A Domain with Homology to Neuronal Calcium Sensors Is Required for Calcium-dependent Activation of Diacylglycerol Kinase α

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Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol produced during stimulus-induced phosphoinositide turnover and attenuate protein kinase C activation. Diacylglycerol kinase α is an 82-kDa DGK isoform that is activated in vitro by Ca2+. The DGKα regulatory region includes tandem C1 protein kinase C homology domains and Ca2+-binding EF hand motifs. It also contains an N-terminal recoverin homology (RVH) domain that is related to the N termini of the recoverin family of neuronal calcium sensors. To probe the structural basis of Ca2+-dependent activation, we expressed a series of DGKα deletions spanning its regulatory domain in COS-1 cells. Deletion of the RVH domain resulted in loss of Ca2+-dependent activation. Further deletion of the EF hands resulted in a constitutively active enzyme, suggesting that sequences in or near the EF hands are sufficient for autoinhibition. Binding of Ca2+ to the EF hands protected sites within both the RVH domain and EF hands from trypsin cleavage and increased the phenyl-Sepharose binding of a recombinant DGKα fragment that included both the RVH domain and EF hands. These observations suggested that Ca2+ elicits a concerted conformational change of these two domains. A cationic amphiphile, octadecyltrimethylammonium chloride, also activated DGKα. As with Ca2+, this activation required the RVH domain. However, this agent did not protect the EF hands and RVH domain from trypsin cleavage. These findings indicate that the EF hands and RVH domain act as a functional unit during Ca2+-induced DGKα activation.

Hydrolysis of phosphatidylinositol 4,5-bisphosphate is a common mechanism of stimulus transduction (1). Diacylglycerol (DAG) released in this reaction activates protein kinase C (PKC) and is then rapidly metabolized back to phosphatidylinositol in a series of reactions initiated by a diacylglycerol kinase (DGK). As such, DGKs attenuate DAG-mediated PKC activation (2). Recent studies indicate that DGKs are also activated by mechanisms independent of phosphoinositide turnover (3, 4). Diacylglycerol kinases catalyze the ATP-dependent phosphorylation of sn-1,2-diacylglycerol to form phosphoatidic acid (PA), which is also a lipid mediator (5, 6). Several DGK isoforms have been cloned (7). All these sequences share a homologous catalytic domain and two or three C1 protein kinase C homology domains (7–9). Some DGKs contain EF hands, which are Ca2+-binding sites (7). These DGKs also have a domain at their N termini to homology to the recoverin family of neuronal calcium sensors (Fig. 1). We term this the recoverin homology (RVH) domain. In S-modulin, the frog orthologue of recoverin, this domain associates with the EF hands to mediate Ca2+-dependent inhibition of rhodopsin kinase (10).

The varied structures of DGK regulatory domains suggest divergent mechanisms of regulation. Several studies have shown variation among DGKs with regard to activation by phospholipids, sphingosine, or Ca2+ (11–15). Kanoh and coworkers (16–18) have studied DGKα, a Ca2+-activated isoform highly expressed in oligodendrocytes and thymocytes. They have shown that Ca2+ binds the EF hand region of the enzyme and that deletion of the EF hands results in constitutive enzyme activation (19, 20). We have now examined a series of DGKα mutants in which the RVH and EF hand domains are sequentially deleted. Our results indicate that the N-terminal RVH domain is required for Ca2+ to activate this enzyme. In contrast to the constitutive activation seen with deletion of the EF hands, DGKs with deletions involving only the RVH domain expressed activity similar to that of wild-type enzyme in the absence of Ca2+. Sites within both the EF hands and RVH domain were protected from trypsin proteolysis by Ca2+, indicating that both domains participate in a Ca2+-induced conformational change. A cationic amphiphile, octadecyltrimethylammonium chloride, markedly stimulated DGKα activity in vitro. This effect, like Ca2+-dependent activation, was dependent on the RVH domain. The DGKα RVH domain does not itself bind Ca2+. However, it does appear to function together with the EF hands to couple Ca2+ binding to release of EF hand-mediated autoinhibition of DGKα.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA-modifying enzymes were from Promega or Life Technologies, Inc. A dideoxy sequencing kit (Sequenase version 2.0) was from U. S. Biochemical Corp. [γ-32P]ATP and [α-32P]dATP were from PerkinElmer Life Sciences. sn-1-Palmitoyl-2-oleoyl phosphatidylethanolamine (PS) and sn-1-palmitoyl-2-oleoyl phosphatidic acid (PA) were from Avanti Polar Lipids, Birmingham, AL. sn-1-Palmitoyl-2-oleoyl glycerol (16:0–18:1 DAG) was prepared by digestion of the corresponding phosphatidylcholine (Avanti) with Bacillus cereus phospholipase C (21). Octyl β-D-glucopyranoside (octyl glucoside), sodium 34092 This paper is available on line at http://www.jbc.org
Amino acids conserved in both EF hands are boxed. Neuronal calcium sensors and DGKs are shaded.

Deoxycholate, N-hexadecyl-N,N-dimethyl-3-ammonio-1-propanol sulfone (C16 sulfobetaine, CBSB), Triton X-100, Triton X-114, dihexadecyl phosphate, diethylenetriaminepentaacetic acid, EDTA, EGTA, leupeptate (C16 sulfobetaine, C16SB), Triton X-100, Triton X-114, dihexadecyl deoxycholate, N,N,N,N-tetraethyl-3-ammonio-1-propane sulfonate (C16SB). Octadecyltrimethylammonium chloride (OTAC) was prepared from Aldrich and recrystallized twice from ethanol/ethyl acetate (1:1, v/v). Octadecyltrimethylammonium bromide was purchased from Aldrich. Improving grade) was from Promega. 2,6-Di-6-agarose beads were from Santa Cruz Biotechnology. Trypsin (sequenc-
250 mM sucrose, 100 mM NaCl, 2.5 mM EGTA, 1 mM MgCl₂, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 50 μM ATP, and 0.02% Triton X-100. After removal of undisrupted cells by brief centrifugation, the lysates were centrifuged at 100,000 × g (Beckman TL-100) for 20 min at 4 °C to pellet membranes. The resultant supernatants were clarified by centrifugation at 10,000 × g and stored at −80 °C until assayed. For immunodetection, 5–10 μl of the 100,000 × g supernatants of lysates from COS-1 cells transiently expressing DGKα or the truncation mutants was applied to SDS-PAGE. Proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). After blocking in TBS-T buffer containing 5% non-fat dry milk, membranes were incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:5000 dilution in TBS-T) for 30 min. After washing 4 times in TBS-T buffer, the horseradish peroxidase conjugates were detected by chemiluminescence. 

Expression of DGKα Regulatory Domain Sequences—Sequences corresponding to conserved domains in the DGKα regulatory region were prepared as GST fusions in Escherichia coli. DNA fragments expressing the selected sequences were prepared by PCR amplification using DGKα-pCDNA3 as a template. Amplification reactions were run at 94 °C for 45 s, 49 °C for 45 s, and 72 °C for 150 s for a total of 28 cycles with a final elongation step at 72 °C for 10 min. Primers GSTN-1 (5’-TACAAAGGAAGGGCGCTG-3’) and GSTC-1 (5’-ACTC-GAGTCATTGTCTTCTGGCCGGCG-3’) amplified DGKα:2-110, which encompasses the RVH domain. Primers GSTN-2 (5’-TGAATTCT-CGTA CTTCCTCCTC-3’) and GSTC-2 (5’-AAGCTGATGATT-GTCCCTGAGCT-3’) amplified DGKα:99-202, which encompasses the EF hands. Primers GSTN-3 (5’-GAAGCTTCTGGAAGACAT-GGGCA-3’) and GSTC-3 (5’-TCTCCGAATCTGATGTGTTGAGAAG-3’) amplified DGKα:198-336, which encompasses the C1 domains. Primers GSTN-1/GSTC-2, GSTN-1/GSTC-3, and GSTN-2/ GSTC-3 were used to amplify DGKα:2-202, DGKα:2-336, and DGKα:99-336, respectively. The DNA fragments were inserted into the PCBlunt vector and sequenced. They were then digested with EcoRI and XhoI and inserted into pGEX-4T-3 (Amersham Pharmacia Biotech). All insertions were in frame with GST.

The GST-fused DGKα-pGEX-4T-3 constructs were expressed in E. coli strain BL21(DE3). Tranformed cells were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin to an A600nm of 0.8 and induced with 0.2 mM isopropyl-1-thio-β-galactopyranoside for 4 h at 37 °C or overnight at 25 °C. Cells were harvested and lysed in 20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 50 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 mM PMSF by two passes through a French press. The lysates were clarified by centrifugation at 10,000 × g for 20 min. The GST fusions were then purified to apparent homogeneity by glutathione-agarose affinity chromatography. When removal of the GST moiety was desired, the recombinant protein adhering to the glutathione-agarose beads was incubated with 2 IU thrombin per mg of protein at 4 °C overnight. The resultant supernatant was dialyzed against 50 mM Tris buffer, pH 7.5, 100 mM NaCl, 250 mM sucrose, and 1 mM DTT. Aliquots were rapidly frozen and stored at −80 °C.

Diacylglycerol Kinase Assays—The standard DGK assay contained in volume of 200 μl the following: 1 mM sodium deoxycholate, 50 mM triethanolamine-HCl, pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM [γ-32P]ATP (100 cpm/pmol), 20 μM sn-1-palmitoyl-2-oleoyl-glycerol (16:0, 18:1 DPG), 1 mM DTT, and enzyme (11, 23). In a typical reaction, an appropriate volume of DAG stock solution (10–20 mM in CHCl₃) was evaporated under a stream of nitrogen in a 16 × 100-mm glass test tube. To the DAG droplet were added the following: 50 μl of 4× assay buffer, 50 μl of 4× detergent, DTT, water, and enzyme to a final volume of 180 μl. Stock solutions of 4× sodium deoxycholate, 10× [γ-32P]ATP, and 4× aqueous buffer were as described previously (21). Reactions were initiated by adding 20 μl of 1× [γ-32P]ATP. Reactions were allowed to proceed for 10 min at 25 °C and terminated by the addition of 3.0 ml of CHCl₃/ethanol (2:1 v/v) containing 1.0 mg dihexadecanoylphosphatide and 1.0 mg of sorbitan triacetate. The organic phase was washed with 1.0 ml HClO₄ and 0.1% H₂PO₄ in H₂O/ethanol (4:1, v/v). The volume of the final organic phase was 2.25 ml. Cerenkov counting 1.2 ml of this organic phase determined incorporation of 32P into PA. For some assays, mixed micelles of octyl glucoside and phosphatidylserine (PS) were employed instead of deoxycholate. In these assays, the total concentration of micelle components, octyl glucoside + PS + DAG, was maintained at 25 mM. Total octyl glucoside added to the assays was the sum of micellar and monomeric octyl glucoside, which was calculated as described (24). For purposes of these calculations, the critical micelle concentration of octyl glucoside was assumed to be 25 mM. The DAG concentration in these assays was 0.5 mM (2 mol%). Other assay components were unchanged. When Ca²⁺ was added to assays, the buffer contained 1 mM EDTA instead of EGTA, and the free Mg²⁺ was maintained at 1 mM. The total Mg²⁺ and Ca²⁺ added were calculated using published stability constants to give the desired levels of free cations (25). Other assays employing Triton and OTAC or Triton and C16SB instead of deoxycholate have been described previously (22). All data reported are averages of at least duplicate determinations that agreed within 10% in all cases. Moreover, all results are representative of two or more independent experiments performed with completely independent enzyme preparations.

Ca²⁺ Overlay Analyses—Calcium binding was assessed by the 45Ca overlay method of Maruyama et al. (62). 1 μg of recombinant proteins or immunoprecipitated DGKα truncation mutants were separated by SDS-PAGE and transferred to nitrocellulose. The membrane was washed free of Ca²⁺ in 75 ml of 5 mM EGTA, pH 7.0. It was rinsed three times with 10 mM imidazole HCl, pH 6.8, 60 mM KCl, and 5 mM MgCl₂, and then incubated for 15 min at 25 °C in 30 ml of the same buffer supplemented with 250 μM 45CaCl₂ (1 μCi/ml). The membrane was then rinsed twice with 45% ethanol, blotted dry, and exposed to x-ray film. The nitrocellulose was stained with Amido Black to verify protein transfer. To prepare immunoprecipitates of the DGKα truncation mutants for these experiments, COS-1 cells transiently transfected with DGKα or the truncation mutants were extracted with buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM sucrose, 100 mM NaCl, 5 mM EGTA, 1 mM NaF, 1 mM MgCl₂, 1 mM DTT, 50 μM ATP, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. The extract was pre-cleared with protein A/G-agarose beads to eliminate nonspecific binding. Anti-FLAG antibody was then added together with fresh protein A/G-agarose beads, and the mixture was incubated at 4 °C overnight. The immune complexes were collected by centrifugation. The precipitate was washed with the above buffer containing only 0.1% Triton X-100, and the bound proteins were eluted into SDS reducing buffer.
Calcium Activation of DGKα

Table I
Activities of wild-type DGKα and deletion mutants

| Expression relative to WT | Specific activity | mmol/min/mg/relative expression |
|--------------------------|------------------|-------------------------------|
| WT DGKα                  | 1.00             | 4.83                          | 0.71 | 5.6 |
| DGKα Δ40                 | 0.25             | 5.44                          | 0.20 | 3.4 |
| DGKα Δ57                 | 0.25             | 13.56                         | 0.88 | 8.0 |
| DGKα Δ196                | 0.125            | 87.84                         | 20.6 | 77.6 |

Assays were performed on 100,000 × g supernatants of lysates from COS-1 cells transiently expressing the DGK constructs. These were assayed for DGK activity by the deoxycholate method and by the octyl glucoside method with both 10 and 20 mol % PS. All activities are corrected by subtracting the background DGK activity, determined under identical assay conditions, of a lysate of COS-1 cells transfected in the same experiment with the pCDNA3 vector. Expression levels of DGKα and its mutants in COS-1 cell were determined by densitometry of immunoblots using anti-FLAG M2 antibody (Kodak). In all cases, the majority of the anti-FLAG immunoreactivity was in the 100,000 × g supernatant. Immunoreactivity associated with the pellets was estimated from immunoblots as follows: WT DGKα, <10%; DGKα Δ40, 15%; DGKα Δ57, 15%; DGKα Δ196, 25% (data not shown). Details of the methods are given under “Experimental Procedures.” All data are averages of duplicate determinations which, in all cases, agreed within 10%. Similar results were observed in two additional independent experiments. WT, wild type.

Fig. 3. Calcium activation of DGKα deletion mutants. The 100,000 × g supernatants (10 μg) of extracts of COS-1 cells transfected with the constructs were assayed for DGK activity in octyl glucoside micelles containing 20 mol % PS. Background DGK activities of COS-1 cells transfected with vector only were determined under identical conditions and subtracted. The activities were normalized for expression level as determined by densitometry of immunoblots. Calcium concentrations were varied from 0 to 50 μM as described under “Experimental Procedures.”

Fig. 4. Ca2+ overlay of DGKα deletion mutants expressed in COS-1 cells. COS-1 cells transfected with the DGKα constructs were extracted and immunoprecipitated with anti-FLAG M2 antibody as described under “Experimental Procedures.” The immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose. A Ca2+ overlay was performed on the nitrocellulose and bound 45Ca detected by autoradiography. Upper panel, Ca2+ overlay of immunoprecipitated DGKα and mutants. Lower panel, immunoblot of immunoprecipitated DGKα and mutants. The arrows indicate the positions of IgG heavy and light chains. Three nonspecific bands between M90 and 120 are also seen in the control lane (CONT). WT, wild type.
Calcium activation of DGK was assessed by 45Ca2+ PAGE and transferred to nitrocellulose. Calcium binding was assessed by 45Ca2+ overlay as described under “Experimental Procedures.” Left, schematic of the DGKα regulatory region polypeptides employed. The RVH domain, EF hands, and C1 domains are indicated. The N-terminal GST moiety is not shown. Upper right, Coomasie Blue stain of expressed polypeptides. Lower right, 45Ca2+ overlay of expressed polypeptides.

Expression of DGKα and Its Truncation Mutants in COS-1 Cells—Regulatory domains of Ca2+-activated DGKs contain several conserved regions. These include EF hands and tandem C1 PKC homology domains (8, 9). EF hand-containing DGKs also contain a 70-amino acid conserved sequence at their N termini (34, 35). Motif searches revealed that this domain is related to the recoverin family of neuronal calcium sensors (Fig. 1). We thus refer to this region as the RVH domain. The homology between DGKs and neuronal calcium sensors extends through the EF hands (Fig. 1).

To investigate the role of the RVH domain in DGKα regulation, a series of N-terminal deletion mutants was prepared (Fig. 2). DGKαΔ40 lacks the first half of the RVH domain and DGKαΔ87 lacks the entire RVH region. DGKαΔ196 lacks both the RVH domain and the EF hands. A FLAG epitope attached to the C termini facilitated detection and quantification of protein expression. These mutants were expressed in COS-1 cells. Cytosol from COS-1 cells expressing DGKα or the deletion mutants showed a marked increase in DGK activity as compared with control cells transfected with vector only. The mutant activities were stable in cell lysates but were only partially recoverable from DEAE-cellulose or Mono Q columns. The 100,000 × g supernatants of COS-1 lysates were thus used for all studies. The presence of protein products with the expected molecular masses was verified by immunoblotting (Fig. 2). Densitometry of the immunoblots indicated that DGKαΔ40, DGKαΔ87, and DGKαΔ196 were expressed at 1/4th, 1/4th, and 1/8th the level of wild-type DGKα. COS-1 cells express an endogenous DGK activity. Transfected cells all expressed activity in this background provides additional confirmation that the mutant DGKs are catalytically competent (23). These results indicate that all the truncated DGKs activated by OTAC and C16SB similarly to several Ca2+-independent DGKs. To obtain further evidence that the mutants express DGK activity, we also expressed the truncated DGKs in S. cerevisiae. All of the mutants expressed DGK activity in the yeast, and in all cases, the phosphatidate product co-migrated with authentic PA on thin layer chromatograms (data not shown). As yeast do not possess an endogenous DGK, expression of activity in this background provides additional confirmation that the mutant DGKs are catalytically competent (23). These results indicate that all the truncated DGKs

RESULTS

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FIG. 5. Calcium binding to recombinant DGKα regulatory domain sequences. Polypeptides encompassing different domains of the DGKα regulatory region were prepared as GST-fused recombinant proteins as described under “Experimental Procedures.” 1 μg of the recombinant proteins was applied to SDS-PAGE and transferred to nitrocellulose. Calcium binding was assessed by 45Ca2+ overlay as described under “Experimental Procedures.” Left, schematic of the DGKα regulatory region polypeptides employed. The RVH domain, EF hands, and C1 domains are indicated. The N-terminal GST moiety is not shown. Upper right, Coomasie Blue stain of expressed polypeptides. Lower right, 45Ca2+ overlay of expressed polypeptides.
express a functional catalytic domain. Activities expressed by full-length DGKα and DGKα Δ196 with the native C termini were identical to those expressed by the corresponding epitope-tagged constructs, indicating that the FLAG tag does not appreciably alter DGKα activity.

**Activation of DGKα Truncation Mutants by Ca\(^{2+}\)**—We examined Ca\(^{2+}\) activation of full-length DGKα and the deletion mutants. Wild-type enzyme showed significant stimulation by Ca\(^{2+}\) and was not required for Ca\(^{2+}\) binding (Fig. 5). To probe for a Ca\(^{2+}\)-induced conformational change, we performed limited trypsin proteolysis of DGKα:2-202 (DGKα-RVH+EF) with and without Ca\(^{2+}\). Aliquots of the reaction were stopped at various times and analyzed by SDS-PAGE (Fig. 6). Calcium protected DGKα:2-202 from proteolysis (Fig. 6). In the absence of Ca\(^{2+}\) (0.1 mM EGTA), the polypeptide was completely digested within 30 min, whereas in 0.1 mM Ca\(^{2+}\), appreciable full-length polypeptide remained after 4 h. These results are not due to an effect of Ca\(^{2+}\) on trypsin. High concentrations of Ca\(^{2+}\) (>1 mM) protect trypsin from autolysis and modestly stimulate its activity, but these effects should be negligible in the 0.1 mM Ca\(^{2+}\) used for these experiments (37, 38). Moreover, if Ca\(^{2+}\) were stimulating trypsin activity, the protection we observed would be even more significant.

To identify those sites particularly susceptible to trypsin, peptide fragments in the 5-min and 4-h digests were analyzed by capillary liquid chromatography-mass spectrometry. Table II shows integrated ion currents of the peptides identified in each digest. In the 5-min digests, Ca\(^{2+}\) had only small effects on the appearance of most peptides derived from sequences between the N terminus and Lys\(^{89}\). However, cleavage at Lys\(^{89}\) was inhibited by Ca\(^{2+}\) (Fig. 5). The RVH domain alone did not bind Ca\(^{2+}\) and was not required for Ca\(^{2+}\) binding (Fig. 5). To probe for a Ca\(^{2+}\)-induced conformational change, we performed limited trypsin proteolysis of DGKα:2-202 (DGKα-RVH+EF) with and without Ca\(^{2+}\). Aliquots of the reaction were stopped at various times and analyzed by SDS-PAGE (Fig. 6). Calcium protected DGKα:2-202 from proteolysis (Fig. 6). In the absence of Ca\(^{2+}\) (0.1 mM EGTA), the polypeptide was completely digested within 30 min, whereas in 0.1 mM Ca\(^{2+}\), appreciable full-length polypeptide remained after 4 h. These results are not due to an effect of Ca\(^{2+}\) on trypsin. High concentrations of Ca\(^{2+}\) (>1 mM) protect trypsin from autolysis and modestly stimulate its activity, but these effects should be negligible in the 0.1 mM Ca\(^{2+}\) used for these experiments (37, 38). Moreover, if Ca\(^{2+}\) were stimulating trypsin activity, the protection we observed would be even more significant.

| Fragment | Calculated mass | Observed mass | 5 min EGTA | 5 min Ca\(^{2+}\) | 4 h EGTA | 4 h Ca\(^{2+}\) |
|----------|----------------|---------------|------------|----------------|----------|------------|
| 4–18     | 1687.9         | 1687.8        | 1.370      | 0.950          | 7.310    | 1.500      |
| 6–18     | 1402.7         | 1402.0        | 7.630      | 7.190          | 140.000  | 28.700     |
| 19–25    | 920.4          | 920.4         | 0.547      | 4.200          | 10.500   | 0.200      |
| 26–32    | 787.5          | 788.0         | 0.570      | 0.117          | 17.200   | 4.910      |
| 27–32    | 659.4          | 659.8         | 7.460      | 1.270          |          |            |
| 33–58    | 2997.4         | 2997.2        | 5.020      | 4.140          | 108.000  | 33.400     |
| 51–58    | 3519.7         | 3520.0        | 0.740      | 8.450          | 6.140    |            |
| 59–90    | 3675.8         | 3676.4        | 2.890      | 0.369          | 30.000   | 9.880      |
| 90–119   | 3451.7         | 3452.0        | 0.046      | 1.740          | 0.420    |            |
| 91–119   | 3295.6         | 3296.0        | 1.460      | 23.200         | 1.410    |            |
| 114–119  | 783.4          | 783.4         | 0.250      | 17.500         | 1.470    |            |
| 120–125  | 781.4          | 781.4         | 14.400     | 0.743          | 11.400   |            |
| 120–136  | 1966.9         | 1966.2        | 13.800     | 0.380          | 11.100   | 1.090      |
| 120–143  | 2852.4         | 2855.6        | 10.600     | 0.380          |          |            |
| 126–136  | 9458.7         | 9459.2        | 9.410      | 3.270          | 1.620    | 7.490      |
| 126–143  | 1203.6         | 1203.4        | 4.270      | 65.500         | 3.300    |            |
| 126–202  | 1209.1         | 1209.0        | 4.440      | 23.200         | 1.410    |            |
| 137–143  | 805.6          | 805.6         | 1.240      | 0.186          | 0.570    | 0.980      |
| 144–164  | 2596.2         | 2596.2        | 5.500      | 0.686          | 662.000  | 24.100     |
| 144–202  | 6624.3         | 6624.4        | 0.615      | 0.294          | 0.800    |            |
| 165–181  | 1895.9         | 1896.8        | 2.390      | 0.246          | 207.000  | 4.400      |
| 182–200  | 1939.1         | 1939.8        | 11.600     | 0.246          | 134.000  | 8.140      |

**Table II**

Recombinant DGKα:2-202 was digested with trypsin as described in the legend to Fig. 6. Samples of the digests from the 5-min and 4-h time points were analyzed by capillary high pressure liquid chromatography-mass spectrometry as described under “Experimental Procedures.” The table shows the fragments identified (sequence numbering as in Fig. 1), the predicted and observed masses, and the integrated ion currents observed in full mass spectrometry mode.
Calcium Activation of DGKα

Ca2+-dependent DGK activities from testis cytosol, salivary cytosol, and NIH 3T3 cells to a similar degree (data not shown). Several Ca2+-independent DGK activities, including an arachidonoyl-DGK from testis membranes, and cysotoxic activities from NIH 3T3 cells and thymus cytosol were only modestly (2–6-fold) activated by OTAC (11, 23). Wild-type DGK activity expressed in COS-1 cells was activated 109-fold by OTAC (Table III). Similar stimulation was seen with the zwitterionic amphiphile, OTAC, although 15-fold higher concentrations were required to achieve the same activation. In lysates of COS-1 cells expressing the truncated DGK activities, OTAC and C16SB had much smaller effects (Table III). Activation of the mutants was comparable to that seen with Ca2+-independent DGKs. In the presence of maximally activating concentrations of OTAC, Ca2+ does not further activate wild-type DGKα (23). These results suggest that OTAC and C16SB have two effects on DGKs, a nonspecific stimulation seen with all isoforms and an additional stimulation seen only with Ca2+-activated DGKs. This latter effect, like Ca2+-dependent activation, required the RVH domains. OTAC and C16SB thus appear to act through RVH and EF hand domains to mimic Ca2+-dependent activation of DGKα. We examined whether OTAC, like Ca2+, protects DGKα:2-202 from trypsin proteolysis. In the absence of Ca2+, OTAC modestly accelerated the proteolysis of DGKα:2-202 (data not shown). In the presence of OTAC, Ca2+ no longer protected DGKα:2-202 (Fig. 8). Triton alone slightly increased proteolysis, but the rate was greatly accelerated by OTAC. Inclusion of OTAC/Triton or C16SB/Triton in the binding and wash buffers of overlay assays had no effect on45Ca2+ binding (data not shown). Overall, these results suggest that DGKα and C16SB inhibit trypsinic cleavage of sites within the RVH domain (Lys305), in the loop between the RVH domain and the EF hands (Lys308/Arg309), and within the EF hands (multiple sites).

Calcium-induced conformational changes of EF hand proteins, including neuronal calcium sensors, can result in altered binding to hydrophobic resins (28, 40, 41). We thus examined whether Ca2+ modulated the binding of DGKα-RVH + EF to phenyl-Sepharose. As shown in Fig. 7, inclusion of Ca2+ caused DGKα-RVH + EF to be retained by the resin. The peak elution in EGTA was between 1 and 1.5 ml, whereas in Ca2+ it was between 2 and 3 ml. The EF hands alone (DGKα:99-202) bound tightly to phenyl-Sepharose, both with and without Ca2+, and were not eluted under the conditions used for this experiment (data not shown). This suggests exposure of a hydrophobic surface upon loss of the RVH domain. Overall, these observations are consistent with a Ca2+-induced conformational change involving both the RVH domain and the EF hands.

**Activation of DGKα by OTAC and C16SB**—We have previously shown that DGKα is markedly stimulated by the cationic amphiphile, OTAC (11). This agent stimulated three other Ca2+-dependent DGK activities from testis cytosol, salivary cytosol, and NIH 3T3 cells to a similar degree (data not shown). Several Ca2+-independent DGK activities, including an arachidonoyl-DGK from testis membranes, and cysotoxic activities from NIH 3T3 cells and thymus cytosol were only modestly (2–6-fold) activated by OTAC (11, 23). Wild-type DGK activity expressed in COS-1 cells was activated 109-fold by OTAC (Table III). Similar stimulation was seen with the zwitterionic amphiphile, C16SB, although 15-fold higher concentrations were required to achieve the same activation. In lysates of COS-1 cells expressing the truncated DGK activities, OTAC and C16SB had much smaller effects (Table III). Activation of the mutants was comparable to that seen with Ca2+-independent DGKs. In the presence of maximally activating concentrations of OTAC, Ca2+ does not further activate wild-type DGKα (23). These results suggest that OTAC and C16SB have two effects on DGKs, a nonspecific stimulation seen with all isoforms and an additional stimulation seen only with Ca2+-activated DGKs. This latter effect, like Ca2+-dependent activation, required the RVH domains. OTAC and C16SB thus appear to act through RVH and EF hand domains to mimic Ca2+-dependent activation of DGKα. We examined whether OTAC, like Ca2+, protects DGKα:2-202 from trypsin proteolysis. In the absence of Ca2+, OTAC modestly accelerated the proteolysis of DGKα:2-202 (data not shown). In the presence of OTAC, Ca2+ no longer protected DGKα:2-202 (Fig. 8). Triton alone slightly increased proteolysis, but the rate was greatly accelerated by OTAC. Inclusion of OTAC/Triton or C16SB/Triton in the binding and wash buffers of overlay assays had no effect on45Ca2+ binding (data not shown). Overall, these results suggest that C16SB and OTAC mimic Ca2+-dependent DGKα activation by disrupting EF hand-mediated autoinhibition but do not compete with Ca2+ binding or mimic the Ca2+-induced conformational change. Loss of OTAC and C16SB stimulation of DGKα activity with deletion of the RVH domain provides independent evidence of a role for this domain in enzyme activation.
dependent inhibition of rhodopsin kinase (10). Neuronal calcium sensors derive from an ancestral 4 EF hand protein but have a unique structure (42). In contrast to the dumbbell-shaped structure of calmodulin and troponin C, neuronal calcium sensors are folded into compact globular structures (36, 43–45). Neuronal calcium sensors also have short helices at the N and C termini and between EF3 and EF4 that are not present in calmodulin (Fig. 1; positions 8–17, 147–153, and 195–200) (36, 43). Guananyl cyclase-activating proteins and calcineurin B subunits are related to neuronal calcium sensors but do not align as well in the regions corresponding to the RVH domain (Fig. 1). Consistent with this, the N-terminal helix of guananyl cyclase-activating protein-2 has a different orientation than that of recoverin (44). Alignment of the first 204 amino acids of DGKα with recoverin suggests that many of its unique features are also present in DGKs (Fig. 1). Nonpolar amino acids involved in the interaction between the N-terminal region of recoverin and its EF hands have homologues in DGKs (Fig. 1; Y70, H73, V74, F78, I100, A101, M104, L120, Y121, I137, and M144, numbering from the figure). Our observation that the DGKα EF hands bind phenyl-Sepharose much more tightly than the combined RVH domain and the EF hands from trypsin cleavage, indicates that it does not elicit the same conformational change as Ca2+-induced unfolding of the entire enzyme. Surprisingly, OTAC, a cationic amphiphile, and C16SB, a zwitterionic amphiphile, also activated DGKα. As with Ca2+-dependent activation, this effect required the presence of the RVH domain. However, OTAC did not protect the RVH/EF hands polypeptide from trypsin proteolysis, indicating that it does not elicit the same conformational change as Ca2+. This result provides additional evidence that the RVH domain plays a critical role in DGKα activation. Overall, our results indicate that functional coupling of the DGKα EF hands to its RVH domain is required to couple Ca2+-induced activation to release of the catalytic domain from EF hand-mediated autoinhibition.

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