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Mobilization of Ca^{2+} from Intracellular Stores in Transfected $Neuro_{2a}$ Cells by Activation of Multiple Opioid Receptor Subtypes

Robert J. Spencer, Wenzhen Jin, Stanley A. Thayer, Sumita Chakrabarti, Ping-Yee Law and Horace H. Loh

Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

ABSTRACT. In neuronal cell lines, activation of opioid receptors has been shown to mobilize intracellular Ca^{2+} stores. In this report, we describe the excitatory actions of opioid agonists on murine neuroblastoma $neuro_{2a}$ cells stably expressing either δ, μ, or κ opioid receptors. Fura-2-based digital imaging was used to record opioid-induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Repeated challenges of δ, μ, or κ opioid receptor expressing cells with 100 nM [D-Ala^2,D-Leu^5]-enkephalin (DADLE), [D-Ala^2,*N*-Me-Phe^4,Gly-ol]-enkephalin (DAMGO), or *trans*-(±)-3,4-dichloro *N*-methyl-*N*-(2-[1-pyrollidinyl] cyclohexyl) benzene acetamide (U-50488H), respectively, elicited reproducible Ca^{2+} responses. Non-transfected $neuro_{2a}$ cells did not respond to opioid agonists. Removal of extracellular Ca^{2+} from the bath prior to and during agonist challenge did not affect significantly the agonist-evoked increase in $[Ca^{2+}]_i$, indicating that the response resulted from the release of Ca^{2+} from intracellular stores. Naloxone reversibly inhibited responses in all three cell lines, confirming that they were mediated by opioid receptors. Expression of cloned opioid receptors in $neuro_{2a}$ cells, coupled with digital $[Ca^{2+}]_i$ imaging, provides a model system for the study of opioid receptors and opioid-activated signaling processes. The fact that all three receptors coupled to the same intracellular signaling mechanism suggests that the primary functional difference between opioid responses *in vivo* results from their selective localization. BIOCHEM PHARMACOL **54**;7:809–818, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. opioid; intracellular Ca^{2+}; $neuro_{2a}$; cloned receptors; Ca^{2+} stores; DADLE; DAMGO; U-50488H

Opioid receptors, via the $G_{i/o}$ family of G proteins, inhibit voltage-sensitive Ca^{2+} channels [1–4], inhibit adenylyl cyclase [5–7], and activate K^+ channels [8, 9]. In addition to these inhibitory actions, opioids have been found to exert excitatory effects [10, 11], suggesting that activation of these receptors affects multiple signaling pathways. Excitatory responses to endogenous opioids may underlie diverse biological functions that include neuronal development [12], induction of long-term potentiation [13], and the potentiation of *N*-methyl-D-aspartate (NMDA) receptor-mediated currents [14, 15]. Activation of the phosphoinositide signaling pathway might account for some of these opioid-mediated effects.

Opioid-induced mobilization of Ca^{2+} from intracellular stores has been described in undifferentiated NG108-15 cells [11, 16, 17]. In this neuronal cell line, δ opioids increase the level of IP_3†, suggesting that these receptors couple to the phosphoinositide signaling cascade [17, 18]. Opioid-induced increases in $[Ca^{2+}]_i$ have also been reported for ND8-47 cells [19], astrocytes [20, 21], ventricular myocytes [22], and other neuronal cell lines [23]. These observations suggest that the mobilization of intracellular Ca^{2+} stores may be a general feature of opioid receptor function.

We explored the possibility that activation of all three opioid receptors might mobilize Ca^{2+} from intracellular stores. Stable expression of δ-, μ-, or κ-opioid receptors in the murine $neuro_{2a}$ cell line enabled the study of these receptors independently within an identical setting. We found that selective activation of each of these receptors evoked increases in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Materials

Chemicals were acquired from the following sources: fura-2 acetoxymethyl ester, Molecular Probes (Eugene, OR); DADLE and DAMGO, National Institute on Drug Abuse

* Corresponding author: Dr. S. A. Thayer, Department of Pharmacology, University of Minnesota Medical School, 3-249 Millard Hall, 435 Delaware Street SE, Minneapolis, MN 55455. Tel. (612) 626-7049; FAX (612) 625-8408; E-mail: thayer@med.umn.edu.

† *Abbreviations*: BK, bradykinin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly-ol]-enkephalin; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HH, HEPES-buffered Hanks' salt solution; IP_3, inositol 1,4,5-triphosphate; and U-50488H, *trans*-(±)-3,4-dichloro *N*-methyl-*N*-(2-[1-pyrollidinyl] cyclohexyl) benzene acetamide.

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(Bethesda, MD); CO_2-independent medium, DMEM, geniticin, and FBS (heat-inactivated), GIBCO Laboratories (Grand Island, NY); all other reagents, Sigma Chemical Co. (St. Louis, MO).

Cell Lines

Mouse DOR-1 subcloned into the *Xho*I site of Bluescript (KS) [24], rat MOR-1 subcloned into the *Hin*dIII site of pCDM8 1 [25–27], and rat KOR-1 subcloned into the *Eco*RI site of pCDNA3 [25] were gifts of Dr. Chris Evans, Dr. Lai Yu, and Dr. L. Y. Lu-Chen, respectively. DOR-1 was subcloned subsequently into the *Eco*RI/*Xba*I sites of pCDNA3 for the transfection of mammalian cells. MOR-1 and KOR-1 were used in their original vectors. Transfection of neuroblastoma $neuro_{2a}$ cells with these plasmid DNAs was carried out by the calcium phosphate DNA precipitation method as described by Chen and Okayama [28] using 30 μg of plasmid DNA. Cell colonies surviving geniticin selection (1 mg/mL) were screened for opioid receptor expression using intact cell binding assays with 1 nM [^{3}H]-diprenorphine as radioactive ligand and 10 μM naloxone to determine the non-specific binding as previously described [5].

Cell Culture

$Neuro_{2a}$ cells (passages 8–20) were cultured in T25 cm^2 flasks in DMEM supplemented with 110 mg/L sodium pyruvate, 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin, and 500 μg/mL geniticin in 10% CO_2 at 37°. Confluent cells were dissociated from the flask by repeated trituration, pelleted, resuspended in fresh medium, and plated onto glass coverslips (25 mm round) at a density of 3×10^4 cells/coverslip in 6-well plates. When the culture was 80% confluent, DMEM was replaced with CO_2-independent medium supplemented with 5 μM forskolin. Growth in CO_2-independent medium (phosphate rather than bicarbonate buffered) increases the frequency of opioid-induced Ca^{2+} responses [16]. Cells were grown in CO_2-independent medium for 4–6 days at 37° and atmospheric gas levels.

Experimental Procedure

$Neuro_{2a}$ cells were loaded with indicator by incubation in HH containing 0.5% bovine serum albumin and 2 μM fura-2 acetoxymethyl ester at 37° for 45 min. HH was composed of the following (in mM): HEPES, 20; NaCl, 137; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. Loading was terminated by incubating in HH for an additional 20–30 min. The coverslip was then placed in a flow-through chamber [29] that was superfused at a rate of 2 to 2.5 mL/min. Changes in superfusate were accomplished with the use of a multiport valve coupled to numerous reservoirs.

Digital Imaging

The chamber containing the fura-2-loaded cells was mounted on the stage of an inverted microscope (Nikon Diaphot) and alternately excited at 340 or 380 nm by rapidly switching optical filters (10 nm band pass) mounted on a computer-controlled wheel (Sutter Instrument, Inc., Novato, CA) placed between a 75 W Xe arc lamp and the epifluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm) through a 70× objective (Leitz; N.A. 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) controlled by an 80486 (DX2/66) computer. Image pairs were collected every 6 sec; exposure to excitation light was between 120 and 200 msec per image, and the interval between paired images was 385 msec.

$[Ca^{2+}]_i$ 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 conclusion of each experiment after removing cells 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 $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, *in which* R is the 340:380 nm fluorescence emission ratio and K_d = 224 nM, the dissociation constant for fura-2 [30]. The maximum ratio (R_{max} = 4.41), the minimum ratio (R_{min} = 0.41), and the constant β (the ratio of the fluorescence measured at 380 nm in Ca^{2+}-free and saturating solution; 6.7) were determined by treating cells with 10 μM ionomycin in Ca^{2+}-free (1 mM EGTA) and saturating (5 mM Ca^{2+}) solution.

RESULTS

In NG108-15 cells, DADLE-induced stimulation of δ-opioid receptors resulted in the release of Ca^{2+} from intracellular stores [16]. We used similar growth and treatment procedures to determine whether opioid-induced increases in $[Ca^{2+}]_i$ could be elicited in $neuro_{2a}$ cells stably expressing various opioid receptor subtypes. $Neuro_{2a}$ cells were grown in serum-free, CO_2-independent medium supplemented with 5 μM forskolin for 4–6 days, and then $[Ca^{2+}]_i$ was recorded with fura-2-based digital imaging as described in Materials and Methods. A response was defined as an $[Ca^{2+}]_i$ increase greater than 50 nM over baseline.

Wild-type $neuro_{2a}$ cells exposed to 10 μM DADLE failed to respond (Fig. 1A, Frame 2, Fig. 1C), whereas 96% (N = 23) of the cells responded to 100 nM BK with an average $[Ca^{2+}]_i$ increase of 474 ± 51 nM (Fig. 1A, Frame 4, Fig. 1C). Thus, δ-opioid receptors are not expressed in this cell line endogenously, although functional BK receptors, phos-

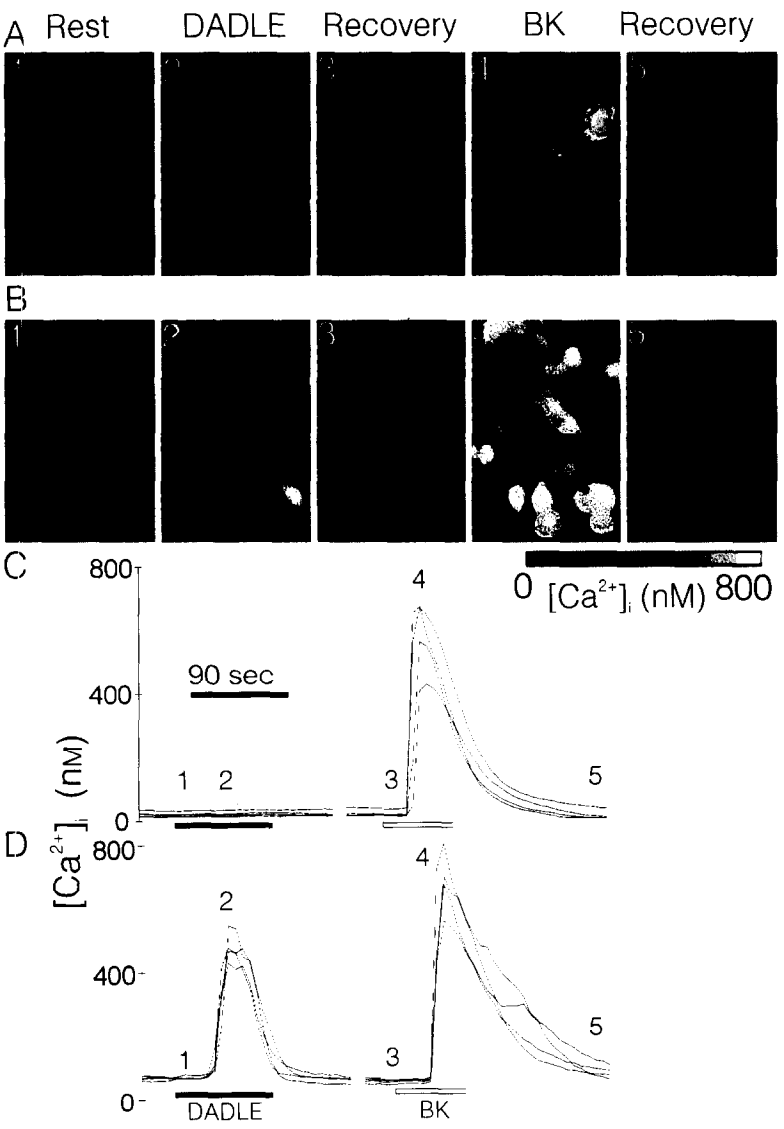

FIG. 1. DADLE-induced $[Ca^{2+}]_i$ increases in neuro$_{2a}$-δOR cells. Pseudocolor representations of $[Ca^{2+}]_i$ were derived from fura-2-based digital images and scaled as shown. (A) Wild-type neuro$_{2a}$ cells failed to respond to a 90-sec application of 10 μM DADLE (frame 2). BK (60 sec, 100 nM) elicited an $[Ca^{2+}]_i$ increase of greater than 50 nM in 96% of the cells treated (N = 23, frame 4). (B) Neuro$_{2a}$ cells transfected with the δ-opioid receptor (N = 77) responded to DADLE (90 sec 10 μM, frame 2), and BK (100 nM, 60 sec) (frame 4). (C) Plot of $[Ca^{2+}]_i$ versus time for wild-type neuro$_{2a}$ cells shown in A. Numbers above the plot correspond to the time points for the frames in A. (D) Plot of $[Ca^{2+}]_i$ versus time for the neuro$_{2a}$ cells transfected with the δ-opioid receptor, as shown in B. In C and D, 10 μM DADLE and 100 nM BK were superfused at the times indicated by the horizontal bars.

phoinositide signaling machinery, and releasable calcium stores are present.

Neuro$_{2a}$ cells were transfected with the δ-opioid receptor (DOR1), as described in Materials and Methods (neuro$_{2a}$-δOR), and stable clones were selected, using geniticin. Radioligand binding studies with [^{3}H]diprenorphine on these neuro$_{2a}$-δOR cells resulted in a B_{max} value of 4.7 pmol/mg protein. The μ and κ opioid receptor-selective ligands did not displace [^{3}H]diprenorphine nor did they inhibit adenylyl cyclase as did δ ligands.* DADLE at 10 μM elicited responses in 43% (N = 77) of these cells (Fig. 1B, Frame 2), with an average response amplitude of 281 ± 40 nM. Ninety-one percent of the cells responded to 100 nM BK with an average increase in $[Ca^{2+}]_i$ of 623 ± 43 nM (Fig. 1B, Frame 4). As represented in the pseudocolor images in panels B and D of Fig. 1, neuro$_{2a}$ cells transfected with the δ-opioid receptor respond to DADLE in synchrony with comparable amplitudes.

For subsequent study of the neuro$_{2a}$-δOR cells, a brief (90 sec) exposure to 100 nM DADLE was used to evoke the increase in $[Ca^{2+}]_i$ because this low concentration produced less desensitization of the receptor and, thus, enabled reproducible responses from the same cell to be elicited. Overall, 65% (N = 343) of neuro$_{2a}$-δOR cells responded to

* Ko JL, Arvidsson U, Law P-Y, Elde R and Loh HH, manuscript submitted for publication.

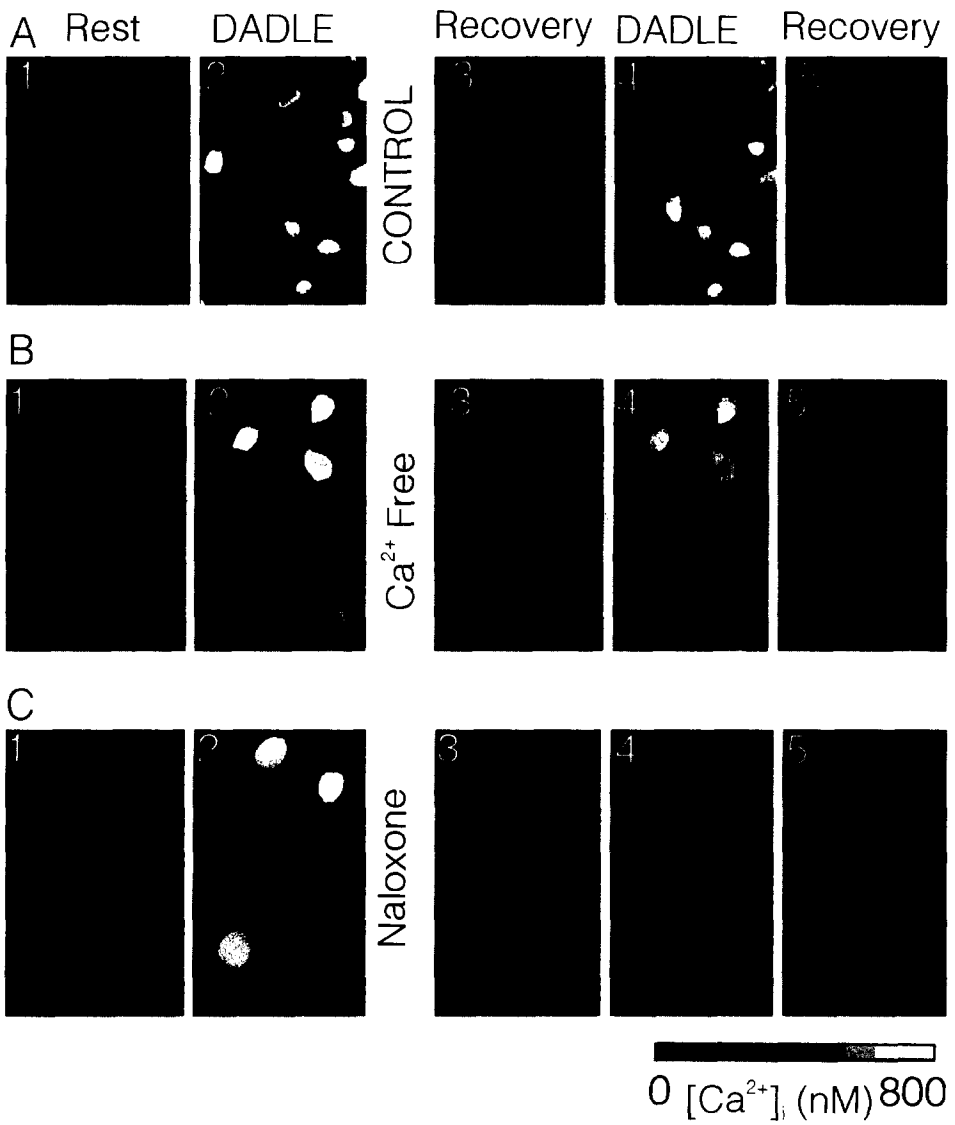

FIG. 2. DADLE-induced mobilization of intracellular Ca^{2+} by an opioid receptor-mediated signal in neuro$_{2a}$-δOR cells. (A) Repeated 90-sec application of 100 nM DADLE (frames 2 and 4) separated by a 25-min wash period (between frames 2 and 4) produced similar increases in $[Ca^{2+}]_i$ (N = 71). (B) HH was replaced with nominally Ca^{2+}-free buffer 3 min prior to and during the second DADLE application (frame 4). (C) Pseudocolor representation showing the inhibition of the 90-sec 100 nM DADLE-induced increase in $[Ca^{2+}]_i$ by 10 μM naloxone (frame 4).

100 nM DADLE with an average $[Ca^{2+}]_i$ increase of 434 ± 26 nM. This response frequency and amplitude are somewhat greater than that seen for 10 μM DADLE. A reduction in the inhibition of adenylyl cyclase produced by high concentrations of leu-enkephalin was described for NG108-15 cells, and may result from a rapid desensitization when cells are exposed to a very high concentration of agonist [31]. When applied at 25-min intervals (Figs. 2A and 3A), the response to the second challenge with DADLE was 94 ± 8% of the first response (N = 71). Thus, because both of the DADLE-evoked $[Ca^{2+}]_i$ increases from a given neuro$_{2a}$-δOR cell were comparable, pharmacologic treatments were applied during the second DADLE application, and the response was normalized to the initial response. This allowed each cell to serve as its own control. To ensure that cells being used on a given day were responding in a consistent manner, at the beginning and end of each series of experiments a control experiment (paired application of opioid agonist) was performed.

We next determined whether the DADLE-evoked increase in $[Ca^{2+}]_i$ recorded in neuro$_{2a}$-δOR cells was due to the release of calcium from intracellular stores, as found for NG108-15 cells [16]. Using the paired protocol, DADLE-evoked responses were collected in the presence and absence of extracellular calcium (Fig. 2A). Superfusion of neuro$_{2a}$-δOR cells with nominally Ca^{2+}-free HH both preceding (3 min) and during the second 100 nM DADLE treatment did not affect the $[Ca^{2+}]_i$ transient significantly. In the absence of extracellular Ca^{2+}, the response was 89 ± 10% (peak 2/peak 1) of the value seen in the first response (N = 74), which was not significantly different from controls. Thus, the source of Ca^{2+} must be intracellular.

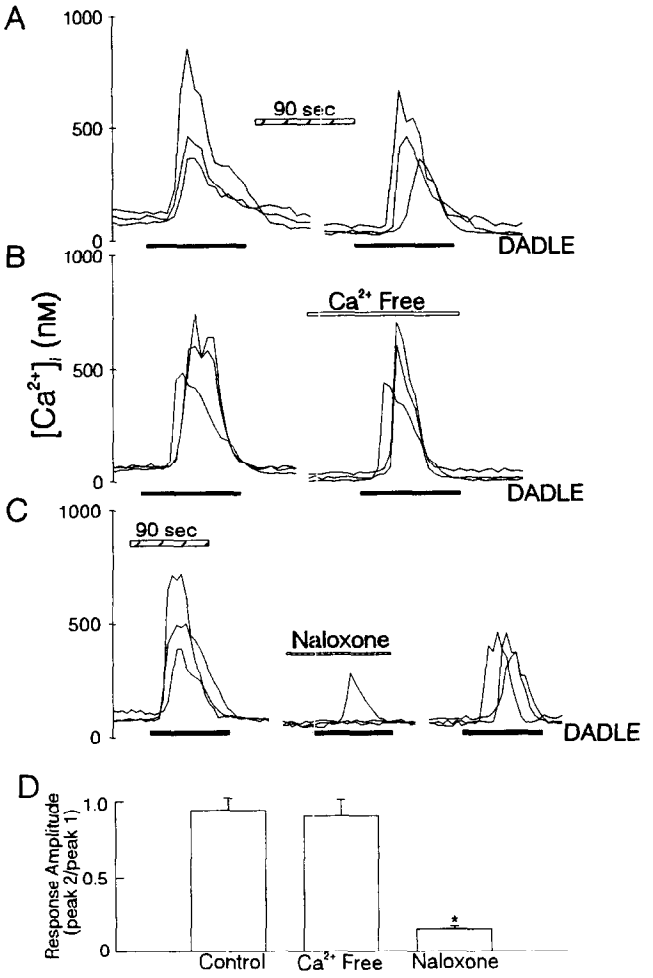

FIG. 3. δ-Opioid receptor-mediated release of Ca^{2+} from intracellular stores. Representative $[Ca^{2+}]_i$ recordings for responding cells shown in Fig. 2. (A–C) A 100 nM concentration of DADLE was applied at the times (90 sec) indicated by the solid bars. Gaps in the recording indicate 25 min of continuous superfusion. (A) Reproducible DADLE-induced $[Ca^{2+}]_i$ increases were elicited in fields containing several $neuro_{2a}$-δOR cells by superfusion for 90 sec (N = 71). This plot presents three representative $[Ca^{2+}]_i$ recordings from the field shown in Fig. 2A. (B) Superfusion of nominally Ca^{2+}-free buffer prior to (3 min) and during the second DADLE application, as indicated by the open bar above the trace, did not affect the $[Ca^{2+}]_i$ increase (N = 74). (C) A 10 μM concentration of naloxone, applied prior to (5 min) and during the second DADLE application, as indicated by the open bar above the trace, inhibited significantly the DADLE-evoked $[Ca^{2+}]_i$ transients (N = 79). Removal of naloxone allowed the DADLE response to recover. (D) Histogram summarizing the effects of calcium-free HH and naloxone (10 μM) on DADLE-induced increases in $[Ca^{2+}]_i$. Key: * $P < 0.0001$ relative to control.

In $neuro_{2a}$-δOR cells, DADLE-evoked responses were due specifically to activation of the opioid receptor, as indicated by the inhibition of the response by the opioid receptor antagonist naloxone (Fig. 2C, Frame 4, Fig. 3C). $Neuro_{2a}$-δOR cells were exposed to 100 nM DADLE to elicit an initial response. Then, prior to (5 min) and during the second exposure to 100 nM DADLE, the cells were superfused with 10 μM naloxone. In the presence of naloxone, the DADLE-elicited increase in $[Ca^{2+}]_i$ was reduced to 15 ± 2% of the initial response (N = 78), a significant decrease relative to control ($P < 0.0001$). The naloxone block was readily reversed, as indicated by a third DADLE-elicited $[Ca^{2+}]_i$ increase following a 25-min wash (Fig. 3C). Thus, stimulation of $neuro_{2a}$-δOR cells by an opioid receptor agonist mobilizes intracellular calcium stores, producing an increase in $[Ca^{2+}]_i$.

We next explored the possibility that other opioid receptor subtypes might functionally couple to intracellular Ca^{2+} stores. Ten micromolar DAMGO failed to elicit a response in wild-type cells, whereas 95% (N = 20) of the cells responded to 100 nM BK with an average $[Ca^{2+}]_i$ increase of 601 ± 91 nM. $Neuro_{2a}$ cells stably transfected with μ-opioid receptors ($neuro_{2a}$-μOR, MOR1) bound [^{3}H]diprenorphine with a B_{max} value of 2.06 pmol/mg [5]. [^{3}H]Diprenorphine was not displaced by δ and κ opioid receptor-selective ligands nor did they inhibit adenylyl cyclase as did μ ligands. When challenged with 10 μM DAMGO, 85% (N = 48) of $neuro_{2a}$-μOR cells responded with an average $[Ca^{2+}]_i$ increase of 581 ± 31 nM. Ninety percent of the same cells responded to 100 nM BK with an average $[Ca^{2+}]_i$ increase of 371 ± 24 nM. Morphine (100 nM) was also an effective agonist eliciting a 634 ± 32 nM $[Ca^{2+}]_i$ increase in 50% of $neuro_{2a}$-μOR cells (N = 154).

In $neuro_{2a}$-μOR cells, reproducible increases in $[Ca^{2+}]_i$ were evoked by challenge with 100 nM DAMGO for 90 sec. Thirty-one percent (N = 449) of the cells responded with an average $[Ca^{2+}]_i$ increase of 373 ± 16 nM. When applied at 25-min intervals (Figs. 4A and 5A), the response to the second challenge with DAMGO was 73 ± 10% of the first response (N = 75). We next used the paired protocol to determine whether the DAMGO-evoked increase in $[Ca^{2+}]_i$ was due to the release of Ca^{2+} from intracellular stores. DAMGO-evoked responses were recorded in the presence and absence of extracellular Ca^{2+}. Superfusion of $neuro_{2a}$-μOR cells with nominally Ca^{2+}-free HH buffer preceding (3 min) and during the second treatment with DAMGO did not affect the $[Ca^{2+}]_i$ transient significantly. In the absence of extracellular Ca^{2+}, the response was 69 ± 8% of the value seen in the first response (N = 64). Thus, challenge of $neuro_{2a}$-μOR cells with DAMGO evokes reproducible $[Ca^{2+}]_i$ transients that result from the release of calcium from intracellular stores.

In $neuro_{2a}$-μOR cells, DAMGO-evoked responses were due specifically to activation of the opioid receptor, as indicated by the inhibition of the response by the opioid receptor antagonist naloxone (Fig. 4C, Frame 4, Fig. 5C). $Neuro_{2a}$-μOR cells were exposed to 100 nM DAMGO to elicit an initial response. Then, prior to (5 min) and during the second exposure to DAMGO, the cells were superfused with 1 μM naloxone. In the presence of naloxone, the DAMGO-elicited increase in $[Ca^{2+}]_i$ was reduced to 8 ± 2% of the initial response (N = 74) ($P < 0.0001$). The naloxone block was readily reversed, as indicated by a third

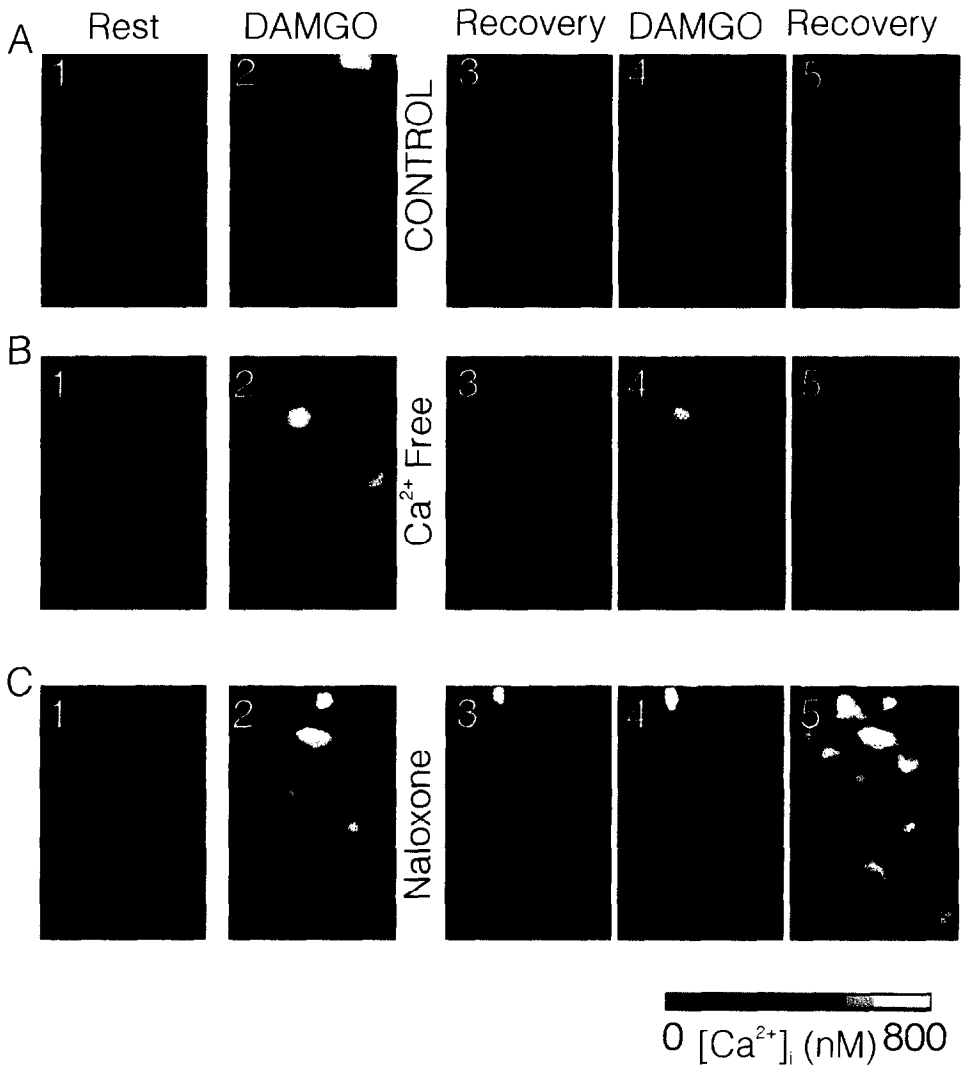

FIG. 4. DAMGO-induced mobilization of intracellular Ca^{2+} by an opioid receptor-mediated signal in neuro$_{2a}$-μOR cells. Pseudocolor representation of fura-2-based $[Ca^{2+}]_i$ were recorded and calculated as described in Materials and Methods. (A) Repeated 90-sec application of 100 nM DAMGO (frames 2 and 4) separated by a 25-min wash period (between frames 2 and 4) produced similar increases in $[Ca^{2+}]_i$ (N = 75). (B) HH was replaced with nominally Ca^{2+}-free buffer 3 min prior to and during the second DAMGO application (frame 4). (C) Naloxone at 1 μM inhibited the DAMGO-induced increase in $[Ca^{2+}]_i$ (frame 4).

DAMGO-elicited $[Ca^{2+}]_i$ increase following a 25-min wash (Fig. 4C, frame 5, Fig. 5C). Thus, stimulation of neuro$_{2a}$-μOR cells by an opioid receptor agonist mobilizes intracellular calcium stores, producing an increase in $[Ca^{2+}]_i$.

We next explored the possibility that κ-opioid receptors might functionally couple to intracellular Ca^{2+} stores. Neuro$_{2a}$ cells were stably transfected with κ-opioid receptors (neuro$_{2a}$-κOR, KOR1) as described in Materials and Methods. Radioligand binding studies with [^{3}H]diprenorphine revealed that in neuro$_{2a}$-κOR cells opioid receptors were expressed at a much lower level than in the neuro$_{2a}$-δOR and -μOR cells with a B_{max} of 0.22 ± 0.06 pmol/mg protein.* The KOR1 clone was shown to be highly selective for κ receptor ligands in radioligand binding assays and for inhibition of adenylyl cyclase when expressed in COS-7 cells [25]. Ten micromolar U-50488H failed to elicit a response in wild-type cells, whereas 100% (N = 26) of the cells responded to 100 nM BK with an average $[Ca^{2+}]_i$ increase of 561 ± 38 nM. When challenged with 10 μM U-50488H, 9% (N = 59) of neuro$_{2a}$-κOR cells responded with an average $[Ca^{2+}]_i$ increase of 338 ± 81 nM. Seventy-six percent of these cells responded to 100 nM BK with an average $[Ca^{2+}]_i$ increase of 275 ± 23 nM. The reason for the decrease in amplitude and frequency of BK responses in the neuro$_{2a}$-κOR cells is unclear.

Neuro$_{2a}$-κOR were challenged with U-50488H (100 nM) for 90 sec. Seventeen percent (N = 365) of the cells responded with an average $[Ca^{2+}]_i$ increase of 230 ± 15 nM. When applied at 25-min intervals (Figs. 6A and 7A), the response to the second challenge with U-50488H was 48 ± 10% of the first response (N = 16). This decrease in amplitude, relative to the responses from neuro$_{2a}$-δOR and neuro$_{2a}$-μOR cells, was due principally to the complete

* Law P-Y and Loh HH, unpublished observations.

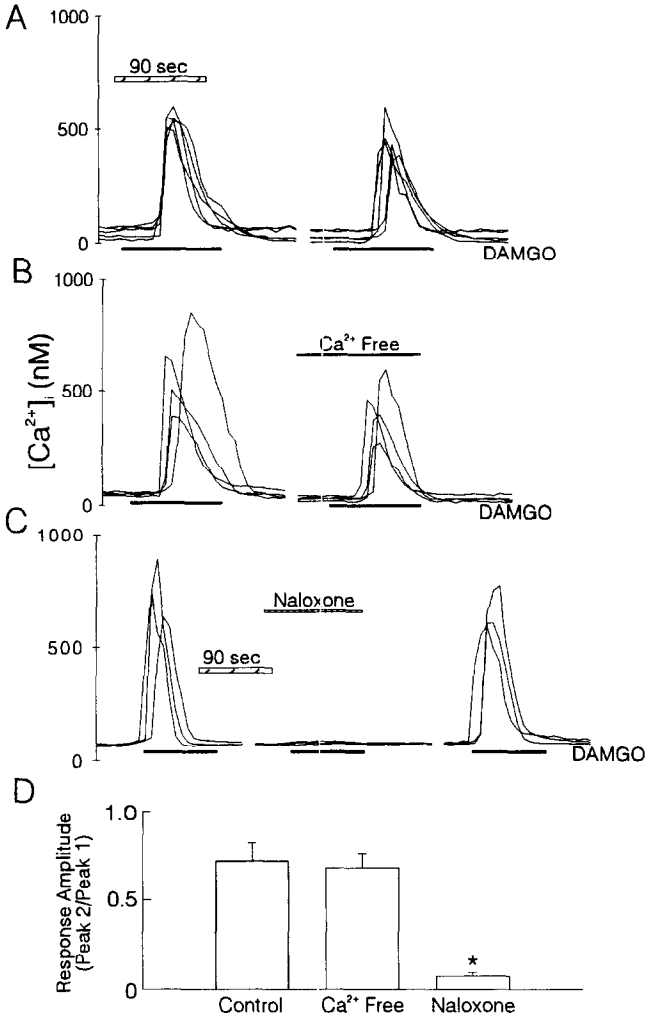

FIG. 5. μ-Opioid receptor-mediated release of Ca^{2+} from intracellular stores. Representative $[Ca^{2+}]_i$ recordings for responding cells shown in Fig. 4. (A–C) DAMGO (100 nM) was applied at the times (90 sec) indicated by the solid bars. Gaps in the recording indicate 25 min of continuous superfusion. (A) Reproducible DAMGO-induced $[Ca^{2+}]_i$ increases were elicited in fields containing several $neuro_{2a}$-μOR cells by superfusion for 90 sec (N = 75). This plot presents five representative $[Ca^{2+}]_i$ recordings from the field shown in Fig. 4A. (B) Superfusion of nominally Ca^{2+}-free buffer prior to (3 min) and during the second DAMGO application, as represented by the open bar above the trace, had no effect on the $[Ca^{2+}]_i$ increase (N = 64). (C) Naloxone (1 μM), applied prior to (5 min) and during the second DAMGO application, as indicated by the open bar above the trace, inhibited significantly the DAMGO-evoked $[Ca^{2+}]_i$ transients (N = 78). Removal of naloxone allowed for the recovery of the DAMGO-induced $[Ca^{2+}]_i$ increase. (D) Histogram summarizing the effects of calcium-free medium and naloxone (1 μM) on DAMGO-induced increases in $[Ca^{2+}]_i$. Key: * $P < 0.0001$ relative to control.

failure of some cells (50%) to respond to the second agonist application.

We next determined whether the U-50488H-evoked increase in $[Ca^{2+}]_i$ was due to the release of Ca^{2+} from intracellular stores. U-50488H-evoked responses were collected in the presence and absence of extracellular Ca^{2+} (Fig. 7A). Superfusion of $neuro_{2a}$-κOR cells with nominally Ca^{2+}-free HH both preceding (3 min) and during the second 100 nM U-50488H treatment did not affect the $[Ca^{2+}]_i$ transient significantly. In the absence of extracellular Ca^{2+}, the response was 50 ± 12% of the value seen in the first response (N = 12), which was not significantly different from controls. Thus, challenge of $neuro_{2a}$-κOR cells with U-50488H evoked $[Ca^{2+}]_i$ transients that resulted from the release of Ca^{2+} from intracellular stores.

In $neuro_{2a}$-κOR cells, U-50488H-evoked responses were due specifically to activation of the opioid receptor, as indicated by the inhibition of the response by the opioid receptor antagonist naloxone (Fig. 6C, Frame 4, Fig. 7C). $Neuro_{2a}$-κOR cells were exposed to 100 nM U-50488H to elicit an initial response. Then, prior to (5 min) and during the second exposure to 100 nM U-50488H, the cells were superfused with 10 μM naloxone. In the presence of naloxone, the U-50488H-elicited increase in $[Ca^{2+}]_i$ was reduced to 7 ± 4% of the initial response (N = 35), a significant decrease relative to control ($P < 0.0001$). The naloxone block was readily reversed, as indicated by a third U-50488H-elicited $[Ca^{2+}]_i$ increase following a 25-min wash (Fig. 7C). Thus, stimulation of $neuro_{2a}$-κOR cells, as well as $neuro_{2a}$-δOR and $neuro_{2a}$-μOR cells, by an opioid receptor agonist mobilizes intracellular Ca^{2+} stores producing an increase in $[Ca^{2+}]_i$.

DISCUSSION

In this report, we demonstrated that δ-, μ-, and κ-opioid receptors, when stably expressed in the neuronal cell line $neuro_{2a}$, each functionally coupled to intracellular Ca^{2+} stores. Wild-type $neuro_{2a}$ cells did not express opioid receptors as indicated by their failure to respond to maximal concentrations of opioid agonists. These results, as well as the significant inhibition of the response by the opioid receptor specific antagonist naloxone, indicate that the opioid-evoked $[Ca^{2+}]_i$ increases described here result from the transfected opioid receptor.

$Neuro_{2a}$-δOR and -μOR cells each expressed an equivalent level of receptors and exhibited comparable responses. The level of expressed receptors was reduced greatly in the $neuro_{2a}$-κOR cells, and both the number of cells that responded and the ability to elicit multiple responses in these cells were decreased. It appears that the relatively low frequency and amplitude of the responses observed in the $neuro_{2a}$-κOR cells were related to reduced expression of the receptors. Because the percentage of cells that responded to U50488H with an increase in $[Ca^{2+}]_i$ was roughly proportional to receptor expression levels, it is tempting to speculate that the limiting component in this opioid response pathway is the receptor itself, as opposed to the G protein or effector components of the pathway. The high failure rate for the second application of agonist in $neuro_{2a}$-κOR cells may result from rapid desensitization, although we cannot distinguish desensitization from the overall poor responsiveness of these cells. The BK responses in the $neuro_{2a}$-κOR cells were also diminished, suggesting that

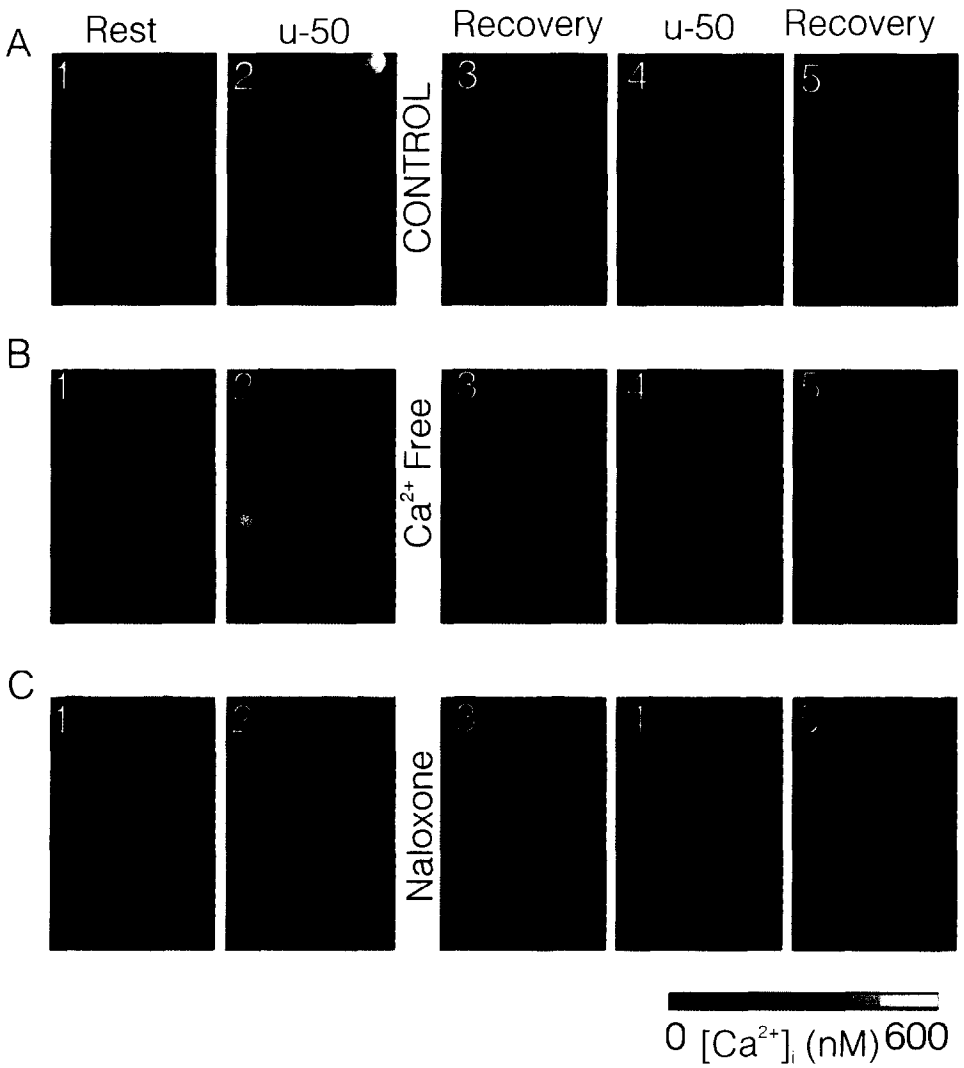

FIG. 6. U-50488H-induced mobilization of intracellular Ca^{2+} by an opioid receptor-mediated signal in neuro$_{2a}$-κOR cells. (A) Repeated 90-sec application of 100 nM U-50488H (frames 2 and 4) separated by a 25-min wash period (between frames 2 and 4) produced similar increases in $[Ca^{2+}]_i$ (N = 16). (B) HH was replaced with nominally Ca^{2+}-free buffer 3 min prior to and during the second U-50488H application (frame 4). (C) Pseudocolor representation showing the inhibition of the U-50488H-induced increase in $[Ca^{2+}]_i$ by 10 μM naloxone (frame 4).

expression of the κ receptor may have impaired the phosphoinositide signaling cascade. Resolution of these issues will require cells that express the κ receptor at high levels, a feat that has proven difficult.

The opioid-induced increase in $[Ca^{2+}]_i$ did not require extracellular Ca^{2+}. We conclude that each of the three opioid receptors, when expressed in the neuro$_{2a}$ cell line, functionally couples to intracellular Ca^{2+} stores, suggesting that the receptors may activate the phosphoinositide cascade. These results are in agreement with those seen by Jin *et al.* [16] in NG108-15 cells, and are consistent with the opioid-induced IP_3 increases measured in NG108-15 cells [17, 18]. Furthermore, activation of μ receptors in SH-SY5Y cells increased IP_3 [32], and activation of μ and κ receptors expressed in *Xenopus* oocytes evoked IP_3-mediated responses [33]. Clearly, opioid receptors couple to phospholipase C in a variety of cell types.

It has been suggested that for the δ-opioid receptor, unique receptor subtypes mediate the various actions of opioids [34]. However, in the experiments described here, a single opioid receptor of known subtype was expressed. Each of the receptors examined had the ability to inhibit adenylyl cyclase activity [5, 24, 25] and the ability to increase $[Ca^{2+}]_i$. Thus, a single receptor is capable of activating two signaling pathways. We speculate that the α subunit of inhibitory G proteins inhibits adenylyl cyclase and that the βγ subunits, by coupling to phospholipase C, may mediate the $[Ca^{2+}]_i$ increase [35].

A neuronal system in which activation of opioid receptors elicits easily measured, reproducible responses to a selective agonist provides a model with which many receptor functions can be studied. Receptor mutagenesis coupled with the functional analysis described here may prove useful for studying receptor desensitization, divergent signaling pathways, and receptor G-protein coupling. Another application of this system is the screening of complex

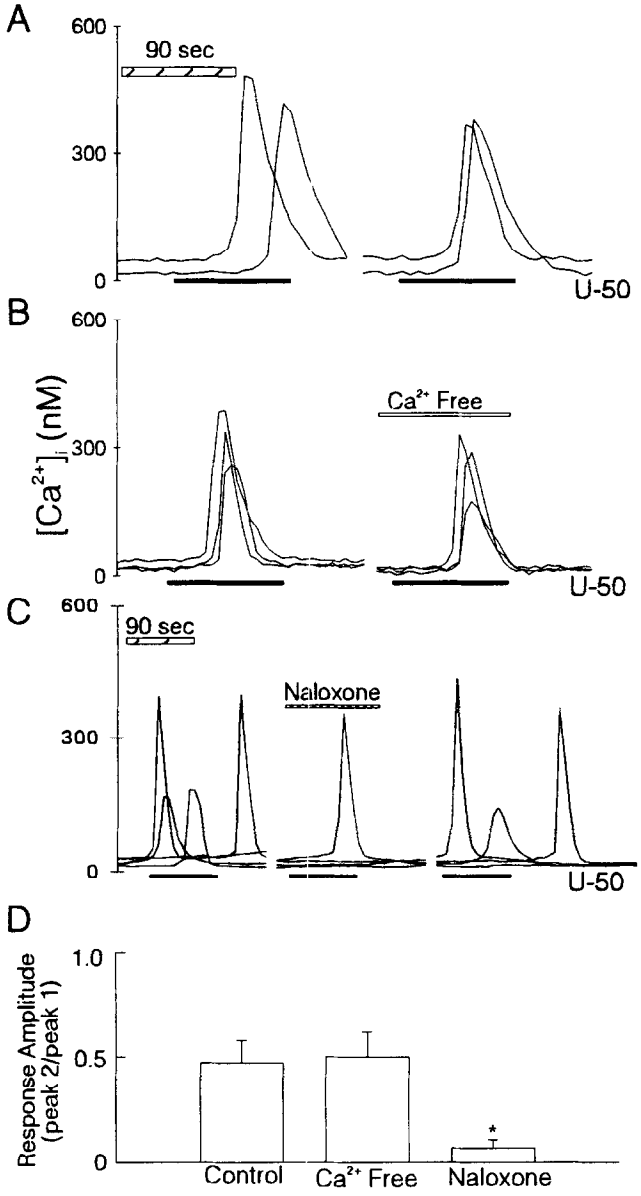

FIG. 7. κ-Opioid receptor-mediated release of Ca^{2+} from intracellular stores. Representative $[Ca^{2+}]_i$ recordings for responding cells shown in Fig. 6. (A–C) U-50488H (100 nM) was applied at the times (90 sec) indicated by the solid bars. Gaps in the recording indicate 25 min of continuous superfusion. (A) Reproducible U-50488H-induced $[Ca^{2+}]_i$ increases were elicited in fields containing several $neuro_{2a}$-κOR cells by superfusion for 90 sec (N = 16). This plot presents two representative $[Ca^{2+}]_i$ recordings from the field shown in Fig. 6A. (B) Superfusion of nominally Ca^{2+}-free buffer prior to (3 min) and during the second U-50488H application, as represented by the open bar above the trace, had no effect on the $[Ca^{2+}]_i$ increase (N = 12). (C) Naloxone (10 μM), applied prior to (5 min) and during the second U-50488H application, as indicated by the open bar above the trace, inhibited significantly the U-50488H-evoked $[Ca^{2+}]_i$ transients (N = 35). Removal of naloxone allowed the U-50488H-induced response to recover. (D) Histogram summarizing the effects of calcium-free medium and naloxone (10 μM) on U-50488H-induced increases in $[Ca^{2+}]_i$. Key: * $P < 0.0001$ relative to control.

mixtures for active components. An increase in $[Ca^{2+}]_i$ seen in response to receptor activation in PC-12 cells has been used effectively to detect biologically active ligands [36].

Because μ, δ, and κ receptors, when expressed in the same environment, appear to couple to the same second messenger systems, the unique spectrum of effects produced by selective agonists *in vivo* must result from their differential localization. Opioid receptors are distributed throughout the CNS; δ receptors (DOR1) are localized to the axons [37, 38], μ (MOR1) to the somatodendritic domain, and κ (KOR1) to the axon and somatodendritic domain [39]. The wide distribution, varied function, and often overlapping expression of individual opioid receptor subtypes have hindered their study. The introduction of cloned opioid receptors into a neuronal cell line enables each receptor to be studied independently within an identical system. In transfected $neuro_{2a}$ cells, each of the three opioid receptors displayed easily measured increases in $[Ca^{2+}]_i$ in response to the appropriate agonist.

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