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CALMODULIN IN INTERFERON PREPARATIONS: EFFECT OF INTERFERON ON CALMODULIN BIOACTIVITY

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SUMMARY Heat-stable calmodulin immunoreactivity and bioactivity were detected in crude preparations of various types of human, murine and chicken interferons (IFNs). Calmodulin containing HuIFN-α was retained on a trifluorophenothiazine-Sepharose column. The two activities were separated by serial elutions with 50 μM Ca^{2+} (HuIFN-α) followed by 2 mM EGTA (calmodulin). While maintaining its full antiviral activity, calmodulin free HuIFN-α inhibited enhancement of Ca^{2+}-ATPase activity *in vitro* by authentic purified eukaryote calmodulin. These results indicate that IFNs are calmodulin-binding proteins and that the secretion of both IFNs and calmodulin occurs from IFN-induced cells.

Calmodulin is an evolutionarily highly conserved intracellular Ca^{2+}-binding protein found in all animal cells that plays a role in regulating the activities of Ca^{2+}-dependent enzymes (1,2). Interferons (IFNs) are a family of multifunctional proteins (5-7) synthesized and released from cells in response to a variety of different inducers (3,4). Recent studies have shown that not only can human leukocyte

Abbreviations:

EGTA, ethylene glycol-bis (β-aminoethyl ether)N,N,N^1N^1-tetra acetic acid); IFN, interferon; HuIFN, human interferon; MuIFN, mouse interferon; ChIFN, chicken interferon; TES-TEA, N-tris [hydroxymethyl] methyl-2-amino-ethane sulfonic acid-triethanolamine.

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interferon (HuIFN-α) bind to calmodulin *in vitro* (8), but that this IFN can affect intracellular levels of calmodulin in human cells (9). This report describes the presence of calmodulin immuno- and bioactivity in IFN preparations from various sources and the separation of immuno- and bioactive calmodulin from bioactive HuIFN-α. Calmodulin-free HuIFN-α is shown to modify calmodulin enhancement of Ca^{2+}-ATPase activity.

MATERIALS AND METHODS

Cell cultures and viruses. Human foreskin (BG-9,BG-27), mouse L-929 and primary chicken embryo cells were grown and maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (10).

A plaque purified clone of vesicular stomatitis virus (VSV, Indiana serotype) was grown and assayed for infectivity in L-929 cells (11). Influenza virus (WS strain), Newcastle disease virus (NDV), and Sendai virus (Cantell strain) were grown in 10-day old embryonated chicken eggs and assayed for hemagglutination activity using chicken erythrocytes (12,13).

Interferons. Human leukocyte interferon (HuIFN-α) was produced by peripheral white blood cells in response to Sendai virus. HuIFN-α was purified to a specific activity of 1×10^7 IU/mg protein (14). HuIFN-β was produced by infection of human foreskin (BG-27) cells with poly rI:rC and purified by the procedure of Mikulski et al. (15). HuIFN-β was also produced in HEp-2 cells by infection with NDV (16). Highly purified preparation of HuIFN-α2, produced in *E. coli* was a gift from Dr. C. Weissmann, University of Zurich, Switzerland.

MuIFN-β was produced in L-929 cells in response to NDV and partially purified as described previously (17). MuIFN-γ, produced in BCG sensitized mice challenged with old tuberculin was a gift from Dr. G. Sonnenfeld, University of Louisville, KY.

ChIFN-β was produced in 10-day-old embryonated eggs by using influenza virus as the inducer (18). ChIFN-α was produced in chicken lymphocytes by infection with Sendai virus (18).

Antiviral activity of IFN preparations was determined in homologous cells by the dye uptake method of Finter (19) using VSV as the challenge virus. Human reference IFN-α obtained from the National Institutes of Health was used as a reference standard for all human IFNs.

Calmodulin assays. Prior to immunoassay, all IFN preparations were heated to 95°C for 5 min., rapidly cooled to 4°C in a methanol dry ice bath, and then centrifuged at 10,000 x *g* for 20 min. The supernatant was collected and immunoassayed for calmodulin activity using a polyclonal, monospecific antibody, as described previously (20). Purified rat testis calmodulin (CAABCO, Houston, TX) was used as a standard.

The IFN preparations were tested for calmodulin bioactivity by their ability to stimulate human red blood cell membrane Ca^{2+}-ATPase activity *in vitro*. The method used in our laboratory

measures enzyme activity as the liberation of phosphate (P_i) from Na_2ATP in the absence and presence of Ca^{2+} (20).

Chromatography of HuIFN-α on trifluorophenothiazine-Sepharose column. Purified HuIFN-α was chromatographed on a trifluorophenothiazine-Sepharose (CAABCO) affinity column. The column was equilibrated with 20 mM TES-TEA buffer, pH 7.4, containing 300 mM NaCl, 1 mM $CaCl_2$ and 1mM β-mercaptoethanol. HuIFN-α (1.7×10^5 IU/0.5ml) was applied to the column and eluted seriatim with equilibrating buffer, then with buffer containing 50 μM Ca^{2+} and finally with buffer containing 2 mM EGTA. Eluates were tested for calmodulin immuno- and bioactivities and for IFN bioactivity.

RESULTS

Calmodulin content in IFN preparations

IFNs produced in different cell types using various inducers were tested for the presence of immunoreactive calmodulin. All types of IFNs tested (human, murine and chicken) contained significant amounts of immunoreactive calmodulin (Table 1). Highly purified cloned HuIFN-α2 contained calmodulin, although the ratio of calmodulin to bioactive interferon was reduced compared to non-purified interferons.

Five IFN preparations were tested for calmodulin bioactivity by direct addition to a human red blood cell membrane Ca^{2+}-ATPase assay. Partially purified as well as crude HuIFN-α, ChIFN-β and cloned HuIFN-α2 enhanced Ca^{2+}-ATPase activity but HuIFN-β from BG-9 and HEp-2 cells did not (Table 1).

Chromatography of HuIFN-α on trifluorophenothiazine-Sepharose column: Separation of calmodulin and IFN antiviral activities

Results presented in Figure 1 show that fractions eluted with 50 μM Ca^{2+} contained 94% of the recoverable HuIFN-antiviral activity. In contrast, 96% of immunoreactive calmodulin was eluted with 2 mM EGTA. The recovery of HuIFN-α and calmodulin activities from the phenothiazine column was 75% and 99%, respectively. Fractions eluted with EGTA containing almost all immunoreactive calmodulin (and 6% of the interferon)

Table 1. Calmodulin content of various interferon preparations

Interferons			IFN titer	Calmodulin	
Type	Source	Inducer	(units/ml)	immuno-reactivity (μg/ml)	bioactivity[1] (μg/ml)
Non-purified					
HuIFN-α	Lymphocytes	Sendai virus	3.0×10^4	4.4	1.2
HuIFN-β	BG-9 cells	Poly rI:rC	8.0×10^3	2.6	0
HuIFN-β	HEp-2 cells	NDV	7.0×10^3	2.1	0
MuIFN-β	L-292 cells	NDV	2.7×10^4	1.3	ND[2]
MuIFN-γ	Mouse	Mycobacterium	2.5×10^3	3.0	ND
ChIFN-α	Lymphocyte	Sendai	6.4×10^2	5.4	5.6
ChIFN-β	Chicken egg	Influenza virus	6.4×10^2	4.1	ND
Partially purified					
HuIFN-α	Lymphocytes	Sendai virus	1.0×10^6	8.1	2.6
HuIFN-β	BG-9 cells	Poly rI:rC	1.0×10^6	1.1	ND
HuIFN-α2	E. coli		1.0×10^5	1.7	1.8
MuIFN-β	L-929 cells	NDV	1.0×10^5	0.1	ND

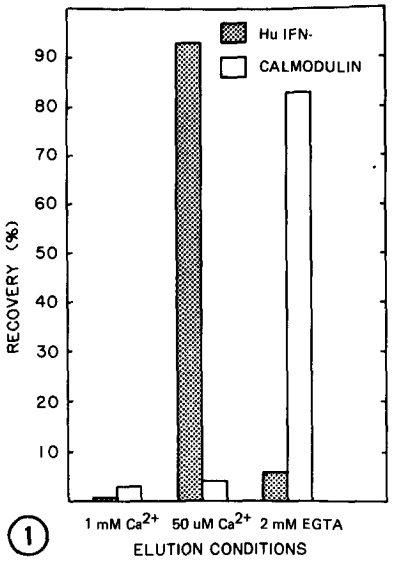
[1]Interferon preparations were directly added to Ca^{2+}-ATPase assay. Stimulatory activity was expressed in μg calmodulin/ml using a simultaneously determined rat testes calmodulin dose response curve as standard. Values are means of three or more determinations.

[2] ND Not done

demonstrated proportional calmodulin bioactivity, stimulating Ca^{2+}-ATPase to the same degree as the rat testis standard (data not shown).

Effect of column purified HuIFN-α on calmodulin bioactivity

HuIFN-α, eluted from the phenothiazine column with TES-TEA buffer containing 50 μM Ca^{2+}, was used to study the effect of interferon on calmodulin bioactivity. This column purified HuIFN-α which contained less than 4% of the total calmodulin originally present in the IFN preparation, inhibited human red



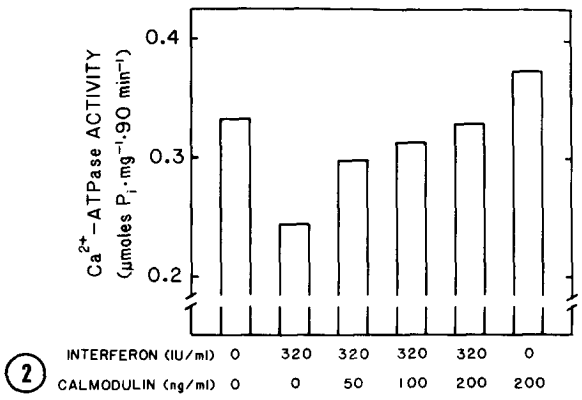


Figure 1. Chromatography of HuIFN-α on triflourophenothiazine-Sepharose column. HuIFN-α (1.7×10^5 IU/0.5 ml) was applied to the column which had been equilibrated with 20 mM TES-TEA buffer, pH 7.4, containing 300 mM NaCl, 1 mM $CaCl_2$ and 1 mM 2-mercaptoethanol. The column was eluted with the equilibrating buffer (1 mM Ca^{2+}), followed by buffer containing 50 μM Ca^{2+} and finally with buffer, containing 2 mM EGTA.

Figure 2. Effect of trifluorophenothiazine column purified HuIFN-α on red cell membrane Ca^{2+}-ATPase activity. Interferon and membranes were incubated with varying concentrations of rat testis calmodulin for one hour at 37°C prior to enzyme assay. Identical results were obtained in four separate experiments.

blood cell membrane Ca^{2+}-ATPase activity (Figure 2). The inhibition by HuIFN-α (320 IU/ml) could be overcome by addition of exogenous rat testis calmodulin to the system.

DISCUSSION

Although the calmodulin-like material detected in the various IFN preparations in this study awaits amino acid analysis, the heat-stability of the material, the ability of that material to stimulate Ca^{2+}-ATPase activity *in vitro* and the nature of the monospecific antibody used in the immunoassay suggests that IFNs contain a moiety very similar, if not identical, to authentic calmodulin. The finding of calmodulin activity in highly

purified cloned HuIFN-α2 produced in E. coli is interesting but not totally surprising. We and others have found calmodulin-like bio- and immunoreactivity in E. coli (21,22).

Results of studies with trifluorophenothiazine affinity chromatography indicate that the antiviral activity of partially purified HuIFN-α was not significantly affected upon removal of over 96% of calmodulin from the IFN preparation. HuIFN-α from which calmodulin was removed inhibited the activity of membrane Ca^{2+}-ATPase in vitro. This inhibition could be overcome by addition of rat testis calmodulin to the assay, suggesting the interferon was acting by binding to the endogenous calmodulin present in our membrane preparation (20) and interfering with its function. The presence of inhibiting and enhancing bioactivities separable by affinity column may explain the variable calmodulin bioactivity relative to immunoreactivity seen in the unfractionated interferons (Table 1).

Eucaryotic cells contain genes for calmodulin and IFNs. The calmodulin gene is expressed at all times; as a result, cells contain significant amounts of calmodulin. In contrast, IFN genes are not expressed in normal cells. Our finding of immuno- and bioactive calmodulin in interferon preparations could be explained by several mechanisms operating in vivo. Intracellular IFNs could bind to cellular calmodulin prior to their release from cells or IFNs could bind extracellularly to calmodulin which had been independently released from IFN-induced cells.

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REFERENCES

1. Cheung, W.Y. (1979). Science 207:19-27.
2. Means, A.R. and Chafouleas, J.G. (1982). Quant. Biol. 46:903-908.

3. Stewart, II, W.E. (1981). The Interferon System. Springer-Verlag. Wien-New York.
4. Pestka, S. (1983). Arch. Biochem. Biophys. 221:1-37.
5. Friedman, R.M. (1977). Bacteriol. Rev. 41:543-567.
6. Gresser, I. (1977). Cellular Immunol. 34:406-415.
7. Friedman, R.M. and Vogel, S.N. (1983). Adv. in Immunol. 34:97-140.
8. Myohanen, T.A. and Alm, G.V. (1983). Affinity chromatography of interferons - A novel application for calmodulin-Sepharose. Affinity Chromatog. and Biol. Recognition. Academic Press, pp. 469-472.
9. Bourgeade, M.F., Besancon, F. and Thang, M.N. (1983). Biochem. Biophys. Res. Comm. 111:430-437.
10. Thacore, H.R., Mount, D.T. and Chadha, K.C. (1982). J. Interferon Res. 2:401-408.
11. Winship, T.R. and Thacore, H.R. (1979). Virology 93:515-526.
12. Giles, R.E. and Ruddle, F.H. (1973). In vitro 9:103-107.
13. Thacore, H.R. and Youngner, J.S. (1971). J. Virol. 7:53-58.
14. Chadha, K.C. and Sulkowski, E. (1984). Method for the production and purification of human leukocyte interferon. U.S. patent 4,485,038, November 1984.
15. Mikulski, A.J., Heine, J.W., Le, H.V. and Sulkowski, E. (1980). Prep. Biochem. 10:102-119.
16. Thacore, H.R. (1976). Infect. Immun. 14:311-314.
17. Knight, Jr., E. (1975). J. Biol. Chem. 250:4139-4144.
18. Thacore, H.R., Kibler, K.K., Gregoria, C.C., Pollina, C.M. and Hudecki, M.S. (1985). J. Interferon Res. 5:279-288.
19. Finter, N.B. (1973). Interferon and Interferon Inducers (Finter, N.B., ed), pp. 135-170, North Holland Publ. Comp., Amsterdam.
20. Davis, F.B., Davis, P.J. and Blas, S.D. (1983). J. Clin. Invest. 71:579-586.
21. Harmon, A.C., Prasher, D. and Cornier, M.J. (1985). Biochem. Biophys. Res. Comm. Vol 127, no. 1:31-36.
22. Mylotte, K.M., Mylotte, J.M., Davis, P.J., Schoenl, M. and Blas, S.D. (1984). Clin. Res. Vol. 32, no. 2:376a.