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δ-Opioid-Induced Liberation of Gβγ Mobilizes Ca^{2+} Stores in NG108-15 Cells

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ABSTRACT

Activation of δ-opioid receptors in NG108-15 cells releases Ca^{2+} from an intracellular store through activation of a pertussis toxin-sensitive G protein. We tested the hypothesis that activation of δ-opioid receptors mobilizes inositol 1,4,5-trisphosphate (IP_3)-sensitive Ca^{2+} stores via liberation of Gβγ. Fura-2-based digital imaging was used to study the mechanism of opioid-induced increases in $[Ca^{2+}]_i$ in NG108-15 cells. Exposure to D-Ala^2-D-Leu^5 enkephalin (100 nM) for 90 s induced increases in $[Ca^{2+}]_i$ that were blocked by microinjection of the IP_3 receptor antagonist heparin (pipette concentration = 100 mg/ml) but not by sham injection. Microinjection of a peptide that binds Gβγ (QEHA, 1 mM) decreased the D-Ala^2-D-Leu^5 enkephalin-evoked response. Microinjection of an inactive peptide (SKEE, 1 mM) that does not bind to Gβγ failed to inhibit the opioid-induced increase in $[Ca^{2+}]_i$. Microinjection of a peptide (QLKK, 15 mM) that binds to free $G\alpha_q$ blocked the increase evoked by 3 nM bradykinin, but microinjection of an inactive peptide (ADRK, 15 mM) did not. Microinjection of QLKK did not significantly affect the opioid-induced increase in $[Ca^{2+}]_i$. Collectively, these data demonstrate that activation of δ-opioid receptors induces the release of Ca^{2+} from IP_3-sensitive stores in NG108-15 cells through activation of the βγ subunits of inhibitory G proteins.

Opioid receptors are members of the G protein-coupled receptor superfamily (Evans et al., 1992; Raynor et al., 1994) and exert their principal effects by coupling to inhibitory G proteins (G_o/G_i). Opioid-induced dissociation of heterotrimeric G proteins produces diverse effects mediated by both α and βγ subunits. $G\alpha_{i/o}$ inhibits adenylyl cyclase (AC) (McKenzie and Milligan, 1990; Murthy and Makhlouf, 1996), whereas Gβγ activates K^+ channels (Logothetis et al., 1987; Clapham and Neer, 1997) and inhibits voltage-dependent Ca^{2+} channels (Herlitze et al., 1996; Ikeda, 1996). Activation of opioid receptors also appears to stimulate phospholipase C (PLC) (Jin et al., 1992; Miyamae et al., 1993; Jin et al., 1994; Tsu et al., 1995; Smart and Lambert, 1996), although how the activated receptor couples to PLC is not clear.

Activation of δ-opioid receptors in NG108-15 cells releases Ca^{2+} from intracellular stores via a process that appears to activate PLC, as indicated by inhibition of the response by the PLC inhibitor U73122 (Jin et al., 1994). Furthermore, Smart and Lambert (1996) have shown that δ-opioids will stimulate the production of 1,4,5-inositol triphosphate (IP_3) in NG108-15 cells. In these studies, both opioid-induced $[Ca^{2+}]_i$ increases and the production of IP_3 were prevented by pertussis toxin (PTX), indicating that the response was mediated by an inhibitory G protein. The α subunits of inhibitory G proteins are not thought to couple directly to PLC. However, Gβγ liberated by the activation of heterotrimeric G_i can stimulate certain isoforms of PLC (Camps et al., 1992; Katz et al., 1992). $G\alpha_q$ may also participate in δ-opioid-induced $[Ca^{2+}]_i$ increases (Okajima et al., 1993), but how this coupling might occur is not clear. In this study, we tested the hypothesis that activation of δ-opioid receptors in NG108-15 cells mobilizes IP_3-sensitive Ca^{2+} stores via liberation of Gβγ.

We used fura-2-based digital imaging to measure opioid-induced $[Ca^{2+}]_i$ responses in single NG108-15 cells. Selective inhibitors that bound IP_3 receptors, Gβγ, and $G\alpha_q$ were microinjected to determine the contribution of these signaling molecules to the $[Ca^{2+}]_i$ increase. We determined that activation of δ-opioid receptors mobilized Ca^{2+} through IP_3 receptors and that Gβγ mediated this response.

Materials and Methods

Reagents and Peptides. Materials used and companies from which they were purchased are as follows: fura-2 acetoxymethyl ester, Molecular Probes, Eugene, OR; D-Ala^2-D-Leu^5 enkephalin (DADLE), Peninsula Laboratories, Inc., Belmont, CA; CO_2-independent media, hypoxanthine/aminopterin/thymidine supplement, Life

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ABBREVIATIONS: PLC, phospholipase C; DADLE, D-Ala^2-D-Leu^5 enkephalin; IP_3, 1,4,5-inositol trisphosphate; PTX, pertussis toxin; AC, adenylyl cyclase; GTPγ*S*, guanosine 5′-O-(3-thiotriphosphate).

Technologies, Inc., Grand Island, NY; all other reagents were purchased from Sigma, St. Louis, MO. QEHA and SKEE peptides were synthesized in the laboratory of Dr. R. P. Elde, using an Applied Biosystems Synergy system (Foster City, CA). The sequences for QEHA peptide and SKEE peptide were QEHAQEPERQYMHIGTMVEFAYALVGK and SKEEKSDKERWQHLADLADFALAMKDT, respectively. QLKK and ADRK peptides were synthesized in the Microchemical Facility at the University of Minnesota, using a Milligen Biosearch BS 9600 system (Novota, CA). The sequences for QLKK peptide and ADRK peptide were QLKKLKEICEKEKKELKKKMDKKRQEKITEAK and ADRKRVETALEACSL, respectively. All peptides were purified by C_{18} reversed-phase HPLC in 0.1% trifluoroacetic acid and eluted with an H_2O-acetonitrile gradient of 0 to 60% in 30 min. Solvents were removed by lyophilization.

Cell Culture. NG108-15 cells (passage 21–30) were grown in T25 cm^2 flasks in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, 0.1 mM sodium hypoxanthine, 0.4 μM aminopterine, 16 μM thymidine, 100 mg/l streptomycin, and 100 I.U./ml penicillin in a humidified atmosphere of 90% air and 10% CO_2. Cells from the stock culture were plated onto glass coverslips (25-mm round) at a density of 3×10^4 cells/coverslip and grown until 80% confluent. The cells then were placed in a CO_2-independent medium (phosphate- rather than bicarbonate-buffered) supplemented with 5 μM forskolin for 3 to 6 days at 37°C and atmospheric gas levels.

Experimental Procedures. The cells were loaded with 2 μM fura-2 acetoxymethyl ester for digital imaging in HEPES-buffered Hanks' salts solution containing 0.5% BSA for 45 min at 37°C. The HEPES buffer was composed of the following: 20 mM HEPES, 137 mM NaCl, 1.26 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 5.0 mM KCl, 0.4 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 3.0 mM $NaHCO_3$, and 5.0 mM glucose. The loading was terminated by washing with HEPES-buffered Hanks' solution for 15 min before starting an experiment. The cover glass was then mounted in a flow-through chamber (Thayer et al., 1988), which was superfused at a rate of 2 ml/min with HEPES-buffered Hanks' solution. Solutions were selected with a multiport valve coupled to several reservoirs. The chamber containing the fura-2-labeled cells was mounted on the stage of an inverted microscope (Nikon Diaphot; Nikon, Melville, NY) and alternately excited at 340 or 380 nm by rapidly switching optical filters (10-nm band pass) mounted in a computer-controlled wheel (Sutter Instrument Co., Novato, CA) placed between a 75 W Xe arc lamp and epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400-nm) through a 90× objective (Leitz, numerical aperture 1.15). Fluorescent images (510, 40-nm band pass) were projected (0.5×) onto a cooled charge-coupled device camera (Photometrics, Inc., Tucson, AZ; 384 × 576 binned to 192 × 288 pixels, 12-bit scale) controlled by an IBM-compatible computer. Image pairs were collected every 6 s; exposure to excitation light was always 120 ms/image and the interval between paired images was 385 ms. $[Ca^{2+}]_i$ was calculated from the ratio of the two background-subtracted digital images. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380-nm image. Background images were collected at the end of each experiment after the cells were removed from the coverslip. Autofluorescence from cells not loaded with the dye was less than 5% and thus, not corrected. Ratio values were converted to free $[Ca^{2+}]_i$ by the equation $[Ca^{2+}]_i = K\,\beta\,(R-R_{min})/(R_{max}-R)$, in which R is the 340/380-nm fluorescence emission ratio and $K = 224$ nM, the dissociation constant for fura-2 (Grynkiewicz et al., 1985). The maximum ratio ($R_{max} = 3.46$), the minimum ratio ($R_{min} = 0.25$), and the constant β (the ratio of the fluorescence measured at 380 nm in Ca^{2+}-free and saturating solution), 5.17, were determined by treating cells with 10 μM ionomycin in Ca^{2+}-free (1 mM EGTA) and saturating (5 mM Ca^{2+}) solution.

Microinjection. Ten minutes after an initial control response to agonist, either DADLE or bradykinin, approximately half of the responding cells were microinjected. Pipettes were pulled from borosilicate glass capillary tubes (TW100F-4; WPI, Sarasota, FL). A microinjector (Eppendorf, Hamburg, Germany) was used to apply a holding pressure of 8 hPa and an injection pressure of 11 to 90 hPa for 0.1 to 0.6 s. Cells were allowed to recover for 35 min after injection. Injection solution was composed of dextran-conjugated tetramethylrhodamine (MW 10,000; pipette concentration = 12.5 mg/ml) combined with test agents in distilled water. A single test agent was examined per experiment. None of the agents microinjected in this study affected the resting fura-2 fluorescence intensity values at the end of the 35-min recovery period. The injection volume was quantified by digital imaging of rhodamine fluorescence at an excitation wavelength of 535 nm (50-nm bandpass) and an emission wavelength of 605 nm (55-nm bandpass). Dilution factors for injected substances were estimated by dividing the rhodamine intensity value from the injected cell by the fluorescence from a droplet comparable in size to a single cell containing a known concentration of rhodamine. The mean fluorescence intensity from 10 droplets containing 0.5 mg/ml tetramethylrhodamine dextran injected into immersion oil was 1953 ± 248 arbitrary units (12-bit scale, 10-ms exposure).

Results

Opioids Increase $[Ca^{2+}]_i$ by Activating IP_3 Receptors. NG108-15 cells were grown in serum-free, CO_2-independent media supplemented with 5 μM forskolin for 4 to 6 days and $[Ca^{2+}]_i$ was recorded with fura-2-based digital imaging as described in *Materials and Methods*. A brief (90-s) exposure to 100 nM DADLE produced a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A). We have shown previously that this response is mediated by an inhibitory G protein and results from Ca^{2+} release from intracellular stores (Jin et al., 1994). In this report we present studies aimed at identification of the signaling pathway that couples inhibitory G proteins to Ca^{2+} mobilization. The general strategy we employed was to elicit an initial control response to 100 nM DADLE (Fig. 1A) and then, after recovery, microinject selective inhibitors into some of the responding cells. Noninjected cells in the same field served as controls. In separate experiments, sham injections or injections of inactive analogs of the inhibitory agents were additional controls. Rhodamine (12.5 mg/ml) was added to the pipette solution and rhodamine fluorescence was quantified by digital imaging to confirm successful microinjection.

Sham injection did not affect the DADLE-induced response (Fig. 1A). DADLE-induced $[Ca^{2+}]_i$ transients elicited 35 min after sham microinjection were comparable in amplitude with noninjected control cells. The second application of DADLE evoked responses that were 77 ± 3% ($n = 175$) and 74 ± 11% ($n = 9$) of the initial response in control and sham-injected cells, respectively (Fig. 1C). We have shown previously that the DADLE-induced $[Ca^{2+}]_i$ increase was blocked by an inhibitor of PLC (Jin et al., 1994) and activation of opioid receptors has been shown to increase IP_3 formation in NG108-15 cells (Smart and Lambert, 1996). These results suggested that the IP_3 receptor might mediate the opioid-induced $[Ca^{2+}]_i$ increase. We tested this possibility directly by microinjecting heparin, which is an antagonist of IP_3 receptors (Ghosh et al., 1988), into DADLE-responsive cells. Microinjection of heparin (pipette concentration = 100 mg/ml) blocked the second DADLE-induced $[Ca^{2+}]_i$ response, whereas reproducible $[Ca^{2+}]_i$ increases in response to the second DADLE stimulation were observed in control cells (Fig. 1B). In heparin-injected cells, the second DADLE-induced response was

23 ± 5% (n = 21) of the initial response, which was significantly different from responses in sham-injected cells (Fig. 1C). The injection volume was smaller in the heparin-injected cells than in sham-injected cells, as indicated by rhodamine fluorescence (Fig. 1D). Based on the fluorescence intensities of known concentrations of rhodamine (see *Materials and Methods*), we estimate that the heparin solution in the pipette was diluted 47-fold. This dilution corresponds to an approximate heparin concentration of 2 mg/ml, a standard concentration applied by patch pipette for intracellular inhibition of IP_3-induced Ca^{2+} release (Willmott et al., 1995; Wu et al., 1995; Hashimoto et al., 1996; Wu and Wang, 1996; Le Grand et al., 1998). These results indicate that IP_3 receptors on intracellular Ca^{2+} stores mediate the opioid-induced $[Ca^{2+}]_i$ increase in NG108-15 cells.

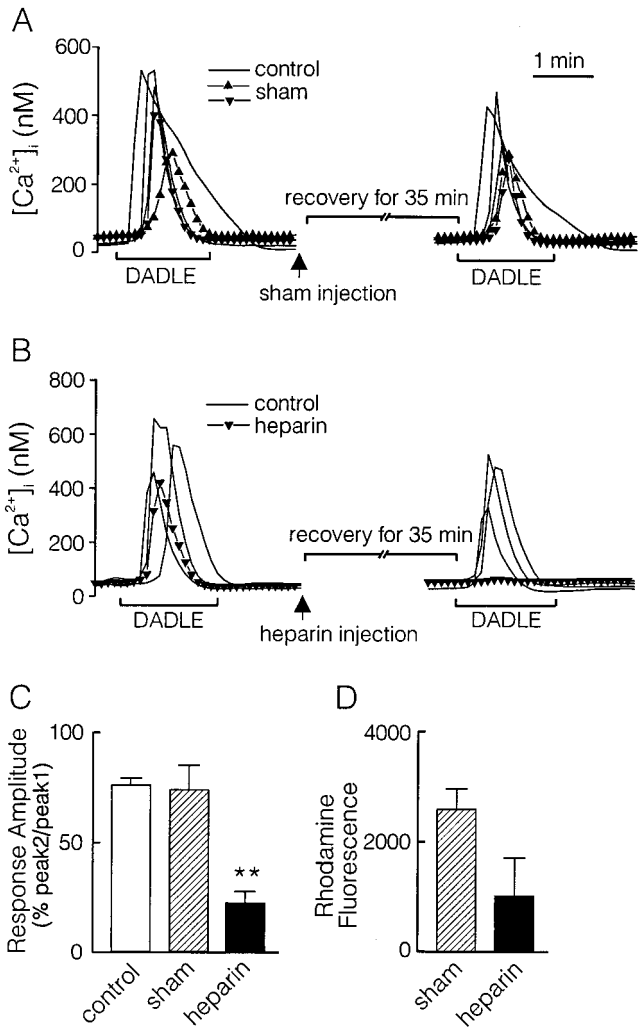

Fig. 1. Microinjection of heparin inhibits the opioid-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in fields of NG108-15 cells, using fura-2-based digital imaging as described in *Materials and Methods*. Image pairs were collected at 6-s intervals. 100 nM DADLE was applied as indicated by horizontal bars. A, sham injection of rhodamine (pipette concentration = 12.5 mg/ml) into two cells (solid symbols) at the time indicated by the arrow did not influence the amplitude of a second opioid-induced $[Ca^{2+}]_i$ increase evoked 35 min after injection. B, microinjection of heparin (pipette concentration = 100 mg/ml) combined with rhodamine blocked the second DADLE-induced $[Ca^{2+}]_i$ transient evoked 35 min after microinjection (solid symbols). C, summary of effects of heparin microinjection on DADLE-induced responses. The DADLE-induced response amplitude is presented as a percentage of the initial control responses (peak2/peak1) for control (n = 175), sham-injected (sham, n = 9), and heparin-injected (heparin, n = 21) cells. D, rhodamine fluorescence (arbitrary units) from injected cells. Data are expressed as mean ± S.E. $^{**}p < .01$, heparin-injected relative to control and sham-injected cells (ANOVA with Bonferroni post-test).

G$\beta\gamma$ Couples Opioid Receptors to IP_3-Sensitive Ca^{2+} Stores. We tested the hypothesis that the opioid-induced $[Ca^{2+}]_i$ increase was mediated through the $\beta\gamma$ subunits of a heterotrimeric G protein. Chen et al. (1995) found that a synthetic peptide (QEHA), which encodes residues 956 to 982 of AC II, binds to G protein $\beta\gamma$ subunits and inhibits G$\beta\gamma$-mediated signaling. Synthetic SKEE peptide represents the cognate region of AC III, which does not bind to G$\beta\gamma$. To determine whether the opioid-induced $[Ca^{2+}]_i$ increases were mediated through G$\beta\gamma$, we microinjected the QEHA peptide (pipette concentration = 1 mM) into NG108-15 cells and subsequently stimulated the cells with DADLE. As shown in Fig. 2B, microinjection of QEHA peptide into NG108-15 cells inhibited the second opioid-induced $[Ca^{2+}]_i$ transient. Injection of 0.1 mM QEHA did not affect the DADLE-evoked response. Pseudocolor images for the experiment plotted in Fig. 2B are displayed in Fig. 2C. The frame numbers correspond to the times indicated along the plot (Fig. 2B) and the two cells injected with 1 mM QEHA are identified by the rhodamine fluorescence shown in the lower left frame (Fig. 2C). The second DADLE-induced $[Ca^{2+}]_i$ increase in the QEHA peptide-injected cells was 24 ± 7% of the initial response, significantly less than the 72 ± 4% response recorded for control cells (Fig. 2D). In contrast, microinjection of SKEE peptide (pipette concentration = 1 mM) into NG108-15 cells did not significantly affect the opioid-induced $[Ca^{2+}]_i$ increase (Fig. 2A). Rhodamine fluorescence in SKEE peptide- and QEHA peptide-injected cells was not significantly different (Fig. 2E), indicating that the amount of peptide injected was similar. Based on the fluorescence intensities of known concentrations of rhodamine (see *Materials and Methods*), we estimate that the pipette solution was diluted 19-fold. This dilution corresponds to an approximate peptide concentration of 53 μM, which is well within the 10 to 200 μM concentration range shown by Chen et al. (1995) to selectively inhibit $\beta\gamma$-stimulated type-2 AC activity. These results demonstrate that free G protein $\beta\gamma$ subunits are involved in the opioid-induced $[Ca^{2+}]_i$ increase in NG108-15 cells.

The Gα_q-Binding Peptide Inhibits Bradykinin-Induced $[Ca^{2+}]_i$ Responses in NG108-15 Cells. Wu et al.(1993) have identified the region of PLCβ1 that interacts with Gα_q. A peptide derived from this region (QLKK) inhibited activation of PLC by Gα_q. We synthesized this peptide and confirmed its activity by testing it against $[Ca^{2+}]_i$ responses elicited by 3 nM bradykinin, which are known to be mediated by Gα_q (Gutowski et al., 1991). Microinjection of QLKK peptide (pipette concentration = 15 mM) inhibited markedly the bradykinin-induced $[Ca^{2+}]_i$ transient (Fig. 3B). The bradykinin-induced response in QLKK peptide-injected cells was 20 ± 11% of the initial control response, significantly smaller than the 72 ± 3% response observed in control cells (Fig. 3C). A pipette concentration of 1 mM QLKK was without effect, and 10 mM peptide produced a partial inhibition. A peptide representing residues 180 to 194 of PLCβ1 (ADRK), which does not interact with Gα_q, did not affect the bradykinin-induced $[Ca^{2+}]_i$ increase when microinjected into NG108-15 cells (Figs. 3, A and C). The response in ADRK

peptide-injected cells was 85 ± 7% of the initial control response. Rhodamine fluorescence intensities in the ADRK peptide- and QLKK peptide-injected cells were not significantly different, indicating that a similar amount of peptide was microinjected in each case. Based on the fluorescence intensities of known concentrations of rhodamine, we estimate that the pipette solution was diluted 17-fold. This dilution corresponds to an approximate peptide concentration of 880 μM. Wu et al. (1993) demonstrated sequence specific inhibition of $G\alpha_q$-mediated guanosine 5′-O-(3-thiotriphosphate)- ($GTP\gamma S$-) dependent activation of PLCβ1 for peptide concentrations up to 1 mM. These results indicate that QLKK peptide acts as an inhibitor of $G\alpha_q$-mediated PLC activation in NG108-15 cells.

DADLE-Induced $[Ca^{2+}]_i$ Responses Are Not Significantly Inhibited by a $G\alpha_q$-Binding Peptide. It has been suggested that the $G\alpha_q$ subunit is also involved in the δ-opioid-induced $[Ca^{2+}]_i$ increase (Okajima et al., 1993). Thus, we determined whether the $G\alpha_q$ subunit was involved in the opioid-induced $[Ca^{2+}]_i$ increase in NG108-15 cells. Microinjection of the QLKK peptide (pipette concentration = 15 mM), which interacts with the $G\alpha_q$ subunit and inhibits $G\alpha_q$-mediated signaling, did not significantly inhibit the opioid-induced $[Ca^{2+}]_i$ transient (Fig. 4B). The DADLE-induced response in QLKK peptide-injected cells was 58 ± 10% (n = 12) of the initial control response, which was not significantly different than the 86 ± 6% (n = 58) and 71 ± 16% (n = 8) observed in control cells and ADRK peptide-injected cells, respectively (Fig. 4, A and C). Rhodamine fluorescence in the ADRK peptide- and QLKK peptide-injected cells was not significantly different, indicating that a similar amount of peptide was microinjected. Based on the fluorescence intensities of known concentrations of rhodamine, we estimate that the pipette solution was diluted 16-fold. This dilution corresponds to an approximate peptide concentration of 940 μM, which is 3-fold greater than the concentration Wu et al. (1993) found to completely inhibit $G\alpha_q$-mediated $GTP\gamma S$-dependent activation of PLCβ1. Thus, even a supramax imal concentration of QLKK failed to significantly inhibit DADLE-induced Ca^{2+} mobilization. These results suggest

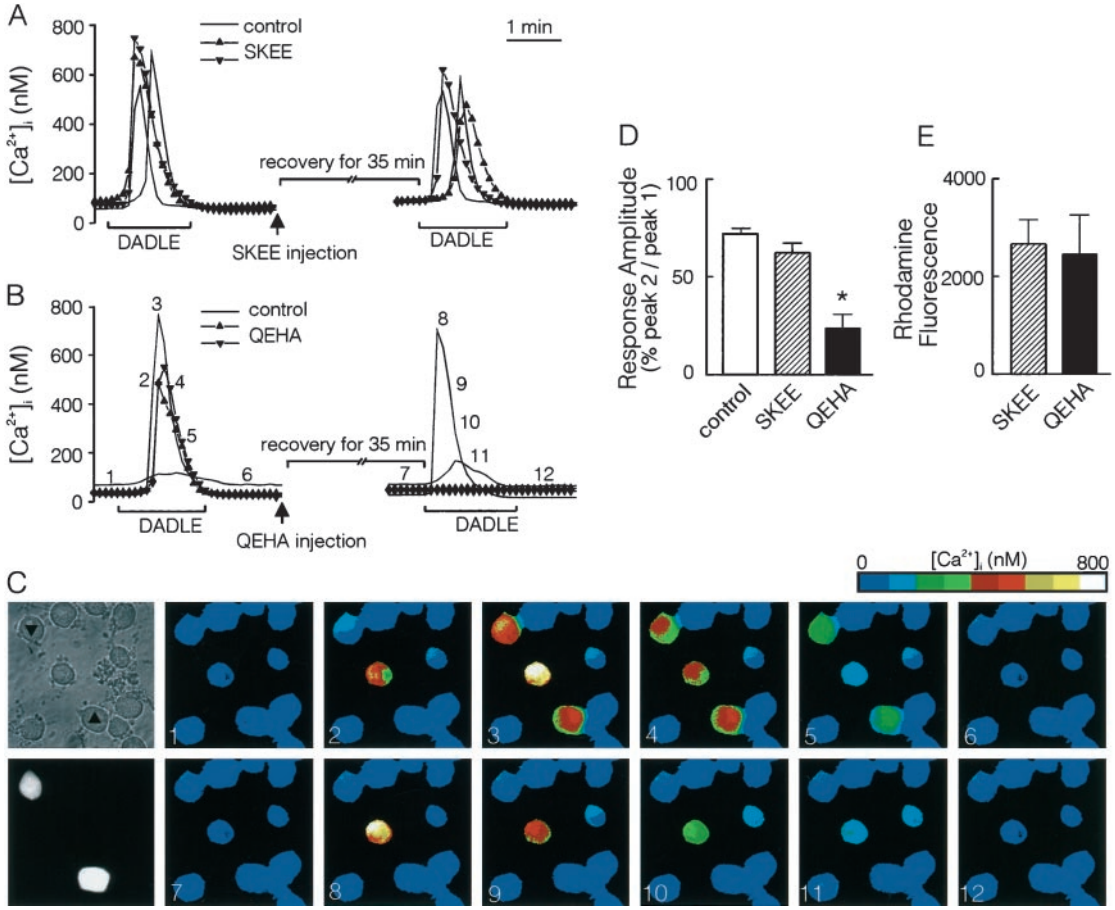

Fig. 2. Microinjection of QEHA peptide inhibits the opioid-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in fields of NG108-15 cells using fura-2-based digital imaging as described in *Materials and Methods*. Image pairs were collected at 6-s intervals. 100 nM DADLE was applied for 90 s as indicated by horizontal bars. A, microinjection of SKEE peptide (pipette concentration = 1 mM) into two cells at the time indicated by the arrow did not decrease the opioid-induced $[Ca^{2+}]_i$ transients (solid symbols). B, microinjection of QEHA peptide (pipette concentration = 1 mM) into NG108-15 cells blocked opioid-induced $[Ca^{2+}]_i$ transients (solid symbols). C, pseudocolor representations of $[Ca^{2+}]_i$ were derived from fura-2-based digital images for the experiment shown in B. Two of the cells in which DADLE induced an increase in $[Ca^{2+}]_i$ were injected with QEHA as indicated by rhodamine fluoresence (lower left frame) and marked with the solid triangles on the bright field image (upper left frame). The images displayed in C were acquired at the times indicated by the frame numbers placed along the plot in B. D, summary of effects of SKEE peptide and QEHA peptide on the DADLE-induced response. The DADLE-induced response amplitude is presented as a percentage of the initial control responses (peak2/peak1) for control (n = 78), SKEE peptide-injected (SKEE, n = 9) and QEHA peptide-injected (QEHA, n = 16) cells. E, rhodamine fluorescence (arbitrary units) from injected cells. There was no significant difference in rhodamine fluorescence between SKEE- and QEHA-injected cells. Data are expressed as mean ± S.E. *$p < .05$, QEHA-injected relative to control and SKEE-injected cells (ANOVA with Bonferroni post-test).

that activation of $G\alpha_q$ is not required for the δ-opioid-induced $[Ca^{2+}]_i$ increase in NG108-15 cells.

Discussion

In this report, we demonstrate through the use of selective antagonists that opioid-induced increases in $[Ca^{2+}]_i$ result from the mobilization of IP_3-sensitive Ca^{2+} stores via the liberation of the $G\beta\gamma$ subunits. Thus, we suggest the signaling pathway outlined in Fig. 5. The essential elements of this model include mobilization of the IP_3-sensitive Ca^{2+} store and opioid-induced liberation of $G\beta\gamma$.

That activation of δ-opioid receptors will mobilize IP_3-sensitive Ca^{2+} stores is consistent with a number of studies including our own (Jin et al., 1994; Smart and Lambert, 1996). We showed previously that DADLE evoked increases in $[Ca^{2+}]_i$ in NG108-15 cells that did not require extracellular Ca^{2+} and were blocked by thapsigargin, an inhibitor of the ATP-dependent Ca^{2+} pump that is responsible for loading Ca^{2+} into intracellular stores. The PLC inhibitor U73122 also inhibited the response. Thus, the inhibition produced by heparin lends further support to the idea that δ-opioid receptors activate PLC to generate IP_3 (Berridge, 1993). Indeed, several laboratories have measured opioid-induced in-

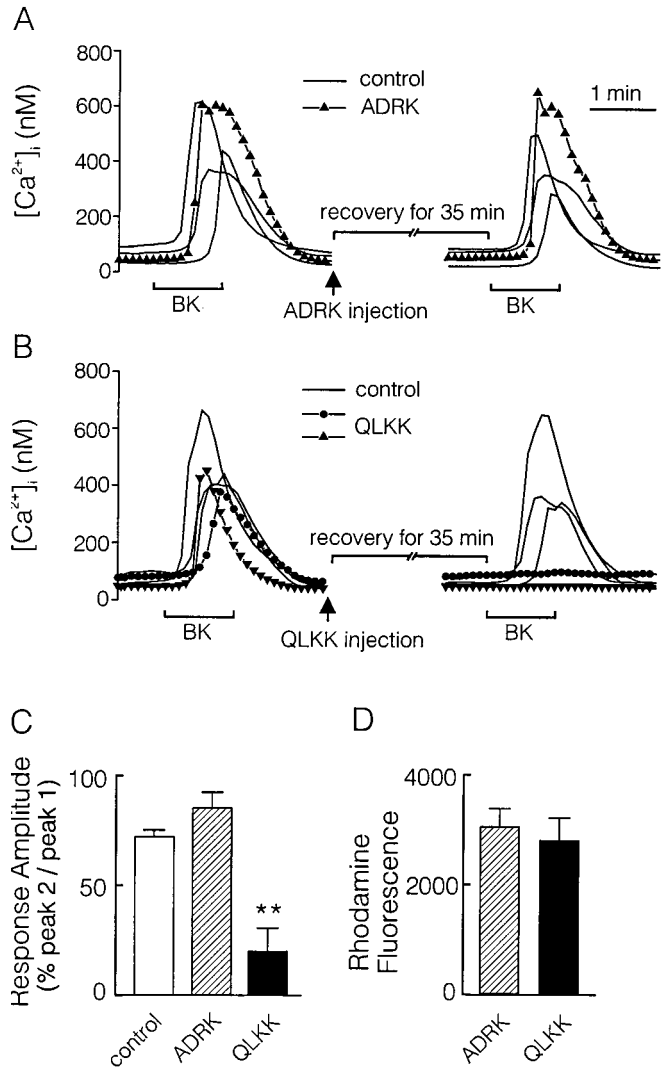

Fig. 3. Microinjection of QLKK peptide inhibits the bradykinin-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in fields of NG108-15 cells using fura-2-based digital imaging as described in *Materials and Methods*. Image pairs were collected at 6-s intervals. 3 nM bradykinin was applied for 60 s as indicated by horizontal bars. A, microinjection of ADRK peptide (pipette concentration = 15 mM) into a single cell at the time indicated by the arrow did not influence the bradykinin-induced $[Ca^{2+}]_i$ transient (solid symbols). B, microinjection of QLKK peptide (pipette concentration = 15 mM) into two cells at the time indicated by the arrow blocked the bradykinin-induced $[Ca^{2+}]_i$ transients. C, summary of effects of QLKK peptide on the bradykinin-induced responses. Bradykinin-induced response amplitude is presented as a percentage of initial control responses (peak 2/peak 1) for control (n = 70), ADRK peptide-injected (ADRK, n = 7), and QLKK peptide-injected (QLKK, n = 6) cells. D, rhodamine fluorescence (arbitrary units) for injected cells was not significantly different between ADRK- and QLKK-injected cells. Data are expressed as mean ± S.E. $^{**}p < .01$ QLKK-injected relative to control and ADRK-injected cells (ANOVA with Bonferroni post-test).

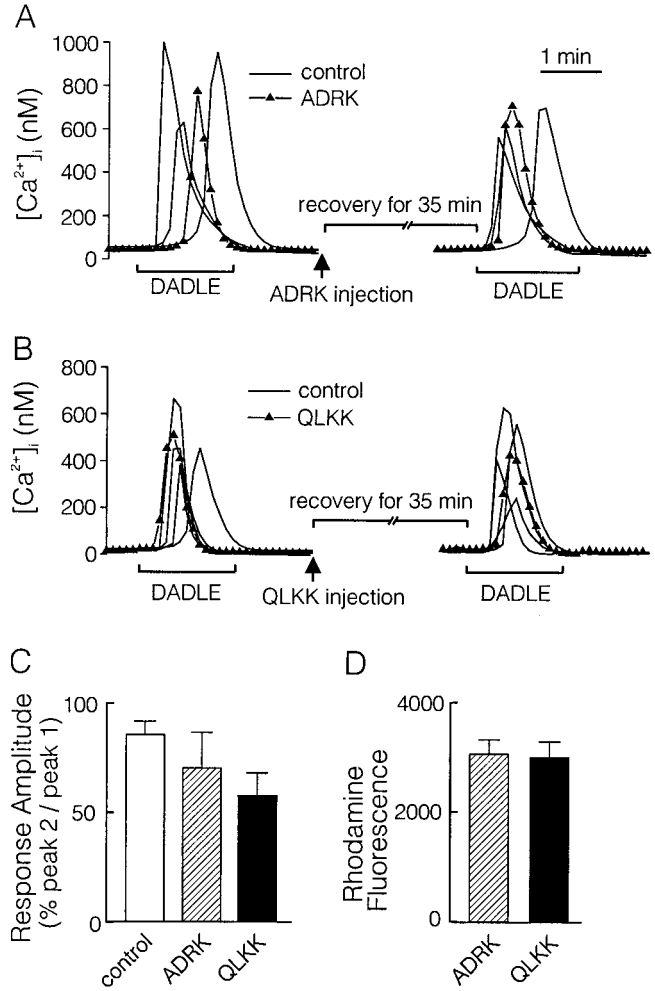

Fig. 4. Microinjection of QLKK did not inhibit the opioid-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was measured in fields of NG108-15 cells using fura-2-based digital imaging as described in *Materials and Methods*. Image pairs were collected at 6-s intervals. 100 nM DADLE was applied for 90-s as indicated by horizontal bars. ADRK peptide (pipette concentration = 15 mM) or QLKK peptide (pipette concentration = 15 mM) was injected into the cells indicated by the solid symbols at the times marked by arrows. Microinjection of ADRK peptide (A) or QLKK peptide (B) did not inhibit DADLE-induced $[Ca^{2+}]_i$ transients. C, summary of effects of QLKK peptide on the DADLE-induced responses. The DADLE-induced response amplitude is presented as a percentage of the initial control response (peak 2/peak 1) for control (n = 58), ADRK-injected (ADRK, n = 8), and QLKK-injected (QLKK, n = 12) cells. D, rhodamine fluorescence (arbitrary units) for injected cells was not significantly different between ADRK- and QLKK-injected cells. Data are expressed as mean ± S.E.

creases in IP_3 directly (Tsu et al., 1995; Smart and Lambert, 1996; Lee et al., 1998).

All previously described δ-opioid receptor-mediated effects in NG108-15 cells, including opioid-induced increases in $[Ca^{2+}]_i$ and IP_3 (Jin et al., 1994; Tsu et al., 1995; Smart and Lambert, 1996), were inhibited by PTX and are thus mediated by inhibitory G proteins. However, there is no precedent for $G\alpha_{i/o}$-type subunits coupling to PLC. Thus, we explored the possibility that the βγ subunits liberated from the dissociation of the $G_{i/o}$ heterotrimer were mediating the opioid-induced increase in $[Ca^{2+}]_i$. It has been shown previously that Gβγ will activate certain β isoforms of PLC (Camps et al., 1992; Katz et al., 1992). The selective inhibition of the DADLE-induced $[Ca^{2+}]_i$ increase by QEHA peptide is consistent with this scenario. There are multiple forms of each of the subunits that make up heterotrimeric G proteins, and in some signaling pathways specific isoforms selectively couple receptor to effector (Kleuss et al., 1991, 1992, 1993). The approach applied in this study could not distinguish the relative contributions of specific Gβγ subunits to opioid-induced Ca^{2+} mobilization, although several Gγ isoforms have been identified in NG108-15 cells (Ueda et al., 1998). $G\gamma_2$, one of the predominant isoforms expressed in NG108-15 cells, has been shown to activate PLCβ2 (Zhang et al., 1996).

In some studies, opioid-induced increases in $[Ca^{2+}]_i$ required costimulation with $G\alpha_q$-generating agonists such as bradykinin or ATP (Okajima et al., 1993). However, the opioid-induced $[Ca^{2+}]_i$ increase described here did not result from opioid-induced liberation of $G\alpha_q$, because microinjection of the QLKK peptide did not significantly inhibit the response, in contrast to the response elicited by bradykinin. This observation is consistent with a similar experiment in smooth muscle cells in which δ-opioid receptor-induced activation of PLCβ was not blocked by an antibody to $G\alpha_q$ (Murthy and Makhlouf, 1996). However, we hesitate to completely rule out a role for $G\alpha_q$ in this response because the QLKK peptide appeared to attenuate the response, although the effect did not reach statistical significance. It was not experimentally feasible to increase the statistical power sufficient to determine whether the small and variable effects of QLKK on the DADLE-evoked response indicated a small contribution from $G\alpha_q$. It is possible that the DADLE-induced $[Ca^{2+}]_i$ increase in NG106-15 cells is mediated by an isoform of PLC that is stimulated by the combined action of $G\alpha_q$ and Gβγ and that in our paradigm $G\alpha_q$ was prebound to the enzyme and, thus, activation of PLC was not readily reversed by the inhibitory peptide. Strassheim et al. (1998) have shown that the β3 isoform of PLC, an isoform activated by both $G\alpha_q$ and Gβγ (Rhee and Choi, 1992; Singer et al., 1997), is present in NG108-15 cells. The idea that PLCβ3 might need to be primed with $G\alpha_q$ to observe an opioid-induced $[Ca^{2+}]_i$ increase would reconcile the results from several laboratories in which the specific culture conditions or the presence of a subthreshold concentration of a $G\alpha_q$-generating agonist was required.

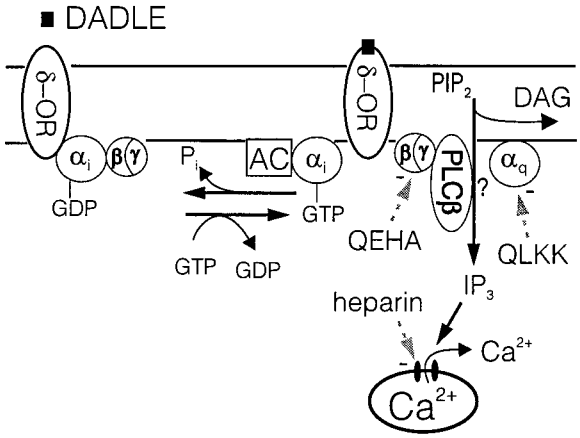

Fig. 5. Hypothetical mechanism of opioid-induced Ca^{2+} release from intracellular stores in differentiated NG108-15 cells. Upon binding of DADLE to the δ-opioid receptor (δ-OR), a PTX-sensitive heterotrimeric G protein dissociates into α_i and βγ subunits. α_i binds to and inhibits AC, and βγ binds to and activates PLCβ. Activation of PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate into IP_3 and diacylglycerol. IP_3 binds to IP_3 receptors located on intracellular stores releasing Ca^{2+} into the cytosol. Heparin is a competitive antagonist for IP_3 receptors. QEHA binds free Gβγ and QLKK binds free $G\alpha_q$.

In summary, we have shown that activation of δ-opioid receptors in NG108-15 cells mobilize IP_3-sensitive Ca^{2+} stores by a Gβγ-dependent mechanism. These results are consistent with an opioid signaling pathway that couples to PLCβ to produce IP_3.

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