Lack of Correlation between Extensive Accumulation of Bisnucleoside Polyphosphates and the Heat-Shock Response in Eukaryotic Cells

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The accumulation in large amounts of bisnucleoside polyphosphates (Ap,X) after heat shock in Xenopus laevis oocytes or cultured hepatoma cells (HTC cells) is observed after exposure to temperatures of 45 °C or higher. The accumulation is a transient phenomenon, with the collapse in cellular ATP concentration severely affecting the rate of synthesis of Ap,X, allowing degrading activities to empty the pool of these compounds under prolonged heat shock. This accumulation of Ap,X to high levels, compared to the basic content, is only observed under conditions leading to irreversible damage, ultimately resulting in the death of the cell. It is shown that the increase in Ap,X after hyperthermia is due to the partial or almost complete inhibition of their degradation pathways, rather than to a stimulation of their rate of synthesis. Finally, the synthesis of heat-shock proteins could be observed under conditions which do not lead to important accumulation of Ap,X, therefore ruling out the possibility that these adenylated nucleotides would behave as chemical signals ("alarmones") triggering the synthesis of heat-shock proteins. Nevertheless, on the basis of our earlier results (Guédon, G., Sovia, D., Ebel, J. P., Befort, D., and Remy, P. (1985) Embo J. 4, 3743–3749), it cannot be excluded that Ap,X might play a role in the regulation of the heat-shock response; this would, however, rely on variations in Ap,X concentrations which do not exceed a factor of 2.

Bisnucleoside polyphosphates, including diadenosine tetra- or triphosphate (Ap,A), have been shown to accumulate in procaryotic cells following heat shock or oxidative stresses (Lee et al., 1983; Bochner et al., 1984). These compounds were proposed to be "alarmones" signaling to the cell the onset of a thermal or oxidative stress and triggering the heat-shock response (Lee et al., 1983). Recently, we showed that microinjection of Ap,A into Xenopus laevis oocytes does not lead to the synthesis of any heat-shock protein (hsp). On the contrary, Ap,A microinjection results in a strong or even complete inhibition of the synthesis of hsps after thermal stress, with the exception of the 70-kDa hsp (Guédon et al., 1985). These results led us to reexamine the relation between heat shock and accumulation of bisnucleoside polyphosphates in Xenopus oocytes as well as HTC cells (hepatoma tissue culture). This paper will show that an extensive accumulation of bisnucleoside polyphosphates in oocytes is only observed above 45 °C, whereas the synthesis of hsps has been shown to be optimal at 33 °C (Bienz and Gurdon, 1982). Likewise, Ap,X accumulation in HTC cells requires temperatures above 45 °C, whereas the heat-shock response is already observed at 43 °C. Furthermore, we observed that Ap,X accumulation after a severe heat shock results mainly from the irreversible denaturation of a specific hydrolase. Finally, Ap,A steady-state concentration in Xenopus oocytes was shown to depend strongly on internal ATP concentration. The biological meaning of variations in the pool of Ap,X (or Ap3X) should therefore be cautiously examined in the light of the variations in the level of their respective precursors, the nucleoside tri- or diphosphates.

MATERIALS AND METHODS

RESULTS AND DISCUSSION

Accumulation of Bisnucleoside Polyphosphates during Heat Shock—Fig. 1 (upper) shows the variations of Ap,X content, as a function of time, in Xenopus oocytes submitted to heat shock at variable temperatures. As can be seen, the accumulation of bisnucleoside polyphosphates in large amounts only occurs above 45 °C. Up to 41 °C, the Ap,X concentration does not vary by more than a factor of 2; but as the mean variation in the measurements from sample to sample is pretty large, this factor may well be within the limits of the experimental uncertainty. When heat shock is carried out at 46 °C, a 8–10-fold accumulation of Ap,X can be observed. If the temperature is further elevated to 50 °C, no significant accumulation of Ap,X can be observed any longer, most likely because the enzymatic system responsible for the synthesis of Ap,X is rapidly heat-inactivated. Likewise, the accumulation of Ap,X in large amounts in HTC cells requires an exposure of the cells at temperatures higher than 45 °C, as shown in Fig. 1 (lower). Even a 1-h exposure at 43 °C only leads to a modification of Ap,X concentration by a factor close to 2.

Relations between Ap,X and ATP Concentrations—The variation in Ap,X concentration after heat shock is clearly biphasic, exhibiting an increase in the first 30 min (oocytes) or 60 min (HTC cells) of the thermal stress, followed by a decrease for longer incubations. This biphasic behavior probably arises from the collapse of the ATP cellular content, as...
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Fig. 2. Upper, variations of ATP and Ap4X concentrations in X. laevis oocytes as a function of heat-shock duration at 45 °C: ○, ATP concentration; ●, Ap4X concentration. Lower, variations of ATP and Ap4X concentrations in HTC cells as a function of heat-shock duration at 50 °C: □, ATP concentration; ■, Ap4X concentration.

Indeed, the only known enzymes capable of synthesizing Ap4X are particular aminoacyl-tRNA synthetases (Blanquet et al., 1983; Goerlich et al., 1982), the $K_m$ of which for ATP usually lie in the millimolar range. With ATP cellular concentration decreasing during heat shock from roughly 1 mM (in the oocyte) or several millimolar (in HTC cells) to a few tens of µM, it can easily be understood that the rate of synthesis of Ap4X will meanwhile drop markedly. Since the size of the Ap4X pool results from an equilibrium between synthesis and degradation reactions (see below), a marked decrease in the rate of synthesis should lead to a decrease in the size of the pool. It should be noticed that the decrease in Ap4X concentration in HTC cells is only observed for much more severe conditions (1 h at 50 °C) than in the oocyte.
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syste(m, suggesting that the ATP-generating system is much more thermostable in the HTC cells, perhaps reflecting substantially different “working” temperatures (20 °C for the oocytes and 37 °C for HTC cells).

The fact that the Ap4X pool is dependent upon ATP concentration is further illustrated in Fig. 3, which shows that microinjection of the oocytes with ATP (2 mM final concentration) prior to hyperthermic treatment leads to the accumulation of Ap4X at higher levels than in control oocytes. It should be noted that even in the absence of heat shock (time 0 in Fig. 3), the Ap4X concentration is 5-6-fold higher when the oocytes are microinjected with ATP.

Of course this dependent relationship between Ap4X and ATP pools requires that the biological meaning of variations in bisnucleoside polyphosphates be interpreted in light of possible modifications in the pool of their precursor nucleotides.

Accumulation of Bisnucleoside Polyphosphates Is Observed Only under Conditions Leading to Irreversible Damage to the Cell—As can be seen in Fig. 4 when hyperthermic treatment remains moderate (up to 5 min at 45 °C), the oocyte remains capable of regaining a normal ATP concentration upon further incubation at 18 °C. But as soon as the incubation is
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FIG. 7. Kinetics of Ap4A synthesis in extracts of X. laevis oocytes previously submitted to variable heat shock. ⊗, control cells; Δ, 15 min of heat shock at 35 °C; ⊘, 60 min of heat shock at 35 °C; ⊙, 15 min of heat shock at 45 °C; □, 60 min of heat shock at 45 °C. In the upper panel, the oocytes were ground in dithiothreitol- and ethylenediamine tetraacetate-containing buffer; in the lower panel, the extract did not contain these compounds (see "Materials and Methods").

prolonged over 5 min, conditions which ensure the accumulation of ApX, irreversible damage starts to occur, as shown by the partial or almost complete lack of recovery of the ATP pool upon return to 18 °C.

HTC cells behave similarly since 30 min of heat shock at

43 °C (allowing an efficient hsp synthesis) are accompanied by a 40% lethality, whereas 30-min exposures to 48 or 50 °C (favoring the ApX accumulation) lead to an almost complete lethality (Fig. 5). This is also reflected by the complete collapse of [35S]methionine incorporation at 37 °C in newly synthesized proteins following heat shock at temperatures higher than 45 °C, as illustrated in Fig. 6.

We also observed similar results when studying the accumulation of ApX in Saccharomyces cerevisiae upon hyperthermia or Cd2+ treatment (Baltzinger et al., 1986). These results suggest that the accumulation of bisnucleoside polyphosphates in eukaryotic cells, in response to stress, is only observed under conditions irreversibly affecting the viability of the cells.

Does Accumulation of Bisnucleoside Polyphosphates Arise from Increase in Their Rate of Synthesis?—The recent observation that bisnucleoside polyphosphates could be accumulated in prokaryotes not only under hyperthermia, but also following exposure to a variety of oxidizing agents led Bohner et al. (1984) to propose that those compounds were alarmones signaling to the cell the existence of an oxidative stress. Aminocyl-tRNA synthetases (or tRNAs) were proposed to be potential sensors ensuring coupling between the onset of oxidative conditions and an increased production of bisnucleoside polyphosphates. It was therefore of interest to investigate whether the accumulation of ApX results from an increased rate of synthesis or from the inhibition of the degradation pathway since degradation enzymes specific for ApX were described in a variety of cells: prokaryotic (Guranowski et al., 1983; Plateau et al., 1986), as well as eukaryotic
(Barnes and Culver, 1982; Hohn et al., 1982; Ogilvie and Antl, 1983; Jakubowski and Guranowski, 1983; Cameselle et al., 1984; Costas et al., 1984). As already stated, the rates of synthesis could not be measured by microinjection of radioactive ATP into the oocytes because the steady state of Ap4X labeling was reached within the time needed for microinjection. The experiments were thus carried out on oocyte extracts prepared as described under Materials and Methods. Fig. 7 shows that no increase in the rate of synthesis of Ap4A can be detected following hyperthermic treatment of the oocyte, neither in the absence nor in the presence of reducing and complexing agents in the cellular extract. On the contrary, the rate of synthesis is slightly decreased (roughly by a factor of 2) upon prolonged incubation at 45 °C. This allows one to rule out a triggering mechanism based either upon oxidative modification of the competent aminoacyl-tRNA synthetases or upon the liberation of activating cations in the cytoplasm. It could be argued that we do not measure the true rate of synthesis of Ap4A due to the existence of both synthesizing and degrading activities in the cellular extract. This is not the case since the degrading activity in Xenopus oocytes is strongly inhibited after severe heat shock, as shown in Fig. 8; the upper panel shows the rate of radioactive Ap4A microinjected in oocytes at a final concentration of 2 mM. Obviously, Ap4A is degraded rapidly, the main degradation product formed being ATP, with the radioactivity then appearing more slowly in ADP and AMP, probably because of secondary reactions. As shown in the lower panel, this lytic activity is not significantly affected by a 60-min treatment at 35 °C, conditions which allow an efficient heat-shock response (see below) but which do not lead to the accumulation of bisnucleoside polyphosphates (see above). On the contrary, incubation at 45 °C, which favors this accumulation, results in a marked inhibition of the Ap4A hydrolyzing activity since 15 or 45 min of exposure at this temperature lead to a 2- or 10-fold reduction in this activity, respectively. It should be emphasized that this residual 10% activity appears to be rather thermostable and could correspond to nonspecific hydrolysis (by a phosphodiesterase-type enzyme).

The above observations show that the accumulation of bisnucleoside polyphosphates is the consequence of inhibition of their degradation pathway, rather than the result of stimulation of their biosynthesis. Relations between Heat-Shock Response and Accumulation of Bisnucleoside Polyphosphates—We therefore observed that the accumulation of Ap4X in large amounts in Xenopus oocytes as well as in HTC cells requires exposure to temperatures of 45 °C or higher. It is worthwhile to compare the above results to the temperature dependence of heat-shock protein synthesis. Bienz and Gurdon (1982) showed that hsp synthesis in the oocyte exhibits a sharp temperature optimum at 33 °C, an increase to 37 °C already leading to a drop by a factor of 2 in the rate of synthesis of the hsps. In the meantime, the overall protein synthesis drops from 50% to less than 10% of its maximum efficiency. Very similarly, Fig. 9 shows that the synthesis of hsps in HTC cells is already efficient at 43 °C.
Although the difference between the temperatures required to trigger the heat-shock response and the accumulation of Ap₄X is less in HTC cells (2°C) than in the oocytes system (12°C), it is clear that high concentrations of Ap₄X are not a prerequisite to hsp synthesis. The higher sensitivity of oocytes to temperature with regard to hsp synthesis is probably due to the fact that these cells have a normal working temperature around 20°C, which means that exposure to temperatures above 30°C may represent a pretty severe thermal stress.

The above results allow one to rule out the hypothesis that Ap₄X could be chemical messengers triggering the synthesis of hsps as postulated by Lee et al. (1983) on the basis that an Escherichia coli htpR mutant which is unable to synthesize hsps still accumulates Ap₄X.

CONCLUSION

Our earlier results (Guedon et al., 1985) have shown that Ap₄A does not trigger the synthesis of heat-shock proteins when microinjected in Xenopus oocytes. The present report shows that, in the two types of eukaryotic cells tested, hsp synthesis, in response to hyperthermic treatment, can occur under conditions where the intracellular content in Ap₄X does not vary by more than a factor of 2. The accumulation of bisnucleoside polyphosphates at high levels, which has already been reported in prokaryotic cells under a large variety of stresses, occurs only under severe heat-shock conditions, apparently resulting in irreversible damage, ultimately leading to cell death.

Furthermore, the transient accumulation of Ap₄X in the cell does not rely on an increased rate of synthesis, but on the inhibition of a specific degradation pathway.

These results do not support the idea that bisnucleoside polyphosphates could play a role of alarmones in the heat-shock response, the synthesis of which would be enhanced upon oxidative damage to the competent aminoacyl-tRNA synthetases (or tRNAs), at least in eukaryotes.

The accumulation of Ap₄X to large extents (compared to the basic levels) appears rather as a nonphysiological by-phenomenon due to the irreversible thermal (or oxidative) inactivation of a degradation enzyme specific for bisnucleoside tetraphosphates. Our results do not allow one to completely rule out participation of bisnucleoside polyphosphates in the regulation of the response to an external stress since we showed that microinjection of Ap₄A into Xenopus oocytes can lead to a decrease of hsp synthesis, with the exception of the 70-kDa hsp (Guedon et al., 1985). If they do play such a role, this will occur for cellular concentrations which, most likely, do not differ from the basal level by more than a factor of 2.

REFERENCES

Alvarez-Gonzales, R., Juarez-Salinas, H., Jacobson, E. L., and Jacobson, M. (1983) Anal. Biochem. 135, 69-77
Baker, J. C., and Jacobson, M. (1984) Anal. Biochem. 141, 451-460
Baltzinger, M., Ebel, J. P., and Remy, P. (1986) Biochimie (Paris) 68, in press
Barnes, L. D., and Culver, C. A. (1982) Biochemistry 21, 6123-6128
Bienz, M., and Gurdon, J. B. (1982) Cell 29, 811-819
Blanquet, P., and Brevet, A. (1983) Mol. Cell. Biochem. 68, 3-11
Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W., and Ames, B. (1984) Cell 37, 225-232
Costas, J. C., Costas, M. J., Siller, M. A. G., and Silero, A. (1984) J. Biol. Chem. 259, 2873-2885
Costas, M. J., Montero, J. M., Cameselle, J. C., Siller, M. A. G., and Silero, A. (1984) Int. J. Biochem. 16, 757-762
Dumont, J. N. (1972) J. Morphol. 136, 153-180
Goerlich, O., Foeckler, R., and Holler, E. (1982) Eur. J. Biochem. 126, 135-142
Guedon, G., Sovia, D., Ebel, J. P., Befort, N., and Remy, P. (1985) EMBO J. 4, 3743-3749
Guranowski, A., Jakubowski, H., and Holler, E. (1983) J. Biol. Chem. 258, 14784-14789
Hohn, M., Albert, W., and Grummt, F. (1982) J. Biol. Chem. 257, 3003-3006
Jakubowski, H., and Guranowski, A. (1983) J. Biol. Chem. 258, 9982-9989
Khym, J. X. (1976) Clin. Chem. 21, 1245-1252
Lee, P. C., Bochner, B. R., and Ames, B. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7406-7400
O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007-4021
Ogilvie, A. (1981) Anal. Biochem. 115, 302-307
Ogilvie, A., and Antl, W. (1983) J. Biol. Chem. 258, 4105-4109
Plateau, P., Fromant, M., Brevet, A., Gesquiere, A., and Blanquet, S. (1986) Biochemistry 24, 914-922
Wielckens, K., Bredehorst, R., Adamietz, P., and Hilz, H. (1981) Eur. J. Biochem. 117, 69-74
were kept

Aliquots were obtained from EGA-Chemie, IAlbuch-FRGI, in 2x10

Calf Embryo Incubator, Steinheim - IRichmond-CA-USA.

in trichloroacetic acid. At the end of the incubation period, the Barth's medium was recovered. Injections were always counted after trichloroacetic acid precipitation to recover the HTC supernatant. The amount of bisnucleotide polyphosphates in the HTC supernatant was determined by the method described by Khym.

Preparation of microinjection solutions: Microinjection solutions were prepared in anhydrous dimethyl sulfoxide. The HTC solution was kept at 19°C in water-jacketed flasks, at 37°C. When the microinjection mixture was seeded into water-jacketed flasks, the temperature of the water-jacket was set at 37°C. After 24 hours, the flasks were seeded with 2x10 cells and incubated for 48 hours at 37°C. The cells were seeded into 2x10 flasks, at 37°C, using a water-jacketed incubator, as described by Baker et al., 1985.

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