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A POTASSIUM CURRENT EVOKED BY GROWTH HORMONE-RELEASING HORMONE IN FOLLICULAR OOCYTES OF *XENOPUS LAEVIS*

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SUMMARY

1. Electrophysiological properties of the growth hormone-releasing hormone (GRH) receptor were studied in *Xenopus* oocytes with an intact follicle cell layer (i.e. follicular oocytes) by measuring whole-cell current using the two-electrode voltage-clamp method.

2. A slow transient outward current was elicited in oocytes, clamped at −60 mV, by the application of rat GRH but not bovine, porcine, or human GRH.

3. The response to GRH was not suppressed by blockers known to inhibit other endogenous receptors present in follicular *Xenopus* oocytes; blockers used were timolol (2 μM; β-adrenergic blocker), theophylline (0·1 mM; purinergic blocker) and atropine (100 nM; muscarinic blocker).

4. The current response evoked by rat GRH occurred in a dose-dependent manner. The concentrations of GRH for threshold and maximum responses were 1 and 100 nM respectively and the estimated EC_{50} (half-maximal effective concentration) was approximately 7 nM. The amplitude and conductance of the response became larger and the latency, time-to-peak and half-decay time were shortened when the concentration of GRH was increased.

5. The GRH response was reversibly inhibited by a K^+ channel blocker, tetraethylammonium$^+$ (TEA^+; 20 mM). The reversal potential for the GRH response was around −100 mV and was compatible with the reported value for a K^+ current in *Xenopus* oocytes. Furthermore, a depolarizing shift of 40 mV in the reversal potential was observed when the external K^+ concentration was increased from 2 to 10 mM, agreeing with the Nernst equation. In contrast, no significant shift in the reversal potential was observed by changing the external concentration of Na^+ or Cl^-.

6. The GRH response was not suppressed in oocytes treated with an acetoxy-

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methyl ester of bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA/AM; 10 μM) which penetrates the cell membrane and chelates internal Ca^{2+}.

7. The GRH response was potentiated by pre-treatment with forskolin (0·4 μM; 5 min), which stimulates adenylate cyclase and increases the internal concentration of adenosine 3′,5′-cyclic monophosphate (cyclic AMP).

8. The GRH response was not obtainable when follicle cells surrounding oocytes were removed mechanically with forceps or enzymically with collagenase (i.e. denuded oocytes). The response was also suppressed when gap junctions, which electrically couple follicle cells and the oocyte, were blocked by 1-octanol (1 mM).

9. The first amino acid is considered to be important for the binding of peptide ligands to their receptors. The first amino acid of rat GRH is histidine while that of the other three GRHs is tyrosine. Human vasoactive intestinal peptide (VIP), whose first amino acid is histidine, however, did not activate the GRH receptor to evoke a K^+ current.

10. It is concluded that: (1) membrane currents evoked by GRH in *Xenopus* oocytes were exclusively dependent on the activation of K^+ channels, whose activity was not dependent on internal Ca^{2+}, but occurred via the cyclic AMP pathway, (2) there is species specificity in evoking current responses, suggesting a specific receptor for GRH, (3) GRH receptors are endogenous and present in follicle cells. The sequence of evolutionary change in the structure of GRH is discussed.

INTRODUCTION

Xenopus oocytes have been widely used to study the structure and mechanisms of function of ion channels and receptors for neurotransmitters and hormones. Oocytes not only possess native receptors but also can express foreign receptors when injected with exogenous mRNAs (for reviews see Soreq, 1985; Dascal, 1987; Snutch, 1988). Endogenous receptors found in *Xenopus* oocytes include those for acetylcholine (ACh) (Kusano, Miledi & Stinnakre, 1977, 1982), catecholamines (Kusano *et al.* 1977, 1982; Sumikawa, Parker & Miledi, 1984; Van Renterghem, Penit-Soria & Stinnakre, 1984), gonadotrophins (Woodward & Miledi, 1987*a*), adenosine (Lotan, Dascal, Cohen & Lass, 1982), angiotensin II (Lacy, McIntosh & McIntosh, 1989), cholecystokinin (CCK), corticotrophin-releasing factor (CRF), arginine vasopressin (AVP) (Moriarty, Gillo, Sealfon & Landau, 1988), vasoactive intestinal peptide (VIP) (Reale, Ashford & Barnard, 1987; Woodward & Miledi, 1987*b*), oxytocin and prostaglandins (Miledi & Woodward, 1989*a*). Endogenous receptors for the majority of these ligands (except for ACh and CCK) are considered to be present in follicle cells which surround oocytes. Macrovilli from follicle cells contact with microvilli of the oocyte via gap junctions, resulting in the direct electrical connection between these cells (Browne, Wiley & Dumont, 1979; Browne & Werner, 1984; Van der Hoef, Dictus, Hage & Bluemink, 1984). Receptors for ACh and CCK are present not only in follicle cells but in the oocyte membrane (Kusano *et al.* 1982; Moriarty *et al.* 1988). The functional significance of these endogenous receptors has not been elucidated, although receptors for gonadotrophins may play a role in regulating follicular development and maturation. Here we report a membrane current response elicited by growth hormone-releasing hormone (GRH) which showed a species specificity

among GRHs derived from different animals (rat, cow, pig and human) in follicular *Xenopus* oocytes.

METHODS

Preparation of follicular and denuded Xenopus oocytes

Mature females of *Xenopus laevis* were obtained from *Xenopus* Ltd (South Nutfield, UK), African *Xenopus* Facility (Noordhoek, South Africa) and Seibu Department Store (Tokyo, Japan). *Xenopus* oocytes (stages V and VI), whose diameters were larger than 1·0 mm (Dumont, 1972), were collected from the ovary of frogs anaesthetized by cooling with ice (Barish, 1983). Oocytes, connected with the covering connective tissue, were freed from each other with watchmakers' forceps. These isolated oocytes are surrounded by a monolayer of follicle cells and therefore called 'follicular oocytes'. When necessary, the follicle cell layer was carefully removed with watchmakers' forceps or by treatment with collagenase (2 mg/ml; Sigma Type I) in Ca^{2+}-free solution (ND96 solution; Boton, Dascal, Gillo & Lass, 1989) for 1·5–2·5 h. These oocytes without the follicle cell layer are called 'denuded oocytes'. Defolliculation was checked under a microscope. Gap junctions, which electrically connect the oocyte and the follicle cell layer (Browne *et al.* 1979; Browne & Werner, 1984; Van den Hoef *et al.* 1984), were blocked by 1 mM-1-octanol (Sigma) (Lacy *et al.* 1989). Oocytes were kept at 19 °C in modified Barth's solution (Barth & Barth, 1959) supplemented with penicillin (100 i.u./ml), streptomycin (100 μg/ml), 0·25 mM-sodium pyruvate and 2% ficoll (Sigma) until electrophysiological study which was performed up to 3 days after isolation of oocytes.

Electrophysiology

Whole-cell currents were measured from *Xenopus* oocytes using the conventional two-electrode voltage-clamp method as described in a previous paper (Yoshida, Plant, Taylor & Eidne, 1989). Glass microelectrodes were filled with 3 M-KCl and had DC resistances of 0·7–1·5 MΩ for current-passing electrodes and 1–3 MΩ for potential-measuring electrodes. Membrane potential and current signals from oocytes were processed by a digital audioprocessor (SONY PCM-701ES, Japan) modified to accept DC signals (Lamb, 1985) and stored on videotapes with a video cassette recorder (Panasonic NV-G12B, Japan). The stored data were displayed on a chart recorder (GRAPHTEC, Model SR 6335-2L) for illustrations. The reversal potential for current responses to GRH was estimated by changing the holding potential or by using the ramp method under voltage-clamp conditions. Ramp voltages of 0·8–1·0 s in duration and slopes of 80–120 mV/s were repetitively applied to oocytes and evoked currents were monitored. The total ionic current across the cell membrane, I_m, which is evoked by a ramp voltage during a GRH response, is described by the following equation:

$$I_m = I_K + I_l = g_K(V - E_K) + g_l(V - E_l), \quad (1)$$

where I_K is the current carried by K^+ which is responsible for the GRH response and I_l is the background leakage current carried by ions other than K^+ (Hodgkin & Huxley, 1952). Their ionic conductances are g_K and g_l, respectively. V and E designate the membrane potential and the reversal potential for a particular ion, respectively. The membrane current before the application of GRH is described by the leakage conductance only, i.e. $I_m = I_l = g_l(V - E_l)$. From eqn (1), when the membrane potential is equal to E_K, the membrane current I_m takes the constant value $g_l(E_K - E_l)$ regardless of the time-dependent change in g_K at E_K; the driving force $(V - E_K)$ for K^+ is zero. Indeed in the present study, a family of membrane currents, which were evoked by repetitive voltage ramps during a response to GRH, met at the same point corresponding to E_K.

In order to measure the total capacitance of oocytes, current pulses of 10–15 ms in duration were applied to the oocyte membrane under the current-clamp conditions. Since the duration of the current pulse was much shorter than the time constant of the oocyte membrane (longer than 100 ms), the membrane potential changed almost linearly during current pulses. The rate of change of the membrane potential (dV/dt) and the amplitude of the applied current were measured and the total capacitance value of oocytes was calculated using the equation (Yamashita, 1982):

$$Q = CV, \quad \text{i.e.} \quad \frac{dQ}{dt} = I = C\frac{dV}{dt} \quad \text{or} \quad C = \frac{I}{dV/dt}.$$

All experiments were carried out at room temperature (20–23 °C).

Solutions

The ionic composition of the standard solution used for electrophysiology was (in mM): 115 NaCl, 2 KCl, 1·8 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES (pH 7·4). The amount of Na^+ used for titrating HEPES to obtain pH 7·4 was measured and compensated for to make the total Na^+ concentration 115 mM. Growth hormone-releasing hormone (GRH) derived from four species (rat, bovine, human and porcine) was purchased from Sigma. Amino acid sequences of these GRHs are shown in Fig. 8. The membrane-impermeant anion methanesulphonate$^-$ (Aldrich) was used to substitute for Cl^-. Sodium (Na^+) was substituted by Tris$^+$ (Sigma) and the pH of the Na^+-free solution was adjusted at 7·4 with HCl. The tonicity of the solution was made equal to that of the standard solution with Tris by considering its dissociation, using the Henderson–Hasselbach equation. This Na^+-free (Tris$^+$) solution was mixed with standard solution to obtain the necessary amount of Na^+. Bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) is a pH-insensitive Ca^{2+} chelator and the membrane-permeant acetoxymethyl ester form (BAPTA/AM) (Calbiochem, San Diego, USA) was used to chelate internal Ca^{2+}. Other drugs were obtained from Sigma. Forskolin (adenylate cyclase activator, Calbiochem), 1-octanol and BAPTA/AM were firstly dissolved in dimethyl sulphoxide (DMSO, Sigma), and diluted to desired concentrations with the final concentration of 0·1 % (v/v) DMSO. DMSO at this concentration showed no effect on its own. The pH of all solutions used for electrophysiology was adjusted to 7·4. Solutions were introduced to and removed from the experimental chamber by perfusion.

Statistical tests

In the present study, the two-tailed Mann–Whitney *U* test, one of the non-parametric tests (or distribution-free statistics), was used for the statistical test for two independent groups, because this test needs only nominal or ordinal scale data and not normal distribution or homogeneity of variance which are required to use *t* tests; both normal distribution and homogeneity of variance are difficult to check in groups consisting of less than thirty data (Phillips, 1978).

RESULTS

Species specificity of responses to GRH

The resting potential of *Xenopus* oocytes is approximately −60 mV when measured with a single microelectrode (Yoshida *et al.* 1989). In the present study, therefore, the holding potential was set at −60 mV unless otherwise mentioned.

Figure 1 shows whole-cell current records upon the application of GRH (100 nM) of four different species (rat, bovine, porcine and human) in a single follicle-enclosed oocyte. Each application of GRH is indicated by a horizontal bar. Note that only rat GRH(1–29), among the four GRH types, was effective in inducing an outward current ($n = 5$). It is also to be noted that the outward current evoked by continuous exposure to rat GRH showed decay after reaching its peak. Other GRH types (bovine, porcine and human) tested in Fig. 1 were complete forms consisting of forty-four amino acids. In general, a complete form of a ligand may fail to evoke a response even if its fragments can induce a response if this response is induced non-specifically. The possibility that the response seen was due to a non-specific response to a short peptide sequence was ruled out when the complete form of rat GRH(1–43) became available and also produced a response from *Xenopus* oocytes ($n = 3$). In addition, a shorter form (1–29) of bovine GRH failed to evoke membrane current ($n = 4$) (not illustrated). This strict species specificity of the response to GRH in *Xenopus* oocytes can be ascribed to the prominent differences in the structures of rat GRH from that of bovine, porcine and human GRHs (see Discussion).

Effects of blockers known to inhibit other endogenous receptors in Xenopus oocytes

As mentioned in the Introduction, a number of ligands are reported to evoke current responses in follicular *Xenopus* oocytes. Most of them are transient outward currents similar to the GRH response shown in Fig. 1. Species specificity of the GRH

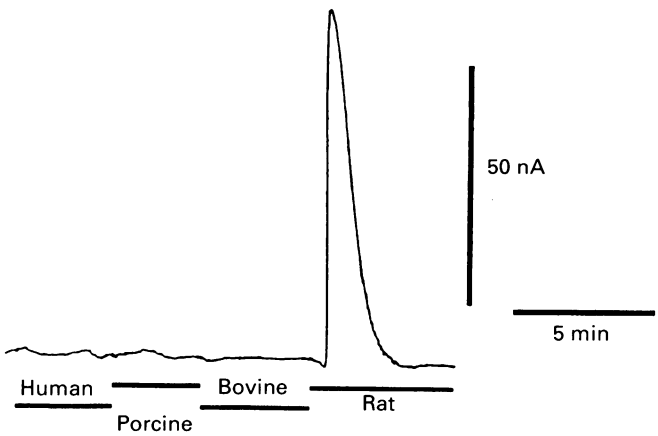

Fig. 1. A current response to growth hormone-releasing hormone (GRH) in a follicular oocyte of *Xenopus laevis*. Whole-cell currents were recorded from the oocyte using the two-electrode voltage-clamp method. The holding potential was −60 mV. GRH (100 nM) from different species (human, porcine, bovine and rat), indicated in the figure, were successively applied to the oocyte. Note that a transient outward current was evoked only by rat GRH. The application of GRH is indicated by horizontal bars under the current trace. Solutions including GRH were introduced to and drained from the experimental chamber by perfusion. The dead time of the perfusion system was less than 2 s in this and in all the following figures. Upward deflections from the baseline indicate outward currents in all figures.

response strongly suggests that the response is mediated by a specific receptor for GRH. In order to further confirm that the response to GRH was not due to the activation of other endogenous receptors, effects of blockers of known endogenous receptors were tested on the response to GRH. Blockers used were timolol (2 μM; β-adrenergic blocker), theophylline (0·1 mM; purinergic blocker) and atropine (100 nM; muscarinic blocker). These concentrations are sufficient to block the receptors in *Xenopus* oocytes (Kusano *et al.* 1982; Lotan *et al.* 1982). In the present study, the application of theophylline ($n = 4$), timolol ($n = 4$) or atropine ($n = 3$) could not exert a blocking action on responses evoked by rat GRH (not illustrated). These data indicate that the response to GRH occurred via a specific receptor for GRH and that rat GRH did not activate receptors other than the GRH receptor through cross-sensitization.

Dose dependence of the GRH response

As is the case with other endogenous responses from *Xenopus* oocytes, the sensitivity of GRH varied considerably from frog to frog. For instance, the amplitude of the response elicited by 100 nM-rat GRH ranged from 14 to 409 nA and the mean value from representative fifteen oocytes was 104·9 ± 99·6 nA (mean ± S.D.).

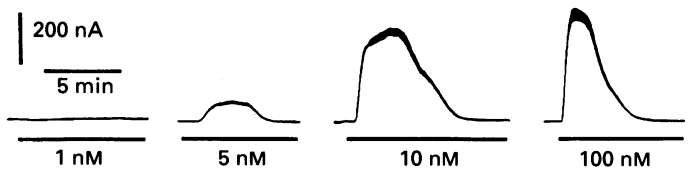

Fig. 2. Dose dependence of current responses elicited by rat GRH in a follicular *Xenopus* oocyte. Different concentrations of GRH were applied consecutively to the same oocyte. Each GRH application was followed by approximately 30 min wash before the next application of GRH. Small voltage pulses of constant amplitude (3 mV, 1 s duration, 0·5 Hz) were applied to the oocyte to monitor changes in the membrane conductance. A thicker line indicates a larger conductance. The application of GRH is indicated by bars. Current and time calibrations apply to all traces.

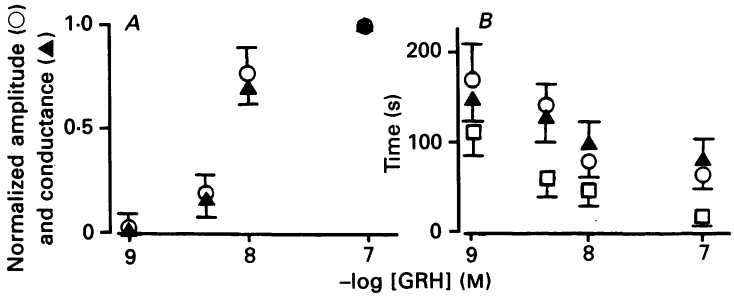

Fig. 3. Dose dependence of responses to GRH. *A*, normalized values of the peak amplitude (○) and the conductance (▲) caused by GRH. *B*, plots of latency (□), time-to-peak (○) and half-decay time (▲) (see text for definition of these parameters). Attached bars indicate S.D. ($n = 4$).

Responses to rat GRH occurred in a dose-dependent manner as shown in Fig. 2. Usually, no current response was evoked in *Xenopus* oocytes when the concentration of rat GRH was lower than 1 nM. The amplitude of the response showed a steep increase with increasing the concentration of GRH and it was saturated at 100 nM. The value of the peak amplitude at a given concentration of GRH was normalized by dividing it with that at 100 nM and its averaged values from four cells are plotted on the semilogarithmic scale (Fig. 3*A*, ○). A concomitant alteration in the membrane conductance was also monitored by applying constant voltage pulses. An increase in the membrane conductance is reflected in the width of the current trace. The GRH response was slow in time course and the change in the conductance occurred gradually and reached a maximum at the peak of the response followed by a decay in the continuous presence of GRH. The membrane conductance before (resting conductance) and during the application of GRH was calculated from the change in the membrane current. The maximum conductance of the GRH response was calculated by subtracting the resting conductance from the maximum total conductance during responses to GRH. For example, the membrane conductance before the application of 100 nM-GRH was 2·3 μS, and this was increased to 14·5 μS at the peak of the response (Fig. 2). Therefore, the peak K^+ conductance activated by GRH was 12·2 μS.

Normalized mean values obtained from four oocytes for the membrane conductance of responses are plotted against concentration of rat GRH (Fig. 3*A*, ▲). The concentrations of rat GRH which elicited threshold and maximum responses were 1 and 100 nM, respectively (although in some oocytes a small response of less than 10 nA was observed at 0·1 nM). An approximate EC_{50} (half-maximal effective concentration) estimated from the amplitude and conductance plots was 7 nM. The latency of the response became shorter when the concentration of GRH was increased (Fig. 3*B*, □). The time course of the GRH response became faster when the concentration of GRH was increased; the time-to-peak (Fig. 3*B*, ○) and the half-decay time (duration between the peak and its half-amplitude on the decay phase) (Fig. 3B, ▲) were shortened with increasing GRH concentration.

Ionic basis of the GRH response

The equilibrium potentials for K^+, Na^+ and Cl^- in *Xenopus* oocytes are approximately −100, +80 and −25 mV, respectively (Dascal, 1987). Currents elicited by rat GRH were outward at the holding potential of −60 mV, suggesting that the GRH response was dependent on K^+. Experiments were therefore carried out to confirm that the GRH response was dependent on K^+.

Tetraethylammonium$^+$ (TEA^+) has been widely used as a K^+ channel blocker in a variety of cells including *Xenopus* oocytes (Lotan *et al.* 1982; Woodward & Miledi, 1987*a*). In the present study, no significant outward current was evoked by rat GRH in the presence of 20 mM-TEA^+ (not shown). This blocking effect of TEA^+ was better demonstrated when TEA^+ was introduced during the GRH response (Fig. 4). Figure 4*A* shows a control response elicited by the application of 100 nM-rat GRH (horizontal bar). On the rising phase of a response to 100 nM-GRH, i.e. before the outward current reached it peak, 20 mM-TEA-Cl was added to the GRH solution (shown by the dashed line) (Fig. 4*B*). The outward current rapidly returned to its original level, i.e. the GRH response was aborted by TEA^+. Recovery of the response to GRH was observed when TEA^+ was washed out with standard solution for 30 min (Fig. 4*C*). Recovered GRH responses were usually slightly smaller in amplitude than control responses. It is concluded that TEA^+ reversibly inhibits the GRH response ($n = 5$).

Further evidence that the GRH response is dependent on K^+ was obtained by measuring its reversal potential. In the present study, the reversal potential was estimated by two methods. The first method involved changing the holding potential to various values, in a step-wise manner, during the GRH response and estimating the reversal potential from the point where the current becomes zero (Kusano *et al.* 1982; Boton *et al.* 1989; Yoshida *et al.* 1989). This method was possible because the GRH response was slow in time course. The average reversal potential for the GRH response obtained by this method, in standard solution (2 mM-external K^+), was $-99{\cdot}7 \pm 3{\cdot}5$ mV ($n = 3$). The E_{rev} determined by the ramp technique (see Methods) was $-101{\cdot}3 \pm 3{\cdot}6$ mV (2 mM-external K^+; $n = 4$), not statistically different from that calculated by the stepping method.

Dependence of the GRH response on K^+ was further ensured by changing the external concentration of K^+. The bathing medium was switched from normal 2 mM to 10 mM in the same oocyte, and a family of membrane currents was obtained by

the ramp method during the GRH response (not illustrated). The average reversal potential for the GRH response in high-K^+ (10 mM) solution was $-61{\cdot}3 \pm 2{\cdot}9$ mV ($n = 4$) giving a depolarizing shift in the reversal potential of 40·0 mV. This is in good agreement with the expected shift of 40·7 mV predicted by the Nernst equation

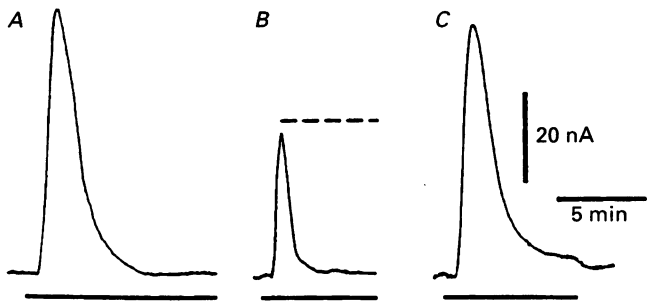

Fig. 4. Blocking effect of tetramethylammonium$^+$ (TEA^+) on a response to rat GRH. *A*, a control response to 100 nM-rat GRH (bar). *B*, a transient outward current was evoked by 100 nM-rat GRH (bar), and it was aborted before reaching the peak by the introduction of 20 mM-TEA^+ (dashed line) in the continuous presence of 100 nM-GRH. *C*, recovery of the response to 100 nM-rat GRH (bar) after the same oocyte had been washed with standard solution for 30 min. Current and time calibrations are the same for all records.

(22 °C). No significant shift was observed when the concentration of other ions was changed. For example, when the external concentration of Na^+ was decreased to one-fifth of that of the normal concentration, by replacement with $Tris^+$, the obtained reversal potential for the GRH response was $-101{\cdot}3 \pm 1{\cdot}7$ mV ($n = 3$). The reversal potential for responses to GRH was $-100{\cdot}7 \pm 3{\cdot}2$ mV ($n = 3$), when the external concentration of Cl^- was lowered to one-third of its standard concentration, by substitution with membrane-impermeant methanesulphonate$^-$ (Sharp & Thomas, 1981).

Mechanism of the GRH response

It is known that Ca^{2+}-activated K^+ channels are widely distributed in various kinds of cells (Latorre, Coronado & Vergara, 1984; Petersen & Maruyama, 1984; Rudy, 1988). In order to check whether Ca^{2+}-activated K^+ channels were activated during the GRH response, oocytes were pre-treated with an acetoxymethyl ester of bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA/AM; 10 μM) for 2–3 h and then challenged with rat GRH. When BAPTA/AM is applied externally, it penetrates the cell membrane and is cleaved by cytoplasmic esterases to yield free BAPTA which remains trapped in the cell and chelates internal Ca^{2+}. This pre-treatment with BAPTA/AM was sufficient to block an increase in the cytoplasmic Ca^{2+}, because ionomycin, a Ca^{2+} ionophore, failed to evoke a current in BAPTA/AM-pre-treated *Xenopus* oocytes. In oocytes which were not treated with BAPTA/AM, ionomycin evoked an inward current via the activation of Ca^{2+}-activated Cl^- channels by increasing the internal concentration of Ca^{2+} (S. Yoshida & S. Plant,

unpublished observation). In the present study, pre-treatment of oocytes with 10 μM-BAPTA/AM for 2–3 h did not obviously inhibit transient outward current responses to GRH. The average peak amplitude of responses to 100 nM-rat GRH(1–29) was 49·8 ± 18·7 nA (n = 4). This mean value is not significantly different

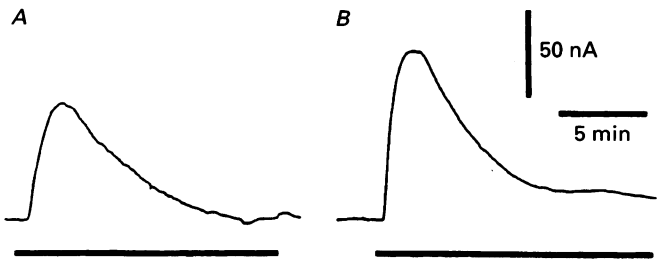

Fig. 5. Facilitatory effect of forskolin on the GRH response. *A*, a response to 100 nM-rat GRH obtained from a *Xenopus* oocyte. *B*, the same oocyte shown in *A* was washed with standard solution for 30 min, pre-treated with 0·4 μM-forskolin for 5 min and was challenged by 100 nM-rat GRH in the continuous presence of forskolin. The application of GRH is indicated by the bars.

from that obtained from the control oocytes mentioned above (104·9 ± 99·6 nA; n = 15). It is concluded that the K^+ channel activated during GRH responses was not dependent on the elevation of the intracellular concentration of Ca^{2+}.

It has been shown that a rise in intracellular Ca^{2+} does not play a predominant role in activating the K^+ channel for the GRH response. Another major pathway known in *Xenopus* oocytes is the adenosine 3′,5′-cyclic monophosphate pathway (cyclic AMP pathway). Ion channels have been shown to be activated by the injection of cyclic AMP into *Xenopus* oocytes (Dascal, Lotan, Gillo, Lester & Lass, 1985; Miledi & Woodward, 1989*b*). In the present study, the internal concentration of cyclic AMP was increased by activating adenylate cyclase by the external application of forskolin. Five minute pre-treatment of oocytes with 0·4 μM-forskolin increased the response elicited by 100 nM-GRH 1·56 ± 0·35 times (n = 3). An example of the effect of forskolin is shown in Fig. 5.

Interaction between GRH and VIP responses

The first amino acid of rat GRH is histidine whereas it is tyrosine for bovine, porcine and human GRHs (see Fig. 8). Since the first amino acid is thought to be important for GRH molecules to bind the GRH receptor (Frohman, Downs, Chomczynski & Frohman, 1989), it is possible that some other peptides whose first amino acid is histidine may activate the GRH receptor or GRH molecules may activate some other receptors whose ligands possess histidine as the first amino acid. Histidine is the first amino acid in several bioactive peptides such as vasoactive intestinal peptide (VIP), glucagon, secretin and porcine intestinal peptide (PHI). VIP is known to evoke a transient K^+ current in follicular *Xenopus* oocytes (Reale *et al.* 1987; Woodward & Miledi, 1987*b*). Therefore human VIP was used in the

present study in order to examine an interaction between GRH and VIP. VIP (human or porcine) consists of the following twenty-eight amino acids: **His**-Ser-**Asp**-**Ala**-Val-**Phe**-**Thr**-Asp-Asn-**Tyr**-Thr-**Arg**-Leu-Arg-Lys-**Gln**-Met-Ala-Val-Lys-**Lys**-Tyr-**Leu**-Asn-Ser-**Ile**-Leu-Asn (the amino acids shared with rat GRH are shown in bold face; 26% homology).

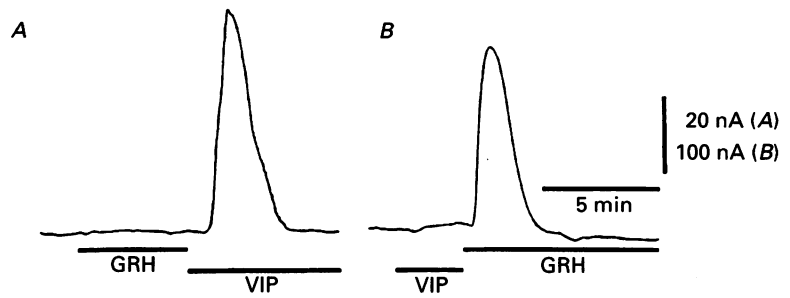

Fig. 6. Effects of human vasoactive intestinal peptide (VIP) and rat GRH in follicular *Xenopus* oocytes. *A*, a transient outward current response to 1 μM-VIP from an oocyte which was not responsive to GRH (100 nM). *B*, records from a different oocyte which responded to 100 nM-rat GRH but did not show a response to VIP (100 nM).

As shown in Fig. 6*A* follicular oocytes which did not show a response to GRH (100 nM) did show a transient K^+ current in response to 1 μM-VIP ($n = 2$). Also, as shown in Fig. 6*B*, some oocytes which did not show a response in VIP (100 nM) responded to the same concentration of rat GRH ($n = 3$). The oocyte in Fig. 6*B* did not respond to a higher concentration of VIP (1 μM) either. These results indicate that VIP and GRH act on distinct receptors.

Abolition of the GRH response by removing the connection between follicle cells and the oocyte membrane

All responses to rat GRH shown above were obtained from follicular *Xenopus* oocytes. In contrast, no responses could be observed in oocytes whose follicle cell layer was removed mechanically with watchmakers' forceps or enzymically with collagenase (see Methods). A representative example is shown in Fig. 7.

A control response to 100 nM-rat GRH(1–29) is illustrated in Fig. 7*A*. When the follicle cell layer, consisting of a monolayer of follicle cells, was removed mechanically with forceps, no change occurred in the membrane current following the application of 100 nM-rat GRH ($n = 4$) (Fig. 7*B*). Similarly, no current responses to 100 nM-rat GRH were observed in oocytes whose follicle cells were removed with collagenase ($n = 6$; not illustrated). Removal of follicle cells from the oocyte membrane was checked by measuring the total membrane capacitance of follicular and mechanically or enzymically denuded oocytes. The average total capacitance value was $469{\cdot}1 \pm 66{\cdot}4$ nF ($n = 20$) for control follicular oocytes (1·2–1·3 mm in diameter) while the value was $245{\cdot}8 \pm 31{\cdot}3$ nF for denuded oocytes ($n = 10$). The decrease in the total membrane capacitance by removing follicle cells indicates that the follicle cell layer was electrically connected to the oocyte membrane. Follicle cells are known to be

connected to the oocyte membrane via gap junctions (Browne *et al.* 1979; Browne & Werner, 1984; Van den Hoef *et al.* 1984). Another method to remove the connection between follicle cells and the oocyte membrane was to treat follicular oocytes with a gap junction blocker, 1-octanol (Lacy *et al.* 1989; Sandberg, Bor, Hong, Marwick,

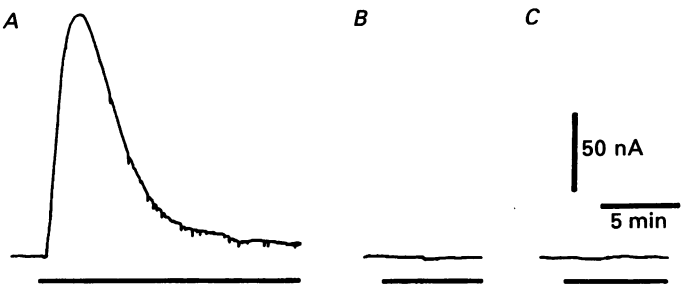

Fig. 7. Effect of removing the connection between the oocyte membrane and the follicle cell layer on the GRH response. *A*, control. An outward current was evoked by 100 nM-rat GRH in a *Xenopus* oocyte with the intact follicle cell layer. Introduction of 100 nM-rat GRH into the experimental chamber is indicated by bars in all records. *B*, an oocyte whose follicle cell layer was mechanically removed with forceps. *C*, an oocyte whose gap junctions, which electrically connect the oocyte and the follicle cell layer, were blocked by the pre-treatment with 1 mM-1-octanol.

Millan & Catt, 1990). The total capacitance value obtained from follicular oocytes which were treated with 1 mM-1-octanol for 10–30 min was 303·8 ± 27·6 nF ($n = 4$). Octanol did not affect the oocyte membrane non-specifically; for example, the resting potential, a good indication of oocyte condition, was not affected. Also, octanol itself did not show any effect on its own when it was applied to oocytes by perfusion. Oocytes were pre-treated with 1 mM-1-octanol and were exposed to 100 nM-rat GRH(1–29) together with 1 mM-1-octanol. No significant change in the membrane current was observed from three oocytes as shown in Fig. 7*C*. A small outward current of 11·5 nA in amplitude was observed from a fourth oocyte. In fact, the average total capacitance value of octanol-treated oocytes was larger than that of mechanically or enzymically denuded oocytes, suggesting that the blocking of gap junctions by octanol was not always complete. These data obtained from denuded and octanol-treated oocytes indicate that surrounding follicle cells play a major role in the response to GRH.

DISCUSSION

Mechanism of the response to growth hormone-releasing hormone in Xenopus oocytes

In the present study, an outward current response was evoked by growth hormone-releasing hormone (GRH) in *Xenopus* oocytes whose follicle cell layer was intact (follicular oocytes). This response to GRH occurred in a dose-dependent manner (Figs 2 and 3). The response to GRH was exclusively dependent on K^+, because it was inhibited by tetraethylammonium$^+$ (TEA^+) and when the external concentration of K^+ was increased a depolarizing shift in the reversal potential for the response was in good agreement with that expected from the Nernst equation.

Altering the external concentration of Na^+ or Cl^- did not induce any significant change in the reversal potential for the GRH response. It has been reported that the application of GRH evoked rhythmic outward currents associated with an increased conductance to K^+ in rat anterior pituitary cells (Nussinovitch, 1988).

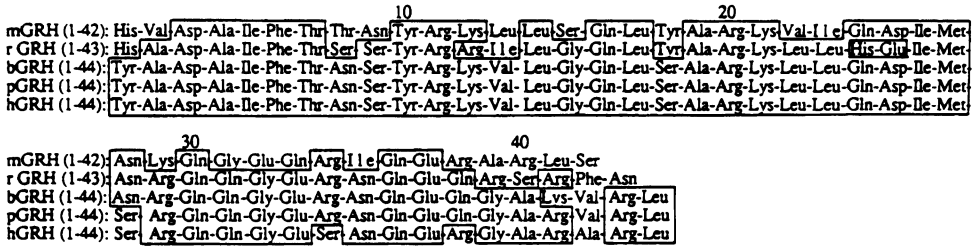

Fig. 8. Amino acid sequences of GRHs derived from four different species (rat, cow, pig and human) used in the present experiment. Mouse GRH is also shown for reference. Amino acids shared by more than two species of GRHs are boxed. Amino acids are numbered for convenience.

It is apparent that the chelation of intracellular Ca^{2+} by BAPTA does not inhibit the response to GRH and therefore the response is not dependent on the release of Ca^{2+} from intracellular stores. In this respect the results are similar to those found for the response to adenosine in *Xenopus* oocytes. The injection of Ca^{2+} has been shown to have no effect on the response to adenosine (Dascal *et al.* 1985) and the injection of a Ca^{2+} chelator EGTA did not decrease the response (Stinnakre & Van Renterghem, 1986).

In *Xenopus* oocytes, it has been suggested that pre-treatment with the adenylate cyclase activator forskolin or with the cyclic AMP phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) causes an increase in intracellular cyclic AMP, probably though activation of cyclic AMP-dependent protein kinases resulting in the activation of K^+ channels, i.e. cyclic AMP-dependent K^+ channels (Van Renterghem, Pénit-Soria & Stinnakre, 1984; Van Renterghem *et al.* 1985; Lotan, Dascal, Oron, Cohen & Lass, 1985; Stinnakre & Van Renterghem, 1986; Smith, Brooker & Brooker, 1987; Woodward & Miledi, 1987*a*, *b*; Moriarty *et al.* 1988). The GRH response was augmented in amplitude when follicular oocytes were pre-treated with forskolin (Fig. 7), indicating that the K^+ channel is cyclic AMP dependent. It is known that the cyclic AMP system is involved in the secretory process of growth hormone (GH) in response to GRH (Brazeau, Ling, Esch, Bohlen, Maugin & Guillemin, 1982; Bilezikjian & Vale, 1983; Labrie, Gagne & Lefevre, 1983; Schettinni, Cronin, Hewlett, Thorner & Macleod, 1984). The involvement of the cyclic AMP pathway in the GRH response is also known in cultured ovine and bovine pituitary cells (Law, Ray & Wallis, 1984; Hart, Ray & Wallis, 1988). These cells respond to GRH in a dose-dependent manner (1 pM–100 nM) to produce a rise in GH release and also in the intracellular level of cyclic AMP (Law *et al.* 1984; Hart *et al.* 1988). A role for cyclic AMP is also supported by the evidence showing that phosphodiesterase inhibitors, cyclic AMP derivatives and activators of adenylate cyclase can cause stimulation of the GH release (Brazeau *et al.* 1982).

Species specificity observed in the GRH response

In the present study, follicular *Xenopus* oocytes responded only to rat GRH, both the fragment (1–29) and the complete form (1–43), but not to porcine, bovine or human GRH.

The amino acid sequences of these four different species of GRH are shown in Fig. 8 and amino acids common to more than two GRHs are boxed (Guillemin, Brazeau, Bohlen, Esch, Ling & Wehrenberg, 1982; Gubler, Monahan, Lomedico, Bhatt, Collier, Hoffman, Bohlen, Esch, Ling, Zeytin, Brazeau, Poonian & Gage, 1983; Spiess, Rivier & Vale, 1983; McCutcheon, Bauman, Murphy, Lance & Coy, 1984; Mayo, Cerelli, Lebo, Bruce, Rosenfeld & Evans, 1985*a*; Mayo, Cerelli, Rosenfeld & Evans, 1985*b*; Frohman *et al.* 1989). Note that these four species of GRH are similar in structure. The overall homology among these GRH types is 65%. When rat GRH is omitted, bovine, porcine and human GRHs show homology as high as 89%. The homology of rat GRH with bovine, porcine and human GRHs is 72, 72, and 67%, respectively. Such a homology is enough for human GRH to evoke electrophysiological responses in rat pituitary cells (Chen, Israel & Vincent, 1989). Considering that human GRH is closer to porcine GRH (93% homology) than bovine GRH (89% homology), we propose a possible evolutionary sequence of change which occurred in the structure of GRH of frog < rat < cow < pig < human. Recently, the complete structure of mouse GRH consisting of forty-two amino acids was deduced from its cloned DNA (cDNA; Frohman *et al.* 1989). It is interesting that the amino acid sequence of mouse GRH shows quite a low homology (51%) with that of rat GRH even though they are both rodents. It is considered that the first amino acid is important for GRH molecules to bind to the GRH receptor (Frohman *et al.* 1989). The first amino acid of mouse GRH, like rat GRH, is histidine and it seems likely that mouse GRH would evoke a response in follicular *Xenopus* oocytes. Unfortunately, however, mouse GRH is not available at the moment. As for the homology, the values of mouse GRH with bovine, porcine and human GRHs are 52, 50 and 50%, respectively. These values are lower than those obtained for rat GRH. Thus, it is suggested that the sequence of the evolutionary change in the structure of GRHs occurred in the order: frog < mouse < rat < cow < pig < human.

Possible location of the GRH receptor

In the present study, the GRH response disappeared when the follicle cell layer was removed mechanically with forceps or enzymically with collagenase (Fig. 7*B*), as has been observed for other endogenous receptors (Kusano *et al.* 1982; Van Renterghem *et al.* 1985; Miledi & Woodward, 1989*b*). Furthermore, the GRH response was suppressed by treatment with a blocker of gap junctions, 1-octanol (Fig. 7*C*), as was the case for endogenous receptors for angiotensin II, adenosine and adrenaline in follicular *Xenopus* oocytes (Lacy *et al.* 1989; Sandberg *et al.* 1990). Responses in follicle cells can be recorded from oocytes because macrovilli of follicle cells contact with microvilli of the oocyte via gap junctions, resulting in direct electrical connection (Browne *et al.* 1979; Browne & Werner, 1984; Van den Hoef *et al.* 1984). Gap junctions are known to be permeable to small ions and molecules including cyclic AMP (Pitts & Sims, 1977; Lawrence, Bees & Gilula, 1978), inositol

trisphosphate and Ca^{2+} (Saez, Connor, Spray & Bennett, 1988). Many reported endogenous receptors in *Xenopus* oocytes are thought to be in follicle cells activating cyclic AMP-dependent K^+ channels (Van Renterghem *et al.* 1984, 1985; Lotan *et al.* 1985; Stinnakre & Van Renterghem, 1986; Smith *et al.* 1987; Woodward & Miledi, 1987*a*, *b*; Moriarty *et al.* 1988). In contrast, CCK and ACh act through the phosphatidylinositol pathway (PI pathway) and are considered to be present in the oocyte membrane (Kusano *et al.* 1982; Moriarty *et al.* 1988). The present data suggest that GRH receptors are predominantly present in follicle cells and activate cyclic AMP-dependent K^+ channels.

Functional significance of GRH receptors in follicle cells

It is possible, if GRH receptors are indeed in follicle cells surrounding the oocyte, that follicle cells play a role in controlling the development of oocytes by secreting GH to oocytes. A further possibility for the role of GRH and the other endogenous receptors which cause a rise in the cyclic AMP level of the oocyte (via gap junctions) has been put forward by Moriarty *et al.* (1988). They suggested that the resulting rise in cyclic AMP, which is known to maintain the *Xenopus* oocyte arrested in the first meiotic prophase (Maller & Krebs, 1980), may regulate this arrest. Further study is required to determine the function of GRH receptors in development of the *Xenopus* oocyte.

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