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Islet Activating Protein Inhibits Physiological Responses Evoked by Cardiac Muscarinic Acetylcholine Receptors. Role of Guanosine Triphosphate Binding Proteins in Regulation of Potassium Permeability†

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ABSTRACT: The involvement of GTP binding proteins in muscarinic acetylcholine receptor (mAChR) mediated responses of cultured chick embryonic cardiac muscle cells was studied by using islet activating protein (IAP) from *Bordetella pertussis*. Incubation of cells for 24 h with IAP resulted in inhibition of subsequent IAP-catalyzed incorporation of [α-^{32}P]ADP-ribose into membrane proteins of M_r 39 000 ($N_{o\alpha}$) and 41 000 ($N_{i\alpha}$); treatment of cultures with 5 ng/mL IAP was sufficient to ADP-ribosylate all available $N_{o\alpha}$ and $N_{i\alpha}$. Inhibition of forskolin-stimulated cAMP accumulation by the muscarinic agonist carbachol was abolished in cultures pretreated with IAP. The affinity of carbachol for the mAChR in membranes from IAP-treated cells was considerably decreased compared to control membranes and was not further decreased by addition of guanyl-5′-yl imidodiphosphate. In contrast, the affinity of carbachol for the mAChR on intact cells was not affected by pretreatment with IAP. To investigate the involvement of N_o and/or N_i in mAChR-mediated increases in K^+ permeability, the effect of IAP treatment on mAChR stimulation of $^{86}Rb^+$ efflux was determined. Treatment of cultures with 5 ng/mL IAP for 24 h completely blocked the stimulation of $^{86}Rb^+$ efflux evoked by carbachol. Because previous work has shown that mAChR regulation of K^+ permeability is independent of changes in cAMP levels, these results suggest a role for N_o and/or N_i in coupling the mAChR directly to K^+ channels in the heart.

Activation of muscarinic acetylcholine receptors (mAChR)[1] in the cardiac muscle membrane causes a decrease in beating rate due to an increase in K^+ permeability (Hutter & Trautwein, 1955). Although muscarinic agonists inhibit the synthesis of cAMP by adenylate cyclase in the heart (Watanabe et al., 1978; Jakobs et al., 1979; Hazeki & Ui, 1981), electrophysiological studies have shown that mAChR-mediated increases in K^+ conductance are independent of changes not only in cyclic nucleotide levels (Trautwein et al., 1982; Nargeot et al., 1983) but also of any cytosolic diffusable second messengers (Sakmann et al., 1983). At present, the molecular mechanism of mAChR coupling to K^+ channels is not known.

Agonist binding to mAChR in the heart results in a guanine nucleotide dependent inhibition of adenylate cyclase activity (Watanabe et al., 1978). Guanine nucleotides also regulate the affinity of the mAChR for agonists in the heart (Berrie et al., 1979). These guanine nucleotide effects can be blocked by treatment with islet activating protein (IAP) from *Bordetella pertussis* (Hazeki & Ui, 1981; McMahon et al., 1985). IAP catalyzes the ADP-ribosylation of 39- and 42-kDa polypeptides in chick heart (Halvorsen & Nathanson, 1984), which correspond in molecular weight to the α subunits of the GTP binding proteins N_o and N_i in bovine brain.[2] Both of these proteins are capable of regulating agonist binding to the mAChR (Florio & Sternweis, 1985). Halvorsen & Nathanson (1984) have demonstrated that the appearance of the mAChR-mediated negative chronotropic response in developing embryonic chick heart correlates well with the physical and functional maturation of the GTP binding proteins N_o and N_i. This suggests a role for guanine nucleotide regulatory proteins in coupling the mAChR to physiological responses in chick heart. In the present study we use IAP to investigate the involvement of N_o and N_i in physiologic responses mediated by the mAChR in cultured chick cardiac cells. In contrast to the recent report of McMahon et al. (1985), we show here that IAP treatment of cells abolishes both guanine nucleotide regulation of agonist binding to the mAChR and mAChR-

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[1] Abbreviations: mAChR, muscarinic acetylcholine receptor(s); IAP, islet activating protein; $N_{o\alpha}$, 39-kDa α subunit of the GTP binding protein ADP-ribosylated by IAP; $N_{i\alpha}$, 41-kDa α subunit of the inhibitory GTP regulatory protein of adenylate cyclase ADP-ribosylated by IAP; N proteins, N_o and N_i; Gpp(NH)p, slowly hydrolyzable GTP analogue guanyl-5′-yl imidophosphate; IC_{50}, concentration that inhibits 50%; EC_{50}, concentration producing 50% effect; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; QNB, quinuclidinyl benzilate; NMS, *N*-methylscopolamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

[2] Also referred to as G_o and G_i by Florio & Sternweis (1985).

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mediated inhibition of cAMP accumulation. In addition, using $^{86}Rb^+$ as a tracer ion to measure K^+ permeability, we demonstrate that treatment of cultures with IAP completely blocks increases in $^{86}Rb^+$ efflux evoked by the muscarinic agonist carbachol. These results suggest that the GTP binding proteins N_o and N_i couple the mAChR to K^+ channels in the heart.

Materials and Methods

Cardiac cell cultures were prepared from 9 day old chick embryos as described by Nathanson (1983) except that the growth medium consisted of 47% M199, 47% K^+-free Earle's salts, 5% fetal calf serum, and 1% penicillin–streptomycin (final concentrations of 100 units/mL and 100 μg/mL, respectively). The medium was changed on day 3 in culture, and experiments were conducted on day 4. Cell culture materials were from previously described sources (Nathanson, 1983).

IAP-catalyzed [α-^{32}P]ADP-ribosylation of membranes and one-dimensional SDS–PAGE were performed as described by Halvorsen & Nathanson (1984), with the following modifications. Cultured cardiac cells (50 μg of membrane protein per 35-mm dish) were washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, 1.5 mM KH_2PO_4, 0.5 mM $MgCl_2$, and 0.9 mM $CaCl_2$, pH 7.4) at 37 °C, scraped off the dish, and homogenized in ice-cold 50 mM sodium phosphate, pH 7.4. After two washes in 50 mM sodium phosphate, membranes were resuspended in 100 mM Tris-HCl, pH 8.0, labeled with [α-^{32}P]NAD in the presence of IAP, and analyzed by SDS–PAGE and autoradiography. IAP was purified from the 24-h culture supernatant of *B. pertussis* (Tahoma phase 1) by the method of Sekura et al. (1983). All other chemicals were obtained from previously described sources (Halvorsen & Nathanson, 1984).

The accumulation of cAMP in cells was assayed as previously described (Nathanson et al., 1978). Cells (0.5 mg of protein per 60-mm dish) were preincubated for 20 min at 37 °C in M199 (25 mM HEPES, pH 7.4) containing 5 mM theophylline. Forskolin (100 μM) and carbachol were then added simultaneously to the medium, and the incubation was continued an additional 5 min. Statistical significance was determined by using a two-sample t test following an F test to ensure equality of variance. For samples with unequal variance ($p > 0.05$), statistical significance was determined by using a two-sample t test for samples with unequal variance (Cochran's method).

Binding assays to intact cells, or membranes from intact cells, were as previously described (Nathanson, 1983). [^{3}H]QNB (35.5 Ci/mmol) and [^{3}H]NMS (79.8 Ci/mmol) were obtained from Amersham.

$^{86}Rb^+$ efflux from cardiac monolayers was measured as described by Hunter & Nathanson (1985). In brief, cells were labeled for 24 h with 2 μCi/mL [^{3}H]leucine (ICN, 58 Ci/mmol), washed, and equilibrated for 3 h at 37 °C with $^{86}Rb^+$ (5 μCi/mL; New England Nuclear, 750 Ci/mmol) containing Earle's salts (118 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.0 g/L D-glucose, and 25 mM HEPES, pH 7.4). External $^{86}Rb^+$ was washed off the cells, fresh Earle's salts with or without carbachol were added, and efflux was measured by removing aliquots for scintillation counting every 30 s for 3 min and immediately replacing with an equal volume of an identical solution (without $^{86}Rb^+$). Cells were solubilized in 1% SDS, and [^{3}H]leucine counts per minute incorporated per microgram of protein was determined. Efflux data were corrected for the amount of protein per well (20–30 μg per well). To determine the half-time of $^{86}Rb^+$ efflux [the time necessary for half of the $^{86}Rb^+$ present in the cells at equilibrium (t = 0) to efflux], $^{86}Rb^+$ remaining (cpm/μg at equilibrium − cumulative cpm/μg effluxed at each time) was plotted as a function of time; half-times of $^{86}Rb^+$ efflux were determined by using a least-squares fit after semilogarithmic analyses of data points with the equation $t_{1/2} = \log(0.5/w)$, where w = the slope of the efflux curve. Lines with correlation coefficients less than 0.99 were discarded. Statistical analyses were performed as for the cAMP accumulation assays. To avoid potential complications due either to short-term densensitization of receptor function (Galper & Smith, 1978) or agonist-induced internalization of receptor (Galper et al., 1982; Feigenbaum & El-Fakahany, 1985), determinations of efflux were not extended for times greater than 3 min.

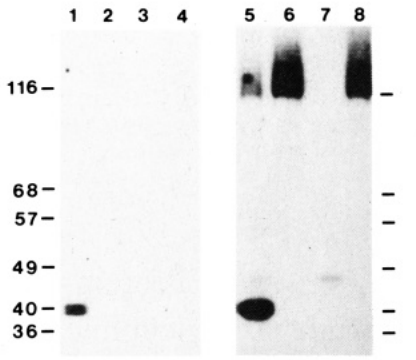

FIGURE 1: Autoradiograph of SDS–polyacrylamide gel of [α-^{32}P]-NAD-labeled membranes. Cells were preincubated for 24 h in the presence or absence of IAP; membranes were prepared and labeled with [α-^{32}P]NAD in the presence of IAP, solubilized in SDS, and subjected to SDS–PAGE and autoradiography. Lanes 1 and 2: membranes from control cells (not preincubated with IAP) incubated with [α-^{32}P]NAD in the presence (lane 1) and absence (lane 2) of IAP. Lanes 3 and 4: Membranes from cells preincubated with 5 ng/mL IAP incubated with [α-^{32}P]NAD in the presence (lane 3) and absence (lane 4) of IAP. Lanes 1–4: Autoradiograph exposed for 6 h. Lanes 5–8 are from an autoradiograph of the same gel as shown in lanes 1–4, respectively, except the autoradiograph was exposed 19 h to demonstrate that treatment of intact cells with IAP fully eliminated subsequent labeling by [α-^{32}P]NAD and IAP.

As noted previously (Hunter & Nathanson, 1985), physiological responsiveness of cultured cardiac cells to muscarinic agonists was highly dependent upon the lot of serum used to supplement growth medium. The cells used in the experiments described here were cultured in Gibco fetal calf serum, lot 31K8843.

Results and Discussion

Cultured cardiac muscle cells were treated for 24 h with increasing concentrations of IAP. Membranes were then prepared, labeled with [α-^{32}P]NAD in the presence of IAP, and subjected to SDS–PAGE (Figure 1). The major polypeptides specifically [α-^{32}P]ADP-ribosylated by IAP in control membranes had M_r's of 39 000 and 41 000 (lanes 1 and 5). A minor labeled 47-kDa polypeptide was also observed; under all conditions, a 116-kDa protein was nonspecifically labeled. Differences in peptide maps indicate that the 39- and 41-kDa polypeptides are not proteolytically related (Martin et al., 1985); immunological distinctions between these two peptides demonstrate that the 39- and 41-kDa proteins in chick heart correspond to the α subunits of N_o and N_i, respectively, from bovine brain.[3] Pretreatment of intact cells in culture for 24 h with 5 ng/mL IAP completely blocked subsequent [α-^{32}P]ADP-ribosylation of the 39- and 41-kDa proteins (lanes

[3] C. Luetje, P. Gierschik, A. Spiegel, and N. M. Nathanson, unpublished observations.

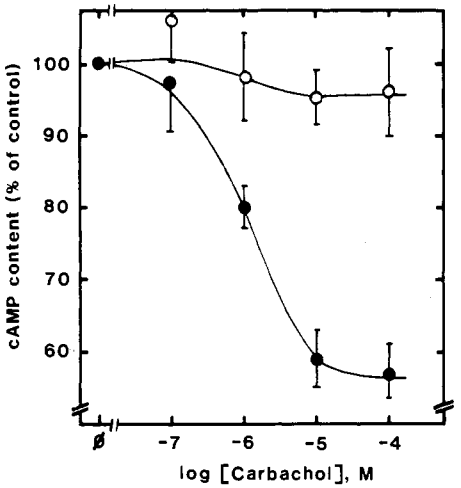

FIGURE 2: Concentration-dependent inhibition of forskolin-stimulated cAMP accumulation by carbachol in cells cultured with or without 5 ng/mL IAP. Cells were incubated with forskolin (100 μM) and carbachol for 5 min, and the cellular content of cAMP was determined and expressed as percent of control (without carbachol added). (●) Non-IAP-treated cells; (○) IAP-treated cells. Each point represents the mean ± SEM from six determinations. Forskolin-stimulated cAMP levels were 2930 ± 95 and 2579 ± 147 pmol/mg of protein (mean ± SEM) for non-IAP-treated and IAP-treated cells, respectively. Values for non-IAP-treated cells are significantly different from values for IAP-treated cells at 10^{-6} M carbachol ($p < 0.025$), 10^{-5} M carbachol ($p < 0.005$), and 10^{-4} M carbachol ($p < 0.005$). (ϕ) No carbachol addition.

3 and 7). For subsequent experiments, cells were treated with 5 ng/mL IAP for 24 h to covalently modify all available N_o and N_i.

The concentration–effect curve for carbachol inhibition of forskolin (100 μM) stimulated cAMP accumulation in control and IAP-pretreated cells is shown in Figure 2. In control cells, carbachol inhibited forskolin-stimulated cAMP accumulation with an IC_{50} of 1 μM; maximal inhibition was 43%. IAP treatment completely blocked inhibition at all concentrations of carbachol tested. McMahon et al. (1985) recently reported that treatment of hatched chicks with IAP resulted in covalent modification of 80–85% of cardiac N_i and abolished guanine nucleotide regulation of agonist binding to cardiac membranes but had no effect on GTP-dependent mAChR-mediated inhibition of adenylate cyclase activity. They conclude that the chick heart mAChR–adenylate cyclase system differs from the classical inhibitory receptor systems with respect to block of function by IAP treatment. However, at a time in development when atria contain low levels of mature N proteins, mAChR-mediated inhibition of adenylate cyclase activity is present, even though guanine nucleotides are relatively ineffective in altering agonist binding (Halvorsen & Nathanson, 1984). It is therefore likely that the remaining 15–20% active N_i observed by McMahon et al. was sufficient to maintain mAChR-mediated inhibition of adenylate cyclase. The results in Figure 2 show that covalent modification of $N_{o\alpha}$ and $N_{i\alpha}$ by IAP completely abolishes the ability of the mAChR to inhibit cAMP accumulation in chick cardiac cells, demonstrating that chick heart does not differ from other cardiac tissues with respect to block of receptor-mediated inhibition of cAMP formation by IAP treatment.

The effects of IAP treatment on muscarinic agonist binding in cardiac membrane homogenates have been well characterized. However, similar binding studies with intact cells have not been reported. We compared the effects of IAP treatment on agonist binding to the mAChR both in intact cells and membrane homogenates. The affinity of the mAChR for

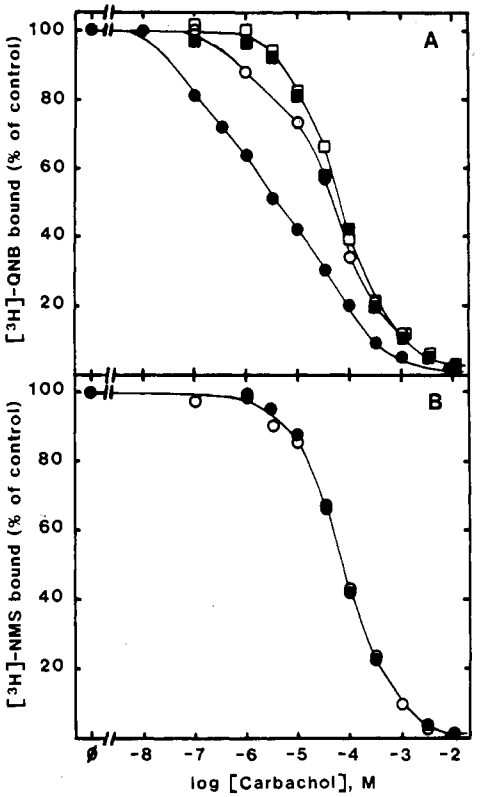

FIGURE 3: Carbachol competition of ^{3}H-labeled antagonist binding to intact cells, or membranes from intact cells, cultured with or without 5 ng/mL IAP. Carbachol competition binding experiments were conducted as described under Materials and Methods. In (A), membranes from cells cultured with (■, □) or without (●, ○) IAP were incubated with [^{3}H]QNB and various concentrations of carbachol. Assays were conducted in the presence (○, □) or absence (●, ■) of 10 μM Gpp(NH)p. In (B), intact cells cultured with (○) or without (●) IAP were incubated with [^{3}H]NMS and increasing concentrations of carbachol. Data are plotted as percent of maximal ^{3}H-labeled antagonist bound and represent the average of two independent experiments, with standard deviations of less than 10% in all cases. (ϕ) No carbachol addition.

agonist was determined by measuring the ability of carbachol to compete with [^{3}H]QNB binding to the mAChR in membrane homogenates. In membranes from control cells (not treated with IAP), carbachol competed for [^{3}H]QNB binding sites with an IC_{50} of 3.2×10^{-6} M (Figure 3A). Addition of Gpp(NH)p to the assay shifted the IC_{50} 14-fold to the right. In membranes from IAP-treated cells, the IC_{50} for carbachol was increased 17-fold with respect to control membranes and was similar to the IC_{50} for carbachol in control membranes in the presence of Gpp(NH)p. Consistent with previous reports in both rat heart (Kurose & Ui, 1983) and chick heart (McMahon et al., 1985), there was no further shift in affinity upon addition of Gpp(NH)p to IAP-treated membranes.

The affinity of the mAChR for carbachol in intact cardiac cells was measured by carbachol competition for [^{3}H]NMS (a membrane-impermeant antagonist) binding sites (Figure 3B). Consistent with previous results (Nathanson, 1983), carbachol bound to the mAChR on intact cells with an IC_{50} (7.9×10^{-5} M) similar to the IC_{50} (4.5×10^{-5} M) for carbachol in membranes in the presence of Gpp(NH)p. Pretreatment with IAP had no effect on mAChR affinity for carbachol in intact cells. The mAChR on intact cells binds agonist with low affinity, presumably because of the presence of high levels of endogenous GTP (Nathanson, 1983). IAP treatment uncouples the receptor from N proteins but has no further effect on agonist binding as the receptor is already in the low affinity state.

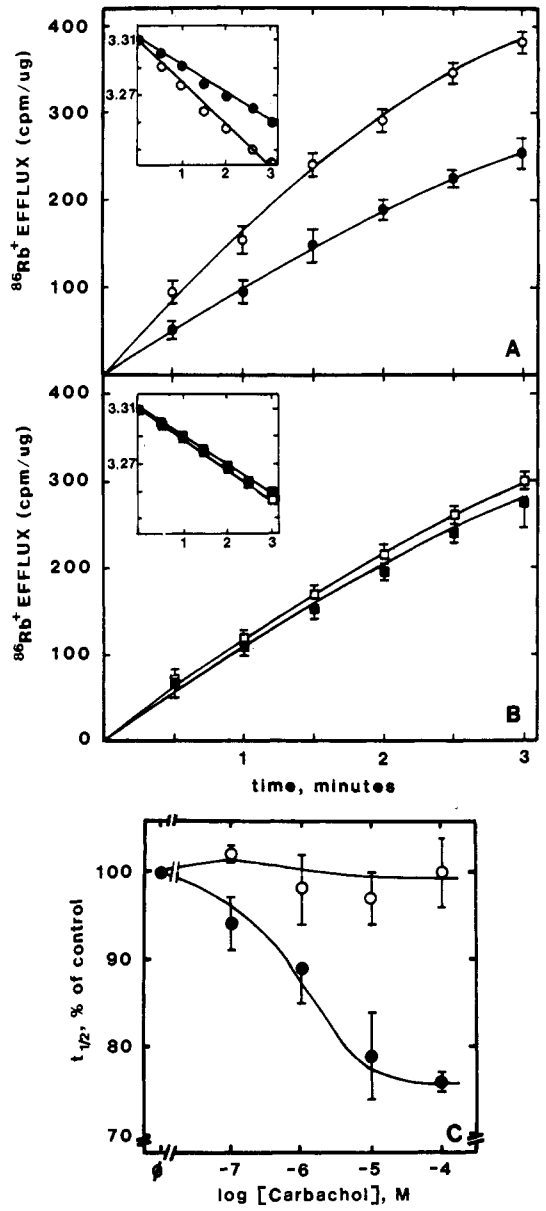

FIGURE 4: Effect of IAP treatment on carbachol-stimulated $^{86}Rb^+$ efflux from cardiac cell monolayers. Medium was replaced on day 3 in culture with medium containing [^{3}H]leucine without or with 5 ng/mL IAP. On day 4, carbachol stimulation of $^{86}Rb^+$ efflux was determined. (A, B) At $t = 0$, fresh medium containing no addition (●, ■) or 10 μM carbachol (○, □) was added to wells containing cells treated without (A) or with (B) IAP. Data are plotted as cpm of $^{86}Rb^+$ efflux/μg of protein (mean ± SD) vs. time. Each curve represents data obtained from triplicate wells. The inset shows data replotted as log $^{86}Rb^+$ remaining vs. time for determination of efflux half-times (see Materials and Methods); correlation coefficients are >0.99. (C) Concentration–effect curve for carbachol stimulation of $^{86}Rb^+$ efflux in non-IAP-treated (●) and IAP-treated (○) cells. Increasing concentrations of carbachol were added to triplicate wells at $t = 0$, and half-times of efflux were determined as described under Materials and Methods. Data represent the average half-times (determined as shown in parts A and B), from five experiments, each done in triplicate ($N = 15$ wells), and are plotted as percent of control half-times for each experiment. Values for non-IAP-treated cells are significantly different from values for IAP-treated cells at 10^{-7} M carbachol ($p < 0.025$), 10^{-5} M carbachol ($p < 0.01$), and 10^{-4} M carbachol ($p < 0.0005$). (ϕ) No carbachol addition.

Activation of cardiac mAChR increases the permeability of the membrane to K^+ ions, resulting in membrane hyperpolarization and a decrease in beating rate (Trautwein & Dudel, 1958). $^{86}Rb^+$ is a convenient K^+ substitute that has been used to study the permeability of K^+ in neuronal cells, glands, and smooth muscle (Arner & Stallcup, 1981; Putney, 1976; Bolton & Clapp, 1984). To investigate whether N_o and/or N_i are (is) involved in mAChR-mediated increases in K^+ permeability, we determined the effect of IAP treatment on the stimulation of $^{86}Rb^+$ efflux by carbachol. Activation of the mAChR in cardiac monolayer cultures by agonist results in the stimulation of $^{86}Rb^+$ efflux, which is both atropine-sensitive and ouabain-insensitive (Hunter & Nathanson, 1985). Parts A and B of Figure 4 show representative efflux curves obtained from triplicate wells containing cells treated without or with IAP, respectively. In non-IAP-treated cells (part A), carbachol (10 μM) caused a 50% increase in $^{86}Rb^+$ efflux; the half-time of $^{86}Rb^+$ efflux in the presence of carbachol was 66% of control, decreasing from 14.8 min in the control to 9.8 min with carbachol (inset). Carbachol had no significant effect on efflux of $^{86}Rb^+$ from IAP-treated cells (part B); the half-times of $^{86}Rb^+$ efflux in the presence and absence of carbachol were 13.7 and 14.7 min, respectively. Figure 4C shows the mean carbachol concentration–effect curve for stimulation of $^{86}Rb^+$ efflux from cells treated with or without IAP. In cells not treated with IAP, carbachol stimulated $^{86}Rb^+$ efflux with an EC_{50} of 0.8 μM. The half-time was decreased to 76 ± 1% (mean ± SEM) of control by carbachol, similar to the maximal effect of carbachol on efflux of $^{42}K^+$ from cultured chick cardiac cells reported by Galper et al. (1982). Treatment with IAP completely blocked the effect of carbachol on $^{86}Rb^+$ efflux. At 100 μM carbachol, the half-time for IAP-treated cells was 100 ± 2% of control (without carbachol). The average half-times measured in the absence of carbachol in untreated and IAP-treated cells were 13.9 ± 0.9 (mean ± SEM) and 13.6 ± 1.0 min, respectively. Thus treatment with IAP does not alter resting $^{86}Rb^+$ efflux. The average equilibrium $^{86}Rb^+$ content in non-IAP-treated and IAP-treated cells was 2010 ± 176 (mean ± SEM) and 2128 ± 105 cpm/μg of protein, respectively, indicating that the absence of a response in IAP-treated cells was not due to a decrease in $^{86}Rb^+$ content, which would reduce the chemical gradient for efflux.

There is considerable electrophysiological evidence that changes in intracellular cAMP levels are not involved in mAChR-mediated increases in K^+ permeability (Trautwein et al., 1982; Nargeot et al., 1983). Our results show that, in addition to inhibition of mAChR-mediated changes in cAMP metabolism and guanine nucleotide regulation of agonist binding, treatment of intact cells with IAP prevents mAChR-mediated increases in K^+ permeability. These biochemical data are consistent with electrophysiological results showing that IAP treatment blocks mAChR-mediated hyperpolarization of the heart (Endoh et al., 1985; Sorota et al., 1985). We have recently demonstrated that intracellular GTP is required for and that IAP treatment blocks acetylcholine-induced increases in inward rectifying K^+ currents as determined by whole cell voltage clamp experiments (Pfaffinger et al., 1985). These results provide evidence that the mAChR is coupled, possibly directly, to K^+ channels in the heart via guanine nucleotide regulatory protein(s) closely related to, or identical with, N_o and/or N_i.

Registry No. cAMP, 60-92-4; K, 7440-09-7; carbachol, 51-83-2; rubidium, 7440-17-7.

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Articles

Effect of Medium Hypertonicity on Reovirus Translation Rates. An Application of Kinetic Modeling in Vivo†

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ABSTRACT: Translation rates were determined for host and virus mRNAs in reovirus-infected SC-1 cells in hypertonic medium. The effect of low doses of cycloheximide on these translation rates was also measured. The results show that hypertonicity selectively stimulates viral translation relative to host translation. Moreover, in hypertonic medium, host translation is slightly stimulated by low doses of cycloheximide, whereas viral translation is markedly inhibited. This effect of cycloheximide is precisely the opposite to what was previously observed in isotonic media [Walden, W. E., Godefroy-Colburn, T., & Thach, R. E. (1981) *J. Biol. Chem. 256*, 11739–11746]. It is shown that both these effects of hypertonicity are predicted by the message competition/discrimination model previously described and thus provide support for the applicability of certain aspects of the model to translation rates in vivo.

Over the past 10 years, considerable evidence has accrued that suggests that message discriminatory initiation factors, in addition to 40S ribosomes, can influence the individual initiation rate for different types of mRNA (Lodish, 1974; Golini et al., 1976; Kabat & Chappell, 1977; DiSegni et al., 1979; Gette & Heywood, 1979; Heywood & Kennedy, 1979; Herson et al., 1979; Parets-Soler et al., 1981; Rosen et al., 1982; Ray et al., 1983; Sarkar et al., 1984; Godefroy-Colburn et al., 1985). It has been suggested that in vivo the concentration of mRNA is higher than that of one such discriminatory factor: thus, mRNAs with the highest affinities for factor outcompete those with lower affinities, and thereby are translated at higher rates (Walden et al., 1981; Godefroy-Colburn & Thach, 1981). While this theory, which we call the "message competition/discrimination (CD)[1] model", is consistent with a large amount of experimental data, further applications and tests of its accuracy in vivo are desirable.

Such an applicability test of the CD model is described in this paper. This is based on the translational response of

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; eIF, eukaryotic initiation factor; TCA, trichloroacetic acid; R*, the 43S ribosomal complex; M*, the complex between mRNA and discriminatory factor; CD, competition/discrimination; MEM, minimal essential medium.

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