Vol. 109, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1982 Pages 588-594

SOMATOCRININ (GROWTH HORMONE RELEASING FACTOR) IN VITRO BIOACTIVITY; Ca^{++} INVOLVEMENT, cAMP MEDIATED ACTION AND ADDITIVITY OF EFFECT WITH PGE_2

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Received October 13, 1982

Summary. The release of GH induced by purified hypothalamic GRF or native or synthetic tumor-derived GRF is antagonized by the presence of $CoCl^2$; it is simulated by 8Br.cAMP, IBMX, cholera toxin, forskolin, with identical maximal effects (E_{max}). Somatocrinin (GRF) stimulates the efflux of cAMP by the pituitary cells in parallel to the release of GH. Addition of either 8Br.cAMP, IBMX, cholera toxin or forskolin to a maximally stimulating dose of GRF does not increase the response which remains GRF-E_{max}. In contradistinction with these results PGE_2 releases GH with a dose-response curve different from that of GRF, and the combination of PGE_2 + GRF produces an E_{max} far greater than that due to either agonist alone; showing a true additivity. The name somatocrinin is proposed to replace the acronym GRF.

We have recently characterized and reproduced by total synthesis a peptide with high intrinsic activity (ED_{50} 15 x 10^{-12} M, in an *in vitro* assay described below) specifically to stimulate secretion of immunoreactive (ir) growth hormone (GH) (1,2). This peptide (hpGRF-44 for human pancreas growth hormone releasing factor composed of 44 amino acid residues), along with two shorter fragments (hpGRF-37 and hpGRF-40) with reduced biological activity, were isolated from a human islet cell carcinoma which had caused acromegaly. We have already reported that GRF in the forms now available is highly specific in stimulating only secretion of GH, *in vitro* (1,3,4) or *in vivo* (5); the statement applies to GRF of hypothalamic origin as a purified material (3), or as native or synthetic hpGRF (1,4,5). The notion of the specificity of hpGRF in regard to the secretion of growth hormone is of importance for

0006-291X/82/220588-07$01.00/0

some of the studies discussed in this note. We report here preliminary findings on some of the mechanisms of action of GRF in its releasing ir-GH *in vitro* using pituitary monolayer cultures. We show that Ca^{2+} is involved in that mechanism; that this is also likely the case for the adenylate cyclase-cAMP system and that comparing results obtained with GRF and the prostaglandin PGE_2 leads to recognize two pools of available GH, each one separately sensitive to each of these two agonists.

MATERIALS AND METHODS

Preparation of cells for the monolayer culture and bioassay: This has been described in details in two recent reports (4,6).

Radioimmunoassays (RIAs): RIAs for rat-GH are conducted using Sinha's monkey-antimurine GH immune serum (7). RIA for cAMP uses Miles-Yeda anti-cAMP immune serum; the antiserum is reconstituted 1:15 and we omit the succinylation reaction; the trace is obtained from New England Nuclear (NEX130). Calculation of standard curves and experimental values are done with the use of the program described in (8).

Synthetic hpGRFs. All synthetic replicates of the tumor derived GRFs were prepared by solid phase synthesis methods as routinely used in this laboratory (9). When we refer to synthetic hpGRF-44 we imply that the molecule is in the amidated form, as is the native material (1,2); on the other hand synthetic hpGRF-40 or hpGRF-37 refer to peptides in the free acid form, as are the native extracted peptides (1,2).

Chemicals. IBMX (3-isobutyl-1-methylxanthine), 8Br.cAMP (Na salt), PGE_2, $CoCl_2$ were purchased from Sigma Chemical Co. Cholera toxin, forskolin were purchased from Calbiochem-Behring Co.

Statistical analyses. Comparisons of the effects of various treatments were conducted by the multiple comparison test of Dunnett following an analysis of variance (program EXBIOL) (10). Multiple dose-response curves in the bioassays were analyzed for simultaneous fitting by the 4-parameter logistic equation of Rodbard et al (11) (program ALLFIT). The same data were also studied by regression analysis and calculations of relative potencies with 95% confidence limits (program BIOPROG) (12).

RESULTS

The data presented in Table 1 show that a blocker of calcium uptake, $CoCl^2$ at 0.2 mM, inhibits partially and at 2.0 mM abolishes completely the response to hypothalamic GRF or synthetic hpGRF-40 or synthetic hpGRF-44.

Increasing cellular content of cAMP by adding 8Br.cAMP in doses ranging from 0.9×10^{-5} M to 2.4×10^{-2} M stimulates release of GH with the same slope of the dose-response curve as that obtained for increasing doses of hypothalamic GRF, native hpGRF-44 or synthetic hpGRF-40 (Fig 1a). The same

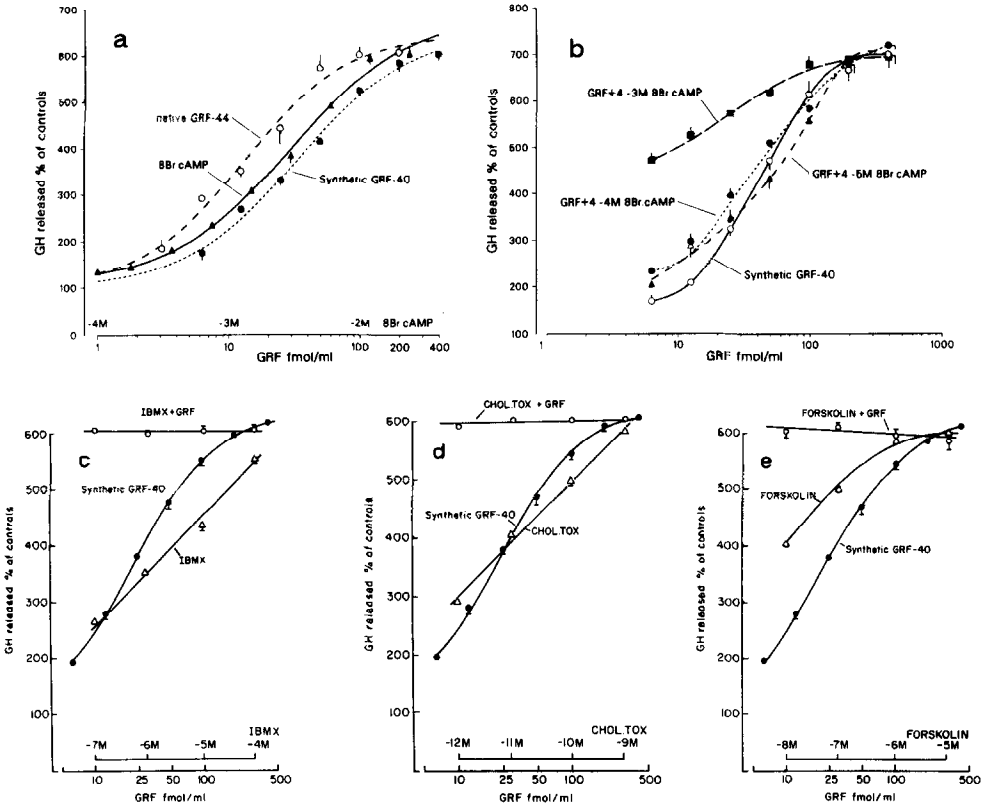

Figure 1: a: Parallelism and identical E_{max} for the log dose-response curves for 8Br.cAMP, native hpGRF-44 and synthetic hpGRF-40. b: Multiple doses of synthetic hpGRF-40 alone and in the presence of three different concentrations of 8Br.cAMP; additivity at the lower dose of both agonists but identical E_{max} for all agonists alone or in combination. c: Multiple doses of synthetic hpGRF-40, multiple doses of IBMX showing dose response curves and identical E_{max}; multiple dose of IBMX with a maximally stimulating dose of GRF (400 fmol/ml) show no increase in the E_{max} value as obtained for GRF alone. d: Same description and conclusion as in c, now for cholera toxin. e: Same description and conclusion as in c, now for forskolin. On all figures, the standard error of the mean value for any treatment is indicated by a vertical bar; when no such bar is shown, the standard error of the mean is smaller than the height occupied by the sign depicting that value of the mean.

maximal effect (E_{max}) is reached (Fig 1a) for all forms of GRF and for 8Br.cAMP. In presence of 8Br.cAMP (4×10^{-5} M to 4.10^{-3}) the GH-secretion stimulated by increasing doses of synthetic hpGRF-40 shows effect additivity of the two agonists, at the lower doses of GRF and at the higher doses of 8Br.cAMP, but in all cases the same E_{max} is reached in presence or absence of 8Br.cAMP (Fig 1b).

Increasing availability of endogenous cAMP by adding the inhibitor of phosphodiesterases, IBMX (10^{-7} M to 10^{-4} M) stimulates secretion of GH as a

Table 1: Effect of $CoCl^2$ on GH-releasing activity of hypothalamic GRF or synthetic GRF-40 and GRF-44

	Hypothalamic GRF GRF reference standard (in GRF units)	$CoCl^2$	GH released ng/ml	$CoCl^2$	GH released ng/ml
a-	0	0	900 ± 23	2 mM	523 ± 6
	0.63 u	0	1856 ± 45	2 mM	513 ± 14
	1.25 u	0	2520 ± 46	2 mM	520 ± 6
	2.5 u	0	3347 ± 44	2 mM	543 ± 14
	5.0 u	0	4673 ± 70	2 mM	550 ± 23
	10.0 u	0	5410 ± 65	2 mM	613 ± 19
	20.0 u	0	5580 ± 51	2 mM	563 ± 22
	40.0 u	0	5623 ± 67	2 mM	560 ± 15
b-	Synthetic hpGRF-40 fmol/ml	$CoCl^2$	GH released ng/ml	$CoCl^2$	GH released ng/ml
	0	0	1530 ±	0.2 mM	1050 ±
	6.3	0	2923 ± 17	0.2 mM	1393 ± 46
	12.5	0	4166 ± 14	0.2 mM	1627 ± 17
	25	0	5397 ± 16	0.2 mM	2033 ± 21
	50	0	6867 ± 46	0.2 mM	2546 ± 13
	100	0	8690 ± 12	0.2 mM	3503 ± 79
	200	0	8973 ± 51	0.2 mM	3677 ± 59
	400	0	9097 ± 14	0.2 mM	3667 ± 67
c-	Synthetic hpGRF-44 fmol/ml	$CoCl^2$	GH released ng/ml	$CoCl^2$	GH released ng/ml
	0	0	323 ± 7	2 mM	150 ± 6
	3.1	0	637 ± 20	2 mM	150 ± 10
	6.3	0	943 ± 27	2 mM	150 ± 10
	12.5	0	1273 ± 22	2 mM	147 ± 18
	25	0	1647 ± 37	2 mM	157 ± 7
	50	0	2017 ± 20	2 mM	166 ± 9
	100	0	2343 ± 20	2 mM	167 ± 9
	200	0	2383 ± 49	2 mM	207 ± 7

function of the dose of IBMX added with a slope of the dose-response curve statistically different from that obtained for hypothalamic GRF or synthetic hpGRF-40 (Fig. 1c). When synthetic hpGRF-40 is added at a maximally stimulating dose (400 fmoles/ml) in presence of IBMX in increasing concentrations (1.10^{-7} M to 10^{-4} M), the value of E_{max} is never higher than that produced by the maximally stimulating dose of GRF alone (Fig. 1c).

Similarly, cholera toxin (10^{-12} M to 10^{-9} M) an activator of the regulatory subunit and forskolin (10^{-6} M to 10^{-5} M), a stimulator of the catalytic subunit of adenylate cyclase stimulate secretion of growth hormone with dose-response curves statistically different from that of synthetic hpGRF-40 (Figs. 1d,e); when the same multiple doses of cholera toxin or forskolin are added, in presence of a maximally effective dose of synthetic hpGRF-40, the maximal response (GH secretion) to all treatments is not different from the E_{max} observed with GRF alone (Fig 1d,e).

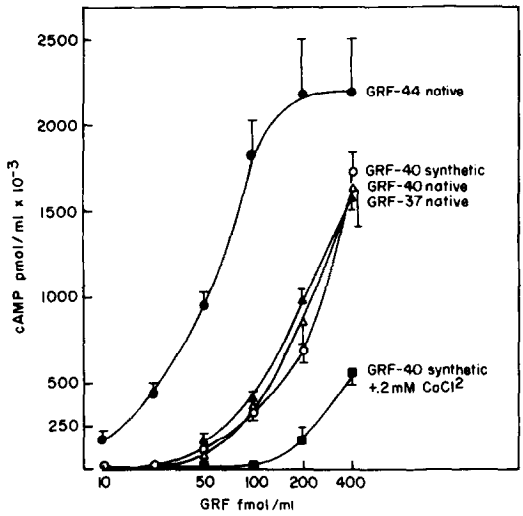

Figure 2: cAMP released 4 hrs in incubation fluids of monolayer pituitary cultures with multiple doses of several preparations of hpGRF, also in presence of 0.2 mM $CoCl^2$ added to GRF-40.

Direct measurement of cAMP released in the culture fluid by the pituitary cells shows an increase as a function of the dose of GRF added to the monolayer pituitary culture. Efflux of cAMP reaches a plateau for the same doses of hpGRF-44 that also yield E_{max} for GH secretion; for the less active forms of GRF, i.e. hpGRF-37, hpGRF-40, the efflux of cAMP is still increasing while the secretion of GH has reached its plateau. The increases in released cAMP parallel the increases in released GH as a function of the potency of GRF added (Fig 2) or available (See for instance cAMP released by the same doses of hpGRF-40 in absence or presence of $CoCl^2$ (Fig 2) comparing with the effects, in the same experiment, of $CoCl^2$ on the release of GH, Table 1, panel b).

In contradistinction to the results obtained with 8Br.cAMP, IBMX, cholera toxin and forskolin, studies of interrelationships between prostaglandin PGE_2 and GRF give entirely different results. The dose response curve to PGE_2 is totally divergent from that observed for hypothalamic GRF or synthetic hpGRF-44 (Fig 3a,b); moreover, PGE_2 E_{max} never reaches that due to GRF (Fig 3a,b) even at the highest tolerable doses of PGE_2 (10^{-5} M). When multiple doses of synthetic hpGRF-44 are studied on GH secretion in presence of PGE_2 (10^{-8} M to 10^{-5} M) a remarkable additivity of effects is observed at all

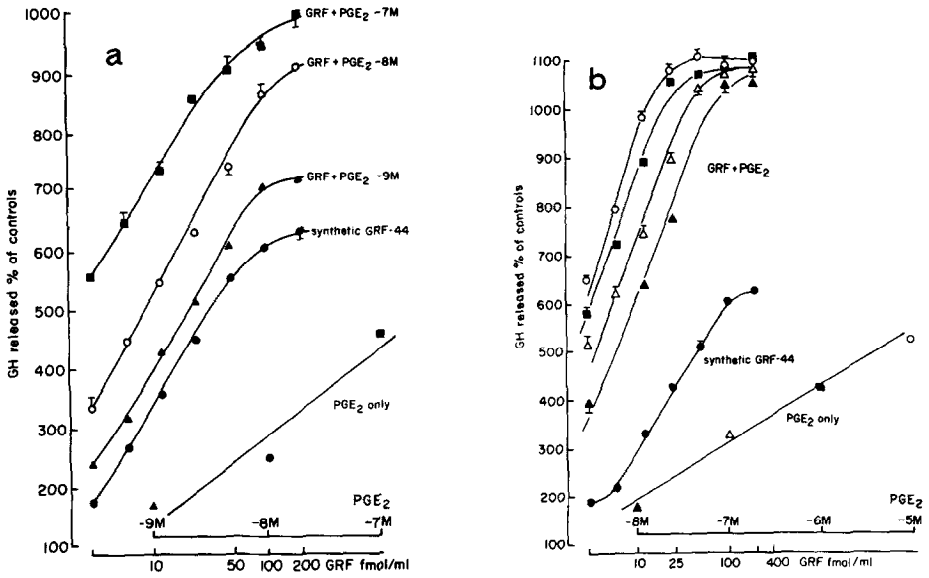

Figure 3: a,b: Results from two independent experiments showing the dose response curves to concentration of PGE_2 as shown, also responses to synthetic hpGRF-44 alone and in presence of multiple concentrations of PGE_2. Note the additivity of the maximal effect due to each agonist. Standard error of the mean shown by a vertical bar when that standard error is greater than the height of the sign showing the value of the mean.

doses, with values for E_{max} of the combined treatments far greater than those regularly observed for GRF alone (Fig 3a,b).

DISCUSSION

The early results reported here show that extracellular Ca^{2+} is necessary in the mechanism of action of GRF.

The evidence presented is best explained by proposing that the adenylate cyclase-cAMP system is involved in the mechanism of action of GRF in stimulating secretion of GH. A finer definition of the involvement of the adenylate cyclase-cAMP system in the expression of effects of GRF remains for further studies with a different biological system than the one available here. The pituitary monolayer culture used here is not a homogenous population of somatotrophs; therefore, the only specificity attributable to the results obtained stems exclusively from the specificity of synthetic GRF in acting only on somatotrophs (since there is no evidence that it stimulates

causes a large increase in the rate of 6-TG incorporation into RNA. Considering the corresponding increase in cytotoxicity and the suppression of 6-TG incorporation into DNA that is also observed, 6-TG incorporation into RNA should be considered as a potentially cytotoxic event in the enhanced 6-TG potency following MTX pretreatment. The suppression of 6-TG incorporation into DNA following MTX pretreatment is probably related to an inhibition of the rate of DNA synthesis that results from MTX (1). Studies are currently being completed to assess both rates of RNA and DNA synthesis in relation to treatment with these agents to understand the changes in 6-TG incorporation. It should also be recognized that 6-TGRP can inhibit several enzymes involved in de novo purine biosynthesis. Our studies demonstrated that 6-TGRP pools were increased with MTX pretreatment, and this may act synergistically with MTX in depleting purine nucleotides and altering DNA synthesis.

In conclusion, our studies demonstrate that MTX can markedly modulate 6-TG cytotoxicity. Pretreatment of cells with MTX will increase 6-TG cytotoxicity, while simultaneous administration of MTX with 6-TG antagonizes 6-TG cytotoxicity. The results suggest that the effect of MTX preexposure is to enhance the activation of 6-TG via PRPP-transferase, and that the increased cytotoxicity is unrelated to 6-TG incorporation into DNA. Incorporation of 6-TG incorporation into RNA, which is enhanced by MTX pretreatment, may contribute to 6-TG cytotoxicity under these conditions. Further biochemical studies are being completed to characterize the mechanism mediating this interesting interaction. These results should be considered with use of MTX and 6-TG in the treatment of leukemia or other neoplastic diseases.

REFERENCES

1. Pratt, W.B. and Reddon, R.W. The Anticancer Drugs, pp. 98-147, Oxford University Press, 1979.
2. Cadman,E., Heimer, R. and Benz, C. J. Biol. Chem. 256: 1695, 1981.
3. Benz, C. and Cadman, E. Cancer Res. 41: 994, 1981.
4. Schneider, W.C. Methods Enzymol. 3: 680-684, 1957.
5. Weiss, J.W. and Pitot, H.C. Biochemistry, 14: 316-326, 1975.
6. Wilkinson, D.J. and Pitot, H.C. J. Biol. Chem. 218: 63-68, 1973.