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Deacetylation of Forskolin Catalyzed by Bovine Brain Membranes

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Summary: Radiolabeled forskolin, 7-(^{3}H-acetyl)-forskolin, was synthesized to explore interactions between forskolin and bovine brain membrane preparations. The radiolabeled derivative was chemically characterized, and found to be indistinquishable from unlabeled forskolin in its ability to stimulate bovine brain adenylate cyclase. Preliminary binding data demonstrated that binding of 7-(^{3}H-acetyl)-forskolin to membranes was concentration dependent. However, competition binding studies using a constant concentration of 7-(^{3}H-acetyl)-forskolin with increasing levels of unlabeled forskolin showed enhanced binding of the labeled derivative. This suggested that 7-(^{3}H-acetyl)-forskolin was degraded by membranes and protected by native forskolin. Incubation of forskolin with membranes and analysis of the products by thin layer chromatography and mass spectroscopy showed the formation of 7-desacetylforskolin. The deacetylation of forskolin was monitored by quantitating the release of [^{3}H]acetate from 7-(^{3}H-acetyl)-forskolin. The reaction was linear with time and protein concentration. These data illustrate that forskolin can be degraded by membranes and indicate that ligand binding studies using labeled forskolin and membrane preparations should be cautiously interpreted.

Forskolin is a naturally occuring diterpene isolated from the roots of *Coleus forskolii* (1). The drug has cardiovascular activity which appears to be related to its ability to activate adenylate cyclase (2). It has been suggested that forskolin acts directly on the catalytic subunit of adenylate cyclase or indirectly through a protein closely associated with the catalytic subunit which is different than the stimulatory guanine nucleotide binding protein (N_S) (3,4,5). However, the stimulation of adenylate cyclase by forskolin can be modified by other adenylate cyclase activators (6) and potentiated by N_S subunit activation (5). In addition, adenylate cyclase of mammalian sperm, which lacks N_S, is not activated by forskolin (7). The mechanism for forskolin activation of adenylate cyclase and its precise site of action are undefined.

Recently, several radiolabeled derivatives of forskolin have been synthesized (8,9) in an effort to study forskolin binding to crude membrane preparations. This work and preliminary work in this laboratory using bovine cerebral cortex membranes showed that there is heterogeneity in forskolin binding to membranes. In addition, the affinities of the various derivatives of forskolin do not correlate well with the potency for forskolin in adenylate cyclase activation. Because of the complexity of the binding isotherms, we examined the possibility that forskolin may be degraded by membranes. In this study, radiolabeled forskolin was prepared with tritium in the acetoxy group at position 7. This derivative was used to detect forskolin esterase activity in crude bovine brain membrane preparations.

Materials and Methods

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the general method of Salomon, *et al.* (10) except that an ATP-regenerating system was unnecessary. Assay mixtures contained 0.25 mM

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[α-^{32}P]ATP(100 cpm/pmol), 5 mM $MgCl_2$, 20 mM Tris·HCl(pH 7.4), 0.1% bovine serum albumin, 5 mM theophylline, 1 mM ß-mercaptoethanol and 1 mM EDTA. Assays were done in triplicate at 30°C for 10 minutes, and recovery was monitored with [^{3}H]cAMP. Protein concentrations were determined by the method of Peterson(11).

Preparation of Bovine Brain Membranes. 250 grams of bovine cerebral cortex was homogenized in an equal volume of homogenization buffer with a Waring blender for 30 seconds. Homogenization buffer contained 20 mM glycylglycine, 5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, pH 7.2, 3 mM dithiothreitol with/without 1 mM phenylmethanesulfonyl fluoride (PMSF). The preparation was then further disrupted with a Polytron homogenizer (30 seconds at maximum setting). The resulting homogenate was centrifuged in a Sorvall RC-3B centrifuge for 30 minutes at 4800 rpm. The pelleted membranes were resuspended in an equal volume of homogenization buffer and the polytron homogenization, centrifugation and resuspension steps repeated three times. The final membrane pellet was aliquoted and stored at -80°C.

Preparation of other Tissues. Rat heart and liver membranes were prepared as stated above for bovine brain membranes. Rat liver cytosol was prepared as per Neville (12) taking the supernatant from the first low speed spin (1500 X g for 10 min.) of homogenized rat liver. This supernatant was then centrifuged at 100,000 X g for 1 hr, the supernatant from this spin was designated as rat liver cytosol.

To prepared human plasma and erythrocytes, whole blood in 0.15% EGTA was centrifuged at 2000 rpm for 5 minutes, the supernatant from this spin was designated human plasma. The erythrocytes were washed two times in PBS and resuspended in a minimal amount of PBS. Sheep whole blood was obtained from Prepared Media Laboratories and sheep plasma obtained as stated above for human plasma.

Partial Purification of Calmodulin (CaM)-Sensitive Adenylate Cyclase. Membranes prepared as above, containing 1 mM PMSF, were used in a further purification of CaM-sensitive adenylate cyclase by the method of Andreasen, *et al.* (13). The adenylate cyclase eluted from the CaM-Sepharose column with EDTA containing buffer is designated F-2.

Synthesis of 7-(^{3}Hacetyl)-forskolin. 7-(^{3}H-acetyl)-forskolin was prepared by the successive base catalyzed deacetylation of native forskolin and then reacetylation with [^{3}H]acetic anhydride. Native forskolin (Calbiochem) was incubated in a solution containing a two-fold excess of NaOH in 80% methanol:water. The reaction was followed by thin layer chromatography (TLC) and was completed within one hour. The reaction mixture was neutralized with acetic acid and then extracted with ethyl acetate. The product, 7-desacetylforskolin, was pure by TLC and it's structure confirmed by IR and NMR. IR($CHCl_3$) - 3610, 3470, 3310, 2940, 1720, 990, and 917 cm^{-1}. NMR(60 MHz, $CDCl_3$, ppm) - five tertiary C-methyl group signals (s at 1.07, 1.27, 1.65, and br s integrates to 2-methyls at 1.42), loss of one acetyl function (s at 2.17) from forskolin spectrum, and loss of one CHOAc proton (d at 5.42).

7-desacetylforskolin was dissolved in 2% pyridine/CH_2Cl_2. [^{3}H]acetic anhydride (ICN) was transfered to the reaction flask in CH_2Cl_2. The reaction was catalyzed by 4-dimethylaminopyridine and followed by TLC. The reaction was allowed to proceed for 8-10 hours and then diluted with 10 mL of CH_2Cl_2 prior to extraction with 2% aqueous HCl. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was chromatographed on a preparative TLC SiO_2 plate (2.0 mm) using 60:40 benzene:ethyl acetate. 7-(^{3}H-acetyl)-forskolin (R_f=0.60, 60:40 benzene:ethyl acetate), separated well from starting material, (7-desacetylforskolin, R_f=0.43) and was eluted from the SiO_2 with ethyl acetate. Product was confirmed by TLC-comigration with native forskolin and mass spectroscopy (Figure 1). 7-(^{3}H-acetyl)-forskolin of specific activities 50 mCi/mmole and 3 Ci/mmole were synthesized.

Isolation of metabolic products of forskolin formed by bovine brain membranes. Forskolin, in ethanol solution, was added to bovine brain membranes (5 mg/mL total protein, final [ethanol] <1%) and incubated at 22°C for 1-24 hrs. After incubation membranes were removed by centrifugation and the supernatant was removed and extracted with $CHCl_3$. The organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated. The concentrated extract was chromatographed on SiO_2 plates (0.25 mm) using ethyl acetate. After drying, the plates were sprayed with a mixture of 15 g $Cu(OAc)_2 \cdot H_2O$, 100 mL H_3PO_4 and 400 mL H_2O and then heated to visualize. Under these conditions forskolin has an R_f=0.60 and 7-desacetylforskolin has an R_f=0.55. In some cases, in order to remove a "phospholipid" contaminant (R_f=0.50), the samples were pre-purified on a preparative TLC plate (2.0 mm) by chromatography using ethyl acetate. Then, the samples were chromatgraphed on TLC plates using 60:40 benzene:ethyl acetate which gave a better separation of forskolin (R_f=0.57) and 7-desacetylforskolin (R_f=0.44).

Centrifugation Binding Assay. The binding assay was based on the assay decribed by Seamon, *et al.* (9). In a typical assay, 200 µL (0.5 mg) of membrane suspension in 50 mM Tris·HCl, pH 7.5 was incubated with 7-(^{3}H-acetyl)-forskolin alone or in combination with unlabeled forskolin (200 µL of 50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$) for 1 hr at 22°C. The tubes were centrifuged for 15 min at 4°C. The supernatant was aspirated and the pellet washed with 1 mL of ice-cold 50 mM Tris·HCl, pH 7.5. The pellets were then dissolved in 1 mL of 0.2 M NaOH and the radioactivity was determined by liquid scintillation counting.

Assay of ^{3}H-acetate release. Deacetylation of forskolin was followed by the release of ^{3}H-acetate from the synthetic derivative 7-(^{3}H-acetyl)-forskolin. In an assay of protein concentration dependence, 800

µL of membrane suspension (increasing total protein concentration) in 50 mM Tris·HCl, pH 7.5 was incubated with 100 µL of 7-(^{3}H-acetyl)-forskolin (5 nmoles/assay) at 20°C for one hour. The mixture was extracted 3 X with 1 mL $CHCl_3$ to remove organic soluble materials, in particular, residual 7-(^{3}H-acetyl)-forskolin. The aqueous phase was added to 15 mL of scintillation fluid and counted. The partitioning efficiency of the extraction was monitored with each experiment by a control extraction of 7-(^{3}H-acetyl)-forskolin from buffer on the same scale. Any residual tritium counts in the aqueous phase (usually 1-5%) were subtracted as blanks.

In the time course, 20 mL of membrane suspension (5 mg/mL) in 50 mM Tris·HCl pH 7.5 was incubated with 500 µL of 7-(^{3}H-acetyl)-forskolin (final concentration 5 µM) at 22°C. At 10 min intervals, three 1 mL aliquots were removed and the reaction stopped by boiling the membranes. Samples were cooled on ice and then maintained at 22°C until the end of the time course. All samples were extracted simultaneously.

All assays of [^{3}H]acetate release were carried out in triplicate, and recovery of water soluble counts by extraction was monitored by the addition of [α-^{32}P]-ATP immediately prior to extraction.

Mass Spectroscopy. Mass spectral measurements were made on a VG-7070H mass spectrometer and associated Data System VG-2035 with 70 electron volts and a source temperature of 220°C for forskolin and 180°C for 7-desacetyl-forskolin. Spectra are low resolution chemical ionization performed with a direct insertion probe in CH_4 reagent gas. A Dupont Vespel probe was used with 7-desacetyl-forskolin.

Results

7-(^{3}H-acetyl)-forskolin was synthesized as described in Materials and Methods. Product structure was confirmed by mass spectroscopy (Figure 1B) and compared with that of native forskolin (Figure 1A). Alcohols, in general, and forskolin, specifically, give a weak parent molecular ion peak but show a pronounced peak resulting from the loss of water (M-18). Chemical ionization was used to increase the probability of locating the molecular ion peak. The use of a methane gas atmosphere gave a quasi-molecular ion of M+1(411). Presence of the M-18 (392, loss of water from forskolin and 393, lose of water from M+1) is taken as confirmation of the molecular ion peak. Forskolin, being a tri-alcohol, continues to lose water to give a predominant 375 (-2 H_2O) and 357 (-3 H_2O). The identical fragmentation patterns for forskolin and 7-(^{3}H-acetyl)-forskolin are taken as confirmation of product structure.

In addition to chemical characterization, the radiolabeled derivative was analyzed for adenylate cyclase stimulatory activity. Forskolin and 7-(^{3}H-acetyl)-forskolin were both tested as activators of

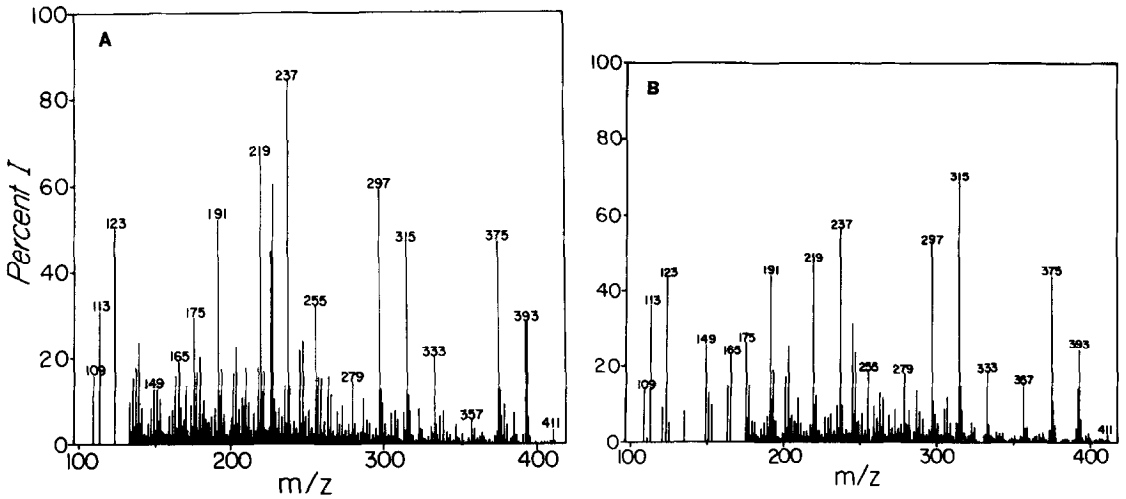

Figure 1 - A) Mass spectrum of native forskolin dissolved in ethanol (20 ng/µL). Spectra is a low resolution chemical ionization performed with direct insertion probe in methane gas atmosphere (220°C). B) Mass spectrum of 7-(^{3}H-acetyl)-forskolin (50 mCi/mmole) synthesized as described in Materials and Methods. Conditions as state in A.

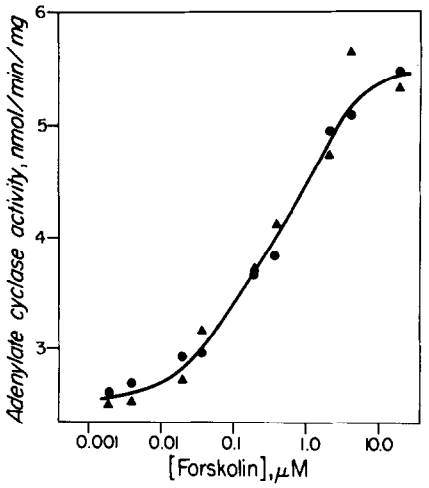

Figure 2 - Forskolin activation of bovine brain adenylate cyclase: Adenylate cyclase activity was assayed as a function of forskolin (●) and 7-(^{3}H-acetyl)-forskolin (▲) concentration as described under Materials and Methods.

partially purified CaM-sensitive adenylate cyclase (F2 fraction, see Materials and Methods). The dose response curves for native and synthetic [^{3}H]forskolin are shown in Figure 2. The synthetic forskolin behaved identically to native forskolin. Their dose response curves extended over more than four log units (native forskolin tested up to 1 mM). The complexity of the dose response curves may be due to heterogeneity of forskolin binding sites, cooperativity between binding sites or degradation of forskolin to less active forms. Nevertheless, [^{3}H]forskolin was indistinquishable from native forskolin by this assay.

The binding of [^{3}H]forskolin to bovine brain membranes was examined. Membranes were incubated with increasing concentrations of 7-(^{3}H-acetyl)-forskolin (10-500 nM) (Figure 3A). As expected, there was increased binding to the membranes with increasing concentrations of forskolin. In addition, membranes were incubated with a constant concentration of 7-(^{3}H-acetyl)-forskolin (100 nM) after a short pre-incubation with increasing amounts of unlabeled forskolin (0.1 nM-10 μM). The binding curve reported (Figure 3B) is the opposite of what would be expected for competition between unlabeled and labeled forskolin at a specific binding site. In this case, there was apparently increased binding (negative inhibition) of the 7-(^{3}H-acetyl)-forskolin in the presence of increasing amount of unlabeled forskolin. These results might be expected if unlabeled forskolin protects labeled forskolin from a degradation process in which the radioactive label is cleaved from the drug. In this case, the presence of an esterase in the crude membrane preparation caused deacetylation of forskolin, producing [^{3}H]acetate and 7-desacetylforskolin.

The deacetylation of forskolin by membranes was examined initially by thin layer chromatography (TLC). Forskolin (10 μM) was incubated with bovine brain membranes using the same conditions as the binding assay, except that the incubation period was extended to 24 hours. The metabolic products were isolated (See Materials and Methods) and chromatographed (Figure 4, lane 2). Forskolin (lane 1) and 7-desacetylforskolin (lane 3) were included as reference. A product was formed which was chromatographically equivalent to 7-desacetylforskolin (R_f=0.43, 60:40 benzene:ethyl acetate). In addition, the two compounds in lane 2 gave the characteristic colors seen for forskolin (purplish-brown)

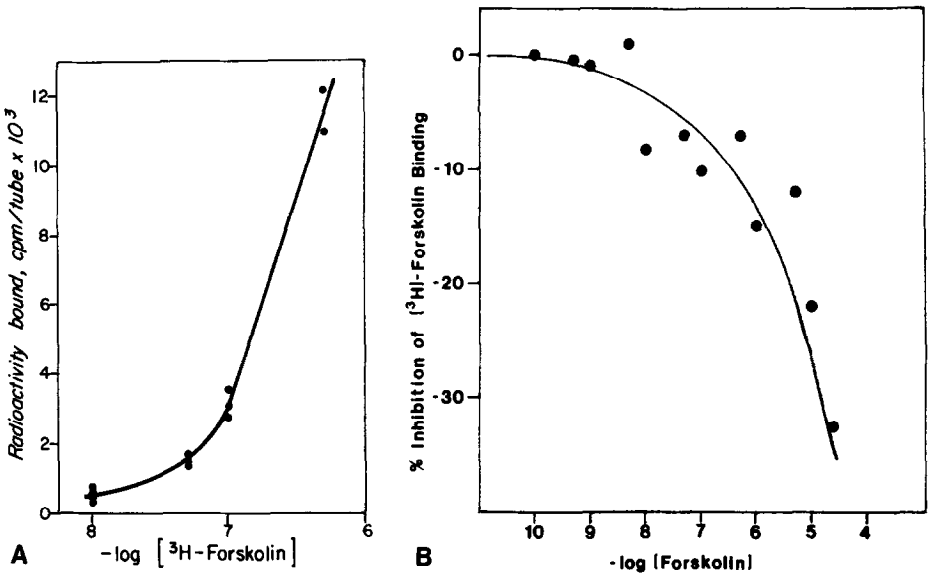

Figure 3 - 7-(^{3}H-acetyl)-forskolin binding to bovine brain membranes: A) Effect of 7-(^{3}H-acetyl)-forskolin (3 Ci/mmole) concentration on radioligand binding. 550 µg of bovine brain membranes were incubated with the indicated amounts of 7-(^{3}H-acetyl)-forskolin as described in Materials and Methods, centrifugation binding assay. B) Binding of 7-(^{3}H-acetyl)-forskolin in the presence of varying concentrations of unlabeled forskolin: Bovine brain membranes (1 mg) were incubated with 100 nM 7-(^{3}H-acetyl)-forskolin (3 Ci/mmole) and increasing concentrations of unlabeled forskolin. Conditions were identical to A, except membranes were pre-incubated with the indicated amounts of unlabeled forskolin for 10 minutes prior to the addition of 7-(^{3}H-acetyl)-forskolin. Each point is a mean of triplicate determinations.

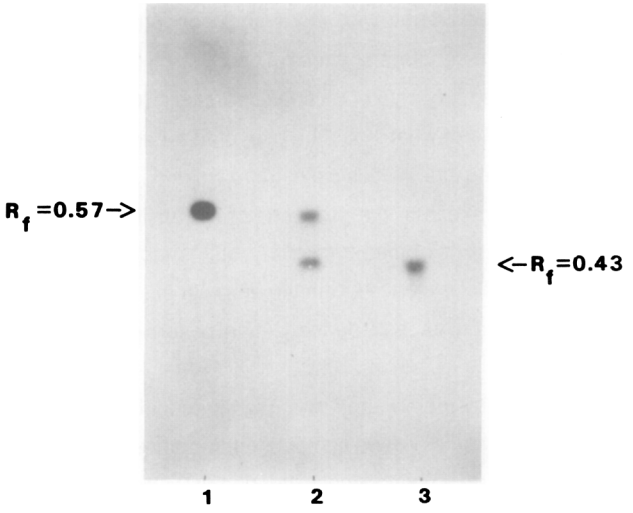

Figure 4 - Thin layer chromatograph of metabolic products of forskolin: Two mg. of forskolin was incubated for 24 hours at 22°C in 50 mL of bovine brain membranes (5 mg/mL 50 mM Tris·Cl, pH 7.5). Forskolin and its degradation product were isolated and pre-purified as described in Materials and Methods. This sample (lane 2) was chromatographed on a SiO_2 plate (0.2 mm) using 60:40 benzene:ethyl acetate along with forskolin standard (lane 1) and 7-desacetylforskolin standard (lane 3).

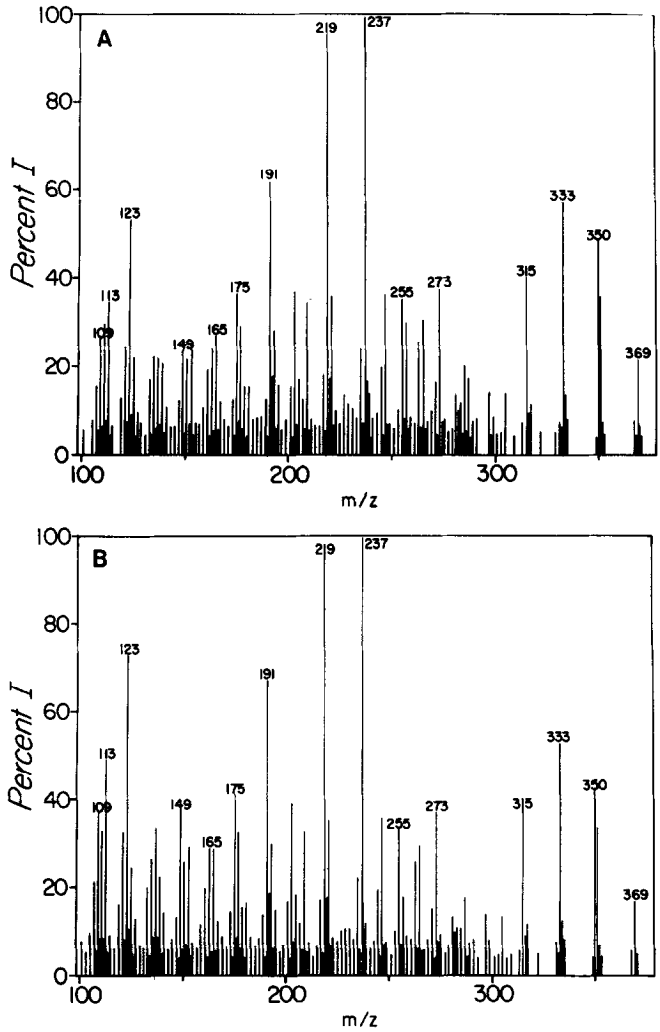

Figure 5 - Mass Spectra of 7-desacetyl-forskolin and forskolin degradation product: A) Mass spectrum of 7-desacetyl-forskolin synthesized as described in Materials and Methods. Spectra is a low resolution chemical ionization performed with a direct insertion probe (Dupont Vespel) in methane gas atmosphere (180°C). B) Mass spectrum of isolated degradation product of forskolin formed during incubation of forskolin with bovine brain membranes.

and 7-desacetylforskolin (light tan) when using a $Cu(OAc)_2$ spray. Boiled membranes showed no production of 7-desacetylforskolin.

The degradation product was isolated using preparative TLC and its structure confirmed by mass spectroscopy (Figure 5). Figure 5A shows the spectra of standard 7-desacetylforskolin. 7-desacetylforskolin is a tetra-alcohol and loses water even more rapidly than forskolin, which increases the difficulty of obtaining a molecular ion peak. By using chemical ionization at a lower source temperature (180°C) in a methane gas atmosphere the M+1 (369) peak was obtained. Again, the predominant M-18 (350) peak was taken as confirmation of the molecular ion peak.

The esterase activity of bovine brain membranes was also monitored by release of [^{3}H]acetate from 7-(^{3}H-acetyl)-forskolin (Figure 6). Release of [^{3}H]acetate was protein concentration dependent (Figure

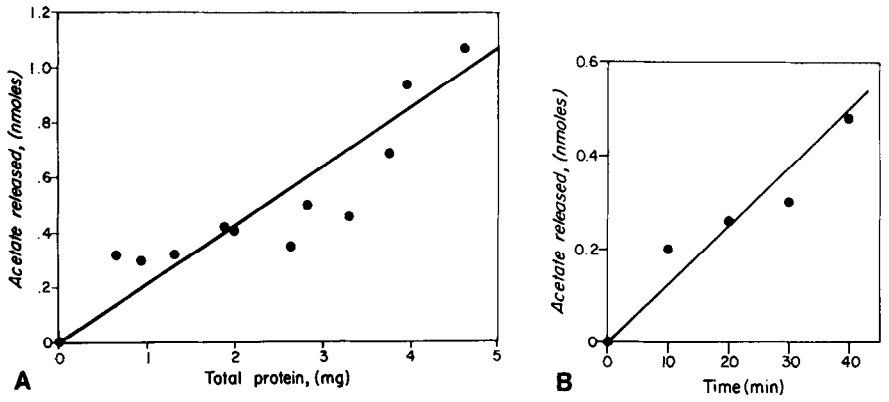

Figure 6 - A) 7-(^{3}H-acetyl)-forskolin (5 nmoles/assay) was incubated with the indicated amounts of bovine brain membrane protein (final volume 900 µL, buffer 50 mM Tris·HCl, 0.5 mM $MgCl_2$, pH 7.5) for 1 hour at 22°C. Each point is a mean of triplicate assays. [^{3}H]acetate release determined as described in Materials and Methods. B) 7-(^{3}H-acetyl)-forskolin (5 µM) was incubated with bovine brain membranes (5 mg/mL Tris·HCl, pH 7.5) at 22°C. One mL aliquots were removed at the times indicated and the reaction stopped by heat inactivation. [^{3}H]acetate release determined as described in Materials and Methods.

6A) with over 20% of total added forskolin deacetylated during the time of the assay. Deacetylation of forskolin was linear with time through 40 minutes (Figure 6B).

Tissues other than brain have been used in binding studies using labeled forskolin (8). To determine if deacetylation might be a problem when using these other tissues in binding studies, several tissues (liver, heart, plasma and erythrocytes) were tested for their ability to deacetylate forskolin. All tissues tested had some level of esterase activity with a wide range of specific activities. The highest activity was seen in liver membranes (731 pmoles/mg/hr) and liver cytosol (520 pmoles/mg/hr). The activity seen in plasma, although it appears low (26 pmoles/mg/hr), is appreciable with approximately 40% of forskolin being deacetylated during the assay. The high level of deacetylation that occurs in plasma should be taken into account in whole animal studies where forskolin is administered intravenously.

Discussion

Forskolin is an important tool for studying adenylate cyclase and the role of c-AMP in regulatory processes. Forskolin stimulation of adenylate cyclase appears to occur directly at the catalytic subunit (or a closely associated protein) and not be receptor mediated. This direct interaction has been used in the development of a 7-O-hemisuccinyldesacetylforskolin-Sepharose affinity column for the partial purification of adenylate cyclase (14). In addition, binding sites for forskolin in membranes have been explored using tritiated derivatives of forskolin (8,9). Seamon, et al. (9), using 12-[^{3}H]forskolin with rat brain membranes reported the presence of two classes of forskolin binding sites. These investigators noted it was difficult to equate the affinity of the high-affinity binding sites with the potency of forskolin for adenylate cyclase stimulation and the amount of low affinity binding sites was much higher than the expected amounts of adenylate cyclase. Furthermore, the dose response curve for forskolin activation of adenylate cyclase extend over more than four log units.

In this study we found that crude bovine brain membranes preparations deacetylate forskolin to form 7-desacetylforskolin. The deacetylation is both time and protein concentration dependent. The product,

7-desacetylforskolin, can activate adenylate cyclase with an EC_{50} of 30 μM and therefore would be expected to bind to adenylate cyclase. The heterogenity seen in forskolin binding may be explained, in part, by this reaction. In previous binding studies using forskolin labeled at a different site, part of the binding seen may have been due to binding of the labeled degradation product. Indeed, Seamon, et al. (9), report binding for the low affinity site of K_d= 0.77 μM and inhibition of [^{3}H]forskolin binding by 7-desacetylforskolin at 0.62 μM. While this correlation may be fortuitous, the possibility that the low affinity binding may be due to 7-desacetylforskolin should be considered. This still leaves unexplained the high amount of these low affinity binding sites. The deacetylation of forskolin catalyzed by bovine brain membranes may also explain why dose response curves for adenylate cyclase stimulation extend over more than four orders of magnitude. The dose response curve seen may be a composite of the activation due to the presence of forskolin and 7-desacetylforskolin.

Deacetylation of forskolin occurs in a variety of tissues and biological fluids. The rate of deacetylation is not high in brain but is significant in liver membranes and cytosol. The esterase is probably not specific for forskolin, although protection was seen by the addition of unlabeled forskolin. It is clear, however, that binding studies in crude membrane preparations must take into account the degradation of forskolin. The inclusion of esterase inhibitors may solve this problem, but the rate of deacetylation was unaffected by the inclusion of the serine protease inhibitor PMSF. Forskolin binding studies using more highly purified preparations of adenylate cyclase lacking esterase activity may lead to less ambiguous data.

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References

1. Bhat, S.V., Bajwa, B.S., Dornauer, H., de Souza, N.J., and Fehlhaber, H.W. (1977) *Tetrahedron Ltrs.* **19**:1669-1672.
2. Metzger, H. and Lindner E. (1981)*IRCS Med. Sci. Libr. Compend.* **9**:99(1981).
3. Seamon, K.B., and Daly, J.W. (1981) *J. Biol. Chem.* **256**: 9799-9801.
4. Barovsky, K., Pendone, C., and Brooker, G. (1984) *Mol. Pharmacol.* **25**: 256-260.
5. Bender, J.L., and Neer, E.J. (1983) *J. Biol. Chem.* **258**: 2432-2439.
6. Daly, J.W. (1984) *Adv. Cyclic Nucleotide Res.* **17**:81-89.
7. Forte, L.R., Bylund, D.B. and Zahler, W.L. (1983) *Mol. Pharmacol.* **24**:42-47.
8. Ho, R. and Shi Q. (1984) *J. Biol. Chem.* **259**:7630-7636.
9. Seamon, K.B., Vaillancourt R., Edwards, M., and Daly, J.W. (1984) *Proc. Natl. Acad. Sci., USA* **81**:5081-5085.
10. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58**:541-548.
11. Peterson, G.L. (1977) *Anal. Biochem.* **83**:346-356.
12. Neville Jr., D.M. (1968) *Biochimica et Biophysica Acta* **154**:540-552.
13. Andreason, T.J., Heideman, W., Rosenberg, G.B., and Storm, D.R. (1983) *Biochemistry* **22**:2757-2762.
14. Pfeuffer, T. and Metzger, H. (1982) *FEBS Lett.* **146**:369-375.