Bovine growth hormone induces oscillations in cytosolic free Ca^{2+} in single rat hepatocytes

Isabel MARRERO, Anne K. GREEN, Peter H. COBBOLD and C. Jane DIXON*

Department of Human Anatomy and Cell Biology, University of Liverpool, Liverpool L69 3BX, U.K.

Single rat hepatocytes microinjected with the photoprotein aequorin generate oscillations in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) when stimulated with agonists acting through the phosphoinositide signalling pathway. We show here that, in single rat hepatocytes, bovine growth hormone (bGH) is able to induce $[\text{Ca}^{2+}]_i$ oscillations which display similarities with oscillations induced by phenylephrine. Thus the rate of rise of

intracellular Ca^{2+} in each oscillation closely resembles that induced by $\text{Ins}(1,4,5)\text{P}_3$ -mediated agonists. However, the duration of bGH-induced oscillations increases with agonist concentration, in contrast to phenylephrine-induced oscillations, which undergo an increase in frequency as the agonist concentration is raised, without any increase in the duration of individual oscillations.

INTRODUCTION

Growth hormone (GH) has a broad range of physiological actions, including major effects on proliferation and differentiation and on intermediary metabolism [1,2]. Thus, in adipocytes, GH induces a transient insulin-like response, stimulating glucose uptake and utilization as well as lipolysis [3,4]. In myocytes GH also stimulates glucose oxidation [5], and in bone GH has been shown to stimulate mineral metabolism [6]. In several other cell types GH stimulates protein synthesis and amino acid transport [7]. A major target organ of GH actions is the liver, where GH regulates the expression of a variety of proteins, including hormone and growth factor receptors, and secretory proteins and enzymes [8]. Acutely, GH increases glucose output by hepatocytes [9].

The action of GH is known to be initiated through binding to specific cell-surface receptors belonging to the cytokine/erythropoietin superfamily [10]. The primary structure of the GH receptor is a single-chain protein of approx. 640 amino acids, with a centrally located hydrophobic transmembrane domain [11]. This structure is clearly different from that of the G-protein-linked receptors, which have seven transmembrane domains [12]. The signalling pathways by which GH mediates its actions are not entirely understood; the GH receptor does not belong to the class of growth factor receptors with intrinsic tyrosine kinase activity. However, GH-stimulated tyrosine kinase activity has been demonstrated in many cell types [13]. It has recently been shown that the GH-liganded GH receptor first dimerizes and then activates JAK2 kinase, a non-receptor protein tyrosine kinase [14], resulting in JAK2 autophosphorylation and phosphorylation of the GH receptor on specific tyrosine residues [15].

A role for the phospholipase C (PLC)-catalysed hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ has been suggested following the demonstration that stimulation by GH of basolateral membranes isolated from canine kidney resulted in $\text{Ins}(1,4,5)\text{P}_3$ formation [16]. Direct evidence for a role for the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in mediating the effects of GH is limited. Ilondo et al. [17] demonstrated that human GH (hGH) specifically and dose-dependently increased $[\text{Ca}^{2+}]_i$ in populations of cultured human IM-9 cells; the rise was slow, progressive and sustained. However,

the hGH-induced $[\text{Ca}^{2+}]_i$ increase was not associated with any detectable increase in $\text{Ins}(1,4,5)\text{P}_3$, nor was it related to tyrosine phosphorylation or JAK2 activation. The authors concluded that the effect was achieved, at least in part, by a stimulation of Ca^{2+} influx. Recently the effect of GH on $[\text{Ca}^{2+}]_i$ has been studied in single Chinese hamster ovary (CHO) cells transfected with GH receptor cDNA [18]. The response was dependent upon the presence of extracellular Ca^{2+} , and consisted of oscillations in $[\text{Ca}^{2+}]_i$ varying in frequency and amplitude. However, using receptor mutants, the investigators determined that binding and activation of JAK2 by the GH receptor was not required to achieve the $[\text{Ca}^{2+}]_i$ rise. Other workers have focused on a central role for protein kinase C (PKC) in mediating the effects of GH. Thus PKC inhibitors have been shown to block the effects of GH on both lipogenesis and lipolysis in rat adipose tissue [19,20]. Furthermore, PKC activation is necessary to transduce the effects of GH on certain genes in primary rat hepatocytes [21]. PLC-catalysed hydrolysis of phosphatidylcholine, which results in diacylglycerol formation and therefore PKC activation in the absence of $\text{Ins}(1,4,5)\text{P}_3$ formation, has been demonstrated in GH-stimulated Ob1771 mouse pre-adipocytes [22].

Here we have studied the effects of bovine GH (bGH) on single aequorin-injected rat hepatocytes. Single hepatocytes, in common with many other cell types, generate oscillations in $[\text{Ca}^{2+}]_i$ in response to agonists acting through the phosphoinositide signalling pathway [23,24]. Previous studies on the effects of GH in rat hepatocytes have used hGH [25]. However, hGH possesses both lactogenic and somatogenic properties in the rat by binding to prolactin receptors and GH receptors [26]. Therefore bovine GH, which is not a ligand for the rat prolactin receptor [27], was used in the studies described here. We show that bGH induces repetitive $[\text{Ca}^{2+}]_i$ oscillations, which are unusual in that the duration of the oscillations increases as the bGH concentration is raised.

MATERIALS AND METHODS

Single hepatocytes were isolated from fed male Wistar strain rats (150–250 g) by collagenase digestion, and prepared for microinjection with the photoprotein aequorin as described previously [28]. Collagenase was from Boehringer. The experimental me-

Abbreviations used: GH, growth hormone; bGH, bovine GH; hGH, human GH; $[\text{Ca}^{2+}]_i$, cytosolic concentration of free Ca^{2+} ; PLC, phospholipase C; PKC, protein kinase C.

* To whom correspondence should be addressed.

dium, Williams medium E (Gibco) gassed with CO₂/air (1:19), was superfused over single hepatocytes at 37 °C. Agonists were added to this medium. Recombinant bGH was purchased from ICN Biochemicals, and dissolved immediately before use in 20 mM Hepes to give a 4 μ M stock. Aliquots were added to the experimental medium to give the required concentration. Data acquisition and analysis were performed as described previously [28].

RESULTS

The effect of bGH, in the concentration range 10 nM–1 μ M, was tested on single aequorin-injected hepatocytes that had responded to phenylephrine (1–4 μ M) by the generation of $[Ca^{2+}]_i$ oscillations as previously observed [29,30]. When stimulated with 100 nM bGH, 18/18 hepatocytes, from 12 separate preparations, responded with the generation of repetitive oscillations in $[Ca^{2+}]_i$ (Figure 1, top panel). This was found to be the most effective dose of bGH; lower concentrations evoked oscillations in a small proportion of cells (1/9 at 10 nM; 2/10 at 20 nM; 8/16 at 50 nM). The threshold concentration for bGH-induced $[Ca^{2+}]_i$ oscillations was therefore between 50 and 100 nM in the majority of cells. We have previously reported for Ins(1,4,5) P_3 -mediated agonists that the latency period between agonist addition and the onset of the cellular response is comparable with the period between oscillations [30]. A similar relationship between the

Figure 1 Comparison of $[Ca^{2+}]_i$ oscillations induced by phenylephrine and bGH in a single hepatocyte

A single rat hepatocyte, micro-injected with aequorin, was superfused with phenylephrine (Phe) and bGH at the concentrations indicated and for the periods indicated. The bottom panel shows in more detail the $[Ca^{2+}]_i$ oscillations induced by 2 μ M phenylephrine and 100 nM bGH. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

Figure 2 The duration of the $[Ca^{2+}]_i$ oscillations is increased as the concentration of bGH is increased

A single aequorin-injected rat hepatocyte was superfused with bGH at the concentrations indicated and for the periods indicated. Before application of 50 nM bGH, the hepatocyte had failed to respond to 25 nM bGH. Time constants: for resting $[Ca^{2+}]_i$, 12 s; for transients, 1 s.

latency period and the frequency of bGH-induced oscillations was observed here, although uncertainty about the time required for the diffusion of the agonist from the superfusate to the cell surface precludes an accurate measurement of the latency period. The $[Ca^{2+}]_i$ oscillations induced by 100 nM bGH were similar to those induced by Ins(1,4,5) P_3 -dependent agonists, rising rapidly from the resting $[Ca^{2+}]_i$ of approx. 200 nM to a peak of greater than 600 nM. Thus the rate of rise of bGH-induced oscillations was 356 ± 129 nM/s (mean $\pm$ S.D., no. of oscillations = 13), compared with 463 ± 220 nM/s (mean $\pm$ S.D., no. of oscillations = 18) for phenylephrine-induced oscillations within the same hepatocyte. No significant difference was revealed by Student's *t* test for the rate of rise between the two sets of oscillations, assuming a significance level of $P < 0.05$. However, the peak $[Ca^{2+}]_i$ of bGH-induced oscillations was greater, by more than 10%, than the peak of phenylephrine-induced oscillations in 12/21 cells.

Figure 1 (bottom panel) compares, in detail, the oscillations induced in a single hepatocyte by 2 μ M phenylephrine and 100 nM bGH. The falling phase of the bGH-induced oscillations is prolonged compared with that of the phenylephrine-induced oscillations. It has previously been reported that the differences in duration of oscillations induced by different agonists result from differences in the duration of the falling phase from the peak $[Ca^{2+}]_i$ to the resting level [30]. Increasing the concentration of phenylephrine leads to an increase in the frequency of $[Ca^{2+}]_i$ oscillations without altering the duration of individual oscillations [30]. The effect of increasing bGH concentration was tested in six hepatocytes, and resulted in the lengthening of individual oscillations in all six cells. The effect of progressively increasing the bGH concentration up to 300 nM was studied in three of these six cells. As the concentration was increased, there was a coincident increase in the duration of $[Ca^{2+}]_i$ oscillations in 3/3 cells, as illustrated in Figure 2. Thus, in the hepatocyte depicted in Figure 2, increasing the bGH concentration from 50 nM to 100 nM resulted in an obvious increase in the duration of the oscillations. Increasing the bGH concentration to 300 nM led to a further increase in the duration of the oscillations, resulting in individual oscillations with durations greater than 1 min in 2/3 cells (the third cell produced a single prolonged oscillation in response to 300 nM bGH). As illustrated in Figure

Figure 3 Application of a high concentration of bGH induces a single $[\text{Ca}^{2+}]_i$ transient of prolonged duration

A single rat hepatocyte was superfused with bGH at the concentrations indicated and for the periods indicated. Time constants: for resting $[\text{Ca}^{2+}]_i$, 12 s; for transients, 1 s.

2, the application of 300 nM bGH was associated with a decrease in peak height in both cells which continued to produce oscillations. The effect of increasing the bGH concentration on the frequency of the oscillations was less obvious. At concentrations just above threshold for oscillation generation (50–100 nM), bGH induced $[\text{Ca}^{2+}]_i$ oscillations at low frequency (approx. 0.25–0.5 min^{-1}). When the concentration was raised, the frequency of oscillations was increased in 5/6 cells (Figure 2); in the final cell the frequency was reduced. As depicted in Figure 2, increasing the bGH concentration further was not associated with a progressive increase in frequency (3/3 cells).

Application of a high concentration of bGH (1 μM) was studied in four cells. At this concentration, bGH induced a single, prolonged transient of approx. 2 min duration, which was not followed by any further changes in $[\text{Ca}^{2+}]_i$ (Figure 3; 4/4 cells).

DISCUSSION

We have shown here that single aequorin-injected rat hepatocytes respond to bGH with series of $[\text{Ca}^{2+}]_i$ oscillations; this is the first demonstration that bGH can evoke $[\text{Ca}^{2+}]_i$ oscillations in single hepatocytes. bGH was as effective as phenylephrine as an agonist, in terms of the percentage of hepatocytes responding. Billestrup et al. [18] have recently demonstrated GH-induced $[\text{Ca}^{2+}]_i$ oscillations in CHO cells expressing GH receptors. However, the oscillations were irregular in terms of both frequency and amplitude, in contrast to the consistency of the responses reported here.

The rate of rise of $[\text{Ca}^{2+}]_i$ within GH-induced oscillations was similar to that of oscillations induced by phenylephrine within the same cell, indicating that a common mechanism is likely to be involved. However, the lengthening of the falling phase of the GH-induced $[\text{Ca}^{2+}]_i$ oscillations as the GH concentration was raised is in contrast to the effect of increasing the concentration of agonists that act at G_q -coupled receptors. Thus increasing the concentration of phenylephrine leads to the generation of

oscillations at an increased frequency without any change in the duration of individual oscillations [29,30].

We surmise that $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization from intracellular stores underlies the GH-induced $[\text{Ca}^{2+}]_i$ oscillations. In erythropoietin-stimulated erythroid cells, the erythropoietin receptor, a member of the same family as the GH receptor [31], has been shown to activate JAK2 [32] and $\text{PLC}\gamma$ [33]. Hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ by $\text{PLC}\gamma$ would result in $\text{Ins}(1,4,5)\text{P}_3$ generation and subsequently to increases in $[\text{Ca}^{2+}]_i$. There is evidence for GH-stimulated PLC-catalysed hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ in basolateral membranes isolated from canine kidney [16]. Conceivably, a similar pathway mediates GH-induced Ca^{2+} signalling in rat hepatocytes. Johnson et al. [25] demonstrated a 1.4-fold increase in diacylglycerol formation when freshly isolated rat hepatocytes were treated with 100 nM hGH, but no evidence was found of inositol phosphate formation, leading the investigators to conclude that the source of diacylglycerol was a phospholipid other than $\text{PtdIns}(4,5)\text{P}_2$. Similarly, the $[\text{Ca}^{2+}]_i$ rise observed in hGH-stimulated cultured human IM-9 cells was not associated with a detectable increase in $\text{Ins}(1,4,5)\text{P}_3$. However, it has previously been shown that vasopressin, at concentrations which induce oscillations in rat hepatocytes, does not result in detectable increases in $\text{Ins}(1,4,5)\text{P}_3$ levels [34,35]. A lack of detectable $\text{Ins}(1,4,5)\text{P}_3$ is not, therefore, a reliable criterion for determining whether $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis is involved in mediating the oscillatory $[\text{Ca}^{2+}]_i$ signalling induced by GH.

Billestrup et al. [18] determined that the $[\text{Ca}^{2+}]_i$ oscillations observed in CHO cells expressing GH receptors were independent of JAK2 activation, and hence tyrosine phosphorylation. The oscillations were dependent upon extracellular Ca^{2+} and were blocked by Ca^{2+} -channel blockers. These investigators therefore concluded that the oscillations reflected Ca^{2+} entry. The involvement of intracellular Ca^{2+} stores, through a process of calcium-induced calcium release, was not ruled out in the maintenance of the oscillations. The rate of rise of the bGH-induced oscillations reported here in single rat hepatocytes resembles that of $\text{Ins}(1,4,5)\text{P}_3$ -mediated phenylephrine-induced oscillations. We believe that this indicates that a common mechanism underlies their production.

In conclusion, bGH induces $[\text{Ca}^{2+}]_i$ oscillations in single aequorin-injected rat hepatocytes. Similarities between these oscillations and those induced by phenylephrine lead us to conclude, tentatively, that such oscillations are mediated by a common, $\text{Ins}(1,4,5)\text{P}_3$ -dependent, mechanism. The generation of $[\text{Ca}^{2+}]_i$ oscillations in response to bGH therefore has important implications for our understanding of the underlying mechanism and eliminates models in which G-proteins play a central role [24].

We are grateful to the Government of the Canary Islands (I.M.) and the Wellcome Trust for funding.

REFERENCES

- Isaksson, O. G. P., Eden, S. and Jansson, J.-O. (1985) *Annu. Rev. Physiol.* **47**, 483–499
- Davidson, M. B. (1987) *Endocr. Rev.* **8**, 115–131
- Goodman, H. M. (1968). *Ann. N.Y. Acad. Sci.* **148**, 419–440
- Goodman, H. M. and Coiro, V. (1981) *Endocrinology* (Baltimore) **108**, 113–119
- Adamafio, N. A., Towns, R. J. and Kostyo, J. L. (1991) *Growth Regul.* **1**, 17–22
- Bouillon, R. (1991) *Horm. Res.* **36** (Suppl. 1), 49–55
- Kostyo, J. L. (1968) *Ann. N.Y. Acad. Sci.* **148**, 389–407
- Zaphiropoulos, P. G., Mode, A., Norstedt, G. and Gustafsson, J.-A. (1989) *Trends Pharmacol. Sci.* **10**, 149–153
- Blake, W. L. and Clarke, S. D. (1989) *J. Endocrinol.* **122**, 457–464

- 10 Kelly, P. A., Djiane, J., Postel-Vinay, M. C. and Edery, M. (1991) *Endocr. Rev.* **12**, 235–251
- 11 Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Bernard, R., Waters, M. J. and Wood, W. I. (1987) *Nature (London)* **330**, 537–543
- 12 Dohlman, H. G., Caron, M. C. and Lefkowitz, R. J. (1987) *Biochemistry* **26**, 2657–2664
- 13 Stred, S. E., Stubbart, J. R., Argetsinger, L. S., Shafer, J. A. and Carter-Su, C. (1990) *Endocrinology (Baltimore)* **127**, 2506–2516
- 14 Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N. and Carter-Su, C. (1993) *Cell* **74**, 237–244
- 15 Carter-Su, C., Argetsinger, L. S., Campbell, G. S., Wang, X., Ihle, J. and Witthuhn, B. (1994) *Proc. Soc. Exp. Biol. Med.* **206**, 210–215
- 16 Rogers, S. A. and Hammerman, M. R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6363–6366
- 17 Ilondo, M. M., De Meyts, P. and Bouchelouche, P. (1994) *Biochem. Biophys. Res. Commun.* **202**, 391–397
- 18 Billestrup, N., Bouchelouche, P., Allevato, G., Ilondo, M. and Nielsen, J. H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 2725–2729
- 19 Smal, J. and De Meyts, P. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 4705–4709
- 20 Gorin, E., Tai, L. R., Honeyman, T. W. and Goodman, H. M. (1990) *Endocrinology (Baltimore)* **126**, 2973–2982
- 21 Tollet, P., Legraverend, C., Gustafsson, J.-A. and Mode, A. (1991) *Mol. Endocrinol.* **5**, 1351–1358
- 22 Catalioto, R.-M., Ailhaud, G. and Negrel, R. (1990) *Biochem. Biophys. Res. Commun.* **173**, 840–848
- 23 Berridge, M. J. (1990) *J. Biol. Chem.* **265**, 9583–9586
- 24 Cobbold, P. H., Sanchez-Bueno, A. and Dixon, C. J. (1991) *Cell Calcium* **12**, 87–95
- 25 Johnson, R. M., Napier, M. A., Cronin, M. J. and King, K. L. (1990) *Endocrinology (Baltimore)* **127**, 2099–2103
- 26 Ranke, M. B., Stanley, C. A., Tenore, A., Rodbard, D., Bongiovanni, A. M. and Parks, J. S. (1976) *Endocrinology (Baltimore)* **99**, 1033–1045
- 27 Yamada, K. and Donner, D. B. (1984) *Biochem. J.* **220**, 361–369
- 28 Cobbold, P. H. and Lee, J. A. C. (1991) in *Techniques in Calcium Research* (McCormack, J. G. and Cobbold, P. H., eds.), pp. 55–81, IRL Press, Oxford
- 29 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1986) *Nature (London)* **319**, 600–602
- 30 Woods, N. M., Cuthbertson, K. S. R. and Cobbold, P. H. (1987) *Cell Calcium* **8**, 79–100
- 31 Bazan, J. F. (1989) *Biochem. Biophys. Res. Commun.* **164**, 788–795
- 32 Todokoro, K., Sugiyama, M., Nishida, E. and Nakaya, K. (1994) *Biochem. Biophys. Res. Commun.* **203**, 1912–1919
- 33 Ren, H. Y., Komatsu, N., Shimizu, R., Okada, K. and Miura, Y. (1994) *J. Biol. Chem.* **269**, 19633–19638
- 34 Thomas, A. P., Alexander, J. and Williamson, J. R. (1984) *J. Biol. Chem.* **259**, 5574–5584
- 35 Charest, R., Prpic, V., Exton, J. H. and Blackmore, P. F. (1985) *Biochem. J.* **227**, 79–90