Glucagon-like peptide-1 receptor expression in *Xenopus* oocytes stimulates inositol trisphosphate-dependent intracellular Ca^{2+} mobilization

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Received 18 February 1998

Abstract The signal transduction pathway of the cloned human glucagon-like peptide-1 (GLP-1) receptor was studied in voltage-clamped *Xenopus* oocytes. Binding of GLP-1(7–36)amide was associated with cAMP production, increased $[Ca^{2+}]_i$ and activation of Ca^{2+}-dependent Cl^- current. The effect of GLP-1(7–36)amide reflects intracellular Ca^{2+} mobilization and was suppressed by injection of the Ca^{2+} chelator BAPTA and the inositol trisphosphate receptor antagonist heparin. The responses were not mimicked by the adenylate cyclase activator forskolin and unaffected by the protein kinase A (PKA) inhibitor Rp-cAMPS. We conclude that GLP-1 receptor expression in *Xenopus* oocytes evokes inositol trisphosphate-dependent intracellular Ca^{2+} mobilization independent of the cAMP/PKA signaling pathway.

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Key words: Glucagon-like peptide-1; Calcium; Inositol trisphosphate; *Xenopus* oocyte; Cyclic adenosine monophosphate

1. Introduction

The glucagon-like peptide-1 (GLP-1) receptor belongs to the glucagon/secretin receptor subfamily of seven-transmembrane G protein-coupled receptors [1]. The GLP-1 receptor couples positively to the adenylate cyclase system and in some cells stimulates an increase in $[Ca^{2+}]_i$. The mechanism of how GLP-1 promotes a rise in $[Ca^{2+}]_i$ is not well understood. In one study it was demonstrated that the GLP-1 receptor following expression in monkey kidney COS cells couples to both the phospholipase C (PLC) and adenylate cyclase signaling systems and that the rise in $[Ca^{2+}]_i$ results from inositol trisphosphate production [2]. In other cell types, GLP-1 receptor expression is either not associated with a rise in the cytoplasmic Ca^{2+} concentration or elevations in $[Ca^{2+}]_i$ are secondary to cAMP-dependent activation of protein kinase A (PKA). For example, stimulation of the recombinant GLP-1 receptor in the human-derived cell line HEK 293 produces a rise in $[Ca^{2+}]_i$ by promoting Ca^{2+}-induced Ca^{2+} release following sensitization of ryanodine receptors by a PKA-dependent mechanism [3]. The pancreatic B-cell is a target cell for GLP-1. Here, GLP-1 promotes PKA-dependent Ca^{2+} mobilization from intracellular stores through Ca^{2+}-induced Ca^{2+} release and Ca^{2+}-stimulated inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) production [4–6].

Xenopus oocytes are widely used to explore the function of recombinant ion channels and seven-transmembrane spanning receptors and are believed to represent a good and convenient model system. We have used *Xenopus* oocytes to gain insight into the cellular mechanism underlying the GLP-1-induced rise in $[Ca^{2+}]_i$. We demonstrate for the first time that activation of the cloned human GLP-1 receptor expressed in *Xenopus* oocytes induces $Ins(1,4,5)P_3$-dependent intracellular Ca^{2+} mobilization and activation of Ca^{2+}-dependent Cl^- current which is *not* mediated by cAMP-induced stimulation of PKA.

2. Materials and methods

2.1. RNA preparation

The human GLP-1 receptor cDNA was obtained from Dr. B. Thorens (Department of Pharmacology and Toxicology, University of Lausanne, Switzerland). For preparation of RNA, the GLP-1 receptor cDNA was linearized and cRNA was synthesized by means of a cRNA Capping kit (Stratagene, La Jolla, CA, USA). The cRNA transcript was extracted twice with phenol/chloroform (1:1) and chloroform/isoamyl alcohol (24:1) and purified by precipitation with ammonium acetate and ethanol. cRNA was washed twice with 70% ethanol, dried, and dissolved in diethyl pyrocarbonate-treated water (final concentration of cRNA was estimated by agarose gel electrophoresis and ethidium bromide staining) and stored at −80°C.

2.2. Electrophysiology

Mature female *Xenopus laevis* obtained from Xenopus I (Ann Arbor, MI, USA) and Xenopus Ltd (Redhill, Surrey, UK) were anesthetized using 0.15% 3-aminobenzoic acid ethyl ester (MS-222) and 3–5 ovarian lobes were surgically removed. Oocytes at developmental stages V–VI were dissected from the ovary and injected with 25 ng (50 nl) cRNA and incubated at 17°C in Barth's medium containing (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.3 $Ca(NO_3)_2$, 0.4 $CaCl_2$, 0.3 $MgSO_4$ and 15 HEPES (pH 7.4 with NaOH) and supplemented with 0.1 mg/ml gentamicin. Two days after injection the oocytes were treated with 0.5 mg/ml collagenase for 20–30 min and the follicle cell layer was removed mechanically with a pair of fine forceps. Recordings of membrane currents were performed under a two-electrode voltage clamp with a holding potential of −60 mV controlled by a Turbo TEC O1C amplifier (npi electronic GmbH, Tamm, Germany) using oocytes incubated for 4–7 days following injection. Current and potential electrodes were filled with 3 M KCl and the pipette resistance was 0.5–1 MΩ. The zero-current potential was adjusted before impaling the electrodes into the oocyte. The oocytes were placed in a small plexiglass chamber and continuously superfused (3 ml/min) with frog Ringer containing (in mM) 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$ and 10 HEPES (pH 7.2 with NaOH) at room temperature (22°C). Drugs were applied through the bath solution and the time required for the test compound to reach the oocyte was < 5 s. Intraoocyte injections were made 30–60 min prior to recordings and injection solutions

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; GLP-1, glucagon-like peptide-1; GLP-1(7–36)amide, glucagon-like peptide-1(7–36)amide; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; PKA, protein kinase A; PACAP, pituitary adenylate cyclase-activating polypeptide; PLC, phospholipase C; VIP, vasoactive intestinal peptide

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PII S0014-5793(98)00254-3

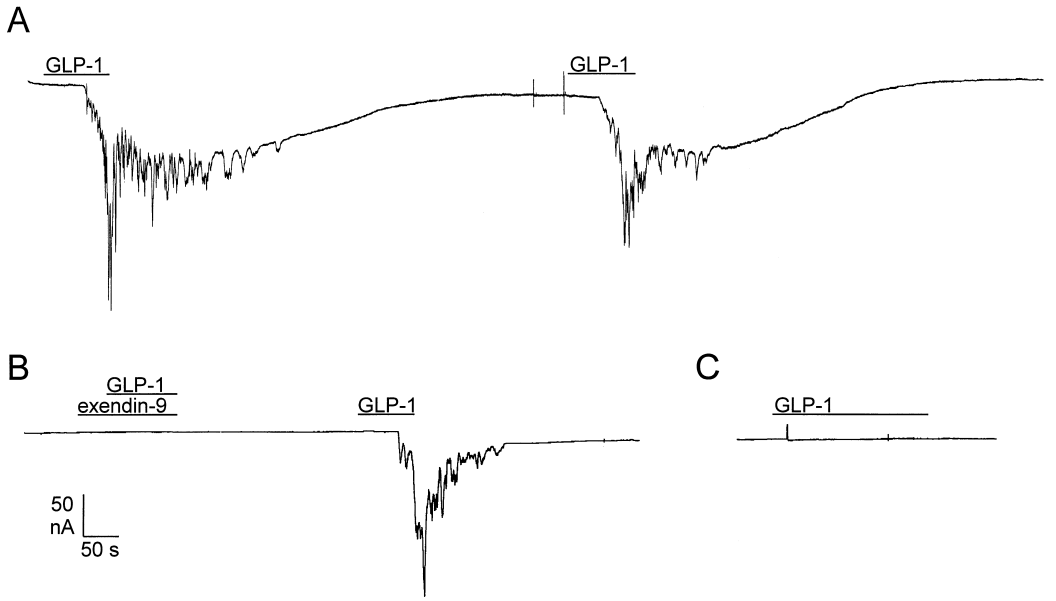

Fig. 1. Cloned GLP-1 receptor activates inward Cl^- current in *Xenopus* oocytes. A: Effect of two GLP-1(7–36)amide (50 nM) applications on Cl^- current in a *Xenopus* oocyte expressing the cloned human GLP-1 receptor. B: Exendin-(9–39) (10 μM) blocks GLP-1(7–36)amide (50 nM)-induced Cl^- current but not a second GLP-1(7–36)amide application 4 min following wash-out of the antagonist from the bath. C: No effect of GLP-1(7–36)amide (50 nM) in non-injected oocytes. In this and the following figures the oocytes were exposed to the compounds during the time indicated by the horizontal lines. The traces are representative of 4–11 experiments.

of BAPTA (100 mM), Rp-cAMPS (2 mM), and heparin (4 mg/ml) were made up in 5 mM HEPES (pH 7.0 KOH). The PKA inhibitor Rp-cAMPS was obtained from BIOLOG Life Science Institute (Bremen, Germany). GLP-1(7–36)amide was synthesized in-house (Novo Nordisk A/S, Bagsvaerd, Denmark). The correctness of structure and purity for GLP-1(7–36)amide was estimated by mass, sequence and HPLC analyses. The GLP-1 receptor antagonist exendin-(9–39) was from Bachem Feinchemikalien (Bubendorf, Switzerland). Ryanodine was from Alomone Labs (Jerusalem, Israel). All other chemicals were purchased from Sigma.

2.3. Measurements of cAMP levels

Intracellular cAMP levels were measured in groups of five oocytes. The oocytes were first incubated in 50 μl frog Ringer for 30 min in the presence of 1 mM IBMX and subsequently stimulated with 50 nM GLP-1(7–36)amide or 10 μM forskolin for 15 min in the continued presence of IBMX. The reaction was terminated by the addition of HCl to the incubation medium (final concentration: 50 mM). The samples were neutralized with NaOH and total cAMP was measured using a cAMP [^{125}I] scintillation proximity assay following the acetylation protocol (RPA 542; Amersham, UK).

2.4. Ca^{2+} measurements

Changes in $[Ca^{2+}]_i$ were estimated by measurements of Fluo-3 fluorescence intensity using an inverted microscope (Zeiss Axiovert 135 TV, Oberkochen, Germany) equipped with a 40× Achrostigmat objective (1.30 numeric aperture, Zeiss) and an IonOptix fluorescence imaging system (IonOptix, Milton, MA, USA). Oocytes were used 1–2 h after injection of Fluo-3 potassium salt (Molecular Probes, Eugene, OR, USA) at a final concentration of 10–20 μM.

2.5. Data analysis

Results are presented as mean values ± S.E.M. for indicated numbers of experiments. Statistical significance was evaluated using Student's *t*-test for unpaired observations.

3. Results and discussion

3.1. Expression of GLP-1 receptor in Xenopus oocytes

Fig. 1A shows that GLP-1(7–36)amide (50 nM) produces an oscillatory inward Cl^- current in *Xenopus* oocytes injected with cRNA for the human GLP-1 receptor. On average, GLP-1(7–36)amide evoked a current response of 162 ± 10 nA following a latency of 65 ± 3 s ($n = 54$ from eight different frogs). Application of 10 nM GLP-1(7–36)amide evoked smaller current responses of 20–30 nA in amplitude and 50 nM of the peptide was therefore used throughout this study. Desensitization of the GLP-1 receptor is not evident and a second application of GLP-1(7–36)amide evoked a current of about the same amplitude as during the first exposure (Fig. 1A). The average responses from a series of four experiments were

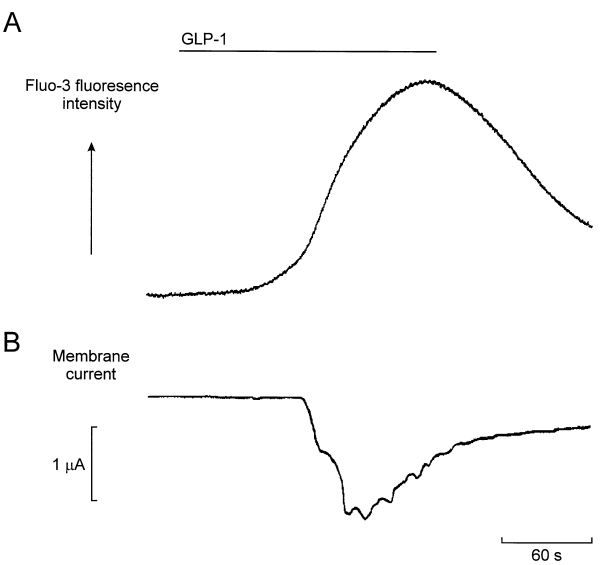

Fig. 2. Parallel measurements of inward Cl^- current and $[Ca^{2+}]_i$ in an oocyte expressing the cloned human GLP-1 receptor. The change in $[Ca^{2+}]_i$ is depicted as an increase in Fluo-3 fluorescence intensity (top trace) whereas the associated inward Cl^- current is shown in the lower trace. The traces are representative of four other experiments.

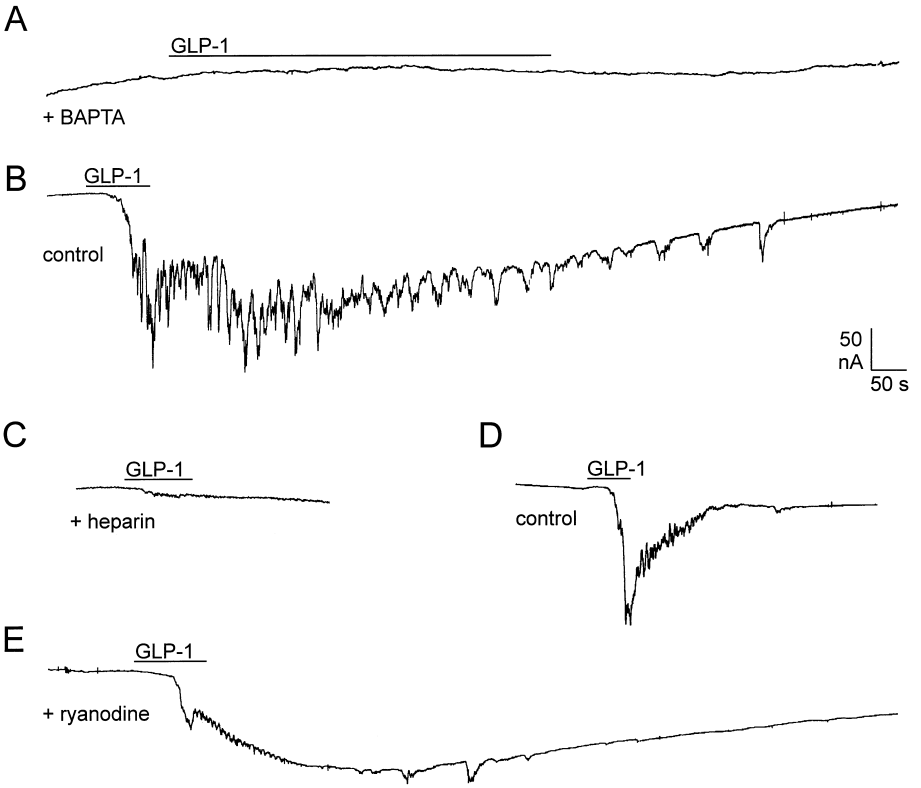

Fig. 3. Activation of GLP-1 receptor evokes Ins(1,4,5)P_3-dependent intracellular Ca^{2+} mobilization. A: Lack of effect of GLP-1(7–36)amide in oocytes injected >0.5 h prior to the experiments with BAPTA (final concentration 100 mM) but not in control injected oocytes (B). C: Microinjection of the inositol trisphosphate receptor antagonist heparin (100 μg/ml) strongly reduced response to GLP-1(7–36)amide (50 nM) stimulation but not in control oocytes (D). E: Current response obtained from oocytes pretreated for >0.5 h with 100 μM ryanodine. The traces are representative of 4–6 experiments.

165 ± 50 nA for the first GLP-1(7–36)amide application and 193 ± 88 nA for the second stimulation. The action of GLP-1(7–36)amide is specific since application of GLP-1(7–36)amide in the presence of the GLP-1 receptor antagonist exendin-(9–39) (10 μM) abolished the stimulatory effect of GLP-1(7–36)amide (Fig. 1B). A second application of GLP-1(7–36)amide 4 min following wash-out of exendin-(9–39) evoked a current response of 200 nA. No effect of GLP-1(7–36)amide was observed in non-injected oocytes ($n = 11$; Fig. 1C).

3.2. GLP-1 receptor activation causes Ins(1,4,5)P_3-dependent intracellular Ca^{2+} release

The above data show that GLP-1 receptors are functionally expressed in *Xenopus* oocytes and stimulation of these receptors by GLP-1(7–36)amide induces activation of an inward Cl^- current. It is generally accepted that oocytes are equipped with Ca^{2+}-activated Cl^- channels and we next investigated whether changes in $[Ca^{2+}]_i$ couple GLP-1 receptor stimulation to activation of the Cl^- current. Fig. 2 shows that stimulation of oocytes expressing the GLP-1 receptor with 50 nM GLP-1(7–36)amide results in a pronounced increase in Fluo-3 fluorescence intensity reflecting an elevation of $[Ca^{2+}]_i$ which was associated with induction of an inward Cl^- current. In all tested oocytes the rise in $[Ca^{2+}]_i$ was transient and with no oscillations ($n = 5$). The oscillatory nature of the Cl^- current observed in many oocytes is likely to reflect *local* changes in $[Ca^{2+}]_i$, whereas the fluorescence measurements report *average* $[Ca^{2+}]_i$ in the oocyte. This is in keeping with previous observations that the membrane current responses do not simply mirror the Ca^{2+} signals [7].

To further substantiate the Ca^{2+} dependence of the Cl^- current oocytes were injected with the Ca^{2+} chelator BAPTA (final concentration 100 μM). In BAPTA-loaded oocytes the stimulatory action of GLP-1(7–36)amide was abolished whereas control injected oocytes responded to an application of this hormone (Fig. 3A,B). On average, BAPTA completely blocked the GLP-1(7–36)amide-evoked current response (n=4) whereas control injected oocytes produced an average current of 188 ± 16 nA ($n = 5$). Injection of the Ins(1,4,5)P_3 receptor antagonist heparin (final concentration 100 μg/ml) into oocytes expressing the GLP-1 receptor strongly reduced the GLP-1(7–36)amide-evoked current (Fig. 3C,D). In this series of experiments, we observed a 97% ($P < 0.025$) block of the Cl^- currents from 252 ± 70 nA in control ($n = 6$) to 18 ± 14 nA ($n = 4$) in heparin-injected oocytes. In contrast (Fig. 3E), pretreating oocytes for >30 min with 50 μM ryanodine, an inhibitor of the process of Ca^{2+}-induced Ca^{2+} release, had no effect on the ability of GLP-1(7–36)amide to evoke a current response: 105 ± 6 nA in ryanodine-treated oocytes ($n = 5$) vs. 124 ± 31 nA in control ($n = 3$). Similar negative results were observed following injection of oocytes with ryanodine (final concentrations: 50–200 μM) >1 h prior to the experiments.

3.3. GLP-1(7–36)amide activates Ca^{2+}-dependent Cl^- current independently of the adenylate cyclase/PKA system

Measurements of cAMP levels revealed that the cloned human GLP-1 receptor associates with endogenous adenylate

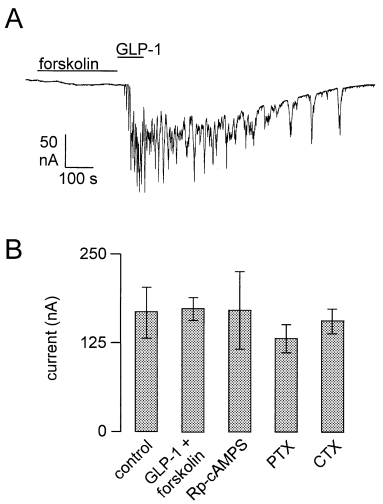

Fig. 4. Stimulatory effect of GLP-1(7–36)amide on Ca^{2+}-activated Cl^- currents does not involve the cAMP/PKA signaling system. A: Consecutive applications of forskolin (10 μM) and GLP-1(7–36)amide (50 nM) to oocytes expressing the cloned human GLP-1 receptor. B: Histograms showing average effects of GLP-1(7–36)amide on Ca^{2+}-activated Cl^- currents in oocytes exposed to forskolin (10 μM) for 3 min; pertussis toxin (PTX) or cholera toxin (CTX) 100 ng/ml for >20 h; or microinjected with the PKA inhibitor Rp-cAMPS (50 μM) for >0.5 h prior to the experiments. Mean values ± S.E.M. of 3–8 experiments in each series.

cyclase in the oocytes since cAMP formation was increased >7-fold from 20 ± 2 nM to 168 ± 24 nM ($P < 0.001$; $n = 4$) following a 15 min GLP-1(7–36)amide (50 nM) stimulation. Fig. 4A shows that application of the adenylate cyclase activator forskolin (10 μM) does not mimic the stimulatory effect of GLP-1(7–36)amide. Furthermore, neither the latency nor the amplitude of the Cl^- current evoked in response to subsequent GLP-1(7–36)amide stimulation was affected (Fig. 4A,B). However, the lack of effect of forskolin on the Cl^- conductance does not reflect its inability to stimulate cAMP production which increased >2.5-fold following a 15 min stimulation. The ability of GLP-1(7–36)amide to stimulate Ca^{2+}-dependent Cl^- currents does not involve activation of PKA as indicated by the observation that intracellular injection of the specific PKA inhibitor Rp-cAMPS (final concentration 50 μM [8]) did not interfere with the stimulatory action of GLP-1(7–36)amide (Fig. 4B).

It is generally accepted that the effect of GLP-1(7–36)amide is mediated by the G-protein subunit $G_{s\alpha}$ [9]. However, this is most likely not the case in *Xenopus* oocytes since pretreatment for >20 h with 100 ng/ml cholera toxin or pertussis toxin did not affect the amplitude of the GLP-1(7–36)amide-evoked Cl^- current (Fig. 4B). These results suggest that the GLP-1 receptor-evoked intracellular Ca^{2+} mobilization occurs independently of cAMP and activation of PKA.

3.4. Conclusions

Activation of the human GLP-1 receptor expressed in *Xenopus* oocytes results in Ins(1,4,5)P_3-mediated intracellular Ca^{2+} mobilization and activation of Ca^{2+}-dependent Cl^- currents. The latency of the GLP-1(7–36)amide-evoked current results mainly from rate-limiting steps in the signal transduction pathway between receptor binding and Ins(1,4,5)P_3 liberation but may also to a lesser extent be a consequence of a slowly rising level of Ins(1,4,5)P_3 towards a threshold required before Ca^{2+} release is triggered [10]. More importantly, the signal transduction pathway utilized by the GLP-1 receptor in oocytes is *independent* of the cAMP/PKA signaling system despite an observed GLP-1- (and forskolin-) stimulated increase in cAMP formation. This contrasts with previous reports on GLP-1 receptor signaling via $[Ca^{2+}]_i$ which is dependent on the receptor's ability to generate cAMP [3–5,11]. In one study it was demonstrated that the GLP-1 receptor coupled not only to adenylate cyclase but also to PLC following transient expression in a monkey kidney cell line [2]. Coupling to both the adenylate cyclase and PLC signaling systems has also been observed for the structurally related glucagon receptor [12]. Interestingly, activation of the PACAP-3 receptor expressed in *Xenopus* oocytes by both VIP and PACAP elicits Ca^{2+}-activated Cl^- currents suggesting that PACAP-3 receptors may also couple to PLC [13]. Some splice variants of the PACAP-2 receptor also couple to PLC [14]. This demonstrates that the cellular signaling mechanism delineated in the present study for the GLP-1 receptor may be operational for a larger group of membrane receptors in the glucagon-secretin family that all signal via increased cAMP formation and changes in $[Ca^{2+}]_i$.

Acknowledgements: We thank Karin Hamborg Albertsen and Lisbet Petri for expert technical assistance.

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