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Anesthetic Requirement in the Quaking Mouse

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Anesthetic requirements for nitrous oxide, cyclopropane, enflurane, and isoflurane were determined in quaking mice (autosomal recessive mutants with a deficiency in central nervous system myelin) and their littermate (non-quaking) controls. Although quaking mice had significantly (15 to 23 per cent) lower nitrous oxide and cyclopropane rolling-response ED_{50}s than did littermate controls, enflurane and isoflurane rolling-response ED_{50}s were not significantly different. Tail-clamp ED_{50}s for cyclopropane, enflurane, and isoflurane in quaking and control mice were not significantly different. Myelin from quaking mice showed higher levels of palmitic, stearic, and docosahexaenoic acids, lower levels of oleic, eicosenoic, and docosatetraenoic (and/or nervonic) acids, and a lower cholesterol/phospholipid ratio. In contrast, the phospholipid, fatty acid, and cholesterol compositions of synaptic plasma membranes isolated from quaking and control mice were essentially identical. It is concluded that gross alterations in the lipid composition of central nervous system myelin have little or no influence on anesthetic requirement. (Key words: Anesthetics, gases; cyclopropane, nitrous oxide. Anesthetics, volatile: enflurane; isoflurane. Brain: synapses. Potency: ED_{50}. Theories of anesthesia: lipid solubility.)

THE CORRELATION between lipid solubility and anesthetic potency suggests a hydrophobic site of anesthetic action in the central nervous system.[1,2] General anesthetics can alter the physical state of phospholipid model membranes,[3–8] and such alterations imply that neuronal membrane lipids may constitute the molecular site of anesthetic action. An alternative approach to defining the importance of brain lipids to anesthetic action is to determine the effects of structural alterations in brain lipids on anesthetic requirements. In this study, an attempt was made to correlate brain lipid composition with anesthetic requirement by measuring anesthetic potency in the "quaking mouse," an autosomal recessive mutant[9] in which the lipid composition of central nervous system myelin differs distinctly from that of heterozygous littermate controls.[10,11] In addition, synaptic plasma membrane phospholipid, fatty acid, and cholesterol compositions in quaking and control mice were quantitated, since synaptic membrane regions are the most likely site of anesthetic action,[12] and because possible differences in synaptic plasma membrane lipid compositions between quaking and control mice had not previously been examined.

Materials and Methods

Male quaking mice and their littermate controls (Jackson Laboratories) were housed with Absorbdri® bedding and provided with Purina Chow® and water *ad libitum*. Mice were exposed to a light:dark cycle of 12 hr:12 hr and were kept in an air-conditioned room at 21–23° C. Average age (±SE) when anesthetic potencies were tested was 76 ± 3 days. Quaking mice weighed (±SE) 24 ± 0.5 g, and their littermate controls, 28 ± 0.6 g (total, 52 pairs).

Nitrous oxide rolling-response ED_{50}s (the dose of nitrous oxide necessary to abolish the righting reflex in 50 per cent of the animals) were measured in quaking mice and controls. For each determination, as many as eight unrestrained mice were placed in individual wire mesh cages that could be rotated at 4 rpm in a 20-1 hyperbaric chamber.[13] Rectal temperatures were monitored in two additional restrained mice and were maintained at 36.5 to 38.0° C by adjusting the chamber temperature through circulating-water heat exchangers. Chamber gases were blown through a soda-lime container to remove carbon dioxide. The chamber initially was flushed with 100 per cent oxygen for 10 min, and approximately 1.15 atm N_2O was subsequently added. After an equilibration period of 30 min, animals rolling over twice during five complete turns of the rotator were considered anesthetized. Further additions of N_2O (usually in increments of 0.11 atm) were made, and the righting reflex was again determined after a 15-min period of equilibration. After the dose at which all animals failed the rolling-response test was reached, the partial pressure of N_2O was lowered in steps, and testing continued until all animals could right themselves. At no time did oxygen content fall below 0.6 atm. Cyclopropane rolling-response ED_{50}s were determined using essentially the same procedure, except that mice were initially exposed to about 0.15 atm cyclopropane, and concentrations were thereafter altered in steps of about 0.03 atm.

Rolling-response ED_{50} determinations for enflurane and isoflurane were similar to that described for nitrous oxide and cyclopropane, except that a continuous flow of oxygen (4 l/min) plus controlled amounts of enflurane or isoflurane delivered from a temperature-compensated vaporizer passed through

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TABLE 1. Anesthetic Requirements in Quaking Mice and Their Littermate Controls

	Method of Testing	Quaking Mouse ED_{50} (±SE) (atm)	Littermate Control ED_{50} (±SE) (atm)
Nitrous oxide	Rolling response	1.11 ± 0.05 (12)*	1.30 ± 0.04 (13)†
Isoflurane	Rolling response	0.00566 ± 0.00015 (12)	0.00560 ± 0.00015 (13)
	Tail clamp	0.0110 ± 0.0006 (12)	0.0119 ± 0.0006 (12)
Enflurane	Rolling response	0.00913 ± 0.00051 (12)	0.00971 ± 0.00042 (12)
	Tail clamp	0.0151 ± 0.0015 (9)	0.0182 ± 0.0013 (12)
Cyclopropane	Rolling response	0.0913 ± 0.0060 (13)	0.118 ± 0.0042 (15)‡
	Tail clamp	0.210 ± 0.0079 (9)	0.221 ± 0.0062 (11)

* Number of animals tested is given in parentheses. One quaking mouse died during the nitrous oxide rolling-response testing; three quaking mice died during enflurane tail-clamp testing; two died during cyclopropane rolling-response testing; four died during cyclopropane tail-clamp testing. Four control mice also died during cyclopropane tail-clamp testing. The responses of these animals were excluded from calculations of ED_{50}.

Values for controls were significantly different from those for quaking mice at † $P < 0.005$ and ‡ $P < 0.001$.

the chamber during the entire experimental period. An initial one-hour equilibration at approximately 0.004 atm isoflurane or 0.0110 atm enflurane was imposed before the righting reflex was tested. The subsequent measurements involved changes of anesthetic concentration in steps of 10 to 20 per cent, and 30-min equilibration times.

The anesthetic potencies of cyclopropane, enflurane, and isoflurane were also measured by use of a tail-clamp procedure. Groups of eight mice were anesthetized with the desired anesthetic in oxygen (4 l/min) in individual Plexiglas® chambers. Rectal temperatures were monitored in each mouse and were maintained at 36.5 to 38.0° C with heat lamps. Animals were initially equilibrated with approximately 0.20 atm cyclopropane for 30 min, or with 0.020 atm enflurane or 0.018 atm isoflurane for one hour. Anesthetic requirement was determined for each mouse by altering anesthetic concentration in 20 per cent steps, equilibrating cyclopropane for 15 min and enflurane and isoflurane for 30 min, and applying a tail clamp that was oscillated for 60 sec. Mice were observed for movement in response to the stimulation.

Nitrous oxide concentration was measured by gas chromatography with a thermal conductivity detector. Cyclopropane concentration was measured with a Beckman LB-1 infrared analyzer. Enflurane and isoflurane concentrations were measured by gas chromatography with a flame ionization detector. Anesthetic requirement (ED_{50}) was determined for each mouse by averaging the concentrations that just abolished and just allowed the righting reflex, or that just prevented and just permitted movement in response to the tail-clamp stimulation. The ED_{50} and standard error for a group of mice were calculated from the individual crossover values. Significance was calculated with an unpaired t test.

Four groups of quaking and control mice (12 to 15 pairs in each group) were examined. Nitrous oxide and isoflurane rolling-response ED_{50}s were determined in the first group, isoflurane tail-clamp ED_{50}s in the second group, cyclopropane rolling-response and tail-clamp ED_{50}s in the third group, and enflurane rolling-response and tail-clamp ED_{50}s in the fourth group. The second determination of anesthetic potency in a given group of animals was performed one to two weeks after the initial measurements of ED_{50}.

Synaptic plasma membranes were prepared using a method of Jones and Matus.[14] Whole brains were homogenized in 10 per cent (w/w) sucrose/5 mM HEPES in a Teflon®–glass homogenizer. The brains from two to four mice were pooled for each preparation. The whole-brain homogenate was centrifuged at 800 × g for 20 min. The supernatant was removed, centrifuged at 9,000 × g for 20 min, and washed once to yield the mitochondrial pellet. The mitochondrial pellet was lysed in 5 ml of 5 mM tris (pH 8.1 at 4° C), incubated at 0° C for 30 min, and homogenized. The lysed suspension was made to equal 34 per cent (w/w) sucrose by addition of 48 per cent (w/w) sucrose. The sample suspension was overlaid with 15 ml of 28.5 per cent (w/w) sucrose and 6 ml of 10 per cent (w/w) sucrose. The discontinuous gradient was centrifuged at 60,000 × g on a Beckman SW27 rotor for 110 min. Myelin was recovered at the 10–28.5 per cent sucrose interface, an enriched synaptic membrane fraction at the 28.5–34 per cent sucrose interface, and mitochondria sedimented to the bottom of the tube. All steps were carried out at a temperature of 0–4° C. All sucrose solutions were buffered with 5 mM HEPES, pH 7.4, and all buffers were bubbled with argon to minimize lipid oxidation. The purity of the synaptic membrane preparations was assessed by measuring the relative activities of $Na^+ + K^+$ ATPase, acetyl-

cholinesterase, lactate dehydrogenase, and succinate dehydrogenase in each of the brain subfractions.[15] Extraction of lipids from membrane pellets and quantitation of fatty acid,[16] phospholipid,[17] and cholesterol compositions were performed as described previously.[15] Myelin and synaptic membrane fractions were isolated from quaking and control mice approximately a month after they were tested for nitrous oxide and isoflurane requirements (first and second groups, 25 pairs of mice); mice were an average of 109 days old when killed for lipid analyses.

Results

The nitrous oxide and cyclopropane rolling-response ED_{50}s of quaking mice were significantly less than those of their littermate controls (table 1). However, enflurane and isoflurane requirements, as measured by both rolling-response and tail-clamp tests, did not differ for the quaking mice and their controls (table 1). In addition, cyclopropane tail-clamp ED_{50}s were not detectably different between control and quaking animals.

The fatty acid compositions of myelin were distinctly different for the quaking mice and the controls. The most notable alterations were higher levels of palmitic, stearic, and docosahexaenoic acids, and lower levels of oleic, eicosenoic, and docosatetraenoic (and/or nervonic) acids in myelin of the quaking mice (table 2). In addition, the myelin cholesterol/phospholipid molar ratio in the quaking mice, 0.956 ± 0.031 (±SE, n = 6), was significantly lower ($P < 0.005$) than was the myelin cholesterol/phospholipid molar ratio in the controls, 1.132 ± 0.018 (n = 5).

In contrast to the myelin lipid compositions, the synaptic plasma membrane lipid compositions in the control and quaking mice were essentially identical. For the five different phospholipid fractions examined, there was at most a 1.4 per cent difference in fatty acid compositions (table 3). Slightly but significantly lower levels of oleic (PE fraction) and eicosenoic acids (PC and PE fractions) were observed in synaptic membranes isolated from quaking mice. The phospholipid compositions and the cholesterol/phospholipid molar ratios for synaptic plasma membranes isolated from quaking mice and their controls were not significantly different (table 4).

TABLE 2. Myelin Fatty Acid Compositions for Quaking Mice and Their Controls*

Fatty Acid	Quaking Mice (n = 6)†	Control Mice (n = 5)
Palmitate 16:0‡	18.8 ± 0.6	14.2 ± 0.2
Palmitoleate 16:1	0.4 ± 0.05	0.7 ± 0.04
Stearate 18:0	32.1 ± 1.0	23.1 ± 0.4
Oleate 18:1 (n-9)	18.5 ± 0.4	28.1 ± 0.1
Linoleate 18:2 (n-6)	0.7 ± 0.05	0.6 ± 0.06
Linolenate and/or arachidate 18:3 (n-6) and/or 20:0	1.1 ± 0.06	0.8 ± 0.05
Eicosenoate 20:1	2.8 ± 0.2	5.8 ± 0.1
Eicosadienoate 20:2		0.2 ± 0.07
Eicosatrienoate 20:3 (n-9)		0.1 ± 0.04
Eicosatrienoate 20:3 (n-6)	1.9 ± 0.2	1.8 ± 0.1
Arachidonate 20:4	10.5 ± 0.4	8.4 ± 0.1
Docosatetraenoate and/or nervonic acid 22:4 and/or 24:1	3.8 ± 0.2	8.3 ± 0.3
Docosapentaenoate 22:5 (n-3)	0.2 ± 0.06	0.3 ± 0.07
Docosahexaenoate 22:6	8.2 ± 0.7	5.8 ± 0.2
Lignocerate 24:0	1.0 ± 0.1	1.9 ± 0.1

* Values are given as weight percentages of the listed fatty acids. Mean values ± SE.

† Numbers of preparations are given in parentheses. The brains from two to four mice were pooled for each preparation.

‡ Fatty acids are expressed in the form x:y (n-z), where x is the number of carbon atoms in the fatty acid chain, y is the number of double bonds, and z is the number of carbon atoms from the terminal methyl group of the fatty acid chain at which the first unsaturation occurs. Thus, linoleic acid, 18:2 (n-6) is 18 carbon atoms long and has two unsaturations occuring between the sixth and seventh and between the ninth and tenth carbon atoms from the terminal methyl group of the fatty acid chain.

Discussion

The goal of this experiment was to determine whether an altered brain lipid composition could influence anesthetic requirement. The results confirm previous reports[10,11,18] that quaking mice have a grossly different myelin fatty acid composition (table 2) and a lower myelin cholesterol content than do their littermate controls. In addition, myelin of quaking mice has a lower cerebroside content and an altered phospholipid composition compared with myelin from control mice.[10] However, the fatty acid, phospholipid, and cholesterol/phospholipid molar ratios were essentially identical for synaptic membranes isolated from quaking mice and from controls. The very slightly lower levels of oleic and eicosenoic acids found in synaptic membranes from quaking mice (table 3) may be due to a slight myelin contamination in the synaptic plasma membrane fraction. This is the first analysis of synaptic membrane lipids in the quaking mouse. The synaptic membrane lipid composition of the quaking mouse is close to that reported for another

TABLE 3. Fatty Acid Compositions of Synaptic Membrane Phospholipids from Quaking Mice and Their Controls*

Fatty Acid	Sphingomyelin		Phosphatidylcholine		Phosphatidylserine		Phosphatidylinositol		Phosphatidylethanolamine	
	Quaking	Control	Quaking	Control	Quaking	Control	Quaking	Control	Quaking	Control
Palmitate 16:0	14.7 ± 1.9	13.6 ± 2.7	53.2 ± 0.3	53.2 ± 0.4	1.5 ± 0.2	1.5 ± 0.2	9.3 ± 0.7	9.4 ± 1.1	8.4 ± 0.3	8.0 ± 0.3
Palmitoleate 16:1	0.2 ± 0.2	0.4 ± 0.2	0.8 ± 0.1	0.9 ± 0.1					0.1 ± 0.0	0.2 ± 0.2
Stearate 18:0	78.8 ± 2.8	79.9 ± 3.4	12.2 ± 0.2	12.2 ± 0.3	46.3 ± 0.6	46.0 ± 0.9	41.5 ± 1.6	41.1 ± 1.7	28.0 ± 0.5	27.2 ± 0.7
Oleate 18:1 (n-9)	5.2 ± 1.7	4.4 ± 0.6	22.8 ± 0.2	23.2 ± 0.3	4.5 ± 0.2	5.1 ± 0.2	4.5 ± 0.2	5.7 ± 0.7	6.1 ± 0.1†	7.5 ± 0.3
Linoleate 18:2 (n-6)			0.5 ± 0.02	0.4 ± 0.05						
Arachidate and/or linolenate 20:0 and/or 18:3 (n-6)	1.1 ± 0.4	1.2 ± 0.4	0.1 ± 0.03				0.3 ± 0.1			
Eicosenoate 20:1			0.7 ± 0.02‡	1.1 ± 0.03		0.1 ± 0.06		0.1 ± 0.1	0.4 ± 0.08§	0.7 ± 0.07
Eicosadienoate 20:2			0.2 ± 0.02	0.2 ± 0.03						
Eicosatrienoate 20:3 (n-6)			0.3 ± 0.03	0.3 ± 0.0	0.3 ± 0.05	0.2 ± 0.06			0.4 ± 0.05	0.4 ± 0.04
Arachidonate 20:4			4.6 ± 0.1	4.3 ± 0.2	1.8 ± 0.07	1.6 ± 0.09	39.6 ± 1.8	38.2 ± 2.0	12.9 ± 0.2	12.7 ± 0.1
Docosatetraenoate and/or nervonic acid 22:4 and/or 24:1			0.4 ± 0.03	0.4 ± 0.08	2.8 ± 0.1	2.9 ± 0.2			4.9 ± 0.2	5.2 ± 0.1
Docosapentaenoate 22:5 (n-6)					0.3 ± 0.07	0.3 ± 0.09			0.3 ± 0.06	0.3 ± 0.0
Docosapentaenoate 22:5 (n-3)					0.1 ± 0.04	0.1 ± 0.05			0.4 ± 0.02	0.3 ± 0.04
Docosahexaenoate 22:6 (n-3)			4.2 ± 0.2	3.9 ± 0.3	42.4 ± 0.7	42.0 ± 0.5	4.8 ± 0.5	5.1 ± 0.4	37.9 ± 0.5	37.2 ± 0.7

* Values are given as weight percentages of the listed fatty acids. Mean values ± SE. Results are from six separate preparations of synaptic membranes from the quaking mice and from five separate preparations from their littermate controls. The brains of two to four mice were pooled for each preparation.

Significantly less than control values at † $P < 0.005$; ‡ $P < 0.001$; § $P < 0.05$.

strain of mice[19] and also to that reported for rat synaptic membrane lipids.[20,21]

The quaking mice had slightly lower N_2O and cyclopropane rolling-response ED_{50}s than did their littermate controls (table 1). However, since the quaking mouse has an ataxic gait, the rolling-response test may not be a valid test of anesthetic requirement in this animal. Therefore, cyclopropane, enflurane, and isoflurane requirements were also measured in quaking mice and controls by use of a tail-clamp procedure. These agents were chosen for their high potencies (which permitted testing at atmospheric pressure) and relatively low blood and tissue solubilities[22] (which allowed for the assumption of identical inspired and alveolar partial pressures). The cyclopropane, enflurane, and isoflurane ED_{50}s obtained for quaking mice by use of the tail-clamp procedure tended to be lower than but were not significantly different from those found in the control mice (table 1). The negative results obtained with the tail-clamp tests support the supposition that the rolling-response test is not a valid measurement for anesthetic potency in quaking mice. However, assuming this is the case, it is unclear why significant differences between enflurane and isoflurane rolling-response ED_{50}s of quaking and control mice could not be detected. Quaking mice showed a trend toward a lower enflurane rolling-response ED_{50}, but isoflurane rolling-response ED_{50}s were essentially identical in quaking and control mice (table 1). An alternative explanation is that the rolling-response and tail-clamp tests demonstrate different sensitivities to anesthetic agents, and that the quaking mouse does have a lower anesthetic requirement for certain (*e.g.*, nitrous oxide and cyclopropane), but not all, anesthetics. At any rate, this decreased anesthetic requirement is at most 23 per cent lower (table 1).

It should be noted that the cyclopropane, enflurane, and isoflurane ED_{50}s obtained by use of the tail-clamp procedure are approximately twice the values obtained using the rolling-response test (table 1). Results of the present experiments agree qualitatively with previously reported isoflurane ED_{50}s obtained for the mouse by the use of tail-clamp[23] and rolling-response[24] tests.

The ability of general anesthetics to disorder[3,4] lipid membranes, to decrease phase transition temperatures,[6–8] and to disrupt lateral phase separations[5] in model lipid membranes has led to the suggestion that an alteration of properties of the membrane lipid bilayer may be sufficient to explain the action of general anesthetics without invoking a direct interaction with membrane proteins. If this were the case, one would expect that an alteration in membrane lipids and in membrane physical state at the site of anesthetic action would influence the potency of the anesthetic. Spin-label studies show a greater membrane disorder for the myelin isolated from quaking mice compared with their controls.[18] However, this greater fluidity and altered lipid composition in the myelin of the quaking mouse has little or no influence on anesthetic potency. These results suggest that the lipids of central nervous system myelin may not constitute a significant site of anesthetic action.

TABLE 4. Phospholipid Compositions and Cholesterol/Phospholipid Molar Ratios of Synaptic Plasma Membranes Isolated from Quaking Mice and Their Littermate Controls*

	Quaking Mice (n = 6)†	Control Mice (n = 5)
Sphingomyelin‡	3.7 ± 0.26	3.6 ± 0.15
Phosphatidylcholine	42.5 ± 0.62	41.8 ± 0.97
Phosphatidylserine	14.2 ± 0.49	13.2 ± 0.83
Phosphatidylinositol	3.4 ± 0.24	3.4 ± 0.10
Phosphatidylethanolamine	36.1 ± 1.11	37.9 ± 0.94
Molar ratio		
Cholesterol/phospholipid§	0.593 ± 0.0160	0.606 ± 0.0093

* Mean values ± SE.
† Number of separate preparations of synaptic plasma membranes is given in parentheses.
‡ Calculated as percentage of phosphate composition.
§ Calculated from the amount of cholesterol recovered and the amount of phosphate recovered in the SPH, PC, PS, PI, and PE fractions from the thin-layer chromatographic plate.

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