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ACCELERATED COMMUNICATION

Antisense Oligodeoxynucleotide to the G_{i2} Protein α Subunit Sequence Inhibits an Opioid-Induced Increase in the Intracellular Free Calcium Concentration in ND8–47 Neuroblastoma × Dorsal Root Ganglion Hybrid Cells

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SUMMARY

In ND8–47 cells, a neuroblastoma × dorsal root ganglion hybrid cell line, activation of δ-opioid receptors induced an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) through dihydropyridine-sensitive calcium channels. This effect was mediated by pertussis toxin-sensitive G proteins. The G protein α subunits α_{i2}, α_{i3}, α_q, and α_s were detected using Western blots, whereas α_o and α_{i1} were not found in ND8–47 cell membranes. To identify the specific G protein α subunit(s) responsible for the increase in $[Ca^{2+}]_i$, we treated ND8–47 cells with antisense oligodeoxynucleotides (AS) complementary to the mRNA for each G protein α subunit (α_{i2}, α_{i3}, or α_s), at a concentration of 10 μM, for up to 6 days and examined their effects on opioid-induced increases in $[Ca^{2+}]_i$ and on the levels of G protein α subunits. $[Ca^{2+}]_i$ was measured in adherent cells using the fluorescent dye fura-2. Treatment of cells with α_{i2}-AS (10 μM, for 6 days) resulted in a 73% inhibition of the [D-Ser^2,Leu^5]-enkephalin-Thr-induced increase in $[Ca^{2+}]_i$. In contrast, pretreatment of cells with α_{i3}-AS (10 μM, for 6 days) or α_s-AS (10 μM, for 6 days) had no effect on the [D-Ser^2,Leu^5]-enkephalin-Thr-induced responses. Western blots indicated that the levels of α_{i2} were decreased when cells were exposed to α_{i2}-AS (10 μM) for 6 days, whereas the levels of α_{i3}, α_s, and α_q were not affected by this treatment. Treatment of the cells with α_{i3}-AS or α_s-AS for 6 days significantly reduced α_{i3} or α_s levels, respectively. These results indicate that the opioid-induced increase in $[Ca^{2+}]_i$ in ND8–47 cells is mediated by $G_{\alpha i2}$.

G proteins, composed of α subunits and βγ dimers, play important roles in mediating opioid-induced cellular responses, including inhibiting adenylyl cyclase activity (1–4), increasing potassium conductance (5), and reducing (6, 7) or increasing (8) calcium conductance. Among G proteins, those most clearly shown to be linked to opioid receptors are PTX-sensitive G proteins, i.e., G_i or G_o. Since many subtypes of these G proteins, including $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o1}$, and $G_{\alpha o2}$, have been identified, purified, and cloned in recent years, it has become possible to independently study the function of each of these G protein subtypes in opioid-induced responses. $G_{\alpha i2}$ has been proposed to be mainly responsible for opioid-induced inhibition of adenylyl cyclase activity (9), because antibodies to $G_{\alpha i2}$, but not those to $G_{\alpha i3}$ or G_o, reduced opioid-induced inhibition of this enzyme. In contrast, G_o is suggested to mediate opioid-induced inhibition of Ca^{2+} channels. Intracellular application of G_o subunits was more effective than application of G_i in reconstitution of the opioid receptor-mediated inhibition of Ca^{2+} current when NG108–15 cells were pretreated with PTX (10). In the same cells, opioid inhibition of Ca^{2+} current was blocked by preinjection of the cells with antibodies against G_o but not by those against G_i (11). Similar assignments of the G protein subtypes to spe-

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ABBREVIATIONS: PTX, pertussis toxin; AS, antisense oligonucleotide(s); $[Ca^{2+}]_i$, intracellular free calcium concentration; DRG, dorsal root ganglion; DSLET, [D-Ser^2,Leu^5]-enkephalin-Thr; APD, action potential duration; SDS, sodium dodecyl sulfate; FBS, fetal bovine serum.

cific effector systems were also made in other hormone-secreting cells, such as GH_3 pituitary cells (12). In addition to opioid-induced inhibitory effects mediated by PTX-sensitive G proteins, it has been proposed that G_s is involved in opioid receptor-induced stimulatory effects by stimulating adenylyl cyclase activity and calcium influx or by inhibiting potassium conductance (13, 14).

In ND8–47 cells, a neuroblastoma × DRG hybrid cell line, our previous studies indicated that activation of δ-opioid receptors induced an increase in $[Ca^{2+}]_i$ by opening dihydropyridine-sensitive calcium channels (8). This effect is mediated by PTX-sensitive G proteins (15). Cholera toxin had no effect on the opioid-induced response. To further understand the role of G_i and G_o or their subtypes in mediating this response, we have examined the presence of known G protein α subunits in ND8–47 cells by Western blot analysis. The G protein α subunits α_{i2}, α_{i3}, α_q, and α_s were detected, whereas α_o and α_{i1} were not found in ND8–47 cell membranes (15). In this study, we used AS complementary to the mRNA for each α_{i2}, α_{i3}, or α_s to treat ND8–47 cells and we examined their effects on the opioid-induced response. Our results indicate that $G_{\alpha i2}$ protein serves as a transducer mediating the opening of calcium channels by δ-opioid receptors.

Materials and Methods

Oligonucleotides and reagents. The strategy for designing the 21–26-mer phosphorothioate oligodeoxynucleotides was adapted from the method of Gollasch *et al.* (16). Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems model 392) and purified by reverse phase high performance liquid chromatography. Each of the oligomers had phosphorothioate groups on the four nucleotides at the 5′ and 3′ ends. The oligomers had the following sequences: α_{i2}-AS, 5′-CGGCAGCACAGGACAGTGCGAACAGC-3′ (corresponding to nucleotides 317–342 of the identical strand of the $G_{\alpha i2}$ gene sequence); α_{i3}-AS, 5′-CAGCACTGCCAGCTAAAACAA-3′ (corresponding to nucleotides 322–342 of the identical strand of the α_{i3} gene sequence); α_{i2}-S, 5′-GCTGTTCGCACTGTCCTGTGCTGCCG-3′; α_s-AS, GCACCAGGTTGCTCATGGCGG.

Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR), DSLET was obtained from Cambridge Research Biochemical Co. (Wilmington, DE), and the ND cell lines were generously supplied by Dr. John Wood (Sandoz, London, UK). For G protein Western blotting, the antisera to G proteins were kindly provided by Dr. Allen Spiegel (National Institutes of Health); second antibody, Vectorstain reagent, and the alkaline phosphatase kit were purchased from Vector Laboratories (Burlingame, CA).

Cell culture. ND8–47 cells were cultured in 175-cm^2 flasks in L-15 medium containing 10% FBS, 2 mM L-glutamine, added 3.3 g/liter $NaHCO_3$, and 3 g/liter glucose. Cultures were maintained in a 5% CO_2 incubator at 37°. Four to 5 days before $[Ca^{2+}]_i$ measurements, cells were transferred to 60-mm culture dishes (Costar, Cambridge, MA), in a volume of 5 ml of cell suspension/dish (about 10^6 cells/dish), and were grown on glass coverslips (9 × 35 mm; Clay Adams, Lincoln Park, NJ).

Oligonucleotide treatment of cells. ND8–47 cells were cultured until 80% confluent in flasks or dishes containing regular L-15 medium with 10% FBS. The regular L-15 medium was then replaced with L-15 medium containing a low concentration (0.5%) of FBS (with 20 ng/ml nerve growth factor), and oligonucleotides were added to the medium as 100× stock solutions, to give a final concentration of 10 μM. Medium was removed and replaced with fresh medium, containing 10 μM oligonucleotides, every 48 hr for up to 6 days.

Measurement of $[Ca^{2+}]_i$. Confluent monolayers of cells grown on coverslips were incubated for 60 min at 37° with 5 μM fura-2 acetoxymethyl ester plus 0.2% pluronic F-127 in Na^+ Hanks' solution containing 5 mM glucose and 0.2% bovine serum albumin. Cells were then washed twice with Na^+ Hanks' solution before fluorescence measurements. Cells on coverslips were placed in a cuvette containing 2 ml of Na^+ Hanks' solution, with maintenance of the temperature at 37°. The fluorescence signal was measured with emission at 510 nm and alternating excitation at 340 nm and 380 nm (slit width, 4 nm), using a PTI Delta Scan spectrofluorometer (Photon Technology International, South Brunswick, NJ). $[Ca^{2+}]_i$ values were calculated according to the following formula (17): $[Ca^{2+}]_i = K_d(R - R_{min})(S_f)/(R_{max} - R)(S_b)$, where R is the ratio of fluorescence intensities at 340 nm and 380 nm, R_{min} and R_{max} are the ratios in the absence of Ca^{2+} and with saturating Ca^{2+}, respectively, K_d is the dissociation constant for fura-2, and S_f and S_b are the fluorescence intensities of the dye measured at 380 nm in the absence of Ca^{2+} and with saturating Ca^{2+}, respectively. Agents were added to the cuvette in 20-μl aliquots. DSLET-induced changes in $[Ca^{2+}]_i$ were calculated by subtracting the base-line $[Ca^{2+}]_i$ value determined immediately before DSLET addition from the value for peak $[Ca^{2+}]_i$ elicited by DSLET.

Gel electrophoresis and immunoblotting. ND8–47 cell membranes were prepared as described previously (15). Membrane proteins were dissolved in sample buffer (100 ml of sample buffer contains 10 ml of 10% SDS, 1 ml of β-mercaptoethanol, 5 ml of 0.5 M Tris·HCl, 50 ml of 50% sucrose, 50 mg of methylene blue, and 34 ml of water) at a concentration of 1 mg/ml. Protein samples (20 μl) were separated on a 12% SDS-polyacrylamine gel at 125 V for 4 hr. The proteins were then transferred from the gel to a nitrocellulose membrane (for 1 hr at 100 V). The nitrocellulose membranes were cut into strips for each lane and placed in strip trays. After incubation in blocking buffer (100 mM Tris·HCl, 0.9% NaCl, 0.1% Tween-20, 5% nonfat milk, pH 7.5) for 1 hr, the nitrocellulose membrane strips were incubated with the following antisera: QL (selective for α_q) at 1/500, AS (selective for α_{i1} and α_{i2}) at 1/4000, GC (selective for α_o) at 1/1000, EC (selective for α_{i3}) at 1/1000, and RM (selective for α_s) at 1/4000. These antisera have been successfully used to detect G_α subunits in other tissues (18–21). The location of each primary antibody was detected with a Vectorstain avidin-biotin complex-alkaline phosphatase kit (catalogue number AK-5001). The presence of alkaline phosphatase was determined with a Vector alkaline phosphatase substrate kit II (catalogue number SK-5200).

Results

After treatment with AS for 6 days, the morphology of the cells, at the level of light microscopy, was not affected. The effects of treatment with AS against α_{i2}, α_{i3}, or α_s on δ-opioid agonist-induced increases in $[Ca^{2+}]_i$ in ND8–47 cells were tested (Fig. 1). Incubation of cells with 10 μM α_{i2}-AS (antisense to α_{i2}) for 6 days resulted in 73% inhibition of DSLET (100 nM)-induced increases in $[Ca^{2+}]_i$. In contrast, α_{i3}-AS (antisense to α_{i3}), α_s-AS (antisense to α_s), and α_{i2}-S (sense to α_{i2}) treatment for the same time had no significant effect on DSLET-induced responses. Treatment of cells with α_{i2}-AS for 4 days induced a 25% inhibition of DSLET (100 nM)-induced increases in $[Ca^{2+}]_i$; however, treatment for 2 days did not influence the DSLET action (Fig. 2).

These results suggested that the inhibitory effect of α_{i2}-AS on the DSLET-induced increase in $[Ca^{2+}]_i$ was due to the inhibition of $G_{\alpha i2}$ expression. To examine the changes in the levels of G proteins after AS treatment, we treated ND8–47 cells with 10 μM α_{i2}-AS for 6 days. The presence of G protein α subunits ($G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha s}$) was examined by Western blot analysis in membranes from untreated cells (Fig. 3A) or from AS-treated cells (Fig. 3B). G protein α subunits α_q (41 kDa), α_{i2} (40 kDa), α_{i3} (41 kDa), and α_s (42 kDa and 45 kDa)

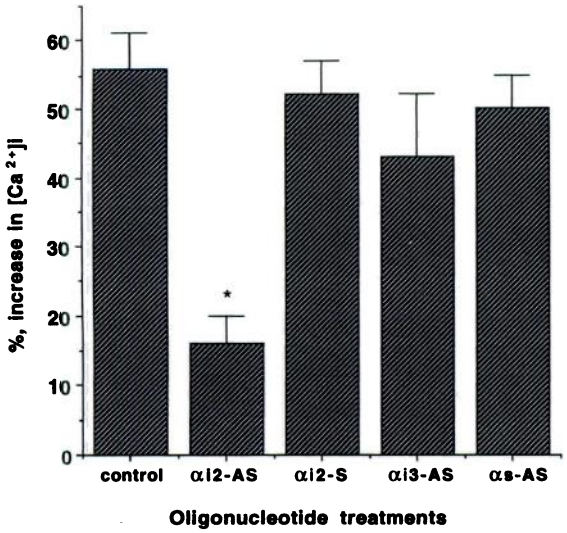

Fig. 1. Changes in $[Ca^{2+}]_i$ induced by 100 nM DSLET in untreated ND8–47 cells (control) or ND8–47 cells treated with 10 μM AS (α_{i2}-AS, α_{i3}-AS, or α_s-AS) or sense oligonucleotide (α_{i2}-S) for 6 days. *Bars*, mean ± standard error of three independent experiments, determined using the statistical program SuperANOVA1.01 (*, $p < 0.05$). The resting $[Ca^{2+}]_i$ value did not change significantly after exposure of cells to oligonucleotides, with the $[Ca^{2+}]_i$ values being 148 ± 12 nM for untreated cells, 155 ± 8 nM for α_{i2}-AS-treated cells, 150 ± 14 nM for α_{i3}-AS-treated cells, 168 ± 13 nM for α_s-AS-treated cells, and 134 ± 16 nM for α_{i2}-S-treated cells.

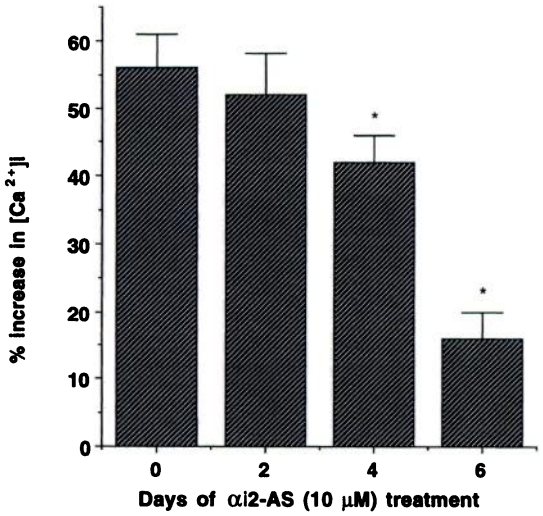

Fig. 2. Effect of pretreatment with 10 μM α_{i2}-AS on 100 nM DSLET-induced increases in $[Ca^{2+}]_i$ in ND8–47 cells. DSLET-induced changes in $[Ca^{2+}]_i$ were measured in untreated cells and cells that had been pretreated with 10 μM α_{i2}-AS for 2, 4, or 6 days. *Bars*, mean ± standard error of three independent experiments. The significance of differences in treatment effects was evaluated by analysis of variance, using the program SuperANOVA1.01 (*, $p < 0.05$).

were detected; α_{i1} (41 kDa) and α_o (39 kDa) were not detected in either treated or untreated ND8–47 cell membranes. In comparison with untreated cells, there was an apparent decline in the level of α_{i2} after the cells were treated with α_{i2}-AS for 6 days, whereas the levels of α_{i3}, α_q, and α_s were not changed. The time course for the change in the level of α_{i2} was also examined (Fig. 4A). The level of α_{i2} declined after 4-day treatment with α_{i2}-AS and was reduced more markedly after treatment for 6 days. This time-dependent inhibition by

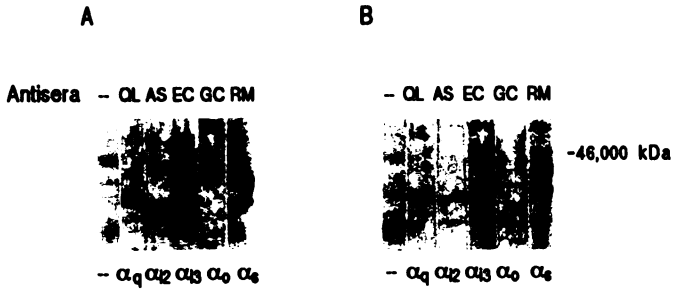

Fig. 3. Western blot of G protein α subunits in membranes of untreated ND8–47 cells (A) and ND8–47 cells treated with 10 μM α_{i2}-AS for 6 days (B). G proteins from cell membranes (20 μg/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with the following antisera: QL (selective for α_q) at 1/500, AS (selective for α_{i1} and α_{i2}) at 1/4000, GC (selective for α_o) at 1/1000, EC (selective for α_{i3}) at 1/1000, and RM (selective for α_s) at 1/4000. To analyze these results, Western blots were scanned with a laser densitomiter. The areas under each α subunit image density peak were measured using the program NIH Image, version 1.55. Mean ± standard error values (square pixels) for each band were calculated from three independent experiments. Differences were analyzed by analysis of variance (SuperANOVA1.01 program). After α_{i2}-AS treatment for 6 days, the intensity of the α_{i2} band was significantly reduced (*, $p < 0.05$), whereas the intensities of the α_q, α_{i3}, and α_s bands were unchanged.

α_{i2}-AS of α_{i2} expression occurred in parallel with the time-dependent inhibition by α_{i2}-AS of the DSLET-induced increase in $[Ca^{2+}]_i$.

Because treatment of ND8–47 cells with AS against $G_{\alpha i3}$ or $G_{\alpha s}$ did not influence the DSLET-induced increase in $[Ca^{2+}]_i$ (Fig. 1), we examined how α_{i3}-AS or α_s-AS affected the expression of either α_{i3} or α_s in the ND8–47 cell membranes (Fig. 4, B and C). After treatment of cells with AS for 4 days, the levels of α_{i3} or α_s began to decline; the reduction was even more apparent after treatment for 6 days. These results indicate that the G proteins $G_{\alpha i3}$ and $G_{\alpha s}$ probably do not participate in opioid regulation of $[Ca^{2+}]_i$ in ND8–47 cells. Because the expression of $G_{\alpha q}$ was unchanged during all AS treatments, including treatment with α_{i2}-AS, which significantly reduced the response to DSLET, it is unlikely that $G_{\alpha q}$ is implicated in the opioid response.

Discussion

Opioid-induced influx through voltage-dependent Ca^{2+} channels has been observed in several studies. In NG108–15 cells, lower concentrations (1–10 nM) of δ receptor agonists induce an increase in $[Ca^{2+}]_i$ both by activating dihydropyridine-sensitive Ca^{2+} channels and by mobilizing Ca^{2+} from intracellular stores (22). In mouse DRGs, lower concentrations (1–10 nM) of κ receptor agonists prolong the APD by directly increasing voltage-sensitive Ca^{2+} conductances (14, 23). In the same cells, lower concentrations of δ receptor agonists prolong the APD by decreasing voltage-sensitive membrane K^+ conductances, which results in delayed repolarization of the neuron, thereby increasing Ca^{2+} influx for each action potential (14, 23).

The mechanisms for the opioid stimulatory effects on Ca^{2+} conductance and $[Ca^{2+}]_i$ were also explored in these studies. In NG108–15 cells, the δ agonist evoked increases in $[Ca^{2+}]_i$ resulting either from Ca^{2+} influx in differentiated cells or from Ca^{2+} release from the inositol-1,4,5-trisphosphate-sensitive stores in undifferentiated cells; these effects were

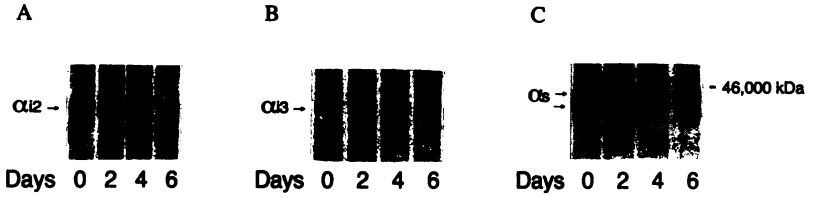

Fig. 4. Western blot of G protein α subunits in membranes of ND8–47 cells treated with 10 μM α_{i2}-AS (A), α_{i3}-AS (B), or α_s-AS (C) for 2, 4, or 6 days. The membrane protein concentrations were analyzed (using the Lowry assay) after each AS treatment. After 6 days of treatment the membrane protein concentrations were as follows: control, 320 μg/flask; α_{i2}-AS, 310 μg/flask; α_{i3}-AS, 280 μg/flask; α_s-AS, 340 μg/flask. The membrane proteins from each treatment group were separated by SDS-polyacrylamide gel electrophoresis (20 μg/lane), transferred to nitrocellulose membranes, and incubated with the following antisera: AS (selective for α_{i1} and α_{i2}) at 1/4000 (A), EC (selective for α_{i3}) at 1/1000 (B), and RM (selective for α_s) at 1/4000 (C). The results were analyzed by scanning the blots using a laser densitometer. The intensity of the α_{i2} and α_{i3} bands began to decline after AS treatment for 4 days; the reduction was even more apparent after treatment for 6 days. The intensity of the α_s band was reduced by almost half after treatment of cells for 6 days with AS against α_s. Note that α_s appears as a doublet of 45 kDa and 42 kDa, with a large amount of the higher molecular mass form. Treatment with AS against α_s reduced the amount of both forms.

blocked by PTX but not cholera toxin, indicating a G_i- or G_o-mediated effect (22, 24). In DRG cells, cholera toxin A or B subunits, as well as the whole toxin, selectively blocked opioid-induced prolongation of the Ca^{2+} component of the action potential (15). Opioid excitatory, but not inhibitory, modulation of the APD was prevented by injection of an inhibitor of cAMP-dependent protein kinase into DRG neurons (25). Furthermore, opioids could stimulate basal adenylyl cyclase activity in these cultures (26). Based upon these observations, it was proposed that opioid-induced APD prolongation in DRG neurons was mediated by opioid receptor subtypes that are positively coupled, via G_s, to adenylyl cyclase/cAMP-dependent voltage-sensitive ionic conductances (14, 15).

A role for G proteins of the G_i family in opioid-induced stimulatory effects on Ca^{2+} channels has not been specifically defined. However, a body of evidence suggests that the G_i protein may play an essential role in hormonal Ca^{2+} channel activation. In adrenal cortical Y1 cells, the stimulatory effect of angiotensin II was PTX sensitive, and the membranes contained PTX-sensitive G proteins of the G_i type but not G_o. PTX-sensitive Ca^{2+} channel activation was also observed in GH_3 cells with thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and angiotensin II and in pheochromocytoma PC-12 cells with endothelin-3 (27). In these cell lines, G_{i2} occurs ubiquitously; additionally, G_{i3} expression was detected in membranes of GH_3 cells, and G_{i1} is expressed in PC-12 cells. The G_i subtypes mediating these effects were determined unambiguously by using AS to suppress the expression of individual G protein α subunits. By microinjection of AS into GH_3 cells, it was shown that stimulation of dihydropyridine-sensitive Ca^{2+} channels by thyrotropin-releasing hormone was mainly mediated by the widely distributed G_{i2}, with a minor contribution by G_{i3} (16).

The composition of G protein α subunits in ND8–47 cells is apparently different from that in other neuronal cell lines that have opioid receptors, such as NG108–15 cells (δ receptor) and 7315c pituitary tumor cells (μ receptor), because α_o and α_{i1} have never been detected in ND8–47 cell membrane extracts. In other experiments, the GC antiserum clearly identified a ~39-kDa band (believed to be α_o) in both NG108–15 membranes (15) and 7315c membranes (18). In addition, antiserum AS (selective for α_{i1} and α_{i2}) clearly identified a doublet (apparent molecular masses of 41 and 40 kDa) in 7315c membranes (19), but in NG108–15 and ND8–47 membranes only the lower 40-kDa band (believed to be α_{i2}) was detected.

Although there are structural differences in the α subunits among various G proteins, the subunits have high levels of conservation. The sequences of α_{i2} and α_{i3} AS used in our study, as used by Gollasch *et al.* (16), are complementary to translated regions of α_{i2} and α_{i3} mRNA showing very limited homology. Considering that unmodified phosphodiester oligodeoxyribonucleotides are unstable in biological fluids and display poor cellular uptake characteristics (28, 29), we modified the AS with phosphorothioate groups at the two ends of each oligonucleotide. The $G_{\alpha i2}$ AS (α_{i2}-AS) markedly inhibited the expression of $G_{\alpha i2}$ and significantly blocked the DSLET-induced increase in $[Ca^{2+}]_i$ in ND8–47 cells. α_{i2}-AS had no effect on the expression of other α subunits. Treatment of the cells with AS to $G_{\alpha i3}$ and $G_{\alpha s}$, or with a sense oligonucleotide for $G_{\alpha i2}$, had no significant effect on the opioid response. The inhibition of opioid responses by the $G_{\alpha i2}$ AS treatment appears to be related to a reduction in the amount of $G_{\alpha i2}$ protein in ND8–47 cell membranes, because this treatment reduced the level of $G_{\alpha i2}$ but had no effect on the levels of $G_{\alpha q}$, $G_{\alpha i3}$, or the two $G_{\alpha s}$ subunits. Treatment of ND8–47 cells with AS against $G_{\alpha i3}$ or $G_{\alpha s}$ reduced the levels of these G proteins but did not influence the DSLET-induced increase in $[Ca^{2+}]_i$. These results, together with our earlier demonstration that nifedipine or verapamil could antagonize the opioid-induced increase in $[Ca^{2+}]_i$ in ND8–47 cells (8), clearly differentiate this action from that reported by Shen and Crain (13, 14) in embryonic mouse spinal cord cultures.

Protein degradation half-life is an important consideration for AS treatment. Even with 100% block of protein synthesis by AS, no depletion of the target protein occurs until the remaining pool of previously synthesized protein is degraded (30). Several days may be required for depletion of a specific target protein (12, 31). In our experiments, a significant reduction in the levels of α subunits did not appear until after treatment of cells with AS for 4 days. This observation probably reflects the slow rate of G protein α subunit turnover.

By treatment of ND8–47 cells with phosphorothioate AS, our studies clearly demonstrate that α_{i2} is responsible for opioid-induced increases in $[Ca^{2+}]_i$ in ND8–47 cells. These results extend the general concept of multiple G protein-mediated regulation of Ca^{2+} channels. In ND8–47 cell membranes, we did not detect α_o subunits using immunoblot analysis, nor did we observe an opioid-induced decrease in $[Ca^{2+}]_i$. These results contrast with the observations in NG108–15 cells, in which both α_{i2} and α_o were detected. In those cells lower concentrations of opioids increase, and higher concentrations of opioids reduce, $[Ca^{2+}]_i$ (22). We sug-

gest, therefore, that it is the G protein coupling of opioid receptors that determines the direction of modulation (opening or closing) of calcium channels by opioids; α_o is mainly responsible for inhibition of Ca^{2+} channels, whereas α_{i2} mediates the opening of Ca^{2+} channels. These results extend the range of G protein-mediated transduction systems influenced by opioid receptor activation. In different systems, it is now established that $G_{\alpha i2}$ mediates opioid inhibition of adenylyl cyclase activity (9) and activation of dihydropyridine-sensitive calcium channels (this paper) and $G_{\alpha o}$ mediates opioid inhibition of N-type calcium channels (12). PTX-sensitive G proteins also mediate opioid-induced mobilization of Ca^{2+} from intracellular stores (24) and the opening of K^+ channels induced by opioids (5), but the specific G protein α subunits implicated in these actions are not yet established.

Using an AS treatment approach, we have demonstrated that the increase in $[Ca^{2+}]_i$ induced in ND8–47 cells by activation of δ-opioid receptors is inhibited by prior treatment of the cells with AS directed against the G protein α_{i2} subunit but not by the complementary sense oligonucleotide or AS to other G protein α subunits present in the membranes. Evidence presented here suggests that opioid receptor activation of dihydropyridine-sensitive calcium channels is mediated by $G_{\alpha i2}$ protein through an as yet undefined mechanism. The physiological relevance of this effect is uncertain. However, our recent studies showed that opioids could induce similar increases in $[Ca^{2+}]_i$ in a subset of mouse DRG neurons in primary culture. The effect was observed predominantly in neurons of larger diameter. This action was blocked by opioid antagonists and nifedipine, suggesting that the mechanism is similar to that observed in the ND8–47 cells used in this study.

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References

1. Sharma, S. K., W. A. Klee, and M. Nirenberg. Opioid-dependent modulation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **74**:3365–3369 (1977).
2. Frey, E. A., and J. W. Kebabian. A μ-opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. *Endocrinology* **115**:1797–1804 (1984).
3. Yu, V. C., M. L. Richards, and W. Sadee. A human neuroblastoma cell line expresses μ and δ opioid receptor sites. *J. Biol. Chem.* **261**:1065–1070 (1986).
4. Konkoy, C. S., and S. R. Childers. Dynorphin-selective inhibition of adenylyl cyclase in guinea pig cerebellum membranes. *Mol. Pharmacol.* **36**:627–633 (1989).
5. North, R. A., J. T. Williams, A. Surprenant, and M. J. Christie. *Mu* and *delta* receptors belong to a family of receptors that are coupled to potassium channels. *Proc. Natl. Acad. Sci. USA* **84**:5487–5491 (1987).
6. Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz. The GTP binding protein, G_o, regulates neuronal calcium channels. *Nature (Lond.)* **325**:445–447 (1987).
7. Seward, L., C. Hammond, and G. Henderson. Opioid-receptor mediated inhibition of the N-type calcium-channel current. *Proc. R. Soc. Lond. B Biol. Sci.* **224**:129–135 (1991).
8. Tang, T., J. G. Kiang, and B. M. Cox. Opioids acting through *delta* receptors elicit a transient increase in the intracellular free calcium concentration in dorsal root ganglion-neuroblastoma hybrid ND8–47 cells. *J. Pharmacol. Exp. Ther.* **270**:40–46 (1994).
9. McKenzie, F. R., and G. Milligan. δ-Opioid receptor mediated inhibition of adenylate cyclase is transduced specifically by the guanine nucleotide binding protein G_{i2}. *Biochem. J.* **267**:391–398 (1990).
10. Hescheler, J., W. Rosenthal, M. Wulfein, M. Tang, M. Yajima, W. Trautwein, and G. Schultz. Involvement of the guanine nucleotide- binding protein, N_o, in the inhibitory regulation of neuronal calcium channels. *Adv. Second Messenger Phosphoprotein Res.* **21**:165–174 (1988).
11. McFadzean, I., I. Mullaney, D. A. Brown, and G. Milligan. Antibodies to the GTP binding protein, G_o, antagonize noradrenaline-induced calcium current inhibition in NG108–15 cells. *Neuron* **3**:177–182 (1988).
12. Liu, Y. F., K. H. Jakobs, M. M. Rasenick, and P. R. Albert. G protein specificity in receptor-effector coupling. *J. Biol. Chem.* **269**:13880–13886 (1993).
13. Shen, K. F., and S. M. Crain. Dual opioid modulation of the action potential duration of mouse dorsal root ganglion neurons in culture. *Brain Res.* **491**:227–242 (1989).
14. Shen, K. F., and S. M. Crain. Cholera toxin-A subunit blocks opioid excitatory effects on sensory neuron action potentials indicating mediation by G_s-linked opioid receptors. *Brain Res.* **525**:225–231 (1990).
15. Tang, T., J. G. Kiang, T. E. Cote, and B. M. Cox. Opioid-induced increase in $[Ca^{2+}]_i$ in ND8–47 neuroblastoma × dorsal root ganglion hybrid cells is mediated through G protein-coupled δ opioid receptors and desensitized by chronic exposure to opioid. *J. Neurochem.*, in press.
16. Gollasch, M., C. Kleluss, J. Hescheler, B. Wittig, and G. Schultz. G_{i2} and protein kinase C are required for thyrotropin-releasing hormone induced stimulation of voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells. *Proc. Natl. Acad. Sci. USA* **90**:6265–6269 (1993).
17. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450 (1985).
18. Izenwasser, S., and T. E. Côté. Inhibition of adenylyl cyclase activity by a homogeneous population of dopamine receptors: selective blockade by antisera directed against G_{i1} and/or G_{i2}. *J. Neurochem.* **64**:1614–1621 (1995).
19. Goldsmith, P., P. Gierschick, C. Milligan, G. Unson, R. Vinitsky, H. L. Malech, and A. M. Spiegel. Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neurophil and brain. *J. Biol. Chem.* **262**:14683–14688 (1987).
20. Goldsmith, P., K. Rossiter, A. Carter, W. Simonds, G. C. Unson, R. Vinitsky, and A. M. Spiegel. Identification of the GTP-binding protein encoded by G_{i3} complememtary DNA. *J. Biol. Chem.* **263**:6476–6479 (1988).
21. Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *J. Biol. Chem.* **266**:9309–9313 (1991).
22. Jin, W., N. M. Lee, H. H. Loh, and S. A. Thayer. Dual excitatory and inhibitory effects of opioids in the neuronal cell line NG108–15. *Mol. Pharmacol.* **42**:1083–1089 (1992).
23. Shen, K. F., and S. M. Crain. Dynorphin prolongs the action potential of mouse sensory ganglion neurons by decreasing a potassium conductance whereas another specific *kappa* opioid does so by increasing a calcium conductance. *Neuropharmacology* **29**:343–349 (1990).
24. Jin, W., N. M. Lee, H. H. Loh, and S. A. Thayer. Opioids mobilize calcium from inositol 1,4,5-trisphosphate-sensitive stores in NG108–15 cells. *J. Neurosci.* **14**:1920–1929 (1994).
25. Chen, G. G., A. Chalazonitis, K. F. Shen, and S. M. Crain. Inhibition of cyclic AMP-dependent protein kinase blocks opioid-induced prolongation of the action potential duration of mouse sensory ganglion neurons in dissociated cell cultures. *Brain Res.* **462**:372–377 (1988).
26. Crain, S. M., K.-F. Shen, and A. Chalazonitis. Opioid excite rather than inhibit sensory neurons after chronic opioid exposure of spinal cord-ganglion culture. *Brain Res.* **455**:99–109 (1988).
27. Koizumi, S., K. Inoue, Y. Kataoka, M. Niwa, and A. Takanaka. Endothelin-3 activates a voltage-gated Ca channel via a pertussis toxin sensitive mechanism leading to dopamine release from PC-12 cells. *Neurosci. Lett.* **166**:191–194 (1994).
28. Stein, C. A., and J. S. Cohen. Oligodeoxynucleotides as inhibitors of gene expression: a review. *Cancer Res.* **48**:2659–2668 (1988).
29. Helene, C., and J. J. Toulme. Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim. Biophys. Acta* **1049**: 99–125 (1990).
30. Albert, P., and S. J. Morris. Antisense knockouts: molecular scalpels for the dissection of signal transduction. *Trends Pharmacol. Sci.* **15**:250–254 (1994).
31. Maxham, C. M., Y. Hod, and C. C. Malbon. Induction of $G_{\alpha i2}$ specific antisense RNA *in vivo* inhibits neonatal growth. *Science (Washington D.C.)* **260**:991–995 (1993).

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