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Stereospecificity of Opiate Receptors in a Synchronized Culture of NG108-15 Cells Probed with Fluorescent Anti-idiotypic Antibodies

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SUMMARY

The stereospecificity of the binding of an anti-opiate receptor, anti-idiotypic antibody to receptors on a synchronized NG108-15 neuroblastoma × glioma cell culture has been examined by fluorescence labeling of the antibodies. We had previously found that unsynchronized NG108-15 cultures showed high specificity (reversibility by receptor-binding ligands) of antibody labeling. However, only a subpopulation additionally showed stereospecific labeling (nonreversibility by non-receptor-binding opiate enantiomers). In the present work we find that the percentage of cells that display stereospecific fluorescence is highly dependent upon position in the cell cycle. Furthermore, the properties of the bound fluorescence, with respect to sensitivity to photobleaching, also depend upon position in the cell cycle. The fluorescence behavior of labeled synchronized cell populations after treatment to inhibit *de novo* glycosylation is also reported. The results reported here may have implications concerning the structure of the opiate receptor complex and efforts to solubilize the binding protein in its native form.

Opiate receptor selectivity for a ligand bound by more than one receptor subclass is defined on the basis of the ratios of the ligand dissociation constants (K_D) values for the different receptors (1). Specificity of binding refers to the competitive inhibition of ligand binding to a receptor either by the same ligand (measured, for example, by excess unlabeled ligand displacing the radioactive form) or by what may or may not be a structural analog known to bind strongly to the same receptor (for example, displacement of DADLE by naloxone) (2). The difference in amounts of labeled ligand bound before and after application of the displacing ligand is the amount bound specifically. Stereospecificity refers to absence of competitive inhibition of binding of specific ligands to opiate receptors by enantiomeric forms of the ligands (or analogs) applied in excess (3). The difference in amounts of labeled ligand bound before and after application of the nonbinding enantiomer is the amount bound stereospecifically. Examples of enantiomeric forms are (+)-morphine (the enantiomer of morphine), dextromethorphan (the enantiomer of the opiate agonist codeine), and dextrorphan (the enantiomer of the agonist levorphanol). Whereas the enantiomers of narcotic analgesics may have pharmacological activity (4), they do not antagonize narcotic analgesic activity by binding to opiate receptors with high affinity. Thus, it was found that for NG108-15 cells dextrorphan binds to opiate receptors with a K_D about 15 times larger than that of naloxone (5). It must be noted that, because the inactive enantiomers do not bind to the ligand site of the receptor with high affinity (whereas specific ligands do), there is a fundamental distinction between the two measurements, specific binding and stereospecific binding. In summary, a ligand with good specificity should be displaced completely by an excess (usually defined as at least 100 times greater concentration) of receptor-binding ligands. The stereospecificity of binding is probed by displacement with excess concentrations of the enantiomers of the receptor-binding ligands.

In a recently completed work,[1] we examined the labeling of opiate receptors on NG108-15 cells by fluorescent rabbit polyclonal α-Ids. These cells express only δ subclass receptors. We had previously shown that our antibodies are specific for μ and δ but not κ opiate receptors (6). In the more recent work, we examined the distribution of fluorescence on the cells and inhibition of labeling by various selective, specific, and stereospecific reagents. When labeled antibody binding specificity was probed with 1 μM naloxone (nonselective opiate antagonist) or DADLE (weakly selective δ agonist), inhibition of labeling

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[1]Ornatowska, M., and J. A. Glasel. Two- and three-dimensional distribution of opiate receptors on NG108-15 cells visualized with the aid of fluorescence confocal microscopy and anti-idiotypic antibodies. Submitted for publication.

ABBREVIATIONS: DADLE, D-Ala^2-D-Leu^5-enkephalin; α-Id, anti-idiotypic antibody; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FITC, fluoroscein isothiocyanate.

TABLE 1

Mitotic (MI) and fluorescence total (FIT) and stereospecific (FIS) indices for live and fixed NG108-15 cells growing in culture after removal of G_1 block, including observations on photobleaching of the cells

Cell type	Time	MI	FIT	FIS	Comments
	hr	%	%	%	
Live	0	4.9	53	0	Patching, very rapid photobleaching
Live	4	12	10	0	Patching, very rapid photobleaching
Live	8	22.3	5	3	Uneven distribution of stain, no photobleaching
Live	12	52	20	14	No photobleaching
Live	16	80	95	34	No photobleaching of total, rapid photobleaching of stereospecific
Live	20	76	100	43	No photobleaching
Fixed	20		100	80	No photobleaching
Live	24	77	86	28	Fast photobleaching of both total and stereospecific
Live	28	62	64	16	No photobleaching
Live, incubated with tunicamycin	20		27	11	Patching, strong photobleaching of both total and stereospecific
Fixed incubated with tunicamycin	20		65	38	Strong photobleaching of both total and stereospecific

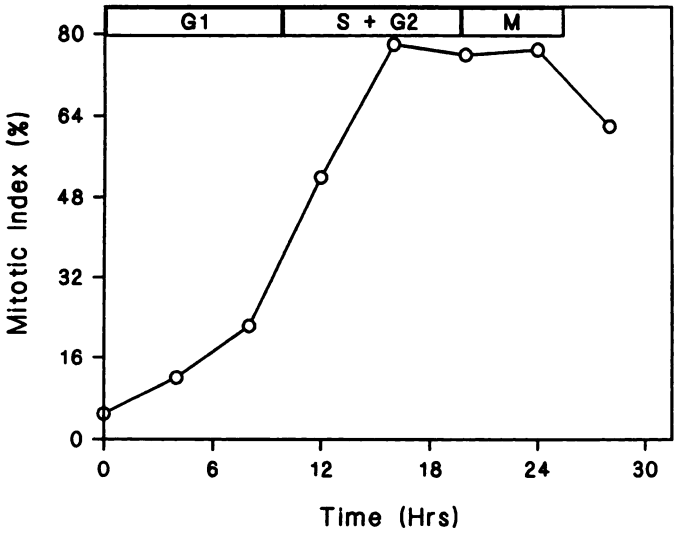

Fig. 1. Mitotic index versus time for NG108-15 cells transferred from isoleucine/glutamine-deficient medium into 10% FBS-DMEM at zero time.

was complete. However, treatment with a highly selective μ agonist, D-Ala^2-NMe-Phe^4-enkephalin-gly-ol, resulted in no inhibition. No labeling was seen on control cells, which express neither μ nor δ receptors. This indicated that nonspecific labeling by the α-Ids was low. However, it was surprising that when, under the same conditions, stereospecificity of labeling was probed with (+)-morphine, dextromethorphan, and dextrorphan the results were that only a subpopulation of cells remained labeled. That is, not all labeled cells in the unsynchronized population were being inhibited by (+)-morphine in the same way; some showed normal fluorescence, whereas others showed none. Hence, we decided to investigate this observation in more detail.

It has been found that NG108-15 neuroblastoma × glioma cells growing after synchronization by the isoleucine/glutamine deprivation method (7) show maximum specific DADLE binding in the S+G_2 phase (≈12–22 hr after release from the G_1 block) (8). It has been suggested that enkephalin receptor coupling to adenylate cyclase (presumably via a GTP-binding protein) is minimal during this phase but reaches a maximum during G_1 phase. The K_D for specific binding did not change with position in the cell cycle.

Tunicamycin is an antibiotic that is a hydrophobic analog of UDP-*N*-acetylglucosamine. It has been widely used as an irreversible inhibitor of *N*-linked glycosylation of proteins. The binding of opiates to membrane fragments from unsynchronized neurotumor cells after growth in the presence of tunicamycin has been reported, along with the accompanying effects due to the presence of guanine nucleotides, divalent cations, and sodium ions in the binding assay mixture (9). The experiments were done by incubating cell cultures in the presence of 0.015–0.5 μg/ml (≈0.02–0.6 μM) tunicamycin for 24 hr. The specific binding of opiates to tunicamycin-treated cells varied with cell line and concentration of the inhibitor. Approximately 50–75% inhibition of binding activity was achieved with concentrations on the order of 0.15–0.3 μg/ml. The combined data suggested that protein glycosylation is required for functional expression of receptors. It was found that incubation with tunicamycin resulted in cells that showed a subsensitivity to GTP and nonhydrolyzable GTP analogs in the radioassay mixture.

Experimental Procedures

Materials. DMEM, FBS, Earle's balanced salts, and the MEM Select-Amine kit were obtained from GIBCO. Sterile 10% FBS was dialyzed against Earle's salts for 7 days (changes every 48 hr) before use in the preparation of the isoleucine/glutamine-depleted medium. The preparation and properties of the α-Id antireceptor antibodies have been previously described (6). Opiate peptide agonists and enantiomers were obtained as gifts from the Office of Technical Assistance, National Institute on Drug Abuse. Tunicamycin (containing a mixture of isomers) and FITC-avidin were obtained from the Sigma Chemical Co.

Rabbit polyclonal α-Id raised in response to the monoclonal antimorphine IgG1 (line 10C3) was biotinylated as we described previously.[1]

Cell culture. The NG108-15 cell line was thawed and grown in regular DMEM, containing 10% FBS, for 3 days. On day 4, cells were transferred to Petri dishes containing glass 25-mm coverslips, washed with synchronizing medium, and left in synchronizing medium for 48 hr. At that time, cells were washed with regular 10% FBS-DMEM and left in 10% FBS-DMEM to begin synchronized growth.

Determination of the viability of the cells and mitotic indices.

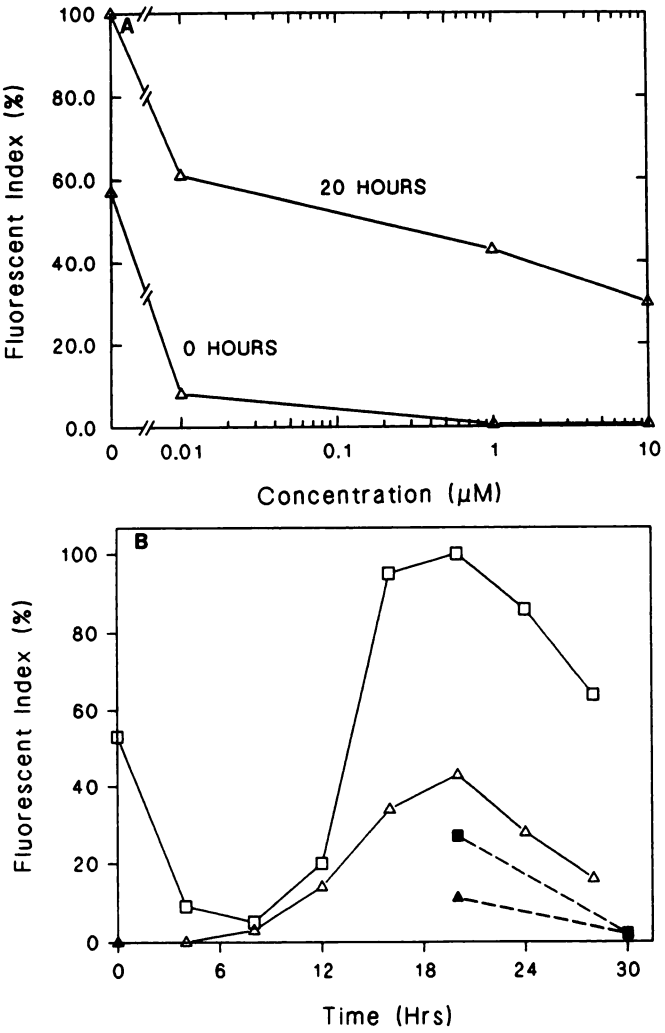

Fig. 2. A, Fluorescence index for live NG108-15 cells versus logarithm of the concentration of (+)-morphine at 0 hr and 20 hr after transfer of the cells from isoleucine/glutamine-deficient medium into 10% FBS-DMEM. Cells were labeled with FITC-avidin after incubation with biotinylated α-Id. B, Fluorescence index versus time for live NG108-15 cells transferred from isoleucine/glutamine-deficient medium into 10% FBS-DMEM at zero time. □, Fluorescence index, with labeling as in A; △, fluorescence index measured in the presence of 1 μM (+)-morphine, with labeling as in A; ■, fluorescence index for cells incubated in tunicamycin and then measured in the presence of 1 μM (+)-morphine, with labeling as in A; ●, fluorescence index of cells incubated in tunicamycin and then fixed, followed by labeling in the presence of 1 μM (+)-morphine.

After removal of the metabolic block, the cells were examined every 4 hr for 28 hr. The viability of the population of cells was determined by examining a sample coverslip after staining with trypan blue. We found there was essentially 100% viability at all times after removal of the block. One milliliter of medium containing cells was centrifuged for 15 min at 4000 rpm in an IEC clinical centrifuge; the supernatant was discarded and a drop of pellet was put on a slide to make a smear. The slide was left to dry, fixed in methanol for 5 sec, and stained in Giemsa stain (GIBCO) for 10 sec, followed by examination with an Olympus CO11 optical microscope at 100× using an oil immersion objective. At each time, 1500 cells were counted. The mitotic index was defined as the percentage of all cells that were in mitosis.

Examination of cells using phase contrast and fluorescence microscopy and determination of fluorescence index. In addition to the determination of mitotic index, the first two of the following experiments were done in parallel every 4 hr for 28 hr. The first experiment consisted of fluorescent antibody labeling of NG108-15 cells. Growth medium was removed from the glass coverslip in a Petri dish by washing with ice-cold Dulbecco's PBS (four times). The cells were then blocked on ice with ice-cold 5% goat serum in PBS for 5 min and stained with FITC-avidinated α-Id for 10 min. They were then washed four times with PBS and examined with an inverted TMD-EF Nikon microscope equipped with epifluorescence and Nomarski optics and a Nikon FE2 camera with an automatic exposure attachment. The second experiment consisted of labeling live NG108-15 cells with fluorescent antibody in the presence of 1 μM (+)-morphine, in order to examine stereospecificity. Medium-free blocked cells, prepared as described above, were stained with FITC-avidinated α-Id in the presence of (+)-morphine for 10 min, washed four times with PBS, and examined microscopically as described above. A third experiment, done separately, consisted of staining fixed NG108-15 cells with FITC-avidinated α-Id, in the presence or absence of 1 μM (+)-morphine, after 20 hr. Medium-free cells were fixed on ice in 3.7% formaldehyde for 1 hr, washed with PBS, blocked with 5% goat serum in PBS, and stained as described above. A fourth experiment consisted of fluorescent antibody-labeling live and fixed NG108-15 cells, incubated for 20 and 40 hr (after release from the metabolic block) in medium containing 0.05 μM tunicamycin, in the presence or absence of 1 μM (+)-morphine. The labeled cells were examined as described above.

To obtain the fluorescence index, 100 cells, counted using phase contrast optics, were scored for any observable fluorescence by each cell in the same field. The fraction that was fluorescent, expressed as a percentage of the total, is the fluorescence index. In the case of tunicamycin treatment, the fluorescence index controls were live or fixed cells grown for 20 or 40 hr, in the absence of tunicamycin, after removal of the block.

Results

Our results are summarized in Table 1 and Figs. 1–3. Fig. 1 shows that the cell population was substantially synchronized. Fig. 2A shows displacement curves of fluorescence index versus concentration of (+)-morphine at two positions in the cell cycle. Fig. 2B shows that the total and stereospecific [measured in the presence of 1 μM (+)-morphine] fluorescence indices are maximal at about 20 hr after release from the G_1 block, corresponding to the maximum in the mitotic index. In agreement with our previous results,[1] fluorescence labeling is completely displaced by 1 μM naloxone at any point in the cell cycle (data not shown). The displacement curve shows that at 20 hr the concentration of (+)-morphine necessary to lower the fluorescence index to its minimal level is ≈1 μM, whereas at 0 hr the corresponding concentration is ≤0.01 μM. Some of the details of fluorescent staining as a function of time after release from the block are shown in Fig. 2B, and the results are summarized in Table 1. Fig. 2B shows that, whereas 50% of the cells show fluorescent labeling at zero time, none of this is stereospecific. At the maximum achieved at 20 hr, approximately 100% of the cells show fluorescent labeling, but only ≈57% of this is stereospecific. Fig. 3 shows typical fluorescence microscopic fields at different positions in the cell cycle.

The fluorescence index for live NG108-15 cells grown in medium containing 0.05 μM tunicamycin, for 20 hr, was 27%, compared with 65% for fixed cells. The presence of 1 μM (+)-morphine during staining lowered the fluorescence index at 20 hr of incubation with tunicamycin to 11% for live cells and to 38% for fixed cells. There was almost no fluorescence after 40 hr (corresponding to a return to G_1 phase) of incubation with tunicamycin for either live or fixed cells. These results are also summarized in Fig. 2B and Table I. Thus, the basic observation is that treatment with 0.05 μM tunicamycin reduces fluores-

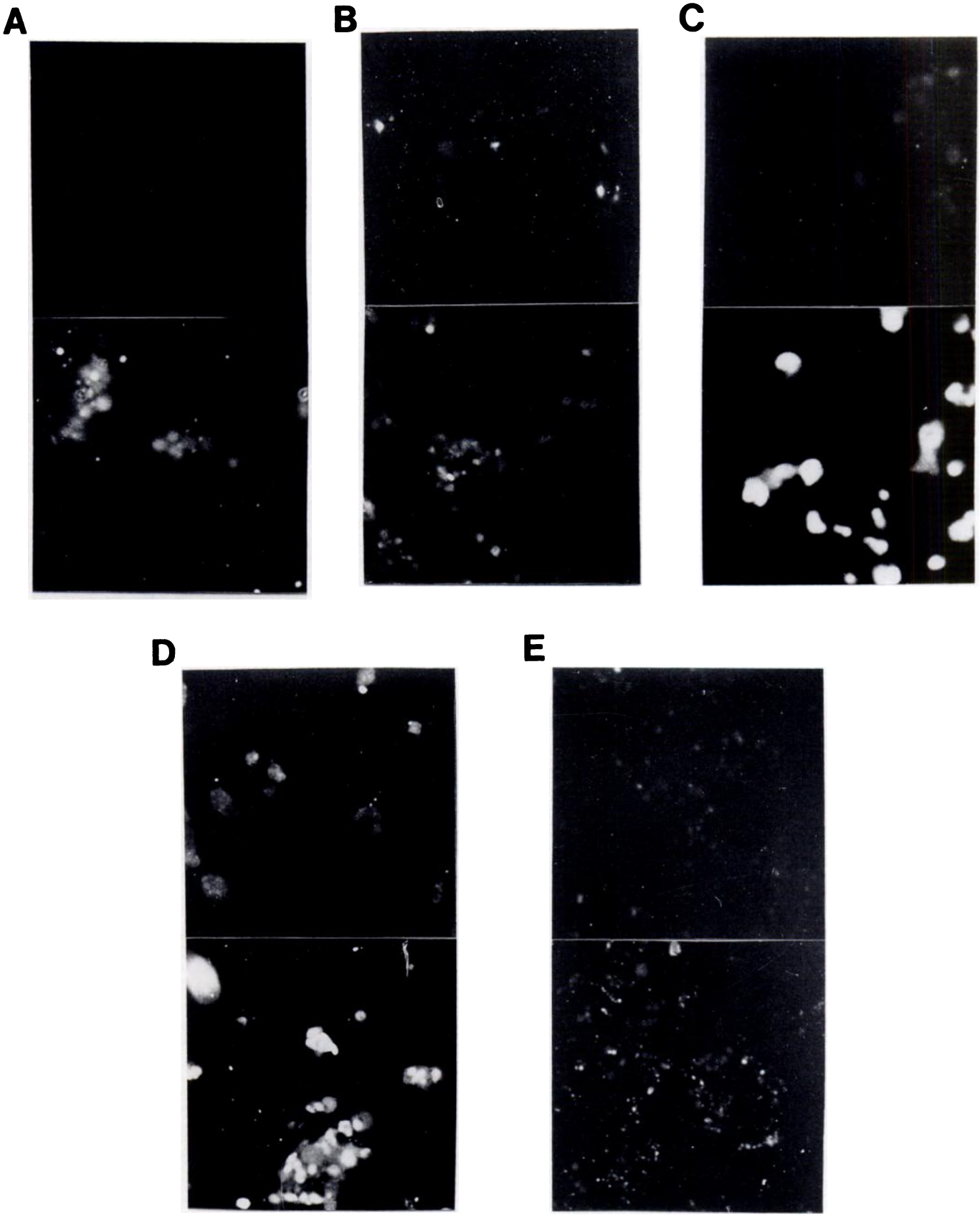

Fig. 3. Distribution of fluorescent staining on stained NG108-15 cells as a function of time after transfer from isoleucine/glutamine-deficient medium into 10% FBS-DMEM at zero time. Staining was done in the absence and in the presence of (+)-morphine (stereospecific fluorescence), as described in the legend to Fig. 2. Cells are live unless described otherwise. A, Time = 0 hr. *Lower*, total fluorescence; *upper*, stereospecific fluorescence. B, Time = 16 hr. *Lower*, total fluorescence; *upper*, stereospecific fluorescence. C, Time = 20 hr. *Lower*, total fluorescence; *upper*, stereospecific fluorescence. D, Time = 20 hr. *Lower*, total fluorescence (fixed cells); *upper*, stereospecific fluorescence of fixed cells. E, Time = 24 hr. *Lower*, total fluorescence; *upper*, stereospecific fluorescence.

cence indices for both live and fixed cells, and both specific and stereospecific components are affected.

Conclusions

We emphasize the distinction between the methods used here and ligand binding assays. We are assaying populations of cells that are being classified as either labeled or not labeled, depending on a visual threshold level. Typically, we find two subpopulations of cells; one is strongly labeled and the other has no visible fluorescence and, therefore, we can treat the phenomenon as all-or-none. In contrast, when a ligand binding assay is performed, a statistical average of binding activity is obtained from a mixture of cells or cell organelles. Thus, reduced binding activity of a ligand to its receptor can only be interpreted as an average decrease of binding activity normalized to quantities such as total number of cells or their total protein content.

The overall conclusions from our experiments are as follows. NG108-15 cells show different α-Id-labeled subpopulations, depending upon which stage of the cell cycle is being observed. The highest fluorescence indices occur between 16 and 24 hr and are ≈100% at 20 hr in the absence of competing drugs. A photobleaching effect has been noticed, which was not found in our previous work on the distribution of opiate receptors in a nonsynchronized culture of NG108-15 cells.[1] Labeling of fixed cells showed ≈100% bright fluorescence at 20 hr, without rapid photobleaching.

Labeling in the presence of (+)-morphine was done to study the stereospecificity of α-Id binding to the δ opiate receptor. The results show that the highest fluorescence indices for live cells in the presence of (+)-morphine occur from 16 to 24 hr, with a maximum stereospecific fluorescence index of ≈43% at 20 hr in the S+G_2 phase. Therefore, at any point in the cell cycle, the population of cells consists of a percentage bearing opiate receptors that are labeled specifically by the α-Ids (naloxone reversible), of which a subpopulation are also stereospecifically labeled by the antibody [not reversible by (+)-morphine]. Fixed cells in the presence of (+)-morphine show approximately double the fluorescence index of live cells at 20 hr. In the synchronized culture, live cells show patching and capping of the fluorescent label at all times, whereas fixed cells have uniformly distributed fluorescence. This observation is in agreement with our previous work on unsynchronized cells.[1]

We observe minimum stereospecific receptor recognition by the labeled α-Ids in the G_1 phase. During this time, it has been found that the δ receptor is maximally coupled to adenylate cyclase (8). However, even at its maximum during S+G_2 phase, stereospecific binding represents only 43% of the total number of cells that are fluorescent. Our results show that even in a synchronous population there is a mixture of nonstereospecific and stereospecific binding to membrane-bound receptors by an antireceptor antibody that has been shown to exhibit 100% specificity for the receptors. In an asynchronous population, the stereospecific binding of this antibody becomes a small fraction of the specific binding. Consequently, the implication is that either different macromolecules exhibit the two modes of binding or one binding site is converted to another by cell cycle-dependent intermolecular interactions. In a strictly structural sense, this phenomenon would be quite unique. The majority of total opiate binding in untreated brain homogenates is stereospecific. This can be altered by a variety of enzymes and other reagents (10). Disregarding enzymes that can destroy stereospecific binding by destroying the binding protein, it is significant that the other reagents act on phospholipids.

The effects on fluorescence index when the cell population is treated with tunicamycin provide further insight into the nature of opiate receptors on NG108-15 cells. We find decreases in fluorescence indices after tunicamycin treatment that are approximately the same as the previously observed percentage decreases in binding activity (9). Under the conditions in both of these experiments, *de novo* glycosylation of proteins is reduced by ≈80%. Addition of (+)-morphine to these treated cells decreases the fluorescence index (27% to 11%) by almost the same factor as observed when (+)-morphine is added to untreated cells (100% to 43%). Furthermore, in other experiments with nonsynchronized populations (data not shown), we have observed the same changes as just described after tunicamycin treatment. We, therefore, conclude that only glycosylated receptors are labeled, that these are present on a subpopulation of cells, and that these are labeled stereospecifically.

The photobleaching results imply that the rate of translational diffusion of the fluorescent labeled antibodies bound to opiate receptors changes several times during the cell cycle. At the start of G_1 rapid bleaching of total (nonstereospecific) fluorescence was observed. Near the beginning of S+G_2 (16 hr) stereospecific photobleaching was rapid but that for total fluorescence was slow, and near the end (24 hr) both total and stereospecific fluorescence showed rapid photobleaching. We can offer no explanation for these observations at this time. However, membrane events involving opiate receptors and their ability to recognize ligands are time dependent and quite complex.

A recent review of efforts to characterize the opiate receptor macromolecular apparatus (11) has commented on the lack of stereospecificity exhibited by the solubilized preparations in which this property has been examined. Our results imply that, even if the native opiate receptor conformation is preserved in the solubilization process, a little more than half of the receptors should exhibit both specificity and stereospecificity when probed with this antibody. The remainder would show only specificity. It remains to be seen whether these phenomena may be generalized to antibody probes derived with methods other than those yielding α-Ids.

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