Stimulation of cloned human glucagon-like peptide 1 receptor expressed in HEK 293 cells induces cAMP-dependent activation of calcium-induced calcium release

Jesper Gromada[a],*, Patrik Rorsman[a], Steen Dissing[b], Birgitte S. Wulff[c]

[a]Islet Cell Physiology, Symbion Science Park, Novo Nordisk A/S, Fruebjergvej 3, DK-2100 Copenhagen, Denmark
[b]Department of Medical Physiology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark
[c]Department of Molecular Pharmocology, Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark

Abstract The actions of glucagon-like peptide-1(7–36)amide (GLP-1(7–36)amide) on cellular signalling were studied in human embryonal kidney 293 (HEK 293) cells stably transfected with the cloned human GLP-1 receptor. The cloned GLP-1 receptor showed a single high-affinity binding site (K_d = 0.76 nM). Binding of GLP-1(7–36)amide stimulated cAMP production in a dose-dependent manner (EC_{50} = 0.015 nM) and caused an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). The latter effect reflected Ca^{2+}-induced Ca^{2+} release and was suppressed by ryanodine. We propose that the ability of GLP-1(7–36)amide to increase $[Ca^{2+}]_i$ results from sensitization of the ryanodine receptors by a protein kinase A dependent mechanism.

Key words: GLP-1 receptor; Calcium; cAMP; HEK 293 cell; CICR

1. Introduction

Glucagon-like peptide 1 (GLP-1) is a gastrointestinal hormone with potent insulinotropic effects on the endocrine pancreas. GLP-1 mediates its effects by binding to specific receptors on the pancreatic β-cells thereby stimulating cAMP formation and insulin secretion [1,2]. Ca^{2+} plays a central role in the stimulus-secretion coupling, and a GLP-1-evoked increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) is one of the decisive events leading to an insulin secretory response in the β-cell. We have previously demonstrated that in the mouse insulin-secreting cell line βTC3, GLP-1(7–36)amide produces a rise in $[Ca^{2+}]_i$ by promoting Ca^{2+}-induced Ca^{2+}-release (CICR) from intracellular stores [3]. The recent cloning of the GLP-1 receptor [4,5] has enabled a more detailed analysis of the cellular processes involved. Here we demonstrate for the first time that expression of the cloned GLP-1 receptor in HEK 293 cells is associated with the induction of later steps in the sequence of events normally activated by GLP-1. These events include generation of cAMP and mobilization of Ca^{2+} from intracellular stores by ryanodine-sensitive CICR.

2. Materials and methods

2.1. Reagents

Rp-cAMPS was obtained from BIOLOG Life Science Institute (Bremen, Germany). Fura-2 acetoxymethyl ester (fura-2/AM), fura-2 pen-

---

*Corresponding author. Fax: (45) (39) 179762.

tapotassium salt, and BAPTA/AM were supplied by Molecular Probes (Eugene, OR, USA). EGTA was from Fluka (Buchs, Switzerland). Ryanodine was obtained from Alomone Labs (Jerusalem, Israel). U73122 was from Biomol (Plumouth Meeting, PA, USA). Ionomycin was provided by Calbiochem (La Jolla, CA, USA). Dulbecco's modified eagle medium (DMEM), Hanks' balanced salt solution (HBSS), trypsin, and Lipofectin transfection reagent were from Gibco BRL, (Life Technologies, Inc, MD, USA). Synthetic GLP-1(7–36)amide was synthesized as described elsewhere [6]. [^{125}I]GLP-1(7–36)amide was prepared by iodinating the peptide with ^{125}I using H_2O_2/lactoperoxidase at neutral pH and purified by reverse-phase HPLC as described elsewhere [7], resulting in a specific activity of 1.76 μCi/pmol. All other chemicals were obtained from Sigma Chemicals (St. Loius, MO, USA).

2.2. Expression of the cloned human GLP-1 receptor

The human GLP-1 receptor cDNA was obtained from Dr. B. Thorens (Department of Pharmacology and Toxicology, University of Lausanne, Switzerland). The cDNA was subcloned as a *EcoRI* fragment into *EcoRI* cut pcDNA3 (expression vector from Invitrogen Corp. San Diego, CA, USA) resulting in pBW302. HEK 293 cells were transfected with 2 μg pBW302 by lipofection using Lipofectin according to the manufacturer's instructions. The cells were grown at 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 50 IU pencillin, 50 μg/ml streptomycin, and 1% non-essential amino acids. Stable clones were selected in culture medium containing 1 mg/ml reagent G418. All data shown in the present study were obtained from one clone with a GLP-1 receptor expression level of 850 fmol/mg protein (i.e. approx. 150000 receptors/cell).

2.3. Receptor binding assay

Receptor binding was analyzed using membranes from HEK 293 cells. Batches of cells were harvested and homogenized with short bursts using an Ultra-turrax T25 homogenizer (Janke and Kunkel, Germany) in a buffer containing (in mM): 25 HEPES, 2.5 $CaCl_2$, 1 $MgCl_2$ and 50 mg/l Bacitracin (pH 7.4). The membranes were centrifuged at 48000 × g and the supernatant was discarded. After resuspension in 5 ml buffer, the pellet was homogenized again and the above steps repeated once. The protein concentration was adjusted to 75 μg/ml and bovine serum albumine (fraction V) was added to a final concentration of 0.1% w/v. Aliquots of 500 μl membranes were incubated with 25 μl [^{125}I]GLP-1(7–36)amide (10^5 cpm) and 25 μl of GLP-1(7–36)amide and incubated for 60 min at 37°C. Bound and unbound peptide was separated by filtration through Whatman GF/C 3607 filters preincubated in 0.1% polyethylenimin. After filtration, the filters were counted for radioactivity in a gamma scintillation counter.

2.4. Measurements of cAMP levels

Intracellular cAMP levels were measured in HEK 293 cells grown to approximately 70% confluency in Nunc 24-well multidishes (1 ml). The cells were first incubated for 15 min in the presence of 1 mM of the phosphodiesterase inhibitor IBMX and subsequently stimulated with GLP-1(7–36)amide for 15 min in the continued presence of IBMX. The reaction was terminated by the addition of HCl to a final concentration of 50 mM. The samples were neutralized with 50 mM NaOH and assayed for cAMP using a cAMP [^{125}I] scintillation proximity assay (RPA 542; Amersham, UK).

2.5. Measurements of $[Ca^{2+}]_i$

Images of $[Ca^{2+}]_i$ in single HEK 293 cells were obtained using an inverted fluorescence microscope (Zeiss Axiovert 135; Oberkochen, Germany) and an Image-1 (Universal Imaging, West Chester, PA, USA) image processing system, as described elsewhere [3]. The cells were loaded with 3 μM fura-2/AM for 30 min at 37°C followed by washing and resuspension in extracellular solution containing (in mM) 140 NaCl, 4 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.3 (NaOH). The Ca^{2+}-free medium was without added Ca^{2+} and supplemented with 100 μM EGTA. $[Ca^{2+}]_i$ was calculated from the measurements of the ratio of fluorescence intensities according to [8]: $[Ca^{2+}]_i = K_d \cdot [(R - R_{min})/(R_{max} - R)] \cdot (S_{f2}/S_{b2})$. The pixel gray values for R_{max} were obtained using ionomycin (1 μM) and was determined as 238. R_{min} and the proportionality constant (S_{f2}/S_{b2}) were 27 and 9.8, respectively. A value for the apparent K_d of Ca^{2+} binding to fura-2 of 224 nM was used [8].

2.6. Statistical analysis

All data are expressed as mean values ± S.E.M. for n tested cells/ experiments.

3. Results and discussion

3.1. GLP-1 receptor binding and cAMP formation

To ensure that the transfected HEK 293 cells express the human GLP-1 receptor, binding analysis was performed with GLP-1(7–36)amide. In HEK 293 cells stably transfected with
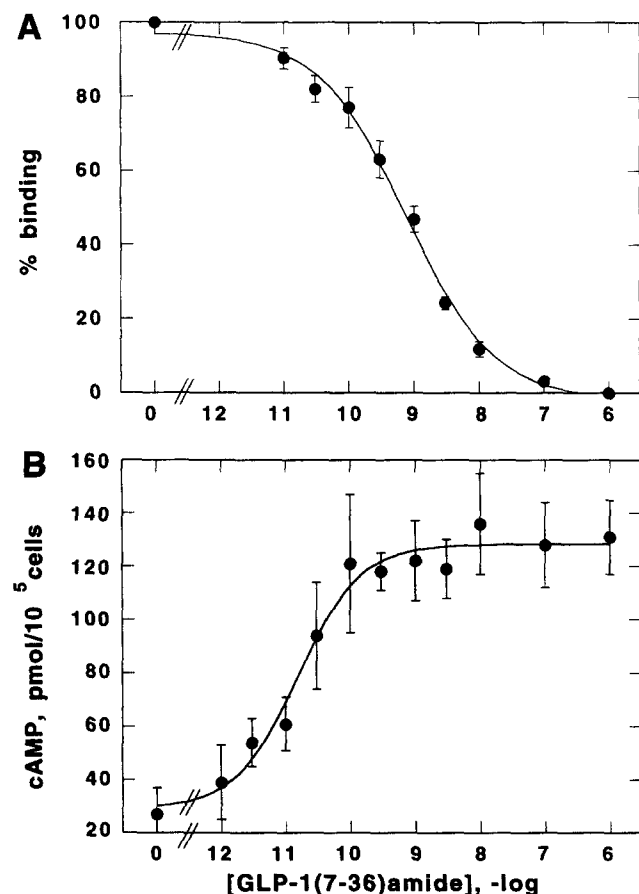

Fig. 1. (A) Displacement of [^{125}I]GLP-1(7–36)amide binding to HEK 293 cells expressing the cloned human GLP-1 receptor by GLP-1(7–36)amide. (B) GLP-1(7–36)amide-evoked cAMP production assessed in intact cells in the presence of 1 mM of the phosphodiesterase inhibitor IBMX.
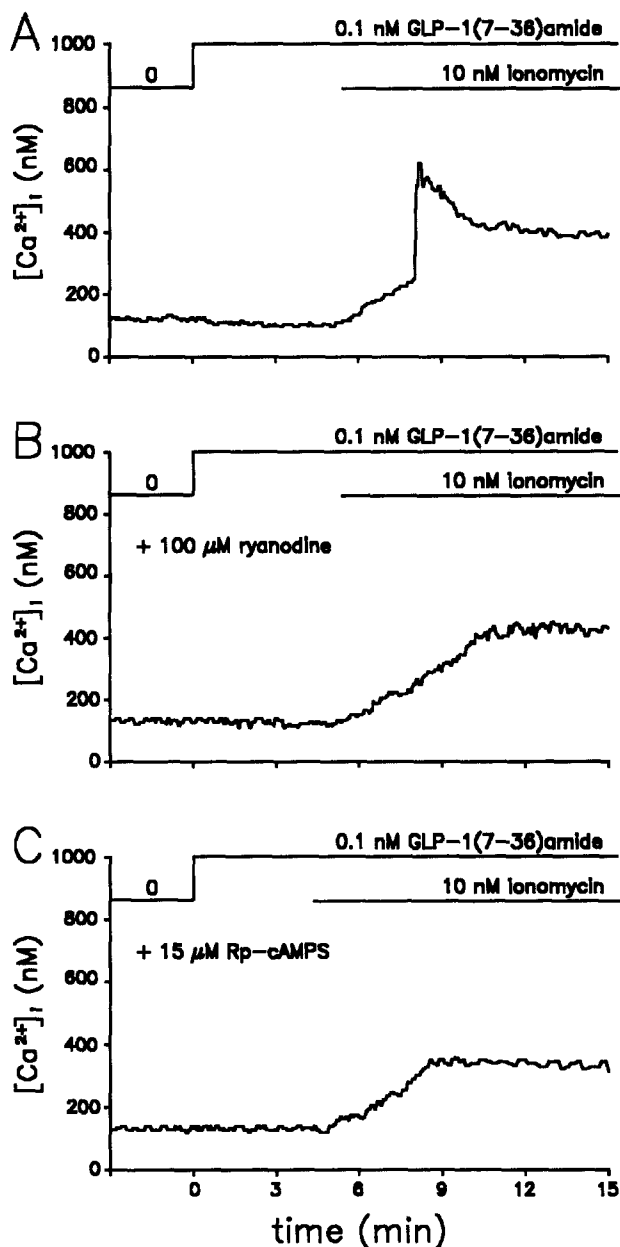

Fig. 2. GLP-1(7–36)amide-evoked changes in $[Ca^{2+}]_i$ in HEK 293 cells expressing the cloned human GLP-1 receptor. (A) HEK 293 cells were stimulated with 10 nM GLP-1(7–36)amide in a Ca^{2+} containing (A) or a Ca^{2+}-free medium with 100 μM EGTA (B). The cells were subsequently stimulated with 10 μM ACh. The traces are representative of 53 and 68 cells from 6 and 8 different experiments.

the GLP-1 receptor specific binding of ^{125}I-labelled GLP-1(7–36)amide was displaced in a concentration-dependent manner using unlabelled GLP-1(7–36)amide. As illustrated in Fig. 1A, the binding data are consistent with the presence of a single high-affinity binding site with K_d of 0.76 ± 0.14 nM ($n = 9$).

Fig. 1B demonstrates that the cloned GLP-1 receptors associate with endogenous adenylate cyclase as indicated by measurements of GLP-1(7–36)amide-induced cAMP formation in intact HEK 293 cells. GLP-1(7–36)amide stimulated cAMP production in a concentration-dependent manner with half-
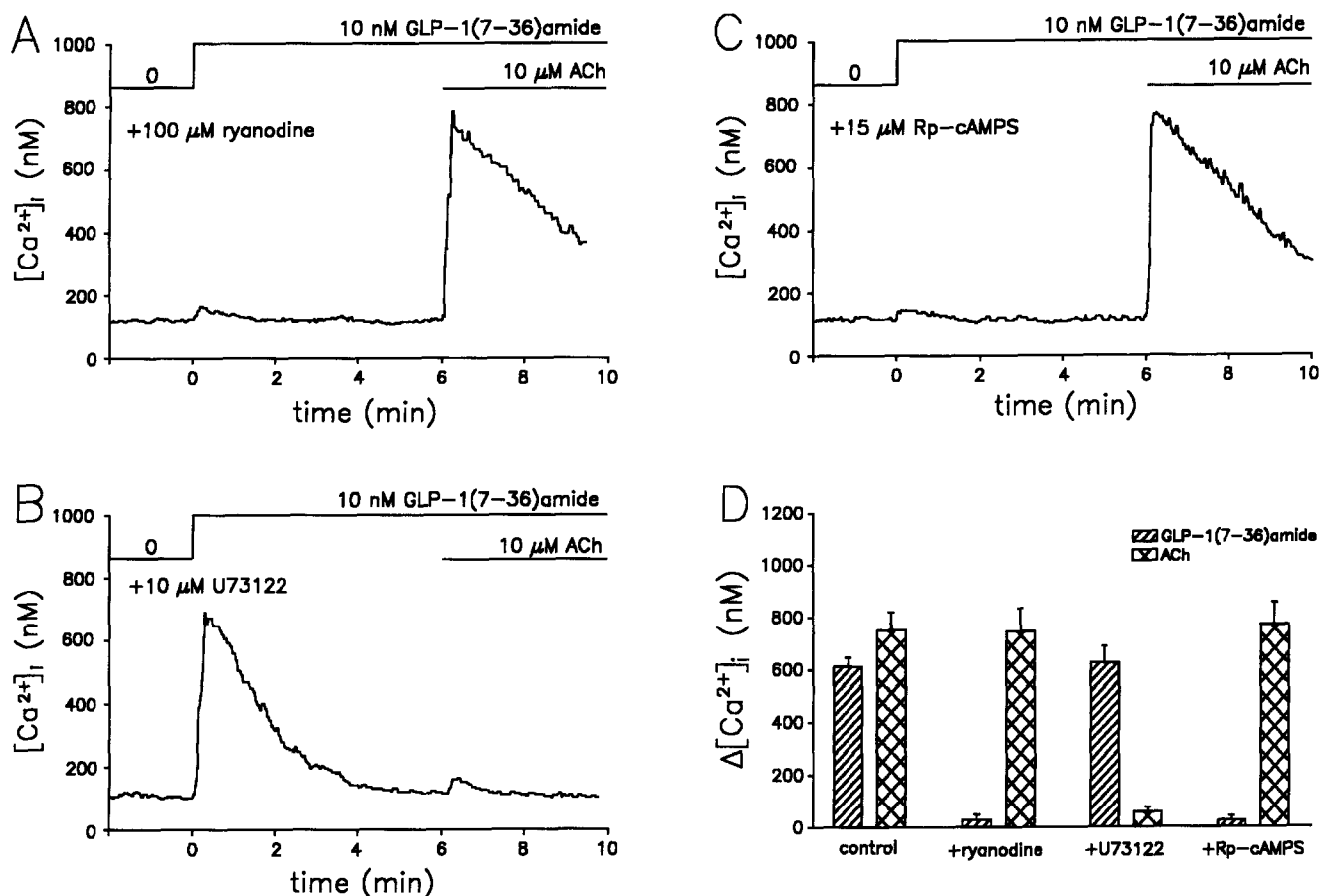
Fig. 3. GLP-1(7–36)amide stimulates Ca^{2+}-induced Ca^{2+}-release by a cAMP-dependent mechanism in HEK 293 cells. (A) 100 μM ryanodine was added to the extracellular medium 3 min before stimulation with 10 nM GLP-1(7–36)amide and subsequently with 10 μM ACh. (B) the cells were exposed to 10 μM of the phospholipase C inhibitor U73122 for 5 min before stimulation with 10 nM GLP-1(7–36)amide followed by 10 μM ACh. (C) The specific PKA inhibitor Rp-cAMPS (15 μM) was added 5 min before stimulation with 10 nM GLP-1(7–36)amide and 10 μM ACh. (D) Summerizes the average changes in $[Ca^{2+}]_i$ under the different experimental conditions. The traces are representative of 23–34 cells from 4 to 7 individual experiments.

maximal effect being observed at 14.9 ± 0.4 pM (n = 6). Similar results for receptor binding and cAMP formation were obtained using GLP-1(7–37) or exendin-4. In 'wild type' HEK 293 cells or in cells transfected with the pcDNA3 vector neither GLP-1(7–36)amide binding nor GLP-1(7–36)amide-induced cAMP production was observed (not shown). The data summarized in Fig. 1 are similar to those previously reported for the cloned rat and human GLP-1 receptor in stably transfected fibroblasts [5,9] and for the endogenoes receptor expressed in insulinoma cell lines [4,9] but lower than those reported for transiently transfected COS-7 cells [10,11].

3.2. The GLP-1 receptor causes mobilization of Ca^{2+} from intracellular stores

Fig. 2A shows that stimulation of HEK 293 cells expressing the GLP-1 receptor by 10 nM GLP-1(7–36)amide results in a rapid increase in $[Ca^{2+}]_i$ from a resting value of 97 ± 12 nM (n = 178) to approximately 650 nM which was followed by a gradual return to the prestimulatory level within 5–6 min despite the continued presence of the agonist. Subsequent stimulation with ACh (10 μM) caused an increase in $[Ca^{2+}]_i$ of the same amplitude as that observed following stimulation with 10 nM GLP-1(7–36)amide. These effects of GLP-1(7–36)amide

and ACh reflect largely mobilization of intracellular Ca^{2+} rather than stimulation of its influx because qualitatively similar responses to the agonists were obtained in the absence of extracellular Ca^{2+} (Fig. 2B). It is generally believed that the effect of GLP-1 in various tissues involves the activation of a G-protein [2]. This also appears to be the case in the transfected HEK 293 cells and pretreatment of the cells with 100 ng/ml pertussis toxin for 24 h reduced the $[Ca^{2+}]_i$-increasing action of GLP-1(7–36)amide by 74 ± 3% (n = 26) whilst not affecting the effect of ACh (not shown).

3.3. GLP-1(7–36)amide stimulates Ca^{2+}-induced Ca^{2+} release

Whereas ACh strongly stimulated the generation of $Ins(1,4,5)P_3$ and its degradation products, as determined by HPLC separation, 10 nM GLP-1(7–36)amide had no effect on the concentrations of the inositol phosphates (data not shown). We conclude therefore that the ability of GLP-1(7–36)amide to mobilize intracellular Ca^{2+} in HEK 293 cells transfected with the human GLP-1 receptor takes place via a mechanism independent of the phospholipase $C/Ins(1,4,5)P_3$ pathway.

We have previously proposed that the capacity of GLP-1 to increase $[Ca^{2+}]_i$ in βTC3 insulinoma cells involves the activation of CICR [3]. We next went on to investigate whether the same
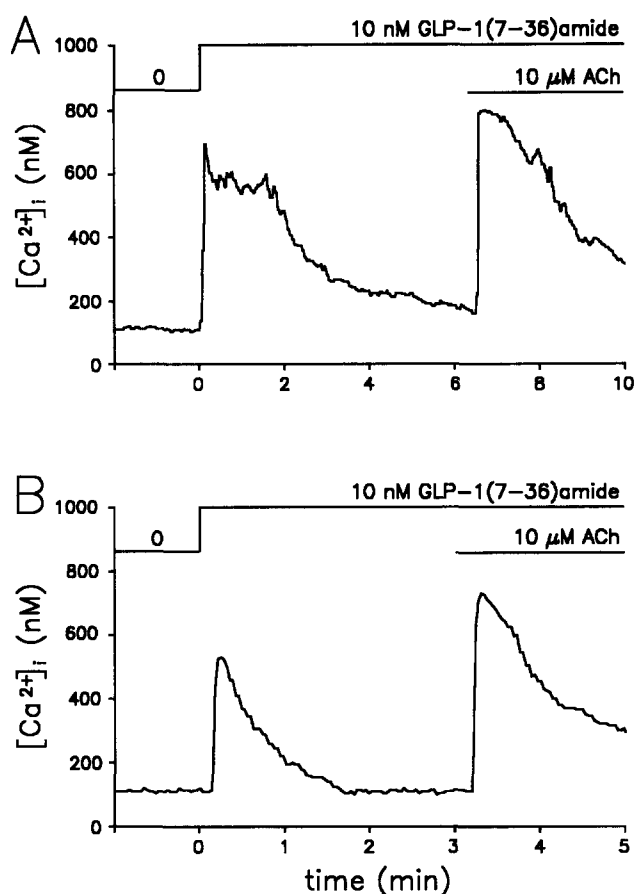
Fig. 4. Elevation of $[Ca^{2+}]_i$ stimulates Ca^{2+}-induced Ca^{2+} release at subthreshold GLP-1(7–36)amide concentrations. (A) stimulation of HEK 293 cells with GLP-1(7–36)amide (0.1 nM) does not evoke changes in $[Ca^{2+}]_i$. Subsequent addition of ionomycin causes a slow rise in $[Ca^{2+}]_i$ which triggers a rapid rise in $[Ca^{2+}]_i$. This latter Ca^{2+} response is not observed in cells pretreated for 5 min with 100 μM ryanodine (B) or with 15 μM Rp-cAMPS (C). The traces are represensative of 34–41 cells from 6–8 individual experiments.

mechanism becomes operational in HEK 293 cells transfected with the human GLP-1 receptors. As shown in Fig. 3A this is likely to be the case as the ability of GLP-1(7–36)amide, but not ACh, to increase $[Ca^{2+}]_i$ in these cells was abolished by ryanodine (100 μM), a potent inhibitor of CICR [12]. By contrast, inclusion of 10 μM U73122, an inhibitor of phospholipase C (PLC)-mediated processes [13,14] selectively inhibited the ACh-evoked $[Ca^{2+}]_i$ response whilst not affecting the response to GLP-1(7–36)amide (Fig. 3B,D). Finally, inhibition of protein kinase A (PKA) by pretreatment of the cells with Rp-cAMPS [15] suppressed the GLP-1(7–36)amide-evoked rise in $[Ca^{2+}]_i$ but did not affect the amplitude of the ACh-induced $[Ca^{2+}]_i$ response (Fig. 3C,D).

3.4. GLP-1(7–36)amide sensitizes Ca^{2+}-induced Ca^{2+} release channels

The above results suggest that GLP-1(7–36)amide promotes Ca^{2+} mobilization from intracellular stores through activation of ryanodine sensitive Ca^{2+} release channels. We next investigated whether this PKA-mediated Ca^{2+}-release process results from sensitization of the intracellular Ca^{2+} release channels to

resting $[Ca^{2+}]_i$. Fig. 4A shows an experiment where a HEK 293 cell was stimulated with 0.1 nM GLP-1(7–36)amide, a concentration which in itself is insufficient to cause a rise in $[Ca^{2+}]_i$. Subsequent addition of a low concentration (10 nM) of the Ca^{2+} ionophore ionomycin induced a slow gradual increase in $[Ca^{2+}]_i$ which triggered a rapid and transient further elevation of $[Ca^{2+}]_i$ which is abolished by pretreatment of the cells with either ryanodine or Rp-cAMPS (Fig. 4B,C).

3.5. Conclusions

Expression of the cloned human GLP-1 receptor in HEK 293 not only results in the appearance of GLP-1(7–36)amide binding sites with properties indistinguishable from those of the native receptors. More importantly, the insertion of these receptors in the HEK 293 cells also leads to the activation of more distal steps in the signal transduction pathway utilized by GLP-1. For example, there is a GLP-1-dependent production of cyclic AMP and mobilization of intracellular Ca^{2+} from ryanodine-sensitive intracellular Ca^{2+}-stores. The effects of GLP-1(7–36)amide on $[Ca^{2+}]_i$-handling in HEK 293 cells expressing the cloned GLP-1 receptor are remarkably similar to those previously reported by us and others in insulin-secreting-cells [3,16]. This suggests that cAMP-dependent modulation of the ryanodine receptors (and Ca^{2+}-release channels) may represent a fundamental cellular property which links receptor activation, via stimulation of adenylate cyclase, to an increase in $[Ca^{2+}]_i$ which in turn accounts for the cellular responses. In this context it is pertinent that all ryanodine receptor isoforms contains PKA consensus phosphorylation sites [17]. The observation that the GLP-1(7–36)amide-evoked Ca^{2+}-response is blocked by Rp-cAMPS finally excludes the possibility that cADP-ribose, a newly discovered Ca^{2+} mobilizing nucleotide of NAD^+ [18,19] is responsible for the observed $[Ca^{2+}]_i$ responses following GLP-1(7–36)amide stimulation.

References

[1] Fehmann, H.C. and Habener, J.F. (1992) Trends Endocrinol. Metab. 3, 158–163.
[2] Thorens, B. and Waeber, G. (1993) Diabetes 42, 1219–1225.
[3] Gromada, J., Dissing, S,, Bokvist, K., Renström, E., Frøkjær-Jensen, J., Wulff, B.S. and Rorsman, P. (1995) Diabetes 44, 767–774.
[4] Thorens, B. (1992) Proc. Natl. Acad. Sci. USA 89, 8641–8645.
[5] Thorens, B., Porret, A., Bühler, L., Deng, S.H., Morel, P. and Widmann, C. (1993) Diabetes 42, 1678–1682.
[6] Adelhorst, K., Hedegaard, B.B., Knudsen, L.B. and Kirk, O. (1994) J. Biol. Chem. 269, 6275–6278.
[7] Drejer, K., Kruse, V., Larsen, U.D., Hougaard, P., Bjørn, S. and Gammeltoft, S. (1991) Diabetes 40, 1488–1495.
[8] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[9] Widmann, C., Bürki, E., Dolci, W. and Thorens, B. (1994) Mol. Pharmacol. 45, 1029–1035.
[10] Dillon, J.S., Tanizawa, Y., Wheeler, M.B., Leng, X.H., Ligon, B.B., Rabin, D.U., Yoo-Warren, H., Permutt, M.A. and Boyd III, A.E. (1993) Endocrinology 133, 1907–1910.
[11] Wheeler, M.B., Lu, M., Dillon, J.S., Leng, X.H., Chen, C. and Boyd III, A.E. (1993) Endocrinology 133, 57–62.
[12] Coronado, R., Morisette, J., Sukhareva, M. and Vaughan, D.M. (1994) Am. J. Physiol. 266, C1485–C1504.

[13] Yule, D.I. and Williams, J.A. (1992) J. Biol. Chem. 267, 13830–13835.

[14] Gromada, J., Jørgensen, T.D. and Dissing, S. (1995) Pflügers Arch. 429, 578–586.

[15] De Wit, J.W., Hekstra, D., Jastroff, B., Stec V.J., Baraniak, J., Van Driel, R. and Van Haastrert, J.M. (1984) Eur. J. Biochem. 142, 255–260.

[16] Cullinan, C.A., Brady, E.J., Saperstein, R. and Labowitz, M.D. (1994) Cell Calcium 15, 391–400.

[17] Furuchi, T., Kohda, K., Miyawaki, A. and Mikoshiba, K. (1994) Curr. Opion. Neurobiol. 4, 294–303.

[18] Galione, A. (1994) Mol. Cell. Endocrinol. 98, 125–131.

[19] Lee, H.C. (1994) News Physiol. Sci. 9, 134–137.