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Modification of G Protein-Coupled Functions by Low-pH Pretreatment of Membranes from NG108–15 Cells: Increase in Opioid Agonist Efficacy by Decreased Inactivation of G Proteins

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SUMMARY

Low-pH pretreatment increases opioid agonist efficacy in inhibiting adenylyl cyclase in brain membranes. The mechanism of this effect was examined in membranes from cultured NG108–15 cells. Pretreatment of NG108–15 membranes at pH 4.5 before assay at pH 7.4 produced the following modifications in G protein-mediated signal transduction: 1) decreased activation of adenylyl cyclase by G_s, 2) increased maximal inhibition of opioid agonist binding by sodium and by guanine nucleotides in the presence of sodium, and 3) increased maximal inhibition of adenylyl cyclase by agonists for G_i-coupled receptors. These results are similar to those previously observed in rat brain membranes. The mechanism by which low-pH pretreatment increased receptor-mediated inhibition of adenylyl cyclase was investigated further by examining low-K_m GTPase activity in low-pH-pretreated NG108–15 cell membranes. Low-pH pretreatment decreased basal and agonist-stimulated low-K_m GTPase activity maximally in the absence of sodium and minimally in the presence of 120 mM NaCl. This change was due to a decrease in the V_{max} of the enzyme, with no change in the K_m for GTP, indicating that GTP hydrolysis was decreased without any decrease in the affinity of the G protein for GTP. Scatchard analysis revealed no decrease in the B_{max} for high affinity opioid agonist binding, and Western blot analysis with a G_i-specific antibody revealed no loss of G_i protein, in low-pH-pretreated membranes. Moreover, concentration-effect curves for GTP in supporting opioid inhibition of adenylyl cyclase showed that low-pH pretreatment increased inhibition by the agonist only at GTP concentrations equal to or greater than the K_m for GTP hydrolysis by the low-K_m GTPase. Taken together, these results indicate that the efficacy of receptor-mediated inhibition of adenylyl cyclase can be increased by decreasing the maximal inactivation rate of G_i subsequent to its activation by the receptor.

The mouse neuroblastoma × rat glioma hybrid cell line NG108–15 has been a useful model system for the study of opioid receptors and the effectors to which they are coupled. These cells contain only δ-type opioid receptors (1), which are coupled to PTX-sensitive G proteins consisting of at least three immunologically identified α unit subtypes, $G_{i2\alpha}$, $G_{o2\alpha}$, and $G_{i3\alpha}$ (2). Opioid agonists inhibit adenylyl cyclase activity in NG108–15 cell membranes (3) in a sodium- and GTP-dependent manner (4), and this action is blocked by prior treatment of the cells with PTX (5). Similarly, opioid agonists stimulate low-K_m GTPase activity in NG108–15 cell membranes in a sodium- and magnesium-dependent (6) and PTX-sensitive manner (7). Both of these effects of δ-opioid receptor activation appear to be mediated by G_{i2}, because they are blocked by antisera specific for this G protein (8). Thus, the NG108–15 cell line provides a homologous system for the study of δ-opioid receptor- and G_{i2}-mediated inhibition of adenylyl cyclase.

The relationship between δ receptors and G proteins has been extensively characterized in NG108–15 cells. The coupling between receptor and G protein appears to be "tight," because progressive alkylation of δ receptors with an irreversible opioid antagonist decreases maximal agonist stimulation of low-K_m GTPase in a manner that correlates with the loss of agonist binding sites (9). However, the coupling between the inhibitory G protein and adenylyl cyclase appears to be "loose," because loss of opioid inhibition of adenylyl cyclase occurs only at concentrations of alkylating agent higher than those required for loss of the GTPase response and occurs as an initial increase in IC_{50} followed by a decrease in maximal responsiveness at higher concentrations of the antagonist (9, 10). These findings are in agreement with indirect evidence for a physical association between δ-opioid receptors and G proteins in NG108–15

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ABBREVIATIONS: PTX, pertussis toxin; NTI, naltrindole; DSLET, D-Ser^2,Thr^6-Leu-enkephalin; DPDPE, D-Pen^{2,5}-enkephalin; DTT, dithiothreitol; BSA, bovine serum albumin; App(NH)p, adenylyl-5′-imidodiphosphate; Gpp(NH)p, guanylyl-5′-imidodiphosphate; GTPγS, guanosine-5′-*O*-(γ-thio)triphosphate; PGE_2, prostaglandin E_2; CTX, cholera toxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.

cell membranes (11, 12) and may explain why desensitization of the opioid response occurs more rapidly for low-K_m GTPase than for adenylyl cyclase upon chronic exposure of NG108–15 cells to opioid agonists (13).

Sodium plays a crucial role in regulating opioid receptor-G protein interactions. Sodium decreases basal low-K_m GTPase activity (14), probably by inhibiting the spontaneous activation of G proteins by unoccupied receptors (15), but does not inhibit agonist-stimulated GTPase. In fact, sodium is required to observe agonist-stimulated GTPase, and the absence of sodium is necessary to observe inhibition of GTPase by opioid antagonists of negative intrinsic activity (15). Another effect of sodium on this system is inhibition of high affinity agonist binding to the receptor. The sodium site responsibe for inhibition of agonst binding is internal to the plasma membrane (16) and may be located on the receptor itself, by analogy with the α_2-adrenergic receptor (17). Whether this same site is involved in regulation of the G protein interactions of the receptor is unknown.

Although the δ receptor-G protein interaction in NG108–15 cell membranes has been extensively characterized, the factors that determine the efficacy of receptor-mediated effects on adenylyl cyclase are poorly understood. One method to explore mechanisms regulating agonist efficacy is low-pH pretreatment of membranes. Previous studies in brain membranes (18–20) showed that low-pH pretreatment 1) decreased stimulation of adenylyl cyclase by G_s, with no change in basal activity, 2) increased opioid receptor-mediated inhibition of adenylyl cyclase, and 3) increased inhibitory effects of sodium and guanine nucleotides on opioid agonist binding, with no change in binding in the absence of these agents. NG108–15 cells offer an excellent model system to examine the mechanism of the low-pH effect, because they contain several G_i-linked receptor systems in addition to δ-opioid receptors and because they are a good system to assay receptor-coupled low-K_m GTPase. The goals of the present study were to 1) determine whether low-pH pretreatment of NG108–15 cell membranes produces effects on G protein function similar to those observed in rat brain membranes, 2) study G_i activation directly by examining low-K_m GTPase activity in low-pH-pretreated NG108–15 cell membranes, and 3) elucidate the fundamental mechanisms by which low-pH pretreatment enhances receptor-mediated inhibition of adenylyl cyclase. These studies indicated that low-pH pretreatment enhanced the efficacy of opioid receptor-mediated inhibition of adenylyl cyclase by attenuating GTPase activity. These investigations may provide insight into the factors that control receptor efficacy in G_i-coupled receptor systems.

Experimental Procedures

Materials. [^{3}H]DPDPE (34.3 Ci/mmol), [^{3}H]NTI (25.9 Ci/mmol), [γ-^{32}P]GTP (30 Ci/mmol), ^{125}I-Protein A (8.81 μCi/μg), and G protein-specific antisera (anti-$G_{\alpha common}$, and anti-$G_i\alpha 2$) were purchased from New England Nuclear Corp. (Boston, MA). [α-^{32}P]ATP (25 Ci/mmol) and Ecolite scintillation fluid were obtained from ICN Radiochemicals (Irvine, CA). D-Ala^2,Met^5-enkephalinamide and DSLET were purchased from Peninsula Laboratories (Belmont, CA). WIN 55212–2 was a kind gift from Dr. S. Ward, Sterling Research Group (Sterling Drug Inc., Rensselaer, NY). Dulbecco's modified Eagle's medium, ATP, App(NH)p, phosphocreatine (di-sodium and di-Tris salts), creatine phosphokinase, ouabain, BSA, hypoxanthine/aminopterin/thymidine medium supplement, forskolin, carbachol, and Tween-20 were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was purchased from GIBCO Laboratories (Grand Island, NY). Acrylamide and Duralose reinforced nitrocellulose were obtained from Stratagene (La Jolla, CA). All unlabeled guanine nucleotides, including GTP, GTPγS, and Gpp(NH)p, were purchased from Boehringer Mannheim (New York, NY). All other chemicals (reagent grade) were obtained from Sigma or from Fisher. NG108–15 cells were a generous gift from Dr. W. Klee (National Institute of Mental Health, Bethesda, MD).

Cell culture and membrane preparation. Cells were cultured at 37° in a humidified atmosphere of 5% CO_2/95% air, in Dulbecco's modified Eagle's medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2.5 mg/ml amphotericin B, 5% fetal bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. For preparation of membranes for binding and adenylyl cyclase assays, cells were harvested and centrifuged at 345 × g for 10 min. The pellet was resuspended in 50 volumes of 50 mM Tris·HCl, pH 7.4, with 1 mM EGTA, and was homogenized with a Polytron. The homogenate was centrifuged at 500 × g for 10 min, and the supernatant was placed on ice. The pellet was resuspended in Tris-EGTA and centrifuged at 500 × g for 10 min. The two supernatants were combined and centrifuged at 26,700 × g for 20 min at 4°. The resulting pellet was then resuspended in Tris-EGTA, and aliquots were stored at −80°. For low-K_m GTPase assays, membranes were prepared as described by Vachon *et al.*, (13), with some modification. Briefly, cells were homogenized in Tris-EGTA containing 1 mM DTT and 0.32 M sucrose (buffer A), with a Dounce homogenizer. The homogenate was centrifuged at 1000 × g for 10 min at 4°, and the supernatant was removed and placed on ice. The pellet was resuspended in buffer A and centrifuged at 1000 × g for 10 min. The two supernatants were combined and centrifuged at 26,700 × g for 20 min at 4°. The membrane pellet was resuspended in buffer A without sucrose (buffer B) and was centrifuged at 26,700 × g for 30 min. Membranes were resuspended in buffer B, homogenized with a Dounce homogenizer, and stored in aliquots at −80°. Membrane protein levels were determined by the method of Bradford (21).

Low-pH pretreatment. Membranes were recovered by centrifugation at 48,000 × g for 10 min at 4° and were pretreated at low pH (18, 20) by resuspension in pH 4.5 buffer (50 mM sodium acetate, 1 mM DTT, 5 mM $MgCl_2$, pH 4.5) (1 ml of buffer/mg of protein) and incubation on ice for 10 min. Control membranes were pretreated in pH 7.4 buffer (50 mM Tris-acetate, 50 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, pH 7.4) as described above. The incubations were terminated by addition of 6–8 volumes of Tris. Membranes were isolated by centrifugation at 48,000 × g for 10 min at 4° and were resuspended in 50 mM Tris, pH 7.4, 3 mM $MgCl_2$, 0.2 mM EGTA (TME buffer).

Adenylyl cyclase assay. Membranes (30–50 μg of protein) were incubated for 15 min at 30°, with or without various drugs, in TME buffer containing 50 μM ATP, [α-^{32}P]ATP (1.5 μCi), 0.2 mM DTT, 0.01% BSA, 50 μM cAMP, 50 μM GTP, 120 mM NaCl, 10 mM theophylline, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase, in a final volume of 100 μl. In some experiments, 120 mM NaCl was replaced by 0–120 mM NaCl and the di-Tris salt of phosphocreatine was used or 50 μM GTP was replaced by 0–100 μM GTP. The reaction was terminated by boiling in a waterbath for 2 min. [^{32}P]cAMP was isolated by the method of Salomon (22). Radioactivity was determined by liquid scintillation counting (35% efficiency for ^{3}H) after 4 ml of the eluate were dissolved in 12 ml of Ecolite scintillation fluid.

Opioid receptor binding. Membranes (30–50 μg of protein) were incubated in TME buffer containing 0.01% BSA and 3 nM [^{3}H]DPDPE or 2 nM [^{3}H]NTI, in a final volume of 1 ml. Nonspecific binding was defined as the difference between total binding and binding in the presence of 5 μM D-Ala^2,Met^5-enkephalinamide. Saturation experiments were performed by addition of 0–100 nM unlabeled DPDPE. Tubes were incubated at 25° for 90 min, and reactions were terminated by rapid filtration through Whatman GF/B glass fiber filters. Bound radioactivity was determined by liquid scintillation counting (50% efficiency) after overnight extraction of the filters in 5 ml of Ecolite scintillation fluid.

Low-K_m GTPase assays. Low-K_m GTPase was assayed as de-

scribed previously (23), with some modification. Membranes (3–5 μg of protein) were incubated for 20 min at 30°, with or without various drugs, in TME buffer containing 0.5 μM GTP (including 0.1 μCi of [γ-^{32}P]GTP), 0.2 mM DTT, 0.01% BSA, 120 mM NaCl, 1 mM ATP, 1 mM App(NH)p, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase, in a final volume of 100 μl. Nonspecific ("high"-K_m) GTP hydrolysis was determined by the inclusion of 50 μM GTP. In some experiments, 120 mM NaCl was replaced by 0–120 mM NaCl and the di-Tris salt of phosphocreatine was used and/or 0.5 μM GTP was replaced by 0.1–2.5 μM GTP. The reaction was terminated by boiling in a waterbath for 2 min, followed by the addition of 100 μl of 40 mM phosphoric acid. After the addition of 800 μl of a 5% activated charcoal suspension in 20 mM phosphoric acid, the tubes were centrifuged at 2600 × *g* for 30 min. Radioactivity was determined after 700-μl aliquots of the supernatant were dissolved in 5 ml of Ecolite scintillation fluid. Nonspecific activity (high-K_m GTPase plus nonenzymatic hydrolysis) accounted for 50–75% of total ^{32}P released.

Electrophoresis and Western blots. Membranes, pretreated at pH 7.4 or pH 4.5, were dissolved in a solution containing (final concentrations) 87.5 mM Tris·HCl, pH 6.8, 25 mM NaCl, 1.5 mM $MgSO_4$, 0.5 mM EGTA, 2% SDS, 5% glycerol, 10 mM DTT, and 0.00125% bromphenol blue and were boiled for 5 min. Samples containing equal amounts of protein (50–75 μg) were placed on a 7–20% linear gradient polyacrylamide slab gel and electrophoresis was conducted at 25 mA for 30 min, followed by 2.5 hr at 50 mA. Proteins were electrophoretically transferred to a reinforced nitrocellulose (Duralose) membrane at 100 V for 1.5 hr. All subsequent procedures were performed at room temperature with gentle agitation. The nitrocellulose was washed for 3 × 5 min in 10 mM Tris·HCl, pH 7.5, with 0.9% NaCl (TBS) and was then immersed in blocking solution (TBS with 0.05% Tween-20 and 1% BSA) for 1 hr. The blocking solution was decanted and the nitrocellulose was washed with TBS for 2 × 5 min. The wash solution was discarded, and the G protein antibody solution was added at a dilution of 1/1000 in TBS-Tween with 1% BSA and was incubated with the nitrocellulose for 18–24 hr. After the primary antiserum solution was decanted, the nitrocellulose was washed for 3 × 5 min in TBS-Tween with 1% BSA, followed by the addition of 10^6 cpm of ^{125}I-Protein A in TBS-Tween with 1% BSA. The incubation continued for 1 hr, after which the membrane was removed from the ^{125}I-Protein A solution and washed in TBS-Tween with 1% BSA for 5 × 5 min. The immunoblot was then dried and exposed to Kodak XAR film, in a cassette with an intensifying screen, for 18–24 hr at −80°. The blot was then aligned with the developed film, for excision of the radiolabeled bands. Bound radioactivity was determined by analysis with a γ counter.

Data analysis. Unless otherwise indicated, data are reported as mean ± standard error values of at least three separate experiments, which were each performed in triplicate. Scatchard analysis of saturation binding data was performed by computer fitting using EBDA and LIGAND. Statistical significance of data (except for GTPase kinetics) was determined by the two-tailed Student *t* test. Nonlinear regression analysis of GTPase kinetics was performed with JMP (SAS), using an iterative model fit to the Michaelis-Menten equation. The resulting K_m and V_{max} values were analyzed by fitting a multivariate analysis of variance model that accounted for the following conditions: control or low-pH pretreatment, presence or absence of NaCl, and the day to day variability of the reaction not related to the experimental interventions. When significance was indicated in the multivariate analysis, the K_m and V_{max} data were fit separately to a univariate analysis of variance model, to determine the significance of each individual experimental condition.

Results

Effects of low-pH pretreatment on G protein and receptor regulation of NG108–15 adenylyl cyclase activity. Previous studies (19, 20) showed that low-pH pretreatment of rat brain membranes decreased G_s-stimulated, increased opioid-inhibited, and had no effect on basal adenylyl cyclase activity. To determine whether similar actions occur in NG108–15 cells, basal and stimulated adenylyl cyclase activities were examined in control and pH 4.5-treated membranes from NG108–15 cells. In these experiments, membranes from NG108–15 cells were pretreated at pH 4.5 before assay of adenylyl cyclase at pH 7.4, whereas control membranes were pretreated in an identical manner at pH 7.4. To differentiate effects on catalytic unit and G_s-stimulated activity, adenylyl cyclase was stimulated with either forskolin (1 μM), which stimulates the catalytic unit directly (24), PGE_2 (10 μM), which elicits prostaglandin receptor-mediated stimulation of G_s, or NaF (10 mM), which activates G_s directly (25). Fig. 1A shows the effect of low-pH pretreatment on adenylyl cyclase activity directly, whereas in Fig. 1B the same data were calculated as percentage inhibition of activity by DSLET. Low-pH pretreatment did not significantly alter basal adenylyl cyclase activity. Although a slight decrease in forskolin-stimulated adenylyl cyclase activity was observed in low-pH-pretreated membranes (from 380% of basal activity in control membranes to 330% in treated membranes), this decrease was nonsignificant. However, low-pH pretreatment significantly reduced the stimula-

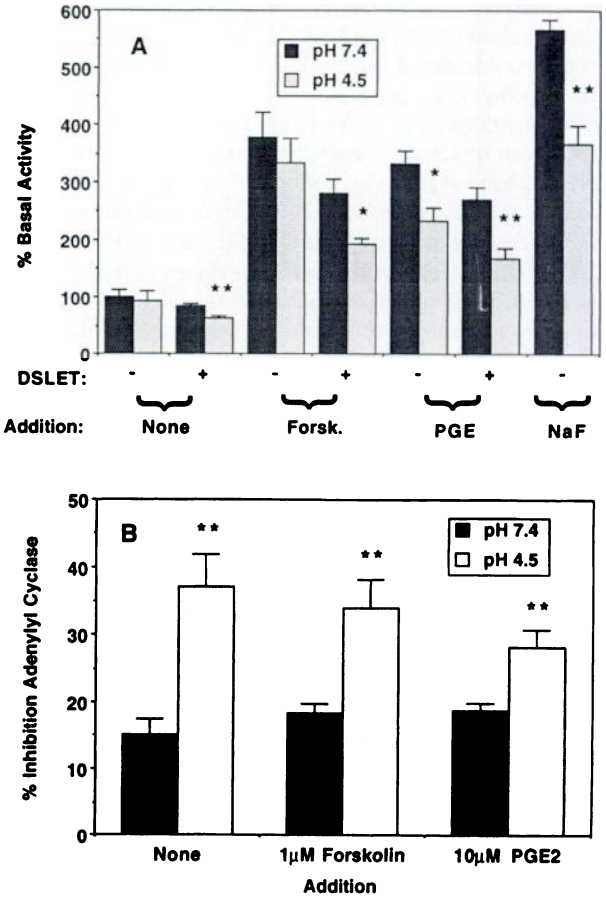

Fig. 1. Effect of low-pH pretreatment on regulation of adenylyl cyclase in NG108–15 cell membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed as described in Experimental Procedures, with or without 1 μM forskolin (*Forsk.*), 10 μM PGE_2, or 10 mM NaF, in the presence or absence of 1 μM DSLET. A, Percentage of basal activity in control membranes; B, percentage inhibition of basal, forskolin-stimulated, or PGE_2-stimulated activity in control or pH 4.5-pretreated membranes. Basal adenylyl cyclase activities were 6.6 ± 0.8 and 6.2 ± 1.2 pmol/mg/min for control and pH 4.5-pretreated membranes, respectively. *, $p < 0.05$; **, $p < 0.01$, different from control membranes.

tion of adenylyl cyclase by PGE_2 (from 330% of basal activity in control membranes to 230% in treated membranes) and NaF (from 570% of basal activity to 370% in treated membranes). The effects of low-pH pretreatment on the inhibition of basal, forskolin-stimulated, and PGE_2-stimulated adenylyl cyclase activity by the δ-opioid agonist DSLET were also examined (Fig. 1). In Fig. 1B, the data were recalculated as percentage inhibition of adenylyl cyclase by 1 μM DSLET under basal, PGE_2-stimulated, and forskolin-stimulated conditions. These results showed that DSLET inhibition was significantly increased in low-pH-pretreated membranes under all three conditions. In each case, low-pH pretreatment approximtely doubled DSLET inhibition, increasing inhibition of basal activity from 15% to 37%, of forskolin-stimulated activity from 19% to 34%, and of PGE_2-stimulated activity from 19% to 28%.

To determine whether the low-pH-induced increase in DSLET inhibition of adenylyl cyclase was produced by an artifactual non-receptor-mediated action of DSLET, adenylyl cyclase was assayed with DSLET in both control and low-pH-pretreated membranes in the presence of 100 nM concentrations of the δ-selective opioid antagonist NTI (data not shown). These results showed that NTI alone had no significant effect on basal activity in either set of membranes. Inhibition of adenylyl cyclase by 100 nM DSLET (34% in low-pH-pretreated membranes, compared with 17% inhibition in control membranes) was completely blocked by addition of 100 nM NTI to either control or low-pH-pretreated membranes.

The effect of low-pH pretreatment on inhibition of basal adenylyl cyclase activity by nonopioid agonists was also investigated. As shown in Fig. 2, neither the muscarinic cholinergic agonist carbachol nor the cannabinoid aminoalkylindole agonist WIN 55212-2 inhibited adenylyl cyclase to the same levels as did DSLET. However, in each case low-pH pretreatment of NG108-15 membranes produced an approximate doubling of agonist-induced inhibition.

Concentration-effect curves for DSLET-inhibited adenylyl cyclase activity were compared in control and low-pH-pretreated NG108-15 cell membranes. As shown in Fig. 3, the inhibitory effect of DSLET was increased in low-pH-pretreated membranes at all concentrations of DSLET examined. The principal effect of low-pH pretreatment was to increase the maximal inhibition by DSLET (18 ± 2% inhibition in pH 7.4-pretreated membranes versus 39 ± 1% inhibition in pH 4.5-pretreated membranes, $p < 0.001$), whereas the IC_{50} value for the agonist was not significantly changed in low-pH-pretreated membranes (4.4 ± 0.8 nM in control versus 7.0 ± 1.1 nM in low-pH-pretreated membranes, $p > 0.05$). This effect of low-pH pretreatment on agonist efficacy was not limited to the opioid system, because carbachol concentration-effect curves were similarly affected by low-pH pretreatment (data not shown).

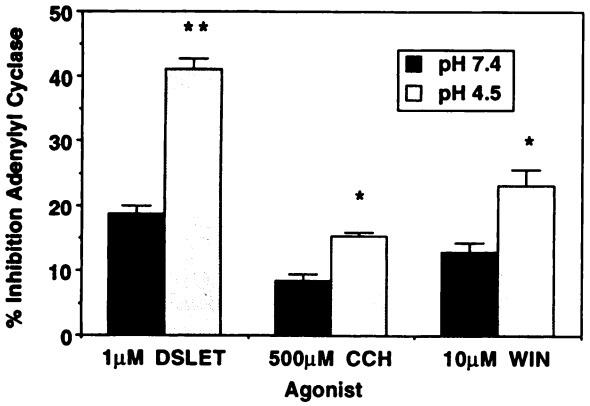

Fig. 2. Effect of low-pH pretreatment on inhibition of adenylyl cyclase by δ-opioid, muscarinic, and cannabinoid agonists. NG108-15 membranes were pretreated at pH 7.4 or pH 4.5 and assayed with or without the indicated concentrations of DSLET, carbachol (*CCH*), or WIN-55,212-2 (*WIN*). Data are expressed as percentage inhibition of basal activity in control or pH 4.5-pretreated membranes. Basal activities were 7.3 ± 0.4 and 8.0 ± 1.1 pmol/mg/min for control and pH 4.5-pretreated membranes, respectively. *, $p < 0.05$; **, $p < 0.01$, different from control membranes.

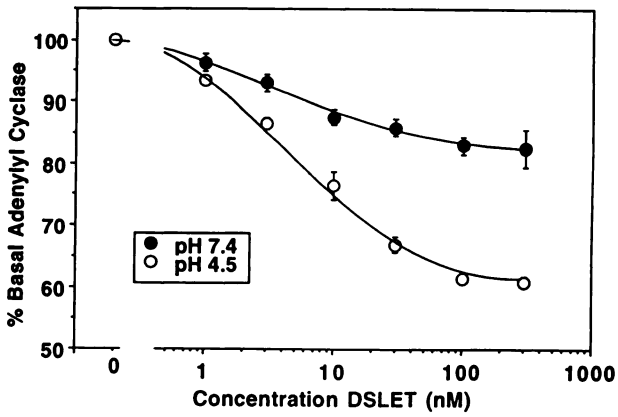

Fig. 3. Concentration-effect curves for DSLET-inhibited adenylyl cyclase in control and low-pH-pretreated NG108-15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with or without DSLET (1-300 nM). Data are expressed as percentage of basal activity in control or pH 4.5-pretreated membranes. Basal activities were 6.8 ± 0.5 and 8.0 ± 1.4 pmol/mg/min for control and pH 4.5-pretreated membranes, respectively.

Because sodium is required for opioid inhibition of adenylyl cyclase, the effect of varying sodium concentrations on adenylyl cyclase activity in control and low-pH-pretreated NG108-15 membranes was examined in the presence and absence of 1 μM DSLET. As shown in Fig. 4A, increasing the NaCl concentration from 0 to 120 mM decreased basal adenylyl cyclase activity by 69% in control membranes. Low-pH pretreatment had no effect on basal activity in the presence of 120 mM NaCl, confirming results obtained previously (Fig. 1) with the NaCl concentration held constant at 120 mM. In contrast, basal adenylyl cyclase activity in the absence of sodium was decreased by 30% in low-pH-pretreated membranes, compared with control. Furthermore, the inhibitory effect of low-pH pretreatment on adenylyl cyclase decreased with increasing sodium, and the ability of sodium to inhibit adenylyl cyclase in low-pH-pretreated membranes was reduced, compared with control membranes (maximal inhibition of 45% in pH 4.5-pretreated membranes versus 69% in control membranes).

The inhibitory effect of 1 μM DSLET on adenylyl cyclase activity as a function of sodium concentration is shown in Fig. 4B, where the percentage of basal activity at each concentration of NaCl in control and low-pH-pretreated membranes is plotted as a function of increasing NaCl concentration. As expected, DSLET-induced inhibition of the enzyme was sodium dependent in both control and low-pH-pretreated membranes. However, the magnitude of inhibition by DSLET was greater in low-pH-pretreated membranes, compared with control membranes, at all NaCl concentrations studied. The effects of sodium and of low-pH pretreatment on maximal inhibition of adenylyl cyclase by DSLET were additive.

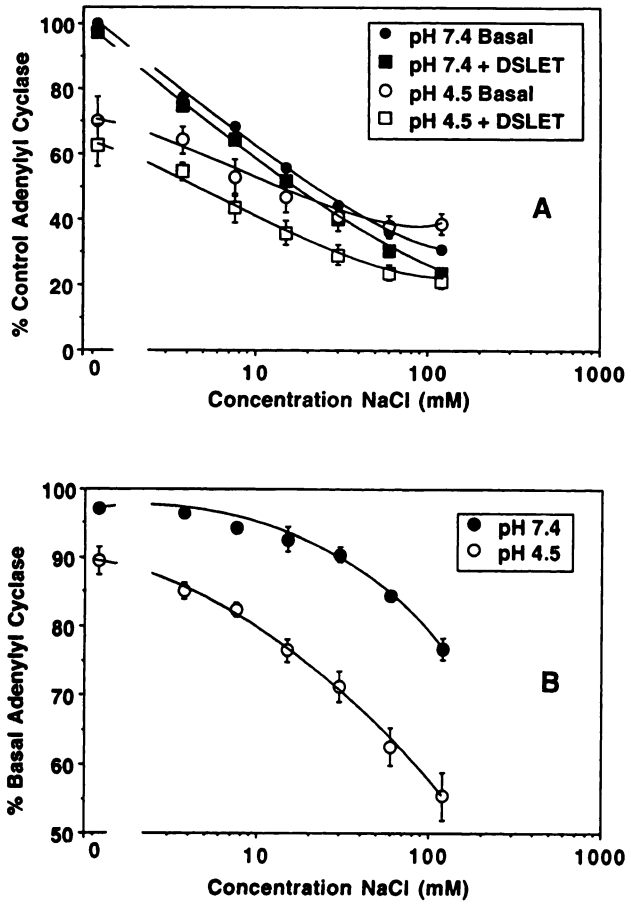

Fig. 4. Effect of sodium on basal and DSLET-inhibited adenylyl cyclase in control and low-pH-pretreated NG108–15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with or without NaCl (3.75–120 mM), in the presence or absence of 1 μM DSLET. A, Percentage of basal activity in control membranes in the absence of NaCl; B, percentage of basal activity in control or pH 4.5-pretreated membranes at each concentration of NaCl. Basal activity was 27.6 ± 2.7 pmol/mg/min in control membranes in the absence of NaCl.

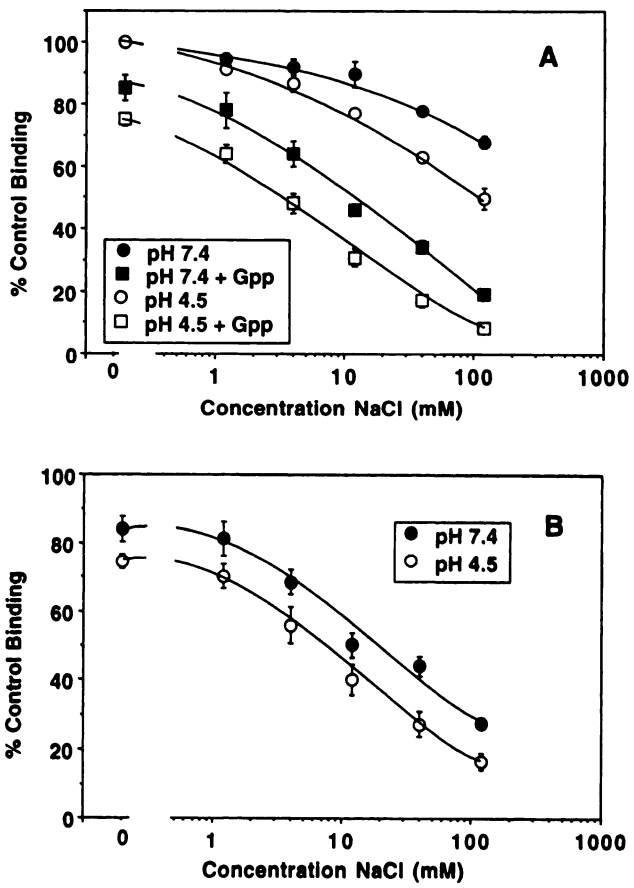

Fig. 5. Effect of low-pH pretreatment on modulation by sodium and Gpp(NH)p (*Gpp*) of opioid agonist binding to NG108–15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with 3 nM [^{3}H]DPDPE, with or without NaCl (1.2–120 mM), in the presence or absence of 50 μM Gpp(NH)p. A, Percentage of binding to control or pH 4.5-pretreated membranes in the absence of NaCl or Gpp(NH)p; B, percentage of binding to control or pH 4.5-pretreated membranes in the presence of NaCl alone at each concentration of NaCl. [^{3}H]DPDPE binding in the absence of NaCl and Gpp(NH)p was 441 ± 67 and 670 ± 117 fmol/mg of protein in control and pH 4.5-pretreated membranes, respectively.

Effects of low-pH pretreatment on modulation of opioid agonist binding by sodium and guanine nucleotides. Previous studies (18) showed that low-pH pretreatment increased sodium and guanine nucleotide regulation of opioid agonist binding in brain membranes. To determine whether similar actions occur in NG108–15 cells, the effect of sodium and Gpp(NH)p on agonist binding to δ-opioid receptors was investigated in control and low-pH-pretreated NG108–15 membranes. Fig. 5 shows the effect of low-pH pretreatment on [^{3}H]DPDPE binding in the presence of various concentrations of NaCl, with and without 50 μM Gpp(NH)p. Results (Fig. 5A) showed that low-pH pretreatment increased the sodium-induced inhibition of [^{3}H]DPDPE binding from 32 ± 2% in control membranes to 50 ± 3% in pH 4.5-pretreated membranes ($p < 0.01$). The potency of NaCl in inhibiting [^{3}H]DPDPE binding was relatively unchanged (IC_{50} value for NaCl was 21 ± 1 mM in control membranes versus 16 ± 1 mM in low-pH-pretreated membranes). In the absence of sodium, the inhibition of [^{3}H]DPDPE binding by 50 μM Gpp(NH)p was slightly increased in low-pH-pretreated membranes (15 ± 4% in control membranes versus 25 ± 2% in pH 4.5-pretreated membranes), although this change was not statistically significant. In independent experiments, the effects of Gpp(NH)p, GTP, and GTPγS on [^{3}H]DPDPE binding were examined in the absence of sodium. Unlike the results of previous experiments in rat brain membranes (18), no significant difference in the maximal inhibition of opioid agonist binding by the nucleotides was observed between control and low-pH-pretreated membranes in the absence of sodium (data not shown).

In Fig. 5B, the binding data obtained in the presence of Gpp(NH)p (from Fig. 5A) have been corrected for the effect of NaCl, at each concentration of NaCl, so that the effect of low-pH pretreatment on guanine nucleotide regulation of binding can be observed as a function of NaCl concentration. These results showed that inhibition of [^{3}H]DPDPE binding by Gpp(NH)p increased with increasing NaCl concentrations in both control and treated membranes. Low-pH pretreatment produced a small increase in Gpp(NH)p inhibition of agonist binding that was relatively constant over the range of NaCl concentrations when the data were corrected for the effect of sodium alone. Therefore, low-pH pretreatment of NG108–15 cell membranes magnified the inhibitory effects of sodium alone and of Gpp(NH)p in the presence of sodium on agonist binding to δ-opioid receptors.

Effects of low-pH pretreatment on NG108–15 low-K_m GTPase activity. To directly measure opioid receptor-mediated stimulation of G protein activity, the effect of DSLET on low-K_m GTPase activity was assayed in control and low-pH-pretreated NG108–15 cell membranes under conditions that favored maximal agonist-induced inhibition of adenylyl cyclase in the previous experiments (i.e., in the presence of 120 mM NaCl). As shown in Fig. 6, low-K_m GTPase activity was stimulated by DSLET similarly in both control and pH 4.5-pretreated membranes. No significant difference was observed in either maximal stimulation (47 ± 5% in pH 7.4-pretreated membranes versus 55 ± 7% in pH 4.5-pretreated membranes) or potency of the agonist (EC_{50} = 3.3 ± 0.7 nM in control membranes and 1.8 ± 0.2 nM in low-pH-pretreated membranes). Therefore, although maximal DSLET-induced inhibition of adenylyl cyclase was increased by low-pH pretreatment, the pretreatment did not alter the maximal percentage stimulation of low-K_m GTPase by DSLET, when assayed under similar conditions.

However, low-pH pretreatment did have an effect on the absolute magnitude of low-K_m GTPase activity. Fig. 7A shows the effect of low-pH pretreatment on basal and agonist-stimulated low-K_m GTPase activity with various concentrations of NaCl. These results show that NaCl decreased basal low-K_m GTPase activity in both control and low-pH-pretreated membranes. Interestingly, low-pH pretreatment also decreased basal low-K_m GTPase activity, with the largest effect of the pretreatment occurring in the absence of NaCl, where basal GTPase activity was decreased by about 19%, compared with control. In the presence of 120 mM NaCl, the difference in basal low-K_m GTPase activity between pH 7.4- and pH 4.5-pretreated membranes was approximately 15%. The effect of low-pH pretreatment was specific to low-K_m GTPase activity, because high-K_m GTPase activity, in the presence or absence of sodium, was not significantly affected by low-pH pretreatment (6.6 ± 0.6 nmol/mg/min versus 7.4 ± 0.7 nmol/mg/min and 6.6 ± 0.8 nmol/mg/min versus 6.8 ± 0.9 nmol/mg/min in control versus low-pH-pretreated membranes in the presence and absence of NaCl, respectively).

Similar results were obtained with DSLET-stimulated low-K_m GTPase activity, as also shown in Fig. 7A. Low-pH pretreatment decreased activity of the enzyme, with the largest decrease in activity, relative to control membranes, being in the absence of sodium. Because low-pH pretreatment decreased basal and agonist-stimulated low-K_m GTPase activity to similar extents, the net agonist-induced stimulation of activity was relatively equivalent between control and low-pH-pretreated membranes, as shown in Fig. 7B. This finding explains why no net change in maximal agonist-stimulated low-K_m GTPase was observed under conditions where maximal agonist-induced inhibition of adenylyl cyclase was increased in low-pH-pretreated membranes.

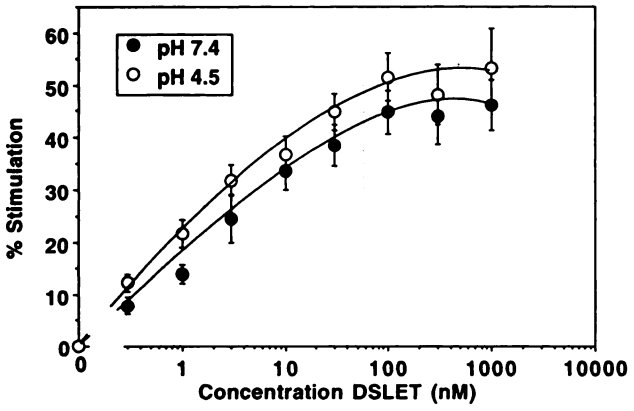

Fig. 6. Effect of low-pH pretreatment on DSLET-stimulated low-K_m GTPase in NG108–15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with or without DSLET (0.3–1000 nM). Data are expressed as percentage stimulation of basal activity in control or pH 4.5-pretreated membranes. Basal low-K_m GTPase activities were 23.1 ± 2.0 and 20.6 ± 1.0 pmol/mg/min in control and pH 4.5-pretreated membranes, respectively.

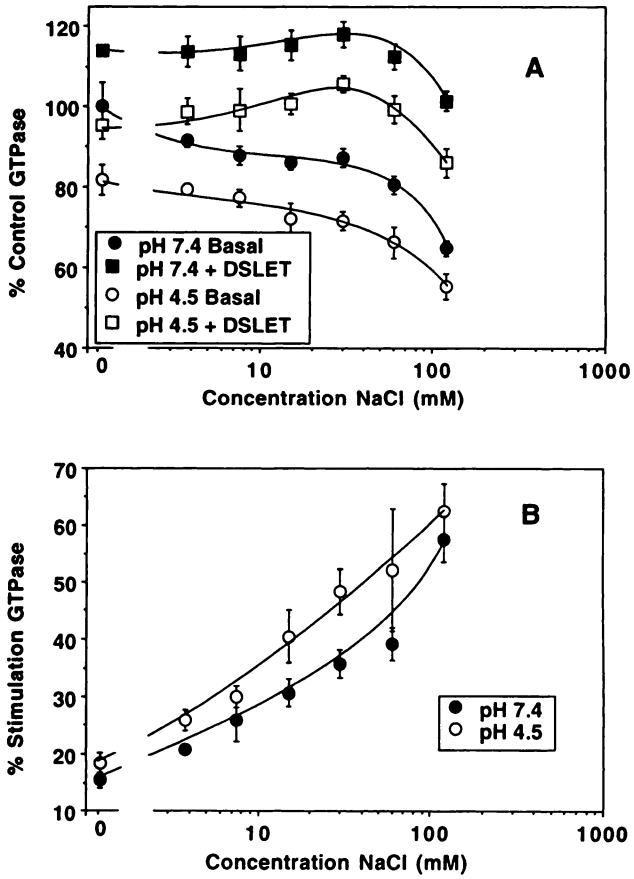

Fig. 7. Effect of sodium on basal and DSLET-stimulated low-K_m GTPase in control and low-pH-pretreated NG108–15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with or without NaCl (3.75–120 mM), in the presence or absence of 1 μM DSLET. A, Percentage of basal activity in control membranes in the absence of NaCl; B, percentage stimulation of basal activity in control or pH 4.5-pretreated membranes at each concentration of NaCl. Basal activity was 40.5 ± 2.4 pmol/mg/min in control membranes in the absence of NaCl.

Role of decreased GTP hydrolysis in increasing agonist efficacy. The finding that low-pH pretreatment decreased the activity of low-K_m GTPase suggests a possible mechanism for low-pH pretreatment-induced increases in receptor-mediated inhibition of adenylyl cyclase. It is possible that decreased hydrolysis maintains G proteins in an active state for a longer period of time, thus providing increased efficacy in inhibiting adenylyl cyclase. Four separate experiments were conducted to test this hypothesis, i.e., 1) Michaelis-Menten kinetic analysis of low-K_m GTPase, 2) assay of G protein levels, 3) assay of high affinity agonist binding sites, and 4) determination of the GTP concentration dependence of agonist-inhibited adenylyl cyclase.

To determine whether the decrease in low-K_m GTPase activity observed in low-pH-pretreated membranes was due to a change in K_m or V_{max}, Michaelis-Menten plots (Fig. 8) were constructed from basal GTPase data obtained in control and low-pH-pretreated membranes in the presence and absence of 120 mM NaCl. As shown in Fig. 8A, the decrease in basal low-K_m GTPase activity in pH 4.5-pretreated membranes in the absence of sodium was due to a decrease in V_{max}, whereas the K_m did not change significantly. In the presence of 120 mM NaCl, low-pH pretreatment decreased the V_{max} to a lesser extent than in the absence of NaCl and the K_m remained unchanged, as shown in Fig. 8B. Table 1 shows mean percentage changes in K_m and V_{max} for low-K_m GTPase produced by low-pH pretreatment and/or 120 mM NaCl, compared with values obtained in control membranes in the absence of sodium. Although statistical analysis of the data was performed on the "raw" K_m and V_{max} values, according to the procedure described in Experimental Procedures, the data in Table 1 were converted to percentage change to highlight the individual effects of low-pH pretreatment and NaCl. Low-pH pretreatment did not significantly alter K_m values in the absence or presence of NaCl but did significantly decrease V_{max} both in the absence (28%) and in the presence (18%) of sodium ($p < 0.005$). NaCl also decreased the V_{max} by 20% and 9% in control membranes and low-pH-pretreated membranes, respectively ($p < 0.05$). The K_m values were increased 22% and 52% by NaCl in control and low-pH-pretreated membranes, respectively ($p < 0.05$). Thus, it appeared that the decreased activity produced by sodium in control and low-pH-pretreated membranes was the result of a combination of changes in K_m and V_{max}, whereas low-pH pretreatment altered only the V_{max}. Because there was no significant interaction between the effects of sodium and low-pH pretreatment on either K_m or V_{max} ($p > 0.5$), the effects of sodium and low-pH pretreatment on low-K_m GTPase activity were considered to be additive. These data explain the findings obtained in the NaCl concentration-effect curves shown in Fig. 7A.

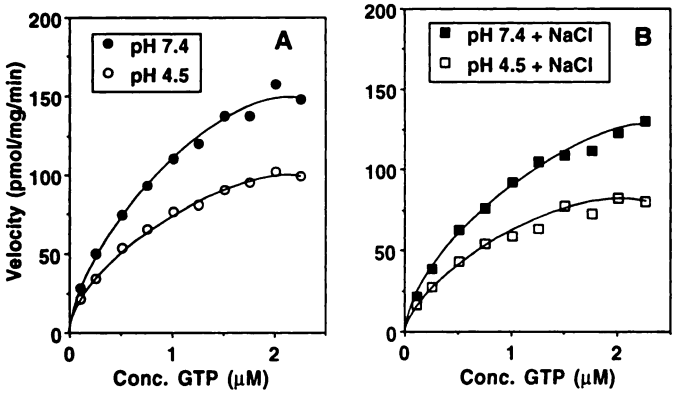

Fig. 8. Michaelis-Menten plots of basal low-K_m GTPase in control and low-pH-pretreated NG108–15 membranes with and without sodium. Membranes were pretreated at pH 7.4 (*closed symbols*) or pH 4.5 (*open symbols*) and assayed with [γ-^{32}P]GTP and various concentrations of unlabeled GTP. A, Comparison of control and low-pH-pretreated membranes without sodium; B, comparison of control and low-pH-pretreated membranes with 120 mM NaCl. Data represent a typical experiment that was assayed in triplicate and was performed five times with similar results.

TABLE 1

Effect of low-pH pretreatment on NG108-15 cell membrane low-K_m GTPase activity in the presence and absence of NaCl

Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with [γ-^{32}P]GTP and various concentrations of unlabeled GTP. K_m and V_{max} data, derived from nonlinear regression analysis of the data, represent percentages of values obtained in control membranes in the absence of NaCl and are means ± standard errors of three to five separate experiments. The K_m and V_{max} values for low-K_m GTPase activity in control membranes in the absence of NaCl were 0.93 ± 0.12 μM and 145 ± 21 pmol/mg/min, respectively.

Pretreatment	No NaCl		120 mM NaCl	
	K_m	V_{max}	K_m	V_{max}
	% of control	% of control	% of control	% of control
pH 7.4	100 ± 12	100 ± 14	122 ± 13[a]	80.2 ± 4.1[a]
pH 4.5	84.3 ± 4.9	72.3 ± 4.7[b]	128 ± 27[a]	65.6 ± 4.8[a,b]

[a] $p < 0.05$, different from membranes without NaCl.
[b] $p < 0.01$, different from control (pH 7.4-pretreated) membranes.

There are two possible mechanisms for the low-pH pretreatment-induced decrease in the V_{max} of low-K_m GTPase, i.e., a reduction in the rate of GTP hydrolysis by G proteins or a diminution in the number of G proteins contributing to the decreased hydrolysis. To determine whether there was a selective loss of G proteins from low-pH-pretreated membranes, Western blot analysis of G protein immunoreactivity was conducted using antisera specific for $G_{i2\alpha}$. The results of a typical experiment (Fig. 9) showed no loss in G_i immunoreactivity between control and low-pH-pretreated membranes. In four separate experiments, G_i immunoreactivity in low-pH-pretreated membranes was 100 ± 14% of that in control membranes. Similar studies with antisera directed nonselectively against G protein α subunits (anti-$G_{\alpha common}$) also showed no change between control and low-pH-pretreated membranes (data not shown). Thus, the most likely explanation for the decrease in GTPase V_{max} was a decrease in the rate of GTP hydrolysis by G_i proteins in low-pH-pretreated membranes.

In the presence of magnesium, G protein-coupled receptors exhibit high affinity agonist binding when guanine nucleotides (and sodium in the case of G_i-coupled receptors) are absent. Thus, the relative number of high affinity agonist binding sites reflects the relative magnitude of G protein coupling of the receptor. To determine whether low-pH pretreatment altered the number of high affinity δ binding sites, saturation analysis

Fig. 9. Western blot from SDS-polyacrylamide gel electrophoresis of G_i proteins in control and low-pH-pretreated NG108–15 membranes. Membranes were pretreated at pH 7.4 (*left lane*) or pH 4.5 (*right lane*). Blots were incubated with G_i-specific antiserum for 18–24 hr and quantified with ^{125}I-Protein A. The immunoblot shown is from one experiment, which was performed four times with similar results.

of [^{3}H]DPDPE binding was conducted in NG108–15 cell membranes (data not shown). In control membranes, [^{3}H]DPDPE bound to a single population of sites with a K_d of 5.9 ± 0.6 nM and a B_{max} of 1480 ± 180 fmol/mg of protein. Low-pH pretreatment had no significant effect on the affinity (K_d = 6.9 ± 1.6 nM) of [^{3}H]DPDPE but actually increased the B_{max} of high affinity δ sites to 2150 ± 282 fmol/mg of protein ($p < 0.05$). These data are additional evidence that the decrease in low-K_m GTPase produced by low-pH pretreatment was not caused by a decrease in the number of receptor-G protein complexes. To determine whether this increase in binding was caused specifically by changes in high affinity (G protein-coupled) sites, antagonist binding to both high and low affinity sites was measured with [^{3}H]NTI. These results (not shown) demonstrated that low-pH pretreatment increased [^{3}H]NTI binding from 2565 ± 65 fmol/mg of protein to 3140 ± 50 fmol/mg of protein ($p < 0.01$). Therefore, the increase in receptor binding produced by low-pH pretreatment was not limited to high affinity agonist sites but was due instead to an increase in total receptor sites.

The most direct test of the role of decreased GTP hydrolysis in increasing agonist efficacy was examination of the GTP concentrations required for agonist-induced inhibition of adenylyl cyclase. This increased efficacy should become most apparent at GTP concentrations approaching those required to achieve V_{max}. In these experiments, NG108–15 membranes, prepared identically to those used in GTPase assays, were pretreated at pH 4.5 or pH 7.4 and assayed for adenylyl cyclase activity in the presence of 120 mM NaCl (Fig. 10). DSLET inhibited the enzyme maximally by 29 ± 1.6% in control membranes versus 41 ± 2.3% in low-pH-pretreated membranes, with no significant difference in the potency of GTP in supporting opioid inhibition between the two membrane preparations. However, at GTP concentrations below the K_m for low-K_m GTPase (about 1 μM), no significant difference in opioid inhibition of adenylyl cyclase was observed between control and low-pH-pretreated membranes. The increase in efficacy of the opioid agonist became apparent only at 1 μM GTP and above, reaching a maximum at approximately 10 μM GTP. These results support the hypothesis that the increase in receptor-mediated inhibition of adenylyl cyclase corresponds to the decrease in V_{max} of low-K_m GTPase in low-pH-pretreated membranes.

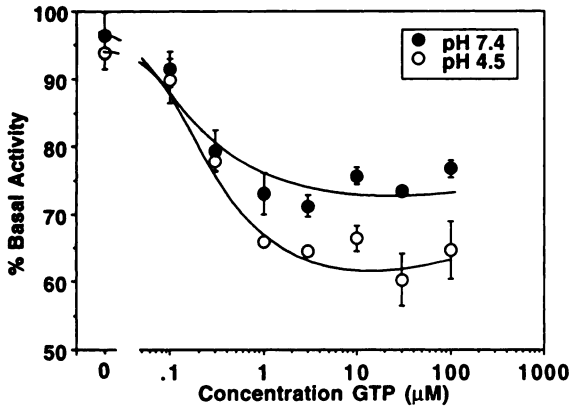

Fig. 10. Effect of varying GTP on DSLET-inhibited adenylyl cyclase in control and low-pH-pretreated NG108–15 membranes. Membranes were pretreated at pH 7.4 or pH 4.5 and assayed with the indicated concentrations of GTP (0.1–100 μM), in the presence or absence of 1 μM DSLET. Data are expressed as percentage of basal activity at each concentration of GTP in control or pH 4.5-pretreated membranes.

Discussion

The previous findings that low-pH pretreatment increased the efficacy of opioid agonists in inhibiting adenylyl cyclase in brain membranes (19, 20) suggested that this treatment may help to define factors important in the regulation of agonist efficacy. The use of NG108–15 cells allowed these studies to be extended to a system where high affinity binding, adenylyl cyclase, and low-K_m GTPase could be studied in the same membranes. The effects of low-pH pretreatment on adenylyl cyclase activity in NG108–15 cell membranes closely paralleled those previously observed in rat brain membranes (19, 20, 26). Low-pH pretreatment selectively decreased G_s-stimulated adenylyl cyclase activity and increased the efficacy of DSLET inhibition of adenylyl cyclase in NG108–15 membranes. Because inhibition of adenylyl cyclase by muscarinic and cannabinoid receptor agonists was also increased by the pretreatment, the effect of low-pH pretreatment on adenylyl cyclase inhibition may be common among G_i-linked receptors.

Studies of sodium and guanine nucleotide modulation of opioid agonist binding revealed both similarities and differences between NG108–15 cell and rat brain membranes. As in rat brain membranes (18), maximal inhibition of agonist binding by sodium was increased by the pretreatment in NG108–15 membranes. In contrast to rat brain membranes, however, low-pH pretreatment did not increase inhibition of agonist binding to NG108–15 membranes by guanine nucleotides in the absence of sodium, although there was a slight increase in Gpp(NH)p inhibition of agonist binding in the presence of NaCl. Because sodium and guanine nucleotides regulate agonist binding by different mechanisms (11, 17), it is not surprising that the effects of low-pH pretreatment may vary between these two parameters.

Although low-pH pretreatment increased DSLET inhibition of adenylyl cyclase, it did not affect DSLET stimulation of low-K_m GTPase in NG108–15 cell membranes. This indicated that low-pH pretreatment did not directly affect opioid receptor-mediated stimulation of G_i activity. The major finding that provided information on the mechanism of action of low-pH pretreatment arose from the examination of sodium and low-pH effects on basal and agonist-stimulated low-K_m GTPase activity in NG108–15 cell membranes. In these experiments, the absolute activity of both basal and agonist-stimulated low-K_m GTPase was decreased by low-pH pretreatment. This result appeared to be paradoxical, because an increase in high affinity agonist binding and an increase in receptor-mediated inhibition of adenylyl cyclase suggested an increase, rather than a decrease, in G protein activity. Thus, the expected result was that agonist-stimulated low-K_m GTPase activity would be increased in low-pH-pretreated membranes.

A plausible explanation for this discrepancy is that the decrease in GTPase activity maintained G_i in an activated state for a longer period of time, i.e., decreased inactivation of G_i without any reduction in GTP binding to the protein. Evidence in favor of this hypothesis was obtained by nonlinear regression analysis of Michaelis-Menten plots of basal low-K_m GTPase. These experiments clearly demonstrated that low-pH pretreatment decreased the V_{max} of basal low-K_m GTPase activity. In fact, the decrease in activity must have been entirely due to a

decrease in V_{max}, because the K_m was not altered by the pretreatment. Thus, it is unlikely that GTP binding to the G protein was affected by low-pH pretreatment. An alternative explanation for a decrease in V_{max} is that there was selective loss of G proteins from low-pH-pretreated membranes. However, Western blots of SDS-polyacrylamide gel electrophoresis of proteins from control and low-pH-pretreated membranes showed no change in G_{i2} immunoreactivity. Taken together, these results provide strong evidence for the hypothesis that low-pH pretreatment decreased the inactivation rate, but not the activation rate, of receptor-coupled G proteins in NG108–15 cell membranes. Unfortunately, Western blots assay only the total amount of G protein α subunits present and do not distinguish between active and denatured proteins. However, alternative methods ([^{32}P]ADP-ribosylation with PTX and [^{35}S]GTPγS labeling of GTP binding sites) present their own difficulties, because [^{35}S]GTPγS binding would have to be performed on purified $G_{i/o}$ proteins and PTX labeling would likely be affected by the change in conformation of the α subunit that produces decreased rates of GTP hydrolysis. Current experiments are underway to explore the relationship between PTX labeling and active G proteins in these membranes. Nevertheless, the conclusion that $G_{i/o}$ proteins are not being denatured is supported by the earlier finding (26) that [^{32}P]azido-anilido-GTP binding to $G_{i/o}$ α subunits was not altered by low-pH pretreatment of rat brain membranes.

Another possible reason for the decrease in low-K_m GTPase V_{max} is that the number of receptor-G protein complexes was decreased by low-pH pretreatment. This could explain the finding that the decrease in V_{max} of basal GTPase was greater in the absence of sodium, where spontaneous receptor activation of G proteins is maximal (15), as well as explaining an increase in sodium inhibition of agonist binding (27). However, two pieces of evidence argue against this possibility. First and most importantly, agonist stimulation of low-K_m GTPase was not decreased in low-pH-pretreated membranes. Second, if the number of receptor-G protein complexes were reduced, a decrease in high affinity agonist binding might be evident. To the contrary, saturation analysis of [^{3}H]DPDPE binding revealed that the apparent number of high agonist affinity binding sites was increased in low-pH-pretreated NG108–15 membranes, although this change was somewhat variable, and a corresponding increase in antagonist binding was also evident. Thus, it is likely that low-pH pretreatment unmasked a population of cryptic binding sites that were inaccessible in control membranes due to sequestration by the membranes, receptor aggregation, or other factors that are as yet undetermined. The receptors "uncovered" by low-pH pretreatment may not be functionally coupled to G proteins, because there was no increase in agonist-stimulated low-K_m GTPase activity in low-pH-pretreated membranes. Examples of the dissociation between high affinity opioid agonist binding and functional G protein coupling in NG108–15 cells can be found in the literature. For example, PTX pretreatment of NG108–15 cells had no effect on high affinity agonist binding to membranes, despite eliminating receptor-mediated inhibition of adenylyl cyclase (27). Moreover, the sodium and guanine nucleotide sensitivity of high affinity agonist binding was retained in membranes prepared from PTX-pretreated NG108–15 cells (27, 28). Thus, G protein-receptor complexes may retain their ability to physically interact and affect agonist binding in NG108–15 membranes, even if the complex is functionally uncoupled.

The low-pH pretreatment-induced increase in opioid binding is in contrast to results obtained in rat brain membranes, where high affinity opioid agonist binding was not altered by low-pH pretreatment. This difference may be explained by the "tightness" of coupling between opioid receptors and G_i in NG108–15 membranes (9, 12, 13), relative to rat brain (29). As discussed above, the guanine nucleotide sensitivity of agonist binding is retained in PTX-pretreated NG108–15 cells (27, 28), whereas this regulation is lost in PTX-treated rat brain membranes (30).

The hypothesis that low-pH pretreatment increased receptor-mediated inhibition of adenylyl cyclase by decreasing the V_{max} of GTP hydrolysis by G_i was further supported by experiments in which the role of GTP in supporting DSLET inhibition of adenylyl cyclase was examined in control and low-pH-pretreated NG108–15 membranes. The data clearly indicate that increases in receptor-mediated inhibition of adenylyl cyclase are maximal at GTP concentrations greater than the K_m for GTP hydrolysis. This would be the expected result if low-pH pretreatment decreased the maximal rate of GTP hydrolysis by inhibitory G proteins. It could be argued that the lack of effect of low-pH pretreatment at low GTP concentrations is an artifact produced by an inability to observe significant increases in agonist activity at less than maximal agonist efficacy. This possibility is unlikely, as demonstrated in several other experiments in this study (see Figs. 3–5), which clearly showed low-pH effects at less than maximal efficacies of agonists. For example, in Fig. 3 low-pH pretreatment increased DSLET inhibition of adenylyl cyclase throughout the agonist dose-response curve, even with concentrations of DSLET that produced little inhibition in control membranes.

The effects of sodium on GTPase were complex, with a decrease in V_{max} and an increase in K_m for GTP, which may indicate sodium-induced inhibition of G protein activation. These results are in agreement with the reported inhibitory effect of sodium on spontaneous activation of PTX-sensitive G proteins by unoccupied receptors in NG108–15 cell membranes (15). Thus, it appears that the inhibitory effect of low-pH pretreatment on low-K_m GTPase and the stimulatory effect of the pretreatment on agonist-induced inhibition of adenylyl cyclase result from a different mechanism of action, compared with that of sodium in producing apparently similar effects. This may explain the additive nature of sodium and low-pH pretreatment in producing these effects.

The effects of low-pH pretreatment on both G_s and G_i function are somewhat similar to those of CTX. Evidence has emerged showing that CTX can ADP-ribosylate the receptor-activated form of G_i (31, 32). Interestingly, the effect of this modification on the functional properties of G_{i2} are strikingly similar to those produced by low-pH pretreatment of NG108–15 cell membranes; both steady state hydrolysis rates and k_{cat} of GTPase were decreased, and high affinity agonist binding was increased (33). Moreover, both low-pH pretreatment and CTX affect G_s function. Finally, Iiri *et al.* (33) predicted that the alterations in G_{i2} function produced by CTX pretreatment should lead to enhancement of the effector response elicited by the modified G protein. This is exactly what was observed with low-pH pretreatment of NG108–15 cell membranes. Another example of this relation between G_s and G_i functions was

provided in a recent study (34) that used low-pH pretreatment of NG108–15 cell membranes to show that enhanced receptor-G_s coupling efficiency occurred in cells chronically treated with opioid agonists.

Less clear in this study is the mechanism of action by which low-pH pretreatment and/or sodium inhibit basal adenylyl cyclase activity in NG108–15 membranes. Although the ability of sodium to inhibit adenylyl cyclase in NG108–15 membranes has been known for some time (4), this effect seems counter-intuitive, in light of more recent findings indicating that sodium inhibits spontaneous activation of G_i by unoccupied opioid receptors (15). By this reasoning, sodium should increase basal adenylyl cyclase activity by decreasing the activity of inhibitory G proteins. Such an increase in adenylyl cyclase activity by sodium has been observed in brain (35–37), as well as in CTX-pretreated 7315c and NG108–15 cell membranes (16). The treatment of NG108–15 cells with CTX may explain the difference in the sodium effect observed in the present study and the work of Blume *et al.* (4), compared with the effect reported by Puttfarcken *et al.* (16). One potential explanation for the inhibitory effect of sodium on adenylyl cyclase activity in membranes from untreated NG108–15 cells is that sodium directly inhibited activity of the catalytic unit. Direct inhibitory effects of monovalent cations on adenylyl cyclase activity have been reported in other tissues (38).

One explanation for the inhibitory effect of low-pH pretreatment on adenylyl cyclase in the absence of sodium arises from the effect of sodium on basal G_i activity in NG108–15 cell membranes. Spontaneous activation of G_i by unoccupied receptors is maximal in the absence of sodium (15) in NG108–15 membranes. Therefore, the inhibitory effect of low-pH pretreatment on G_i inactivation (i.e., GTP hydrolysis) may result in greater inhibition of adenylyl cyclase under "basal" conditions in the absence of sodium. Because basal adenylyl cyclase in the presence of 30–120 mM sodium is not decreased by low-pH pretreatment, it is probable that low-pH pretreatment disproportionately decreases the inactivation rate of receptor-stimulated G_i or that basal G_i activity in the absence of receptor activation is very low in NG108–15 membranes.

In conclusion, low-pH pretreatment produced alterations in G protein function in NG108–15 cell membranes that were similar to those previously observed in rat brain membranes (18–20, 26). These changes included decreased stimulation of adenylyl cyclase by G_s, increased inhibition of opioid agonist binding by sodium, and increased receptor-mediated inhibition of adenylyl cyclase. An explanation for this latter effect may lie in the apparent ability of the pretreatment to decrease GTP hydrolysis by G_i. Future research on the mechanisms by which low-pH pretreatment modifies receptor-G protein-effector interactions may provide additional insights into the factors that determine inhibitory agonist efficacy and into the coordinate regulation of adenylyl cyclase by G_i and G_s in this signaling cascade.

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