Agonist-Induced Desensitization and Down-Regulation of δ Opioid Receptors Alter the Levels of Their ^{125}I-β-Endorphin Cross-Linked Products in Subcellular Fractions from NG108-15 Cells†

Mariana M. Belcheva, Elena G. Ignatova, Eric C. Young, and Carmine J. Coscia*

E. A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104

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ABSTRACT: The δ opioid binding sites in subcellular fractions from NG108-15 cells were characterized with respect to their relative molecular size and levels under conditions of receptor adaptation. ^{125}I-β-Endorphin was cross-linked to preparations enriched in plasma membranes (P_{20}), nuclear membranes or nuclear matrices. Five cross-linked bands appear in all subcellular fractions. The largest molecular size reaction product in nuclear matrix preparations (∼72 kDa) differed from that in the other two fractions-(∼83 kDa). Immunoblot analyses with an antibody to the δ opioid receptor gave a P_{20} band pattern similar to that for the corresponding cross-linked products. To determine which cross-linked products in P_{20} are glycoproteins, labeled membranes were solubilized and purified by wheat germ agglutinin chromatography. The absence of a ∼36 kDa band after purification suggests that this product is not a glycoprotein. The remaining four bands were present in *N*-acetyl-D-glucosamine eluates, although their % distribution changes in favor of the largest molecular size band (∼83 kDa). Immunoblotting of the eluate gave a single diffuse band at ∼73 kDa, suggesting the native glycoprotein has a molecular size in the 70−80 kDa range. Etorphine-induced desensitization of cell surface receptors increased the amount of some cross-linked products associated with nuclear membranes. The same treatment did not affect the relative density of the four larger molecular size bands in P_{20}, but increased the density of the ∼26 kDa product two fold. Etorphine-induced down-regulation evoked an elevation of cross-linked products in nuclear matrix preparations, while all band densities of P_{20} were diminished. These results suggest that nuclear matrix associated opioid binding sites represent internalized, truncated forms of the glycosylated δ opioid receptor found in P_{20}.

Since the discovery of opioid receptors in brain, it has been recognized that a fraction of the total cellular binding is present within cells rather than on the cell surface (Simantov et al., 1976; Smith & Loh 1976; Roth et al., 1981; Klein et al., 1986). In the course of subcellular localization studies on brain opioid receptors, we found that synaptic plasma membrane-enriched fractions contained about 70% of total opioid binding (Roth et al., 1981). Of the remaining sites about half were shown to be in microsomal fractions enriched in ER/Golgi with lesser amounts in coated vesicles (Bennett et al., 1985).

Similarly, plasma membrane-enriched fractions from NG108-15 cells comprised much of the δ opioid binding, whereas the remainder was found associated with preparations containing ER/Golgi and purified nuclei (Belcheva et al., 1991, 1993). Data to support the notion of nuclear localization of about 15% of opioid binding were gained by subcellular fractionation and immunohistochemical approaches. Subsequent subnuclear fractionation revealed that binding sites were distributed between nuclear membrane and nuclear matrix preparations (Belcheva et al., 1995). The origin and role of nuclear opioid binding sites remain unknown. Nevertheless, there are data to suggest that nuclear membrane associated opioid receptors are newly synthesized sites en route to the cell surface, possibly on ER which is contiguous with the outer nuclear membrane (Belcheva et al., 1993, 1995). Moreover, the possibility exists that nuclear matrix opioid binding sites may represent internalized cell surface receptors.

Independent evidence for nuclear associated opioid binding has been reported. Naloxone binding has been discovered in rat hypothalamic and uterine low speed sediments enriched in nuclei (Vertes et al., 1993, 1995). Furthermore, the "ζ opioid growth factor receptor" has been reported to be localized in nuclei of rat cerebellar cells (Zagon et al., 1993).

EM-autoradiographic evidence for intracellular opioid sites was provided by Beaudet et al. (1989). In subsequent experiments using a photoaffinity probe (azido-DTLET), the same group discovered only 44% of the labeled δ binding sites were on neuronal plasma membranes in rat neostriatum while the remainder was associated with the cytoplasm of axons (Pasquini et al., 1992). Some nuclear binding was also discerned, comprising 2.5−7.7% of μ, δ, and κ sites in neostriatum and an unspecified number of μ sites in locus coeruleus (Beaudet et al., 1989; Jomary et al., 1992; Pasquini et al., 1992).

Recent immunohistochemical localization of δ opioid receptors in brain, spinal cord and cultured cells has also corroborated the existence of significant amounts of intracellular opioid receptors in some cells (Arvidsson et al., 1995;

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* To whom correspondence should be addressed at St. Louis University, Dept. of Biochemistry and Molecular Biology, 1402 South Grand Blvd., St. Louis, MO 63104.

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Bausch et al., 1995; Cheng et al., 1995). EM data revealed localization of δ opioid receptor immunoreactivity in numerous cytoplasmic organelles including large dense core vesicles and smaller clear vesicles (Cheng et al., 1995). Nuclear staining by anti-opioid receptor antibodies was also detected in NG108-15 cells (Belcheva et al., 1993), μ transfected COS cells (Surratt et al., 1994), and spinal cord (Gastard et al., 1995). Thus, immunohistochemical and EM autoradiographic findings complement subcellular fractionation results.

To understand the origin and fate of intracellular populations of opioid receptors, their characterization is essential. An important property of intracellular opioid receptors is their molecular size relative to cell surface sites. Cross-linking of δ opioid receptors with ^{125}I-β-endorphin has yielded several distinct molecular entities in brain and cultured cells (Howard et al., 1985; Keren et al., 1988; McLean et al., 1989, 1990; Ko et al., 1992). An immunoblot of a partially purified δ opioid receptor has also been reported (Dado et al., 1993). Thus, it was of interest to determine the relative sizes of δ opioid receptors associated with nuclear membranes and nuclear matrix fractions and the changes in their levels upon agonist-induced desensitization and down-regulation.

MATERIALS AND METHODS

Chemicals. Most chemicals were purchased from Sigma Chemical Co., St. Louis, MO with the exception of β-endorphin (Multiple Peptide Systems, San Diego, CA), naltrindole (Research Biochemical International, Natick, MA), etorphine (NIDA Drug Supply, Research Triangle Park, NC), WGA-agarose beads (E-Y Laboratories, San Mateo, CA), and BSOCOES, bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone (Pierce, Rockford, IL).[1]

Cell Culture Growth and Subcellular Fractionation. NG108-15 cells were grown at 37 °C in a humidified CO_2 (10%) incubator in Dulbecco's modified Eagle's medium (DMEM) and Ham's Nutrient Mixture F12 containing calf serum (10%) as described (Belcheva et al., 1993). Cells were harvested and stored at −70 °C before subcellular fractionation. Homogenization of cells was achieved by gentle disruption in a "cell cracker" (Belcheva et al., 1993). Fractions enriched in nuclear membranes, nuclear matrix, and P_{20} (from cell homogenates by sedimenting a 1000*g* supernatant at 20000*g*) were prepared according to previously developed procedures (Belcheva et al., 1993, 1995). In most experiments nuclear membranes, nuclear matrix, and P_{20} fractions were isolated in the presence of protease inhibitors. A cocktail containing 10 μg of leupeptin/mL, 2 μg of pepstatin A/mL, 200 μg of bacitracin/mL, and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added to buffers used for preparation of the subcellular fractions.

Treatment of NG108-15 Cells with Etorphine To Induce Desensitization and Down-Regulation. Desensitization of opioid receptors was attained by supplementing cell culture media with 10 nM etorphine for 2 h, whereas down-regulation was elicited using 10 nM etorphine for 24 h as previously described (Belcheva et al., 1995). Desensitization and down-regulation was ascertained previously by binding and cAMP measurements (Belcheva et al., 1995). Membrane and nuclear matrix preparations from control and etorphine-treated cells were washed at least 5 times with 50 mM Tris-HCl, pH 7.4. On the basis of binding data, this procedure proved sufficient to remove most residual drug (Belcheva et al., 1995).

Cross-Linking with ^{125}I-β-Endorphin. The method of Howard et al. (1985) was adopted with some modifications. Membrane preparations from control and etorphine-treated cells were suspended in 50 mM phosphate buffer, pH 7.4 (50 mM K_2HPO_4, 1 mM EDTA, 10 μM leupeptin, and 0.01% bacitracin) and incubated with ^{125}I-β-endorphin (2 nM, 2000 Ci/mmol, Multiple Peptide Systems) in the presence and absence of 1 μM unlabeled β-endorphin for 1 h at room temperature. For nuclear matrix preparations, samples were incubated with 5−10 nM ^{125}I-β-endorphin and 1 μM naltrindole was applied to determined nonspecific binding. For all subcellular fractions, the incubation was halted by centrifugation for 5 min in a tabletop centrifuge (Beckman microfuge) at the maximum speed. After resuspension of the pellets in phosphate buffer, they were incubated with the cross-linker BSOCOES (1 mM) for 15 min on ice. The reaction was terminated by addition of 50 mM Tris-HCl, pH 7.4, and centrifugation. To remove labeled β-endorphin that was not cross-linked, samples were resuspended in 50 mM NaCl and incubated for 30 min at room temperature.

WGA Affinity Chromatography of Solubilized ^{125}I-β-Endorphin Cross-Linked P_{20} Membranes. P_{20} fractions were cross-linked with 2 nM ^{125}I-β-endorphin in the presence (nonspecific binding) and absence (total binding) of 1 μM β-endorphin as described above. Solubilization and WGA chromatography of the cross-linked membranes were performed according to the procedure developed by Liu-Chen and co-workers (Chen et al., 1995), using 2% Nonidet P-40 as a detergent. *N*-Acetyl-D-glucosamine eluates of the retained glycoproteins (0.5 mL) were collected from two (for total and nonspecific binding) wheat germ agglutinin-agarose (WGA) columns and after concentration, peak fractions were run on 10% SDS−PAGE.

Immunoblotting. P_{20} membranes (50 μg/lane) or *N*-acetyl-D-glucosamine eluates (30 μg/lane) from the WGA columns were resuspended in SDS sample buffer and electrophoresed on 10% SDS−PAG. Proteins were transferred overnight to Immobilon-P membranes (Millipore) using transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol). Membranes were blocked with 1% casein in TBST (50 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20) for 1 h. After three 5 min washes with TBST, membranes were blotted with primary antibody (1:2000 to 1:4000 dilution in TBST) for 1 h. Rabbit antisera used was generated against a sequence in the N-terminal (PVPSARAELQFSLLA) of δ opioid receptors (Signal Transduction Inc., San Diego, CA). In immunoblot assays both preimmune and peptide-preabsorbed antisera served as negative controls. After removal of antisera the blot was washed 5 times with TBST. Secondary antiserum (goat anti-rabbit horseradish peroxidase conjugate, Sigma) was added at 1:7000 dilution and incubation for 1 h followed. Membranes were washed 5 times, and bands were visualized using isoluminol as a substrate for the horseradish peroxidase.

Electrophoresis and Densitometry. SDS−PAGE of subcellular fractions was performed as described by Laemmli (1970). Samples were adjusted to equal protein content (100−200 μg) and electrophoresed on SDS−PAG (10%).

[1] Abbreviations: BSOCOES, bis[2-(succinimidooxycarbonyloxy)-ethyl]sulfone; PMSF, phenylmethylsulfonyl fluoride; P_{20}, membrane fractions obtained from cell homogenates by sedimenting a 1000*g* supernatant at 20000*g*; TBST, Tris-buffered saline containing Tween 20; WGA, wheat germ agglutinin.

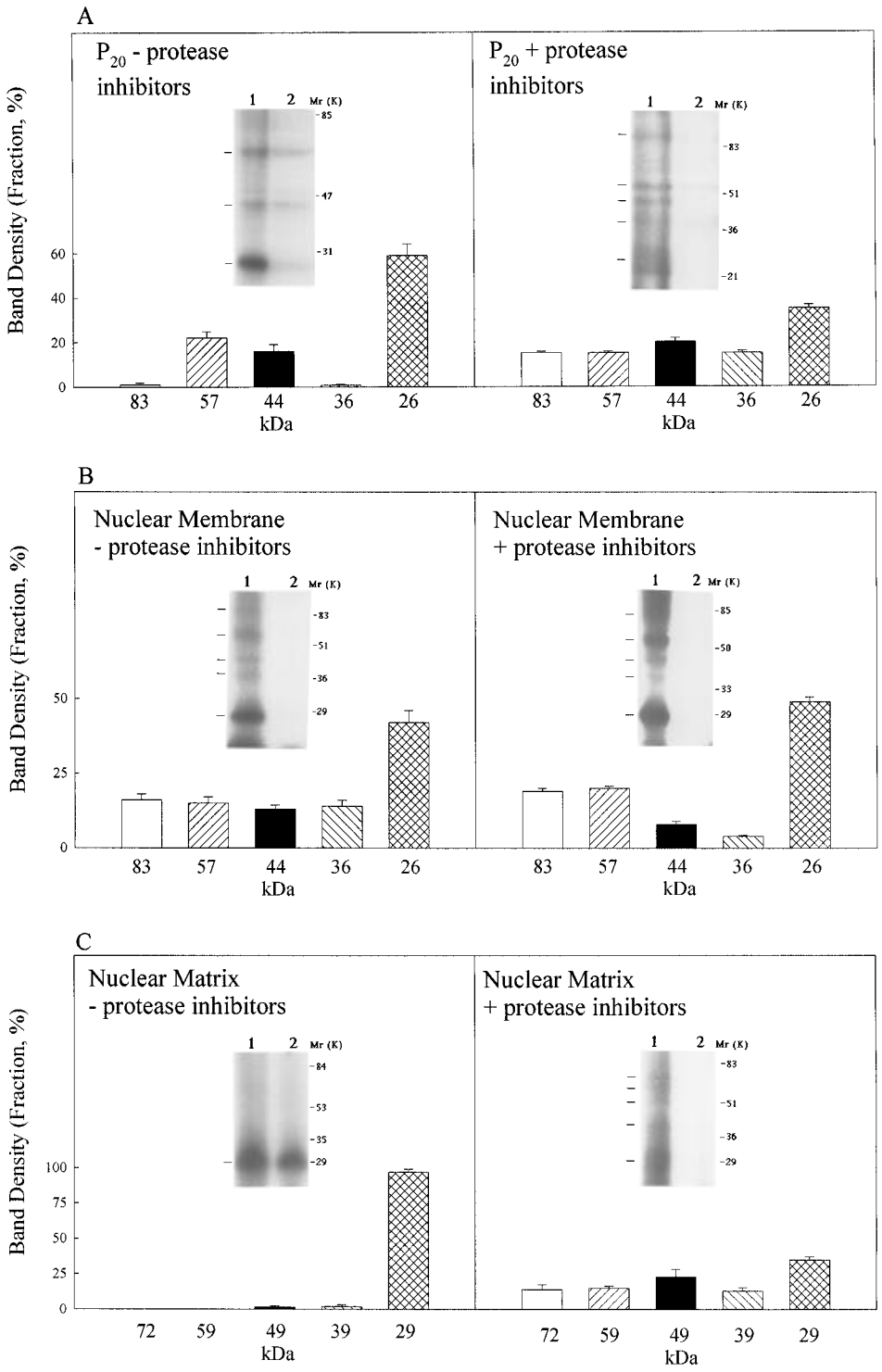

FIGURE 1: Representative autoradiographs and densitometric analysis of ^{125}I-β-endorphin cross-linked bands from P_{20} (A), nuclear membrane (B), and nuclear matrix (C) prepared from NG108-15 cells in the absence or presence of protease inhibitors. P_{20} membranes, nuclear membranes, and nuclear matrix preparations ± a cocktail of protease inhibitors were cross-linked with ^{125}I-β-endorphin and subjected to SDS−PAGE (100 μg of protein/lane). In the autoradiographs, lane 1 shows total binding, while lane 2 displays nonspecific binding. Specific densities were determined as the difference between the density of total and nonspecific binding. Background level was estimated for each lane and subtracted out before determining specific density. Observed densities are expressed as a fraction of the total density (100%) detected for all products in each preparation. Data are the mean ± SEM of three to eight experiments.

After being stained with Coomassie blue to ascertain uniform protein loading, gels were dried and initially exposed to diagnostic films (Kodak X-Omat, XRP-1) for a period of 5−10 days. Subsequently, gels were exposed to Kodak Scientific Imaging films (Biomax MS), which provide maximum sensitivity for ^{125}I, and the exposure time was 1−2 days. The intensity of the bands was quantified with an image analysis program (Phosphorimager, Molecular Dynamics). In each experiment, samples of total and nonspecific binding for controls and etorphine-treated cells were run on the same gel. Background was estimated for each lane and subtracted out before determining specific density. Molecular weights of the bands were estimated using molecular weight standards and GelReaderLCsi program.

Protein Assays. Protein concentrations were determined by either the method of Lowry et al. (1951) or Bradford (1976) with bovine serum albumin as standard.

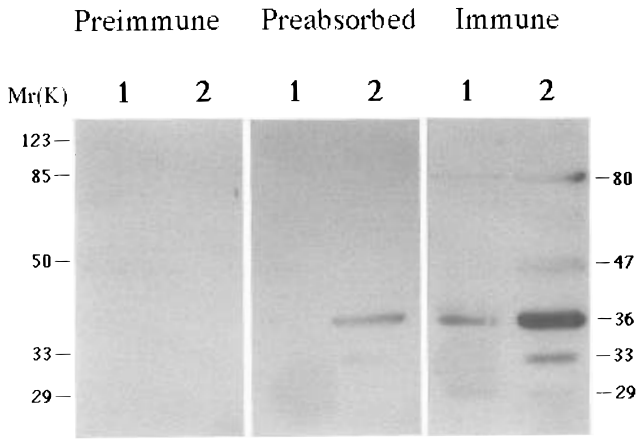

FIGURE 2: Immunoblot analysis of subcellular fractions from NG108-15 cells. Membrane preparations from rat brain and NG108-15 cells (50 μg/lane) were resolved by 10% SDS−PAGE, transferred to Immobilon-P membranes, and blotted with preimmune, peptide (50 μg) pre-absorbed and immune serum (1:4000 dilution for each). Lanes 1, rat brain membranes; lane 2, NG108-15 P_{20}. This is a representative immunoblot that was replicated.

RESULTS

^{125}I-β-Endorphin Cross-Linking and Immunoblot Analyses of δ Sites in Subcellular Fractions from NG108-15 Cells. To determine the relative molecular size of their δ opioid sites, subcellular fractions were prepared from NG108-15 cells and subjected to cross-linking with ^{125}I-β-endorphin. SDS−PAGE yielded reaction products that differ in number and % distribution depending on the presence or absence of protease inhibitors during the isolation of the fractions. Three bands were found in P_{20} membranes in the absence of inhibitors, and five products were present when the membranes were prepared in the presence of inhibitors. Somewhat diffuse bands centered at ∼83 (76−90), ∼57 (55−61), ∼44 (41−48), ∼36 (35−38), and ∼26 (24−28) kDa were detected in the presence of inhibitors (Figure 1A).

In nuclear membrane preparations five bands were detected with mobilities similar to those seen for P_{20} products (Figure 1B). The effect of protease inhibitors on the distribution of reaction products from nuclear membranes was not as profound as that observed for P_{20} products.

In cross-linking experiments with nuclear matrix fractions, omission of protease inhibitors from the isolation procedure afforded a single major (97%) band of ∼29 kDa (Figure 1C). A substantial decrease in the density of the ∼29 kDa band was observed in the presence of the protease inhibitors as seen for P_{20} products. Inclusion of protease inhibitors yielded five nuclear matrix cross-linked products: ∼72 (67−76), ∼59 (56−61), ∼49 (46−50), ∼39 (37−40), and ∼29 (27−29) kDa, four of which were similar to those of other subcellular fractions. However, the largest molecular size band was found to be less than that from P_{20} and nuclear membranes.

An N-terminal peptide-directed anti-δ opioid receptor antibody was used as a probe in immunoblotting experiments on P_{20} membranes (Figure 2). The absence of a signal with preimmune serum and the elimination or substantial reduction of bands by preabsorbtion with the N-terminal peptide attested to its specificity. The brain membranes gave a major band at 36 kDa. The N-terminal antibody gave a pattern of bands in P_{20} preparations somewhat similar to that seen for the cross-linked products from the P_{20} fraction of NG108-15 cells (Figure 2). Some differences may be attributable to the different methodologies and the presence of β-endorphin in cross-linked products which elevates their apparent size over that seen in the immunoblots, presumably by ∼3 kDa/receptor molecule. Bands in immunoblots were sharper than those obtained by autoradiography of cross-linked products which could also account for differences in mobility.

WGA Chromatography of Cross-Linked P_{20} Membranes. To determine which of the P_{20} cross-linked products are glycoproteins, the labeled membranes were solubilized and purified by WGA chromatography. *N*-Acetyl-D-glucosamine eluates of the retained glycoproteins (0.5 mL) were collected from two columns (total and nonspecific binding). The absence of a cross-linked band at ∼36 kDa after the purification step suggests that this product is devoid of a glycosyl moiety. The other relatively diffuse bands were present in the eluates, although their % distribution is skewed in favor of the higher molecular size band (∼83 kDa) after WGA chromatography (Figure 3A).

The N-terminal antibody detected a single diffuse band at 72−73 kDa in *N*-acetyl-D-glucosamine eluates of the β-endorphin cross-linked receptor (Figure 3B). The mobility of this band is within the range of the 78−86 kDa cross-linked product given the variations in the methods used. These results are consistent with the interpretation of cross-linking data that the larger molecular size band represents glycosylated native receptor which may undergo some limited proteolysis after elution from the WGA column. Relatively small amounts of the ensuing smaller molecular size products may not be detectable by immunoblotting which is less sensitive than cross-linking analyses.

Changes in Levels of Cross-Linking Products Upon Opioid Receptor Desensitization. NG108-15 cells were treated with etorphine under conditions previously established to induce opioid receptor desensitization as evidenced by binding and cAMP measurements (Belcheva et al., 1995). Subcellular fractionation of membranes from desensitized cells was performed in the presence of protease inhibitors. However, since [^{3}H]naltrindole binding to nuclear matrix preparations was abolished completely under desensitization conditions in the presence or absence of protease inhibitors [Belcheva et al. (1995) and unpublished observations], cross-linking was not performed on this fraction. Etorphine-induced desensitization did not affect the relative density of the four larger molecular size bands in P_{20} membranes, but the density of the ∼26 kDa product increased 2-fold (Figure 4). The same desensitization conditions enhanced levels of nuclear membrane cross-linked products by 28−68%.

Changes in Levels of Cross-Linking Products upon Down-Regulation of Cell Surface Receptors. Upon etorphine-induced down-regulation, the density of the nuclear matrix associated bands was increased significantly (142−245%), while levels of the reaction products associated with either P_{20} or nuclear membrane fractions were diminished by 30−76% (Figure 5). Interestingly, the ∼36 kDa band associated with the nuclear membranes becomes the most predominant under down-regulation conditions.

DISCUSSION

The present study provides additional characterization of the molecular properties of the δ opioid sites associated with plasma membrane, nuclear membrane and nuclear matrix preparations from NG108-15 cells under conditions of receptor adaptation. The ^{125}I-β-endorphin cross-linking experiments suggest that comparable amounts of five reaction

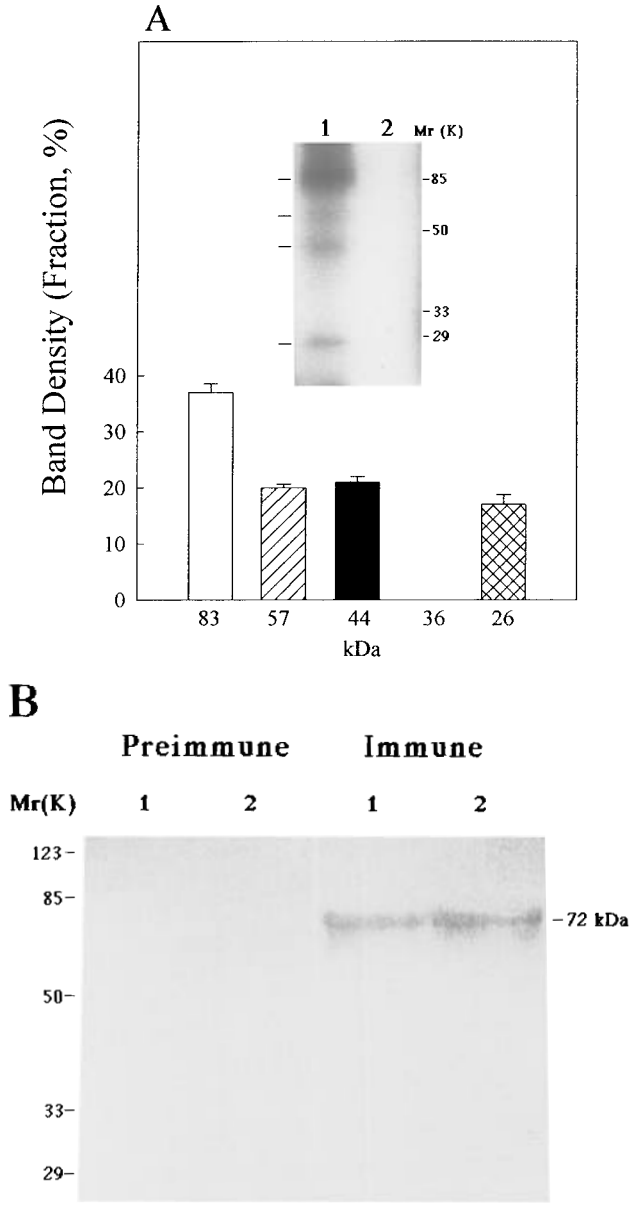

FIGURE 3: Representative autoradiograph, densitometric and immunoblot analysis of ^{125}I-β-endorphin cross-linked bands in P_{20} membranes after WGA chromatography. (A) Cross-linking data. P_{20} membranes were cross-linked with 2 nM ^{125}I-β-endorphin in the absence (lane 1) or in the presence of 1 μM unlabeled β-endorphin (lane 2). Labeled membranes were solubilized and purified with WGA columns as described in Materials and Methods. Specific densities and background measurements were determined as described in the legend to Figure 1. Data are the mean ± SEM of 4 experiments. (B) Western blot analysis. *N*-Acetyl-D-glucosamine eluates from the cross-linking experiment described in (A) were concentrated, separated by 10% SDS−PAGE (12 μg of protein/lane) and transferred to Immobilon-P membranes. The membranes were blotted with 4 μg/mL of ammonium sulfate-precipitated IgG fractions from preimmune serum or N-terminal-directed anti-DOR1 antibody. Lanes 1 and 2 are two different WGA column eluates.

products were seen in P_{20} or nuclear membrane fractions when isolated in the presence of protease inhibitors. Immunoblotting analyses of P_{20} gave a somewhat similar band pattern in light of the differences contributed by the presence of β-endorphin and by the different methodologies entailed. Two different effects of β-endorphin cross-linking can be anticipated to impact on the molecular size. Namely, the molecular weight will increase, presumably by ~3 kDa/receptor molecule. Moreover, conformational changes may ensue upon binding of β-endorphin to the receptor to alter the shape of the molecule and possibly its gel mobility. The cross-linking methodology also seems to promote a greater amount of smaller molecular size fragments than the immunoblotting procedure despite the use of the same protease inhibitor cocktail in membrane preparations. Thus, it is more likely that the five cross-linked products contain fragments of the receptor rather than complexes of β-endorphin covalently bound to protein adjacent to the opioid receptor. Reciprocally, the cross-linking data corroborate the specificity of the antibodies used.

The nuclear matrix preparation also generated five cross-linked products, but the largest of which (~72 kDa) is smaller in size than the slowest moving specific bands detected in P_{20} or nuclear membranes. This finding may be due to the presence of a novel δ binding site in nuclear matrix preparations that is not found in the other two subcellular fractions. However, immunoblotting of nuclear matrix preparations revealed the presence of a ~68 kDa band (data not shown) which suggests that a putative novel binding site would have to share some homology at the N-terminal with the δ opioid receptor. On the other hand, the nuclear matrix preparation may contain a truncated form of the δ opioid receptor. Limited proteolysis of nuclear matrix preparations may occur at a much higher rate than in other fractions. For example, in the absence of protease inhibitors, the nuclear matrix reaction product is enriched (97%) in a single band of ~29 kDa. Since the nuclear matrix is the only preparation exposed to detergents, proteolysis may be exacerbated during its isolation. Moreover, this fraction may be enriched in proteases that readily cleave the opioid receptor. Analysis of the crude nuclear pellet for specific lysosomal enzyme activities (β-glucuronidase and β-hexoseamindase) reveals they are 10−12% of that found in the ER/Golgi-enriched fraction (0.97 ± 0.09 vs 9.0 ± 0.3 and 15 ± 1.2 vs 123 ± 8.2 nmol/mg/min, respectively). Since nuclei occupy a relatively large proportion of the intracellular space of NG108-15 cells, the fraction of nuclear lysosomal total activity is about 40% of the sum detected in all membrane preparations (Belcheva et al., 1991).

On the basis of the the amino acid sequence of the cloned δ opioid receptor the predicted molecular weight of the polypeptide chain alone is estimated to be 40.5 kDa. Cross-linked products and immunoblot bands of larger size that were detected in this study would be expected to be glycosylated molecules. Results of the WGA chromatography experiments suggested that the ~36 kDa band which was absent in the *N*-acetyl-D-glucosamine eluate is a non-glycosylated cross-linked product. This is consistent with data from cross-linking and immunoblotting of the WGA eluates of the P_{20} fractions wherein the predominant product is ~83 kDa in the former and only a ~73 kDa band was evidenced in the latter. If the apparent molecular weight of the native δ opioid receptor is in this range, then the two carbohydrate moieties believed to be present would account for 30−40 kDa. This number is relatively high compared to that reported for μ opioid receptors, which upon removal of the N-linked carbohydrates from five potential glycosylation sites on ^{3}H-β-FNA-labeled μ receptors expressed in COS-1 or CHO cells, yielded a 40 kDa smaller band. (Chen et al., 1995). Moreover, carbohydrate moieties of a large number of other G protein-coupled receptors were estimated to range anywhere from 4 to 30 kDa (Sawutz et al., 1987; Plantner et al., 1991). Alternative explanations can be posited. For example, there may be posttranslational modi-

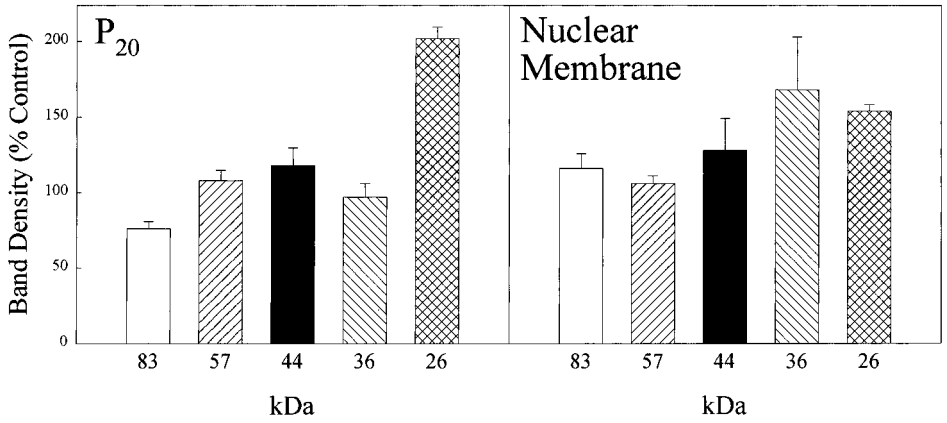

FIGURE 4: Densitometric analysis of ^{125}I-β-endorphin cross-linked bands in subcellular fractions from control and opioid-desensitized NG108-15 cells. NG108-15 cells were treated with etorphine (10 nM, 2 h) and subcellular fractions were isolated in the presence of protease inhibitors as described in Materials and Methods. Specific densities and background measurements were determined as described in the legend to Figure 1. In each experiment samples of total and nonspecific binding for control and treated cells were run on the same gel. Observed density changes induced by etorphine treatment are expressed as % control which were normalized to 100%. Data are the mean ± SEM of three or four experiments.

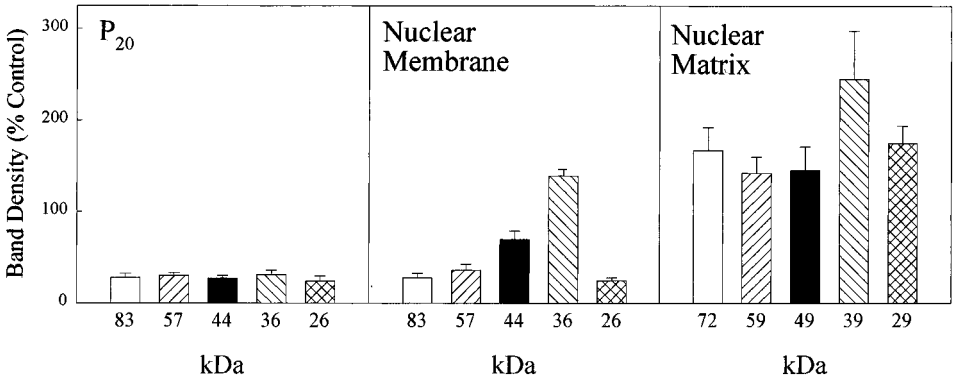

FIGURE 5: Densitometric analysis of ^{125}I-β-endorphin cross-linked bands in subcellular fractions from opioid agonist down-regulated NG108-15 cells. NG108-15 cells were treated with etorphine (10 nM, 24 h) and subcellular fractions were isolated in the presence of protease inhibitors as described in Materials and Methods. Densitometric analyses of the observed density changes induced by etorphine in P_{20}, nuclear membrane and nuclear matrix fractions were performed as described in Figures 1 and 4. Observed density changes induced by etorphine treatment are expressed as % control which were normalized to 100%. Data are the mean ± SEM of three to eight experiments.

fications of the δ polypeptide chain other than glycosylation. Also it has been reported that a G protein-coupled receptor can dimerize under the SDS denaturing conditions adopted for PAGE (Papermaster & Dreyer 1974).

If the smaller molecular size bands at ~57 and ~44 kDa found after the WGA chromatography represent limited proteolysis products, they have retained their binding ability. This is supported by the protease inhibitor data (Figure 1) and structure−binding activity studies (Kong et al., 1994). In the latter investigation, a truncated δ opioid receptor, devoid of the N-terminal and first transmembrane region, that was engineered and expressed in COS cells retained δ agonist-mediated inhibition of forskolin-stimulated cAMP production. On the basis of their elution and immunoblot data, these lower molecular size bands may be fully or partially glycosylated fragments that contain covalently labeled ^{125}I-β-endorphin but not the N-terminal stretch recognized by the antibody used in these studies. However, we cannot rule out the possibility that the higher molecular size glycosylated fragments undergo some limited proteolysis to form the ~57 or ~44 together with ~26 kDa product after elution from the WGA column. At present this appears to be the only viable explanation for the presence of the ~26 kDa product which is too small a fragment to contain both carbohydrate and a binding site for β-endorphin.

The predominance of a cross-linked 26−29 kDa product in all subcellular fractions isolated especially in the absence of protease inhibitors suggests that the proteolysis reaction which generates this fragment can occur readily. The fact that 97% of the nuclear matrix product was found to be a ~26 kDa band also supports our previous binding data (Belcheva et al., 1995). Agonists bind with low affinity, if at all, to this fraction and neither sensitivity of this agonist binding to the GTP analog Gpp(NH)p nor adenylate cyclase activity was detected suggesting the presence of G protein/effector uncoupled sites.

The origin of the 26−29 kDa band has been investigated in several ^{125}I-β-endorphin cross-linking studies. Keren et al. (1988) suggested that a 25 kDa band is a breakdown product of a higher molecular weight peptide, since omission of protease inhibitors from their assay buffers led to greater levels of 25 kDa product as seen in these studies. Harada et al. (1992) detected only a single 29 kDa band derived from NG108-15 cells or membrane preparations. The authors speculated that this protein may not be a proteolytic product of a larger labeled protein, but it represented the δ opioid receptor in NG108-15 cells, which is functionally coupled to pertussis toxin-sensitive G protein. Ko et al. (1992) proposed that only a 28 kDa band fulfilled the requirements for an opioid binding site, in part because its intensity alone was reduced under opioid receptor down-regulation conditions in NG108-15 cells. Here we also detected a selective increase in the P_{20} and nuclear membrane ~26 kDa reaction products upon desensitization. In contrast,

all five cross-linking P_{20} reaction products were diminished upon down-regulation. Moreover, cross-linking of recombinant μ, δ, and κ receptors transfected in CHO, COS or PC12 cells yielded a major ~28 kDa band along with larger molecular size products, consistent with the notion that the ~28 kDa protein is a proteolytic degradation product (Coscia et al., 1995).

An important achievement of this study was the discovery that changes in cross-linking product(s) from nuclear matrix and nuclear membrane fractions occur during agonist-induced cell surface receptor adaptation in NG108-15 cells. The cross-linking experiments revealed that nuclear matrix preparations display an increase in the density of the five cross-linked products upon cell surface receptor down-regulation. The correlation between the loss of P_{20} binding sites and elevation of the levels of nuclear matrix associated product suggest that the nuclear matrix sites are internalized cell surface receptors. These results complement previously reported binding data (Belcheva et al., 1995). The increase in the nuclear membrane ~36 kDa band of the receptor seen upon agonist-induced down-regulation may possibly be due to greater limited proteolysis. An increase in lysosomal enzyme activity has been documented under these conditions (Belcheva et al., 1992).

Upon desensitization most of the P_{20} bands remain the same or are slightly decreased. Similarly total binding of this fraction does not change appreciably (Belcheva et al., 1995). The singular increase of the ~26 kDa P_{20} product under these conditions may again be possibly attributable to increases in lysosomal enzyme activity (Belcheva et al., 1992). Upon desensitization the increase in density of some nuclear membrane bands is in good agreement with the up-regulation of nuclear membrane agonist binding observed under the same conditions (Belcheva et al., 1995). The greater increase in smaller nuclear membrane products is consistent with the trend seen in the P_{20} fraction reflecting elevated limited proteolysis. The finding of this selective increase without compensatory decreases in the remaining bands may reflect the initiation of new protein synthesis in nuclear membranes which ultimately results in the restoration of levels depleted by subsequent down-regulation of cell-surface receptors (Collins et al., 1992). Taken together these findings further support the hypothesis that nuclear membrane receptors are newly-synthesized molecules en route to the cell surface, whereas nuclear matrix contains internalized, truncated δ opioid sites. The acquisition of this information represents one of the first steps in determining the function of the subnuclear sites.

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