methiodide (50 µM); 5, TIC methiodide (67 µM).*

Investigation of possible mechanisms of 5-HT regulation of intracellular Ca^{2+}-level.

To evaluate the possible mechanisms of action of 5HT-antagonists and agonists on Ca^{2+}-levels in the cells of the sea urchin embryo, two additional series of ratio imaging experiments were performed. Replacement of normal ASW with Ca^{2+}-free ASW caused a significant decrease in the TIC methiodide (100 µM) effect (Table 2). Addition of L-type Ca^{2+}-channel blocker nifedipine (20 µM) to normal ASW 10-40 s before TIC methiodide (100 µM) also decreased the effect of the latter (Table 2, Fig. 3); a D-600 (Ca^{2+}-channel antagonist, 40 µM) also had a similar but weaker effect in 4 experiments.

Morphological alterations

In 10 experiments with CG-1/DX imaging we found that IM methiodide (100 µM) caused regression of the cleavage furrow (Fig. 2a). The first signs of regression were observed 2 min after IM methiodide administration, and regression was complete at 10 min.

Preloading embryos with BAPTA/AM (1 µM) increased the frequency of regression when methiodides of IM (100 µM) and KYuR-14 (100 µM) were administered. Such embryos were able later to normalise their development (10-15 min after furrow regression), i.e., the regression of the cleavage furrow was a transient and reversible phenomenon. 5-HT (100 µM) and 5-HTQ (100 µM) prevented in many cases the furrow regression evoked by methiodides of IM and KYuR-14.

In all of the experiments (31) with TIC methiodide, cleavage furrow regression was also observed. Within 5-10 min after washing out the 5-HT-antagonist, reassembly of the cleavage furrow occurred; spontaneous reformation was never observed in these experiments. As a consequence, assembly-disassembly of the cleavage furrow could be repeated up to 5 times with repeated 5HT-antagonist administration and subsequent washings.

Effects of 5-HT-antagonists before the first cleavage division

In Fura-2 ratio imaging experiments, 5 HT-antagonists IM (EPM, 40-70 µM, 5 experiments), IM methiodide (40-70 µM, 5 experiments), and imipramine (70 µM, 2 experiments) had no significant effects on the development of Ca^{2+}-peak at fertilisation.

From 18 min after fertilisation until the first cleavage division, IM and IM methiodide were able to cause changes of intracellular Ca^{2+}-levels (see Table 3). The effects of these antagonists were rather variable in size and shape. The cytostatic effects of 5-HT-antagonists were often absent in Ca^{2+}-probe microinjected embryos (20–70 µM), although these antagonists were used in the concentrations that fully block the cleavage divisions in usual pharmacological experiments. Control pharmacological experiments where embryos were preloaded with BAPTA/AM showed decreased cytostatic effects of IM and ionomycin.

Influence of 5-HT-antagonists and agonists on intracellular Ca^{2+}-levels

The main finding of the present work is the direct evidence that some 5-HT-antagonists specifically influence Ca^{2+}-levels in the cells of early sea urchin embryos. It is suggested that addition of 5-HT-antagonists blocks the corresponding receptors and signal pathways. The relatively short latent period of the poorly penetrant 5-HT-antagonists suggests that 5-HT-receptors are localised on the surface membrane of the embryonic cell (see Buznikov *et al.*, 1993, Shmukler, 1993). Weakening of these effects of 5-HT-antagonists by the addition of poorly penetrant 5-HT-agonist 5HTQ, confirmed the specificity of the effect and the surface membrane localisation of the corresponding receptors.

Judging from the results of the experiments with Ca^{2+}-free ASW and nifedipine, these effects are linked with Ca^{2+}-influx from the external medium via L-type Ca^{2+}-channels. The presence of such channels was shown earlier in the surface membrane of early embryos of sea urchin *Paracentrotus lividus* (Yazaki *et al.*, 1995).

5-HT-antagonists block specifically cleavage divisions via intracellular receptors (Buznikov, 1984, 1990; Buznikov *et al.*, 1996). This specific action in the present experiments was diminished by Ca^{2+}-buffering by means of Ca^{2+}-fluorescent probes or BAPTA/AM. Therefore, this suggests that certain Ca^{2+}-transients in the cytoplasm are necessary for the realisation of cytostatic action of the 5-HT-antagonists tested. This is supported by evidence that, in our experiments, administration of Ca^{2+}-fluorescent probes also decreased the cytostatic activity of ionomycin, an activity certainly caused by an increase in cytoplasmic Ca^{2+}. There are other examples of the influence of Ca^{2+}-probes on cellular events connected with Ca^{2+}-transients (Bolsover *et al.*, 1993).

Effects of 5-HT-antagonists on the cytoskeleton

The phenomenon of cleavage furrow regression under the action of 5-HT-antagonists appears to be specific because the addition of 5-HT-agonists inhibited or prevented it. It could be caused by the action of 5-HT-

TABLE 1
EFFECTS OF 5HT-ANTAGONISTS ON CA^{2+}-LEVEL DURING 1ST CLEAVAGE DIVISION

Substance	Concentration (µM)	Area of peak (µM Ca^{2+} x min)	Increase of Ca^{2+}-level (µM)	Latent period (min)	Time to peak maximum (min)	Number of experiments*
TIC	100	0.323±0.169	0.094±0.051	0.76±0.49	2.89±1.46	3 (4)
TIC methiodide	200	1.108±0.163	0.248±0.023	1.38±0.21	3.74±0.31	13 (13)
	100	0.601±0.142	0.145±0.028	1.26±0.47	3.68±0.68	20(32)
	67	0.303±0.079	0.066±0.015	2.36±0.75	7.31±1.58	7(7)
	40	0.151±0.043	0.044±0.015	2.50±0.73	7.52±1.76	3(3)
IM methiodide	100	0.462±0.153	0.121±0.037	1.50±0.69	3.78±0.82	7(7)
DMSO	0.5%	-0.043±0.039	-0.018±0.013	0.68±0.20	3.15±0.90	6

* In brackets, number of drug administrations

antagonists on surface membrane 5-HT-receptors because PPM 5-HT-antagonists had the most significant and reproducible effect. This is a novel result. We thought earlier that only intracellular 5HT-receptors are coupled to the process of cleavage division itself (Buznikov, 1984, 1990); we supposed that surface membrane 5-HT-receptors were only regulators of blastomere interactions and cleavage pattern formation (Shmukler, 1993). This point of view should be changed now.

The regular sequence of events under consideration is: i) administration of 5-HT-antagonist, ii) increase in intracellular Ca^{2+}-levels, iii) regression of the cleavage furrow. We suppose that these events involve a Ca^{2+}-level increase evoked by 5-HT-antagonists which leads to some changes of the state of the contractile ring, which in turn evokes regression of cleavage furrow. Evidently, the presence of Ca^{2+}-fluorescent probes or BAPTA/AM, i.e., the substances possessing Ca^{2+}-buffering properties, in the cytoplasm is an important or even necessary condition for cleavage furrow regression.

The cause of cleavage furrow regression promoted by the action of 5-HT-antagonists may be the specific sensitivity of elements of the contractile ring to intracellular Ca^{2+}-levels. Probably, they include the calmodulin link, judging from similar effects of 5-HT- and calmodulin antagonists (Buznikov, 1967). The role of Ca^{2+}-buffers in this case might be to maintain increased Ca^{2+}-levels (and counteracting cellular Ca^{2+}-sequestering systems) long enough to evoke contractile ring disassembly. Possibly, the targets of Ca^{2+}-signalling, in this case, are components of the cytoskeleton that are not involved in triggering furrow formation (Schatten, 1994; Wong *et al.*, 1996). These processes probably have different regulatory pathways and spatial-temporal organisation also (Shmukler *et al.*, 1986; Buznikov *et al.*, 1996; Whalley *et al.,* 1992).

The results obtained present the opportunity for new hypotheses and further experiments. In particular, the possibility of repeated cleavage furrow assembly-disassembly will allow us to develop a pseudostationary model useful for the investigation of the processes of early embryogenesis and will help in further analysis of the role of 5-HT and other prenervous neurotransmitters in cleavage divisions.

Experimental Procedures

Gametes

Gametes of the sea urchin *Lytechinus pictus* (Pacific Biomarine Laboratories, Venice, CA, USA) were obtained by injecting females and males with 0.05 ml of 0.5 M KCl. ASW (430 mM NaCl, 27 mM $MgCl_2$, 28 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$, 1 mM EDTA, pH 8.0) was used as the incubation medium. All components of ASW were analytical grade from BDH (Poole, UK). The jelly coat was eliminated by 2-3-fold passage of eggs through nylon mesh (calcium measuring experiments) or left intact (all other experiments).

Calcium measurement experiments

Eggs used in experiments with microinjection of fluorescent dyes (Calcium molecular probes) were lightly attached to coverslips pre-treated with poly-L-lysine

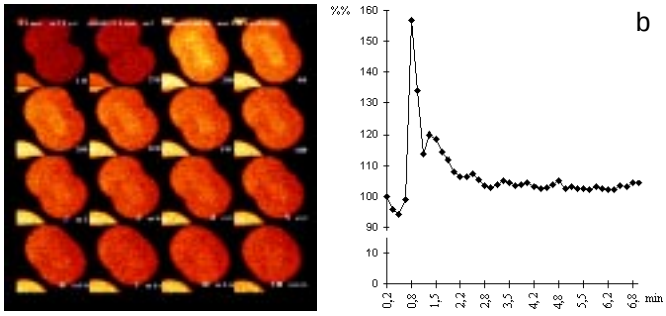

Fig. 2. Effect of IM methiodide (100 μM) on $Ca^{2+}_{[i]}$-level in *L.pictus* during 1st cleavage division. (a) *Confocal image (Calcium Green-1/DX). Time from the moment of IM methiodide administration - in the right lower corner of each image.* **(b)** *Graph of $Ca^{2+}_{[i]}$-level in the same experiment (frequency of record 1 per 10 sec). Abscissa, time (min); ordinate, Ca^{2+} (% from starting level); starting point: administration of IM methiodide (66 min after fertilisation).*

(0.02 mg/ml) according with the procedure described earlier (Swann and Whitaker, 1986). Fertilisation was performed by adding a small drop of diluted sperm directly to the experimental bath.

In experiments with ratio imaging measurement of intracellular Ca^{2+}, Fura-2 and Fura-2/DX were used (Molecular Probe Inc., Eugene, USA). The procedures of measurements were standard (Swann and Whitaker, 1986). Solutions of drugs were added to the experimental bath (total volume 2 ml) in 20 μl aliquots.

Changes of $Ca^{2+}_{(i)}$- level were evaluated by peak square (μM Ca^{2+} x min), amplitude (μM Ca^{2+}), latent period (time to 10% increase as compared to the starting level before the drug administration) and full time to peak of the developed effect. Experimental data were plotted and processed using SigmaPlot 5.0 and estimated by Student and Fisher paired tests.

Confocal microscope experiments

CG-1/DX (potassium salt, mw. 10 000, Molecular Probe Inc. Eugene, USA) was diluted in the following solution: 0.5 M KCl, 20 mM PIPES, pH 6.7 in confocal Ca^{2+} imaging experiments. The dye was injected into the eggs to a final concentration of 5 μM using pulses from a pressure injection system. Optical slices of loaded eggs were obtained by confocal laser scanning microscopy (CLSM, Leica Lasertechnik, Heidelberg, Germany). Excitation wavelength was 488 nm, then fluorescent signal was filtered with a 530±15 nm band pass filter. Variations in $Ca2+_{(i)}$ were monitored and analysed using a Leica CLSM analysis program. The signal intensities of whole eggs were measured and plotted. The neurochemicals used in these experiments were added to the medium at the moment when the cleavage furrow began to form (i.e., from 65 to 70 min after fertilisation). Data were compared to those obtained from Fura-2 experiments.

Pharmacological experiments

Early embryos used in pharmacological experiments were obtained and handled according to standard procedure (Buznikov and Podmarev, 1991). Suspension of fertilised eggs was placed into wells of cluster "Costar 3524" (150-

TABLE 2

EFFECTS OF 5-HTQ, NIFEDIPINE AND CA2+-FREE ASW ON CA2+-LEVEL AS COMPARED TO THE EFFECT OF TIC METHIODIDE PER SE

TIC methiodide 100 μM	Peak area (μM Ca2+ x min)	TIC methiodide 100 μM per se (control) (μM Ca2+ x min)	Difference between peak areas (μM Ca2+ x min)**	Significance of difference
+5-HTQ 100 μM (14)*	0.473±0.083	1.088±0.178	0.615±0.196 (-56.5)	p< 0.01
+5-HT 200 μM (4)	0.452±0.062	0.741±0.106	0.289±0.123 (-37.8)	p< 0.05
+ Nifedipine 20 μM (12)	0.346±0.104	1.010±0.115	0.664±0.155 (-65.7)	p< 0.01
+ D-600 40 μM (4)	1.023±0.045	1.409±0.102	0,386±0.111 (-27.4)	p< 0.05
In Ca2+-free ASW (7)	0.074±0.024	0.463±0.135	0.388±0.137 (-83.8)	p< 0.05

* - Number of experiments in brackets; ** - In brackets, %% to the effect of TIC methiodide per se

TABLE 3
EFFECTS OF 5HT-ANTAGONISTS ON CA2+-LEVEL BEFORE 1ST CLEAVAGE DIVISION

Substance	Concentration (μM)	Area of peak (μM Ca2+ x min)	Increase of Ca2+-level (μM)	Latent period (min)	Time to peak maximum (min)	Number of experiments*
IM	40	0.624±0.293	0.685±0.408	1.0±0.71	2.18±1.08	6 (6)
IM methiodide	40	0.167±0.050	0.073±0.019	0.79±0.38	2.56±0.51	11 (18)
TIC methiodide	100	0.120±0.094	0.031±0.027	1.2±0.68	3.11±1.44	4 (4)

* In brackets, number of drug administrations

200 embryos in 1 ml of medium per well). In some cases eggs were preloaded with BAPTA/AM (RBI, USA) (60 min treatment with 1 μM solution of BAPTA/AM in ASW following by 3-fold washing by ASW before fertilisation). Neurochemicals were added to the cells during the formation of the 1st cleavage division furrow (controls to the calcium measurement experiments) or immediately after fertilisation. Results of all pharmacological experiments were recorded visually and by means of photomicrography.

Chemicals

Beside the above-mentioned chemicals, the following substances were used: 5-HT, cyproheptadine, propranolol, metoclopramide, D-600, ionomycin (Sigma, USA), 5-HTQ, TIC, TIC methiodide, 5-HT-antagonists IM and IM methiodide (Buznikov, 1990) were kindly supplied by Prof. V.A. Zagorevsky (Institute of Pharmacology and Chemotherapy, Russian Acad. Med. Sci., Moscow, Russia). The other pair of 5-HT-antagonists (KYuR-14 hydrochloride and KYuR-14 methiodide) were synthesised by Dr. M.A. Yurovskaya (Moscow State University, Dept. of Chemistry, Russia). DMSO (Sigma, USA) was used as a solvent for stock solutions of neurochemicals; its final concentration in experimental bath or cluster cells did not exceed 0.5%.

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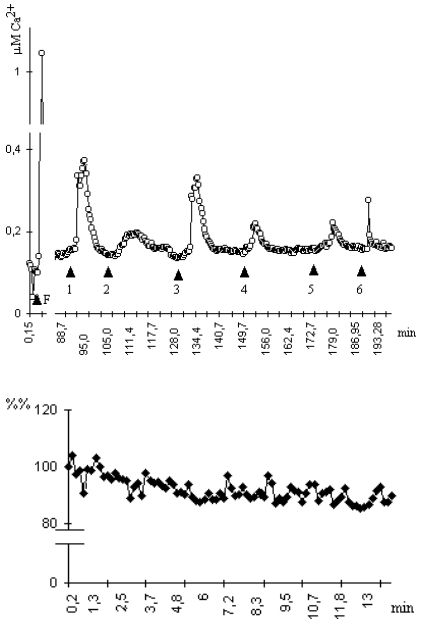

Fig. 3. Specificity of the effects of 5HT-antagonists. **(a)** *Changed effect of TIC methiodide (100 μM) by timely administered 5-HTQ (100 μM) and nifedipine (20 μM) in ratio imaging experiment. Abscissa, time (min); ordinate, Ca^{2+} (μM). Arrows: F, fertilisation; 1, TIC methiodide (100 μM); 2, TIC methiodide (100μM) + 5HTQ (100 μM); 3, TIC methiodide (100 μM); 4, TIC methiodide (100μM) + nifedipine (20μM); 5, TIC methiodide (100 μM); 6, TIC methiodide (100 μM).* **(b)** *Prevention of the effect of IM methiodide by timely administered 5-HTQ (100 μM) in a confocal experiment.*

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