Unique Structural and Functional Properties of the ATP-binding Domain of Atypical Protein Kinase C-\(\gamma\)*

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Atypical protein kinase C-\(\gamma\) (aPKC-\(\gamma\)) plays an important role in mitogenic signaling, actin cytoskeleton organization, and cell survival. Apart from the differences in the regulatory domain, the catalytic domain of aPKC-\(\gamma\) differs considerably from other known kinases, because it contains a modification within the glycine-rich loop motif (GXGXXG) that is found in the nucleotide-binding fold of virtually all nucleotide-binding proteins including PKCs, Ras, adenylate kinase, and the mitochondrial F1-ATPase. We have used site-directed mutagenesis and kinetic analysis to investigate whether these sequence differences affect the nucleotide binding properties and catalytic activity of aPKC-\(\gamma\). When lysine 274, a residue essential for ATP binding and activity conserved in most protein kinases, was replaced by arginine (K274R mutant), aPKC-\(\gamma\) retained its normal kinase activity. This is in sharp contrast to results published for any other PKC or even distantly related kinases like phosphoinositide 3-kinase \(\gamma\), where the same mutation completely abrogated the kinase activity. Furthermore, the sensitivity of aPKC-\(\gamma\) for inhibition by GF109203X, a substance acting on the ATP-binding site, was not altered in the K274R mutant. In contrast, replacement of Lys-274 by tryptophan (K274W) completely abolished the kinase activity of PKC-\(\gamma\). In accordance with results obtained with other kinase-defective PKC mutants, in cultured cells aPKC-\(\gamma\)-K274W acted in a dominant negative fashion on signal transduction pathways involving endogenous aPKC-\(\gamma\), whereas the effect of the catalytically active K274R mutant was identical to the wild type enzyme. In summary, aPKC-\(\gamma\) differs from classical and novel PKCs also in the catalytic domain. This information could be of significant value for the development of specific inhibitors of aPKC-\(\gamma\) as a key factor in central signaling pathways.

Protein kinase C (PKC)1 (1) is a family of Ser/Thr kinases involved in signal transduction pathways triggered by numerous extracellular and intracellular stimuli. PKC isoenzymes have been shown to play an essential role in a broad range of cellular functions including mitogenic signaling (1–5), cytoskeleton rearrangement (6, 7), glucose metabolism (8–12), differentiation (13–15), and regulation of cell survival and apoptosis (16–22). At least 11 different members of the PKC family have been identified so far. Based on structural similarities and cofactor requirements they have been grouped into three subfamilies: 1) the classical or conventional PKCs (cPKC\(\alpha\), \(\beta_1\), \(\beta_2\) and \(\gamma\), activated by \(\mathrm{Ca}^{2+}\), diacylglycerol and phosphatidylserine); 2) the novel PKCs (nPKC\(\delta\), \(\epsilon\), \(\eta\), and \(\theta\), independent of \(\mathrm{Ca}^{2+}\) but still responsive to diacylglycerol; and 3) the atypical PKCs (aPKC\(\zeta\) and \(\lambda\), where PKCA is the mouse homologue of human PKC\(\zeta\)). aPKCs differ significantly from all other PKC family members in their regulatory domain in that they lack both the calcium-binding domain and one of the two zinc finger motifs required for diacylglycerol binding (reviewed in Ref. 23). These differences lead to a different requirement of cofactors for activation; aPKCs are insensitive to \(\mathrm{Ca}^{2+}\) and diacylglycerol and exhibit an elevated basal enzymatic activity. Instead of \(\mathrm{Ca}^{2+}\) and lipids there exist several protein regulators like \(\lambda\)-interacting protein and \(\gamma\)-interacting protein, both activators of aPKCs, and PAR-4 (prostate apoptosis response), an inhibitor of aPKCs (24, 25).

Despite the fact that the regulatory domain of PKCs seems to serve essential regulatory functions, constructs with mutations within the regulatory domain rendering the PKC isoenzymes constitutively active still maintain their in vivo selectivity that must in part reside within the catalytic domain. However, although the isoenzyme-specific differences in the regulatory domain and their role in the activation of the three different PKC subfamilies are well understood, little is known about the functional implication of the subtle structural differences in the catalytic domain. The catalytic domain is characteristic for all protein kinases, with a remarkable degree of sequence identity between the more than 180 known mammalian kinases, and highly conserved from yeast to man. The 200–300 amino acid residues of the catalytic core region are predicted to fold into a common three-dimensional structure, as revealed by crystallization of several protein kinases (26–29). Some protein kinase motifs are extremely well conserved. One of these motifs is the glycine-rich loop with the consensus sequence GXGXXG located in the first of 12 subdomains of the protein kinase catalytic domain. The three glycine residues of the glycine-rich loop are conserved in over 95% of the known human kinases known so far and even in other nucleotide-binding proteins such as the small G proteins Ras, Rac, and Rho. They fold into a \(\beta\)-strand-turn-\(\beta\)-strand structure and form a flexible clamp that covers and anchors the nontransferrable phosphates of ATP (reviewed in Ref. 30). The glycine residues provide the flexibility necessary for anchoring the ATP molecule and excluding the water; for this reason they are essential both for effective catalytic activity and low ATPase activity because of phosphate transfer.

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§ The abbreviations used are: PKC, protein kinase C; aPKC, atypical PKC; FSBA, fluorosulfonylbenzoyladenosine; WT, wild type; NTA, nitritotriacetic acid.

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on water molecules. The third glycine also forms a hydrogen bond with the ATP $\beta$-phosphate oxygen. Point mutations in any of the conserved glycines generally lead to a loss of enzymatic activity and have been shown to be responsible for enzymatic defects leading to human diseases. Examples include a form of diabetes where the third glycine in the ATP-binding site of the insulin receptor is substituted by valine, leading to insulin resistance (31), and the Ras-V12 oncogene involved in tumor development, where the second glycine is replaced by valine (32, 33). It is of special interest that in aPKCs the third glycine is substituted by alanine, suggesting a unique mechanism of ATP binding with respect to other PKCs and unrelated kinases.

The most conserved and probably best characterized residue of protein kinases is the so-called “invariant lysine” in subdomain II of the catalytic domain, corresponding to Lys-274 in human PKC\(\alpha\). It is directly involved in the phosphotransfer reaction and interacts with the $\alpha$- and $\beta$-phosphates of ATP, thereby anchoring and orienting the nucleotide. Additionally, for the catalytic subunit of PKA this residue has been shown to form a salt bridge with the carboxyl group of the nearly invariant Glu-91 in subdomain III (corresponding to Glu-293 in aPKC\(\alpha\)). Replacement of the invariant lysine by any other amino acid, including arginine, generally leads to a catalytically inactive kinase, and site-directed mutagenesis of this residue has almost become a standard approach to generate kinase-dead mutants. This has been shown for PKC\(\zeta\), $\theta$, $\alpha$, and $\epsilon$ (1, 7, 34), Src (35), the epidermal growth factor receptor (36), the insulin receptor (37), Mos (38), Fps (39), and many other kinases. Interestingly, the invariant lysine is also conserved in the lipid kinase phosphoinositide 3-kinase, where a conservative K799R mutation not only completely abolishes ATP binding and kinase activity but also blocks the interaction with the phosphoinositide 3-kinase inhibitor wortmannin (40, 41).

Despite the fact that the ATP-binding site in the catalytic domain belongs to the most conserved sequences within the protein kinase family, the most specific inhibitors of PKC bind to this part of the molecule and act by blocking ATP binding to the enzyme. This is the case for the bisindolylmaleimide GF109203X and other homologues of the potent PKC inhibitor staurosporine. It is intriguing that even within the PKC family these inhibitors exhibit clear isoenzyme specificity, affecting atypical isoforms more than 100-fold less than classical or novel PKCs (7). This fact together with the remarkable difference within the glycine-rich loop compared with other kinases suggests that, surprisingly, atypical PKCs differ significantly in the structure of their catalytic domain from other PKC isoforms and protein kinases in general. Because such a difference on one hand could in part explain the in vivo isoenzyme specificity and, on the other hand, should be useful for the development of specific aPKC inhibitors, we decided to exactly characterize the enzymatic properties of atypical PKCs. To proof the assumption of a unique ATP-binding domain of aPKCs and to see whether this is functionally reflected in the mode of ATP binding, we used site-directed mutagenesis to replace the central ATP-binding residue, the invariant lysine (Lys-274), by the chemically similar arginine or by tryptophan and measured the enzymatic activity of the resulting mutant proteins. The PKC\(-\alpha\)K274R mutant exhibited normal enzymatic activity, and kinetic properties are very similar to the wild type enzyme. In contrast, the substitution of Trp for the invariant Lys-274 (aPKC\(-\alpha\)K274W) resulted in a catalytically inactive enzyme. The same behavior could be observed when the PKC\(\alpha\) mutants were studied in intact cells. This is in sharp contrast to virtually all other protein kinases, where the invariant lysine seems to be essential for correct folding of the kinase domain and interaction with ATP. To our knowledge, similar results have only been published for the very distantly related CDK-activating kinase from budding yeast (42). These results confirm that aPKC\(\alpha\) possesses a unique ATP-binding domain, and they show that this results in an unusual mechanism of ATP binding and probably catalysis. This knowledge should be useful for the development of specific inhibitors of atypical PKCs as central mediators of essential cellular signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents—**The plasmids PKC\(-\alpha\)WT, PKC\(-\alpha\)A120E, and PKC\(-\alpha\)K274R were kindly provided by T. Biden (Garvan Institute of Medical Research, Sydney, Australia) (8). All PKC cDNAs were C-terminally tagged with RGS-His\(_6\), using standard polymerase chain reaction procedures and subcloned into the expression vector pEF-Neo. The plasmids PKC\(-\alpha\)AE, RasL61, and fos-Luc have been described previously (1). GF109203X was obtained from Calbiochem, and FSBA was from Sigma.

**Construction of the PKC\(\alpha\) Mutants—**All point mutations were generated using polymerase chain reaction with the PKC\(-\alpha\)wild type construct pEF-PKCA-\(\alpha\)WT as a template, except for PKC\(-\alpha\)K274W/T555E. The mutagenesis primers were 5′-GCC AGA TCC TAT TTA TGC AAT GTG TGT GGT GAA AGA AGC TGT TGA TTA-3′ and 5′-CAT TAA CAA CAA CTT TTT TCA CAA CCC ACCA TTG CAT AAA TAC GAT CTG CC-3′ for PKC\(-\alpha\)K274W, 5′-CTA ATG AAC CGT TCC AGC TGC AGC CAG CAT AGC ATG ATC ATA TTG CAT CGC TCT CTC GAG CAT AAC TTG ATC ATC TCG CC-3′ for PKC\(-\alpha\)K274W/T555E, 5′-CAC AAT GTC ATC ATC TTC CGT CCA GAG CAT AAC TTG ATC AGC ATG GGA TCC CC-3′ for PKC\(-\alpha\)K274W/T555E as a template, and the T555E mutagenesis primers. The anchor primers for all polymerase chain reactions were 5′-AGT CAG GAT GAG CAG ACC CAG AGC AGG-3′ and 5′-TCT AGA TCA TCA ATG ATG ATG GTG AGA TCC CGG ACT AGT GAC ACA TTC TGC GAG CAT-3′. The polymerase chain reaction products were inserted into the pEFneo vector using the restriction enzymes EcoRI (5′) and SpeI (3′).

**Cell Culture and Transfection Protocols—**COS-7 cells were grown in Dulbecco’s minimal essential medium (Biochrom KG, Berlin, Germany) containing 1.028 g/liter N-acetyl-l-alanyl-l-glutamine and 4.5 g/liter D-glucose, supplemented with 10% heat-inactivated fetal calf serum and 50 $\mu$g/ml gentamycin. HC-11 cells were cultivated in RPMI 1640 medium containing 25 mm HEPES and 446 mg/liter l-alanyl-l-glutamine supplemented with 10% fetal calf serum, 50 $\mu$g/ml gentamycin, 5 $\mu$g/ml insulin (Sigma), and 10 ng/ml epidermal growth factor (Sigma). For transfection, cells were seeded at a density of 200,000 cells/well. After 6 h, the medium was replaced by 1 ml of Optimem medium (Life Technologies, Inc.). 1.5 $\mu$g of transfection plasmid/well was mixed with 0.5 $\mu$g of OptiMEM and 4.5 $\mu$g of Lipofectin (Life Technologies, Inc.) according to the manufacturer’s protocol, added to the cells and incubated overnight. After removal of the transfection mix, cells were washed with phosphate-buffered saline, 1 ml of fresh medium was added, and the cells were incubated for additional 24 h.

**Purification of RGS-His\(_6\)-tagged PKC—**Transiently transfected cells were scraped off in phosphate-buffered saline and centrifuged at 1,000 $\times$ g for 5 min. The pellet was resuspended in 500 $\mu$l of lysis buffer containing 150 mm NaCl, 50 mm HEPES, pH 7.5, 1% Nonidet P-40, 50 $\mu$g/ml leupeptin, 50 $\mu$g/ml aprotinin, and 1 mm phenylmethylsulfonyl fluoride and incubated on ice for 10 min. Cell lysates were centrifuged at 10,000 $\times$ g for 10 min, and the supernatant was transferred to a fresh tube. 100 $\mu$l of Ni\(^{2+}\)-NTA agarose (Qiagen) equilibrated in lysis buffer was added, and the tubes were rotated for 1 h to allow binding of RGS-His\(_6\)-tagged PKCs to Ni\(^{2+}\)-agarose. The purified PKCs were then washed four times with lysis buffer supplemented with 50 mm imidazole and eluted with 500 mm imidazole in 20 mm Tris-Cl, pH 7.5.

**Measurement of In Vitro PKC Activity—**100 ng of purified PKC were added to 100 $\mu$l of assay buffer containing 20 mm Tris-Cl, pH 7.5, 20 mm MgCl\(_2\), 50 $\mu$M substrate peptide PKC\(-\alpha\)–19/31/37/55/38/58 (Alexis), 40 $\mu$M ATP (Roche Molecular Biochemicals), and 1 $\mu$M of $\gamma$-\(^{32}\)P/ATP (PerkinElmer Life Sciences). The reaction was incubated at 30°C for 10 min and stopped on ice. 50 $\mu$l of the reaction mix were transferred to phosphocellulose disc sheets (Life Technologies, Inc.). The phosphocellulose sheets were washed three times with 1% phosphoric acid and twice with distilled water and then transferred to a scintillation vial with 4 ml of scintillation fluid. The bound radioactivity was then measured in a liquid scintillation counter.

**Detection of PKC Levels by Western Blot Analysis—**20 ng of purified PKC was separated on a 10% polyacrylamide gel and transferred to a...
polyvinylidene fluoride membrane (Immobilon-P™, Millipore) by electroblotting. The membrane was equilibrated with Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) and blocked with Tris-buffered saline containing 1% Tween-20 and 1% skim milk powder overnight. The first antibody was added in a concentration of 0.1 µg/ml for the anti-RGS-His6 antibody (Qiagen) and 0.25 µg/ml for the anti-PKCi antibody (BD Transduction Laboratories), and the membranes were incubated for 1 h. After washing with Tris-buffered saline containing 1% Tween-20, the blot was incubated for 1 h with the secondary antibody (anti-mouse horseradish peroxidase, 0.5 µl/ml; Amersham Pharmacia Biotech). Detection of the protein bands was performed using SuperSignal Reagent (Pierce).

Detection of PKC Levels by Slot-blot Analysis—The indicated amounts of protein were directly pipetted onto a polyvinylidene fluoride membrane (Immobilon-P™, Millipore) using a slot-blot apparatus (Amersham Pharmacia Biotech). Detection of PKC was performed as described above for the Western blot analysis.

Luciferase Assays—HC-11 mouse mammary epithelial cells were seeded into 6-well plates. 24 h later they were transfected for 1 h with 1.5 µg of DNA and 6 µl/well of Transfast (Promega) in 1 ml of OptiGerm medium. 24 h after transfection, the cells were serum-starved with RPMI 1640 medium containing 0.5% fetal calf serum and 5 µg/ml insulin (Sigma) for additional 24 h. Following this incubation, the cells were collected and lysed according to the manufacturer’s prescription. Luciferase activity was measured using the Dual-Luciferase™ Reporter Assay (Promega) in a MicroBeta Trilux 1450 counter (PerkinElmer). The fos-SRE-luciferase activity values were standardized by the activity of a cotransfected pRL-SV40 Renilla luciferase (Promega).

RESULTS

Effects of the Substitution of the Invariant Lysines in cPKCa and aPKCi on the in Vitro Enzymatic Activity—To characterize specific properties of the catalytic domain of atypical PKCi, we have used site-directed mutagenesis to replace the invariant lysine in the ATP-binding pocket by the chemically similar amino acid residue arginine or by the unrelated tryptophan residue (K274R and K274W mutants, respectively), as described under “Experimental Procedures.” All constructs were inserted into the eukaryotic expression vector pEF-neo containing a COOH-terminal RGS-His6 tag to allow protein purification from whole cell lysates. The constructs were transiently transfected into COS-7 cells using Lipofectin. 48 h later, cells were lysed, and RGS-His6 tagged cPKCa was purified from the lysates by addition of Ni2+-agarose. After elution with imidazole the protein concentration was measured, and the samples for the in vitro kinase assay were adjusted to equal amounts of protein. The activity of the purified enzymes was measured in vitro by phosphorylation of a standard PKC substrate peptide derived from the cPKCa pseudosubstrate sequence (PKCa-19–31/Ser-25). The in vitro activity of the aPKCi mutants was compared with active and kinase-dead mutants of cPKCa purified in the same way. Fig. 1A shows that in contrast to the Lys → Arg mutant of cPKCa, aPKCi-K274R exhibits in vitro enzymatic activity similar to the wild type enzyme. Additionally, like the wild type aPKCi enzyme, aPKCi-K274R shows a very high basal activity in the absence of cofactors that is comparable with the activity of constitutively active cPKCa-AE or the corresponding Ala → Glu mutant of aPKCi (aPKCi-A120E). The results also show that the high basal enzymatic activity of aPKCi cannot be further increased by this mutation of the pseudosubstrate domain.

To control the expression of the different mutants, the purified proteins were separated on a polyacrylamid gel, blotted onto nitrocellulose membrane, and detected with a RGS-His6-specific antibody. Fig. 1B shows that all proteins were expressed in the COS-7 cells, although the expression level of kinase-dead aPKCi-K274W is significantly lower than that of the other constructs.

Complete Absence of Enzymatic Activity in aPKCi-K274W—The difference in the expression level between wild type aPKCi and aPKCi-K274W was quantified by a slot-blot analysis using different amounts of purified protein. Fig. 2A shows that the expression level of wild type aPKCi is about 10-fold higher than that of the kinase-inactive aPKCi-K274W mutant. Because equal amounts of purified protein were used for both wild type aPKCi and aPKCi-K274W in the slot-blot analysis, these results also show that the Ni2+-agarose used for purification exhibits significant unspecific binding to His-rich proteins, although these proteins do not have significant kinase activity (Fig. 1A, vector control). We wanted to confirm that the absence of kinase activity in the aPKCi-K274W samples was not caused by a lower PKC content in the final reaction mix because of the lower expression level. For this purpose, different amounts of purified aPKCi-WT and aPKCi-K274W were adjusted to equal protein concentrations with Ni2+-agarose and measured in vitro kinase activity. Fig. 2B shows that aPKCi-K274W is unable to phosphorylate the peptide substrate even at high concentrations, whereas wild type aPKCi leads to a concentration-dependent increase in enzymatic activity.

aPKCi-K274W Is Enzymatically Inactive in Intact Cells—In contrast to other PKC isoforms, aPKCi exhibit high basal enzymatic activity in vitro without the addition of cofactors. Although other PKCs have been shown to require diacylglycerol and acidic phospholipids like phosphatidylserine for optimal activity and the maintenance of their active conformation, for atypical PKCs a basal activation by protein activators like λ-interacting protein (43) or ζ-interacting protein (25) seems to be sufficient. The enzymatic activation of aPKCs is dependent on the autophosphorylation of a threonine residue in the C

![Image](https://via.placeholder.com/150)
terminals that is conserved in all PKCs (44). Introduction of a negative charge in this position (T555E) leads to an enzyme with elevated basal enzymatic activity (Fig. 3). To verify whether aPKC-K274W retains any residual activity possibly activable in vivo, the same point mutation was introduced into the aPKC-K274W protein. Fig. 3 shows that this double mutant is still completely inactive when measured in vitro. Because the T555E is capable to effectively increase the enzymatic activity of the wild type enzyme, the lack of activation of aPKC-K274W indicates that this mutant is missing any residual activity.

Because a kinase-dead mutant of aPKC is a valuable tool for studying signal transduction pathways in vivo, we next confirmed its enzymatic inactivity using a biological readout system. HC-11 mouse mammary epithelial cells were transiently transfected with a construct containing luciferase reporter gene under the control of the fos-SRE promoter element, together with different combinations of Ras-L61 and aPKC. Induction of luciferase expression was measured with a luminometer and standardized for expression by a constitutively expressed Renilla luciferase gene construct. The bars represent the means ± S.E. of triplicate experiments.

ATP Dependence of PKC\textsubscript{i}-WT and -K274R—Because the replacement of the invariant lysine by arginine generally leads to a complete abolishment of ATP binding and thus enzymatic activity of many protein kinases, e.g. PKC\textalpha, it was of interest whether the kinetic properties of the PKC\textsubscript{i}-K274R mutant differed from the wild type enzyme. For this purpose, the kinase activity of purified PKC\textsubscript{i}-WT and -K274R was measured at different ATP concentrations. As shown in Fig. 5A, both the wild type and the K274R mutant of aPKC show very similar Michaelis-Menten kinetics for ATP. The Lineweaver-Burk plot shown in Fig. 5B illustrates that the affinity of aPKC-K274R for ATP is comparable with that of the wild type enzyme, with $K_m^{(ATP)}$ values of $11.4 \pm 0.6$ and $13.5 \pm 4.25 \mu M$, respectively. By comparison, the ATP affinity of the classical PKC isoenzyme PKC\textalpha is lower even in the constitutively active form, with a $K_m$ value of $38.8 \mu M$ (data not shown).

In contrast to the $K_m$ value, the $V_{max}$ of the phosphorylation reaction is significantly decreased with aPKC-K274R ($7.4 \pm 1.6$ versus $14.0 \pm 2.3$ pmol-min$^{-1}$-mg protein$^{-1}$) with wild type...
A) Michaelis-Menten plot of enzymatic activity versus ATP concentration. B, Lineweaver-Burk plot of the same results.

![Graph A](image1)

![Graph B](image2)

**Fig. 5. Biochemical characterization of aPKC-i WT and K274R.** Wild type aPKC and the aPKC-iK274R mutant were purified from transiently transfected COS cells. The enzymatic activity of PKC was measured with equal amounts of purified proteins and increasing ATP concentrations. A, Michaelis-Menten plot of enzymatic activity versus ATP concentration. B, Lineweaver-Burk plot of the same results.

- Graph A shows a plot of enzymatic activity against ATP concentration for wild type aPKC (PKC-i WT) and aPKC-iK274R (PKC-i KR). The x-axis represents the ATP concentration in micromolar, while the y-axis shows the activity in pmol/min/mg protein. The line for PKC-i WT is above that for PKC-i KR, indicating lower activity.
- Graph B is the Lineweaver-Burk plot of the same data, with the x-axis representing the reciprocal of the ATP concentration and the y-axis representing the reciprocal of the activity.

The data suggest that the invariant lysine plays a less central role for ATP binding than in other kinases and is essential for the interaction with diacylglycerol, a lipid cofactor of all other PKC isoenzymes. Also the pseudosubstrate domain, a conserved sequence motif shown to bind to and block the substrate-binding domain before activation, differs significantly in aPKCs. Much less pronounced are the differences in the primary structure of the catalytic domain. Nevertheless, these subtle differences are supposed to be at least partly responsible for the in vivo selectivity of different PKC isoenzymes, because mutations within the regulatory domain rendering the PKC isoenzymes constitutively active do not affect their biological selectivity of different PKC isoenzymes.

**DISCUSSION**

The atypical PKC isoforms aPKC-i and aPKC-ii play essential roles in signaling pathways involved in mitogenesis, differentiation, malignant transformation, and resistance to apoptosis (1, 7, 13, 46). Malfunction of these kinases has been shown to be implicated in the development of several human diseases, e.g., Alzheimer disease, chronic lymphatic leukemia, and diabetes (8, 16, 47, 48). The main structural differences between aPKCs and the classical and novel isoforms reside predominantly in the regulatory domain; the atypical isoforms lack the calcium-binding region and one of two zinc finger motifs responsible for the interaction with diacylglycerol, a lipid cofactor of all other PKC isoenzymes. Also the pseudosubstrate domain, a conserved sequence motif shown to bind to and block the substrate-binding domain before activation, differs significantly in aPKCs. Much less pronounced are the differences in the primary structure of the catalytic domain.
specificity (1, 6, 7). Like for other PKCs, the catalytic domains of aPKC\(_i\) and aPKC\(_a\) share the main structural motifs of all protein kinases, e.g. the invariant lysine responsible for correct folding of the ATP-binding pocket and directly interacting with ATP, the invariant glutamate in subdomain III forming a salt bridge with the invariant lysine and stabilizing its interaction with ATP, and a phosphorylation site in the activation loop (Lys-274, Asp-373, and Thr-403 in aPKC\(_i\), respectively) (30, 44, 49). Intriguingly, one structural hallmark of virtually all protein kinases and other nucleotide-binding proteins, the GXGXXG motif, is only partially conserved in atypical PKCs; they contain an alanine residue instead of the third conserved glycine (GrGsyA) (Fig. 7). The glycine-rich sequence is an integral part of the ATP-binding site, it serves as a lid to anchor the ATP and to exclude water from the catalytically active site. Consistently, mutation of one of these conserved glycines normally leads to an enzyme with impaired catalytic activity, as has been shown for the insulin receptor, the nonreceptor tyrosine kinase Fyn, and the Ras oncogene, the latter with respect to GTPase activity (31–33). This indicates that the GXGXXG motif is essential for correct binding of the nucleotide. An altered glycine-rich motif in aPKCs therefore may be a hint to a slightly different mode of ATP binding compared with other kinases. Intriguingly, aPKCs are quite insensitive to specific PKC inhibitors acting at the ATP-binding site and competing with ATP for binding, like the bisindolylmaleimide GF109203X. This led us to the assumption that these unique properties of aPKCs are caused by a unique ATP-binding domain that may also be reflected in the function of its central residue, the so-called invariant lysine. This residue is conserved throughout the whole kinase family, and even a conservative mutation like a Lys-to-Arg exchange almost inevitably abolishes the enzymatic activity of the protein (35–39). For the characterization of the aPKC\(_i\) ATP-binding domain we have used a combination of site-directed mutagenesis and in vitro enzymatic analysis. We have replaced the conserved Lys-274 by the chemically similar amino acid Arg or by Trp, which has different chemical and structural properties. The proteins have been expressed in COS cells as RGS-His\(_{16}\)-tagged forms and purified with Ni\(^{2+}\)-agarose, and their activity was measured in vitro with a pseudosubstrate-derived peptide as substrate. Surprisingly and in contrast to virtually all other reports of protein kinases, an aPKC\(_i\) with an Arg exchange of the invariant lysine retained almost full enzymatic activity (Fig. 1A). There exist only very few reports in the literature about kinase that are insensitive for mutations of the most conserved amino acid in the ATP-binding pocket, e.g. one concerning the very distantly related CDK-activating kinase 1p from *Saccharomyces cerevisiae*.

Another conspicuous property of aPKC\(_i\) is its high basal activity in vitro. Although other PKC isoforms require cofactors like phosphatidylserine and diacylglycerol for full activity, even if they have been “preactivated” by an Ala \(\rightarrow\) Glu mutation in the pseudosubstrate region, wild type aPKC\(_i\) and even the KR mutant show full enzymatic activity without addition of any cofactor. It has been shown previously that for full activation PKCs have to be phosphorylated on at least three different positions: the first phosphorylation is introduced on a conserved Thr residue in the activation loop by other kinases like the phosphoinositide-dependent kinase (44, 49–51). Following activation, two other phosphorylations are introduced by autophosphorylation at two Ser/Thr residues near the C terminus. These activating phosphorylations can be mimicked by introduction of a negative charge through mutational exchange of the phosphorylatable Ser/Thr residues for glutamic acid (51). Interestingly, wild type atypical PKCs already contain a glutamate residue instead of the second autophosphorylatable residue (Ser). Therefore, the negatively charged glutamic acid residue could confer a state of constitutive activation and explain the high basal activity of these kinases.

One remarkable phenomenon was the correlation between protein level and activity of aPKCs. Although aPKC-WT, -A120E, and -K274R were expressed at comparably high levels (Fig. 1B), the protein level of the inactive K274W mutant was much lower. When quantified by slot-blot analysis, the concentration of aPKC\(_i\)-K274W in extracts from transiently transfected COS cells was about \(\frac{1}{5}\) of the wild type protein. Because the wild type gene and both mutants had been cloned into the same expression vector (pEF-neo) and the integrity of the promoter has been controlled by sequencing (data not shown), all proteins are expected to be synthesized in the cell at the same level. Thus, different aPKC\(_i\) concentrations can only be explained by variations in protein stability. It has been shown previously that PKCs are degraded by proteasomes by the ubiquitylation pathway (52). To see whether inactive aPKC\(_i\) is more susceptible for proteasomal degradation than active forms and therefore present at a lower protein level in the cell, MG-132, an inhibitor of the proteasomal degradation pathway, was used. This substance had no effect on the level of either wild type or kinase-dead aPKC\(_i\) (data not shown). This indicates that at least degradation by the proteasome pathway is not directly linked to enzyme activity. Yet, other degradation pathways not affected by MG-132 could be responsible for this effect.

One other possibility for how the lack of enzymatic activity could cause a reduced protein stability could be the inability of kinase-dead aPKC\(_i\) to autophosphorylate. It has been shown previously for cPKC\(_\alpha\) that activation by phorbol esters can induce its own phosphorylation and that this phosphorylated form is resistant to the degradation process normally induced by phorbol ester treatment (53). To test this hypothesis for aPKC\(_i\), we replaced the autophosphorylatable threonine by glutamate to mimic autophosphorylation. The potency of this mutation to mimic an autophosphorylated state of the enzyme and thus to stimulate enzymatic activity is shown in Fig. 3. Nevertheless, the protein level of the aPKC\(_i\)-K274W/T555E double mutant is not increased with respect to the K274W mutant (data not shown). Because the second position known to be accessible for autophosphorylation in classical and novel PKCs in aPKCs is already replaced by glutamic acid, autophosphorylation seems not to influence the stability of atypical PKC isoenzymes.

For the use of kinase-dead mutants as dominant negative inhibitors of endogenous kinases in the study of signal transduction pathways, it is essential that the mutants lack any residual enzymatic activity. The K274W/T555E double mutant of aPKC\(_i\) was also used to confirm this property for aPKC\(_i\)-K274W mutant. Because the T555E mutation is able to
strongly activate wild type aPKC. (Fig. 3), the same mutation would be expected to increase a potential residual enzymatic activity of aPKC-K274R. As shown in Fig. 3, the K274W/T555E double mutant completely retained its kinase-dead state when measured in vitro. This confirms the finding that the K274W mutant really lacks any residual protein kinase activity. The same result could also be achieved when increasing amounts of purified protein where used for the in vitro measurement of kinase activity (Fig. 2B). Although wild type aPKC exhibited a clear dependence in the enzymatic activity on the protein concentration in the sample, the enzymatic activity aPKC-K274W samples never rose over the background level of samples from vector control transfected cells.

The fact that the replacement of the invariant lysine in the catalytic domain of aPKC by arginine does not abolish its enzymatic activity is in sharp contrast to findings with several other protein kinases like the epidermal growth factor receptor, the insulin receptor, Src, Mos, Fps, and even the lipid kinase phosphoinositide 3-kinase (35–40). It is of special interest that for phosphoinositide 3-kinase this Lys-to-Arg mutant not only is unable to bind ATP and thus phosphorylate its substrate phosphoinositide but also lost the capability to bind the inhibitor wortmannin that covalently binds to the invariant lysine (Lys-802), thus inhibiting the activity of the wild type enzyme (40). This mutant also lost its affinity to FSBA, another ATP analogue that covalently binds to and blocks the invariant lysine within the ATP-binding pocket. Because the mutation of the invariant lysine in aPKC only marginally affected the enzymatic activity of the kinase, it was of special interest to see whether the affinity to ATP or its analogues was affected. By biochemical characterization of both the wild type aPKC and the K274R mutant, we could show that the affinity of the mutated protein for ATP is not altered (Fig. 5); the Km value of atypical PKCs and probably also the mode of ATP binding properties of the catalytic domain of aPKC and for the development of specific inhibitors of atypical PKCs blocking the important signaling pathways mediated by this enzyme.

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Unique Catalytic Domain of aPKC

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