Mechanism by which cAMP activates PI3-kinase and increases bile acid secretion in WIF-B9 cells

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Kagawa, Tatehiro, Lyuba Varticovski, Yoshimichi Sai, and Irwin M. Arias. Mechanism by which cAMP activates PI3-kinase and increases bile acid secretion in WIF-B9 cells. Am J Physiol Cell Physiol 283: C1655–C1666, 2002. First published August 7, 2002; 10.1152/ajpcell.00041.2002.—Previous studies in rat bile canalicular membrane vesicles and WIF-B9 cells revealed that cAMP-induced trafficking of ATP-binding cassette (ABC) transporters to the canalicular membrane and their activation require phosphoinositide 3-kinase (PI3-K) products. In the present studies, canalicular secretion of fluorescein isothiocyanate-glycocholate in WIF-B9 cells was increased by cAMP and a decapeptide that enhances PI3-K activity; these effects were inhibited by wortmannin. To determine the mechanism(s) whereby cAMP activates PI3-K, we examined signal transduction pathways in WIF-B9 and COS-7 cells. cAMP activated PI3-K in both cell lines in a phosphotyrosine-independent manner. PI3-K activity increased in association with p110β in both cell lines. The effect of cAMP was KT-5720 sensitive, suggesting involvement of protein kinase A. Expression of a dominant-negative β-adrenergic receptor kinase COOH terminus (β-ARKct), which blocks Gβγ signaling, decreased PI3-K activation in both cell lines. cAMP increased GTP-bound Ras in COS-7 but not WIF-B9 cells. Expression of dominant-negative Ras abolished cAMP-mediated PI3-K, which suggests that the effect is downstream of Ras and Gβγ. These data indicate that cAMP activates PI3-K in a cell type-specific manner and provide insight regarding mechanisms of PI3-K activation required for bile acid secretion.

bile secretion; heterotrimeric G protein; Gβγ; protein kinase A; Ras

Administration of dibutyryl cAMP (DBcAMP) to rats increases biliary secretion of taurocholate, which results from increased trafficking of bsep, the canalicular ATP-dependent bile acid transporter, from intracellular sites to the canalicular membrane (11, 24). The concentration of bsep in the canalicular membrane increased approximately threefold, concomitant with a similar increase in PI3-K activity and ATP-dependent transport of taurocholate (21). Administration of wortmannin in vivo or after addition to canalicular membrane vesicles inhibited each response to DBcAMP; however, addition of phosphoinositide products of PI3-K but not of phosphatidylinositol (PI)4-P, the enzyme’s substrate, to canalicular membrane vesicles restored bsep activity in the presence of wortmannin (21). In studies using a polarized rat hepatoma-human fibroblast hybrid cell line (WIF-B9), administration of a decapeptide (PI3-K peptide; Refs. 15, 18), which enters the cell and activates PI3-K, increased canalicular bile acid secretion in a wortmannin-inhibitable manner (32, 42, 53). These previously published studies in rat liver, canalicular membrane vesicles, and WIF-B9 cells suggest interactions between cAMP and PI3-K signal transduction pathways as related to bsep trafficking and activity in the canalicular membrane. Studies in other systems suggest similar cross talk. For example, wortmannin inhibits cAMP activation of Akt (2, 3, 13, 28, 54, 56) in a cell type-specific manner, as well as serum- and glycocorticoid-induced protein kinase (Skg) (35).

The mechanism(s) responsible for cAMP and PI3-K cross talk have not been identified in any system and were the objective of the present studies. WIF-B9 and COS-7 cells were chosen for study on the basis of the following rationale. Preliminary studies in freshly isolated rat hepatocytes confirmed that cAMP increased PI3-K activity (54); however, primary cultures lack cellular polarity, which prevents correlation with bsep function or trafficking. Hepatocytes that retain tight junctions during preparation (so-called hepatocyte doublets) were not studied because they retain a hemicanaliculus from adjacent cells that complicates investigation of canalicular membrane trafficking (24). Furthermore, transient transfection in primary hepatopo...
cytes is unpredictable for biochemical studies. Because transport studies in WIF-B9 cells yielded results similar to those observed in rat liver (42, 53), we chose to study the relation between cAMP administration and PI3-K activation in polarized WIF-B9 cells and in non-polarized COS-7 cells as a control.

**MATERIALS AND METHODS**

**Materials.** DBcAMP, wortmannin, pertussis toxin (PTX), phosphatidylserine (PS), PI, PI 3,4-bisphosphate (PI 3,4-P2), and glycocholate were purchased from Sigma (St. Louis, MO). KT-5720, PP2, and forskolin were obtained from Calbiochem (San Diego, CA).

**Cell culture.** COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Life Technologies, Rockville, MD). WIF-B9 cells, an immortalized cell line obtained from a rat hepatoma cell line-human fibroblast fusion that forms functional bile canaliculus (20, 45), were grown in F-12 medium (Life Technologies) supplemented with 10% FBS. All cells were cultured at 37°C in a humidified atmosphere with 5% (COS-7 and Jurkat) or 7% (WIF-B9) CO2. Cells were starved after 24 h and subjected to the experiments as described in

**Expression of dominant-negative constructs.** COS-7 cells were transfected with RasN17 (gift of Dr. L. A. Feig, Tufts University, Boston, MA) by using Lipofectamine Plus (Life Technologies) according to the manufacturer's instructions and cultured for 24 h. Cells were induced to quiescence and subjected to treatment with DBcAMP. For expression of dominant-negative Gpl, cells were infected with adenovirus vector alone or adenovirus expressing the β-adrenergic receptor kinase COOH terminus (β-ARKct) (gift of Dr. R. J. Lefkowitz, Duke University Medical Center, Durham, NC) at 100 multiplicities of infection (MOI) (7, 26). Medium was replaced with complete medium 2 h after infection. Cells were starved after 24 h and subjected to the experiments as indicated. The expression of β-ARKct was tested by immunoblotting with a β-ARK antisemur that recognizes the COOH terminus of β-ARK2 (gift of Dr. R. J. Lefkowitz).

**PI3-K activity assay.** Cells were washed with ice-cold phosphate-buffered saline (PBS) three times and lysed in lysis buffer (50 mM HEPES, pH 7.5, 0.5 mM EDTA, 5 mM sodium orthovanadate, 10% glycerol, and 1% NP-40) with protease inhibitors (0.5 mM PMSF, 5 μg/ml pepstatin A, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were incubated on ice for 5 min and spun at 4°C for 10 min at 12,000 g to remove insoluble fractions. The supernatant was frozen immediately in aliquots by immersion in liquid nitrogen and stored at −80°C until being used. PI3-K activity assay was performed in immunoprecipitates by using 200 μg of protein from cell lysates after incubation with anti-p85 antibody (no. 06–497; Upstate Biotechnology, Lake Placid, NY) or 350 μg of protein incubated with anti-phosphotyrosine (anti-P-tyr) antibody (4G10; Upstate Biotechnology) or anti-p110α, β, or γ antibodies (H-201, H-198, and H-199, respectively; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 2 h followed by incubation with 30 μl of 50% suspension of protein A beads (Sigma) for 1 h. After the beads were washed once with lysis buffer and three times with PBS, they were sedimented by brief centrifugation and PI3-K activity was quantitated as previously described (32, 50). Briefly, the reaction was performed in a total volume of 50 μl containing 150 μM ATP, 25 mM MOPS (pH 7.0), 5 mM MgCl2, 1 mM EGTA, and 0.2 mg/ml sonicated lipids: PS-PI-PI 4,5-bisphosphate (PI 4,5-P2) (1:1:1 vol/vol/vol) in sonication buffer (25 mM MOPS, pH 7.0, 1 mM EGTA) with 25 μCi of [γ-32P]ATP per assay (NEN, Boston, MA). After incubation at 37°C for 20 min, the reaction was stopped with 90 μl of methanol-1 N HCl (1:1 vol/vol) and lipids were extracted twice in 100 μl of chloroform. The organic layer was combined, dried under nitrogen gas flow, and analyzed by TLC (Whatman, Clifton, NJ). Polyphosphoinositides were resolved in water-acetic acid-n-propanol (34:1:65 vol/vol/vol), and 32P label was detected by autoradiography. TLC spots corresponding to PI 3,4-trisphosphate (PIP3) products were scraped and eluted in scintillation liquid, and 32P incorporation into PIP3 was quantified by liquid scintillation counting. To compare the results obtained from different experiments, whole cell lysates from HEK-293 cells were diluted 1,000-fold (50) and used as control (designated as 1 unit of PI3-K activity).

**Immunoblotting.** Twenty micrograms of cell lysates obtained as described in PI3-K activity assay were separated by SDS-PAGE, transferred onto polycryllyden fluoride (PVDF) membranes (Millipore, Bedford, MA), and probed with antibodies to Akt and phospho-Akt (pAkt) (Cell Signaling Technology, Beverly, MA), total ERK1/2 (Upstate Biotechnology), and phospho-ERK1/2 (pERK1/2, Santa Cruz Biotechnology). The membranes were probed with appropriate horseradish peroxidase-coupled secondary antibodies (Cell Signaling) followed by enhanced chemiluminescence (ECL) reaction (NEN, Boston, MA). The densitometric analysis was performed with the NIH Image program, and the relative intensity was calculated relative to total protein content. Normal rabbit IgG (Santa Cruz Biotechnology) was used as a negative control.

**Ras-GTP assay.** GTP-bound Ras was quantified as described previously (12). Briefly, the Ras-binding domain of Raf was expressed as a glutathione S-transferase (GST)-fusion protein in Escherichia coli and affinity purified on glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ). Ten micrograms of immobilized fusion protein were incubated with four hundred micrograms of whole cell lysates for 2 h at 4°C. The beads were washed once with lysis buffer and three times with PBS. After addition of 30 μl of sample buffer, the beads were heated to 95°C for 5

**Table 1. Canalicular secretion of FITC-GC in WIF-B9 cells and effects of cAMP, PI3-K peptide, and wortmannin**

<table>
<thead>
<tr>
<th>FITC-GC Secretion, gray scale units</th>
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<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>DBcAMP (500 μM)</td>
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<tr>
<td>PI3-K peptide (20 μM)</td>
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<td>Control + wortmannin (100 nM)</td>
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<td>DBcAMP (500 μM) + wortmannin (100 nM)</td>
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<td>PI3-K peptide (20 μM) + wortmannin (100 nM)</td>
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Results are expressed as means ± SE for 4–6 individual studies performed in duplicate. See MATERIALS AND METHODS for procedural details. DBcAMP, dibutyryl CAMP; PI3-K, phosphoinositide 3-kinase; FITC-GC, fluorescein isothiocyanate-glycocholic acid conjugate. † P < 0.01 compared with control. * P < 0.01 compared with results minus wortmannin (one-way ANOVA).
min and centrifuged for 2 min at 12,000 g in a microcentrifuge column (Bio-Rad Laboratories, Hercules, CA) to remove the beads. The flow-through was separated by SDS-PAGE followed by immunoblotting with anti-Ras antibodies (Upstate Biotechnology). To confirm that an equal amount of total Ras protein was present in each sample, cell lysates were also immunoblotted with anti-Ras antibodies.

**Bile acid transport in WIF-B9 cells.** With a previously described technique (42), monolayers of WIF-B9 cells were grown on glass coverslips, mounted in a Dvorak-Stotler cul-

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**Fig. 1.** cAMP activates phosphoinositide 3-kinase (PI3-K) in a time- and dose-dependent manner. COS-7 (A, C, E, and G) and WIF-B9 (B, D, F, and H) cells were induced to quiescence and treated with cAMP (100 μM) for indicated times (A–D); with cAMP (100 μM) for 60 min, forskolin (50 μM) for 30 min, EGF (10 ng/ml) for 15 min, and insulin (10 ng/ml) for 15 min (E and F); or with cAMP with different concentrations of cAMP for 60 min (G and H). Whole cell lysates (200 μg) were immunoprecipitated with anti-p85 antibodies followed by PI3-K assay, and radioactivity incorporated into the phosphatidylinositol (PI) 3,4,5-trisphosphate (PIP₃) was quantified. Representative autoradiographs (A for COS-7 and B for WIF-B9 cells) are shown. Data (C–H) were obtained from 3–8 independent experiments; each value represents the mean ± SE. *P < 0.05 vs. basal levels (repeated-measure 1-way ANOVA).
ture chamber that was thermostabilized at 37°C, and promptly viewed by confocal laser scanning microscopy. Cells were perfused with modified F-12 supplemented with 5% FBS and 12 mM HEPES (pH 7.0) with a perfusion pump (model 351; Sege Instruments, Cambridge, MA) at 0.3 ml/min. Images were obtained with an Odyssey XL confocal system (Noran Instruments, Middletown, WI) and an inverted Nikon Diaphot microscope equipped with a Nikon ×60 oil-immersion Planachromat lens (NA 1.4). After a baseline fluorescent image was acquired, the chamber was perfused for 20 min with a second solution containing 0.5 μM glycocholic acid conjugated with fluorescein isothiocyanate (FITC-GC), which exhibits the requisite structural features of conventional bile acids and is a substrate for the bile acid transporter (16, 25). Excitation was at 488 nm with a krypton-argon laser. The microscope was calibrated daily by adjusting the gain and offset to obtain constant values for an intensity calibration curve. Linearity of the signal as a function of the concentration of the fluorescent probe was verified. Quantification was performed with Inter Vision computer software by measuring fluorescence intensity in the perinuclear cytoplasmic and canalicular regions, which were delimited by boundaries drawn on the first image of the series. Similar studies were performed in WIF-B9 cells that were preincubated for 10 min at 37°C with DBcAMP (300 μM) or rhodamine-linked PI3-K peptide (5, 20 μM) (15, 18, 42) and washed twice with PBS before perfusion with FITC-GC. Cells were also incubated in wortmannin (100 nM) for 10 min at 37°C before addition of DBcAMP, PI3-K peptide, or FITC-GC. Control cells for experiments using wortmannin were exposed to DMSO (0.01%).

RESULTS
cAMP and PI3-K peptide increased FITC-GC secretion in a wortmannin-inhibitable manner in WIF-B9 cells. As shown in Table 1, cAMP and PI3-K peptide significantly increased FITC-GC secretion into bile canaliculi of WIF-B9 cells 2.0- and 2.1-fold, respectively; both responses were inhibited by prior incubation with wortmannin (100 nM). In a previous study (42), canalicular secretion of FITC-GC by WIF-B9 cells was time dependent, saturable, stimulated by preincubation of cells with taurocholate (100 μM) or PI3-K peptide (5 μM), and decreased by preincubation with wortmannin (100 nM). The present study demonstrated that, like taurocholate, cAMP and PI3-K peptide significantly increased FITC-GC secretion in WIF-B9 cells. The stimulatory effects of cAMP and PI3-K peptide were both inhibited by wortmannin (100 nM), which suggests that cAMP may enhance bile acid

![Fig. 2. cAMP activates PI3-K in a phosphotyrosine-independent manner.](image)

A: immunoblot of tyrosine-phosphorylated proteins. COS-7 or WIF-B9 cells were stimulated with EGF (10 ng/ml) or insulin (10 ng/ml) for 15 min or with cAMP (100 μM) for indicated times. Whole cell lysates (20 μg) were separated by SDS-PAGE and probed with anti-phosphotyrosine (anti-P-tyr) antibodies. B: effect of cAMP on anti-P-tyr-associated PI3-K activity. PI3-K activity was determined in immunoprecipitates from 400 μg of protein with anti-P-tyr antibodies. Data represent means ± SE from 3 independent experiments.

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secretion through activation of PI3-K. These studies provided the impetus to identify the biochemical mechanism(s) whereby cAMP and PI3-K interact with one another as regards bile acid secretion.

**cAMP activated PI3-K in a time- and dose-dependent manner in COS-7 and WIF-B9 cell lines.** PI3-K activity was quantified before and after stimulation with the cell-permeant cAMP analog DBcAMP. Within 15 min, cAMP activated p85-associated PI3-K in both cell lines (Fig. 1, A and C for COS-7 and B and D for WIF-B9 cells). Growth factors such as PDGF produced maximal activation within 10 min (50); in contrast, the maximal response (2.0- to 2.5-fold) to cAMP was observed at 60 min; p85-associated PI3-K activation returned to basal levels after 4 h in both cell lines. Stimulation with forskolin (50 μM, 30 min) also activated PI3-K by 2.8 ± 0.5- and 2.3 ± 0.2 (mean ± SE)-fold in COS-7 and WIF-B9 cells, respectively (Fig. 1E for COS-7, Fig. 1F for WIF-B9 cells). The increase in association with p85 was not smaller than that observed by growth factors; EGF (10 ng/ml, 15 min) in COS-7 and insulin (10 ng/ml, 15 min) in WIF-B9 induced 2.4 ± 0.5- and 2.6 ± 0.4 (mean ± SE)-fold increase, respectively. The cAMP effect on PI3-K was dose dependent (Fig. 1G and H). The calculated ED50 was 67.0 and 56.2 μM in COS-7 and WIF-B9 cells, respectively. The increase in PI3-K activity resulted from modification in intrinsic catalytic activity, because the amount of immunoreactive p110 subunits associated with p85 did not vary after cAMP stimulation (data not shown). In whole lysates,
PI3-K activity increased 1.5-fold in both cell lines within 1 h (data not shown).

cAMP-mediated PI3-K activation was independent of phosphotyrosine. cAMP stimulation did not generate tyrosine-phosphorylated proteins, in contrast to the effect of EGF and insulin in COS-7 and WIF-B9 cells, respectively (Fig. 2A). Phosphotyrosine-associated PI3-K activity did not change after cAMP stimulation at any time period, in contrast to the effect of EGF and insulin, which induced robust activation in COS-7 and WIF-B9 cells (Fig. 2B). These observations indicate that cAMP-mediated PI3-K activation is phosphotyrosine independent. The rapidity of PI3-K activation by cAMP suggests that transcription is unaffected. Therefore, on the basis of evidence from other pathways that involve cAMP, we investigated the role of PKA, Gβγ, and Ras in cAMP-mediated activation of PI3-K in COS-7 and WIF-B9 cells.

**p110 Catalytic subunit-specific PI3-K activity and expression of each subunit.** To determine why cAMP did not activate Akt in WIF-B9 cells despite cAMP activation of p85-associated PI3-K, we characterized expression of PI3-K catalytic subunits by measuring catalytic subunit-associated PI3-K activity and protein expression by Western blotting. cAMP increased p110α-associated PI3-K activity in COS-7 cells (3-fold) and in WIF-B9 cells (2.5-fold) (Fig. 4A). p110β-associated PI3-K activity increased 1.7-fold in COS-7 cells, whereas basal and cAMP-stimulated activities in immunoprecipitates were substantially lower in WIF-B9 cells. PI3-K activity associated with p110γ was undetectable in both cell lines even in the presence of cAMP. These results are not due to an inhibitory effect of anti-p110γ antibodies on PI3-K because p110γ-associated PI3-K activity was detected in Jurkat cells. Immunoblotting revealed p110α expression in cell lysates and p85 immunoprecipitates in COS-7 and WIF-B9 cells (Fig. 4B). In contrast, p110β was expressed only...
in COS-7 cells in concordance with the activity assay data described above. No protein was detected in immunoprecipitates with normal rabbit IgG, which confirmed specificity of the results. Expression of p110γ was detected only in Jurkat cells, an acute T cell leukemia cell line, confirming that expression of p110γ, originally cloned from a bone marrow cDNA library (49), is relatively restricted to hematopoietic cells. The class 1A PI3-K catalytic subunit, p110α, was not tested because its expression is restricted to leukocytes (52). These results reveal that COS-7 cells express p110α and p110β, whereas WIF-B9 cells only express p110α.

*cAMP-mediated PI3-K activation was sensitive to an inhibitor of PKA, KT-5720.** KT-5720, an inhibitor of PKA, did not affect basal PI3-K activity but decreased cAMP-mediated PI3-K activity by 74% in COS-7 and 79% in WIF-B9 cells (Fig. 5A). Addition of PKA catalytic subunit to PI3-K in vitro did not change PI3-K activity (data not shown). Src kinase can be activated by cAMP and bind to and activate PI3-K (14). We found that cAMP induces activation of Src in COS-7 cells (data not shown). However, PP2, a Src inhibitor, had no effect on PI3-K activity (Fig. 5B). Because several reports indicate cross talk between G₁ and G₂ signaling (4), we also investigated the involvement of G₂ signaling with PTX. PTX also failed to block cAMP-mediated PI3-K activation (Fig. 5B), suggesting that neither Src nor G₂ signaling is involved.

**Role of Ras in cAMP-mediated PI3-K activation.** Ras induces PI3-K activation by direct binding to the p110 catalytic subunits (41) in a phosphotyrosine-independent manner. Addition of cAMP to COS-7 cells increased GTP-bound Ras in 15–30 min [2.2 ± 0.4 (mean ± SE)-fold increase at 30 min; \( P < 0.05 \) compared with basal level; Fig. 6A]. To determine whether Ras activation in COS-7 cells is upstream of PI3-K, we determined the effect of wortmannin on cAMP-induced Ras activation. Wortmannin did not affect cAMP-mediated Ras activation (Fig. 6B). In WIF-B9 cells, cAMP did not activate Ras; however, in the presence of cAMP, wortmannin reduced Ras activation to below control levels (Fig. 6B). These data suggest that, in COS-7 cells, Ras participates in PI3-K activation and is either upstream or independent. In contrast, cAMP-mediated increase in PI3-K activity in WIF-B9 cells does not involve activation of Ras.

To determine whether cAMP-induced PI3-K activation is dependent of Ras, we transfected COS-7 cells with dominant-negative Ras (Ras17N). Figure 6C, right, shows overexpression of immunoreactive Ras in WIF-B9 cells treated with cAMP and PTX. Whole cell lysates (200 μg) were immunoprecipitated with anti-p85 antibodies, and PI3-K activity was assayed as in Fig. 1. Each value represents the mean ± SE from 3–6 independent experiments. \( *P < 0.05 \) vs. cAMP alone (paired t-test).

![Fig. 5. cAMP-induced PI3-K activation is KT-5720 sensitive.](http://ajpcell.physiology.org/)
transfected cells. Transfection of Ras17N did not affect EGF-mediated PI3-K activation, which is consistent with recruitment of p85 to tyrosine-phosphorylated proteins in EGF-mediated activation of PI3-K (Ref. 55; Fig. 6C, left). In contrast, transfection of Ras17N abolished cAMP-mediated PI3-K activation, suggesting that Ras is upstream of PI3-K after stimulation with cAMP in COS-7 cells. Similar experiments could not be performed in WIF-B9 cells because, as we (42) and others have observed, conventional techniques for transient transfection of WIF-B9 cells produce insufficient yield for biochemical studies.

**Gβγ involvement in cAMP-mediated PI3-K activation.** Gβγ is reported to stimulate PI3-K in many cell types (27, 47, 49, 51). We used an adenoviral vector that expresses β-ARKct to inhibit signaling through Gβγ. β-ARKct specifically binds to and sequesters Gβγ complexes (7). Cells were infected with the adenovirus, and the effect of cAMP on PI3-K activation was quantified. The expression of β-ARKct was confirmed by Western blotting (Fig. 7A, top). Whereas control adenovirus infection had no effect, β-ARKct infection attenuated cAMP-mediated PI3-K activation by 68% and 70% in COS-7 and WIF-B9 cells, respectively (Fig. 7A, bottom), suggesting involvement of Gβγ in this process. We also determined whether Gβγ is required for cAMP-induced Ras activation in COS-7 cells (Fig. 7B). Expression of β-ARKct blocked cAMP-mediated activation of Ras, whereas infection with control adenovirus did not affect Ras activation [fold increase: 1.8 ± 0.3 (mean ± SE) for cAMP + empty adenovirus and 0.9 ± 0.2 for cAMP + β-ARKct; P < 0.05]. Similar results were observed with pERK1/2 [fold increase: 2.5 ± 0.5 (mean ± SE) for cAMP + empty adenovirus and 1.2 ± 0.4 for cAMP + β-ARKct; P < 0.05; Fig. 7B], which provides a positive control for attenuation of Ras function in response to β-ARKct. These observations suggest that Gβγ is upstream of Ras in response to cAMP in COS-7 cells.

**DISCUSSION**

cAMP activated class IA PI3-K in a time- and dose-dependent manner in COS-7 and WIF-B9 cells. There have been few studies determining PI3-K activity in response to cAMP. PI3-K activation was reported in
FRTL-5, a rat thyroid follicular cell line (33), and PC12, a pheochromocytoma cell line (37). In our study, increased PI3-K activity was detected at 15 min, remained elevated for 2 h, and declined at 4 h. This time course of response differs from effects observed after addition of growth factors such as PDGF, in which PI3-K activity peaks at 10–15 min and returns to baseline within 1 h (6, 48, 50). On stimulation with growth factors, p85 subunit SH2 domains interact with specific phosphotyrosine motifs (46), resulting in activation of PI3-K activity (8). Tyrosine phosphorylation can be removed quickly by tyrosine phosphatases, which leads to dissociation of p85 from target proteins. In contrast to the response induced by growth factors, cAMP activation of p85-associated PI3-K was independent of phosphotyrosine in both cell lines.

Akt is a downstream target of PI3-K and, in some studies, cAMP-induced Akt activation was blocked by a PI3-K inhibitor, wortmannin (13, 37, 54). However, in 293-EBNA cells, forskolin activation of Akt was PI3-K independent (10), suggesting that Akt response to cAMP may differ by cell type. Although cAMP increased PI3-K activity in both COS-7 and WIF-B9 cells, Akt activation was detected only in COS-7 cells and was inhibited by nanomolar concentrations of wortmannin. In WIF-B9 cells, insulin but not cAMP activated Akt, which suggests that different pools of PI3-K are utilized. Because cAMP activates Akt in hepatocytes (28, 54, 56), WIF-B9 cells may lack intermediate molecules required for cAMP-mediated Akt activation in response to PI3-K. Additional observations suggest that PI3-K activation by cAMP may not be directly linked to Akt. In both cell lines, maximal PI3-K activation was observed at 60 min (Fig. 1, C and D) whereas maximal phosphorylation of Akt occurred at 15 min and declined to baseline levels by 60 min (Fig. 3, A and B).

To examine mechanisms of activation based on PI3-K catalytic subunits, we determined expression of p110 isoforms in COS-7 and WIF-B9 cells. In some studies, cAMP induction of PI3-K, which suggests that different pools of PI3-K are utilized. Because cAMP activates Akt in hepatocytes (28, 54, 56), WIF-B9 cells may lack intermediate molecules required for cAMP-mediated Akt activation in response to PI3-K. Additional observations suggest that PI3-K activation by cAMP may not be directly linked to Akt. In both cell lines, maximal PI3-K activation was observed at 60 min (Fig. 1, C and D) whereas maximal phosphorylation of Akt occurred at 15 min and declined to baseline levels by 60 min (Fig. 3, A and B).

Fig. 7. cAMP-induced PI3-K activation is Gβγ dependent. A: COS-7 and WIF-B9 cells were infected with adenovirus expressing β-adrenergic receptor kinase COOH terminus (β-ARKct) (β-ARKct AV) at 100 multiplicities of infection (MOI) and incubated for 2 days in complete medium. Empty adenovirus (empty AV) was used as a control. After starvation, cells were stimulated with cAMP (100 μM) for 15 min. PI3-K activity was assayed in whole cell lysates (200 μg) immunoprecipitated with anti-p85 antibodies. Each value represents the mean ± SE from 3 independent experiments. *P < 0.05 vs. cAMP alone (paired t-test). Whole cell lysates (20 μg) were immunoblotted with a β-ARK antiserum that recognizes the carboxyl terminus of β-ARK2 (top). B: activation of Ras and ERK1/2 is Gβγ dependent in COS-7 cells. COS-7 cells infected with β-ARKct AV were stimulated with cAMP (100 μM) for 30 min, and GTP-bound Ras and ERK1/2 phosphorylation were determined. To confirm an equal amount of total Ras in each sample, 20 μg of total cell lysates were immunoblotted with anti-Ras antibodies. Similar studies were performed with antibodies to ERK1/2 or pERK1/2. The blot shown is representative of 3 independent experiments.
of this protein in colonic epithelium (43). Lyso phosphaticid acid- and carbachol-induced Akt activation require p110", which suggests that p110\(\beta\) expression may be essential for transducing cAMP signaling to Akt in WIF-B9 cells.

\(\gamma\) specifically activates p110\(\gamma\) (47) and p110\(\beta\) (19, 27, 30). Blocking downstream signaling from heterotrimeric G proteins by expression of \(\beta_{12}\)-ARKct, which specifically binds to and sequesters \(\gamma\) complexes (7), impaired activation of PI3-K by cAMP in both cell lines. Because WIF-B9 cells express only p110\(\alpha\), it is likely that \(\gamma\) involvement is indirect. PI3-K activation was also sensitive to KT-5720, suggesting involvement of PAK. The molecular mechanism whereby PKA leads to release of \(\gamma\) is not well understood; a signal switch to G from G has been proposed in response to \(\beta_{2}\)-adrenergic receptor stimulation (4). Because PTX did not block cAMP-induced PI3-K activation, other pathways probably exist. For example, reactive oxygen species can induce \(\gamma\) liberation without activating G protein-coupled receptors (34).

An additional mechanism for PI3-K activation is activation of Ras. GTP-bound Ras can directly bind to PI3-K catalytic subunits, resulting in conformational changes and increased catalytic activity (40). Ras can also be a downstream target of PI3-K (17, 55). cAMP induced an increase in GTP-bound Ras in COS-7 and melanocytes (1) but not in WIF-B9 cells. Activation of Ras in COS-7 cells was not blocked by wortmannin, and expression of the dominant-negative Ras construct (RasN17) abolished PI3-K activation by cAMP. These data suggest that Ras activation is upstream of PI3-K in COS-7 cells and is required for cAMP-mediated PI3-K activation. These observations are also consistent with activation of ERK1/2 by cAMP, which was not sensitive to wortmannin (data not shown). Several mechanisms have been proposed for Ras activation in response to cAMP. cAMP-GEFs can directly activate Rap1/2 (5, 22), and a cyclic nucleotide Ras-GEF, CDS-25Mm, which is phosphorylated by PKA, has also been described (36). However, expression of these GEFs has been found specifically in brain. Other, as yet unidentified, molecules may function as GEFs for Ras in response to cAMP in other cells. Ras and ERK1/2 activation were blocked by expression of \(\beta_{12}\)-ARKct, which suggests that \(\gamma\) release is required for Ras activation in COS-7 cells. Although the molecular mechanisms are not well defined, \(\gamma\) release may activate Ras by initiating assembly of a multiprotein complex including \(\beta\)-arrestin or c-Src in clathrin-coated pits (29, 38).

A recent report showed that cAMP had an opposite effect in COS cells that overexpress PI3-K and Akt (23). Under these conditions, cAMP blocked PI3-K and Akt activation. The difference between our data and these results may be due to the use of different clones of COS cells or to the fact that overexpression of these proteins leads to a different response.

In conclusion, cAMP activated PI3-K in COS-7 and WIF-B9 cells and the pathway leading to PI3-K activation was cell type specific. PKA and \(\gamma\) were required in both cell lines; however, the response in COS-7 cells required Ras activation whereas the response in WIF-B9 cells did not. Our study indicates that there are different pathways by which cAMP activates PI3-K. How these individual mechanisms relate to specific cellular responses, such as bile acid secretion, requires additional studies, which are in progress.

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