Aging of Rat Heart Myocytes Disrupts Muscarinic Receptor Coupling That Leads to Inhibition of cAMP Accumulation and Alters the Pathway of Muscarinic-Stimulated Phosphoinositide Hydrolysis†

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ABSTRACT: The biochemical responses to muscarinic stimulation (inhibition of isoproterenol-stimulated cAMP accumulation and stimulation of phosphoinositide turnover) were investigated in intact myocyte cultures prepared from the hearts of newborn rats. The studies employed young (5 days after plating) and aged (14 days old) myocyte cultures. Aging of the myocyte cultures was accompanied by marked alterations in both the inhibition of cAMP accumulation and the stimulation of the phosphoinositide metabolism via the muscarinic receptors. However, the effects on the two muscarinic responses were different. The first response was disrupted at the level of the coupling of the muscarinic receptors with adenylate cyclase through G_i. On the other hand, muscarinic stimulation of phosphoinositide hydrolysis still occurred in the aged myocyte cultures; however, the inositol trisphosphate generated was not converted to inositol 1-phosphate as in young cultures or as in aged cultures stimulated by norepinephrine. This raises the possiblity that muscarinic activation of aged myocyte cultures shifts the metabolic state of the cells and alters the pathway of phosphoinositide hydrolysis. Treatment of aging cultures with phosphatidylcholine liposomes under conditions that yielded aged myocyte cultures with a lipid composition resembling that of young ones restored the muscarinic effect on cAMP accumulation, where the impairment in aged cultures was at the coupling stage (which takes place in the plasma membrane). This treatment had no effect on the response of the phosphoinositide metabolism to muscarinic stimulation.

Muscarinic receptors play a major role in the control of cardiac function. Agonist binding to these receptors exerts a negative chronotropic response through activation of K^+ channels (Giles & Noble, 1976; Renaud et al., 1980). Cardiac muscarinic receptors inhibit catecholamine-stimulated cAMP formation (Harden et al., 1982; Dunlap & Brown, 1984; Liang et al., 1986) and are coupled to the phosphoinositide metabolism (Quist, 1982; Brown & Brown, 1984; Brown et al., 1985). The inhibition of catecholamine-stimulated cAMP formation through cardiac muscarinic receptors requires the interaction of GTP or its stable analogue Gpp(NH)p[1] with G_i [reviewed by Gilman (1987)]. This inhibition is blocked by pertussis toxin (IAP), which specifically ADP-ribosylates the α-subunit of G_i (Halvorsen & Nathanson, 1984; Liang et al., 1986). Interactions of cardiac muscarinic receptors with G-protein(s) are also manifested by the shift of agonist binding sites from high to low affinity in the presence of guanyl nucleotides (Berrie et al., 1979; Sokolovsky et al., 1980, 1983; Galper et al., 1984; Moscona-Amir et al., 1986).

The coupling of cardiac muscarinic receptors to phospholipase C activation and phosphoinositide metabolism also appears to involve G-protein(s), as indicated by the effect of GTP on phosphoinositide hydrolysis in permeabilized chick heart cells (Brown & Jones, 1986). The nature of the G-protein(s) involved in this coupling is not yet clear; several G-proteins (G_i, G_o, G_p, G_c) were reported to be involved in effects on the phosphoinositide metabolism depending on the type of tissue [reviewed in Lo and Hughes (1987)].

We have previously employed cultures of primary myocytes prepared from the hearts of newborn rats to investigate the properties of the muscarinic receptors. The ligand-binding characteristics of the receptors, and especially their mode of coupling with G-proteins (as reflected in the effects of guanyl nucleotides on agonist binding), were strongly dependent on the cultures' age and on the lipid composition of the myocytes (Moscona-Amir et al., 1986). Parallel age-dependent changes were observed in the G-protein population (G_i and two additional IAP substrates) of the cultured myocytes (Moscona-Amir et al., 1988). All of these alterations were eliminated when the aging cultures were treated with egg PC liposomes under conditions that prevent the changes in the cellular lipid composition and in the organization of the plasma membrane lipids in lateral domains (Yechiel et al., 1985; Moscona-Amir et al., 1986, 1988).

Since the function of the muscarinic receptors in the myocytes is exerted through the activation of specific biochemical responses (inhibition of cAMP accumulation and the stimulation of phosphoinositide turnover), it was important to explore how the coupling between the muscarinic receptors and these biochemical responses is affected by the culture's age and lipid composition. The results presented here demonstrate that the two responses are affected differently by the above parameters. While the coupling of muscarinic receptors through G_i with the inhibition of cAMP accumulation is highly

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[1] Abbreviations: Gpp(NH)p, 5′-guanylyl imidodiphosphate; IAP, *Brodetella pertussis* toxin; PC, phosphatidylcholine; [^{3}H]4NMPB, *N*-[^{3}H]methyl-4-piperidyl benzilate; IBMX, isobutylmethylxanthine; 8-bromo-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; [^{3}H]-inositol, *myo*-[2-^{3}H]inositol; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; $InsP_1$, inositol 1-phosphate; $InsP_2$, inositol 1,4-bisphosphate; $InsP_3$, inositol trisphosphate; (1,4,5)-$InsP_3$, inositol 1,4,5-trisphosphate; (1,3,4)-$InsP_3$, inositol 1,3,4-trisphosphate; (1,2c,4,5)-$InsP_3$, inositol 1,2-cyclic,4,5-trisphosphate; (1,3,4,5)-$InsP_4$, inositol 1,3,4,5-tetrakisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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sensitive to the lipid composition and organization (it is lost in aged cultures and is restored by the PC liposome treatment), stimulation of phosphoinositide hydrolysis by muscarinic agonists persists in aged cultures, but the $InsP_3$ is not converted to $IsnP_1$. This alteration is not prevented by the PC liposome treatment.

Materials and Methods

Reagents. [^{3}H]4NMPB (45 Ci/mmol) was prepared as described earlier (Kloog & Sokolovsky, 1978) in over 97% purity. Atropine sulfate, carbamylcholine chloride, collagen, L-isoproterenol, IBMX, forskolin, 8-bromo-cAMP, and cholera toxin were from Sigma (St Louis, MO). Egg PC (>99% pure) was from Avanti (Birmingham, AL). IAP was purchased from List Biochemicals (Campbell, CA). [^{3}H]Inositol (17 Ci/mmol) and [U-^{14}C]leucine (342 mCi/mmol) were from Amersham. [^{3}H]cAMP (31 Ci/mmol) was from New England Nuclear (Boston, MA). Medium (F-10) and antibiotics were from Biological Industries (Beth Haemek, Israel). Fetal calf serum and horse serum were from Sera-Lab (Sussex, England).

Preparation of Myocyte Cultures. Myocyte-enriched cultures (>95% myocytes) were prepared from the hearts of newborn (1–3 days old) rats (CD strain) by a slight modification (Moscona-Amir et al., 1986) of the method of Kasten (Kasten, 1973; Yechiel et al., 1985). The cells were grown on collagen-coated dishes in F-10 (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 medium change every 2 days. The cells are plated at a density that allows about two divisions; under these conditions, confluency is attained after 2–3 days, and the ratio of fibroblasts in the cultures does not increase with the culture's age (Yechiel & Barenholz, 1985).

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 et al., 1979), sterilized by filtration through 0.22-μm millipore filter, and added to the medium in the dish to give a final concentration of 1.2 mM. This procedure was repeated with every medium change.

Binding of Muscarinic Agonists to Intact Cells. The binding of unlabeled muscarinic agonists (carbamylcholine and oxotremorine) to intact myocytes was measured by competition against [^{3}H]4NMPB, a modification of the procedure of Galper et al. (1982) being employed. One day prior to the binding experiment, cells growing in 16-mm multiwells were fed with fresh medium containing [U-^{14}C]leucine (0.01 μCi/mL). The cells were washed twice with a warm modified Krebs–Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11 mM glucose, pH 7.4) and incubated (1 h, 37 °C) with 0.5 mL of the same buffer containing a constant concentration (2.5 nM) of [^{3}H]4NMPB together with varying concentrations of the unlabeled agonists. No significant internalization of [^{3}H]4NMPB occurred during this period, since after the incubation over 90% of the cell-associated [^{3}H]-4NMPB could be removed by excess of unlabeled 4NMPB or of the more hydrophylic ligands *N*-methylscopolamine, oxotremorine, and carbamylcholine. Each well was rinsed three times with 3 mL of ice-cold buffer (total washing time was 12 s). NaOH (1 N, 0.3 mL) was added. After 30 min, the cells were collected, and 0.2 mL of 1 M Tris-HCl (pH 7.4) was added. The tritium and ^{14}C counts were determined by liquid scintillation spectrometry (Packard Tri-carb 300). In some wells, aliquots were also taken for protein determination (Lowry et al., 1951), to enable the calculation of the protein content per ^{14}C counts. This factor permitted calculation of the protein content in each well from the ^{14}C counts (Galper et al., 1982). Nonspecific binding of [^{3}H]4NMPB was determined in the presence of 5 μM unlabeled atropine and subtracted.

Competition curves were analyzed by a nonlinear curve fitting procedure using the simplest possible model of two noninteracting classes of sites with high and low affinities for the agonist and an equal affinity for [^{3}H]4NMPB (Moscona-Amir et al., 1986).

Inhibition of cAMP Accumulation. Cells plated onto 16-mm multiwells were washed 3 times with the Krebs–Henseleit buffer and incubated (20 min, 37 °C) with 1 mL of F-10 medium (without sera and antibiotics) supplemented with 20 mM HEPES and 100 μM IBMX. Isoproternol (10^{-6} M) and varying concentrations of muscarinic agonists (carbamylcholine or oxotremorine) were then added (alone or together). After 2 min at 37 °C, the reaction was terminated by addition of hot sodium acetate buffer (50 mM, pH 4). Cells were scraped off and boiled (5 min). The level of cAMP was determined by a modification (Naor et al., 1975) of the competition protein binding assay of Gilman (1970).

Phosphoinositide Hydrolysis Assay. Phosphoinositide hydrolysis was monitored by measuring the levels of the tritiated inositol phosphates in the presence of LiCl, an inhibitor of the enzyme that converts $InsP_1$ to inositol (Berridge et al., 1982). Cells in 35-mm dishes were incubated with 1 mL of growth medium containing [^{3}H]inositol (1.0 μCi/mL) for 18–20 h. They were washed three times with the Krebs–Henseleit buffer and incubated (20 min, 37 °C) with 1 mL of F-10 devoid of sera and supplemented with 20 mM HEPES and 10 mM LiCl (pH 7.4).

Assays were initiated by the addition of the indicated drugs, and incubation (at 37 °C) lasted for the time periods specified. Reactions were stopped by aspiration of the medium and addition of 1 mL of chloroform–methanol (1:2). Extraction and separation of water-soluble products of phosphoinositide hydrolysis by ion-exchange chromatography were done by the method of Berridge et al. (1982). Samples of the chloroform phase were dried and counted in parallel with the phosphoinositol fractions eluted from the columns. Results were expressed as the ratio of the dpm in each inositol phosphate fraction to the total dpm incorporated (Gonzales & Crews, 1984). In those experiments where the levels of the phosphoinositide hydrolysis products were measured after a very brief exposure to carbamylcholine, the reaction was terminated by the addition of 1 mL of cold methanol (instead of chloroform–methanol). The cells were scraped and introduced into vials containing chloroform–HCl (100:1 v/v). Separation of the aqueous and chloroform phases and ion-exchange chromatography proceeded as described above.

Measurement of (1,4,5)-$InsP_3$. Cells were washed three times with the Krebs–Henseleit buffer and incubated (20 min, 37 °C) with HEPES-buffered F-10 containing 10 mM LiCl. Phosphoinositide hydrolysis was stimulated by addition of carbamylcholine or norepinephrine. The reaction was terminated by addition of cold 15% trichloroacetic acid. Samples were centrifuged at 2000*g* (15 min, 4 °C), and $InsP_3$ was extracted from the supernatant with 10 volumes of water-saturated diethyl ether. Specific determination of the (1,4,5)-$InsP_3$ level in each sample was carried out with a kit of D-*myo*-inositol (1,4,5)-trisphosphate ^{3}H assay system supplied by Amersham, following the manufacturer's instructions.

Table I: Binding of Muscarinic Agonists to Intact Myocytes[a]

agonist	culture	R_H (%)	R_L (%)	R_{SL} (%)	K_H (μM)	K_L (μM)	K_{SL} (μM)
carbamylcholine	young		56 ± 6	44 ± 6		30 ± 4	430 ± 100
	aged		ND[b]	90 ± 5		ND	560 ± 50
oxotremorine	young	47 ± 3	53 ± 3		0.03 ± 0.01	0.9 ± 0.2	
	aged	49 ± 4	51 ± 4		0.03 ± 0.01	1.0 ± 0.3	

[a] In view of the different affinities of oxotremorine and carbamylcholine to the receptors, the two sites and the respective dissociation constants were given different designations for the two agonists (K_H and K_L for oxotremorine binding to the high- and low-affinity sites versus K_L and K_{SL} for the binding of carbamylcholine, which is characterized by low and superlow affinities). The binding of carbamylcholine and oxotremorine to intact myocytes in young (5 days old) and aged (14 days after plating) cultures was measured by competition with 2.5 nM [^{3}H]4NMPB, as described under Materials and Methods. This concentration saturated 67% and 60% of the sites in young and aged cultures, respectively. The K_d value of [^{3}H]-4NMPB was similar in young and aged cultures (1.4 nM), and the B_{max} values varied between 80 and 120 fmol/mg of protein. The parameters were obtained by fitting each curve to a model assuming two noninteracting sites which differ in their affinities toward the agonists but bind [^{3}H]4NMPB with the same affinity. The parameter values shown are the means ± SD of four separate experiments (each fitted separately to the above model) in each case. [b] ND = not determined.

Determination of Muscarinic Subtypes. Total RNA was extracted from young or aged myocytes by guanidinium thiocyanate and precipitated in LiCl as described by Cathala et al. (1983). Total RNA (25 μg/lane) was fractionated by formaldehyde/agarose (6.5% and 1%, respectively), blotted onto a Gene Screen (New England Nuclear) (Maniatis et al., 1982), and assayed for hybridization with ^{32}P-labeled probes specific for the M1, M2, M3, and M4 muscarinic receptor subtypes, described in detail elsewhere (Pinkas-kramarski et al., 1989).

Results and Discussion

Characterization of Muscarinic Receptors in Intact Myocytes. The purpose of this study was to investigate the muscarinic receptor mediated biochemical responses (inhibition of cAMP accumulation and stimulation of phosphoinositide hydrolysis) in rat heart myocytes as a function of culture age and lipid composition. Since these biochemical responses are mediated by muscarinic agonists *in intact cells*, it was necessary to characterize their binding to the muscarinic receptors in intact myocytes (rather than in homogenates or membrane preparations) and to identify the subtypes of the receptors in these cells.

Binding studies were conducted in intact cultured myocytes, 5 (young cultures) or 14 days (aged cultures) after plating. The binding of the muscarinic agonists carbamylcholine and oxotremorine was measured by competition with the tritiated antagonist. The results of four separate experiments with each agonist are summarized in Table I. For oxotremorine, similar results were obtained with young and aged cultures (Table I). The competition curves were flattened, and the data were well fitted by the simple model specifying two classes of binding sites (R_H and R_L) which bind oxotremorine with high and low affinities, respectively. On the other hand, carbamylcholine binding was markedly affected by the culture's age. In young cultures, flattened competition curves fitting binding to two sites were obtained; in view of the low affinities of these sites for carbamylcholine, they were designated R_L and R_{SL} (low and superlow affinities, rather than R_H and R_L as in the case of oxotremorine). In aged cultures, the competition curve of carbamylcholine with [^{3}H]4NMPB underwent a clear shift to the right; in terms of the two-sites model, this shift fitted conversion of R_L to R_{SL} (Table I). Since the superlow-affinity sites (in both young and aged cultures) are special to intact myocytes and are not observed in homogenates prepared from the same cultures (Moscona-Amir et al., 1986), it appears that the agonist binding characteristics in intact myocytes differ from those in homogenates.

The shift to R_{SL} in the aged cultures is not due to changes in the muscarinic subtypes expressed by the cells, since only M2 could be detected in both young and aged cultures following RNA extraction and blotting using probes specific to the various muscarinic receptor subtypes M1, M2, M3, and M4 [the probes are described in detail elsewhere—Pinkas-Kramarski et al. (1989)].

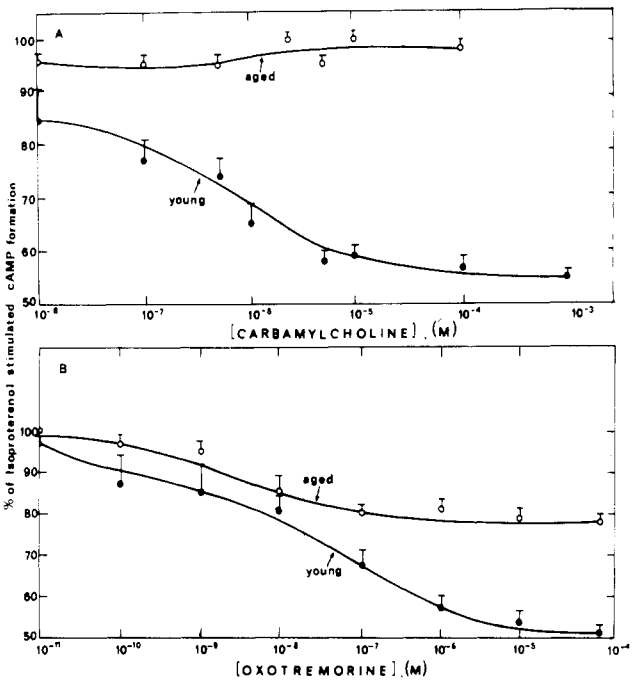

FIGURE 1: Effect of carbamylcholine (A) and oxotremorine (B) on the inhibition of isoproterenol-stimulated cAMP formation in intact myocytes. Experiments were conducted with young (●) and aged (○) cultures as described under Materials and Methods. Data are expressed as percent inhibition of isoproterenol-stimulated cAMP formation. The isoproternol stimulation was 35 ± 5-fold and 40 ± 5-fold above basal in young and aged cultures, respectively; the basal levels of cAMP accumulation were 2.3 ± 0.3 and 2.5 ± 0.3 pmol/16-mm well in young and aged cultures, respectively. Values shown are means ± SD of five and three separate experiments for carbamylcholine and oxotremorine, respectively.

Inhibition of Isoproterenol-Stimulated cAMP Accumulation. Young or aged myocyte cultures were exposed (2 min, 37 °C) to various concentrations of muscarinic agonists (carbamylcholine or oxotremorine), and their effect on the basal and on the isoproterenol-stimulated accumulation of cAMP was measured as described under Materials and Methods. Isoproterenol (10^{-6} M) stimulated cAMP accumulation above the basal level to almost the same extent in young [mean stimulation ± SE was (35 ± 5)-fold in 10 measurements] and in aged myocytes [(40 ± 6)-fold, $n = 6$]. The inhibition of this stimulation by carbamylcholine differed dramatically between young and aged cultures. In young cultures, carbamylcholine inhibited isoproterenol-stimulated cAMP accumulation in a dose-dependent manner (Figure 1A).

Maximal inhibition of 45 ± 5% was obtained at a concentration of 10^{-4} M carbamylcholine, and the K_{act} (agonist concentration required to achieve half-maximal response) was 8×10^{-7} M. Atropine at 5 μM completely abolished the carbamylcholine effect, further reinforcing the notion that this inhibition is mediated through muscarinic receptors. In aged cultures, the ability of carbamylcholine to inhibit isoproterenol-stimulated cAMP accumulation was completely lost and was not detected even at 10^{-4} M agonist. In both young and aged cultures, carbamylcholine (up to 10^{-4} M) had no effect on the basal level of cAMP.

The effect of oxotremorine on the isoproterenol-stimulated cAMP accumulation is shown in Figure 1B. In young cultures, a picture similar to that observed with carbamylcholine was obtained. Maximal inhibition of 50 ± 4% was achieved at 10^{-5} M oxotremorine, and the K_{act} was 2×10^{-8} M. In aged cultures, the ability of oxotremorine to inhibit isoproterenol-stimulated cAMP accumulation was considerably lower, but unlike the situation with carbamylcholine, it retained part of its inhibitory effect (maximal inhibition of 24% and K_{act} around 5×10^{-9} M). Oxotremorine had no effect on the basal level of cAMP in both young and aged cultures. These findings are in accord with the report on reduced cholinergic control of adenylate cyclase in the hearts of aging rats (Narayanan & Tucker, 1986).

Comparison between the agonist binding parameters (Table I) and the respective K_{act} values for the inhibition of cAMP accumulation shows a good correlation in the case of oxotremorine (the K_H values are compatible with K_{act} for oxotremorine in both young and aged cultures); however, in the case of carbamylcholine, the K_{act} measured in young cultures is several orders of magnitude below its dissociation constants. This apparent discrepancy can be explained by the existence of spare receptors coupling carbamylcholine binding with the inhibition of adenylate cyclase, resulting in saturation of the carbamylcholine response at low receptor occupancy. This explanation was offered by Brown and Brown (1984) for the carbamylcholine-mediated inhibition of cAMP accumulation in chick myocytes. As in the studies presented here, this phenomenon was much weaker in the case of oxotremorine, most likely due to its lower efficacy (Fisher et al., 1983), which requires a higher receptor occupancy to elicit response (Brown & Brown, 1984). The residual inhibiting effect of oxotremorine on cAMP accumulation in aged cultures may reflect the fact that oxotremorine continues to bind to aged myocytes with high affinity, as opposed to the situation with carbamylcholine (Table I). Indeed, the K_{act} value for oxotremorine in aged cultures still correlates with K_H for this ligand, although the extent of the inhibition is attenuated.

In order to examine whether the effects described above are mediated through G_i, young myocytes were treated with IAP (25 ng/mL) overnight (Moscona-Amir et al., 1988). As shown in Figure 2, the IAP treatment completely abolished the ability of both carbamylcholine and oxotremorine to inhibit isoproterenol-stimulated cAMP accumulation. It should be noted that pretreatment with this dose of the toxin (25 ng/mL) had almost completely prevented subsequent ^{32}P-ADP-ribosylation of the 40-kDa α_i (common to both young and aged cultures) but had no effect on ADP-ribosylation of the two other bands detected only in young cultures (28 and 42 kDa) (Moscona-Amir et al., 1988). These findings indicate that the coupling of the myocyte muscarinic receptors with adenylate cyclase is mediated through G_i.

Since the aged myocyte cultures contain α_i (Moscona-Amir et al., 1988) and have fully active isoproterenol-stimulated

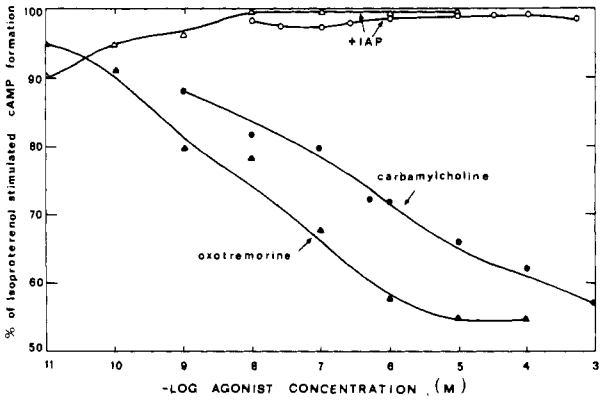

FIGURE 2: Effect of IAP on the ability of carbamylcholine and oxotremorine to inhibit isoproterenol-stimulated cAMP formation. Young (5 days after plating) myocyte cultures were incubated overnight with 25 ng/mL IAP. The ability of carbamylcholine (○) and oxotremorine (△) to inhibit isoproterenol-stimulated cAMP formation is compared with their inhibitory effects [(●) carbamylcholine; (▲) oxotremorine] on young myocyte cultures which were not treated with IAP. Typical representative curves (one out of three, which varied only within 7–12%) are shown. The basal cAMP accumulation levels were 2.3 and 2.4 pmol/16-mm well for untreated and IAP-treated cultures; isoproterenol (1 μM) stimulation was 33-fold in untreated cultures and 30-fold in the IAP-treated cultures.

adenylate cyclase, the most reasonable explanation for the defect in the muscarinic inhibition of cAMP accumulation in the aged cultures is an impairment in the coupling between the muscarinic receptors and adenylate cyclase through G_i. This notion is in accord with studies on homogenates prepared from the myocyte cultures, which demonstrated dramatic alterations in the coupling of the muscarinic receptors with G-proteins in aged cultures (Moscona-Amir et al., 1986). An impaired coupling may also explain the reduction in the ability of oxotremorine to inhibit cAMP accumulation in aged cultures in spite of the lack of alteration in its binding (Table I, Figure 1). In this context, it should be noted that age-dependent reduction in the coupling of G-proteins with adenylate cyclase in rat brain cortex was observed (Nomura et al., 1986), and the muscarinic regulation of adenylate cyclase in embryonic chick hearts was reported to depend on development, in correlation with the level of muscarinic receptors and α_o (Liang et al., 1986; Luetje et al., 1987).

In order to examine whether the cellular lipid composition affects the muscarinic control of adenylate cyclase activity, aging myocyte cultures were treated with PC liposomes (described under Materials and Methods). This treatment results in aged cultures with a lipid composition similar to that of young ones (Yechiel & Barenholz, 1985). The results shown in Figure 3 clearly demonstrate that this treatment restored the ability of carbamylcholine to inhibit isoproterenol-stimulated cAMP accumulation in the aged myocyte cultures. Maximal inhibition of 48 ± 4% was achieved at 10^{-4} M carbamylcholine, and the K_{act} was 6×10^{-7} M. These values are in good agreement with those observed in young cultures; moreover, the binding parameters of carbamylcholine in the liposome-treated aged cultures were close to those observed in young cultures (R_L = 53 ± 5%, K_L = 58 ± 8 μM, K_{SL} = 800 ± 200 μM, according to the nomenclature of Table I). As in the case of young myocyte cultures, IAP treatment of the liposome-treated aged cultures (as in Figure 2) abolished the carbamylcholine-mediated inhibition of cAMP accumulation.

These findings suggest a role for lipid composition and organization in coupling the muscarinic receptors with adenylate cyclase. Indeed, the same liposome treatment was

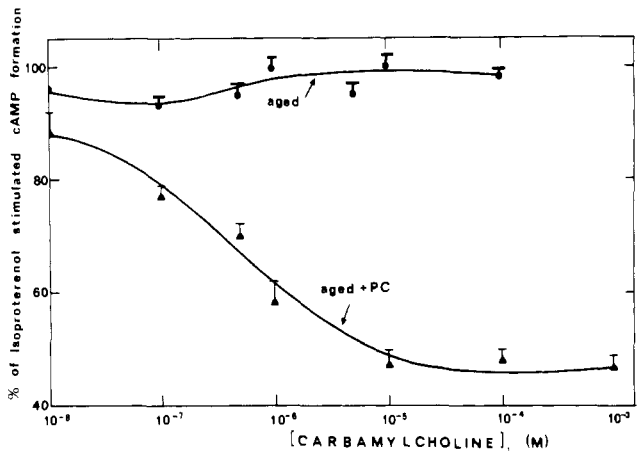

FIGURE 3: Effect of PC liposome treatment on the ability of carbamylcholine to inhibit isoproterenol-stimulated cAMP formation in aged myocytes. Myocyte cultures were treated with PC liposomes as described under Materials and Methods. The inhibition of isoproterenol-stimulated cAMP formation by carbamylcholine was measured in parallel in aged cultures treated with PC liposomes (▲) and in untreated aged cultures from the same preparation (●). Values shown are means ± SD of three different experiments. The isoproterenol stimulation was 40 ± 5-fold and 38 ± 4-fold in untreated and liposome-treated aged cultures, respectively. The basal levels of cAMP accumulation were 2.5 ± 0.3 (untreated aged cultures) and 2.4 ± 0.3 pmol/16-mm well (liposome-treated aged cultures).

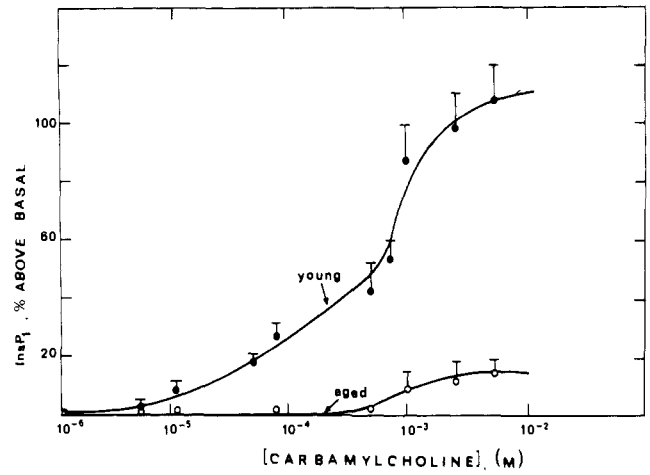

FIGURE 4: Effect of carbamylcholine on the accumulation of [^{3}H]$InsP_1$ in intact myocytes. Myocyte cultures were prelabeled with [^{3}H]inositol, washed, and incubated (60 min, 37 °C) in growth medium containing 10 mM LiCl and various concentrations of carbamylcholine (see Materials and Methods). Accumulation of [^{3}H]$InsP_1$ was measured in young [(●) 5-day old] and aged [(○) 14 days after plating] intact myocytes. Percent above basal of [^{3}H]$InsP_1$ is the increase (in percent) of [^{3}H]$InsP_1$ accumulation above the basal level. The basal levels of [^{3}H]$InsP_1$ formed (expressed as the percentage of total ^{3}H-labeled inositol lipids) were 3.2 ± 0.9 and 4.5 ± 1.2 in young and aged cultures, respectively. Values shown are means ± SD of five separate experiments.

shown to restore normal muscarinic–G-protein(s) interactions in homogenates of the aged myocyte cultures (Moscona-Amir et al., 1986). The altered coupling of the muscarinic receptors with the inhibition of cAMP accumulation in the aged cultures could stem directly from changes in the lipid composition and the organization of the plasma membrane lipids in lateral domains; these parameters are markedly altered with the age of the cultured myocytes and are restored following the PC liposome treatment (Yechiel et al., 1985). However, possible alterations in direct interactions of specific lipids with one or more of the interacting components (muscarinic receptors, G_i, or adenylate cyclase) cannot be ruled out. In this context, it should be noted that the lipid composition may affect β-adrenergic receptors and their coupling with G_s (Limbird & Lefkowitz, 1976; Kirilovsky et al., 1985) and the cholesterol level was reported to be critical for adenylate cyclase activation (Whetton et al., 1983a,b).

Stimulation of Phosphoinositide Hydrolysis. The ability of muscarinic agonists to stimulate phosphoinositide hydrolysis was determined in young and aged cultures by measuring the accumulation of [^{3}H]$InsP_1$ with the technique of Berridge et al. (1982). Cells were prelabeled overnight with 1 μCi/mL [^{3}H]inositol, washed, and exposed (60 min, 37 °C) to various concentrations of carbamylcholine or oxotremorine in the presence of LiCl. A 60-min incubation was chosen since preliminary experiments indicated that 30–60 min are required to achieve maximal accumulation of [^{3}H]$InsP_1$ above the basal level. Under these conditions, carbamylcholine induced a 2-fold maximal increase (100% above the basal level) in [^{3}H]$InsP_1$ accumulation in young myocyte cultures. The maximal response was achieved at 2.5–5.0 mM agonist, and the K_{act} value was 6×10^{-4} M (Figure 4). Atropine (5×10^{-7} M) completely blocked this effect. In aged cultures, the carbamylcholine effect was markedly reduced: the maximal increase in [^{3}H]$InsP_1$ accumulation above the basal level was only 15 ± 4% (Figure 4). The K_{act} value appears to be in the same range as in young cultures but cannot be determined accurately due to the low signal. As for oxotremorine, its ability to elevate the [^{3}H]$InsP_1$ level was extremely weak already in young cultures (a 20% increase at millimolar concentrations of the drug). This is in accord with the previous reports on the partial agonist nature of oxotremorine regarding the stimulation of the phosphoinositide metabolism (Brown & Brown, 1984; Brown & Goldstein, 1986; Brown & Jones, 1986). Comparison with the agonist binding parameters (Table I) shows a good agreement between the K_{act} for carbamylcholine-mediated [^{3}H]$InsP_1$ accumulation and its dissociation constant from the superlow-affinity sites (K_{SL}). This may indicate that the R_{SL} state of the receptors, which is induced by carbamylcholine binding to intact myocytes but not by oxotremorine binding (Table I), is the one coupled with the phosphoinositide metabolism. The correlation between K_{SL} for carbamylcholine and its K_{act} for [^{3}H]$InsP_1$ accumulation suggests that, unlike inhibition of cAMP accumulation by carbamylcholine, activation of the phosphoinositide response requires full receptor occupancy. Differences between coupling of receptors to adenylate cyclase (where "spare receptors" are detected) and to activation of the phosphoinositide response (where full occupancy is required) have been noted in several systems (Fisher et al., 1983; Brown & Brown, 1984).

The low sensitivity of aged myocyte cultures to stimulation of [^{3}H]$InsP_1$ accumulation by carbamylcholine could stem either from reduced phosphoinositide hydrolysis (reduced activation of phospholipase C) or from an impairment at a later stage in the conversion of the hydrolysis products to $InsP_1$. To distinguish between these possibilities, the level of [^{3}H]$InsP_3$, [^{3}H]$InsP_2$, and [^{3}H]$InsP_1$ was measured as a function of the exposure time to carbamylcholine (2.5 mM) in young and aged cultures. Figure 5 clearly demonstrates that carbamylcholine stimulates the formation of $InsP_3$ in both young and aged cultures, as indicated by the quick rise (within 1 min) in the level of [^{3}H]$InsP_3$ in both cultures (Figure 5). The similarity between young and aged cultures disappears when one examines the time scale and level for the appearance of [^{3}H]$InsP_1$. While in young cultures the typical pattern of [^{3}H]$InsP_1$ accumulation following the appearance of [^{3}H]$InsP_3$ and [^{3}H]$InsP_2$ is observed, the rise in [^{3}H]$InsP_1$ in aged cultures is minute, and this product does not accumulate with time

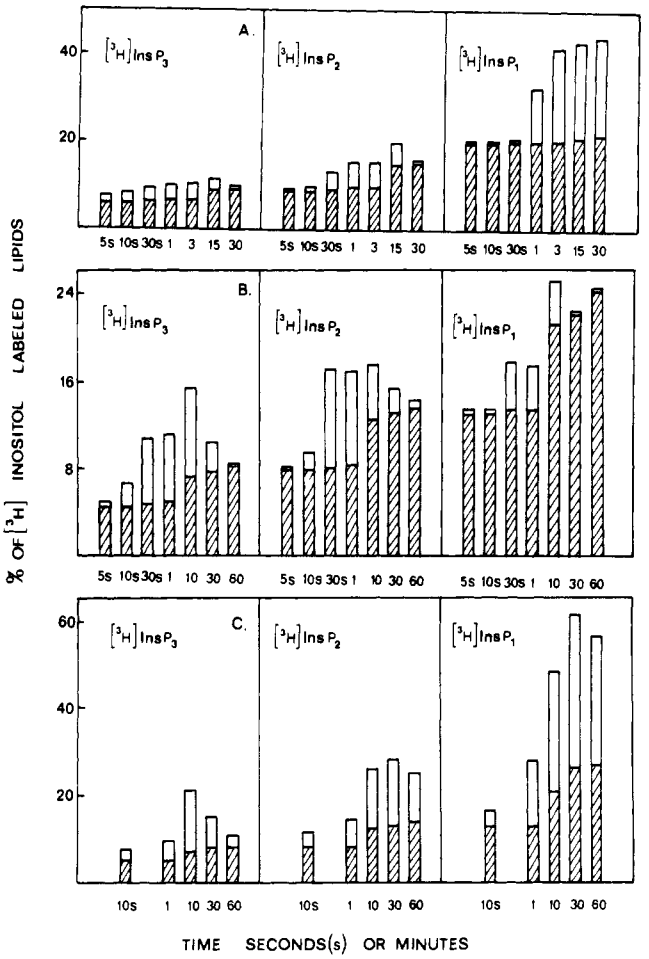

FIGURE 5: Time course of the levels of phosphoinositide hydrolysis products during exposure to carbamylcholine and norepinephrine. The accumulation of $[^3H]InsP_3$, $[^3H]InsP_2$, and $[^3H]InsP_1$ was measured as a function of the exposure time of young (5 days old) (A) and aged (14 days old) (B) cultures to carbamylcholine (2.5 mM) and of aged cultures to norepinephrine (500 μM) (C). The measurements were performed by the phosphoinositide hydrolysis assay of Berridge et al. (1982), as described under Materials and Methods. The results shown are those of representative experiments (one out of three experiments in each case, which varied within 15–20%). Data are presented as the percentage of $[^3H]InsP_3$, $[^3H]InsP_2$, or $[^3H]InsP_1$ of the total ^3H-labeled inositol lipids (blank bars). The basal levels are shown as superimposed crosshatched bars.

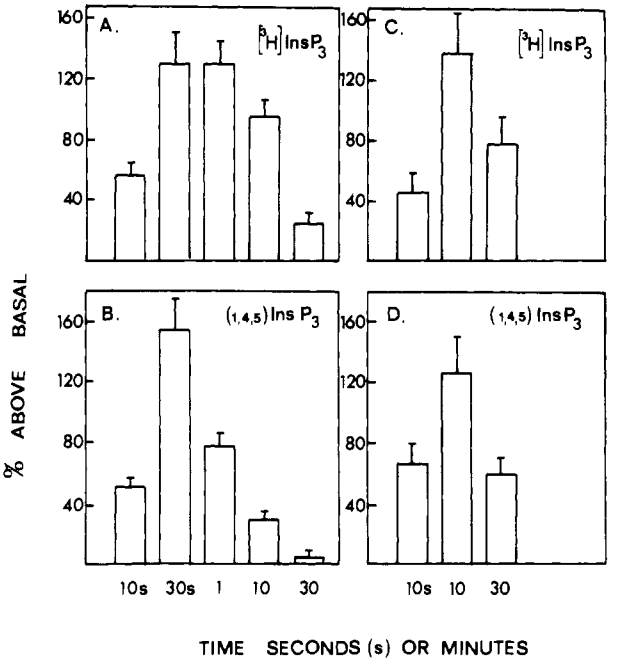

FIGURE 6: Comparison between the levels of $[^3H]InsP_3$ and (1,4,5)-$InsP_3$ as a function of the exposure time to carbamylcholine and norepinephrine in aged cultures. (A and B) Cultures exposed to 2.5 mM carbamylcholine. (C and D) Cultures exposed to 500 μM norepinephrine. In (A) and (C), the cultures were prelabeled with [^3H]inositol, and the level of $[^3H]InsP_3$ was determined by the phosphoinositide hydrolysis assay (Materials and Methods; see Figure 5). The $[^3H]InsP_3$ fraction eluted from the Dowex column in this assay includes all $[^3H]InsP_3$ isomers and also $[^3H]InsP_4$. In (B) and (D), cells from the same preparation were employed to determine exclusively the level of (1,4,5)-$InsP_3$ with the D-*myo*-inositol (1,4,5)-trisphosphate ^3H assay system (Amersham; see Materials and Methods). The results shown are means ± SD of three experiments in each case. In (A) and (C), the basal levels of $[^3H]InsP_3$ (as percentage of total ^3H-labeled insoitol lipids) were 4.8 ± 0.2 (10 s to 1 min), 8.5 ± 1.5 (10 min), and 9 ± 1 (30 min). In (B) and (D), the basal levels of (1,4,5)-$InsP_3$ were (in pmol/35-mm dish) 0.6 ± 0.1 (10 s to 1 min), 0.8 ± 0.1 (10 min), and 1 ± 0.15 (30 min). In the case of carbamylcholine, Student's t test indicated that the differences between (A) and (B) are only statistical up to 30 s ($p > 0.25$ for 10 s and $p > 0.1$ for 30 s) but become significant at longer times ($p < 0.005$ for 1 min, $p < 0.001$ for 10 min, and $p < 0.005$ for 30 min). On the other hand, in the case of norepinephrine, the differences between (C) and (D) are statistical in all cases ($p > 0.05$ for 10 s, $p > 0.25$ for 10 min, and $p > 0.1$ for 30 min).

(Figure 5). These results suggest that the defect in the response of the aged myocyte cultures to carbamylcholine is at a stage beyond PIP_2 hydrolysis. The altered response of the aged cultures is not due to a general defect in the phosphatases that convert $InsP_3$ to $InsP_2$ and $InsP_1$, since activation of phosphoinositide hydrolysis in aged cultures through α_1-adrenergic receptors (using norepinephrine) resulted in a comparable elevation in $[^3H]InsP_3$, which was further processed in the normal pattern leading to $[^3H]InsP_1$ accumulation (Figure 5).

The other alternative is that the $[^3H]InsP_3$ fraction generated in aged cultures in response to carbamylcholine is highly enriched in isomers other than (1,4,5)-$InsP_3$, the initial hydrolysis product of PIP_2 (Irvine, 1986). Such isomers could have a different metabolic fate. There are indications that the metabolism of (1,4,5)-$InsP_3$ is more complex than originally thought since other isomers such as (1,2c,4,5)-$InsP_3$ (Wilson et al., 1984, 1985) and (1,3,4)-$InsP_3$, as well as (1,3,4,5)-$InsP_4$, were recently detected (Irvine et al., 1986; Batty et al., 1985). Neither the biological function nor the metabolic fate of these isomers has been fully established.

In order to explore whether isomers other than (1,4,5)-$InsP_3$ are generated in aged cultures following stimulation by carbamylcholine, the D-*myo*-inositol (1,4,5)-trisphosphate ^3H assay system (described under Materials and Methods) was employed to determine the (1,4,5)-$InsP_3$ level as a function of the exposure time to the agonist. This assay enables exclusive quantitation of the level of (1,4,5)-$InsP_3$, unlike the procedure employed in Figure 5 where the $[^3H]InsP_3$ fraction may also contain other $[^3H]InsP_3$ isomers as well as $InsP_4$. The two procedures were performed in parallel on the same aging cultures; incompatibility between the levels of the triphosphoinositides determined by the two methods would indicate the existence of other $InsP_3$ isomers in addition to (1,4,5)-$InsP_3$. The results (Figure 6A,B) demonstrate that this indeed is the situation. It should be noted that the differences become apparent only at longer exposure times, in accord with the notion that (1,4,5)-$InsP_3$ is formed initially and only later it is converted to other isomers—most likely (1,3,4,5)-$InsP_4$ and (1,3,4)-$InsP_3$ (Taylor, 1987). On the other hand, the differences between the two methods were not significant in aged cultures stimulated by norepinephrine (Figure 6), in

accord with the normal accumulation of [^{3}H]$InsP_1$ under these conditions.

A plausible explanation for all of the above results is that the coupling of the muscarinic receptors with phospholipase C persists in the aged cultures, but the (1,4,5)-$InsP_3$ initially formed after muscarinic stimulation is quickly converted into other $InsP_3$ isomers, which are metabolized by a pathway different from the one leading to $InsP_1$. The alternate pathway could be the one involving conversion of (1,4,5)-$InsP_3$ to (1,3,4)-$InsP_3$ (Batty et al., 1985; Irvine et al., 1986; Erneaux et al., 1986; Hawkins et al., 1986; Taylor, 1987). The normal conversion of [^{3}H]$InsP_1$ in aged myocyte cultures stimulated by norepinephrine suggests that this alternate pathway must be induced by the muscarinic agonist, probably by activating another metabolic path for processing (1,4,5)-$InsP_3$. Different metabolic states could form in response to carbamylcholine as compared with norepinephrine since, in addition to stimulation of PIP_2 hydrolysis, the two agonists activate different physiological responses (e.g., the opening of K^+ channels through the muscarinic receptors or the stimulation of adenylate cyclase the β-adrenergic receptors in the case of norepinephrine). An alternative explanation for the lack of phosphoinositide response to carbamylcholine in aged cultures is that muscarinic agonists fail to stimulate a phospholipase C that acts on PIP_2 in aged myocytes. However, the fact that the initial generation of [^{3}H]$InsP_3$ is comparable in young and aged cultures stimulated by carbamylcholine and in carbamylcholine vs norepinephrine-stimulated aged cultures (Figure 5) suggests that PIP_2 hydrolysis is stimulated by carbamylcholine in aged cultures. Moreover, comparison of panels B and D of Figure 6 demonstrates that the initial (1,4,5)-$InsP_3$ levels generated in aged cultures following carbamylcholine and norepinephrine stimulation are comparable, suggesting that the differences in the response of aged myocytes to the two agonists lie at a stage beyond the activation of phospholipase C.

In order to investigate the involvement of G-proteins in the stimulation of the phosphoinositide metabolism in the cultured myocytes, young myocyte cultures were treated by IAP or by cholera toxin, which induce ADP-ribosylation of the α subunits of different G-proteins (Kurose et al., 1983; Bokoch et al., 1984; Sternweis & Robishaw, 1984; van Dop et al., 1984). These experiments were conducted with young cultures, where the coupling with the muscarinic receptors can be clearly measured by following [^{3}H]$InsP_1$ accumulation. Preincubation (overnight, 37 °C) of these cultures with 25 ng/mL IAP had no effect on the carbamylcholine-stimulated [^{3}H]$InsP_1$ accumulation: the increases in the percentage of [^{3}H]$InsP_1$ accumulation above the basal level were 90 ± 10% and 85 ± 8% before and after IAP treatment. The resective basal levels of [^{3}H]$InsP_1$ formed, expressed as percentage of total ^{3}H-labeled inositol lipids, were 3.2 ± 0.9 and 3.4 ± 0.8. This lack of effect is contrasted with the complete blockade of the muscarinic inhibition of cAMP accumulation under the same conditions (Figure 2). Preincubation of young myocyte cultures with cholera toxin (overnight, 37 °C, with 1 μg/mL toxin) significantly inhibited the carbamylcholine-mediated [^{3}H]$InsP_1$ accumulation (Figure 7). However, this effect appears to be induced indirectly through the increase in the cellular cAMP level following the persistent activation of adenylate cyclase by cholera toxin, as indicated by the similar reduction in the ability of carbamylcholine to stimulate [^{3}H]$InsP_1$ accumulation following other treatments that elevate cAMP. Thus, treatment of the cells with 8-bromo-cAMP, forskolin, or IBMX reduced significantly the muscarinic-mediated [^{3}H]$InsP_1$ accumulation (Figure 7). It should be noted that such an indirect effect through the cAMP level does not play a role in our measurements on the effects of muscarinic agonists on the phosphoinositide metabolism (Figure 4), since these measurements were conducted under conditions where cAMP formation is not stimulated.

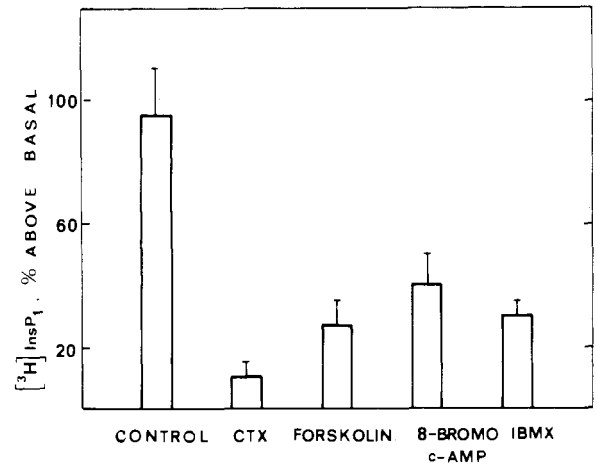

FIGURE 7: Effect of cholera toxin and treatments that elevate the cAMP level on the accumulation of [^{3}H]$InsP_1$ stimulated by carbamylcholine in young cultures. The accumulation of [^{3}H]$InsP_1$ was measured in young (5 days old) myocyte cultures following exposure (60 min, 37 °C) to 2.5 mM carbamylcholine. Prior to the exposure to carbamylcholine, the cultures were treated as follows: untreated (control); 1 μg/mL cholera toxin (CTX) (14–18 h, 37 °C); 10 μM forskolin (10 min, 37 °C); 1 mM 8-bromo-cAMP (60 min, 37 °C); 200 μM IBMX (60 min, 37 °C). Values shown are means ± SD of three different experiments.

In view of the finding that treatment of aging cultures with PC liposomes restored the ability of carbamylcholine to inhibit isoproterenol-stimulated cAMP accumulation (Figure 3), the effects of this treatment on the carbamylcholine-stimulated accumulation of [^{3}H]$InsP_1$ were examined. While 2.5 mM carbamylcholine induced a maximal increase of 2.1-fold in the accumulation of [^{3}H]$InsP_1$ in young cultures, it caused only a minor increase (1.2-fold; see Figure 4) in this parameter in aged cultures. A similar effect (a 1.1-fold increase) was observed in aged cultures treated with PC liposomes. Thus, the latter treatment did not restore the accumulation of [^{3}H]$InsP_1$ in the cultured myocytes in response to muscarinic stimulation. In this context, it is intriguing to note that (1,4,5)-$InsP_3$, the initial product of PIP_2 hydrolysis, is water soluble and most of its metabolism occurs in the cytosol; this may be the reason for the failure of the PC liposome treatment (which converts the lipid composition and domain structure in aged cultures to resemble those of young myocyte cultures) to restore the accumulation of $InsP_1$ in response to muscarinic stimulation.

In summary, the present work demonstrates that aging of the myocyte cultures is accompanied by marked alterations in the biochemical responses of the cells to muscarinic stimulation (inhibition of cAMP accumulation and stimulation of the phosphoinositide metabolism). However, while in the first case it is the coupling between the muscarinic receptors and the biochemical response which is disrupted, the alteration in the phosphoinositide metabolism appears to occur at stages beyond the activation of PIP_2 hydrolysis (i.e., the coupling remains intact). The different sensitivities of the two responses to the lipid composition and organization in the myocytes (only the muscarinic inhibition of cAMP accumulation in aged cultures is restored following the PC liposome treatment) may reflect the purely membranal environment in which the coupling of the muscarinic receptors with adenylate cyclase takes

place, while the hydrolysis products of PIP_2 are water soluble.

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