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THE INFLUENCE OF SECOND MESSENGERS AND RELATED SUBSTANCES ON THE SENSITIVITY OF EARLY EMBRYOS TO CYTOSTATIC NEUROCHEMICALS

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Abstract—1. "Prenervous" neurotransmitters interact with second messengers in the regulation of early embryogenesis of sea urchins and the clawed frog *Xenopus laevis*.

2. Propranolol and atropine inhibit cleavage divisions of *X. laevis* only after their intracellular administration.

3. The level of the membrane potential (including a zero level) of the embryos studied is not essential for the cleavage divisions.

4. Possible mechanisms of action of the "prenervous" neurotransmitters at the intracellular level and their coupling with second messengers are discussed.

INTRODUCTION

Substances identical to synaptic transmitters of adult organisms (acetylcholine, catecholamines, indolylalkylamines) have been discovered in early embryos of all animal groups studied (Buznikov, 1967, 1981, 1984). The functional activity of these "prenervous" neurotransmitters, in particular their participation in the regulation of cleavage divisions and early cell interactions, was shown in the experiments with early embryos of Echinodermata (sea urchins and starfishes) (Buznikov, 1967, 1981; Buznikov and Shmukler, 1978, 1981; Toneby, 1977, Renaud *et al.*, 1983; Shmukler, 1981). Penetration of neurochemicals (antagonists of "prenervous" neurotransmitters) into the cells was the prerequisite of their cytostatic and intercellular activities (Buznikov, 1967; Buznikov and Shmukler, 1978, 1981). It has been suggested that: (a) "prenervous" neurotransmitter receptors are characterized by intracellular localization and (b) they are functionally coupled with the second messengers—cyclic nucleotides and calcium ions (McMahon, 1974; Buznikov, 1977; Buznikov and Shmukler, 1981; Shmukler, 1981). The main purpose of the present work was to check on these suggestions. We also hoped to obtain other evidence permitting comparison between the characteristics of "prenervous" and neuronal neurotransmitter systems.

MATERIALS AND METHODS

The experiments were carried out on early embryos of *Xenopus laevis* and the sea urchins *Strongylocentrotus intermedius* and *Scaphechinus mirabilis*. Early embryos were obtained and incubated by a standard procedure (Buznikov and Podmarev, 1975; Detlaf and Rudneva, 1975).

Microinjection of the chemicals studied into *X. laevis* blastomeres was made by means of a microinjector constructed on the basis of a standard Hamilton microsyringe with a dosing accuracy within ± 0.1 nl. The microsyringe was hermetically connected to a glass micropipette with a tip diameter of about 3–5 μm. The injected substances were diluted in a salt solution the ionic composition of which mainly corresponded to the content of the predominant ions of the intracellular medium (Gillespie, 1982). Such a solution without neurochemicals was used during microinjections as a control. The effects of microinjection were estimated according to the time of formation of two successive cleavage furrows in the blastomeres after injection, the intact blastomere of the same embryo serving as an additional control.

Measurements of the membrane potential and voltage clamp were carried out by means of glass microelectrodes according to a standard procedure. In the experiments with sea urchin embryos with microelectrodes filled with 3 M KCl, 1 mM EGTA was added which allowed prolonged (for several hours) continuous recording of the membrane potential.

When investigating the role of the ionic balance in the cells of sea urchin embryos "isoionic water" was used: 150 mM KCl, 50 mM NaCl, 6.5 mM $MgCl_2$, 150 mM Tris and 2 mM EGTA; the ionic composition of the latter corresponded to the intracellular medium (Rothschild and Barnes, 1953). The embryos were placed into "isoionic water" (IIW) several minutes after fertilization simultaneously with the administration of the neurochemicals. The effect was estimated by the relative number of embryos passing the 1st cleavage division normally. In other respects the procedure was standard (Buznikov *et al.*, 1979).

The following chemicals were used: propranolol (Sigma, USA), adrenaline, serotonin, atropine, dibutyryl-cAMP, dibutyryl-cGMP, cAMP, the calcium ionophore A 23187 (Calbiochem-Boehring, USA), tryptamine (Reanal, Hungary), imipramine (Gideon Richter, Hungary) and sodium fluoride (Soyuzkhimreaktiv, USSR). Neurochemicals (indocarb and 5-bromotryptamine) revealing antiserotonin activity in sea urchin embryos were also applied.

RESULTS

a. *Microinjection of neurochemicals into blastomeres of* Xenopus laevis *embryos*

The beta-adrenergic blocking drug propranolol was injected into one of the blastomeres of early

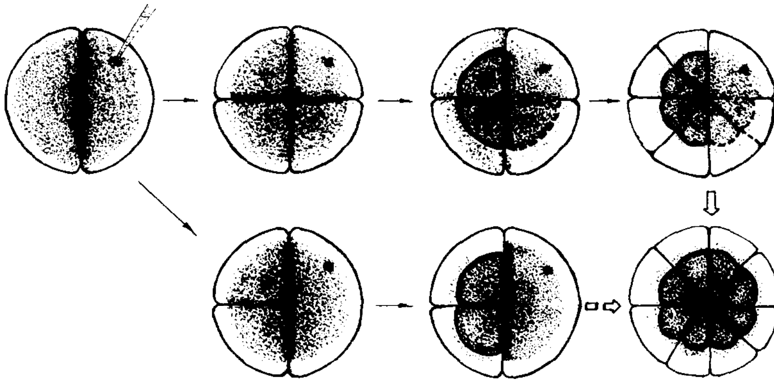

Fig. 1. Effect of propranolol microinjection on the development of *X. laevis* early embryos. The broken line designates cleavage furrows which can be formed in one of the daughter blastomeres of the injected cell. White arrows show possible restoration of normal cleavage pattern.

X. laevis embryos, the total number of experimental embryos amounting to 324. The final concentration of propranolol calculated for the volume of injected cell was 66 μM.

It was shown earlier that much higher (up to 1 mM) concentrations of propranolol dissolved in the surrounding water did not affect the development of early *X. laevis* embryos. At the same time the division of propranolol-injected blastomeres was inhibited in 82.2 ± 3.0%, 78.0 ± 3.1 or 77.8 ± 9.8% of the cases (with injections performed at the stage of 2, 4 or 8 blastomeres, respectively). It is evident that the sensitivity to the cytostatic action of propranolol did not change significantly during the stages investigated. Microinjection of control solution without propranolol caused inhibition or disturbances of cleavage divisions in 20.6 ± 6.9% of cases. A two-fold decrease in the propranolol concentration abruptly reduced the cytostatic effect.

The propranolol effect could be observed immediately after injection or could be delayed for one cell cycle. Development of one as well as both daughter blastomeres can be blocked (Fig. 1). The inhibitory effect of propranolol was reversible; it was preserved only during several cell cycles; after that cleavage divisions started again. Sometimes the disappearance of the cytostatic effect of propranolol was followed by a "cleavage explosion" pattern. This means that all or almost all previously lacking cleavage furrows were formed simultaneously.

To check on the specificity of the propranolol cytostatic effect, the bastomeres were injected with this drug mixed with adrenaline, tryptamine or serotonin. These experiments showed a pronounced protective effect of adrenaline (final concentration 100 μM). The number of cleavage divisions decreased practically to the control level (Table 1).

Injection of adrenaline without propranolol inhibited cleavage divisions in the same number of cases as in the control experiments. In some cases the cleavage furrow was formed faster in the injected blastomere as compared to other blastomeres of the experimental embryo. Serotonin (final concentration 130 μM) and tryptamine (final concentration 280 μM) did not weaken the propranolol induced inhibition of cleavage divisions (Table 1).

Preliminary experiments with microinjection of atropine (final concentration 50 μM) also showed a strong cytostatic effect reduced by simultaneous administration of acetylcholine.

Table 1. Effects of microinjection of neurochemicals and related substances into the blastomeres of *X. laevis*

Substance (μM)	Protector (μM)	Inhibition of cleavage division (%)	Difference compared to control	Significance	Difference compared to effect of propranolol (%)	Significance
Control	—	20.5 ± 6.4	—	—	—	—
Propranolol (66)*	—	80.2 ± 2.2	+59.7	>0.999	—	—
Propranolol (66)	Adrenaline (100)	27.5 ± 4.4	+7.0	<0.95†	−52.7	>0.999
Propranolol (66)	Serotonin (130)	70.0 ± 13.8	+49.5	>0.99	−10.2	<0.95
Propranolol (66)	Tryptamine (280)	80.6 ± 5.0	+60.1	>0.999	+0.4	<0.95
Propranolol (66)	A 23187 (32)‡	22.2 ± 13.8	+1.7	<0.95	−58.0	>0.999
Propranolol (66)	A 23187 (32)§	85.7 ± 7.6	+65.2	>0.999	+5.5	<0.95
Propranolol (66)	$CaCl_2$ (10)	34.4 ± 8.4	+13.9	<0.95	−45.8	>0.999
Propranolol (66)	dBcAMP (2000)‖	48.7 ± 5.6	+28.2	>0.99	−31.5	>0.999
Propranolol (66)	cAMP (2000)‖	85.7 ± 7.6	+65.2	>0.999	+5.5	<0.95
Adrenaline (100)	—	27.8 ± 7.5	+7.3	<0.95	−52.4	>0.999

*Average inhibition of stage 2–8 blastomeres.
†Non-significant result.
‡A 23187 was administered into the medium with Ca^{2+} level at 100 μM.
§A 23187 was administered into the medium with Ca^{2+} level about 1 μM.
‖Dibutyryl-cAMP and cAMP were added to the medium.

b. *Effect of calcium ions and cyclic nucleotides on the sensitivity of early embryos to neurochemicals*

If calcium ions and the calcium ionophore A 23187 (32 μM) were present in the incubation medium the cytostatic effect of propranolol injected into *X. laevis* blastomeres was expressed much less; in the absence of calcium ions A 23187 did not reduce the propranolol effect (Table 1). Reduction of the propranolol cytostatic effect was also observed when the drug was injected into the blastomeres together with calcium (final concentration of Ca^{2+} 10 μM) (Table 1). In some cases several minutes after Ca^{2+} injection the division furrow was formed in the injected blastomeres much earlier than in the intact ones. Corresponding experiments on the early sea urchin embryos were carried out previously (Buznikov *et al.*, 1984).

In the next part of the present study injection of propranolol into the blastomeres of *X. laevis* was accompanied by addition of dibutyryl-cAMP (2 mM) to the surrounding water. The inhibition of cleavage division in the injected blastomeres then decreased as compared to the propranolol effect alone. If instead of dibutyryl-cAMP the same concentration of cAMP was added to the medium, the cytostatic effect of propranolol was not reduced. It was also found that cyclic nucleotides and the adenylate cyclase activator sodium fluoride reduced the cytostatic effect of antiserotonin drugs on early sea urchin embryos (Table 2).

c. *Sensitivity of early embryos to neurochemicals and the membrane potential*

A study of the effect of antagonists of "prenervous" serotonin, indocarb and imipramine, on the membrane potential (MP) value in embryos of the sea urchin *S. mirabilis* during the first three cleavage divisions was carried out. At these developmental stages MP is between −40 and −80 mV (average MP −51.1 ± 3.5 mV). No appreciable (above 2–4 mV) and regular MP changes (those related to the cell cycle included) were found (Fig. 2). After a marked latent period (20–30 min) indocarb (50 μm) and imipramine (60 μM) induced strong depolarization of the membrane decreasing MP up to between −10 and −15 mV (Fig. 3).

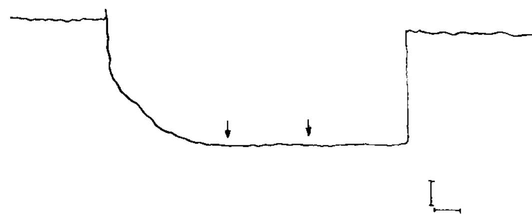

Fig. 2. Absence of MP changes in the course of cell cycle of *S. mirabilis* embryos. Arrows show beginning and end of cytotomy. Calibration: time, 3 min; potential, 10 mV.

When early embryos of the sea urchins *S. mirabilis* and *S. intermedius* were placed into isoionic water (IIW), as expected, MP practically decreased to zero (Fig. 4). Nevertheless successive cleavage divisions were observed in these embryos. Neither the rate nor type of these divisions differed from those in the control embryos. The only peculiarity in the development of such embryos was disturbance of early intercellular communications which was outwardly

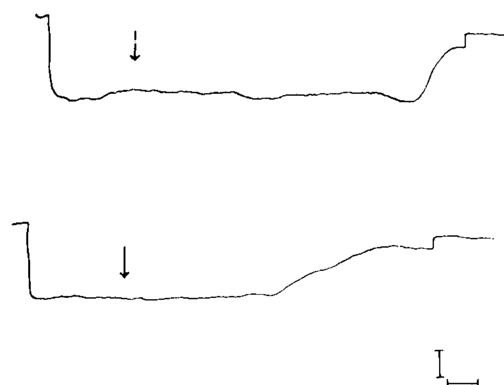

Fig. 3. Effect of indocarb and imipramine on MP of *S. mizabilis* embryos. Arrows show administration of substances into the medium. Upper curve—indocarb effect (50 μM), lower curve—imipramine effect (60 μM). Calibration: time, 2 min; potential, 20 mV.

Table 2. Effect of cyclic nucleotides and sodium fluoride on sensitivity of sea urchin *S. intermedius* early embryos to neuropharmacological drugs

Substance (μM)		Protector (μM)		Protective effect (%)	Significance
Indocarb	25	cAMP	300	0*	—
		Dibutyryl-cAMP	200	+25 ± 6.2†	>0.999
		Dibutyryl-cGMP	180	+‡	>0.95
		Sodium fluoride	10,000	+33 ± 10.6	>0.99
			5000	0	—
Imipramine	120	Dibutyryl-cAMP	200	+	>0.95
		Sodium fluoride	10,000	0	—
	60	cAMP	300	+30 ± 11.2	>0.95
		Dibutyryl-cAMP	200	+38 ± 8.3	>0.999
		Dibutyryl-cGMP	180	+35 ± 13.5	>0.95
		Sodium fluoride	10,000	+34 ± 15.0	>0.95
			5000	+	>0.95
5-Bromotryptamine	120	cAMP	300	+60 ± 9.1	>0.999
		Dibutyryl-cAMP	200	0	—
		Dibutyryl-cGMP	180	0	—

*Absence of significant effect of the protector.
†Increase in the number of embryos having passed 1st cleavage division upon the administration of protector as compared to the control (without protector).
‡Presence of protective effect found by alternative analysis qualitatively.

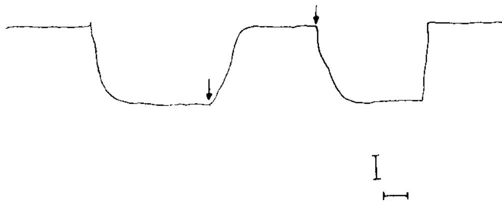

Fig. 4. MP changes in *S. mirabilis* embryo when seawater is substituted by IIW. Arrows show the beginning and end of perfusion of the embryo with IIW. Second cleavage division took place when the embryo was kept in IIW. Calibration: time, 2 min; potential, 10 m V.

very much like that observed when the Ca^{2+} level in the incubation medium was decreased. In *X. laevis* embryos unlike those of the sea urchin cyclic changes of MP (successive de- and repolarization) were observed, which correlated with the cell cycle. However, in each cycle these changes could be distinguished only after the beginning of cleavage furrow formation, i.e. they did not precede cytokinesis. Under a voltage clamp of one of the *X. laevis* blastomeres at different levels, zero level included, the cleavage divisions in such blastomeres proceeded normally at the same rate as in other blastomeres of the same embryo. Consequently, as in early sea urchin embryos, complete depolarization of the membrane did not affect the cell division.

Early sea urchin embryos incubated in IIW completely preserved their sensitivity to the specific cytostatic action of the antiserotonin chemical indocarb. The minimal concentration of this chemical needed for complete blockage of the cleavage divisions was not changed reliably when early embryos were transferred from normal seawater to IIW, i.e. when MP was reduced to zero. Another chemical tested, 5-bromtryptamine ($120 \mu M$), revealed its blocking activity in IIW as well; however, sensitivity to this chemical decreased (the number of embryos with completely blocked cleavage divisions decreased by $29.9 \pm 12.9\%$, $P > 0.95$). A decreased sensitivity to 5-bromtryptamine was also observed in experiments with IIW when higher concentrations of this chemical were applied.

DISCUSSION

The idea that the "prenervous" neurotransmitters are universally involved in the regulatory process of early embryogenesis was up to now mainly based on the presence of these substances in early embryos of various groups of invertebrates and vertebrates (Buznikov, 1967, 1981). Pharmacological experiments which discovered specific sensitivities of early embryos to neurochemicals were mostly carried out only on marine invertebrates, firstly on echinoderms. The basic evidence which allowed the conclusion concerning the intracellular localization of receptor structures as a specific peculiarity of "prenervous" neurotransmitter systems was also obtained on these subjects. Experiments on *X. laevis* with microinjection of neurochemicals proved the functional activity of "prenervous" neurotransmitters and the intracellular localization of corresponding receptors in early embryos of vertebrates as well, i.e. they presented a real basis for the generalization advanced. By the way, no microinjections were carried out in the experiments with the early embryos of echinoderms. The conclusion about the intracellular localization of receptors was based on the fact that the specific sensitivity to neurotransmitter antagonists decreased or disappeared, if penetration of these neurochemicals into blastomeres was suppressed or blocked (Buznikov *et al.*, 1977). Experiments with microinjection of neurochemicals into the embryonic cells undoubtedly present direct evidence.

As a result of the experiments performed other similar features of "prenervous" neurotransmitter systems were discovered in echinoderms and amphibians. Thus several functionally active neurotransmitters can be present in the early embryos of both animal groups. In both cases the specific cytostatic effect of neurochemicals is reversible. In addition, in the early embryos of both echinoderms and amphibians (Buznikov, 1967), the neurochemicals first of all suppress cytokinesis; kariokinesis can proceed. This is indicated specifically by the phenomenon of "cleavage explosion" revealed in sea urchins and in amphibians. It should also be noted that exogenous neurotransmitters (in the case of sea urchins dissolved in the incubation medium, and in case of *X. laevis* microinjected into the blastomeres) in both cases either weaken or eliminate the cytostatic effect of related neurochemicals.

On the other hand certain differences were found in the "prenervous" neurotransmitter system. Thus the cytostatic effect of propranolol injected into *X. laevis* blastomeres seems to be more specific, it is reduced or eliminated only by adrenaline, while with sea urchin early embryos serotonin and tryptamine also produce a protective action against propranolol (Buznikov, 1967). According to the results of preliminary experiments on *X. laevis* atropine also produces a cytostatic effect: even at high concentration it does not affect the cleavage divisions of sea urchins (Buznikov, 1967). It is unclear as yet to what extent these differences reflect the peculiarities of "prenervous" neurotransmitter systems of echinoderms and amphibians and to what extent they are the result of different experimental techniques.

The data obtained in the present study favor the participation of cyclic nucleotides and Ca^{2+} ions in the functional activity of "prenervous" neurotransmitters. It is in terms of these data that the ability of cyclic nucleotides to reduce the cytostatic effect of neurochemicals should be considered in early embryos of sea urchins and *X. laevis*, i.e. to some extent remove the consequences of disturbed functioning of "prenervous" neurotransmitters and imitate the action of exogenous serotonin and adrenalin. Finally the after-effects of disturbed functions of "prenervous" neurotransmitters can be reduced by increasing the Ca^{2+} level in the blastomeres of sea urchins (Buznikov *et al.*, 1984) and amphibians. Functional coupling of "prenervous" neurotransmitters with intracellular messengers was suggested rather long ago (McMahon, 1974; Buznikov, 1977, 1980; Toneby, 1977; Buznikov and Shmukler, 1981; Shmukler, 1981; Sadokova, 1983). Besides the evidence obtained in this work some other data also

favor this suggestion, in particular those concerning a decrease in the level of cyclic nucleotides under the cytostatic action of serotonin antagonists on early sea urchin embryos (Sadokova, 1983).

Coupling of "prenervous" neurotransmitters with cyclic nucleotides and Ca^{2+} seems to be made at the intracellular level without the direct participation of the cellular membrane. It is shown by the independence or limited dependence of the specific cytostatic effect of neurochemicals on the MP and ionic balance of the embryonic cells. Moreover the data obtained completely exclude models inferring any participation of the cellular membrane and its potential in the regulation of cleavage divisions and consequently in the "prenervous" neurotransmitter process.

Functionally active complexes of neurotransmitter receptors with the enzymes of cyclic nucleotide synthesis at early embryogenesis must be localized intracellularly, i.e. named "interoreceptosomes". Evidently, these complexes must contain catalytic and receptor components connected and at the same time divided by the plasma membrane, the intracellular one in this case. This concept about the peculiar localization of "prenervous" interoreceptosomes enables a conclusion concerning the non-trivial (intracellular) localization of adenylate cyclase in the cells of early embryos. Our next study is aimed at checking this conclusion.

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