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Purification and characterization of three types of protein kinase C from rabbit brain cytosol

(phorbol ester receptors/Ca^{2+} regulation)

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ABSTRACT **Three types of protein kinase C were purified from rabbit brain cytosol. Each type has a molecular mass of ≈80 kDa and serves as a receptor for phorbol esters. Polyclonal antibodies produced to two protein kinase C types were relatively type-specific, indicating that these proteins have unique antigenic determinants. We, therefore, characterized the enzymatic activities to determine if these proteins also had distinct biochemical properties. Type 1 protein kinase C was relatively less Ca^{2+}-dependent than types 2 and 3. The addition of Ca^{2+} increased V_{max} approximately 40% for type 1, 600% for type 2, and 1400% for type 3 as compared to the V_{max} measured at lower Ca^{2+} conditions. These results suggest that differences in primary structure can confer type-specific biochemical properties, and this in turn may provide the basis for protein kinase C type-specific stimulus–response coupling.**

Protein kinase C has recently been shown to be a gene family that codes for several closely related but distinct polypeptides (1–4). Comparison of the predicted primary structures of the coded proteins indicates an overall sequence homology in which four constant regions are separated by five variable regions. The spacing of the variable sequences is conserved among the three known protein kinase C structures (2, 3), which suggests that these variable regions may have important functional properties rather than merely being structural spacers. Furthermore, variable region 3 in one protein kinase C has significant sequence homology with a Ca^{2+} binding region of calmodulin (1). Thus, a potential difference in Ca^{2+} modulation of protein kinase C activity may be predicted from the deduced primary structures. Identification and characterization of the protein products is required to test this prediction.

The relationship between protein kinase C activation and cytosolic Ca^{2+} levels is not clear. In some cases, phorbol ester-directed responses appear to be nearly Ca^{2+}-independent (5). In other cases, a clear requirement for Ca^{2+} is indicated (6, 7). One explanation for these discrepancies is that different forms of protein kinase C have different requirements for Ca^{2+}. Possibly, differences in biochemical properties of the protein kinase C types may provide the basis for type-specific stimulus–response coupling. To test this hypothesis, we have characterized the three types of protein kinase C that we have purified with respect to modulation of activity by Ca^{2+} and PtdSer.

MATERIALS AND METHODS

Protein kinase C was measured using histone III-S as the substrate and the assay conditions described (8). Phorbol ester binding was measured using [^{3}H]phorbol 12,13-dibutyrate (PBt_2; 12.5 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) as previously described (9). Histone type III-S, PBt_2, and PtdSer were all purchased from Sigma. Electrophoresis reagents and hydroxylapatite were from Bio-Rad. Protein was determined by the method of Bradford (10) using reagents from Bio-Rad. Rabbit brains were purchased from Pel-Freez. Antibodies to types 2 and 3 protein kinase C were prepared after purifying the proteins by $NaDodSO_4$/PAGE and electrophoretically transferring them to nitrocellulose. The protein kinase C bands (≈150 μg) were identified by staining with amido black, dissolved in dimethyl sulfoxide, emulsified with complete Freund's adjuvant, and given to goats by multiple subdermal injections. After 5 weeks, animals were boosted with ≈100 μg of the purified proteins. Subsequent boosts were with native protein which was >90% pure as judged by $NaDodSO_4$/PAGE.

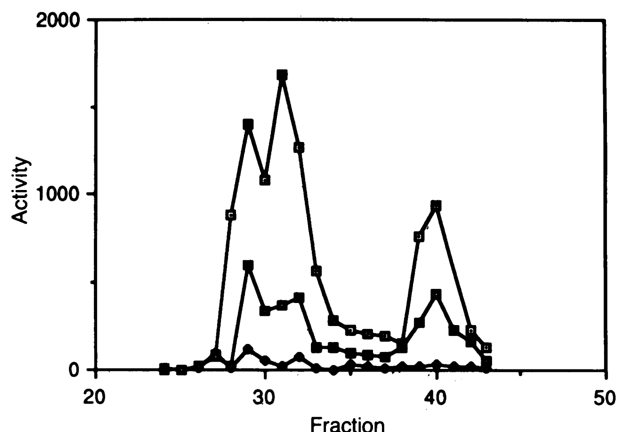

FIG. 1. Hydroxylapatite chromatography of rabbit brain protein kinase C. Protein kinase C from rabbit brain cytosol was prepared as described in the text and then chromatographed on hydroxylapatite. Fractions from the leading edge of the first peak (type 1), the trailing edge of the second peak (type 2), and the third peak (type 3) were pooled. Fractions between peaks 1 and 2, referred to as type 2a, were pooled and are considered to contain a mixture of types 1 and 2. Aliquots of fractions were assayed for PBt_2 binding (⊡) using 10 nM [^{3}H]PBt_2, 100 μg of PtdSer per ml, 0.33 mM EDTA, 0.5 mM Ca^{2+}, 0.5 mg of bovine serum albumin per ml, and 7.5 mM magnesium acetate (9). Kinase activities were assayed with 0.5 mM EDTA, 0.5 mM Ca^{2+}, 0.5 mg of bovine serum albumin per ml, 0.33 mg of histone III-S per ml, 2.5 mM ATP, and 7.5 mM Mg acetate (8) in the presence (◘) or absence (◆) of 100 μg of PtdSer per ml. Units for binding are [^{3}H]PBt_2 bound per μl of fraction. Units for kinase are nmol of ^{32}P transferred per μl in 5 min.

RESULTS

Resolution of Three Types of Protein Kinase C by Hydroxylapatite Chromatography. Frozen rabbit brains (25 brains)

Abbreviations: PBt_2, phorbol dibutyrate; PtdSer, phosphatidylserine.

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Table 1. Purification of PBt_2 binding and protein kinase C activities from rabbit brain cytosol

		$^3H[PBt_2]$ binding				Protein kinase C activity			
Fraction	Protein, mg	pmol/mg	Fold purification	Total	% recovery	nmol/mg	Fold purification	Total	% recovery
HAP load	600	88	1.0	52,800	100	49	1.0	29,400	100
Type 1									
HAP pool	30	176	2.0	5,280	10	33	0.7	990	3.4
PtdSer–EGTA	0.19	12,800	145	2,430	4.6	3,870	79	735	2.5
Type 2									
HAP pool	31	144	1.6	4,460	8.5	75	1.5	2,380	8.1
PtdSer–EGTA	0.16	10,800	123	1,730	3.3	3,030	62	485	1.7
Type 3									
HAP pool	24	299	3.4	7,180	14	125	2.6	3,000	10
PtdSer–EGTA	0.24	6,000	68	1,440	2.7	2,590	53	622	2.1

Data are representative of four separate preparations of each type of protein kinase C. HAP pool refers to peak fractions from hydroxylapatite (HAP) chromatography (see Fig. 1). PtdSer–EGTA refers to material specifically eluted with EGTA from the PtdSer-affinity column as described in the text. Units of binding activity are pmol of $^3H[PBt_2]$ bound per mg of protein. Units of kinase activity are nmol of ^{32}P transferred per mg of protein in 5 min in the presence of Ca^{2+} without PtdSer (independent kinase) or with PtdSer (protein kinase C activity). Independent kinase activity has been subtracted from the protein kinase C activity.

were homogenized in ≈300 ml of 20 mM Tris Cl, pH 7.4/0.25 M sucrose/5 mM EDTA/1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride/leupeptin (50 μg/ml). The 100,000 × g supernatant was applied to a 150-ml DEAE-Sephacel column, and protein kinase C was eluted with a 750-ml gradient of 20 mM Tris Cl, pH 7.5/1 mM EDTA/1 mM dithiothreitol (buffer A) with 0–0.3 M NaCl. Peak fractions were pooled, precipitated with 85% ammonium sulfate, and dialyzed against buffer A. The sample was then applied to a 1.7 × 25 mm column of hydroxylapatite equilibrated in 40 mM phosphate/2 mM EDTA. Activities were eluted with a 250-ml gradient containing up to 500 mM phosphate plus 2 mM EDTA. Fractions were assayed for kinase and PBt_2 binding activities (Fig. 1). Three peaks of Ca^{2+}/PtdSer-dependent kinase activity were found, each of which cochromatographed with phorbol ester binding activity as measured with PBt_2.

Peak fractions were pooled as described in the legend to Fig. 1 and adjusted to 100 μg of leupeptin per ml/7.5 mM Mg^{2+}/0.2 mM Ca^{2+} before applying them to a 3–4 ml PtdSer-affinity column (11) equilibrated in 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5/0.2 M KCl/1 mM dithiothreitol/10% glycerol (buffer B) containing 0.2 mM Ca^{2+}. Columns were washed with 25 ml of buffer B containing 0.2 mM Ca^{2+}. Protein kinase C was then eluted with 10 ml of buffer B containing 2 mM EGTA. The protein peak was concentrated in a Centricon-30 (Amicon) and stored at 4°C. Activities were stable for ≈1 month.

The relative amounts of protein kinase C types 1, 2, and 3 that are recovered by this procedure are similar (Table 1).

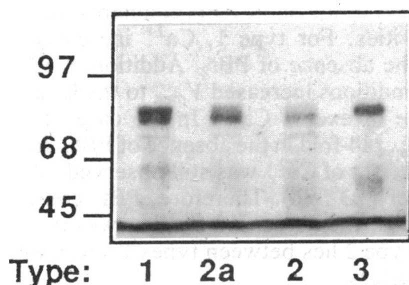

FIG. 2. $NaDodSO_4$/PAGE of PtdSer-affinity purified protein kinase C activities. Samples (1 μg) were electrophoresed on 7.5% $NaDodSO_4$/ polyacrylamide gels and stained with Coomassie blue. The doublets apparent in this preparation were reproducibly observed in four different preparations of protein kinase C and were not observed in 10% gels of the same thickness (0.75 mm). Numbers on the left refer to molecular mass (in kDa) of standard proteins.

The ratios of binding to kinase activities were also similar for each type under the standard assay conditions (i.e., saturating levels of Ca^{2+} and PtdSer). The purity of the final preparations was assessed by $NaDodSO_4$/PAGE (Fig. 2). Each sample contained a major band at ≈80 kDa. Lower molecular mass contaminants of ≈67 kDa in types 1 and 2 and 50 kDa in type 3 were apparent. Antibodies prepared to the protein kinase C bands react relatively poorly with these lower molecular mass bands (see below). Therefore, we cannot address the possibility that these bands represent protein kinase C fragments such as protein kinase M (12).

Immunochemical Characterization of Protein Kinase C Types. The immunoreactivity of each type of protein kinase C with antibodies prepared to type 3 protein kinase C is shown in Fig. 3*A*. A strong reaction was observed with 1 pmol of type 3. In contrast, the reaction with 10 pmol of types 1 and 2 was barely detectable. Similarly, antibodies prepared to type 2 protein kinase C were highly specific for type 2 as compared with types 1 and 3 (Fig. 3*B*). The production of type-specific antibodies to proteins that have similar enzymatic and binding properties suggests that they are related but not identical polypeptides. The fact that they each have

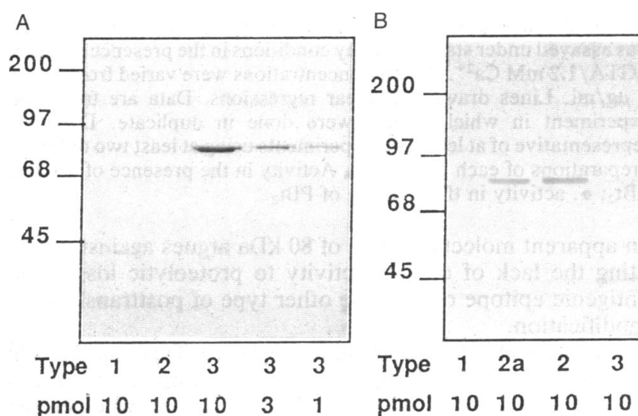

FIG. 3. Immunoreactivity of three types of protein kinase C. The indicated amounts of each isoform were electrophoresed through a 7.5% $NaDodSO_4$/polyacrylamide gel. The specific activity of each sample was approximately 10 pmol of $[^3H]PBt_2$ per μg of protein. Proteins were electrophoretically transferred to nitrocellulose. Immunoreactivity with antisera prepared to type 3 protein kinase C (1:100 dilution) (*A*) or type 2 protein kinase C (1:100 dilution) (*B*) was assessed using rabbit anti-goat IgG, which was coupled to horseradish peroxidase. Numbers on left refer to molecular mass (in kDa) of standard proteins.

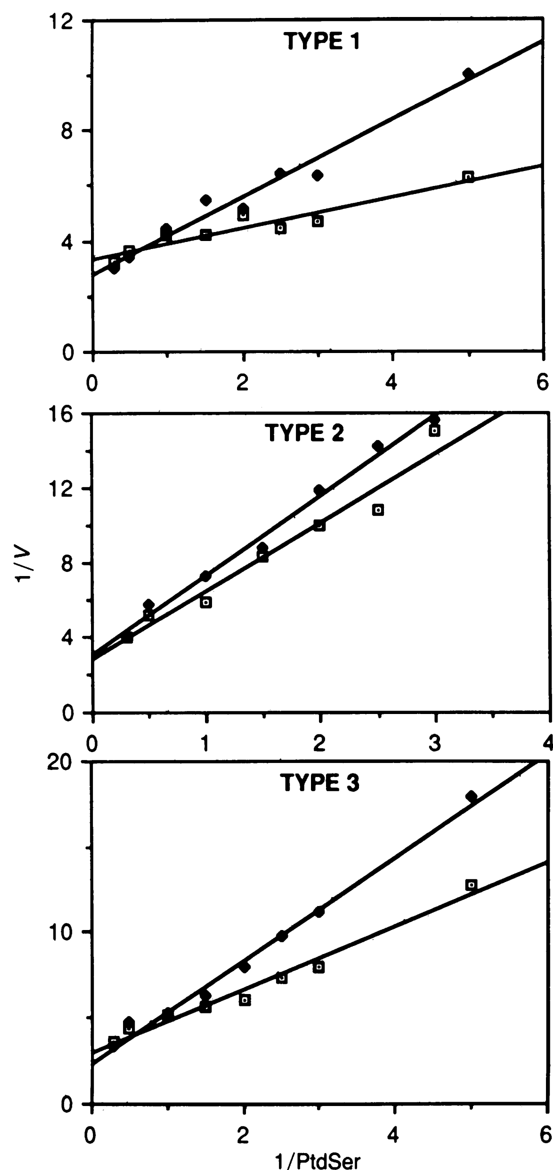

FIG. 4. Double-reciprocal plots of PtdSer requirement for kinase activity at saturating Ca^{2+}. Kinase activity of each isoform (16 ng) was assayed under standard assay conditions in the presence of 1 mM EGTA/1.2 mM Ca^{2+}. PtdSer concentrations were varied from 0.2 to 3 μg/ml. Lines drawn are linear regressions. Data are from one experiment in which assays were done in duplicate. Data are representative of at least two experiments using at least two different preparations of each isoform. ⊡, Activity in the presence of 200 nM PBt_2; ◆, activity in the absence of PBt_2.

an apparent molecular mass of 80 kDa argues against attributing the lack of cross-reactivity to proteolytic loss of an antigenic epitope or to some other type of posttranslational modification.

Biochemical Characterization of Three Types of Protein Kinase C. The Ca^{2+}/PtdSer requirements for the three types of protein kinase C were determined to test whether the associated kinase activities may be regulated differently. Protein kinase C activity is sensitive to Ca^{2+}, phospholipid, and diacylglycerol or phorbol ester. For example, the Ca^{2+} requirement is modified by the PtdSer concentration used in the assay and vice versa. Diacylglycerol and PBt_2 decrease both the Ca^{2+} and PtdSer requirements (13, 14). In order to identify differences among the protein kinase C activities, assay conditions were systematically varied. First, the concentration dependence for PtdSer was measured either at saturating Ca^{2+} (1.0 mM EGTA/1.2 mM Ca^{2+}) or limiting

Table 2. PtdSer dependence of protein kinase C activity at saturating Ca^{2+}

Protein kinase C	K_m, μg/ml − PBt_2	K_m, μg/ml + PBt_2	V_{max} − PBt_2	V_{max} + PBt_2
Type 1	0.58 ± 0.15	0.30 ± 0.10	3380 ± 808	3440 ± 1140
Type 2	1.9 ± 0.32	1.6 ± 0.37	3710 ± 153	3580 ± 540
Type 3	1.7 ± 0.1	0.40 ± 0.2	4370 ± 130	4780 ± 500

Data are from experiments performed as described in the legend to Fig. 4. All values are means ± SEM and were determined in at least two different protein kinase preparations. For all the K_m determinations and the V_{max} determination for type 3 protein kinase C without PBt_2, $n = 3$. For all other determinations, $n = 4$. PBt_2 significantly decreased the K_m for types 1 and 3 ($P < 0.05$) only. For each type of protein kinase, PBt_2 did not significantly increase V_{max}. The V_{max} values, with or without PBt_2, for each type of protein kinase C were not significantly different from one another. Statistical comparisons were by one-way analysis of variance.

Ca^{2+} (1.0 mM EGTA/0.6 mM Ca^{2+}). The limiting Ca^{2+} condition was calculated to produce a free Ca^{2+} concentration of ≈200 nM Ca^{2+} using published activity coefficients (15). Double-reciprocal plots for each protein kinase C type under excess Ca^{2+} conditions are shown in Fig. 4. In each case the plots were linear. Addition of PBt_2 did not increase the apparent V_{max} but did modestly decrease the K_m for PtdSer for types 1 and 3 ($P < 0.05$) (Table 2). The K_m for PtdSer for type 2 in the presence of PBt_2 was relatively higher than for types 1 and 3 ($P < 0.05$).

Under conditions of limiting Ca^{2+}, the dependence of protein kinase C activity on PBt_2 was more apparent. The saturation curves emphasize the differences (Fig. 5). Under these conditions, type 3 activity was highly dependent on added PBt_2. In contrast, type 1 activity was stimulated ≈30% by PBt_2. Type 2 was intermediate between types 1 and 3. The kinetic constants were estimated by double-reciprocal plots of data obtained by using 20–200 μg of PtdSer per ml and are summarized in Table 3. For each form, limiting Ca^{2+} conditions caused a 100-fold increase in the K_m for PtdSer as compared with the higher Ca^{2+} conditions (Table 2). Under the limiting Ca^{2+} assay conditions, PBt_2 did not significantly decrease the apparent K_m values for PtdSer. PBt_2 significantly increased V_{max} for each type of protein kinase C under these assay conditions ($P < 0.05$). The V_{max} value for type 1 was significantly larger than the V_{max} for types 2 and 3 in both the absence and presence of added PBt_2 ($P < 0.05$ in all cases).

The quantitative differences in Ca^{2+} and PBt_2 effects on V_{max} are summarized in Table 4. On the left, the effect of PBt_2 on the activities is compared. PBt_2 did not affect V_{max} under the high Ca^{2+} assay conditions. Under low Ca^{2+} conditions, PBt_2 increased V_{max} for type 3 more than it did for types 1 and 2. The right-hand side of the table compares the effect of Ca^{2+} on the activities. For type 1, Ca^{2+} increased the activity 1.4-fold in the absence of PBt_2. Addition of PBt_2 under the low Ca^{2+} conditions increased V_{max} to the level measured in the presence of excess Ca^{2+}. In the case of type 3, Ca^{2+} increased V_{max} 14-fold in the absence of PBt_2. In the presence of PBt_2, an effect of Ca^{2+} was still observed, although it was much smaller (3.3-fold). Therefore, PBt_2 could not entirely compensate for the decreased Ca^{2+} as was the case for type 1 activity. Type 2 lies between types 1 and 3 with respect to these parameters.

The activities were also compared with respect to the amount of Ca^{2+} required for maximal protein kinase C activity. For the purpose of illustrating differences among the protein kinase C types, a PtdSer concentration of 40 μg/ml was found to be suitable (Fig. 6). In each case, maximal activity in the absence of PBt_2 was achieved with 0.8–0.9 mM Ca^{2+} and 1 mM EGTA. Under these assay conditions, PBt_2

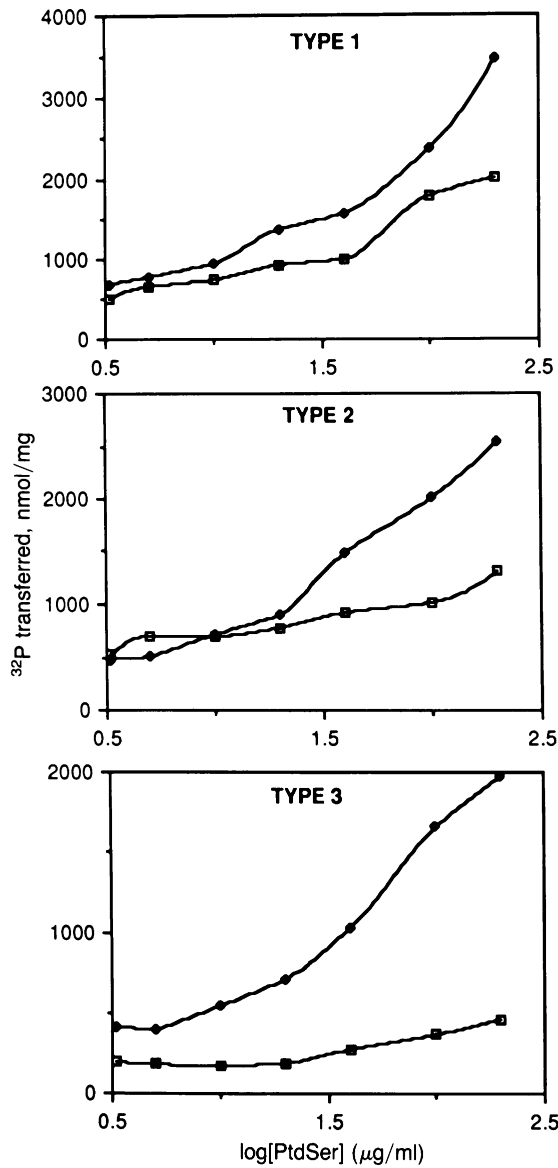

FIG. 5. PtdSer concentration dependence for kinase activity at limiting Ca^{2+}. Kinase activity of each isoform (16–50 ng) was assayed under standard assay conditions in the presence of 1 mM EGTA/0.6 mM Ca^{2+}. Data are from one experiment in which kinase activity was determined in duplicate. Data are representative of at least two experiments, which included assays of a different preparation of each isoform. ⊡, Activity in the presence of 200 nm PBt_2; ◆, activity in the absence of PBt_2.

Table 3. PtdSer dependence of protein kinase C activity at limiting Ca^{2+}

Protein kinase C	K_m, μg/ml		V_{max}	
	− PBt_2	+ PBt_2	− PBt_2	+ PBt_2
Type 1	58 ± 12*	72 ± 21†	1960 ± 318†	3830 ± 450†
Type 2	133 ± 39†	131 ± 40†	997 ± 129*	2390 ± 179†
Type 3	90 ± 25‡	44 ± 1‡	426 ± 103*	1470 ± 220*

Data are from experiments performed as described in the legend to Fig. 5. All values are means ± SEM and were determined in at least two separate protein kinase C preparations. For each type of protein kinase C, no statistically significant difference in K_m values was noted with or without PBt_2.
*$n = 3$.
†$n = 4$.
‡$n = 2$.

Table 4. Quantitative differences in Ca^{2+} and PBt_2 effects on V_{max}

Protein kinase C	V_{max} (+ PBt_2)/ V_{max} (− PBt_2)		V_{max} (high Ca^{2+})/ V_{max} (low Ca^{2+})	
	Low Ca^{2+}	High Ca^{2+}	− PBt_2	+ PBt_2
Type 1	1.8*	0.9	1.4	1.0
Type 2	2.6*	0.9	6.3*	1.5*
Type 3	3.5†	0.9	13.8*	3.3†

Data are from double-reciprocal plots of two separate preparations of each isoform. For each preparation, the double-reciprocal plots were reproduced two or three times. Statistical comparisons were by one-way analysis of variance.
*$P < 0.05$.
†$P < 0.01$.

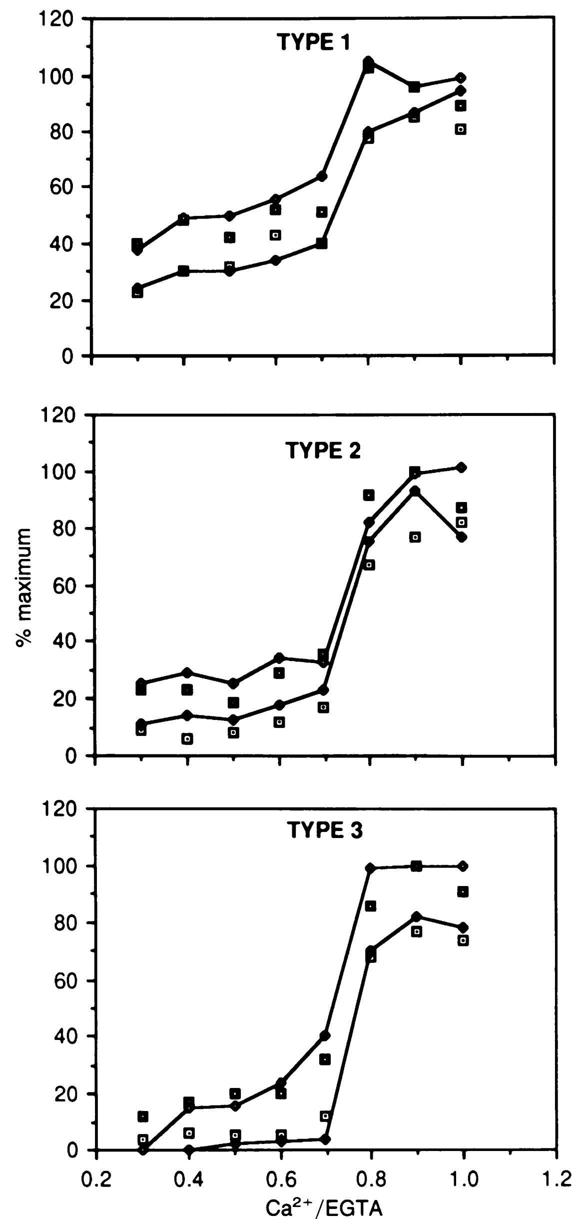

FIG. 6. Ca^{2+}-concentration dependence of kinase activity in the presence or absence of PBt_2. Kinase activity of each isoform (16 ng) was assayed under standard assay conditions in the presence of 40 μg of PtdSer per ml, 1 mM EGTA, and Ca^{2+} concentrations as indicated. Data shown are for two separate preparations of each isoform, each of which was assayed in duplicate. ◆ and ⊡, activity in the absence of PBt_2; ◘ and ◇, activity in the presence of 200 nm PBt_2.

did not substantially decrease the Ca^{2+} requirement; however, when assayed with lower PtdSer concentrations, an effect of PBt_2 on decreasing the Ca^{2+} requirement was observed for each protein kinase C type (data not shown). The most striking difference among the three protein kinase C types was the level of activity measured with excess EGTA versus higher Ca^{2+}. In each case, the activities measured with excess EGTA were still PtdSer-dependent and, therefore, are not considered to be due to independent kinase activity. In the absence of PBt_2, Ca^{2+} increased the activity of type 1 protein kinase C ≈5-fold. The effect of Ca^{2+} was larger for type 2 (≈10-fold) and type 3 (15- to 20-fold). At 1 mM EGTA and 0.3 mM Ca^{2+}, PBt_2 increased the activity of each type of protein kinase C; however, the percentage of maximal activity achieved was in the order type 1 > type 2 > type 3.

DISCUSSION

Recent evidence from several laboratories has demonstrated that there is a family of protein kinase C genes (1–4). Comparison of the genes indicates that the coded proteins are homologous but that structural differences in the variable regions may account for biochemical differences among the coded proteins. These biochemical differences may provide the basis for type-specific stimulus–response coupling *in vivo*. Differences in potential Ca^{2+} binding regions among the three bovine clones have already been described (1). This result presents the strong possibility that the coded proteins may have distinct Ca^{2+}-regulatory features. This would help to explain some of the difficulties encountered in defining the relationship between protein kinase C activation by diacylglycerol or phorbol esters and the required level of cytosolic Ca^{2+}. In some cases, phorbol ester action can be demonstrated with vanishingly small Ca^{2+} concentrations (5), perhaps due to a type 1-like protein kinase C activity. Results of other experiments indicate that protein kinase C is a Ca^{2+}-dependent enzyme and that cytosolic Ca^{2+} levels are required to support the activity in the presence of diacylglycerol or phorbol esters (6). These responses seem more similar to a type 3 protein kinase C activity. The identification of specific Ca^{2+} requirements for the three isoforms is an important clue for sorting out the relationship between cytosolic Ca^{2+} levels and protein kinase C activation.

At present, the evidence that the three proteins from rabbit brain represent different gene products is based on the production of protein kinase C type-specific antibodies. Separation of three protein kinase C types from rat brain cytosol by using hydroxylapatite has also been reported recently (16). Evidence that these are isozymes was based on the identification of unique autophosphorylated peptides and the production of type-specific monoclonal antibodies. The presence of doublets in our type 1 preparation may also suggest additional protein kinase C heterogeneity. Sequence analysis is required to define unique primary sequences and to determine the relationship between these proteins and genes that have already been cloned. In collaboration with Karen Leach (Upjohn), we have recently produced several monoclonal antibodies against each protein kinase C type. These will be valuable for structure–function analysis of protein kinase C types and will help in identifying sequences that are involved in regulation by Ca^{2+}, PtdSer, and PBt_2.

In conclusion, these results document biochemical differences among the protein kinase C activities associated with three apparently different proteins. The three enzymes also differ in $[^3H]PBt_2$ binding properties (unpublished results). The differences in enzymatic activity bear out the predictions from analyses of the isolated genes, which suggest that type-specific sequences may result in type-specific biochemical properties. The knowledge obtained from these biochemical analyses must be applied to cell culture model systems in order to fully address the functional significance of the observed biochemical differences among protein kinase C types.

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1. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. & Ullrich, A. (1986) *Science* **233**, 853–858.
2. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T., Chen, E., Waterfield, M. D., Fraacke, U. & Ullrich, A. (1986) *Science* **233**, 859–866.
3. Knopf, J. L., Lee, M., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. W. & Bell, R. M. (1986) *Cell* **46**, 491–502.
4. Ono, Y., Kurokawa, T., Kawahara, K., Nishimura, O., Marumoto, R., Igarashi, K., Sugino, Y., Kikkawa, U., Ogita, K. & Nishizuka, Y. (1986) *FEBS Lett.* **203**, 111–115.
5. DiVirgilio, F., Lew, D. P. & Pozzan, T. (1984) *Nature (London)* **310**, 691–693.
6. Drust, D. S. & Martin, T. F. J. (1985) *Biochem. Biophys. Res. Commun.* **128**, 531–536.
7. Wolf, M., Cuatrecasas, P. & Sayhoun, N. (1985) *J. Biol. Chem.* **260**, 15718–15722.
8. Jaken, S. (1985) *Endocrinology* **117**, 2293–2300.
9. Jaken, S. (1987) *Methods Enzymol.* **141**, 275–287.
10. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
11. Uchida, T. & Filburn, C. (1984) *J. Biol. Chem.* **259**, 12311–12315.
12. Inoue, M., Kishimoto, A., Takai, Y. & Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7610–7616.
13. Nishizuka, Y. (1986) *Science* **233**, 305–311.
14. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851.
15. Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W. (1983) *J. Biol. Chem.* **258**, 15336–15345.
16. Huang, K. P., Nakabayashi, H. & Huang, F. L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8535–8539.