The role of the protein kinase C (PKC) family of serine/threonine kinases in cellular differentiation, proliferation, apoptosis, and other responses makes them attractive therapeutic targets. The activation of PKCs by ligands in vivo varies depending upon cell type; therefore, methods are needed to screen the potency of PKCs in this context. Here we describe a genetically encoded chimera of native PKCδ fused to yellow- and cyan-shifted green fluorescent protein, which can be expressed in mammalian cells. This chimeric protein kinase, CY-PKCδ, retains native or near-native activity in the several biological and biochemical parameters that we tested. Binding assays showed that CY-PKCδ and native human PKCδ have similar binding affinity for phorbol 12,13-dibutyrate. Analysis of translocation by Western blotting and confocal microscopy showed that CY-PKCδ translocates from the cytosol to the membrane upon treatment with ligand, that the translocation has similar dose dependence as that of endogenous PKCδ, and that the pattern of translocation is indistinguishable from that of the green fluorescent protein-PK Cδ fusion well characterized from earlier studies. Treatment with phorbol ester of cells expressing CY-PKCδ resulted in a dose-dependent increase in FRET that could be visualized in situ by confocal microscopy or measured fluorometrically. By using this construct, we were able to measure the kinetics and potencies of 12 known PKC ligands, with respect to CY-PKCδ, in the intact cell. The CY-PKCδ chimera and the in vivo assays described here therefore show potential for high throughput screening of prospective PKCδ ligands within the context of cell type.

The members of the protein kinase C (PKC) family of serine/threonine kinases represent critical signaling molecules in the cell. PKCs are activated by the second messenger sn-1,2-diacylglycerol (DAG) or by their ultrapotent analogues, the phorbol esters. Because PKCs activate downstream cellular pathways that regulate cell proliferation, apoptosis, differentiation, and other responses, they are important therapeutic targets. Indeed, a number of compounds targeting protein kinase C are currently at different stages of drug development. Examples include LY333531, being evaluated for diabetic retinopathy and anti-angiogenesis (1, 2), prostratin and dPP, being evaluated for AIDS chemotherapy (3, 4), and bryostatin 1 and ingenol 3-angelate, being evaluated as cancer chemotherapeutic agents (5–7).

The known isoforms of protein kinase C include the classical PKCs (α, β, γ, and δ), which are calcium-dependent; the novel PKCs (ε, η, and θ), which are calcium-independent, and the atypical PKCs (ζ and η), which are not activated by DAG or the phorbol esters (8, 9). The classical and novel PKCs each contain one or more highly conserved C1 domains, zinc finger motifs that act as the binding site for DAG and the phorbol esters (10, 11).

Biological assays of PKC activation have limited predictive value for the understanding of structure-activity relationships because of extensive modulation of PKC behavior by the cellular environment, which includes contributions from lipids, calcium, and cellular binding proteins and differences in the ability of different potential drugs to penetrate the cell membrane and activate the kinases (12, 13). Therefore, assays that can be performed with intact cells to measure PKC activation are urgently needed.

Genetically encoded fluorescent proteins that undergo changes in fluorescent resonance energy transfer (FRET) are a powerful tool for in situ visualization of intracellular events and are increasingly finding application in high throughput screening (HTS) of potential drugs. These include indicators for the second messengers calcium (14, 15), cAMP, and cGMP (16) among others. Sensors that are activated by protein kinases, and therefore indicative of protein kinase activity, have also been developed. These sensors generally consist of portions of a kinase substrate fused to a fluorescent protein in an arrangement such that a conformational change occurs upon phosphorylation, resulting in a change in FRET. These sensors include phoceses (17) as well as downstream pseudosubstrates for cAMP-dependent protein kinase (18), PKC (19), Rac (20), Src, Abl, and epidermal growth factor (21) among others. In several instances, sensors utilize the full protein kinase fused to fluorescent proteins. Examples are modified protein kinase B/Akt (22) and modified MAPK2 (23).

Here we describe a genetically encoded fusion of native protein kinase Cδ with flanking CFP and YFP peptides. This
chimeric protein kinase retains near-native binding affinity and cellular translocation activity; treatment of cells expressing this chimeric protein kinase results in a dose-dependent change in FRET that can be visualized in situ or measured fluorimetrically in a high throughput format. By using this chimera, we were able to analyze the in vivo pharmacokinetics and pharmacodynamics of 12 well characterized PKC ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—PMA, dPP, thymeleatoxin, prostratin, phorbol 12,13-di-octanoate (diC8), phorbol 12,13-dihexanoate (diC6) were synthesized as described previously (26) and cloned into the XhoI and BamHI sites of Escherichia coli DH5α-MCR was grown at 37 °C in Luria-Bertani (LB) medium either in broth or on agar. Kanamycin was used in LB medium at a concentration of 50 μg/ml. Recombinant DNA transformation of E. coli was done according to the heat-shock protocol (25).

**Construction of Plasmids**—The mouse PKCγ gene was excised from pGFP-PKCγ (26) and cloned into the XhoI and BamHI sites of pECFP-N1 (Clontech), creating pCFP-PKCγ mixture (250 μg/ml phosphatidyl choline, 100 μg/ml bovine IgG, and variable concentrations of [3H]-PDBu. CytoScint ES (ICN Biomedicals, Aurora, OH) was added to all concentration of free [3H]PDBu; verified by triplicate sequencing of the insert (DNA minicore, Center for Cancer Research, NCI, National Institutes of Health, Bethesda).

**Molecular Biology Methods**—Plasmid DNA was purified chromatographically using commercial kits from Qiagen (Valencia, CA). PCR's were performed using PfuUltra high fidelity DNA polymerase (Strategenes, La Jolla, CA) according to the manufacturer's directions. All experiments were performed the day following transfection. Escherichia coli DH5α-MCR was grown at 37 °C in Luria-Bertani (LB) medium either in broth or on agar. Kanamycin was used in LB medium at a concentration of 50 μg/ml. Recombinant DNA transformation of E. coli was done according to the heat-shock protocol (25).

**Construction of Plasmids**—The mouse PKCγ gene was excised from pGFP-PKCγ (26) and cloned into the XhoI and BamHI sites of pECFP-N1 (Clontech), creating pCFP-PKCγ. The EYFP gene was amplified from pEYFP-C1 (Clontech) using forward and reverse primers that incorporated NheI and XhoI sites, respectively, and the amplicon was introduced into pCFP-PKCγ, creating pCFP-PKCγ. The clone was verified by triplicate sequencing of the insert (DNA minicore, Center for Cancer Research, NCI, National Institutes of Health, Bethesda).

**Binding of [3H]PDBu—[3H]PDBu binding was measured by using the polyethylene glycol precipitation assay developed by our laboratory (27). Recombinant human PKCγ was obtained from Invitrogen, whereas CY-PKCγ was expressed in CHO-K1 cells and purified by immunoprecipitation with mouse anti-GFP monoclonal antibody (Roche Applied Science) and protein A/G-agarose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's directions. The assay mixture (250 μl) contained 50 mm Tris-Cl, pH 7.4, 100 μg/ml phosphate-buffered saline, 4 μg/ml bovine IgG, and variable concentrations of [3H]-PDBu. Incubation was carried out at 37 °C for 5 min. Samples were chilled on wet ice for 5 min followed by the addition of 200 μl of 35% polyethylene glycol in 50 mm Tris-HCl, pH 7.4. The samples were incubated on wet ice for another 10 min. The tubes were centrifuged at 12,400 × g for 15 min at 4 °C. 100-μl aliquots of the supernatant were pipetted into scintillation vials for quantitation of the total bound [3H]-PDBu. CytoScint ES (ICN Biomedicals, Aurora, OH) was added to all scintillation vials prior to counting. Nonspecific binding was determined by using an excess (40 μM) of nonradioactive PDBu. Specific binding was calculated as the difference between total and nonspecific binding.

In a typical binding assay, seven concentrations of [3H]PDBu were used. The Kd value for the ligand was calculated via nonlinear multiple regression to the binding Equation 1,

\[
B_L = \frac{B_{max}L}{K_d + L} 
\]

where \(B_L\) represents the concentration of bound [3H]PDBu; \(L\) is the concentration of free [3H]PDBu; \(B_{max}\) is the theoretical maximum receptor occupancy, and \(K_d\) is the dissociation constant. Values represent the mean of triplicate experiments, as indicated, with triplicate determinations of each point in each experiment.

**Dose-response Curves as Measured Fluorimetrically**—CHO-K1 cells were grown in T-75 flasks and transfected as described above. The day after transfection, cells were washed in Dulbecco's phosphate-buffered saline, trypsinized for <1 min, and collected in Dulbecco's phosphate-buffered saline supplemented with 10% fetal bovine serum and 10 mm glucose. The suspension was centrifuged at 700 × g for 10 min; the supernatant was aspirated, and the cells were resuspended in the same buffer. The cell suspension was pipetted into a cuvette for each series of measurements at each ligand concentration. Ligand was diluted in dimethyl sulfoxide and added to the cell suspension in a 1:100 dilution. Fluorescence was measured using a FluoroMax-3 fluorimeter (Jobin Yvon Horiba, Edison, NJ) that was kept at 37 °C with a circulating water bath. Samples were excited at 435 nm, and the emission ratio was measured as the ratio of emission at 474 nm to emission at 527 nm after correction for background fluorescence by the ligand. The change in emission ratio for each concentration of ligand was determined as the emission ratio following exposure to drug minus the emission ratio prior to exposure. Dimethyl sulfoxide alone was demonstrated to have no effect on the emission ratio. The fractional response, \(Y\), for each time point was calculated as the change in emission ratio at that concentration of ligand divided by the change in emission ratio after addition of the highest concentration of ligand used.

These data were fit to two different equations. First, the fractional response, \(Y\), for the assay end points was plotted as a function of free ligand and fitted by Levenberg-Marquardt nonlinear regression to the Hill Equation 2,

\[
Y = \frac{1}{1 + (\frac{ED_{50}}{L})^{n}} 
\]

where \(Y\) is the fractional response as described above; \(L\) is the concentration of free ligand, and \(n\) is the Hill coefficient. Values represent the mean of triplicate experiments as indicated.

Second, the kinetic data, consisting of fractional response, \(Y\), as a function of time \(t\) and of ligand concentration \(L\), were fitted by Levenberg-Marquardt nonlinear regression to the association kinetic Equation 3,

\[
Y = B_L \frac{ED_{50}}{ED_{50} + L} (1 - e^{-t/ED_{50}^{1/n}}) 
\]

where \(Y\) represents the fractional effect; \(L\) represents the free ligand concentration; \(ED_{50}\) is the ligand concentration yielding half-maximal effect, and \(k\) is a rate constant (28). Because we are describing the rate and potency of biological effect, we have used the term \(ED_{50}\) in place of \(K_d\) and \(k\) in place of \(k_a\).

**Analysis of Membrane Translocation**—Western blotting and subsequent analysis were performed as described previously (29). In summary, cultured CHO-K1 cells were treated with varying concentrations of PMA diluted in MeSO. Following treatment, cells were washed, harvested into 20 mm Tris-HCl, pH 7.4, containing protease inhibitor mixture (500 μM aminothiolbenzenesulfonfyl fluoride hydrochloride, 500 μM EDTA, 1 μM E-64, 1 μM leupeptin, and 1 μg/ml aprotinin) from Calbiochem, collected with a cell scraper, and lysed by sonication. Total cell lysates were fractionated by ultracentrifugation. The soluble (cytosolic) fraction represents the supernatant after a 1-h centrifugation at 200,000 × g. The Triton X-100 soluble (membrane) fraction was prepared by a 1-h extraction of the pellet with the same buffer containing 1% Triton X-100 followed by sonication and centrifugation for 1 h at 200,000 × g.

The protein samples were subjected to SDS-PAGE and were transferred to nitrocellulose membranes (Invitrogen). Blotting of membranes was performed using rabbit anti-PKCγ polyclonal (Santa Cruz Biotechnology), rabbit anti-PKCα polyclonal (Santa Cruz Biotechnology), or mouse anti-GFP monoclonal antibody (Roche Applied Science). Following washing, membranes were incubated with horseshadish peroxidase-conjugated goat secondary antibody (Bio-Rad), developed with ECL Western blot detecting reagent (Amersham Biosciences), and exposed to film. Bands were quantitated densitometrically using Scion Image (Scion Corp., Frederick, MD). In a typical membrane translocation assay, five concentrations of ligand were used. Values for each point represent the average value for that concentration obtained from triplicate experiments. The \(ED_{50}\) for the ligand was calculated using nonlinear multiple regression to the Hill equation (Equation 2).
RESULTS

Construction of CY-PKCb—The docking of ligand with PKC is thought to lead to a change in PKC tertiary structure such that the pseudosubstrate region of PKC is released from the catalytic site of the enzyme. Provided this conformational change sufficiently altered the spatial alignments of, or distance between the N and C termini, we might be able to monitor the ligand binding in the cellular environment by a change in FRET using a chimeric fusion of PKC flanked by YFP and CFP at the N and C termini, respectively. Therefore, we cloned the PKC clone of pCFP-PKC into pECFP-N1, forming pCFP-PKC. The resulting plasmid, pCY-PKC, was analyzed by restriction digestion and sequencing to verify that the construction was successful.

Verification That CY-PKCb Behaves Like Wild-type PKCb in Intact CHO Cells—A potential problem with the PKC fusion construct is that the addition of the CFP and YFP at the N and C termini might affect the cellular behavior of the PKC or its binding of phorbol ester or DAG. Three approaches were used to assess this issue. First, we compared the in vitro binding affinities of [3H]PDBu to CY-PKCb and human PKCb (Invitrogen). CY-PKCb was isolated from transfected CHO cells by immunoprecipitation; binding assays were performed as described under “Experimental Procedures.” The $K_d$ value obtained for CY-PKCb was 0.98 ± 0.37 nM ($n = 3$) and for human PKCb it was 0.52 ± 0.05 nM ($n = 4$) (Fig. 1). These $K_d$ values indicate that CY-PKCb exhibits a binding affinity similar to that of wild-type PKCb.

Second, we qualitatively and quantitatively examined the dose-dependent translocation of CY-PKCb and the endogenous PKCb from the soluble fraction to the membrane fraction. CHO cells transfected with CY-PKCb were treated with five concentrations of PMA, incubated for 20 min, harvested, and fractionated into cytosolic and membrane fractions as described under “Experimental Procedures.” A 20-min incubation time with PMA had been shown to be sufficient to observe translocation of PKCb but not so long as to induce down-regulation of PKCb expression (29). The fractions were analyzed via SDS-PAGE and Western blotting with anti-PKCb or anti-GFP antibodies. Treatment with PMA induced roughly similar translocation of endogenous PKCb and the CY-PKCb from the cytosolic fraction to the membrane fraction, as illustrated for a representative experiment (see Fig. 2). There was, however, more CY-PKCb present in the membrane fraction of the control (Me2SO-treated) cells than was the case for the endogenous PKCb, and the highest concentration of PMA caused full translocation of endogenous PKCb but not of CY-PKCb (see Fig. 2A). A possible explanation for this difference is the level of overexpression of the exogenous CY-PKCb.

In order to compare quantitatively the sensitivity of translocation of the CY-PKCb and the endogenous PKCb in response to ligand, the Western blots were analyzed densitometrically, and the average extents of translocation (three experiments) were plotted as a function of PMA concentration, and $E_{D50}$ values were determined using the Hill equation as described under “Experimental Procedures” (see Fig. 2). The $E_{D50}$ values agreed with a factor of 2, and for endogenous PKCb, $E_{D50} = 328 ± 59$ nM, and for CY-PKCb, $E_{D50} = 637 ± 183$ nM.

Third, we examined the pattern of translocation of CY-PKCb. The kinetics of translocation and final cellular destination of transfected PKC vary depending upon the ligand and the PKC isozyme; this is especially noted with PKCb (24, 30). We wished to confirm that the kinetics of translocation of CY-PKCb were similar to that of the GFP-PKCb protein that is typically used in translocation studies. By using confocal laser scanning microscopy, we found that the real time translocation kinetics and cellular localization of GFP-PKCb and CY-PKCb following treatment with PMA were indistinguishable ($n = 4$ experiments) (see Fig. 3).

Characterization of FRET Signal in Intact Cells by Fluorimetry—Binding of ligand to CY-PKCb and its subsequent activation is expected to induce a change in protein conformation, potentially altering the spatial relationship between the N-terminal YFP and C-terminal CFP fluorophores. This would be measurable as a change in FRET. We wished to quantify the change, if any, in FRET and to determine whether a relationship could be drawn between change in FRET and ligand concentration.

We performed spectral analysis of CHO cells expressing CY-PKCb prior to and 10 min following treatment with 1 μM PMA, using an excitation wavelength of 433 nm. A noticeable decrease in the CFP peak ($\lambda = 474$ nm) and an increase in the YFP peak ($\lambda = 527$ nm) indicated an increase in FRET (see Fig. 4).

We determined real time changes in FRET by continually measuring the ratio of emission at 527 nm over emission at 474 nm during treatment of the cells with 1 μM PMA. The emission ratio increased quickly after addition of PMA and reached a

FIG. 1. The binding properties of human PKCb and CY-PKCb. Assays were performed using [3H]PDBu as described under “Experimental Procedures.” A Scatchard plot is shown within each binding curve. Results shown are from single, representative experiments. $K_d$ values were 0.98 ± 0.37 nM (mean ± S.E., $n = 3$ experiments) for CY-PKCb and 0.52 ± 0.05 nM ($n = 4$ experiments) for human PKCb.

Visualization by Confocal Microscopy—Cultured cells were grown on 0.17-mm Delta T dishes (Biotec Inc., Butler, PA) and transfected the next day as described above. Prior to observation, the cells were washed twice in Dulbecco’s modified Eagle’s medium without phenol red, supplemented with 1% fetal bovine serum. The temperature was kept steady at 37 °C throughout all experiments. Fluorescent cells were examined with a Zeiss LSM 510 imaging system (Carl Zeiss Inc, Thornwood, NY) with an Axiosvert 100M inverted microscope operating with a 25-milliwatt argon laser tuned to 458 nm. Cells were imaged with a 63 × 1.4 NA Zeiss Plan-Apochromat oil immersion objective. Images were collected using a single track configuration where the yellow and cyan emission was split with an NFT 515 dichroic filter and collected in PMT 1 with a LP 530 filter and PMT 2 with a BP 465-505 filter, respectively. FRET was visualized as the ratio of the image collected through PMT1 over that collected through PMT2. Prior to the calculation of the ratio, pixels from both images with intensities less than 4% of saturation were removed by a threshold function.
plateau within 5 min (see Fig. 4). Measurement of FRET over a time period of up to 30 min did not yield a further change in the emission ratio (data not shown).

We next determined the extent of the change in FRET as a function of PMA concentration. The changes in FRET were measured after 5 min at various PMA concentrations, and, based on end point measurements, a dose-response curve was generated (Fig. 5A) that fit the Hill equation and yielded a value for ED50 of 283 ± 59 nM (n = 3).

Because we were using whole cell assays, binding of free ligand to surfaces, as well as partitioning to cellular membranes, could deplete the concentration of free ligand and cause artifacts in our results. To verify that this was not occurring, we performed assays with radioactive [3H]PDBu under comparable conditions. In these assays, the concentration of free ligand, determined by scintillation counting, dropped by 1% or less after incubation with cells (data not shown), indicating that the free ligand concentration remained in excess of the receptor concentration.

Characterization of the FRET Signal by Confocal Microscopy—Visualization of changes in FRET within the cell may potentially allow us to resolve the sequence of events associated with PKCδ activation. CHO cells transfected with the CY-PKCδ plasmid were used for these experiments. We examined the ratio of emission through the YFP channel over emission through the CFP channel when cells were excited via a 458-nm laser line. Our results confirmed FRET activity, which initially was most apparent at the cytoplasmic membrane (Fig. 3, B and C, 5-min time points) and subsequently became evident throughout the internal membranes (Fig. 3, B and C, 15- and 30-min time points). These results are consistent with published data showing that PMA traverses cell membranes slowly (12, 31).

Kinetic Analysis of CY-PKCδ-Ligand Interaction in Vivo—The CY-PKCδ construct allowed us to investigate the pharmacokinetics and pharmacodynamics of phorbol esters in CHO cells. We chose a total of 12 PKC ligands for this analysis. Six of these ligands were chosen because either they were well characterized or were in clinical trials; these were prostratin, dPP, bryostatin 1, ingenol 3-angelate, PMA, and thymeleatoxin. The remaining six ligands comprised a homologous series of symmetric phorbol 12,13-diesters that provided a range of lipophilicities with which we could investigate the influence of lipophilicity on the rate of the FRET response and on potency.
for CY-PKCδ; these were diC2, diC3, PDBu, diC6, diC8, and diC9.

For each ligand, the ED_{50} was determined by two different methods. First, we produced dose-response curves based on end point measurements of the change in FRET, fitting these to the Hill equation, thus obtaining values for ED_{50} (see Fig. 5 and Table I). Second, we took the total kinetic data consisting of the change in FRET as a function of time and ligand concentration, and we regressed these data to a pharmacokinetic/pharmacodynamic model (see “Experimental Procedures”), which seeks to describe the relationship between potency, ED_{50}, and a rate of effect, k (see Fig. 6 and Table I). The end point data successfully fit the Hill equation for all 12 ligands. We were unable to obtain kinetic data for bryostatin 1 as it emitted autofluorescence to the extent that it made real time measurement of change in FRET unreliable. We were unable to achieve a satisfactory curve fit for the kinetic data for PMA and diC9. These drugs are among the most lipophilic derivatives examined (log p = 6.38 and 8.34), and we assume that their more complicated kinetics arise from their low aqueous solubility.

All 12 ligands induced a sharp increase in FRET following addition of drug. The mean change in FRET relative to that induced by PMA across different ligands was 98% (mean ± S.E.), suggesting that there were no markedly different conformational changes induced by any of the ligands examined. The rate of change in FRET as represented by k was dependent upon lipophilicity in a consistent fashion, with the calculated t_{1/2} ranging from 22 s for prostratin to 108 s for diC8 (Fig. 7 and Table I); the highly lipophilic phorbol ester diC9 appeared to have an additionally slower rate of effect (data not shown). These rate data are consistent with data from studies that measured the rate at which phorbol esters traverse the cyto-

Fig. 4. PMA induces a change in FRET in CHO cells expressing CY-PKCδ. A shows the emission spectrum (excitation λ = 433 nm) of CHO cells expressing CY-PKCδ prior to and 5 min after treatment with 1 μM PMA. Following treatment with PMA, a decrease in the CFP peak (maxima at 474 nm) and an increase in the YFP peak (maxima at 527 nm) are shown. Emission is measured in relative fluorescence units (RFU). B shows the change in FRET as a function of time after addition of 1 μM PMA. FRET is measured as the ratio of emission at 527 nm over emission at 474 nm. PMA was added at t = 0 s. Similar results were obtained with a total of four experiments.

Fig. 5. Dose dependence of FRET changes. Dose-response curves from representative experiments with PMA (A), PDBu (B), dPP (C), and prostratin (D) are shown. The change in FRET of cells expressing CY-PKCδ was determined as the relative change in the ratio of emission at 527 nm over emission at 474 nm; ED_{50} values were calculated by curve fitting to the Hill equation (see “Experimental Procedures”). Similar results were obtained for triplicate experiments. Mean ± S.E. values for each of the drugs tested are reported in Table I.
Potencies and kinetic parameters of ligands

Values indicate the mean ± S.E. of three or more individual experiments. Values listed under “Kinetic data” represent values obtained by fitting the total kinetic data to the kinetic equation shown under “Experimental Procedures.” The t½ was calculated for $L = ED_{50}$. Values listed under “End point data” represent values obtained by fitting end point measurements of change in FRET to the Hill equation.

| Ligand             | Log $P$ | $ED_{50}$ (nM) | n | $ED_{50}$ (nM) | $k$ (nm$^{-1}$ s$^{-1}$) | $t_{1/2}$ (s) | $r^2$ |
|--------------------|---------|----------------|---|----------------|-------------------------|---------------|-------|
| Prostratin         | -0.38   | 8440 ± 1300    | 1.18 ± 0.10 | 6960 ± 480    | 2.23 ± 0.17 × 10$^{-6}$ | 22.3          | 0.95  |
| dPP                | 1.46    | 322 ± 38       | 1.06 ± 0.13 | 369 ± 58      | 2.00 ± 0.00 × 10$^{-6}$ | 47.0          | 0.96  |
| Ingenol 3-angelate | 3.89    | 52.7 ± 2.1     | 1.51 ± 0.29 | 26 ± 11       | 1.67 ± 0.09 × 10$^{-4}$ | 79.7          | 0.95  |
| Bryostatin 1       | 4.14    | 264 ± 53       | 1.35 ± 0.13 | 490 ± 120     | 3.33 ± 0.33 × 10$^{-5}$ | 21.3          | 0.82  |
| Thymeleatoxin      | 6.01    | 225 ± 27       | 0.85 ± 0.02 | 11,900 ± 4300 | 4.53 ± 1.3 × 10$^{-7}$ | 64.3          | 0.88  |
| PMA                | 6.38    | 283 ± 27       | 1.38 ± 0.23 | 758 ± 150     | 8.38 ± 0.79 × 10$^{-6}$ | 54.6          | 0.90  |
| Symmetric phorbol 12,13-diester | 1.47 | 16,600 ± 2300 | 1.40 ± 0.14 | 11,900 ± 4300 | 4.53 ± 1.3 × 10$^{-7}$ | 64.3          | 0.88  |
| diC3               | 2.45    | 980 ± 190      | 1.18 ± 0.09 | 173 ± 24      | 5.25 ± 0.63 × 10$^{-5}$ | 38.2          | 0.95  |
| PDBu               | 3.43    | 215 ± 22       | 1.27 ± 0.05 | 31.5 ± 6.9    | 3.27 ± 0.17 × 10$^{-4}$ | 33.7          | 0.93  |
| diC6               | 5.39    | 30.4 ± 6.3     | 1.41 ± 0.23 | 325 ± 36      | 9.87 ± 0.07 × 10$^{-4}$ | 108           | 0.87  |
| diC8               | 7.36    | 419 ± 33       | 0.95 ± 0.13 | 6108 ± 0.87   | 4.53 ± 1.3 × 10$^{-7}$ | 64.3          | 0.88  |
| diC9               | 8.34    | 700 ± 200      | 1.03 ± 0.13 |

**Fig. 6.** Pharmacokinetic/pharmacodynamic analysis of ligand potency and rate of association in vivo. Data from individual experiments, representing the change in FRET as a function of ligand concentration and time, were fitted by nonlinear regression to the kinetic equation given under “Experimental Procedures,” thus determining values for $ED_{50}$ and $k$. This was performed for each experiment with each ligand. Single, representative experiments showing the three-dimensional fit for diC8 (A) and prostratin (B) are shown. At least three experiments were performed for each ligand. Mean calculated values for $ED_{50}$ and $k$ for each ligand are reported in Table I.

**Fig. 7.** The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.

The measured potencies of these ligands were dependent upon lipophilicity. Measurement by regressing end point values to the Hill equation and by regressing the total kinetic data to the kinetic equation described under “Experimental Procedures” yielded similar values for $ED_{50}$ and $k$. The 2 orders of magnitude difference in the measured $ED_{50}$ values between dPP and prostratin are consistent with the values obtained in other in vivo studies (32, 33).

The Kubinyi model has been used to describe the relationship between biological effect and lipophilicity across a homologous series of ligands (34). This equation was initially used to describe this relationship for a model of mouse ear reddening induced by a series of symmetric phorbol 12,13-diester very similar to the series being used in this study. We fitted our data to the Kubinyi equation (Fig. 7), and we found that the homologous series of phorbol 12,13-diester fit the equation differently than did the asymmetric ligands (Fig. 7). Among the asymmetric ligands, ingenol 3-angelate was closest to the peak in potency predicted by the Kubinyi relationship and was more undoubtedly a simplification; nonetheless, it still gives a fairly good fit ($r^2 = 0.96$ to 0.82) and provides an approximation for the rate of response.

The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.

The measured potencies of these ligands were dependent upon lipophilicity. Measurement by regressing end point values to the Hill equation and by regressing the total kinetic data to the kinetic equation described under “Experimental Procedures” yielded similar values for $ED_{50}$ and $k$. The 2 orders of magnitude difference in the measured $ED_{50}$ values between dPP and prostratin are consistent with the values obtained in other in vivo studies (32, 33).

The Kubinyi model has been used to describe the relationship between biological effect and lipophilicity across a homologous series of ligands (34). This equation was initially used to describe this relationship for a model of mouse ear reddening induced by a series of symmetric phorbol 12,13-diester very similar to the series being used in this study. We fitted our data to the Kubinyi equation (Fig. 7), and we found that the homologous series of phorbol 12,13-diester fit the equation differently than did the asymmetric ligands (Fig. 7). Among the asymmetric ligands, ingenol 3-angelate was closest to the peak in potency predicted by the Kubinyi relationship and was more undoubtedly a simplification; nonetheless, it still gives a fairly good fit ($r^2 = 0.96$ to 0.82) and provides an approximation for the rate of response.

The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.

The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.

The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.

The influence of lipophilicity on binding potency and rate of association in vivo. The mean values for $ED_{50}$, calculated by using end point measurements of change in FRET, were plotted against lipophilicity. This relationship was fitted to the Kubinyi equation (34) separately for the symmetric phorbol 12,13-diester (dashed line) and the asymmetric phorbol esters and related PKC ligands (solid line). Bryo 1, bryostatin 1; THX, thymeleatoxin; IA, ingenol-3-angelate.
potent than PMA. Similarly, we had reported previously that ingenol 3-angelate was more potent than PMA for inducing translocation of GFP-PKC\(\delta\) expressed in CHO cells (6).

**DISCUSSION**

The published literature reports that the measured *in vitro* binding affinity of ligands to protein kinase C is not necessarily predictive of the potencies of those ligands to activate PKC-mediated responses in intact cells. Likewise, selectivity of ligands for PKC isoforms may depend upon cell type (29, 35). To develop PKC isomorph-selective ligands, it is therefore imperative to establish methods for determining the structure-activity relationships of ligands in the biological context of the appropriate cell type.

PKC\(\delta\) is perhaps the most challenging PKC for such analysis. It shows a complex pattern of distribution upon phorbol ester treatment of cells, where both the rate of translocation as well as localization depend upon the nature of the ligand (24). In many but not all contexts, PKC\(\delta\) is pro-apoptotic (36) and anti-proliferative, thus being a functional antagonist of PKC\(\alpha\) and PKC\(\epsilon\) (37, 38). It is subject to co-regulation by tyrosine phosphorylation (39) and often shows complicated dose-response curves in response to bryostatin 1 (35, 40, 41), a natural product currently being evaluated for cancer chemotherapy.

To evaluate the feasibility of using FRET as an indicator of *in vivo* interaction of PKC with ligands, we prepared a PKC\(\delta\) fusion to CFP and YFP, which we named CY-PKC\(\delta\). Several initial issues needed to be evaluated. First, did the construct respond to phorbol esters in intact cells in a fashion similar to unmodified PKC\(\delta\)? Second, did the construct give a measurable FRET signal?

We report here that the fluorescent CY-PKC\(\delta\) chimera indeed showed biological and biochemical activity representative of native PKC\(\delta\) for all of the parameters that we tested. *In vitro* binding assays with partially purified CY-PKC\(\delta\) protein demonstrated that the CY-PKC\(\delta\) protein bound PDBu with affinity similar to that of the human PKC\(\delta\) (0.98 ± 0.37 and 0.52 ± 0.04 nM, respectively). Western blotting experiments, which examined the ability of PMA to induce translocation of PKC\(\delta\) and CY-PKC\(\delta\) in cells, showed that both proteins translocated from the cytosol to the membrane within 20 min following treatment with drug. Dose-response curves, generated by densitometric analysis of these Western blots, provided ED\(_{50}\) values that differed by 2-fold between PKC\(\delta\) and CY-PKC\(\delta\) (328 ± 59 and 637 ± 183 nM, respectively). Finally, observation of translocation in real time with a laser scanning confocal microscope showed no visible difference in the rate of translocation between GFP-PKC\(\delta\) and CY-PKC\(\delta\) or in the complex pattern of distribution. On the other hand, we did observe minor differences between PKC\(\delta\) and CY-PKC\(\delta\) in the level of membrane association in the absence of stimulation.

We wished to characterize the FRET signal generated by CY-PKC\(\delta\) and to determine whether it varied with ligand concentration. By using fluorimetry, we examined CHO cells expressing CY-PKC\(\delta\). Spectral analysis of these CHO cells showed that treatment of these cells with the potent phorbol ester PMA resulted in a significant increase in the 527-nm emission peak and decrease in the 474-nm emission peak, an indicator of increased FRET between CFP and YFP. This increase in FRET varied with the concentration of added ligand, allowing us to produce dose-response curves representing *in vivo* response of CY-PKC\(\delta\) as a function of ligand concentration. These dose-response curves yielded ED\(_{50}\) values for PMA that were within 2-fold of the ED\(_{50}\) values obtained from the Western blotting experiments for PMA-induced translocation from the cytosol (283 ± 27 and 637 ± 183 nM, respectively). Indeed, this mean ED\(_{50}\) value more closely matches that obtained from the Western blotting experiments with endogenous PKC\(\delta\) (328 ± 59 nM). The actual mechanism underlying the change in FRET signal is not yet known. Although a conformational change of the CY-PKC\(\delta\) upon ligand binding is the most straightforward explanation, a change in intermolecular interaction(s) is also possible.

By confocal microscopy we were able to determine the localization of the change in FRET of CY-PKC\(\delta\) over time. Treatment of cells expressing CY-PKC\(\delta\) with PMA showed a FRET response at the plasma membrane at early time points that progressed to internal membranes over a period of 15 min. Because the *in vivo* assays had shown no increase in FRET beyond 5 min following treatment with PMA, the continuing change in the pattern of response seen under the confocal microscope beyond the 5-min time point likely represented the translocation of already responsive CY-PKC\(\delta\) molecules as opposed to an increase in total FRET response.

We extended the *in vivo* analysis to 11 other ligands, many of which had been extensively studied and some of which are currently in clinical trials. Six of these ligands comprised a series of symmetric phorbol 12,13-diesters, thus providing a range of lipophilicities. These *in vivo* data allowed us to perform two types of analyses. First, the end point measurements of change in FRET were plotted as a function of ligand concentration and fitted to the Hill equation, obtaining values for ED\(_{50}\) and n. Second, the total kinetic data, consisting of a change in FRET as a function of time and of ligand concentration, were fitted to a pharmacokinetic/pharmacodynamic model, thus obtaining values for ED\(_{50}\) and an effect rate constant k (Table I).

All of the 12 ligands induced a sharp increase in FRET in CY-PKC\(\delta\)-expressing cells that occurred at a rate dependent upon the lipophilicity of the ligand. In addition, the measured potencies of each of the ligands with respect to CY-PKC\(\delta\) were also dependent upon lipophilicity (Table I and Fig. 7). Indeed, this relationship neatly fit the Kubinyi bilinear model (34), which seeks to describe the relationship between biological effect and lipophilicity.

The role of the PKCs in signal transduction makes them attractive therapeutic targets. Already, a number of PKC ligands are being exploited as pharmaceuticals. Bryostatin 1 is currently in clinical trials for melanoma, multiple myeloma, renal cell carcinoma, and B-cell chronic lymphocytic leukemia (5). Ingenol 3-angelate is in clinical trials for non-melanotic skin cancer. LY333531 is in clinical trials for diabetic retinopathy and renopathy caused by vascular hyperproliferation (1, 2). Prostratin, a polar phorbol ester derived from a Samoan plant extract, arrests virus replication of human immunodeficiency virus in T-cells and monocyte/macrophage cultures (3), and both it and dPP induce proliferation of latent human immunodeficiency virus and are therefore being examined as an adjuvant for highly active antiretroviral therapy (4). Further investigation into the pharmacology of phorbol esters and other PKC ligands may yield additional drugs for cancer or other therapeutic targets.

We have described the creation of a genetically encoded, fluorescent chimera of native PKC\(\delta\) with a FRET signal that can be measured in the laboratory. This chimeric protein kinase retains native or near-native activity among the several biological and biochemical parameters that we tested. Its FRET response to added ligand can be measured fluorimetrically or visualized *in situ* by confocal microscopy. By using this construct, we were able to determine the kinetics and potencies of 12 known PKC ligands in the intact cell. This genetically encoded CY-PKC\(\delta\) chimera and the *in vivo* assays described herein therefore show potential for HTS of prospective PKC\(\delta\) ligands.
Acknowledgments—We thank Stephen Wincovitch (CCR Confocal Microscopy Core Facility, NCI, National Institutes of Health) for assistance with confocal microscopy. We also thank Paul Randazzo (Laboratory of Cellular Oncology, NCI, National Institutes of Health) for aid with fluorimetry.

REFERENCES

1. Ishii, H., Jirousek, M. R., Koya, D., Takagi, C., Xia, P., Clermont, A., Bursell, S. E., Kern, T. S., Buss, L. M., Heath, W. F., Stramm, L. E., Feener, E. P., and King, G. L. (1996) Science 272, 728–731

2. Ishii, H., Koya, D., and King, G. L. (1998) J. Mol. Med. 76, 21–31

3. Gustafson, K. R., Cardellina, J. H., McMahon, J. B., Gulakowski, J. R., Ishtiyak, J., Szallasi, Z., Le, N. E., Blumberg, P. M., Weislow, O. S., and Beutler, J. A. (1992) J. Med. Chem. 35, 1978–1986

4. Bocklandt, S., Blumberg, P. M., and Hamer, D. H. (2003) Antiviral Res. 59, 89–98

5. Clamp, A., and Jayson, G. C. (2002) Anti-Cancer Drugs 13, 673–683

6. Kedei, N., Lundberg, D. J., Toth, A., Welburn, P., Garfield, S. H., and Blumberg, P. M. (2004) Cancer Res. 64, 3243–3255

7. Ogborne, S. M., Subhier, A., Jones, B., Cozi, S. J., Boyle, G. M., Morris, M., McAlpine, D., Johns, J., Scott, T. M., Sutherland, K. P., Gardner, J. M., Le, T. T., Lenarczyk, A., Aylward, J. H., and Parsons, P. G. (2004) Cancer Res. 64, 2833–2839

8. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498

9. Mochly-Rosen, D., and Kauvar, L. M. (2000) Mol. Pharmacol. 55–61

10. Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1990) Curr. Biol. 5, 973–976

11. Newton, A. C. (1995) Semin. Immunol. 12, 55–61

12. Szallasi, Z., Smith, C. B., and Blumberg, P. M. (1994) J. Biol. Chem. 269, 27159–27162

13. Dokas, L. A., Pisano, M. R., Schrama, L. H., Zwiers, H., and Gispen, W. H. (1996) Brain Res. Bull. 34, 321–329

14. Miyawaki, A., Llopis, J., Heim, R., McCaffery, J. M., Adams, J. A., Ikura, M., and Tsien, R. Y. (1997) Nature 388, 882–887

15. Honda, A., Adams, S. R., Sawyer, C. L., Lei, R., and Dostmann, W. R. (2001) Nat. Protoc. 3, 208–217

16. Nagai, Y., Miyazaki, M., Aoki, R., Zama, T., Inouye, S., Hirose, K., Iino, M., and Hagiwara, M. (2000) Nat. Biotechnol. 18, 313–316

17. Violin, J. D., Zhang, J., Tsien, R. Y., and Newton, A. C. (2003) J. Cell Biol. 161, 899–909

18. Ohmori, S., Shirai, Y., Sakai, N., Fujii, M., Konishi, H., Kikkawa, U., and Saito, N. (1998) Mol. Cell. Biol. 18, 5263–5271

19. Brodie, C., Bogi, K., Acs, P., Lorenzo, P. S., Biro, T., Szallasi, Z., Mushinski, J. F., and Blumberg, P. M. (1997) J. Biol. Chem. 272, 28793–28799

20. Graham, D. L., Lowe, P. N., and Chalk, P. A. (2001) Anal. Biochem. 296, 221–229

21. Ting, A. Y., Kain, K. H., Klemke, R. L., and Tsien, R. Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 15003–15008

22. Calleja, V., Ameer-Beg, S. M., Wojcik, B., Waschutski, R., Downward, J., and Larrij, B. (2003) Biochem. J. 372, 33–40

23. Neininger, A., Thielemann, H., and Gaestel, M. (2001) EMBO Rep. 2, 703–708

24. Wang, Q. J., Fang, T. W., Fenick, D., Garfield, S., Bienfait, K., Marquez, V. E., and Blumberg, P. M. (2000) J. Biol. Chem. 275, 12136–12146

25. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., pp. 1.116–1.118, Cold Spring Harbor Laboratory Press, NY

26. Wang, Q. J., Bhattacharyya, D., Garfield, S., Nacro, K., Marquez, V. E., and Blumberg, P. M. (1999) J. Biol. Chem. 274, 37233–37239

27. Lewin, N. E., and Blumberg, P. M. (2003) Methods Mol. Biol. 233, 129–156

28. Larijani, B. (2003) Apoptosis: Models for Binding, Trafficking, and Signaling, pp. 153–180, Oxford University Press, New York

29. Szallasi, Z., Smith, C. B., Pettit, G. R., and Blumberg, P. M. (1994) J. Biol. Chem. 269, 2118–2124

30. Ohmori, S., Shirai, Y., Sakai, N., Fujii, M., Konishi, H., Kikkawa, U., and Saito, N. (1998) Mol. Cell. Biol. 18, 5263–5271

31. Murphy, T. V., Pruntonz, C., Kotsonis, P., Iannazzo, L., and Majewski, H. (1999) Eur. J. Pharmaco. 381, 77–84

32. Szallasi, Z., Kruus, K. W., and Blumberg, P. M. (1992) Carcinogenesis 13, 2161–2167

33. Szallasi, Z., Krsmanovic, L., and Blumberg, P. M. (1993) Cancer Res. 53, 2907–2912

34. Kuhiny, H. (1976) Arzneim. Forsch. 26, 1991–1997

35. Szallasi, Z., Denning, M. F., Smith, C. B., Dlugosz, A. A., Yuspa, S. H., Pettit, G. R., and Blumberg, P. M. (1994) Mol. Pharmacol. 46, 840–850

36. Brodie, C., and Blumberg, P. M. (2003) Apoptosis 8, 19–27

37. Brodie, C., Kuperstein, I., Acs, P., and Blumberg, P. M. (1998) Brain Res. Mol. Brain Res. 56, 108–117

38. Acs, P., Wang, Q. J., Bogi, K., Marquez, A. M., Lorenzo, P. S., Hiro, T., Szallasi, Z., Mushinski, J. F., and Blumberg, P. M. (1997) J. Biol. Chem. 272, 28793–28799

39. Brodie, C., Bogi, K., Acs, P., Lorenzo, P. S., Baskin, L., and Blumberg, P. M. (1998) J. Biol. Chem. 273, 30713–30718

40. Lorenzo, P. S., Bogi, K., Acs, P., Pettit, G. R., and Blumberg, P. M. (1997) J. Biol. Chem. 272, 33338–33343

41. Lorenzo, P. S., Bogi, K., Hughes, K. M., Beheshiti, M., Bhattacharyya, D., Garfield, S. H., Pettit, G. R., and Blumberg, P. M. (1999) Cancer Res. 59, 6137–6144
Analysis by Fluorescence Resonance Energy Transfer of the Interaction between Ligands and Protein Kinase Cδ in the Intact Cell
Derek C. Braun, Susan H. Garfield and Peter M. Blumberg

J. Biol. Chem. 2005, 280:8164-8171.
doi: 10.1074/jbc.M413896200 originally published online December 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M413896200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 18 of which can be accessed free at http://www.jbc.org/content/280/9/8164.full.html#ref-list-1