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The Guanine Nucleotide Exchange Factor RasGRP Is a High - Affinity Target for Diacylglycerol and Phorbol Esters

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ABSTRACT

RasGRP is a recently described guanine nucleotide exchange factor (GEF) that possesses a single C1 domain homologous to that of protein kinase C (PKC). The phorbol ester [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) bound to this C1 domain (C1-RasGRP) with a dissociation constant of 0.58 ± 0.08 nM, similar to that observed previously for PKC. Likewise, the potent PKC activator bryostatin 1, a compound currently in clinical trials, showed high affinity binding for C1-RasGRP. Structure activity analysis using several phorbol ester analogs showed both similarities and differences in ligand selectivity compared with PKC; the differences were comparable in magnitude to those between different PKC isoforms. Similarly, the potency of the PKC inhibitor calphostin C to inhibit [^{3}H]PDBu binding to C1-RasGRP was similar to that observed for PKC. In contrast to the relative similarities in ligand recognition, the lipid cofactor requirements differed between RasGRP and PKC. The C1 domain plus the EF-hand motif of RasGRP (C1EF-RasGRP) was markedly less dependent on acidic phospholipids than was PKCα. The differences in lipid requirements were reflected in differential ligand selectivity under conditions of limiting lipid. Despite the presence of twin EF-hand like motifs, calcium did not affect the binding of [^{3}H]PDBu to C1EF-RasGRP. We conclude that RasGRP is a high affinity receptor for phorbol esters and diacylglycerol. RasGRP thus provides a direct link between diacylglycerol generation or phorbol ester/bryostatin treatment and Ras activation.

The Ras-related GTPases are essential elements in the signal transduction pathways in the cell, playing a pivotal role in the control of cell proliferation and cytoskeletal rearrangements (Macara et al., 1996). These proteins cycle between an inactive form bound to GDP and an active GTP-bound state (Macara et al., 1996; Bos, 1997). Guanine nucleotide exchange factors (GEFs) positively modulate the small GTPases by catalyzing the dissociation of the bound GDP to allow the association of GTP. Several mammalian GEFs have been identified so far, which include Sos (Chardin et al., 1993), RasGRF (Shou et al., 1992), and RalGDS (Albright et al., 1993). Recently, a Ras-specific GEF called RasGRP was identified by a cDNA cloning approach from rat brain mRNA (Ebinu et al., 1998).

One interesting feature of RasGRP is the presence of a diacylglycerol (DAG) binding motif, which possesses strong homology to the DAG-binding site, or C1 domains, of protein kinase C (PKC) (Hurley et al., 1997). The importance of this DAG motif in the RasGRP signaling has been demonstrated by using deletion mutants. Although prolonged exposure to phorbol 12-myristate-13-acetate (PMA) induces a transformed morphology in rat2 cells expressing RasGRP, the C1-deletion mutant does not confer any substantial PMA-induced change in cell morphology (Ebinu et al., 1998). In addition, all deletions that removed the C1 domain from RasGRP eliminate the transforming activity of this protein in NIH 3T3 cells, and the transforming capacity of this protein is restored by attaching to it either the RasGRP-C1 domain or the C1 domain of PKC (Tognon et al., 1998).

The C1 domain consists of a cysteine-rich motif, or zinc finger, which coordinates two zinc ions in its structure. Binding of DAG as well as phorbol esters to PKC occurs at the C1 domains (Kaibuchi et al., 1989; Ono et al., 1989). This domain is present in tandem in the novel (isoforms δ, ϵ, η, and θ) and conventional (isoforms α, β, and γ) PKCs, whereas it is found only once in the atypical (isoforms ζ and λ/ι) PKCs (Newton, 1995). For the novel and conventional PKCs, binding of DAG/phorbol esters to the C1 domains induces activation and membrane translocation of the enzyme. Similarly, the C1 motif of RasGRP mediates cell membrane localization on

ABBREVIATIONS: GEFs, guanine nucleotide exchange factors; DAG, diacylglycerol; PKC, protein kinase C; [^{14}C]DPPC, L-α-[1-^{14}C]dipalmitoylphosphatidylcholine; [^{3}H]PDBu, [^{3}H]phorbol 12,13-dibutyrate; [^{3}H]Bryo, [26-^{3}H]bryostatin 1; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; POPA, 1-palmitoyl-2-oleoylphosphatidic acid; PMA, phorbol 12-myristate-13-acetate; OAG, 1-oleoyl-2-acetoyl-*sn*-glycerol; TRP, transient release protein; C1EF-RasGRP, C1 domain plus the EF-hand motif; LUV, large unilamellar vesicle.

phorbol ester stimulation (Ebinu et al., 1998; Tognon et al., 1998).

For many years, DAG was believed to act solely through the PKC family of isozymes. More recently, new structural classes of DAG receptors have been discovered, including the Munc13 (Brose et al., 1995), chimaerin (Ahmed et al., 1993), and PKD families (Valverde et al., 1994). For the Ras signaling pathways, indirect PKC-mediated modulation by DAG has been observed (Marais et al., 1998). Now, RasGRP, expressed mainly in brain and lymphoid tissues, provides a direct link between DAG generation and Ras activation.

In this study we analyzed the properties of RasGRP as a DAG/phorbol ester receptor. We found that the C1 domain of RasGRP bound phorbol esters and other ligands known to bind to C1 domains with high affinity. Moreover, this binding is dependent on phospholipids, as has been observed for PKC (Newton and Johnson, 1998) and other C1-domain proteins (Kazanietz et al., 1995a; Caloca et al., 1997) but showed substantial differences in phospholipid selectivity. Although RasGRP has two calcium binding sites similar to the EF-hands, calcium did not affect the phorbol ester binding, suggesting that RasGRP is calcium-independent like the novel PKCs.

We conclude that RasGRP is a novel, high-affinity target for phorbol esters with structure-activity requirements generally resembling those of PKC. Its differences compared with PKC in lipid selectivity provide a mechanism for differential control, whether by phospholipids or pharmacological agents.

Experimental Procedures

Expression and Purification of RasGRP Proteins from *Escherichia coli*. The cDNA coding for RasGRP was cloned from rat brain mRNA (Ebinu et al., 1998). The C1 domain of RasGRP (C1-RasGRP), containing residues 538 through 598 of RasGRP, was subcloned into a pGEX vector to produce a GST-fusion protein in *E. coli* strain BL21. The protein was then purified using glutathione-Sepharose 4B beads according to the recommendations of Amersham Pharmacia Biotech (Piscataway, NJ). The C1 domain plus the EF-hand motif of RasGRP (C1EF-RasGRP), corresponding to residues 471 through 598 of RasGRP, and the whole RasGRP protein were subcloned into a pMal vector to produce maltose-binding fusion proteins. These proteins were expressed in *E. coli* BL21 and purified on an amylose resin according to the manufacturer's guidelines (New England Biolabs, Beverly, MA). Similar recombinant RasGRP proteins expressed in *E. coli* have been used for biochemical analysis as GEF proteins (Ebinu et al., 1998). The analysis demonstrates enhancement of the Ras-GDP complex dissociation and association with GTP, and the in vitro complex formation with Ras.

Expression and Purification of PKCα and the C1b Domain of PKC δ. Recombinant PKCα was expressed in Sf9 insect cells and partially purified as described elsewhere (Kazanietz et al., 1993). Recombinant C1b domain of PKCδ was expressed and purified from *E. coli* as a GST-fusion protein (Kazanietz et al., 1995b).

Preparation of Lipid Vesicles. Sonicated dispersions of phosphatidylserine were prepared in 50 mM Tris-HCl, pH 7.4. For preparation of large unilamellar vesicles (LUV), mixtures of lipids in chloroform containing added traces of L-α-[1-^{14}C]dipalmitoylphosphatidylcholine ([^{14}C]DPPC) were dried under nitrogen. Lipids were then resuspended in 170 mM sucrose, 20 mM Tris-HCl, pH 7.4. Aliquots of lipid (500 μl) were vortexed for 2 min, subjected to three freeze-thaw cycles and then extruded 40 times through two-stacked 0.1-μm pore polycarbonate filters using a Liposofast microextruder (Avestin, Ottawa, Canada) to form LUVs. The final lipid concentration was calculated from the amount of [^{14}C]DPPC included in the lipid mixture.

Binding of [^{3}H]Phorbol 12,13-Dibutyrate. Binding of [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) was measured using the polyethylene glycol precipitation assay as described elsewhere (Sharkey and Blumberg, 1985). The assay mixture contained 50 mM Tris-HCl, pH 7.4, 1 mg/ml IgG, 0.1 mM $CaCl_2$, PKC, or RasGRP, and the corresponding lipid mixture or sonicated phosphatidylserine dispersion. Incubations were carried out at 18°C for 5 min. Nonspecific binding was measured using an excess of nonradioactive PDBu (30 μM). Values of specific binding were determined in triplicate at each ligand concentration in each experiment. Nonspecific binding was typically less than 20% of the total binding observed in the assays either for PKC or RasGRP.

Binding of [26-^{3}H]Bryostatin 1. Binding of [26-^{3}H]bryostatin 1 ([^{3}H]Bryo) was determined as described using a filtration assay (Kazanietz et al., 1994). Briefly, the binding assays were performed with seven concentrations of [^{3}H]Bryo (specific activity, 481 GBq/mmol) ranging from 0.5 to 32 nM. C1-RasGRP was incubated with [^{3}H]Bryo at 37°C for 5 min in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mg/ml IgG, 1 mM $CaCl_2$, and lipid micelles containing 300 μg/ml phosphatidylserine and 1.5 mg/ml Triton X-100. After incubation, samples were chilled on ice for 5 min and then two 50-μl aliquots were removed and applied onto Whatman ion exchange paper disks (DE-81) for determination of bound ligand. After 30-s absorption, the paper disks were washed rapidly with 25 ml of ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, and 55% (v/v) methanol. For determination of total ligand, two additional 50-μl samples were applied onto paper disks and measured directly for radioactivity. Nonspecific binding was determined in the absence of added protein.

Data Analysis. Equilibrium dissociation constants (K_d) and inhibition constant (K_i) were determined using Origin 5.0 software (Microcal Software, Northampton, MA). All values are expressed as the mean ± S.E.M. Data were analyzed using one-way ANOVA followed by Dunnett's test. For paired data, statistical significance was determined by Student's t test (GraphPad Prism 2.01; GraphPad Software Inc., San Diego, CA).

Calculation of Free Calcium Concentrations. Concentrations of free calcium were calculated using a computer program generously provided by Claude Klee (National Cancer Institute, National Institutes of Health, Bethesda, MD) that takes into account pH, Mg^{2+}, K^+, Na^+, EGTA, EDTA, and Ca^{2+} concentrations present in the sample. According to the free calcium concentration calculated by the program, EGTA or calcium chloride was added to each sample to reach the desired final free calcium concentration. Calcium contamination by different components of the binding sample was taken into account in the calculations.

Materials. Bovine brain phosphatidylserine and PDBu were obtained from Sigma Chemical Co. (St. Louis, MO). Calphostin was purchased from Calbiochem (San Diego, CA). Sucrose Ultra Pure (99.9%) was obtained from ICN Biomedicals (Costa Mesa, CA). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-oleoylphosphatidylserine (POPS), 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG), 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE), and 1-palmitoyl-2-oleoylphosphatidic acid (POPA) were purchased from Avanti Polar Lipids (Alabaster, AL). DE-81 ion exchange disks were obtained from Whatman Ltd. (Clifton, NJ). [^{3}H]PDBu (777 Gbq/mmol) was purchased from New England Nuclear (Boston, MA). [^{3}H]Bryo was prepared as previously described for [^{3}H]Bryostatin 4 (Lewin et al., 1992). [^{14}C]DPPC (4.0 Gbq/mmol) was obtained from Amersham Pharmacia Biotech.

Results

The isolated C1-RasGRP was expressed as a GST-fusion protein in *E. coli* and partially purified as described under *Experimental Procedures*. Using C1-RasGRP in the presence

of phosphatidylserine, we analyzed [^{3}H]PDBu binding as a function of phorbol ester concentration. As shown in Fig. 1, C1-RasGRP bound [^{3}H]PDBu with high affinity; the dissociation constant (K_d) observed in the presence of 100 μg/ml phosphatidylserine was 0.58 ± 0.08 nM (n = 4). Similar K_d values were obtained using the whole RasGRP protein (K_d = 0.49 ± 0.09, n = 4). This affinity was similar to what we have observed previously for PKCα (0.2 nM) (Kazanietz et al., 1993).

To determine the structure-activity relations for ligand recognition by C1-RasGRP, we performed competition binding studies using a range of structurally diverse, high-affinity ligands for other C1 domains. The ligands included 12-deoxyphorbol esters, daphnane derivatives, indole alkaloids, and DAG. The results are shown in Table 1. Compared with PKC, C1-RasGRP bound these other ligands with generally similar structure-activity relations, but with noteworthy differences. Thus, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) was more potent than had been observed with any of the PKC isoforms (Kazanietz et al., 1993) or β-chimaerin (Caloca et al., 1997); compared with PKCα, OAG was 20-fold more potent for binding to C1-RasGRP. In addition, (−)-octylindolactam V showed enhanced potency compared with (−)-indolactam V, as expected from its greater lipophilicity. But that increase (4.6-fold) was less than for other receptors (e.g., 21-fold for PKCα) (Kazanietz et al., 1993).

The macrocyclic lactone Bryo is an ultrapotent PKC activator with antitumor biological activity, currently in clinical trials as a cancer chemotherapeutic agent (Pluda et al., 1996). Bryo is of further interest because of its functional antagonism of a subset of phorbol ester-mediated biological responses (Blumberg and Pettit, 1992). [^{3}H]Bryo binding was measured in the presence of phosphatidylserine/Triton X-100 micelles rather than phosphatidylserine alone because of methodological constraints with this ligand (Kazanietz et al., 1994). Under these conditions, [^{3}H]Bryo bound C1-RasGRP with a K_d of 1.01 ± 0.10 nM (n = 4). This value is similar to that of PKCα (1.6 nM) (Kazanietz et al., 1994) and of higher affinity than β2-chimaerin (8.5 nM) (Caloca et al., 1997).

Calphostin C has been suggested to be a selective PKC inhibitor, which acts at the regulatory domain (Bruns et al., 1991). It is now clear that calphostin C is not selective for PKC but also targets other C1 domains, such as those of the chimaerins and Unc-13 (Areces et al., 1994; Caloca et al., 1997). We have found here that calphostin C additionally inhibited PDBu binding to the C1-RasGRP, with an ED_{50} of 2.00 ± 0.30 μM (Fig. 2). Because calphostin C inhibition depends on photoactivation, the absolute potency can be affected by changes in exposure to fluorescent light during the course of the binding experiment. Therefore, we ran in parallel inhibition experiments with two other well known targets of calphostin C: PKCα and the isolated C1b domain of PKCδ. We incubated samples in the presence of calphostin C under fluorescent light for 15 min before addition of [^{3}H]PDBu. Under these experimental conditions, the C1b domain of PKCδ was inhibited by calphostin C with potency similar to that for C1-RasGRP (ED_{50} = 1.47 ± 0.31 μM), whereas PKCα displayed modestly weaker sensitivity (ED_{50} = 5.6 ± 1.6 μM).

Acidic phospholipids such as phosphatidylserine are important cofactors in the phorbol ester/DAG binding to PKC (Newton and Johnson, 1998). The membrane binding affinity of PKC is conferred not only by the C1 domains but also by other structures in the protein such as the C2/C2′ domain and the pseudosubstrate region (Newton and Johnson, 1998). To further explore the role of phospholipids in the interaction of phorbol esters with C1-RasGRP, we examined the effect of different phospholipids on the reconstitution of the RasGRP/PDBu binding. Because it has been reported that calcium increases the affinity of some PKCs for phospholipids, we

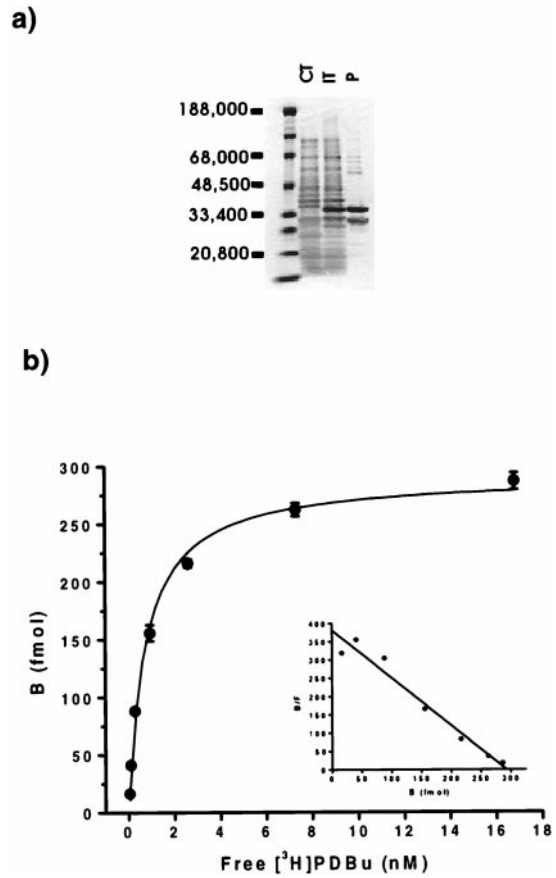

Fig. 1. Saturation curve and Scatchard plot (inset) for [^{3}H]PDBu binding to C1-RasGRP. In a, C1-RasGRP was affinity-purified with glutathione-Sepharose 4B as described under *Experimental Procedures*. Samples were subjected to electrophoresis, and then the gels were stained with Coomassie Blue. Positions of the molecular weight marker (in daltons) are shown on the left. CT, total lysate control; IT, total lysate after induction; P, purified protein. In b, binding assays were performed on the purified protein (a) using sonicated dispersions of phosphatidylserine (100 μg/ml). Binding (B) is expressed in femtomoles of [^{3}H]PDBU. Data shown are from a single experiment. Three additional experiments gave similar results.

TABLE 1
Structure-activity analysis of binding to the C1 domain of RasGRP
The ID_{50} values were determined from competition curves, and the corresponding K_i values were calculated as indicated under *Experimental Procedures*. Values represent the mean ± S.E. of n experiments per group.

Compound	K_i	n
	nM	
DPP	0.50 ± 0.10	3
Prostratin	16.2 ± 2.4	3
Mezerein	1.01 ± 0.04	4
(−)Indolactam V	8.1 ± 0.7	4
(−)octylindolactam V	1.75 ± 0.32	3
Thymeleatoxin	4.7 ± 0.5	3
OAG	42.8 ± 1.8	3

wanted to include the calcium responsive element of RasGRP in these studies. Therefore, we used the C1 domain plus the EF-hand motif (C1EF-RasGRP). We varied the phospholipid composition in the binding assay using liposomes consisting of 5 or 20 mol% of one of the following phospholipids: POPS, POPA, POPE, and POPG. The remaining phospholipid was neutral POPC, which yields a 1000 μM final phospholipid concentration. Figure 3 summarizes the results. POPC alone reconstituted binding to 33% of that observed in the presence of 20 mol% POPS. POPE produced no additional enhancement beyond that supported by POPC alone. The anionic phospholipids POPS, POPA, and POPG at 5 mol% caused similar enhancements of PDBu. At 20 mol%, POPS further increased this binding, whereas no additional increment was observed for POPA or POPG.

We performed similar reconstitution experiments on PKCα to permit direct comparison with our results for RasGRP. The molar concentrations of binding sites for PKCα used in these experiments were similar to the concentrations used for C1EF-RasGRP (Fig. 3). Figure 4 shows the reconstitution of PDBu binding to PKCα for lipid vesicles of different compositions. Maximal reconstitution of binding to PKCα was observed in the presence of 20 mol% POPS. Reducing the content of POPS in the lipid vesicles to 5 mol% decreased the binding level by 70%, indicating a greater POPS requirement for PDBu binding to PKCα than to C1EF-RasGRP (Fig. 4). In the presence of POPA, the level of PDBu binding reconstitution also showed a strong dependence on the lipid content in the vesicles. At 5 mol% POPA, binding levels reached only 47% of the binding reconstituted in the presence of 20 mol% POPA. For POPG, weak reconstitution of PDBu binding was observed, with no dependence on the mol% content of the vesicles at the concentrations tested. For 20 mol% POPG, maximum binding reached 31% of the full reconstitution observed in the presence of 20 mol% POPS. POPE produced only 7 to 8% of the level of PDBu binding compared with POPS, and POPC alone supported only 4% of this level. Together, these results emphasize the greater dependence of PKCα on anionic phospholipids.

For POPS and POPA, the two phospholipids that induced high levels of PDBu binding for both C1EF-RasGRP and PKCα, we determined the concentration dependence for reconstitution. Fixing the content at 5 or 20 mol%, we varied the molar concentration from 10 to 3000 μM (Fig. 5). The results were normalized to the maximal level of reconstitution, which for both C1EF-RasGRP and PKCα was provided by 20 mol% phospholipid at 3000 μM. The concentration of 20 mol% POPS required to reconstitute PDBu binding by 50% (EC_{50}) for C1EF-RasGRP was 266 $\pm$ 25 μM. PKCα showed a substantially lower EC_{50} (21.4 $\pm$ 1.4 μM) and reached saturation at about 300 μM. Although reducing the POPS content of the lipid vesicles to 5 mol% caused a 300-fold decrease in its ability to reconstitute binding to PKCα (EC_{50} at 5 mol% = 6500 $\pm$ 1200 μM), it only produced a 3-fold reduction for C1EF-RasGRP (EC_{50} at 5 mol% = 797 $\pm$ 89 μM). These results clearly demonstrate the higher dependence of PKCα compared with C1EF-RasGRP on POPS. Similar patterns of sensitivity were observed for PKCα and C1EF-RasGRP in the presence of 5 or 20 mol% POPA. At 20 mol% POPA, the

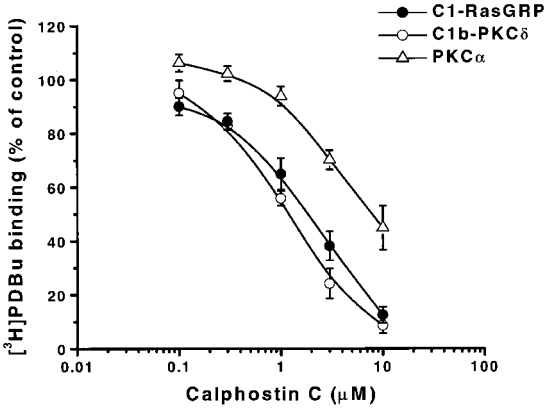

Fig. 2. Inhibition of [^{3}H]PDBu binding by calphostin C. Increasing concentrations of calphostin C were used to compete 1 nM [^{3}H]PDBu. Samples were preincubated in the presence of calphostin under ordinary fluorescent light for 15 min before starting the binding assay. Values represent the mean $\pm$ S.E. of three experiments per group, with triplicate determinations in each experiment.

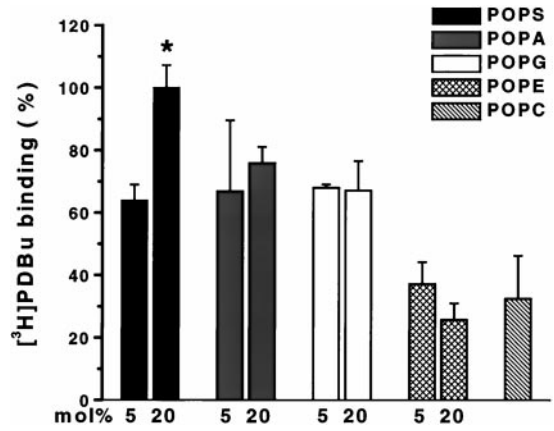

Fig. 3. Effect of phospholipid composition on [^{3}H]PDBu binding to C1EF-RasGRP. Binding of [^{3}H]PDBu was performed using 1 nM radioligand in the presence of 0.1 mM $CaCl_2$ and the phospholipid vesicles (LUVs) at a final concentration of 1000 μM. LUVs were composed of either 5 or 20 mol% of the indicated phospholipid (POPS, POPA, POPE, and POPG) and POPC as the remainder. The results are expressed as percentage of the binding at 100 mol% POPS (123 $\pm$ 9 fmol of [^{3}H]PDBu bound). Data represent the mean $\pm$ S.E. of three experiments, with triplicate determinations in each experiment. *$P < .05$, compared with paired 5 mol% values.

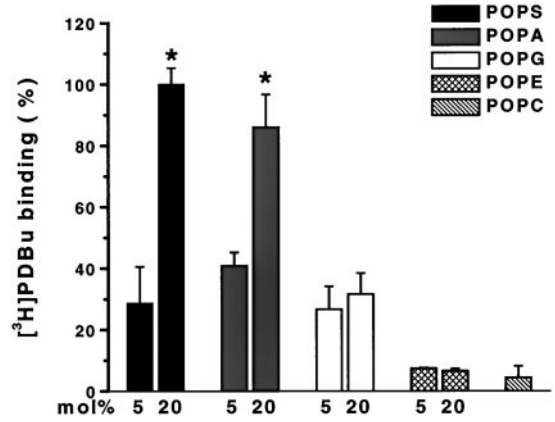

Fig. 4. Effect of phospholipid composition on [^{3}H]PDBu binding to PKCα. Binding of [^{3}H]PDBu was performed using 1 nM radioligand in the presence of 0.1 mM $CaCl_2$ and the phospholipid vesicles (LUVs) at a final concentration of 1000 μM. LUVs were composed of either 5 or 20 mol% of the indicated phospholipid (POPS, POPA, POPE, and POPG) and POPC as the remainder. The results are expressed as percentage of the binding at 100 mol% POPS (112 $\pm$ 6 fmol of [^{3}H]PDBu bound). Data represent the mean $\pm$ S.E. of three experiments, with triplicate determinations in each experiment. *$P < .05$, compared with paired 5 mol% values.

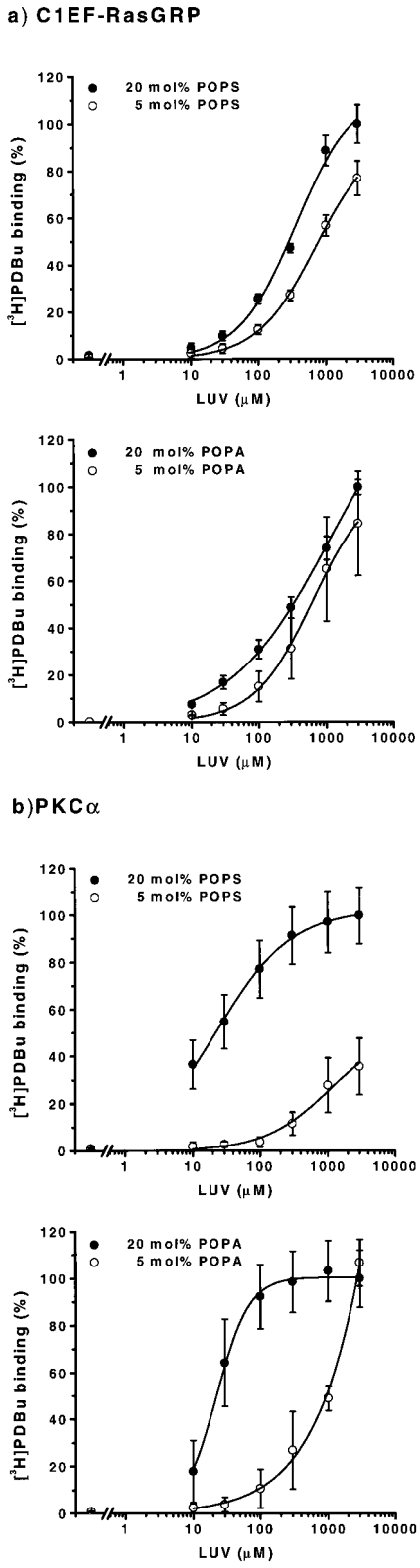

Fig. 5. Reconstitution of [^{3}H]PDBu binding to C1EF-RasGRP (a) and PKCα (b) by POPS and POPA. Binding of [^{3}H]PDBu was performed in the presence of 1 nM radioligand and 0.1 mM $CaCl_2$. Dose-response curves were performed at either 5 or 20 mol% anionic phospholipid (POPS or POPA) with POPC as the remainder. The results are expressed as percentage of the binding at 3000 μM of either 20 mol% of POPS (C1EF-RasGRP = 138 ± 11 fmol of [^{3}H]PDBu bound; PKCα = 114 ± 14 fmol of [^{3}H]PDBu bound) or 20 mol% POPA (C1EF-RasGRP = 126 ± 4 fmol of [^{3}H]PDBU bound; PKCα = 92 ± 11 fmol of [^{3}H]PDBu bound). Values represent the mean ± S.E. of four experiments per group.

EC_{50} levels for binding reconstitution were similar to those observed at 20 mol% POPS. PKCα had an EC_{50} of 22.3 ± 3.6 μM, whereas the EC_{50} for C1EF-RasGRP was 258 ± 33 μM. The EC_{50} value at 5 mol% POPA was 2.2-fold higher than that at 20 mol% POPA for C1EF-RasGRP. For PKCα it was 33-fold higher. Binding to PKCα was therefore somewhat better reconstituted by 5 mol% POPA than by 5 mol% POPS. Thus, 5 mol% POPA at 3000 μM total lipid fully reconstituted PKCα, whereas 5 mol% POPS did not (Fig. 5b).

A prediction derived from these studies was that the observed differences in binding reconstitution under different lipid conditions should reflect, among other factors, a change in the affinities of both RasGRP and PKCα for PDBu. To test this hypothesis, we determined the extent of [^{3}H]PDBu binding as a function of PDBu concentration under two different conditions of reconstitution: 5 mol% POPS at 1000 μM and 20 mol% POPA at 100 μM. For comparison with our usual binding conditions, saturation experiments were also performed in the presence of bovine brain phosphatidylserine. Figure 6 summarizes the results. In the presence of saturating concentrations of bovine brain phosphatidylserine, PKCα bound PDBu with 5.8-fold higher affinity than did C1EF-RasGRP. However, changes in the lipid composition induced substantial changes in the relative affinities. Thus, at 5 mol% POPS, C1EF-RasGRP preferentially bound PDBu with 2.0-fold stronger affinity than did PKCα. In contrast, at 20 mol% POPA, PKCα bound PDBu with 10-fold stronger affinity than did C1EF-RasGRP. The overall shift in selectivity between these two receptors was thus changed by 20-fold as a function of lipid composition.

RasGRP possesses a pair of calcium binding sites, similar to EF-hands, which represent the calcium binding motif in the molecule. Recombinant GST-RasGRP fusion proteins expressed in *E. coli* have been shown to bind ^{45}Ca in vitro (Ebinu et al., 1998). In order to elucidate whether this domain serves as a calcium-responsive element modulating phorbol ester binding, we determined dose response curves for PDBu binding as a function of free calcium under two different lipid conditions. For comparison, we included as a control the response of PDBu binding to PKCα on variations in the calcium level. Figure 7 shows that, for PKCα, 100 mol% POPS resulted in almost full

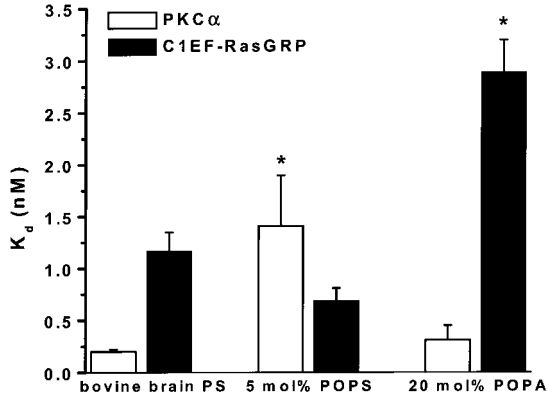

Fig. 6. Dissociation constants (K_d) of C1EF-RasGRP and PKCα for [^{3}H]PDBu binding in the presence of different phospholipids. K_d values for each protein were calculated from saturation binding experiments using [^{3}H]PDBu as ligand in the presence of 0.1 mM $CaCl_2$. Three different lipid mixtures were used: 100 μg/ml bovine brain phosphatidylserine, 5 mol% POPS at 1000 μM, and 20 mol% POPA at 100 μM. Values represent the mean ± S.E. of three to five experiments per group. $^*P < .05$, compared with bovine brain phosphatidylserine.

reconstitution of PDBu binding, even in the absence of calcium. At 5% mole fraction of POPS, PKCα showed a concentration-dependent response with an EC_{50} of 3.2 ± 2.1 μM calcium. At the highest concentration of calcium tested (1000 μM), the level of binding reached only a 18% of the reconstitution observed at 100 mol% POPS. For C1E-RasGRP, both lipid compositions induced binding reconstitution that was calcium-independent (Fig. 7). The failure of calcium to increase the level of binding under conditions of partial reconstitution (5 mol% POPS) argues against a role of the EF-hands of RasGRP in this process.

Discussion

Our emerging understanding of signal transduction is that many receptors are coupled to multiple, branched pathways. The biological outcome of a plasma membrane signaling event reflects a complex integral of these pathways in the context of the individual cell. Thus, the lipophilic second messenger *sn*-1,2-DAG interacts with the C1 domains found in five families of proteins—PKC, PKD, chimaerin, Munc13, and RasGRP—with a total of 20 members identified in these families so far. Two members of the transient release protein (TRP) family (Hofmann et al., 1999) have also been described as being responsive to DAG, although this response must reflect interaction at a site other than a C1 domain.

Previous studies have established the DAG modulation of RasGRP mediated by the C1 domain motif. For example, in rodent fibroblasts, PMA treatment induced membrane partitioning of ectopically expressed RasGRP (Ebinu et al., 1998; Tognon et al., 1998), and the isolated C1 domain of RasGRP was shown to bind phorbol esters in vitro (Ebinu et al., 1998). In the present work we have characterized the C1 domain of RasGRP as a high affinity receptor for phorbol esters/DAG and have explored the phospholipid requirements for this binding.

Our studies on the structure-activity relations for the C1 domain of RasGRP showed both similarities and differences in phorbol ester ligand selectivity compared with PKCs. The differences are comparable in magnitude to those between the novel and conventional subfamilies of PKCs. Likewise, the PKC inhibitor calphostin C showed comparable potency to inhibit phorbol ester binding to the C1 domain of RasGRP and to PKC, reflecting their homologous C1 domains. In contrast to these similarities, the phospholipid cofactor requirements showed considerable differences between C1-RasGRP and PKC. Typical of the PKCs, PKCα displayed a strong dependence on acidic phospholipids. In contrast, RasGRP showed much reduced requirements. Indeed, even in the absence of any acidic phospholipid, there was considerable reconstitution of PDBu binding to the C1 domain of RasGRP. These differences in lipid requirement between RasGRP and PKC imply that the cellular lipid environment could modify DAG responses at the cellular level. Thus, not only the level of DAG generation or phorbol ester treatment but also the lipid cofactors might play a role in the modulation of RasGRP and Ras.

Although a requirement for phosphatidylserine (Orr and Newton, 1992) or other acidic phospholipids (Lee and Bell, 1989) in PKC activation has been demonstrated, the phospholipid binding motifs on the PKC molecule are still being characterized. The C2 domain, or calcium binding site of the conventional PKC, has been suggested as one of the sites for anionic phospholipid binding (Newton and Johnson, 1998;

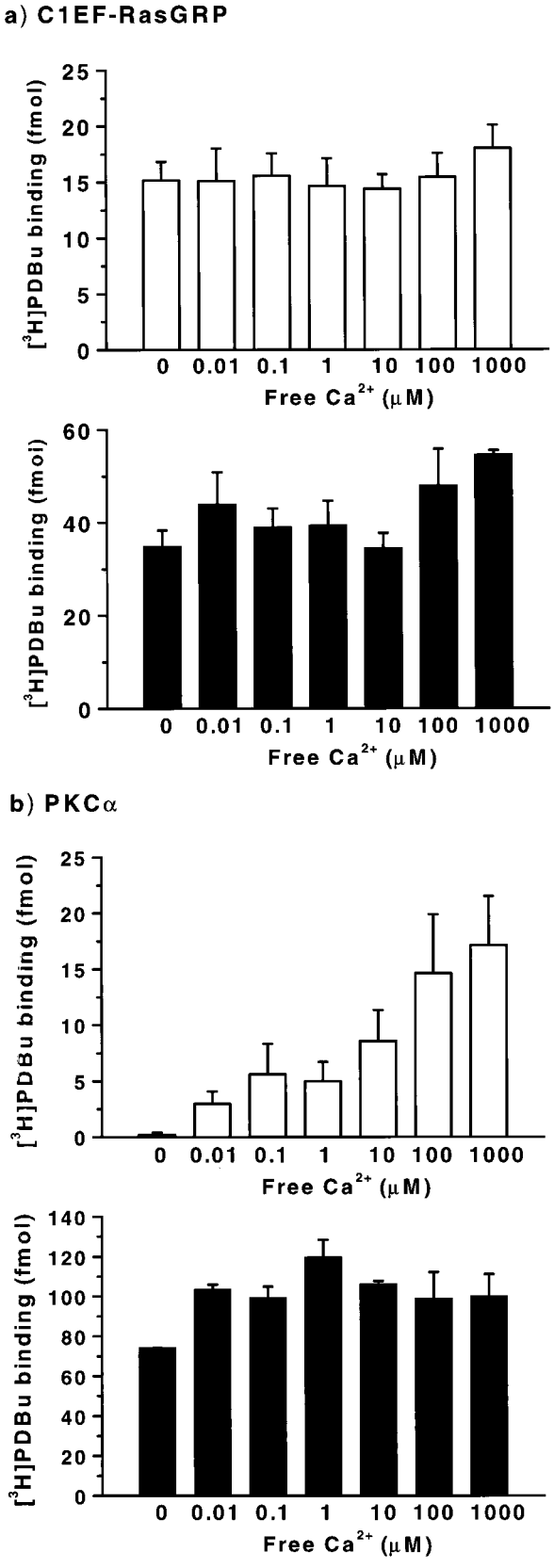

Fig. 7. Influence of the concentration of free calcium on [^{3}H]PDBu binding to C1EF-RasGRP (a) and PKCα (b). Binding to [^{3}H]PDBu was performed at 1 nM radioligand concentration in the presence of either 5 (□) or 100 (■) mol% POPS and increasing concentrations of free calcium. The results are expressed as femtomoles of [^{3}H]PDBu bound. Data represent the mean ± S.E. of three to four experiments per group.

Medkova and Cho, 1999). For the novel PKCs, which lack the C2 domain, a C2-like region or E motif exists (Sossin and Schwartz, 1993), and this domain has been shown to be responsible for binding to phospholipids independently of calcium (Sossin et al., 1996). Neither of these domains, C2 or C2-like motif, has a homolog on the RasGRP molecule. Thus, the differences between RasGRP and PKC in phospholipid requirement are not surprising. On the other hand, RasGRP possesses a paired EF-hand-like motif (Ebinu et al., 1998), which could be a calcium-responsive element in the protein, playing a qualitatively similar role to the C2 domain of the conventional PKCs. Studies using mutants of RasGRP affecting the first or the second EF-hand motif pointed to the second EF-hand as the higher affinity site for binding calcium (Ebinu et al., 1998). On the other hand, the EF-hands were not required for the transforming activity of RasGRP (Tognon et al., 1998). Our studies showed that the EF-hand motif does not confer calcium dependence to the phorbol ester-RasGRP binding interaction.

For the Ras pathways, at least three classes of DAG receptors are positioned to play a modulatory role: PKC, chimaerins, and RasGRP. PKC has been implicated in the activation of the Ras effector Raf-1 and thereby the MAP kinase cascade (Sozeri et al., 1992; Marais et al., 1998). Chimerins are GTPase-activating proteins that specifically act on Rac (Kozma et al., 1996), a member of the Ras-like small GTPases thought to function in concert with Ras (Khosravi-Far et al., 1995; Qiu et al., 1995). Finally, RasGRP, which is mainly expressed in brain and lymphoid tissues, provides a direct couple between DAG generation and Ras activation (Ebinu et al., 1998; Tognon et al., 1998).

The multiplicity of DAG signaling pathways highlights the difficulty of functionally linking different DAG populations within the cell to particular biochemical or biological processes. Likewise, it is difficult to ascribe a particular cellular response to a given pharmacological treatment. Clearly, many effects of PMA that have been attributed to PKCs need to be re-examined in light of the emerging novel targets for the phorbol esters. However, it is not clear that all C1 domain proteins have similar access to different pools of DAG within the cell. Furthermore, subtle structural differences between C1 domains, as well as the evidence presented here showing unique cofactor preferences, argue that the different DAG-responsive systems might be regulated differentially in normal cells. It also seems likely that differential regulation might be achieved using pharmacological approaches in either the experimental or clinical settings.

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