Signal transduction mechanism of the seabream growth hormone secretagogue receptor

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Abstract **We have recently cloned the full-length cDNAs of the two growth hormone secretagogue receptor (GHSR) subtypes from a teleost species, the black seabream (*Acanthopagrus schlegeli*) [Mol. Cell. Endocrinol. 214 (2004) 81], namely sbGHSR-1a and sbGHSR-1b. Functional expression of these two receptor constructs in human embryonic kidney 293 (HEK293) cells indicated that stimulation of sbGHSR-1a by growth hormone secretagogues (GHS) could evoke increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), whereas sbGHSR-1b appeared to play an inhibitory role on the signal transduction activity of sbGHSR-1a. In the present study, we have further investigated the signal transduction mechanism of sbGHSR-1a. The peptide GHS GHRP-6 and the non-peptide GHS L163,540 were able to trigger a receptor specific and phospholipase C (PLC)-dependent elevation of $[Ca^{2+}]_i$ in HEK293 cells stably expressing sbGHSR-1a. This GHS-induced calcium mobilization was also dependent on protein kinase C activated L-type calcium channel opening. It was found that sbGHSR-1a could function in an agonist-independent manner as it exhibited a high basal activity of inositol phosphate production in the absence of GHS, indicating that the fish receptor is constitutively active. In addition, the extracellular signal-regulated kinases 1 and 2 (ERK1/2) were found to be activated upon stimulation of sbGHSR-1a by GHRP-6. This observation provides direct evidence in the coupling of sbGHSR-1a to ERK1/2 activation. Neither G_s nor G_i proteins are coupled to the receptor, as GHS did not induce cAMP production nor inhibit forskolin-stimulated cAMP accumulation in the sbGHSR-1a bearing cells. Furthermore, the ability of the GHSR antagonist D-Lys(3)-GHRP-6 to inhibit basal PLC and basal ERK1/2 activity suggests that this compound is an inverse agonist. In summary, the sbGHSR-1a appears to couple through the $G_{q/11}$-mediated pathway to activate PLC, resulting in increased IP_3 production and Ca^{2+} mobilization from both intracellular and extracellular stores. Moreover, sbGHSR-1a may trigger multiple signal transduction cascades to exert its physiological functions.**
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1. Introduction

Growth hormone secretagogues (GHS) are small molecules that can stimulate growth hormone (GH) secretion in a number of vertebrate species [1–6]. These GHS exert their physiological actions via activation of a G-protein coupled receptor (GPCR) called growth hormone secretagogue receptor (GHSR) [7–9]. In mammals, GHSR is mainly located in the pituitary and hypothalamus [10], but significant levels are also found in peripheral tissues [11]. It was, therefore, hypothesized that GHS could take part in diverse physiological actions apart from controlling GH secretion. For example, it has now been shown that the GHS system is involved in a number of physiological functions including the control of energy expenditure [12,13], the quality of sleep [14], pancreatic functions and glucose metabolism [15], gastric acid secretion [16], and cardiovascular activity [17].

In view of its diverse physiological roles, the signaling mechanism of GHSR has attracted much research interest and large species difference in the signal transduction mechanism of the receptor has been noticed. In rat somatotrophs, GHS could induce the accumulation of inositol triphosphate (IP_3) and activate protein kinase C (PKC) leading to an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) [18,19]. On the other hand, the action of GHS on ovine somatotrophs was found to depend mainly on the production of cAMP in which activation of PKC potentiates the protein kinase A (PKA) cascade causing GH secretion [20]. PKC activation and cAMP production have also been observed in human acromegalic tumor cells by GHRP-2, but the cAMP-PKA pathway does not seem to be essential for the GHS-induced GH secretion in these cells [21]. In all these reports, the experiments have been performed in primary culture of cells obtained from pituitary glands or pituitary tumors. It is, therefore, not certain whether the activation of both the cAMP-PKA and the phospholipase C (PLC)-PKC pathways by GHS is the result of direct GHSR activation or via cross-talk with other receptors or with other signaling pathways in the cells. In order to better elucidate the signaling events in a cleaner system, cultured cell lines expressing specific receptor constructs are necessary.

We have previously identified two transcripts of GHSR from a teleostean species, the black seabream *Acanthopagrus schlegeli*, namely sbGHSR-1a which is a classical GPCR with 7-transmembrane domains and GHSR-1b which is a truncated receptor containing 5-transmembrane domains with no known signaling activity [22]. The sbGHSR-1a shares a low degree of

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homology with the mammalian counterparts. Also different from the mammalian situation, the expression of these receptors in seabream is restricted to the pituitary and some other brain parts only, with expression in the peripheral tissues being extremely low. If species difference in the GHSR signaling events is apparent even among the mammalian GHSRs in which the sequences are more than 90% identical with each other, the elucidation of the signaling mechanism of the fish GHSR would be highly warranted from a phylogenetic point of view, since the fish receptor shares only about 60% identity with the mammalian GHSRs. Moreover, we found that the functional activity of sbGHSR-1a could be attentuated by the co-expressed sbGHSR-1b, but it is not yet known whether the sbGHSR-1b exerts its modulatory role by interfering with the signal transduction pathway of sbGHSR-1a. Further investigations on how the two receptors interact will only be possible when we have a better understanding on how the functional sbGHSR-1a couples to cell signaling pathways. The present report thus aims to study the pharmacological profile and receptor-effector coupling system of the sbGHSR-1a in cultured human embryonic kidney 293 (HEK293) cells. Results obtained would shed light on the signaling mechanism of the teleostean GHSR and would also provide the basis for further studies on the molecular mechanism of how sbGHSR-1b regulates sbGHSR-1a activity.

2. Materials and methods

2.1. Materials

All the materials used in cell culture were obtained from Invitrogen (Carlsbad, CA, USA) unless otherwise stated. GHRP-2, GHRP-6 and ipamorelin were purchased from Neosystem (Strasbourg, France). The GHSR antagonist, D-Lys(3)-GHRP-6, was purchased from Bachem (Bubendorf, Switzerland). The non-peptide GHS L163,540 was a gift from Dr. Roy Smith of the Merck Research Laboratories (Rathway, USA). Nitrendipine was purchased from Calbiochem (La Jolla, CA, USA). U73122, phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). U0126 and all the antibodies used were purchased from Cell Signaling Technology (Beverly, MA, USA). The [^{3}H]*myo*-inositol was purchased from NEN Life Sciences Products (Boston, MA, USA). [^{3}H]adenine was purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Cell culture and development of a clonal cell line stably expressing sbGHSR-1a

A clonally selected HEK293 cell line stably expressing sbGHSR-1a (HEK-sbGHSR-1a) was developed as previously described [22] and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 500 μg/ml G418.

2.3. Intracellular calcium mobilization assay

Intracellular calcium mobilization in HEK-sbGHSR-1a cells induced by various GHS was measured as described previously [22], using Fluo-4 AM (Molecular Probes, Eugene, OR, USA) on a Wallac Victor^2 multilabel plate reader (Perkin–Elmer Life Sciences, Boston, MA, USA) at $\lambda_{Ex} = 485$ nm and $\lambda_{Em} = 535$ nm. The values of the fluorescence increase were calculated as the peak fluorescence value minus the basal level.

2.4. [^{3}H]inositol phosphate accumulation assay

HEK-sbGHSR1a cells were seeded in 12-well culture plates pretreated with 0.01% (w/v) poly-D-lysine (Sigma–Aldrich). After an overnight incubation (16–20 h) with [^{3}H]*myo*-inositol (1 μCi/ml; specific activity 10–25 Ci/mmol), the medium was aspirated and the cells washed with HEPES-buffered saline (HBS) (15 mM HEPES, pH 7.4, 140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, and 11 mM glucose) followed by 10 min incubation at 37 °C in HBS containing 20 mM LiCl. The buffer was removed and replaced with fresh buffer and the cells were incubated, in duplicates, with HBS or 100 μM D-Lys(3)-GHRP-6 for 15 min. Then, the cells were incubated with different concentrations of agonists for a further 60 min, and the reaction was terminated by aspiration and addition of ice-cold 20 mM formic acid. The plates were left on ice for at least 60 min before separating the [^{3}H]inositol phosphates from the total radioactive inositol fraction by column chromatography as described previously [23].

2.5. [^{3}H]cAMP accumulation assay

[^{3}H]cAMP accumulation was measured by seeding HEK-sbGHRS-1a cells in 12-well culture plates pre-treated with 0.01% (w/v) poly-D-lysine. After an overnight incubation (16–20 h) with [^{3}H]adenine (1 μCi/well; specific activity 27 Ci/mmol), the medium was aspirated and the cells were washed with HBS. They were then incubated with the test compounds dissolved in HBS containing 1 mM 3-isobutyl-1-methyl xanthine at 37 °C for 30 min. The reaction was terminated by aspiration and addition of ice-cold 5% trichloroacetic acid containing 1 mM ATP. [^{3}H]cAMP accumulation was assessed by determining the ratio of [^{3}H]cAMP to total [^{3}H]adenine nucleotides as described previously [24].

2.6. Measurement of ERK1/2 activity using Western blots

Twenty hours before the assay, the HEK-sbGHSR1a cells were serum-starved in 6-well plates. The cells were then pre-treated with either control buffer (HBS) for 30 min, 100 μM D-Lys(3)-GHRP-6 for 15 min or 10 μM U0126 for 30 min at 37 °C. Control buffer or 5 μM GHRP-6 was then added and the cells were incubated for a further 15 min before the assay was terminated by cell lysis on ice for 30 min (Cell lysis buffer: 50 mM Tris base, pH 7.5, 0.1 M NaCl, 5 mM EDTA, 67 mM sodium pyrophosphate, 0.01% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 1 mM dithiothreitol, 4 μM leupeptin and 4 μg/ml aprotinin). The cell lysates were then centrifuged at $12\,000 \times g$ for 15 min at 4 °C. Equal amounts of lysates (30–50 μg/lane) were separated by SDS–PAGE on a 12% gel and electrophoretically transferred onto Hybond nitrocellulose membranes (Amersham Biosciences). The membranes were then incubated with anti-phospho-ERK1/2 (anti-pERK1/2) antibody or anti-ERK1/2 antibody overnight at 4 °C. Immunoblots were developed by horseradish peroxidase-linked goat anti-rabbit immunoglobulin and an ECL chemiluminescence kit (Amersham Biosciences). The band intensity was quantified using the computer program Scion Image (Scion Corporation, Frederick, USA).

2.7. Data analysis

pEC_{50} values were calculated by fitting the log concentration–response curves by standard non-linear regression analysis using Prism 3.0 (GraphPad Software, San Diego, USA). The production of [^{3}H]inositol phosphates was determined as the ratio of radiolabeled inositol phosphates to total inositol and was expressed as percentage conversion [(inositol phosphates)/(total inositol) × 100%]. [^{3}H]cAMP accumulation was also calculated as percentage conversion and is expressed as fold induction relative to the control (HBS treatment). All data are expressed as mean values ± SEM from three or more independent experiments. The data were considered statistically significant at $P < 0.05$ using either unpaired *t* test, or one-way ANOVA followed by Tukey's multiple comparison test or Newman–Keuls test.

3. Results

3.1. Activation of sbGHSR-1a leads to changes in $[Ca^{2+}]_i$

GHRP-6 evoked an elevation of $[Ca^{2+}]_i$ in HEK-sbGHSR-1a cells (Fig. 1), which was typified by a rapid initial peak (maximal at approximately 20 s after the addition of the agonist) followed by a gradual decline to a plateau level for up to at least 3 min. The peak response represented an almost doubling of the basal unstimulated level.

The ability of sbGHSR-1a to elevate $[Ca^{2+}]_i$ was characterized using the three synthetic GHS (Table 1). All the agonist-induced $[Ca^{2+}]_i$ elevations were log concentration-dependent with the potency in the following order: ipamore-

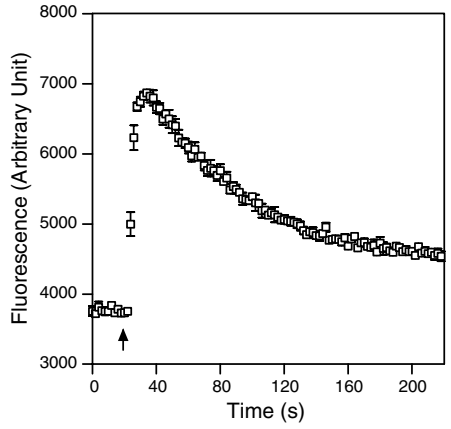

Fig. 1. A time-course study of $[Ca^{2+}]_i$ elevation in HEK-sbGHSR-1a cells stimulated by 30 μM GHRP-6. The arrow indicates the time when GHRP-6 was introduced.

lin > L163,540 > GHRP-6. Taking the cellular response induced by GHRP-6 as the standard, the relative efficacy of L163,540 was the highest, followed by GHRP-6 and ipamorelin.

3.2. Extracellular Ca^{2+} and L-type Ca^{2+} channels are involved in sbGHSR-1a activation

Although GHRP-6 and L163,540 could still elicit some increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 2, panels A2 and B2), the magnitudes of the responses were much reduced when compared with the situation in which extracellular Ca^{2+} was present. Furthermore, the responses were more transient in nature and returned to the basal level within 90 s, as opposed to a more sustained response in the presence of extracellular Ca^{2+}.

The above results indicated that calcium channels were activated during the course of sbGHSR-1a signaling. This was substantiated by the fact that pre-treatment of HEK-sbGHSR-1a cells by the L-type calcium channel blocker nitrendipine for 30 min significantly inhibited the GHRP-6 and L163,540-induced increases in $[Ca^{2+}]_i$ (Fig. 2C).

3.3. Activation of sbGHSR-1a leads to changes in [^{3}H]inositol phosphate production

Both GHRP-6 and L163,540 produced log concentration-dependent increases in [^{3}H]inositol phosphate production in HEK-sbGHSR-1a cells (Fig. 3A) with EC_{50} values of 3.63 and 0.17 μM, respectively (Table 2). Pre-treatment of HEK-sbGHSR-1a cells for 15 min with the GHSR-1a antagonist D-Lys(3)-GHRP-6 shifted the log concentration-response curves of GHRP-6 and L163,540 to the right (Fig. 3A), giving estimated pA_2 values of 4.33 ± 0.12 and 4.29 ± 0.15, respectively.

Table 1
Ability of various synthetic GHS to increase $[Ca^{2+}]_i$ in HEK-sbGHSR-1a cells

Agonist	pEC_{50}	Intrinsic activity	Hill coefficient
GHRP-6	5.48 ± 0.09	100	1.82 ± 0.34
L163,540	6.24 ± 0.08	145 ± 14	1.71 ± 0.19
Ipamorelin	6.50 ± 0.07	76 ± 8	1.81 ± 0.26

Basal fluorescence in the absence of agonist was 3990 ± 102 U. Maximum response to GHRP-6 was 7422 ± 113 U. Values are mean values ± SEM ($n = 6$).

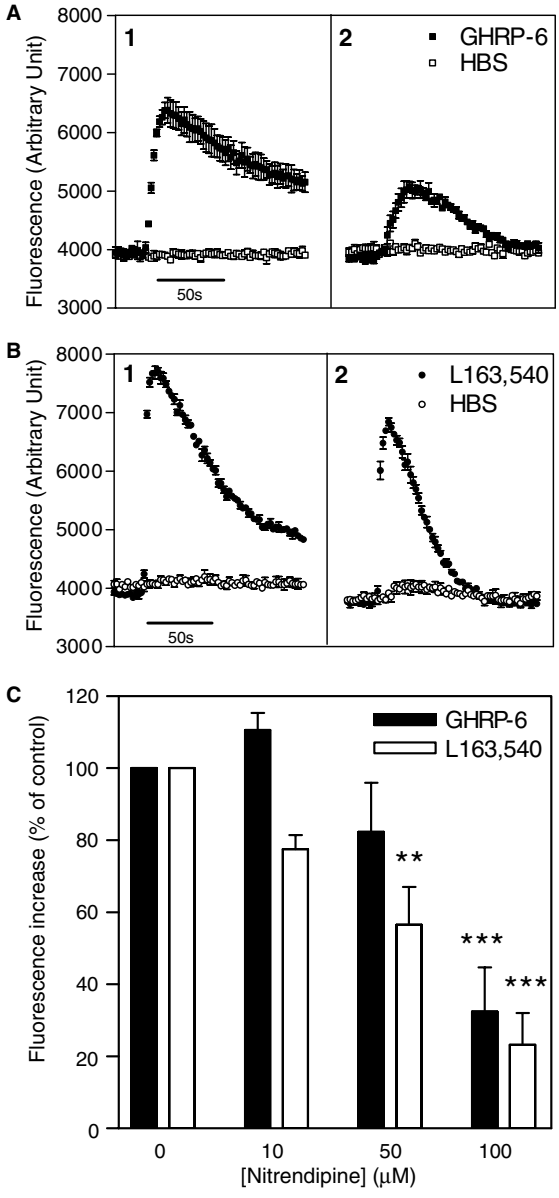

Fig. 2. The role of extracellular Ca^{2+} in GHS-induced calcium mobilization in HEK-sbGHSR-1a cells. Panel A is the time-course of $[Ca^{2+}]_i$ elevation upon stimulation by 30 μM GHRP-6 in the presence (Panel A1) or absence (Panel A2) of extracellular Ca^{2+}. Panel B is the time-course of $[Ca^{2+}]_i$ elevation upon stimulation by 30 μM L163,540 in the presence (Panel B1) or absence (Panel B2) of extracellular Ca^{2+}. Panel C is the effect of nitrendipine on the GHRP-6- and L163,540-induced $[Ca^{2+}]_i$ elevation. $^{**}P < 0.01$; $^{***}P < 0.001$.

Notably, the basal PLC activity was significantly higher in HEK-sbGHSR-1a cells than that of the parental HEK293 cells, suggesting that the sbGHSR-1a is a constitutively active receptor (Table 2). This high basal PLC activity in the HEK-sbGHSR-1a cells could also be significantly decreased by the GHSR antagonist D-Lys(3)-GHRP-6.

3.4. GHS-induced $[Ca^{2+}]_i$ elevation is PLC- and PKC-dependent

The GHS-mediated PLC activation was further demonstrated to be crucial for the agonist-induced $[Ca^{2+}]_i$ elevation, as pre-treatment of HEK-sbGHSR-1a cells with the PLC

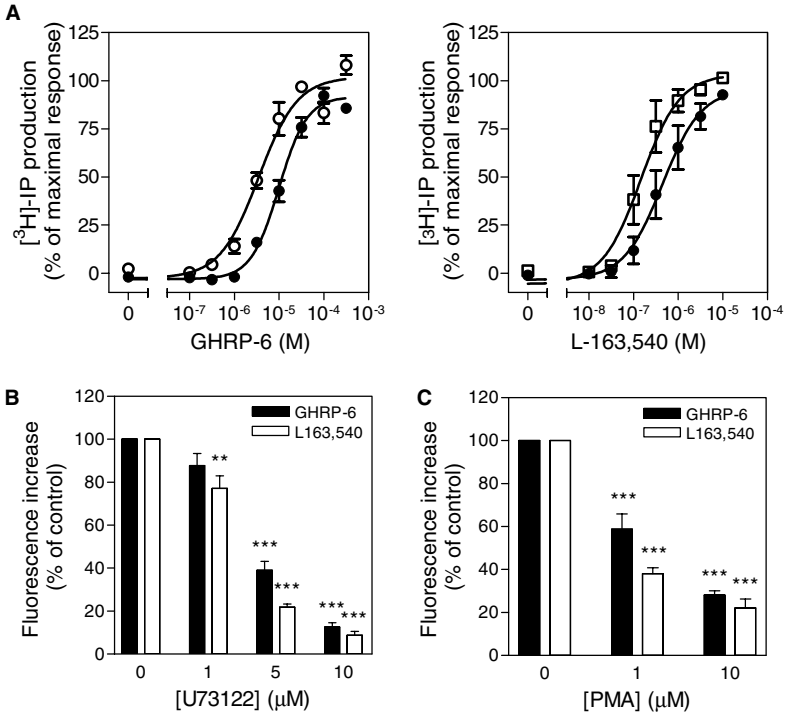

Fig. 3. GHS-induced $[Ca^{2+}]_i$ elevation in HEK-sbGHSR-1a cells is PLC-PKC-dependent. Both GHRP-6 (○) and L163,540 (□) cause a log concentration-dependent increase in [^{3}H]inositol phosphate ([^{3}H]IP) production in HEK-sbGHSR1a cells. Pre-treatment of the cells with 100 μM D-Lys(3)-GRHP-6 (●) shifted the log concentration-responsive curves to the right (Panel A). Inhibition of PLC by U73122 (Panel B) and inhibition of PKC by PMA (Panel C) decreased the GHS (30 μM)-induced $[Ca^{2+}]_i$ increase in HEK-sbGHSR-1a cells, $^{**}P < 0.01$; $^{***}P < 0.001$.

Table 2
GHS-stimulated increases in [^{3}H]inositol phosphate production in HEK-sbGHSR-1a cells

GHS agonist ± antagonist	pEC_{50}	Hill coefficient	Basal activity (% conversion)	Maximum response (% conversion)
GHRP-6 alone	5.44 ± 0.06	1.47 ± 0.47	6.50 ± 1.29	69.72 ± 11.14
GHRP-6 + D-Lys(3)-GHRP-6	4.94 ± 0.07	1.27 ± 0.27	4.35 ± 1.98*	67.68 ± 7.09
L163,540 alone	6.78 ± 0.16	2.31 ± 0.61	5.29 ± 2.32	70.10 ± 11.60
L163,540 + D-Lys(3)-GHRP-6	6.29 ± 0.25	1.35 ± 0.35	3.35 ± 1.49*	68.54 ± 9.41

The peptide GHS agonist (GHRP-6) and the non-peptide GHS agonist (L163,540) were tested, either alone or after pre-treatment with 100 μM GHSR antagonist (D-Lys(3)-GHRP-6). Values are mean values ± SEM ($n = 6$). $^*P < 0.05$ vs basal activity. The basal activity of the parental HEK293 cells without the expressed receptor was 0.69 ± 0.05% conversion ($n = 5$).

inhibitor U73122 for 30 min inhibited GHRP-6 and L163,540-induced increases in $[Ca^{2+}]_i$ (Fig. 3B). To investigate the role of PKC in mediating the GHS-stimulated increases in $[Ca^{2+}]_i$, HEK-sbGHSR-1a cells were incubated for 24 h with PMA to downregulate the PKC activity. Calcium mobilization in response to GHRP-6 and L163,540 was significantly inhibited by PMA pre-treatment (Fig. 3C). However, the profile of the GHS-induced $[Ca^{2+}]_i$ elevation was not changed in the presence of either inhibitor (data not shown).

3.5. Activation of sbGHSR-1a does not lead to changes in [^{3}H]cAMP production

Neither GHRP-6 nor L13,540 produced any change in [^{3}H]cAMP production in HEK-sbGHSR-1a cells, indicating a lack of coupling of sbGHSR-1a to G_s proteins (Fig. 4). The adenylate cyclase activator forskolin increased [^{3}H]cAMP accumulation to 24.9 ± 3.0 fold basal. This increase in adenylate cyclase activity was neither affected by GHRP-6 nor L163,540, indicating a lack of coupling of sbGHSR-1a to G_i-proteins (Fig. 4).

3.6. Phosphorylation of extracellular signal-regulated kinases (ERK1/2) is a signaling event of GHS activation of HEK-sbGHSR-1a cells

GHRP-6 produced a 4-fold activation of ERK1/2 in HEK-sbGHSR-1a cells (Fig. 5). Pre-treatment of cells with the GHSR antagonist D-Lys(3)-GHRP-6 inhibited both basal and GHRP-6-stimulated ERK1/2 activity by about 40% and 55%, respectively. The MEK inhibitor U0126 abolished the GHRP-6-induced phosphorylation of ERK1/2 (Fig. 5).

4. Discussion

Receptor interaction was demonstrated in the two previously cloned GHSRs from the seabream pituitary, in which

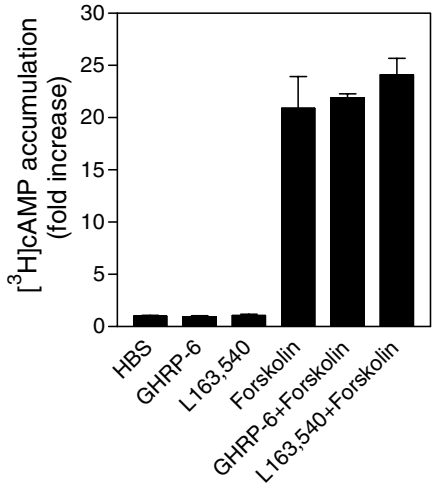

Fig. 4. Effect of HBS, forskolin (100 μM), GHRP-6 (30 μM) and L163,540 (30 μM) on [^{3}H]cAMP accumulation in HEK-sbGHSR-1a cells.

expression of sbGHSR-1b in HEK-sbGHSR-1a cells attenuated GHS induced $[Ca^{2+}]_i$ [22]. Because of this receptor interaction and because of the co-existence of both receptors in the seabream pituitary, the use of seabream pituitary cells is therefore not preferable for studying the signaling of sbGHSR-1a due to the complexities involved. Thus, the use of a recombinant expression system in transfected cultured cells, which has been demonstrated to be a powerful tool in a wide variety of species [25–28], provides a cleaner system to perform the signaling studies. The HEK293 cell was chosen because this cell line contains no endogenous GHSR-1a [22].

Stimulation by both peptide and non-peptide GHS elicited a log concentration-dependent increase in $[Ca^{2+}]_i$ in HEK-sbGHSR-1a cells, but the potency of these GHS was less than

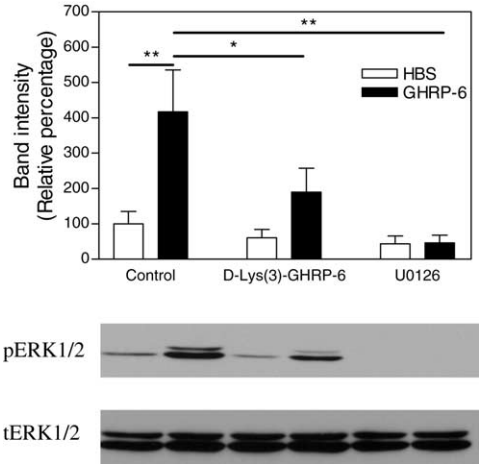

Fig. 5. Effect of GHRP-6 on ERK1/2 phosphorylation in HEK-sbGHSR1a cells. Both the GHSR antagonist D-Lys(3)-GHRP-6 (100 μM) and the MEK inhibitor U0126 (10 μM) significantly decreased the GHRP-6 (5 μM)-induced ERK1/2 phosphorylation. The Western blot shown underneath the graph is a representative result from three independent experiments (pERK1/2: phosphorylated ERK1/2; tERK1/2: total ERK1/2). $^*P < 0.05$; $^{**}P < 0.01$.

that shown in previous human GHSR-1a studies [8]. This is not surprising as the synthetic GHS were developed to act best on the human GHSR. The endogenous ligand for human GHSR-1a, ghrelin, was also demonstrated to be a weak stimulator of HEK-sbGHSR-1a cells, as detectable signals could only be recorded when 10 μM ghrelin (data not shown) was used. Attempts to quantify the receptor density of sbGHSR-1a in HEK-sbGHSR-1a cells using commercially available iodinated human ghrelin failed, confirming that human ghrelin has a very low affinity towards sbGHSR-1a. Further investigations on the activity of the natural ligand await identification of the endogenous seabream ghrelin.

Both the peptide and non-peptide GHS stimulated a biphasic elevation of $[Ca^{2+}]_i$ in HEK-sbGHSR-1a cells. The sbGHSR-1a thus appears to couple through the $G_{q/11}$-mediated pathway to activate PLC, resulting in increased IP_3 production and Ca^{2+} mobilization from intracellular stores (the initial transient elevation in $[Ca^{2+}]_i$) and from extracellular sources via L-type Ca^{2+} channels (the plateau phase of the elevated $[Ca^{2+}]_i$). Similar results have been observed in rat and human pituitary cells showing that the L-type Ca^{2+} channel was involved in GHSR-1a-induced Ca^{2+} mobilization [29,30]. This L-type Ca^{2+} channel is in fact found in a wide range of cell types and is particularly involved in endocrine cells for hormone secretion [31]. In teleosts, the activation of L-type Ca^{2+} channels was reported to be a pre-requisite for GH secretion from the somatotrophs [32,33]. It is thus postulated that the GHS-mediated GH secretion in seabream pituitary probably also involves activation of the L-type Ca^{2+} channels. On the other hand, the GHS-induced $[Ca^{2+}]_i$ response was slow in onset in rat skeletal muscle and was not blocked by nifedipine or the removal of external Ca^{2+} [34], suggesting that the L-type Ca^{2+} channels may not be essential for GHSR signaling in non-pituitary tissues.

In rat somatotrophs, PLC and PKC activation is essential for the GHRP-6 induced GH secretion [35]. GHS also increased phosphoinositide turnover, causing the translocation of PKC in rat and human pituitary cells [19,36]. In the present study, we have demonstrated that inhibition of either PLC or PKC in HEK-sbGHRS-1a cells attenuated GHS-stimulated increases in $[Ca^{2+}]_i$. Furthermore, activation of sbGHSR-1a appears to cause a PKC-dependent opening of L-type Ca^{2+} channels. If the Ca^{2+} channels were activated by Ca^{2+} released from intracellular stores, then inhibition of PKC would not decrease the GHS-stimulated increase in $[Ca^{2+}]_i$ as shown in Fig. 3C. Recently, Camina et al. [37] showed that ghrelin-induced calcium mobilization was unchanged after PKC inhibition by PMA treatment in HEK293 cells expressing human GHSR-1a. This result is very different from our observations shown in the present study as well as those reported by Bresson-Bepoldin and Dufy-Barbe [18] in rat pituitary. It is not known at present what accounts for this species difference in the role of PKC in GHS-induced calcium mobilization, and further investigations are warranted.

In ovine somatotrophs, GHRP-2 stimulation caused an accumulation of cAMP [38] and it has been suggested that adenylate cyclase-PKA is the major signal transduction pathway for GHS in the ovine pituitary [20]. A similar situation was reported in human acromegalic tumor cells in which GHRP-2 increased cAMP production [21]. However, it is uncertain whether the GHS-mediated cAMP accumulation in the above studies is GHSR-specific, since there are other receptor

types in the pituitary. The possibility that receptor subtypes other than GHSR-1a and GHSR-1b [39] are activated by GHS to trigger the observed cAMP accumulation cannot be ruled out. Studies on sbGHSR-1a signal transduction reported herein show no evidence for the activation or inhibition of adenylate cyclase activity, providing direct evidence, at least in seabream, that GHSR-1a does not affect adenylate cyclase. During the preparation of this manuscript, Carreira et al. [40] reported a similar finding that human GHSR-1a expressed in HEK293 cells did not trigger cAMP production when stimulated by ghrelin. In view of the fact that previously obtained results on the species differences in GHSR signaling have been conducted in primary cells from tissues, a revisit of the signaling mechanism by transfection of the receptor constructs in cultured cell lines is highly warranted.

Interestingly, the HEK-sbGHSR-1a cells display a relatively high level of basal [^{3}H]inositol phosphate production when compared with the parental HEK293 cells. This basal PLC activity could be significantly inhibited by the GHSR-1a antagonist D-Lys(3)-GHRP-6 (Table 2), suggesting that sbGHSR-1a can function in an agonist-independent manner and that D-Lys(3)-GHRP-6 is an inverse agonist. This high GHSR-1a basal activity found in seabream, together with the recent finding that the human GHSR-1a is also a constitutively active receptor [41], indicates that some aspects of GHSR have been conserved during evolution, in terms of the receptor–effector coupling systems.

Previous studies have demonstrated that receptors which stimulate PLC via coupling to $G_{q/11}$ proteins can activate extracellular signal-regulated kinases (ERK) [42,43]. Although studies on H9c2 cardiomyocytes showed that both ghrelin and des-ghrelin inhibited doxorubicin-induced apoptosis via ERK and Akt serine kinase, it has been suggested that the anti-apoptosis effect of ghrelin is mediated through a novel GHSR subtype rather than the classical GHSR-1a [44]. It is also not clear whether the ability of ghrelin to activate insulin receptor substrate-1, phosphatidylinositol 3-kinase and mitogen-activated protein kinase (MAPK) in hepatoma cells is GHSR-1a specific or not [45]. The effect of GHS on the phosphorylation of ERK1/2 in HEK-sbGHSR-1a cells was therefore studied. It is evident from our results that the GHRP-6-induced ERK1/2 phosphorylation reported herein is a GHSR-1a specific response, as the presence of the GHSR antagonist could significantly inhibit the ERK1/2 phosphorylation. These results suggest that sbGHSR-1a has a potential to trigger multiple signal transduction pathways of which one is the MAPK cascade. However, it remains unknown whether there is any cross-talk between the PLC-PKC pathway and the MAPK cascade. Moreover, the biological consequences of the ERK1/2 phosphorylation after stimulation of GHSR still remain to be elucidated.

In summary, sbGHSR-1a is a constitutively active receptor which triggers a PLC-PKC-dependent calcium mobilization in HEK293 cells when activated by either peptide or non-peptide GHS. This calcium mobilization also depends on the opening of L-type calcium channels activated by PKC. Seabream GHSR-1a activation also leads to ERK1/2 phosphorylation for which the biological significance remains to be elucidated.

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