Vol. 202, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1994 Pages 391-397

HUMAN GROWTH HORMONE INCREASES CYTOSOLIC FREE CALCIUM IN CULTURED HUMAN IM-9 LYMPHOCYTES: A NOVEL MECHANISM OF GROWTH HORMONE TRANSMEMBRANE SIGNALLING[1]

Mapoko M. Ilondo&*, Pierre De Meyts& and Pierre Bouchelouche#

&Hagedorn Research Institute, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark
#Department of Clinical Chemistry, Herlev Hospital, University of Copenhagen, DK-2730 Herlev, Denmark

Received May 25, 1994

SUMMARY. Cytosolic free calcium ions concentration ($[Ca^{2+}]_i$) was measured in cell suspensions of cultured human IM-9 lymphocytes by dual wavelength fluorescence spectrometry using the calcium probe fura-2. Human GH (0.2-50 nM) induced a slow, progressive and sustained increase in $[Ca^{2+}]_i$. The GH effect was specific and exhibited a biphasic pattern, presumably reflecting GH receptor dimerization, typical of some other GH actions. The hGH effect depended on extracellular calcium, suggesting that at least part of the $[Ca^{2+}]_i$ increase was due to a stimulation of calcium influx. GH did not increase IP_3. Somatostatin-14 in the range 10^{-10} to 10^{-8} M, while having no effect of its own on $[Ca^{2+}]_i$, inhibited the effect of hGH. This inhibition by somatostatin was prevented by pretreatment of the cells with pertussis toxin. The hGH-induced $[Ca^{2+}]_i$ increase was not related to either protein tyrosine phosphorylation or protein kinase C activation, thus suggesting a novel mechanism of GH transmembrane signalling. © 1994 Academic Press, Inc.

Growth hormone (GH) causes a large variety of biological effects in numerous tissues, such as stimulation of cell proliferation and differentiation, induction of numerous genes and complex effects on protein, lipid and carbohydrate metabolism (1,2). GH initiates its actions by binding to specific cell surface receptors belonging to the cytokine/erythropoietin receptor superfamily (3). However, the different steps linking GH binding to biological effects are still poorly understood, although interesting clues are slowly emerging. There is evidence that both tyrosine phosphorylation (4) and protein kinase C activation (5) are involved in GH action, but it is not yet clear how these two intracellular pathways eventually relate to any specific effects of GH.

Cytosolic free calcium ions have been shown to act as second messengers for many extracellular signals, including peptide hormones and growth factors (6). For what concerns GH,

1. Part of this work was presented at the Fourth Joint Meeting of the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology, San Francisco (CA), June 3-7, 1993.

* Corresponding author. Fax: (+45) 31 68 05 02.

Abbreviations: $[Ca^{2+}]_i$: cytosolic free calcium ions concentration; (h)GH: (human) growth hormone; IGF I: insulin like growth factor I; IP_3: inositol 1,4,5-triphosphate; PTX: pertussis toxin; SMS: somatostatin-14.

0006-291X/94 $5.00
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no such evidence has been provided so far. Recently, Schwartz et al. (7,8) reported that primary rat adipocytes made refractory to the insulin-like effects of GH by previous GH stimulation had higher basal $[Ca^{2+}]_i$ than GH-sensitive adipocytes and responded to a new GH challenge by a further increase in $[Ca^{2+}]_i$. However, these effects were thought to be particular to the refractory adipocytes and were not considered as a general feature of GH stimulation (7,8).

We studied the effects of GH on $[Ca^{2+}]_i$ in various cell lines expressing typical GH receptors, including cultured human IM-9 lymphocytes (9). The IM-9 cell line has been shown previously to respond to GH stimulation by an increase in cell proliferation (10) and IgG production (11). We here report that human GH (hGH) specifically and dose-dependently increased the $[Ca^{2+}]_i$ in the IM-9 cells. At variance with some other *in vitro* effects of GH, the hGH-induced $[Ca^{2+}]_i$ increase in the IM-9 lymphocytes was not related to either tyrosine phosphorylation of cellular proteins or protein kinase C activation, thus suggesting a novel mechanism of GH transmembrane signalling.

MATERIALS AND METHODS

Reagents: The acetoxymethylester of fura-2 (fura-2/AM), staurosporin, phorbol 12-myristate 13-acetate, sphingosine, acridine orange and pertussis toxin were purchased from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, tyrphostin-25 and herbimycin were purchased from Gibco (Gaithersburg, MD). All other reagents were of chemical grade and purchased from Sigma.

Buffer: The buffer used for fluorescence measurements was modified Krebs-Ringer buffer with Hepes (KRH), pH 7.4 at 37°C, containing 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose and 20 mM HEPES. In experiments performed in Ca^{2+}-free medium, 1 mM EGTA was substituted for $CaCl_2$ and the pH of the buffer was adjusted to 7.4 at 37°C with NaOH.

Hormone and cell preparations: Recombinant human GH, insulin and IGF I were from Novo Nordisk (Gentofte, Denmark). Somatostatin-14 was purchased from Sigma. The IM-9 lymphocytes, an established line of human lymphoblastoid cells (12), were cultured and prepared as previously described (9,13). The cells were kept for at least 4 h at 37° C in serum-free medium (RPMI 1640 with 20 mM Hepes, pH 7.4) before use. When used, protein kinase inhibitors were incubated for 30 min at 37° C with the cells prior to calcium measurements.

Measurement of $[Ca^{2+}]_i$: Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was measured with the calcium sensitive fluorescent probe fura-2 as previously described (14). To improve dye loading, the dye was diluted with an equal volume of the non-ionic detergent Pluronic F-127 (0.02 %, final) and briefly sonicated to obtain fine emulsions. Briefly, cells (5 x 10^6/ml) were first loaded with 1 mM fura-2/AM for 30 min at 37°C in RPMI 1640 with 20 mM Hepes. After centrifugation, the cells were left for another 30 min at room temperature to complete deesterification of fura-2/AM. They were then successively washed twice with RPMI 1640 with 20 mM Hepes, pH 7.4, and once with the buffer (KRH) used for the fluorescence measurements, and resuspended in KRH buffer. An aliquot (10 x 10^6 cells) was transferred to a 4-ml quartz cuvette (Hellma, Mullhein-Baden, Germany) and the sample volume was adjusted to 2 ml with KRH buffer. Fluorescence was measured by dual wavelength spectrometry with excitatory wavelengths of 340 nm and 380 nm and emission wavelength of 510 nm, as previously described (14). The $[Ca^{2+}]_i$ was derived from the 340/380 nm ratio, after correcting for autofluorescence, by the method of Grynkiewicz (15). The dissociation constant of the Ca^{2+}-fura-2 complex was assumed to be 224 nM (15).

Measurements of IP_3 and cAMP: Cells (2x10^7/ml) were incubated at 37° C with 5 nM hGH in a total volume of 10 ml. At time zero and at regular time intervals thereafter, duplicate 0.5-ml aliquots were removed and centrifuged (10,000 g, 15 sec.). The supernatant was kept and frozen at -70° C until use. The concentrations of IP_3 and cAMP were determined in the supernatant using commercial kits (Amersham International) according to the instructions from the manufacturer.

RESULTS AND DISCUSSION

Effects of hGH on $[Ca^{2+}]_i$ in cultured human IM-9 lymphocytes

In medium with 1 mM $CaCl_2$, resting $[Ca^{2+}]_i$ remained unchanged for up to 1 h in unstimulated cells (Fig. 1a). When 5 nM hGH was added to the cells, $[Ca^{2+}]_i$ first remained stable for 5 min, then increased steadily and continuously during the 60-min observation period. We observed the same pattern in two other cell lines -i.e. RIN-5AH (16) and the mouse LB T-cell lymphoma (17,18)- expressing typical somatogenic receptors (M.M.Ilondo and P. Bouchelouche, unpublished observations). Both cell lines have been previously shown to respond to hGH stimulation by increased insulin biosynthesis (16) and cell proliferation (18), respectively. In other systems, agonist-induced $[Ca^{2+}]_i$ increase is usually rapid, being observed within minutes after agonist treatment (6). The slow, progressive and sustained increase in $[Ca^{2+}]_i$ observed after stimulation with GH is rather unusual, and could possibly explain why this effect of GH has been overlooked until now.

Human GH stimulates Ca^{2+} entry

The sources of calcium for agonist-induced changes in $[Ca^{2+}]_i$ may involve both external calcium and mobilization from intracellular stores. To determine the origin of the hGH-induced $[Ca^{2+}]_i$ increase in the IM-9 lymphocytes, we studied the response to hGH in a Ca^{2+}-free medium. Resting $[Ca^{2+}]_i$ was not modified when extracellular calcium was omitted, indicating that there is no basal entry of calcium into these cells. When 5 nM hGH was added in these conditions, no $[Ca^{2+}]_i$ increase was observed (Fig.1b), suggesting that at least part of the hGH effect on $[Ca^{2+}]_i$ was due to a stimulation of Ca^{2+} entry. In many cell types, calcium influx can be triggered by depletion of intracellular calcium stores, the signal being one of the mediators generated in cells after stimulation with agonists, e.g. IP_3 (19). We therefore measured IP_3 levels in the supernatant of the IM-9 cells in the conditions used for $[Ca^{2+}]_i$ measurements and we did

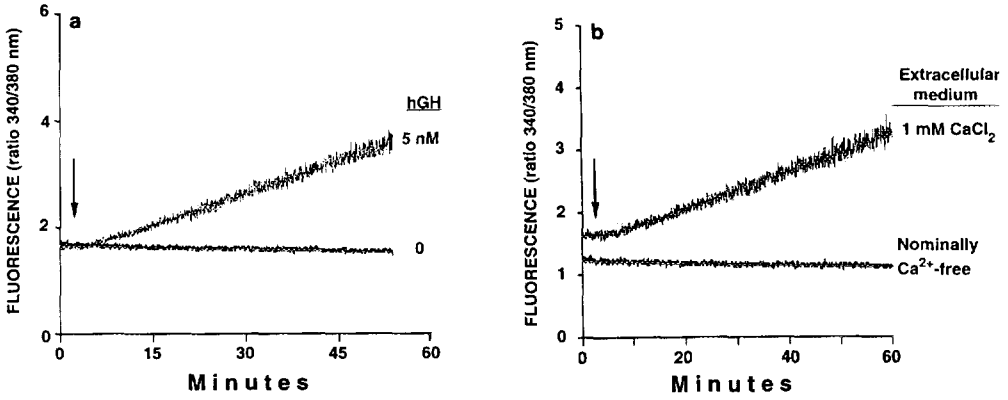


Figure 1. Changes in $[Ca^{2+}]_i$ in response to hGH.
IM-9 cells were loaded with fura-2 and changes in $[Ca^{2+}]_i$ were monitored by measuring fura-2 fluorescence at 340 and 380 nM, as described in *Methods*. Fluorescence is plotted as a function of time. **a.** Cells were incubated with or without 5 nM hGH as indicated by the arrow in the presence of 1 mM extracellular calcium. **b.** Cells were stimulated with 5 nM hGH (*arrow*) in the presence and absence of extracellular medium, as indicated.

Table 1: Lack of effect of insulin and IGF I on $[Ca^{2+}]_i$ in IM-9 lymphocytes

	$[Ca^{2+}]_i$ (nM)		
	Control	Insulin	IGF I
No hGH	93 ± 6	96 ± 8	106 ± 14
+ 5 nM hGH	186 ± 12	180 ± 8	197 ± 23

IM-9 cells were treated with 5 nM insulin or IGF I, with or without 5 nM hGH, and $[Ca^{2+}]_i$ was measured after 40 min.

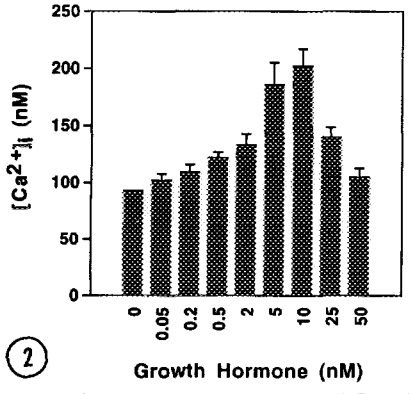
not observe any change after stimulation with GH (data not shown). The lack of IP_3 increase in the IM-9 cells is in agreement with some previous reports. Indeed, although accumulation of diacylglycerol has been demonstrated in various cell lines after stimulation with GH (20-22), no increase in IP_3 has ever been reported (21) and the available evidence suggests that the diacylglycerol originates from phosphatidylcholine breakdown (22).

Specificity of the hGH effect

Apart from GH receptors, the IM-9 cells also express specific receptors for insulin and IGF I. So, to determine the specificity of the hGH effect, we measured $[Ca^{2+}]_i$ after stimulation with these two hormones. Neither insulin nor IGF I at the same molar concentration as hGH (5 nM) induced any change in $[Ca^{2+}]_i$ in the IM-9 cells. Moreover, when added together with hGH, neither insulin nor IGF I affected the increase in $[Ca^{2+}]_i$ induced by hGH (Table 1).

Dose-dependence of the hGH effect

The effect of hGH concentration on the $[Ca^{2+}]_i$ increase was studied 40 min after hGH stimulation. The dose-response curve exhibited a biphasic pattern (Fig. 2). $[Ca^{2+}]_i$ first increased



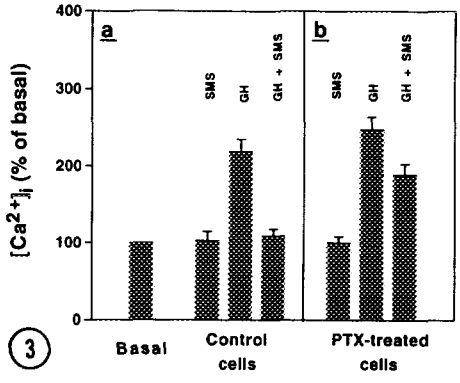


Figure 2. Dose-dependence of the hGH effect.
IM-9 cells were treated with varied concentrations (0-50 nM) of hGH and $[Ca^{2+}]_i$ was measured. The $[Ca^{2+}]_i$ after 40 min stimulation is shown as a function of the hGH concentration.

Figure 3. Effects of SMS and pertussis toxin on the hGH-induced increase in $[Ca^{2+}]_i$.
IM-9 cells were treated with or without hGH (5 nM), SMS (10^{-8} M) or both, and $[Ca^{2+}]_i$ was measured after 40 min. a. Control cells. b. Cells were pretreated for 3h at 37° C with 120 ng/ml pertussis toxin prior to hormone stimulation and calcium measurements. Results were normalized for unstimulated cells (basal $[Ca^{2+}]_i$).

with the hGH concentration from a basal value of 93 ± 15 nM up to a maximum of 202 ± 20 nM (2-fold increase above basal) at 10 nM hGH (ED_{50}: 0.5 nM). Then, $[Ca^{2+}]_i$ decreased continuously at higher hGH concentrations, returning to near basal levels (96 ± 16 nM, 103 % of basal) at 50 nM hGH. GH binding to its cell surface receptors results in the formation of a complex comprised of one molecule of hormone per two molecules of receptor (23,24). It has been shown that this receptor dimerization is essential for GH action in various cell lines, including the IM-9 cells (25,26). We recently proposed a mathematical formulation of the dimerization concept (27) and the predicted patterns of hormonal response confirm the experimental data available so far. The biphasic dose-response curve of the hGH effect on $[Ca^{2+}]_i$ is consistent with the predictions of the dimer model and suggests that, like some other GH effects, e.g. cell proliferation (25), the GH-induced increase in $[Ca^{2+}]_i$ requires receptor dimerization.

Effects of somatostatin-14 on hGH-induced $[Ca^{2+}]_i$ increase

Membrane ion channels in numerous tissues have been reported to be regulated by G-proteins (28). We addressed whether somatostatin-14 (SMS), which is known to act through an inhibitory, pertussis toxin-sensitive, G-protein (29), might interfere with the effect of GH on $[Ca^{2+}]_i$. When SMS in the range 10^{-10} - 10^{-8} M was added to the IM-9 cells, no effect on $[Ca^{2+}]_i$ was observed (Fig. 3a). However, when added together with 5 nM hGH, SMS completely inhibited the $[Ca^{2+}]_i$ increase induced by hGH. To examine whether the inhibitory effect of SMS occurred through cAMP, we measured cAMP levels in the IM-9 cells and did not find any change in cAMP levels after stimulation with hGH, either alone or with SMS (data not shown). Next, we pretreated the IM-9 cells with 120 ng/ml pertussis toxin for 3h prior to $[Ca^{2+}]_i$ measurements. As shown in Fig. 3b, the $[Ca^{2+}]_i$ increase after hGH stimulation was maintained and even increased as compared to control cells, but the inhibition of the hGH effect by SMS was prevented by pretreatment with pertussis toxin. This might suggest that hGH stimulated calcium entry by operating a calcium-permeable cation channel which is negatively regulated by a pertussis toxin-sensitive G-protein.

Relation of hGH-induced $[Ca^{2+}]_i$ increase to other cellular effects of GH

GH stimulation in various cell lines, including the IM-9 cells, has been shown to cause the binding of the cytoplasmic tyrosine kinase JAK2 to the GH receptor dimer (4), resulting in the tyrosine phosphorylation of several cellular proteins (4,26). It has been proposed that tyrosine phosphorylation is an early and essential step leading to the expression of a biological response to GH (4,30). To address the relation of the hGH-induced $[Ca^{2+}]_i$ increase to tyrosine phosphorylation, we preincubated the cells for 1 h at 37°C, prior to $[Ca^{2+}]_i$ measurements, with various inhibitors of protein tyrosine kinases, i.e. herbimycin (0.5 μM), tyrphostin-25 (10 μM) and staurosporin (1 μM). All three have previously been shown to inhibit the GH receptor-associated tyrosine kinase (JAK2) both *in vitro* and *in vivo* (30). As shown in Figure 4, none of these inhibitors affected the hGH-induced $[Ca^{2+}]_i$ increase in the IM-9 cells. This suggested that, unlike some other effects of GH, the GH effect on $[Ca^{2+}]_i$ is not related to tyrosine phosphorylation and JAK2, but rather constitutes a different and novel pathway of GH action. Similarly, two protein kinase C inhibitors, i.e. sphingosine (50 μM) and acridine orange (5 μM)

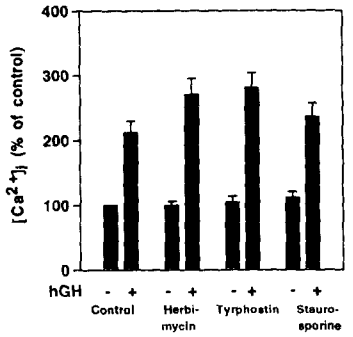


Figure 4. Effects of tyrosine kinase inhibitors on the hGH-induced increase in $[Ca^{2+}]_i$.
IM-9 cells were treated for 30 min at 37° C with herbimycin, tyrphostin and staurosporin at the concentrations indicated. They were then incubated with or without 5 nM hGH and $[Ca^{2+}]_i$ was measured after 40 min.

(5,31), had no effect on the hGH-induced $[Ca^{2+}]_i$ increase, whereas phorbol 12-myristate 13-acetate (100 nM), a direct activator of protein kinase C (32), did not induce any increase in $[Ca^{2+}]_i$ in the IM-9 cells (data not shown).

The kinetics of $[Ca^{2+}]_i$ increase reported here are not typical of calcium release from intracellular stores, but the possibility that calcium influx might trigger an intracellular release of calcium, i.e. calcium-induced calcium release (33), cannot be ruled out at present. Studies are in progress, using calcium video-imaging of single cells, to address this issue and to further characterize the mechanisms responsible for the GH-stimulated calcium entry.

In conclusion, we addressed in this paper whether GH regulates the concentration of cytosolic free calcium ions ($[Ca^{2+}]_i$) in target cells. We found that human GH at physiological concentrations increased $[Ca^{2+}]_i$ in the human IM-9 lymphocyte line, a classical *in vitro* model of GH action, and in some other cell lines responsive to GH. This suggested for the first time that $[Ca^{2+}]_i$ might act as an intracellular messenger of GH action. The effect of hGH on $[Ca^{2+}]_i$ was not related to some other previously described mechanisms of GH action, thus suggesting a novel pathway of GH transmembrane signalling. Cytosolic free calcium ions play a major role in many cellular functions, including DNA and protein synthesis, cell proliferation and differentiation, and gene expression (6). Although the relation of the GH effect on $[Ca^{2+}]_i$ to any of these effects remains to be established, measurements of $[Ca^{2+}]_i$ provide an additional and promising tool for investigating *in vitro* the mechanisms of GH action, and open new and so far unsuspected perspectives in GH signalling.

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