EF-hand motifs of α, β and γ isoforms of diacylglycerol kinase bind calcium with different affinities and conformational changes

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The three diacylglycerol kinase isoenzymes (DGKα, DGKβ and DGKγ) cloned so far contain in common a tandem repeat of EF-hand motifs. However, the Ca^{2+} dependences of the DGK activities are known to be variable between isoenzymes, and the Ca^{2+}-binding activities of these motifs have not been tested except for those present in DGKα. We therefore attempted to define the intrinsic properties of EF-hands occurring in the DGK isoenzymes. For this purpose we bacterially expressed and purified the EF-hand motifs (termed DKE forms) of the three DGKs. Equilibrium dialysis with the purified DKE forms showed that all of the expressed proteins could bind approx. 2 mol of Ca^{2+} per mol. However, the apparent dissociation constant (K_d) for calcium binding to α-DKE (9.9 μM) was an order of magnitude greater than those estimated for β-DKE (0.89 μM) and γ-DKE (0.40 μM). Experiments with 2-*p*-toluidinyl-naphthalene 6-sulphonate, a probe for hydrophobic regions of proteins, showed that the binding of Ca^{2+} to β-DKE resulted in the exposure of hydrophobic amino acids, whereas hydrophobic regions of α-DKE and γ-DKE were masked by the addition of Ca^{2+}. Taken together, these results indicate that DGKα, DGKβ and DGKγ possess EF-hand structures with intrinsic properties different from each other with respect to affinities for Ca^{2+} and Ca^{2+}-induced conformational changes.

INTRODUCTION

Diacylglycerol kinase (DGK) converts diacylglycerol to phosphatidic acid. Diacylglycerol is an activator of protein kinase C [1], and phosphatidic acid is a bioactive lipid exhibiting several biological activities such as stimulation of GTPase-inhibiting protein [2] and many important enzymes. Phosphatidic acid and its metabolite, lysophosphatidic acid, are also known to act as mitogens when administered to a variety of cells [3]. DGK is thus probably important in the regulation of cellular functions. Recently, cDNAs coding for DGK isoenzymes (DGKα [4], DGKβ [5] and DGKγ [6]) have been cloned and the primary structures of these isoenzymes were commonly found to contain two consecutive EF-hand motifs [4]. Thus DGK isoenzymes cloned so far have been revealed to be additional members of the EF-hand type Ca^{2+}-binding proteins. In addition to two sets of Ca^{2+}-binding EF-hand motifs (C2), all of these DGK isoenzymes possess basically the same domain structures, i.e. the N-terminal conserved region (C1), two cysteine-rich zinc finger-like structures (C3) and the C-terminal C4 region [4–6]. However, these isoenzymes have been shown to exhibit different tissue- and cell-specific modes of expression despite their similar basic structures. That is, DGKα is most abundant in pig thymus [7,8] and oligodendrocytes of rat brain [9], and DGKβ is particularly enriched in rat neurons [5]. DGKγ, in contrast, is highly expressed in the human retina [6] and rat cerebellar Purkinje cells [10]. It thus seems likely that the DGK species are involved in highly differentiated cellular functions.

It is well known that several cellular signal transductions mediated by calcium have important roles in the regulation of physiological functions in various cell types [11]. Many Ca^{2+}-binding proteins represented by calmodulin have EF-hand structures in common. Most of the EF-hand proteins such as calmodulin, troponin C and calcineurin B are relatively small molecules (10–20 kDa) [12]. Such proteins play a specialized role as Ca^{2+}-sensitive regulatory units for many target enzymes, and most of their amino acid sequences are occupied by EF-hand motifs. In contrast, DGK is a relatively large EF-hand protein (80–90 kDa) and represents a fusion protein composed of EF-hands and other functional domains [4]. This type of fusion protein with a proven capacity for Ca^{2+}-binding has so far been demonstrated only for calpain [13].

All EF-hand sequences in DGK isoenzymes α, β and γ adequately conform to the criteria proposed to be required for Ca^{2+} binding [11]. We have already found that DGKα purified from pig thymus cytosol indeed binds 2 mol of Ca^{2+}/mol of the enzyme with an apparent dissociation constant, K_d, of 0.3 μM. Moreover, the addition of Ca^{2+} in the presence of phosphatidylserine markedly activates the enzyme purified or expressed in COS-7 cells [14,15]. In contrast, DGKγ unexpectedly exhibits the maximal activity without added Ca^{2+} [6]. The Ca^{2+}-dependence of DGKβ activity was not tested in detail, although some stimulatory effects of Ca^{2+} are noted for this isoenzyme [5]. Such discrepant effects of Ca^{2+} on the activities of DGK isoenzymes led us to investigate whether the EF-hands of the three DGK isoenzymes had different properties with respect to Ca^{2+}-binding and regulatory functions. In this study, using the EF-hand regions expressed in *Escherichia coli*, we found that the intrinsic properties of EF-hands of DGK isoenzymes were distinctly different from each other.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP and $^{45}CaCl_2$ (27–37 mCi/mg) were from DuPont–New England Nuclear. Phosphatidylserine and *sn*-1,2-dioleoyl-

Abbreviations used: DGK, diacylglycerol kinase; DKE, EF-hand region of DGK; ED_{50}, effective dose; GST, glutathione S-transferase; TNS, 2-*p*-toluidinyl-naphthalene-6-sulphonate.

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glycerol were the products of Sigma. β-Octylglucoside was purchased from Calbiochem. 2-*p*-Toluidinylnaphthalene-6-sulphonic acid (TNS; potassium salt) was purchased from Nakalai Tesq. Glutathione–Sepharose 4B was from Pharmacia Fine Chemicals. Restriction enzymes, DNA-modifying enzymes and linkers were purchased from Takara Shuzo Co., Toyobo Co. and New England BioLabs. Calmodulin purified from bovine brain was a gift from Dr. H. Yoshino (Sapporo Medical University, Sapporo, Japan). Oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. All other chemicals were of the highest quality commercially available.

COS-7 cell transfection and assay of DGK activity

General manipulation of DNA was performed with standard procedures [16]. The cDNAs encoding the full length of open reading frames of pig DGKα [15], rat DGKβ [5] and human DGKγ [6] were subcloned into the *Eco*RI site of the simian virus 40-based expression vector pSRE [14], which was derived by modification of the original pcDL-SR α-296 vector [17]. Approx. 10 μg of each of the resultant constructs, pSRE–DGKα [15], pSRE–DGKβ, pSRE–DGKγ, and pSRE vector alone were transfected into COS-7 cells with DEAE-dextran [18]. The supernatant of the cell lysate was assayed for DGK activity with the octylglucoside mixed-micellar method as described [14]. The free Ca^{2+} concentrations in the assay mixtures were calculated with a computer program (Free Calcium Software) on the basis of the reported data [19].

Construction of expression plasmids and purification of α, β and γ EF-hand regions of DGK (DKE forms)

PCR was performed to amplify the cDNA sequences corresponding to amino acid positions 103–196 in rat DGKα (termed α-DKE) [4], 142–236 in rat DGKβ (β-DKE) [5] and 169–262 in human DGKγ (γ-DKE) [6]. The sense primers (S1 for α-DKE, 5′-GGAGATCTCCCTTCTGGAGGGCGGCCGG-3′; S2 for β-DKE, 5′-GGAGATCTCTCTTCTGGAAAGAGGAAGA-3′; S3 for γ-DKE, 5′-GGAGATCTCCCTGCTGGAGACGGG-GAGG-3′) were derived from the sequences SLLEGGR (positions 103–109) [4], SLLERGR (positions 142–148) [5] and SLLETGR (positions 169–175) [6] respectively. The anti-sense primers (A1 for α-DKE, 5′-GGAGATCTCTCCAGACCCAGCAACA-CAAG-3′; A2 for β-DKE, 5′-GGAGATCTTTCCAAGCCCA-GCAAGACTAG-3′; A3 for γ-DKE, 5′-GGAGATCTATCCA-TCCCCAGGAGCACCAG-3′) were derived from the sequences ELGLLVL (positions 196–190) [4], ELGLLVL (positions 236–230) [5] and DMGLLVL (positions 262–256) [6] respectively. To facilitate subcloning, *Bgl*II endonuclease sites (underlined) were added to the 5′ end of each primer. pBluescript-DGKα, pBluescript-DGKβ and pBluescript-DGKγ were used as templates for PCR amplification with the primers S1 plus A1 (for the α-DKE cDNA fragment), S2 plus A2 (for the β-DKE cDNA fragment) and S3 plus A3 (for the γ-DKE cDNA fragment) respectively. Amplification was performed as described [20]. Amplified PCR products were digested with *Bgl*II and subsequently separated by preparative agarose-gel electrophoresis. Bands at the predicted positions were recovered and inserted into the *Bam*HI site of pET-3c [21] to obtain in-frame fusion products with the first 11 residues of the T7 gene φ10 product.

To express the protein products, *E. coli* BL21 (DE3)/pLysS harbouring each constructed plasmid were induced with isopropyl β-D-thiogalactoside [21] and the cells were harvested. Cells expressing high levels of the expressed proteins were suspended in lysis buffer containing 20 mM Tris/HCl (pH 7.4)/0.25 M sucrose/1 mM EDTA/4 mM EGTA/1 mM dithiothreitol/1 % (v/v) Triton X-100/1 μg/ml leupeptin/1 μg/ml pepstatin/1 μg/ml aprotinin/20 μg/ml soybean trypsin inhibitor/1 mM PMSF. After freeze–thawing, the cells were lysed by sonication. The inclusion bodies were pelleted by centrifugation (10000 ***g*** for 30 min) and washed once with 1 M sucrose. The proteins were solubilized with a minimal volume of 8 M urea in buffer A [30 mM Tris (pH 7.5)/30 mM NaCl/1 mM dithiothreitol] [22], and then were refolded by dialysis against 4 M urea in buffer A for 12 h followed by three successive dialyses against buffer A. After centrifugation at 100000 ***g*** for 30 min, the soluble proteins were used for the Ca^{2+} binding assay and analysis by alkaline urea/PAGE. At this stage the purity of the EF-hand preparations determined by densitometric analysis after SDS/PAGE was approx. 70 %, and the ^{45}Ca overlay assay detected no other Ca^{2+}-binding proteins in the preparations. For further purification of expressed proteins used for the assay of interaction with TNS, preparative alkaline urea/PAGE was performed. In this case, proteins were eluted from the gel by homogenization in 8 M urea/buffer A, and were then refolded as described above.

Expression plasmids for DKE proteins as fusions with glutathione S-transferase (GST), termed GST–DKE, were obtained by inserting the amplified PCR products into the *Bam*HI site of pGEX-3X (Pharmacia). To express the protein products, *E. coli* XL1-Blue were induced with isopropyl β-D-thiogalactoside [21]. Cells expressing high levels of the expressed proteins were harvested and suspended in lysis buffer. After freeze–thawing, the cells were lysed by sonication. Fusion proteins with GST were purified from the 100000 ***g*** supernatant of cell lysate by affinity chromatography with glutathione–Sepharose 4B (Pharmacia) in accordance with the protocol supplied. The purity of fusion proteins with GST determined by densitometric analysis after SDS/PAGE was approx. 90 %.

Ca^{2+} binding assay

The $^{45}Ca^{2+}$ overlay assay with electrophoretically transferred α-DKE, β-DKE and γ-DKE was done in accordance with Maruyama et al. [23] as described previously [15]. The Ca^{2+} binding to the expressed α-DKE, β-DKE and γ-DKE was quantitatively measured by dialysing them against buffer B [100 mM KCl/10 mM imidazole (pH 6.5)/0.1 mM EGTA] [24]. Before use, glass and plastic wares and dialysis tubes were treated to obtain Ca^{2+}-free conditions by the method of Potter et al. [24]. The free Ca^{2+} concentration was adjusted by adding EGTA/Ca^{2+} buffer, as calculated with the computer program (Free Calcium Software). Demineralization of the DKE forms in buffer B was performed by dialysis overnight at 4 °C against the same buffer to minimize the level of Ca^{2+}. The DKE solutions (250 μl, 60 nmol/ml) in buffer B were dialysed at 22 °C overnight against the same buffer containing between 10 nM and 500 μM free Ca^{2+} calculated as described above. Once equilibrium had been reached, duplicate 100 μl samples of the solutions inside and outside the dialysis bags were taken for ^{45}Ca measurements, and the rest of the solution inside was used for protein determination. Protein was determined with the Pierce protein assay kit, with BSA as the standard. The amounts of Ca^{2+} bound to the DKE forms were calculated by the method of Potter et al. [24]. The Ca^{2+} binding to the expressed GST–α-DKE and GST–γ-DKE was measured as described above. Competition experiments were performed by dialysing DKE forms against buffer B containing 100 μM Ca^{2+} and 1 mM Mg^{2+}.

Alkaline urea/PAGE

Alkaline (pH 8.6) urea/PAGE (5 % acrylamide) was performed to detect mobility shifts of DKE forms caused by the Ca^{2+}

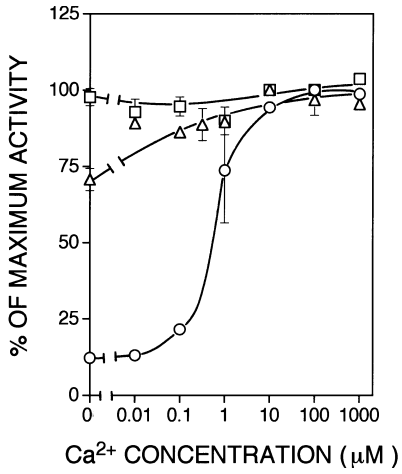

Figure 1 The effects of Ca^{2+} on the activities of DGK isoforms

The 100 000 ***g*** supernatants were prepared from COS-7 cells transfected with pSRE–DGKα ($\bigcirc$), pSRE–DGKβ ($\triangle$) and pSRE–DGKγ ($\square$) and dialysed extensively against Ca^{2+}-free buffer. The enzyme activity was measured with 5 μg of protein of the supernatants at various Ca^{2+} concentrations. The free Ca^{2+} concentrations indicated were achieved by varying the Ca^{2+}/EGTA ratio. The EGTA concentration was kept at 2 mM in the assay. The results are expressed as percentages of maximal activities obtained in the presence of 1 mM Ca^{2+}. Results are means $\pm$ S.D. for four incubations.

binding [25]. Before PAGE analysis, DKE forms were pre-incubated at 30 °C for 30 min in the presence of Ca^{2+} (0.1 mM) or its absence (1 mM EGTA).

Interaction with TNS

Measurements in the presence of 15 μM TNS were performed as described by McClure and Edelman [26] with calmodulin (5 μM, equivalent to 20 μM EF-hands) and DKE forms (10 μM, equivalent to 20 μM EF-hands). In this case, EGTA (750 μM) and $CaCl_2$ (1.0 mM) were added to obtain Ca^{2+}-free and loaded forms respectively, and the measurements were made with an excitation wavelength of 365 nm and slit set at 2 nm. Fluorescence spectra were recorded by scanning from 380 to 550 nm with a Hitachi spectrofluorimeter equipped with a high-stability xenon lamp.

RESULTS

Effects of calcium on the enzyme activities of DGK isoenzymes expressed in COS-7 cells

We have already reported that DGKα purified from pig thymus or expressed in COS-7 cells is markedly activated by Ca^{2+} [14,15]. However, DGKβ expressed in COS-7 cells shows considerable activity without added Ca^{2+} and is only slightly activated in the presence of Ca^{2+} [5]. It was also demonstrated that DGKγ exhibits maximal activity without added Ca^{2+} [6]. These results were obtained in separate experiments; the exact modes of Ca^{2+} dependence have not been examined for the activities of DGKβ and DGKγ. To confirm the variable effects of Ca^{2+} on their activities, the Ca^{2+} concentrations in the enzyme assay mixture were varied from 0 to 1000 μM in the presence of phosphatidylserine (Figure 1). As reported previously, the activity of DGKα was fully activated with 10 μM calcium, with an effective dose (ED_{50}) of 0.34 μM, whereas that of DGKγ was maximal without added Ca^{2+}. The activity of DGKβ was nearly maximal in the absence of Ca^{2+} and only slightly activated with a very low Ca^{2+} concentration (approx. 0.01 μM). It was thus confirmed that DGKβ and DGKγ are apparently independent of Ca^{2+}, in contrast with DGKα. The discrepant effects of Ca^{2+} on the DGK isoenzymes thus led us to investigate the intrinsic properties of the three sets of EF-hand motifs of DGK isoenzymes. For this purpose we expressed in *E. coli* the two consecutive EF-hand structures of DGK isoenzymes α, β and γ.

The expression of α-DKE, β-DKE and γ-DKE in *E. coli*

As shown in Figure 2, the recombinant plasmids produced the EF-hand domains as fusion proteins (termed α-DKE, β-DKE and γ-DKE) with short sequences of bacteriophage T7 ϕ10 derived from pET-3c vectors at their N-and C-termini. Figure 3(A) shows the SDS/PAGE analysis of α-DKE, β-DKE and γ-DKE prepared for fluorescence measurements. The molecular masses of α-DKE, β-DKE and γ-DKE estimated from the results of SDS/PAGE were consistent with their calculated values (12.4, 12.5 and 12.6 kDa respectively).

Ca^{2+} binding to α-DKE, β-DKE and γ-DKE

Despite the apparent lack of Ca^{2+}-dependence observed for DGKβ and DGKγ (Figure 1), the two EF-hand sequences of

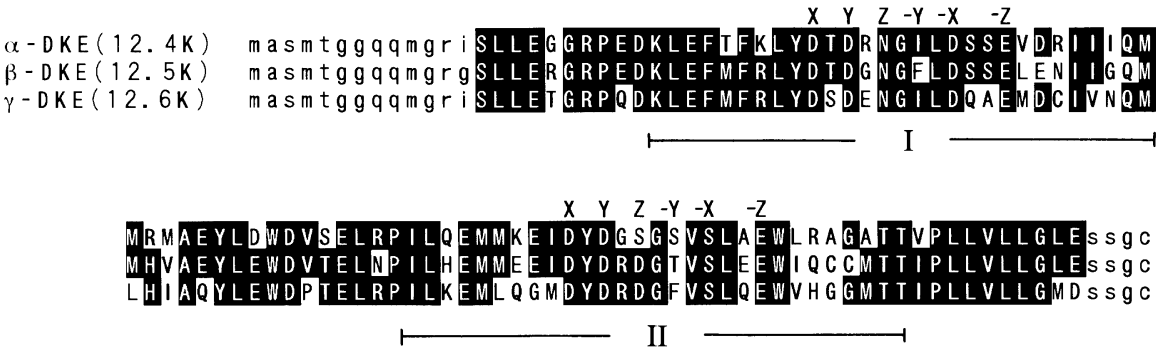

Figure 2 Amino acid sequences of DKE forms

Amino acid residues with lowercase letters were derived from bacteriophage T7 ϕ10 encoded by pET-3 vectors. Identical amino acids are indicated in reverse type. The two Ca^{2+}-binding loop regions (I and II) of the EF-hand structures are indicated below the sequences. X, Y, Z, —Y, —X and —Z denote the Ca^{2+}-co-ordinating positions [37]. The expected relative molecular masses of α-DKE, β-DKE and γ-DKE are also shown in parentheses.

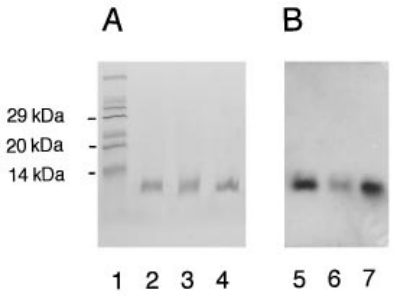

Figure 3 SDS/PAGE analysis of the DKE forms and ^{45}Ca autoradiography of electrophoretically transferred DKE forms

(**A**) SDS/PAGE analysis of molecular mass marker proteins (lane 1), the expressed α-DKE (lane 2), β-DKE (lane 3) and γ-DKE (lane 4). Purified proteins (2 μg) were subjected to electrophoresis in a 15% gel and stained with Coomassie Brilliant Blue. The marker proteins were, from the top: carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). (**B**) ^{45}Ca autoradiography of electrophoretically transferred DKE forms. The purified α-DKE (lane 5), β-DKE (lane 6) and γ-DKE (lane 7) (100 pmol of each) were separated by SDS/PAGE and transferred to a nitrocellulose membrane. The blots were incubated with $^{45}CaCl_2$ and autoradiographed for 18 h.

these DGK isoenzymes adequately conformed to the criteria proposed to be required for Ca^{2+} binding [11]. Although we have already shown that DGKα does indeed bind Ca^{2+} [15], the Ca^{2+}-binding activities of DGKβ and DGKγ have not yet been investigated. First we performed a Ca^{2+} overlay assay with purified DKE forms. The results, given in Figure 3(B), clearly showed that all of the EF-hand regions of DGK isoenzymes could bind Ca^{2+}. The different signal intensities of $^{45}Ca^{2+}$ in Figure 3(B) were due to the different transfer efficiencies of DKE forms to nitrocellulose membranes (results not shown).

Next we determined the stoichiometry and affinities of Ca^{2+}-binding activities of different DKE forms. In this case we used partly purified DKE forms (approx. 70% pure) obtained from the inclusion bodies, because the equilibrium dialysis method [27] requires large amounts of the expressed proteins. We confirmed that the partly purified proteins contained no other Ca^{2+}-binding proteins by using the Ca^{2+} overlay method assay (results not shown). The amounts of each DKE form were calculated from the protein bands in Coomassie Blue-stained SDS/PAGE with BSA as a standard. We also tested calmodulin purified from bovine brain as a control. Figure 4(A) clearly shows that all DKE forms bound approx. 2 mol of Ca^{2+}/mol. Calmodulin was confirmed to bind 4 mol of Ca^{2+}/mol as reported [28,29]. However, the affinities for Ca^{2+} of the DKE forms were clearly different from each other (Figure 4A). The K_d values (means $\pm$ S.D.) for calcium binding to α-DKE, β-DKE and γ-DKE were estimated as $9.9 \pm 2.1\ \mu M$ ($n = 6$), $0.89 \pm 0.15\ \mu M$ ($n = 4$) and $0.40 \pm 0.04\ \mu M$ ($n = 3$) respectively. We noted that the K_d for α-DKE thus obtained was 25-fold higher than that for γ-DKE. It was also noted that the K_d determined for α-DKE deviated markedly from that previously determined for the intact DGKα ($K_d = 0.3\ \mu M$) [14].

Although the present analysis disclosed a marked difference in the affinities for Ca^{2+} exhibited by the EF-hands of DGK isoenzymes, there remained a possibility that the denaturation and refolding process involved in the DKE purification might have affected their K_d values. To test this possibility we expressed the α- and γ-EF-hands in *E. coli* as soluble GST fusion proteins. For unknown reasons we could not prepare adequate amounts of β-EF-hands in this experiment. As shown in Figure 4(B), we obtained similar K_d values (means $\pm$ S.D.) for the GST–α-DKE

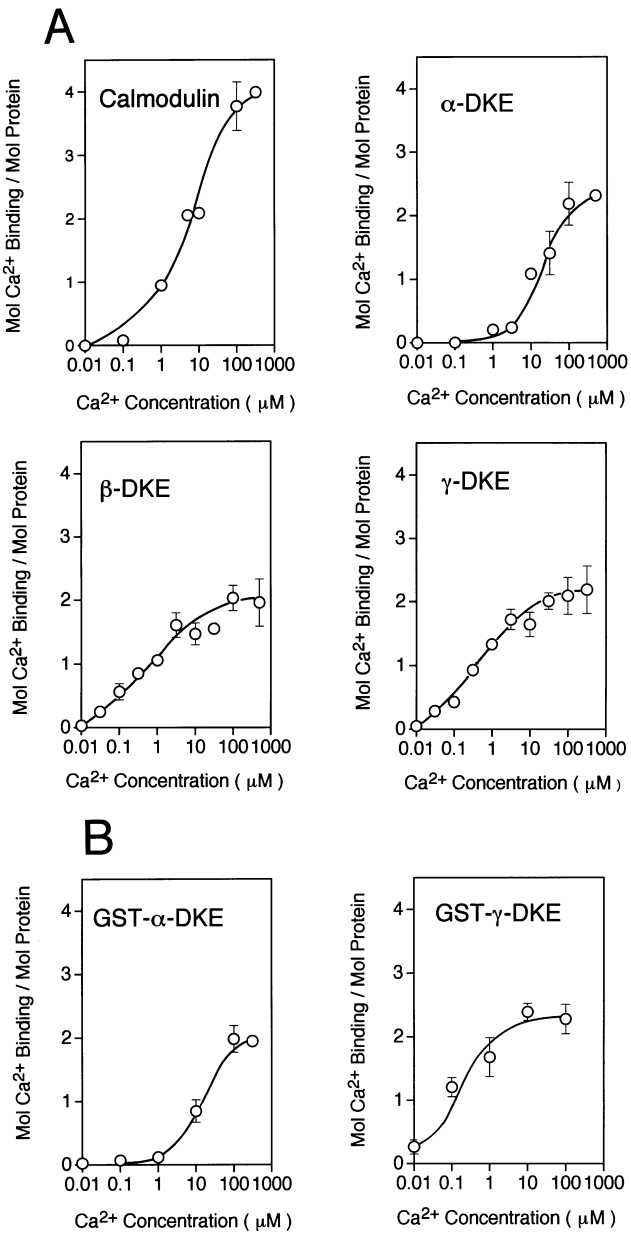

Figure 4 Ca^{2+}-binding to DKE forms

Ca^{2+}-binding to calmodulin, α-DKE, β-DKE or γ-DKE (**A**) and GST–α-DKE or GST–γ-DKE (**B**) was measured by equilibrium dialysis at various $^{45}CaCl_2$ concentrations as described in the Materials and methods section. Values are means $\pm$ S.D. ($n = 2$–6). The K_d values for DKE forms and GST–DKE forms were obtained from the Ca^{2+} concentration required to give half-maximal binding. For calmodulin, the Ca^{2+} concentration needed to bind 2 mol of Ca^{2+}/mol of calmodulin was taken as the K_d value.

($10.0 \pm 0.10\ \mu M$, $n = 3$) and GST–γ-DKE ($0.17 \pm 0.02\ \mu M$, $n = 3$). We therefore considered that the K_d values obtained for DKE forms (Figure 4A) represented the intrinsic properties of EF-hands present in the three DGK isoenzymes.

The enzyme assay mixture for DGK isoenzymes inevitably contained Mg^{2+}. We considered the possibility that the binding of Mg^{2+} instead of Ca^{2+} to DGK isoenzymes might have resulted in their apparently different responses to Ca^{2+} shown in Figure 1, because troponin C and myosin light chain are known to bind not only Ca^{2+} but also Mg^{2+} with high affinities [27,30]. However,

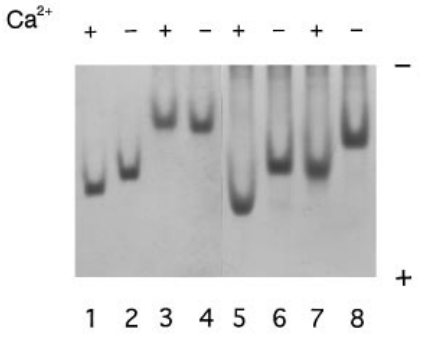

Figure 5 Conformational changes of α-DKE, β-DKE and γ-DKE examined by alkaline urea/PAGE

After preincubation of α-DKE, β-DKE or γ-DKE and calmodulin (5 μg of protein in each) in the presence of Ca^{2+} (0.1 mM) or its absence (1 mM EGTA), the samples were subjected to electrophoresis in an alkaline urea gel (5%) and stained with Coomassie Brilliant Blue. Lanes 1 and 2, calmodulin; lanes 3 and 4, α-DKE; lanes 5 and 6, β-DKE; lanes 7 and 8, γ-DKE.

the addition of 1 mM Mg^{2+} to the dialysis buffer did not affect the K_d values in the present analysis (results not shown). This indicated that the EF-hands of DGK isoenzymes did not bind Mg^{2+} with significant affinity.

Conformational changes induced by Ca^{2+} binding

We next investigated the conformational changes of the three EF-hand regions induced by Ca^{2+} binding. Calmodulin and other small EF-hand proteins are known to undergo conformational changes on Ca^{2+} binding that can be detected as a mobility shift in alkaline urea/PAGE analysis [25]. As shown in Figure 5, when compared with the Ca^{2+}-free form, the migration of β-DKE and γ-DKE became faster in the presence of Ca^{2+}, as did calmodulin, whereas that of α-DKE became slower in the presence of Ca^{2+}. These results suggested that the binding of Ca^{2+} to DKE forms caused variable conformational changes. The difference in mobility shift observed between α-DKE and other DKE forms might reflect the different extents of electrically charged amino acids that are exposed by the Ca^{2+} binding [31].

It is well known that the Ca^{2+} binding to calmodulin induces a conformational change that results in the exposure of hydrophobic amino acids [31]. Thus, using TNS, we investigated the changes in fluorescence intensity of the DKE forms caused by the addition of Ca^{2+} (Figure 6). The high basal fluorescence intensity observed for all DKE forms indicated that, in contrast with calmodulin, the hydrophobic regions of the DKE forms were already exposed in the absence of Ca^{2+}. This was probably due to the presence of eight hydrophobic residues immediately following the second EF-hand loops of the three DGK isoenzymes (Figure 2). When compared with other EF-hand-type Ca^{2+}-binding proteins such as calmodulin and troponin C [31], the EF-hands of DGK isoenzymes are unique in that the two Ca^{2+}-binding loops are separated by a relatively long insertion of 16 amino acids that contains eight hydrophobic residues (Figure 2). This insertion might also be responsible for the basal fluorescence intensity observed. Despite the relatively high basal absorbance, we detected distinct effects of Ca^{2+} on the hydrophobicities of the DKE forms. It was shown that the Ca^{2+} binding to β-DKE resulted in the exposure of hydrophobic amino acids in contrast with the smaller hydrophilicities exhibited by the Ca^{2+}-loaded α-DKE and γ-DKE. The EF-hands of DGKβ and DGKγ were thus demonstrated to undergo conformational changes different from each other despite their similar affinities for Ca^{2+} (Figure 3) and behaviours in alkaline urea/PAGE analysis (Figure 5).

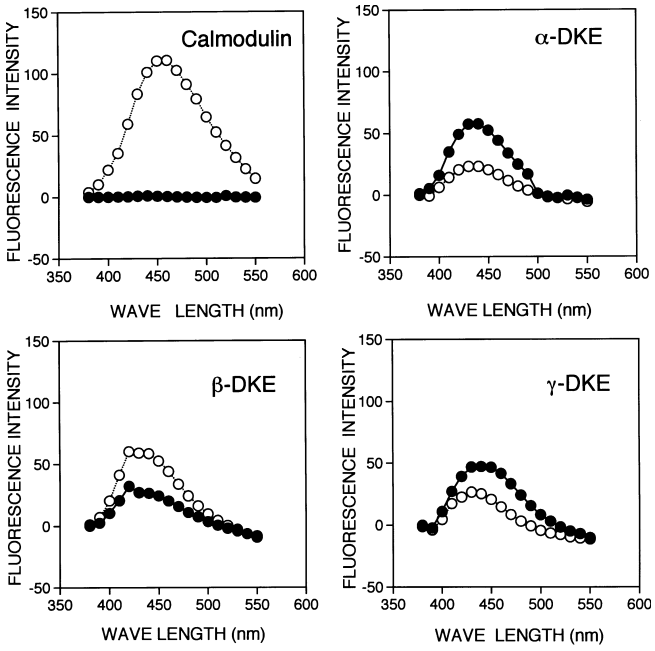

Figure 6 Effects of Ca^{2+} on TNS fluorescence of DKE forms

Emission spectra of 15 μM TNS in 50 mM Tris/HCl (pH 7.6) containing 5 μM calmodulin and 10 μM α-DKE, β-DKE or γ-DKE were measured in the presence (○) or absence (●) of 1 mM Ca^{2+}. Samples were excited at 365 nm. Excitation and emission bandpasses were 2 and 10 nm respectively.

DISCUSSION

Our work has shown that the EF-hand motifs of all of three DGK isoenzymes could bind 2 mol of Ca^{2+}/mol. This is the first direct evidence that the EF-hands of DGKβ and DGKγ are domains possessing biological functions. In addition we found that their affinities for Ca^{2+} differed. In particular there was a marked difference between the affinity of α-DKE and those of β-DKE and γ-DKE. γ-DKE had the highest affinity for Ca^{2+}, with its K_d being 1/25 of that determined for α-DKE. We compared the amino acid sequences of Ca^{2+}-binding loops of three sets of EF-hands (Figure 2). We noted that the fifth position (Z) in the second EF-hand loops of β-DKE and γ-DKE was occupied by an Asp residue, whereas the corresponding residue in α-DKE was Ser (Figure 2). Because position Z is one of the co-ordination sites of a positively charged ion, Ca^{2+}, these differences might account for α-DKE's having the lowest affinity for Ca^{2+}.

We have already reported the K_d for Ca^{2+} binding to intact DGKα to be 0.3 μM [14]. The K_d for Ca^{2+} binding to α-DKE described here (9.9 μM) was thus exceedingly high (33-fold) compared with that for the intact enzyme. The enzyme activity of intact DGKα was activated by Ca^{2+} with an ED_{50} value of approx. 0.3 μM (Figure 1). This observation also accounts for the higher affinity for Ca^{2+} of the intact enzyme compared with that determined for the EF-hands expressed. It was also suggested that DGKα was activated in parallel with the extents of Ca^{2+} binding to its EF-hands. At present it is difficult to account for the discrepant K_d values obtained for the intact enzyme and for the expressed EF-hands. In this respect it is known that the

affinity for Ca^{2+} binding to calmodulin increases markedly in the presence of target proteins, as has been found with mastoparan (16-fold increase) and with caldesmon (2.9-fold) [33]. It is thus possible that the affinity for Ca^{2+} of EF-hands in intact DGKα might be enhanced 33-fold through its intramolecular interactions with other domains. If this is so, it can be speculated that the already high affinities for Ca^{2+} exhibited by β-DKE and γ-DKEs would be further increased when functioning in the intact enzyme molecules. These possibilities are interesting subjects for further study.

Before the experiments we had assumed four possibilities to account for the variable effects of Ca^{2+} on the enzyme activities of DGK isoenzymes: (1) the EF-hands of DGKβ and DGKγ cannot bind Ca^{2+}; (2) Mg^{2+} in the enzyme assay mixture competes with Ca^{2+} for binding to the EF-hands; (3) DGKβ and DGKγ can be activated by low concentrations of Ca^{2+} attained even in the presence of 1 mM EGTA because of an extremely high affinity of their EF-hands for Ca^{2+}; and (4) the EF-hands of DGKβ and DGKγ are not directly involved in the regulation of the enzyme activities measured *in vitro*, in contrast with those contained in DGKα. The first two possibilities could be ruled out in the present study because of the high affinity for Ca^{2+} demonstrated for β-DKE and γ-DKE and the effects of Mg^{2+} on Ca^{2+} binding. As already discussed, the affinities for Ca^{2+} of EF-hands in DGKβ and DGKγ might be increased in intact enzymes through an intramolecular interaction. It is also difficult to assume different roles of the EF-hands present in isoenzymes with basically the same structure. These considerations seem to support the third possibility. In this respect it should be noted that it was difficult to obtain a strictly Ca^{2+}-free condition in the enzyme assay mixture because of the presence of phosphatidylserine and other additions. However, variable effects of Ca^{2+} on the electrophoresis patterns in alkaline urea/PAGE and on the hydrophobicities of the three DKE forms might indicate different functions of these EF-hands even though they are present in the members of the same enzyme family. Further work is required to determine which of the two remaining possibilities can account for the apparent Ca^{2+}-independences of the two DGK isoenzymes. For calmodulin [31] and troponin C [34], binding of Ca^{2+} to the EF-hands induces conformational changes, resulting in the exposure of hydrophobic amino acids. We showed that the EF-hands of DGK isoenzymes also underwent conformational changes on Ca^{2+} binding and that the conformation of the Ca^{2+}-loaded EF-hands was variable depending on the isoenzyme. The variable conformational changes probably represent different regulatory mechanisms operating for DGK isoenzymes, which are known to exhibit tissue- and cell-specific modes of expression. The changes in fluorescence in the presence of Ca^{2+} are relatively small for the EF-hand fusion proteins when compared with calmodulin. Calmodulin inevitably contacts target proteins through intermolecular interactions. In contrast, the EF-hand region of DGK seems to take effect through intramolecular interactions. Thus, compared with calmodulin, the smaller conformational changes induced by Ca^{2+} binding might be sufficient for activating DGK.

Recently we cloned a fourth member of the DGK gene family termed DGKδ [20]. Human DGKϵ [35] and DGKζ [36] have also been cloned by Prescott's group. These novel isoenzymes are lacking in EF-hands, suggesting the presence of regulatory mechanisms different from those operating for the EF-hand-containing DGK isoenzymes. The fact that the presence of EF-hands is limited to a certain subfamily of DGK isoenzymes further suggests significant regulatory roles for the Ca^{2+}-binding domains. We confirmed that DGKβ and DGKγ are apparently independent of Ca^{2+} despite the presence of functional EF-hands. Although we could not fully elucidate the mechanisms of variable Ca^{2+}dependences of DGK isoenzymes, the different intrinsic properties of EF-hands of DGK isoenzymes might reflect their distinct functions.

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