[Frontiers in Bioscience 9, 967-974, January 1, 2004]

THE WNT/CALCIUM PATHWAY: BIOCHEMICAL MEDIATORS, TOOLS AND FUTURE REQUIREMENTS

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1. ABSTRACT

Wnt proteins represent a family of secreted, lipid-modified glycoproteins that can activate different intracellular pathways. Upon binding to certain members of the Frizzled family of Wnt receptors some Wnts like Wnt-4, Wnt-5A or Wnt-11 are able to elicit an intracellular release of calcium ions. This calcium signaling acitivity is sufficient to activate calcium sensitive enzymes like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII) or calcineurin (CaCN). This so-called Wnt/calcium pathway plays important roles during dorso-ventral patterning of the embryo, regulating cell migration, as well as heart development, and might play a role during tumor suppression. The foci of this review are the biochemical aspects of Wnt/calcium signaling, the tools that are available to study Wnt/calcium signaling, and the open questions that need to be addressed in the future to validate this signaling pathway.

2. WNT PROTEINS AND SIGNALING PATHWAYS

Recently, the developmental function of Wnt/calcium signaling has been discussed in several reviews in great detail (1-2). The focus of this review will thus be concentrated on biochemical aspects of this pathway. The biochemical analysis of Wnt-Frizzled signaling starts with the ligands themselves, the Wnts. Analysis of the human genome data indicate the existence of most likely 19 members of the Wnt gene family. These genes code for secreted glycoproteins and recently published purification protocols indicate the presence of a lipid modification (3). This is likely the reason that purification of active Wnt proteins failed for many years. To activate intracellular signaling, Wnt proteins interact with receptors of the Frizzled family (4). These are receptors with seven transmembrane segements (7TMS) that are distant relatives of the G-protein coupled receptors (GPCRs). The rather high number of Wnt ligands and their corresponding receptors implicate the existence of several signaling pathways. Early work has subdivided the Wnt gene family into two classes. Whereas the Wnt-1/wingless class activates the co-called canonical Wnt/beta-catenin pathway (see Figures 1-3, and especially Figure 3, left side), induces secondary axis formation in *Xenopus*, and transforms C57mg mammary epithelial cells, the Wnt-5A class failed in all these assays. In contrast, these Wnts, including Wnt-5A, Wnt-4, and Wnt-11, activate non-canonical Wnt signaling and inhibit the axis inducing activity of the Wnt-1/wingless class indicating some inhibitory effect on the Wnt/beta-catenin pathway. The canonical Wnt/beta-catenin pathway involves proteins like dishevelled, adenomatous poliposis coli (APC), and axin and leads to the stabilization of cytoplasmic beta-catenin which can influence gene expression by interaction with transcription factors of the TCF/LEF family. Non-canonical Wnt pathways do not utilize beta-catenin as a signal transducer, but involve either intracellular release of calcium ions (5) or activation of Rho GTPases and Jun-N-terminal kinase (6). In addition, recent work indicates the regulation of cGMP signaling by Wnts (7), discussed elsewhere in this issue. Within this review I will focus only on the biochemical properties of the non-canonical Wnt/calcium signaling pathway.

3. INTRACELLULAR RELEASE OF CALCIUM IONS

Ionized calcium (Ca^{2+}) is a widely used intracellular mediator in signal transduction (8). The cytoplasmic calcium concentration is very low (100 nM), in contrast to the calcium concentration found in the extracellular space (2 mM). As calcium cannot be degraded or metabolized, the cell has evolved mechanisms to achieve cytoplasmic calcium at these lower concentrations. These mechanisms comprise ATP-driven calcium pumps that shift cytoplasmic calcium into the endoplasmatic reticulum as well as calcium binding proteins that serve as calcium

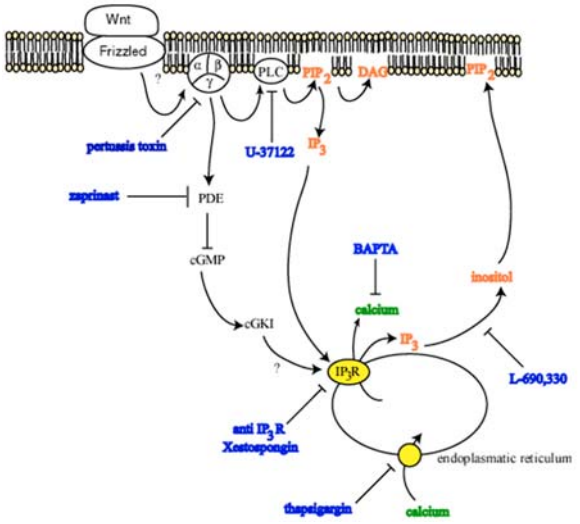

Figure 1. Wnt-mediated intracellular calcium release. The inositol phosphate cycle is highlighted in red. Inhibitors available to study calcium signaling are indicated in blue. Regulation of calcium release by cGMP via cGKI is hypothetical.

buffers. Release of calcium of the endoplasmatic reticulum is achieved by opening specific ion channels. In non-excitable cells, calcium release occurs through the IP_3 receptor (IP_3R).

Binding of Wnts to Frizzled leads to an activation of heterotrimeric G-proteins and subsequent activation of phospholipase C by the G-protein beta/gamma dimer (9,10). This enzyme cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is released from the membrane, binds to the IP_3 receptor which subsequently releases calcium ions from intracellular stores. This calcium release is locally restricted and calcium ions can subsequently activate calcium-sensitve proteins like protein kinase C (PKC) (11), calcium-calmodulin dependent kinase II (CamKII) (12), and/or calcineurin (CaCN) (13) . IP_3 is degraded to inositol by specific phosphatases and recycled to PIP_2 (i.e., the PI cycle) (Figure 1).

Although the model of Wnt/calcium signaling as depicted in Figure 1 seems to be easy and convincing, there is a major pitfall which needs to be resolved, reflecting the question whether this calcium release by Wnts is a direct response. This model of Wnt/calcium was primarily based on RNA overexpression studies in zebrafish and *Xenopus* embryos (9-12). However, calcium represents a quick response upon ligand/receptor interaction. These data obtained by overexpression are based on a rather long time frame and thus might be an indirect response. This problem was circumvented by the use of inducible Frizzled chimeric receptors that consist of the intracellular Frizzled domains and extracellular/transmembrane segments of the $beta_2$-adrenergic receptor (12, 14, 15). Stimulation of the $beta_2$-adrenergic/Rfz-2 chimeric receptor stimulates Wnt signaling and is sufficient to trigger intracellular calcium release within minutes (12). Further support for the existence of a Wnt/calcium pathway is given by the observation that Wnt-11 conditioned medium, but not control conditioned medium, leads to activation of calcium-calmodulin dependent kinase II within 10 minutes (16). However, further experiments need to be performed using the newly purified active Wnt ligands to test the direct interdependence of calcium release and Wnt binding.

Experiments using purified Wnt ligands will also provide some insight into the kinetics of Wnt-induced calcium signaling. These experiments need to address a dose-response relation between Wnts and calcium signaling, the frequency and amplitude of triggered calcium transients, and the question whether sustained G-protein coupled Wnt signaling leads to a desensitation of the Frizzled, as recently suggested (17).

Specific tools are available to interfere with the PI cycle: L-690,330 is an inhibitor of inositol monophosphate, Xestospongin (XeC) blocks the function of the IP_3R, and U-37122 is an inhibitor of phospholipase Cbeta. All of these reagents interfere with the PI cycle and thus block calcium within a cell. Thapsigargin is an inhibitor of Ca-ATPases that pump calcium back into the endoplasmatic reticulum and thus serve to deplete intracellular calcium stores. Used in early *Xenopus* or zebrafish embryos, these reagents clearly indicate the requirement of calcium signaling for ventral development (18,19). These reagents will be very useful to show the involvement of calcium signaling driven by Wnts in other contextes. Recent work has indicated that the intracelluar calcium release triggered by Frizzleds and Wnts can be modulated by inhibiting cGMP specific phosphodieasterases (7). Indeed, treatment of mouse F9 cells with Wnt-5A or overexpressing Frizzled-2 not only leads to increased numbers of calcium transients but also to a decrease in intracellular cGMP concentration suggesting a cross-talk between cGMP and calcium. Blocking a cGMP specific phosphodiesterase in zebrafish embryos blocked the Wnt/Frizzled induced intracellular calcium release. Interestingly, in smooth muscle cells a direct interaction between the IP_3R, a cGMP activated protein kinase (cGKI) and a protein named IRAG was shown. IRAG is a substrate for cGKI and phosphorylation of IRAG blocks IP_3R mediated calcium release (20, 21). It will be of great interest to see whether this link can be proven for Wnt signaling as well. If so, the intracellular cGMP concentration modulated by Wnts (and other molecules) can influence the release of intracellular calcium by the same Wnt.

4. ACTIVATION OF PHOSPHOLIPASE C: MORE THAN G-PROTEIN ACTION?

A still open question in the field of non-canonical Wnt signaling is the question whether there are several beta-catenin independent pathways, *i.e.* Wnt/calcium, Wnt/cGMP, Wnt/JNK pathways *etc.*, or whether these pathways show identical or overlapping properties commonly observed in a network. This issue was recently investigated and Sheldahl et al. (22) were able to show that a dishevelled construct, dshdeltaDIX, which was thought to

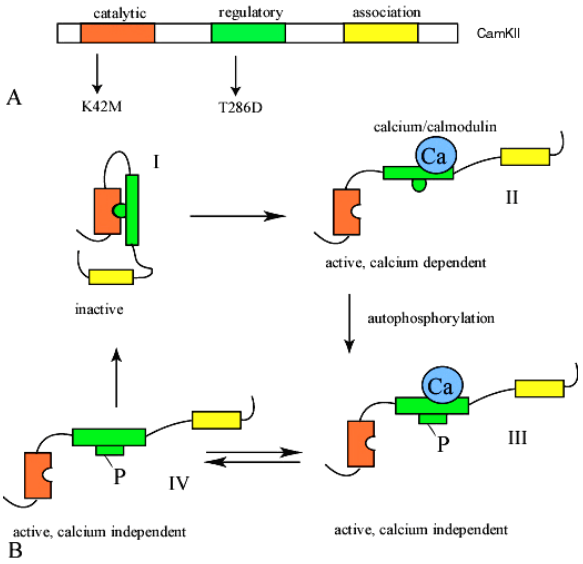

Figure 2. A: Domain structure of monomeric CamKII. K42M and T286D indicate two CamKII mutants that are kinase-dead and constitutively active, respectively. B: Activation of CamKII by calcium/calmodulin. Autophosphorylation leads to a calcium-independent, but active CamKII.

be specific for Wnt/JNK signaling (23) also induces calcium release. In contrast to Wnt or Frizzled induced-calcium transients, dshdeltaDIX triggered calcium release showed to be independent of G-proteins as adding pertussis toxin had no effect. More supprisingly, however, was the observation that Wnt/Frizzled mediated, G-protein coupled calcium release also requires the action of dishevelled. In embryos depleted for dishevelled protein, Wnt-5A and Rfz-2 failed to activate calcium signaling. This observation basicaly implicates two different explanations. First, dishevelled can activate RhoGTPases (*e.g.,* Rac) which can activate phospholipase C in other contextes (24). Within this scenario full activation of PLCbeta by Wnts would require two separate signaling branches downstream of Frizzled. Alternatively, dishevelled might act as a scaffolding protein to establish a higher order protein complex of Frizzeld receptor, G-proteins, and PLCbeta. Further work will be required to solve this question.

5. CALCIUM-CALMODULIN DEPENDENT KINASE II (CAMKII)

CamKII is a ubiquitous, multifunctional protein kinase which is activated by calcium-loaded calmodulin (25, 26). What assays are available to monitor activation of CamKII by Wnts? To answer this question it is very helpful to study the molecular nature of CamKII and its activation by calcium/calmodulin. CamKII is a multimeric enzyme consisting of eight to twelve subunits. Each subunit has an autoinhibitory, a catalytic, and an association domain required for multimerization (Figure 2A). In absence of calcium/calmodulin, the autoinhibitory domain blocks the active site of the enzyme, thereby inactivating it. Upon binding of calcium/calmodulin a conformational change releases the active site from the inhibitory domain (Figure 2B). Subsequent to calcium/calmodulin binding, an autophosphorylation occurs and the active enzyme now is independent of calcium/calmodulin regulation and thus is active well beyond the initial calcium signal. Thus, demonstrating an activation of CamKII can be achived by two different methods: first, an enzymatic activity assay can measure the calcium-independent CamKII acitivity (that is after autophosphorylation of the enyme); and second, one can probe for the increase of the phosphorylated form of CamKII on a Western blot using a phospho-specific antibody. Both assays have been employed to show activation of CamKII by different Wnt ligands or Frizzleds (12, 27, 28). Loading controls for unaltered amounts of total CamKII are performed using Western blots with enzyme-specific antibodies. In addition, one can add exogenous calcium ions to CamKII activity assays, resulting in full activation of the enzyme. Together with measuring the calcium-independent activity, it is possible then to calculate the fraction of active CamKII and to see whether an increase in enzymatic activity really reflects an activation of CamKII or rather an upregulation of CamKII expression.

CamKII has been shown to function during early ventral development in *Xenopus* (12, 27). During limb muscle fiber development, CamKII activation (like Wnt-5A) favors the development of slow muscle fibers (29). At least for ventral development, identification of TAK-1 / NLK as downstream targets of CamKII (27) revealed a molecular mechanism of CamKII action (Figure 3). These findings are consistent with findings that NLK is an inhibitor of the Wnt/beta-catenin pathway which is required for dorsal development (30, 31). CamKII has also been shown to block the Wnt/beta-catenin pathway at the level of LEF/beta-catenin (32). Consistent with a role for Wnts and CamKII in ventral development, Wnt-5A mutant fish (called *pipetail* mutant) show a disturbed calcium signaling pattern and a ventralized phenotype. This phenotype could be at least partially reverted by a constitutively active CamKII (18). In addition there is a long list of intracellular CamKII targets including several transcription factors (*e.g.,* CREB, C/EBPbeta, SRF, ATF-1, Elk-1) (33-36) and cytoskeletal proteins (*e.g.,* Vimentin, Desmin, Keratin) (26) as indicated in Figure 3. Further work will determine whether or not these factors are also targets of CamKII during Wnt signaling.

What tools do we have in hand to study CamKII function and to link CamKII function to these potentail downstream factors? A kinase-dead mutant of CamKII has been engineered (K42M according to the rat nomenclature), which unfortunately is not a very strong dominant negative mutant (12, 25, 26). Several constitutively active mutants have been reported. Substitution of threonine 286 to aspartate (T286D) mimics autophosphorylation (12, 25, 26). Other mutants just carry the catalytic domain, but lack the autoregulatory and association domain (18). All of these constructs were successfully employed to study CamKII function during Wnt/calcium signaling. Several pharmacological drugs, such as KN93 inhibitor, also are known that inhibit CamKII.

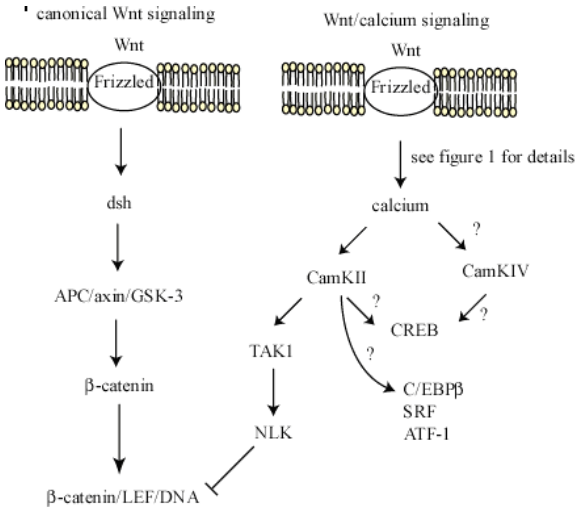

Figure 3. Activation of CamKII by Wnts (right side). Activation of TAK1 and NLK by CamKII leads to inhibition of canonical Wnt signaling (left side). Activation of CamKIV by Wnts is hypothetical. The modifications displayed for the other known targets of Cam Kinases, such as CREB, C/EBPbeta, SRF or ATF-1, are hypothetical.

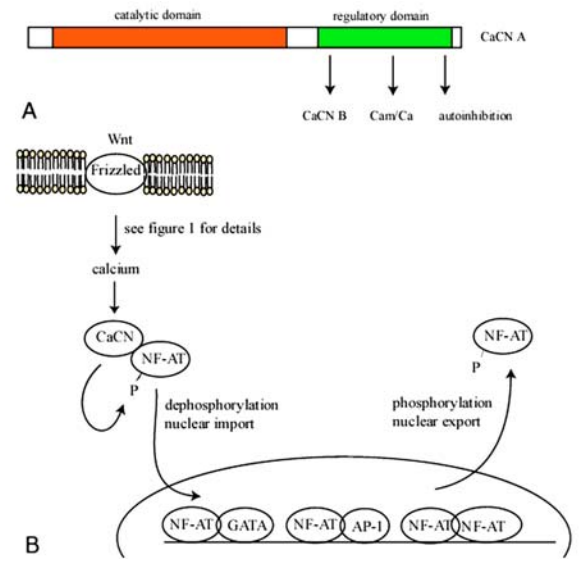

Figure 4. A: Domain structure of subunits of calcineurin A. B: Activation of calcineurin/NF-AT signaling by Wnts. Wnts can interact with GATA and AP-1 transcription factors. The phosphorylation status of NF-AT determines the cellular localization.

Experiments to decipher the function of Wnt-mediated CamKII activation also must take into account that CamKII is encoded by four genes (alpha, beta, gamma, delta). Homo- and hetero-multimers are known and differential splicing increases the number of potential CamKII holoenzyme variants. Furthermore, it will be important to see whether Wnt signaling is able to activate other calmodulin-dependent kinases, such as CamKI, III or IV, as these kinases might have more restricted expression patterns and substrate specificities (37). CamKIV has been shown to be required for blood development which is a ventral, mesodermal derivative (38), this observation provides support for a role for Wnt/calcium signaling in ventral development.

6. CALCINEURIN

Calcineurin, also called protein phosphatase 2B (CaCN, PP2B), is a serine/threonine protein phosphatase which is controlled by calcium/calmodulin (39). The enzyme consists of two subunits, one of which is the catalytic subunit regulated by calcium/calmodulin (Figure 4A) and a second regulatory domain comprising additional calcium-binding domains. Furthermore calcineurin requires Fe^{3+} and Zn^{2+} ions for activity. Dephosphorylation of substrates requires a consensus sequence and, in addition, some higher order structure. The enzyme is regulated in a cooperative manner resulting in a narrow threshold for enzyme activation (39). Several target proteins that are regulated by CaCN are known, among them the transcription factors Elk-1 and nuclear factors of activated T-cells (NF-AT). NF-AT factors form a family of 5 members, four of which are regulated by calcium/calcineurin (NF-AT 1-4). Dephosphorylation of NF-AT leads to an activation of the transcription factor, which then enters the nucleus to regulate target gene expression (Figure 4B). Activation of NF-AT-driven target genes or the translocation of NF-AT into the nucleus provide valuabe and tested read outs with which to monitor activation of calcineurin. Studies making use of both assays have show regulation of CaCN by Wnts (13), although this cannot be said for an enzymatic calcineurin activity assay which is available, but has not been used in this context. Assay of Wnt-regulated CaCN activity will be of primary importance, if one wants to compare dose response curves of calcineurin activation by Wnt/calcium signaling.

NF-AT factors can bind DNA as monomers or dimers, and also functionally interact with AP-1 (Jun/fos) or GATA transcription factors (40, 41). AP-1 itself is a target of several signaling pathways. Nuclear export of NF-AT is mediated by phosphorylation through different kinases like glycogen synthase kinase 3beta (GSK3beta), jun N-terminal kinase (JNK) or p38 (42). Thus, NF-ATs are able to integrate several signaling inputs like calcium signaling (through calcineurin) or kinase signaling (through AP-1 or nuclear export of NF-AT). Interestingly, this integration of different signaling pathways might be responsible for the fact that different NF-AT members might be localized either in the nucleus or the cytoplasm within the same cell (41). Through calcineurin, NF-AT factors can sense the frequency and the amplitude of intracellular calcium transients. NF-AT transcription factors thus represent very versatile regulators of transcription. Identification of NF-AT as a Wnt mediator opens a wide field of future research.

As for other components of the Wnt/Calcium pathway, CaCN/NF-AT signaling has been linked to ventral development although the mechanism is not fully understood yet (13). NF-AT proteins have been described to interact with GATA transcription factors during skeletal

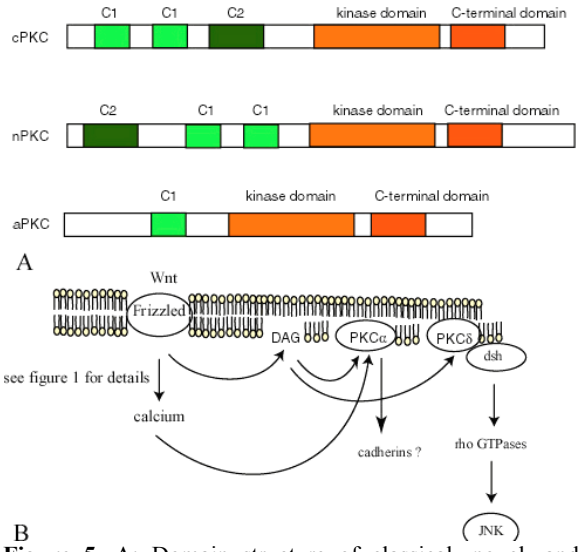

Figure 5. A: Domain structure of classical, novel, and atypical protein kinase C (PKC). B: Activation of PKCalpha and PKC delta by Wnts. PKCdelta is specifically involved in activating Jun N-terminal kinase (JNK). This Wnt/JNK pathway resembles the planar cell polarity pathway in *Drosophila*.

muscle and heart development during embryogenesis. Hypertrophic changes of skeletal or cardiac muscle during adulthut also involve signals mediated by calcineurin (43-47). As non canonical Wnt ligands are involved in skeletal and heart muscle development it might be worth to analyze whether Wnt signaling requires the action of calcineurin and NF-AT in these contextes. Different tools are availble for this purpose. A dominant negative NF-AT has been used to study NF-AT function during ventral Xenopus development. Furthermore, the immunosuppresive drugs cyclosporin A and FK506 act in concert with intracellular binding partners cyclophilin and FKBP12 as inhibitors for calcineurin.

7. PROTEIN KINASE C

Diacylglycerol (DAG) is produced within cells by the action of phospholipase C and represents an important second messenger in mammalian cells. Intracellular effects of DAG are mainly mediated by members of the protein kinase C family, although other target molecules for DAG are known as well (see below). Members of the protein kinase C family can be subdivided into three functional classes. Members of the classical PKCs (cPKCs, *e.g.,* PKCalpha, beta, gamma) are activated by DAG in a calcium-dependent manner. All these members share two N-terminal C1 domains that mediate interaction with DAG and a C2 domain that is essential for calcium binding (Figure 5A). Calcium binding to this C2 domains mediates phospholipid interactions and indeed full activation of cPKCs also requires the presence of phosphatidylserine. At the C-terminus, a catalytic domain is located that can be subdivided into a serine/threonine kinase domain and a substrate binding domain. Novel PKCs (delta, epsilon, tau) require DAG for activation, but do not depend on calcium. These members lack a classical C2 domain, but have a N-terminal C2 like domain (Figure 5A). Presence of phosphatidylserine is still required for activation of the nPCKs. Finally, atypical PKCs (zeta, lamda) require neither DAG nor calcium for activation, but do depend upon the presence of phosphatidlyserine (Figure 5A). Both, C1 and C2 domains are motifs that are required for membrane targeting of PKCs. Binding of DAG to the C1 domain of nPKC and Ca^{2+}-dependent phospolipid binding to the C2 domain (as well as DAG/C1 interactions in cPKCs) lead to the translocation of PKCs to the membrane. The translocation of PCKs to the membrane has proven to be a valuable and tested read out with which to monitor PKC activation. Beside lipid and calcium binding, phosphorylation of the enzyme is involved in PKC regulation. Several lines of evidence point towards PDK-1 as the upstream PKC activating kinase (55). However, it is not clear yet whether or not this phophorylation is constitutive or whether it is dependent on the presence of other ligands/agonists.

Given the dependence on calcium, classical PKCs were the first PKC members that have been shown to be activated by Wnts (11). For read outs of Wnt activation two different assays were used: first, membrane translocation of PKCalpha; and second, an enzymatic activity assay. Activation of classical PKCs by Wnts were linked to regulation of cell adhesion (48-49). Recently, PKCdelta, which is a member of the nPKC family, also was shown to be regulated by Wnts (50). PKCdelta was placed upstream of Dsh in the Wnt/JNK pathway that regulates JNK activity, earlier suggested by others (16). Interestingly, Dsh has been shown to be a substrate for phosphorylation of PKCalpha *in vitro* (12). Given the similar substrate specificity of PKC members, Dsh is likely a substrate for phosphorylation by several PKCs.

Several tools are available with which to study PKC function: the PKC activator phorbol ester (e.g., TPA) are widely used to mimic DAG and activate PKCs. The C1 domain is not found exclusively in PKCs, however, and this domain also can be found in other proteins like chimaerins, rasGRPs, PKD1, and Munc13s which both bind DAG and are regulated through DAG or TPA (51). Inhibitors are known for PKCs, both directed against the C1 or the catalytic domain, respectively. Given the argument above, inhibitors against the catalytic domain are preferable for use in this line of investigation. These inhibitors include indolocarbazoles and bisindolylmaleimides. The bis compounds, in particular, has significant activivity as an inhibitor of MEKs. Furthermore, PKC mutants or isotype specific antisense oligonucleotides have been used (50, 52, 53) to distinguish between different PKC forms.

Wnt-mediated regulation of PKC has been linked to cell-cell adhesion and tissue separation during embryogenesis (49), as well as to cell movements during gastrulation (12, 50). It will be of primary importance to identifiy additional roles for this Wnt/PKC branch and to identify downstream effectors of PKCs in this context. PKC knock-out mice have been generated for PKC beta, gamma, epsilon, delta, zeta, but only mild phenotypes were

reported that suggests some redundancy in PKC function (51). Indeed, all PKCs prefer similar phosphorylation motifs like RXXS/TXRX (with X for any amino acid). More N-terminal, basic amino acids are preferred for optimal phosphorylation by PKC. Many proteins have been shown to be phosphorylated by PKCs, both *in vivo* and *in vitro*. Since Wnt signaling and PKC function have been linked mainly with respect to cell adhesion and cytoskeletal reorganization, early experiments might focus on prominent substrate proteins such as MARCKS (myristoylated alanine-rich C-kinase substrate) or pleckstrin (platelet and leukocyte C-kinase substrates) (54, 55). Raf-1, shown to be phosphorylated and activated by PKC, is an additional intriguing possible target in development, given that Wnts have been shown to be linked both to MAP kinase signaling and to nuclear events (55).

Specificity of PKC signaling may include regulation by the assembly of higher order complexes through the action of anchor proteins. The best known examples are so called RACKs (receptors of activated C-kinase). It will be important to probe these anchor proteins and scaffolds, such as Dishevelled or AKAPs, in understanding the fuller spectrum of PKC mediation of Wnt signaling (56). Although evidence clearly has established a link between Wnt and PKC signaling, our knowledge of the details of this signaling pathway is still very rudimentary.

8. CALPAIN

Calpain is a calcium-activated protease (57). Although regulation of this enyzme by Wnt signaling has not been reported so far, it will be of particular interest to test this provocative hypothesis. Calpain has been shown to degrade beta-catenin in SW480 cells and the function of Wnt-5A has been repeatedly shown to negatively regulate beta-catenin signaling (57). Thus it will be of interest to test whether Wnt-5 stimulated calcium release can provoke an activation of calpain and catalysis of a calpain-mediated decrease of intracellular beta-catenin concentrations.

9. FUTURE REQUIREMENTS

As multiple downstream meditors have been identified, several interesting question need to be addredssed to fully interrogate the details of the Wnt/calcium pathway. Most importantly, testing the kinetics of the response of the non-canonical Wnt/Ca2+ pathway of cells to the activation by purified, active Wnts will resolve the linger issue of direct versus indirect effects of Wnts on calcium signaling. Only the results from these experiments can adequately address the possibility of indirect effects. Careful quantification of these experimental systems and read outs will reveal how Wnts modulate frequency and amplitudes of calcium oscillations. In parallel, enzymatic activity assays should be performed to link calcium transients to enzyme acitivities, seeking to establish unambiguous dose-response relationships for all responses. Furthermore, how broad/narrow is the specificity of Wnt/calcium signaling, *i.e.,* are CamKII, PKC, and/or CaCN activated simultanously in the same cell? If not, what is the significance and benefit of the signal divergence? Finally, downstream effectors of calcium-sensitive enzymes and downstream target genes must be more fully explored to resolve existing gaps in our current scheme of Wnt/calcium signaling. As highlighted by the case of the potential cGMP/cGKI/IP_3R signal linkage map or for the regulation of NF-AT transcription factors, these experiments also will shed light on the exciting topic of cross-talk between the non-canonical Wnt signaling pathways and other signal transduction pathways.

10. ACKNOWLEDGEMENTS

The work in my group is supported by the Deutsche Forschungsgemeinschaft (SFB 497, Tp A6).

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Key words: Wnt, Frizzled, calcium, PKC, CamKII, CaCN, Review

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