In the present report, we demonstrate that Tb$^{3+}$ binds to protein kinase C and serves as a luminescent reporter of certain cationic metal-binding sites. Tb$^{3+}$ titration of 50 mM protein kinase C results in a 20-fold enhancement of Tb$^{3+}$ luminescence which is half-maximal at 12 µM Tb$^{3+}$. A $K_d$ of ~145 nM was determined for Tb$^{3+}$ binding to the enzyme. The excitation spectrum of bound Tb$^{3+}$ exhibits a peak at 280 nm characteristic of energy transfer from protein tryptophan or tyrosine residues. The luminescence of this complex can be markedly decreased by other metals, including Pb$^{2+}$ ($IC_{50} = 25$ µM), La$^{3+}$ ($IC_{50} = 50$ µM), Hg$^{2+}$ ($IC_{50} = 300$ µM), Ca$^{2+}$ ($IC_{50} = 6$ mM), and Zn$^{2+}$ ($IC_{50} > 10$ mM), and chelation of Tb$^{3+}$ by 2 mM EGTA. Tb$^{3+}$ binding to protein kinase C is correlated with its inhibition of protein kinase activity ($IC_{50} = 8$ µM, $r = 0.99$) and phorbol ester binding ($IC_{50} = 15$ µM, $r = 0.98$). Tb$^{3+}$ inhibition of protein kinase C activity cannot be overcome by excess Ca$^{2+}$, but can be partially overcome with excess phosphatidylserine or by chelation of Tb$^{3+}$ with EGTA. Tb$^{3+}$ noncompetitively inhibits phorbol ester binding by decreasing the maximal extent of binding without significantly altering binding affinity. The results suggest that the Tb$^{3+}$-binding site is at or allosterically related to the enzyme’s phosphatidylinerine-binding site, but is distinct from the phorbol ester-binding domain and the Ca$^{2+}$-binding site that regulates enzyme activity.

EXPERIMENTAL PROCEDURES

Materials—Dioleoyl phosphatidylserine (disodium salt) and dioleoylglycerol were obtained from Avanti. [γ-32P]ATP and [125I]PDBu were from Amersham and Du Pont-New England Nuclear, respectively. TbCl$_3$, LaCl$_3$, and HgCl$_2$ were from Aldrich. PhCH$_2$CO$_2$H and ZnCl$_2$ were obtained from Mallinckrodt. Histone type HI, ATP, nonradioactive phorbol esters, and other reagents were obtained from Sigma.

Methods—Protein kinase C was isolated from outdated human platelets obtained from the Red Cross. Platelets were washed, sonicated in 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 5 mM EGTA, 5 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonfuryl fluoride, and 10 µg/ml leupeptin. The lysate was centrifuged at 140,000 x g for 60 min. Protein kinase C was purified from the crude supernatant by DEAE-cellulose and phenyl-Sepharose chromatography as described by Walton et al. (14), followed by high performance liquid chromatography on phenyl-5PW (Beckman) and DEAE-5PW (Watson). The enzyme was concentrated and stored at ~90°C in 10 mM Tris (pH 7.5), 100 µM EDTA, 100 µM EGTA, 60% glycerol, and 5 µg/ml leupeptin for use in luminescence studies. Cruder fractions (typically 0.2 µmol/min/mg) were used in protein kinase activity and phorbol ester-binding studies.

The isoenzyme used in this study, one of two present in human platelets, was highly dependent on Ca$^{2+}$ and phospholipid, and was presumed to be similar to brain type III protein kinase C. The present study demonstrates that Tb$^{3+}$ is a sensitive reporter of metal binding domains in protein kinase C, and that Tb$^{3+}$ binding to the enzyme can be correlated with inhibition of its protein kinase activity and phorbol ester binding.
In the presence of 5 μg/ml phosphatidyserine, its activity was stimulated at least 5-fold by 200 μM Ca\(^{2+}\).

The Bradford method (16) was used for protein determinations. Protein kinase C activity was assayed essentially as described by Kikkawa et al. (17). The reaction was initiated by the addition of enzyme, incubated at 30 °C for 2 min, and terminated by the addition of 30% trichloroacetic acid. Phosphoproteins were isolated by filtration (Millipore HAWP) and quantified by liquid scintillation counting. A modification of the method of Tanaka et al. (15) was used to assay phorbol ester binding. The assay mix was incubated at 30 °C for 30 min, and terminated by the addition of 3 ml of cold 0.5% dimethyl sulfoxide. The mixture was filtered through Whatman GF/C filters soaked with 0.3% polyethylenimine, washed twice with two 3-ml volumes of 0.5% dimethyl sulfoxide, and dried for scintillation counting. Nonspecific [\(\text{H}\)]pD Bu binding (assayed in the presence of 30 μM cold pD Bu) was found to be slightly less than the value obtained in the absence of protein kinase C. The latter value was subtracted from the measured total bound [\(\text{H}\)]pD Bu to derive the specific binding data.

Prior to titration with Tb\(^{3+}\), protein kinase C aliquots were desalted with Spectra/Gel HW-40F (Spectrum) to remove EDTA and EGTA. Luminescence studies were conducted with a Perkin-Elmer LS-5B luminescence spectrometer, using gate and delay times of 1 ms. Excitation and emission wavelengths were 280 and 543 nm, respectively. Titrations were conducted in buffers made from high purity water containing 10% glycerol (Boehringer Mannheim enzyme grade) to stabilize the dilute enzyme. The reported luminescence values were corrected for minor background luminescence attributable to interaction of terbium with the buffer.

**RESULTS AND DISCUSSION**

Tb\(^{3+}\) titrations of 50 nM protein kinase produced an increase in Tb\(^{3+}\) luminescence which was half-maximal near 12 μM and maximal at 20 μM Tb\(^{3+}\) (Fig. 1). To determine the \(K_d\) of Tb\(^{3+}\) for protein kinase C, we conducted Tb\(^{3+}\) titrations of various concentrations of the enzyme (5–59 nM). A plot of the apparent \(K_d\) (estimated from [Tb\(^{3+}\)] required for half-maximal luminescence enhancement) versus [protein kinase C] yielded a linear plot (\(r = 0.99\)) which extrapolated to a \(K_d\) of ~145 nM at zero enzyme concentration. This suggested that Tb\(^{3+}\) binds to protein kinase C with a \(K_d\) of approximately 145 nM.

The inset in Fig. 1 shows the excitation spectrum of 20 μM Tb\(^{3+}\) and 50 nM protein kinase C (spectrum 3). The excitation peak near 280 nm results from resonance energy transfer from aromatic amino acid residues of the enzyme to bound Tb\(^{3+}\).

The luminescence increase could be 67% reversed by chelation of Tb\(^{3+}\) by 2 mM EGTA (Fig. 1, inset, spectrum 4). The luminescence of Tb\(^{3+}\) was enhanced 20-fold by the presence of protein kinase C (compare spectra 2 and 3). In the absence of Tb\(^{3+}\), protein kinase C did not contribute significantly to luminescence (spectrum 1). The increase in Tb\(^{3+}\) luminescence produced by protein kinase C was not observed after heat denaturation of the enzyme (100 °C for 5 min). Further, proteins which do not have metal binding sites (including bovine serum albumin and α-chymotrypsin) produced no increase in Tb\(^{3+}\) luminescence under the same conditions used to study protein kinase C. This is consistent with Tb\(^{3+}\) binding to a specific metal binding site which exists on the native conformation of protein kinase C.

Diacetyl glycerol or phorbol esters increase the affinity of protein kinase C for Ca\(^{2+}\), permitting full activation at resting cytosolic Ca\(^{2+}\) concentrations (19). We conducted Tb\(^{3+}\) titrations of protein kinase C in the presence of PMA and phosphatidyserine to determine whether these regulatory ligands affect Tb\(^{3+}\) binding. The titration curve was unchanged by the addition of 150 nM PMA, but was shifted to the right (half-maximal ~30 μM, maximal ~70 μM) by addition of 20 μg/ml phosphatidyserine (data not shown). Titrations were conducted in the presence of both 150 nM PMA and 20 μg/ml phosphatidyserine were not significantly different from those conducted in the presence of phosphatidyserine alone. Thus, whether in the presence or absence of phosphatidyserine, PMA did not appear to alter the affinity of protein kinase C for Tb\(^{3+}\). Phosphatidyserine alone did, however, decrease the apparent affinity of the enzyme for Tb\(^{3+}\).

We examined the ability of Pb\(^{2+}\), Hg\(^{2+}\), Zn\(^{2+}\), La\(^{3+}\), Ca\(^{2+}\), and Mg\(^{2+}\) to displace Tb\(^{3+}\) from its binding site in protein kinase C and reduce its luminescence. Fig. 2 shows the effects of these metals on Tb\(^{3+}\)-protein kinase C luminescence. Lead was most effective in displacing Tb\(^{3+}\), causing a 50% luminescence decrease at 25 μM and an 84% decrease at 200 μM. La\(^{3+}\), Mg\(^{2+}\), and Ca\(^{2+}\) were progressively less effective, causing 50% decreases at 50 μM, 300 μM, and 6 mM, respectively. Zn\(^{2+}\) decreased luminescence by only 35% at 10 mM, and Mg\(^{2+}\) (1–20 mM) had minimal ability to displace Tb\(^{3+}\). Thus, Pb\(^{2+}\), La\(^{3+}\), Hg\(^{2+}\), and, to a lesser extent, Ca\(^{2+}\) and Zn\(^{2+}\), appeared to compete with Tb\(^{3+}\) for binding sites on the enzyme. The enzyme’s Mg\(^{2+}\)-binding site was clearly distinct from the site which binds Tb\(^{3+}\). Pb\(^{2+}\) was the only metal examined that appears to bind with similar or higher affinity to the lanthanide-binding site of protein kinase C than Tb\(^{3+}\) and La\(^{3+}\) themselves.

We examined the effects of Pb\(^{3+}\) on protein kinase C...
activity and [3H]PDBu binding to determine whether alterations in these parameters are correlated with Tb3+ binding to the enzyme (as monitored by luminescence). In addition, we examined other metals in this context to permit comparison with Tb3+. Fig. 3 shows the effect of Tb3+ and other metals on protein kinase C activity in the presence of 20 μM Ca2+. Each of the metals tested inhibited protein kinase C activity as follows: Tb3+ and La3+ with IC50 = 8 μM, Hg2+ with IC50 < 1 μM, Pb2+ with IC50 = 15 μM, and Zn2+ with IC50 = 20 μM. In agreement with the report of Speizer et al. (8), we saw no evidence of heavy metal stimulation of protein kinase C activity. Interestingly, the concentration dependence of Tb3+ inhibition of enzyme activity displayed a strong positive correlation (r = 0.99) with Tb3+ luminescence enhancement caused by its binding to the enzyme over the range of 1–20 μM. The inhibition of protein kinase C activity by 20 μM Tb3+ appeared to be partially reversible, since 200 μM EGTA was capable of restoring activity by 28%. This is consistent with the observation that EGTA chelation of Tb3+ partially reversed its binding to the enzyme, as monitored by Tb3+ luminescence (Fig. 1, inset). Tb3+ binding and inhibition of protein kinase C activity could not be fully reversed even by 2 mM EGTA, consistent with its high affinity for the enzyme. Inhibition of protein kinase C by Hg2+ occurred well below its IC50 for Tb3+ displacement from protein kinase C (300 μM), suggesting that Hg2+ inhibition was caused by interaction with sites distinct from those which bind Tb3+. The cysteine-rich regions of protein kinase C have been shown to be particularly susceptible to interaction with Hg2+ (8).

In the assay system utilized, maximal protein kinase C activity was produced by 200 μM added Ca2+, while 20 μM Ca2+ was sufficient to produce half-maximal activation. We examined metal inhibition of protein kinase C activity in the presence of both 20 μM and 200 μM Ca2+. The pattern of inhibition by all the metals was similar at 20 μM and 200 μM Ca2+, suggesting that inhibition was not due to metal competition with Ca2+ at the Ca2+-binding site which controls activity.

Inhibition of [3H]PDBu binding by Hg2+, La3+, and Tb3+ (Fig. 4) was closely related to their inhibitory effects on protein kinase C activity. The IC50 values for Hg2+ (2 μM), Tb3+ (15 μM), and La3+ (15 μM) inhibition of [3H]PDBu binding were reasonably close to their respective IC50 values for inhibition of protein kinase activity. Tb3+ binding to protein kinase C (as monitored by luminescence over the range of 1–20 μM) was positively correlated with its inhibition of [3H]PDBu binding (r = 0.98). Pb2+ and Zn2+ were relatively ineffective inhibitors of [3H]PDBu binding, decreasing it to 70% and 55% of control, respectively, at 100 μM.

Inhibition of protein kinase C activity by Tb3+, La3+, Hg2+, Pb2+, and Zn2+ could not be reversed by a 10-fold increase in Ca2+, suggesting that they did not compete for Ca2+-binding sites on the enzyme. We therefore examined the possibility that Tb3+ binding might be competitive with the binding of phosphatidylserine or phorbol esters. Speizer et al. (8) have shown that the phospholipid dependence of protein kinase C activation is altered by certain heavy metals, including Cu2+. As shown in Fig. 5, Tb3+ had a similar effect. Increasing concentrations of phosphatidylserine were able to overcome Tb3+ inhibition of protein kinase C activity. In the absence of Tb3+, 4 μg/ml phosphatidylserine caused half-maximal activation of protein kinase C. In the presence of 10 μM and 20 μM Tb3+, half-maximal activation required 10 μg/ml and 20 μg/ml phosphatidylserine, respectively. Lineweaver-Burk analysis (shown in the inset to Fig. 5) suggested that Tb3+ is apparently a competitive inhibitor with respect to phosphatidylserine (K = 3 μM). Thus, Tb3+ binds to protein kinase...
protein kinase C. Tb³⁺ and La³⁺ were unable to substitute for phorbol ester binding sites by competition with the phosphatidylserine (3H)PDBu binding assay used in this study is almost that cannot be significantly reversed by a 10-fold excess of competitive inhibition of phorbol ester binding may be secondary to its inhibition of phosphatidylserine binding, since the Tb³⁺-binding site (18). The primary structure of the regulatory domain of type III protein kinase C contains two potential metal-binding sites which lie in close proximity, are nonidentical, and are distinct from the cysteine-rich region (3, 4).

Tb³⁺ appears to interact with protein kinase C at the phospholipid-binding site and apparently acts as a competitive inhibitor with respect to phosphatidylserine. While it is possible that Tb³⁺ could interfere with activation of the enzyme by altering the conformation of phospholipid vesicles, this would probably not occur in the 5-20 µM concentration range over which Tb³⁺ produces inhibition. Further, Tb³⁺ binding inhibits protein kinase C activity over the same range in which it bound to the enzyme in our luminescence studies suggesting a Tb³⁺-protein kinase C interaction in the absence of phosphatidylserine. Moreover, the ability of phosphatidylserine to decrease the apparent Tb⁺⁺-binding affinity of protein kinase C is consistent with the competitive model of inhibition suggested by Lineweaver-Burk analysis.

The mechanism of protein kinase C inhibition by Tb⁺⁺ is apparently similar to that of certain heavy metals, including Cu⁺⁺. Similar to Tb⁺⁺, Cu⁺⁺ inhibits activity by altering the phosphatidylserine dependence of protein kinase C activation and noncompetitively inhibits (3H)PDBu binding (8). Pb⁺⁺ and Hg⁺⁺ appear to be able to displace Tb⁺⁺ from its binding site, suggesting proximity or overlap between the heavy metal- and lanthanide-binding sites of protein kinase C. Like La³⁺, Pb⁺⁺ inhibits protein kinase C activity over nearly the same range in which it displaces Tb⁺⁺ from the enzyme, suggesting that Pb⁺⁺ inhibition could be mediated by interaction with the Tb⁺⁺-binding site. It is likely that Hg⁺⁺ inhibits protein kinase C activity by interacting with a different site (perhaps a cysteine-rich region), since its IC₅₀ for displacement of Tb⁺⁺ is 300-fold greater than its IC₅₀ for inhibition of enzyme activity, and Hg⁺⁺ is known to interact with cysteine residues on proteins.

In conclusion, inhibition of protein kinase C by Tb⁺⁺, La³⁺, and certain heavy metals (including the environmental pollutants Pb⁺⁺, Cd⁺⁺, and Cu⁺⁺) is mediated by a metal-binding site on the native enzyme that is distinct from the Ca²⁺-binding site which regulates enzyme activity. Metals binding to this site compete with phosphatidylserine binding and noncompetitively inhibit phorbol ester binding to activate the enzyme. Our studies suggest that if these metals were to interact with protein kinase C in vivo, they could impair its activation and translocation by inhibiting its binding to membrane phosphatidylserine. Many Ca²⁺- and dicylglycerol-dependent cellular processes could be dramatically altered by the disruption of this essential protein kinase.

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