Suppression of Cyclic GMP-dependent Protein Kinase Is Essential to the Wnt/cGMP/Ca\textsuperscript{2+} Pathway*

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Novel downstream effectors sensing changes in intracellular concentrations of Ca\textsuperscript{2+} and cyclic GMP in response to activation of the Wnt/Frizzled-2 pathway were sought. Activation of Frizzled-2 suppressed protein kinase G activity while activating NF-AT-dependent transcription. Each of these responses was abolished by pertussis toxin and by knock-down of the expression of either G\textsubscript{alpha}i\textsubscript{3} or G\textsubscript{alpha}\textsubscript{q}. Activation of NF-AT-dependent transcription in response to Wnt5a stimulation was suppressed by activation of protein kinase G and by buffering intracellular Ca\textsuperscript{2+}. Elevation of intracellular cyclic GMP either by inhibition of cyclic GMP phosphodiesterase or by addition of 8-bromo-cyclic GMP was shown to activate protein kinase G, to block Ca\textsuperscript{2+} mobilization, as well as to markedly attenuate activation of NF-AT-dependent transcription in response to Wnt5a stimulation. Chemical inhibition of protein kinase G by Rp-8-pCPT-cGMP, conversely, was shown to provoke increased NF-AT gene transcription and Ca\textsuperscript{2+} mobilization in the absence of Wnt stimulation. Protein kinase G is shown to be a critical downstream effector of the noncanonical Wnt-Frizzled-2/cGMP/Ca\textsuperscript{2+} pathway.

Wnts are secreted, palmitoylated glycoprotein ligands that regulate development, acting via the heptahelical, G protein-coupled Frizzleds (1, 2). The Frizzled subfamily of G protein-coupled receptors is highly conserved and is composed of 10 members in mammals (3). The first Wnt-sensitive pathway to be elucidated, mediated by Frizzled-1, regulates the cellular stability of \beta-catenin (i.e. the Wnt/\beta-catenin or "canonical" pathway) and thereby the transcriptional activation of developmental genes that are sensitive to members of the lymphocyte enhancer factor/T cell factor (Lef/Tcf) transcription factors (4, 5). The Wnt/Ca\textsuperscript{2+}/cyclic GMP pathway constitutes a noncanonical pathway that is mediated by Frizzled-2 (6–8). Activation of Frizzled-2 by Wnt5a leads to activation of the phosphatidylinositol pathway (9) and activation of Ca\textsuperscript{2+}-sensitive enzymes, including the protein kinases, protein kinase C, and calcium/calmodulin-sensitive protein kinase II (10), as well as the phosphatase calcineurin (11). Ca\textsuperscript{2+} imaging with Fura-2 dye in whole embryos of zebrafish has revealed Wnt5a-stimulated Ca\textsuperscript{2+} mobilization (12). More recently this Wnt/Ca\textsuperscript{2+} pathway has been expanded by the discovery of G protein-mediated activation of a cyclic GMP phosphodiesterase (i.e. PDE6)\textsuperscript{2} that results in a Wnt-induced, sharp decline in the intracellular concentration of cyclic GMP (7, 13, 14).

Unlike the situation for downstream effectors sensitive to Ca\textsuperscript{2+} mobilization, elucidation of the downstream effector(s) for Wnt-regulated changes in the intracellular concentration of cyclic GMP has remained elusive. It is not known, for example, whether the signals from Wnt5a mediated via Frizzled-2 to Ca\textsuperscript{2+} mobilization and to cyclic GMP PDE operate in concert or act independently of each other in the Wnt/Ca\textsuperscript{2+}/cGMP pathway. Although probed in zebrafish embryos and mouse F9 cells, Ca\textsuperscript{2+} mobilization in response to Wnt signaling has not been investigated in the context of immediate upstream regulatory element. In the current study, we identify the cyclic GMP-dependent, serine/threonine protein kinase G (PKG) as a primary upstream effector of Ca\textsuperscript{2+} mobilization in response to Wnt5a, essential for signaling of the noncanonical Wnt/cGMP/Ca\textsuperscript{2+} pathway.

MATERIALS AND METHODS

Cell Culture—Mouse F9 teratocarcinoma cells were obtained from the ATCC collection (Manassas, VA). The cells were propagated and maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% heat-inactivated fetal bovine serum (Hyclone, South Logan, UT) at 37 °C in a 5% CO\textsubscript{2} incubator. Clones stably transfected with pcDNA3 harboring either Rfz2 or \beta\textsubscript{2}AR/Rfz2 or co-transfected with NF-AT-sensitive luciferase reporter gene (NFAT-Luc) (Stratagene, La Jolla, CA) were selected in medium containing neomycin analogue, G418 (0.4 mg/ml; Invitrogen, Carlsbad, CA). At least three independent clones were propagated for each transfection and maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.1 mg/ml G418. The H7 clone of human embryonic stem cells (hESC) was obtained from the WiCell Research Institute. H7 cells were cultured on mytomycin C-treated mouse embryonic fibroblasts in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 2% knockout serum replacement, 1% nonessential amino acids, 1 mM l-glutamine, 0.1 mM \beta-mercaptoethanol, and 4 mg/ml basic

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* This work was supported by Grant GM 069375 from the NIGMS, National Institutes of Health. 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.

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2 The abbreviations used are: PDE, phosphodiesterase; PKG, protein kinase G; hESC, human embryonic stem cell(s); WT, wild-type; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N′,N″,N‴-tetraacetic acid tetra(acetoxyethyl) ester; 8-Br-cGMP, 8-bromo-cyclic GMP.
fibroblast growth factor (all from Invitrogen). Purified mouse recombinant Wnt5a (645-WN) and anti-Wnt5a antibody (AF645) were purchased from R & D Systems (Minneapolis, MN).

Assay of PKG Activity—PKG activity was determined as described previously with modifications (15). Briefly, supernatants (20 μl) from the crude whole cell fractions were employed with 200 mg/ml PKG-specific heptapeptide substrate (RKRSRAE; Bachem, San Siego, CA), 50 μM ATP, and 2.5 μCi of [γ-32P]ATP in TMG buffer containing 20 mM Tris-HCl, pH 7.4, 20 mM magnesium acetate, 10 mM glyceraldehyde, 100 mM okadacid, and a protease inhibitor mixture (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride). The reaction mixtures were incubated at 37 °C for 20 min, and the reaction was terminated by the addition of 4 μl of 2 N HCl. Preboiled or HCl-treated supernatants were used as blank samples. Twenty microliters of the resultant mixture were spotted onto P-81 phosphocellulose filters. Air-dried P-81 filters were washed with H3PO4 (75 mM) three times. Incorporated [γ-32P] in substrates were measured by liquid scintillation spectrometry. The samples were assayed in triplicate. Kinase activity is normalized with protein concentration and presented in percentages of PKG activity of treatment group to that of the control group.

Cytoplasmic Calcium Measurement—Mouse F9 cells were plated on collagen-coated coverslips inserted in 35-mm dishes (MatTek Corporation, Ashland, MA) and cultured overnight. hESC free of contaminating mouse embryonic fibroblasts were cultured on Matrigel-coated (BD Biosciences) coverslips in medium conditioned by mouse embryonic fibroblasts and supplemented with basic fibroblast growth factor. The intracellular Ca2+ was measured as described (16). Briefly, coverslip-attached cells were loaded with 2 μM Fura-2 acetoxyethyl ester (Molecular Probes, Inc., Eugene, OR) for 40 min at 37 °C in the dark. The cells were then washed three times with Krebs buffer composed of 128 mM NaCl, 5 mM KCl, 77.5 mM NaH2PO4, 1.3 mM MgSO4, and 1.3 mM CaCl2 and kept in the same buffer at room temperature for 10 min in the dark. Fura-2-loaded cells were treated with isoproterenol (10 μM) or with Wnt5a (25 ng/ml) or buffer alone. The cells were immediately monitored via a Hamamatsu ORE-ER AG digital CCD camera coupled to a Nikon inverted microscope. Excitation at 340 and 380 nm were generated using a 175-watt ozone-free xenon lamp and a Fura-2 fluorescence filter set coupled to a Sutter Lambda DG-4 high speed filter switcher. The emitted light was channeled into the CCD camera via an ultraviolet dichroic mirror and an interference filter of 510 nm. Measurement of fluorescence intensity was performed at a rate of 200 points/s, and the ratio of absorbance at 340/381 nm (A340/A380) was computed using Dynamic Intensity Analysis (Compx Inc., Cranberry Township, PA).

Treatment of Cells with Morpholino Antisense Oligonucleotides—Morpholino phosphorodiamidate antisense oligonucleotides targeting the translational initiation sites of Goα12, Goα13, and Goαq were purchased from Gene Tools (Corvallis, OR). The sequences of morpholinos for Goα12, Goα13, and Goαq are as follows: CACTCCCCATTTCTGCTGTCTCCTC is for Goα12, CTCCCCGCGCTCCTCAGACGCC is for Goα13, and CCTCTCGGTCCAGGTACATCCCAT is for Goαq. F9 cells (wild-type or stably transfected with the desired genes according to the requirements of the experiments) were treated with morpholino antisense oligonucleotides according to the manufacturer’s Special Delivery protocol. Briefly, the cells were seeded onto 60-mm culture dishes. The cells were 80% confluent on the day morpholino treatment was applied. Growth medium was replaced by the serum-free medium containing 1.4 μM morpholino antisense oligonucleotides and 0.6 μM ethoxylated polyethylenimine delivery reagent. After 3 h of incubation at 37 °C, the cells were grown in standard culture medium with serum, but free of morpholinos, for an additional 72 h before the expression levels of target proteins were assessed by immunoblotting. For cultures that were subsequently subjected to further PKG or NF-AT activity assays, the cells were treated either with isoproterenol or Wnt5a and not (control).

Immunoblotting—Whole cell lysates were used for detecting PKG and Go protein subunits. The samples (30 μg of protein/lane) were subjected to SDS-PAGE for separation. The resolved proteins were transferred onto nitrocellulose membranes. The blots were probed with antibodies against PKGβ, PKGαβ/γ (Stressgen Biotechnologies, Victoria, Canada), or the indicated Go protein subunits (anti-Goαi, anti-Goα12 from Santa Cruz Biotechnology, Santa Cruz, CA; anti-Goαq from Chemicon, Temecula, CA) and followed by incubation with a corresponding peroxidase-conjugated secondary antibody (anti-mouse from Santa Cruz Biotechnology; anti-rabbit from Kirkegaard and Perry Laboratories, Gaithersburg, MD). Immune complexes were detected by enhanced chemiluminescence method, as per the manufacturer’s instructions. Equal loading was verified by probing with antibodies to β-actin (Sigma-Aldrich).

NF-AT-sensitive Luciferase Assay—F9 clones stably co-transfected with NF-AT reporter gene and with expression vector harboring either Rfz1 or βAR/Rfz1 chimera were cultured on 12-well plates. The cells were starved from serum for overnight and then were stimulated with Wnt5a (25 ng/ml) or isoproterenol (10 μM) for 5 h or indicated periods. The cells were lysed with 1× luciferase cell culture lysis reagent (Promega, Madison, WI). Supernatants from cell lysates were subjected to luciferase assay according to the manufacturer’s instruction (Stratagene, La Jolla, CA). Briefly, 20 μl of supernatant were mixed with 100 μl of luciferase assay buffer (40 mM Tricine, pH 7.8, 0.5 mM ATP, 10 mM MgSO4, 0.5 mM EDTA, 10 mM 1,4-dithiothreitol, 0.5 mM coenzyme A, and 0.5 mM luciferin), and the intensity of luminescence was immediately measured using a luminometer (Lumat LB 9507; Berthold Technologies, Oak Ridge, TN). Samples were assayed in triplicate, and the luciferase activity was normalized based on protein concentrations of the cell lysate. The results are presented as ratios of relative light units of treatment groups to control groups.

Statistical Analysis—The experiments were conducted at least in triplicate. All of the data are expressed as the means ± S.E. from at least three separate experiments. Comparisons of data among groups were performed with one-way analysis of variance followed by the Newman-Keuls test. Statistical significance (p value of less than 0.05) is denoted with asterisks or pound symbols.
RESULTS

Ca\textsuperscript{2+} Mobilization in Response to Frizzled-2 Activation in Mouse Embryonic F9 Cells—The mouse F9 embryonic teratocarcinoma (F9) cells are totipotent cells that were shown earlier to be capable of being transfected with \(\beta_2\)-adrenergic receptor/Frizzled chimeras or authentic Frizzleds that, when activated, promote these embryonic cells in culture to form a primitive endoderm-like phenotype characteristic of early stages in mouse development (2, 13). These cells express a modest number of \(\beta_2\)-adrenergic receptors. Frizzled-2 expression by wild-type F9 cells, if any, is insufficient to enable Wnt5a-stimulated activation of the cyclic GMP/Ca\textsuperscript{2+} pathway.

Mouse F9 cells stably expressing the \(\beta_2\)-adrenergic/rat Frizzled-2 chimera (\(\beta_2\)AR/Rfz2), a chimera that binds \(\beta\)-adrenergic agonist but stimulates the Frizzled-2 pathway (17), were loaded with the Ca\textsuperscript{2+}-sensitive dye Fura-2 and treated with the isoproterenol (10 \(\mu\)M; Fig. 1A). In these embryonic cells, activation of the Frizzled-2 chimera provokes an increase in intracellular concentrations of Ca\textsuperscript{2+} within 60 s that can be visualized by fluorescence microscopy and monitored in real time as the change in the ratio of absorbance of light at 340 nm \textit{versus} that at 380 nm, that is characteristic for Ca\textsuperscript{2+}-activated Fura-2. The kinetics of the Ca\textsuperscript{2+} response agrees well with earlier measurements assayed by spectroscopy (17). The Frizzled-2 stimulation of Ca\textsuperscript{2+} mobilization plateaus within 3 min and is maintained for up to 10 min in the continued presence of agonist stimulation. Stimulating zebrafish embryos expressing \(\beta_2\)AR/Rfz2 chimera with isoproterenol leads to Ca\textsuperscript{2+} transients (13), identical in character to those stimulated by Wnt5a alone (12). Wild-type (WT) and F9 cells stably transfected with an “empty” expression vector display no Ca\textsuperscript{2+} response to activation of the Frizzled-2 pathway (Fig. 1A, inset).

The pharmacological properties of the Ca\textsuperscript{2+} response of F9 cells expressing the Frizzled-2 chimera to \(\beta\)-adrenergic agonist was probed further. The increase in intracellular Ca\textsuperscript{2+} stimulated by activation of the Frizzled-2 chimera with agonist could be abolished by simultaneous treatment with the \(\beta\)-adrenergic antagonist propranolol (+ Pro, 10 \(\mu\)M) (Fig. 1B). Expression of this Frizzled-2 chimera in zebrafish embryos by-passed the need for purified Wnt5a, allowing the use of isoproterenol to activate the noncanonical pathway, a response that also was sensitive to antagonism by propranolol (13). The Frizzled-2 activation of Ca\textsuperscript{2+} mobilization was sharply attenuated by the pretreatment of the F9 cells with pertussis toxin (Fig. 1B), an agent that catalyzes the ADP-ribosylation and inactivation of G protein \(\alpha\)-subunits of the Go family, including Go, G\(\alpha_2\), and G\(\alpha_4\) (18–20).

We compared the Ca\textsuperscript{2+} response stimulated by purified Wnt5a using F9 cells expressing the authentic rat Frizzled-2 (Fig. 1C) to the response stimulated by the activation of the Frizzled-2 chimera (Fig. 1B). Wnt5a stimulates a mobilization of intracellular Ca\textsuperscript{2+} in the F9 clones expressing Frizzled-2 (Fig. 1C). Application of Wnt3a, which can activate Frizzled-1 but not Frizzled-2 receptors, in contrast, did not stimulate the Ca\textsuperscript{2+} response (data not shown). Differences in the kinetics of the stimulated Ca\textsuperscript{2+} mobilization between the clones expressing Frizzled-2 versus the \(\beta_2\)AR/Rfz2 chimera are apparent (Fig. 1, B and C). First, the kinetics of the Ca\textsuperscript{2+} response to Wnt5a stimulation was biphasic, whereas the response to stimulation of the chimeric receptor was not. Second, the kinetics of Ca\textsuperscript{2+} response to Wnt5a stimulation was not as rapid as that of F9 cells expressing the chimera and stimulated by \(\beta\)-adrenergic agonist. The slower and atypical kinetics of the Wnt5a-stimulated response are likely to be a reflection of the palmitoylated, glycoprotein nature of the Wnt5a, which tends to form micelles in slow equilibrium with the bulk solution and Frizzled receptors (21). Pertussis toxin treatment nearly abolished Wnt5a-stimulated Ca\textsuperscript{2+} mobilization for F9 cells expressing the Frizzled-2, as it did for clones expressing the \(\beta_2\)AR/Rfz2 chimera stimulated by isoproterenol (Fig. 1, B and C). These experiments are the first to demonstrate a \textit{bona fide}, pertussis toxin-sensitive Ca\textsuperscript{2+} mobilization of mammalian embryonic cells in culture, following activation of the Frizzled-2 pathway. With this model system, detailed biochemical analysis of the downstream signaling of the Wnt/Ca\textsuperscript{2+}/cGMP that is not possible in zebrafish embryos was enabled in F9 cells. Finally, we tested in another embryonic cell line, H7 hESC in culture, whether purified Wnt5a regulated the intracellular Ca\textsuperscript{2+} concentration, using the same approach adopted for F9 cells. Although not fully characterized, hESC do express Frizzled-2 receptor (data not shown). Treating H7 hESC with Wnt5a stimulated an increase in intracellular Ca\textsuperscript{2+} levels (Fig. 1D), which agrees with the response observed in F9 cells (Fig. 1C). The addition of excess anti-Wnt5a antibody to the Wnt5a mixture neutralized the ligand, blocking the Wnt5a-stimulated Ca\textsuperscript{2+} response (Fig. 1D).

Frizzled-2 Regulates NF-AT-dependent Transcriptional Activation—Whereas the canonical Wnt/\(\beta\)-catenin pathway signals to the level of Lef/Tcf-sensitive transcriptional activation (5), the noncanonical Wnt/Ca\textsuperscript{2+} pathway has been reported in \textit{Xenopus} embryos to activate a group of developmentally important genes, including those dependent upon the nuclear transcription factor of activated T-cells (NF-AT) (11). Using a luciferase-based reporter gene construct to assay NF-AT-dependent gene transcription, we set out to test whether the Frizzled-2 signaling downstream of Ca\textsuperscript{2+} mobilization and cGMP extended in mammalian cells to the level of NF-AT-sensitive transcription. NF-AT-dependent transcription indeed was activated in F9 clones expressing the Frizzled-2 chimera treated with \(\beta\)-adrenergic agonist (Fig. 2A). The transcriptional response to Frizzled-2 activation was first observed at 2 h post-stimulation, with a peak response noted within 5 h. The NF-AT transcriptional response remained elevated for more than 5 h, thereafter declining to near basal levels within 20 h. For F9 cells expressing the \(\beta_2\)AR/Rfz2 chimera, treatment with \(\beta\)-adrenergic antagonist propranolol effectively blocks the Frizzled-2-mediated activation of the NF-AT-dependent transcription in response to isoproterenol, as it does the Ca\textsuperscript{2+} mobilization (Fig. 1B).

If the results obtained from cells expressing the \(\beta_2\)AR/Rfz2 chimera accurately reflected Frizzled-2 action, then treatment of F9 clones stably expressing authentic rat Frizzled-2 (Rfz2) with purified Wnt5a would be expected also to stimulate NF-AT-dependent transcription (Fig. 2B). Wnt5a stimulates a tran-
The NF-AT-dependent transcriptional response to Wnt5a stimulation displays kinetics (Fig. 2B) in good agreement with those obtained in F9 cells expressing the β2AR/Rfz2 chimera (Fig. 2A).

The NF-AT-dependent transcriptional response was tested for specificity. Wnt5a antibody, which neutralizes the ligand,
abolishes Wnt5a-stimulated activation of NF-AT-dependent transcription in rat Frizzled-2-expressing cells (Fig. 2C). F9 clones expressing the β2AR/Rfz1 chimera were compared with the β2AR/Rfz2 chimera-expressing clones for NF-AT transcriptional activation in response to stimulation with β-agonist (Fig. 2D). Treatment with the β-adrenergic agonist isoproterenol stimulates activation of the NF-AT-dependent transcription in clones expressing the β2AR/Rfz2 chimera. The F9 clones expressing the β2AR/Rfz1 chimera, in sharp contrast, displayed little NF-AT-sensitive transcriptional activation, i.e. the same level of NF-AT-dependent transcription observed in the WT or empty vector transfected F9 cells (data not shown). These data agree with the observations that Wnt5a, but not the Frizzled-1 agonist ligand Wnt3a, stimulates the intracellular Ca\(^{2+}\) response in the F9 clones expressing authentic Rfz2 (data not shown).
TABLE 1
Efficiency of knockdown of G-protein subunits targeted by antisense morpholinos
The abundance of each subunit in the absence of antisense morpholino treatment is set to a value of 1.00. The results displayed are the mean values ± S.E. from four or more separate experiments. An additional control, measurement of β-actin, provided an independent measure of the loading uniformity in the immunoblotting of whole-cell lysates from F9 cells. See the text for details. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

| Antisense Protein expression | Gα1 | Gα2 | Gαo |
|-----------------------------|-----|-----|-----|
| Gα2                         | 1.00 ± 0.08 | 1.17 ± 0.06 | 1.00 ± 0.15 | 0.22 ± 0.01* | 1.00 ± 0.09 | 1.18 ± 0.06 |
| Gα1                         | 1.00 ± 0.18 | 0.21 ± 0.03* | 1.00 ± 0.06 | 1.01 ± 0.03 | 1.00 ± 0.07 | 1.07 ± 0.04 |
| Gαo                         | 1.00 ± 0.02 | 1.09 ± 0.07 | 1.00 ± 0.06 | 1.10 ± 0.08 | 1.00 ± 0.04 | 0.25 ± 0.05*** |
| β-actin                     | 1.00 ± 0.07 | 0.94 ± 0.05 | 1.00 ± 0.09 | 0.96 ± 0.09 | 1.00 ± 0.03 | 1.05 ± 0.08 |

We explored whether or not G proteins were mediating the Frizzled-2 signaling to the level of NF-AT transcriptional activation. As was observed for the Wnt5a/Ca²⁺ response, pertussis toxin pretreatment largely abolished the NF-AT-dependent transcriptional response (Fig. 2E). We used morpholino antisense oligonucleotides to suppress the expression of the two pertussis toxin G protein α-subunits, Gα₁₂ and Gα₁₆, previously shown to mediate Wnt regulation of Ca²⁺ mobilization and activation of cyclic GMP PDE (13). The antisense morpholinos were able to knock down the expression of the targeted G protein subunit by more than 75% (Fig. 2E, insets, and Table 1). Knock-down of either Gα₁₂ or Gα₁₆, but not Gα₁₃ (control), with antisense morpholinos attenuates the Frizzled-2-mediated activation of NF-AT-dependent transcription (Fig. 2E). Taken together these data demonstrate that NF-AT transcriptional activation by Frizzled-2 is a G protein-mediated downstream readout of the Wnt/Ca²⁺/cGMP pathway.

We performed similar diagnostic tests on the role of intracellular Ca²⁺ on Frizzled-2 regulation of NF-AT-sensitive transcriptional activation. We made use of the membrane-permeable form of a Ca²⁺-buffering agent, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA), to neutralize Frizzled-2 stimulated changes in intracellular Ca²⁺ levels. Preloading the cells with the acetoxymethyl ester of BAPTA buffers intracellular Ca²⁺ and was found to abolish the Frizzled-2 activation of the NF-AT transcription (Fig. 2F). The levels of “basal” NF-AT transcription, in contrast, were largely unaffected by buffering intracellular Ca²⁺ with BAPTA. Thus, Ca²⁺ mobilization in response to activation of Frizzled-2 is upstream to and obligate for the expression of NF-AT transcriptional regulation by the Wnt/Ca²⁺/cGMP pathway.

Noncanonical Wnt/Ca²⁺/cGMP PDE Signaling Regulates Protein Kinase G—Earlier it was shown that the Wnt/Frizzled-2 pathway includes not only Ca²⁺ mobilization but also activation of cyclic GMP-sensitive PDE6 (14), catalyzing a sharp decline in intracellular concentrations of cyclic GMP (13). The intracellular sensor(s) for cyclic GMP in the Wnt/Ca²⁺/cGMP pathway is (are) not known. Likewise, whether or not the regulation of the Ca²⁺ mobilization and of the intracellular concentrations of cyclic GMP reflects common or distinct signaling pathways downstream of Wnt5a/Frizzled-2 is unknown. A prime candidate effector worthy of analysis is the cyclic GMP-dependent serine/threonine protein kinase, PKG. PKG was tested as a possible downstream effector by measuring its activity directly. F9 clones stably expressing the Frizzled-2 chimera were stimulated with β-adrenergic agonist, and PKG activity was assayed. If PKG is an effector for the Frizzled-2 pathway, activation of Frizzled-2/PDE signaling would be expected to reduce PKG activity. PKG activity was found to be reduced in response to activation of the Frizzled-2 pathway (Fig. 3A). The decline in PKG activity mimics the decline of the intracellular concentration of cyclic GMP provoked by activation of the Wnt/Ca²⁺/cGMP pathway (13). The decline in PKG activity in response to Frizzled-2 activation was not reflected at the level of PKG mRNA, because reverse transcription-PCR amplification of the cellular RNA revealed no change in PKG mRNA abundance over this time frame (data not shown). At the protein level, the expression of Iα and Iβ subunits of PKG measured by immunoblotting of whole cell lysates of F9 cells also revealed no change in PKG subunit abundance (Fig. 3A). Thus, activation of Frizzled-2 stimulates a decline in PKG activity, but not in its abundance. We examined whether the regulation of PKG activ-
ity by Frizzled-2 activation was readily reversible, making use of the β2AR/Rfz2 chimera-expressing cells, in which isoproterenol can activate and propranolol antagonist the Frizzled-2 pathway (2, 13, 17). The activation of Frizzled-2 chimera by isoproterenol was maintained for 30 min, with a corresponding decline in PKG activity (Fig. 3B). At that point, propranolol was added to half of the tubes to block β-adrenergic agonist stimulation of Frizzled-2 chimera, and the incubation continued for 90 additional min. The addition of propranolol antagonizes and fully reverses within 90 min the decline in PKG activity caused by the stimulation of the Frizzled-2 pathway (Fig. 3B).

As noted above, inhibition of PKG activity for F9 clones expressing the Frizzled-2 chimera in response to the β2-adrenergic agonist propranolol is sensitive to blockade by the β2-adrenergic antagonist propranolol (Fig. 3C). The role of G proteins in development was first detected by the ability of pertussis toxin, which inactivates members of the Gi family of heterotrimeric G proteins, to block key pathways. We tested whether pertussis toxin treatment also blocks the ability of Frizzled-2 chimera to activate the downstream events that culminate in the inhibition of PKG activity (Fig. 3C). Pertussis toxin abolishes the ability of Frizzled-2 to reduced PKG activity, much like it did for the Ca2+ mobilization response stimulated by the activation of Frizzled-2 (Fig. 1B).

We tested these primary observations in F9 clones expressing authentic rat Frizzled-2 and stimulated with purified Wnt5a. Wnt5a treatment stimulates a frank decrease in PKG activity (Fig. 3D), in good agreement with the data derived from cells expressing the Frizzled-2 chimera (Fig. 3, A and C). Furthermore, Frizzled-2-mediated suppression of PKG activity in response to Wnt5a was blocked by pretreatment of the cells with pertussis toxin (Fig. 3D). These observations suggest that members of the Gi family mediate Wnt5a signaling downstream to PKG. If this premise is correct, then knock-down of G proteins implicated in Frizzled-2 signaling (as shown in Fig. 2E) should also abolish the ability of Frizzled-2 signaling to regulate PKG activity. Knock-down studies of G proteins involved in the Wnt/Frizzled-2/Ca2+/cGMP pathway revealed that Ga1o and Ga27, but not Ga11 (control), were essential for the reduction of PKG activity in response to activation of Frizzled-2 (Fig. 3E).

To further test these observations, we investigated the ability of knock-down of selective G protein α subunits to block Wnt5a-induced reduction in PKG activity in F9 cells stably expressing the authentic Rfz2 (Fig. 3F). Knock-down with antisense morpholinos specific for the endogenous Ga1o blocks the ability of Wnt5a to reduce PKG activity. Having attenuated the ability of Wnt5a to regulate PKG by knock-down of endogenous Ga27, we performed an experiment designed to try to rescue the Wnt5a-stimulated response by expressing Ga27 exogenously in the knock-down cells. The Ga27 mRNA expressed by the expression vector lacks the 5′-untranslated region targeted by the antisense morpholinos. Expression of exogenous Ga27 rescued the Wnt5a-stimulated regulation of PKG (Fig. 3F). Expression of exogenous Ga1o, in sharp contrast, does not rescue the PKG response lost in the Ga27-deficient cells. Parallel rescue experiments were performed on F9 cells made deficient in Ga1o (Fig. 3G). In F9 cells stably expressing Rfz2 and treated with morpholinos antisense to Ga1o, the ability of Wnt5a to regulate PKG is lost. Expression of exogenous Ga1o rescued the Wnt5a-stimulated regulation of PKG in these cells (Fig. 3G). Expression of exogenous Ga1o, however, does not rescue the PKG response lost in the Ga1o-deficient cells.

If the decline of the intracellular concentration of cyclic GMP is primarily responsible for the Frizzled-2-stimulated decline in PKG activity, then increasing the intracellular concentration of cyclic GMP would be expected to block Wnt-stimulated action on PKG activity. Treating F9 clones either with the PDE6-selective inhibitor Zaprinast or with a cell-permeable, nonhydrolyzable cyclic GMP analogue, 8-bromo-cyclic GMP (8-Br-cGMP), elevates intracellular concentrations of this cyclic nucleotide (22). Inhibition of cyclic GMP PDE with Zaprinast elevates basal PKG activity and abolished Frizzled-2-stimulated reduction of PKG activity (Fig. 3H). Increasing intracellular cyclic GMP levels with cyclic GMP analogue 8-Br-cGMP also elevates PKG activity and, like Zaprinast treatment, blocks Frizzled-2-stimulated reduction of PKG activity.

**FIGURE 3. Protein kinase G activity is regulated by the noncanonical Wnt/cGMP/Ca2+/cGMP pathway.** A, F9 clones expressing the β2AR/Rfz2 chimera were treated with isoproterenol (10 μM), and the activity of PKG was measured in whole cell lysates prepared at the time period indicated (upper panel). Major isoforms of PKG, PKGα1a and β as well as β-actin in each sample were probed by immunoblotting (middle) with specific antibodies (lower panel). *, p < 0.05. B, F9 cells stably expressing β2AR/Rfz2 chimera receptor were stimulated by isoproterenol alone (+/+) or in the presence of the β2-adrenergic antagonist propranolol after 30 min of incubation of isoproterenol (+/Iso + Pro). PKG activity was measured as described under "Materials and Methods." **, p < 0.05; ***, p < 0.001 versus the propranolol alone (+/Iso) or in the presence of propranolol (+/Iso + Pro); or these clones were pretreated with pertussis toxin for 2 h prior to the stimulation by isoproterenol (+/Iso + PTX).** PKG activity was measured. *, p < 0.05 versus the +/Iso at 0 min; #, p < 0.05, versus the corresponding + Iso groups. C, clones expressing the β2AR/Rfz2 were stimulated by isoproterenol alone (+/Iso) or in the presence of pertussis toxin for 2 h prior to the stimulation by isoproterenol (+/Iso + PTX). PKG activity was measured. **, p < 0.05 versus the +/Iso at 0 min; #, p < 0.05, versus the corresponding + Iso groups. D, F9 clones stably expressing rat Frizzled-2 were stimulated by Wnt5a and PKG activity in whole cell lysates collected at the indicated time was determined. PKG activity from Frizzled-2-expressing cells pretreated with pertussis toxin (+PTX) prior Wnt5a stimulation for 1 h was compared. **, p < 0.01 versus the + Wnt5a at 0 min; ##, p < 0.01 versus the −Wnt5a at 60 min. E, F9 cells expressing β2AR/Rfz2 chimera were treated with morpholino antisense oligonucleotides (Antisense) for 72 h to knock down Ga1o, Ga1o, or Ga27 individually prior to addition of isoproterenol (+/Iso, 10 μM). The cells were lysed after 1 h of incubation of isoproterenol, and PKG activity was measured. The protein levels of Ga1o, Ga1o, and Ga27 were assessed by immunoblotting (Fig. 2C). *, p < 0.05 versus control. F, expression of Ga1o, but not Ga27, rescued the effect of knock-down of Ga27 on PKG activity. F9 cells expressing rat Frizzled-2 receptor were treated with morpholino antisense oligonucleotides to Ga27 (Antisense-Ga27) for 72 h to knock down Ga27. Twenty-four hours after morpholino treatment, the cells were transfected with expression vectors harboring the cDNA of either Ga1o, or Ga27 (versions not sensitive to the antisense morpholinos directed to the 5′-untranslated region of the native G protein counterparts) for 2 days. The cells were stimulated by Wnt5a (+ Wnt5a) or without stimulation (− Wnt5a) for 20 min, and PKG activity in whole cell lysates was measured. ***, p < 0.001. G, expression of Ga1o, but not Ga27, rescued the effect of knock-down of Ga1o on PKG activity. F9 cells expressing rat Frizzled-2 were treated with morpholino antisense oligonucleotides to Ga1o (Antisense-Ga1o) for 72 h to knock down Ga1o. Twenty-four hours after morpholino treatment, the cells were transfected with Ga1o, or Ga27, for 2 days. The cells were stimulated by Wnt5a (+ Wnt5a) or without stimulation (− Wnt5a) for 30 min, and PKG activity in whole cell lysates was measured. **, p < 0.01. H, F9 clones stably expressing β2AR/Rfz2 chimera were treated with Zaprinast (1 μM), 8-Br-cGMP (100 μM), or Rp-8-pCPT-cGMP (2 μM) for 30 min. The F9 clones were then challenged by isoproterenol (+/Iso, 10 μM) for 1 h, and the activity of PKG was measured in whole cell lysates. PKG activity is presented as a percentage by setting the activity at time 0 or without stimulation as 100%. The results are presented as the mean values ± S.E. of three or more separate experiments. ***, p < 0.01; ***, p < 0.001.
**Wnt Signaling via Protein Kinase G**

FIGURE 4. PKG and Ca^{2+} mobilization converge in Frizzled-2 signaling with NF-AT-dependent gene transcription. A, F9 cells stably expressing β_{1}AR/Rfz2 chimera were loaded with Fura-2 and co-incubated without or with either Zaprinast (+Zap, 1 μM) or 8-Br-cGMP (100 μM) for 45 min prior stimulation by isoproterenol (+Iso). The concentration of intracellular free Ca^{2+} measurements were performed as described under "Materials and Methods." B, F9 cells stably expressing rat Frizzled-2 were loaded with Fura-2 and co-incubated without or with either 8-Br-cGMP (100 μM) or Zaprinast (+Zap, 1 μM) for 45 min prior to stimulation by Wnt5α. The intracellular free Ca^{2+} measurements were performed as described. C, wild-type F9 cells were loaded with Fura-2 and cytoplasmic Ca^{2+} was monitored by Fura-2 imaging. The addition of either 8-Br-cGMP (100 μM) or Rp8-pCPT-cGMP (2 μM) commenced 1 min after the monitoring started. The results are presented as ratios of intensity of absorbed fluorescence at excitation 340 over 380 nm monitored from one single experiment, representative of more than three separate experiments with similar results. D, F9 clones stably expressing β_{1}AR/Rfz2 chimera were incubated with Zaprinast, or 8-Br-cGMP, or Rp8-pCPT-cGMP for 30 min and then stimulated by isoproterenol (10 μM, +Iso) for 6 h. The cells were lysed and assayed for NF-AT-dependent luciferase activity. **, p < 0.01; ###, p < 0.001 versus the control; ##, p < 0.01; ####, p < 0.001 versus the +Iso alone group. E, F9 clones stably expressing Frizzled-2 were treated with either buffer (−) or BAPTA for 30 min prior stimulation by Wnt5α. The activity of PKG was measured in whole cell lysates after 30 min of stimulation. PKG activity was presented as a percentage by setting the activity from control as 100%. *, p < 0.05.

PKG activity (Fig. 3H). Thus, Frizzled-2-stimulated inhibition of the cellular activity of PKG is sensitive to the inactivation of Gα_{q} protein family members by pertussis toxin as well as to suppression of either Gα_{q} or Gα_{q2}. Conversely, elevation of intracellular cyclic GMP levels can both activate PKG activity and abolish Frizzled-2-stimulated inhibition of PKG activity.

PKG and Ca^{2+} Converge in Frizzled-2 Signaling at NF-AT-dependent Gene Transcription—What, then, is the relationship between the Ca^{2+} mobilization and the changes in intracellular concentration of cyclic GMP downstream of Frizzled-2 signaling? The effect of increasing intracellular concentration of cyclic GMP on the ability of Frizzled-2 activation to mobilize intracellular Ca^{2+} was investigated. F9 clones were treated with either the PDE inhibitor Zaprinast or with the 8-Br-cGMP (Fig. 4A). Treatment with either Zaprinast or with 8-Br-cyclic GMP attenuates the ability of Frizzled-2 to stimulate Ca^{2+} mobilization, i.e. increasing PKG activity can suppress the ability of Frizzled-2 to provoke Ca^{2+} mobilization. Based upon these observations, PKG appears to function upstream of Ca^{2+} mobilization in response to Frizzled-2 activation. This hypothesis was tested further by making use F9 clones expressing authentic rat Frizzled-2 and stimulated with purified Wnt5α (Fig. 4B). The Wnt5α-stimulated mobilization of intracellular Ca^{2+} was nearly abolished by treating the clones with 8-Br-cGMP and Zaprinast, in good agreement with the observations derived with the F9 clones expressing the Frizzled-2 chimera.

We next tested our hypothesis by comparing the effects of activation versus inhibition of protein kinase G on the Ca^{2+} mobilization response. F9 cells were treated with 8-Br-cGMP to activate and with Rp8-[(4-chlorophenylthio)-guanosine 3’,5’-cyclic monophosphorothioate (Rp8-pCPT-cyclic GMP) to inhibit PKG (23), and Ca^{2+} mobilization was monitored (Fig. 4C). Elevation of intracellular cyclic GMP activates
PKG (Fig. 3H) but has little effect on the intracellular Ca\(^{2+}\) levels, as imaged with Fura-2 (Fig. 4C). Direct inhibition of PKG activity with Rp-8-pCPT-cyclic GMP (Fig. 3H), in sharp contrast, provokes an increase in intracellular concentration of Ca\(^{2+}\) (Fig. 4C). A 20-min lag in the time course of intracellular concentrations of Ca\(^{2+}\) was observed between the addition of Rp-8-pCPT-cyclic GMP and a mobilization of Ca\(^{2+}\). The observed time lag likely reflects the time required for diffusion of the inhibitor to the intracellular compartment responsible for PKG-mediated Ca\(^{2+}\) mobilization.

If a relationship between cyclic GMP and Ca\(^{2+}\) impacts the regulation of NF-AT-dependent transcription, one would expect that 1) the activation of PKG, like the inhibition of cyclic GMP-sensitive PDE, would attenuate the effect of Frizzled-2 activation and 2) the inhibition of PKG, like the activation of cyclic GMP-sensitive PDE, would mimic the effects of Frizzled-2 activation. NF-AT-dependent transcription was measured in F9 clones expressing the Frizzled-2 chimera and then stimulated with isoproterenol (Fig. 4D). Activation of Frizzled-2 chimera leads to increased cyclic GMP PDE activity, a decrease in intracellular cyclic GMP levels, yet increased Ca\(^{2+}\) mobilization (13). Zaprinast inhibits PDE6, elevates cyclic GMP levels (13), and increases PKG activity (Fig. 3H). If the hypothesis is correct, Zaprinast treatment should provoke an attenuation of Frizzled-2 activation of NF-AT-dependent transcription. Zaprinast indeed attenuates Frizzled-2 activation of NF-AT-dependent transcriptional response (Fig. 4D), as it does the Ca\(^{2+}\) mobilization (Fig. 4A). Treating cells with the 8-Br-cGMP should increase PKG activity and, if the hypothesis is correct, should likewise attenuate transcription of the NF-AT-dependent transcription in response to Frizzled-2 activation. As shown for Ca\(^{2+}\) mobilization (Fig. 4A), treating F9 clones with 8-Br-cyclic GMP (and thereby directly activating PKG) does attenuate the ability of Frizzled-2 activation to stimulate NF-AT-dependent transcription (Fig. 4D). Finally, the hypothesis suggests that treating the F9 clones with the PKG inhibitor Rp-8-pCPT-cyclic GMP would be expected to mimic the activation of Frizzled-2. Treating cells with Rp-8-pCPT-cyclic GMP indeed stimulates NF-AT-dependent transcription in the absence of Frizzled-2 activation (Fig. 4D). Surprisingly, this level of inhibition of PKG activity by Rp-8-pCPT-cyclic GMP not only elevated basal transcription of the NF-AT gene but still also enabled an activation of NF-AT-dependent transcription by Frizzled-2 (Fig. 4D). Higher concentrations of the PKG inhibitor, which would likely more strongly activate PKG activity and preclude further activation in the presence of Frizzled-2 activation, were not tolerated by the cells, so this point remains speculative. Cyclic GMP PDE, cyclic GMP, and PKG appear to function upstream of the signaling from Frizzled-2 to the level of Ca\(^{2+}\) mobilization.

Although we show that Ca\(^{2+}\) mobilization and NF-AT-sensitive transcription in response to Frizzled-2 activation requires inhibition of PKG, we sought to test whether the inhibition of PKG by Frizzled-2 activation required Ca\(^{2+}\) mobilization. We preloaded the cells with the acetoxyethyl ester of BAPTA to buffer intracellular Ca\(^{2+}\). Buffering of intracellular Ca\(^{2+}\) with BAPTA in F9 cells stabilizes expressing rat Frizzled-2 chimera indeed blocks activation of NF-AT-dependent gene transcription in response to isoproterenol (Fig. 2F). We tested the effects of BAPTA loading on Frizzled-2 activation of PKG activity in F9 clones stably expressing Frizzled-2 and stimulated with purified Wnt5a. Wnt5a stimulates inhibition of PKG activity, as shown earlier (Fig. 3D). If the mobilization of Ca\(^{2+}\) is obligate for PKG activation by Wnt5a, then buffering intracellular Ca\(^{2+}\) with BAPTA might be predicted to block Wnt5a-stimulated inhibition of PKG. Loading F9 clones with BAPTA, however, did not alter the character of the suppression of PKG activity in response to Wnt5a (Fig. 4E).

**DISCUSSION**

The current studies illuminate several novel facets of the Wnt/cGMP/Ca\(^{2+}\) pathway mediated by Frizzled-2 (Fig. 5). In the Xenopus embryo, activation of the Wnt/Ca\(^{2+}\) pathway promotes ventral cell fate by interfering with the canonical Wnt/\(\beta\)-catenin pathway (24). In the zebrafish embryo, Wnt5a stimulates phosphatidylinositol signaling and Ca\(^{2+}\) transients essential to normal development (9, 12). The mouse embryonic F9 cells were exploited in the current work to elucidate the signal linkage map from a proximal step (i.e. activation of Frizzled-2) downstream to the activation of the developmentally regulated, luciferase reporter gene sensitive to NF-AT. A key, cyclic GMP sensor downstream of Frizzled-2 was identified, and the interplay between the two dominant cellular signaling responses from the noncanonical pathway, Ca\(^{2+}\) mobilization and decreased cellular concentrations of cyclic GMP, was uncovered.

Protein kinase G is shown to be a key sensor for cyclic GMP in the Wnt/cGMP/Ca\(^{2+}\) pathway (Fig. 5), although additional cyclic GMP sensors may be participating to a lesser extent. PKG activity is reduced, and NF-AT-sensitive transcription is
increased in response to stimulation of Frizzled-2 by Wnt5a. Decreasing PKG activity with Rp-8-pCPT-cyclic GMP mimics the effect of Wnt5a stimulation on PKG activity, induces Ca^{2+} transients, and increases NF-AT-sensitive transcription, even in the absence of Wnt. Furthermore, elevating the intracellular concentration of cyclic GMP either by inhibition of cyclic GMP phosphodiesterase (PDE6) or by addition of the 8-bromo analogue of cyclic GMP increases PKG activity and effectively blocks Wnt5a-stimulated NF-AT transcriptional activation. Thus, the Wnt/Ca^{2+}/NF-AT signaling pathway is mediated downstream of cyclic GMP, in part, by protein kinase G. Recently, a genome-wide *Drosophila* RNA interference screen also indicated PKG as a potential regulator of NF-AT (25).

The immediate upstream regulator of cyclic GMP (and thereby PKG) is a cyclic GMP phosphodiesterase that is sensitive to inhibition by either dipryridamole or Zaprinast (7, 13). Zaprinast is a selective inhibitor of the phosphodiesterase PDE6 (26), identified in mouse F9 cells and a well known effector of the heterotrimeric G protein G_{12} (27). We show that each aspect of the signal linkage map shown in Fig. 5 is sensitive to inactivation of G proteins by pertussis toxin, a toxin that catalyzes the ADP-ribsylation of members of the Gi protein family, including Ga_{s2} and Ga_{o}. The G protein Ga_{o} has been shown to function in Wnt signaling in both the canonical and noncanonical Wnt pathways in a variety of systems, from mouse F9 cells (2, 7, 13, 17) to *Drosophila* embryos (28). Finally, as was observed for zebrafish embryos injected with RNA encoding a unique chimera composed of the exofacial and transmembrane domains of the human β_{2}-adrenergic with the cytoplasmic domains of rat Frizzled-2 (i.e. β_{2}AR/Rfz2 chimera), isoprotrenol stimulates the noncanonical pathway mimicking the action of Wnt5a binding to Rfz-2, again reflecting upon the G protein-coupled nature of Frizzleds (13, 29).

The interplay between Ca^{2+} mobilization and cyclic GMP regulation at a macroscopic level is revealed in the current study. Wnt5a stimulates a decline in intracellular cyclic GMP, attenuates PKG activity, and enables robust Ca^{2+} mobilization (Fig. 5). The increase in Ca^{2+} mobilization appears to be dependent upon a decline in intracellular cyclic GMP and a corresponding reduction in the activity of PKG. Rp-8-pCPT-cyclic GMP inhibits PKG activity, mimicking Wnt5a action, and increases Ca^{2+} mobilization, even in the absence of Wnt5a. Elevation of intracellular cyclic GMP either by inhibition of the cyclic GMP phosphodiesterase or by addition of cell-permeable cyclic GMP analogue, 8-Br-cyclic GMP, effectively blocks the Ca^{2+} mobilization in response to Wnt5a. These observations provide compelling evidence that Wnt5a-stimulated Ca^{2+} mobilization is regulated downstream of cyclic GMP and its sensor protein kinase G (Fig. 5). This is not to imply that some initial component of the Ca^{2+} mobilized in response to Wnt5a cannot escape or be linked to Wnt signaling outside of the schematic shown here. Changes in Ca^{2+} levels, which can be readily measured directly by Fura-2 imaging, appear to occur in advance of the measured changes in PKG activity. Lacking information on the “gain” possible in Wnt signaling pathway, we cannot rule out a Ca^{2+} mobilization in response to a rapid activation of phosphatidylinositol signaling by Wnt, as observed elsewhere (9). The bulk of Ca^{2+} mobilized, however, appears to require signaling from Wnt via cyclic GMP PDE/cyclic GMP/PKG (Fig. 5).

Interestingly, blocking the Wnt-stimulated Ca^{2+} mobilization by buffering intracellular Ca^{2+} with BAPTA precludes Wnt5a-stimulated activation of NF-AT-sensitive transcription, but not the ability of Wnt to inhibit PKG activity. Because basal NF-AT transcriptional activity is observed in the cells treated with BAPTA under these conditions, the buffering of intracellular Ca^{2+} does not appear so severe as to preclude basal activity of the transcriptional response. These data help to clarify earlier results in which PDE inhibitors (either Zaprinast or dipryridamole) were found to block Wnt5a stimulation of Ca^{2+} mobilization in zebrafish embryos (13). Blockade of the cyclic GMP PDE activity not only attenuated the Ca^{2+} mobilization in response to Wnt5a, but also stunted normal development of the zebrafish embryos (13). The Wnt5a-stimulated increase in intracellular Ca^{2+} regulates the activity of downstream Ca^{2+}-sensitive enzymes, such as the phosphoprotein phosphatase calcineurin and Ca^{2+}/calmodulin-dependent protein kinase II. It is the activation of calcineurin that largely is responsible for the activation of NF-AT-dependent transcription. Thus, signals from the Wnt/cGMP/Ca^{2+} pathway are required for the regulation of NF-AT-sensitive gene transcription, a major element in the Wnt5a response mediated by Frizzled-2. The signals downstream of cyclic GMP and PKG enable the Wnt-stimulated Ca^{2+} mobilization, although molecular description of this interplay and identification of PKG substrates essential for signaling of the Wnt/cGMP/Ca^{2+} pathway are not known.

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