β-Arrestin 1 Is Required for PAC₁ Receptor-mediated Potentiation of Long-lasting ERK1/2 Activation by Glucose in Pancreatic β-Cells*Ś

Christophe Broca¹, Julie Quoyer¹,², Safia Costes, Nathalie Linck, Annie Varrault, Pierre-Marie Deffayet, Joël Bockaert, Stéphane Dalle, and Gyslaine Bertrand³

From the Institut de Génomique Fonctionnelle, CNRS, Unité Mixte de Recherche 5203, INSERM, U661, Université Montpellier I, and Université Montpellier II, 34094 Montpellier Cedex 5, France

In pancreatic β-cells, the pituitary adenylate cyclase-activating polypeptide (PACAP) exerts a potent insulin secretory effect via PAC₁ and VPAC receptors (Rs) through the Gα_s/cAMP/protein kinase A pathway. Here, we investigated the mechanisms linking PAC₁R to ERK1/2 activation in INS-1E β-cells and pancreatic islets. PACAP caused a transient (5 min) increase in ERK1/2 phosphorylation via PAC₁Rs and promoted nuclear translocation of a fraction of cytosolic p-ERK1/2. Both protein kinase A- and Src-dependent pathways mediated this transient ERK1/2 activation. Moreover, PACAP potentiated glucose-induced long-lasting ERK1/2 activation. Blocking Ca²⁺ influx abolished glucose-induced ERK1/2 activation and PACAP potentiating effect. Glucose stimulation during KCl depolarization showed that, in addition to the triggering signal (rise in cytosolic [Ca²⁺]), the amplifying pathway was also involved in glucose-induced sustained ERK1/2 activation and was required for PACAP potentiation. The finding that at 30 min glucose-induced p-ERK1/2 was detected in both cytosol and nucleus while the potentiating effect of PACAP was only observed in the cytosol, suggested the involvement of the scaffold protein β-arrestin. Indeed, β-arrestin 1 (β-ar1) depletion (in β-ar1 knock-out mouse islets or in INS-1E cells by siRNA) completely abolished PACAP potentiation of long-lasting ERK1/2 activation by glucose. Finally, PACAP potentiated glucose-induced CREB transcriptional activity and IRS-2 mRNA expression mainly via the ERK1/2 signaling pathway, and likewise, β-ar1 depletion reduced the PACAP potentiating effect on IRS-2 expression. These results establish for the first time that PACAP potentiates glucose-induced long-lasting ERK1/2 activation via a β-ar1-dependent pathway and thus provide new insights concerning the mechanisms of PACAP and glucose actions in pancreatic β-cells.

The neuropeptide, Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP)⁴ plays an important role in the regulation of pancreatic islet functions. PACAP is expressed in islet parasympathetic nerve terminals and strongly potentiates insulin secretion in a glucose-dependent manner both in vitro (1–5) and in vivo in rodent (6) and humans (7). The physiological functions of PACAP are mediated by three receptor subtypes that belong to the class II G-protein-coupled receptors (GPCRs): PAC₁, VPAC₁ and VPAC₂ receptors (Rs). PAC₁R is selective for PACAP, whereas VPAC₁R and VPAC₂R bind to PACAP and Vasoactive Intestinal Peptide (VIP) with equal high affinity (8). Class II GPCRs, which include also receptors for glucagon and the incretin glucagon-like peptide 1 (GLP-1), are coupled to the heterotrimeric G-protein Gₛ, which stimulates the adenylate cyclase (AC), (9). Besides its insulinotropic action, PACAP has been recently reported to exert long term beneficial effects on β-cell mass in various experimental mouse models of diabetes (10, 11). However, the receptor(s) and the mechanism(s) involved in these long term effects are yet unknown.

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are important effectors of GPCRs and regulate cell growth, survival, and differentiation (12). In pancreatic β-cells, cross-talk between the cAMP-protein kinase A (PKA) and ERK1/2 signa-

⁴ The abbreviations used are: PACAP, pituitary adenylate cyclase-activating polypeptide; AC, adenylate cyclase; β-ar1, β-arrestin 1; CREB, cAMP-response element-binding protein; ERK, extracellular signal-regulated kinase; GLP-1, glucagon-like peptide-1; GPCR, G-protein-coupled receptor; IRS-2, insulin receptor substrate-2; Kᵥ1 channel, ATP-sensitive K⁺ channel; K⁺-ATP channel, ATP-sensitive K⁺ channel; KᵢR, Krebs-Ringer-Bicarbonate HEPES buffer; MEK, mitogen-activated protein kinase/ERK kinase 1/2; PKA, protein kinase A; p90 RSK, 90-kDa ribosomal S6 kinases; VDCC, voltage-dependent calcium channel; VIP, vasoactive intestinal peptide; siRNA, small interfering RNA.

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1 Both authors contributed equally to this work.

2 Supported by INSERM and the Région Languedoc-Roussillon.

3 To whom correspondence should be addressed. Tel.: 33-4-67-14-29-38; Fax: 33-4-67-54-24-32; E-mail: gyslaine.bertrand@igf.cnrs.fr.
GPCRs desensitization. Interestingly, G-protein- and β-arrestin-dependent activations of the ERK1/2 pathway are both temporally and spatially distincts in cells and thus modulate differently the functions of ERK1/2. Hence, whereas G-protein-dependent activation of ERK1/2 signal is transient and nuclear, ERK1/2 activation through the β-arrestin-dependent pathway is usually sustained and retained in the cytosol.

Glucose is the major physiological stimulus of pancreatic β-cells. It exerts a tight control on insulin secretion through its metabolism via two major, hierarchical signaling pathways: a triggering pathway, i.e. the ATP-sensitive K⁺ (K~ATP~) channel-dependent pathway, and an amplifying pathway, i.e. the K~ATP~ channel-independent pathway (22). The triggering pathway results from oxidative glycolysis that increases the ATP/ADP ratio, leading to closure of K~ATP~ channels, and, subsequently, membrane depolarization, opening of voltage-dependent Ca²⁺ channels (VDCCs) and a rise in intracellular calcium ([Ca²⁺]₅), which is the triggering signal. The amplifying pathway does not further increase [Ca²⁺]₅, but rather enhances the response to the triggering Ca²⁺ signal. In pancreatic β-cells, glucose, at the physiological concentration range, stimulates ERK1/2 by a rise in [Ca²⁺]₅ (15, 17, 18, 23–25). A major physiological relevance of the activation of ERK1/2 by glucose in β-cells is the stimulation of both transcription through phosphorylation of various transcriptional factors (26, 27) and secretion (28) of insulin.

To further explore the role of PACAP in β-cells, we investigated the mechanisms linking PACAP receptors and ERK1/2 activation in the INS-1E cell line and mouse pancreatic islets, using pharmacological and molecular approaches. PACAP alone induces transient ERK1/2 activation via PAC₁R through using pharmacological and molecular approaches. PACAP activation in the INS-1E cell line and mouse pancreatic islets, gated the mechanisms linking PACAP receptors and ERK1/2 pathway does not further increase [Ca²⁺]₅; the response to the triggering Ca²⁺ (La Jolla, CA). Lipofectamine™ 2000, stealth siRNATM double-stranded duplexes, random hexamer oligonucleotides, and MoMuLV-RT were from Invitrogen. All other chemical reagents were purchased from Sigma-Aldrich. Pharmacological inhibitors were dissolved in DMSO and then diluted in incubation medium to a final concentration of 0.1% DMSO. PathDetect CRE trans-Reporting system was from Stratagene (La Jolla, CA). The Enhanced Luciferase Assay kit was from BD Biosciences Pharmingen (San Diego, CA). BCA™ Protein Assay kit was from Pierce. Anti-Phospho-p44/p42 MAPK (p-ERK1/2) (which selectively recognizes the doubly phosphorylated active forms of these kinases), anti-phospho-(Ser/Thr) PKA substrate, anti-α/β-tubulin, anti-IRS-2, horseradish peroxidase-linked anti-rabbit IgG antibodies, and RNAnow reagent were from Cell Signaling Technology (Ozyme, France). Anti-β-arrestin 1 and anti-ERK1 antibodies were from BD Transduction Laboratories (Lexington, KY). Horseradish peroxidase-linked anti-mouse IgG and anti-β-arrestin 2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Animals—β-Arrestin 1 knockout (β-ar1 KO) mice were generated on C57BL/6 background as previously described (29). Wild-type (WT) littermate controls were C57BL/6 mice (5 weeks old) purchased from Charles River, France.

Pancreatic Islet Isolation—Pancreatic islets were isolated from male mice (15–18 weeks old) by hand-picking after collagenase digestion of whole pancreas (5). Briefly, mice were killed by cervical dislocation, the pancreas was filled by injection of collagenase solution, excised, digested at 37 °C, and mechanically disrupted with a 14-gauge needle in cold PBS supplemented with 1.2 mM CaCl₂, 1 mM MgCl₂, and 5 mM glucose. After selection by hand-picking under a microscope, the islets were distributed in batches of 120–150 and preincubated for 2 h in a Krebs-Ringer Bicarbonate (KRB) buffer containing (mM): NaCl 120; KCl 4.7; KH₂PO₄ 1.2; MgSO₄ 1.2; CaCl₂ 2.5; NaHCO₃ 24, and supplemented with 0.1% bovine serum albumin and 1.1 mM glucose. Batches were then incubated for 30 min at 37 °C in KRB supplemented with 16.7 mM glucose with or without PACAP as described in the figure legends. Thereafter, islets were washed with cold PBS, frozen in N₂ liquid, and harvested in lysis buffer (50 mM HEPES, 1% Nonidet P-40, 1 mM Na₃VO₄, 100 mM NaF, 10 mM pyrophosphate, 4 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin) (16) and lysed by sonication.

INS-1E Cell Line Culture—INS-1E cells were grown in RPMI 1640 (11 mM glucose) containing, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES, and 50 µM β-mercaptoethanol as described (30) and supplemented with 7.5% fetal calf serum. INS-1E cells (passages 72–90) at ~70% confluence, were preincubated for 2 h in glucose-free Krebs-Ringer Bicarbonate HEPES buffer (KRBH) (30), with or without inhibitors. Afterward, cells were incubated in KRBH supplemented with different glucose concentrations and other test agents for various times as indicated in the figure legends. Cells were then washed once with cold PBS and harvested in lysis buffer (50 mM HEPES, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 mM pyrophosphate, 100 mM NaF, and 1 mg/ml bacitracin) as described previously (16).

Western Blotting—Cell lysates were clarified by centrifugation (15,000 × g for 30 min at 4 °C). Protein concentrations were determined by the bicinchoninic acid (BCA) method. Equal amounts of proteins were denatured in Laemmli sample buffer, separated through 10% SDS-PAGE, and transferred to nitrocellulose membranes. After blocking, membranes were probed with the appropriate antibody at 4 °C overnight and
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then incubated with horseradish peroxidase-linked secondary antibody followed by enhanced chemiluminescence detection. Autoradiographs were digitized, and the band density was analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

Silencing β-Arrestin 1 Expression Using RNA Interference—Three different 25-nucleotide stealth siRNA double-stranded duplexes were designed to specifically knock down rat β-arr1 expression. These siRNAs and a non-silencing RNA duplex (control siRNA), were used in deprotected and desalted forms. INS-1E cells were transiently transfected with 40 nM siRNA duplex using Lipofectamine™ 2000 according to the manufacturer’s instructions. Briefly, INS-1E cells (400,000 cells per well) were seeded in 6-well plates in antibiotic-free culture medium 1 day before transfection. Then at ~40–50% confluency, cells were transfected in Opti-MEM with Stealth™ RNAsi-Lipofectamine™ 2000 complexes. Six hours after transfection, medium was replaced with complete RPMI medium. All assays were performed at least 70 h after siRNA transfection.

Subcellular Fractionation—After a 2-h preincubation in KRHBH without glucose, INS-1E cells plated in 15-cm dishes (70–80% confluency) were stimulated, or not, with glucose and/or PACAP for 5 or 30 min. Cells were then washed twice with ice-cold PBS and scraped in 1 ml of hypotonic buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCl2, 1 mM MgCl2, 5 mM EDTA, Proteases Inhibitors Mixt). After 15-min incubation, cells were Dounced 40 times on ice and centrifuged at 1000 × g for 5 min at 4 °C. Supernatants containing cytosol and membranes were collected for protein determination before denaturation in Læmmli buffer and Western blot analysis by SDS-PAGE. Pellets containing nuclei were Dounced 30 times in Tris-buffered saline-sucrose/EDTA buffer (10 mM Tris (pH 7.5), 300 mM sucrose, 1 mM EDTA (pH 8), 0.02% Nonidet P-40, Proteases Inhibitors Mixture), centrifuged at 2500 × g for 5 min at 4 °C, and pellets were rinsed twice in 1 ml of TSE buffer. Finally, pellets containing pure nuclei were dissolved in Tris-buffered saline-sucrose/EDTA buffer for protein determination before Western blot analysis by SDS-PAGE.

Immunostaining for p-ERK1/2 in INS-1E Cells—INS-1E cells were grown on glass coverslips for 3 days until 50% confluency. After a 2-h preincubation in KRHBH without glucose, cells were stimulated, or not, with glucose and/or PACAP. Cells were then fixed in 3.7% paraformaldehyde in PBS for 30 min at 4 °C, washed twice with ice-cold PBS and scraped in 1 ml of hypotonic buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCl2, 1 mM MgCl2, 5 mM EDTA, Proteases Inhibitors Mixt). After 15-min incubation, cells were Dounced 40 times on ice and centrifuged at 1000 × g for 5 min at 4 °C. Supernatants containing cytosol and membranes were collected for protein determination before denaturation in Læmmli buffer and Western blot analysis by SDS-PAGE. Pellets containing nuclei were Dounced 30 times in Tris-buffered saline-sucrose/EDTA buffer (10 mM Tris (pH 7.5), 300 mM sucrose, 1 mM EDTA (pH 8), 0.02% Nonidet P-40, Proteases Inhibitors Mixture), centrifuged at 2500 × g for 5 min at 4 °C, and pellets were rinsed twice in 1 ml of TSE buffer. Finally, pellets containing pure nuclei were dissolved in Tris-buffered saline-sucrose/EDTA buffer for protein determination before Western blot analysis by SDS-PAGE.

Luciferase Reporter Gene Assay—The transcriptional activity of CREB (cAMP-response element-binding protein) was evaluated using the PathDetect trans-reporting system, which consists in a fusion trans-activator plasmid expressing the activation domain of CREB fused with the yeast GAL4 DNA binding domain (pFA2-CREB) and a luciferase reporter plasmid containing a synthetic promoter with five tandem repeats of GAL4 binding sites that control expression of the luciferase gene (pFR-Luc). Transient transfections in INS-1E cells were carried out using Lipofectamine™ 2000. Briefly, INS-1E cells (250,000 cells per well) were seeded in 12-well plates with antibiotic-free culture medium 1 day before transfection. Cells at ~50–60% confluency were transfected in Opti-MEM with the reporter (pFR-Luc) and the fusion transactivator (pFA2-CREB) plasmids. After 6-h transfection, medium was replaced with complete culture medium. The following day, after 2 h of quiescence in glucose-free KRHBH buffer with or without 10 μM U0126, transfected cells were incubated for 6 h as indicated in the legend of Fig. 7A. Following the treatment, cells were washed in PBS and harvested in active reporter lysis buffer for 15 min with shaking. Cell debris was removed by centrifugation (12,000 × g for 2 min at 4 °C), and supernatants were stored at −80 °C until luciferase activity assay and protein determinations. Luciferase activity was measured using the enhanced Luciferase Assay kit and the ANALYST™ fluorometer (Molecular Devices). Protein concentrations were determined with the BCA method and used to normalize luciferase activity.

Real-time Quantitative RT-PCR—Total RNA from INS-1E cells was extracted using RNAnow reagent and DNase-treated according to the manufacturer’s instructions. RT-PCR analysis of total RNA was carried out using random hexamer oligo-nucleotides and MoMuLV-RT for reverse transcription. Real-time PCR amplification was performed in duplicate, using the 7500 System (Applied Biosystems) and according to the manufacturer’s instructions. The sequences of the primers used are as follows: rArrb1, TGCGCCAGTATG-CAGACATC/GGCGACTTGTGTACGTGATG; rArrb2, TGTGCCCTTCCGATATG/GCGAAAGACAGCGCCA-GTAC; Irs2, GAAGGTTGACATCTTTGACAT/GAG-AAAAAGCCTTTTGCACATCTGA; Tbp, GTTGACCCACCA-GGCGTTCAG/ATTCGACGGAAATATTCTGGCTACA; B2M, CGTGTGCTTGGATGAAAGAGAGATGGGCACA-GTACAGCTGAAATT; and Tubb2, CAAGGGTTTCTCTGAC-TGG/GAATCCATCGTCCAGGCC.

The selection of the appropriate housekeeping genes was performed using geNorm (31). The level of expression of each gene X was normalized to the geometric mean of the expression levels of three housekeeping genes R (B2M, Tbp, and Tubb2) according to the formula, X/geometric mean (R1, R2, and R3) = 2(Ci/X)− arithmetic mean (Ci, R1, R2, and R3), where Ci is the threshold cycle.

Expression of Data and Statistics—Data were presented as mean ± S.E. of n independent experiments. Statistically significant differences between groups were assessed by Student’s t test or by analysis of variance, followed by the Newman-Keuls test in the case of multiple comparisons. Differences were considered significant at p < 0.05.
**RESULTS**

**PACAP via PAC1R Induces a Rapid and Transient Activation of ERK1/2 and Its Nuclear Translocation**—To investigate the mechanism(s) by which PACAP activates ERK1/2, we assessed ERK1/2 phosphorylation status by Western blotting with phospho-specific antibodies that recognize only Thr-202 and Tyr-204 by ERK1/2 kinases (MEK1/2). To determine the intracellular mechanisms involved in PACAP activation of ERK1/2, we first evaluated the role of the upstream kinases MEK1/2 by using two selective inhibitors, PD98059 (20 μM) and U0126 (10 μM). They completely abolished the PACAP effect on ERK1/2 (supplemental Fig. S1, A and B), indicating the involvement of the upstream MEK1/2 in the PACAP-induced ERK1/2 activation.

On the other hand, it is known that [Ca\(^{2+}\)] is critical to mediate ERK1/2 activation in pancreatic β-cells by physiological stimuli, i.e. glucose and GLP-1 (13, 18, 26). Furthermore, PACAP has been shown to increase [Ca\(^{2+}\)], in a glucose-dependent manner via opening of the VDCCs (1, 33). To determine whether Ca\(^{2+}\) played a role in PACAP-induced ERK1/2 activation, we used the VDCCs blocker, nifedipine. In line with the observation that PACAP had no effect on [Ca\(^{2+}\)], in the absence of glucose (1), the transient activation of ERK1/2 by PACAP was not significantly inhibited by nifedipine (supplemental Fig. S1, C and D). These results suggest that Ca\(^{2+}\) influx via VDCCs is not essential in the PACAP-induced transient activation of ERK1/2.

Spatiotemporal regulation of ERK1/2 activation plays an important role in determining the functions of these kinases (32). To investigate the subcellular distribution of p-ERK1/2, subcellular fractionation and immunofluorescence observed by confocal microscopy analysis were performed. In quiescent INS-1E cells, the majority of ERK1/2 was in the cytosolic fraction (Fig. 1C). However, there was a low but detectable amount of ERK2 in the nuclear fraction. Addition of PACAP induced an accumulation of both ERK1 and ERK2 in the nucleus and also an increase in p-ERK1/2 in both the cytosolic and nuclear fractions suggesting a translocation to the nucleus of a fraction of the cytosolic p-ERK1/2. No effect of PACAP was observed at 30 min (data not shown). In addition, although quiescent INS-1E cells exhibited no staining for p-ERK1/2 (Fig. 1D), we found that ~14 ± 3% of cells were responsive to PACAP and displayed p-ERK1/2 immunofluorescence in both the cytoplasm and the nucleus. Thus, PACAP induces transient ERK1/2 activation in both the cytosol and nucleus.
PAC₁Rs have been shown to preferentially interact with Goᵦ, but several studies have suggested that they could also interact with Goᵦ (8). However, in line with the observation that PAC₁Rs are not coupled to PLC in rat and mouse pancreatic islets (5), PACAP activation of ERK1/2 activity did not depend on the Goᵦ/PLC/PKC pathway. Indeed, PACAP failed to increase inositol phosphate production, and pretreatment with GF109203X (10 μM), a broad spectrum PKC inhibitor, had no significant effect on PACAP activation of ERK1/2 in INS-1E cells (data not shown).

Furthermore, several Goᵦ-coupled receptors, notably the β₂-adrenergic receptor (35), were shown to promote ERK1/2 activation following a switch of coupling from Goᵦ to Goᵦ₁₀. Nevertheless, selective inhibition of Goᵦ₁₀ signaling by overnight treatment with pertussis toxin did not inhibit ERK1/2 activation by PACAP (supplemental Fig. S2, A and B). As a control, pertussis toxin efficiently blocked the inhibition of glucose-stimulated ERK1/2 activation by the Goᵦ₁₀-coupled α₁-adrenergic receptor agonist UK14304 as recently reported (25). Taken together, these results suggest that the PAC₁R mediates ERK1/2 phosphorylation in a Goᵦ/PKA- but not a Goᵦ-coupled or a Goᵦ₁₀-dependent manner.

Receptor tyrosine kinases and non-receptor tyrosine kinases also have been implicated in the ERK1/2 activation by GPCRs (12, 19). We therefore explored whether activation of Src, a non-receptor tyrosine kinase, represented a critical link between PAC₁R activation and ERK1/2 phosphorylation. Pretreatment with 10 μM PP2, a selective inhibitor of the Src family of tyrosine kinases, reduced PACAP-induced ERK1/2 phosphorylation by ~50% (p < 0.01) (Fig. 2, C and D). This inhibition was specific, because pretreatment with PP3, a negative control for PP2, had no effect. Src can activate ERK1/2 by several mechanisms, notably by transactivation of EGFR (12, 19). Here, EGFR transactivation was not involved, because PAC₁R activation was insensitive to the EGFR intrinsic kinase inhibitor AG1478, which completely abolished EGF effects on ERK phosphorylation in parallel control experiments (supplemental Fig. S2, C and D). Remarkably, PACAP activation of ERK1/2 was totally abolished by the co-pretreatment with H89 and PP2 (Fig. 2, E and F), indicating that PAC₁R transiently stimulates ERK1/2 phosphorylation through both a PKA and a tyrosine kinase Src-dependent pathways.

**PAC₁R Acts as a β-Arrestin-coupled Receptor with Glucose**

![Image](https://example.com/image.png)

**FIGURE 2. Molecular mechanisms of PACAP-induced transient ERK1/2 activation.** The indicated inhibitors were added during the last 30 min of the 2-h quiescent period and during the 5 min of PACAP (100 nM) stimulation. A and B, effects of the PKA inhibitors H89 and CMIQ. C and D, effects of the specific Src kinase inhibitor PP2 (10 μM) and its negative control PP3 (10 μM). E and F, additive effect of the PKA inhibitor H89 (10 μM) and the Src kinase inhibitor PP2 (10 μM). Equal amounts of total cellular lysates (30 μg) were separated by SDS-PAGE and probed for phosphorylated substrates of PKA (p-SPKA), p-ERK1/2, or total ERK1/2 as described under "Experimental Procedures." Representative immunoblots (A, C, and E) are shown, and graphs (B, D, and F) show the quantitative analysis of the increase in ERK2 activation over basal. Data are means ± S.E. of 4–8 independent experiments. ***, p < 0.01; ***, p < 0.001 compared with the control treatment (PACAP alone).

We and others have previously demonstrated that PAC₁R induces cAMP production through AC activation in pancreatic islets cells (4, 5, 33), which in turn can lead to PKA activation. Because the cAMP/PKA pathway has been shown to activate the ERK1/2 signaling cascade in β-cells (13–16), we first attempted to evaluate the contribution of the Goₛ/cAMP/PKA pathway in PACAP activation of ERK1/2 by using the PKA inhibitor H89. Western blot analysis with antibodies against phospho-PKA substrates showed that pretreatment with 10 μM but not with 3 μM was necessary to completely inhibit PACAP activation of PKA (Fig. 2A). Moreover, 10 μM H89 reduced PACAP activation of ERK1/2 by only ~50% (p < 0.001) after 5 min of stimulation (Fig. 2B). A similar inhibition was recorded with 3 μM 4-cyano-3-methylisouquinoline, another selective PKA inhibitor (34). In addition, the specificity of H89 for PKA was demonstrated by the inability of 10 μM H89 to alter EGF-induced ERK1/2 phosphorylation (data not shown). The partial suppression of the PACAP-induced ERK1/2 activation by the PKA inhibitors clearly indicates that PACAP activation of ERK1/2 requires PKA but suggests also the involvement of additional pathway(s).
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Under glucose stimulation, PACAP induces a sustained ERK1/2 activation in the cytosol. Cells were submitted to a 2-h quiescent period in KRH buffer without glucose, and then 8.3 mM glucose with or without 100 nM PACAP or VIP was added for various times as indicated. A and B, time course of PACAP-induced phosphorylation of ERK1/2 in the presence of 8.3 mM glucose. Equal amounts of total cellular lysates (30 µg) were separated by SDS-PAGE and probed for p-ERK1/2 or total ERK1/2 as described under "Experimental Procedures." A representative blot (A) is shown, and the graph (B) illustrates the quantitative analysis of the increase in ERK2 activation over basal. Data are means ± S.E. of 4–8 independent experiments. C and D, subcellular distribution of activated ERK1/2 after 5 or 30 min in the absence (−) or presence of 8.3 mM glucose (G) and with 100 nM PACAP (G+P). C, for subcellular fractionation, cytosolic and nuclear fractions were prepared as described under "Experimental Procedures." Equal amounts of nuclear and cytosolic fractions lysates (50 µg) were separated by SDS-PAGE and probed for p-ERK1/2 or total ERK1/2. CREB and α/β-tubulin were used as controls of nuclear and cytosolic fraction purity, respectively. Representative immunoblots of five independent experiments are shown. D, for immunofluorescence studies, cells were fixed, permeabilized, and immunostained for p-ERK1/2, and Hoechst was used to identify nuclear structure as described under "Experimental Procedures." Scale bar = 10 µm.

biphasic activation, we performed experiments at early (5 min) and late (30 min) time points to follow PACAP effect in the presence of 8.3 mM glucose.

Glucose induced a sustained increase of p-ERK1/2 in both cytosolic and nuclear fractions (Fig. 3C), as well as a translocation of a fraction of p-ERK1/2 in the nucleus as previously reported (16, 23, 24). PACAP amplified the glucose-induced p-ERK1/2 in both cytosolic and nuclear fractions at 5 min. In contrast, at 30 min, PACAP potentiated the glucose stimulation only in the cytosol. Similarly, at 30 min, PACAP mainly increased glucose-induced p-ERK1/2 immunofluorescence in the cytoplasm (Fig. 3D). Thus, PACAP alone induces both cytosolic and nuclear transient ERK1/2 activation, whereas in the presence of glucose, it potentiates the cytosolic and long-lasting ERK1/2 activation by glucose.

PACAP Potentiates the Amplifying Pathway of Glucose Involved in Long-lasting ERK1/2 Activation—Glucose exerts its control on β-cells via two major, hierarchical signaling pathways (22): the triggering pathway (K⁺-ATP channel-dependent Ca²⁺ influx with rise in [Ca²⁺]i) and the amplifying pathway (K⁺-ATP channel-independent without further increase of [Ca²⁺]i). In pancreatic β-cells, glucose-mediated ERK1/2 activation depends on glucose metabolism and subsequently on Ca²⁺ influx via VDCCs (15, 17, 18, 23, 24). Indeed, pretreatment with nifedipine, a VDCC blocker (Fig. 4, A and B) decreased glucose-induced ERK1/2 activation at both 5 and 30 min. Nifedipine markedly reduced PACAP potentiation of glucose-induced ERK1/2 activation at 30 min (~70%, p < 0.01), whereas only slightly inhibited the PACAP response at 5 min (~25%, p < 0.05). These results suggest that Ca²⁺ influx generated by glucose metabolism (which corresponds to the triggering pathway) is required for glucose-induced ERK1/2 activation and, subsequently, for PACAP potentiation of the long-lasting ERK1/2 activation. In contrast, the early and transient ERK1/2 activation by PACAP is scarcely dependent on Ca²⁺ influx in agreement with the results we obtained with PACAP alone (supplemental Fig. S1, C and D).

We then tested the possible involvement of the amplifying pathway of glucose. To this aim, we used one classic experimental approach that consists to hold K⁺-ATP channels open with diazoxide and to depolarize β-cells with KCl to induce Ca²⁺ influx and a rise in [Ca²⁺]i (22, 36). Under these conditions diazoxide prevents the K⁺-ATP closure by glucose and therefore its triggering pathway. Treatment with 30 mM KCl and 250 µM diazoxide induced only an early (5 min) increase in ERK1/2 phosphorylation (Fig. 4, C and D). Addition of 8.3 mM glucose did not significantly affect the early stimulation but in contrast, caused a clear ERK1/2 activation at 30 min (lane a versus b: ~3-fold increase, p < 0.001). PACAP caused early ERK1/2 activation independently of the presence of glucose, whereas the late sustained potentiation occurred only in the presence of the sugar (lane a versus c: ~2-fold increase of glucose response, p < 0.01, Fig. 4, C and D). These results show that the glucose-induced long-lasting ERK1/2 activation requires both the triggering pathway (i.e. a rise in [Ca²⁺]i), which is necessary but not sufficient, and the amplifying pathway. In addition, PACAP potentiates the amplifying pathway of glucose involved in long-lasting ERK1/2 activation.

β-Arrestin 1 Is Required for PACAP to Potentiate Glucose-induced Long-lasting ERK1/2 Activation—It is well documented that activation of ERK1/2 by several GPCRs can occur...
mRNA levels via the ERK1/2 signaling pathways using U0126, a specific MEK1/2 inhibitor. To assess the ability of PACAP to enhance CREB transcriptional activity in INS-1E cells, we used a luciferase reporter system to determine CREB activity. As expected, treatment for 6 h with 8.3 mM glucose induced a ~9-fold increase in the activation of the CREB reporter luciferase activity and GLP-1 caused a ~3.5-fold amplification of this response (Fig. 7A). PACAP, similarly to GLP-1, caused an amplification of the glucose response (~3-fold). U0126 (10 μM) significantly decreased the response to glucose alone and with either PACAP or GLP-1 by 30% (p < 0.05), 50% (p < 0.01), and 45% (p < 0.01), respectively. These results demonstrate that, in the presence of glucose, PACAP induces CREB transcriptional activity mainly via the ERK1/2 pathway.

Real-time quantitative RT-PCR was used to measure IRS-2 mRNA levels in INS-1E cells incubated in the presence of 2.8 or 8.3 mM glucose with or without PACAP or GLP-1 for 20 h. Incubation with 8.3 mM glucose significantly increased IRS-2 mRNA levels (~1.5-fold, p < 0.01 (Fig. 7B)). PACAP and GLP-1, ineffective per se at low glucose (not shown), induced comparable potentiation of the glucose-induced increase in IRS-2 mRNA levels by ~1.5-fold (p < 0.01). U0126 blocked these potentiating effects of PACAP and GLP-1. Western blot analysis (Fig. 7C) confirmed that PACAP and GLP-1 increased glucose-induced IRS-2 protein expression (~1.5-fold, p < 0.05). Furthermore, β-arr1 silencing reduced both GLP-1 and PACAP potentiation (p < 0.05), without significantly affecting the effect of glucose. Taken together these results suggest that, through β-arrestins, which are scaffold proteins linking receptors to downstream signaling cascades, including the ERK1/2 pathway (19–21). Compared with G-protein-dependent ERK1/2 activation, which is rapid, transient, and leads to nuclear translocation, the β-arrestin-dependent pathway is usually slower but sustained and confined to the cytosol (19–21). To test whether β-arr1 was involved in PACAP potentiation of glucose-induced long-lasting ERK1/2 activation, we depleted by siRNA the expression of endogenous β-arr1 in INS-1E cells. Among the three β-arr1 siRNA duplexes tested, two (2 and 3) used at 40 nM specifically decreased β-arr1 mRNA and protein expression by ~80% without affecting β-arrestin 2 expression (Fig. 5, A and B). The β-arr1 depletion did not affect ERK1/2 expression level (Fig. 5C). Upon stimulation with 8.3 mM glucose and PACAP, we observed that, whereas β-arr1 depletion had no significant effect on the early phase (5 min) of ERK1/2 activation, it completely abolished PACAP potentiation of the long-lasting ERK1/2 activation by glucose at 30 min (p < 0.01). Similarly, transient ERK1/2 activation (5 min) by PACAP alone was not significantly modified by β-arr1 depletion (supplemental Fig. S3, A and B). Thus, β-arr1 is involved in PACAP potentiation of the long-lasting ERK1/2 activation by glucose.

Biological Relevance of β-Arrestin 1 Involvement in PACAP Potentiation of Glucose-Induced Long-lasting ERK1/2 Activation in Mouse Pancreatic Islets—To strengthen our observations from the INS-1E cell line, experiments were also performed in pancreatic islets isolated from WT and β-arr1 KO mice. As shown in Fig. 6, raising glucose concentration from 1.1 to 16.7 mM induced at 30 min a clear stimulation of ERK1/2 (~2-fold increase) in both WT and β-arr1 KO mouse islets. PACAP (100 nM) amplified the glucose-induced p-ERK1/2 (~1.7-fold increase, p < 0.01) in WT mouse islets but had no effect in β-arr1 KO mouse islets. These results confirm the data obtained in the INS-1E cell line and give evidence that PACAP potentiation of glucose-induced long-lasting ERK1/2 activation via a β-arrestin 1-dependent pathway is physiologically relevant.

Functional Role of β-arr1-dependent Signaling in PACAP-induced IRS-2 Expression—In pancreatic β-cells, the transcription factor CREB can be activated by glucose and GLP-1 either directly through PKA or indirectly through ERK1/2 via p90RSK (37, 38). In addition, it is known that IRS-2 is a CREB target gene, which is crucial for β-cell survival (39). Thus, we next investigated whether PACAP could increase CREB activity or IRS-2 mRNA levels in the presence of glucose, PACAP induces CREB transcriptional activity mainly via the ERK1/2 pathway.
in the presence of glucose, PACAP increases IRS-2 expression through the β-arrestin 1-dependent sustained ERK1/2 activation.

**DISCUSSION**

In pancreatic β-cells, PACAP is a parasympathetic neuropeptide, which potentiates insulin secretion in a glucose-dependent manner through PAC1Rs and VPACRs (33). These receptors are GPCRs mainly coupled to Gs protein and AC activation resulting in strong activation of the cAMP/PKA signaling pathway. Several members of the glucagon/GLP-1 superfamily, including PACAP, have been shown to activate the MEK/ERK pathway (13–18). ERK1/2 activation resulting in strong activation of the cAMP/PKA signaling pathway (42). In pancreatic β-cells, PAC1Rs and VPACRs in pancreatic β-cells (2, 5, 40). Both peptides are equipotent in increasing glucose-induced insulin secretion and cAMP production, but PACAP is more efficient than VIP on the latter (5). In the current study, we show that PACAP interacts only with PAC1R to stimulate the MEK/ERK1/2 cascade. In pancreatic β-cells, PAC1R is highly expressed, and binding studies indicate that PAC1R prevails over VPACR (41). The functional role of PAC1R has been confirmed in PAC1R-deficient mice, and we have shown that PAC1R deficiency is associated with decreased glucose-induced insulin secretion (40).

PACAP alone induces a fast and transient (5 min) ERK1/2 activation that is partially PKA-dependent. Most of the neurotrophic effects of PACAP are preferentially mediated by PAC1Rs through the cAMP/PKA/ERK1/2 signaling pathway (42). In pancreatic β-cells, several others members of the PACAP superfamily such as GLP-1, gastric inhibitory polypeptide, and glucagon, have been shown to activate the MEK/ERK pathway (13–18). ERK1/2 activation by either gastric inhibitory polypeptide or glucagon has been reported to be fully dependent on PKA (14, 16), whereas...
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FIGURE 7. Depletion of β-arrestin 1 blocks PACAP-induced IRS-2 expression. A, measurement of luciferase activity in INS-1E cells transiently co-transfected with pFR-Luc and pFA2-CREB as described under “Experimental Procedures.” 18 h after transfection, cells were submitted to a 2-h quiescent period in KRBH buffer with or without U0126 (10 μM) and then treated with 8.3 mM glucose with or without 100 nM PACAP or GLP-1 for 6 h. Data are means ± S.E. of five independent experiments. *, p < 0.05; **, p < 0.01. B, measurement of IRS-2 mRNA levels in INS-1E cells. After a 2-h quiescent period with or without U0126 (10 μM), cells were treated in the presence of 2.8 or 8.3 mM glucose with or without PACAP or GLP-1 (100 nM) for 20 h. IRS-2 mRNA level was determined by quantitative RT-PCR analysis and normalized to the geometric mean of the expression levels of three housekeeping genes (B2M, TBP, and Tubb2) as described under “Experimental Procedures.” Data are means ± S.E. of four independent experiments. *, p < 0.05; **, p < 0.01. C and D, INS-1E cells were transiently transfected with the β-arrestin 1 siRNA duplex 2 or the control siRNA duplex. After 70 h following transfection and a 2-h quiescent period, INS-1E cells were treated in the presence of 2.8 or 8.3 mM glucose for 20 h with or without 100 nM PACAP or GLP-1. Equal amounts of total cellular lysate (30 μg) were separated by SDS-PAGE and probed for IRS-2 or α/β-tubulin as described under “Experimental Procedures.” Representative blots (C) are shown, and the graph (D) represents the quantitative analysis of the increase in IRS-2 protein expression over basal. Data are means ± S.E. of four independent experiments. *, p < 0.05.

Glucose has been previously shown to activate ERK1/2 in the cytosol and to cause their nuclear accumulation (23, 24). Here, subcellular fractionation and immunofluorescence studies show that glucose leads to changes in the spatiotemporal activation of ERK1/2 by GLP-1 is partially or not all linked to PKA.

It is acknowledged that GPCRs activate the Raf/MEK1/2/ERK1/2 cascades through G-protein- and β-arrestin-depent pathways (19–21). The G-protein-dependent pathway is mediated by classic G-protein-stimulated production of their respective second messenger-dependent kinases (Gα via cAMP/PKA, Gαq via PLC and PKC) or by other pathways, including non-receptor tyrosine kinases such as Src and/or receptor tyrosine kinases such as EGFRs. Because PAC₃Rs are not coupled to PLC in rodent pancreatic islets (5) and in INS-1E cells (not shown), we rule out any implication of the Gα/PLC/PKC. Indeed, PKC inhibition has no effect on the PACAP-stimulated ERK1/2 activation in INS-1E cells. In contrast to some Gα-coupled receptors, notably β₂-adrenergic receptor (35), the PAC₃R-induced ERK1/2 activation is also independent from a pertussis-toxin-sensitive Gαᵣ₁ protein. On the other hand, we show that besides the Gα/cAMP/PKA pathway, PACAP activation of the ERK1/2 cascade also depends on the Src family of protein-tyrosine kinase. The involvement of Src is not due to transactivation of EGFR, because PACAP activation is insensitive to inhibition of the EGFR intrinsic kinase. Src has been shown to associate with either β-arrestin recruited to phosphorylated GPCRs by G-protein-coupled receptor kinases (43), or with the α subunit of Gs and Gi (44), or directly with GPCRs (45). Important roles of Src have been reported in ERK1/2 activation by various GPCRs (46, 47) through G-proteins and/or β-arrestin-dependent or -independent mechanisms. In our study, a β-arrestin 1-dependent mechanism in the Src recruitment could be ruled out because β-arrestin depletion by siRNA did not affect PACAP-induced transient ERK1/2 activation (supplemental Fig. S3, A and B). Further studies are therefore required to determine the mechanisms involved in Src recruitment.

Pancreatic β-cells are tightly controlled by glucose, which acts via its oxidative metabolism by generating a triggering pathway that consists of K⁺-ATP channels closure, depolarization, and rise in [Ca²⁺]ᵣ, and an amplifying pathway that is characterized by increased efficacy of Ca²⁺ (22). The amplifying pathway serves the action not only of glucose but also of other stimuli. Whereas the second messengers involved in the triggering pathway are well characterized, the underlying messengers involved in the amplifying pathway are still unknown (48). The fine mechanism of activation of ERK1/2 by glucose is still indeterminate (27). After confirming that glucose causes a slow, progressive, and sustained ERK1/2 phosphorylation (17, 18, 24), we show that calcium is essential, because this sustained ERK1/2 activation is prevented by inhibition of Ca²⁺ influx through VDCCs in agreement with previous studies (17, 18, 23, 24). However, we clearly show that calcium is not sufficient, because the sustained glucose action was not reproduced by KCl depolarization (which produces the triggering signal). Finally, we demonstrate that glucose-induced sustained ERK1/2 activation requires the amplifying pathway, because it is observed also during KCl depolarization even when glucose is unable to close K⁺-ATP channels in the presence of diazoxide. Then we show that PACAP potentiation of glucose-induced long-lasting ERK1/2 activation requires not only Ca²⁺ influx through VDCCs, as expected, but also the amplifying pathway of glucose. Thus, PACAP clearly potentiated the amplifying pathway of glucose-induced long-lasting ERK1/2 activation.

The kinetics and subcellular localization of activated ERK1/2 are the major factors determining their cellular responses (32). Glucose has been previously shown to activate ERK1/2 in the cytosol and to cause their nuclear accumulation (23, 24). Here, subcellular fractionation and immunofluorescence studies show that glucose leads to changes in the spatiotemporal...
mediated ERK1/2 activation is rapid and transient and results in nuclear translocation. In contrast, β-arrestin-mediated ERK1/2 activation is slower, sustained, and sequestered in the cytosol. Here we show that β-arrestin 1 is required for PACAP potentiation of the long-lasting ERK1/2 activation by glucose in both INS-1E cells and, more interestingly, in mouse pancreatic islets. In contrast, the early (5 min) ERK1/2 activation by PACAP alone or in the presence of glucose is insensitive to β-arrestin knockdown. In addition to their role as signaling scaffold proteins, β-arrestins were initially identified to be involved in the desensitization of GPCRs by facilitating their internalization by endocytosis. However, in our study β-arrestin 1 depletion did not clearly enhance the early (5 min) ERK1/2 activation by PACAP suggesting that β-arrestin 1 does not play a major role in the desensitization of PAC1R. GPCRs are divided into two classes according to the type of interaction they have with β-arrestins (49). Class A receptors exhibit a transient association with β-arrestins and dissociate rapidly after internalization. By contrast, class B receptors stay associated with β-arrestins after internalization promoting the formation of stable receptor-β-arrestin-ERK1/2 complexes and thus sustained ERK1/2 activation. The stability of receptor-β-arrestin complexes is differentially regulated depending on the type of G-protein-coupled receptor kinases that mediate the interaction of β-arrestin with the activated GPCRs, on the ubiquitylation, or on Ser-412 phosphorylation of β-arrestin (20, 46, 50). The potential role of glucose in the control of such devices in the PAC1R-mediated sustained ERK1/2 activation should be addressed in future studies.

Finally, we assessed a functional role of β-arrestin 1 in PACAP effect on ERK1/2 activation. In pancreatic β-cells, in addition to directly activate nuclear transcription factors, ERK1/2 can target numerous cytoplasmic substrates such as p90RSK (51), which after phosphorylation is translocated to the nucleus and subsequently activates transcription factors such as CREB (52). In pancreatic β-cells, CREB plays an essential role in the control of β-cell survival and growth by glucose or GLP-1 in part by regulating IRS-2 gene expression (39). In addition, we previously showed that ERK1/2 control CREB transcriptional activity (37). Here, we show that PACAP, like GLP-1, potentiates glucose-induced CREB transcriptional activity and increases IRS-2 mRNA largely via the MEKs/ERK1/2 cascade. In addition, both PACAP- and GLP-1-induced IRS-2 protein expression are markedly reduced by β-arrestin 1 depletion. Thus, the β-arrestin 1-dependent pathway of ERK1/2 activation plays a key role in PAC1R and GLP-1Rs signaling leading to IRS-2 expression. Interestingly, it has been recently reported that β-arrestin 1 modulates GLP-1 functions such as ERK1/2 and CREB activation and insulin secretion, in the pancreatic β-cell line INS-1 (53). However, in this study, basal IRS-2 protein level was directly down-regulated by β-arrestin 1 silencing through an unknown mechanism. Because IRS-2 plays a pivotal role in the maintenance of mass and function in pancreatic β-cells (54, 55), the β-arrestin 1-dependent pathway of PAC1R potentiation of glucose-induced sustained ERK1/2 activation could be relevant in the PAC1R action required for a normal glucose-stimulated insulin secretion (40) as well as in the long term protective effects of PACAP on β-cell mass reported in various mouse models of diabetes (10, 11).

In summary, our study shows that PAC1R-mediated ERK1/2 activation is tightly controlled by glucose in the INS-1E cell line and pancreatic islets. So, independently of the presence of glucose, PACAP induces a rapid and transient ERK1/2 activation in both cytosol and nucleus. In contrast, in the presence of glucose, PACAP potentiates the glucose-induced long-lasting ERK1/2 activation in the cytosol. Moreover, we demonstrate that the β-arrestin 1-dependent pathway mediates PAC1R potentiation of both the cytosolic sustained ERK1/2 activation and IRS-2 expression induced by glucose. This emerging mechanism of GPCRs action, which is dependent on the amplifying pathway of glucose, could be important for the preservation of β-cell functional mass and may represent a new therapeutic approach for the treatment of diabetes.

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