Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 340-345

IDENTIFICATION OF A CALCIUM- AND PHOSPHOLIPID- DEPENDENT PHORBOL ESTER BINDING ACTIVITY IN THE SOLUBLE FRACTION OF MOUSE TISSUES

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Received January 19, 1983

A soluble cytosolic protein which specifically and with high affinity binds tumor promoting phorbol ester tumor promoters was found in mouse tissues. Calcium and phosphatidyl serine had to be included in the binding assay to obtain maximal binding activity by the soluble receptor. Soluble binding activity levels paralleled the levels of membrane associated binding activity, with the soluble activity accounting for 27% to 80% of the total. Except the calcium and phospholipid requirement, no difference was found between the soluble and membrane associated receptors, suggesting that they are the same protein which can move between the soluble cytosol and cell membranes.

Early studies of the molecular mechanism of action of the tumor promoting 12-O-tetradecanoylphorbol-13-acetate (TPA)[2] suggested an effect on cellular membrane constituents(1,2,3). More recently methods for measuring specific and high affinity binding of tritiated TPA and its less lipophilic analog, phorbol-12,13-dibutyrate, by cells or cell membranes have been reported (4,5). Although investigators utilizing [^{3}H]phorbol-12,13-dibutytrate have used numerous methods for separating bound from free ligand, no phorbol ester binding activity was detected in the soluble cytosol of cells or tissues (6,7). Using the cold acetone assay (4) a small amount of [^{3}H]TPA binding activity was detected in the soluble cytosol of mouse epidermis that could not be accounted for by contamination with membrane protein. This was not pursued until recently when it was found that the binding of [^{3}H]TPA by mouse brain soluble cytosol was greatly enhanced by the conditions used for reconstitution of the

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[2]Abbreviations used: TPA, 12-O-tetradecanoylphorbol-13-acetate; PK-C, protein kinase C.

0006-291X/83/040340-06$01.50/0

TPA receptor during its purification from a detergent extract of brain particulate protein. A preliminary characterization of this soluble phorbol ester receptor protein is described below. The possibility that this soluble receptor is the same protein as a calcium- and phospholipid-dependent protein kinase (PK-C)[2] was also investigated.

MATERIALS AND METHODS

Mouse tissues were excised from 10 to 18 week old female CD-1 mice (Charles River Breeding Labs) after sacrifice by cervical dislocation and were placed in 1 to 30 ml of homoginizing buffer (20 mM Tris, 10 mM EDTA, 2 mM ethyleneglycol-bis(2-aminoethyl ether) N,N´-tetraacetic acid, 2 mM phenyl methylsulfonyl fluoride, pH 7.4). The tissues were homogenized with a Brinkman polytron homogenizer and were sedimented at 100,000 x g for 1 h or 78,000 x g for 1.5 h. Floating lipid was discarded and the soluble supernatent fluid was removed and saved. A volume of assay buffer (20 mM Tris - HCl, pH 7.4) equal to the original homogenate was added to the pelleted protein and the protein was suspended by brief treatment with the polytron homogenizer. Epidermis was removed from the dermis, homogenized, and filtered as previously described (4) except the homogenizing buffer described above was used. Before homogenization the stomach and intestines were rinsed with sufficient homogenizing buffer to remove their contents. All procedures were performed at 2° to 4°.

Specific binding of [^{3}H]TPA (6.5 Ci/mmol from New England Nuclear) to protein samples was quantitated using the cold acetone method (4) modified as follows. Samples (5 to 40 µl containing 1 to 1000 µg of protein) were added to 1 ml of assay buffer containing 10 µg phosphatidyl serine (Sigma Chemical Co.) and 1 mM $CaCl_2$. Phosphatidyl serine was omitted from the particulate protein assays. [^{3}H]TPA (10 pmol) was added with and without TPA (5 nmol) in 5 µl dimethylsulfoxide and the 1 ml mixtures were incubated for 1.5 to 6 h at 2° to 4°. Five 2.5 ml washes of cold acetone were used to separate bound from free [^{3}H]TPA.

Calcium- and phospholipid-dependent protein kinase activity was determined by addition of 2.5 nmol γ-^{32}P ATP (0.2 to 0.8 Ci/mmol from New England Nuclear) to 30 µg histone (type 3 from Sigma Chemical Co.), 500 nmol magnesium acetate, 6.25 µmol 2-mercaptoethanol, 10 µg phosphatidyl serine, 125 nmol $CaCl_2$, and 1 to 10 µl of protein sample in 125 µl of assay buffer. After incubation at 30° for 3 min the reactions were stopped by individually transferring two 50 µl aliquots onto 2.5 cm square pieces of phosphocellulose paper (PC81 from Whatman) and washing with water, acetone, and ether as described (8). The radioactivity on each piece of paper, determined by scintillation counting, was averaged for each assay and the results from control assays which did not contain phosphatidyl serine or calcium were subtracted.

Protein was determined by the method of Lowry (11) using bovine serum albumin as the standard protein

RESULTS AND DISCUSSION

The most comprehensive published studies of the subcellular (6) and tissue distributions (7) of phrobol ester receptors have not revealed the presence of any binding activity in the soluble cytosolic fraction of mouse tissues. TPA

Table 1. Cofactor requirements of the soluble cytosolic phorbol ester receptor

Calcium	Phosphatidyl serine	[^{3}H]TPA Specifically Bound (pmol)
0	0	0.056 ± 0.008
0	3 μg/ml	0.086 ± 0.010
0.1 mM	3 μg/ml	0.242 ± 0.096
1 mM	0	0.446 ± 0.078
1 mM	10 μg/ml	0.916 ± 0.078

Specific [^{3}H]TPA binding to 15 μg of soluble cytosolic protein prepared from mouse brain was determined in triplicate assays and the means ± S.D. are shown.

binding activity has been extracted in soluble form from nuclei prepared from mouse epidermis (9) and from mouse brain with detergent (10). After chromatographic fractionation of the detergent extract the TPA binding activity was

Table 2. [^{3}H]TPA binding activity and protein kinase activity in fractions of mouse tissues.

Tissue	[^{3}H]TPA binding activity			Soluble PK-C activity
	Soluble (pmol/mg protein)	Particulate (pmol/mg protein)	Fraction soluble(%)	(pmol/min/mg protein)
Forebrain	104.04	25.37	40	6688
Cerebellum	75.94	17.64	44	4742
Spleen	22.84	11.32	79	784
Spinal Cord	16.19	8.90	40	712
Stomach	15.30	5.84	52	422
Thymus	12.06	4.58	76	392
Ovaries	6.48	1.46	40	98
Small Intestine	4.49	5.68	27	84
Colon	3.38	0.76	64	152
Epidermis	3.38	3.35	48	110
Lung	3.27	4.17	66	155
Adrenal	2.88	1.97	56	63
Heart	2.54	0.15	69	69
Kidney	2.42	0.89	57	53
Fat	1.64	2.33	68	82
Skeletal Muscle	1.34	0.30	80	51
Liver	0.80	0.15	78	16

Assays were done as described on duplicate preparations of each tissue. The mean results for the two samples are listed.

greatly enhanced when phospholipid and calcium were included in the binding assay (10). This led to the finding that calcium and phospholipid, particularly phosphatidyl serine, also greatly enhanced the TPA binding aactivity present in the soluble cytosol prepared from mouse brain as shown in Table 1. The amounts of calcium and phosphatidyl serine utilized yielded maximal binding of [^{3}H]TPA. Specific [^{3}H]TPA binding activity has been found in all the mouse tissues listed in Table 2, which shows the TPA receptor concentrations in the soluble and particulate fractions of those tissues. The rank orders of tissues based on the soluble and particulate receptor concentrations are approximately the same. The neural tissues, spleen, stomach, and thymus contain the highest concentrations and the liver, muscle, heart, fat, kidney, and adrenals the least. With the exception of the small intestine, the soluble receptor represented 40% to 80% of the total [^{3}H]TPA binding activity present in the tissue fractions. These data show that the soluble cytosol contains a major pool of TPA receptor that may be quite related to the cellular membrane associated pool of TPA receptor.

The degree of similarity between the soluble and membrane associated TPA receptors was investigated next. The ability of various chemical analogs of TPA to inhibit the binding of [^{3}H]TPA to mouse brain soluble cytosol and

Table 3. Specificity of soluble and particulate [^{3}H]TPA receptors from mouse brain for binding phorbol esters and mezerein.

Inhibitor	Concentrations Tested (M)	Concentration which inhibits by 50% (nM)	
		Particulate	Soluble
TPA	10^{-10} to 10^{-7}	2.1	2.5
Mezerein	10^{-9} to 10^{-6}	37.2	14.8
Phorbol-12,13-dibenzoate	3×10^{-6} to 3×10^{-9}	100	158.5
Phorbol-12,13-dibutyrate	10^{-7} to 10^{-4}	794	1479
Phorbol-12,13-diacetate	3×10^{-7} to 3×10^{-4}	15850	60260

Inhibitors were added in 5 µl dimethylsulfoxide to the standard binding assay (using 4nM [^{3}H]TPA) to give final concentrations in the range indicated. The concentrations of inhibitors which inhibited [^{3}H]TPA binding by 50% of the maximal inhibition were determined by graphical analysis.

particulate protein were not markedly different, as shown by the data in Table 3. Preliminary results from a detailed biochemical comparison of the soluble and particulate TPA receptors (to be published elsewhere) have failed to demonstrate any differences between the two. This suggests that the TPA receptor may be able to move between the soluble and membrane associated pools.

It was recently reported (12) that TPA could activate a calcium- and phospholipid-dependent protein kinase known as PK-C. Since this was demonstrated with a soluble protein preparation it raises the possibility that the soluble TPA receptor reported here may in fact be this protein kinase. To further investigate this possibility, PK-C activity was determined for the soluble fractions of all the tissues listed in Table 2. The results show that the distribution of PK-C activity in the soluble cytosol of mouse tissues parallels the distribution of the soluble TPA receptor. The PK-C specific activities vary over 550-fold yet the ratio of PK-C to [^{3}H]TPA binding activities only varies over a four-fold range. These data along with the existence of a particulate form of PK-C (13) and the very broad phylogenetic distribution for both the TPA receptor (7) and PK-C (14) strongly support the conclusion that at least one biological role of the TPA receptor is as a calcium- and phospholipid-dependent protein kinase.

ACKNOWLEDGEMENT

This research was supported by NIH Grants CA 22484 and CA 07175.

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