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Dual Excitatory and Inhibitory Effects of Opioids on Intracellular Calcium in Neuroblastoma × Glioma Hybrid NG108–15 Cells

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SUMMARY

The intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured in single NG108–15 cells using indo-1-based microfluorimetry. In cells differentiated for 6–14 days in serum-free, forskolin (5 μM)-supplemented medium, application of micromolar concentrations of [D-Ala^2,D-Leu^5]-enkephalin (DADLE) inhibited Ca^{2+} influx mediated by voltage-gated Ca^{2+} channels. DADLE, at concentrations ranging from 1 nM to 1 μM, also produced rapid transient increases in $[Ca^{2+}]_i$ (EC_{50} = 10 nM). The $[Ca^{2+}]_i$ increases elicited by DADLE did not correlate with the inhibitory effects of the peptide. DADLE-induced $[Ca^{2+}]_i$ increases were blocked by naloxone. In single cells, sequential application of selective opioid agonists (30 nM) evoked responses of the rank order DADLE = [D-Pen^2,D-Pen^5]-enkephalin > (*trans*)-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide > [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, consistent with activation of a δ-opioid receptor. The response was completely blocked by removal of extracellular Ca^{2+} or application of 1 μM nitrendipine, indicating that the increase in $[Ca^{2+}]_i$ results from Ca^{2+} influx via dihydropyridine-sensitive, voltage-gated Ca^{2+} channels. Substitution of *N*-methyl-D-glucamine for extracellular Na^+ or application of 1 μM tetrodotoxin greatly reduced, and in some cases blocked, the DADLE-induced $[Ca^{2+}]_i$ increase, consistent with amplification of the response by voltage-gated Na^+ channels. The $[Ca^{2+}]_i$ increase was mimicked by both dibutyryl-cAMP and phorbol 12,13-dibutyrate. These findings are consistent with a δ-opioid-induced depolarization, possibly mediated by a second messenger, that subsequently recruits voltage-sensitive Ca^{2+} channels. In contrast to differentiated cells, undifferentiated cells responded to DADLE with a modest $[Ca^{2+}]_i$ increase that was not sensitive to nitrendipine. In these cells, activation of the same second messenger system may elevate $[Ca^{2+}]_i$ by mobilization from intracellular stores rather than influx. In addition to previously described inhibitory coupling to adenylyl cyclase and Ca^{2+} channels in NG108–15 cells, these results suggest that a novel, excitatory, effector system may also couple to opioid receptors.

Opioids exert inhibitory actions on several effector systems, for example, μ and δ opioids activate K^+ conductances in guinea pig submucous plexus and rat locus coeruleus neurons, respectively (1), κ opioids inhibit voltage-gated Ca^{2+} channels in sensory neurons (2), and δ opioids inhibit adenylyl cyclase in neuronal cell lines (3–5) and locus coeruleus neurons (6). Thus, the predominant effects of opioids released at excitatory synapses are decreased excitability and firing rate (7).

In the hippocampal slice preparation opioids have been shown to produce excitatory effects (8) that are usually ascribed to inhibition of inhibitory interneurons (9, 10). However, excitatory effects of opioids have been described *in vivo* that are not readily explained by this disinhibition mechanism (11, 12). Peripheral opiate-induced hyperalgesia may be mediated by a direct action of opiates (13). Indeed, Crain and Shen (14) have described an opioid-induced broadening of the action potential in single sensory neurons in culture, suggesting that opioids can produce direct excitatory effects.

The δ-opioid receptor present in the neuronal cell line NG108–15 couples via guanine nucleotide-binding proteins to multiple effector molecules, including adenylyl cyclase (3–5) and Ca^{2+} channels (15, 16). While studying these inhibitory responses, we observed small opioid-induced increases in $[Ca^{2+}]_i$, suggesting that δ-opioid receptors may be coupled to yet another effector in NG108–15 cells, one capable of eliciting excitatory effects.

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Materials and Methods

Cell culture. NG108–15 cells (passages 20–30) were grown in culture as previously described (5). Briefly, cells were plated onto glass

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; U50488H, (*trans*)-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl)benzeneacetamide; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; dBucAMP, dibutyryl-cAMP; PdBu, phorbol 12,13-dibutyrate; NMDG, *N*-methyl-D-glucamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IP_3, inositol 1,4,5-trisphosphate.

coverslips (25 mm, round) at a density of 3×10^4 cells/coverslip and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 0.1 μM hypoxanthine, 10 μM aminopterine, and 17 μM thymidine, in a humidified atmosphere of 95% air/5% CO_2 at 37°. After 1–2 days in growth medium, undifferentiated cells were either used for experiments or placed in serum-free medium containing 5 μM forskolin to induce cellular differentiation. Differentiated cells were used after 6–14 days.

Instrumentation. $[Ca^{2+}]_i$ was monitored in single cells using indo-1 and a dual-emission microfluorimeter. For excitation of indo-1, light from a 75-W xenon arc lamp was passed through a monochromator (Photon Technologies Inc.) set to 350 nm (slit width, 2 nm) and collimated with a parabolic mirror. For epifluorescence excitation, light was reflected from a dichroic mirror (380 nm; Omega Optical) through a 70× phase-contrast oil-immersion objective (Leitz; numerical aperture, 1.15). Emitted light was sequentially reflected off dichroic mirrors (440 and 516 nm) through band pass filters (405/20 and 495/20 nm, respectively) to photomultiplier tubes operating in photon-counting mode (Thorn EMI). Cells were illuminated with transmitted light (610-nm long pass) and visualized with a video camera placed after the second emission dichroic mirror. Recordings were defined spatially with a rectangular diaphragm. The 5-V photomultiplier outputs were integrated by passing signals through eight-pole Bessel filters at a cut-off frequency of 2.5 Hz. These signals were then input to two channels of a direct-memory access analog-to-digital converter (Indec Systems) continuously sampling at 200 Hz. Data points were averaged every second, resulting in a final sampling rate of 1 Hz.

Calibration. After completion of an experiment, the microscope stage was adjusted so that no cells or debris occupied the field of view defined by the diaphragm, and background light levels were determined (typically <5% of cell counts). Autofluorescence from cells not loaded with indo-1 was undetectable. Records were later corrected for background and ratio values were recalculated. 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 405/495-nm fluorescence emission ratio and $K = 250$ nM, the dissociation constant for indo-1 (17). The maximum ratio (R_{max}), the minimum ratio (R_{min}), and the constant β (the ratio of the fluorescence measured at 495 in the absence and presence of saturating Ca^{2+}) were determined in neurons by treatment with ionomycin in the absence (1 mM EGTA) and presence of saturating Ca^{2+} (5 mM Ca^{2+}). The system was recalibrated after any adjustment to the apparatus. Values for R_{min}, R_{max}, and β ranged from 0.22 to 0.23, from 2.43 to 2.82, and from 3.46 to 3.54, respectively.

Experimental procedure. NG108–15 cells were incubated with 2 μM indo-1/acetoxymethyl ester in HEPES-buffered Hanks' salt solution containing 0.5% bovine serum albumin for 45–60 min at 37°. Hanks' 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; $NaHPO_4$, 0.6; $NaHCO_3$, 3.0; and glucose, 5.6. The coverglass was then mounted in a flow-through chamber (18) and placed on the stage of the microfluorimeter. Loading was terminated by washing with Hanks' buffer for 20–30 min before an experiment was started. The chamber was superfused at a rate of 2 ml/min and solutions were selected with a multiport valve coupled to several reservoirs. $CaCl_2$ was replaced with 20 μM EGTA for Ca^{2+}-free experiments.

Results

NG108–15 cells were grown in serum-free, forskolin-supplemented medium for 6–14 days, during which time flat polygonal cells differentiated into rounded cells that extended fine processes and expressed voltage-sensitive Ca^{2+} channels. NG108–15 cells differentiated for 6 days displayed robust depolarization-induced $[Ca^{2+}]_i$ transients mediated entirely by dihydropyridine-sensitive Ca^{2+} channels (19). These responses could be inhibited by δ opioids, as previously described (15, 16). The inhibition of Ca^{2+} influx did not occur in every cell (Fig. 1) and

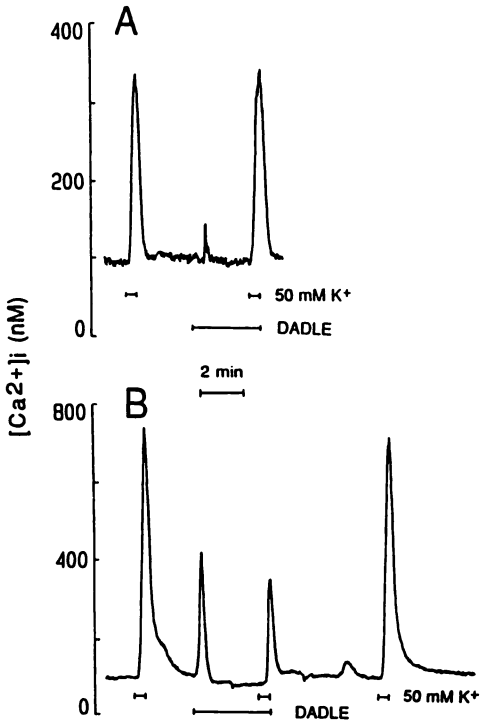

Fig. 1. DADLE-induced $[Ca^{2+}]_i$ increases do not correlate with opioid inhibition of Ca^{2+} channels. NG108–15 cells were treated with a depolarizing (50 mM K^+) stimulus or 1 μM DADLE, as indicated by the *horizontal bars*. A, In some cells in which DADLE elicited a $[Ca^{2+}]_i$ increase, it failed to inhibit the depolarization-induced response ($n = 15$). B, Other cells displayed both the DADLE-induced $[Ca^{2+}]_i$ increase and attenuation of the depolarization-evoked response ($n = 10$). Many cells responded to DADLE with an inhibitory effect without an excitatory response ($n = 61$), and the majority of cells did not respond to DADLE with either an excitatory or inhibitory effect ($n = 122$).

required micromolar concentrations of DADLE for significant inhibition. In carrying out these studies, we observed occasional $[Ca^{2+}]_i$ increases coincident with the application of DADLE to the bath. These DADLE-induced $[Ca^{2+}]_i$ transients did not correlate with the sensitivity of the Ca^{2+} channel response to inhibition by DADLE. The cell shown in Fig. 1A responded with a $[Ca^{2+}]_i$ increase when DADLE was applied, but the depolarization-induced response was not inhibited by DADLE ($n = 15$). However, cells such as the one described in Fig. 1B responded to DADLE with both an immediate $[Ca^{2+}]_i$ increase and a significant inhibition of the depolarization-induced $[Ca^{2+}]_i$ transient ($n = 10$). Many cells responded to DADLE with an inhibitory effect without an excitatory response ($n = 61$), and the majority of cells did not respond to DADLE with either an excitatory or inhibitory effect ($n = 122$). Thus, DADLE-induced $[Ca^{2+}]_i$ increases and DADLE-induced inhibition of Ca^{2+} channels were neither correlated (Fig. 1A) nor mutually exclusive (Fig. 1B). A total of 208 cells were examined for both excitatory and inhibitory effects of DADLE. In the remainder of this report, we will describe in more detail the nature of the small opioid-induced $[Ca^{2+}]_i$ increases observed in NG108–15 cells. We found that a slightly greater percentage of cells responded to DADLE when they were differentiated for longer than 6 days. A group of 369 cells were differentiated for 7–14 days and subsequently challenged with DADLE. Inhibitory effects were not determined for this group of cells. Ninety-three cells responded with an increase in $[Ca^{2+}]_i$, of which only 50 cells displayed $[Ca^{2+}]_i$ increases of sufficient amplitude (>50

nM increase above baseline) to enable pharmacological characterization. Because of this cell to cell variation in response, all experiments were internally controlled.

The $[Ca^{2+}]_i$ increase elicited by DADLE displayed a concentration dependence similar to that of other opioid-mediated responses in NG108–15 cells. Nanomolar concentrations of DADLE produced increases in $[Ca^{2+}]_i$, as shown in the single-cell concentration-response curve in Fig. 2. The mean EC_{50} was 10 ± 7 nM (n = 3), in good agreement with the 2.9 nM IC_{50} reported for DADLE inhibition of adenylyl cyclase (5) and the 8.8 nM K_d for Leu-enkephalin inhibition of Ca^{2+} channels in these cells (15). In our studies, DADLE-induced inhibition of Ca^{2+} channels required micromolar concentrations to produce detectable inhibition of depolarization-induced Ca^{2+} influx, in contrast to the results of Tsunoo *et al.* (15) but in agreement with the high opioid concentrations used by others (2, 16). Thus, DADLE-induced $[Ca^{2+}]_i$ increases could be differentiated from inhibition of Ca^{2+} channels by a potency difference of >2 orders of magnitude. Nanomolar concentrations of opioids prolong, and micromolar concentrations shorten, action potential duration in sensory neurons (20).

Multiple opioid-induced $[Ca^{2+}]_i$ increases could be elicited from a single cell, allowing characterization of the receptor that mediates this effect (Fig. 3A). The receptor was completely desensitized immediately after a 90-sec exposure to 100 nM DADLE but, as shown in Fig. 3A, after a 25-min wash period another response of comparable amplitude could be elicited. In all of the experiments described in this report, opioids were applied at 25-min intervals. Naloxone at 1 μM inhibited the $[Ca^{2+}]_i$ increase evoked by 100 nM DADLE by 87 ± 6% (Fig. 3B; n = 3), and 100 μM naloxone completely blocked the response (n = 5), indicating that the effect was mediated by an opioid receptor. To determine which opioid receptor type mediated the $[Ca^{2+}]_i$ increase, we performed agonist selectivity studies. Fig. 3C shows the $[Ca^{2+}]_i$ increases elicited by sequen-

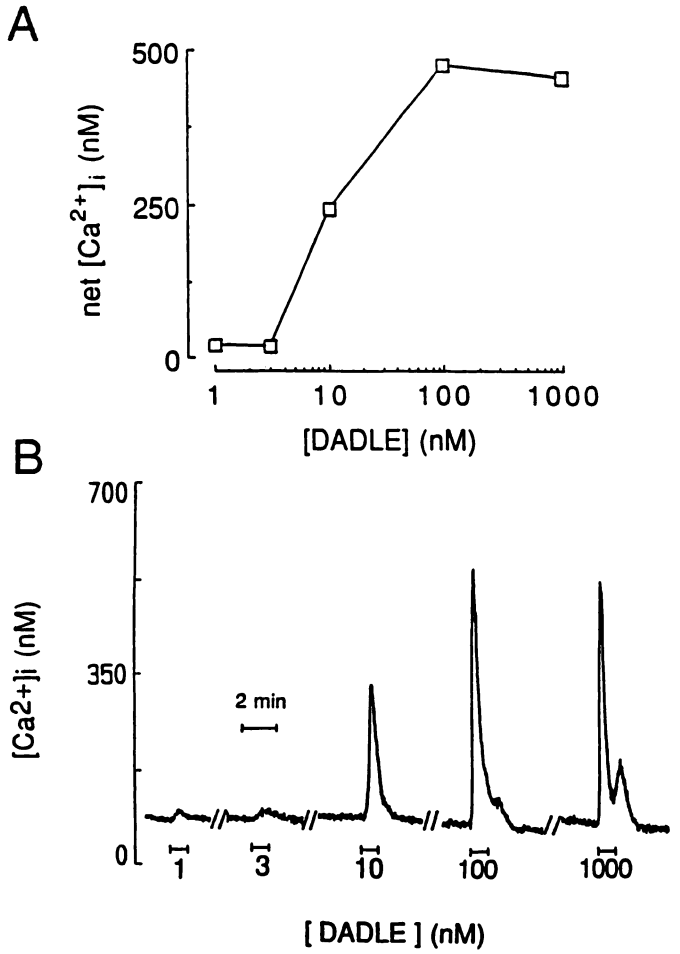

Fig. 2. DADLE elicits concentration-dependent increases in $[Ca^{2+}]_i$ in single NG108–15 cells. A, A concentration-response curve for DADLE-induced $[Ca^{2+}]_i$ increases was determined for a single cell (EC_{50} = 10 ± 7 nM; n = 3). B, Representative $[Ca^{2+}]_i$ traces from the experiment plotted in A. DADLE was superfused onto the cells at the concentrations shown, at the times indicated by the *horizontal bars*. *Diagonal lines*, gaps in the recording; DADLE was applied at 25-min intervals.

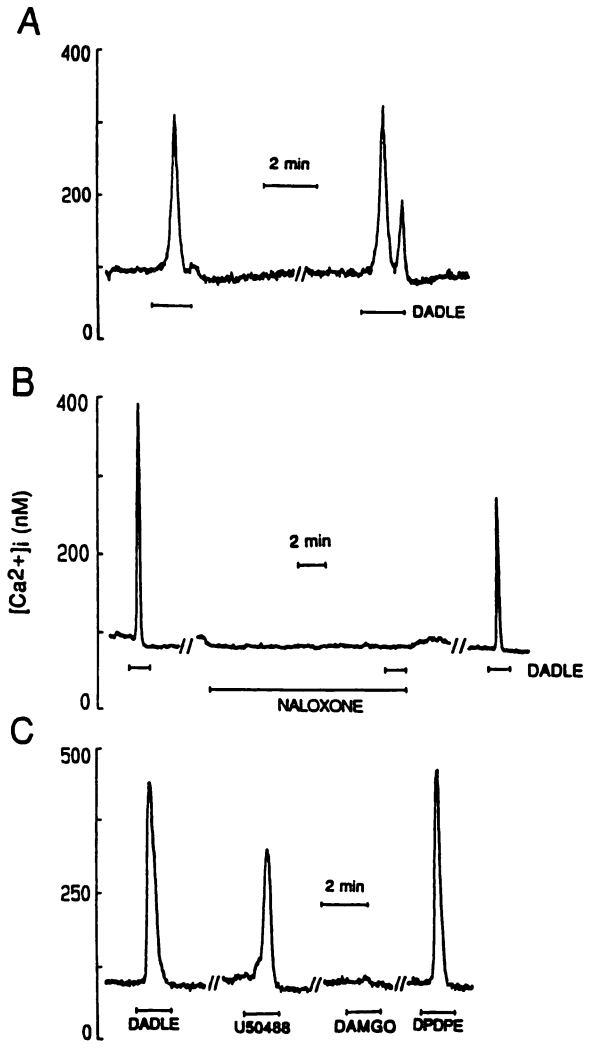

Fig. 3. DADLE-induced $[Ca^{2+}]_i$ increases are mediated by opioid receptors. A, Reproducible opioid-induced $[Ca^{2+}]_i$ increases were elicited in single cells by superfusion with 100 nM DADLE for 90 sec, as indicated by the *horizontal bars*. B, The response to 100 nM DADLE was reversibly blocked by 1 μM naloxone (n = 3). The decreased amplitude of the third response may be due to either rundown of the evoked response or incomplete washout of the naloxone. C, Opioid agonists (30 nM) were sequentially applied to an NG108–15 cell, resulting in the following rank order of potency: DPDPE = DADLE > U50488H > DAMGO. Complete testing of all four agonists on a single cell was successfully completed for only the cell shown because the response often declined before application of DPDPE. Each relative ranking, for example DAMGO relative to DADLE, was replicated at least three times. In some experiments, dynorphin A(1–13) was used as a κ agonist instead of U50488, with similar results. *Diagonal lines*, gaps in the recording; Agonists were applied at 25-min intervals.

tial application of selective opioid agonists (30 nM) to the same cell. The observed rank order of potency, DPDPE = DADLE > 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 (21). Complete characterization of the receptor will require complete concentration-response curves for the agonists.

Previously described effects of opioids in these cells include inhibition of adenylyl cyclase and inhibition of Ca^{2+} channels which would not be expected to produce an increase in $[Ca^{2+}]_i$. Therefore, determination of the source of Ca^{2+} was of interest. In the absence of extracellular Ca^{2+}, DADLE failed to elevate $[Ca^{2+}]_i$ (Fig. 4A). Thus, the $[Ca^{2+}]_i$ increase resulted from Ca^{2+} influx, raising the possibility that Ca^{2+} might enter the cell via voltage-sensitive Ca^{2+} channels. In these cells, the majority of voltage-dependent Ca^{2+} influx is dihydropyridine-sensitive (19). Nitrendipine at 1 μM completely blocked the DADLE-induced $[Ca^{2+}]_i$ increase (Fig. 4B), indicating that nanomolar concentrations of DADLE activate voltage-sensitive Ca^{2+} channels. Because activation of dihydropyridine-sensitive Ca^{2+} channels requires membrane depolarization, we explored the possibility that a Na^+-dependent depolarization might be involved. When extracellular Na^+ was replaced with the impermeant cation NMDG, the DADLE-induced response was completely blocked in three of five responding cells (Fig. 4C). This result is consistent with the idea that Na^+ influx depolarizes the cell, leading to subsequent recruitment of voltage-gated Ca^{2+} channels. We next considered the possibility that a small membrane depolarization might be amplified by activation of tetrodotoxin-sensitive, voltage-gated, Na^+ channels. Tetrodotoxin (1 μM) completely blocked the DADLE-induced $[Ca^{2+}]_i$ transient in two of four responding cells. However, in two cells the response, although greatly diminished, was in part resistant to tetrodotoxin blockade (Fig. 4D). Tetrodotoxin attenuation of the $[Ca^{2+}]_i$ increase suggests that voltage-gated Na^+ channels play a significant role in amplifying a depolarization sufficient to recruit voltage-gated Ca^{2+} channels. The failure of tetrodotoxin and Na^+ removal to consistently block the entire DADLE-induced $[Ca^{2+}]_i$ increase implies that Na^+ influx amplifies but does not initiate membrane depolarization.

In summary, our data are consistent with DADLE producing a modest depolarization, which is then amplified by activation of voltage-gated Na^+ channels; this larger depolarization then recruits voltage-sensitive Ca^{2+} channels, leading to Ca^{2+} influx. Second messenger-induced depolarizing responses have been previously described in neurons (22–24). To explore the possibility that opioids were generating second messengers that then modulated ion channels, we applied analogs of diacylglycerol and cAMP to determine whether these agents would mimic the DADLE-induced $[Ca^{2+}]_i$ increase. Both PdBu (1 μM) and dBucAMP (1 mM) elicited $[Ca^{2+}]_i$ transients similar in form to those elicited by DADLE (Fig. 5). A larger percentage of cells responded to PdBu (73%) than to dBucAMP (13%). Experiments with kinase inhibitors have been inconclusive to date, due to nonspecific effects of these agents on $[Ca^{2+}]_i$ homeostasis (25).

Because opioid receptors are present in undifferentiated NG108–15 cells but voltage-sensitive Ca^{2+} channels are not significantly expressed (26), we investigated the effects of opioids on $[Ca^{2+}]_i$ in undifferentiated cells (Fig. 6). Surprisingly, DADLE produced $[Ca^{2+}]_i$ increases in undifferentiated cells (Fig. 6A). This effect was blocked by naloxone (Fig. 6B). However, the pharmacological profile of the response in undifferentiated NG108–15 cells was different from that determined for differentiated cells. Removal of extracellular Ca^{2+} reduced but did not block the DADLE-induced $[Ca^{2+}]_i$ increase (Fig. 6C), suggesting that all or part of the Ca^{2+} was released from intracellular Ca^{2+} stores. Consistent with the idea that DADLE mobilized Ca^{2+} from intracellular stores, but in contrast to observations in differentiated cells, nitrendipine failed to block the DADLE-induced response in undifferentiated NG108–15 cells (Fig. 6D).

Discussion

Superfusion of single NG108–15 cells with opioids produced rapid transient increases in $[Ca^{2+}]_i$, as detected with indo-1 microfluorimetry. This novel response to opioids occurred at

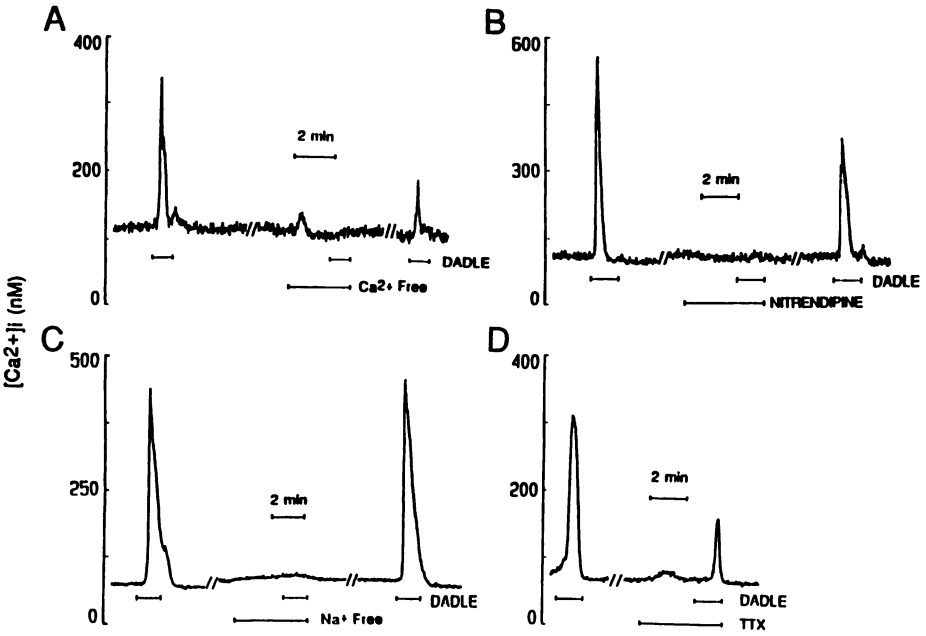

Fig. 4. DADLE recruits voltage-sensitive Ca^{2+} channels. DADLE (100 nM) was superfused onto single NG108–15 cells during the time indicated by the *horizontal bar*. The DADLE-induced $[Ca^{2+}]_i$ increase resulted from Ca^{2+} influx via dihydropyridine-sensitive Ca^{2+} channels, as indicated by the complete block of the response by removal of extracellular Ca^{2+} (n = 4) (A) or treatment with 1 μM nitrendipine (n = 4) (B). C, Replacing extracellular Na^+ with the impermeant cation $NMDG^+$ blocked the DADLE-induced response in some cells (three of five cells) and greatly reduced it in others. D, Similarly, tetrodotoxin (*TTX*) (1 μM) reduced but did not necessarily block the DADLE-induced $[Ca^{2+}]_i$ increase (two of four cells). In two cells, tetrodotoxin completely blocked the response. *Diagonal lines*, gaps in the recording; DADLE was applied at 25-min intervals.

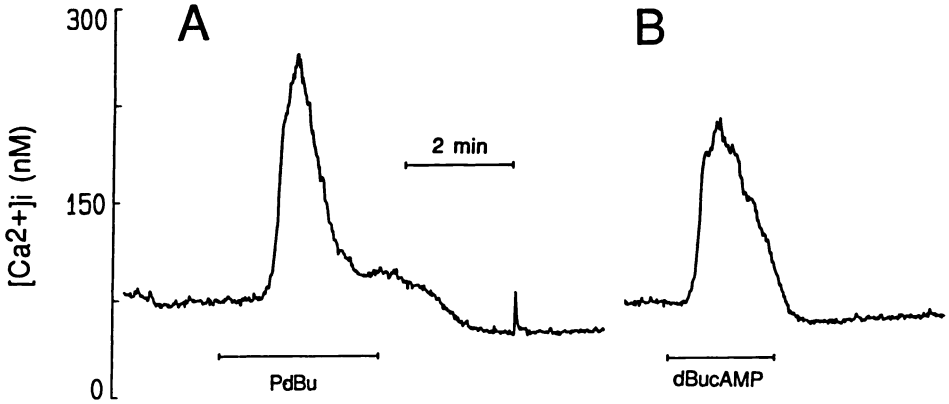

Fig. 5. dBucAMP and PdBu mimic the opioid-induced Ca^{2+} influx. PdBu (1 μM) (A) or dBucAMP (1 mM) (B) was superfused onto single NG108–15 cells as indicated by the *horizontal bars*. The $[Ca^{2+}]_i$ increases were similar in form to those elicited by opioids. A larger percentage of the cells responded to PdBu (8 of 11 cells), relative to dBucAMP (2 of 15 cells).

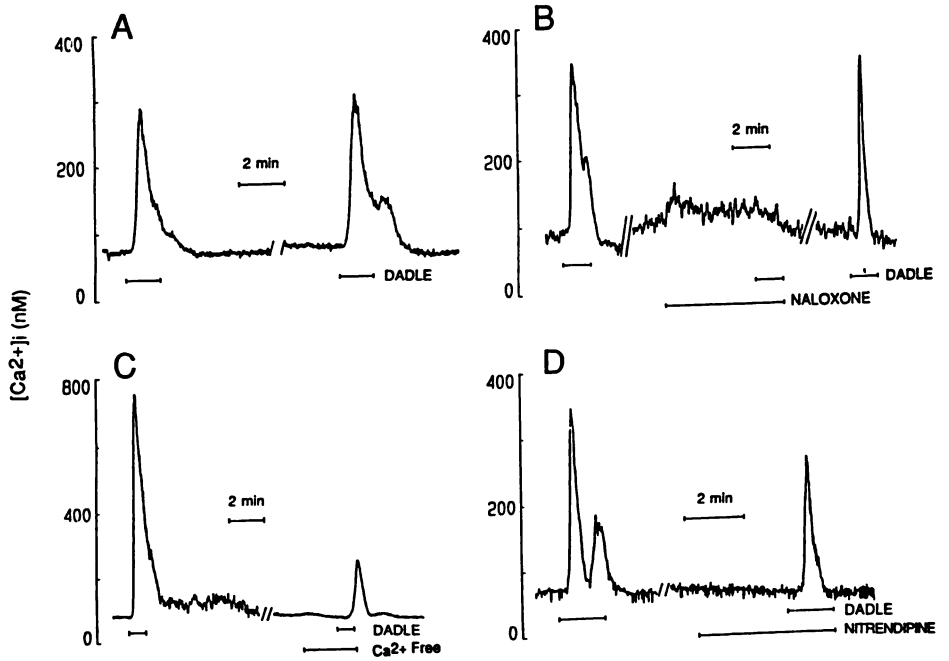

Fig. 6. DADLE mobilizes Ca^{2+} from intracellular stores in undifferentiated NG108–15 cells. A, DADLE (100 nM) elicits a rapid and transient increase in $[Ca^{2+}]_i$ in undifferentiated NG108–15 cells (n = 28). B, This $[Ca^{2+}]_i$ increase was blocked by 100 μM naloxone (n = 4). C, The DADLE-induced $[Ca^{2+}]_i$ increase was attenuated but not blocked by removal of extracellular Ca^{2+} (n = 3). D, In contrast to differentiated cells, 1 μM nitrendipine failed to block the DADLE-induced $[Ca^{2+}]_i$ increase (n = 3).

DADLE concentrations (EC_{50} = 10 nM) well below those required to elicit inhibitory effects on Ca^{2+} channels. The excitatory response was neither correlated with nor mutually exclusive of the inhibitory effect of DADLE on Ca^{2+} channels. The $[Ca^{2+}]_i$ increases were mediated by opioid receptors, as indicated by naloxone block and agonist selectivity studies. The $[Ca^{2+}]_i$ increase produced by opioids was the result of Ca^{2+} influx through dihydropyridine-sensitive Ca^{2+} channels. Although a direct effect of the opioid on Ca^{2+} channels could not be ruled out, the response was dependent on extracellular Na^+ and inhibited by tetrodotoxin, suggesting that an inward Na^+ current depolarized the cell sufficiently to recruit voltage-sensitive Ca^{2+} channels. The membrane-permeant second messenger analogs dBucAMP and PdBu mimicked the opioid-induced $[Ca^{2+}]_i$ increase, indicating that perhaps an opioid-induced change in second messenger concentration underlies the response. Naloxone-reversible, opioid-induced $[Ca^{2+}]_i$ increases were also elicited in undifferentiated NG108–15 cells. These responses were not blocked by dihydropyridine drugs or removal of extracellular Ca^{2+}, consistent with the release of Ca^{2+} from intracellular stores.

In NG108–15 cells, nanomolar concentrations of opioids evoked an excitatory response that was manifest as an increase in $[Ca^{2+}]_i$. Yet, opioids are generally thought to produce inhibitory responses through inhibition of adenylyl cyclase (3–5), activation of K^+ channels (1), or inhibition of Ca^{2+} channels (2, 15, 16). Any one of these inhibitory effects might result in an excitation of a network of neurons if inhibitory interneurons were selectively attenuated (9, 10). Disinhibition has been demonstrated previously in hippocampal slices (8). In the neuronal clonal cultures described in this report synaptic effects are minimal, and indeed we have recorded DADLE-induced $[Ca^{2+}]_i$ increases from cells grown in sparse cultures from which no interconnections were observed. Thus, the opioid-induced $[Ca^{2+}]_i$ increase we have observed in NG108–15 cells appears to be a direct excitatory response. Direct excitatory effects of opioids have been described in sensory neurons (20). Nanomolar concentrations of opioids prolonged the duration of the action potential, whereas micromolar concentrations shortened the action potential in mouse dorsal root ganglion neurons. How that direct excitatory effect relates to the one described here is not clear, although it is interesting that in both cases the excitatory effects occur at opioid concentrations some 2 orders of magnitude lower than those required to produce maximal inhibitory effects. Indeed, the potency of the direct excitatory response suggests that it is distinct mechanistically

from inhibitory responses. The idea that high affinity excitatory effects are distinct from low affinity inhibitory effects is supported by the observation that nanomolar concentrations of opioids enhance enkephalin release from the guinea pig myenteric plexus via a cholera toxin-sensitive process, whereas higher concentrations (10–100 nM) inhibit release by a pertusis toxin-sensitive pathway (27). Excitatory responses have been recently described for other receptors previously categorized as inhibitory neuromodulators. Activation of Y1 receptors for neuropeptide Y inhibits adenylyl cyclase and mobilizes Ca^{2+} from an intracellular store in the neuroblastoma cell line SK-N-MC (28). Additionally, in rat sensory neurons Y1 receptor activation has been shown to enhance Ca^{2+} currents; in contrast, activation of Y2 receptors was inhibitory (29). The excitatory response was ascribed to a unique receptor subtype. Interestingly, δ receptor subtypes have been suggested, based on *in vivo* data (30), although no clear distinction has been made at the molecular or cellular level.

The opioid-induced $[Ca^{2+}]_i$ increase we have described in NG108–15 cells may be explained by several potential mechanisms. There could be a direct coupling, possibly via a guanine nucleotide-binding protein, between the opioid receptor and the Ca^{2+} channel. This linkage has been described previously for the inhibition of Ca^{2+} channels (31) and may couple stimulatory Y1 receptors to Ca^{2+} channels as well (29). However, the dependence of the DADLE-induced $[Ca^{2+}]_i$ increase on extracellular Na^+ is difficult to reconcile with this mechanism. The Na^+ dependence suggests that dihydropyridine-sensitive Ca^{2+} channels that mediate the Ca^{2+} influx are not modulated directly by the opioid but are recruited by an opioid-induced depolarization. In this scenario, the depolarization is likely to be modest and not of sufficient strength to activate the high-threshold, L-type Ca^{2+} channels that predominate in differentiated NG108–15 cells (19). Thus, the small depolarization is amplified by voltage-gated Na^+ channels; this process would explain the sensitivity of the response to tetrodotoxin, a Na^+ channel blocker. In some cells the response was not completely blocked by tetrodotoxin, consistent with voltage-gated Na^+ channels playing a role in amplification, not initiation of the depolarization. The initial depolarization could result from any of several depolarizing mechanisms previously described for neurons. Inhibition of a K^+ conductance could occur either directly, as shown for glutamatergic suppression of I_m in hippocampal neurons (32), or indirectly, as described for modulation of a Ca^{2+}-activated K^+ conductance by kinases (23). Alternatively, activation of both protein kinases A and C has been shown to activate tetrodotoxin-insensitive Na^+ channels (22, 24). Indeed, in the neuroblastoma cell lines NG108-CC15 and N1E-115 morphine has been shown to produce membrane depolarization and Ca^{2+} influx (33). Opioid stimulation of the phosphoinositide cascade has been observed in rat spinal cord and brainstem (34), although not in neuroblastoma cells (35). We have shown that NG108–15 cells possess the cellular machinery necessary to respond to cAMP and diacylglycerol analogs with an increase in $[Ca^{2+}]_i$ (Fig. 5). The supposition that the $[Ca^{2+}]_i$ increase is mediated by an opioid-induced change in second messenger concentration is further supported by observation of opioid-induced $[Ca^{2+}]_i$ responses in undifferentiated NG108–15 cells.

In undifferentiated NG108–15 cells, DADLE elicited increases in $[Ca^{2+}]_i$ that were not the result of Ca^{2+} influx. Removing extracellular Ca^{2+} attenuated but did not block the response, suggesting that the Ca^{2+}-free buffer depleted an intracellular Ca^{2+} store, as has been reported previously for IP_3-mediated responses (36). If the $[Ca^{2+}]_i$ transient resulted from Ca^{2+} influx, then removing extracellular Ca^{2+} would have completely blocked the response. Thus, the $[Ca^{2+}]_i$ transients in undifferentiated cells must result from mobilization of Ca^{2+} from intracellular Ca^{2+} stores. Presently, the only mechanism of this type known to exist is agonist-stimulated breakdown of phosphatidylinositol to IP_3 and subsequent IP_3-induced Ca^{2+} release (37). Undifferentiated NG108–15 cells possess a robust agonist/IP_3-mediated $[Ca^{2+}]_i$ response.[1] We speculate that in undifferentiated NG108–15 cells activation of opioid receptors stimulates the phosphoinositide cascade, resulting in IP_3-mediated Ca^{2+} mobilization; in differentiated cells, diacylglycerol activates a depolarizing conductance that leads to Ca^{2+} influx.

The scarcity of excitatory responses (25%) in these cells has made detailed mechanistic studies difficult and required that all pharmacological studies be paired with a control and drug-treated DADLE response measured on the same cell. We have considered two possible explanations for the scarcity of responses in what is thought to be a clonal cell line. One possibility is that the line is no longer homogeneous, although we limited our studies to cells from passages 20–30. Alternatively, the differentiation of the cells produced by forskolin is not necessarily uniform, because differentiation is dependent on cell density and other local environmental factors, although undifferentiated cells showed a similar variability. Finally, if the response is mediated by an opioid-induced membrane depolarization, as we hypothesize, a threshold would have to be reached in order to activate voltage-sensitive Na^+ channels. It is possible that only a small percentage of responses are of sufficient strength to cross this threshold and hence result in a significant increase in $[Ca^{2+}]_i$.

Excitatory effects observed *in vivo* include euphoria in humans, gross excitation in the cat, and hyperalgesia produced by low concentrations of opiates (11, 12, 38). The hyperalgesic effects are not adequately explained by disinhibition, which appears consistent with central excitatory effects of opioids. Opioid receptors on primary sensory neurons may mediate a peripheral induction of irritation, inflammation, and pain (13). Hyperalgesic versus analgesic effects might be conferred by either the dose dependence of the responses (low dose = hyperalgesic) or specific localization of receptors (peripheral = hyperalgesic).

The DADLE-induced $[Ca^{2+}]_i$ increases described here may account for these excitatory effects, and they suggest that a novel, excitatory, effector system may couple to opioid receptors. Clearly additional work is needed to detail the mechanism of the opioid-induced $[Ca^{2+}]_i$ increase as well as to determine whether our observations made in a neuronal cell line can be extended to include primary neuronal tissue.

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