Biochimica et Biophysica Acta, 697 (1982) 170–173
Elsevier Biomedical Press

BBA 91073

PRECIPITATION OF NUCLEOTIDES BY CALCIUM PHOSPHATE

KJELL STENBERG [a], BO ÖBERG [a,*] and JYOTI B. CHATTOPADHYAYA [b]

[a] *Department of Antiviral Research, Research and Development Laboratories, Astra Läkemedel AB, S-151 85 Södertälje and* [b] *Department of Microbiology, Biomedical Centre, Box 581, University of Uppsala, S-751 23 Uppsala (Sweden)*

(Received December 15th, 1981)

Key words: Nucleotide precipitation; Calcium phosphate

Earlier it has been shown that nucleic acids of high molecular weight can be introduced into cells by coprecipitation with calcium phosphate. We have studied the requirements for calcium phosphate coprecipitation of shorter nucleotides. The degree of coprecipitation of dodecanucleotides lacking terminal phosphate varied between 25 and 72%. Tetramers with a 5′-monophosphate were coprecipitated to 29–87% by calcium phosphate. A high content of guanosine residues and an increased number of terminal phosphate groups increased the degree of coprecipitation of nucleotides. The trinucleotide pppA2′p5′A2′p5′A was effectively precipitated by calcium phosphate but the monophosphate and the core structure were not.

Introduction

Studies on the biological effects of large or highly charged molecules are hindered by their inability to penetrate freely into cells. An increased penetration has been achieved by hypotonic treatment [1], hypertonic treatment [2], and treatment with lysolecithin [3], dextran sulfate [4], Tween-80 [5] and Nonidet P-40 [6]. The calcium phosphate precipitation technique introduced by Graham and van der Eb [7] for the enhancement of DNA infectivity has been used extensively to facilitate the penetration of DNA into cells. Recently this method has also been used to introduce the oligonucleotide 2′,5′-A into cells [8–10] and thus make possible a determination of its role in the mechanism of action of interferon.

The limitations and quantitation of the calcium phosphate method in the precipitation of shorter nucleotides to enhance cellular penetration have not been described. We have determined the influence of charge of phosphate residues, base composition and size on the ability of nucleotides to be precipitated by calcium phosphate.

Methods

Calcium phosphate precipitation. The precipitation was performed essentially as described by Hovanessian et al. [9]. The nucleotides were dissolved to an A_{260nm} of 0.3 in 21 mM Hepes, 137 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 6 mM glucose at pH 7.05. $CaCl_2$ was added to a final concentration of 125 mM. The mixtures were incubated for 45 min at 20°C [9] and then centrifuged for 10 min at $3000 \times g$ to sediment calcium phosphate and coprecipitated nucleotides. The A_{260nm} of the supernatants were determined and used to calculate the extent of precipitation of nucleotides. Addition of a concentrated (2 M) solution of calcium chloride to an incubation medium covering cell monolayers in order to precipitate nucleotides with calcium phosphate should be avoided in order to avoid extensive cell damage.

* To whom correspondence should be addressed

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Chemicals. The mononucleotides and calf thymus DNA were from Sigma Co. St. Louis MS, the deoxydinucleotide 5'-monophosphates dpApG, dpGpG, dpGpC, and dpCpG from Collaborative Research Inc., MA. and dpApC, dpApT, dpGpA, dpGpT, dpCpA, dpCpT, dpTpA, dpTpG, dpTpC and dpTpT were from PL Biochemicals, WI. The nucleotides ApGpU, ApApG, GpApC, pA2'p5'A2'p5'A and pppA2'p5'A2'p5'A were from PL Biochemicals Inc., and ApApA, ApGpC, d(pApGpC) and d(GpCpA) were from Collaborative Research Inc. A2'p5'A2'p5'A was synthesized as described previously [11]. The deoxytetramers, T_8 and poly dT, were from Collaborative Research Inc. The dodecamers, d(CpTpCpCpCpApCpCpApCpCpT), d(CpTpCpCpCpApCpCpApTpCpT), d(TpTpCpCpCpApCpCpApCpCpT), and d(GpGpCpGpCpGpTpTpTpCpApT) were synthesized following a published procedure [12]. Hydroxyapatite was from Bio-Rad Laboratories, CA., and DEAE-dextran was from Pharmacia, Uppsala. All internucleotide linkages are 3'-5' unless otherwise specified.

Results

Precipitation of mononucleotides. The extent of precipitation of a mononucleotide by calcium phosphate was influenced by the number of phosphate groups as shown in Table I. Monophosphates could not be precipitated, diphosphates only partly (31–77%), while triphosphates were precipitated more efficiently (51–100%). When the precipitations of nucleotides of different base composition were compared, the following order of decreasing ability to be precipitated was observed; guanine, cytosine, adenine, uracil, thymine.

Precipitation of dinucleotide 5'-monophosphates. The precipitation of monophosphorylated deoxydinucleotides by calcium phosphate showed (Table II) that precipitation of the dinucleotide monophosphates was favoured by guanosine residues. The total amount of material precipitated was, however, rather low for all dinucleotides (6–37%).

Precipitation of trimers. As shown in Table III trimers with free 3'- and 5'-hydroxyl groups were not precipitated by calcium phosphate. Introduction of a 5'-monophosphate increased the ability to form a precipitate and a 5'-triphosphate group resulted in an almost total precipitation of the trinucleotide pppA2'p5'A2'p5'A. Exchange of Ca^{2+} for Mg^{2+}, Mn^{2+} or Zn^{2+} did not lead to any precipitation of A2'p5'A2'p5'A, nor did addition of 10 μg/ml calf thymus DNA, 0.1 μg/ml poly dT, 10 μg/ml hydroxyapatite or 10 μg/ml DEAE-dextran during the calcium phosphate precipitation (not shown).

Precipitation of tetramers. The base composition of tetranucleotides influenced the precipitation by calcium phosphate as shown in Table IV. The tetramer containing four guanosine residues was more easily precipitated than adenosine and thymidine tetranucleotides. A 5'-nonphosphorylated tetramer did not precipitate.

Precipitation of oligo and polynucleotides. An

TABLE I

PRECIPITATION OF MONONUCLEOTIDES

Nucleoside	Percent precipitated		
	5'-Monophosphate	5'-Diphosphate	5'-Triphosphate
Adenosine	0	31	76
Guanosine	9	70	100
Cytidine	10	38	81
Uridine	10	52	61
Thymidine	7	27	51
Deoxyadenosine	6	32	88
Deoxyguanosine	7	77	91
Deoxycytidine	5	32	76

TABLE II

PRECIPITATION OF DEOXYDINUCLEOTIDE 5′-MONOPHOSPHATES BY CALCIUM PHOSPHATE

5′-Nucleotide	Percent precipitated			
3′-Nucleotide:	dA	dG	dC	dT
pdA	–	21	15	9
pdG	15	37	22	21
pdC	16	20	–	17
pdT	9	19	6	7

efficient precipitation of 5′-nonphosphorylated nucleotides required a chain length of at least 12 base residues (Table V). In addition to this, the base composition of dodecamers had a considerable influence on the precipitation by calcium phosphate. Calf thymus DNA was used as a control and was completely precipitated.

TABLE III

PRECIPITATION OF TRIMERS BY CALCIUM PHOSPHATE

Trimer	Percent precipitated
A2′p5′A2′p5′A	0
pA2′p5′A2′p5′A	11
pppA2′p5′A2′p5′A	79
ApGpU	3
ApApG	12
GpApC	5
ApApA	0
ApGpC	2
d(pApGpC)	46
d(GpCpA)	2

TABLE IV

PRECIPITATION OF TETRAMERS BY CALCIUM PHOSPHATE

Tetramer	Percent precipitated
d(pGpGpGpG)	87
d(pApApApA)	41
d(pTpTpTpT)	29
d(pApApApG)	53
d(pApGpCpT)	70
d(GpGpCpC)	4

TABLE V

PRECIPITATION OF OLIGO AND POLYNUCLEOTIDES BY CALCIUM PHOSPHATE

Oligo and polynucleotides	Percent precipitated
d(TpTpTpTpTpTpTpT)	10
d(CpTpCpCpCpApCpCpApCpCpT)	25
d(CpTpCpCpCpApCpCpApTpCpT)	48
d(TpTpCpCpCpApCpCpApCpCpT)	48
d(GpGpCpGpCpGpTpTpTpCpApT)	72
d(pApApApApApA)	67
$d(pT)_{12-18}$	98
DNA, calf thymus	98

Discussion

Calcium phosphate precipitation of DNA [7] has recently been used to facilitate cellular uptake of pppA2′p5′A2′p5′A [8–10]. The use of this method to study cellular effects of biologically active nucleotides depends on a coprecipitation of the nucleotides with calcium phosphate. Some limitations of this method were defined in the present investigation. The precipitation of polynucleotides with a chain length of 12 or more nucleotides did not depend on 5′-terminal phosphate groups for precipitation.

To obtain an appreciable precipitation of oligonucleotides with a chain length of less than 12 bases, 5′-terminal phosphate groups had to be introduced (Tables II, III and IV) to assist in the binding to Ca^{2+} and hence the precipitation. In addition, the base composition had a strong influence on the amount of nucleotide monophosphate precipitated together with calcium phos-

phate. It was evident that a high guanosine content favoured precipitation of monophosphorylated dinucleotides (Table II), tetramers (Table IV) as well as dodecamers (Table V).

Mononucleotides required two or three phosphate groups for precipitation with calcium phosphate (Table I). This observations might suggest a mechanism involving chelating of the Ca^{2+} by the phosphates resulting in a decreased hydration. The mechanism is probably different from that operating in the precipitation of longer polynucleotides.

Quantitative coprecipitation of trinucleotides with calcium phosphate was not possible with one terminal monophosphate but a terminal 5′-triphosphate resulted in a complete precipitation (Table III). Precipitation of pppA2′p5′A2′p5′A (2′,5′-A) by calcium phosphate has been reported by Hovanessian et al. [9] but no quantitative data were given. The failure of A2′p5′A2′p5′A to inhibit cellular protein synthesis after calcium phosphate precipitation [13] could probably be explained by a lack of precipitation as indicated in Table III.

Furthermore, attempts to increase the precipitation of A2′p5′A2′p5′A by addition of hydroxyapatite, DEAE-dextran, calf thymus DNA, and poly dT or exchanging Ca^{2+} with Mg^{2+}, Mn^{2+} and Zn^{2+} were not successful, indicating that a 5′-phosphorylation to a di or triphosphate might be necessary to precipitate this type of nucleotide. It was also clear that a concentrated $CaCl_2$ solution could not be added directly to cells when precipitating nucleotides without extensive cell damage.

The present results indicate that the calcium phosphate technique can be used to precipitate nucleotides containing at least 12 bases. However, shorter nucleotides require 5′-terminal phosphate groups. There is also a dependency on base composition for succesful precipitation with this technique.

Acknowledgement

We thank the National Swedish Board for Technical Development for financial support.

References

1 Berger, N.A. (1978) Methods Cell Biol. 20, 325–340
2 Castellot, J.J., Jr., Miller, M.R. and Pardee, A.B. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 351–355
3 Miller, M.R., Castellot, J.J., Jr. and Pardee, A.B. (1978) Biochemistry 17, 1073–1080
4 McCoy, G.D. and Racker, E. (1976) Cancer Res. 36, 3346–3349
5 Billen, D. and Olson, A.C. (1976) J. Cell. Biol. 69, 732–736
6 Rozengurt, E. and Heppel, L.A. (1975) Biochem. Biophys. Res. Commun. 67, 1581–1588
7 Graham, F.L. and Van Der Eb, A.J. (1973) Virology 52, 456–467
8 Hovanessian, A.G. and Wood, J.N. (1980) Virology 101, 81–90
9 Hovanessian, A.G., Wood, J., Meurs, E. and Montagnier, L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3261–3265
10 Baglioni, C., D'Alessandro, S.B., Nielsen, T.W., den Hartog, J.A.J., Crea, R. and van Boom, J.H. (1981) J. Biol. Chem. 256, 3253–3257
11 Gioeli, C., Kwiatkowski, K., Öberg, B. and Chattopadhyaya, J.B. (1981) Tetrahedron Letters 22, 1741–1744
12 Balgobin, N., Josepson, S. and Chattopadhyaya, J.B. (1981) Acta Chem. Scand. B 35, 201–212
13 Panet, A., Czarniecki, C.W., Falk, H. and Friedman, R.M. (1981) Virology 114, 567–572