Antagonism of Rat β-Cell Voltage-dependent K⁺ Currents by Exendin 4 Requires Dual Activation of the cAMP/Protein Kinase A and Phosphatidylinositol 3-Kinase Signaling Pathways

Received for publication, July 15, 2003, and in revised form, October 3, 2003
Published, JBC Papers in Press, October 16, 2003, DOI 10.1074/jbc.M307612200

Patrick E. MacDonald‡‡, Xiaolin Wang‡‡, Fuzhen Xia†, Wasim El-kholy††‡‡, Elisha D. Targonsky‡, Robert G. Tsushima‡‡, and Michael B. Wheeler‡‡††
From the Departments of ‡Physiology and †Medicine, University of Toronto, Toronto, Ontario M1H 1E6, Canada

Antagonism of voltage-dependent K⁺ (Kv) currents in pancreatic β-cells may contribute to the ability of glucagon-like peptide-1 (GLP-1) to stimulate insulin secretion. The mechanism and signaling pathway regulating these currents in rat β-cells were investigated using the GLP-1 receptor agonist exendin 4. Inhibition of Kv currents resulted from a 20-mV leftward shift in the voltage dependence of steady-state inactivation. Blocking cAMP or protein kinase A (PKA) signaling (R-8-cAMP and H-89, respectively) prevented the inhibition of currents by exendin 4. However, direct activation of this pathway alone by intracellular dialysis of cAMP or the PKA catalytic subunit (cPKA) could not inhibit currents, implicating a role for alternative signaling pathways. A number of phosphorylation sites associated with phosphatidylinositol 3 (PI3)-kinase activation were up-regulated in GLP-1-treated MIN6 insulinoma cells, and the PI3 kinase inhibitor wortmannin could prevent antagonism of β-cell currents by exendin 4. Antagonists of Src family kinases (PP1) and the epidermal growth factor (EGF) receptor (AG1478) also prevented current inhibition by exendin 4, demonstrating a role for Src kinase-mediated trans-activation of the EGF tyrosine kinase receptor. Accordingly, the EGF receptor agonist betacellulin could replicate the effects of exendin 4 in the presence of elevated intracellular cAMP. Downstream, the PKCζ pseudosubstrate inhibitor could prevent current inhibition by exendin 4. Therefore, antagonism of β-cell Kv currents by GLP-1 receptor activation requires both cAMP/PKA and PI3 kinase/PKCζ signaling via trans-activation of the EGF receptor. This represents a novel dual pathway for the control of Kv currents by G protein-coupled receptors.

Voltage-dependent K⁺ (Kv) channels are important regulators of membrane potential in excitable tissues where they generally mediate action potential repolarization (1). In pancreatic islet β-cells, Kv channels repolarize glucose-stimulated action potentials, limit entry of Ca²⁺ through voltage-dependent Ca²⁺ channels, and therefore act as negative regulators of insulin secretion (2). Recent work by us and others (3–5) demonstrates the importance of Kv channels, particularly Kv2.1, in the regulation of β-cell excitability and insulin secretion. Importantly, because β-cell Kv channels are closed under resting conditions, the excitatory and insulinotropic effects of Kv channel antagonists are glucose-dependent (6, 7).

Glucagon-like peptide-1 (GLP-1) is secreted by intestinal L-cells in response to nutrient ingestion (8). Although known to exert effects on cell growth and proliferation, satiety, and intestinal motility, the most well recognized action of GLP-1 is to enhance insulin secretion from pancreatic islet β-cells (9). GLP-1 and its analogues are under intense investigation as potential treatments for type-2 diabetes, because their insulinotropic effect is dependent upon elevated glucose, avoiding the potentially dangerous complication of hypoglycemia (10). The actions of GLP-1 are mediated by the G protein-coupled GLP-1 receptor and result from effects on many targets within the β-cell, the most well characterized being the cAMP and PKA-dependent inhibition of KᵥATP channels (9). Recent evidence also demonstrates a cAMP-dependent and PKA-independent component to the insulino-tropic effect of GLP-1, mediated by elevated Ca²⁺-induced Ca²⁺ release from the endoplasmic reticulum via activation of cAMP guanine nucleotide exchange factor II (Epac2) (11, 12).

Recently (13), we reported that GLP-1 and the GLP-1 receptor agonist exendin 4 antagonizes Kv currents in rat β-cells (13). Because this likely contributes to the insulino-tropic effect of GLP-1, particularly the glucose dependence, we investigated the mechanism of Kv current reduction and the signal transduction pathway(s) involved. We show that exendin 4 antagonizes Kv currents in rat β-cells by causing a hyperpolarizing shift in the voltage dependence of steady-state inactivation. We further demonstrate that antagonism of Kv channels by exendin 4 depends on activation of both the cAMP/PKA and phosphatidylinositol 3 (PI3)-kinase/PKCζ signaling pathways. Activation of PI3 kinase can result from an Src kinase-mediated trans-activation of the EGF receptor. This study provides

* This work was supported in part by research grants from the Canadian Institutes of Health Research (CIHR; MOP-49521) and the Canadian Diabetes Association (to M. B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† CIHR Fellow. Present address: Dept. of Molecular and Cellular Physiology, Lund University, Lund 22184, Sweden.
‡ Supported by a CIHR New Emerging Team Grant in Diabetes Complications.
‡‡ Supported by a Novo Nordisk Studentship from the Banting and Best Diabetes Centre.
§§ CIHR Investigator. To whom correspondence should be addressed: Dept. of Physiology, University of Toronto, 1 Kings College Circle, Rm. 3352, Toronto, Ontario MSS IA6, Canada. Tel.: 416-978-6737; Fax: 416-978-4940; E-mail: michael.wheeler@utoronto.ca.

1 The abbreviations used are: Kv, voltage-dependent K⁺; GLP-1, glucagon-like peptide-1; PKA, protein kinase A; PI3, phosphatidylinositol 3; PKC, protein kinase C; EGF, epidermal growth factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; MOPS, 4-morpholinepropanesulfonic acid; MAP, mitogen-activated protein; MEK, MAP kinase/extracellular signal-regulated kinase.

This paper is available on line at http://www.jbc.org
a novel dual-signal pathway mechanism for the regulation of Kv currents. Additionally, the present work suggests a potential mechanism contributing to the glucose dependence of the insulinotropic effect of GLP-1.

EXPERIMENTAL PROCEDURES

Regents—Exendin 4 and GLP-1 were from Bachem (Torrance, CA). AG1428 and PP1 were from Biomol (Plymouth Meeting, PA). Betacelulvin, cAMP, GMP-PNP, H-89, insulin, MgATP, DP890859, Rp-cAMPS, and tetrathyreolumium were from Sigma-Aldrich. Bisindolylmaleimide, calphostin C, rapamycin, TPA, wortmannin, the constitutively active PKA catalytic subunit, and the PKA_2 pseudosubstrate were from Calbiochem, S-ppCys-2'-O-Me-cAMP was from BIOLOG Life Science Institute (Bremen, Germany). When necessary, reagents were dissolved in dimethyl sulfoxide (Sigma-Aldrich). Final solutions contained no greater than 0.01% dimethyl sulfoxide, and control solutions contained the same when appropriate. Anti-V5 antibody (1:10000) was from Sigma-Aldrich, anti-Kv2.1 antibody (1:1000) was from Upstate (Charlottesville, VA), and anti-EGF receptor antibody (1:1000) was from Cell Signaling Technology (Beverly, MA). EGF receptor (erbB1)-specific primers were as follows: forward, 5'-ACCTGTTGAGAAAGTGGCC-3'; and reverse, 5'-ACCTGCCAGATGTAAGC-3'.

Iletod Isolation and Cell Culture—Male Wistar rats (250–300 g), p110–/– mice (from J. M. Penninger and P. H. Backx, University of Toronto), and P19 cells (GLP-1 receptor /–/- mice (from D. J. Drucker, University of Toronto) were anesthetized by intraperitoneal injection of ketamine hydrochloride and xylazine (100 and 20 mg/kg) and sacrificed by exsanguination according to University of Toronto guidelines. Ilets of Langers were isolated by collagenase digestion and dispersed to single cells as described previously (5, 14). Cells were plated on glass coverslips in 35-mm dishes in EMPI 1640 medium with 25 mm glucose, 0.25% HEPES, 7.5% fetal bovine serum, 100 units/ml penicillin G sodium, 100 ppm/ml streptomycin sulfate (penicillin-streptomycin from In Vitro) and cultured for 1–3 days prior to electrophysiological recordings. MIN6 insulinoma cells (passage 35–40), from S. Seino (Chiba University, Chuo-ku, Japan), were cultured in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 ppm/ml streptomycin sulfate, and β-mercaptoethanol (2 ppm/500 ml) at 37°C and 5% CO_2. HIT-T15 cells (passage 80–95), from R. P. Robertson (Pacific NW Research Institute, Seattle, WA), were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% l-glutamine, 100 units/ml penicillin G sodium, and 100 ppm/ml streptomycin sulfate (penicillin-streptomycin from In Vitro) at 37°C and 5% CO_2.

Electrophysiology—Cells were patch-clamped in the whole-cell configuration using an EPC-9 amplifier and PULSE software (HEKA Electronik, Lambrecht, Germany). Patch pipettes were prepared from 1.5-mm thin walled borosilicate glass tubes using a two-stage micropipette puller (Sutter, Novato, CA). The mixture was centrifuged for 10 s at 15000 x g, and the pellet was washed twice with washing buffer (50 mm NaHPO_4, pH 8.0, 150 mm NaCl, 0.5% Triton X-100, 100 mm imidazole). Fifty ml of lysate was saved as the non-immunoprecipitated sample, whereas 25 ml of a 50% slurry of nickel-nitritriacetic acid resin (QIAGEN) was added to the remainder and mixed gently for 2 h at 4°C. The mixture was centrifuged at 10 s at 15000 x g, and the pellet was washed twice with washing buffer (50 mm NaHPO_4, pH 8.0, 150 mm NaCl, 0.5% Triton X-100, 250 mm imidazole).

Immunoprecipitation and Western Blotting—HIT-T15 cells, plated in 10-cm culture dishes, were transfected with a construct expressing a V5-His-tagged GLP-1 receptor in the pcDNA3.1-V5-His vector (Invitrogen) using LipofectAMINE 2000 (Invitrogen). Thirty h after transfection, cells were washed with ice-cold phosphate-buffered saline and lysed with 0.3 ml of lysis buffer (50 mm NaHPO_4, pH 8.0, 150 mm NaCl, 0.5% Triton X-100, 100 mm imidazole). Fifty ml of lysate was saved as the non-immunoprecipitated sample, whereas 25 ml of a 50% slurry of nickel-nitritriacetic acid resin (QIAGEN) was added to the remainder and mixed gently for 2 h at 4°C. The mixture was centrifuged at 10 s at 15000 x g, and the pellet was washed twice with washing buffer (50 mm NaHPO_4, pH 8.0, 150 mm NaCl, 0.5% Triton X-100, 250 mm imidazole). Protein was eluted with 3 x 35 ml of elution buffer (50 mm NaHPO_4, pH 8.0, 150 mm NaCl, 0.5% Triton X-100, 250 mm imidazole).

Immunoblotting was performed as described previously (5). Twenty-five pg of protein from each sample was separated on a 10% polyacrylamide gel and transferred to PVDF-Plus™ membrane (Fish). Primary antibodies (see above) were detected with appropriate secondary antibodies (goat anti-rabbit, 1:3000 and donkey anti-rabbit, 1:7500; Amersham Biosciences) for 1 h at room temperature. Visualization was by chemiluminescence (ECL; Amersham Biosciences) and exposure to Kodak film (Eastman Kodak Co.) for 5 to 10 min.

Phospho-site Screen—MIN6 cells were grown in monolayer to 70–80% confluence in 10-cm dishes, and the cells were washed twice with phosphate-buffered saline and pre-incubated for 30 min with a Krebs-Ringer bicarbonate buffer containing the following (in mM): NaCl, 115; KCl, 5; NaHCO_3, 24; CaCl_2, 2.5; MgCl_2, 1; HEPES, 10; glucose, 5; and 0.1% (w/v) bovine serum albumin at 37°C and 5% CO_2. The cells were washed twice again with Krebs-Ringer bicarbonate buffer and incubated in the same buffer, or with or without 7 x 10^-5 M GLP-1 for 10 min. Whole cell protein lysates were extracted from MIN6 cells treated with GLP-1 using lysis buffer containing the following (in mM): MOPS, 20; EGTA, 2; EDTA, 5; sodium fluoride, 30; β-glycerophosphate, 40; sodium pyrophosphate, 10; sodium orthovanadate, 2; phenylmethylsulfonyl fluoride, 1; benzamidine, 3; pepstatin A, 0.005; leupeptin, 0.01; and 0.5% Nonidet P-40 and 0.5% Triton X-100 at pH 7.0. The phosphorylation levels of 40 sites were screened by Kinetics™ KPPS 1.1 (Rikenzex, Vancouver, Canada).

RESULTS

Antagonism of β-Cell Kv Currents by GLP-1 and Exendin 4—We have shown recently (4, 5) that Kv channels are important glucose-dependent regulators of insulin secretion and can be regulated by GLP-1 receptor activation in rat β-cells (13). Because GLP-1 antagonizes Kv currents in β-cells, we investigated whether the GLP-1 receptor could physically associate with Kv2.1, a prominent β-cell Kv channel (3–5). A V5-His-tagged GLP-1 receptor construct was expressed in HIT-T15 insulinoma cells and immunoprecipitated by binding to a nickel-nitritriacetic acid resin. HIT-T15 cells were used, because these express low levels of endogenous GLP-1 receptor (17) but abundant Kv2.1 protein (5). Western blotting for Kv2.1 and V5-His-tagged GLP-1 receptor demonstrated the abundant expression of Kv2.1 and no expression of the GLP-1 receptor construct in whole lysates from untransfected HIT-T15 cells (Fig. 1A, lane 1). Whole lysates from HIT-T15 cells transfected with the GLP-1 receptor construct showed abundant expression of both Kv2.1 and the GLP-1 receptor (Fig. 1A, lane 2).

Immunoprecipitation of lysates from untransfected HIT-T15 cells did not pull down Kv2.1 protein (Fig. 1A, lane 3), whereas Kv2.1 could be pulled down from lysates of cells expressing the tagged GLP-1 receptor (Fig. 1A, lane 4).

Rat β-cells were voltage-clamped in the whole-cell configuration at near-physiological temperatures (32–35°C). Outward voltage-dependent K⁺ currents were similar to those we have reported previously (4, 13, 18) (Fig. 1B) and could be blocked with the general Kv channel antagonist tetraethylammonium.
Fig. 1. Kv2.1 binding by the GLP-1 receptor and antagonism of rat β-cell Kv currents by GLP-1. In panel A, Western blot for Kv2.1 (anti-Kv2.1 antibody) and V5-His tagged GLP-1 receptor (anti-V5 antibody) was performed on whole cell lysates (lanes 1 and 2) and immunoprecipitates of a V5-His-tagged GLP-1 receptor construct (lanes 3 and 4). Lane 1, whole cell lysates from untransfected HIT-T15 cells. Lane 2, whole cell lysates from HIT-T15 cells transfected with the tagged GLP-1 receptor construct. Lane 3, immunoprecipitates from untransfected HIT-T15 cells. Lane 4, immunoprecipitates from HIT-T15 cells transfected with the GLP-1 receptor construct. In panel B, outward voltage-dependent K+ currents were elicited from rat β-cells voltage-clamped in the whole cell configuration at 32–35°C. Currents could be antagonized by the general Kv channel antagonist tetraethylammonium (TEA) (20 mM) or by GLP-1 (10–8 M). The time course for the effect of GLP-1 is shown in panel C, where GLP-1 (10–8 M) was added at 0 min, * p < 0.05; ** p < 0.001 compared with time = 0.

(15 mM) (Fig. 1B). Maximum steady-state outward currents were blocked by 40.8 ± 4.1% (n = 9, p < 0.001) upon perfusion of GLP-1 (10–8 M) (Fig. 1B). To ensure that currents were stable, control currents were recorded every min for ~5 min prior to addition of GLP-1. Kv currents were maximally inhibited at ~6 min after exposure to GLP-1 (Fig. 1C). Partial washout of this effect was observed (not shown). However, because of technical difficulties in recording from cells at 32–35°C for prolonged periods (>20 min), experiments were not of sufficient duration to observe complete washout.

Subsequent experiments were performed using the GLP-1 receptor agonist exendin 4, which has a recently demonstrated therapeutic relevance in clinical trials (19). Confirming our previous studies (18), exendin 4 (10–8 M) inhibited voltage-dependent outward K+ currents from rat β-cells by 43.4 ± 6.3% (n = 7, p < 0.001) (Fig. 2A). At a concentration two orders of magnitude lower (10–10 M), exendin 4 antagonized rat β-cell voltage-dependent K+ currents by 20.8 ± 3.9% (n = 4, p < 0.05). The current activation time constant in the presence of 10–8 M exendin 4 (τa = 3.14 ± 0.27 ms, n = 7) was not significantly different from controls (τa = 2.69 ± 0.22 ms, n = 7) (Fig. 2B). We reported previously (18) that rat β-cell Kv currents inactivate with both fast (τf) and slow (τs) time constants at 32–35°C (18). Neither τf nor τs in the presence of 10–8 M exendin 4 (τf = 303.4 ± 45.1 ms and τs = 2.00 ± 0.20 s, n = 7) were different from controls (τf = 258.5 ± 54.5 ms and τs = 2.52 ± 0.42 s, n = 7) (Fig. 2B). The contribution of fast inactivation to total inactivation was also not changed (41.2 ± 5.9 versus 36.7 ± 2.9%, n = 7). Also, the voltage dependence of current activation under control conditions (V50a = −7.57 ± 4.31 mV, n = 7) was not significantly different after treatment with 10–8 M exendin 4 (V50a = −9.77 ± 5.15 mV, n = 7) (Fig. 2C). Interestingly, the reduction in Kv currents results from a ~20-mV hyperpolarizing shift in the voltage dependence of steady-state inactivation from −43.4 ± 3.2 to −66.6 ± 5.1 mV (n = 4 and 3, p < 0.01) (Fig. 2C).

cAMP/PKA Signaling Is Necessary but Not Sufficient for Antagonism of Kv Currents by Exendin 4—the importance of cAMP/PKA signaling for the insulinotropic effect of GLP-1 receptor activation is well known (9). To investigate the involvement of cAMP and PKA signaling in the inhibitory effect of exendin 4 on Kv currents, β-cells were pre-treated (30 min) with either the cAMP antagonist Rp-cAMPs (100 μM, n = 5) or the PKA antagonist H-89 (1 μM, n = 6). Voltage-dependent
Regulation of β-Cell Kv Currents

Phospho-site Screening Identifies PI3 Kinase as an Additional Candidate Pathway—To identify candidate signaling pathways that may be involved in the GLP-1 receptor-mediated reduction of voltage-dependent K⁺ currents, MIN6 insulinoma cells were treated with GLP-1 (10⁻⁸ M) for 10 min, and protein lysates were screened with multiple phospho-site-specific antibodies using the Kinetronics™ KPPS 1.1 screen. MIN6 insulinoma cells were used in this case, because they allow for preparation of the necessary amount of protein lysates for the phospho-screen, and in our hands they exhibit good insulin secretory responses to GLP-1 (~1.5-fold).

Two separate experiments were conducted, and the fold increases in phosphorylation of 40 sites on 30 different proteins are shown in Table I. Interestingly, a number of known PDK1 phosphorylation sites are up-regulated. These include Akt ser-473 and Thr-308, PKCαβ Thr-638/Thr-641, and PKCδ Thr-505 (Table I). Because PDK1 is a direct effector of PI3 kinase signaling, these results may be taken to suggest that this pathway is activated by GLP-1. Activation of PI3 kinase by GLP-1 is supported by the work of others (22–24). Additionally, PI3 kinase signaling may be involved in activation of the MAP kinase pathway either through the ability of PI3 kinase itself to phosphorylate proteins or through the recruitment of small G-proteins (25, 26). Notably the MEK1/2 Ser-221/225 site was phosphorylated, whereas the MEK3 Ser-189/Thr-197 site was paradoxically dephosphorylated. This may be significant, because MEK1 phosphorylation, through the activation of the p110y isoform of PI3 kinase, has been implicated in the activation of MAP kinase (26), although it is not clear whether PI3 kinase can phosphorylate MEK1 directly. Although the present phospho-screen was unable to detect phosphorylated MAP kinase, others have implicated the activation of this pathway as the mechanism for the effects of GLP-1 on β-growth and differentiation (22, 24, 27). In the experiments below, the activation of the PI3 kinase signaling pathways is confirmed and investigated further using pharmacological antagonists.

PI3 Kinase and PKCζ Activity Are Necessary for Regulation of Kv Channels by Exendin 4—In support of a role for PI3 kinase signaling, the effect of exendin 4 on Kv currents was completely abolished by wortmannin (100 nM, n = 6), a PI3 kinase inhibitor (Fig. 4). Confirmation of this result with the structurally unrelated PI3 kinase antagonist LY294002 was not possible, because this compound is also a potent and direct antagonist of Kv channels (28). The downstream effectors of PI3 kinase responsible for regulation of Kv currents by GLP-1 receptor activation were investigated using a number of pharmacological antagonists. Antagonism of p70 S6 kinase (rapamycin, 10 nM, n = 6), MEK1 (PD98059, 20 μM, n = 6), and conventional PKC isofoms (calphostin C, 500 nM, n = 7) failed to prevent the reduction of Kv currents in rat β-cells by exendin 4 (10⁻⁸ M) (Fig. 4). Possible downstream effectors of PI3 kinase include atypical PKC isofoms, because the effect of exendin 4 could also be prevented by bisindolylmaleimide (100 nM, n = 7) (Fig. 4), which antagonizes PKC at the ATP binding site (29, 30). However, it is important to note that this compound alone was capable of inhibiting β-cell Kv currents (Fig. 4). We investigated a role for PKCζ, because this isofom is known to be activated by GLP-1 (22) and associate with Kv channel regulatory subunits (31), and phosphorylation at a conserved PKC

Regulation of β-Cell Kv Currents

**Fig. 3.** Signaling through cAMP and PKA is necessary but not sufficient for the effect of exendin 4 on rat β-cell Kv currents. Maximum sustained outward K⁺ current was measured as the average current during the last 25 ms of a 500-ms depolarizing pulse from −70 to +70 mV in rat β-cells voltage-clamped in the whole cell configuration at 32–35 °C and normalized to controls. In panel A, the reduction in current caused by treatment with exendin 4 (10⁻⁸ M; black bars) could be prevented by pre-treatment of cells with Rp-cAMPs (100 μM) or the PKA inhibitor H-89 (1 μM). In panel B, activation of G-proteins with GMP-PNP (10 nM) could replicate the effect of exendin 4 treatment, whereas cAMP (100 μM), the constitutively active PKA catalytic subunit (200 units/ml), and a selective Epac activator (50 μM) could not. The small effect of forskolin (5 μM) and isobutylmethylxanthine (IBMX) (100 μM) can be attributed to a direct effect of forskolin on Kv channels. *, p < 0.05; ***, p < 0.001 compared with pre-exendin 4 and control.

outward K⁺ currents in cells pre-treated with either antagonist were not different from untreated cells and could not be antagonized significantly by treatment with exendin 4 (10⁻⁸ M) (Fig. 3A). These results demonstrate the necessity of the cAMP/PKA signaling pathway for Kv current inhibition by exendin 4.

Because activation of the cAMP/PKA pathway by GLP-1 is mediated by G-protein activation of adenylyl cyclase (9), we investigated whether general G-protein activation was sufficient to reproduce the inhibitory effect of GLP-1 receptor activation on β-cell Kv channels. Inclusion of the non-hydrolysable GTP analogue GMP-PNP (10 nM) in the pipette solution decreased outward K⁺ current (by 55.7 ± 6.0%, n = 8, p < 0.001) similar to exendin 4 (Fig. 3B). We next investigated whether direct activation of the cAMP/PKA pathway could replicate this inhibitory effect. Addition of cAMP (100 μM) to the pipette solution was unable to decrease voltage-dependent outward K⁺ currents in rat β-cells (n = 7) (Fig. 3B). Also, intracellular dialysis of the constitutively active PKA catalytic subunit (200 units/ml) had no effect on currents compared with dimethyl sulfoxide controls (n = 6), which were themselves not different from untreated cells (Fig. 3B). Forskolin (5 μM) and isobutylmethylxanthine (100 μM) together blocked currents by 35.6 ± 7.5% (n = 5, p < 0.05) (Fig. 3B). However, forskolin (and its inactive analogs) directly inhibit Kv currents in insulin secretory cells (20, 21). Additionally, β-cell Kv currents could not be antagonized by treatment with the Epac-selective cAMP analog 8-pCPT-2′-O-Me-cAMP (50 μM, n = 7) (Fig. 3B). Our inability to antagonize voltage-dependent K⁺ channels in β-cells by activating the cAMP/PKA pathway is in agreement with previous reports (20, 21) and suggests that additional pathways are also required for the inhibitory action of GLP-1.

**Table I.**

| Antagonist          | % of Control Current | p-value |
|---------------------|----------------------|---------|
| Control             | 100                  |         |
| Rp-cAMPs            | 50                   | <0.001  |
| H-89                | 50                   | <0.001  |
| cAMP                | 50                   | <0.001  |
| Forskolin (5 μM)    | 50                   | <0.001  |
| IBMX (100 μM)       | 50                   | <0.001  |
| GMP-PNP (10 nM)     | 50                   | <0.001  |
| PKCζ (1 μM)         | 50                   | <0.001  |

The downstream effectors of PI3 kinase responsible for regulation of Kv currents by GLP-1 receptor activation were investigated using a number of pharmacological antagonists. Antagonism of p70 S6 kinase (rapamycin, 10 nM, n = 6), MEK1 (PD98059, 20 μM, n = 6), and conventional PKC isofoms (calphostin C, 500 nM, n = 7) failed to prevent the reduction of Kv currents in rat β-cells by exendin 4 (10⁻⁸ M) (Fig. 4). Possible downstream effectors of PI3 kinase include atypical PKC isofoms, because the effect of exendin 4 could also be prevented by bisindolylmaleimide (100 nM, n = 7) (Fig. 4), which antagonizes PKC at the ATP binding site (29, 30). However, it is important to note that this compound alone was capable of inhibiting β-cell Kv currents (Fig. 4). We investigated a role for PKCζ, because this isofom is known to be activated by GLP-1 (22) and associate with Kv channel regulatory subunits (31), and phosphorylation at a conserved PKC
Although bisindolylmaleimide (100 nM) prevented the effect of exendin 4 (10 nM), MEK1 (PD98059; 20 μM), and p70 S6 kinase (rapamycin; 10 nM) did not. Although inhibitors of conventional PKC isoforms (calphostin C; 500 nM) also prevented the effect of exendin 4 (10 nM), this broad spectrum PKC inhibitor alone blocked current. The PKCζ pseudosubstrate inhibitor (25 μM) also prevented the effect of exendin 4 (10 nM). ***, p < 0.001 compared with pre-exendin 4.

Regulation of β-Cell Kv Currents

**Table I**

| Protein (abbreviation) | Epitope | -Fold change |
|------------------------|---------|--------------|
| Cyclin-dependent kinase 1 (CDK1) | Ser-133 | N.D. * |
| Cyclin-dependent kinase 2 (CDK2) | Thr-15 | 1.12 |
| dsnRNA-dependent protein kinase (PKR) | Thr-451 | N.D. |
| Extracellular regulated kinase 1 (ERK1) | Thr-202/Tyr-204 | 0.96 |
| Extracellular regulated kinase 2 (ERK2) | Thr-185/Tyr-187 | N.D. |
| Glycogen synthase kinase 3α (GSK3α) | Ser-21 | N.D. |
| Glycogen synthase kinase 3β (GSK3β) | Tyr-216 | 1.38 |
| Glycogen synthase kinase 3β (GSK3β) | Thr-183/Tyr-185 | N.D. |
| MAP kinase kinase 1/2 (MEK1/2) | Ser-221/Ser-225 | 2.95 |
| MAP kinase kinase 3 (MEK3) | Ser-180/Thr-183 | 0.54 |
| MAP kinase kinase 6 (MEK6) | Ser-207/Thr-211 | N.D. |
| Oncogene JUN (JUN) | Ser-73 | N.D. |
| Oncogene Src (Src) | Ser-73 | 1.18 |
| Oncogene Src (Src) | Tyr-529 | 2.78 |
| Oncogene Src (Src) | Thr-418 | N.D. |
| p38 MAP kinase (p38 MAPK) | Ser-457 | 1.18 |
| Protein kinase B1 (Akt1) | Ser-473 | 1.37 |
| Protein kinase B1 (Akt1) | Thr-308 | 2.17 |
| Protein kinase Cα (PKCα) | Ser-657 | 0.90 |
| Protein kinase Cα (PKCα/β) | Thr-638/Thr-641 | 1.22 |
| Protein kinase Cα (PKCβ) | Thr-505 | 1.71 |
| Protein kinase Cε (PKCe) | Ser-719 | 0.97 |
| Retinoblastoma 1 (RB) | Ser-780 | 4.80 |
| Retinoblastoma 1 (RB) | Ser-807/Ser-811 | N.D. |
| Ribosomal S6 kinase 1 (RSK1) | Thr-360/Ser-364 | N.D. |
| 70-kDa S6 kinase p70 (70 S6K) | Thr-389 | N.D. |
| 77-kDa S6 kinase p70 (70 S6K) | Thr-389 | 1.11 |
| Signal transducer and activator of transcription 1 (STAT1) | Ser-701 | N.D. |
| Signal transducer and activator of transcription 2 (STAT3) | Ser-727 | 1.11 |
| Signal transducer and activator of transcription 3 (STAT5) | Tyr-694 | N.D. |
| SMA- and MAD-related protein 1 (SMAD1) | Ser-463/Ser-465 | N.D. |
| 40-kDa stress-activated protein kinase (JNK) | Thr-183/Tyr-185 | N.D. |
| 44-kDa stress-activated protein kinase (JNK) | Thr-183/Tyr-185 | N.D. |

* N.D., not detected.

**Fig. 4. Activity of PI3 kinase and the atypical PKCζ isoform is required for the effect of exendin 4 on rat β-cell Kv currents.** Maximum sustained outward K⁺ current was measured in rat β-cells as in Fig. 3. Pre-treatment of cells with the PI3 kinase inhibitor wortmannin (100 nM) abolished the effect of exendin 4 (10⁻⁸ M; black bars), whereas inhibitors of conventional PKC isoforms (calphostin C; 500 nM), MEK1 (PD98059; 20 μM), and p70 S6 kinase (rapamycin; 10 nM) did not. Although bisindolylmaleimide (100 nM) prevented the effect of exendin 4 (10⁻⁸ M), this broad spectrum PKC inhibitor alone blocked current. The PKCζ pseudosubstrate inhibitor (25 μM) also prevented the effect of exendin 4 (10⁻⁸ M). ***, p < 0.001 compared with pre-exendin 4.

**Transactivation of PI3 Kinase via the EGF Receptor Is Necessary but Not Sufficient for Antagonism of Kv Channels by Exendin 4.** The p110γ isoform of PI3 kinase is the only isoform known to be directly activated by G-protein-coupled receptors, and this occurs through an interaction with Gi/γ (34). However, exendin 4 could inhibit Kv currents equally in β-cells from p110γ+/- (47.1 ± 4.9%, n = 4, p < 0.001) and -/- (53.7 ± 5.9%, n = 4, p < 0.001) mice (Fig. 5A). To investigate whether exendin 4 activates PI3 kinase through the direct activation of a receptor other than the GLP-1 receptor, we studied β-cells from GLP-1 receptor -/- mice. Exendin 4 had no effect on Kv currents in β-cells lacking the GLP-1 receptor, even in the presence of 100 μM cAMP (Fig. 5A). Because one recent study (35) demonstrates that a Src kinase-mediated trans-activation of EGFR receptors and subsequent activation of PI3 kinase is involved in the effects of GLP-1 on β-cell proliferation, we examined whether this was the mechanism of PI3 kinase activation in the present study. Both the EGFR receptor tyrosine kinase antagonist AG1428 (250 nM, n = 6) and the Src kinase antagonist PP1 (10 μM, n = 7) prevented exendin 4-mediated reductions in β-cell Kv currents (Fig. 5B). We detected EGFR receptor (erbB-1) mRNA by reverse transcriptase-PCR and EGFR protein by Western blotting in INS-1 and MIN6 site is known to reduce current through cloned Kv channels (32, 33). A role for the atypical PKCζ isoform is supported as intracellular dialysis of the PKCζ pseudosubstrate inhibitor (25 μM), which had no effect alone, also prevented Kv current reduction by exendin 4 (Fig. 4).
Regulation of β-Cell Kv Currents

Fig. 5. Src tyrosine kinase and EGF receptor trans-activation is required for the effect of exendin 4 on rat β-cell Kv currents. Maximum sustained outward K+ current was measured in rat β-cells as in Fig. 3. In panel A, exendin 4 (10^(-8) M; black bars) antagonized Kv currents in β-cells from mice lacking (-/-) or heterozygous (+/−) for the G-protein-activated p110γ isoform of PI3 kinase but had no effect on currents in β-cells from mice lacking the GLP-1 receptor. To activate the cAMP/PKA pathway in the GLP-1 receptor −/− cells, cAMP (100 μM) was included in the pipette. In panel B, pre-treatment of cells with the Src family tyrosine kinase antagonist PP1 (10 μM) or the EGF receptor tyrosine kinase antagonist AG1428 (250 nM) prevented the effect of exendin 4 (10^(-8) M; black bars). ***, p < 0.001 compared with pre-exendin 4.

We further examined whether activation of PI3 kinase alone could replicate the effect of exendin 4 on β-cell Kv currents. The EGF receptor agonist betacellulin (5 ng/ml), which activates PI3 kinase in INS-1 β-cells (35), reduced β-cell Kv currents somewhat (by 20.2 ± 4.5, n = 5, p < 0.05) (Fig. 6A) but could not completely replicate the effect of exendin 4. Activation of PI3 kinase with insulin (200 nM) was without effect (n = 5) (Fig. 6A). Also, β-cell Kv currents were not antagonized by activation of conventional PKC isoforms (200 nM TPA, n = 6) (Fig. 6A). These results suggest that Src kinase-mediated trans-activation of the EGF receptor and subsequent activation of PI3 kinase/PKCγ signaling is required, but not sufficient, for antagonism of β-cell Kv currents by exendin 4.

Betacellulin Can Fully Replicate the Effect of Exendin 4 on β-cell Kv Currents in the Presence of Increased cAMP. Because cAMP/PKA signaling and EGF receptor activation are necessary, but not each sufficient in themselves for Kv current regulation by GLP-1 receptor activation in β-cells, we investigated the effect of activating both signaling pathways in parallel. To activate PKA, 100 μM cAMP was included in the patch pipette. Under these conditions, insulin (200 nm) now tended to antagonize β-cell Kv currents (by 28.3 ± 4.4%, n = 5) although this did not reach statistical significance (Fig. 6A). However, betacellulin (5 ng/ml) was now able to antagonize Kv currents by 40.5 ± 3.7% (n = 6, p < 0.001) (Fig. 6A and B). Betacellulin and cAMP together also caused a hyperpolarizing shift in the voltage dependence of steady-state inactivation (V_{50} = −62.0 ± 2.0 mV, n = 6, p < 0.001), entirely replicating the effect of exendin 4 (Fig. 6C). Betacellulin alone resulted in an intermediate shift (V_{50} = −55.7 ± 2.3 mV, n = 5, p < 0.05) (Fig. 6C). TPA (200 nM, n = 6) did not reduce β-cell Kv currents in the presence of increased cAMP, which is consistent with a role for the atypical PKCζ isoform as opposed to conventional PKC isoforms activated by phorbol esters. These and the above results suggest that the GLP-1 receptor antagonizes β-cell Kv currents by activating both the cAMP/PKA and EGF receptor signaling pathways.

Discussion

Regulation of voltage-dependent K+ channel activity in pancreatic β-cells by GLP-1 may contribute significantly to the to the excitatory and insulinoetropic effects of this incretin hormone (13). Importantly, because antagonism of β-cell Kv current stimulates insulin secretion in a glucose-dependent manner (4–7), block of these channels may contribute to the well known glucose-dependent effects of GLP-1. Recently, alternative mechanisms for the glucose dependence of GLP-1 have also been proposed, including the ADP-dependent inhibition of K_{ATP} channels by PKA (37) and the sensitization of Ca^{2+}−induced Ca^{2+} release by activation of the guanine-nucleotide exchange factor Epac2 (38). However, the exact mechanism of glucose dependence remains to be established and may result from a complex interaction of the many known targets of GLP-1 signaling (9).

Rat β-cell Kv currents were antagonized by both GLP-1 and the GLP-1 receptor agonist exendin 4, confirming our previous results (13). Indeed, immunoprecipitation of a tagged GLP-1 receptor over-expressed in HIT-T15 cells was able to pull down the Kv2.1 channel α-subunit, demonstrating an association that may be direct or mediated by intermediate protein-protein interactions. GLP-1 blocked currents by −6 min, whereas washout of the effect occurs over a longer time period (>20 min). These relatively slow time courses are consistent with the complex nature of the signaling pathway involved. Current block resulted from a leftward shift in the voltage dependence of steady-state inactivation. Functionally this means that after GLP-1 treatment a greater number of Kv channels will already be inactivated at a given membrane potential and therefore unavailable for action potential repolarization. This is expected to prolong the action potential, leading to greater influx of Ca^{2+} and a greater insulin secretory response (13).

The G-protein-coupled GLP-1 receptor regulates the majority of its known targets through the classic cAMP/PKA signaling pathway (9). β-Adrenergic stimulation reduces Kv currents in lymphocytes (39) and enhances Kv currents in cardiac myocytes (40), and in both these tissues cAMP/PKA signaling has been implicated (41, 42). Antagonism of rat β-cell Kv currents by exendin 4 is dependent on the cAMP/PKA signaling pathway as demonstrated by the ability of Rp-cAMPs and H-89 to block this effect (Fig. 3A). We were unable to replicate the effect of exendin 4 with activators of this pathway including cAMP and the constitutively active PKA catalytic subunit (Fig. 3B), which is consistent with previous reports in mouse β-cells (20) and the INS-1 insulinoma cell line (21). We were also not able to block Kv currents with the Epac-selective cAMP analog. The small but significant effect of forskolin and isobutylmethylxanthine cannot be taken as evidence that activation of cAMP signaling alone can block currents, because forskolin (and inactive analogs) is known to block Kv currents directly (20, 21). It therefore seems clear from these results that GLP-1 receptor-mediated block of Kv currents requires an additional signaling pathway.
Recent work has identified additional pathways activated by the GLP-1 receptor, including the PKA-independent activation of Epac2 (38) and EGF receptor trans-activation and subsequent activation of PI3 kinase (35). Phospho-site screening of MIN6 cells treated acutely with GLP-1 indicates that the PI3 kinase signaling pathway is activated, because a number of phosphorylation sites downstream of the PI3 kinase-activated PDK1 were up-regulated. This is not surprising, because signaling through the GLP-1 receptor results in phosphorylation of Akt (43) and activation of PKCζ (22) in INS-1 insulinoma cells. Additionally, the MEK1/2 Ser-221/Ser-225 phosphorylation site was up-regulated, and this may also be downstream of PI3 kinase (26). Our inability to detect up-regulation of ERK phosphorylation at 10 min following GLP-1 treatment may be expected, because ERK phosphorylation in response to GLP-1 is transient (detectable at 5 but not 15 min) in the absence of high glucose concentrations (44). These results suggest that GLP-1 treatment causes the phosphorylation of key activation sites of numerous downstream targets of PI3 kinase.

Functionally, activation of the PI3 kinase pathway was determined to be necessary for the ability of exendin 4 to antagonize β-cell Kv currents, because the effect could be completely abolished by the PI3 kinase inhibitor wortmannin (Fig. 4). Direct activation of the G-protein-regulated p110y isoform of PI3 kinase is not involved, because exendin 4 could still antagonize currents in β-cells from mice lacking p110y. PI3 kinase was also not activated by a direct effect of exendin 4 on an alternate receptor, because currents in β-cells lacking the GLP-1 receptor were not blocked by exendin 4, even when the cAMP/PKA pathway was activated. A recent study (35) demonstrates that, in the INS-1 insulinoma cell line, GLP-1 receptor activation leads to PI3 kinase activation through a mechanism involving Src kinase-mediated trans-activation of the EGF receptor. The involvement of this pathway in exendin 4-mediated Kv current block was demonstrated using antagonists of Src family protein tyrosine kinases and EGF receptor tyrosine kinase activity, which were each able to prevent current inhibition by exendin 4. Although the phospho-site screen demonstrates a down-regulation of Src Tyr-418 phosphorylation, an effect expected to reduce activity, it should be noted that the antibody used also recognizes conserved sites on related kinases (45), and the Src family tyrosine kinase inhibitor used here (PP1) is non-selective among different Src family members (46).

Because activation of either cAMP/PKA signaling or PI3 kinase signaling (with insulin or betacellulin) alone was insufficient to replicate the effect of exendin 4 on β-cell Kv currents, we examined the effect of concomitant activation of both pathways. In the presence of increased intracellular cAMP, betacellulin could completely replicate the inhibitory action of exendin 4. TPA was still without effect, confirming no role for the typical PKC isoforms. Importantly, the mechanism of current block by exendin 4, an ~20-mV hyperpolarizing shift in the steady-state voltage dependence of inactivation, was completely replicated by treatment with cAMP and betacellulin. Despite the fact that both insulin and EGF receptor signaling activate PI3 kinase, we did not observe a significant reduction in Kv current upon treatment with insulin, even in the presence of high intracellular cAMP. This functional separation of the two signaling pathways is consistent with the inability of
EGF receptor ligands to replicate the effect of insulin on glucose uptake in adipocytes (47, 48) and may result from the differential activation of PI3 kinase isoforms (49) or the subcellular compartmentalization of signaling molecules (50, 51). These results provide further evidence that both cAMP/PKA and EGF receptor-mediated PI3 kinase signaling are necessary for Kv current block in pancreatic β-cells.

Downstream of PI3 kinase, the effect of exendin 4 on β-cell Kv currents does not involve MAP kinase, P70 S6 kinase, or conventional isoforms of PKC, because inhibitors of these did not prevent antagonism of currents. Bisindolylmaleimide, which can inhibit both conventional and atypical PKC isoforms, though antagonism of currents by exendin 4 could be prevented by bisindolylmaleimide, this PKC inhibitor alone caused a 40% reduction in Kv currents. A role for the atypical PKCζ isoform is supported, because the PKCζ pseudosubstrate, which, in contrast to bisindolylmaleimide, did not affect control currents, prevented antagonism of currents by exendin 4 (Fig. 4). The involvement of PKCζ in mediating current reduction is also supported by studies demonstrating that this isoform is known to associate with Kv channel regulatory subunits (31), and phosphorylation at a conserved PKCζ site is known to reduce current through cloned Kv channels (32, 33).

Here we investigated the mechanism and signal transduction pathways involved in Kv current block by the GLP-1 receptor agonist exendin 4 in primary rat β-cells. Kv currents were antagonized as the result of a ~20-mV hyperpolarizing shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The downstream of PI3 kinase, the effect of exendin 4 on glucose-dependent insulinotropic effect of GLP-1, because antagonists of PKCβ1 and EGF receptor ligands to replicate the effect of insulin on glucose uptake in adipocytes (47, 48) and may result from the differential activation of PI3 kinase isoforms (49) or the subcellular compartmentalization of signaling molecules (50, 51). These results provide further evidence that both cAMP/PKA and EGF receptor-mediated PI3 kinase signaling are necessary for Kv current block in pancreatic β-cells.

Downstream of PI3 kinase, the effect of exendin 4 on β-cell Kv currents does not involve MAP kinase, P70 S6 kinase, or conventional isoforms of PKC, because inhibitors of these did not prevent antagonism of currents. Bisindolylmaleimide, which can inhibit both conventional and atypical PKC isoforms, though antagonism of currents by exendin 4 could be prevented by bisindolylmaleimide, this PKC inhibitor alone caused a 40% reduction in Kv currents. A role for the atypical PKCζ isoform is supported, because the PKCζ pseudosubstrate, which, in contrast to bisindolylmaleimide, did not affect control currents, prevented antagonism of currents by exendin 4 (Fig. 4). The involvement of PKCζ in mediating current reduction is also supported by studies demonstrating that this isoform is known to associate with Kv channel regulatory subunits (31), and phosphorylation at a conserved PKCζ site is known to reduce current through cloned Kv channels (32, 33).

Here we investigated the mechanism and signal transduction pathways involved in Kv current block by the GLP-1 receptor agonist exendin 4 in primary rat β-cells. Kv currents were antagonized as the result of a ~20-mV hyperpolarizing shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The downstream of PI3 kinase, the effect of exendin 4 on glucose-dependent insulinotropic effect of GLP-1, because antagonists of PKCβ1 and EGF receptor ligands to replicate the effect of insulin on glucose uptake in adipocytes (47, 48) and may result from the differential activation of PI3 kinase isoforms (49) or the subcellular compartmentalization of signaling molecules (50, 51). These results provide further evidence that both cAMP/PKA and EGF receptor-mediated PI3 kinase signaling are necessary for Kv current block in pancreatic β-cells.

Downstream of PI3 kinase, the effect of exendin 4 on β-cell Kv currents does not involve MAP kinase, P70 S6 kinase, or conventional isoforms of PKC, because inhibitors of these did not prevent antagonism of currents. Bisindolylmaleimide, which can inhibit both conventional and atypical PKC isoforms, though antagonism of currents by exendin 4 could be prevented by bisindolylmaleimide, this PKC inhibitor alone caused a 40% reduction in Kv currents. A role for the atypical PKCζ isoform is supported, because the PKCζ pseudosubstrate, which, in contrast to bisindolylmaleimide, did not affect control currents, prevented antagonism of currents by exendin 4 (Fig. 4). The involvement of PKCζ in mediating current reduction is also supported by studies demonstrating that this isoform is known to associate with Kv channel regulatory subunits (31), and phosphorylation at a conserved PKCζ site is known to reduce current through cloned Kv channels (32, 33).

Here we investigated the mechanism and signal transduction pathways involved in Kv current block by the GLP-1 receptor agonist exendin 4 in primary rat β-cells. Kv currents were antagonized as the result of a ~20-mV hyperpolarizing shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The shift in the voltage dependence of inactivation, whereas voltage-dependent activation and current kinetics were unaffected. The downstream of PI3 kinase, the effect of exendin 4 on glucose-dependent insulinotropic effect of GLP-1, because antagonists of PKCβ1 and EGF receptor ligands to replicate the effect of insulin on glucose uptake in adipocytes (47, 48) and may result from the differential activation of PI3 kinase isoforms (49) or the subcellular compartmentalization of signaling molecules (50, 51). These results provide further evidence that both cAMP/PKA and EGF receptor-mediated PI3 kinase signaling are necessary for Kv current block in pancreatic β-cells.