Mechanism of Persistent Protein Kinase D1 Translocation and Activation

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Summary

The specificity of many signal transduction pathways relies on the spatiotemporal features of each signaling step. G protein-coupled receptor-mediated activation of protein kinases leads to diverse cellular effects. Upon receptor activation, PKD1 and several C-type protein kinases (PKCs), translocate to the plasma membrane and become catalytically active. Here we show that, unlike PKCs, PKD1 remains active at the membrane for hours. The two DAG binding C1 domains of PKD1 have distinct functional roles in targeting and maintaining PKD1 at the plasma membrane. C1A achieves fast, maximal, and reversible translocation, while C1B translocates partially, but persistently, to the plasma membrane. The persistent localization requires the C1B domain of PKD1, which binds Gαq. We incorporate the kinetics of PKD1 translocation into a three-state model that suggests how PKD1 binding to DAG and Gαq uniquely encodes frequency-dependent PKD1 signaling.

Introduction

Receptor-mediated signal transduction processes employ a small number of second messengers to generate a great variety of cellular responses. The spatial and temporal localization of the signaling molecule, combined with its substrate specificity, lead to specific responses. A major question of current biology is how the spatial and temporal specificity of these signal responses are accomplished. Here we investigate the translocation and activation mechanism of protein kinase D (PKD, PKK, and PKD1; Lemmon et al., 2002) in response to G protein Gαq receptor-mediated activation.

Many cellular receptors initiate the release of the second messenger, DAG. In particular, Gαq G protein-coupled and tyrosine kinase receptors stimulate phospholipase C (PLC) catalysis of plasma membrane phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] to form DAG and inositol 1,4,5-trisphosphate (IP3). Biochemical assays (Liscovitch, 1992) and studies employing DAG binding domains (Oancea et al., 1998) suggest that the DAG signal is transient (~200 s) in spite of sustained receptor stimulation. The subsequent intracellular calcium (Ca2+) elevation by IP3, as well as the activation of protein kinase C, lasts seconds to minutes. However, the effect of these signals lasts much longer; in minutes to hours, these signals are translated into profound cellular changes. How do transient DAG signals lead to prolonged downstream signaling events? In this study, we investigate how PKD1 is maintained active at the plasma membrane for much longer durations than DAG. We show that, after DAG-mediated translocation, PKD1 is held at the membrane through binding to Gαq, greatly prolonging its activation.

DAG accumulates in the plasma membrane after receptor activation. Subsequently, proteins that contain DAG binding C1 (cysteine-rich) domains translocate to the plasma membrane (Oancea and Meyer, 1998), become activated, and initiate their specialized functions. PKD1 remains active for hours after plasma membrane translocation (Matthews et al., 2000) and affects such apparently diverse biological processes as proliferation (Zhukova et al., 2001) and apoptosis (Asada et al., 1998; Endo et al., 2000). PKD1 initiates Rho-mediated cytoskeletal changes (Yuan et al., 2001) as well as reorganization of the Golgi apparatus (Jamora et al., 1999; Liljedahl et al., 2001). These disparate functions suggest that localization is critical to PKD1’s function.

PKD1’s domain structure is unlike that of any other protein kinase. PKD1 contains a complex regulatory domain and a catalytic domain (Figure 1A). The regulatory domain contains an N-terminal alanine/proline-rich variable region (V1) and two C1 domains (C1A and C1B) analogous to similar domains in the PKC family. However, PKD-C1B differs from the C1 consensus profile (Hurley et al., 1997). The regulatory domain also contains a pleckstrin homology (PH) domain reminiscent of the PKB family of kinases. The catalytic domain (Ser/Thr kinase) is most similar (41%) to the myosin light chain kinase (MLCK) of Dictostelium (Valverde et al., 1984). Deletion of the tandem C1 domains (C1) or the PH domain of the regulatory region renders the kinase fully active (Iglesias and Rozengurt, 1998; Valverde et al., 1994). It is not clear whether domain deletion removes the domain’s ability to inhibit the catalytic site or whether it otherwise alters the protein’s quaternary structure.

The tandem C1 domain of PKD1 (C1) has been implicated in localizing PKD1 to the plasma membrane in response to activation of receptors that lead to generation of DAG or to phorbol ester (Yuan et al., 2001). As evidence that there are C1A- and C1B-specific functions, PKD1’s C1A domain is required for PKD1 localization to the Golgi apparatus (Baron and Malhotra, 2002; Maeda et al., 2001), while the C1B domain is required for nuclear import (Rey and Rozengurt, 2001). The relative affinity of C1A and C1B for DAG or phorbol ester is controversial. Iglesias et al. (1998) found that the two C1 domains were dissimilar, with the C1B domain responsible for the majority of phorbol ester binding. In contrast, Irie et al. (1999) found that C1A and C1B bound phorbol ester equally and in a similar manner to the analogous domains of PKCγ. The PH domain of PKD1 does not appear to bind any particular lipid with high specificity. However, the PKD-PH domain participates in interactions with PKCγ (Waldron et al., 1999) and the
Figure 1. Irreversible Translocation to the Plasma Membrane and Activation of PKD1-GFP in Response to Stimulation of Gq-Coupled Receptors

(A) The domain structure of PKD1. The regulatory domain of PKD1 contains a tandem C1 domain (C1A + C1B), a PH domain, and the V1, V2, and V3 variable regions. GFP was fused to the carboxyl terminus.

(B) PKD1-GFP-expressing HM1 cells before and after addition of 10 μM carbachol. Sequential confocal images were recorded every 5 s to monitor the translocation of the GFP-tagged proteins to the plasma membrane. Calibration bar, 10 μm.

(C) PKC-γ-GFP-expressing HM1 cells before and after addition of 10 μM carbachol. Note that PKC-γ translocation is complete by 50 s and is reversible. Calibration bar, 10 μm.

(D) Carbachol-induced PKD1-GFP translocation to the plasma membrane was not reversed within 90 min. Calibration bar, 20 μm.

(E) The kinase activity of PKD1-GFP expressed in HM1 cells stimulated with carbachol was determined with a phospho-PKD1-specific antibody. PKD1-GFP was immunoprecipitated before and at different time intervals after carbachol stimulation with an anti-GFP antibody. Equal amounts of protein were analyzed either with a GFP-specific antibody (lower panel) or with the phospho-PKD1-specific antibody (upper panel). The intensity of the bands in the upper panel indicates what fraction of the PKD1-GFP detected in the lower panel was catalytically active. The activity of the kinase, as detected by the phospho-PKD1 antibody, remains elevated even after 60 min and is correlated with the plasma membrane translocation of the protein.

G protein βγ subunit (Jamora et al., 1999). Finally, the PKD1-PH domain also appears to be required for the nuclear export of PKD1 (Rey et al., 2001). Here we investigate the mechanism by which Gq receptor stimulation initiates the long-term translocation of PKD1 to the plasma membrane. Our results show
that only the membrane-associated PKD1 is catalytically active and that its activation is a multistep process. First, in response to DAG production, PKD1 is targeted to the plasma membrane via its C1A domain. Membrane-associated PKD1 binds to GTP-Gαq via its C1B and V1 domains and remains at the membrane, even after the DAG levels return to baseline. In this state PKD1 is active. The unique combination of a transient DAG signal and binding to Gαq allows PKD1 to translate a short hormonal stimulus into a long-lasting cellular response. As a consequence, periodic hormonal signals that stimulate the cell at time intervals shorter than 1000 s will be integrated by PKD, while signals that occur at time intervals longer than 1000 s will be decoded by PKD1 as independent signals.

Results

PKD1 Translocates and Remains Active at the Plasma Membrane in Response to Gαq

Receptor Activation

Carbachol elicits transient Ca2⁺ and DAG responses in HEK cells stably transfected with the Gαq-linked muscarinic type 1 receptor (HM1) (Peralta et al., 1988). Using GFP-tagged PKD1, we sought to determine the time course of PKD1 translocation from the cytosol to the plasma membrane. Moreover, we investigated the relationship between translocation and activation of the kinase. As shown previously, the GFP tag does not interfere with DAG binding or the catalytic activity of the kinase (Matthews et al., 2000).

Before stimulation, the fluorescent PKD1-GFP protein was primarily cytosolic, with a small fraction localized to intracellular structures. After M1 receptor activation, the amount of fluorescence translocated from cytosol to the plasma membrane increased gradually to a plateau in ~400 s (Figure 1B). As shown in Figure 1D, translocation persisted for more than 90 min. A similar time course of PKD1 translocation was observed after stimulation of endogenous Gαq-linked purinergic receptors in HEK cells, histamine receptors in HeLa cells, and PAF receptors transfected into HEK cells (data not shown). PKD1-GFP translocation was not detected after stimulation of Gαi-linked M2 or FMLP receptors in HEK cells (data not shown).

We first compared PKD1 and PKC translocation in a model system. PKCβ1 was used because it is a Ca2⁺-independent PKC isoform containing a DAG binding C1z domain, and, like PKD, translocates in response to Gαz receptor stimulation. PKCβ1-GFP translocation was detected in response to the same carbachol stimulus protocol used for PKD1-GFP. Unlike PKD1-GFP translocation, PKCβ1-GFP translocation was transient, reaching a peak in ~50 s and reversing within 400 s (Figure 1C). A higher fraction of the PKCβ1-GFP was associated with intracellular structures than for PKD1-GFP, but this fraction did not translocate to the plasma membrane. Moreover, lysophosphatidic acid (LPA) receptor activation of Gαz in primary rat cardiac fibroblasts persistently translocated endogenous PKD, as shown by immunostaining with a PKD-specific antibody and cellular fractionation (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/4/4/561/DC1).

To answer the question of how PKD1 translocation correlates to kinase activation, we immunoprecipitated PKD1-GFP from HM1 cells, unstimulated and 15, 30, and 60 min after carbachol stimulation. Catalytically active PKD1 was detected with an anti-phospho PKD1 (Ser916) antibody that detects only the active form of the kinase (Matthews et al., 1999). Equal amounts of immunoprecipitated PKD1-GFP were detected either with a GFP-specific antibody or with the phospho-PKD1-specific antibody. Our results show that PKD1 becomes activated in response to stimulation of the M1 receptor and remains active for longer than 60 min (Figure 1E). We conclude that the persistent translocation of PKD1 to the plasma membrane in response to Gαq receptor stimulation is tightly correlated with persistent kinase activation.

The intensity of fluorescence within individual cells was quantified and normalized as shown in Figure 2A (see Experimental Procedures). Carbachol-induced translocations of PKD1-GFP, PKCβ1-GFP, and PKCβ-GFP (a Ca2⁺- and DAG-dependent PKC) are compared in Figure 2B. The most striking difference measured between these three molecules was the persistence of PKD1-GFP at the plasma membrane. Moreover, the rate for PKD1 translocation to the membrane was much slower than that for the two PKC isoforms (Figure 2B; t1/2 = 54.0 ± 12.5 s, n = 12 for PKD1; t1/2 = 15.4 ± 2.6 s, n = 8 for PKCβ1; and t1/2 = 11.60 ± 1.6 s, n = 15 for PKCβ). These rate differences could reflect the relative speeds and complexities of the protein conformational changes required for translocation or simply the differences in their binding affinities for DAG. For PKD1, phosphorylation of two sites in the activation loop (Waldron et al., 2001) might initiate a conformational change necessary for DAG binding. Alternatively, its unique regulatory (V1) domain may undergo rearrangement. To determine the relative role of these domains in the translocation process, we constructed PKD1s with truncated individual or tandem domains.

Deletion and truncation mutants of PKD1 are summarized in Figure 2C. The translocation of expressed mutant PKD1-GFPs was monitored after carbachol addition. The mutant lacking the V1 domain (PKD1V1) translocated to the plasma membrane slightly faster than wild-type (wt) PKD1 (t1/2 = 34.1 ± 9.5 s; n = 15). Unlike that of wt PKD1, PKD1V1’s translocation was slowly reversible. Further truncation of the C1z domain of PKD1 abrogated translocation to the plasma membrane, suggesting that the C1z domain is necessary for receptor-induced PKD1 translocation.

To determine whether DAG binding to the C1z domain was not only necessary, but also sufficient, for PKD1 translocation, we bypassed the muscarinic receptor to directly increase DAG’s concentration in the membrane. The Clostridia perfringens PC-PLC enzyme cleaves plasma membrane phosphatidylcholine to form DAG and IP3. When PC-PLC was added to HM1 cells, PKD1-GFP was translocated to the membrane over the same time course as for muscarinic receptor stimulation (Figure 2D). These results suggest that binding of the C1z domain to DAG is both necessary and sufficient for translocation of PKD1 to the plasma membrane.
Figure 2. The C12 Domain of PKD1 and DAG Production Are Necessary and Sufficient for PKD1 Translocation to the Plasma Membrane

(A) For quantitation of translocation, confocal images of HM1 cells expressing PKD1-GFP or other GFP-tagged proteins were acquired as time series of one hundred images, taken every 4–6 s. The fluorescence intensity of a cytosolic area (red circle) representing more than 10% of the total cytosolic surface area was measured for each image in a time series. The ratio of the relative fluorescence intensity at each time point [Icyt(t)] and the fluorescence intensity of the same area before stimulation [Icyt(0)] was determined as a function of time. After the ratio was normalized, plasma membrane (PM) translocation was calculated as 1 - R(t)norm.

(B) Comparison of GFP-tagged PKD1, PKC\(\alpha\), and PKC\(\gamma\) translocation to the membrane. PKC\(\alpha\) translocated most rapidly (t1/2 = 11.6 ± 1.6 s) but also reversed within 2 min. PKC\(\gamma\) translocation was less rapid (t1/2 = 15.4 ± 2.6 s), while PKD1 translocation was slowest (t1/2 = 54.0 ± 12.5 s) and did not reverse during the time frame of the experiment. Each trace represents the average of 12–18 cells from at least three different experiments.

(C) The C12 domain of PKD1 is required for the carbachol-induced PM translocation of PKD1. Deletion of the V1 domain slightly slowed its PM translocation. In the absence of the C12 domain, PKD1-V1C12 did not translocate to the plasma membrane. Each trace represents the average of 12–18 cells from at least three different experiments.

(D) Exogenous phosphatidylcholine-PLC (PC-PLC) produces plasma membrane DAG and closely mimics the effect of carbachol on PKD1-GFP translocation. The response was averaged from ten cells from three different experiments.

PKD1 and PKC\(\gamma\) Translocations Have Similar Sensitivities to Receptor Activation, but Distinct Time Courses

In our experiments, PKD1 remained localized to the plasma membrane, even when DAG’s concentration returned to baseline. This persistent PKD1 localization may be due either to a high binding affinity to the plasma membrane DAG or to binding to a membrane-associated protein. Since stimulation of the HM1 cells with decreasing concentrations of carbachol produced correspondingly less DAG, differences in the PKD1 or PKC-DAG binding affinity should shift the carbachol dose-response curve.

To determine the dose response to carbachol, cells expressing either PKD1-GFP or PKC\(\gamma\)-GFP were stimulated with increasing concentrations of carbachol. Subsequent addition of the phorbol ester, PDBu (1 \(\mu\)M), was assumed to elicit maximal plasma membrane translocation and was therefore used to calibrate the amplitude of the carbachol signal. When PKD1- or PKC\(\gamma\)-expressing cells were stimulated with submaximal concentrations of carbachol, the translocation response maintained the
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Figure 3. PKD1 and PKCγ Have Similar Dose Responses to Carbachol, but Different Translocation Kinetics

(A) Representative traces of individual HM1 cells used to determine the amplitude and kinetics of the response to carbachol. Cells expressing either PKD1-GFP or PKCγ-H9257-GFP were stimulated first with carbachol and, after 250 s, with PDBu to elicit maximal translocation. The traces were normalized to the maximal PDBu response. The amplitude of responses to 0.1 μM carbachol was 58% and 66% of maximum for PKD1-GFP and PKCγ-GFP, respectively.

(B) Averaged amplitudes of the plasma membrane translocation of PKD1-GFP or PKCγ-GFP as a function of carbachol concentration (Figure 3B). Twelve to 18 cells from three independent experiments were averaged for each point.

(C) Kinetics of translocation of PKD1-GFP or PKCγ-GFP as a function of carbachol concentration (Figure 3C). For each cell, the time corresponding to the half-maximal amplitude (t1/2) of the carbachol response was determined from graphs similar to those shown in A. Twelve to 18 cells from three independent experiments were averaged for each point.

The C1A and C1B Domains of PKD1 Have Distinct Functional Roles

PKD1’s C1 domain differs from conventional and novel PKCs in the length of the linker (V2 domain) between the two C1 domains (Figure 1A). In contrast to conventional and novel PKCs with only 15 or 22 amino acids linking the C1 domains, PKD1 has a V2 domain containing 82 amino acids. This longer and more flexible linker might allow the two C1 domains to act as separate modules and to serve different roles in PKD1’s function.

To test the role of the individual C1 domains in the translocation of PKD, we GFP-tagged truncated mutants containing the C1A domain or the C1B domain. When GFP fusion proteins of the C1A or C1B domains alone were expressed in HM1 cells, no translocation was observed in response to receptor activation or to phorbol ester. However, the C1A and C1B domains translocated when the variable regions flanking them were present. No measurable translocation was detected for the flanking regions alone or for the GFP-tagged PH domain of PKD1 in response to receptor activation. These experiments suggest that the domains flanking C1A and C1B may play a role in stabilizing PKD1’s binding to the plasma membrane.

Carbachol stimulation of HM1 cells expressing C1A-GFP (Figure 4A) induced maximal C1A translocation to the plasma membrane after 20 s. Surprisingly, this translocation was fully reversible in less than 300 s. In contrast to C1A, C1B-GFP was only partially translocated to the plasma membrane after 100 s, and its translocation persisted for more than 300 s (Figure 4B).

To compare the translocation amplitude and kinetics of the two C1 domains, we plotted the averaged plasma membrane translocation in response to carbachol (normalized to subsequent PDBu-induced translocation; Figure 4C). The C1A-containing mutant translocated rapidly, reaching 66% of the maximal response by 20 s, and fully reversed within 300 s. In contrast, the C1B-containing mutant reached only 16% of the maximal amplitude and did not reverse in up to 300 s. One interpretation of this result is that the C1A domain targets PKD1 to the plasma membrane, while C1B maintains PKD1 at the plasma membrane.

To test the hypothesis that the two C1 domains have distinct functional roles, we constructed a chimeric form of PKD1 in which the C1B domain was replaced by a second C1A domain (PKD(2×C1A)-GFP). When expressed in HM1 cells, PKD(2×C1A)-GFP had the same distribution as wt PKD1-GFP (data not shown). But, unlike wt PKD1-GFP, PKD(2×C1A)-GFP translocates...
Figure 4. The C1A and C1B Domains Have Distinct Roles in PKD1 Translocation: C1A Targets PKD1 to the Plasma Membrane, while C1B Stabilizes PKD1 at the Plasma Membrane

(A) The C1A-containing PKD1-GFP mutant was reversibly translocated to the plasma membrane after HM1 cell stimulation with 10 μM carbachol. Calibration bar, 10 μm.

(B) The C1B-containing PKD1-GFP mutant was partially, but irreversibly, translocated to the plasma membrane after HM1 cell stimulation with 10 μM carbachol. Calibration bar, 10 μm.

(C) Comparison of the time course and amplitude of C1A-GFP, C1B-GFP, and PKD1-GFP translocation to the plasma membrane. Each trace represents the average of 12-18 cells from three independent experiments.

(D) A chimeric form of PKD1-GFP containing two C1A domains (PKD1(2×C1A)-GFP) translocates to the plasma membrane rapidly, but transiently, upon stimulation with 10 μM carbachol as compared with wt PKD1-GFP.

PKD1 Interaction with Gαq* Stabilizes PKD1 at the Plasma Membrane

Once translocated by DAG to the plasma membrane, PKD1 remains associated with the membrane for hours. One potential mechanism for locking PKD1 into its membrane-associated state is by binding to a plasma membrane protein. In early studies we noticed that cotransfection of PKD1-GFP with the constitutively active form
Figure 5. PKD1-GFP is localized to the plasma membrane in the presence of constitutively active Goq (Goq*)
(A) Confocal images of HM1 cells coexpressing Goq* and GFP-tagged PKD1, γC1A, PKCγ, or PKCγ. Only PKD1-GFP localized at the plasma membrane. Calibration bar, 10 μm.
(B) Quantitation of plasma membrane localization induced by Goq*. Confocal images of HM1 cells transfected with PKD1-GFP (only) or with both Goq* and either PKD1-GFP, γC1A-GFP, PKCγ-GFP, or PKCγ-GFP. For each individual cell the ratio R was calculated as the fraction of fluorescence localized at the plasma membrane (Flumembrane/Fcytosol). Each bar represents the average of 12–18 cells from at least three different experiments.
(C) PKD1-GFP is localized to the plasma membrane by Goq*, but not by Goq or constitutively active Goq13*, Gγ, or Gjγ. Confocal images of HM1 cells transfected with PKD1-GFP and either Goq*, Goq, Gjγ and PLCγ2, Goq13*, or Gjγ were recorded from at least two independent experiments. The averaged ratio (R) was calculated for 12–18 individual cells.
(D) Both the V1 and the C1B domains of PKD1 are required for the Goq*-induced localization of PKD1-GFP to the plasma membrane. Confocal images of HM1 cells coexpressing Goq* and the GFP-tagged constructs shown on the left were recorded and analyzed as above. Removal of the V1 domain from PKD1 reduced the plasma membrane localization by ~30%, while removal of the C1B domain reduced localization by ~80%.

of Goq (Goq*, the Q209L mutant of Goq that locks the protein in the GTP-bound state) localized PKD1 almost exclusively to the membrane (Figure 5A). This may have been a consequence of either constitutive generation of DAG or the result of PKD1 binding to a membrane protein. To detect DAG in the plasma membrane, we fused the PKCγ-C1A domain to GFP (γC1A-GFP) and used it as a DAG indicator. Upon coexpression with Goq* (Figure 5A), no change in the cellular distribution of the γC1A-GFP was observed when compared with
PKD1's cellular localization induced by Gαq* was quantitated as the fraction of the total PKD1-GFP fluorescence localized at the plasma membrane compared with that localized at the cytoplasm (R = Fplasm/Fcyt; Figure 5B). In the absence of Gαq*, PKD1-GFP was mainly cytosolic (R = 0.001 ± 0.009) but became almost entirely plasma membrane-localized in Gαq*-expressing cells (R = 0.78 ± 0.07). For controls, PKD1 membrane localization was also measured in cells expressing inactive Gαq, Gβγ (which can generate DAG only in the presence of PLC(β)2), or constitutively active Gαi (Gαi*) or Gα13 (Gα13*) subunits. As shown in Figure 5C, PKD1-GFP translocation to the membrane was only correlated with Gαq*-cotransfection. The lack of PKCγ or PKCη translocation in the presence of Gαq*, combined with the strong association between Gαq* (but not Gβγ) expression and PKD1 plasma membrane localization, raises the possibility that PKD1 and Gαq* directly interact.

To determine the domains of PKD1 that are important for Gαq*-induced PKD1 membrane localization, we tested various GFP-tagged PKD1 truncation mutants cotransfected with Gαq* (Figure 5D). In order to preserve the tertiary structure of the protein, we deleted either the N- or C-terminal PKD1 domains, but not intramolecular domains. In the absence of the catalytic domain, the localization of PKD1 was not significantly changed (R = 0.74 ± 0.13, n = 13) compared with that of wt PKD1 (R = 0.78 ± 0.07, n = 17). Furthermore, removal of the PH domain from the regulatory domain did not alter the distribution of the protein (R = 0.7 ± 0.09, n = 14). However, removal of the entire V1 domain significantly reduced the R value (0.45 ± 0.12, n = 27), and this effect was not dependent on the presence or absence of the catalytic domain (R = 0.48 ± 0.13, n = 19). Removal of the C1B domain also significantly decreased membrane localization (R = 0.15 ± 0.09, n = 10). These results suggest that the V1 domain together with the C1B domain mediates the plasma membrane localization of PKD1 in the presence of Gαq*. The involvement of C1B in persistent plasma membrane localization is consistent with the results in Figure 4. We conclude that C1B, which partially, but irreversibly, translocates to the plasma membrane, is capable of stabilizing PKD1 at the plasma membrane.

We next sought to determine the activation state of PKD1-GFP in the presence of the inactive or the constitutively active form of Gαq. PKD1-GFP coexpressed with Gαq or Gαq* was immunoprecipitated from HM1 cells with a GFP-specific antibody and then probed either with an anti-GFP antibody or anti-phospho PKD1 (Ser916) antibody (Figure 6A). A much higher fraction of PKD1-GFP was active upon PKD1-GFP constitutive translocation to the plasma membrane in the presence of Gαq* than in the presence of Gαq. These results suggest that Gαq* is sufficient for PKD1 translocation to the plasma membrane and activation.

The interaction between PKD1 and Gαq* was directly tested by coimmunoprecipitation (Figures 6B–6D). First, a GFP-specific antibody immunoprecipitated PKD1-GFP, while an anti-HA antibody immunoprecipitated HA-Gαq*. Although both the N and the C termini of Gαq* are known to be functionally important, Qian et al. showed that an N-terminal HA tag did not interfere with Gαq activation of PLC (Qian et al., 1993). We tested whether the N-terminal HA-tagged Gαq* was capable of localizing PKD1-GFP to the plasma membrane. As shown in Figure 6B, the HA tag did not interfere with the ability of Gαq* to localize PKD1-GFP to the plasma membrane. Furthermore, PKD1-GFP's cellular distribution was not changed by HA tagging of the inactive Gαq. The interaction between PKD1-GFP and HA-Gαq* was evaluated by immunoprecipitating PKD1-GFP from HEK cells expressing both proteins and testing for the presence of HA-Gαq* (Figure 6C). HA-Gαq* also communoprecipitated with the C1B-containing fragment of PKD1 (Figure 4B), suggesting that the C1B domain is not only necessary, but also sufficient, for the PKD1-Gαq* interaction.

The interaction between PKD1 and Gαq* was also tested in rat primary cardiac fibroblasts, since high levels of both PKD1 and Gαq are expressed endogenously (Figure 6D). In fibroblasts Gαq communoprecipitated with a PKD1-specific antibody (Figure 6D, lane 3), but not with a PKCβ-specific antibody (data not shown). These results show that both expressed and endogenous Gαq* and PKD1 directly interact.

The length of time that Gαq spends in the GTP-bound state controls the activity of PLCβ, in turn determining the amount of DAG produced. PKD1 itself could modulate the GTPase activity of Gαq. To address this question, we determined whether expression of PKD1 affected the time course of DAG production in response to receptor activation. Relative changes in single-cell DAG levels were estimated from the translocation of the C1A domain of PKCγ (γC1A-GFP) in response to receptor activation (Figure 7A). The translocation time course of γC1A-GFP to the plasma membrane has been shown to be closely correlated with the accumulation of DAG in the plasma membrane (Oancea et al., 1998). When HM1 cells were transfected with γC1A-GFP alone or with γC1A-GFP and PKD-dSRed2 and stimulated with carbachol, the presence of PKD-dSRed2 did not significantly change the amplitude and time course of the γC1A-GFP response to carbachol in the absence of PKD1 (t½ = 157.9 ± 40.7 s; t½ = 136.1 ± 34.6 s in the presence of PKD). This suggests that PKD1 is not likely to modulate the GTP-bound state of Gαq. However, a direct test for PKD1 modulation of the GTPase activity of Gαq requires both purified Gαq and PKD1 proteins. Because of PKD1's large molecular size and poor solubility, we could not obtain sufficient purified PKD1 protein to directly test this possibility.

PKD1 Decodes Different Frequencies of Hormonal Stimuli

The results described above were obtained for persistent hormonal stimulation. However, in many physiological systems, hormonal stimulation occurs in short and periodic pulses. The frequency as well as the duration of the pulses can determine the specificity of the cellular response. Hence, we sought to determine how PKD1 responds to stimuli of different frequencies. First, we analyzed the response of PKD1-GFP to a 60 s carbachol pulse applied to HM1 cells (Figure 7B). The plasma membrane translocation of PKD1-GFP was compared with
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Figure 6. PKD1-GFP Is Activated by Directly Interacting with Gαq*
(A) The kinase activity of PKD1-GFP was elevated in the presence of Gαq*, but not Gαq. PKD1-GFP was expressed in HM1 cells in combination with either Gαq* or Gαq and immunoprecipitated with an anti-GFP antibody. Equal amounts of protein were analyzed either with a GFP-specific antibody (lower panel) or with a phospho-PKD1 (Ser916)-specific antibody (upper panel).
(B) HA-tagged constitutively active Gαq* (HA-Gαq*), but not HA-Gαq, localized PKD1-GFP to the plasma membrane. An HA tag fused to the N terminus of Gαq* or Gαq did not interfere with the Gαq*-induced localization of PKD1-GFP to the plasma membrane. HA-Gαq did not alter the intracellular distribution of PKD1-GFP. Calibration bar, 10 μm.
(C) Gαq* and PKD1 directly interact via the C1B domain of PKD1. HEK cells were transfected with HA-Gαq* and GFP, HA-Gαq* and PKD-GFP, or PKD-C1B-GFP. Monoclonal anti-HA was the primary antibody used in lanes 1–6 and 9–11, and anti-GFP was the primary antibody used in lanes 7 and 8. Lanes 3 and 6 contain eight times more total protein than lanes 2 and 5, respectively. HA-Gαq* (lane 6) coimmunoprecipitated with PKD-GFP with the anti-GFP antibody (representative of six gels). HA-Gαq* (lane 11) also coimmunoprecipitated with PKD-C1B-GFP, with the same GFP-specific antibody.
(D) Endogenous Gαq* and PKD1 directly interact in primary cardiac ventricular fibroblasts. Cultured ventricular fibroblasts stimulated with LPA (lane 1) were used to immunoprecipitate Gαq* (lane 2) or PKD1 (lane 3). The samples were analyzed with a polyclonal Gαq-specific antibody. As shown in lane 3, PKD1 coimmunoprecipitates Gαq*. Lane 3 contains four times more total protein than lane 2. The result is representative of four independent experiments.

The translocation of the DAG indicator, γC1A-GFP. While γC1A-GFP translocation was fast and completely reversible within 200 s (t1/2 = 77.0 ± 10.9 s), the translocation of PKD1-GFP was slow and reversed to baseline only after 1000 s (t1/2 = 439.8 ± 109.8 s). A similar time course was obtained for PKD1 activation measured with the phospho-PKD1-specific antibody after a carbachol pulse (data not shown). This suggests that translocation to the plasma membrane and activation of PKD1 are closely related.

The time course for PKD1 translocation and activation in response to a short hormonal stimulus predicts that PKD1 will respond differently to periodic stimuli that occur at intervals shorter than 1000 s than to stimuli that occur at longer time intervals. To test this prediction, we measured PKD1’s translocation response to 30 s-long carbachol pulses at 400 s intervals (Figure 7C, left panel). Each pulse evoked a submaximal PKD1 response. Recovery was minimal during the 400 s interpulse interval, resulting in a cumulative response. For the same pulse
protocol, the translocation of the γC1A-GFP was maximal and fully recovered between pulses (data not shown). When 60 s-long carbachol pulses were used at 1000 s intervals, the response of PKD1 was markedly different (Figure 7C, right panel). While γC1A-GFP showed the same type of response as for the previous protocol (data not shown), PKD1-GFP responded maximally and fully recovered between carbachol pulses.

PKD1 has a uniquely long residence at the plasma membrane as a result of being “handed off” from DAG to Gαq. Stimuli that occur at time intervals shorter than 1000 s are integrated in the PKD1 response, while lower-frequency pulses elicit the same PKD1 response as an independent pulse. The range of stimulation intervals that can be decoded by PKD1 is dramatically different from members of the PKC family. PKD1 stabilization at the plasma membrane by Gαq converts a short and transient DAG signal into prolonged PKD1 activation.

Kinetic Model for PKD1 Signal Transduction

The results described above can be incorporated into a three-state model for PKD1 signal transduction (Figure 8A). The three observable states are: cytosolic (inactive) PKD1, DAG-bound PKD1, and Gαq-bound PKD1. The first state is the equilibrium state in the absence of receptor activation: PKD1 is cytosolic and catalytically inactive. As DAG is produced by hydrolysis of PIP2, PKD1 is bound and translocated to the membrane. This step probably involves rearrangement of the C1 domains, the time course of which we cannot observe. Once at the plasma membrane, PKD1 binds Gαq*. Although DAG levels may subsequently change, PKD1 is locked into its activated state by its association with active Gαq.

From our experimental data we can determine the rates for the transitions that involve a translocation from cytosol to plasma membrane or vice versa. From the PKD1 translocation curve (Figure 7A), kαq = 1/42 s⁻¹ (0.024 s⁻¹, or 1.4/min). From the PKD1 off kinetics (Figure 7B), kαq = 1/417 s⁻¹ (0.0024 s⁻¹, or 0.14/min). Using our model, we then estimated the rate for the transition between the DAG- and the Gαq-bound states by fitting to a three-state nonequilibrium model. The best fit was obtained for kαq = 1/7 s⁻¹ (0.14 s⁻¹, or 8.6/min). For this particular value of kαq and for the indicated DAG pulses (Figure 8B), the calculated time course of the plasma membrane-bound PKD1 (PKD1-DAG and PKD1-Gαq) matches the experimental data. This suggests that the DAG-bound state is relatively short lived and that the transition to the Gαq-bound state occurs before PKD1 can dissociate from DAG and return to the cytosol. This intermediate may be important for inducing a conformational change that allows PKD1 to bind to Gαq*. In the presence of Gαq*, but in the absence of DAG, we predict that the transition between the cytosolic state and the Gαq*-bound state occurs very slowly, presumably because cytosolic PKD1 has neither the conformation nor proximity for Gαq* binding.

Discussion

We have shown that translocation of PKD1 from the cytosol to the cell membrane and subsequent activation translates short-lived Gαq activation into a prolonged cellular signal. The unique structure of PKD1 distinguishes its kinetics from other DAG-translocated PKC enzymes; the C1A domain enables fast, maximal, and reversible translocation, while the slow reversibility of the translocation is effected by PKD1’s N-terminal variable domain (V1) and C1B domains directly binding to
Persistent PKD1 Translocation and Activation

Figure 8. Model for PKD1-Mediated Signal Transduction

(A) Three-state kinetic model for PKD1 translocation and activation.
(B) Predicted time course for PKD1 translocation (blue trace) compared with an experimental trace of a single cell (red trace). The time courses of all three PKD1 states were derived from the solution of the full kinetic model. The blue trace represents the calculated change in occupancy of the cytosolic state (PKD) in response to the simulated DAG pulses (green).
(C) Stepwise model for PKD1-mediated signal transduction. In resting cells, catalytically inactive protein kinase D (PKD1) is cytosolic, while G\textsubscript{q} is bound to G\textsubscript{q} at the plasma membrane (first panel). In the presence of extracellular agonists, receptors are activated, and G\textsubscript{q}-GTP functionally dissociates from G\textsubscript{q}. Activated phospholipase C (PLC) produces diacylglycerol (DAG) at the plasma membrane. In the presence of DAG, PKD1 gradually translocates from the cytosol to the plasma membrane as a result of its C12 domain binding to DAG (second panel). The membrane-localized PKD1 binds G\textsubscript{q} (third panel). The G\textsubscript{q}-bound PKD1 remains catalytically active (fourth panel).

G\textsubscript{q}. In effect, PKD1 is latched into its active state by G\textsubscript{q} binding.

Model for PKD1 Signal Transduction
Prior to receptor stimulation, the majority of the PKD1 is soluble and spread diffusely throughout the cytosol. Upon activation of G\textsubscript{q}-coupled receptors, GTP-bound G\textsubscript{q} activates PLC\textsubscript{z}, which, in turn, hydrolyzes PIP\textsubscript{2} into IP\textsubscript{3} and DAG. DAG remains associated with the plasma membrane and does not appear to redistribute into the cell. The soluble arm of this signal transduction pathway (IP\textsubscript{3}) initiates a rapid increase in Ca\textsuperscript{2+}. Over the next few minutes, PKD1 becomes associated with the plasma membrane DAG via its C12 domains. This process is called translocation, since the end result is a net increase in membrane-bound PKD1 and a decrease in cytosolic PKD1. Our results support the hypothesis that PKD1 is stabilized at the membrane by also binding to G\textsubscript{q}, theoretically requiring a conformational change induced by the C12 domain binding to DAG. Although PKD1-G\textsubscript{q} binding might occur in the absence of DAG, such binding would be much slower. Long-term stabilization of PKD1 in the plasma membrane is associated with the long-term activation of the kinase, resulting in a long-lasting cellular response.

PKD1 does not have an obvious pseudosubstrate that can shield its active site. Thus, the point at which the kinase becomes active is less clear than for conventional protein kinase C (Oancea and Meyer, 1998). PKD1 is cleaved by caspase, releasing the active catalytic domain of PKD1. Moreover, in the absence of its PH or C12 domain, PKD1 is constitutively active (Iglesias and Rozengurt, 1998; Valverde et al., 1994). This suggests that PKD1, like PKCs, has a two-domain structure (Pears-G\textsubscript{q} binding).

Functional Implications of PKD1 Binding to G\textsubscript{q}*
G\textsubscript{q} is attached to the plasma membrane mainly through palmitoyl modifications of its C-terminal cysteines
that connect PKD1 activation with downstream cellular cytosol, was calculated for each time point as the ratio between studies will be necessary to elucidate the signaling steps representing the fraction of fluorescence that disappeared from the rescence due to prolonged laser illumination was a minor fraction of the total fluorescence. The unique feature of the PKD1 translocation/activation chemoluminescence kit (Amersham Pharmacia Biotech) was used for detection of protein bands.

Experimental Procedures

Cell Culture, Molecular Biology, and DNA Transfection
HEK and HM1 cells were grown as described (Strubing et al., 2001). Primary ventricular fibroblasts were dissociated from ventricles of postnatal p1 rat pups with the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical). Fibroblasts were grown in DMEM media supplemented with 10% fetal bovine serum and penicillin/streptomycin. All cells used for imaging studies were from passages 2-5. GFP fusion proteins were constructed with the pEFGP-N or pEFGP-C vectors (Clontech). N-terminal HA-tagged Gβq was constructed with pCMV-HA (Clontech). Proteins or protein fragments were amplified by PCR and ligated into their respective vectors.

For imaging purposes, cells were transfected with DNA by electroporation according to the procedure described in Teruel and Meyer (1997). Cells at 70%-80% confluency were transfected 14–30 hr prior to experiments. For biochemical assays, HEK cells were transfected with LipofectAMINE 2000 (Invitrogen) and assayed 30–50 hr after transfection.

Immunoprecipitation and Western Blotting
HEK cells plated on 35 mm petri dishes were transfected with one or more DNA constructs containing either GFP or HA tags. Cells were lysed with PBS with 1% Triton X-100 and protease inhibitors, and the lysates were incubated overnight at 4°C with either ImmunoPure Immobilized Protein A/G (Pierce) and GFP antibody (Molecular Probes) or with anti-HA affinity matrix (Roche Molecular Biochemicals) or with anti-Gβγ matrix (Roche Molecular Biochemicals) and polyclonal anti-GFP antibody (Molecular Probes) were used as primary antibodies to detect the HA- or GFP-tagged proteins. An enhanced chemoluminescence kit (Amersham Pharmacia Biotech) was used for detection of protein bands.

Confluent cardiac ventricular fibroblasts were serum-starved for 24 hr and then stimulated with 20 μM lypo-phosphatidic acid (LPA) (Sigma) for 10 min. Cells were then harvested with PBS with 1% Triton X-100 and protease inhibitors, and the lysates were incubated overnight at 4°C with either ImmunoPure Immobilized Protein A/G (Pierce) and anti-Gβγ antibody specific for the C terminus of Gβγ (gift from Dr. Paul Sternweis, UTSW, Dallas) or with agarose-conju-gated anti-PKD1 antibody C-20 (Santa Cruz Biotechnology). The immunoprecipitated samples were analyzed as described above, with the Gβγ-specific antibody for blotting.

Confocal Imaging and Image Analysis
Scanning confocal microscopy was used to monitor the translocation of GFP, RED2, or YFP fusion constructs in response to different stimuli. Cells expressing GFP fusion proteins were excited by 488 nm laser illumination, and the emission was bandpass filtered from 512 to 527 nm (Zeiss LSM 410). Images were analyzed in NIH Image 1.62. Time series of 80–100 images were recorded, and the fraction of fluorescence translocated to the plasma membrane was calculated for each condition. In each time series, the first two images were recorded as a reference point before the stimulus. Images were not corrected for photobleaching, since the decrease in fluorescence due to prolonged laser illumination was a minor fraction of the total fluorescence. For each cell and each image of a time series, we measured the fluorescence of a homogenous area of the cytosol [Icyt(t)], representing more than 10% of the total cytosolic area. The ratio R(t), representing the fraction of fluorescence that disappeared from the cytosol, was calculated for each time point as the ratio between Icyt(t) and the value determined from the first two reference images.
[\text{Icyt}(0)], \text{R}(t)\text{ was normalized between 0 and 1 [\text{R}(t)_{\text{norm}}]} in order to compare cells with different levels of expression. Assuming that the total fluorescence of the cell remained constant and that the translocation occurred exclusively between the cytosol and plasma membrane, the increase in plasma membrane fluorescence, or PM translocation, can be calculated as 1 - \text{R}(t)_{\text{norm}}. Half-times ($t_{1/2}$) for translocation were analyzed with MatLab. The ratio $R$ in Figure 5 was calculated as $\text{I}_{\text{PM}} - \text{I}_{\text{cyt}}(t_{\text{in}}) / \text{I}_{\text{PM}} - \text{I}_{\text{cyt}}(t_{\text{out}})$, where $t_{\text{in}}$ is the fluorescence intensity in a representative region of the plasma membrane and $t_{\text{out}}$ is the fluorescence intensity of a representative region of the cytosol.

Modeling and Simulation

A three-state kinetic model was developed to simulate the occupancy and rates of transition between the three states of PKD1: cytosolic (state A), DAG bound (state B), and G-Ca$^+$ bound (state C). A closed loop was assumed, given that, on the timescale of our experiments, synthesis or degradation of protein was negligible. The following differential equations were used to create the model:

$$\frac{dA}{dt} = -k_{AB}A(B) + k_{BA}B(t) - k_{AC}A(t) + k_{CA}C(t)$$

$$\frac{dB(t)}{dt} = +k_{AB}A(t)B(t) - k_{BA}B(t) + k_{CB}C(t) - k_{BC}B(t)$$

$$\frac{dC(t)}{dt} = +k_{AC}A(t) - k_{CA}C(t) + k_{BC}B(t) - k_{CB}C(t)$$

The following rate constants were calculated from the experimental data: $k_{AB} = 1/42.7 \text{ s}^{-1}$, $k_{BA} = 1/74.1 \text{ s}^{-1}$, and $k_{AC} = 1/416.6 \text{ s}^{-1}$. $k_{CB}$ (direct binding of cytoplasmic PKD1 to G-Ca$^+$) was assumed to be insignificant. $\text{DAG}(t)$ is a time-dependent function that represents the DAG signal produced in response to activation of the M1 receptor. The $\text{DAG}(t)$ function was determined by fitting the experimental data obtained for the G-Ca$^+$-GFP translocation in response to a carbachol pulse. For a given $\text{DAG}(t)$ function, the time-dependent occupancy of all three states was determined as a function of the $k_{CB}$ and $k_{BC}$ rates.

The set of coupled differential equations was solved with the ordinary differential equation solver in Matlab, which uses the explicit Runge-Kutta formula, with a relative tolerance of $10^{-5}$. The least-squares fit was performed between the time course of state C and the experimental data. We then determined the rate constants for the $k_{CB}$ and $k_{BC}$ rates that minimized the error. The best fit was obtained for a $k_{CB}$ of 1/67 s$^{-1}$ and for very small values of $k_{BC}$ (~1/1000 s$^{-1}$).

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