Cell Differentiation 2, 151–156 (1973). © North-Holland Publishing Company

PHA-INDUCED INHIBITION OF DNA SYNTHESIS AND CLEAVAGE IN *LIMNAEA* EGGS

R.L. BRAHMACHARY and Anjana DE
Indian Statistical Institute, Calcutta, India

Accepted 4 April 1973

The effect of phytohaemaglutinin, which reversibly inhibits early cleavage in *Limnaea* embryo, was studied at the level of RNA and DNA synthesis. PHA significantly inhibits incorporation of ^{3}H-thymidine into acid-insoluble fraction. This is one of the earliest effects of PHA treatment. By contrast, incorporation of ^{32}P into RNA is not affected. Incorporation of ^{35}S is also normal.

Phytohaemaglutinin (PHA) exerts many curious effects on various kinds of cells, as has been documented in a recent spate of literature on this subject. Of the numerous papers we can cite the reviews by Pogo et al. (1966) and Joachim (1966) which seem to establish that PHA stimulates mitosis in lymphocytes and other cells, a spectacular case being that of soil amoebae studied by Ågrell (1966). While PHA-induced mitosis has been recorded in most cases, Wayne Smith et al. (1971) report the formation of multinucleated, giant cells in cultures of human colostral macrophages; multiplication of these cells by mitosis was minimal. Brahmachary et al. (1969) observed complete but reversible inhibition of cleavage in *Limnaea* eggs reared in PHA. The spectrum of actions exerted by PHA is thus wide and merits closer study. The early events, following stimulation by PHA include alterations in DNA, RNA, lipid and protein metabolism and have been reviewed by Loeb (1968; 1970) and Loeb et al. (1973). According to these authors DNA polymerase activity seems to increase tremendously; this results from a de novo synthesis of the enzyme followed by a proportional increase in DNA synthesis or replication. PHA thus offers a good system for studying the events of mitosis such as DNA replication, the interrelationships between DNA synthesis and RNA synthesis, between DNA synthesis and protein synthesis etc.

In the present investigation we have studied the effect of PHA on the metabolism of DNA, RNA and certain sulphur containing substances in *Limnaea* eggs while early cleavage is arrested.

MATERIAL AND METHODS

Eggs of *Limnaea* during early cleavage stages were used. Treatment with PHA (Difco) was carried out as in the previous report (Brahmachary et al., 1971a). Of the two forms of PHA, namely PHA-P and PHA-M, the former was used. Taking the standard concentration (i.e. one vialful of dry material dissolved in 5 ml of water) as n, various dilutions up to $n/20$ can be used. $n/5$ was used in the present experiments. Eggs from the same egg mass were kept in glass planchettes containing an equal amount of water (control) and PHA-P.

After a certain period of time, varying from 30–120 min, when microscopic observation showed that PHA-treated cells were arrested, 10–20 μc of ^{32}P (phosphoric acid neutralized to about pH 7.2) was added to the medium of the control and treated batch. Counts in TCA-insoluble material were determined as previously described (Brahmachary et al., 1968). RNA fractionation was carried out on agarose gels, following the earlier procedure employing ion agar (Brahmachary et al., 1971). ^{35}S (25 μc) was incorporated and counts in TCA-insoluble material was measured as reported (Greaves et al., 1972). ^{3}H-thymidine dissolved in water was added to the medium in order to study the synthesis of DNA. 20 μc in 0.2 ml water was used in each experiment. 1 ml of water (control) or PHA solution was used. Times of incubation were from 15 min to 1 hr. Incubation with thymidine was for 15 min in table 6, and 30 min in other cases. The incubation period with ^{35}S was for 1 hr. Incubation with ^{32}P was for 30 min. The composition of scintillation fluid was: paraterphenyl, 0.4 g; napthalene, 5 g; popop, 0.02 g, (dissolved in 100 ml dioxane), 10 ml of this solution was taken and mixed with the tritium-labelled material dissolved in 0.3 ml ammonia (0.88) and made up to 1 ml.

RNA was fractionated by electrophoresis, in agarose gel and the corresponding veronal buffer (instant gel and instant buffer, Serva, Heidelberg) at a voltage of 350 and current of 20–22 mA. The agar plate had a size of 75 X 25 mm and electrophoresis was carried out for about 30 min. Rat liver 28S, 18S and 4S RNA were used as markers. Intracellular pools of ^{3}H-thymidine were measured following earlier methods (Brahmachary et al., 1971b).

RESULTS

The incorporation of ^{32}P into TCA-insoluble fraction is shown in table 1. The eggs arrested by PHA before the first cleavage or at 2-cell or 4-cell stage do not exhibit any inhibition in the incorporation as compared to the normal eggs. In one case even a stimulation is detectable.

In order to detect significant changes, if any, in the pattern of RNA synthesis following addition of PHA which arrests cleavage, phenol-extracted RNA from eggs inhibited at the first cleavage was separated by agarose-gel electrophoresis. As the patterns of RNA synthesis in normal developmental stages have already been stud-

Table 1
Incorporation of ^{32}P into TCA-insoluble fraction in normal and PHA-treated eggs, after incubation in ^{32}P for 30 min.

Expt. no.	No. of eggs in		cpm (in all eggs) in	
	Control	PHA	Control	PHA
1	30	31	2235	3455
2	33	33	1418	1443
3	40	54	2007	2507

Table 2
Incorporation of ^{32}P into RNA fractionated by gel electrophoresis, after incubation in ^{32}P for 30 min.

	cpm PHA-treated	cpm control
above 28S	3700	680
28S	705	101
28 – 18S	1147	57
18S	1179	141
18 – 4S	565	60
4S	1145	135

Table 3
The effect of PHA on incorporation of ^{35}S, after incubation in ^{35}S for 1 hr.

Expt. no.	No. of eggs in		cpm (in all eggs) in	
	Control	PHA	Control	PHA
1	30	31	2235	3455
2	33	33	1418	1443
3	40	54	2007	2507

Table 4
Thymidine incorporation into DNA during successive periods of the cleavage cycle. cpm indicates counts in the acid insoluble part of all eggs.

No. of eggs		Time of incubation in ^{3}H-thymidine	Stage	cpm
1.	20	80 min	Uncleaved extrusion of two polar bodies	3397
	20	73 min	From the above stage to cleavage	2211
2.	14	30 min	Just-cleaving to mid cleavage	2068
	12	30 min	mid-cleavage to 4-cell	3120

Table 5
The thymidine-incorporation pattern in PHA-treated eggs and control.

Expt. no.	No. of eggs		cpm (in all eggs)	
	Control	PHA	Control	PHA
1	31	32	9,962	3,124
2	24	22	1,510	393

ied (Brahmachary et al., 1968; 1971a) any striking change in PHA-treated eggs would be recognizable. Table 2 shows incorporation into various RNA fractions.

It is seen from the table that all fractions of RNA have been synthesized in the PHA-arrested eggs. The pattern is not strikingly different from that of normal eggs at this stage. (The absolute values of counts cannot of course be compared, because different amounts of radioactive RNA were put in the grooves of the gels).

Table 3 shows the extent of incorporation of ^{35}S into TCA-insoluble fraction. Here again there is no reduction in case of the PHA-treated, arrested cells, in one example even stimulation can be marked.

Thymidine incorporation, presumably into DNA, is known to be a nearly continuous process in early cleaving eggs (see Discussion). Table 4 shows the rates of incorporation into normal eggs during the successive periods of the cleavage cycle.

Incorporation of thymidine into PHA arrested eggs was then investigated. There seems to be a significant inhibition of DNA synthesis in the arrested eggs, as evident from incorporation of ^{3}H-thymidine into the acid-insoluble fraction (table 5). Eggs in early cleavage stages were used; incubation in ^{3}H-thymidine was for 30 min.

As DNA synthesis is likely to be inhibited in all circumstances that inhibit cell division, it was necessary to note the incorporation not merely after the arrest of cell division, but immediately following PHA treatment. As evident from table 6 incorporation into DNA is very significantly reduced in all the cases. The intracellular pool of ^{3}H-thymidine decreases slightly in the PHA-treated eggs but incorporation into DNA decreases far more (up to about 80%).

Table 6
Incorporation in the first 15 minutes of PHA-treatment. Eggs were at 2-cell stage; incubation in ^{3}H-thymidine for 15 min.

Expt. no.	No. of eggs		Total cpm	
	Control	PHA	Control	PHA
1	16	18	139	59
2	24	26	271	71
3	18	16	629	110

DISCUSSION

The results reported here shed light on the nature of action exerted by PHA. As was known earlier, *Limnaea* eggs in the early cleavage stages are reversibly inhibited, i.e. mitosis is arrested so long as PHA is present. The PHA molecule thus acts as a reversible blockage at some level. The reversibility indicates the possibility that the molecule is loosely bound. Greeves et al. (1972) showed that PHA which was bound to sepharose beads and therefore in an insoluble form, can activate lymphocytes, probably at the outer membrane level. The apparent stimulation in RNA (and in ^{35}S-containing substance) as evident in one or two cases may well be due to an effect of PHA on the cell membrane. The present results indicate, however, that inhibition of cleavage is not due to a blocking of RNA synthesis. Furthermore, no particular class of RNA is very strongly inhibited as seen from table 2. It thus seems that arrested mitosis probably cannot be correlated with a blocking of or inhibition of any particular class of RNA, let alone the inhibition of over-all RNA synthesis.

The results show that the metabolism of ^{35}S which is a marked feature during cleavage (Brahmachary et al. 1971b; and Ghosal, in preparation) is synthesized in PHA-treated, arrested eggs apparently to the same extent as in the normal egg.

By contrast, there is a very marked inhibition of DNA synthesis due to PHA. As has been found in the early cleavage stage of sea-urchin eggs, DNA synthesis is a continuous process, that is G_1 and G_2 phases are short or missing (Hinegardner et al., 1964; and Kedes et al., 1969). Our results also show that there are no long resting phases. As stimulation of DNA synthesis due to stimulation of DNA polymerase synthesis is concomitant with mitosis (Loeb, 1968, 1970 and Loeb et al., 1973), the considerable inhibition of DNA synthesis now revealed (see tables 5 and 6) may be the agent causing arrest in mitosis. In fact the inhibition is detectable not only after arrest of cleavage, but also immediately after addition of PHA which apparently binds to the membrane (Goldstein et al., 1965; Pogo et al., 1966; So et al., 1970; Aubrey et al., 1972). Furthermore, a decoupling of RNA and DNA synthesis as well as of the synthesis of sulphur containing substances and DNA synthesis is evident. The PHA-arrested egg therefore offers a system for exploring some of the mitotic events from new angles as also the problem of DNA synthesis.

ACKNOWLEDGEMENTS

One of us (R.L.B.) is most grateful to Dr. U.E. Loening and to the referee of Cell Differentiation for valuable suggestions.

This research project was supported by an ICMR Scheme.

Thanks are also due to Dr. Biren Biswas and his staff for helping with the liquid scintillation counter.

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