ACTIVATION OF A NUCLEASE BY pppA2'p5'A2'p5'A IN INTACT CELLS

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1. Introduction

A series of 2'–5'-linked oligoadenylic acid triphosphate (2-5A) inhibitors of cell-free protein synthesis are synthesised in extracts from interferon-treated cells or reticulocyte lysates in response to double-stranded RNA [1–5]. Although the natural occurrence of these inhibitors has yet to be demonstrated in intact cells, they are active when deliberately introduced into hypertonically treated BHK 21-cells [6]. In cell-free systems subnanomolar concentrations of 2-5A inhibit protein synthesis by activating an endonuclease which degrades both cellular and viral RNA [7–13]. In the absence of a regenerating system 2-5A is rapidly broken down, the 2-5A activated endonuclease is also unstable and the inhibition of protein synthesis is transient [11,12]. We show here that a similar series of events occurs in response to 2-5A in the intact cell. The introduction of 2-5A into BHK-21 cells by permeabilisation in hypertonic medium followed by resealing, results in an enhanced level of endonuclease activity concurrent with an inhibition of protein synthesis. This inhibition is transient and on incubation for a further period, which depends on the concentration of 2-5A added, protein synthesis returns to the control level. Virus production can also be inhibited by the similar introduction of 2-5A into virus-infected cells.

Abbreviations: EMC, encephalomyocarditis virus; 2-5A, a mixture of 5'-triphosphorylated, 2'–5'-linked, oligoadenylic acids in which pppA2'p5'A2'p5'A is the predominant component but which also contains the corresponding tetramer, pentamer and higher oligomers in decreasing amounts and lesser amounts of the corresponding 5'-diphosphates and 5'-monophosphates

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2. Materials and methods

2.1. *Cells and virus*

BHK-21 cells were grown in monolayer culture in Sterilin flasks and tissue culture dishes in Dulbecco's modified Eagle's medium containing 10% calf serum. Ehrlich ascites tumour cells were grown in Eagle's spinner medium [14] and EMC virus was grown in Krebs ascites tumour cells as in [15].

2.2. *Cell permeabilisation and treatment with 2-5A*

Cells were permeabilised as described [6] in medium minus serum containing 4% NaCl. After 45 min at 37°C (when 95% of the cells could be stained by Trypan blue) the hypertonic medium was removed and the cells allowed to reseal in complete medium containing 10% serum. > 90% of the cells resealed after 10 min of incubation at 37°C. Routinely, 2-5A was added with the hypertonic medium to the cells. The preparation of 2-5A used for the experiments described here was purified trimer (pppA2'p5'A2'p5'A) and the concentrations referred to are in AMP equivalents.

2.3. *Assay of [^{3}H]uridine-labelled polyadenylated RNA*

20 h monolayers of BHK-21 cells in 60 mm dishes were incubated for 30 min in 1 ml medium containing 2% calf serum and 50 μCi [^{3}H]uridine (Radiochemical Centre, Amersham; spec. act. 29 Ci/mmol) at 37°C,

washed once with medium minus serum then permeabilised and treated with 2-5A. The cells were incubated in resealing medium for 40 min at 37°C, washed 3 times with cold saline and solubilised in buffer (0.1 M NaCl, 0.01 M Tris–HCl (pH 8.8), 2 mM EDTA, 1% 2-mercaptoethanol) containing 1% Triton N 101 and 0.5% sodium deoxycholate [16]. The lysate was spun at 2000 × *g* for 10 min adjusted to 0.4 M NaCl, 0.5% SDS and chromatographed on oligo(dT)-cellulose [17].

2.4. *Preparation of ^{32}P-labelled polyadenylated RNA*

Ehrlich ascites tumour cells (1 l) at 10^6 cells/ml were incubated in spinner medium minus phosphate containing 10% dialysed calf serum and 5 mCi [^{32}P]orthophosphate (Radiochemical Centre, Amersham; carrier free) for 18 h at 37°C. Post-mitochondrial supernatant fractions were prepared as in [18], NaCl added to 0.5 M, proteinase K to 200 μg/ml and incubated for 30 min at 25°C then chromatographed on oligo(dT)-cellulose. The polyadenylated RNA was ethanol precipitated and resuspended in buffer (0.02 M Tris–HCl (pH 7.6), 0.05 M KCl) at 150 μg/ml. The specific activity of the RNA was ~170 000 cpm/μg.

2.5. *Detection of endonuclease activity*

Post-mitochondrial supernatant fractions from BHK 21 cells (fig.3) or rabbit reticulocyte lysates (fig.4) were incubated with ^{32}P-labelled mRNA at 30°C and samples analysed by SDS–polyacrylamide gel (7.5%) electrophoresis as in [19].

3. Results and discussion

3.1. *Inhibition of protein synthesis by 2-5A in the intact cell is transient*

Protein synthesis in BHK-21 cells can be inhibited by 2′–5′-linked adenine oligonucleotides if these cells are first permeabilised by incubation in hypertonic medium [6]. Exposure of the cells to the oligonucleotides for as little as 5 min before resealing by incubation in normal medium is sufficient for the subsequent inhibition to be observed. This inhibition can be detected within 30 min after resealing, reaches a maximum in < 1 h, but is reversed on prolonged incubation. The time taken for control levels of protein synthesis to be regained is dependent on the concentration of 2-5A employed (fig.1). After overnight incubation the cells have fully recovered and are sensitive to inhibition by re-exposure to 2-5A in hypertonic medium (data not shown).

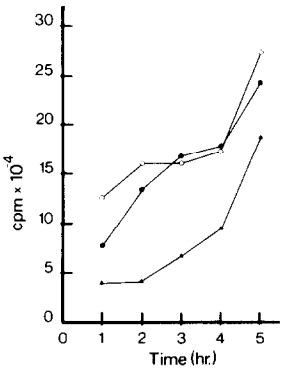

Fig.1. Effect of 2-5A on the kinetics of protein synthesis in permeabilised BHK-21 cells. Cell monolayers grown on 35 mm Sterilin multiwell dishes were permeabilised (section 2) in the absence (○) or presence of 2-5A at final concentration of 1 μM (●) or 10 μM (▲). After resealing for 10 min by incubation in normal medium, triplicate monolayers from each group were washed once in medium minus serum and methionine, then pulsed for 1 h in 1 ml of this medium containing 1 μCi [^{35}S]methionine (750–900 Ci/mmol). The cells were washed and assayed for trichloroacetic acid-precipitable radioactivity as in [6]. Further sets of triplicates were similarly processed each hour for 4 h.

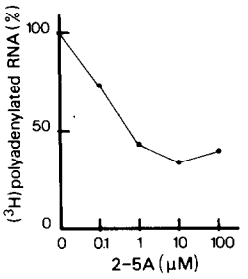

Fig.2. Effect of 2-5A on prelabelled [^{3}H]polyadenylated RNA content of permeabilised BHK-21 cells. [^{3}H]polyadenylated RNA content in permeabilised cells treated with 2-5A was estimated (section 2) at 30 min after resealing. The results are given as a percentage of the [^{3}H]polyadenylated RNA present in control cells (67, 746 c.p.m.) permeabilised in the absence of 2-5A.

Table 1
Inhibition of virus growth by 2-5A

Treatment	Multiplicity of infection	EMC virus yield	
		at 10 h	at 25 h
10 μM 2-5A permeabilised cells	0.2	1.15×10^3	1.25×10^5
control permeabilised cells	0.2	1.30×10^4	1.40×10^7
control cells	0.2	1.15×10^4	2.75×10^7
10 μM 2-5A permeabilised cells	20	2.2×10^6	1.5×10^7
control permeabilised cells	20	7.0×10^6	1.9×10^7
control cells	20	1.0×10^7	1.6×10^7

Monolayers of BHK-21 cells were infected with EMC then permeabilised and treated with 2-5A 1.5 h post infection. Tissue culture fluids were collected and assayed for virus yield by plaquing on BHK-21 cells

3.2. *Activation of a nuclease by 2-5A in intact cells*

In cell-free systems prepared from mouse, chick or HeLa cells or in reticulocyte lysates, 2-5A activates an endogenous endonuclease which degrades mRNA resulting in the inhibition of protein synthesis [7–13]. Experiments were carried out, therefore, to determine if an endonuclease is similarly involved in the inhibition of protein synthesis by 2-5A in the intact cell. Messenger RNA in BHK-21 cells was prelabelled by incubation in medium containing [^{3}H]uridine. These cells were then permeabilised and treated with different concentrations of 2-5A. After resealing and incubating for a period sufficient to allow inhibition of protein synthesis to become established, extracts were prepared from the cells and the ^{3}H-labelled polyadenylated RNA content determined. Cells, permeabilised and treated in this way showed a 2-5A concentration-dependent decrease in prelabelled polyadenylated RNA (fig.2). Such a decrease can be satisfactorily explained only by the action of an endonuclease on this RNA. There is no immediate effect of 2-5A on RNA synthesis per se measured after permeabilisation 2-5A treatment and resealing (unpublished observation). The breakdown of polyadenylated RNA in response to 2-5A appears to plateau at 10 μM 2-5A. This concentration of 2-5A usually gave the maximum inhibition of protein synthesis [6] (see also fig.1). The inhibitory activity of 2-5A in the cell-free system is limited by the level of activatible endogenous endonuclease present in the system [11].
In the intact cell, increasing 2-5A to 100 μM does not result in a greater reduction in polyadenylated RNA content. This suggests that the level of activatible endogenous endonuclease may also limit the activity of 2-5A in the intact cell.

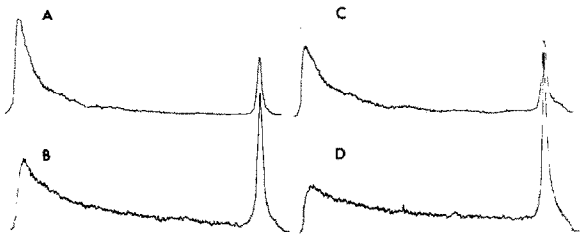

Fig.3. Enhanced endonuclease activity in extracts from permeabilised cells treated with 2-5A. Post-mitochondrial supernatants were prepared from cells permeabilised in the absence (A,B) or presence (C,D) of 1 μM 2-5A, 1.5 h after resealing. Samples (10 μl) were incubated with ^{32}P-labelled EAT mRNA (section 2) at 30°C and 4 μl analysed for mRNA breakdown at 10 min (A,C) and 20 (B,D) of incubation. Scans of appropriate channels of an autoradiograph of the dried gel are presented. Electrophoresis was from left to right.

3.3. *Enhanced endonuclease activity in extracts from 2-5A-treated permeabilised cells*

In accord with the increased breakdown of polyadenylated RNA in the intact cell treated with 2-5A, an enhanced endonuclease activity could be detected in extracts prepared from these cells. Extracts prepared at different times after permeabilisation, 2-5A treatment and resealing were incubated with ^{32}P-labelled

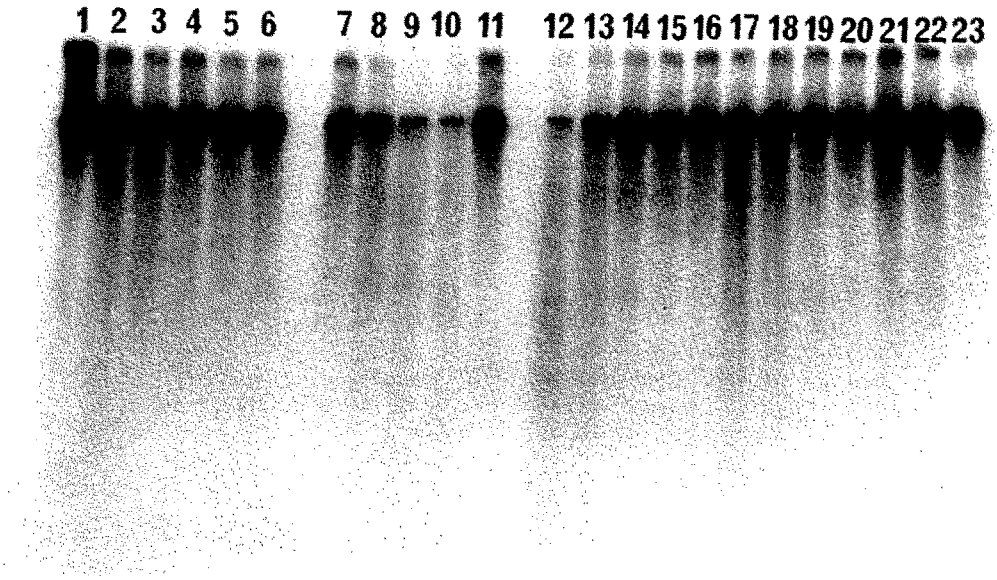

Fig.4. Detection of 2-5A in extracts prepared from BHK-21 cells permeabilised in the presence of 2-5A. Cells permeabilised in the presence or absence of 2-5A were resealed for 10 min, washed 4-times with cold medium minus serum scraped into 0.2 ml of this medium and centrifuged at 1500 × *g* for 5 min. The cell pellets were lysed into an equal volume (30 μl) of buffer (10 mM Hepes (pH 7.6), 90 mM KCl, 2.5 mM magnesium acetate) containing 0.5% NP40, centrifuged at 2000 × *g* for 10 min and the supernatant from this heated at 90°C for 5 min. The denatured protein was removed by a further centrifugation and supernatants assayed for 2-5A using the reticulocyte lysate gel endonuclease assay (section 2). An autoradiograph of the dried gel is shown. Track 1, ^{32}P-labelled mRNA substrate alone; tracks 2–6, extract from control cells at final dilutions of 1: 5, 15, 50, 150, 500; tracks 7–11, control extract diluted 1: 6.25 plus 2-5A at final concentrations of 2, 6, 20, 60 nM and minus 2-5A (track 11). Tracks 12–16, extracts from cells treated with 250 μM 2-5A diluted 1: 5, 15, 50, 150, 500. Tracks 17–20, extracts from cells treated with 25 μM 2-5A diluted 1: 5, 15, 50, 150. Tracks 21–23, extracts from cells treated with 2.5 μM 2-5A diluted 1: 5, 15, 50.

mRNA and samples removed for analysis by electrophoresis on polyacrylamide gels [19]. In the extracts prepared at 1.5 h after resealing, an enhanced rate of degradation of the ^{32}P-labelled mRNA was observed (fig.3). No enhanced degradation could be detected in extracts prepared 3 h after resealing (data not shown). These results are consistent with a transient activation of an endonuclease in the intact cell similar to that observed previously in cell-free systems [11].

3.4. *Inhibition of virus replication by 2-5A*

In the interferon-treated virus-infected cell, both the accumulation of viral RNA and viral protein synthesis may be inhibited [20]. Whether either of these phenomena are 2-5A mediated has yet to be established. It was of interest, in this respect, to test if 2-5A deliberately introduced into the virus-infected cell is capable of inhibiting virus replication. In cells infected with vaccinia or EMC virus prior to exposure to 2-5A in hypertonic medium, there is a reduction in both total EMC viral RNA accumulation and in vaccinia polyadenylated RNA (data not shown). In accord with this there is also a reduction in virus yield from EMC virus-infected cells permeabilised and treated with 2-5A (table 1). This reduction is dependent on the multiplicity of infection of the virus. At low multiplicities of infection the yields are reduced by > 2 logs by treatment with 10 μM 2-5A. At high multiplicities of infection virus growth is delayed, but by 25 h post-infection both the control and 2-5A-

treated cells show similar yields. It is perhaps worth noting here that the extent of the antiviral effect of interferon is also dependent on the multiplicity of infection of the virus [20].

3.5. *Recovery of 2-5A from intact cells*

To date, there is no evidence for the natural occurrence of 2-5A in the intact cell. Although it is reasonable to assume that the enhanced endonuclease activity detected in extracts from permeabilised, 2-5A-treated cells is mediated by 2-5A, it was not possible to follow the fate of 2-5A directly in these extracts. The reason for this was the sensitivity of the cell-free protein synthesising systems to non-specific inhibitory material present in the extracts from permeabilised cells. The dilution required to avoid this inhibition also dilutes out the 2-5A. Non-specific inhibition is not a problem in the gel endonuclease assay but an unavoidable dilution of the extract is still required [19]. However, when the cells were treated with higher concentrations of 2-5A, it was possible to detect 2-5A by the gel endonuclease assay in extracts prepared from these cells after resealing (fig.4, tracks 12,13). Since 2-5A (250 μM) added to control cells (not permeabilised) either during the first 45 min incubation or immediately before washing and resealing, could not be detected in extract subsequently prepared from these cells, the degradation shown in tracks 12,13 (fig.4) could not be the result of carry over of 2-5A from the permeabilisation incubation. The incubations run in tracks 7–11 (fig.4) make it clear that the failure to detect 2-5A in extracts prepared from cells incubated with 25 or 2.5 μM 2-5A (tracts 17–20 and 21–23, respectively) was not the result of inactivation of the endogenous reticulocyte endonuclease by unidentified factors in the extract, nor degradation of 2-5A during the in vitro incubation prior to the gel assay. It remains possible, however, that only a small proportion of the 2-5A added to the cells may actually enter the permeabilised cell and inhibit protein synthesis.

In conclusion, the results presented here demonstrate that on introduction of 2-5A into intact cells there is a transient activation of an endogenous nuclease activity and inhibition of protein synthesis. The response to 2-5A is similar to that described for cell-free systems [11]. Moreover, by introducing 2-5A into virus-infected cells, virus growth can be inhibited. This confirms that it is at least possible that the 2-5A system activated by double-stranded RNA produced in interferon-treated virus-infected cells, may operate to reduce virus growth [21]. The transient activation of the endonuclease could result in the degradation of both host and viral RNA effectively eliminating the virus and allowing the host cells to recover. Alternatively, 2-5A mediated endonuclease may indeed show some selectivity for the degradation of viral RNA [22]. Whether 2-5A is involved remains to be established, but the demonstration here that 2-5A deliberately introduced into cells can be recovered from these cells provides a basis for a more intensive investigation of the possible natural occurrence of 2-5A in the interferon-treated virus-infected cell. This approach may also prove useful to determine the significance of the recently reported wide distribution and variation of 2-5A synthetase activity in cells and tissues not treated with interferon [23].

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References

[1] Hovanessian, A. G., Brown, R. E. and Kerr, I. M. (1977) Nature 268, 537–540.
[2] Kerr, I. M., Brown, R. E. and Hovanessian, A. G. (1977) Nature 268, 540–542.
[3] Hovanessian, A. G. and Kerr, I. M. (1978) Eur. J. Biochem. 84, 149–159.
[4] Kerr, I. M. and Brown, R. E. (1978) Proc. Natl. Acad. Sci. USA 75, 256–260.
[5] Ball, L. A. and White, C. N. (1978) Proc. Natl. Acad. Sci. USA 75, 1167–1171.
[6] Williams, B. R. G. and Kerr, I. M. (1978) Nature 276, 88–90.
[7] Clemens, M. J. and Williams, B. R. G. (1978) Cell 13, 565–572.
[8] Baglioni, C., Minks, M. A. and Maroney, P. A. (1978) Nature 273, 684–687.
[9] Ratner, L., Wiegand, R. C., Farrell, P. J., Sen, G. C., Cabrer, B. and Lengyel, P. (1978) Biochem. Biophys. Res. Commun. 81, 947–954.

[10] Schmidt, A., Zilberstein, A., Shulman, L., Federman, P., Berissi, H. and Revel, M. (1978) FEBS Lett. 95, 257–264.
[11] Williams, B. R. G., Kerr, I. M., Gilbert, C. S., White, C. N. and Ball, L. A. (1978) Eur. J. Biochem. 92, 455–462.
[12] Minks, M. A., Benvin, S., Maroney, P. A. and Baglioni, C. (1979) Nucl. Acids Res. 6, 767–770.
[13] Ball, L. A. and White, C. N. (1979) Virology 93, 348–356.
[14] Friedman, R. M., Metz, D. H., Esteban, R. M., Tovell, D. R., Ball, L. A. and Kerr, I. M. (1972) J. Virol. 10, 1184–1198.
[15] Kerr, I. M. and Martin, E. M. (1972) J. Virol. 9, 559–561.
[16] Collins, P. L., Hightower, L. E. and Ball, L. A. (1978) J. Virol. 28, 324–336.
[17] Ball, L. A. and White, C. N. (1978) Virology 84, 479–495.
[18] Kerr, I. M., Olshevsky, U., Lodish, H. F. and Baltimore, D. (1976) J. Virol. 18, 627–635.
[19] Williams, B. R. G., Gilbert, C. S. and Kerr, I. M. (1979) Nucl. Acids Res. 6, 1335–1350.
[20] Friedman, R. M. (1977) Bacteriol. Rev. 41, 543–567.
[21] Kerr, I. M., Brown, R. E. and Ball, L. A. (1974) Nature 250, 57–59.
[22] Nilsen, T. W. and Baglioni, C. (1979) Proc. Natl. Acad. Sci. USA in press.
[23] Stark, G. R., Dower, W. J., Schimke, R. T., Brown, R. E. and Kerr, I. M. (1979) Nature 278, 471–473.