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CONCENTRATION-DEPENDENT EFFECTS OF AY-9944 AND U18666A ON STEROL SYNTHESIS IN BRAIN

VARIABLE SENSITIVITIES OF METABOLIC STEPS

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Abstract—The possibility that the effects of inhibitors of sterol biosynthesis in brain are concentration dependent was examined. The drugs 3β-(2-diethylaminoethoxy) androst-5-en-17-one·HCl (U18666A) and *trans*-1,4-bis (2-chlorobenzylaminomethyl) cyclohexane·2HCl (AY-9944) were evaluated, because both substances markedly affect brain sterol metabolism and have been reported to alter the structure and/or function of developing brain. Incorporation of [2-^{14}C]-DL-mevalonate by cell-free homogenates of rat brain into squalene and individual sterol fractions was measured in the absence and presence of a wide range of inhibitor concentrations. The qualitative and quantitative effects of U18666A and, to a lesser extent of AY-9944 on the formation of radiolabeled brain sterols were highly dependent on inhibitor concentration. The results indicate that there are two categories of metabolic steps in the pathways for sterol synthesis that were sensitive to inhibition by U18666A and AY-9944. The first category comprises steps that are located before lanosterol formation and were suppressed only by relatively high concentrations of inhibitors. At these higher inhibitor concentrations essentially all sterol synthesis was eliminated and drug effects at sites after lanosterol were therefore obscured. U18666A was over 6000 times more active than AY-9944 in inhibiting at category one sites. The second category of steps comprises those that are located late in cholesterol biosynthesis and were very sensitive to inhibition. Inhibitor effects at these late steps appeared to be drug-specific.

The drugs U18666A [3β-(2-diethylaminoethoxy) androst-5-en-17-one·HCl] and AY-9944 [*trans*-1,4-bis (2-chlorobenzylaminomethyl) cyclohexane·2HCl] have recently been shown to produce pronounced effects upon the structure and/or function of developing brain and upon biosynthesis of sterols. Treatment of neonatal rats with U18666A results in development of chronic epileptiform activity [1] and treatment of rats with AY-9944 leads to grossly abnormal myelin structure [2]. Both drugs have been shown to decrease cholesterol formation by inhibiting enzymes that act late in cholesterol biosynthesis. U18666A and AY-9944, respectively, inhibit the reduction of desmosterol [3] and 7-dehydrocholesterol [4–7] to cholesterol and can cause tissue accumulation of these sterol intermediates.

In addition to altering brain chemistry, structure and function, U18666A and AY-9944 have recently been reported to induce permanent cataracts in rats [8, 9]. Prior to the onset of lens opacities in rats treated with U18666A, the cholesterol concentrations of the lenses were observed to be greatly decreased, but desmosterol levels were, surprisingly, not increased [8]. Desmosterol did not appear in the opaque lens until long after onset of the cataracts. These observations suggested the possibility that the effects of U18666A upon sterol synthesis might be variable. Perhaps the net effect of inhibitors on sterol synthesis is dependent on the tissue concentration of the inhibitor. The present investigation examined this possibility; the results show that the effects of U18666A and AY-9944 upon synthesis *in vitro* of sterols in rat brain can be dependent upon the inhibitor concentration.

MATERIALS AND METHODS

Animals and preparation of brain homogenates. Male Sprague–Dawley rats, 22–24 days of age (Hilltop Lab Animals Inc., Scottdale, PA) were decapitated. The brains were quickly removed and washed in ice-cold saline. Brains from four to five rats were pooled, blotted dry, minced, and hand-homogenized in 2 vol. of 67 mM sodium potassium phosphate buffer (pH 7.4) as described by Kelley *et al.* [10]. Three pools of homogenized brains were prepared in each experiment. Homogenates were centrifuged at 1500 *g* for 20 min at 5°. The supernatant fraction from each homogenate was removed and saved for incubations *in vitro*. The protein concentrations in the supernatant fractions were determined [11] and they ranged between 12 and 14 mg/ml.

Incubations in vitro. All incubations were conducted in 25-ml Erlenmeyer flasks. Each flask contained 1.0 ml of a given supernatant pool, 2.0 ml of a buffered cofactor solution [12], 0.10 ml of water containing 2.2 μCi of [2-^{14}C]-DL-mevalonate (40.8 mCi/mmole as the dibenzylethylene diamine salt, DBED; New England Corp., Boston, MA), and 0.10 ml of a propylene glycol–water solution (1:1, v/v) containing U18666A or AY-9944 at thirty-two times the final desired concentration. Final concentrations of U18666A ranged between 10^{-3} M and 10^{-9} M; AY-9944 ranged between 10^{-3} M and

10^{-7} M. Control incubations received the aqueous propylene glycol solution without drugs. Incubations were conducted in triplicate at 37° for 5 hr in a metabolic shaker. Each of the three cell-free homogenate pools prepared was tested with each drug concentration examined.

Recovery and separation of ^{14}C *labeled lipids.* Incubations were terminated by adding reaction mixtures to 20 vol. of chloroform–methanol (2:1, v/v). Total lipids were recovered and washed according to Folch *et al.* [13]. Digitonide precipitable sterols were prepared from 10% aliquots of the total lipid extracted [14]. These precipitates were solubilized in methanol, and the ^{14}C-content was measured by standard scintillation counting techniques. Counting efficiencies were determined by addition of [^{14}C]toluene as an internal standard.

The remaining total lipids were dissolved in chloroform and fractionated by thin-layer chromatography (t.l.c.) on silica gel G plates (20 × 20 cm) using a solvent of *n*-hexane–diethyl ether–glacial acetic acid (73:25:2, by vol.). Two samples were applied to each plate along with standards. Unlabeled carrier desmosterol (200 μg) (Sigma Chemical Co., St. Louis, MO), 7-dehydrocholesterol (200 μg) (Aldrich Chemical Co., Milwaukee, WI) and lanosterol (100 μg) (Serdary Research Laboratory, London, Ontario) were added before chromatography. Plate zones corresponding to free sterols ($Rf = 0.17$), lanosterol ($Rf = 0.24$) and sterol esters, squalene included, ($Rf = 0.70$) were separately recovered, and the lipids were extracted into anhydrous diethyl ether. Extraction of ^{14}C-lipids from the various silica gel zones was over 98 per cent complete. One-half of the recovered lipids from each of these three fractions was rechromatographed as described by Ramsey and Fredericks [12] on silica gel G containing 7% $AgNO_3$ using a solvent of chloroform–acetone (95:5, v/v). Unlabeled carrier cholesterol oleate (400 μg) (K & K Laboratories, Plainview, NY) and squalene (400 μg) (Sigma Chemical Co.), along with additional unlabeled cholesterol (200 μg) (Sigma Chemical Co.), desmosterol (400 μg), 7-dehydrocholesterol (400 μg) and lanosterol (400 μg), were added to the respective samples prior to the second chromatography. Rechromatography of the sterol ester–squalene fraction and the lanosterol fraction was done at room temperature; separation of the free sterols, however, was conducted at 5° (this improved the separation of desmosterol from cholesterol). The various lipids separated with *Rf* values similar to those reported by Ramsey and Fredericks [12]. The plate zones corresponding to cholesterol, desmosterol, 7-dehydrocholesterol, lanosterol, squalene, and sterol esters were individually recovered, and the lipids extracted into anhydrous diethyl ether. Extraction of ^{14}C-labeled lipids from the silver nitrate bearing silica gel zones was 97–99 per cent complete. About 80 per cent of the radiolabel recovered from the total sterol fraction of the first chromatography was accounted for in the cholesterol, desmosterol and 7-dehydrocholesterol fractions that were separately recovered from the silver nitrate-thin-layer chromatogram.

The ^{14}C-content of the various fractions was measured by liquid scintillation counting. Incorporation of [^{14}C]mevalonate into the individual lipid fractions was expressed as dpm × 10^{-2}/mg protein. The values for the triplicate incubations were averaged and the variation was expressed as the mean ± the standard error.

We have assumed that a ^{14}C-labeled sterol that comigrated on t.l.c. with a given added carrier was identical to the carrier. Using radioactivity-monitored gas–liquid chromatography, Ramsey and Fredericks [12] characterized the ^{14}C-labeled sterols (formed from incubation of homogenates of rat brain with [^{14}C]mevalonate in the absence of inhibitors) that were separately recovered from the lanosterol, cholesterol, desmosterol and 7-dehydrocholesterol zones of the silver nitrate-t.l.c. system. The ^{14}C in the lanosterol region was found to be about 80% [^{14}C] lanosterol and the remainder radiolabeled 4α-methyl-5β-cholesta-8,24-dien-3β-ol. The cholesterol zone contained about 90% [^{14}C] cholesterol and 10% [^{14}C]-5α-cholesta-8,24-dien-3β-ol. The desmosterol and 7-dehydrocholesterol regions were found to contain only radiolabeled desmosterol and 7-dehydrocholesterol respectively.

RESULTS

Concentration-dependent effects of U18666A on formation of brain sterols. In the presence of more than 10^{-7} M U18666A, there was little incorporation of [^{14}C]mevalonate into lanosterol or digitonide-precipitable sterols (Fig. 1 and Table 1), yet incorporation into squalene was greater than in the control homogenates (Table 1). This suggested a block in the conversion of squalene to lanosterol. The increased incorporation into squalene, however, poorly compensated for the greatly decreased incorporation of ^{14}C into lanosterol plus other sterols (Table 1). This would indicate that most of the inhibition of total sterol synthesis at high levels of U18666A occurred prior to squalene. In the presence of 10^{-9}M U18666A there was little or no significant decrease in the incorporation of [^{14}C]mevalonate into squalene, lanosterol, or into total digitonide-precipitable sterols, although incorporation into cholesterol was still depressed (Fig. 1 and Table 1). The decreased incorporation into cholesterol was balanced by an increased incorporation into desmosterol. The patterns of sterol biosynthesis seen in the presence of 10^{-7} and 10^{-8} M U18666A reflect inhibitions both at sites prior to lanosterol and in the conversion of desmosterol to cholesterol. Incorporation of [^{14}C]mevalonate into total digitonide-precipitable sterols was decreased at both of these concentrations, although only slightly at 10^{-8} M (Fig. 1).

Concentration-dependend effects of AY-9944 upon brain sterol formation. At concentrations of AY-9944 above 5×10^{-4} M there was virtually a complete block in the incorporation of [^{14}C]mevalonate into squalene, lanosterol, and digitonide-precipitable sterols (Fig. 1 and Table 2). Thus, absence of incorporation into cholesterol at these higher concentrations is apparently explained by inhibition at metabolic steps prior to the formation of squalene. The ability of AY-9944 to inhibit formation of total digitonide-precipitable sterols was quickly lost once the

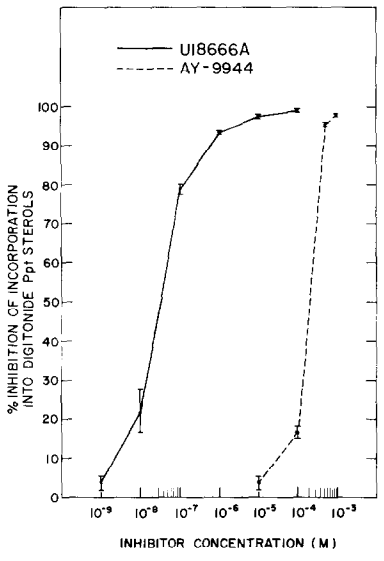

Fig. 1. Concentration-dependent effects of U18666A and AY-9944 upon incorporation of [2-^{14}C]-DL-mevalonate into digitonide-precipitable sterols by brain. Cell-free homogenates of brains from young rats (22 to 24-days-old) were incubated for 5 hr at 37° with [2-^{14}C]mevalonate (2.2 μCi as the DBED salt; sp. act. = 40.8 mCi/mmole) in the absence or presence of various concentrations of U18666A or AY-9944. Total lipids were subsequently extracted into chloroform–methanol (2:1, v/v) and the digitonide-precipitable sterols were isolated from aliquots of the extract. Each value is the mean ± S.E. (bars) of three or more incubated homogenates of pooled brains (four to five whole brains per pool) except for 10^{-4} M and 5×10^{-4} M AY-9944, where two pools were used. Fifty per cent inhibition of incorporation into digitonide-precipitable sterols was produced by approximately 3.2×10^{-8} M U18666A and 2.1×10^{-4} M AY-9944. Control homogenates incorporated an average of approximately 39,000 dpm/mg protein into total digitonide-precipitable sterols.

drug concentration was lowered to 10^{-4} M (Fig. 1). Decreased incorporation of [^{14}C]mevalonate into cholesterol, however, was still apparent at 10^{-4} M to 10^{-7} M AY-9944. The decreased incorporation into cholesterol at these inhibitor concentrations was coupled with an increased incorporation into 7-dehydrocholesterol, such that the sum of radioactivity incorporated into cholesterol plus 7-dehydrocholesterol in treated homogenates was similar to that in control homogenates.

DISCUSSION

The results of the present study indicate that there are two categories of metabolic steps in sterol synthesis that are sensitive to inhibition by U18666A and AY-9944. The first category comprises steps that are located prior to formation of lanosterol and are inhibited only by relatively high concentrations of drugs. The second category comprises steps that are located late in cholesterol biosynthesis and are very sensitive to inhibition. When inhibitor levels were high, inhibition of the steps in category one resulted in a virtually complete block of the formation of all sterols and, as a result, drug effects late in the pathway were, of course, not observed. As inhibitor levels were decreased, these early steps were no longer inhibited and, thus, drug effects further down the pathway could be detected. Inhibition at these later steps resulted in a selective decrease in the formation of cholesterol. The concept that the effects of sterol inhibitors can be dependent upon the concentration of these drugs is also supported by recent findings of Field *et al.* [15]. Using yeast cultures, they demonstrated that 3β-(β-dimethylaminoethoxy)-androst-5-en-17-one at 10^{-4} M caused the appearance of 2,3,22,23-dioxosqualene but ergosta-5,7,22,24 (28)-tetraen-3β-ol accumulated at 3×10^{-6} M. They concluded that the Δ24 (28) reductase was more sensitive to inhibition by this drug than the 2,3-oxidosqualene cyclase.

Several different category one steps may exist for a given inhibitor. For example, U18666A apparently blocked incorporation of [^{14}C]mevalonate into lanosterol by decreasing both conversion of squalene

Table 1. Concentration-dependent effects of U18666A on incorporation of [2-^{14}C]-DL-mevalonate into squalene and sterols by brain

Lipid fraction	Disintegrations per minute × 10^{-2} incorporated/mg protein*,†						
	Experiment 1				Experiment 2		
	Control	10^{-4} M	10^{-6} M	10^{-7} M	Control	10^{-8} M	10^{-9} M
Squalene	10.8 ± 0.9	27.1 ± 2.9	16.2 ± 1.8	22.0 ± 2.2	14.2 ± 3.0	9.6 ± 2.0	14.8 ± 4.2
Lanosterol	46.7 ± 0.4	2.0 ± 0.3	3.1 ± 0.3	9.2 ± 1.0	39.4 ± 5.6	23.0 ± 1.0	52.0 ± 5.4
7-Dehydrocholesterol	19.3 ± 0.8	4.1 ± 0.6	7.9 ± 1.3	6.4 ± 1.0	15.7 ± 0.5	23.5 ± 2.6	14.3 ± 1.0
Desmosterol	9.2 ± 1.6	0.7 ± 0.1	1.8 ± 0.3	21.8 ± 3.1	8.1 ± 0.2	16.9 ± 2.5	39.1 ± 9.4
Cholesterol	106.7 ± 7.9	0.6 ± 0.1	3.7 ± 0.4	7.7 ± 1.4	154.4 ± 8.9	83.1 ± 12.6	101.9 ± 10.2
Sterol ester	1.1 ± 0.1	5.4 ± 0.8	1.4 ± 0.0	2.3 ± 0.4	1.4 ± 0.0	1.6 ± 0.1	2.0 ± 0.1
Totals	193.9 ± 10.9	39.9 ± 4.6	34.2 ± 0.6	69.3 ± 7.5	233.3 ± 10.2	157.5 ± 13.5	224.2 ± 8.5

* Cell-free homogenates of brain were incubated as described in the legend to Fig. 1. Total lipids were recovered and fractionated by two separate thin-layer chromatographies; the second chromatography used silica gel G prepared with 7% $AgNO_3$.

† Each value is the mean ± S.E.M. of incorporation by three homogenates of pooled brains (four to five whole brains per pool) incubated for 5 hr at 37°.

Table 2. Concentration-dependent effects of AY-9944 upon incorporation of [2-^{14}C]DL-mevalonate into squalene and sterols by brain

Lipid fraction	Disintegrations per minute × 10^{-2} incorporated/mg protein*,†					
	Control	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M
Squalene	13.8 ± 1.2	3.5 ± 0.7	7.3 ± 1.0	11.5 ± 0.8	24.8 ± 0.6	25.1 ± 4.0
Lanosterol	38.3 ± 3.2	2.3 ± 0.3	31.6 ± 6.7	19.6 ± 1.7	21.7 ± 0.9	35.2 ± 1.6
7-Dehydrocholesterol	19.5 ± 1.4	7.9 ± 3.0	66.1 ± 8.4	58.2 ± 8.2	67.6 ± 12.7	36.9 ± 2.7
Desmosterol	8.4 ± 0.9	1.4 ± 0.5	10.9 ± 2.4	12.2 ± 0.5	6.3 ± 1.6	4.4 ± 0.3
Cholesterol	72.6 ± 7.4	1.4 ± 0.4	14.5 ± 2.4	8.9 ± 0.0	23.4 ± 0.5	44.2 ± 7.5
Sterol esters	0.6 ± 0.1	2.5 ± 0.5	0.3 ± 0	0.5 ± 0.1	0.4 ± 0	0.7 ± 0
Totals	153.3 ± 11.5	19.0 ± 3.3	130.7 ± 8.7	110.9 ± 9.5	144.2 ± 14.8	146.5 ± 5.8

* Cell-free homogenates of brain were incubated as described in the legend to Fig. 1. Total lipids were recovered and fractionated by two separate and consecutive thin-layer chromatographies; the second employed silica gel G prepared with 7% $AgNO_3$.

† Each value is the mean ± S.E. of incorporation by three homogenates of pooled brains (four to five whole brains per pool) incubated for 5 hr at 37°.

to lanosterol and formation of squalene. Decreased conversion of mevalonate to squalene could have been the main effect of U18666A at the higher concentrations. AY-9944 at high levels totally blocked sterol synthesis apparently by inhibiting at steps prior to formation of squalene. Great differences were seen between the quantitative abilities of U18666A and AY-9944 to inhibit category one steps. Based upon inhibition of [^{14}C]mevalonate incorporation into total digitonide-precipitable sterols, U18666A was over 6000 times more potent an inhibitor than AY-9944.

The category two steps at which U18666A and AY-9944 inhibit are different. U18666A inhibited reduction of desmosterol and AY-9944 inhibited the reduction of 7-dehydrocholesterol. The ability of these drugs to inhibit these particular reductions has long been recognized [3–7]. However, as with inhibition at category one sites, there appear to be clear differences in the quantitative abilities of U18666A and AY-9944 to inhibit at category two steps. U18666A is perhaps at least 100 times more potent than AY-9944, since 10^{-9} M U18666A and 10^{-7} M AY-9944 produced similar percentage reductions in ^{14}C-incorporation into cholesterol.

The importance of inhibition of steps in category one compared with steps in category two in explaining the mechanism of action of sterol inhibitors in a given tissue obviously could depend upon the local concentration of the drug. Unfortunately, information is not available on tissue levels of AY-9944 or U18666A. Why certain metabolic steps in cholesterol biosynthesis appear especially sensitive to inhibition by these agents is unknown. Perhaps it reflects differences in the K_i values between the drugs and given enzymes or differences in the accessibility of the drugs to various enzymes. For example, in cholesterol biosynthesis, the metablic steps between mevalonic acid and farnesyl pyrophosphate are catalyzed by soluble enzymes, whereas those that follow occur in the microsomes [16]. Presumably drugs added to incubations *in vitro* would be more accessible to soluble enzymes, yet evidence for inhibition of the soluble enzymes was obtained only at the higher drug concentrations. Since both U18666A and AY-9944 are lipid soluble compounds, it is also conceivable that they are concentrated in the lipid matrix of microsomal membranes and thus the enzymes located there, that act late in cholesterol synthesis, could be more sensitive to inhibition.

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