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Enhancement of the Responsiveness of the Rat Diaphragm by L-Methionine and Phospholipid Methylation and Their Relationships to Aging[1]

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ABSTRACT

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The functional efficiency of the diaphragm to stimulation by acetylcholine or electrical stimulation may decline in old age. Therefore, the contractions of hemidiaphragms of Fischer 344 rats of ages 2 to 33 months were measured after electrical stimulation of the phrenic nerve or the muscle *in vitro*. S-Adenosyl-L-methionine-mediated methylation of membrane phospholipids may increase membrane fluidity and responsiveness of the muscle. An increase in the intracellular levels of S-adenosyl-L-homocystein inhibits phospholipid methylation. The intracellular levels of S-anenosyl-L-methionine were increased by incubating the hemidiaphragm in L-methionine (500 μM), L-homocysteine thiolactone (100 μM), adenosine (100 μM) and erythro-9-(2-hydroxy-3-nonyl)adenine, an inhibitor of adenosine deaminase (10 μM). The following results were obtained: 1) Microsomes from hemidiaphragm contained phospholipid methyltransferases. 2) L-Methionine increased muscle tension developed by electrical stimulation of the muscle or the nerve. This increase in the tension is dependent upon the concentration of L-methionine (125–500 μM). 3) Labeled methyl groups were incorporated from labeled L-methionine into phospholipids of the hemidiaphragm. 4) Presence of adenosine, L-homocysteine thiolactone and erythro-9-(2-hydroxy-3-nonyl)adenine inhibited the effect of L-methionine to increase muscle tension and incorporation of methyl groups into phospholipids. 5) Muscle tension developed by electrical stimulation of the phrenic nerve or the muscle decreased with increasing age. 6) The methionine effect was erratic or insignificant in hemidiaphragms of old rats (>15 months). These observations indicate that S-adenosyl-L-methionine-mediated membrane phospholipid methylation and fine regulation of membrane fluidity in the diaphragm may become defective with advancing age, and may contribute partly to the functional deficits of the diaphragm. However, these investigations do not exclude the possibility that other methyltransferases besides phospholipid methyltransferase may contribute to functional deficits of the diaphragm during aging.

Cell membranes consist of a lipid bilayer in which receptors, enzymes and other biochemically active proteins are embedded. The lipid bilayer is mainly composed of phospholipids and provides a fluid matrix for proteins and their movement (Singer and Nicolson, 1972). The membrane phospholipids are mainly composed of PE and PC. Large proportions of the total PE and PC are asymmetrically distributed in the membrane, PE facing the interior of the cell and PC facing the exterior of the cell (Chap *et al.*, 1977; Rothman and Lenard, 1977). PE N-methyltransferase (methyltransferase I), which is located in the interior bilayer of the membrane, converts PE to PME in the presence of SAM (Hirata *et al.*, 1978; Sastry *et al.*, 1981a,b). Methyltransferase I has a low K_m (<1.0 μM) for SAM, and PME accumulates transiently within the membrane during this first methylation. PME is subsequently converted into PMME and PC by phosphatidyl-N-methylethanolamine N-methyltransferase (methyltransferase II) in the presence of SAM (Hirata *et al.*, 1978; Sastry *et al.*, 1981a). Methyltransferase II has a high K_m for SAM (~100 μM) and PC formed is translocated to the exterior of the membrane (Hirata and Axelrod, 1978a).

The occurrence of the two phospholipid methyltransferases is widely distributed. They are present in the plasma membranes of reticulocytes (Hirata *et al.*, 1979) mitochondrial, nuclear and microsomal membranes in the rat liver (Sastry *et*

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ABBREVIATIONS: PE, phosphatidylethanolamine; PC, phosphatidylcholine; PME, phosphatidyl-N-methylethanolamine; SAM, S-adenosyl-L-methionine; PMME, phosphatidyl-N,N-dimethylethanolamine; ACh, acetylcholine; SAH, S-adenosyl-L-homocysteine; L-HCT, L-homocysteine thiolactone; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; TLC, thin-layer chromatography; ATPase, adenosine triphosphatase.

al., 1981a), microsomal membranes from adrenal glands (Hirata *et al.*, 1978), islet cells of pancreas (Landon *et al.*, 1980), human placental villi (Barnwell and Sastry, 1981a,b) and spermatozoa (Janson and Sastry, 1981). Therefore, the occurrence of stepwise methylation is a common phenomenon and may have implications in the transfer or spread of biological signals across or along membranes.

Transient accumulation of PME within the membrane due to the differences in the affinities of SAM to the two phospholipid methyltransferases has been found to decrease membrane microviscosity and increase fluidity (Hirata and Axelrod, 1978b, Sastry *et al.*, 1981b). This increase in membrane fluidity has been shown to be involved in the coupling of the *beta* adrenergic receptor and adenylate cyclase (Hirata *et al.*, 1979); ribosomal insertion of cytochrome P-450 into liver endoplasmic reticulum during induction by phenobarbital and 3-methylcholanthrene (Sastry *et al.*, 1981b); and liver regeneration (Jaiswal *et al.*, 1982). It may be involved in Ca^{++} influx during skeletal muscle contraction to ACh. However, the role of phospholipid methylation in cholinergic function is not known. Therefore, we have investigated the role of phospholipid methylation in cholinergic function using rat phrenic nerve hemidiaphragm preparation as a model. As SAM does not enter the cells efficiently (Stramentinoli and Pezzoli, 1979; Hoffman *et al.*, 1980), it was formed *in situ* by incubating the hemidiaphragm with L-methionine (fig. 1). Similarly, increased intracellular levels of SAH, an inhibitor of SAM-mediated methylations, were formed by incubating the hemidiaphragm with adenosine, L-HCT and EHNA, an inhibitor of ado-deaminase (Pike *et al.*, 1978) (fig. 1).

The present investigations indicate that increasing the cellular formation of SAM increases phospholipid methylation and the contraction heights of the hemidiaphragm upon electrical stimulation. Both of these effects are inhibited by increasing levels of SAH. The effect of L-methionine or hemidiaphragm decreased as a function of age.

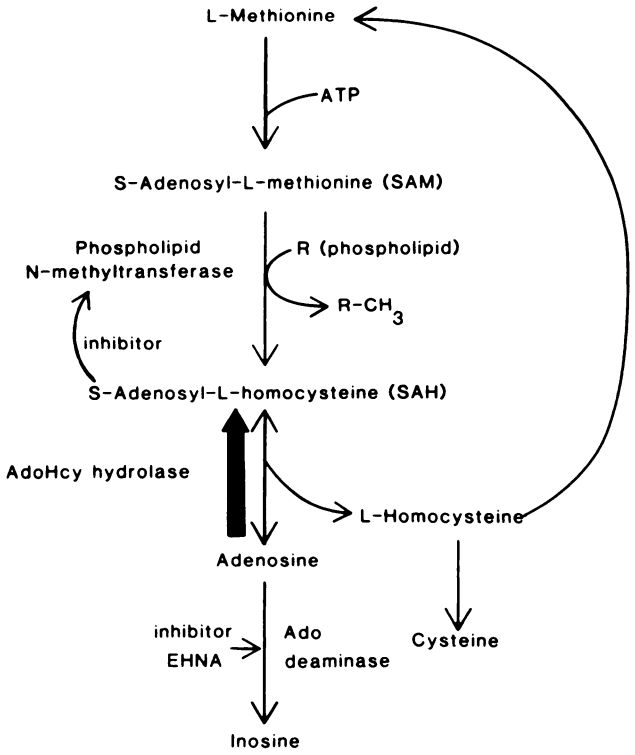

Fig. 1. Metabolic pathways for the formation of SAM and its hydrolysis.

Materials and Methods

Materials. EHNA was supplied by Burroughs Wellcome Company (Research Triangle Park, NC). L-Methionine, L-HCT and adenosine were obtained from Sigma Chemical Co. (St. Louis, MO).

Several phospholipids were collected for identifying the methylated phospholipids. PE, phosphatidyl-N-methylethanolamine and phosphatidyl-N,N-dimethylethanolamine were obtained from Grand Island Biological Company (Grand Island, NY). These phospholipids were derivatives of egg phosphatidylcholine by the exchange of bases in the presence of phospholipase D. Synthetic β, γ-dipalmitoyl-α-phosphatidylethanolamine and its N-methylated derivatives were obtained from Calbiochem-Behring Corp. (La Jolla, CA).

S-adenosyl-L-[*methyl*-^{3}H]methionine (64 Ci/mmol) was purchased from New England Nuclear (Boston, MA). This preparation of SAM was diluted with 100 mM Tris-glycylglycine buffer to obtain concentrations lower than 1.0 μM. Unlabeled SAM was purchased from Boehringer Mannheim (Indianapolis, IN) and was added to labeled SAM to obtain concentrations higher than 2 μM. L-[*methyl*-^{14}C]Methionine (50 mCi/mmol) was supplied by Amersham Corp. (Arlington, Heights, IL). Unlabeled L-methionine was purchased from Calbiochem-Behring Corp. All other reagents were of analytical grade.

Animals. The experiments in figures 2 to 4 were done using Fischer 344 rats purchased from Harlan Laboratories (Indianapolis, IN). They were 2 months of age.

The Animal Resources Program of the National Institute of Aging has maintained colonies of aged cohort Fischer 344 rats under a contract with Charles River Breeding Laboratories, Inc. (Willmington, MA). The animals for our studies on aging were transported from these colonies and were acclimatized in the animal rooms of Vanderbilt University School of Medicine for 5 days before they were used in the study.

Phrenic nerve-hemidiaphragm preparations and recording of contractions. These preparations were dissected from Sprague-Dawley or Fischer 344 rats according to the procedures described by Bülbring (1946) and Chiou and Sastry (1970). Each final preparation was a strip of the diaphragm with an intact phrenic nerve. The strip had a fanlike shape with the narrow margin at the tendinous end and the wide margin at the coastal end. The former was tied with a thread connected to a Microdisplacement Myograph Transducer (F-50, Narco Biosystems Inc., Houston, TX) and the latter was fixed by a stainless-steel hook to the bottom of an organ bath (capacity 50 ml). The bathing fluid has a pH of 7.4 and the following composition (in millimolar): NaCl, 137.2; $NaHCO_3$, 11.9; dextrose, 11.1; KCl, 2.7; $CaCl_2$, 1.2; NaH_2PO_4, 0.4; $MgCl_2$, 0.1. The bathing solution was oxygenated with a mixture of 95% oxygen and 5% carbon dioxide. The bathing medium was kept at 37 ± 0.5°C. The contractions were recorded with 0.5 g of tension on the muscle. The phrenic nerve or the hemidiaphragm was stimulated by a pair of platinum electrodes with a frequency of 6 shocks/min. An S88 stimulator (Grass Instrument Co., Quincy, MA) and an SIU 5 Stimulus Isolation Unit were used.

Effects of L-methionine on the contractions induced by electrical stimulation of the phrenic nerve or the hemidiaphragm. Different doses of L-methionine (50–500 μM) were added to the diaphragm, and the contractions were continuously recorded for 1 hr by stimulation of the nerve or the muscle. Each hemidiaphragm preparation was used for a single challenge with only one dose of L-methionine. The contractions of the control hemidiaphragm preparations, which were not challenged with L-methionine, were run under similar conditions. The pulse duration was 0.3 and 5 msec for the stimulation of the nerve and the muscle, respectively. The maximum voltage was 5 and 120 V for the nerve and the muscle, respectively. The preparation was equilibrated in the bathing solution for 20 min before the addition of drugs to the bath. Linear contractions of the muscle were recorded by using a physiograph (DMP-4B, Narco Biosystems). The maximum effect of L-methionine was observed within 30 min.

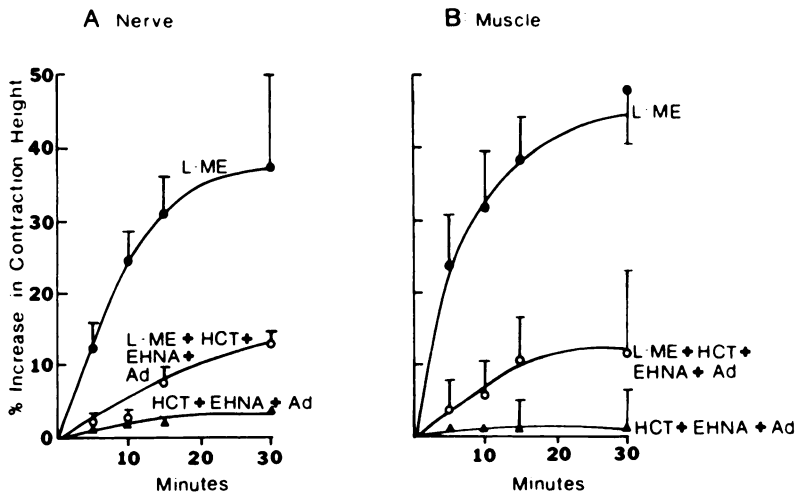

Fig. 2. Alterations in contraction heights of the rat hemidiaphragm upon electrical stimulation of the phrenic nerve (A) or the muscle (B) in the presence of L-methionine (L-ME, 500 μM) only, and L-ME (500 μM), L-HCT (100 μM), EHNA (10 μM) and adenosine (Ad, 100 μM). The addition of L-ME to the bath increases the formation of SAM by the muscle, and this, in turn, increases phospholipid methylation. The addition of Ad, L-HCT and EHNA (an inhibitor of ado-deaminase) increases the formation of SAH which inhibits phospholipid methylation. All values are means ± S.E. from five experiments. Average age: 2 months.

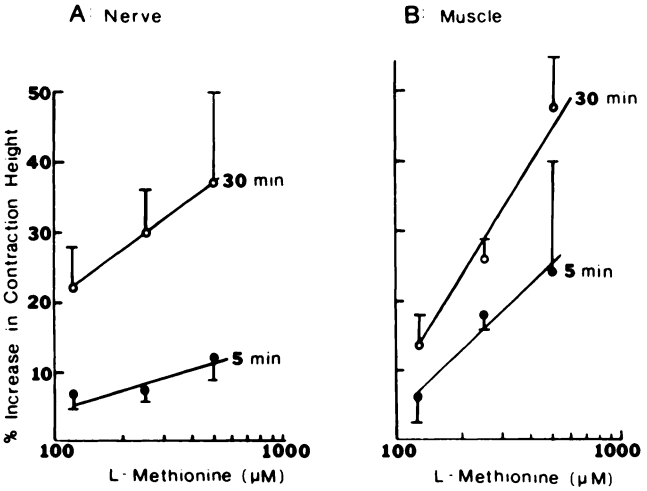

Fig. 3. Dose-response relationships in the contraction heights of phrenic nerve-hemidiaphragm when the nerve (A) or the muscle (B) were electrically stimulated in the presence of varying doses of L-methionine for different time periods. Each point is a mean ± S.E. from five values.

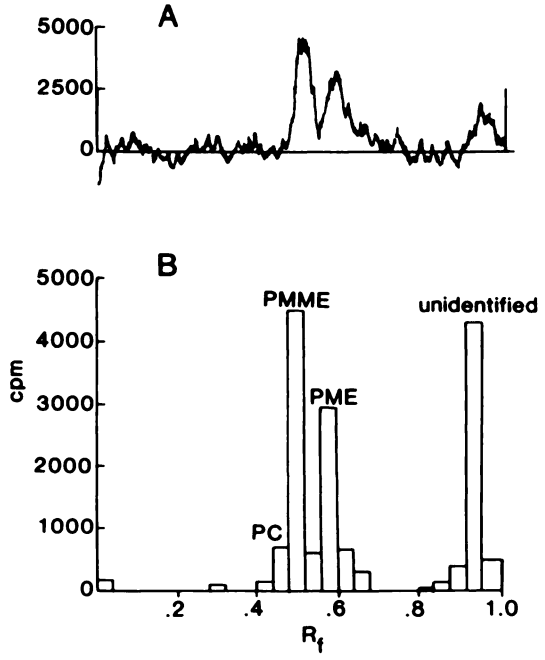

Fig. 4. Thin-layer chromatogram of methylated phospholipids formed when microsomes from hemidiaphragm were incubated with [^{3}H]SAM (2 μM). A, thin-layer chromatogram obtained using a Packard chromatogram scanner. All three ^{3}H-methylated phospholipids, PME, PMME and PC, were formed when diaphragm microsomes were incubated with low concentrations of SAM (2 μM). The peak at 0.94, which is very close to the solvent front, was not identified. When diaphragm microsomes were incubated with 202 μM SAM, PC (R_f 0.44) and an unidentified peak (R_f 0.96) were found in the chromatogram of the reaction products. The radioactivities of the blank samples fall below the horizontal line marked O. B, the radioactivities were measured by counting 0.25-inch sections of the chromatogram. SAM concentration: 2 μM. Blank samples were chromatographed under identical conditions. The values of the blanks were subtracted from the corresponding values of SAM-mediated methylation in plotting the thin-layer chromatograms. The R_f values of the peaks of the methylated phospholipids agree with those in fig. 4A.

Effects of L-methionine in the presence of adenosine, L-HCT and EHNA. Continuous recordings of the contractions of hemidiaphragm upon electrical stimulation of the nerve or the muscle were obtained in the presence of 1) a mixture of L-methionine (500 μM), adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM); 2) a mixture of adenosine, L-HCT and EHNA; and 3) the components of the above mixture individually.

Incorporation of ^{14}C-methyl groups into phospholipids of the rat diaphragm during incubation with L-[*methyl*-^{14}C]methionine and its inhibition by phospholipid methylation. Two hemidiaphragms were mounted in two muscle baths and stabilized as described above. L-Methionine (500 μM) containing L-[*methyl*-^{14}C]methionine (0.025 μCi) was added to one muscle bath. The hemidiaphragm in this bath was used as a control. L-Methionine (500 μM) containing L-[methyl-^{14}C]methionine (0.025 μCi), adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM) were added to the second bath. Both hemidiaphragms were stimulated simultaneously with platinum electrodes for 30 min as described before. At the end of 30 min, the hemidiaphragms were washed thoroughly with fresh Krebs' solution, and were used for the extraction of membrane phospholipids as described below.

Each hemidiaphragm was sliced with a McElwain Slicer and homogenized in 2 ml of isotonic sucrose (0.25 M) at 4°C using a Teflon hand homogenizer (size C) at 1200 rpm six strokes). An aliquot (0.1 ml) of the homogenate was used for the assay of protein. A second aliquot (0.4 ml) was pipetted into a 15-ml plastic tube and 6 ml of chloroform-methanol-hydrochloric acid (2.1:0.02, v/v) was added. Tubes were stoppered and shaken for 10 min. The mixture was allowed to settle, and the top aqueous layer was siphoned off. The lower chloroform layer was washed twice with 4 ml of 0.1 M KCl in 50% methanol. The methylated phospholipids were recovered and identified as described in the section on the assay for phospholipid N-methyltransferases.

Preparation of microsomes from hemidiaphragms. Hemidiaphragms (six) were dissected from Sprague-Dawley rats and washed

with isotonic sucrose (0.25 M) at 4°C. They were sliced on a McElwain Tissue Slicer and homogenized with 19 ml of isotonic sucrose using a Teflon hand homogenizer (size C) and 1200 rpm (six strokes). The tissue was further homogenized with three strokes of a serrated Teflon pestle. The homogenate was centrifuged at 12,100 × *g* for 10 min. The supernatant was decanted into prechilled centrifuge tubes and recentrifuged at 20,200 × *g* for 10 min. At the end of the spin, the supernatant was again centrifuged, this time at 105,000 × *g* for 60 min. The supernatant was decanted and discarded. The walls of the centrifuge tubes were cleaned of fatty substances with a gauze sponge. The microsomal pellet was resuspended in 1.0 ml of 0.15 M KCl and rehomogenized using a Teflon hand homogenizer at 1200 rpm (15 strokes) to obtain a uniform suspension of microsomes.

The above preparation of microsomes consisted mainly of membranes from sarcoplasmic reticulum and plasma membrane. An average preparation has a Ca^{++} uptake activity of 100 nmol/mg of protein per 30 min as determined by the method of Binder *et al.* (1976). There is a 6- to 7-fold enrichment of 5'nucleotidase activity, a marker for plasma membrane, as determined by the method of Avruch and Wallach (1971).

Assay for phospholipid N-methyltransferases. These enzymes were assayed by ^{3}H-methyl groups transferred from [^{3}H]SAM to the endogenous PE in the membrane. The total methylation was measured by the incorporation of ^{3}H-methyl groups into phospholipids at 202.24 μM SAM. Methyltransferase I was assayed by the amount of phosphatidyl-N-[^{3}H-*methyl*]ethanolamine formed at 2.24 μM SAM. Methyltransferase II was assayed by the amount of phosphatidyl-[^{3}H]choline formed. The procedures have only minor modifications from those described by Sastry *et al.* (1981a,b).

The total volume of the reaction medium (50 μl) was composed of a mixture of three aliquots (20, 10 and 20 μl) in Tris-glycylglycine buffer (50 mM) at pH 8 (unless otherwise specified). The first aliquot contained the membrane preparation (100 μg of protein); the second was deionized water; and the third aliquot (20 μl) contained the substrate, [^{3}H]SAM. The reaction was started by the addition of the third aliquot to the mixture of the first two in a 6-ml tube. The reaction medium was incubated at 37°C for 30 min. The reaction was stopped by the addition of 3 ml of chloroform-methanol-hydrochloric acid (2:1:0.02, v/v) and the reaction mixture was shaken for 10 min. The chloroform-methanol-HCl extract was washed twice by shaking with 2 ml of 0.1 M KCl in 50% methanol. The aqueous phase was aspirated each time and rejected. One milliliter of the chloroform phase was transferred to a counting vial, and the solvent was evaporated to dryness at 80°C. The residue of phospholipids was dissolved in 10 ml of Aquasol (Amersham Corp.) and the total radioactivity was measured. The remaining chloroform phase was dried over anhydrous Na_2SO_4 and used for the determination of the ratios of ^{3}H-methylated phospholipids in the chloroform extracts as described below.

The chloroform extracts of phospholipids were concentrated under a stream of nitrogen and chromatographed on Silica Gel G plates (Uniplate, Analtech Inc., Newark, DE). Chromatograms were developed in a solvent system comprised of chloroform-propionic acid-*n*-propylalcohol-water (1:2:2:1, v/v). The solvent was allowed to move 6.5 inches, and 0.25 inch sections were transferred to counting vials. Aquasol was added and radioactivity was counted. Authentic samples of phospholipids were chromatographed simultaneously and their spots were visualized by iodine vapor. The positions of the ^{3}H-methylated phospholipids on the TLC plates were also detected by the peaks in the chromatogram obtained using a Packard Radiochromatogram Scanner and a gaseous medium of helium (98.7%) and butane (1.3%). The radioactivities in the TLC peaks corresponding to PME, PMME and PC were determined and expressed as ratios of the total radioactivity on the TLC plates. These ratios were used to estimate the quantities of the three methylated phospholipids formed during the enzymatic reactions.

Protein determinations. Protein content in the homogenates was established according to the method of Lowry *et al.* (1951).

Statistics. Results are expressed as means and standard errors wherever possible. The significance of the difference between the mean values was calculated by Student's *t* test. A result was considered significant if its P value was less than .05.

Results

Effects of L-methionine on responses of the rat phrenic nerve-hemidiaphragm to electrical stimulation. Incubation of cell cultures or tissues with L-methionine increases the formation of SAM (Schlenk, 1979; Finkelstein, 1979). Therefore, in order to evaluate the effects of increased formation of SAM on cholinergic transmissions *in situ*, we have studied the effects of L-methionine on the phrenic nerve-hemidiaphragm.

L-Methionine increased the responsiveness of the hemidiaphragm to the phrenic nerve stimulation (fig. 2A, top curve) or direct electrical stimulation of the muscle (fig. 2B, top curve). In both cases, maximal increase in the contraction height of the muscle was obtained within 20 to 30 min. No further increase in the contraction height was obtained after incubation of the preparation for more than 30 min. In control experiments without L-methionine, this preparation gave consistent and equal responses to the electrical stimulation of the nerve or the muscle for more than 2 hr.

An increase in the contraction height of the hemidiaphragm in the presence of L-methionine will be referred to in the following pages as the L-methionine effect.

The L-methionine effect is dose-dependent in both the electrical stimulation of the nerve (fig. 2A) and the muscle.

Effects of adenosine, L-HCT and EHNA on the L-methionine effect. Incubation of the cells and tissues with methionine increases the formation of SAM in the cells. Similarly, incubation of the cells and tissues with a mixture of adenosine, L-HCT and EHNA increases the formation of SAH, which is an inhibitor of SAM-mediated enzyme reactions. In order to evaluate the methionine effect, we have measured the contractions of the hemidiaphragm during incubation with a mixture of L-methionine (500 μM), adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM) upon electrical stimulation of the phrenic nerve or the muscle. The presence of adenosine, L-HCT and EHNA abolished about 66% of the L-methionine effect both during electrical stimulation of the phrenic nerve (fig. 2A, middle curve) and the hemidiaphragm (fig. 2B, middle curve). In the absence of L-methionine, the mixture of adenosine, L-HCT and EHNA has no effect upon the electrical stimulation of the nerve (fig. 2A, lower curve) or the muscle (fig. 2B, lower curve).

Incorporation of ^{14}C-methyl groups from L-[*methyl*-^{14}C] methionine into phospholipids during electrical stimulation of the hemidiaphragm. Incubation of tissues with L-[*methyl*-^{14}C]methionine should form [^{14}C]SAM, which should methylate membrane phospholipids, which increase ^{14}C-methyl group incorporation into phospholipids. This incorporation into phospholipids should be inhibited when the hemidiaphragms are incubated in the presence of labeled L-methionine, adenosine, L-HCT and EHNA, because SAH formed *in situ* would inhibit methylation of membrane PE by phospholipid methyltransferases. When the hemidiaphragm was stimulated in the presence of L-[*methyl*-^{14}C]methionine (500 μM) for 30 min by direct electrical stimulation, ^{14}C-methyl groups incorporated into membrane phospholipids were equal to 1938 ± 215 dpm/mg of protein (M_1 ± S.E.; n = 8). In the presence of adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM), along with labeled L-methionine, ^{14}C-methyl groups incorporated into membrane phospholipids were equal to 1055 ± 215 dpm/mg of protein (M_2 ± S.E.; n = 8). The difference between means M_1

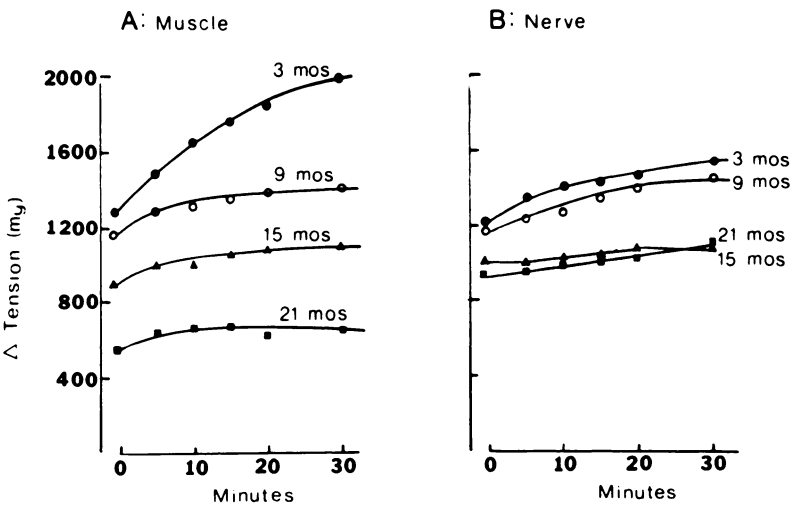

Fig. 5. Effect of L-methionine (500 μM) on the contractions of the rat hemidiaphragm by direct electrical stimulation of the muscle or the phrenic nerve from male Fischer 344 rats of different age groups as a function of time. The first point in each curve is a control Δ tension just before the addition of L-methionine. Each point is a mean from five values. Standard errors were omitted to prevent confusion. A, Δ tension developed by direct electrical stimulation of the muscle. The mean at 3 months was significantly different from the corresponding means at 9, 15 and 21 months. B, Δ Tension developed by the electrical stimulation of the nerve. There were no significant differences in the curves at 3 and 9 months and the curves at 15 and 21 months. The means on the curve at 3 and 9 months were significantly different from the corresponding means at 15 and 21 months at $P < .05$.

and M_2 was significant at $P < .02$. Therefore, about 46% of the incorporation of ^{14}C-methyl groups into membrane phospholipids during stimulation was inhibited in the presence of SAH in the hemidiaphragm *in situ*.[2]

Occurrence of phospholipid methyltransferases in the rat diaphragm. If the L-methionine effect can be explained by increased membrane phospholipid methylation, there should be evidence that SAM-mediated stepwise phospholipid methylation could occur in the membranes of the hemidiaphragm. Therefore, microsomes were prepared from the rat hemidiaphragm and analyzed for phospholipid methyltransferases. When microsomal membranes were incubated with low concentrations of [^{3}H]SAM (2 μM), two methylated phospholipids, PME and PMME were formed (fig. 4). Inasmuch as the K_m of SAM for methyltransferase I was about 0.86, this enzyme should be saturated at 2 μM SAM. Therefore, the total PME formed was used as a criterion to calculate methyltransferase I activity. The microsomes of the hemidiaphragm have a methyltransferase I activity of 10.95 ± 0.47 fmol of PME formed per microgram of protein per 30 min.

At high SAM concentrations (202 μM), methyltransferase II (K_m, 87 μM) was also saturated. When the microsomal membranes were incubated with [^{3}H]SAM, only one major product, PC, was formed. Therefore, methyltransferase II activity was calculated from the [^{3}H]PC formed at high SAM concentrations. The diaphragm microsomes have methyltransferase II activity of 39.7 ± 3.08 fmol of PC formed per microgram of protein per 30 min.

L-Methionine-induced responsiveness of the phrenic nerve-hemidiaphragm of rats of different age groups. Tension developed upon electrical stimulation of the muscle decreased gradually as a function of age from 3 to 21 months (means at time 0 in fig. 5A). L-Methionine increased the tension developed in the hemidiaphragm of 3-month-old rats as a function of time of incubation. This L-methionine effect decreased as a function of age (fig. 5A).

The tension developed in the hemidiaphragm upon electrical stimulation of the phrenic nerve in 15- and 21-month-old rats was lower than that in 3- or 9-month-old rats (fig. 5B). The effect of L-methionine to increase the tension of the hemidiaphragm was lower in 15- and 21-month-old rats than in 3- or 9-month-old rats.

[2] The nonstimulated hemidiaphragms incorporated 902 ± 226 dpm/mg of protein (M_3 ± S.E.; $n = 8$). The difference between M_2 and M_3 was not significant.

Effect of adenosine, L-HCT and EHNA on the responsiveness of hemidiaphragm to L-methionine as a function of age. L-Methionine (500 μM) increased the Δ tension in hemidiaphragms of 3-month-old rats upon electrical stimulation from 1100 to 2100 mg. When adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM) were present in the bath along with L-methionine, the enhancement of Δ tension was only 1100 to 1400 mg in 30 min (fig. 5A). This indicates that about 57% of the enhancement of Δ tension by L-methionine was inhibited by the mixture of adenosine, L-HCT and EHNA (fig. 6A).

The enhancement of Δ tension by L-methionine upon electrical stimulation of hemidiaphragms of 21-month-old rats was from 900 to 1100 mg, indicating a 22% increase over the control in 30 min (fig. 5B). The presence of adenosine, L-HCT and EHNA decreased this effect to the control level in 30 min (fig. 6B).

There was only a slight increase in the enhancement of Δ tension in the hemidiaphragms of the 3-month-old rats upon stimulation of the phrenic nerve. This effect was not observed when adenosine, L-HCT and EHNA were present in the bath (fig. 7, A and B).

Discussion

In the present investigations, L-methionine increased the contraction heights of hemidiaphragm upon electrical stimulation of the nerve or the muscle. Incubation of the hemidiaphragm in the presence of labeled L-methionine incorporated methyl groups into the phospholipids of the muscle. The presence of adenosine, L-HCT and EHNA in the bath inhibited the enhancement of the contraction heights as well as the incorporation of methyl groups into membrane lipids. Phospholipid methyltransferases are present in the microsomes from hemidiaphragm, which mainly consist of membranes from sarcoplasmic reticulum and plasma membrane. These observations indicate that phospholipid methylation plays a significant role in the functional activity of the rat diaphragm.

Enhancement of the contraction heights of the hemidiaphragm to the electrical stimulation of the nerve could be explained by facilitation of several steps in the stimulation-contraction coupling: 1) increased accessibility of cholinergic nicotinic receptors to ACh released from the nerve terminal, 2) unmasking of nicotinic receptors and thus increasing the number of nicotinic receptors, 3) improved communication between the nicotinic receptor and the contractile system and 4) im-

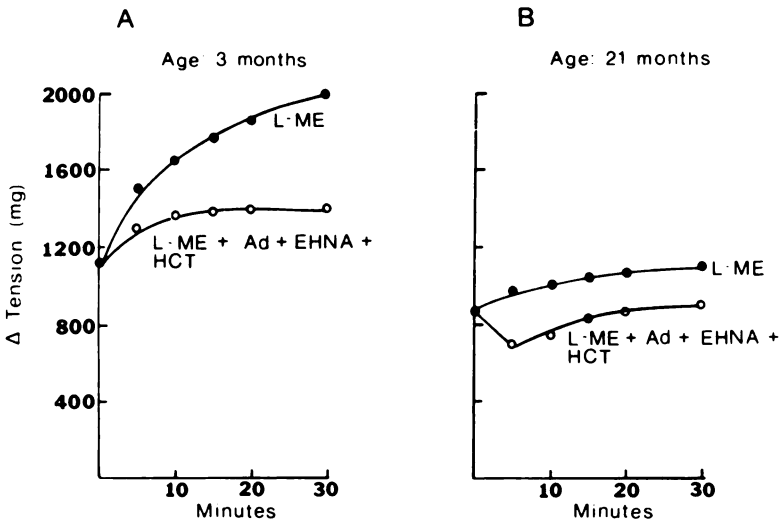

Fig. 6. Effect of L-methionine (L-ME, 500 μM) on the contractions of Fischer 344 rat hemidiaphragm by direct electrical stimulation of the muscle from different age groups. The effect induced by L-ME was inhibited by adding adenosine (Ad, 100 μM), EHNA (10 μM) and L-HCT (100 μM) to the bath. Each point is a mean from five values. A, hemidiaphragms from 3-month-old rats. B, hemidiaphragms from 21-month-old rats.

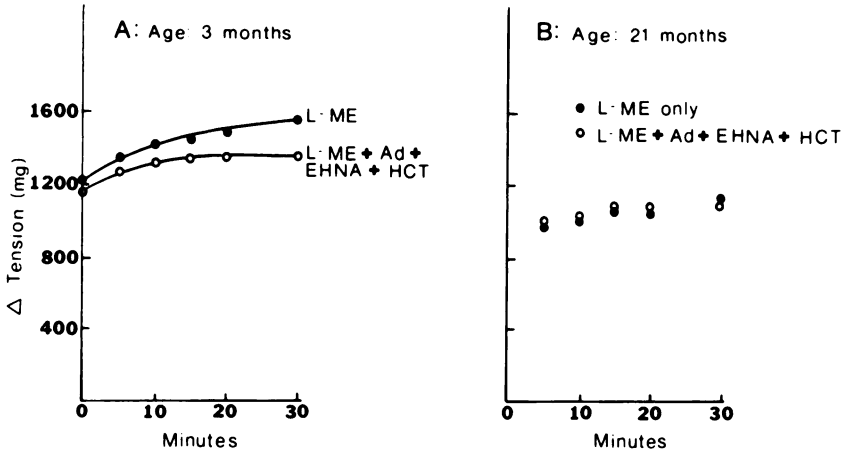

Fig. 7. Effect of L-methionine (L-ME, 500 μM) on the contractions of hemidiaphragm by stimulation of the phrenic nerve from Fischer 344 rats of different age groups. Details are given in the legend to figure 5. Ad, adenosine.

proved transduction in which information from the nicotinic receptors triggers the effector. In the hemidiaphragms of young animals (2 months old), there were no significant differences in the enhancement of contraction heights between the electrical stimulation of the phrenic nerve or direct stimulation of hemidiaphragm. Therefore, the site of the L-methionine effect must be beyond the stimulation of the nicotinic receptor by ACh.

One of the common steps in the contraction induced by ACh and electrical stimulation is the depolarization of the cell membrane and Ca^{++} movements. For skeletal muscle contraction, the two main Ca^{++} sources for contraction in the muscle tissues are the release of Ca^{++} from sarcoplasmic reticulum and the entry of Ca^{++} (Hurwitz *et al.*, 1973, Rosenberger and Triggle, 1978). Specific entry mechanisms in the plasma membranes include Ca^{++} channels and Na^{++} channels. It is possible that phospholipid methylation facilitates the formation of Ca^{++} channels. Although there is no direct evidence with skeletal muscle, it has been shown that there is a direct relationship between phospholipid methylation and Ca^{++} entry in mast cells (Ishizaka *et al.*, 1980; Hirata and Axelrod, 1980).

Sarcoplasmic reticulum is an important source of Ca^{++} utilized in skeletal muscle contraction (Sandow, 1970). There is no direct evidence as to whether phospholipid methylation influences transfer of information from the receptor to the Ca^{++} stores in sarcoplasmic reticulum. During the relaxation process, the Ca^{++} ions have to be extruded from the cytoplasm. This is brought about by active uptake in the sarcoplasmic reticulum, active extrusion across the plasma membrane and a Ca^{++}/Na^{+} exchange process (Rosenberger and Triggle, 1978). The first two processes are represented by Mg^{++}- and Ca^{++}-activated ATPases. Although there is no direct evidence that phospholipid methylation effects these processes in the skeletal muscle, it has been demonstrated that phospholipid methylation affects Ca^{++} efflux in erythrocytes (Strittmatter *et al.*, 1979). The efflux of Ca^{++} is regulated by Ca^{++}-ATPase. This enzyme is enclosed by an annulus of phospholipids which is necessary for its function (Bennett *et al.*, 1978). Phospholipid methylation increased Ca^{++} efflux and Ca^{++}-ATPase activity in erythrocytes. The maximum increase in ATPase activity was achieved at a low concentration of SAM, when PME accumulated in the membrane. Accumulation of PME in the membrane decreased microviscosity and increased membrane fluidity in erythrocyte membranes (Hirata and Axelrod, 1978b). These observations are consistent with the report that a decrease in membrane fluidity decreased ATPase activity in sarcoplasmic reticulum (Hidalgo *et al.*, 1978). All of these observations are consistent with the suggestion that the enhancement of responses of hemidiaphragm by a low concentration of L-methionine are due to transient increases of PME in the membrane. These transient increases in PME cause transient increases in membrane fluidity. Changes in membrane fluidity will facilitate processes of Ca^{++} entry and exit from the cytoplasm. Due to changes in

Ca^{++} movements, signal transfer and transduction are possibly enhanced in the hemidiaphragm by incubation with L-methionine.

Increasing intracellular levels of L-SAH inhibited the effect of L-methionine on the tension developed in the hemidiaphragm as well as the incorporation of methyl groups into the membrane phospholipids. These observations suggest that the methionine effect is, in part, due to increased phospholipid methylation. The extent of this part could not be assessed from the available data, because SAH inhibits all SAM-mediated methylations. SAH also inhibits protein and nucleic acid methyltransferases (Pugh *et al.*, 1978; Rollins and Dahlquist, 1980; Woodson *et al.*, 1981). Methylation of —COOH, —SH and >NH groups in proteins can alter surface charges and affect the structure and function of membranes. Therefore, besides phospholipids, methylation of these groups may partly contribute to the methionine effect.

The effect of L-methionine to increase the contraction height of hemidiaphragm upon direct electrical stimulation of the muscle decreases as a function of age in rats from 3 to 21 months. This means that the loss of the L-methionine effect as a function of age is gradual. When the nerve to the hemidiaphragm was stimulated, the loss of the methionine effect was not gradual; there was a large change between the preparations from 9- and 15-month-old rats. This difference could be attributed to changes in the phrenic nerves between the ages of 9 and 15 months. Although phospholipid methyltransferases are known to occur in nervous tissues (Crews *et al.*, 1980), the methionine effect in phrenic nerves cannot be explained by phospholipid methylation alone. It is possible that deficits may develop in all SAM-mediated methylations in phrenic nerves of rats between 9 and 15 months. Protein carboxymethylation has been implicated in neurosecretory processes, especially in neutralization of charges on the vesicular membrane for fusion with plasma membrane (Diliberto *et al.*, 1979; Eiden *et al.*, 1979). It is possible that deficits will develop in the stimulation-secretion coupling for release of ACh from phrenic nerves during aging between 9 and 15 months. These deficits in stimulation-secretion coupling will superimpose on stimulation-contraction coupling during aging. Our studies on stimulation-secretion coupling of ACh and the role of SAM-mediated methylations are in progress.

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