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Guanosine 5′-Triphosphate Binding Protein (G_i) and Two Additional Pertussis Toxin Substrates Associated with Muscarinic Receptors in Rat Heart Myocytes: Characterization and Age Dependency†

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ABSTRACT: The coupling of muscarinic receptors with G-proteins was investigated in cultured myocytes prepared from the hearts of newborn rats. The coupling was investigated in both young (5 days after plating) and aged (14 days after plating) cultures, in view of the completly different effects of 5′-guanylyl imidodiphosphate [Gpp(NH)p] on muscarinic agonist binding to homogenates from young vs aged cultures [Moscona-Amir, E., Henis, Y. I., Yechiel, E., Barenholz, Y., & Sokolovsky, M. (1986) *Biochemistry 25*, 8118–8124]. Pretreatment of cultures from both ages by *Bordetella pertussis* toxin (IAP) was found to eliminate any Gpp(NH)p effect on carbamylcholine binding. IAP by itself induced a rightward shift in the carbamylcholine competition curve in homogenates from aged cultures, but no such effect was observed in homogenates from young cultures. IAP-catalyzed [^{32}P]ADP-ribosylation of membrane preparations from young and aged cultures revealed major differences between them. Young cultures exhibited a major IAP substrate at 40 kDa, which was also recognized by anti-α_i antibodies, and two novel IAP substrates at 28 and 42 kDa, which were weakly ADP-ribosylated by the toxin and were not recognized with either anti-α_i or anti-α_o antibodies. In aged cultures, only the 40-kDa band (ribosylated to a lower degree) was detected. The parallel age-dependent changes in the three IAP substrates (28, 40, and 42 kDa) and in the interactions of the G-protein(s) with the muscarinic receptors strongly suggest close association between the two phenomena. All of these age-dependent changes in the G-protein related parameters were prevented by phosphatidylcholine–liposome treatment of the aged cultures. The role of the membrane lipid composition in these phenomena is discussed.

Many cell surface receptors exert their action through specific guanine nucleotide regulatory proteins (G-proteins) which couple receptors to signal generating systems in the plasma membrane. A growing body of evidence suggests that a family of G-proteins is involved in different cellular functions: inhibition (G_i) and stimulation (G_s) of adenylate cyclase in the heart (Sternweis et al., 1981; Endoh et al., 1985; Liang et al., 1986), control of K^+ channels (G_k) in mammalian and avian heart (Martin et al., 1985; Logothetis et al., 1987; Yatani, 1987), mediation (via G_o) of muscarinic receptor–Na^+ channel interactions in rat brain and heart (Cohen-Armon & Sokolovsky, 1986; Cohen-Armon et al., 1988), inhibition of inward Ca^{2+} currents (probably via G_o) (Halvorsen & Nathanson, 1984), stimulation of phosphoinositide metabolism (via G_i or G_p) (Nakamura & Ui, 1985; Hepler & Harden, 1986), stimulation of the activity of membrane-bound phospholipase A_2 (Burch et al., 1986; Jelsema, 1987; Jelsema & Axelrod, 1987; Axelrod et al., 1987), and stimulation of cGMP-dependent phosphodiesterase (G_T) (Fung, 1983). While functionally diverse, G-proteins share several structural and mechanistic features. Each G-protein consists of α, β, and γ subunits and is most clearly distinguished from other G-proteins by the biological and biochemical attributes of its GTP binding α subunit (Michel et al., 1985; Roof et al., 1985). The α subunit of some G-proteins, for example, G_i (Kurose et al., 1983; Bokoch et al., 1984), G_o (Sternweis & Robishaw, 1984), and G_T (van Dop et al., 1984), is specifically ADP ribosylated by *Bordetella pertussis* toxin (IAP).[1]

The binding of guanine nucleotides to G-proteins strongly affects the binding of agonists to a wide variety of receptors, particularly those linked to adenylate cyclase. Muscarinic receptors mediate inhibition of adenylate cyclase in heart (Watanabe et al., 1978; Endoh et al., 1985; Liang et al., 1986) and brain (Onali et al., 1983), and the effect of guanine nu-

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[1] Abbreviations: IAP, *Bordetella pertussis* toxin; PC, phosphatidylcholine; [^{3}H]4NMPB, *N*-[^{3}H]methyl-4-piperidyl benzilate; Gpp(NH)p, 5′-guanylyl imidodiphosphate; DTT, dithiothreitol.

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cleotides on the binding of muscarinic agonists is well documented [as reviewed in Sokolovsky et al. (1983), Sokolovsky (1984), and McKinney and Richelson (1984)].

In most tissues, classical muscarinic antagonists bind to a homogeneous population of sites, while agonist binding yields curvilinear Scatchard plots interpreted to suggest site heterogeneity (two or three affinity states toward agonists) (Birdsall et al., 1984; Sokolovsky, 1984; Mattera et al., 1985). Guanine nucleotides convert high-affinity muscarinic agonist binding sites into the low-affinity state (Berrie et al., 1979; Sokolovsky et al., 1980, 1983; Galper et al., 1982; Dunlap & Brown, 1984; Martin et al., 1985; McMahon et al., 1985; Moscona-Amir et al., 1986); on this basis, it was suggested that coupling between the muscarinic receptors and G-proteins (G_i or G_o) is responsible for their different affinity states toward agonists.

Employing primary myocyte cultures prepared from the hearts of newborn rats, we have recently demonstrated that the properties of cardiac muscarinic receptors and their interactions with G-proteins are strongly affected by the membrane lipid composition (Moscona-Amir et al., 1986). Upon aging of the myocytes in culture, the cholesterol level of the cells is elevated, and the PC/sphingomyelin ratio is reduced (Yechiel & Barenholz, 1985). These changes resulted in significant alterations in the organization of the plasma membrane lipids in lateral domains (Yechiel et al., 1985). Concomitant alterations were observed in the binding characteristics of the muscarinic receptors, and especially in their mode of coupling with G-proteins, as reflected in the effects of guanine nucleotides on agonist binding (Moscona-Amir et al., 1986). All the above changes were eliminated when the cultured myocytes were treated with egg PC liposomes during aging in culture (Yechiel & Barenholz, 1985; Yechiel et al., 1985; Moscona-Amir et al., 1986).

The pronounced differences between young and aged myocyte cultures in the coupling of muscarinic receptors with G-proteins and the ability to manipulate the lipid composition of the cultured myocytes make this system especially suitable for detailed studies on the molecular mechanism of G-protein–muscarinic receptor interactions in cardiac tissue. In the present work we investigated this subject by using pertussis toxin catalyzed ADP-ribosylation and immunoblotting to identify the G-protein species and levels in young vs aged myocyte cultures and to explore the effects of manipulation of the cellular lipid composition by treatment with PC liposomes on these parameters.

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, ATP, Gpp(NH)p, lima bean trypsin inhibitor, soybean trypsin inhibitor, leupeptin, collagen, and DTT were from Sigma (St. Louis, MO). Egg PC (>99% pure) was from Avanti (Birmingham, AL). Pertussis toxin was purchased from List Biochemicals (Campbell, CA). [^{32}P]NAD^+ was from New England Nuclear (Boston, MA). 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). Affinity-purified peroxidase-conjugated goat IgG against rabbit IgG (H + L chains) was obtained from Bio-Yeda (Rehovot, Israel). Antibodies against G_i and G_o components were a generous gift from Dr. Allen M. Spiegel of the National Institutes of Health (Bethesda, MD). Two antibody preparations were used: (i) RV/3, affinity-purified rabbit antibodies against the α and β subunits of G_o from bovine brain (Gierschik et al., 1986); (ii) AS/6, rabbit antiserum against a synthetic peptide representing the C-terminus of transducin α. The antiserum labels α_T and multiple forms of α_i but not α_o (Goldsmith et al., 1987).

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 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 a 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.

Preparation of Cell Homogenates. Cultured myocytes were washed 3 times with 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, Dottingen, West Germany) with three 15-s bursts separated by 30-s pauses. Protein was determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

Agonist Binding. The binding of the unlabeled muscarinic agonist carbamylcholine to myocyte homogenates was measured by competition experiments against [^{3}H]4NMPB. Aliquots (0.5 mL) of the homogenates were incubated 30 min at 37 °C with various concentrations of carbamylcholine (0.1 μM to 5 mM) and a constant concentration (2.5 nM) of [^{3}H]4NMPB. The reaction was terminated by rapid filtration through Whatman GF/C filters followed by washing 3 times with the cold modified Krebs buffer. Radioactivity was counted 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. Competition curves were analyzed by a nonlinear curve fitting procedure using a model of two noninteracting classes of binding sites with high (R_H) and low (R_L) affinities for the agonist and equal affinities for the labeled antagonist [^{3}H]4NMPB (Kloog et al., 1979; Moscona-Amir et al., 1986).

Pertussis Toxin Treatment. Cultures of intact myocytes were preincubated with pertussis toxin (which does not need preactivation in the case of intact cells) in order to (i) assay the extent of ADP-ribosylation by endogenous NAD^+ in the living cells (in combination with the procedure described for [^{32}P]NAD^+ ribosylation) and (ii) determine the effect of the toxin treatment on muscarinic agonist binding and its modulation by guanine nucleotides. The incubation was performed for 12 h (37 °C, 5% CO_2, in the growth medium) with 25 or 100 ng/mL pertussis toxin added directly to the dish.

[^{32}P]ADP-Ribosylation with Pertussis Toxin. For [^{32}P]-ADP-ribosylation of G-proteins we have combined procedures that were detailed by Liang et al. (1986) and Gierschik et al. (1986). Myocyte cultures which were or were not treated with toxin, as described above, were washed 3 times with the

Krebs–Henseleit solution and scraped off the dish in 1 mL of TMSD buffer (50 mM Tris-HCl, 1 mM EDTA, 0.2 M sucrose, 0.6 mM DTT, 75 mM NaCl, 16 μg/mL soybean trypsin inhibitor, 16 μg/mL lima bean trypsin inhibitor, 9 μg/mL leupeptin, pH 7.4). After homogenization and centrifugation (30000*g*, 15 min), the pellet containing partially purified membranes was resuspended in the TMSD buffer to give 4 mg of protein/mL. Each sample containing 200 μg of membrane protein was treated for 1 h at 37 °C with 1 μg of pertussis toxin (preactivated by incubation for 1 h at 37 °C in 330 mM Tris-HCl containing 14 mM DTT, pH 7.4) in a buffer containing 2.5 mM $MgCl_2$, 2.3 mM ATP, 75 mM Tris-HCl, and 1 μCi of [^{32}P]NAD^+ (pH 7.4). The final volume was 120 μL. The membranes were pelleted and resuspended in sample buffer (Laemmli, 1970). After being boiled for 1 min, the samples were subjected to SDS–PAGE (Laemmli, 1970) performed on a resolving gel gradient of 5–15% polyacrylamide (200 μg of protein/lane). The gels were dried and exposed to AGFA-CURIX RP2 X-ray film with an enhancing screen for 5 days at −70 °C. Densitometry was performed with a Quick Scan R&D densitometer (Helena Laboratories, Beumont, TX).

Immunoblotting. Myocyte membranes were prepared and subjected to SDS–PAGE as described above (except for the lack of [^{32}P]ADP-ribosylation). The gels were electroblotted (14 h, 10 °C, 150 mA) onto nitrocellulose paper as described by Towbin et al. (1979). Nitrocellulose strips were first immersed in a buffer (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) containing 3% gelatin and then incubated with anti-α_i (1:100 dilution) or anti-α_o (directed also against the β subunit; 1:20 dilution) in 1% gelatin according to the procedure of Dr. A. M. Spiegel (Gierschik et al., 1986). The bands labeled with the antibodies were visualized by peroxidase-conjugated goat anti-rabbit IgG (Towbin et al., 1979).

RESULTS

Effects of IAP on Agonist Binding to Muscarinic Receptors in Homogenates of Myocyte Cultures. We have recently demonstrated that, in homogenates of young myocyte cultures (5 days old), Gpp(NH)p (50 μM) induced a rightward shift in carbamylcholine competition curves, whereas in aged myocyte cultures (14 days old) a leftward shift was observed upon addition of Gpp(NH)p (Moscona-Amir et al., 1986). Figure 1 demonstrates that both of these Gpp(NH)p effects on carbamylcholine binding were abolished by pretreatment of the cultures with 25 ng/mL IAP. This indicates that in both young and aged myocyte cultures the Gpp(NH)p-mediated modulation of muscarinic agonist binding is conducted through IAP-sensitive G-protein(s).

The toxin had no effect on [^{3}H]4NMPB binding to muscarinic receptors of young and aged cultures. However, pretreatment of aged cultures with IAP caused by itself a significant rightward shift in the carbamylcholine competition curve; the IC_{50} values were 15 ± 7 μM and 120 ± 30 μM for nontreated and IAP-treated cultures, respectively, $n = 4$ (see Figure 1B). Such an effect of IAP was not observed in young cultures (Figure 1A).

Identification of IAP Substrates in Young and Aged Myocyte Cultures. The alteration in the coupling of the myocyte muscarinic receptors with IAP-sensitive G-protein(s) upon aging of the myocyte cultures could reflect changes in the population of G-protein species in the cells. To investigate this subject, we performed ADP-ribosylation of membrane preparations from young and aged myocyte cultures which contained the same mass of protein (200 μg), using [^{32}P]NAD^+ and preactivated pertussis toxin. The results (Figure 2A,A′)

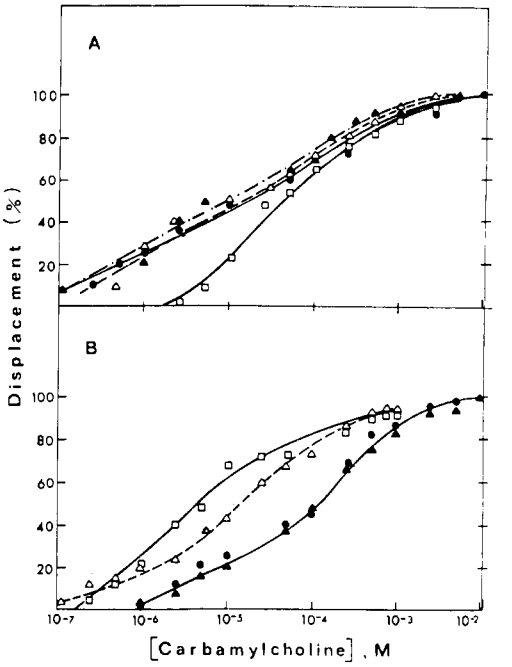

FIGURE 1: Effect of pertussis toxin on carbamylcholine binding to homogenates of myocyte cultures. Data from representative experiments are shown. Similar results were obtained in three additional experiments. The experiments were performed with homogenates prepared from young (A) and aged (B) cultures, as described under Materials and Methods. Cultures were pretreated with 25 ng/mL IAP where indicated. Data are plotted as the percentage of [^{3}H]-4NMPB displaced by the agonist. Each point was determined in triplicate (deviations between the triplicates were below 10%). The lines represent computerized fit to the model of two noninteracting classes of sites [see also Moscona-Amir et al. (1986)]. The parameters obtained for the experiments conducted on cultures pretreated with IAP are as follows: young culture, $R_H = 53 \pm 5\%$, $K_H = 0.6 \pm 3$ μM, and $K_L = 42 \pm 11$ μM; young culture in the presence of Gpp-(NH)p, $R_H = 60 \pm 5\%$, $K_H = 0.7 \pm 0.3$ μM, and $K_L = 60 \pm 20$ μM; aged cultures, $R_H = 35 \pm 5\%$, $K_H = 1.0 \pm 0.4$ μM, and $K_L = 102 \pm 40$ μM; aged cultures in the presence of Gpp(NH)p, $R_H = 38 \pm 5\%$, $K_H = 1.5 \pm 0.9$ μM, and $K_L = 130 \pm 50$ μM. (△) Binding to homogenates prepared from untreated cultures (control); (□) binding to homogenates of untreated cultures in the presence of 50 μM Gpp(NH)p (added to the reaction mixture along with the agonist); (●) binding to homogenates of cultures pretreated with IAP; (▲) binding to homogenates of cultures pretreated with IAP [in the presence of 50 μM Gpp(NH)p].

demonstrate the specific labeling of a major band at about 40 kDa in membranes from both young and aged cultures. Densitometer scans of the autoradiograms (Figure 2B) indicated that the intensity of this band was lower by a factor of 1.2–2.1 (three experiments) in the aged cultures. It should be noted that the labeling intensity in aged cultures was decreased relative to that in young cultures on both a per milligram of protein basis (since the same mass of protein, i.e., 200 μg, was loaded on each lane) and on a per cell basis (in spite of the fact that each 200-μg sample contained a larger number of cells in young cultures than in the aged ones, thus compensating for the fact that aged cultures contained 20–30% more protein per cell). In addition, two weakly labeled bands (42 and 28 kDa) were observed in membranes from young myocytes but were absent in aged cultures (Figure 2A,A′). The 28-kDa band was typically labeled to 7–10% of the intensity of the 40-kDa band, while the 42-kDa band had 3–8% of the intensity of the 40-kDa band. Since protease inhibitors were present during membrane preparation and incubation, it is unlikely that the 28-kDa polypeptide is a proteolytic product of other IAP substrate(s). The susceptibility of the proteins labeled by [^{32}P]NAD^+ and IAP to ADP-ribosylation

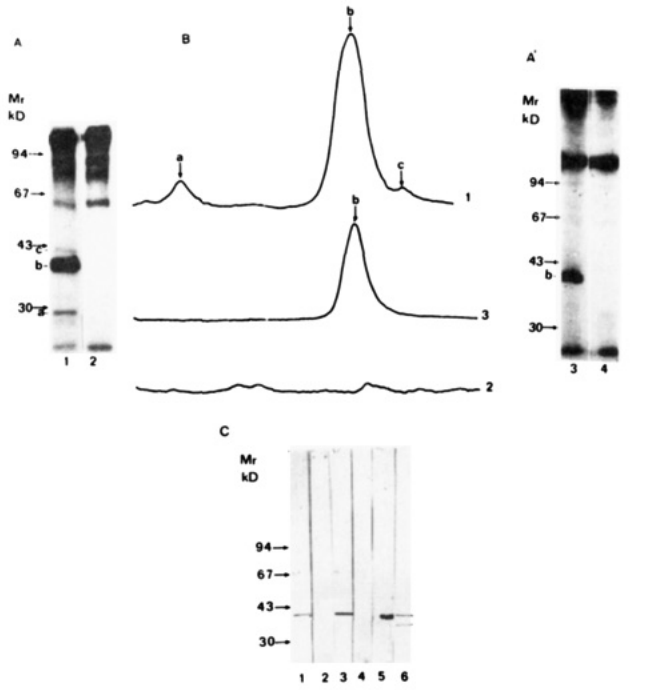

FIGURE 2: [^{32}P]ADP-ribosylation and immunoblotting of membrane preparations from young and aged myocyte cultures. ADP-ribosylation by [^{32}P]NAD$^+$ and IAP and immunoblotting using antibodies against α_i or α_o were performed as detailed under Materials and Methods. In both assays, 200 μg of protein was loaded on each lane of the gel. (A and A') Autoradiograms of [^{32}P]ADP-ribosylated membrane preparations from young [(A) lanes 1 and 2] and aged [(A') lanes 3 and 4] cultures. The lanes numbered 1 and 3 contain membranes incubated with [^{32}P]NAD$^+$ and IAP. The lanes numbered 2 and 4 show nonspecific labeling (membranes incubated with [^{32}P]NAD$^+$ without toxin). The bands marked a, b, and c were specifically labeled in the young cultures, while the only specific labeling in the aged cultures was in band b. (B) Densitometer tracing of the autoradiograms shown in (A) and (A'). The scans began slightly below band a (28 kDa) and ended at 43 kDa. The scans are identified by the numbers of the appropriate lanes in (A) and (A'). In the specific experiment shown, the intensities of bands a and c relative to that of band b in the young cultures were about 8% and 5%, respectively. (C) Immunoblots of myocyte membranes. Lanes 1 and 2 contained membrane preparations from young myocyte cultures; lanes 3 and 4, membranes from aged myocyte cultures; lanes 5 and 6, a cholate extract from bovine brain. Labeling of the blots was performed with anti-α_i (lanes 1, 3, and 5) or anti-α_o (lanes 2, 4, and 6) antibodies. Note that in lane 6 the 35-kDa band corresponds to the β subunit of G_o.

in the intact cells was studied in young and aged cultures by preincubation of intact myocytes with pertussis toxin; membrane preparations from the toxin-treated cultures were then subjected to [^{32}P]ADP-ribosylation by [^{32}P]NAD$^+$ and IAP, to examine the reduction in radiolabeling due to ADP ribosylation of proteins in the intact cells with endogenous NAD$^+$. Figure 3 demonstrates that pretreatment of the cultures with 25 ng/mL IAP induced a strong reduction in the [^{32}P]ADP-ribosylation of the 40-kDa band (10–25-fold in young cultures and 5–15-fold in aged cultures; three separate experiments). Pretreatment with 100 ng/mL toxin induced nearly total ADP-ribosylation of this band, as indicated by the almost complete disappearance of the labeling (30–100-fold reduction of the labeling in the young cultures and 50–100-fold reduction in aged cultures; three separate experiments). The additional two weakly labeled bands observed upon [^{32}P]ADP-ribosylation of membrane preparations from young cultures were significantly less susceptible to ADP-ribosylation in the intact cells (Figure 3). Thus, the labeling of the 42-kDa band was reduced by a factor of 1.3–1.6 following pretreatment with 25 ng/mL IAP, and 3–5-fold by pretreatment with 100 ng/mL

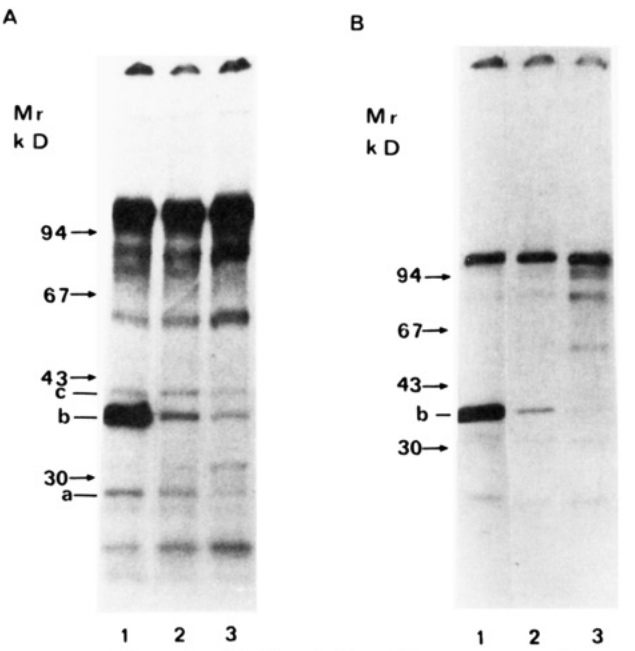

FIGURE 3: Effect of ADP-ribosylation of intact myocytes by pretreatment with IAP on subsequent [^{32}P]ADP-ribosylation. Intact young (A) and aged (B) myocyte cultures were either untreated (1) or treated with 25 ng/mL IAP (2) or 100 ng/mL IAP (3) (see Materials and Methods). Membranes were subsequently prepared, [^{32}P]ADP-ribosylated, and subjected to SDS–PAGE and autoradiography (Materials and Methods). The bands marked a, b, and c are those labeled with the same notation in Figure 2.

toxin. The labeling of the 28-kDa band was diminished 1.4–2-fold after pretreatment with 25 ng/mL IAP and by a factor of 3–6 following pretreatment with 100 ng/mL toxin (three experiments in each case).

In order to identify the ADP-ribosylated proteins, we performed immunoblotting of membrane preparations from young and aged myocyte cultures, using anti-α_o and anti-α_i antibodies. These antibodies were shown to specifically react with α_o and α_i (Gierschik, 1986; Goldsmith, 1987; see also immunoblots of brain extract, Figure 2C, lanes 5 and 6). As shown in Figure 2C, anti-α_i antibodies reacted with a polypeptide which coincided with the 40-kDa ADP-ribosylated band. No reaction was observed with anti-α_o antibodies.

Effects of Liposome Treatment on the IAP Substrates in the Myocyte Cultures. The lipid composition of the myocyte cultures as well as their organization in domains is altered dramatically between young (5 days old) and aged (14 days old) cultures (Yechiel & Barenholz, 1985; Yechiel et al., 1985). In order to explore whether the changes in the lipid composition and organization are involved (directly or as a trigger for other processes) in the alterations of the G-protein population in the aged myocyte cultures, we have treated the aging cultures (from day 6 and on) with egg PC liposomes (see Materials and Methods). This treatment elevates the PC/sphingomyelin ratio and reduces the cholesterol level back to the values observed in the young myocyte cultures (Yehiel & Barenholz, 1985), resulting in aged cultures whose lipid composition is similar to that of young cultures. We have formerly shown (Moscona-Amir et al., 1986) that all of the muscarinic binding characteristics [including the Gpp(NH)p modulation of agonist binding] of aged cultures treated with PC liposomes are similar to those of young myocyte cultures. In order to correlate these findings with the changes in the IAP substrate population in the aged cultures, we employed [^{32}P]NAD$^+$ and IAP to achieve [^{32}P]ADP-ribosylation of membrane preparations from aged cultures treated with PC liposomes. The

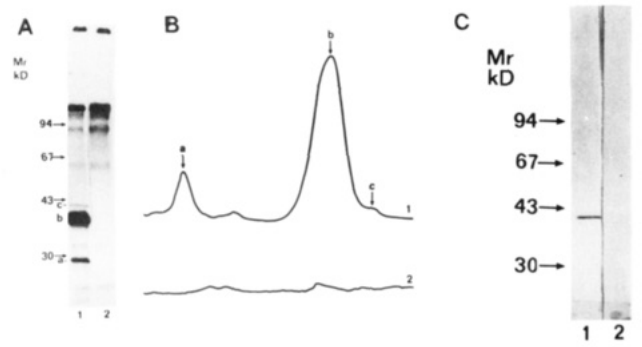

FIGURE 4: [^{32}P]ADP-ribosylation and immunoblotting of membrane preparations from aged myocyte cultures treated with PC liposomes. Aged cultures were treated with PC liposomes, and membrane preparations from the treated cultures were subjected to [^{32}P]ADP-ribosylation or immunoblotting (using anti-α_i or anti-α_o antibodies) as described under Materials and Methods. In both assays, each lane of the gel was loaded with 200 μg of protein. Note that the labeling observed after [^{32}P]ADP-ribosylation in aged untreated cells (control experiments) is shown in Figure 2A′,B. (A) Autoradiograms of membrane preparations incubated with [^{32}P]NAD$^+$ in the presence (lane 1) or in the absence (control; lane 2) of IAP. The lettering a–c refers to the same bands marked by these letters in Figure 2A. (B) Densitometer tracing of the autoradiograms shown in (A). The area from slightly below the band marked a to up to 43 kDa was scanned. The numbers (1 and 2) refer to lanes 1 and 2 in (A). (C) Immunoblots of membrane preparations. Labeling was performed with anti-α_i (lane 1) or anti-α_o (lane 2) antibodies.

results (Figure 4A,B) demonstrate that the [^{32}P]ADP-ribosylation pattern of the liposome-treated aged cultures is different from that of aged untreated cultures (Figure 2A′,B) and resembled that of young cultures (Figure 2A,B). Thus, the 40-kDa band (marked b) is labeled with a significantly higher intensity than that in aged cultures; in fact, the intensity of this band was even somewhat higher (by a factor of 1–1.3; three experiments) than that in young cultures. Moreover, the two bands with weaker labeling (28 and 42 kDa), which were absent in untreated aged cultures (Figure 2A′), have reappeared following PC liposome treatment. The 28-kDa band was somewhat stronger than that in membrane preparations from young myocyte cultures (1–2.3-fold; three experiments), while the 42-kDa band was somewhat weaker (by a factor of 1–0.5 relative to that of young cultures; three experiments).

Immunoblotting assay of the PC liposome treated aged cultures revealed a positive response with anti-α_i antibody which coincided with the 40-kDa ribosylated band (Figure 4C, lane 1). No reaction was observed with anti-α_o antibody (Figure 4C, lane 2).

Discussion

The present study demonstrates that rat heart myocytes contain three IAP substrates (28, 40, and 42 kDa). The 42- and 28-kDa ones are not ADP-ribosylated by this toxin in aged myocyte cultures (Figure 2A,A′). These two IAP substrates are neither α_i nor α_o as they were not recognized by specific anti-α_o and anti-α_i antibodies (Figure 2C). The 40-kDa IAP substrate which reacted with the α_i antibodies (Figure 2C, lanes 1 and 3) is the major [^{32}P]ADP-ribosylated band of both young and aged cultures (Figure 2A,A′). The extent of IAP-catalyzed ADP-ribosylation of the 40-kDa substrate was significantly higher in young myocytes as compared with the aged myocytes.

In agreement with earlier reports (Kurose & Ui, 1983; Martin et al., 1985; McMahon et al., 1985), IAP had blocked the effects of Gpp(NH)p on agonist binding to muscarinic receptors in both young and aged cultures. IAP treatment by itself induced a rightward shift in the carbamylcholine binding curve in homogenates from aged cultures but not in homogenates from young cultures (Figure 1A,B). The different age-dependent effects of IAP treatment on carbamylcholine binding provide additional evidence for the alteration in the mode of coupling between muscarinic receptors and G-protein(s) which occurs upon aging of the myocytes in culture, as suggested in our previous paper (Moscona-Amir et al., 1986).

The parallel age-dependent changes in the 28-, 40-, and 42-kDa IAP substrates and in the G-protein interactions with the muscarinic receptors strongly suggest close association between the two phenomena. This suggestion is supported by the observed effects of PC liposome treatment on aged myocyte cultures. This treatment, which was shown to prevent the age-dependent alterations in the coupling of the myocyte muscarinic receptors with G-protein(s) (Moscona-Amir et al., 1986), also prevented the age-dependent disappearance of the 28- and 42-kDa IAP substrates (Figure 4A).

In our previous paper (Moscona-Amir et al., 1986) we have shown that in young rat myocyte cultures (5 days after plating) Gpp(NH)p induced a shift of the high-affinity agonist binding sites to the low-affinity form. In aged cultures (14 days after plating) an opposite effect of Gpp(NH)p was observed. The complete neutralization of any Gpp(NH)p effect on carbamylcholine binding achieved by pretreatment of the cultured myocytes with IAP (Figure 1) demonstrated that IAP-sensitive G-protein(s) mediate both of the effects of the guanine nucleotides. Our findings suggest that these proteins include G_i and presumably two additional G-proteins that would contain α_{42} and α_{28} components.

This suggestion is consistent with previous reports on the family of G-proteins. Immunological estimates of the total G-protein content of cells show that there may still be many unidentified G-proteins (Faloon et al., 1986). It was also reported that resolution of ADP-ribosylation proteins by two-dimensional gel electrophoresis revealed that they consist of at least six distinct isoelectric forms (Deery et al., 1987). The existence of a 43-kDa IAP substrate was reported by Iyengar et al. (1987). This protein, which exists in trace amounts in comparison to the major IAP substrates G_{41} and G_{40}, was isolated from erythrocyte membranes and was demonstrated to exist also in several other cell types. A 40-kDa IAP substrate distinct from α_i or α_o was demonstrated in brain tissue (Katada et al., 1987) and rabbit heart (Malbon et al., 1985). Another G-protein with an α subunit of about 21 kDa was also found in human placental membranes by photoaffinity labeling with a GTP analogue (Evans et al., 1986).

In the absence of specific antibodies, the quantity of the two novel IAP substrates of the rat heart myocytes cannot be estimated. Their appearance as weakly ADP-ribosylated bands in comparison with the major labeled 40-kDa protein could indicate that they are present in smaller amounts or that they are less susceptible to IAP. Data on [^{32}P]ADP ribosylation of membrane preparations from toxin-pretreated cultures demonstrated that the 28- and 42-kDa IAP substrates were less susceptible to IAP than the 40-kDa substrate, since only pretreatment with a higher dose of the toxin (100 μg/mL) could significantly diminish the subsequent [^{32}P]ADP-ribosylation of these two proteins (Figure 3). These data, however, do not rule out the possibility of their presence in low amounts in the rat myocytes.

Interestingly, Neer et al. (1984) have isolated three IAP substrates from bovine cerebral cortex and found that the 40- and the 39-kDa substrates were less susceptible to ADP-

ribosylation by IAP than the 41-kDa substrate. In rat heart myocytes the major IAP substrate reacts with anti-α_i antibodies but not with anti-α_o antibodies (Figure 2C). It appears then to be the α_i subunit of the G_i protein. In rat cardiac membranes IAP catalyzed ADP-ribosylation of a single 40-kDa protein (Kurose & Ui, 1983). However, in chick heart myocytes two polypeptides with molecular masses of 39 and 41 kDa were identified, whose relative levels were dependent on the embryonic development (Liang et al., 1986; Halvorsen & Nathanson, 1984).

The 40-kDa polypeptide was [^{32}P]ADP-ribosylated to a lower extent in aged rat myocyte cultures as compared with young cultures. The 28- and the 42-kDa polypeptides were lost in aged myocyte cultures. These changes in the IAP substrate population which occurred upon aging of myocytes in culture could be due to reduction in their content or could be a result of a decrease in susceptibility to IAP.

Manipulation of the cellular lipid composition and organization of aged cultures by treatment with PC liposomes has been shown to prevent the age-dependent changes in various biochemical and physiological properties of the rat heart myocytes (Yechiel & Barenholz, 1985; Yechiel et al., 1985). The role of lipid composition and organization in the age-dependent alterations in the coupling of myocyte muscarinic receptors with G-protein(s) was studied by treating the aging myocyte cultures with PC liposomes from the sixth day after plating and on. The data presented in Figure 4 demonstrate that all of the parameters of the labeled proteins in aged cultures treated with PC liposomes were similar to those of young myocyte cultures. Thus, the two additional ADP-ribosylated bands (28 and 42 kDa) typical of young cultures appear also in aged cultures treated with PC liposomes, and the ribosylation of the 40-kDa band was increased. Note that the PC liposome treatment prevented the age-dependent alterations in the coupling of myocyte muscarinic receptors with G-protein(s) (Moscona-Amir et al., 1986). These results clearly indicate a major role for the lipid composition and organization of the myocytes in determining the nature of the coupling of their muscarinic receptors with G-proteins. The changes in the composition and organization of the lipids may lead to altered localization (e.g., sequestration) or to changes in the conformation of the muscarinic receptors and/or G-proteins in the plasma membrane, thus having a direct effect on the coupling between the two entities. Alternatively, the lipid composition changes may serve as a primary trigger to various physiologic processes, whose ultimate result is alteration of the species distribution within the G-protein population, leading to different interactions with the muscarinic receptors. The role of the membrane lipids in determining the properties and coupling of cellular receptor systems may be rather general, in view of the effects of the lipid composition on the interaction of β-adrenergic receptors with G_s (Kirilovsky et al., 1985) and the dependence of the conformation and function of the nicotinic acetylcholine receptor on the nature of the lipid bilayer (McNamee et al., 1986).

The physiological role of the two novel IAP substrates found in the rat heart myocytes is yet unknown. It is possible that they may be associated with the muscarinic receptor mediated activation of phospholipase C (Brown & Brown, 1984; Masters et al., 1984) or with the activation of membrane-bound phospholipase A_2 (Axelrod et al., 1987; Burch et al., 1986).

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Activated Conformers of *Escherichia coli* Sulfite Reductase Heme Protein Subunit†

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ABSTRACT: The heme protein subunit of *Escherichia coli* sulfite reductase shows enhanced reactivity with its substrate and a number of other ligands after a cycle of reduction and reoxidation at alkaline pH. At pH 9.5 this variant of the enzyme possesses at least four EPR-detectable, chloride-sensitive high-spin conformers, in contrast to the single chloride-insensitive species observed in the oxidized, resting enzyme at pH 7.7. Quantitative reversal of the spectral and ligand-binding properties of the "activated" enzyme to those of the resting enzyme is observed on reacidification to pH 7.7. At intermediate pH values, there occurs an acid-catalyzed relaxation of the activated enzyme to the resting form. This reaction is distinct from the one responsible for the accelerated ligand binding and production of multiple EPR conformers, which appears to be regulated by a process with a pK of 8.5.

Many metalloproteins are activated for catalysis and/or ligand binding by reduction of their metal-containing prosthetic groups. In the case of hemoglobin and myoglobin, it is the ferrous state of the iron, per se, that is the factor of prime importance. The mechanism of activation for heart cytochrome *c* oxidase is more complex and appears to involve the sustained rupture of the bond between cytochrome a_3 and a bridging, sulfur-containing amino acid that occurs after reduction and turnover of O_2 (Antonini et al., 1977). A spin-state change and increased ligand affinity of the substrate-binding heme are coupled to the reduction of the metal centers in *Pseudomonas putida* cytochrome P-450 (Sligar, 1976) and *Pseudomonas aeruginosa* nitrite reductase (Walsh et al., 1979). Reoxidation reverses the activation in all of these proteins, but with cytochrome *c* oxidase the reversion to the starting species is slow enough (minutes) to be followed by using room temperature optical spectroscopy.

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