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Peripheral-Type Benzodiazepine Receptors in the Regulation of Proliferation of MCF-7 Human Breast Carcinoma Cell Line

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ABSTRACT. Peripheral-type benzodiazepine receptors (PBR) have been implicated in cell proliferation. The aim of the present study was to test the effect of the PBR ligands PK 11195 and Ro 5-4864 and the central-type benzodiazepine receptor ligand clonazepam on breast carcinoma cell proliferation, using [^{3}H] thymidine incorporation. We then carried out a study to identify where the PBR-specific ligands Ro 5-4864 and PK 11195 act in the cell cycle, using flow cytometric analysis. We found PBR expression in the malignant breast cancer tumors, representing various levels of estrogen and/or progesterone receptors, as well as in the MCF-7 breast carcinoma cell line. PK 11195 and Ro 5-4864 inhibited cell proliferation at concentrations of 10^{-5} to 10^{-4} M, while clonazepam (the central-type benzodiazepine receptor-specific ligand) had no effect. In this same concentration range, PK 11195 and Ro 5-4864, in contrast to clonazepam, induced an accumulation of MCF-7 cells in both the G_0-G_1 and G_2-M phases of the cell cycle. The present study demonstrates that PBR ligands play a role in regulating cell proliferation in the human breast carcinoma cell line MCF-7. BIOCHEM PHARMACOL **58**;2:273–278, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. Ro 5-4864; PK 11195; MCF-7; cell cycle; cell proliferation; peripheral-type benzodiazepine receptor

The PBR‡‡ is an intracellular protein receptor located mainly on the outer mitochondrial membrane [1], although other localizations have been reported [2–4]. The PBR is composed of three subunits: the isoquinoline binding protein, the voltage-dependent anion channel, and the adenine nucleotide transporter [5]. These receptors can bind the benzodiazepine ligand Ro 5-4864 (4′-chlorodiazepam) [6] and the non-benzodiazepine ligand PK 11195 (an isoquinoline carboxamide derivative) [7]. It has been reported that PK 11195 can bind to different mammalian species with nanomolar affinity. In contrast, although Ro 5-4864 binds rodent PBR with nanomolar affinity, it binds to human PBR with 2-fold lower affinity [8, 9]. A number of functions have been suggested for PBR, for example in steroidogenesis [10–12], cell proliferation [13], and cell respiration [14]. However, its true function has not been conclusively shown. As an indication of PBR involvement in cell proliferation, we and others have found increased PBR density in different tumors and transformed cell lines [15–18]. Specifically, we have observed alterations in the normal pattern of PBR densities in tissue cancers in human, e.g. ovarian carcinoma [16] and adenocarcinoma of the colon [17]. Additionally, it has been reported that PK 11195 inhibits cell proliferation of the C6 glioma [19] and adenopituitary [20] cell lines.

Breast cancer, a particularly deadly women's malignancy, has seen no significant improvement in its treatment over the last 25 years [21]. Breast cancer is known to fall into different categories, depending upon its hormonal responsiveness. The most aggressive of these tumors are those that have totally lost steroid receptor responsiveness [22]. In the current study, we focused on a human breast carcinoma cell line to see if there was a correlation between cell replication and the signal transduction pathway. Using the hormone-dependent human breast carcinoma cell line MCF-7 [23], we found that PBR-specific ligands did affect cell proliferation in this breast cancer cell line at the level of

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‡‡ *Abbreviations:* PBR, peripheral-type benzodiazepine receptor(s); FACS, fluorescence-activated cell sorter; CBR, central-type benzodiazepine receptor(s).

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thymidine incorporation, as well as changing its cell-cycle profiles.

MATERIALS AND METHODS

Materials

The pharmacological agents used were PK 11195 (Pharmuka Laboratories), Ro 5-4864 and clonazepam (Hoffmann-La Roche). Stock solutions and dilutions of the drugs (10^{-2} M) were prepared in DMSO. Ham's F-12 medium containing L-glutamine, fetal bovine serum, penicillin/streptomycin, and trypsin were purchased from Biological Industries. Propidium iodide was obtained from Sigma Chemical Co. [*Methyl*-^{3}H] thymidine was obtained from Rotem Industries Ltd. Lumax was purchased from Lumac.

Preparation of Membranes for Binding Assay

MCF-7 cells and primary human fibroadenomas were homogenized separately in 50 mM phosphate buffer (pH 7.4) at 4°, using a Brinkmann polytron (setting 10) for 30 sec. The homogenate was centrifuged at 49,000 *g* for 20 min, and the pellet was homogenized and spun again as above. The final pellet was suspended in 50 mM ice-cold phosphate buffer (pH 7.4) to achieve about 100 to 250 μg protein per mL and used for binding assay.

[^{3}H] PK 11195 Binding Assay

In order to detect the density and affinity of [^{3}H] PK 11195 to MCF-7 and primary human fibroadenomas, we conducted saturation curves of [^{3}H] PK 11195 binding. Membranes (40–100 μg protein) were incubated with 25 μL [^{3}H] PK 11195 (0.4–12 nM, final concentration) in the absence (total binding) or presence (non-specific binding) of 75 μL unlabeled PK 11195 (1 μM, final concentration) in 500 μL final volume. After incubation for 60 min at 4°, samples were filtered under vacuum over Whatman GF/B filters and washed three times with 50 mM ice-cold phosphate buffer (pH 7.4). Filters were placed in vials containing 4 mL xylene/Lumax (3:1, v/v) and counted for radioactivity after 12 hr. Protein concentrations were determined according to the method of Lowry *et al.* [24] using BSA as standard.

Cells and Cell Culture

MCF-7 cells were maintained in Ham's F-12 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM glutamine, and human insulin (100 IU) containing 10% fetal bovine serum at 37° in a humidified atmosphere of 5% CO_2/95% air. Cells were passaged every 7–8 days and seeded at 1×10^6 per plate in a 25-cm^3 flask.

Cell Proliferation Assays

[^{3}H] THYMIDINE INCORPORATION ASSAY. Cells were cultured in 24-well plates (5×10^4 per well) in 0.5 mL medium. One hour later, drugs were added to the cells at final concentrations of 10^{-8} to 10^{-4} M for 0, 18, 26, and 30 hr. [*Methyl*-^{3}H] thymidine at a final concentration of 1 μM was also added to the cells. After the incubation was terminated at the appropriate hour(s), the cells were washed 3 times with 1 mL of ice-cold PBS (pH 7.4) to remove the unincorporated thymidine. DNA was precipitated by the addition of 0.5 mL of iced 10% trichloroacetic acid solution. After 1 hr, the trichloroacetic acid solution was removed, and the cells were solubilized overnight in 0.5 mL of 1 M sodium hydroxide. The next day, the cells were counted in scintillation fluid containing xylene/Lumax (3:1, v/v).

CELL COUNTING. Cells were plated in 24-well dishes (5×10^4 cells per well), trypsinized, and counted visually using a hemocytometer, at time points parallel to thymidine incorporation.

Flow Cytometry

Analysis by FACS (FACScan, Becton Dickinson) was performed following the method of Vindelov and Christensen [25]. The intensity with which a cell's propidium iodide-stained nucleus emits light is directly proportional to its DNA content.

Cells were cultured in 12-well plates (5×10^5 per well). One hour later the cells were exposed for 24 hr to different drug concentrations dissolved in DMSO, ranging from 10^{-8} to 10^{-4} M. Briefly, cells were trypsinized, washed twice in ice-cold PBS, centrifuged at 800 *g* for 5 min, and resuspended in 0.5 mL PBS. Propidium iodide (20 μL, 2.5 mg/mL) was added, followed by the addition of 10 μL 20% Triton X-100 prior to gentle mixing. FACS analysis was performed on a Becton Dickinson FACScan apparatus collecting 5000 cells, using the CellFit cell-cycle test.

Statistical Analysis

The proportion of cells in the different phases of the cell cycle was determined using DNA histogram analysis within the CellFit program. Data are expressed as the mean percentage of control ± SE of multiple replicates for each time period and drug concentration. Statistical analysis was determined by ANOVA with the Dunnett post hoc test for Scatchard analysis and the [^{3}H] thymidine incorporation study and ANOVA with the Newman-Keuls post hoc test for FACS analysis. Results of the Scatchard analysis are expressed as means ± SE.

RESULTS

PBR in Human Breast Cancer

The hormone-dependent human breast cancer cell line MCF-7 bound PK 11195 (Fig. 1). Representative Scatchard analysis indicated a maximal number of binding sites of approximately 4000 fmol/mg protein and a K_d value of 2.9

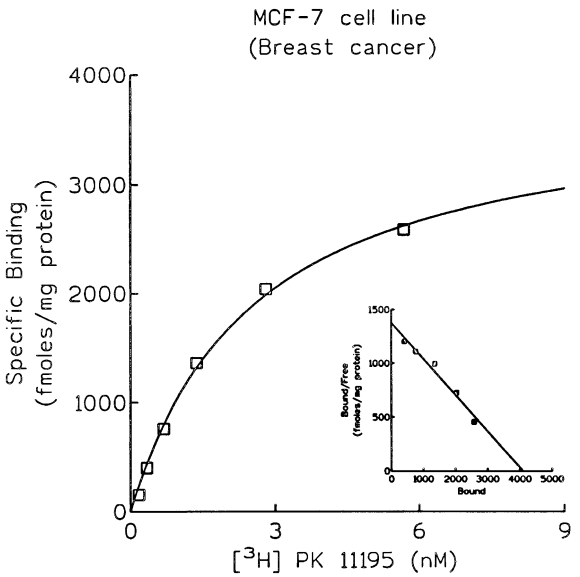

FIG. 1. Saturation curve and Scatchard analysis of [^{3}H] PK 11195 binding by the human breast cancer cell line MCF-7.

nM. For this breast carcinoma cell line, the maximal number of binding sites was approximately 3-fold greater than that found for the primary fibroadenoma tissue (1371 ± 139 fmol/mg protein).

Effect of PBR Ligands on MCF-7 Cell Proliferation

To measure the effects of benzodiazepine ligands on the proliferation of the MCF-7 breast carcinoma cell line, the incorporation of [^{3}H] thymidine was followed (Fig. 2). Changes in cell proliferation, as measured by [^{3}H] thymidine uptake, were also confirmed by the visual counting of cells. We found that the PBR ligands PK 11195 and Ro 5-4864 (10^{-8} to 10^{-4} M, final concentrations) induced inhibition of cell proliferation, in a concentration-dependent manner. More specifically, these ligands produced a significant inhibition (>50%; $P < 0.001$) of [^{3}H] thymidine uptake within the micromolar concentration range (7×10^{-5} M for PK 1195 and 7×10^{-6} M for Ro 5-4864) at different time points (18, 26, and 30 hr). It should be noted that at zero time no incorporation was observed in the presence or absence of the ligands. Therefore, these counts were used as baseline values.

Clonazepam, a CBR-specific ligand, did not affect [^{3}H] thymidine incorporation at concentrations of 10^{-8} to 10^{-4} M at the different time points (0, 18, 26, and 30 hr) (Fig. 2). This reinforced our understanding that we were studying PBR-specific effects.

As PBR-specific ligands affected MCF-7 cell proliferation, we then set out to identify at which point in the cell cycle these drugs might exert their effect. Determination of the cellular DNA content was carried out using flow cytometric analysis (Fig. 3). The results are expressed by showing the percentage of cells present in each of three cell-cycle categories, i.e. the cell-cycle divisions corresponding to cell growth (G_0-G_1 phase), DNA synthesis (S phase), and cell growth and mitosis (G_2-M phase). Each result represents the mean percentage of its respective total cell number for at least seven experiments.

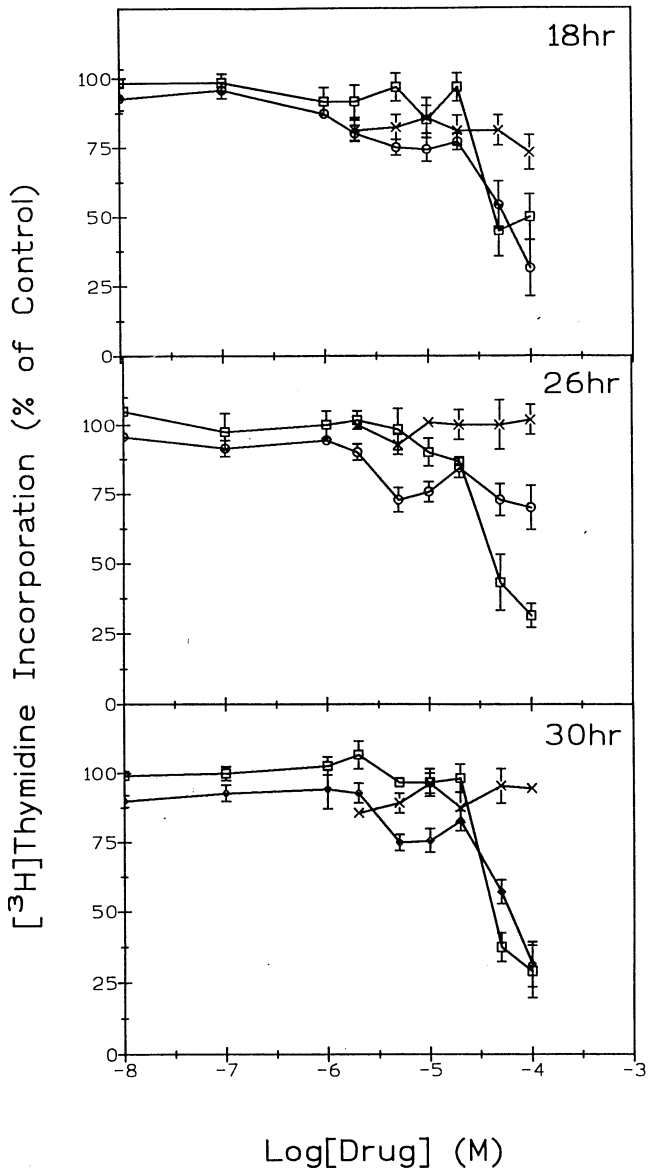

FIG. 2. Inhibition of MCF-7 cell proliferation by PBR-specific ligands. Proliferation was measured as [^{3}H] thymidine incorporation into cells incubated for 18, 26, or 30 hr with various concentrations of PK 11195 (□), Ro 5-4864 (○), and clonazepam (×). Data are expressed as the mean percentage of the control ± SE of 14 experiments. Statistical significance was evaluated by ANOVA (Dunnett test), and the results are detailed in the text.

At zero time, no significant change was observed at concentrations of 10^{-6} to 10^{-4} M of all ligands tested during the various cell-cycle phases compared to control. At 24 hr, we found that the PBR-specific ligands PK 1195 and Ro 5-4864 induced a significant dose-dependent change in the MCF-7 cell-cycle profile (Fig. 3, B and C). That is, both PK 1195 and Ro 5-4864 induced an accumulation of MCF-7 cells in both the G_0-G_1 and the G_2-M

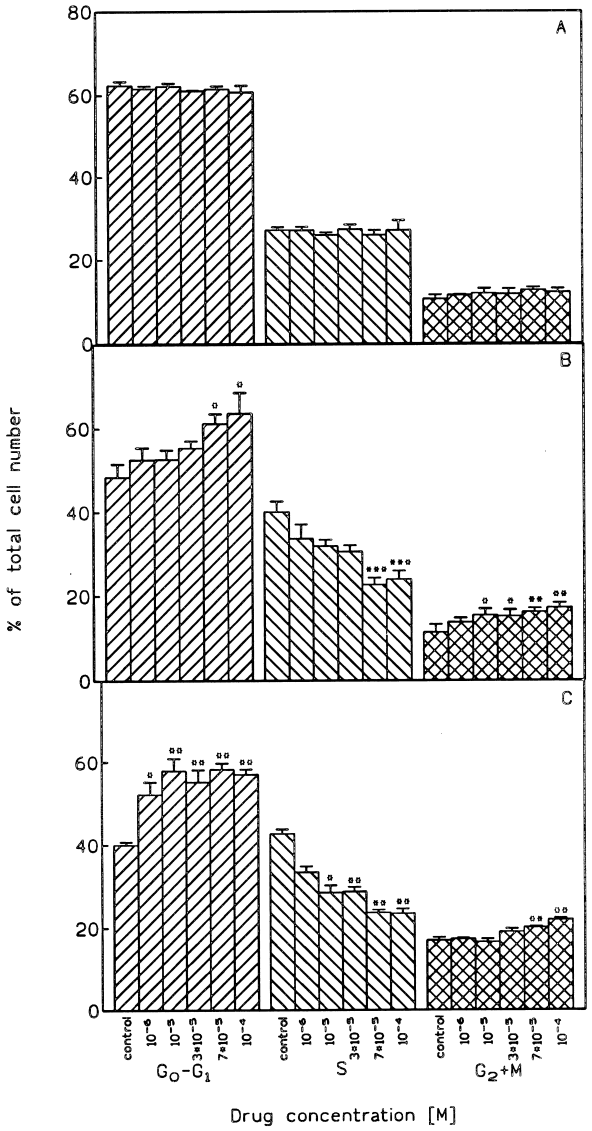

FIG. 3. Effects of all ligands on the MCF-7 cell-cycle profile, tested at zero time (A), and the PBR ligands PK 11195 (B) and Ro 5-4864 (C) at 24 hr. It should be noted that clonazepam (control) gave a similar profile at zero time and at 24 hr, and that both of these profiles resembled (A). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control.

phases. To complement this, a coordinate decrease in the percentage of total cells in the S phase was observed. In contrast, when the CBR ligand clonazepam was used over 24 hr, no significant change in the percentage of MCF-7 cells was observed between the various stages of the cell cycle (data not shown).

DISCUSSION

In the current study, we observed an elevation of PBR density in the MCF-7 human breast carcinoma cell line compared to human breast fibroadenoma. These observations may point to underlying mechanisms of PBR involvement in the development of breast cancer.

The [^{3}H] thymidine incorporation results obtained in the current study demonstrated a significant antiproliferative effect of the MCF-7 cells after treatment with the PBR-specific ligands PK 11195 and Ro 5-4864 at micromolar concentrations. This was a true antiproliferative effect, as the effect of PBR-specific ligands on MCF-7 cells was also confirmed by the physical counting of the cells (data not shown). This effect was observed followed 18, 26, and 30 hr of treatment. Interestingly, the effect of Ro 5-4864 was one order of magnitude more potent than PK 11195 in inhibiting [^{3}H] thymidine incorporation into the cells. That is, where PK 11195 caused a significant inhibitory effect of the MCF-7 cells at 7×10^{-5} M, Ro 5-4864 already caused an inhibitory effect at 7×10^{-6} M.

These antiproliferative effects can be specifically associated with PBR, as there was no significant dose-dependent change in the presence of the CBR-specific ligand clonazepam, which has a structure very similar to that of the PBR ligand Ro 5-4864. Wang *et al.* [13] were the first to show a strongly positive correlation between the affinity of PBR-specific ligands and their effect on cell proliferation of thymoma cells. This raised the possibility of PBR involvement in the control of cellular growth and proliferation of malignant cells. Similar effects have also been observed in Chinese hamster lung cells [26], C6 glioma cells [19], rat pituitary tumors [20], and Swiss 3T3 cells [27].

Accumulating data indicate that the antiproliferative effect of PBR ligands in malignant cell lines occurs at micromolar concentrations [13, 19, 20, 26]. As PBR ligands bind to their known receptor(s) with nanomolar affinity, it has been suggested that these ligands might function via different binding sites associated with "low- and high-affinity" receptors [19, 20]. We have confirmed the presence of these low-affinity peripheral benzodiazepine binding sites in rat, calf, rabbit, and guinea pig [28]. We know from other systems that there might be a few orders of magnitude difference between the affinity of ligand binding to their receptors and their physiological effect [29].

It is known that calcium can cause stimulation of cell proliferation and that high cytoplasmic levels of calcium are a prerequisite for mitosis. Therefore, it has been suggested that the antiproliferative effect of PBR ligands might be connected with the blockade of voltage-dependent anion channels [19]. In another study, it was found that calcium chloride could reverse inhibition of rat pituitary tumor cell proliferation caused by PBR-specific ligands *in vitro* [20]. Moreover, Cantor *et al.* [30] showed that nifedipine, a dihydropyridine calcium channel blocker, displaces Ro 5-4864 from membranes of the heart, kidney, and brain. More detailed structure–function studies are therefore needed.

After showing the antiproliferative effect of PBR-specific ligands on the MCF-7 cell line, we initiated experiments to better characterize the location within the cell cycle where PBR ligand binding might act. We found that there was a dose-dependent change in the restriction of the cells throughout the cell cycle. After 24 hr of treatment with the

PBR-specific ligand, there were changes in accumulation of cells in the G_0-G_1 and G_2-M phases. This indicated that the cells stayed in these phases for a longer time. As for the S phase, there was a coordinate decrease in the percentage of the cells remaining following 24 hr of treatment. This indicated that the cells stayed in this phase for a shorter time during each cell cycle. Again, this was a PBR-specific effect, as the CBR-specific ligand clonazepam caused no changes at all.

In a study on Chinese hamster lung cells, Camins *et al.* [26] found that not only did Ro 5-4864 and PK 11195 have an effect on these cells' cell-cycle profiles, but also that the mixed PBR/CBR drug diazepam also inhibited cell proliferation. The proliferation-inhibitory effect of diazepam may have been due to the PBR component of the drug. Moreover, only in the latter study was an increase in the accumulation of lung cells in the G_2-M phase of the cell cycle measured in response to PBR ligand [26]. This difference between lung cells and breast carcinoma cells may highlight a tissue-specific difference in PBR signal transduction. In cultured brain cells, clonazepam has also been found to inhibit [^{3}H] thymidine incorporation at very high (micromolar) concentrations [20, 31]. The fact that this was not found in the present study for breast carcinoma cells is further reinforcement for the tissue specificity of PBR function.

In the cell-cycle experiments, as in the [^{3}H] thymidine incorporation studies, we found that Ro 5-4864 was one order of magnitude more potent than PK 11195. These changes might have been due to a specific influence of PBR ligands on the antiproliferative effect in MCF-7 cells. An explanation of this difference in potency between these two drugs may be that Ro 5-4864 binds to a different site or conformation of the receptor. An alternate explanation may be that, although PK 11195 binds with higher affinity to PBR of MCF-7 cells compared to Ro 5-4864, the latter ligand may have a higher biological efficacy in the inhibition of MCF-7 cell proliferation.

The changes in cell-cycle profiles seen in the current study suggest that both of the PBR-specific ligands act not only in a similar way, but also at both major restriction points of the cell cycle (at the G_2-S and G_2-M junction points in breast carcinoma cells). It would be of interest to determine if this is a direct or secondary effect of PBR-specific ligand treatments. Moreover, it would be of interest to study the signal transduction pathway that mediates the PBR cell-cycle changes at the functional level (knockout studies) as well as at the structural level.

A number of groups of investigators have studied the PBR at the structural and functional levels in different tissues and cell types. While differences in effects of the PBR on functions such as cell proliferation in various tissues are now being found, resolution of the primary role of this receptor is still not understood. As steroidogenesis is not found in the MCF-7 cell line, although it strongly expresses PBR, alternate tissue-specific primary functions may be relevant for this gene's function. In the final analysis, the role and regulation of this increasingly interesting gene still requires further investigation.

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