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DIRECT MEASUREMENT OF SPECIFIC BINDING OF HIGHLY LIPOPHILIC PHORBOL DIESTER TO MOUSE EPIDERMAL MEMBRANES USING COLD ACETONE

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SUMMARY

Using acetone cooled to $-78^{\circ}C$ to wash a mouse epidermal membrane preparation after incubation with [^{3}H]12-0-tetradecanoylphorbol-13-acetate, nonspecific binding of the ligand is reduced by 100 fold allowing specific binding to be easily quantitated. This technique may be useful in binding studies of other highly lipid soluble chemicals to membrane constituents.

Investigations of specific binding of the highly lipophilic tumor promoting phorbol diester [^{3}H]12-0-tetradecanoylphorbol-13-acetate (^{3}H-TPA)[1] to membrane preparations have been largely unsuccessful (1,2,3). This has been because the majority of the ^{3}H-TPA partitions into the lipids of the membranes and the assay methods used to date have not separated the ^{3}H-TPA partitioned in the membrane from the ^{3}H-TPA specifically bound by one or more membrane proteins. One solution to this problem is to reduce the amount of membrane to very low levels to limit the nonspecific partitioning. In crude particulate preparations of mouse epidermis (the target tissue for TPA in skin tumor promotion), this would result in unmeasurably small amounts of specific binding. However, ^{3}H-TPA binding to minute amounts of crude particulate preparations of the brain, which have more specific binding activity, has been successfully measured (4). Another solution has been to use a less lipophilic analog

[1] Abbreviations used: ^{3}H-TPA = [^{3}H]12-0-tetradecanoylphorbol-13-acetate, ^{3}H-PDB = [^{3}H]phorbol-12,13-dibutyrate.

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of TPA, ^{3}H-PDB[1], which apparently is bound at the same site as TPA in brain (4) but which has only weak mouse skin tumor promoting activity (5). An alternative which avoids these drawbacks is to reduce the partitioning into the membrane lipids by reducing the polarity of the medium in which the membrane is suspended. This is accomplished by the addition of water miscible organic solvents. Unfortunately, with the exception of some polyhydroxylated compounds, these solvents generally denature most proteins by their ability to reduce the polarity of the solution (6). Denaturation is slowed considerably when the temperature of the solvent water mix is reduced below 0°C as evidenced by the generally accepted method of solvent fractionation of proteins at reduced temperatures (6). The assay described here employs the addition of -78°C acetone to buffered solutions or suspensions of the test sample. It is demonstrated that this reduces nonspecifically bound ^{3}H-TPA to acceptably low levels, and that the use of cold acetone does not denature the molecule responsible for the binding ^{3}H-TPA or certain membrane associated enzymes.

METHODS AND MATERIALS

Crude epidermal membranes were prepared from epidermal material scraped by razor blade from pieces of depiliated dorsal skin from 100 female 16 to 26 week old CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA). The scrapings were placed in 30 ml of 20 mM Tris-HCl, pH 7.4 buffer containing 2 mM 2-mercaptoethanol and 0.25 M sucrose, homogenized for 60 sec with a polytron homogenizer and filtered through nylon mesh (63 μm pore size). The residue was again placed in 30 ml of buffer, homogenized, and filtered. This was repeated until the last filtrate was nearly clear, or 5 to 7 times in all and then the pooled filtrate was centrifuged for 30 min at 1000 x g. After discarding the floating lipid layer the supernatant was decanted and saved. An additional 30 ml of buffer were added, the pellet resuspended by vortex mixing, and centrifuged again. This process was repeated and all 3 supernatants were combined and centrifuged for 45 min at 88,000 x g. The membrane pellets were resuspended by sonicating in 100 ml of 20 mM Tris-HCl pH 7.4 buffer with 2 mM 2-mercaptoethanol. Yields of protein ranged from 20 to 50 mg.

Specific binding of ^{3}H-TPA was assayed using 1 ml of resuspended crude epidermal membranes in dichlorodimethylsilane-treated 13 x 100 mm glass test tubes. ^{3}H-TPA (New England Nuclear, Boston, MA, specific radioactivity of 17.2 Ci/mmol) dissolved in dimethylsulfoxide with

or without a 500 fold excess amount of unlabeled TPA (Lifesystems, Newton, MA) was added to each sample in a total volume of 5 µl, and vigorously mixed. ^{3}H-TPA was added to give the final total concentrations specified. After 2 to 6 hrs incubation at 4°C, each sample was filtered as follows: 2.5 ml of acetone cooled in a dry ice-ethanol bath was added to the sample and the contents immediately decanted into a Millipore glass microanalysis filter holder equipped with a screen support and a 2.4 cm diameter Whatman GF/C glass fiber filter, fitted into a side arm flask attached to a vacuum. The tube and filter were washed with 2.5 ml cold acetone for a specified additonal number of times. The filter was placed in a scintillation vial with 5 ml toluene based scintillation fluid for determination of radioactivity bound to the filter. Specific binding was calculated as the average amount bound without excess unlabeled TPA less the average amount bound in the presence of excess unlabeled TPA (nonspecifically bound).

Unless specified otherwise all chemicals were reagent grade and all the above operations were done at 0 to 4°C. Protein was assayed by the method of Bradford (7) after dissolving the protein in 0.1% sodium dodecylsulfate. Phospholipid was determined as described by Hess and Derr (8).

RESULTS AND DISCUSSION

It is evident from the data in Figs. 1A and 1C that acetone at any temperature is effective at reducing non-specifically bound ^{3}H-TPA while buffer is not. Specifically bound ^{3}H-TPA was not dependent upon the number of rinses but was reduced considerably with 0° or 21° acetone compared to -78° acetone (Fig. 1B). This is consistent with slower protein denaturation at -78°C than 0° or 21°C. Rinsing with buffer (Fig. 1D) did not result in reproducible amounts of specifically bound ^{3}H-TPA. Saturable specific binding of high affinity (K_D = 1.1 nM based on total TPA concentration) is shown in Fig. 2A. Fig. 2B shows that specific binding is linear with respect to protein for concentrations between 20 and 200 µg/ml, when using a total ^{3}H-TPA concentration of 13.1 nM. In contrast, Figures 2C and 2D show that when buffer is used in the binding assay in place of -78°C acetone, the nonspecifically bound ^{3}H-TPA does not saturate at high ^{3}H-TPA concentrations but does saturate at protein concentrations above 10 µg/ml when using 13.1 nM total ^{3}H-TPA concentration. This is consistent with simple partitioning of ^{3}H-TPA into the hydrophobic portions of the membranes. By calculating

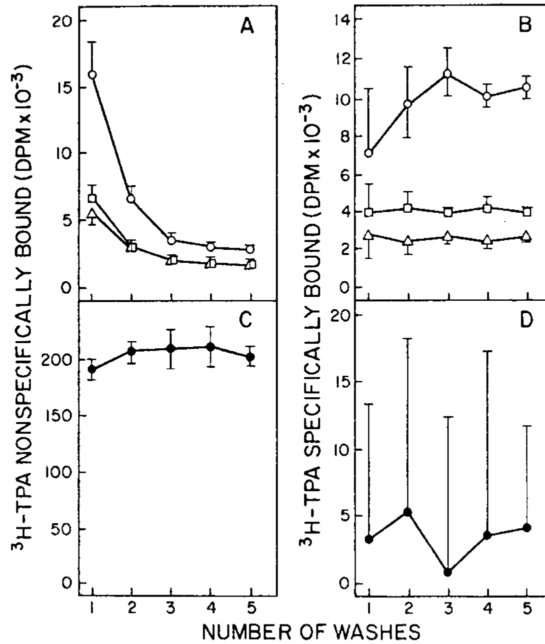

Fig. 1. Nonspecifically bound (Figs. A and C) and specifically bound (Figs. B and D) ^{3}H-TPA after incubation with 71 µg crude mouse epidermal membranes with 15 nM ^{3}H-TPA and rinsing the tube and filter with the indicated number of 2.5 ml volumes of rinsing agent: -78°C acetone (○), 0°C acetone (□), 21°C acetone (△) or 0°C buffer (●). Brackets indicate standard deviations (N=4).

the ratio of nonspecific binding in Figure 2A to that in Figure 2C, it is evident that the nonspecific binding of ^{3}H-TPA was reduced 100 fold. This means that by using acetone cooled to -78°C, the ratio of specific to nonspecific binding at the ^{3}H-TPA concentration which gave half maximal binding (1 nM) was 25.3 instead of 0.33. In fact, using buffer only, the amounts of nonspecifically bound ^{3}H-TPA were so high at the protein concentration used that specific binding of ^{3}H-TPA could not be measured accurately at any ^{3}H-TPA concentration.

Three objections could be made to the use of acetone. First is the question of derangement of membrane structure, including dissolution of membrane lipid and denaturation of proteins, resulting in reduction of the amount of specifically bound ^{3}H-TPA. Under typical assay conditions 51±5% of the phospholipid and 13±2% of the protein in epidermal membrane

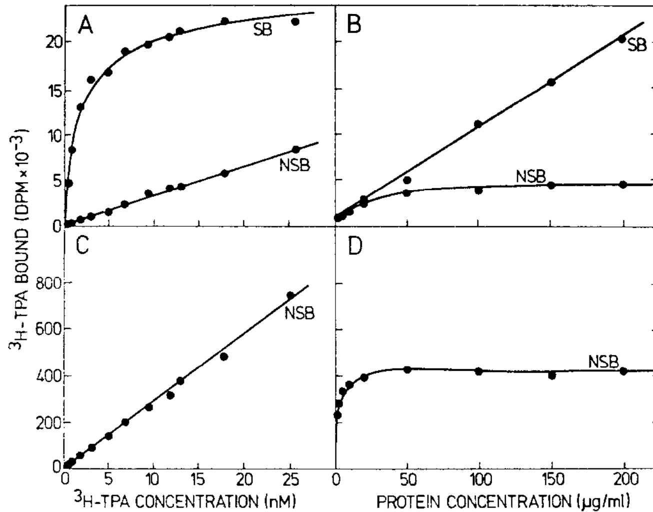

Fig. 2. Binding of ^{3}H-TPA to 220 μg/ml crude mouse epidermal membranes at various total ^{3}H-TPA concentrations (Figs. A and C) and to various concentrations of membrane protein using a total ^{3}TPA concentration of 13.1 nM (Figs. B and D). Specifically bound ^{3}H-TPA (SB) and non-specifically bound ^{3}H-TPA (NSB) were measured as described for Figs. A and B but not for Figs. C and D in which 0°C buffer was used in place of -78°C acetone as a rinsing agent. Five 2.5 ml volumes of the rinsing agent were used for each assay. Each point is the mean of quadruplicate assays.

preparations passes through the filter, suggesting that cold acetone dissolves some but not all of the membrane lipid. In another experiment, the activity per mg protein of phosphodiesterase I, 5' nucleotidase, and ^{3}H-TPA binding were not affected by addition of -78°C acetone to epidermal membranes and sedimentation of the protein, indicating protein denaturation did not occur (data not shown). Secondly, acetone may alter the energetics of the binding reaction by reducing the polarity of the medium. At present there is no evidence that bears on this point, although "lipophilic interaction" energies are reportedly reduced in water-solvent mixtures, especially at low temperatures (6). However, if the binding equilibrium is not appreciably temperature dependent, but the association and dissociation reactions are, as suggested in preliminary experiments (not shown) then at -78°C it is unlikely that the amount of specifically bound ^{3}H-TPA changes in the few seconds

when acetone is present. Thirdly, it might be argued that acetone induces aggregation and precipitation of proteins and that ^{3}H-TPA might be nonspecifically trapped in such aggregates. If this occurs, any ^{3}H-TPA bound by aggregated protein would be displaced by excess unlabeled TPA and so could be distinguished from specifically bound ^{3}H-TPA.

Unfortunately, this method has the limitation that a number of agents interfere with the assay. Detergents reduce the binding considerably, while salts and sucrose at concentrations at or above 60 mM often form precipitates which slow the filtration rate.

There are several advantages to the use of the cold acetone method to directly measure the binding of a highly lipophilic molecule to membrane constituents as contrasted with the use of a less lipophilic analog or the use of very low membrane concentrations. First, there may be no analog available which is less lipophilic, or as in the case of phorbol diesters, less lipophilic analogs may be virtually inactive. The cold acetone method can be used to accurately quantitate specific binding in the presence of much larger amounts of membrane lipid than can be tolerated when cold acetone is not employed, allowing detection of specific binding by tissues or tissue fractions with very low amounts of binding activity. The use of acetone also makes filtration assays possible, since without acetone the large surface area of most filters would probably result in large amounts of nonspecific binding. Filter assays are much more rapid than sedimentation assays, and require very little specialized equipment. This method is also useful for binding assays with protein solutions and cell suspensions. Perhaps most importantly, this method could be used in the study of membrane binding of other highly lipophilic agents for which there is only indirect evidence of specific high affinity binding. This might include studies with phenobarbital type inducers of microsomal mixed function oxidases (9), tetrahydrocannabinol, the fat soluble vitamins, certain chemical carcinogens, and plant and microbial toxins.

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REFERENCES

1. Lee, L.-S. and Weinstein, I.B. (1978) J. Environ. Pathol. and Toxicol., 1, 627-639.
2. Mastro, A.M. and Pepin, K.G. (1980) Chem.-Biol. Interact., 30, 171-179.
3. Schimmel, S.D., Grotendorst, G.R., and Grove, R.I. (1980) Cancer Lett., 9, 229-236.
4. Dunphy, W.G., Delclos, K.B., and Blumberg, P.M. (1980) Cancer Res., 40, 3635-3641.
5. Thielmann, H.W. and Hecker, E. (1969) Fortschritte der Krebsforschung (Schmidt, G.G. and Wetter, O., eds), Vol. 7, 171-179, Shattauer, New York.
6. Douzou, P. and Balny, C. (1979) Adv. Protein Chem., 32, 77-189.
7. Bradford, M. (1976) Anal. Biochem., 72, 248-254.
8. Hess, H.H. and Derr, J.E. (1975) Anal. Biochem., 63, 607-613.
9. Poland, A., Mak, I., Glover, E., Boatman, R.J., Ebetino, F.H., Kende, A.S. (1980) Molecular Pharmacol., 18, 571-580.