Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner

Laird C. Sheldahl*, Maiyon Park*, Craig C. Malbon† and Randall T. Moon*

In studies of developmental signaling pathways stimulated by the Wnt proteins and their receptors, *Xenopus* Wnt-5A (Xwnt-5A) and a prospective Wnt receptor, rat Frizzled 2 (Rfz2), have been shown to stimulate inositol signaling and Ca^{2+} fluxes in zebrafish [1–3]. As protein kinase C (PKC) isoforms can respond to Ca^{2+} signals [4], we asked whether expression of different Wnt and Frizzled homologs modulates PKC. Expression of Rfz2 and Xwnt-5A resulted in translocation of PKC to the plasma membrane, whereas expression of rat Frizzled 1 (Rfz1), which activates a Wnt pathway using β-catenin but not Ca^{2+} fluxes [5], did not. Rfz2 and Xwnt-5A were also able to stimulate PKC activity in an *in vitro* kinase assay. Agents that inhibit Rfz2-induced signaling through G-protein subunits blocked Rfz2-induced translocation of PKC. To determine if other Frizzled homologs differentially stimulate PKC, we tested mouse Frizzled (Mfz) homologs for their ability to induce PKC translocation relative to their ability to induce the expression of two target genes of β-catenin, *siamois* and *Xnr3*. Mfz7 and Mfz8 stimulated *siamois* and *Xnr3* expression but not PKC activation, whereas Mfz3, Mfz4 and Mfz6 reciprocally stimulated PKC activation but not expression of *siamois* or *Xnr3*. These results demonstrate that some but not all Wnt and Frizzled signals modulate PKC localization and stimulate PKC activity via a G-protein-dependent mechanism. In agreement with other studies [1–3,6,7], these data support the existence of multiple Wnt and Frizzled signaling pathways in vertebrates.

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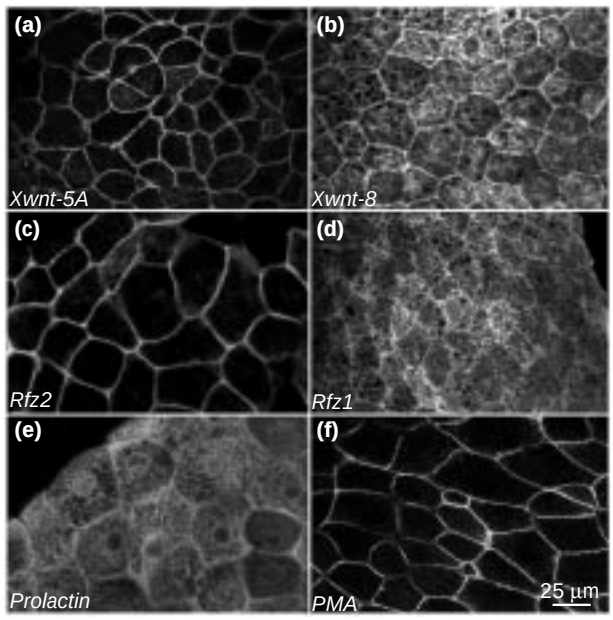
Results and discussion

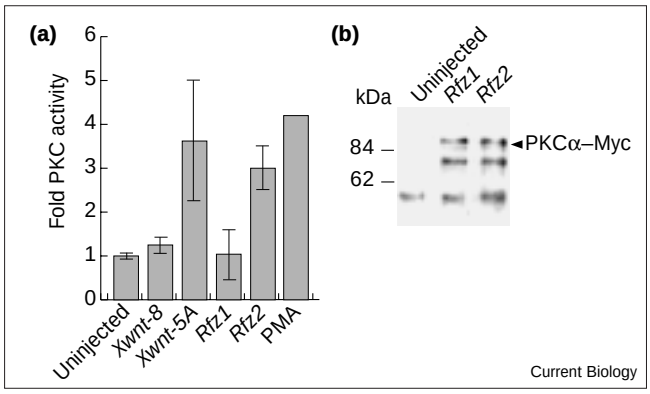
To test whether PKC activity is modulated by Wnt or Frizzled signaling, *Xenopus* embryos were injected with RNAs encoding various Wnt and Frizzled homologs in conjunction with RNAs encoding PKC isoforms tagged with green fluorescent protein (GFP) or a Myc epitope. The tags allowed us to monitor the subcellular distribution of the PKC as an assay of its activation. The assay relies on evidence that translocation of PKC to the plasma membrane generally correlates with enzyme activation [8,9]. Ectopic expression of Xwnt-5A (Figure 1a, $n = 4$ experiments) was able to induce the translocation of Myc-tagged PKC to the plasma membrane to a much greater extent than expression of either Xwnt-8 (Figure 1b, $n = 4$ experiments) or Xwnt-3A (data not shown). In addition, expression of Rfz2 (Figure 1c, $n = 10$ experiments) induced translocation of PKC, whereas expression of Rfz1 (Figure 1d, $n = 4$ experiments) did not. Injection of as little as 40 pg *Xwnt-5A* RNA ($n = 3$ experiments) or *Rfz2*

Figure 1



Xwnt-5A and Rfz2, but not Xwnt-8 nor Rfz1, elicit PKC translocation. (a) Following co-injection of *Xwnt-5A* RNA, Myc-tagged PKC is localized at the plasma membrane. (b) Co-injection of *Xwnt-8* RNA has a negligible effect upon PKC localization. (c) Following co-injection of *Rfz2* RNA, PKC is localized at the plasma membrane. (d) Co-injection of *Rfz1* RNA has no apparent effect upon PKC localization. (e) Injection of control (prolactin) RNA shows PKC is present throughout the cytoplasm in the absence of exogenous Wnt signals. (f) Treatment for 30 min with PMA, a potent PKC activator, is sufficient to relocalize PKC to the plasma membrane.

Figure 2

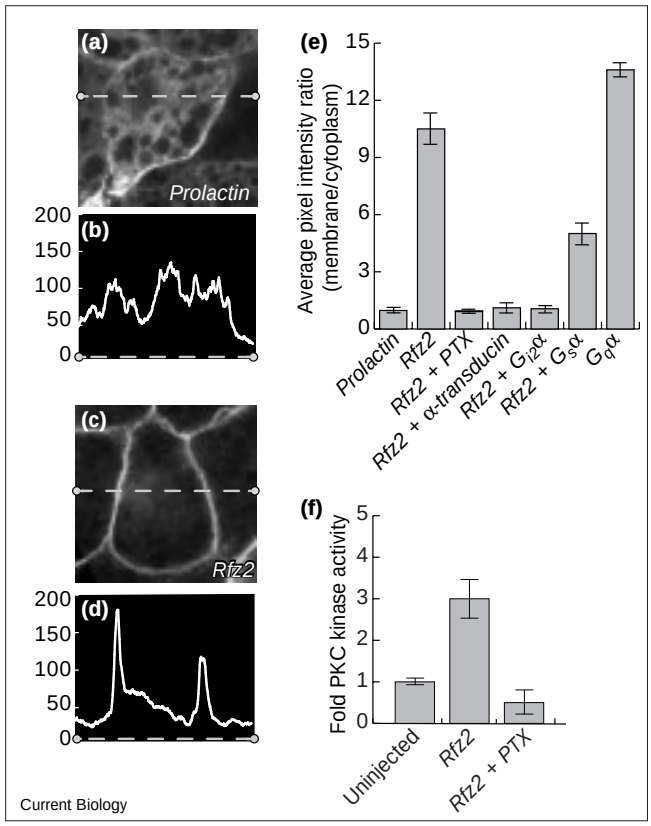


RNAs that induce PKC translocation also increase PKC activity in an *in vitro* kinase assay. (a) Relative phosphorylation of a PKC substrate peptide by immunoprecipitated XPKCα–Myc from embryos co-injected with RNA encoding either Xwnt-8 (n = 3 experiments), Xwnt-5A (n = 3), Rfz1 (n = 3) or Rfz2 (n = 6). As we wanted to measure relative kinase activities, uninjected embryos (n = 8) were measured for basal kinase activity, and this level was set to 1. PMA was added to the reaction tubes as a positive control. (b) Embryo lysates from panel (a) were immunoblotted with an anti-Myc monoclonal antibody, 9E10, to confirm equal expression of PKC after Frizzled injection.

RNA (n = 3 experiments) was able to induce translocation of PKC, while injection of 100-fold more *Xwnt-8* or *Rfz1* RNA did not. We therefore conclude that Wnt and Frizzled homologs differ in their ability to induce translocation of PKC to the plasma membrane. Injection of prolactin RNA (Figure 1e) and acute treatment with phorbol-12-myristate-13-acetate (PMA) (Figure 1f) were used as negative and positive controls respectively for PKC translocation.

Translocation of PKC to the membrane can be observed in the presence of PKC inhibitors [10] and in the absence of diacylglycerol [11], suggesting that PKC translocation is not always directly linked to kinase activation. We therefore directly measured PKC activity using an *in vitro* kinase assay [12]. Confirming the translocation data, injection of *Xwnt-5A* or *Rfz2* RNAs resulted in an increase in total PKC activity in stage 8 embryos compared with embryos injected with *Xwnt-8* or *Rfz1* RNA (Figure 2a). *Xwnt-5A*-injected embryos showed a 2.9-fold increase in PKC activity over *Xwnt-8*-injected embryos ($p < 0.01$ in Student's t-test), and *Rfz2*-injected embryos showed a 2.9-fold increase over *Rfz1*-injected embryos ($p < 0.05$). Western blots confirm equal levels of expression of Myc-tagged *Xenopus* PKCα (XPKCα–Myc) in the injected embryos (Figure 2b). Increased endogenous PKC activity has also been observed in embryos injected with *Xwnt-5A* RNA (M. Kühl and R.T.M., unpublished observations). These data support the hypothesis that Wnt and Frizzled homologs differ in their ability to stimulate a signaling pathway that leads to activation of PKC.

Figure 3



Agents that block G_i or G_o-βγ subunit signaling block Rfz2-induced PKC translocation. Two-cell *Xenopus* embryos were injected with RNAs encoding XPKCα–GFP, Rfz2, and either pertussis toxin, α-transducin, $G_{i2}\alpha$, $G_s\alpha$ or prolactin. (a,c) Examples of confocal images used. (b,d) Images (17 different cells from 4 different experiments for each condition in (e); see also Supplementary material published with this paper on the internet) were analyzed using NIH Image software to plot the pixel intensity profile (y-axis: 0 = black, 255 = white) over a linear section of the cell (dashed lines in (a,c) as representative cells). (e) The average pixel intensity of membrane regions divided by the average pixel intensity of intracellular regions was used as a relative measure of PKC membrane localization. (f) Confirming the results in (e), injection of PTX was also able to reduce PKC activity in *Rfz2*-injected embryos, as measured directly by the independent *in vitro* kinase assay.

Because intracellular release of Ca^{2+} in response to Rfz2 and Xwnt-5A is dependent upon G proteins [2], we asked whether agents that block G-protein signals would block the ability of Rfz2 to induce PKC translocation. The assay used was the translocation of tagged PKC to the membrane, coupled with quantitative image analysis (Figure 3a–d). We first co-injected RNA encoding the A protomer of pertussis toxin (PTX) and RNAs encoding XPKCα–Myc and Rfz2. PTX catalyzes the ADP-ribosylation of G_i and G_o α subunits and subsequently blocks GDP–GTP exchange, thereby blocking Gα and Gβγ signals. Expression of PTX blocked Rfz2-induced translocation of PKC (Figure 3e). Using the independent *in vitro*

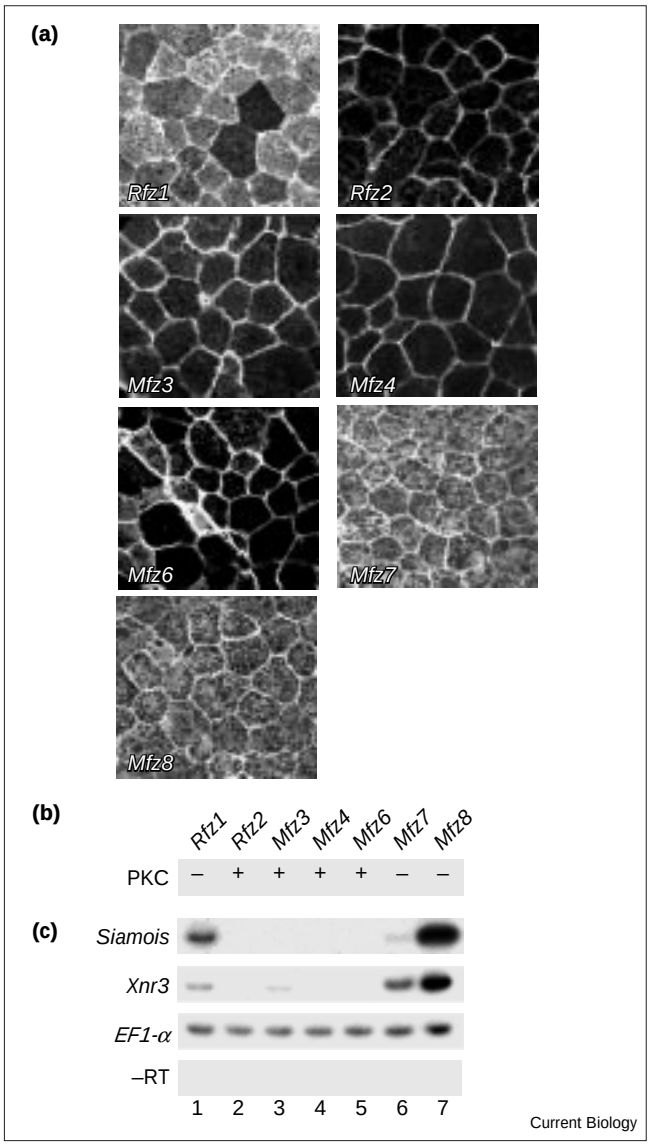
kinase assay, injection of PTX RNA reduced Rfz2-induced PKC activity to control levels (Figure 3f), confirming that Rfz2 induces activation of PKC in a PTX-sensitive manner.

α-Transducin, which sequesters βγ subunits [13], was then tested for its ability to block Rfz2-induced translocation of PKC. Injection of α-transducin RNA attenuated the translocation of PKC to the membrane in response to Rfz2 (Figure 3e). As an independent test of Gβγ signaling we injected RNA encoding $G_{i2}\alpha$ subunits, which have been shown to inhibit Gβγ-induced activation of *Xenopus* phospholipase C-β (PLC-β) [14]. We found that expression of $G_{i2}\alpha$ attenuated Rfz2-induced translocation of PKC to a much greater extent than did $G_s\alpha$, which was used as a control (Figure 3e). RNA encoding $G_q\alpha$, injected as a positive control for Gα-induced activation of PLC-β/phosphoinositol signaling [14], induced PKC translocation in the absence of Rfz2 (Figure 3e). Collectively, these results suggest that Rfz2 functions upstream of βγ subunits of PTX-sensitive G proteins, though it remains unclear whether G proteins are directly or indirectly involved.

We used assays for PKC translocation and induction of β-catenin target genes to test the activities of various Frizzled homologs [15]. Ectopic expression of Rfz2, Mfz3, Mfz4 and Mfz6 promoted membrane translocation of PKC in *Xenopus* animal caps (Figure 4a,b) and did not appreciably induce the expression of *siamois* and *Xnr3* (Figure 4c). Mfz3 did, however, weakly induce expression of *Xnr3* in a minority of trials (4 out of 9; Figure 4c). Conversely, ectopic expression of Mfz7 and Mfz8, like that of Rfz1, did not elicit PKC translocation (Figure 4a), but did induce expression of *siamois* and *Xnr3* (Figure 4c). The data suggest that each member of the vertebrate Frizzled family of putative Wnt receptors activates one of two apparently distinct signaling pathways in these assays.

We have shown that Xwnt-5A, Rfz2, Mfz3, Mfz4 or Mfz6 can elicit the translocation of a typical PKC isoform from the cytoplasm to the plasma membrane, and to a greater extent than do Xwnt-8, Rfz1, Mfz7 or Mfz8. Furthermore, both Rfz2 and Xwnt-5A lead to an increase in total PKC activity measured by independent *in vitro* kinase assays. The ability of Rfz2 to activate PKC is sensitive to agents that block signaling by βγ subunits from PTX-sensitive G proteins, further supporting the hypothesis [2] that G proteins are involved in the transduction of at least some Wnt signals. The activation of PKC is likely to occur downstream of phosphatidylinositol metabolism and Ca^{2+} fluxes [1,2] and is probably distinct from events initiated by Wnt and Frizzled homologs that work through β-catenin and the homologs of the transcription factors Lef and Tcf. As PKC activity has been implicated in cellular responses to Wnt-1 signals [16,17] an attractive hypothesis is that PKC provides cross-talk between distinct Wnt signaling pathways. Our demonstration that Wnt and Frizzled homologs differ in their ability to stimulate PKC activity should contribute to an understanding of the diversity of Wnt signaling pathways.

Figure 4



Frizzled homologs display a differential ability to increase PKC activity versus *siamois* and *Xnr3* expression. (a) Both cells of two-cell *Xenopus* embryos were injected with *XPKCα–Myc* RNA along with RNA encoding Rfz1, Rfz2, Mfz3, Mfz4, Mfz6, Mfz7 or Mfz8 as indicated. The subcellular localization of ectopic PKC is shown. (b) PKC localization in animal cap cells from experiments as in (a) was imaged by confocal microscopy and quantified as in Figure 3. Frizzled homologs which showed less than 1.1-fold relative membrane versus cytoplasmic staining are denoted '–', while Frizzled homologs which showed fivefold or greater membrane versus cytoplasmic staining are denoted '+'. (c) Frizzled homologs were assayed for the ability to increase *siamois* and *Xnr3* expression in *Xenopus* embryos by RT–PCR amplification. EF1-α expression was measured as a loading control and –RT denotes this reaction without reverse transcriptase.

Materials and methods

Embryological methods and RNA injections

Xenopus embryos were injected at the 2–4 cell stage in the animal pole with the following synthetic RNAs: *Xwnt-3A*, *Xwnt-5A*, *Xwnt-8* [3], rat *Frizzled 1* (*Rfz1*) [5], rat *Frizzled 2* (*Rfz2*) [2], mouse *Frizzled 3* (*Mfz3*), mouse *Frizzled 4* (*Mfz4*), mouse *Frizzled 7* (*Mfz7*), mouse *Frizzled 8* (*Mfz8*) or mouse *Frizzled 6* (*Mfz6*) [15]. In a given experiment, the RNA doses used (40 pg to 4 ng) were the same for all the RNAs being compared. GFP-tagged (S65T mutant [18]) or Myc-tagged [19] *Xenopus PKCα* (*XPKC–Myc*) DNAs were constructed from *PKCα* cDNA [20] using standard methods, then injected at 0.5–1 ng at a constant dose within a given experiment. Prolactin RNA was co-injected to equalize RNA levels. RNAs (0.5–1 ng) encoding α-transducin, pertussis toxin [2], $G_s\alpha$, $G_{i2}\alpha$ or $G_q\alpha$ [21,22] were co-injected where noted. Animal caps from injected embryos were dissected at stage 8 and cultured in 1× modified Barth's solution until sibling embryos reached stage 11–12, whereupon the caps were fixed and processed for confocal microscopy (minimum 10 animal caps per condition). PMA (1 μM; Sigma) was added 30 min before fixation as a positive control.

Kinase activity assays

Embryos at the 2–4 cell stage were injected as described above with *XPKCα–Myc* plus *Rfz1*, *Rfz2*, *Xwnt-8* or *Xwnt-5A* mRNA. Forty stage-8 embryos were homogenized in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Triton X-100, 50 μM NaF, 10 μG/ml aprotinin, 10 μg/ml leupeptin); XPKCα–Myc was isolated by immunoprecipitation and assayed for activity using the following reagents: [γ-^{32}P]ATP (NEN Life Science Products), a substrate peptide derived from myelin basic protein (Ac-MBP(4–14); Gibco BRL), phosphatidyl-L-serine (Avanti Polar Lipids) as a cofactor and GF109203X (Bio-Mol) as a specific inhibitor. PMA (10 μM) was added to the reaction tubes as a positive control. GF109203X blocked over 85% of ^{32}P-incorporation into the substrate peptide. Standard assay conditions were as follows: 20 mM Tris pH 7.5, 10 μM ATP, 20 mM $MgCl_2$, 1 μM $CaCl_2$, 50 μM Ac-MBP(4–14), 0.3% mixed micelles (3% Triton X-100 and 50 μg/ml phosphatidyl-L-serine), for a final volume of 25 μl incubated for 5 min at 30°C, then spotted onto phosphocellulose discs (Gibco BRL), which were washed before counting in a scintillation counter.

Immunoblotting

Immunoprecipitates were separated by SDS–PAGE, electrophoretically transferred onto nitrocellulose membranes, and probed with the 9E10 anti-Myc antibody [19].

Gene induction assays

The induction of *Xnr3* and *siamois* were measured by reverse transcriptase–PCR (RT–PCR) in several independent experiments as previously described [5] and a representative experiment is shown.

Supplementary material

A supplementary figure showing representative images of cells that were analyzed for Figure 3 is published with this paper on the internet.

Acknowledgements

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Supplementary material

Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner

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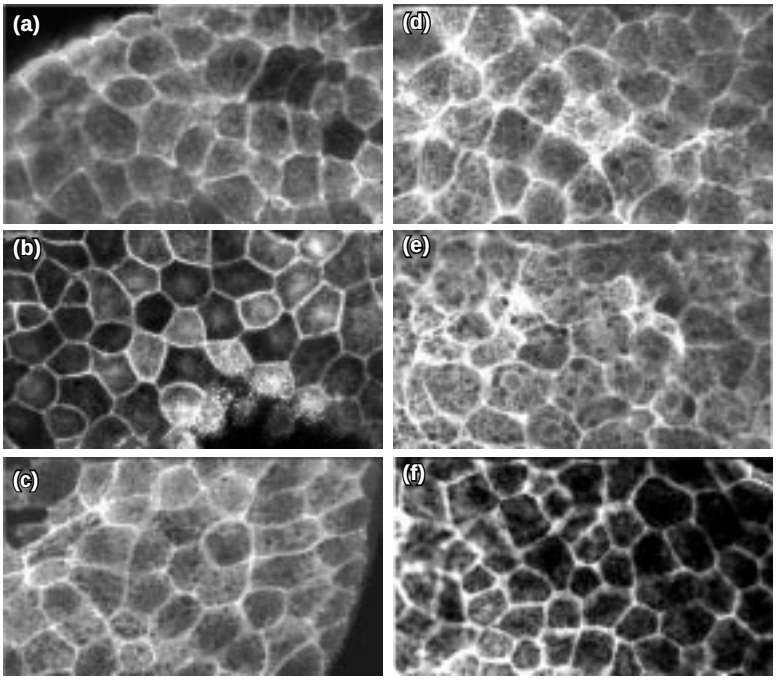
Figure S1

Representative images of cells that were analyzed for Figure 3. XPKCα–GFP localization is imaged in each case, after co-injection of PKC–GFP RNA along with the following RNAs: (a) control (*prolactin*); (b) *Rfz2*; (c) *PTX* plus *Rfz2*; (d) *α-transducin* plus *Rfz2*; (e) $G_i\alpha$ plus *Rfz2*; and (f) $G_s\alpha$ plus *Rfz2*. See Figure 3e for quantitation.



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