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Protein Kinase C Activation Increases the Rate and Magnitude of Agonist-Induced δ-Opioid Receptor Down-regulation in NG108-15 Cells

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SUMMARY

Protein kinase C (PKC) activation was examined for its role in δ-opioid receptor down-regulation in the neuroblastoma × glioma hybrid cell line NG108-15. Incubation of NG108-15 cells for 2 hr at 37° with up to 1 μM 4β-phorbol 12β-myristate 13α-acetate (PMA), a phorbol ester that activates PKC, had no effect on opioid binding to membranes prepared from these cells. However, as little as 3 nM PMA incubated with an opioid agonist and NG108-15 cells potentiated the decrease and the rate of decrease of opioid binding, compared with agonist alone. Scatchard analysis of [^{3}H][D-Ala^2,D-Leu^5]enkephalin (DADLE) binding revealed that NG108-15 cells incubated for 3 hr with 1 nM DADLE and 30 nM PMA displayed a >50% reduction in the number of [^{3}H]DADLE binding sites with no affinity change at the remaining sites, compared with cells treated with DADLE alone. The antagonist naloxone blocked both DADLE-induced and PMA-enhanced DADLE-induced down-regulation. The agonists morphine and cyclazocine, which alone were unable to induce δ receptor down-regulation, did so in the presence of PMA. The PKC inhibitor staurosporine and down-regulation of PKC by chronic PMA treatment blocked PMA potentiation of DADLE-induced down-regulation, but not "normal" DADLE-induced down-regulation. The enhancement of down-regulation by PMA was unaffected by either metabolic inhibitors or incubations at 20°, conditions that blocked down-regulation by DADLE alone. NG108-15 cells incubated with [^{3}H]DADLE and PMA retained more [^{3}H]DADLE than cells incubated with [^{3}H]DADLE alone, suggesting that PMA enhanced receptor internalization instead of merely inhibiting membrane binding. The diacylglycerol 1-oleoyl-2-acetyl-glycerol and bradykinin substituted for PMA but not carbachol, indicating that PKC activated physiologically may play a role in δ receptor down-regulation.

The mouse neuroblastoma × rat glioma hybrid cell line NG108-15 has been used as a model tissue culture system to study the δ-opioid receptor and its biochemical and physiological function at the cellular level. Opioid agonists, acting through the δ receptor in this cell line, have been shown to inhibit AC activity (1, 2). This inhibition of AC activity is transduced from the δ receptor to AC by a PTX-sensitive G_i (3). Upon chronic opioid agonist occupation of the δ receptor and concurrent inhibition of AC activity, several cellular adaptations occur. The first adaptation to occur is a decreasing effectiveness, over time, of an opioid agonist to inhibit AC activity (2). This desensitization, and the degree of desensitization produced, depends on the efficacy of the opioid agonist in inhibiting AC activity (4). After desensitization, opioid receptor down-regulation may occur in NG108-15 cells (5). Down-regulation involves a loss of opioid binding sites on the cell surface, which is brought about by the internalization and subsequent degradation of δ receptors (6). The time courses of desensitization and down-regulation are different, and they appear to be separate processes (4). In addition, not all opioid agonists that cause desensitization cause receptor down-regulation; only those opioids with high intrinsic activity cause receptor down-regulation (4).

Investigations of opioid receptor down-regulation in NG108-15 cells have been unable to elucidate the mechanism by which it occurs. The inhibition of AC activity has been investigated for its possible role in down-regulation. Down-regulation of receptors that stimulate AC activity, such as the β-adrenergic receptor, may be decreased by genetically altered receptor-G protein interaction (7). To determine whether a receptor-G_i interaction existed for down-regulation of receptors that inhibit AC activity, PTX was used to inactivate G_i by ADP-ribosyla-

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ABBREVIATIONS: AC, adenylyl cyclase; PTX, pertussis toxin; PKC, protein kinase C; PI, phosphatidylinositol; PMA, 4β-phorbol 12β-myristate 13α-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; OAG, 1-oleoyl-2-acetylglycerol; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DAMGO, [D-Ala^2,(Me)Phe^4,Gly(ol)^5]enkephalin; Me_2SO, dimethylsulfoxide; G protein, guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide-binding protein.

tion of its α_1 subunit (8). This inactivation results in a functional uncoupling of G_i-linked receptors from G_i. Opioid receptors in PTX-treated NG108–15 cells, or membranes prepared from them, can no longer inhibit AC activity in the presence of agonists (3). However, PTX-treated NG108–15 cells display no differences in either the rate or magnitude of opioid agonist-induced down-regulation, compared with untreated cells (9). This finding has been extended to the muscarinic and α_2-adrenergic receptors in NG108–15 cells (10). Both of these G_i-linked receptors can be desensitized to AC and down-regulated in response to chronic agonist exposure, both can be uncoupled from AC inhibition by PTX, and both show no changes in the amount of down-regulation they undergo after PTX treatment. Clearly, either the inactivated substrates of PTX (G_i and several other related G proteins) can somehow mediate opioid receptor down-regulation, while their effect on AC is blocked, or down-regulation of opioid and other G_i-linked receptors proceeds by another mechanism.

PKC is a Ca^{2+}/phospholipid-dependent enzyme activated *in vivo* by receptors linked to PI turnover (11). Activated PKC undergoes intracellular recompartmentalization whereby it translocates from the cytoplasm to the plasma membrane of the cell (12). At this point, PKC can act on membrane receptors, or other membrane components, to influence receptor-mediated events such as ion fluxes, receptor-second messenger coupling, and down-regulation (13–15). Phorbol esters, such as PMA, are the most potent known activators of PKC. The effects of phorbol esters on a variety of cell membrane receptor functions have been examined and correlated with their ability to stimulate PKC translocation and activity. PMA can inhibit the responses of PI-coupled receptor systems that activate PKC physiologically, thus producing negative feedback inhibition (16). However, it is not necessary for a receptor to be coupled to PI turnover for it to be modulated by PKC activity. The β-adrenergic and the nicotinic cholinergic receptor are not linked to PI turnover, but both of these receptors display increased phosphorylation of their subunits in response to PMA activation of PKC (17, 18). Given the broad range of effects of PMA-activated PKC on receptors and receptor-modulated events and the lack of effect of PTX on opioid receptor down-regulation, an investigation was initiated to determine whether PKC activation could modulate δ-opioid receptor down-regulation in NG108–15 cells.

Materials and Methods

Cell culture. NG108–15 cells were cultured at 37° in a humidified 5% CO_2/95% air atmosphere in Ex-cell 300 medium containing 5% fetal bovine serum, 1 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine. Cells were grown to confluence in either 100-mm × 20-mm Petri dishes or 75-cm^2 flasks. They were collected by mechanical agitation, followed by centrifugation at 200 × *g* for 10 min at 4° and resuspension in fresh medium. Aliquots of the cell suspension were added to 75-cm^2 flasks containing 50 ml of the growth medium, and the cells were cultured overnight. Cell viability was routinely monitored by trypan blue dye exclusion using a hemacytometer and was consistently determined to be >90%. Experiments were performed directly in the tissue culture flask after the overnight adaptation period, with reagents being added directly to the medium in the flask. By allowing the cells to attach and proliferate in the flask overnight, more consistent results were obtained between and within experiments.

In experiments examining the effects of different temperatures on down-regulation, incubation temperatures were maintained by either a water bath or Dewar's flask. After the cells were resuspended in medium, 1-ml aliquots of the cell suspension were added to 40-ml centrifuge tubes containing 4 ml of Krebs-Ringer-HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, and 10 mM HEPES, at pH 7.4) and the appropriate experimental reagents. Subsequent steps in the preparation of membranes from cells used in temperature experiments were the same as those in other experiments.

Dinitrophenol and sodium azide were added 30 min before other reagents to maximize their inhibition of down-regulation (19). PMA, staurosporine, and OAG were added from stock solutions in 100% Me_2SO and, therefore, an equivalent volume of Me_2SO was always used in the control (<1% of the final incubation volume). Unless otherwise noted, all other reagents were added from concentrated solutions using either medium or water, except dinitrophenol, which was dissolved in 0.05 N NaOH; an equivalent amount of NaOH was added to the control.

Radioligand binding assays in membrane homogenate. After a 37° incubation period with the appropriate experimental reagents, cells were centrifuged at 200 × *g* for 10 min at 4°, the medium was aspirated, 5 ml of ice-cold 50 mM Tris·HCl, pH 7.5, were added to the cells, which were disrupted by a Brinkmann homogenizer (setting 4, 10 sec), and the volume was brought to 10 ml with buffer. The homogenate was centrifuged at 39,600 × *g* for 20 min and resuspended in 10 ml of 50 mM Tris·HCl, pH 7.5, and the washing step was repeated. The final membrane homogenate was resuspended in 3 ml of 50 mM Tris·HCl, pH 7.5. Protein concentration was determined by the method of Bradford (20), with bovine serum albumin as the standard. Radioligand binding assays were initiated by addition of 450 μl of membrane homogenate (100–200 μg of protein) to each binding assay tube containing radioligand at 22°, in a final volume of 1 ml of 50 mM Tris·HCl, pH 7.5. Nonspecific binding was determined in the presence of 10 μM naloxone. Incubations were terminated after 60 min by filtration over Schleicher & Schuell no. 32 glass fiber filters, using a Brandel 48-well cell harvester. The filters were immediately rinsed three times with 4 ml of ice-cold 50 mM Tris·HCl, pH 7.5, and were quantitated by scintillation counting in 5 ml of Ecoscint A.

Data on δ-opioid receptor down-regulation, induced by nanomolar concentrations of opioids, were expressed as percentage of control binding, which was binding to membranes from NG108–15 cells incubated for 60 min with the opioid. Artifacts from incomplete removal of the opioid under investigation during membrane preparation were, therefore, avoided. No down-regulation was reported in NG108–15 cells treated with 10 nM etorphine for < 3 hr (5), so minimal, if any, down-regulation was thought to occur in the control cells incubated with agonist for 60 min. However, membranes from cells treated for 60 min with agonist showed small reductions in binding, compared with untreated cells.

[^{3}H]DADLE retention assays with intact cells. NG108–15 cells were harvested, and aliquots of the cell suspension were added to six-well tissue culture plates, with each well containing 5 ml of medium. Cells were allowed to attach and proliferate at 37° overnight. The following day, the medium was gently aspirated from the wells, and a final volume of 1 ml of fresh medium was added. In experiments using chloroquine, the chloroquine was added to each well 30 min before the addition of other reagents to maximize its potentiation of [^{3}H]DADLE retention (6). Nonspecific retention of [^{3}H]DADLE was determined in the presence of 10 μM naloxone. After incubation at 37° for 15–180 min, the medium was rapidly aspirated, and the cells received two 1-ml washes with ice-cold medium. The cells were then solubilized in 1 ml of 1 N NaOH, which was added to scintillation vials. The wells were then rinsed with 1 ml of 1 N HCl, and the rinse was added along with the previous 1-ml aliquot to the scintillation vials. Aquasol-2 scintillation fluid (15 ml) was added to each scintillation vial, and the radioactivity was determined by scintillation counting.

Materials. [^{3}H]DADLE (39 Ci/mmol) and [^{3}H]diprenorphine (48

Ci/mmol) were purchased from Amersham. Ex-cell 300 medium was purchased from JR Scientific. Fetal bovine serum was purchased from Hyclone Laboratories. Penicillin and streptomycin were purchased from GIBCO Laboratories. Unlabeled DADLE, human β-endorphin-1-31, and bradykinin were purchased from Bachem, Inc. OAG was purchased from Avanti Polar Lipids, Inc. Staurosporine was purchased from Kamiya Biomedical Co. Naloxone was obtained from Endo Labs. Morphine sulfate was purchased from Mallinckrodt Chemical Co. Etorphine and cyclazocine were obtained from the National Institute on Drug Abuse. Dinitrophenol and Me_2SO were purchased from Fisher Scientific Co. Ecoscint A was purchased from National Diagnostics. Aquasol-2 was purchased from New England Nuclear. All other reagents were purchased from Sigma Chemical Co. The NG108-15 cell hybrid was a gift from Dr. W. Klee (National Institute of Mental Health).

Data analysis. Linear regression, curve-fitting, and statistical analysis were performed by the data analysis software package RS/1 (BBN Software Products, Corp.) on a DEC PDP11/73. Analysis of variance and Dunnett's or Newman-Keuls multiple-range tests were performed where appropriate to determine statistically significant differences.

Results

PMA increased agonist-induced down-regulation of δ-opioid receptors in NG108-15 cells. PMA reduced opioid binding to NG108-15 membranes from cells incubated with PMA, but only when the cells were also incubated with an opioid agonist. As shown in Fig. 1, incubation of NG108-15 cells with 0.01-1 μM PMA for 2 hr had no effect on specific [^{3}H]DADLE binding to membranes prepared from the treated cells, compared with Me_2SO vehicle-treated controls. Even when the cells were simultaneously exposed to the Ca^{2+} ionophore A23187 together with PMA, no effect was observed (data not shown). Therefore, the lack of response of the NG108-15 cells to PMA treatment was not due to an inadequate intracellular Ca^{2+} concentration. In sharp contrast, when 10 nM etorphine was included along with varying concentrations of PMA for 2 hr, a subsequent decrease in [^{3}H]DADLE binding was detected in membranes, compared with membranes from cells incubated with the Me_2SO vehicle and etorphine alone (Fig. 1). The decrease in binding was dependent on the concentration of PMA, with 3 nM PMA producing the smallest significant binding inhibition of 20 ± 3% ($p < 0.05$) and a maximal inhibition of 36 ± 4% ($p < 0.01$) being reached at 30 nM PMA. PMA concentrations greater than 30 nM did not further reduce [^{3}H]DADLE binding. When 4α-phorbol, which cannot activate PKC, was used with etorphine in place of PMA, no decrease in opioid binding was observed (data not shown).

A Scatchard plot comparing [^{3}H]DADLE binding to membranes from NG108-15 cells treated with 1 nM DADLE and from cells treated with 1 nM DADLE and 30 nM PMA is shown in Fig. 2. In both cases, specific binding was to a single class of sites, as was also observed with [^{3}H]DADLE binding to membranes from untreated NG108-15 cells, which had an average K_d value of 0.48 ± 0.04 nM and a B_{max} value of 620 ± 40 fmol/mg of protein. In the DADLE-only-treated cells, the average K_d and B_{max} values were 0.73 ± 0.06 nM and 600 ± 71 fmol bound/mg of protein, respectively. In the DADLE- plus PMA-treated cells, the corresponding values were 0.96 ± 0.11 nM and 280 ± 58 fmol bound/mg of protein. Under these experimental conditions, DADLE- plus PMA-treated cells displayed a 53 ± 5% reduction in the number of [^{3}H]DADLE binding sites ($p < 0.05$) with no significant change in affinity at the remaining sites, compared with the DADLE-only-treated cells. Similar

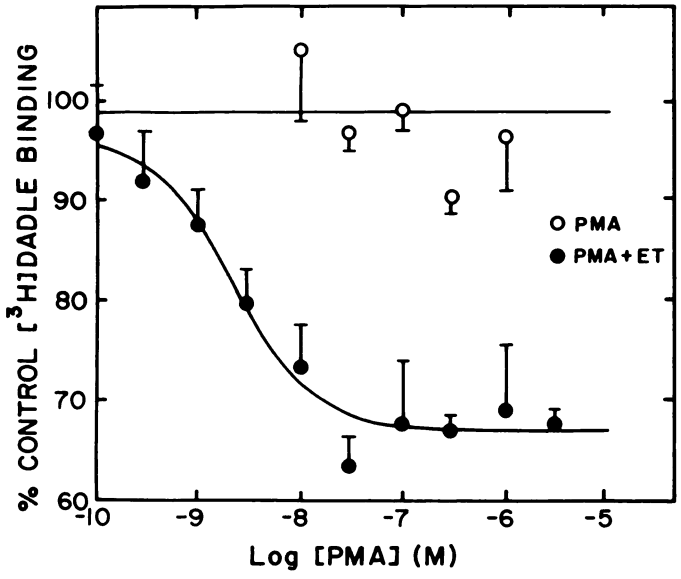

Fig. 1. Effect of either PMA or PMA and etorphine on specific [^{3}H]DADLE binding to membranes. NG108-15 cells were cultured at 37° with increasing concentrations of either PMA (O) or PMA and 10 nM etorphine (*ET*) (●). Cells were harvested at the end of the 2-hr treatment, and 1 nM [^{3}H]DADLE binding to membranes was determined as described in Materials and Methods. Control binding was regarded as specific [^{3}H]DADLE binding to membranes from cells treated for 2 hr with either Me_2SO vehicle (800 ± 170 cpm) or Me_2SO vehicle and 10 nM etorphine (1020 ± 300 cpm) for the PMA or PMA and etorphine conditions, respectively. *Points* represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

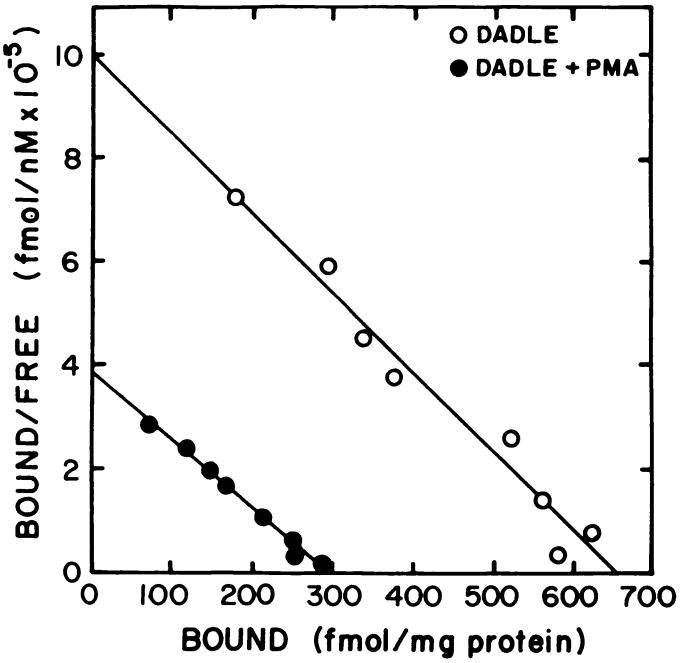

Fig. 2. Scatchard analysis of [^{3}H]DADLE binding to membranes from NG108-15 cells incubated with either DADLE or DADLE and PMA. NG108-15 cells were incubated for 3 hr with either 1 nM DADLE (O) or 1 nM DADLE and 30 nM PMA (●). Cells were harvested at the end of the 3-hr treatment, and [^{3}H]DADLE binding to membranes was determined using final [^{3}H]DADLE concentrations ranging from 0.25 nM to 16 nM, as described in Materials and Methods. A representative experiment that was replicated three times is shown.

results were obtained with both alkaloids, such as etorphine, and opioid peptides, such as DADLE. The affinity and efficacy of the opioid for the δ receptor were the determinants of the opioid concentration needed to observe PMA-enhanced down-regulation.

The time course for the decrease in opioid binding induced by 30 nM PMA and/or 1 nM etorphine was similar regardless of whether a radiolabeled opioid agonist or antagonist was used to measure binding sites. As shown in Fig. 3, [^{3}H]DADLE binding was significantly reduced in etorphine- plus PMA-treated cells, compared with etorphine-treated cells, at all times points ($p < 0.01$) except 24 and 48 hr. This is in sharp contrast to binding in the etorphine-only group, which was not significantly different from control binding until 6 hr after the addition of etorphine ($p < 0.01$). Similarly, when [^{3}H]diprenorphine binding was used to examine binding sites, as shown in Fig. 4, almost the same results were obtained, demonstrating that PMA-enhanced down-regulation was observed with both [^{3}H] DADLE, an agonist that measures high affinity opioid binding sites, and an antagonist, which recognizes both high and low affinity binding sites. Similar to agonist binding, antagonist binding was significantly reduced in the etorphine- plus PMA-treated cells, compared with the etorphine-treated cells, by 2 hr ($p < 0.01$) but not at 24 and 48 hr, and the etorphine-treated cells were not significantly different from control cells until 24 hr after the addition of etorphine ($p < 0.05$). Interestingly, both [^{3}H]DADLE and [^{3}H]diprenorphine binding did not show any changes induced by PMA alone, except for an increase in binding at 48 hr ($p < 0.05$), which was not due to differences in protein concentration (data not shown). This finding again emphasizes that enhanced opioid binding reductions were a function of PMA and agonist working together and not PMA acting on its own. The increase in binding at 48 hr may result from some slow receptor up-regulation process initiated by activated PKC, or it may result from a lack of PKC activity induced by chronic PMA exposure. After long term exposure to PMA, the PKC of many different cell types undergoes proteolytic degradation, thereby down-regulating PKC (21–24).

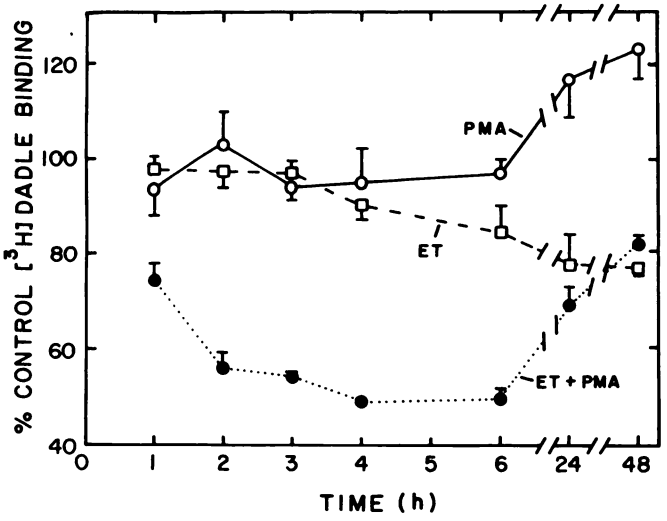

Fig. 3. Time course for the changes in [^{3}H]DADLE binding to membranes from NG108–15 cells induced by PMA, etorphine, or PMA and etorphine. NG108–15 cells were incubated with 30 nM PMA (○), 1 nM etorphine (*ET*) (□), or 30 nM PMA and 1 nM etorphine (●) for the times indicated. Cells were harvested at the designated times and 1 nM [^{3}H]DADLE binding to membranes was determined as described in Materials and Methods. Control binding was to membranes from cells treated for 5 min with PMA (2510 ± 280 cpm) for the PMA-only group or for 5 min with etorphine, either with or without Me_2SO vehicle (2440 ± 1040 cpm), for the etorphine plus PMA and etorphine-only groups, respectively. *Points* represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

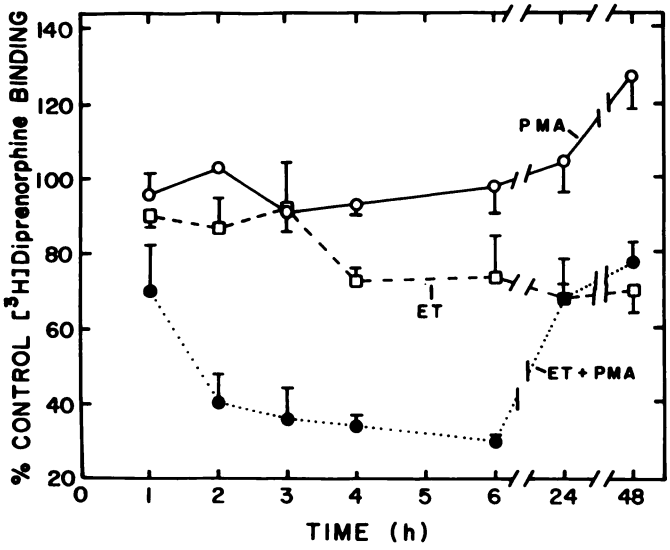

Fig. 4. Time course for the changes in [^{3}H]diprenorphine binding to membranes from NG108–15 cells induced by PMA, etorphine, or PMA and etorphine. NG108–15 cells were cultured at 37° with 30 nM PMA (○), 1 nM etorphine (*ET*) (□), or 30 nM PMA and 1 nM etorphine (●) for the times indicated. Cells were harvested at the designated times and 1 nM [^{3}H]diprenorphine binding to membranes was determined as described in Materials and Methods. Control binding was to membranes from cells treated for 5 min with PMA (2640 ± 560 cpm) for the PMA-only group or for 5 min with etorphine, either with or without Me_2SO vehicle (1960 ± 630 cpm), for the etorphine plus PMA and etorphine groups, respectively. *Points* represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

This distinction of PMA and agonists working together was examined further, as shown in Table 1. Like the alkaloid etorphine, the peptide agonists DADLE and human β-endorphin-1–31, which induced down-regulation of δ receptors in NG108–15 cells (4), also had this effect amplified by PMA. In contrast, the antagonist naloxone did not induce down-regulation by itself or with PMA, and naloxone blocked the effects of DADLE alone and PMA-enhanced down-regulation. A concentration of 10 nM β-endorphin-1–31 was required to observe a significant decrease in binding. This β-endorphin concentration is relatively high, considering its affinity and efficacy at δ-opioid receptors (25). Proteolytic degradation of the peptide during the 3-hr incubation, as noted in another investigation (4), probably accounts for the need to use a higher concentration of β-endorphin than would be needed with a proteolysis-resistant peptide. Intriguingly, morphine, a μ-selective agonist, and cyclazocine, a nonselective benzomorphan partial agonist, did not induce down-regulation of opioid binding sites even after 24 hr but did after 3 hr if incubated with PMA. High concentrations of morphine and cyclazocine were chosen to demonstrate that, in the absence of PMA, these two agonists absolutely could not induce down-regulation, confirming another investigation (4). The effect of PMA on down-regulation suggested that δ receptor occupation by an opioid agonist, and not necessarily an agonist that can stimulate down-regulation by itself, was a requirement.

TABLE 1

Effect of PMA on [^{3}H]diprenorphine binding to membranes from NG108-15 cells treated with opioid agonists and the antagonist naloxone

NG108-15 cells were cultured for 3 or 24 hr with the ligands shown, with or without 30 nM PMA. Specific 1 nM [^{3}H]diprenorphine binding to membranes prepared from treated cells was measured as described in Materials and Methods. Control binding was to membranes from cells treated for 60 min with each compound, except for the 3-hr morphine condition, which used a 5-min morphine treatment control. Average specific control [^{3}H]diprenorphine binding was 3300 ± 1150 cpm. Data represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

Culture treatment	[^{3}H]Diprenorphine binding	
	−PMA	+PMA
	% of control	
3-hr DADLE, 10^{-9} M	83 ± 3[a]	38 ± 3[b,c]
3-hr β-endorphin, 10^{-8} M	88 ± 6	71 ± 6[a]
3-hr morphine, 10^{-5} M	98 ± 3	65 ± 8[b,d]
3-hr cyclazocine, 10^{-5} M	104 ± 8	73 ± 3[a,d]
3-hr naloxone, 10^{-6} M	98 ± 2	100 ± 8
3-hr DADLE, 10^{-9} M, + naloxone, 10^{-6} M	95 ± 3	90 ± 3
24-hr DADLE, 10^{-9} M	43 ± 8[a]	
24-hr morphine, 10^{-5} M	86 ± 5	
24-hr cyclazocine, 10^{-5} M	109 ± 12	

[a] Value significantly lower than control, $p < 0.05$.
[b] Same as *a*, $p < 0.01$.
[c] Value significantly lower than corresponding condition without PMA, $p < 0.01$.
[d] Same as *c*, $p < 0.05$.

TABLE 2

Effect of staurosporine on the loss of [^{3}H]diprenorphine binding to membranes from cells treated with either DADLE or DADLE and PMA

NG108-15 cells were incubated for 3 hr with 1 nM DADLE with or without 30 nM PMA, in the absence and presence of 1 μM staurosporine. Specific 1 nM [^{3}H] diprenorphine binding to membranes prepared from the treated cells was measured as described in Materials and Methods. [^{3}H]Diprenorphine binding to membranes from cells treated for 60 min with 1 nM DADLE was regarded as control binding (4280 ± 480 cpm), except for the no-DADLE or -PMA condition, which used a 3-hr Me_2SO vehicle control (6260 ± 890 cpm). Incubating cells for 60 min with 1 nM DADLE and 1 μM staurosporine did not result in [^{3}H]diprenorphine binding significantly different from the 60-min DADLE control (data not shown). Data represent the mean ± standard error of percentage of control binding from three experiments, performed in triplicate.

Culture treatment	[^{3}H]Diprenorphine binding	
	−Staurosporine	+Staurosporine
	% of control	
DADLE	80 ± 3[a]	76 ± 2[a]
DADLE + PMA	42 ± 2[b]	77 ± 4[a]
Control (no DADLE or PMA)		87 ± 3[c]

[a] Value significantly lower than control, $p < 0.01$.
[b] Value significantly lower than corresponding condition without PMA, $p < 0.01$.
[c] Same as *a*, $p < 0.05$.

PMA-augmented down-regulation by PKC activation was separable from "normal" agonist-induced down-regulation. To determine whether PMA was acting through PKC to produce its effect, the PKC inhibitor staurosporine was used to block PKC activity. Additionally, the effect of staurosporine on "normal" (without PMA) DADLE-induced down-regulation was also investigated. Staurosporine had no effect in blocking down-regulation induced by DADLE alone but was effective in blocking the augmentation induced by PMA, as shown in Table 2. Membranes from cells treated with staurosporine in the absence of PMA and DADLE showed a 13% inhibition of [^{3}H]diprenorphine binding, in comparison with untreated cells. The [^{3}H]diprenorphine binding to membranes from staurosporine-treated cells did not differ significantly from that to membranes from cells treated with DADLE, or DADLE plus PMA, and staurosporine. Although the slight inhibition of [^{3}H]diprenorphine binding by staurosporine complicates the interpretation of these results, staurosporine blocked PMA-augmented DADLE-induced down-regulation, suggesting a direct PKC effect. Staurosporine may also reduce [^{3}H]diprenorphine binding by another mechanism.

As indicated above, chronic activation of PKC by PMA down-regulates PKC activity by proteolysis of the PKC itself. NG108–15 cells pretreated for 24 hr with PMA were subsequently examined for their ability to undergo normal and PMA-enhanced down-regulation, as depicted in Table 3. Chronic pretreatment of the cells with PMA completely abolished the ability of PMA to potentiate down-regulation. Furthermore, DADLE-induced down-regulation was the same between cells chronically treated with PMA and those treated with Me_2SO vehicle. PMA alone again produced a significant increase in opioid binding after a 48-hr incubation.

Metabolic inhibitors, such as dinitrophenol and sodium azide, block δ receptor down-regulation in NG108–15 cells (6). Down-regulation is also blocked in these cells by sufficiently low temperatures (4). As shown in Table 4, dinitrophenol and sodium azide completely blocked the down-regulation induced by DADLE alone but were ineffective in reducing the down-regulation induced by DADLE and PMA. In addition, performing the incubation with DADLE, with or without PMA, at 30° did not prevent down-regulation in either case, but at 20° DADLE with PMA was still capable of inducing down-regulation, whereas DADLE alone was ineffective. These findings suggest that DADLE- plus PMA-induced down-regulation may be produced by a different mechanism than down-regulation produced by DADLE alone.

PMA-augmented down-regulation involves increased receptor internalization. One of the key features of δ-opioid receptor down-regulation in NG108–15 cells is that the δ receptor internalizes upon agonist exposure and subsequently is delivered to lysosomes for degradation (6). Chloroquine, a lysosomotropic agent, can be used to prevent internal digestion of the ligand-receptor complex and thus promote accumulation of a radiolabeled agonist that would otherwise be quickly released back into the extracellular compartment (26). To determine whether the loss of opioid binding potentiated by PMA

TABLE 3

Effect of long term PMA treatment on the loss of [^{3}H]diprenorphine binding to membranes from cells incubated with DADLE

NG108-15 cells were incubated for 48 hr with 30 nM PMA, with the last 3 or 24 hr of PMA treatment concurrent with 1 nM DADLE treatment. Specific 1 nM [^{3}H] diprenorphine binding to membranes was determined at the end of the 48-hr treatment, as described in Materials and Methods. [^{3}H]Diprenorphine binding to membranes from cells treated for 48 hr with 30 nM PMA (or Me_2SO vehicle), with the last 60 min of treatment concurrent with 1 nM DADLE treatment, was regarded as control binding, except for the no-DADLE condition, which used a 48-hr Me_2SO vehicle treatment control. The average control [^{3}H]diprenorphine binding for all conditions was 3500 ± 580 cpm. Data represent the mean ± standard error of percentage of control binding from three experiments, performed in triplicate.

Culture treatment	[^{3}H]Diprenorphine binding	
	−PMA	+PMA
	% of control	
3-hr DADLE	88 ± 7	85 ± 2
24-hr DADLE	59 ± 1[a]	51 ± 4[b]
Control (no DADLE)		126 ± 5[c]

[a] Value significantly lower than control, $p < 0.01$.
[b] Same as *a*, $p < 0.05$.
[c] Value significantly higher than control, $p < 0.05$.

TABLE 4

Effect of metabolic inhibitors and lowered temperature on loss of [^{3}H]diprenorphine binding to membranes from cells treated with either DADLE or DADLE and PMA

NG108-15 cells were incubated at 37° for 3 hr with the agents shown and with either DADLE or DADLE and 30 nM PMA. DADLE at a final concentration of 1 nM was used with the metabolic inhibitors, and 10 nM DADLE was used in the temperature experiments, performed at the stated temperatures for the 3-hr incubation. Specific 1 nM [^{3}H]diprenorphine binding to membranes was determined at the end of the 3-hr treatment, as described in Materials and Methods. [^{3}H] Diprenorphine binding to membranes from cells treated for 60 min with 1 nM or 10 nM DADLE at the appropriate temperature was regarded as control binding. The average control [^{3}H]diprenorphine binding was 2810 ± 560 cpm. Data represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

Culture treatment	[^{3}H]Diprenorphine binding	
	DADLE	DADLE + PMA
	% of control	
No inhibitors	78 ± 3[a]	37 ± 5[b,c]
1 mM Dinitrophenol	103 ± 6	43 ± 4[b,c]
10 mM Sodium azide	95 ± 3	36 ± 5[b,c]
30° Incubation	69 ± 5[a]	56 ± 5[b,c]
20° Incubation	110 ± 8	72 ± 6[a,c]

[a] Value significantly lower than control, $p < 0.05$.
[b] Same as *a*, $p < 0.01$.
[c] Value significantly lower than corresponding condition without PMA, $p < 0.01$.

was due to receptor internalization and not receptor inactivation, the specific retention of [^{3}H]DADLE by NG108–15 cells was examined in the absence and presence of chloroquine and PMA. The specific retention of [^{3}H]DADLE is the sum of [^{3}H] DADLE specifically bound to the cell surface and [^{3}H]DADLE specifically internalized. The specific retention of [^{3}H]DADLE is differentiated from the nonspecific retention by the inclusion of 10 μM naloxone. Two concentrations of [^{3}H]DADLE were used in the retention experiments, because it has been shown that the rate of internalization of [^{3}H]DADLE in NG108–15 cells is concentration dependent (4).

When 10 nM [^{3}H]DADLE was incubated with NG108–15 cells for 60 and 180 min, 100 μM chloroquine produced a significant increase in specific [^{3}H]DADLE retained at both time points ($p < 0.01$), compared with time-matched cells without chloroquine, as shown in Fig. 5A. Likewise, the combination of 100 μM chloroquine and 30 nM PMA produced significantly greater [^{3}H]DADLE retention at 60 and 180 min ($p < 0.01$), compared with time-matched PMA-containing cells without chloroquine. Thus, chloroquine increased the [^{3}H]DADLE retention of the cells undergoing down-regulation by agonists alone and agonist with PMA, consistent with the hypothesis that both groups of cells were internalizing δ receptors. PMA alone produced a small, but significant, increased retention of [^{3}H]DADLE at 15 ($p < 0.05$) and 60 min ($p < 0.01$), compared with untreated cells, but this increase disappeared by 180 min. Again, this result suggests that PMA in concert with an agonist was increasing δ receptor internalization, but in the absence of chloroquine [^{3}H]DADLE was probably being released by exocytosis by 180 min. For cells incubated with chloroquine, the [^{3}H]DADLE retention between 60 and 180 min was not significantly different, indicating that chloroquine was probably preventing a loss of internalized [^{3}H] DADLE through disruption of lysosomal function. However, incubation of cells with 10 nM [^{3}H]DADLE, PMA, and chloroquine did not produce significantly greater retention, compared with the chloroquine without PMA group, at any time point. A possible reason for this finding would be that 10 nM

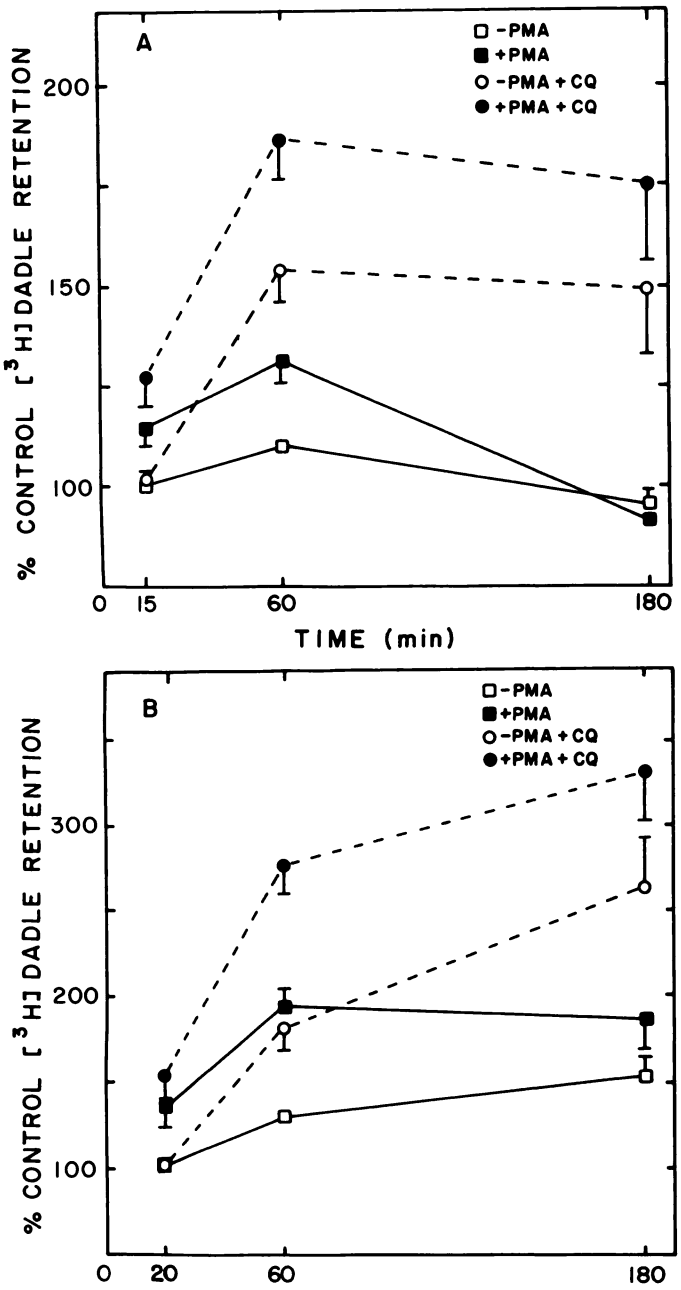

Fig. 5. Retention of [^{3}H]DADLE in NG108–15 cells as modulated by PMA, chloroquine, or PMA and chloroquine. NG108–15 cells were pretreated for 30 min with 100 μM chloroquine (*CQ*) (○, ●) or vehicle (□, ■). Cells were then exposed to 10 nM (A) or 3 nM (B) [^{3}H]DADLE, with (●, ■) or without (○, □) 30 nM PMA, for the times indicated. The culture medium was aspirated at the designated times, and the intact cells were rinsed with medium and solubilized with 1 N NaOH as described in Materials and Methods. Control retention was defined as the amount of [^{3}H]DADLE retained at the shortest time point without PMA and chloroquine for the without-PMA and with-PMA groups or without PMA but with chloroquine for the groups containing chloroquine. Control retention in the presence of 10 nM [^{3}H]DADLE (A) without PMA or chloroquine was 4800 ± 1020 cpm, whereas in the absence of PMA but with chloroquine control retention was 3450 ± 390 cpm. Control retention in the presence of 3 nM [^{3}H]DADLE (B) without PMA or chloroquine was 2020 ± 260 cpm, whereas in the presence of chloroquine control retention was 1710 ± 100 cpm. *Points* represent the mean ± standard error of percentage of control [^{3}H]DADLE retention from at least three experiments, performed in triplicate.

[^{3}H]DADLE with chloroquine was producing too large an amount of [^{3}H]DADLE retention by itself (by a high rate of receptor internalization) and PMA was unable to augment this receptor internalization further. If this were true, a lower concentration of [^{3}H]DADLE would be expected to produce a lower rate of internalization that could be significantly augmented by PMA, thus avoiding a "ceiling" effect.

When 3 nM [^{3}H]DADLE was used instead of 10 nM, 100 μM chloroquine alone did not produce a significant increase in [^{3}H]DADLE retained at 60 min but did at 180 min ($p < 0.01$), compared with time-matched cells without chloroquine (Fig. 5B). This is consistent with a lower receptor internalization rate than was produced by 10 nM [^{3}H]DADLE, where significant increases were obtained at both time points. In contrast, the combination of chloroquine and PMA produced significantly greater [^{3}H]DADLE retention at 60 and 180 min ($p < 0.01$), compared with the time-matched PMA-containing cells without chloroquine. This result would be expected if PMA was increasing the rate of receptor internalization, producing significant increases in [^{3}H]DADLE retention earlier, and the effect of chloroquine was limited to maintaining internalized ligand. PMA alone produced a significant increase in [^{3}H]DADLE retention at 60 min ($p < 0.01$), compared with controls, but like the 10 nM [^{3}H]DADLE retention experiments this increase was not significantly different by 180 min. For cells incubated with chloroquine, [^{3}H]DADLE retention between 60 and 180 min was not significantly different for the PMA group or significantly increased ($p < 0.05$) for the non-PMA group. However, unlike the 10 nM [^{3}H]DADLE retention experiments, PMA with chloroquine did produce a significantly greater retention, compared with the chloroquine without PMA group, at 60 min when 3 nM [^{3}H]DADLE was used ($p < 0.05$).

Other activators of PKC mimicked the effect of PMA on δ receptor down-regulation. To determine whether agents other than a potent phorbol ester, such as PMA, could enhance agonist-induced δ receptor down-regulation, OAG, bradykinin, and carbachol were tested. OAG is a synthetic diacylglycerol, which can activate PKC similarly to endogenous diacylglycerol produced by PI turnover (27). NG108–15 cells contain bradykinin receptors that use PI turnover as a second messenger system, activating PKC by increasing diacylglycerol production and increasing the internal Ca^{2+} concentration (28, 29). Muscarinic cholinergic receptors on NG108–15 cells are coupled to inhibition of AC activity, similar to δ-opioid receptors. Like δ receptors, these muscarinic receptors desensitize and down-regulate in response to chronic agonist exposure (30, 31). If a common biochemical pathway exists for the down-regulation of G_i-coupled receptors, then activation of muscarinic receptors with the muscarinic agonist carbachol might be expected to modulate the down-regulation of δ-opioid receptors induced by DADLE. As shown in Fig. 6, 100 μM OAG and 10 μM bradykinin did not induce δ receptor down-regulation in the absence of DADLE. In the presence of 1 nM DADLE, OAG ($p < 0.05$) and bradykinin ($p < 0.01$) did induce a significant decrease in [^{3}H]diprenorphine binding, compared with cells treated with DADLE alone. However, carbachol had no effect on δ receptor down-regulation induced by 1 nM DADLE over a 6-hr incubation period.

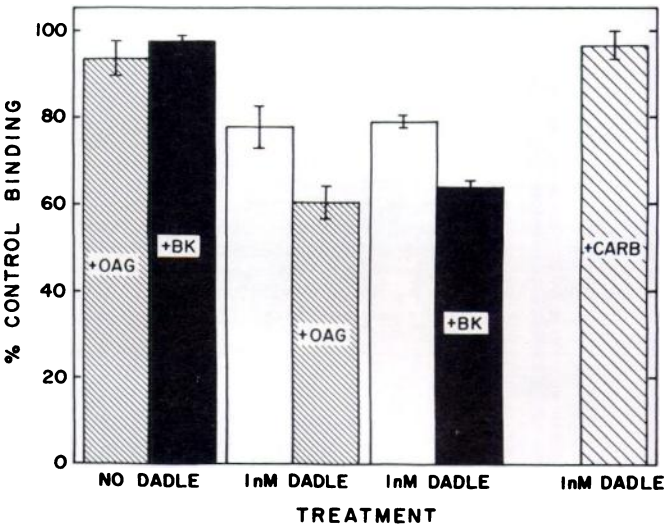

Fig. 6. OAG and bradykinin, but not carbachol, mimicked the effect of PMA in augmenting opioid agonist-induced down-regulation. NG108–15 cells were treated either with 100 μM OAG, 10 μM bradykinin (*BK*), 1 nM DADLE, or 1 nM DADLE with either 100 μM OAG or 10 μM bradykinin for 3 hr or with 1 nM DADLE with 1 μM carbachol (*CARB*) for 6 hr. Cells were harvested and 1 nM [^{3}H]diprenorphine binding to membranes was determined, except for the DADLE with carbachol group, which used 1 nM [^{3}H]DADLE binding, as described in Materials and Methods. Control binding was to membranes from cells treated with Me_2SO or medium vehicle for 3 hr (2680 ± 670 cpm) for OAG and bradykinin-only groups or 1 nM DADLE with Me_2SO or medium vehicle for 60 min (3240 ± 640 cpm) for 1 nM DADLE groups, except for the DADLE with carbachol group, which used 1 nM DADLE for 6 hr as control binding (1010 ± 240 cpm). *Points* represent the mean ± standard error of percentage of control binding from at least three experiments, performed in triplicate.

Discussion

The results presented here provide evidence that PMA can increase opioid agonist-induced down-regulation of δ-opioid receptors in NG108–15 cells. The evidence that PMA activates PKC to produce this effect is as follows: 1) the effect of PMA was blocked by staurosporine, an inhibitor of PKC; 2) the effect of PMA was lost upon chronic treatment with PMA, which down-regulates PKC; and 3) OAG and bradykinin, which activate PKC in NG108–15 cells, substituted for PMA to produce similar effects, whereas carbachol, which presumably does not activate PKC in NG108–15 cells, did not substitute for PMA. Additionally, 4α-phorbol, which cannot activate PKC, did not increase δ receptor down-regulation when added with etorphine, and PMA added with DADLE produced no δ receptor down-regulation when incubated with NG108–15 cells at 5° (data not shown). Therefore, PMA is probably increasing down-regulation not through nonspecific chemical action but by the specific activation of PKC at temperatures consistent with enzymatic activation.

A key feature of δ-opioid receptor down-regulation induced by PMA was its requirement for agonist-occupied receptors. PMA did not reduce agonist or antagonist opioid binding when incubated with NG108–15 cells in the absence of agonists. Chronic treatment (48 hr) with PMA alone actually led to a slight increase in [^{3}H]DADLE and [^{3}H]diprenorphine binding. Mere occupation of the δ receptor was also ineffective in inducing PMA-enhanced down-regulation, as determined by the addition of the antagonist naloxone instead of an agonist. Naloxone added with PMA did not affect opioid binding, but naloxone blocked PMA-enhanced DADLE-induced down-regulation of δ receptors. PMA increased the down-regulation produced by etorphine, DADLE, and β-endorphin, all opioid agonists previously reported to induce δ receptor down-regula-

tion in NG108–15 cells. Even opioid agonists, such as morphine and cyclazocine, that failed to induce δ-receptor down-regulation in NG108–15 cells reduced opioid binding to cells treated with these drugs and PMA. As determined by their ability to inhibit [^{3}H]diprenorphine binding to NG108–15 cell membranes, cyclazocine and morphine had K_d values of 52 nM and 5340 nM, respectively (25). In comparison, the K_d values for DADLE and etorphine were 10 nM and 22 nM, respectively (25). Thus, morphine may be weak in inducing down-regulation because of its poor affinity for the δ-opioid receptor. However, the affinity for cyclazocine at the δ receptor is 100-fold greater than that for morphine, but the benzomorphans have been shown to have low intrinsic activity, as measured by their ability to inhibit adenylyl cyclase activity (25). The effect of PMA on down-regulation cannot be a generalized enhancement of metabolic rates, because PMA can promote the down-regulation of δ receptors in response to agonists that cannot induce down-regulation by themselves.

Because of the selectivity of PMA in enhancing δ-receptor down-regulation solely in the presence of opioid agonists, investigations were directed at determining whether PKC activation was responsible for normal down-regulation. Although staurosporine abolished the augmentation of down-regulation induced by PMA and DADLE together, it did not affect the normal amount of down-regulation induced by DADLE, suggesting that PKC activation may not be the sole mechanism involved in receptor down-regulation. Also, a 24-hr pretreatment of NG108–15 cells with PMA prevented PMA-enhanced δ receptor down-regulation from occurring but had no effect on DADLE-induced down-regulation. It is very likely that chronic PMA pretreatment, as observed with other cells (21–24), down-regulated PKC itself in these cells, preventing PMA from enhancing receptor down-regulation but, again, having no effect on normal agonist-induced receptor down-regulation. The down-regulation of PKC by PMA is probably also why, in the time course experiments, δ receptor down-regulation induced by etorphine and PMA was not significantly different from that induced by etorphine alone at 24 and 48 hr. Additionally, under conditions that totally blocked DADLE-induced down-regulation, PMA- plus DADLE-induced down-regulation still occurred. Dinitrophenol and sodium azide, metabolic inhibitors, had no effect on the down-regulation induced by DADLE and PMA, while preventing any down-regulation induced by DADLE alone. Lowering of the incubation temperature to 30° still permitted DADLE alone and DADLE plus PMA to induce down-regulation, but at 20° only DADLE plus PMA induced down-regulation, whereas DADLE alone was ineffective. It is possible that the PMA treatment may simply accelerate an agonist-mediated process that occurs very slowly at 20°. However, this possibility would not explain the permissive effect PMA had on morphine and cyclazocine, which did not induce down-regulation by themselves even with a 24-hr incubation but did induce down-regulation in the presence of PMA after only 3 hr. The evidence indicates that activation of PKC is not the only underlying endogenous mechanism by which δ receptor down-regulation occurs and that normal opioid agonist-induced and PMA-enhanced down-regulation are distinct and separate processes in NG108–15 cells.

Because PMA-enhanced receptor down-regulation seems to occur by a distinct and separate process from down-regulation produced by agonist alone, is it "true" down-regulation, in the sense that the ligand-receptor complex is internalized and degraded, and not receptor inactivation in the plasma membrane? Scatchard analysis revealed a loss of binding sites without a decrease in receptor affinity at the remaining sites in PMA-enhanced down-regulation, but this result would be obtained if the receptors were being internalized or if the receptors were being inactivated at the cell surface. To differentiate between these two possibilities, [^{3}H]DADLE retention in intact NG108–15 cells was measured. The retention of [^{3}H]DADLE (the sum of cell surface and internalized agonist) was measured instead of [^{3}H]DADLE uptake (internalized agonist only) because of the difficulty of washing NG108–15 cells, which attach very loosely to culture plate surfaces. More extensive procedures to remove [^{3}H]DADLE specifically bound to the cell surface would have detached the cells from the culture plate, leading to their subsequent loss. As mentioned previously, NG108–15 cells in suspension display reduced tendencies to undergo down-regulation, as has been reported for N4TG1 mouse neuroblastoma cells (19). Consequently, approaches that would require the use and recovery of suspended cells were not attempted.

The specific [^{3}H]DADLE retention experiments clearly demonstrated the ability of PMA to increase retention at certain time points. If PMA were simply inactivating surface receptors (destroying their ability to bind [^{3}H]DADLE), then a decrease in retention would be expected. Conversely, if PMA were increasing true down-regulation, then increased retention would be expected as the ligand-receptor complex was internalized. If PMA were producing irreversible binding of [^{3}H]DADLE to the δ receptor, without it undergoing internalization, then there should be no decrease in [^{3}H]DADLE retained between 60 and 180 min in the cells treated with PMA in the absence of chloroquine, but this did occur (Fig. 5A). Furthermore, chloroquine had a marked effect in increasing the [^{3}H]DADLE retained in PMA- and non-PMA-treated cells, particularly at 180 min (Fig. 5). This finding suggests that lysosomal degradation of receptor and release of internalized [^{3}H]DADLE occurred in PMA-treated cells, as has been reported for NG108–15 cells treated with [^{3}H]DADLE only (6).

From the data presented in this report, the activation of PKC is not the only mechanism responsible for δ receptor down-regulation in NG108–15 cells. Nevertheless, it is possible to activate PKC by means other than phorbol esters, such as through receptors that are coupled to PI turnover. This is significant because of the possibility that naturally occurring PKC activation in a cell could produce or increase δ receptor down-regulation if an opioid agonist was present at the same time. Activation of PKC by OAG is closer to the *in vivo* activation of PKC than is the use of PMA, because OAG is more similar to endogenous DAG than are the phorbol esters. Still more representative of the *in vivo* situation is the use of bradykinin to activate PKC through receptor-driven PI turnover to produce diacylglycerol and a rise in internal Ca^{2+}. Similar to PMA treatment, OAG and bradykinin did not induce δ receptor down-regulation in the absence of an agonist, but they induced a greater reduction of opioid binding in the presence of DADLE than that observed with DADLE alone. The effect of bradykinin was not due to a general desensitization/down-regulation mechanism of agonist-occupied receptors being increased by another receptor agonist, because carbachol, which can desensitize and down-regulate muscarinic cholin-

ergic receptors in NG108–15 cells, did not mimic the reduction of opioid binding by bradykinin. This is particularly interesting because the muscarinic receptor subtypes in NG108–15 cells are coupled to inhibition of AC activity, like the δ-opioid receptor, and not to PI turnover, like the bradykinin receptor (28, 29, 32). The half-maximal concentration of carbachol needed to inhibit cAMP formation in NG108–15 cells has been reported to be approximately 1 μM (32), the concentration used in δ receptor down-regulation experiments reported here. Thus, there is no suggestion of some unknown common down-regulation mechanism shared by agonist-occupied receptors coupled to the same second messenger system (G_i/inhibition of AC) in NG108–15 cells, because activation of muscarinic receptors did not increase the down-regulation of DADLE-occupied δ receptors. Cross-talk between receptors (opioid and bradykinin) of different second messenger systems does appear to exist. This is true even though δ receptors in NG108–15 cells are not linked to PI turnover, and in the mouse neuroblastoma × Chinese hamster embryonic brain cell line NCB-20 δ receptor activation does not produce PI turnover or modulate PI turnover elicited by bradykinin (33, 34). Cross-talk between bradykinin and δ-opioid receptors exists for select, but not all, receptor functions. Additional evidence for receptor cross-talk comes from the finding that the μ-selective peptide DAMGO caused a sustained increase in glutamate-activated currents, mediated by the *N*-methyl-D-aspartate receptor, in rat spinal trigeminal neurons (35). Intracellularly applied PKC mimicked the effect of DAMGO, whereas a PKC inhibitor blocked the sustained increase in glutamate-activated currents induced by DAMGO. PKC may play a role in mediating the actions of both μ- and δ-opioids.

Activation of PKC by phorbol esters has been shown to affect many different types of receptors. PMA-activated PKC can desensitize and/or down-regulate receptors coupled to PI turnover, producing negative feedback inhibition (13, 14). This is not the case for the δ receptor, which is not coupled to PI turnover in NG108–15 cells (33). PMA-activated PKC can desensitize receptors coupled to the stimulation or inhibition of AC activity (36–38), and this does include the δ opioid receptor. Louie *et al.* (39) reported that PMA treatment of NG108–15 cells reduced the B_{max} value of opioid binding sites, with no change in the K_d value of the remaining sites, in the absence of an opioid agonist. However, their binding experiments were performed in the presence of 100 mM NaCl, so it is unclear whether this or other differences in methodology were responsible for divergent results. After PMA treatment, 45% of opioid inhibition of cAMP accumulation in intact NG108–15 cells was abolished (39). The locus of the PMA attenuation of opioid inhibition of AC was believed to be G_i, and G_i is known to be phosphorylated by PKC in human platelets (40). In DDT_1MF-2 cells, Cowlen and Toews (41) reported that PMA-enhanced internalization of α_1-adrenergic receptors occurred only in the presence of an α_1-adrenergic agonist; PMA alone did not induce receptor internalization. This finding is analogous to the evidence presented here on the effect of PMA on δ-opioid receptors, except that the α_1-adrenergic receptor is coupled to PI turnover (41). Toews *et al.* (42) have also reported that, in 1321N1 cells, agonists alone and PMA alone desensitized β-adrenergic receptors by different mechanisms. A similar situation for δ receptor down-regulation exists for agonists and agonists with PMA; they induce down-regulation by different mechanisms. However, these studies are the first to demonstrate that PKC activated by phorbol ester enhanced agonist-induced δ receptor down-regulation and that PKC activated by receptors coupled to PI turnover can do the same, suggesting a possible physiological role for PKC in regulating δ receptor number. It is possible that the δ receptor may act as a substrate for PKC but it can only be phosphorylated in its agonist-occupied conformation, leading to the selective effects of PMA on agonist-induced down-regulation. These studies are also the first to demonstrate that opioid agonists, such as morphine and cyclazocine, that do not normally down-regulate δ receptors did down-regulate δ receptors in the presence of PMA. If PKC activation is required for down-regulation of opioid receptors in neurons, this may explain conflicting literature accounts of the ability of opioid agonists to produce opioid receptor down-regulation in the brain (43–47).

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