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Zinc as a Second Messenger of Mitogenic Induction

Effects on Diadenosine Tetraphosphate (Ap_4A) and DNA Synthesis

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DNA synthesis and adenosine(5′)tetraphosphate(5′)adenosine (Ap_4A) levels decrease in cells treated with EDTA. The inhibitory effect of EDTA can be reversed with micromolar amounts of $ZnCl_2$. $ZnCl_2$ in micromolar concentrations also inhibits Ap_4A hydrolase and stimulates amino acid-dependent Ap_4A synthesis, suggesting that Zn^{2+} is modulating intracellular Ap_4A pools. Serum addition to G1-arrested cells enhances uptake of Zn, whereas serum depletion leads to a fivefold decrease of the rates of zinc uptake. These results are discussed by regarding Zn^{2+} as a putative 'second messenger' of mitogenic induction and Ap_4A as a possible 'third messenger' and trigger of DNA synthesis.

Mitogenic stimulation of quiescent mammalian cells is characterized by progression through the G1 period and initiation of DNA synthesis at the G1/S phase boundary. The primary mitogenic signal triggering these events is the interaction between extracellular growth factors (PDGF, EGF, FGF etc.) with their cognate membrane receptors. Receptor binding is followed by patching and internalization of clustered mitogen–receptor complexes. After endocytosis the complexes are transported by clathrin-coated vesicles to lysosomes and degraded. No recycling of receptors or growth factors is known. The link between these events and stimulation of DNA synthesis, which usually occurs 8–12 h after exposure of cells to growth factors, is unknown. But it is generally accepted that the initiation of DNA replication in the nucleus after entry into S phase is under the control of cytoplasmic components [1, 2]. The cytoplasmic factor seems to act as a positive regulatory signal, i.e., as an inducer of replication [3].

Previous studies [4] indicated that the component inducing DNA synthesis is produced after mitogenic stimulation by the cell itself and that it is accumulated gradually during progression through the G1 phase until a critical threshold concentration is reached which determines entry into S phase. A favorite candidate for such a signal molecule is adenosine(5′)tetraphospho(5′)adenosine (Ap_4A). This purine nucleotide has been suggested, on the basis of its high metabolic lability and the correlation of its pool sizes with the growth- and cell cycle state of eukaryotic cells, to be part of the intracellular signal chain of mitogenic induction [5–8]. This hypothesis was strongly supported by the obser-

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vation that the site of its intracellular activity is the DNA replicative machinery. Addition of Ap_4A to permeabilized G1-arrested baby hamster kidney (BHK) cells yielded initiation of DNA replication in the quiescent cells [9, 10]. Ap_4A has been demonstrated to bind as a specific ligand to calf thymus DNA polymerase α (deoxynucleosidetriphosphate: DNA deoxynucleotidyl transferase) holoenzyme (M_r 404000) and to affinity label a subunit of M_r 57000 [11]. Also the high molecular weight (HMW) (660000) and low molecular weight (LMW) (145000) forms of DNA polymerase α of human HeLa cells were shown to possess highly specific, non-covalent, Ap_4A-binding activity [12]. The dissociation constants were determined to be 0.27 μM for the calf thymus enzyme and 16–22 μM for the HeLa DNA polymerase α.

There is accumulating evidence that Ap_4A could be involved in the priming reaction of mammalian DNA polymerase α holoenzyme. Ap_4A was shown by Rapaport et al. [13] to act as a primer for DNA synthesis in vitro by DNA polymerase α from HeLa cells with poly(dT) and by Zamecnik et al. [14] with a double-stranded (ds) synthetic polymer as a template. Our recent results demonstrate that Ap_4A primes DNA replication by calf thymus DNA polymerase α in vitro with circular single-stranded (ss) M13 phage DNA as a template at concentrations of about 0.1 μM [15].

To further substantiate the hypothesis of Ap_4A being a putative positive signal molecule triggering onset of DNA synthesis, we studied factors influencing both intracellular Ap_4A levels and the replicative machinery. Here we describe experiments suggesting that Zn^{2+} plays an important role in the transmission of the primary, extracellular mitogenic signal, i.e., growth factor–receptor binding, into intracellular events, expansion of Ap_4A pools and onset of DNA synthesis.

MATERIALS AND METHODS

[^{3}H]Ap_4A (20 Ci/mmole) was synthesized as described [9]. ATP, Ap_4A, alkaline phosphatase and snake venom phosphodiesterase were from Boehringer, Mannheim; ^{65}Zn and [^{3}H]thymidine (30 Ci/mmole) were from Amersham.

Cell Culture and in vivo Measurement of DNA Synthesis and Zn Uptake

Baby hamster kidney (BHK) cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 15-cm dishes as described [16]. G1-arrested cells were obtained by culturing cells with 0.3% serum for 48 h. For measuring DNA synthesis and Zn uptake 5×10^4 BHK cells were plated in 3½-cm dishes with 2 ml medium. DNA synthesis was measured by adding [^{3}H]thymidine (final concentration, 1 μM) for 1 h. The medium was removed, the cultures washed, and trichloroacetic acid (TCA)-precipitable radioactivity determined. Zn uptake was determined by adding 0.1 μCi ^{65}Zn to each of two triplicate sets of 3½-cm dishes. One triplicate set was incubated for 30 min at 37°C, the second set was precooled for 15 min at 0°C and incubated after ^{65}Zn addition for 30 min on ice. After incubation cells were quickly washed three times with 2 ml ice-cold saline, scraped off the dishes with 1 ml 0.5% Triton X-100 and the radioactivity was determined in a toluene/Triton-based scintillation cocktail. Uptake values were calculated by background (0°C values) subtraction.

Ap_4A Assay

For Ap_4A determination 10^8 cells were used per assay. After medium removal cultures were washed twice with cold saline, then 2 ml 5% formic acid were added to the cultures and the cells scraped off. The suspensions were centrifuged (10 min, 10000 g), the pellets were washed once with 2 ml formic acid, the supernatants were combined, 0.1 μCi [^{3}H]Ap_4A was added as internal standard, and the nucleotide extracts were lyophilized. The nucleotide extracts were dissolved in 2 ml 20 mM Tris-formate, pH 8.0, 1 mM $MgCl_2$, and digested with 1 unit of alkaline phosphatase (purified on Sephadex G-75) at 37°C until no significant ATP moiety was observed with the luciferin/luciferase system. The digest was diluted with 20 ml 0.12 M ammonium bicarbonate buffer, pH 8.2, applied on a (15×0.7 cm) Whatman DE52 column after equilibration with the same buffer. The column was washed with 50 ml of the equilibration buffer and Ap_4A was eluted with 30 ml 0.3 M ammonium bicarbonate. After lyophilization, Ap_4A was solved in 100 μl H_2O and 700 μl sample buffer (0.1 M Tris-acetate, pH 7.75, 2 mM EDTA) and 200 μl luciferin/luciferase (Lumit PM, Lumac A.G.) were added. After determination of the ATP background, 5 μl snake venom phosphodiesterase (0.15 units) were added and the Ap_4A content measured as expressed by ATP generation after phosphodiesterase digestion. The amount of Ap_4A generated was calculated from an ATP and Ap_4A standard curve. The method is suitable to determine unlabelled Ap_4A content even in cells from quiescent cultures with Ap_4A levels as low as 0.01 μM. Ap_4A values are given in molar concentrations. Cell volumes were routinely measured by flow cytophotometry.

The assay applied here is not absolutely specific for Ap_4A since other adenosine(5′)tetraphospho(5′)nucleosides (Ap_4N) can be measured with 50% yield in comparison to Ap_4A as well. However, the Ap_4A levels determined in randomly growing cultures by the method applied here are similar to those determined previously by a more specific method [5].

Ap_4A Hydrolase Assay

10^7 BHK cells were washed twice with saline, scraped off the dishes and suspended in buffer E (20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 1% Nonidet P40, 0.7% sodium deoxycholate). After swelling for 10 min at 0°C the cells were homogenized and centrifuged (15 min, 10000 rpm) and the supernatant dialysed for 16 h. Fifty μl samples containing 10 μl extract or purified Ap_4A hydrolase, 12 μl 10 μM Ap_4A solution, 1 μl [^{3}H]Ap_4A (20 Ci/mmole), 17 μl buffer E and 10 μl $ZnCl_2$ at various concentrations were incubated for 5 min at 37°C. The reaction was stopped by adding 5 μl of concentrated formic acid and the reaction products were analysed by chromatography on polyethyleneimine thin-layer plates. For quantification, thin-layer plates were cut into pieces and the radioactivity determined.

Ap_4A-Synthetase Activity Assay

Cell extracts were prepared as described for Ap_4A hydrolase assays. To determine Ap_4A synthetase activity 100 μl samples containing 50 μl extract, 20 mM Tris-HCl, pH 7.8, 12.5 mM $MgCl_2$, 1 mM ATP, $ZnCl_2$ at various concentrations were incubated for 1 h at 37°C in the presence or absence of a mixture of all 20 L-amino acids at 1 mM final concentration. The reaction was stopped by addition of 5 μl concentrated formic acid and the Ap_4A synthesized in the reaction mixture determined as described above.

RESULTS

Effects of EDTA and Zn^{2+} on DNA Synthesis in BHK Cells

Addition of EDTA to the tissue culture medium at concentrations significantly lower than those of the Mg^{2+} and Ca^{2+} ions in the medium (together approx. 2.6 mM) resulted in more than 95% inhibition of DNA synthesis (fig. 1). 95% inhibition of [^{3}H]thymidine incorporation was observed 13 h after application of 1 mM EDTA (fig. 2). The inhibitory effect of EDTA on DNA synthesis could be fully reversed by addition of Zn^{2+} to the culture medium in a dose-dependent

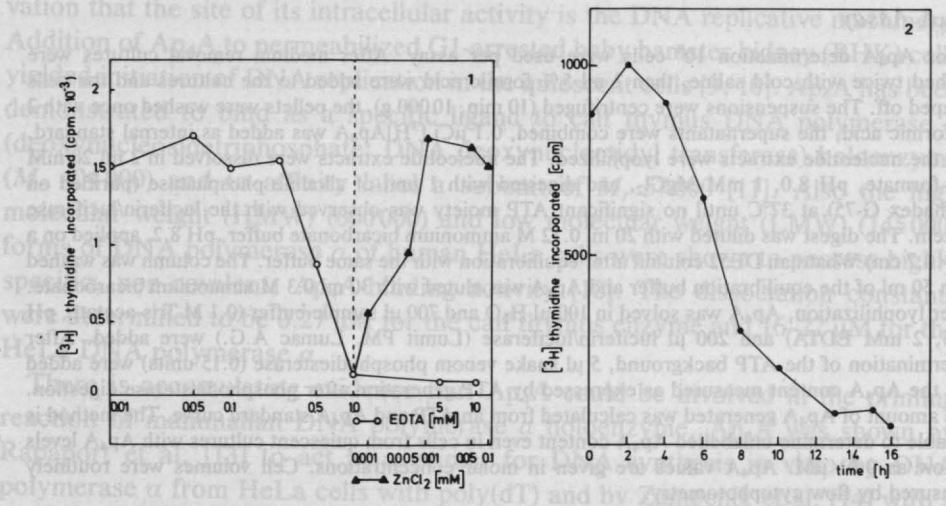

Fig. 1. Inhibition of DNA synthesis in BHK fibroblasts by increasing concentrations of EDTA (○). EDTA was added to growing cultures for 16 h and [^{3}H]thymidine incorporation into TCA-precipitable material determined by pulse-labelling for 30 min with [^{3}H]thymidine, (▲) reversal of the inhibitory effect of EDTA on DNA synthesis by addition of increasing amounts of $ZnCl_2$ in the presence of 1 mM EDTA. Exponentially growing BHK cells were preincubated for 16 h with 1 mM EDTA, then various amounts of $ZnCl_2$ were added for 8 h.

Fig. 2. Time course of inhibition of DNA synthesis in BHK fibroblasts by EDTA. One mM EDTA was added to exponentially growing cultures and DNA synthesis measured by pulse-labelling for 30 min with [^{3}H]thymidine as described in the caption to fig. 1.

manner. These observations are consistent with previously reported data concerning the effects of chelating agents and Zn^{2+} on DNA synthesis in animal cells [17]. Fig. 1 demonstrates that addition of 10 μM Zn^{2+} fully restores DNA synthesis within 8 h in BHK cells pretreated with 1 mM EDTA for 16 h. Fig. 3 shows that this effect is specific for Zn^{2+} ions, Fe^{3+}, Mg^{2+} or Ca^{2+} ions cannot prevent or reverse the inhibitory effect of EDTA.

Effect of EDTA and Zn^{2+} on Ap_4A Levels in BHK Cells

Since Ap_4A levels fluctuate correlated with the growth and cell cycle state of BHK cells [6] we addressed the question whether the effects of EDTA and Zn^{2+} on the DNA-synthesizing activity could be mediated by Ap_4A. Therefore, we studied the effects of Zn^{2+} withdrawal by EDTA and its reversal by exogenous Zn^{2+} on intracellular Ap_4A levels. Zn^{2+} depletion by addition of 1 mM EDTA to the culture medium leads to a decrease of the intracellular Ap_4A concentration of exponentially growing BHK cells from 1.7 to 0.14 μM (fig. 4). Addition of $ZnCl_2$ to these cells results in an increase to 1.4 μM Ap_4A at 10 μM $ZnCl_2$, and 1.9 μM Ap_4A at 50 μM $ZnCl_2$. Inhibition of DNA synthesis parallels the decrease of Ap_4A levels, and the time of onset of DNA synthesis upon application of Zn^{2+} is correlated with the concentration of cellular Ap_4A. These results suggest that

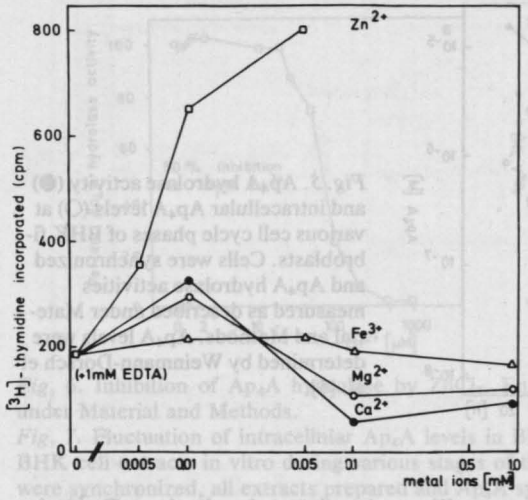

Fig. 3. Effects of metal ions on DNA synthesis of BHK fibroblasts inhibited by 1 mM EDTA. Treatment as described in the caption to fig. 1.

Ap_4A is involved in the regulation of DNA synthesis and that its concentration in living cells is controlled by Zn^{2+}.

Ap_4A Hydrolase Activity Changes Inversely to the Ap_4A Concentration during Cell Cycle

The dramatic fluctuations of Ap_4A pools in correlation with growth rate and cell cycle state led to the question how those changes are brought about in

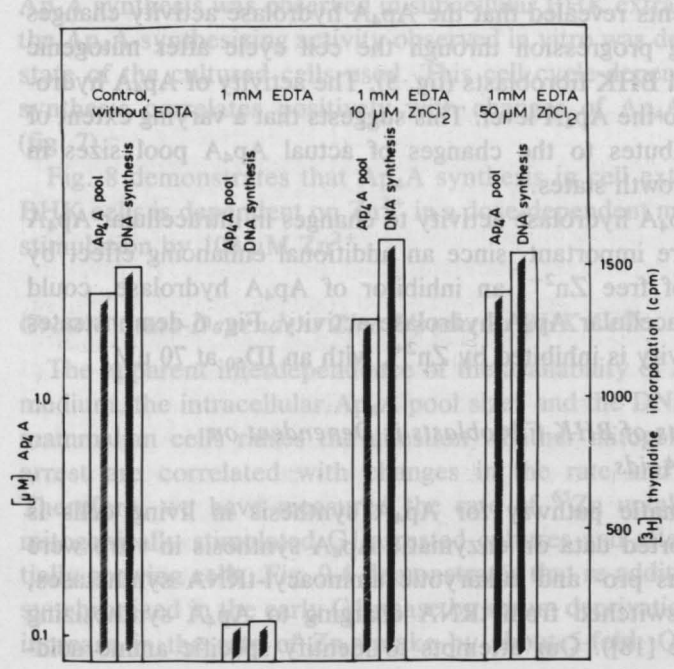

Fig. 4. Effects of EDTA and $ZnCl_2$ on intracellular Ap_4A pools and on DNA synthesis in BHK fibroblasts. Exponentially growing cultures were treated by 1 mM EDTA $\pm$10 or 50 µM $ZnCl_2$, respectively, for 16 h and DNA synthesis measured as described under Material and Methods.

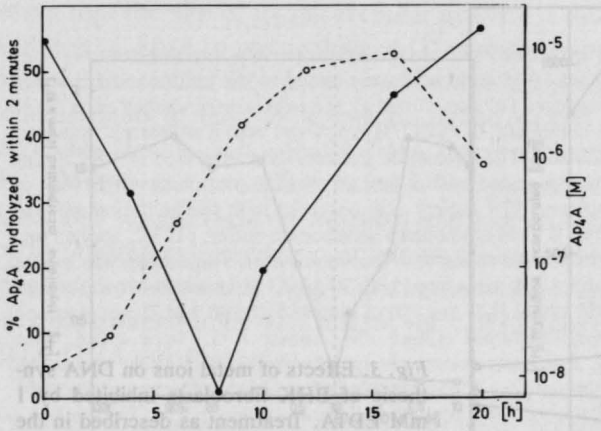

Fig. 5. Ap_4A hydrolase activity (●) and intracellular Ap_4A levels (○) at various cell cycle phases of BHK fibroblasts. Cells were synchronized and Ap_4A hydrolase activities measured as described under Material and Methods. Ap_4A levels were determined by Weinmann-Dorsch et al. [6].

response to growth and cell cycle-controlling signals and whether Zn^{2+} is involved as a mediator. Intracellular Ap_4A concentrations could be controlled by influencing the biosynthesis or the degradation of Ap_4A. Therefore we studied whether changes of the activity of the specific enzyme for Ap_4A catabolism, i.e., of Ap_4A hydrolase, correlate with fluctuations of intracellular Ap_4A pools at different growth and cell cycle states. In the course of these experiments we made use of the fact that the specific Ap_4A hydrolase activity in cell extracts can be inhibited by 100 μM $ZnCl_2$ (see below), whereas unspecific phosphodiesterase activities cannot. On subtracting the basal level of unspecific phosphodiesterase activities, the measurements revealed that the Ap_4A hydrolase activity changes drastically 30-fold during progression through the cell cycle after mitogenic stimulation of G1-arrested BHK fibroblasts (fig. 5). The activity of Ap_4A hydrolase fluctuates inversely to the Ap_4A level. This suggests that a varying extent of Ap_4A degradation contributes to the changes of actual Ap_4A pool sizes in different cell cycle and growth states.

This contribution of Ap_4A hydrolase activity to changes in intracellular Ap_4A pools could be even more important, since an additional enhancing effect by varying concentrations of free Zn^{2+}, an inhibitor of Ap_4A hydrolase, could further modulate the intracellular Ap_4A hydrolase activity. Fig. 6 demonstrates that Ap_4A hydrolase activity is inhibited by Zn^{2+}, with an ID_{50} at 70 μM.

Ap_4A Synthesis in Extracts of BHK Fibroblasts is Dependent on Zn^{2+} and Free Amino Acids

Up to now the enzymatic pathway for Ap_4A synthesis in living cells is unknown. All so far reported data on enzymatic Ap_4A synthesis in vitro were obtained by using various pro- and eukaryotic aminoacyl-tRNA synthetases, some of which can be switched from tRNA charging to Ap_4A synthesizing enzymes (for review, see [18]). Our attempts to identify specific amino acid-

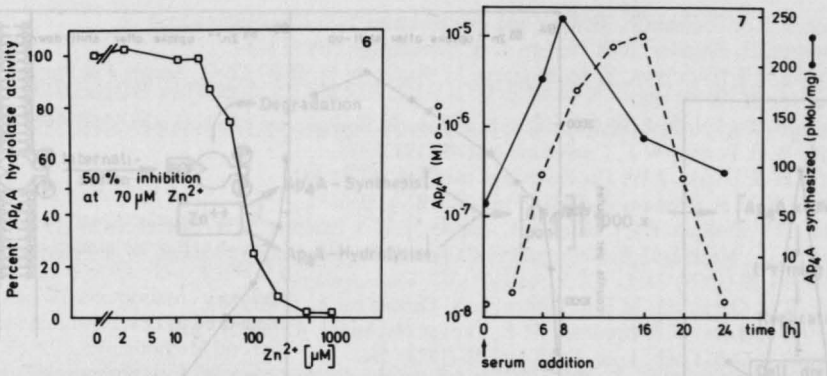

Fig. 6. Inhibition of Ap_4A hydrolase by $ZnCl_2$. Enzyme activities were determined as described under Material and Methods.

Fig. 7. Fluctuation of intracellular Ap_4A levels in BHK cells and of Ap_4A synthesizing activity in BHK cell extracts in vitro during various stages of the cell division cycle. BHK fibroblast cultures were synchronized, all extracts prepared and Ap_4A synthesis activity determined as described under Material and Methods.

independent Ap_4A synthetases in mammalian cell extracts led to negative results. Incubation of extensively dialysed cell extracts from BHK cells of various cell cycle phases, together with 1 mM ATP and 12.5 mM $MgCl_2$ at pH 7.8 and 37°C, yielded significant amounts of newly synthesized Ap_4A, but only when both an amino acid mixture and $ZnCl_2$ was added (data not shown).

In the presence of 50 µM Zn^{2+} and of a 1 mM amino acid mixture a significant Ap_4A synthesis was observed in subcellular BHK extracts in vitro. The extent of the Ap_4A-synthesizing activity observed in vitro was dependent on the cell cycle state of the cultured cells used. This cell cycle-dependent fluctuation of Ap_4A synthesis correlates positively with changes of Ap_4A levels in BHK in vivo (fig. 7).

Fig. 8 demonstrates that Ap_4A synthesis in cell extracts from mid-G1 phase BHK cells is dependent on Zn^{2+} in a dose-dependent manner with a maximum of stimulation by 100 µM Zn^{2+}.

Growth State-Dependent Zinc Uptake in BHK Cells

The apparent interdependence of the availability of Zn^{2+} in the tissue culture medium, the intracellular Ap_4A pool sizes and the DNA synthesizing activity in mammalian cells raises the question whether mitogenic stimulation or growth arrest are correlated with changes in the rate and extent of Zn^{2+} uptake. Therefore, we have measured the rate of ^{65}Zn uptake in BHK cells both in mitogenically stimulated G1-arrested cultures and after shift-down in exponentially growing cells. Fig. 9*A* demonstrates that re-addition of 10% serum to cells synchronized in the early G1 phase by serum deprivation results in an immediate increase in the rate of Zn uptake by about 5-fold. On the other hand, serum

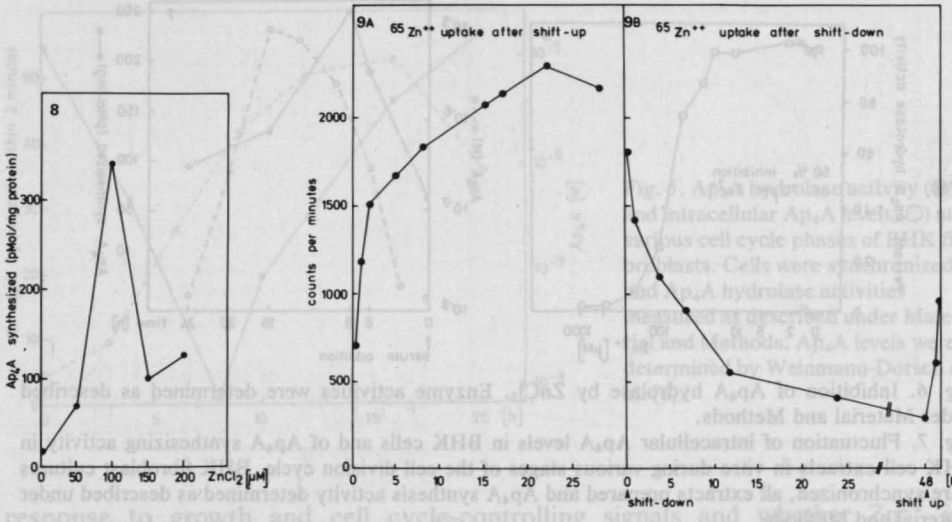

Fig. 8. Zn^{2+} dose dependency of Ap_4A synthesis activity in BHK fibroblast extracts. Cells were synchronized, extracts prepared 8 h after serum addition and assayed for Ap_4A synthesis at various $ZnCl_2$ concentrations as described under Materials and Methods.

Fig. 9. Rate of Zn uptake in BHK fibroblasts under shift-up and shift-down conditions. (*A*) BHK fibroblasts were arrested in early G1 phase by serum deprival as described under Materials and Methods, 10% serum was added at time zero and ^{65}Zn uptake measured by pulse labelling for 60 min as described in Methods. (*B*) Serum was withdrawn from the medium of exponentially growing BHK cells at time zero and the medium replenished at 48 h.

withdrawal causes a 5-fold decrease of uptake rates for ^{65}Zn (fig. 9*B*). Thus, the intracellular zinc content could be controlled either by mitogenic stimulation or by growth arrest.

DISCUSSION

Taking together the data on Ap_4A that have been accumulated during the last 6–8 years it is very suggestive that Ap_4A acts as a chemical messenger or 'metabolic symbol' [19] of mitogenic induction similar to cAMP in hormone induction. The interaction between growth factors and their cognate receptors in cellular membranes could trigger signals which are transmitted into a central intracellular response, i.e. 1 000-fold expansion of the Ap_4A pool. Whether Ap_4A pools are regulated primarily on the level of biosynthesis, degradation or on a combination of both, is still a matter of speculation. Ap_4A may interact directly with the replicative machinery, presumably after binding to its highly specific binding site on DNA polymerase α (Ap_4A acceptor protein), acting either as an allosteric effector of the polymerase or by being involved in the priming reaction. These reactions of Ap_4A could then cause the eventual intracellular response of mitogenic induction, i.e., onset of DNA replication at the G1/S-phase boundary of the cell division cycle.

Increasing evidence suggests that Zn^{2+} plays an important role in the trans-

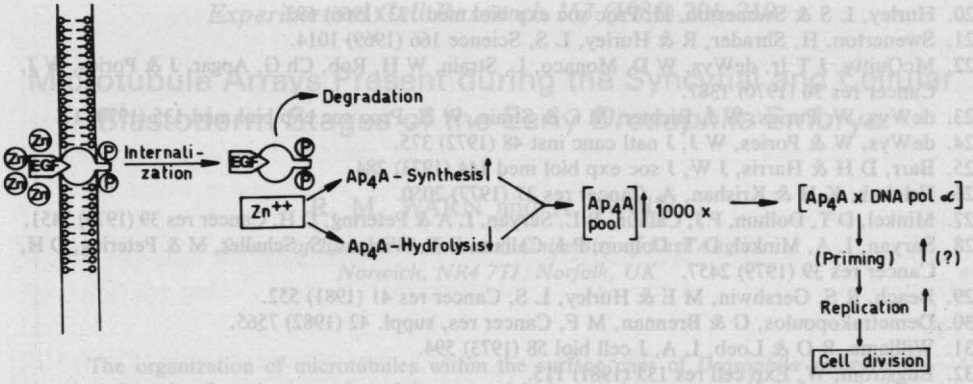

Fig. 10. Simplifying scheme of putative signal transmission upon mitogenic stimulation.

mission of the primary, extracellular mitogenic signal, i.e., growth factor-receptor binding, into intracellular events of mitogenic induction (see fig. 10). The observation that mitogenic induction leads to increased Zn^{2+} uptake and that Zn^{2+} is essential for the rising of intracellular Ap_4A concentrations and DNA replication suggests that Zn^{2+} is a 'second messenger' of mitogenic induction and that Ap_4A is a 'third messenger'. This assumption is supported by previously published observations indicating that zinc deficiency prevents cell and tumor growth [20–30] and/or inhibits DNA replication [31, 32].

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REFERENCES

1. Graham, C F, Arms, K & Gurdon, J B, Dev biol 14 (1966) 349.
2. Johnson, R T & Harris, H, J cell sci 5 (1969) 625.
3. Rao, P N & Johnson, R T, Nature 225 (1970) 159.
4. Rao, P N, Wilson, B A & Sunkara, P S, Proc natl acad sci US 75 (1978) 5043.
5. Rapaport, E & Zamecnik, P, Proc natl acad sci US 73 (1976) 3984.
6. Weinmann-Dorsch, Ch, Hedl, A, Grummt, I, Albert, W, Ferdinand, F-J, Friis, R R, Pierron, G, Moll, W & Grummt, F, Eur j biochem 138 (1984) 179.
7. Weinmann-Dorsch, Ch, Pierron, G, Wick, R, Sauer, H & Grummt, F, Exp cell res 155 (1984) 171.
8. Weinmann-Dorsch, Ch & Grummt, F, Exp cell res 160 (1985) 47.
9. Grummt, F, Proc natl acad sci US 75 (1978) 371.
10. — Cold Spring Harbor symp quant biol 43 (1978) 649.
11. Grummt, F, Waltl, G, Jantzen, H M, Hamprecht, K, Huebscher, U & Kuenzle, C C, Proc natl acad sci US 76 (1979) 6081.
12. Rapaport, E, Zamecnik, P C & Baril, E F, Proc natl acad sci US 78 (1981) 838.
13. — J biol chem 256 (1981) 12148.
14. Zamecnik, P C, Rapaport, E & Baril, E F, Proc natl acad sci US 79 (1982) 1791.
15. Grummt, F, Albert, W, Zastrow, G & Schnabel, A, Proteins involved in DNA replication (ed U Hübscher & S Spadari) p. 373. Plenum, New York (1984).
16. Grummt, F, Paul, D & Grummt, I, Eur j biochem 76 (1977) 7.
17. Rubin, H, Proc natl acad sci US 69 (1972) 712.
18. Grummt, F, Plant mol biol 2 (1983) 41.
19. Tomkins, G, Science 189 (1975) 760.

20. Hurley, L S & Swenerton, H, Proc soc exp biol med 123 (1966) 692.
21. Swenerton, H, Shrader, R & Hurley, L S, Science 166 (1969) 1014.
22. McQuitty, J T Jr, deWys, W D, Monaco, L, Strain, W H, Rob, Ch G, Apgar, J & Pories, W J, Cancer res 30 (1970) 1387.
23. deWys, W, Pories, W J, Richter, M C & Strain, W H, Proc soc exp biol med 135 (1970) 17.
24. deWys, W & Pories, W J, J natl canc inst 48 (1972) 375.
25. Barr, D H & Harris, J W, J soc exp biol med 144 (1973) 284.
26. Falchuk, K H & Krishan, A, Cancer res 37 (1977) 2050.
27. Minkel, D T, Dolhun, P J, Calhun, B L, Saryan, L A & Petering, D H, Cancer res 39 (1979) 2451.
28. Saryan, L A, Minkel, D T, Dolhun, P J, Calhun, B L, Wielgus, S, Schaller, M & Petering, D H, Cancer res 39 (1979) 2457.
29. Beach, R S, Gershwin, M E & Hurley, L S, Cancer res 41 (1981) 552.
30. Demetrakopoulos, G & Brennan, M F, Cancer res, suppl. 42 (1982) 7565.
31. Williams, R O & Loeb, L A, J cell biol 58 (1973) 594.
32. Engström, W, Exp cell res 135 (1981) 115.

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