Role of Lipids in Age-Related Changes in the Properties of Muscarinic Receptors in Cultured Rat Heart Myocytes†

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ABSTRACT: The effects of the culture's age and of liposome treatments on the properties of muscarinic receptors in cultured rat heart myocytes prepared from the hearts of newborn (1–3 days old) rats were investigated. In these studies we investigated the binding characteristics of antagonists and agonists to the myocyte muscarinic receptors in young (5 days after plating) vs. older (14 days after plating) cultures. Our findings demonstrate that the aging of the cells in culture is accompanied by a reduction in the muscarinic binding capacity and by alterations in the proportion of high- and low-affinity states toward muscarinic agonists, as well as by striking changes in the mode of coupling of the receptors with guanine nucleotide binding protein(s) [G protein(s)]. The above effects of the culture's age occur concomitantly with alterations in the lipid composition of the cultured myocytes (in 14-day old cultures, the phosphatidylcholine/sphingomyelin ratio is reduced, and the cholesterol level is elevated). In order to explore whether the lipid composition is involved in the mechanism that alters the properties and coupling of the muscarinic receptors, we treated aging cultures with liposomes containing egg phosphatidylcholine. This treatment resulted in 14 day old cultures with a lipid composition similar to that of young cultures, and the treated myocytes demonstrated muscarinic receptor properties similar to those of young myocyte cultures. The implications for the role of membrane lipid composition and organization in determining the properties of the muscarinic receptors and their coupling with G proteins are discussed.

Primary myocyte cultures prepared from hearts of newborn rats provide a convenient cellular model system for studies on age-related effects in heart cells. The minimal cell division (which ends within 3 days) in these cultures enables studies on the same cells for an extended time without the complications arising in cell lines from continuous cell division and from changes related to the number of passages. In a recent study (Yechiel & Barenholz, 1985) it was demonstrated that the cultured myocytes undergo changes in the lipid composition as a function of culture age. These changes, which include elevation in the cholesterol level and reduction of the PC/SM[1] ratio, parallel those reported for several tissues upon aging in the whole animal (Yechiel & Barenholz, 1985; Barenholz, 1984; Shinitzky, 1984; Rivnay et al., 1979; Rouser et al., 1972). The altered lipid composition of the aged myocyte cultures was accompanied by alterations in the level of both membrane and cytoplasmic enzymes, in the lateral mobility and organization of plasma membrane lipids and proteins, and in the beating rate of the cultured myocytes (Yechiel & Barenholz, 1985; Yechiel et al., 1985). All the above changes were eliminated when the cultured myocytes were treated with liposomes containing egg PC during aging in culture, suggesting that manipulations of the lipid composition may restore biochemical and physiologic responses damaged by the process of aging in culture (Yechiel & Barenholz, 1985; Yechiel et al., 1985).

Since the control of both the biochemical and physiologic responses of the cells is receptor-mediated, it was of special interest to examine whether age-dependent alterations occur in the properties of specific myocyte membrane receptors. The muscarinic receptors provide excellent candidates for such studies, in view of their important role in the control of cardiac function. On the physiologic level, muscarinic agonists were shown to provoke a negative chronotropic response (Renaud et al., 1980; Carmeliet et al., 1975). On the biochemical level, cardiac muscarinic receptors were shown to inhibit catecholamine-stimulated cyclic AMP formation (Harden et al., 1982; Dunlap & Brown, 1984) and to couple with phosphoinositide metabolism (Brown & Brown, 1984; Brown et al., 1985; Quist, 1982) in both heart slices and chick heart cells. Cardiac muscarinic receptors were also shown to interact with a guanine nucleotide binding protein (Harden et al., 1982; Berrie et al., 1979), most likely of the inhibitory type (G_I) (Dunlap & Brown, 1984; Mattera et al., 1985), which is required for the receptor-mediated inhibition of cyclic AMP formation.

Binding of muscarinic ligands has been extensively studied in both brain and heart [reviewed in Sokolovsky (1984) and Sokolovsky et al., (1983)]. In most tissues, classical muscarinic antagonists bind to a homogeneous population of sites, while agonist binding yields curvilinear Scatchard plots, suggesting either site heterogeneity (two or three affinity states toward agonists) (Mattera et al., 1985; Sokolovsky, 1984; Sokolovsky et al., 1983; Kloog & Sokolovsky, 1977; Birdsall et al., 1984; Moscona-Amir et al., 1985) or cooperative interactions (Henis & Sokolovsky, 1983). Since guanine nucleotides convert high-affinity muscarinic agonist binding sites into the low-affinity state (Dunlap & Brown, 1984; Mattera et al., 1985; Gurwitz & Sokolovsky, 1980; Galper et al., 1982), it was

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[1] Abbreviations: PC, phosphatidylcholine; SM, sphingomyelin; G protein, guanine nucleotide binding protein; [^{3}H]4NMPB, *N*-[^{3}H]-methyl-4-piperidyl benzilate; [^{3}H]AcCh, acetyl[*methyl*-^{3}H]choline chloride; Gpp(NH)p, 5′-guanylyl imidodiphosphate.

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proposed that coupling of muscarinic receptors with effector proteins (e.g., G_I or G_o), may be at least partially responsible for the different affinity states toward agonists.

In this paper we report studies on the binding characterisitics of agonists and antagonists of the myocyte muscarinic receptors in young vs. aged cultures and on the effects of alterations in the cellular lipid composition through treatments with liposomes on the above parameters. Our findings demonstrate that the aging of the cells in culture is accompanied by changes in the level and in the binding properties of their muscarinic receptors, as well as in the mode of coupling of the receptors with G proteins. These changes can be eliminated by treatment with PC liposomes, demonstrating a direct correlation between the properties of the receptors and the composition and organization of the lipid in the myocyte membranes.

Materials and Methods

Reagents. [^{3}H]4NMPB (45 Ci/mmol) was prepared as described elsewhere (Kloog & Sokolovsky, 1978); the purity of the compound was over 97%. [^{3}H]AcCh (86 Ci/mmol) was purchased from Amersham (Amersham, England). Atropine sulfate, carbamylcholine chloride, cholesterol (>99% pure), and Gpp(NH)p were from Sigma (St. Louis, MO). Egg PC (>99% pure) was from Avanti (Birmingham, AL). Oxotremorine was obtained from Aldrich (Milwaukee, WI). Medium (F-10) and antibiotics were from Biological Industries (Beth Haemek, Israel). Sera (fetal calf serum and horse serum) were from Sera-Lab (Sussex, England). All other chemicals were of the highest purity available.

Preparation of Myocyte Cultures. Myocyte-enriched cultures (>95% myocytes) were prepared from the hearts of newborn (1–3 days old) rats (CD strain) by the method of Kasten (Yechiel & Barenholz, 1985; Kasten, 1973). An important modification was that the culture dishes were coated with 50 μg of collagen/plate (Sigma, St. Louis, MO) prior to the seeding of the myoctes; elimination of this step resulted in detachment of cells from the dish after more than a week in culture. The cells were grown in F-10 medium supplemented with additional calcium (1.2 mM final concentration), 10% fetal calf serum, 10% horse serum, and antibiotics (200 000 IU/L penicillin, 200 mg/L streptomycin). They were grown at 37 °C, 100% relative humidity, and 5% CO_2, with a medium change every 2 days.

Treatment of Cells with Liposomes. Six days after plating, the cells were treated with liposomes prepared from egg PC. Liposomes (small unilamellar vesicles) were prepared by ultrasonic irradiation (Estep, 1979), sterilized by filtration through 0.22-μm millipore filter, and added to the medium in the dish to give a final lipid concentration of 2.0 mM. This procedure was repeated with every medium change.

Preparation of Cell Homogenates. Cultured myocytes were washed 3 times with a modified Krebs–Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11 mM glucose, pH 7.4) and scraped off each dish in 1 mL of the above solution. After a 3-fold dilution in the same buffer, they were homogenized at setting 5 on an ultra Torrax (Ika-Werk Instruments, West Germany) with three 15-s bursts separated by 30-s pauses. Aliquots of the homogenates, which were employed in the various binding assays, were taken for the determination of DNA and protein content. DNA was determined by the method of Richards (1974) with salmon sperm DNA as a standard. Protein was determined according to Lowry et al. (1950) with bovine serum albumin as a standard.

Antagonist Binding Assay. [^{3}H]4NMPB binding to homogenates of myocyte cultures was measured essentially as described by us earlier (Kloog et al., 1979). Aliquots (0.5 mL) of the homogenates were incubated 30 min at 37 °C with various concentrations of [^{3}H]4NMPB. Further incubation did not result in additional binding, indicating that equilibrium has been reached. The reaction was terminated by rapid filtration through Whatman GF/C filters, followed by washing 3 times with the cold-modified Krebs buffer and counting the radioactivity by liquid scintillation spectrometry (Packard Tri-carb 300). Nonspecific binding was determined in the presence of 5 μM unlabeled atropine and subtracted from the total binding to obtain the specific binding.

Agonist Binding Assays. The binding of unlabeled muscarinic agonists (carbamylcholine and oxotremorine) to myocyte homogenates was measured as described in the former section, by competition against 2.5 nM [^{3}H]4NMPB (Kloog et al., 1979). The binding of the agonist AcCh was measured directly employing [^{3}H]AcCh, following the procedure described by us earlier (Gurwitz et al., 1984, 1985). Briefly, the cells were collected and homogenized in a hypotonic solution (50 mM Tris-HCl, pH 7.4) and incubated 30 min at 25 °C to allow complete lysis and removal of endogenous AcCh and guanine nucleotides. The homogenates were centrifuged (30 000 *g*, 15 min) and resuspended in the same buffer, and the whole procedure (30-min incubaton and centrifugation) was repeated. The pellet was suspended in the Krebs-Henseleit solution and treated with diisopropyl fluorophosphate (200 μM, Sigma, St. Louis, MO) for 15 min at 25 °C to inactivate AcCh esterase. [^{3}H]AcCh was then added, and the mixture was incubated 1 h at 25 °C and filtered through Whatman GF/C filters, which were washed once more by the buffer. The time between the addition of cold buffer and the termination of the filtration was 3–4 s. Nonspecific binding was determined as described for the antagonist binding assay and subtracted. Since the [^{3}H]AcCh binding assay detects only the high-affinity AcCh binding sites, the total concentration of muscarinic binding sites in the same membrane preparation was determined by measuring [^{3}H]4NMPB binding at saturation (Gurwitz et al., 1984, 1985).

Binding Data Analysis. Binding isotherms and competition curves were analyzed by a nonlinear curve fitting procedure using a model for either one or two binding sites, as detailed in previous reports (Moscona-Amir et al., 1985; Gurwitz & Sokolovsky, 1980; Kloog et al., 1979), employing the curve fitting program BMDPAR (November 1978 revision) developed at the Health Science Computing Facility (University of California, Los Angeles, CA). In the case of [^{3}H]4NMPB binding, which yields linear Scatchard plots, the analysis was performed with a one-site model (Moscona-Amir et al., 1985; Gurwtiz & Sokolovsky, 1980; Kloog et al., 1979) and so was the case also for [^{3}H]AcCh binding, which detects only one class of sites (high affinity) under the experimental conditions employed (Gurwitz et al., 1985, 1984). The binding of unlabeled agonists by competition against [^{3}H]4NMPB was analyzed by a two-site model incorporating high- and low-affinity states, to which antagonists bind with an equal affinity (Kloog et al., 1979). This model fitted well the experimental data, and the use of a three-site model did not significantly improve the fit; we have therefore routinely performed the analysis employing the two-site model, which involves fewer parameters.

Results

Antagonist Binding to Muscarinic Receptors in Homogenates of Myocyte Cultures. In order to explore alterations in the binding properties of the muscarinic receptors during the process of aging in culture, we investigated [^{3}H]4NMPB

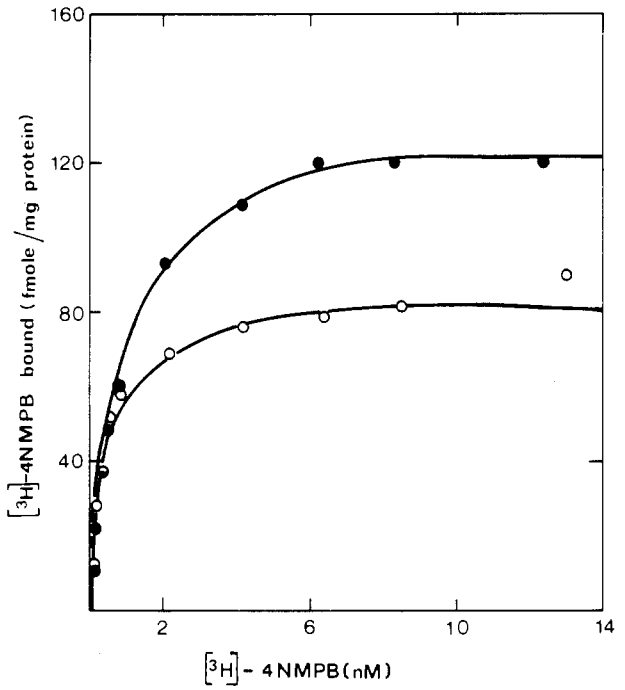

FIGURE 1: Binding of [^{3}H]4NMPB to homogenates prepared from young [5 days after plating (●)] and old [14 days after plating (○)] myocyte cultures. Specific [^{3}H]4NMPB binding was determined as described under Materials and Methods. The experiments were performed at 37 °C in modified Krebs–Henseleit solution (pH 7.4). Each point was determined in triplicate. Nonspecific binding (in the presence of 5 μM atropine) was subtracted.

binding to homogenates prepared from young (3 days after plating) and aged (14 days after plating) myocyte cultures. These dates after plating were chosen since after 5 days the cells have already recovered from the culture-preparation procedure (Yechiel & Barenholz, 1985) and since major changes are observed between myocyte cultures of these ages in the lipid composition, enzyme levels, dynamics of membrane components, and beating rate (Yechiel & Barenholz, 1985; Yechiel et al., 1985).

The results of a typical experiment of [^{3}H]4NMPB binding are shown in Figure 1. It is evident that aging of the myocyte cultures is accompanied by a concomitant decrease in the muscarinic binding capacity (B_{max}), although the dissociation constant (K_d) of [^{3}H]4NMPB is not significantly altered (1.4 ± 0.3 nM vs. 1.0 ± 0.2 nM in young vs. aged cultures; n = 3). The reduction in the muscarinic binding capacity in aged cultures is not simply due to changes in the toal protein content of the cultured myocytes, since it is observed both per milligram of protein (34 ± 4%) and per microgram of DNA (44 ± 4%). Since the cellular DNA content in these cultures does not change between days 5 and 14 (Yechiel & Barenholz, 1985), these results suggest that the amount of muscarinic receptors per cell decreases upon aging in culture.

Binding of Agonists to Muscarinic Receptors in Homogenates of Myocyte Cultures. Agonist binding is considerably more sensitive to the state of the muscarinic receptors than that of antagonists, since in most cases only agonists bind differently to the high (R_H) and low (R_L) affinity states and can therefore detect interconversion between the two receptors states [reviewed in Sokolovsky (1984) and Sokolovsky et al. (1983)]. We have therefore examined the binding of agonists to the muscarinic receptors in homogenates prepared from young and aged myocyte cultures. In these studies, the binding of carbamylcholine and oxotremorine was measured by competition with [^{3}H]4NMPB, while [^{3}H]AcCh binding was measured in a direct binding assay.

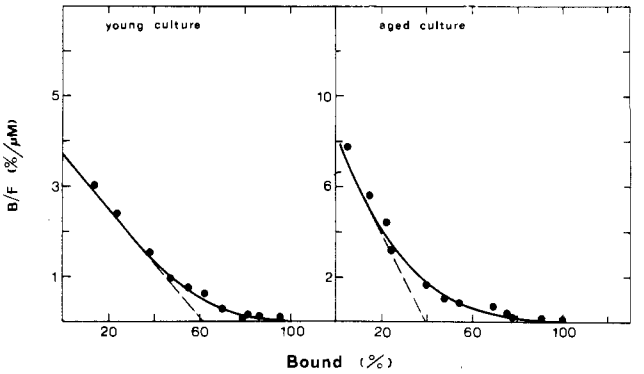

FIGURE 2: Scatchard plots of carbamylcholine binding to homogenates of myocyte cultures. The experiments were performed with homogenates prepared from Young (5 days old) and aged (14 days old) cultures, as described under Materials and Methods. B/F (bound/free carbamylcholine) is expressed as percent bound/free carbamylcholine. Each point was determined in triplicate. Full lines represent computerized fit to the two-state receptor model while the dashed lines represent binding to the high-affinity agonist binding site (R_H) alone.

Table I: Binding of Agonists to Muscarinic Receptors in Homogenates of Myocyte Cultures[a]

agonist	culture	R_H (%)	K_H (μM)	K_L (μM)
carbamylcholine	young	60 ± 5	4.0 ± 2	42 ± 11
	aged	40 ± 2	0.7 ± 0.5	11 ± 5
oxotremorine	young	77 ± 6	0.2 ± 0.1	1 ± 0.5
	aged	75 ± 2	0.4 ± 0.2	2 ± 0.7
acetylcholine	young	65 ± 10[b]	0.012 ± 0.002	
	aged	50 ± 6[b]	0.011 ± 0.003	

[a] The binding of carbamylcholine and oxotremorine to homogenates of young (5 days after plating) and aged (14 days in culture) myocyte cultures was measured by competition with [^{3}H]4NMPB, while AcCh binding was determined directly with [^{3}H]AcCh (see Materials and Methods). Percent R_H represents the percentage of high-affinity agonist binding sites in the total receptor population. K_H and K_L are the dissociation constants for agonist binding to R_H and R_L, respectively. The results are mean ± SE of three separate experiments in each case, with nonlinear regression and a two-site model (for carbamylcholine and oxotremorine) or linear regression to a one-site model (for AcCh) to analyze each curve. In the case of [^{3}H]AcCh, each curve was constructed of 10 points, and the Scatchard plots (not shown) were linear (correlation coefficients around 0.98). Student's t test indicated that the differences between young and aged cultures are significant in the case of carbamylcholine for all the parameters measured ($p < 0.005$ for percent R_H, $p < 0.05$ for K_H, and $p < 0.01$ for K_L) and in the case of acetylcholine for percent R_H only ($p < 0.05$). In all other cases, the differences between young and aged cultures were only statistical ($p > 0.1$; for percent R_H of oxotremorine and K_H of acetylcholine, $p > 0.2$). [b] Since the [^{3}H]AcCh binding assay measures only R_H, the percentage of R_H was obtained by dividing the maximal [^{3}H]AcCh binding capacity by the [^{3}H]4NMPB binding capacity of the same membrane preparation.

The competition experiments yielded flattened inhibiting curves for both carbamylcholine and oxotremorine. The results of a typical experiment are depicted in Figure 2. The data were well fitted by a two-site model specifying two classes of sites—R_H and R_L—which bind agonist with high and low affinities, respectively. In the case of carbamylcholine, aging of the cultured myocytes was accompanied by a reduction in the percentage of R_H and by an increase in the affinities of both R_H and R_L toward the agonist (Table I). The altered affinities for carbamylcholine are not the result of lower affinities toward the competitor [^{3}H]4NMPB, since the dissociation constant of the latter was not significantly altered upon aging of the cultures (see former section).

Unlike the situation with carbamylcholine, oxotremorine binding was similar in homogenates of young and aged myocyte cultures. The percentage of R_H was rather high in the

Table II: Effect of Gpp(NH)p on Agonist Binding to Muscarinic Receptors in Homogenates of Myocyte Cultures[a]

agonist	culture	R_H (%)	K_H (μM)	K_L (μM)
carbamylcholine	young	*b*	ND	20 ± 7
	aged	75 ± 4	3.3 ± 0.5	37 ± 13
oxotremorine	young	77 ± 8	0.5 ± 0.3	3 ± 2
	aged	90 ± 4	0.7 ± 0.4	ND
acetylcholine	young	50 ± 5[c]	0.013 ± 0.007	
	aged	80 ± 4	0.017 ± 0.004	

[a] Agonist binding to homogenates of young and aged myocyte cultures was measured as described under Materials and Methods, except that 50 μM Gpp(NH)p was added to the reaction mixture along with the agonists. All the controls in the absence of Gpp(NH)p are given in Table I. The results are mean ± SE of three separate experiments in each case. R_H, K_H, and K_L are defined as in Table I. Young and aged stand for myocete cultures 5 and 14 days after plating, respectively. ND = not determined due to either the lack of high-affinity sites (carbamylcholine) or the very low proportion of low-affinity sites (oxotremorine). Student's *t* test indicated that the differences between young and aged cultures in the presence of Gpp(NH)p were highly significant for percent R_H of carbamylcholine ($p < 0.0005$) and AcCh ($p < 0.005$) but only on the verge of significance for percent R_H of oxotremorine ($p < 0.1$). The differences between the K_H values were only statistical ($p > 0.25$) in all cases, except for the marginal ($p < 0.1$) significance observed for K_L of carbamylcholine. [b] In homogenates prepared from young cultures only low-affinity binding sites were detected for carbamylcholine. [c] The percentage of R_H for [^{3}H]AcCh binding was determined as in Table I.

case of oxotremorine (77%) and remained unaltered upon aging. Similarly, only minor changes (2-fold at most) were detected in the dissociation constants of R_H and R_L toward oxotremorine (Table I).

AcCh binding was measured directly by employing the labeled neurotransmitter itself. Due to the low affinity of [^{3}H]AcCh to R_L and the high nonspecific binding at high concentrations of the neurotransmitter, only the binding of [^{3}H]AcCh to the high-affinity sites (R_H) is measured (Gurwitz et al., 1984; 1985). Thus, a separate determination of the total concentration of muscarinic binding sites ($R_H + R_L$) was performed with a saturating concentration (20 nM) of [^{3}H]-4NMPB, in order to evaluate the percentage of R_H in the total receptor population. The results (Table I) demonstrate that the percentage of R_H as detected by direct binding of [^{3}H]-AcCh decreased upon aging of the myocyte cultures, as observed for carbamylcholine binding in the competition experiments. On the other hand, no age-dependent changes in the affinity of AcCh to R_H were detected (Table I).

It should be noted that the reduction in the proportion of R_H in the case of carbamylcholine and AcCh upon aging does not necessarily reflect interconversion of R_H to R_L, since at the same time the total receptor capacity (B_{max} for [^{3}H]-4NMPB) decreased by 34%. If the sites lost upon aging in culture are mostly high-affinity sites, a reduction in the proportion of R_H would be observed.

Effect of Guanine Nucleotides on Binding of Agonists to Homogenates of Myocyte Cultures. The interaction of cardiac muscarinic receptors with G protein(s) is most readily detected by the effects of guanine nucleotides on the binding characteristics of muscarinic agonists. In order to explore alterations in these interactions upon aging of the myocyte cultures, we examined the effects of Gpp(NH)p on the binding of carbamylcholine, oxotremorine, and [^{3}H]AcCh to the muscarinic receptors in homogenates prepared from young and aged cultures. The results of these experiments are summarized in Table II. The data demonstrates striking differences between young and aged cultures in terms of the Gpp(NH)p-induced interconversion of the muscarinic receptors, as detected by carbamylcholine or [^{3}H]AcCh binding. While in young cultures the Gpp(NH)p effect was in the direction observed normally in both cardiac and brain preparations (conversion of R_H to R_L), an opposite effect was observed in aged cultures (conversion of R_L to R_H). These effects appear to be exerted directly on agonist binding, since the binding of the antagonist [^{3}H]4NMPB (employed as a competitor in the measurement of carbamylcholine binding) was not affected by Gpp(NH)p under the experimental conditions employed [in homogenates of young cultures, the K_d value was 1.4 ± 0.2 nM in either the absence or the presence of Gpp(NH)p, and the respective B_{max} values were 125 ± 15 and 121 ± 14 fmol/mg of protein; in homogenates from aged cultures, the K_d values were 1.0 ± 0.2 and 1.1 ± 0.2 in the absence and presence of Gpp(NH)p, and the respective B_{max} values were 95 ± 10 and 97 ± 11 fmol/mg of protein]. Moreover, the Gpp(NH)p effects were detected also for [^{3}H]AcCh binding, which was determined directly (in the absence of 4NMPB). The results obtained with [^{3}H]AcCh, whose binding was measured in washed membrane preparations devoid of endogenous guanine nucleotides (see Materials and Methods), also provide an important control which demonstrates that contaminations due to the presence of endogenous guanine nucleotides are not involved in the Gpp(NH)p effects measured. Indeed, displacement experiments with carbamylcholine or oxotremorine performed on washed membrane preparations (prepared as for [^{3}H]AcCh binding) gave results essentially similar to those obtained with the homogenates. Thus, it appears that there are fundamental changes in the coupling of the myocyte muscarinic receptors with G protein(s) upon aging of the myocyte cultures.

Unlike the marked Gpp(NH)p effects on carbamylcholine and AcCh binding, very minor effects of Gpp(NH)p were observed on oxotremorine binding. In fact, the only effect that was marginally significant was on the percentage of R_H in homogenates of aged myocyte cultures, which increased from 75 % to 90% upon treatment with Gpp(NH)p (compare Table I, line 4, with Table II, line 4). Thus, the lack of sensitivity of oxotremorine binding to the culture's age (Table I) is also reflected in its low sensitivity to Gpp(NH)p-induced interconversion in both young and aged myocyte cultures (compare lines 3 and 4 in Tables I and II).

It should be noted that the marked differences in the response of young and aged myocyte cultures to Gpp(NH)p-induced modulation in the binding of carbamylcholine and AcCh are not accompanied by similar changes in the affinity of the agonists toward R_H or R_L (compare the K_H and K_L values of the different agonists in Tables I and II). The only difference that was above the experimental error was in the case of carbamylcholine binding to homogenates of aged myocyte cultures, where Gpp(NH)p induced a small (3–4-fold) increase in the values of both K_H and K_L (compare line 2 in Tables I and II). Thus, the effect of Gpp(NH)p on the binding of carbamylcholine is to induce interconversion between R_H and R_L in both young and aged cultures; however, the direction of the interconversion is reversed in aged cultures.

Effects of Liposome Treatments on Properties of Muscarinic Receptors in Aged Myocyte Cultures. The myocyte cultures undergo major age-dependent changes in the composition of their lipids. Young cultures have 0.6, 10, and 0.42 nmol of sphingomyelin, PC, and cholesterol per microgram of DNA, respectively. Old cultures display 100% more sphingomyelin, 20% less PC, and 50% more cholesterol (Yechiel & Barenholz, 1985). In order to explore whether the lipid composition is involved in the mechanism that alters the properties and the coupling with G proteins of the myocyte muscarinic receptors upong aging, our approach was to treat

Table III: Effects of Liposome Treatment on Carbamylcholine Binding to Homogenates of Aged Myocyte Cultures[a]

agonist	culture	R_H (%)	K_H (μM)	K_L (μM)
carbamylcholine	young	60 ± 5	4.0 ± 2	42 ± 11
	aged	40 ± 2	0.7 ± 0.5	11 ± 5
	aged + PC	67 ± 4	4.7 ± 1.7	38 ± 6
carbamylcholine + Gpp(NH)p (50 μM)	young		ND[b]	20 ± 7
	aged	75 ± 4	3.3 ± 0.5	37 ± 13
	aged + PC		ND[b]	21 ± 3

[a] Carbamylcholine binding to homogenates of young (5-days old) or aged (14-days old) myocyte cultures was measured as described under Materials and Methods, in the absence or presence of 50 μM Gpp(NH)p. Aged + PC represent aged cultures treated with PC liposomes. R_H, K_H, and K_L are defined as in Table I. The results are mean ± SE of three separate experiments in each case. Note that in the presence of Gpp(NH)p no high-affinity carbamylcholine binding was detected in homogenates of young cultures or of aged cultures treated with PC liposomes. Student's t test indicated that aged cultures treated with PC liposomes were similar to young untreated cultures in all the muscarinic parameters tested; the differences between young and PC-treated aged cultures were only statistical in all cases ($p > 0.25$; except for percent R_H of carbamylcholine, where $p > 0.1$ was obtained). On the other hand, significant differences were observed between untreated aged cultures and PC-treated aged cultures: in the absence of Gpp(NH)p, $p < 0.005$ was obtained for all the parameters; while in the case of carbamylcholine plus Gpp(NH)p, $p < 0.0005$ was determined for percent R_H and $p < 0.1$ was determined for K_L.

the aging cultures with specific liposomes in order to prevent the aging-related changes in the cellular lipid composition. If the latter determines the properties and coupling of the muscarinic receptors, then treatments that result in the lipid composition of aged cultures being similar to that of young cultures should also abolish the age-dependent changes in the muscarinic receptors. Such a situation is obtained by treating the aging cultures with egg PC liposomes (Yechiel & Barenholz, 1985; Yechiel et al., 1985). Young cultures were treated from day 6 in culture to day 14 by the PC liposomes as described under Materials and Methods; this treatment elevates the PC/SM ratio (increasing the PC and decreasing the SM content of the cultured myocytes) with a concomitant decrease in the cholesterol level back to the values observed in young cultures (Yechiel & Barenholz, 1985). The effect of this treatment on the properties of the muscarinic receptors in aged cultures was determined with respect to the parameters that showed the highest susceptibility to the culture's age—namely, B_{max} of [^{3}H]4NMPB and the binding characteristics of carbamylcholine [with and without Gpp(NH)p].

Unlike the reduction in B_{max} for [^{3}H]4NMPB observed upon aging of the myocyte cultures (34% and 44% reduction when calibrated per milligram of protein and per microgram of DNA, respectively), the [^{3}H]4NMPB binding capacity was not reduced in aged cultures treated with PC liposomes; the latter cultures displayed increases of 38 ± 2% (per milligram of protein) and 49 ± 3% (per microgram of DNA) in B_{max} for [^{3}H]4NMPB relative to untreated aged cultures. The liposome treatment had no significant effect on K_d for [^{3}H]4NMPB, in analogy to the lack of effect of the culture's age on this parameter.

The effects of the PC liposome treatment on the binding characteristics of carbamylcholine (dissociation constants and percent R_H) to homogenates of aged myocyte cultures are summarized in Table III, along with the alterations in the effects of Gpp(NH)p treatment. It is apparent that the PC liposome treatment prevented the reduction in the proportion of R_H upon aging of the cultured myocytes; in fact, homogenates of aged cultures treated with PC liposomes displayed a somewhat higher percentage of R_H than homogenates prepared from young myocyte cultures (Table III). The striking effects of the liposome treatment were observed also in the Gpp(NH)p-induced interconversion of the carbamylcholine high- and low-affinity binding sites: while in homogenates of untreated aged cultures Gpp(NH)p induced a "reversed" interconversion (i.e., converted R_L to R_H), aged cultures treated with PC liposomes displayed only low-affinity carbamylcholine binding sites in the presence of Gpp(NH)p, a response similar to that of homogenates of young myocyte cultures.

Discussion

Dependence of Properties of Muscarinic Receptors in Myocyte Cultures on Culture's Age. Studies on cultures of heart cells can provide important information on the properties of cardiac muscarinic receptors and the responses elicited through them. Several groups have used cultures of chick embryo heart cells to investigate cardiac muscarinic receptors (Galper et al., 1982, 1984a,b; Nathanson, 1983); by use of cultures prepared from embryos of different ages in ovo, changes in the properties of cardiac muscarinic receptors during embryonal development were explored, and a correlation was demonstrated between the appearance of guanine nucleotide responsiveness in the muscarinic receptors and the development of physiologic responses (Galper et al., 1984b). An analogous approach can be applied to study aging-like processes that occur in culture, providing that cell division is not applicable, as is the case with the rat heart myocyte cultures employed in this study.

The age-dependent alterations in the lipid composition of the myocyte cultures (Yechiel & Barenholz, 1985) are accompanied by clear changes in the properties of their muscarinic receptors. These changes include reduction in B_{max} for [^{3}H]4NMPB (Figure 1), alterations in the percentage of high- and low-affinity agonist binding sites (Table I, Figure 2), and changes in the Gpp(NH)p-induced interconversion between R_H and R_L (Table II). It is interesting to note that the binding characteristics of various muscarinic agonists change differently with the culture's age. Thus, the percentage of R_H was reduced with the culture's age in the case of carbamylcholine and AcCh, but not in the case of oxotremorine. The different nature of oxotremorine is in line with former studies that demonstrated that the kinetics of its interaction with high-affinity muscarinic binding sites resemble antagonists rather than AcCh or carbamylcholine (Schreiber et al., 1985) and with the earlier demonstration that oxotremorine differs from carbamylcholine in the effects on cyclic AMP formation and phosphoinositide hydrolysis in embryonic chick heart cells (Brown & Brown, 1984). The latter study also proposed that the high-affinity state of the muscarinic receptor is associated with inhibition of adenylate cyclase and the low-affinity state is coupled with the phosphoinositide response. The alteration in the proportions of R_H with the culture's age may therefore have important physiological implications.

The effects of aging in cultue on the Gpp(NH)p-induced interconversion of the myocyte muscarinic receptors deserve special attention. The changes in the Gpp(NH)p effect upon aging of the cultures are striking: while in young cultures a conversion from R_H to R_L is observed, a reverse effect (conversion from R_L to R_H) is obtained in homogenates of aged cultures (Table II). The reversed effect of Gpp(NH)p, which is in the opposite direction to that observed normally in either heart or brain preparations (Dunlap & Brown, 1984; Berrie et al., 1979; Mattera et al., 1985; Gurwitz & Sokolovsky, 1980), demonstrates that the coupling between the muscarinic receptors and G protein(s) (most likely G_i, but possibly also G_o) is subject to changes with the culture's age. It should be noted that that such a reversed effect of Gpp(NH)p on agonist

binding to muscarinic receptors was observed by us earlier in the adenohypophysis of female rats: R_H was converted to R_L in the proestrous and diestrous stages, while a conversion of R_L to R_H was observed at the estrous stage (Avissar & Sokolovsky, 1981).

The ability of Gpp(NH)p to convert R_L to R_H in aged cultures has important implications on the mode of coupling of the muscarinic receptors with G-protein(s): such a phenomenon would not be possible according to the mechanism proposed, for example, in the case of β-adrenergic receptors [for reviews, see Lefkowitz et al. (1983) and Helmreich and Pfeuffer (1985)], where the binding of Gpp(NH)p or GTP to the guanine nucleotide binding protein(s) is supposed to uncouple the receptor from the G protein, resulting in a low-affinity agonist binding state. Apparently, the situation is more complex in the case of the muscarinic receptors in the cultured myocytes, since it is unlikely that uncoupling would lead to opposite effects on the same receptor system in young and aged cultures. A mechanism involving direct allosteric interactions between the muscarinic receptors and G protein(s) is one reasonable possibility that is currently under study in our laboratory.

In view of the relatively high GTP concentrations within cells, the age-dependent alteration in the Gpp(NH)p effect on the myocyte muscurinic receptors can be important for their coupling with biochemical and physiologic responses in the intact cells. The sensitivity of the Gpp(NH)p-induced interconversion of the myocyte muscarinic receptors is in accord with the extreme sensitivity of this parameter in heart preparations to the experimental conditions. Thus, at normal ionic strength, guanine nucleotides have no effect on antagonist binding to cardiac muscarinic receptors (Dunlap & Brown, 1984; Galper et al., 1982), while under nonphysiologic conditions (low ionic strength) a reciprocal effect on antagonist binding (increased affinity, and in one case the appearance of positive cooperativity) was reported (Mattera et al., 1985; Burgisser et al., 1982; Martin et al., 1984).

Role of Lipids in Determining Properties and Coupling of Myocyte Muscarinic Receptors. The age-dependent alterations in the properties of the myocyte muscarinic receptors occur concomitantly with changes in the cellular lipid composition. If the latter changes are responsible for the altered properties of the muscarinic receptors, their prevention should also eliminate the aging-related changes in the receptors. In order to determine whether the changes in the cellular lipid alterations are involved in the mechanism that leads to the age-related alterations in the binding characteristics and coupling with G protein(s) of the muscarinic receptors in the cultured myocytes, we altered the lipid composition of aged myocyte cultures by treatment with PC liposomes. The data in Table III demonstrate that cultures treated with PC liposomes [a treatment that reduced the level of SM and cholesterol relative to untreated aged cultures by 50% and 40%, respectively, and increased the PC level by 25% (Yechiel & Barenholz, 1985)] showed carbamylcholine binding properties and Gpp(NH)p-induced interconversion similar to those of young myocyte cultures. The reversal of the properties of the muscarinic receptors back to those of young myocyte cultures by the PC liposome treatment is of special interest in view of the fact that the changes in the lipid composition that occur in the aging cultures (and which are prevented by the liposome treatment) parallel those reported for several tissues upon aging in the whole animal (Yechiel & Barenholz, 1985; Barenholz, 1984; Shinitzky, 1984; Rivnay, et a., 1979; Rouser et al., 1972). Thus, basic aging-related processes in the cellular model system may also be relevant to aging in the whole animal.

The findings on the effects of changes in the cellular lipid composition (due to aging in culture or due to liposome treatment) on the properties of the myocyte muscarinic receptors suggest that the latter are strongly dependent on the membrane lipid composition. This notion is in line with a former report on altered binding characteristics of muscarinic receptors in neuronal membranes following tratment with phospholipase C (Parthasarathy et al., 1984). Moreover, in chick cardiac cells cultured under different conditions (with normal vs. lipoprotein-deficient serum), differences in the physiologic (beating rate) and biochemical (half-life) properties of the muscarinic receptors were correlated with changes in the cholesterol content (Renaud et al., 1982). Interestingly, a role of lipids in the interaction of another receptor system (β-adrenergic receptors from turkey erythrocytes) with G_S (stimulatory G protein) was reported recently on the basis of reconstitution studies (Kirilovsky et al., 1985); thus, the role of lipid composition in determining the properties and coupling of cellular receptor systems may be rather general and not limited to the case of the muscarinic receptors.

We have demonstrated earlier that age-dependent alterations in myocyte membrane lipid composition produce marked effects on the organization of membrane lipids and proteins in domains (Yechiel et al., 1985). Thus, although we cannot completely rule out specific lipid effects on the muscarinic receptors (e.g., direct interaction with specific lipids), it is much more likely that the changes in the state of the muscarinic receptors and in their coupling to G protein(s) occur due to alterations in the membrane lipid organization. This notion is strengthened by the finding (Yechiel et al., 1985) that the myocyte lipid composition affects the dynamic properties of succinylconcanavalin A receptors, which represent a rather wide group of membrane glycoproteins.

Registry No. Gpp(NH)p, 34273-04-6; carbamoylcholine, 462-58-8; oxotremorine, 70-22-4; acetylcholine, 51-84-3; cholesterol, 57-88-5.

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CORRECTION

Mucidin and Strobilurin A Are Identical and Inhibit Electron Transfer in the Cytochrome bc_1 Complex of the Mitochondrial Respiratory Chain at the Same Site as Myxothiazol, by Gebhard Von Jagow, Gordon W. Gribble, and Bernard L. Trumpower*, Volume 25, Number 4, February 25, 1986, pages 775–780.

The conclusion that strobilurin A (mucidin) reacts at a binding or inhibition site different from that of antimycin A has also been derived from the study of strobilurin A (mucidin) resistant mutants of *Saccharomyces cerevisiae* [Subik, J., Briquet, M., & Goffeau, A. (1981) *Eur. J. Biochem. 119*, 613–618].