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Comparative Biochemistry and Physiology Part A 120 (1998) 457–462

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Serotonin and acetylcholine modulate the sensitivity of early sea urchin embryos to protein kinase C activators

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Received 4 June 1996; received in revised form 22 January 1998; accepted 26 February 1998

Abstract

The protein kinase C activators 1-oleoyl-2-acetyl-rac-glycerol (OAG) and phorbol 12-myristate 13-acetate (PMA) evoke similar developmental anomalies in early embryos of sea urchins, that is, block of cleavage divisions, formation of giant polyploid nuclei with nucleolus-like inclusions, damage of cortical microfilaments and extrusion of small cytoplasts (mini-cells). Protein kinase inhibitors belonging to two different chemical groups (derivatives of isoquinoline and naphthalene) protect the embryos against OAG and PMA action. Some of these inhibitors (H-7, H-8, and H-9) also weaken the effects of 5-HT antagonists. 5-HT weakens and acetylcholine (ACh) potentiates the developmental anomalies evoked by PMA and OAG. It is suggested that endogenous 5-HT and ACh of early sea urchin embryos are functionally coupled with second messenger systems acting through the regulation of protein kinase C activity.

Keywords: Serotonin; Serotonin antagonists; Acetylcholine; Protein kinase C activators protein kinase C inhibitors; Sea urchin embryos; Cleavage divisions; Cytotoxicity

1. Introduction

Classical neurotransmitters such as serotonin (5-HT), acetylcholine (ACh) and catecholamines are functionally active throughout ontogenesis. In particular, these multifunctional regulators take part in the triggering and control of cleavage divisions and in early blastomere interactions [3,4,6,7,9,31,37]. Cytoskeleton components, especially, cortical microfilaments, serve as the effectors of these prenervous neurotransmitter functions [4]. At the same time the molecular machinery of the latter remains rather unclear.

There are good reasons for thinking that second messengers participate in such neurotransmitter functions to no smaller degree than in synaptic neurotransmission [3,9,37]. Data already published concerned mainly cyclic nucleotides and calcium ions [3,10,11,36].

We wondered if other second messengers, for example, activators of protein kinase C (PKC), may be also involved in prenervous functions of neurotransmitters. We begin a test of this assumption by confirming the effects of PKC activators themselves—phorbol 12-myristate 13-acetate (PMA) and diacylglycerols—on the echinoderm oocytes, eggs and early embryos [8], finding effects of PMA that were described in early sea urchin embryos by other authors. However, no attempt had been made prior to this to connect these effects with functional activity of prenervous neurotransmitters [2,12,14,16,20,21,23,24,30,38,40]. In this paper we study the effects of PKC activators on early sea urchins embryogenesis and the modulation of PKC activators by 5-HT and ACh.

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1095-6433/98/$19.00
PII S1095-6433(98)10045-4

2. Materials and methods

The experiments were performed on early embryos of the sea urchins *Paracentrotus lividus*, *Strongylocentrotus intermedius*, *Arbacia lixula*, *Lytechinus pictus* and *Sphaerechinus granularis*. The techniques for obtaining and handling embryos were standard [5]. The chemicals used were added to the medium (artificial sea water, ASW) 10–20 min after fertilization or immediately after the first or second cleavage divisions. Control embryos and treated embryos, including their viability were observed in the light microscope and recorded by means of photomicrography. In addition, the *P. lividus* embryos were fixed in methanol-glacial acetic acid (1:1) for preparing squashes, treated with 1% solution of Triton X-100 and stained using silver nitrate to show up the nucleoli [19].

Total proteins of embryos preincubated in ASW with [^{3}H]lysine (Amersham, UK) 1 mCi/ml for 60 min and simultaneously treated with PMA, 1 μM, were separated by 7.5% SDS-PAGE. Dried gels were exposed to X-ray films. Total RNA samples were isolated by guanidinum thiocyanate–phenol–chloroform extraction, as described by Chomczynski and Sacchi [13]. After separation on a 1% denaturing gel, RNA samples were blotted onto Amersham Hybond-N membrane and exposed to X-ray film. Routine methods were used for labelling of newly synthesized RNAs [26]. Control embryos were incubated without PMA. The demonstration of Feulgen-DNA content was estimated by cytophotometry, as previously described [8].

Preliminary experiments were carried out to determine incorporation of labelled amino acids into proteins as described by Buznikov et al. [8].

Chemicals used: protein kinase C activators 1-oleoyl-2-acetyl-rac-glycerol (OAG) and PMA; protein kinase inhibitors H-7, H-8, H-9 (Sigma, USA), HA-156, and ML-9 (ICN Flow, USA); and neurochemicals 5-HT hydrochloride (5-HT), ACh iodide (Sigma) and 5-HT antagonists inmecarb hydrochloride (IHC) and inmecarb methiodide (IMI) synthesized by Prof. V.A. Zagorevsky, Moscow, and indolopyridoazepine hydrochloride (IPH) synthesized by Dr. S.A. Pogosyan, Yerevan.

3. Results

The developmental anomalies that arise after PMA or OAG treatment are very similar in all the sea urchin species studied. These anomalies were described in detail previously [8]. Here we add the results obtained on two further sea urchin species (*S. intermedius*, 31 experiments, and *L. pictus*, 24 experiments). It was found that OAG (150–200 μM) or PMA (0.02–0.03 μM) block cleavage divisions in all experiments (Figs. 1 and 2). Only a few embryos (no more than 1–2%) passed into the next cleavage after adding of OAG or PMA. Pigment granules were concentrated near the cell surface as one or more spots. This effect, together with cell deformation, implies the relaxation and disruption of cortical microfilaments.

Numerous anuclear mini-cells (small cytoplasts) arose at one pole of the uncleaved embryo or near the cleavage furrow of two-cell embryo (Figs. 1 and 2(A)).

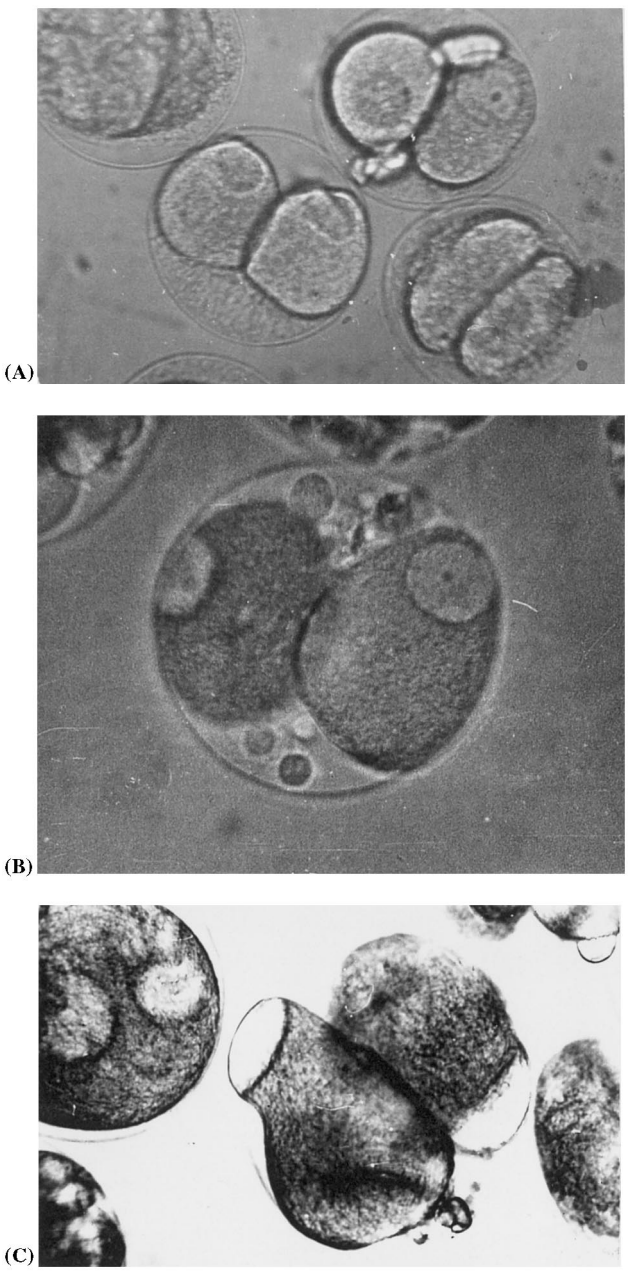

Fig. 1. The effects of OAG 200 μM (A) and PMA 0.1 μM (B,C) on the two-cell embryos of sea urchin Paracentrotus lividus. A and B—3 h after addition of OAG or PMA to ASW; typical syndrome (block of cleavage divisions, extrusion of mini-cells near cleavage furrow, formation of giant nuclei with nucleolus-like inclusions). C—5 h after addition of PMA to ASW; beginning of extrusion of nuclei (formation of karyoplasts).

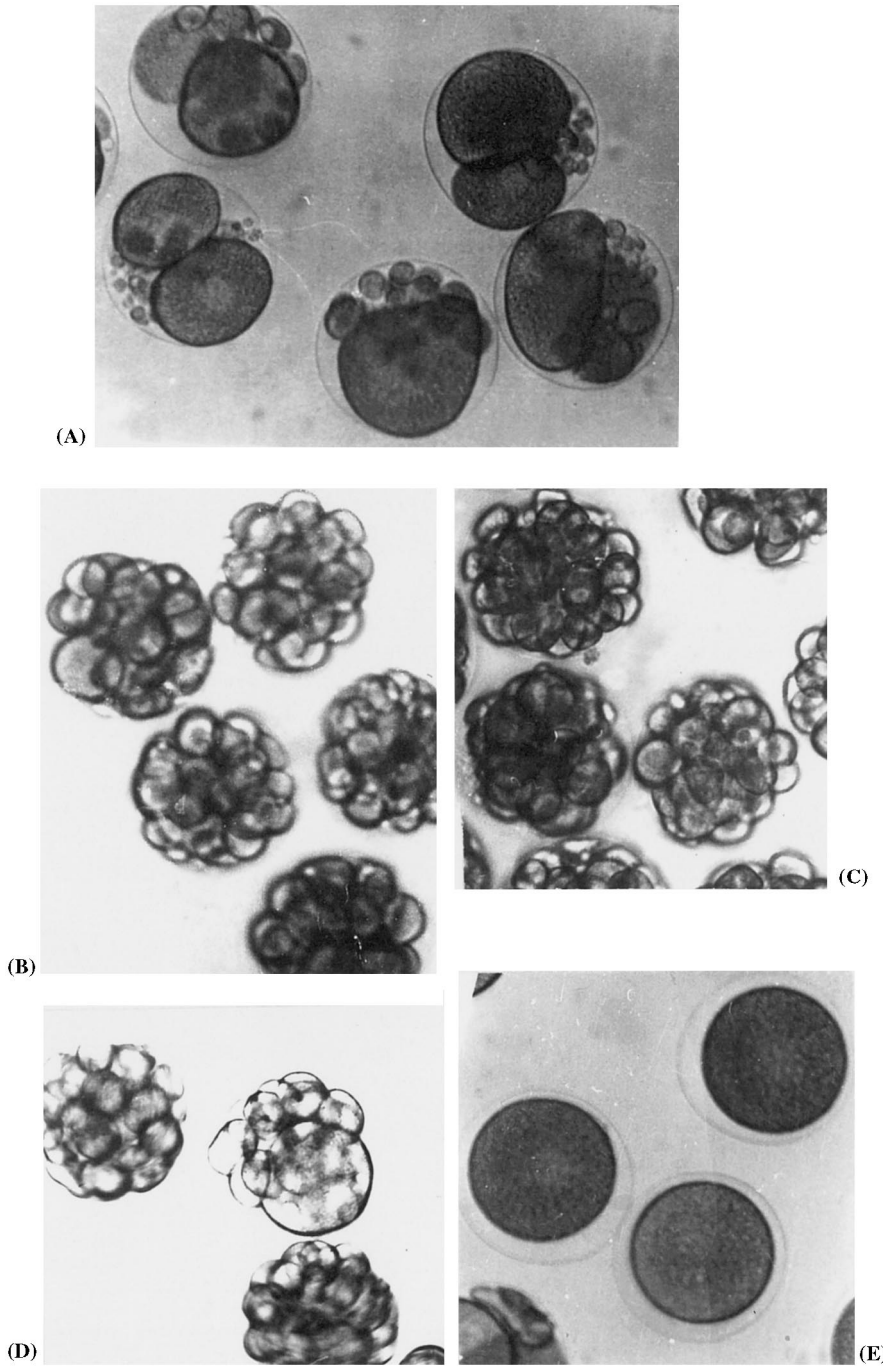

Fig. 2. The action of protein kinase C inhibitors and neurochemicals on the sensitivity of early embryos of sea urchin *Sphaerechinus granularis* to phorbol 12-myristate 13-acetate (PMA) 0.5 μM. A. PMA alone; typical appearance. B. PMA + H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride, 10 μM); almost normal early blastulae with greatly reduced PMA effects; C. PMA + ML-9 (1-(5-chloronaphthalene-1-sulfonyl-1H-hexahydro-1,4-diazepine, 10 μM); the protective action is the same, as in the case of H-7. D. PMA + 5-hydroxytryptamine, 50 μM; the protective action is weaker then in the case of protein kinase inhibitors, but is quite evident. E. PMA + acetylcholine, 100 μM; strong potentiation of PMA effect.

Giant cell nuclei with 1–2 nucleolus-like inclusions were formed 50–80 min after OAG or PMA addition (Figs. 1 and 2). These giant nuclei persisted for 5–10 h or longer without a sign of nuclear envelope breakdown. In certain cases giant nuclei were extruded from cells (Fig. 1(C)). This extrusion implied the disruption of cortical microfilaments too. Repeated washing of OAG-treated (but not PMA-treated) embryos with ASW resulted in resumption of development and step-by-step disappearance of the above-mentioned anomalies (sometimes as far as full normalization of development). The embryos incubated in OAG or

PMA-containing ASW remained alive for 6–12 h after the beginning of experiment.

It was demonstrated by cytophotometric estimation of Feulgen-DNA content that the giant nuclei were more often tetra-or octaploid. Nuclei with ploidies of 2, 16 or 32 n were rare as was aneuploidy. The nucleolus-like inclusions were stained by silver nitrate as were the true nucleoli. It is possible that these inclusions are identical to nucleoli.

Preliminary experiments showed that PMA-treated embryos retained the ability to incorporate labelled amino acids in proteins (not shown). The rate of incorporation was 50–80% greater than in the control embryos. On the other hand, we did not find any reliable differences between the pattern of newly-synthesized [^{3}H]-labelled proteins or RNAs in control and PMA-treated embryos.

Increasing OAG or PMA concentrations up to 500–800 or 100–200 μM, respectively, did not enhance their effects or accelerate the death of embryos. When concentrations of OAG and PMA were decreased to 50–150 μM and 0.01 μM, respectively, cleavage was not stopped immediately, and giant nuclei were only detected in a smaller proportion of embryos. At the same time, other anomalies were seen which suggested the perturbations of early cell–cell interactions. There was weakening of interblastomere adhesion, irregular orientation of blastomeres and inhibition of micromere formation (See for comparison, [2,6,35,40]) OAG 10–20 μM or PMA 0.005–0.01 mM did not effect the development at least up to the mid blastula stage.

Protein kinase inhibitors H-7, H-8, H-9, HA-156, (isoquinoline derivatives), A-3 and ML-9 (naphthalene derivatives) at concentrations up to 100 μM did not act on the early embryogenesis of sea urchins but protected embryos against OAG or PMA action (Fig. 2). Each inhibitor added to ASW simultaneously with OAG or PNA fully prevented all the above mentioned effects of these protein kinase C activators (Fig. 2).

Though some anomalies of development (light deformations of embryos, giant nuclei with nucleolus-like inclusions) appeared in these experiments during the first cleavage division, they then disappeared. The most effective concentration of protein kinase C inhibitors was 5–20 μM. The effects of neurotransmitters, their agonists, and antagonists on the sensitivity to PMA were studied on the early embryos of *P. lividus* and *L. pictus*. All neurochemicals were used at a concentration which had no effect on development during the early cleavage divisions. When added in the absence of PKC agonists, we found that 5-HT (20–100 μM) decreased the sensitivity of embryos to PMA. This protective action was usually weaker then the corresponding effect of protein kinase inhibitors: the block of cleavage divisions was prevented but the damage to cell–cell interactions were not reversed in some embryos (Fig. 2(D)).

5-HT antagonists (tertiary amines) inmecarb hydrochloride (IHC) (10 mM) and indolopyridoazepine hydrochloride (IPH) (15 mM) did not affect the expression of PMA's effects. At the same time, 20–50 mM inmecarb methiodide (IMI) (quarternary ammonia base) sharply enhanced the cytostatic effect of PMA but blocked the formation of non-nuclear mini-cells; giant nuclei with nucleolus-like inclusions were seen in all PMA-treated embryos. Very similar effect was found in the experiments with ACh (50–200 μM) too (Fig. 2(E)). All the protein kinase C inhibitors we used (10–20 μM) fully prevented the enhancement of the PMA effect by ACh or IMI.

Finally, we found that the protein kinase inhibitors H-7, H-8 and H-9 decreased the sensitivity of *P. lividus* and *A. lixula* embryos to the cytostatic effects of the both 5-HT antagonists tested—IHC (Fig. 3) and IPH. Other protein kinase inhibitors were not used in this group of experiments.

4. Discussion

Protein kinase C is an important regulatory enzyme in membrane and intracellular signal transduction [1,18,27]. This enzyme is found in the cytoplasm of the eggs and early embryos of sea urchins where it is needed in particular for the triggering and regulation of cleavage divisions [15,23,29,34,38,39]. Protein kinase C in early sea urchin embryos is sensitive to the activators and inhibitors used here both in vivo and in vitro [25,29,34]. Our experiments show that: i) protein kinase C activators PMA and OAG evoked quite similar developmental anomalies; ii) these anomalies are prevented by protein kinase inhibitors, belonging to two different chemical groups. Therefore, it can be considered as proven that it is activation of protein kinase C in early sea urchin embryos that caused the anomalies of development observed.

The same considerations lead to the conclusion that 5-HT, the 5-HT-antagonist IMI and ACh influence just those effects of PMA and OAG caused by the activation of protein kinase C. In other words, the data show the possible functional coupling of prenervous neurotransmitters 5-HT and ACh with one additional group of second messengers—diacylglycerols—through diacylglycerol-dependent protein kinase C, 5-HT negatively modulating PKC and ACh having a positive effect. The ability of protein kinase C inhibitors to decrease the sensitivity of cleaving embryos to the specific cytostatic action of anti-5-HT drugs leads to the same conclusion. It may be added that 5-HT is also functional antagonist of PMA during oocyte maturation [8,22,28]. In contrast, ACh potentiates the effect of PMA on the oocyte maturation [32].

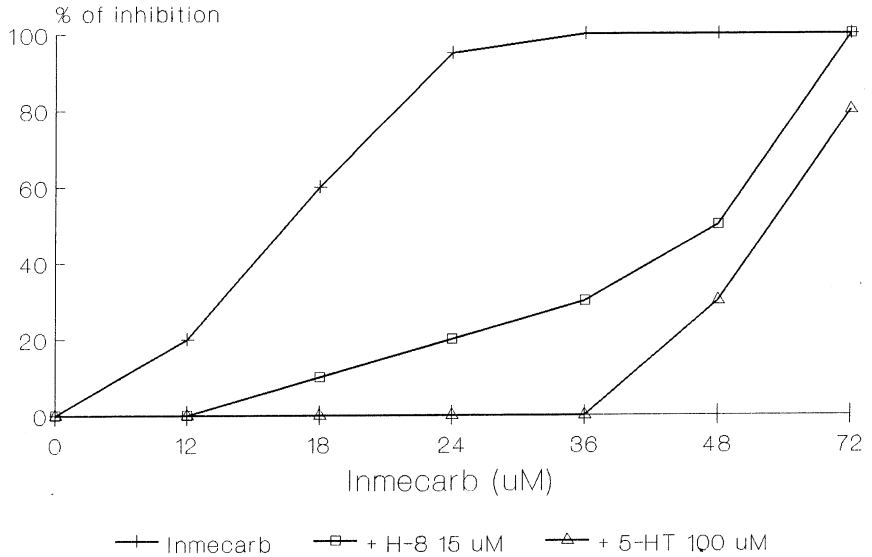

Fig. 3. The action of 5-hydroxytryptamine antagonist inmecarb hydrochloride (IHC) on the first cleavage division in embryos of sea urchin Lytechinus pictus. 1. IHC alone; 2. IHC + H-8 (*N*-[2-(methylamino) ethyl]-5isoquinoline sulfonamide hydrochloride, 15 μM); 3. IHC + 5-hydroxytryptamine, 100 μM. All the substances tested were added to ASW 10 min after fertilization.

As to the developmental anomalies evoked by PMA or OAG, they can be divided into two groups. First, there are anomalies most likely due to loss of cortical cytoskeleton (blockade of cleavage divisions, extrusion of non-nuclear mini-cells and karyoplasts, redistribution of pigment granules). Therefore, it is possible that endogenous 5-HT and ACh act on the state of cortical microfilaments during cleavage divisions (see, [4]) via protein kinase C. Second, there are anomalies which result in formation of the giant polyploid cell nuclei with long-lived envelopes and nucleolus-like inclusions (possibly, true nucleoli). They could be partially caused by the block of chromatin condensation and chromosome movement in mitosis after normal DNA replication, i.e. by a block to formation of the mitotic apparatus. However, such a block cannot itself explain the precocious appearance of nucleoli (they appear at the mid blastula stage in normal sea urchin embryos, coincident with a marked increase in RNA synthesis—see [33]).

We suggest therefore that prenervous neurotransmitters, functionally coupled to protein kinase C, take part not only in the regulation of cytoplasmic processes but also in nucleo-cytoplasmic interactions during the cell division cycle. There are other indirect data that support a role for prenervous neurotransmitters both in such interactions and in the control of cytoskeleton: Emanuelsson [17] has shown that [^{3}H] 5-HT and its antagonist [^{3}H] mesulergine bind both to the microfilaments and to the nuclear envelope in early embryos of polychaete *Ophryotrocha labronica*.

It was rather unexpected that such striking morphological changes in the cell nucleus and the precocious appearance of nucleoli were not accompanied by any noticeable alterations in macromolecular syntheses. We do not exclude the possibility that such modifications occur rapidly and transiently and so have escaped detection. We plan to test this idea using more sensitive molecular biology techniques. In this way, we should be able to obtain further information (positive or negative) related to the possible participation of prenervous neurotransmitters in the cytoplasmic influences on cell nuclei during early embryonic development.

Acknowledgements

This work was supported by the Russian Fund for Basic Investigations, grant 96-04-49, the firm ICN Galenika and a Joint Project Grant from the Royal Society.

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