Opioids Mobilize Calcium from Inositol 1,4,5-Trisphosphate-Sensitive Stores in NG108-15 Cells

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Opioids elicit an increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in neuroblastoma $\times$ glioma hybrid NG108-15 cells, which, depending upon growth conditions, results from either Ca^{2+} influx in differentiated cells or Ca^{2+} release from internal stores in undifferentiated cells (Jin et al., 1992). In this report we describe fura-2-based digital imaging studies that demonstrate that opioid-evoked Ca^{2+} release in these cells results from the activation of phospholipase C (PLC) and subsequent mobilization of the inositol 1,4,5-trisphosphate (IP_3)-sensitive store. D-Ala²-D-Leu⁵-enkephalin (DADLE) evoked concentration-dependent increases in $[\text{Ca}^{2+}]_i$ ($\text{EC}_{50} \cong 4$ nM). The response was blocked by naloxone (1 μM). In single cells, sequential application of selective opioid agonists (10 nM) evoked responses of the rank order DADLE = D-Pen², D-Pen⁵-enkephalin (DPDPE) > *trans*-(+) 3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl] cyclohexyl) benzeneacetamide (U50488) > D-Ala², *N*-Me-Phe⁴, Gly⁵-ol-enkephalin (DAMGO), consistent with activation of a δ -opioid receptor. Forty percent ($n = 198$) of the cells responded to 100 nM DADLE with a net $[\text{Ca}^{2+}]_i$ increase of 483 ± 40 nM. Bradykinin (100 nM) elicited a response in 91% of the cells with a mean net amplitude of 707 ± 36 nM. The DADLE-evoked responses were not blocked by removal of extracellular Ca^{2+} ; instead, they were abolished by treatment with 10 nM thapsigargin, an agent that depletes and prevents refilling of IP_3 -sensitive Ca^{2+} stores. A 1 μM concentration of U73122, an aminosteroid inhibitor of PLC, completely blocked the DADLE-evoked $[\text{Ca}^{2+}]_i$ increase, while an inactive analog, U73433, was without effect. To explore the possible role of G-proteins in mediating opioid-induced $[\text{Ca}^{2+}]_i$ increases in NG108-15 cells, we pretreated cells with pertussis or cholera toxin; pertussis toxin blocked the opioid-induced response while cholera toxin was without effect, consistent with a G_i - or G_o -mediated effect. Activation of the opioid inhibitory pathway previously described for these cells appears to stimulate the phosphoinositide (PI) cascade as well. Including the PI cascade among the multiple second messenger systems modulated

by opioids may be key to understanding the biochemical events that underlie acute and chronic opioid action.

[Key words: intracellular calcium, opioids, NG108-15, phospholipase C, thapsigargin, U73122]

Opioid receptors couple via G-proteins to inhibit voltage-sensitive Ca^{2+} channels (Gross and Macdonald, 1984; Hescheler et al., 1987; Surprenant et al., 1990; Jin et al., 1993) and to activate K^+ channels (Werz and Macdonald, 1983; Christie et al., 1987). These inhibitory actions underlie the decrease in neurotransmitter release and reduced cellular excitability produced by opioids (Duggan and North, 1984). Opioid receptors also negatively couple to adenylyl cyclase (Sharma et al., 1975a), though the resultant effects on neuronal excitability are less clear (Wang and Aghajanian, 1987). To our knowledge, opioid receptor coupling to the phosphoinositide (PI) cascade has not been demonstrated, though recent reports suggest that such a coupling may exist. Opioids potentiate NMDA currents via a mechanism blocked by a selective inhibitor of protein kinase C (Chen and Huang, 1991), yet attempts to measure opioid-induced increases in PI breakdown products have generally been negative (Yu and Sadee, 1986; but see Periyasamy and Hoss, 1990).

Recent studies indicate that many neurotransmitters previously thought to play purely inhibitory roles, such as GABA (Hahner et al., 1991), adenosine (Okada et al., 1992), calcitonin gene-related peptide (Wiley et al., 1992), and neuropeptide Y (Wiley et al., 1991), also exert excitatory effects. These effects have been ascribed to either of two mechanisms. In some cases an additional receptor subtype has been discovered; one subtype selectively produces inhibition and the other produces excitation. Alternatively, a single receptor may couple to multiple second messenger systems exerting excitatory or inhibitory effects depending on the state of the cell (Bourne and Nicoll, 1993).

Both excitatory and inhibitory effects of opioids have been observed. At the cellular level, opioid effects on cell excitability are generally inhibitory, though neuronal networks, depending on the circuitry, can be disinhibited by opioids producing gross excitation (Zieglansberger et al., 1979; Madison and Nicoll, 1988; Neumaier et al., 1988). In sensory neurons, opioids exert both inhibitory as well as direct excitatory effects (Crain and Shen, 1990). We have found that opioids elicit a direct excitatory effect in NG108-15 cells that is manifest as an increase in the $[\text{Ca}^{2+}]_i$. Depending upon growth conditions, the $[\text{Ca}^{2+}]_i$ increase results from either Ca^{2+} influx in differentiated cells or Ca^{2+} release from internal stores in undifferentiated cells (Jin et al., 1992). In this report we have studied the opioid-induced Ca^{2+} mobilization in more detail. We find that opioids activate phos-

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Figure 1. DADLE-induced $[Ca^{2+}]_i$ increases are mediated by opioid receptors. *A*, Reproducible DADLE-induced $[Ca^{2+}]_i$ transients were elicited by superfusion with 100 nM DADLE for 90 sec at 25 min intervals ($n = 6$). *B*, The DADLE-induced response was blocked reversibly by 1 μ M naloxone ($n = 3$). Drugs were applied as indicated by the horizontal bars. $[Ca^{2+}]_i$ was measured in single NG108-15 cells by indo-1-based photometry.

pholipase C (PLC), resulting in a subsequent release of Ca^{2+} from an inositol 1,4,5-trisphosphate (IP_3)-sensitive store.

Materials and Methods

Materials. Materials were purchased from the following companies: fura-2 acetoxymethyl ester (AM) and indo-1 AM, Molecular Probes, Eugene, OR; U73122 and U73433, Biomol Research Laboratories, Inc., Plymouth Meeting, PA; pertussis toxin (PTX) and cholera toxin (CTX), List Biological Laboratories, Inc., Campbell, CA; D-Ala²-D-Leu⁵-enkephalin (DADLE), D-Pen², D-Pen⁵-enkephalin (DPDPE), and D-Ala², N-Me-Phe⁴, Gly⁵-ol-enkephalin (DAMGO), Peninsula Laboratories, Inc., Belmont, CA; U50488, Upjohn Laboratories, Kalamazoo, MI; thapsigargin, LC Service Corp., Woburn, MA; CO_2 -independent media, GIBCO Labs, Grand Island, NY; all other reagents, Sigma, St. Louis, MO.

Cell culture. NG108-15 cells (passages 20–30) were grown in culture as previously described (Jin et al., 1992). Stock cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 0.1 μ M hypoxanthine, 10 μ M aminopterin, 17 μ M thymidine in a humidified atmosphere of 90% air and 10% CO_2 . Cells from the stock cultures were plated onto glass coverslips (25 mm round) at a density of 3×10^4 cells/coverslip and grown for 6–9 d in CO_2 -independent media (phosphate rather than bicarbonate buffered) supplemented with 5 μ M forskolin.

Experimental procedure. NG108-15 cells were incubated in either 2 μ M indo-1 AM, for photometry, or 2 μ M fura-2 AM, for digital imaging, in HEPES-buffered Hank's salt solution containing 0.5% bovine serum albumin for 45–60 min at 37°C. Hank's buffer 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. The cover glass was then mounted in a flow-through chamber (Thayer et al., 1988) that was superfused at a rate of 2 ml/min and placed on the stage of the microfluorimeter. Loading was terminated

Figure 2. The DADLE-induced $[Ca^{2+}]_i$ increase is mediated by δ -opioid receptors. Opioid agonists (10 nM) were sequentially superfused onto an NG108-15 cell during the times indicated by the horizontal bars. Recording is representative of four experiments in which the rank order of potency, DADLE = DPDPE > U50488 > DAMGO, was observed. Diagonal lines indicate gaps in the recording; agonists were applied at 25 min intervals.

by washing with Hank's buffer for 20–30 min prior to starting an experiment. Solutions were selected with a multiport valve coupled to several reservoirs. $CaCl_2$ was replaced with 20 μ M EGTA for Ca^{2+} -free experiments.

Photometry. We have previously described our instrumentation for indo-1-based dual emission microfluorimetry and its calibration (Jin et al., 1992).

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 in a computer-controlled wheel (Sutter Instrument, Inc.) 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 90 $\times$ objective (Leitz; NA 1.15). Fluorescent images (510, 40 nm band pass) were projected (0.5 $\times$) onto a cooled CCD camera (Photometrics, Inc.; 384 $\times$ 576 binned to 192 $\times$ 288 pixels) controlled by an 80486 (DX2/66) computer. Image pairs were collected every 6.2 sec; exposure to excitation light was always less than 100 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 $[Ca^{2+}]_i = K\beta(R - R_{min})/(R_{max} - R)$, in which R is the 340:380 nm fluorescence emission ratio and $K = 224$ nM, the dissociation constant for fura-2 (Gryniewicz et al., 1985). The maximum ratio ($R_{max} = 2.93$), the minimum ratio ($R_{min} = 0.16$), 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 μ M ionomycin in Ca^{2+} -free (1 mM EGTA) and saturating (5 mM Ca^{2+}) solution.

Results

NG108-15 cells were grown in serum-free, CO_2 -independent media supplemented with 5 μ M forskolin for 6–9 d. Elevating cAMP levels in NG108-15 cells grown in DMEM will induce the cells to differentiate. In contrast, the cells used in this study extend very short, if any, processes and lack a significant depolarization-induced increase in $[Ca^{2+}]_i$, and thus by these criteria are undifferentiated. Treatment of these cells with 100 nM DADLE for 90 sec evoked a $[Ca^{2+}]_i$ increase that rose at least 50 nM above the resting level in 33% of the cells ($n = 114$). In previous studies (Jin et al., 1992) we grew cells in standard bicarbonate-based media (DMEM) and found that only 10% of

the undifferentiated cells responded with an increase in [Ca²⁺]_i. While attempting to enhance this low frequency of response we experimented with CO₂-independent growth media, which, in combination with forskolin, reproducibly generated cultures with a response frequency greater than 30%. In responding cells, [Ca²⁺]_i increased from an average basal level of 83 ± 4 nM to a net amplitude of 648 ± 87 nM as indicated by indo-1-based dual emission photometry (Fig. 1A). Reproducible responses could be elicited by applying DADLE at 25 min intervals (peak 2/peak 1 = 95 ± 16%). We employed this paradigm to determine the nature of the receptor mediating these responses. Naloxone (1 μM) blocked completely and reversibly the DADLE-evoked responses (Fig. 1B; *n* = 3), indicating that the DADLE-induced increases in [Ca²⁺]_i were mediated by opioid receptors. To determine which opioid receptor type mediated the [Ca²⁺]_i increase, we performed agonist selectivity studies. Figure 2 shows the [Ca²⁺]_i increases elicited by sequential application of selective opioid agonists (10 nM) to the same cell. The observed rank order of potency, DADLE = DPDPE > U50488H > DAMGO, is consistent with activation of the δ-opioid receptor. This finding is consistent with radioligand binding studies in undifferentiated NG108-15 cells in which a homogeneous population of δ-opioid receptors was found (Law et al., 1985). Complete characterization of the receptor will require complete concentration-response curves for the agonists.

In order to study the DADLE-induced [Ca²⁺]_i increases more efficiently, we employed fura-2-based digital imaging in order to record responses from a population of cells simultaneously (Fig. 3). Fields of 6–12 cells were selected for recording (Fig. 3A). When challenged with 100 nM DADLE for 90 sec, 40% (79 of 198) of the cells responded with a [Ca²⁺]_i increase of at least 50 nM, in good agreement with the frequency of responding cells recorded individually with photometry (Fig. 3B, frames 1–7). Subsequent superfusion of the cells with 100 nM bradykinin for 1 min induced a large increase in [Ca²⁺]_i in 91% (242 of 267) of the NG108-15 cells (Fig. 3B, frames 8–14). Cells responded to DADLE with an average increase in [Ca²⁺]_i of 483 ± 40 nM, which is only 68% of the average peak response to bradykinin (707 ± 36 nM). Both DADLE- and bradykinin-evoked peak [Ca²⁺]_i values recorded with digital imaging were somewhat smaller than those recorded with photometry, presumably due to the slower sampling rate employed for imaging studies. In Figure 3C plots of [Ca²⁺]_i versus time are shown for cells representing those that responded to both DADLE and bradykinin (green squares) as well as cells that failed to respond to DADLE but did respond to bradykinin (red squares). It appears that the PLCβ1/IP₃ pathway is functional in virtually all of these cells while only a subset respond to DADLE. The amplitude of the bradykinin-induced [Ca²⁺]_i increase in those cells that responded to DADLE may have been attenuated because of insufficient time to refill Ca²⁺ stores between stimuli.

DADLE elicited increases in [Ca²⁺]_i over the concentration range of 1–30 nM (Fig. 4). Maximal responses were typically obtained at 30 nM (*n* = 19; four experiments), in good agreement

with opioid-induced direct excitatory effects previously described in these (Jin et al., 1992) and other cell types (Crain and Shen, 1990; Gintzler and Xu, 1991). The excitatory effect described here occurs at opioid concentrations several orders of magnitude lower than those required for inhibition of Ca²⁺ channels in these cells (Hescheler et al., 1990; Kasai, 1992; Jin et al., 1993). The EC₅₀ for DADLE was approximately 4 nM (Fig. 4D), which is in good agreement with the EC₅₀ for DADLE-evoked Ca²⁺ influx in differentiated NG108-15 cells.

We have previously described opioid-induced Ca²⁺ influx in differentiated NG108-15 cells (Jin et al., 1992); however, the DADLE-induced increases in [Ca²⁺]_i described in this report result from the release of Ca²⁺ from an intracellular store. As shown in Figure 5A, removal of extracellular Ca²⁺ had little effect on the amplitude of the DADLE-induced [Ca²⁺]_i transient (representative of six of seven cells). In one experiment the response was inhibited significantly such that in the absence of extracellular Ca²⁺ the average response amplitude was 62 ± 16% (peak 2/peak 1; *n* = 7), which is smaller than control responses (Fig. 1A; 95 ± 16%). Clearly, the majority of the response results from Ca²⁺ derived from an intracellular pool. Since the IP₃-sensitive store is a likely candidate for Ca²⁺ mobilization resulting from activation of plasmalemmal receptors (Berridge, 1993), and NG108-15 cells possess large IP₃-sensitive Ca²⁺ stores (Lo and Thayer, 1993), we explored the possibility that opioids might be releasing this store. Thapsigargin inhibits the Ca²⁺/Mg²⁺-ATPase responsible for sequestering Ca²⁺ into IP₃-sensitive stores (Thastrup et al., 1990). In NG108-15 cells, 10 nM thapsigargin will deplete and prevent irreversibly the refilling of the IP₃-sensitive Ca²⁺ store (Lo and Thayer, 1993). In Figure 5B we show that thapsigargin mobilized Ca²⁺ from an intracellular store and prevented all subsequent DADLE-elicited responses (90 ± 8% inhibition; *n* = 5), consistent with the idea that DADLE is releasing Ca²⁺ from the IP₃-sensitive Ca²⁺ store.

DADLE-induced mobilization of the IP₃-sensitive store suggests that opioids stimulate PI hydrolysis in NG108-15 cells. To test this hypothesis we treated NG108-15 cells with the PLC inhibitor U73122. We have shown previously that a 20 min incubation with 1 μM U73122 will completely block the PLC-mediated Ca²⁺ mobilization stimulated by bradykinin in NG108-15 cells (W. Jin, T.-M. Lo, H. H. Loh, and S. A. Thayer, unpublished observations). A close structural analog, U73343, was without effect on bradykinin-induced Ca²⁺ mobilization. Superfusion of 1 μM U73122 for 20 min completely blocked DADLE-induced Ca²⁺ mobilization (Fig. 6A; *n* = 4). In contrast, the inactive analog U73343 did not affect the DADLE-induced [Ca²⁺]_i transient (Fig. 6B; *n* = 3).

DADLE, acting with high affinity on opioid receptors, apparently activates PLC, leading to the subsequent mobilization of the IP₃-sensitive Ca²⁺ store. Yet opioid receptors in NG108-15 cells are thought to couple to G_o and G_i, which do not activate PLCβ1 via their α subunits (Hepler and Gilman, 1992). Inhibitory heterotrimeric G-proteins have been shown to stimulate

Figure 3. DADLE and bradykinin increase [Ca²⁺]_i in NG108-15 cells. *A*, Bright-field image of NG108-15 cells grown in CO₂-independent media supplemented with 5 μM forskolin for 6 d. Areas used for calculating [Ca²⁺]_i were defined by a mask determined from the 380 nm fluorescence image and are delimited by the yellow border. *B*, Pseudocolor representations of [Ca²⁺]_i were derived from fura-2-based digital images and scaled as shown in *C*. The images displayed in *B* were acquired at the times indicated by the frame numbers along the plot in *C*. *C*, [Ca²⁺]_i values from the areas circumscribed by the rectangles in *A* are plotted versus time. Representative responses are shown for a cell that responded to both DADLE and bradykinin (green squares) and a cell that responded to bradykinin only (red squares). Sequential ratio pairs were collected every 6.2 sec. The cells were superfused with 100 nM DADLE for 90 sec or 100 nM bradykinin (BK) for 60 sec as indicated by the horizontal bars in *C*.

PLC β 2 via their $\beta\gamma$ subunits (Camps et al., 1992; Katz et al., 1993). We examined DADLE- and bradykinin-induced responses after inactivation of specific populations of G-proteins with CTX or PTX (Fig. 7). The frequency of responses greater than 50 nM in this cohort of untreated cultures was 42% ($n = 106$) for DADLE and 93% for bradykinin. Pretreatment with 1 μ g/ml CTX for 4 hr failed to inhibit the [Ca²⁺]_i increase evoked by either DADLE or bradykinin (Fig. 7A). In contrast, pretreatment of the cultures for 24 hr with 100 ng/ml PTX blocked the DADLE-evoked [Ca²⁺]_i increase, but the bradykinin-induced response remained intact (Fig. 7B), consistent with G_o or G_i mediating the opioid response and G_q mediating the bradykinin response. These data are summarized in Figure 7C. After treatment with CTX, 38% and 87% of the cells responded to DADLE and bradykinin, respectively. Treatment with PTX significantly reduced the number of cells responding to DADLE to 10% ($p < 0.005$), while the percentage of cells responding to bradykinin remained unaffected (88%). δ opioid receptors in NG108-15 cells have been shown previously to inhibit adenylyl cyclase via G_i and to inhibit Ca²⁺ channels via G_o (Hsia et al., 1984; Hescheler et al., 1987). Neither CTX nor PTX treatment affected the bradykinin-induced response, consistent with the idea that the bradykinin receptor couples to PLC β 1 by α_q ; G_q is not sensitive to PTX (Hepler and Gilman, 1992).

Discussion

We have found that activation of opioid receptors by DADLE activates a PTX-sensitive G-protein resulting in a subsequent stimulation of PLC and mobilization of Ca²⁺ from an IP₃-sensitive store. These data provide a biochemical explanation for the opioid-induced increases in [Ca²⁺]_i we (Jin et al., 1992) and others (Okajima and Kondo, 1992) have described previously in NG108-15 cells. Activation of the PI cascade may also underlie the opioid-induced enhancement of NMDA-activated currents in spinal neurons (Chen and Huang, 1991), but probably not the CTX-sensitive prolongation of the action potential by opioids in sensory neurons (Shen and Crain, 1990).

The DADLE-induced increase in [Ca²⁺]_i was blocked by naloxone, which is consistent with a response mediated by an opioid receptor. The rank order of potency for selective agonists, DADLE = DPDPE > U50488 > DAMGO, is consistent with activation of a δ receptor. Additionally, the effect was elicited by nanomolar concentrations of DADLE, suggesting that the [Ca²⁺]_i increase results from an interaction of high affinity. The EC₅₀ of approximately 4 nM is in good agreement with the 1.9 nM K_d for DADLE in radioligand binding assays (Law et al., 1985) and the 2.9 nM IC₅₀ for inhibition of adenylyl cyclase (Law et al., 1982) in these cells. Inhibition of Ca²⁺ channels by opioids has generally required micromolar concentrations (Hescheler et al., 1990; Kasai, 1992; Jin et al., 1993; but see Tsunoo et al., 1986). Extensive concentration-response data for opioid inhibition of Ca²⁺ currents are not available and the rather high concentrations of opioids used to inhibit whole-cell Ca²⁺ currents may be necessitated by the need to elicit rapid and maximal inhibition to discern modulation of the currents from channel

rundown. Interestingly, opioid-induced excitatory effects, manifest as action potential broadening in dorsal root ganglion neurons, were detected at opioid concentrations 2 orders of magnitude lower than those required to elicit inhibitory effects in the same cells (Shen and Crain, 1989). Excitatory effects have been reported for a number of neurotransmitters previously thought to elicit purely inhibitory actions. In the cases of neuropeptide Y and adenosine the excitatory effect has been clearly attributed to the discovery of an additional, excitatory receptor subtype (Wiley et al., 1991; Okada et al., 1992). NG108-15 cells are thought to possess only δ opioid receptors (Law et al., 1985). The existence of multiple δ receptor subtypes has been suggested (Sofuoglu et al., 1990), but at present subtype-selective drugs are not available to test the hypothesis that a novel δ receptor subtype mediates the excitatory effects of opioids in NG108-15 cells.

In NG108-15 cells, opioid receptor coupling to Ca²⁺ channels and adenylyl cyclase has been shown previously to be mediated by G_o and G_i type G-proteins (Hescheler et al., 1987; Costa et al., 1988; Beech et al., 1992). The effective inhibition of the opioid-induced Ca²⁺ mobilization by PTX suggests that opioid coupling to PLC may employ one of these same previously characterized inhibitory G-proteins. This result is in contrast to the opioid-induced excitatory responses in sensory neurons (Shen and Crain, 1990) and F11 cells (Fan et al., 1993), which were blocked by CTX. To our knowledge, there is no evidence suggesting that α_i is capable of activating PLC directly. However, $\beta\gamma$ subunits liberated by the activation heterotrimeric G_i could stimulate PLC β 2 (Katz et al., 1993). The small amplitude of the DADLE-induced [Ca²⁺]_i transients is consistent with less robust IP₃ production observed for G_i- and G_o-mediated versus G_q-mediated PLC activation. In *Xenopus* oocytes expressing muscarinic m3 receptors, which couple to G_q, stimulation with agonist resulted in increases in [Ca²⁺]_i that were large, rapid, and transient. In contrast, stimulation of m2 receptors, which couple to G_i, produced modest increases in [Ca²⁺]_i that were slow, oscillatory, and focal (Lechleiter et al., 1991). Calcitonin gene-related peptide and GABA_B receptors provide additional examples of receptors that activate inhibitory G-proteins to produce excitatory effects in addition to their inhibitory actions (Hahner et al., 1991; Wiley et al., 1992).

There is circumstantial evidence suggesting a link between opioid receptors and PLC. For example, in spinal trigeminal neurons opioid-induced increases in NMDA currents can be blocked by a selective protein kinase C inhibitor (Chen and Huang, 1991). This effect may underlie opioid-mediated induction of long-term potentiation (Derrick et al., 1992). In NG108-15 cells the PLC inhibitor U73122 (Bleasdale et al., 1990) effectively blocked DADLE-induced increases in [Ca²⁺]_i. We have shown previously that U73122 will completely block bradykinin-induced [Ca²⁺]_i increases in these cells (Jin, Lo, Loh, and Thayer, unpublished observations), a response previously shown to be mediated by PLC (Brown and Higashida, 1988). The selectivity of U73122 for PLC has not been fully established. Our confidence in the conclusion that U73122 blocked

Figure 4. Nanomolar DADLE concentrations elicit [Ca²⁺]_i increases in NG108-15 cells. Pseudocolor representations of [Ca²⁺]_i were derived from fura-2-based digital images and scaled as shown. The same field of NG108-15 cells was treated with 1 nM (A), 3 nM (B), and 10 nM (C) DADLE. DADLE was applied for 90 sec at 25 min intervals. Resting [Ca²⁺]_i levels were determined (frame 1), then the cells were treated with DADLE and [Ca²⁺]_i, and images were recorded 32 sec (frame 2), 38 sec (frame 3), 57 sec (frame 4), and 82 sec (frame 5) later. Bath exchange required approximately 10 sec. D, Data from four experiments are summarized; the response at each concentration is the mean $\pm$ SE of at least 18 cells.

0 [Ca²⁺]_i (nM) 400

Figure 5. DADLE releases Ca^{2+} from the IP₃-sensitive store. *A*, The DADLE-induced (100 nM) $[Ca^{2+}]_i$ transient was not significantly diminished in the absence of extracellular Ca^{2+} ($n = 6$). *B*, Thapsigargin at 10 nM released Ca^{2+} from an intracellular store and prevented a subsequent response to DADLE ($n = 5$). Drugs were applied at the times indicated by the horizontal bars. $[Ca^{2+}]_i$ was measured in single NG108-15 cells by indo-1-based photometry.

DADLE-induced responses by inhibition of PLC is bolstered by failure of the inactive structural analog U73343 to inhibit the opioid response, as well as the results of our thapsigargin and Ca^{2+} -free experiments that implicate the IP₃-sensitive store. Thapsigargin has been widely used and has proven very selective for inhibition of Ca^{2+}/Mg^{2+} -ATPase isoforms found only on intracellular Ca^{2+} stores (Thastrup et al., 1990; Lo and Thayer, 1993).

Attempts to measure PI breakdown products have generally been negative (Yu and Sadee, 1986; but see Periyasamy and Hoss, 1990). Previous attempts to detect opioid-induced increases in IP₃ may have failed because biochemical methods lack the sensitivity to detect a modest increase produced by opioids. The $[Ca^{2+}]_i$ increases we observed were both small, approximately 32% smaller than those elicited by bradykinin, and heterogeneous: only 33–40% of the cells responded with an increase in $[Ca^{2+}]_i$. Thus, techniques that average across a population of cells are at a distinct disadvantage for detecting the response. In addition, we have used unique cell culture conditions to optimize the detection of opioid-induced $[Ca^{2+}]_i$ increases. The observation that growth conditions can increase the number of responding cells argues against the idea that the heterogeneity results from divergence of what is thought to be a clonal cell line.

In previous studies we found that DADLE-induced $[Ca^{2+}]_i$ increases resulted from Ca^{2+} influx in differentiated cells and resulted from Ca^{2+} mobilization in undifferentiated cells. The cells used in this study extend very short, if any, processes and lack a significant depolarization-induced increase in $[Ca^{2+}]_i$, and thus by our criteria are undifferentiated. We speculate that during differentiation, which in some respects models neuronal development (Hamprecht et al., 1985), the opioid-stimulated PI hydrolysis switches from a response dominated by Ca^{2+} release from the IP₃-sensitive store to one predominantly resulting

Figure 6. DADLE activates PLC to mobilize Ca^{2+} from the IP₃-sensitive store. The PLC inhibitor U73122 ($n = 4$; *A*) and its inactive analog U73343 ($n = 3$; *B*) were superfused onto single NG108-15 cells during the times indicated by the horizontal bars. DADLE (100 nM), U73122 (1 μ M), and U73343 (1 μ M) were applied during the times indicated by the horizontal bars. $[Ca^{2+}]_i$ was measured in single NG108-15 cells by indo-1-based photometry.

from a depolarization-induced response mediated by diacylglycerol activation of protein kinase C and subsequent Ca^{2+} influx. We have not, as yet, determined the ionic currents that underlie Ca^{2+} influx in differentiated cells, but a dependence on extracellular Na^{+} is consistent with C kinase activation of an Na^{+} conductance. Activation of P₂ purinoceptors increases a depolarizing conductance carried primarily by Na^{+} , providing precedent for such a mechanism (Shen and North, 1993). Protein kinase C will also modify voltage-gated Ca^{2+} channels directly (Yang and Tsien, 1993). The growth conditions that produce the greatest yield of responding cells, whether differentiated (DMEM) or undifferentiated (CO₂-independent media), included chronic elevation of cAMP by treatment with forskolin. Thus, cross talk between the cAMP and PI second messenger systems may be required to permit opioid-induced $[Ca^{2+}]_i$ increases. There is a precedent for state-dependent activation of adenylyl cyclase by G_i-linked receptors (Federman et al., 1992) and the importance of such biochemical coincidence detectors has been reviewed (Bourne and Nicoll, 1993). One of the results of chronic treatment of NG108-15 cells with opioids is a compensatory increase in adenylyl cyclase activity (Sharma et al., 1975b), raising the interesting possibility that during chronic treatment with opioids, opioid receptor coupling to the PI cascade may become more pronounced.

Indeed, chronic treatment of dorsal root ganglion neurons with opioids increases the number of cells with opioid-induced excitatory responses and greatly decreases the threshold for eliciting opioid-induced excitatory responses in these cells (Crain et al., 1988; Shen and Crain, 1992). The molecular and cellular changes that result from chronic treatment with opioids have not presented a clear picture. In NG108-15 cells chronic opioids will produce a downregulation of receptors and a corresponding increase in the activity of adenylyl cyclase (Smith et al., 1988),

Figure 7. DADLE-induced $[Ca^{2+}]_i$ increases are mediated by a PTX-sensitive G-protein. Cultures were pretreated with either CTX (1 $\mu g/ml$, 4 hr; A) or PTX (100 ng/ml, 24 hr; B). Resting $[Ca^{2+}]_i$ levels were determined in the cells (frame 1), which were then superfused with 100 nM DADLE for 90 sec (frame 2), allowed to recover for 1 min (frame 3), and finally challenged with 100 nM bradykinin for 60 sec (frame 4). Pseudocolor representations of $[Ca^{2+}]_i$ were derived from fura-2-based digital images and scaled as shown. Data from untreated ($n = 106$), CTX-treated ($n = 71$), and PTX-treated ($n = 96$) cultures are summarized in C. *, The frequency of DADLE-evoked responses in PTX-treated cells was significantly different from untreated sister cultures ($p < 0.005$, χ^2 test).

yet *in vivo*, gross changes in receptor number have not been detected and relating cAMP levels to electrical activity has not been straightforward. We believe that the possibility that chronic exposure to opiates unmasks excitatory actions of these drugs warrants testing.

We have shown that activation of opioid receptors in the neuronal cell line NG108-15 will activate PLC resulting in subsequent release of Ca²⁺ from intracellular stores. We speculate this effect can be generalized to primary tissue. Including the PI cascade among the multiple second messenger systems modulated by opioids may be helpful in clarifying the biochemical events which accompany acute and chronic opioid action.

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