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Modulation of Nb2 cell mitogenesis by peripheral benzodiazepine ligands

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The University of Arizona, 1989
Modulation of Nb2 Cell Mitogenesis by Peripheral Benzodiazepine Ligands

by

Kevin Edward Gerrish

A Thesis Submitted to the Faculty of the COMMITTEE ON PHARMACOLOGY AND TOXICOLOGY (Graduate) In Partial Fulfillment of the Requirements For the Degree of MASTER OF SCIENCE WITH A MAJOR IN TOXICOLOGY In the Graduate College THE UNIVERSITY OF ARIZONA 1989
STATEMENT BY AUTHOR

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ACKNOWLEDGEMENTS

First, I must thank my adviser Dr Hugh Laird and my committee members Dr David Nelson and Dr Charles Putnam for their help and support in writing this thesis. I would also like to thank John Barr and Tony Parola for their critical perspective of my work as well as those rip roaring poker nights. Special thanks must go to Patricia Bauman whose love, understanding, and encouragement helped me through the process of my research and writing of this thesis.

This thesis is dedicated to my family who have supported me in everything I have done. Without that support none of this would have been possible.
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ABSTRACT

In this study, we investigated the effects of the peripheral benzodiazepine ligands, Ro5-4864 (putative agonist) and PK 11195 (putative antagonist) on prolactin stimulated mitogenesis in Nb2 cells. Ro5-4864 and PK 11195 at 10^{-9} M maximally enhanced prolactin stimulated mitogenesis. At 10^{-6} M Ro5-4864 inhibited prolactin stimulated mitogenesis. Clonazepam, a ligand for the central benzodiazepine receptor had no effect on mitogenesis. Interaction studies were undertaken to determine if Ro5-4864 and PK 11195 act on the same site. The ability of each ligand to enhance the mitogenic action of prolactin was blocked by a 10^{-6} M concentration of the other ligand. Finally, simultaneous addition of 10^{-9} M of the ligands resulted in no additive effect over each ligand alone. These data show that peripheral benzodiazepine ligands modulate prolactin-stimulated mitogenesis and suggests they interact at the same binding site.
INTRODUCTION

Use of Cultured Cells in Research

The ability to grow cells in culture has provided science with a powerful research tool. It allows for the study of biological and biochemical behavior of cells and their response to factors in their environment, such as hormones and growth factors (Sato and Ross, 1979; Sato et al., 1982). The use of isolated cultured cells has simplified the investigation of biological events that take place in the whole animal. Cells may be watched under a microscope and monitored regularly which allows for a quantitative estimate of viable cells to be determined. Cultured cells offer a homogeneous population of cells growing in a constant environment, this environment may be changed, within limits, to determine effects on growth. Normal cells usually have only a limited lifespan in vitro, cultured cancer cells can often be maintained for indefinite periods, and some lines have been propagated in continuous culture for decades (Pollack, 1981). Much of the cancer research today involves the study of the biochemistry of cell proliferation and mechanisms by which compounds may affect it. In these type of studies the researcher can apply compounds that are being tested in known concentrations, as well as known time periods. The specific growth response in the presence of these compounds can be measured over a short time period and significant results can be obtained with very few cells.
History of the Nb2 Node Lymphoma Cell

Nb rat lymphoma cells were first described by Robert L. Noble in rats that had been implanted with estrogen pellets (Noble et al, 1975). These tumors could be maintained by serial transplantation of the cells in estrogenized Noble rats. Since there are many complexities inherent in studies with tumors in vivo, an isolated cell system for continuous culture was needed. In 1980 Gout et al. defined the conditions necessary for continuous culture of Nb2 cells. He found that these cells grew readily in Fischer's medium supplemented with 10% fetal calf serum (FCS), 10% horse serum (HS), 10^-4 M 2-mercaptoethanol (2-ME), penicillin (50 U/ml), and streptomycin (50 ug/ml). The cells were maintained in an atmosphere of 95% Air/5% CO2 at 37°C. These cells could be induced to enter a stationary growth phase by omitting FCS, and could be used to evaluate "lymphoma cell growth-promoting activity" of hormones and other factors (Noble et al, 1980).

Hence, studies were undertaken to determine what factor(s) was important to stimulate these cells to grow. Many different types of serum were tested for their ability to induce Nb2 cell growth. It was determined that the activity of the sera paralleled the ability to stimulate growth. Normal rat serum was moderately active, estrogenized rat serum had 10x more activity and serum from rats bearing pituitary tumors was extremely active (1:10^5 dilution). Serum from hypophysectomized rats was devoid of growth activity even if the animal was estrogenized. This data showed: (1) that growth of these cells was stimulated by factors in peripheral blood which were subject to control by the pituitary and (2) that estrogen stimulated growth indirectly, through the pituitary (Noble et al,
1980). It was finally determined that stationary cultures could be induced to grow by addition of the pituitary hormone, prolactin (ovine and rat); with concentrations as low as 10 pg/ml having an effect (Gout et al, 1980). Other pituitary hormones or estrogens had little or no effect. These cells are now used as a specific and sensitive bioassay for lactogenic hormones, which is comparable with radioimmunoassay (Tanaka et al, 1980).

Nb2 cells possess receptors (approximately 12000 per cell) that bind specific lactogenic hormones such as prolactin and growth hormone (Shiu et al., 1983). Receptor binding kinetics are biphasic i.e. they saturate at 1 hour (37°C) followed by a rapid decline. The affinity of these receptors for lactogens is approximately 20 fold higher than any other cell types. Maximal growth can occur when only 35% of the receptors are occupied suggesting the presence of spare receptors (Shiu et al., 1983). Divalent, but not monovalent antigen binding fragments (Fabs) abolish the prolactin response and mimic prolactin action when added alone (Shiu et al., 1983). This suggests that the antibodies or hormone initiate a response by cross-linking that receptor and that entry of the prolactin molecule, or fragments of it into the cell is not necessary for its biological action.

The lactogen receptor on the Nb2 cells may also prove to be markedly different than lactogen receptors found in other organs, such as the liver. Webb and Wallis (1988) found that the apparent molecular weight (Mr) of the labelled receptor protein in Nb2 cells is greater than that of rat liver membranes. The Nb2 cell receptor appears to exist as a disulphide-linked oligomer whereas the receptor in the rat liver membrane does not. It is likely that possession of this
unusual receptor underlies the cell's dependence on lactogenic hormones for growth and that it may be involved in the malignancy of this line.

Nb2 cells are also useful for studying biochemical events involved in cellular mitogenesis. Growth arrested cells incubated in medium without FCS displayed a decreased rate of DNA synthesis as well as decreased ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (SAMD) levels (Richards et al., 1982). Within a few hours after the addition of ovine prolactin, ODC and SAMD levels rapidly increased followed by a resumption of DNA synthesis and eventually cell division. These findings suggested that time related events set in motion by addition of prolactin to the stationary cultures reflect the activation of cells in the G0/G1 phase and their subsequent passage through the cell cycle (Richards et al., 1982).

Other studies have been carried out in order to further define the possible sequence of events that occur when prolactin interacts with the Nb2 cell. Offenstein and Rillema (1987) determined that ongoing phospholipase activity is essential for prolactin to express mitogenic actions in the Nb2 cell. Russell et al. (1987) also found that quinacrine, an inhibitor of phospholipase A2 and C activities, inhibited prolactin-stimulated mitogenesis in Nb2 cells. A large number of cellular processes may be switched on through phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5 bisphosphate. Two products from this hydrolysis, diacylglycerol and inositol trisphosphate regulate the two parallel signal pathways that switch on a large number of cellular responses, which includes mitogenesis (Berridge, 1984).
Diacylglycerol, is known to stimulate protein kinase C (Takai et al., 1982), while inositol trisphosphate induces release of intracellular calcium stores (Streb et al., 1983; Burgess et al., 1984; Joseph et al., 1984). The calcium that is released reacts with a calmodulin dependent kinase that also regulates cellular responses. Buckley et al. (1986) studied two cellular responses believed to be regulated by this dual pathway, prolactin-stimulated ornithine decarboxylase induction and cellular proliferation in Nb2 node lymphoma cells. They found that in the presence of a protein kinase C antagonist, polymyxin B, a calmodulin inhibitor, R24571 (Geitzen et al., 1981) and tamoxifen, an inhibitor of both enzymes (Lam, 1984; O'Brian et al., 1985) prolactin-stimulated ornithine decarboxylase induction and proliferation were inhibited. In addition, calcium chelation or cation channel antagonism also inhibited both of these responses. These data suggested that protein kinase C activation and calcium mobilization are requisite events for the prolactin-stimulated induction of ornithine decarboxylase as well as cellular proliferation in Nb2 node lymphoma cells.

The diacylglycerol limb of the signal pathway may also be responsible for regulating the activation of a neutral Na+/H+ exchanger which may play an important role in growth control (Moolenaar et al., 1983). Too et al. (1987a) identified a Na+/H+ exchanger that was stimulated by 12-O-tetradecanoyl-phorbol-13-acetate (TPA), a phorbol ester known to activate protein kinase C (Castagna et al., 1982; Nishizuka, 1984). They also observed that 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine dihydrochloride (H-7) a selective inhibitor of protein kinase C inhibited prolactin-stimulated mitogenesis as well as
the Na+/H+ exchange. In another study Too et al. (1987b) found that this response was specific for stimulation by prolactin since rat growth hormone which is not mitogenic did not have any effect. Taken together with the previously described data strongly suggests that protein kinase C is a very important link in the possible sequence of events involved in prolactin signal transduction.

Phosphoinositide turnover may also lead to stimulation of cyclic AMP, which is stimulated by prostaglandins released from the phosphoinositide turnover (Rozengurt et al., 1983). Buckley et al. (1986) found that 8-bromo-cyclic AMP, a cyclic AMP analog inhibited prolactin-stimulated mitogenesis in the Nb2 cells. They suggested that cyclic AMP may not be positive modulator of Nb2 cell mitogenesis. Several groups have explored this possibility in more detail. Larsen et al. (1988) and Pines et al. (1988) found that cholera-toxin which ADP ribosylates a stimulatory G-protein (Gs), inhibits prolactin-stimulated Nb2 cell mitogenesis in a dose dependent manner. ADP-ribosylation of a Gs leaves it "turned on" so high levels of cyclic AMP would accumulate, a possibility which Pine's group investigated. They found that this was indeed the case since the cells treated with cholera toxin had four times the cyclic AMP that was found in the control cells. They also supported their hypothesis by using dibutyryl cyclic AMP a non-hydrolyzable cyclic AMP analog and forskolin an activator of adenylate cyclase which produced the same result, inhibition of mitogenesis. However, both groups found that phorbol esters could counteract these effects suggesting the presence of a cyclic AMP independent pathway. Protein kinase C is a non cyclic AMP dependent kinase, providing more evidence which implicates protein kinase C participation in the control of Nb2 cell mitogenesis.
Peripheral Benzodiazepine Receptor

The anxiolytic and anticonvulsant activity of benzodiazepines has been attributed to binding of these compounds to a receptor coupled to the gamma-aminobutyric (GABA)/chloride ionophore complex in the central nervous system (Costa et al., 1975; Costa and Guidotti, 1979). A distinct population of diazepam binding sites has been described in peripheral tissues (Braestrup and Squires, 1977; Mohler and Okada, 1977). These peripheral sites possess a different rank order of binding potency for the benzodiazepine ligands compared to the central receptor. They have been designated the peripheral-type benzodiazepine receptor (PBZR) (Marangos et al., 1982; Shoemaker et al., 1983).

The PBZR binds select benzodiazepines (Wang et al., 1984), isoquinoline carboximides (Benavides et al., 1985; Dubroeucq et al., 1986), and pyrethroids (Devaud et al., 1986; Devaud and Murray, 1988) with high affinity. The rank order of benzodiazepine binding affinity to the PBZR is Ro5-4864>Diazepam>>Clonazepam (Wang et al., 1984). The central type receptor binds these in the opposite rank order (Wang et al., 1984).

Peripheral Benzodiazepine Receptor: Possible Physiological Role

The biological role of the peripheral benzodiazepine receptor has not been determined as of yet. Several lines of evidence suggest a role for this receptor in the modulation of cellular mitogenesis. Clarke and Ryan (1980) found that
micromolar concentrations of diazepam, a ligand with moderate affinity for the peripheral benzodiazepine receptor, blocked mitogenesis in Swiss 3T3 cells and that this effect was cytostatic not cytotoxic. Wang et al. (1984a) observed that there was a strong correlation (r=0.85) between binding to the peripheral benzodiazepine receptor and the compounds ability to inhibit AKR mouse thymoma cell proliferation. They also determined that even in micromolar concentrations the effect was not cytotoxic (as determined by trypan blue staining). A recent report by Steplen et al. (1987) demonstrated an enhancement of estradiol-induced DNA syntheses in the anterior pituitary gland by Ro5-4864. In their study Ro15-1788 a central-type benzodiazepine receptor ligand and sodium valproate, a GABA-transaminase inhibitor had no effect. Diazepam and its metabolite oxazepam have also been shown to be tumor promoters in mouse liver (Diwan et al., 1986).

Another possible area in which the peripheral benzodiazepine receptor may modulate mitogenesis is the ability of peripheral ligands to induce differentiation of cells. Several groups have suggested a hypothetical inverse relationship between the probabilities of proliferation and terminal differentiation (Gusella et al., 1976; Konigsberg et al., 1978; Brooks et al., 1980). According to this theory if a cell is somehow restricted from proliferating, it will then terminally differentiate. Clarke and Ryan (1980) found that diazepam could induce Friend erythroleukemia cells to differentiate. Matthew et al. (1981) found that B16/C3 melanoma cells possessed a high affinity binding site for diazepam, a ligand with moderate affinity for the peripheral benzodiazepine receptor. They found that Ro5-4864 was the most potent inhibitor of diazepam binding and that it along with
diazepam and flunitrazepam were the most potent inducers of melanogenesis, a marker of differentiation. Wang et al. (1984b) observed that there seemed to be a direct structure activity relationship with the ability to induce differentiation and this induction was concentration (micromolar) and time dependent (5 days) for full expression in Friend erythroleukemia cells. This body of evidence, both in vitro and in vivo strongly suggests that the peripheral benzodiazepine receptor can modulate cellular mitogenesis.

Peripheral benzodiazepine ligands have also been shown to interact with distinct cellular functions. In 1979 Strittmatter et al. demonstrated that Ro5-4864 stimulated phospholipid methylation in C6 astrocytoma cells. This may be an important effect since synthesis and translocation of methylated phospholipid affects lymphocyte mitogenesis (Toyashima et al., 1979). DeLorenzo et al. (1981) demonstrated that diazepam and Ro5-4864, ligands which bind to the peripheral benzodiazepine receptor, inhibited a calcium-calmodulin protein kinase system in the brain. The calcium-calmodulin kinase represents an identifiable biochemical membrane system that can be modulated by these benzodiazepines (DeLorenzo et al., 1981). Both of these events demonstrate membrane system regulation by the peripheral benzodiazepines which may play an important role in cellular function.

Peripheral benzodiazepine ligands have also been found to influence other cellular biochemical events. In 1985 Morgan et al. discovered that these ligands affected two distinct activities, nerve growth factor-induced neurite outgrowth and ornithine decarboxylase induction in rat pheochromocytoma cells (PC12). These ligands inhibited neurite outgrowth while many increased induction of
ornithine decarboxylase activity. Another study by Curran and Morgan in 1985 discovered a novel action of peripheral benzodiazepine ligands at the level of gene expression. They found that c-fos expression induced by nerve growth factor was enhanced more than 100 fold in the presence of the peripheral benzodiazepine ligand, Ro5-4864. Curran and Morgan correlated this observation with an earlier work (Morgan et al., 1985) to suggest that peripheral benzodiazepine ligands may modify a specific biological response at the level of gene expression.

Anholt (1986) suggested that peripheral benzodiazepine receptors may represent important control sites for the modulation of intermediary metabolism. Since this receptor is thought to be associated with the outer mitochondrial membrane this role was investigated by two separate groups. Hirsch et al. (1988) observed that the peripheral benzodiazepine ligands, Ro5-4864 and PK 11195 mediated the inhibition of mitochondrial respiratory control. They found that the ability to inhibit mitochondrial respiratory control correlated extremely well with their binding affinity to the receptor (r=0.97). In 1989 Larcher et al. reported that a dose dependent decrease of O2 consumption in neuroblastoma cells occurred in the presence of Ro5-4864 and PK 11195. These studies provide evidence for a possible role of the peripheral benzodiazepine receptor in regulating cellular metabolism.

Potential Endogenous Ligands: Possible Role of Porphyrins

If the peripheral benzodiazepine receptor has a physiological function, one would postulate that an endogenous modulator of the receptor would exist. The
first evidence of an endogenous modulator was presented by Beaumont et al. (1983). He described competitive inhibitors specific for peripheral benzodiazepine receptors present in urine and plasma ultrafiltrates. His group partially purified this inhibitor but did not identify it. In 1984, Mantione et al. observed that acidified methanol extracts from several tissues (e.g. stomach, kidney, lung) inhibited $[^3H] \text{Ro}5-4864$ binding to peripheral sites. This extract did not significantly affect $[^3H] \text{Diazepam}$ binding to the central benzodiazepine site. Ultrafiltration or gel filtration yielded high and low molecular weight fractions which competitively inhibited $[^3H] \text{Ro}5-4864$ binding. Once again the structure of this compound was not determined.

Schoemaker et al. (1985) reported that benzodiazepine binding in the kidney was augmented by perfusion. Verma et al. (1987) explored the possibility that blood might contain an endogenous ligand for the receptor. They isolated and purified potent inhibitors from human blood and rat organs. Thin layer chromatography of these compounds displayed a peak with absorbance identical to hemin. They also determined by Scatchard analysis that pure Hemin and Protoporphyrin IX competitively inhibited $[^3H] \text{Ro}5-4864$ binding to the mitochondrial peripheral benzodiazepine receptor. Structure activity relationship profiles of porphyrins and metalloporphyrins indicated that the number of carboxylic acids is critical for affinity for the peripheral type receptor (dicarboxylic=nM affinity, tetracarboxylic=inactive, and precursors=weak or inactive). In 1988 Verma and Snyder further explored the role of porphyrins. They established that structural requirements determining affinity were fairly strict and that the most potent (Hemin and Protoporphyrin IX) were also the most
prominent physiologically. Therefore, they concluded from their studies that porphyrins were the major endogenous compound that interacted potently with the peripheral benzodiazepine receptor. They suggested three possibilities to support their hypothesis: 1) Endogenous concentrations of porphyrins are quite ample to provide substantial occupancy of the receptor under normal conditions (DelBatelle, et al., 1979), 2) Fluctuations in porphyrin levels noncovalently modulate the enzymatic activity of tryptophan pyrrolase (Litman and Correia, 1985), guanylate cyclase (Ignarro et al., 1984) and glutathione-S-transferase (Smith et al., 1985) and could similarly regulate the peripheral benzodiazepine receptor and 3) Involvement of the porphyrins with several mitochondrial proteins as well as the role of mitochondria in porphyrinogenesis also coincide with mitochondrial localization of the peripheral benzodiazepine receptor.

A recent report by Hirsch et al. presented evidence for an interaction between peripheral benzodiazepine ligands and porphyrins with respiratory control in mitochondria. The peripheral benzodiazepine ligands, Ro5- 4864 and PK 11195 as well as Mesoporphyrin IX and Deuteroporphyrin IX decreased respiratory control in isolated mitochondria. The relative potencies of the agents seems to correlate with their affinity for the peripheral benzodiazepine receptor. Clonazepam, a central agonist had no effect. Verma and Snyder (1988) as well as Hirsch et al. (1988) conclude that this regulation of mitochondrial function may underlie the wide spectrum of actions produced by benzodiazepines and porphyrins.
Purpose of Study

Preliminary evidence has shown that a high affinity binding site for Ro5-4864 can be identified on Nb2 cells. Ro5-4864 modulates prolactin-stimulated thymidine incorporation and ornithine decarboxylase induction in the Nb2 cell. The first goal of this study was to further explore the role of the peripheral benzodiazepine receptor and ligands which bind to it in the modulation of prolactin-stimulated mitogenesis of the Nb2 cell. The preliminary work was done with Ro5-4864, a putative peripheral benzodiazepine receptor agonist. It was determined that more detailed studies were required to complete this work. Thus, more extensive dose response studies were performed using a wider range of ligand concentrations. PK 11195, the putative antagonist for the peripheral benzodiazepine receptor used in addition, to complete the characterization of the peripheral benzodiazepine receptor ligands effects on prolactin- stimulated mitogenesis of the Nb2 cell. Verma and Snyder (1987) have proposed that porphyrins are the endogenous ligands for the peripheral benzodiazepine receptor. They have determined that Protoporphyrin IX was the most potent inhibitor of \( ^{3}\text{H}-\text{Ro5-4864} \) binding to the peripheral benzodiazepine receptor (Verma and Snyder, 1988). The second goal of this study was to determine if Protoporphyrin IX acted as an endogenous ligand in the Nb2 cell. The third goal of the study was to determine if the rank order of affinity of selected ligands for the peripheral benzodiazepine receptor found on the Nb2 cell was similar to those previously reported in other tissues (Wang et al., 1984a). The IC\(_{50}\) value for each of these ligands can be calculated and these values can be used to determine if a correlation exists between binding affinity and ability to modulate mitogenesis.
Taken together these studies using Nb2 cells can help to elucidate the mechanism by which peripheral benzodiazepine ligands exert their modulatory effects on growth.
MATERIALS AND METHODS

I. Cell Culture Procedures

A. Preparation of Cell Culture Medium

The Nb2 node lymphoma cells are routinely cultured in Fischer's medium (Gibco Laboratories). The medium is prepared by adding 850 ml of deionized distilled water to 100 ml of 10x concentrated medium in a volumetric flask. Sodium bicarbonate (Mallinckrodt) which acts as the buffering system is added to the stock medium and the pH is adjusted to 7.4. The volume is adjusted to 1 liter with deionized distilled water and is filter sterilized using a 0.2 μM disposable filter unit (Nalgene). The medium is then aliquoted into sterile 100 ml bottles for subsequent supplementation and use. These aliquots are supplemented with 10% (v/v) fetal calf serum (Hyclone Laboratories), 10% (v/v) horse serum (Crowpher Laboratories), 2% (v/v) 10^-4 M 2-mercaptoethanol (Sigma Chemical), 1% (v/v) 7.5% Sodium Bicarbonate (Mallinckrodt) and 1% (v/v) Penicillin/Streptomycin (Gibco Laboratories).

The medium which contains all of the supplements is known a growth medium, since fetal calf serum provides the source of prolactin necessary for Nb2 cell growth. The medium used for experimentation contains all of the supplements with the exception of fetal calf serum, this is called stationary medium. The removal of fetal calf serum from the medium removes the prolactin source, allowing the cells to enter a quiescent phase.
B. Thawing of Frozen Cells for Use

Nb2 cells in logarithmic growth may be frozen in a medium containing 8% DMSO. These frozen cells can be used to re-establish Nb2 cell cultures. These cells are frozen at -80°C until used. To revive the cells the vial is immersed in warm water and the outside of the vial is washed with ethanol. The contents of the vial are transferred to a sterile 50 ml centrifuge tube containing growth medium which has been pre-gassed with 95% Air/5% CO2. This allows for an equilibration of the medium with the proper atmospheric conditions. After a 30 minute incubation to allow for removal of the DMSO, the cells are centrifuged for 5 minutes at 300 x G and the pellet is resuspended in 10 ml of fresh growth medium. The cells are allowed to incubate overnight at 37°C in a 75 cm² culture flask (Falcon). At this time the cells are centrifuged at 300 x G, the pellet resuspended, and an aliquot of the cells is counted. The cells are then placed back in the same flask and are incubated again for 24 hours. From this point on growth is monitored daily and aliquots of growth medium (2-5 ml) are added until the volume is 20 ml. The cells are allowed to grow to a density (approximately 1x10⁶ cells/ml) from which routine cell subculturing can be performed. This period of time is approximately one week.

C. Cell Subculturing, Use and Storage

Nb2 node lymphoma cells are maintained as suspension cultures in 75 cm² tissue culture flasks at 37°C in an atmosphere of 5% CO2/95% Air and 70% humidity. The medium used for maintenance of exponentially growing cultures
has been previously described. Cells were routinely subcultured twice a week (Tuesday and Friday) to a density of 1.8x10^4 cells/ml. The cells are removed from the culture flask and placed in 50 ml centrifuge tubes. These are centrifuged at 300 x G for 5 min, the medium is aspirated off and the pellet resuspended in 10 ml of medium. An aliquot of this suspension is counted to determine cell density and the appropriate calculation is done in order to determine the amount of this suspension needed to "reseed" the cultures. Cells remaining after subculturing are: 1) frozen for future use in freezing medium (Fischer's + 8% DMSO) at a concentration of 2x10^6 cells per ml, 2) frozen in .32 M Sucrose for use in receptor binding experiments or 3) aliquoted for use in mitogenic studies.

D. Preparation of Cells for Experimentation

Cells which are to be used for mitogenic studies must be induced to enter a quiescent state (ie. enter the G0/G1 phase of the cycle). The use of stationary cells allows for experiments to be conducted with a synchronous population of cells. Cells are prepared for experimentation by centrifugation of cells in log phase growth and removal of the growth medium. The cell pellet is resuspended in stationary medium and is washed twice with this medium to insure removal of the remaining growth medium. The cells are recounted to determine cell density and a 1.94 ml aliquot containing 1x10^4 cells/ml is added to each well of a 24 well culture dish and is placed in the incubator. These cells are incubated for 48 hours to ensure all cells have become stationary. After 48 hours the cells may be used for experimentation.
Experimental Procedures

A. Prolactin Dose Response Experiments

In order to determine if the Nb2 cell to be used for further experimentation are prolactin-dependent, a prolactin dose response experiment must be performed. Ovine prolactin (oPRL) (NIADDK-NIH, Bethesda, MD) is dissolved in 1.7 ml of 0.01 N NaOH and 300 ul of .05 M KH2PO4 is added to neutralize the solution, the final concentration of prolactin being 2 mg/ml. Fifty ul aliquots are added to sterile eppendorf microcentrifuge tubes and are frozen at -20° C until used. To prepare prolactin stocks for experiments, 950 uls of stationary medium is added to a thawed prolactin aliquot giving a solution which contains 100,000 ng/ml. Ten fold serial dilutions of the stock are made with stationary medium to provide the concentrations needed for the prolactin dose response experiment. All concentrations made up are 100 times the desired final concentration since a 100 fold dilution occurs when dosing the 2 ml cell suspension. The prolactin concentrations range from 0.001 ng/ml to 10 ng/ml for a typical dose response curve. As mentioned previously, the cells are placed in stationary medium for two days and are dosed with the appropriate concentration of prolactin. After dosing, the cells are allowed to incubate 4 days at 37° C and are counted by hemocytometry. There is approximately a 10% error in estimation of cell numbers by this method. Viability is determined by Trypan Blue exclusion method. Each concentration is run in triplicate. The average of the counts from the triplicates are used to calculate cells/ml for that concentration. The calculation used to determine cells/ml can be found below.
B. Drug Dose Responses: Modulation of Mitogenesis Studies

These experiments were performed in order to determine the ability of benzodiazepines to modulate prolactin-stimulated mitogenesis in the Nb2 cell. Clonazepam (a central acting benzodiazepine), PK 11195 (a putative peripheral antagonist) and RO5-4864 (a putative peripheral agonist) were dissolved in 95% ethanol at concentrations that the particular compounds solubility would allow for. These were stored at -20° C until they were used. On the day of the experiment a 1 mM stock solution was made up and 10 fold serial dilutions were made with stationary medium so that the stock solutions ranged from concentrations of 10^-4 M to 10^-13 M. Each stock was 100 fold greater than the desired final concentration since a 100 fold dilution occurs when adding the drug to the well. Actual final concentrations used in each well of these experiments ranged from 10^-6 to 10^-15 M. Each drug concentration was tested in triplicate with the counting procedure identical to that used for the prolactin dose response. The highest concentration of drug added (10^-6 M) contained the highest concentration of ethanol per well. To account for any change due to ethanol alone the concentration of ethanol was held constant throughout all concentrations of drugs. The concentration of ethanol never exceeded 0.02%. An appropriate vehicle control (ethanol) was used to determine whether or not ethanol had a measurable effect of its own. The prolactin dose used in these experiments was 0.1 ng/ml which had been determined from previous experiments to be a sub-optimal mitogenic dose. This prolactin concentration permitted for evaluation of positive and negative effects of these compounds on prolactin-
stimulated mitogenesis. Each dose was done in triplicate and the experiments were repeated three times.

C. Drug Interaction Experiments

Two compounds were determined to have an effect on prolactin-stimulated mitogenesis, Ro5-4864 and PK 11195. Since these compounds are peripheral benzodiazepine ligands the possibility existed that they interacted at the same binding site. These experiments were carried out in order to determine this. Based on previous observations from individual dose responses it was decided that the concentrations to be used would be 10\(^{-6}\), 10\(^{-9}\), and 10\(^{-15}\) M for each drug. When dosing the cells the concentration of PK 11195 was held constant and the concentrations of Ro5-4864 were varied. For example, PK 11195 was added as 10\(^{-6}\) M while Ro5-4864 was added at 10\(^{-6}\), 10\(^{-9}\), and 10\(^{-15}\) M. The same was done for 10\(^{-9}\) and 10\(^{-15}\) M PK 11195. The prolactin concentration used was 0.1 ng/ml and the ethanol concentration was held constant in each well. The ethanol concentration never exceeded 0.05%. Each interaction study was done in triplicate and the experiments were repeated three times.

D. Porphyrin Studies

The proposed endogenous ligand for a binding site should produce the same effects when it interacts at the binding site if it has a true physiological function. These experiments were carried out to determine if this was true for Protoporphyrin IX which is a proposed endogenous ligand for this binding site.
Protoporphyrin IX (Aldrich) was solubilized in 0.1 M Tris base (pH 10.40) to a concentration of 1 mM. Ten fold dilutions were made so that the stock concentrations ranged from $10^{-4}$ to $10^{-13}$ M. Each stock was made up so that it was 100 fold higher than the desired final concentration since a 100 fold dilution is made when adding the solution to the well. The final concentrations in the wells for these experiments ranged from $10^{-6}$ to $10^{-15}$ M. At the end of the 96 hour incubation period the porphyrin concentration remaining in the wells was measured by the method of Granick et al. (1972). The highest dose of Protoporphyrin IX contained the highest concentration of Tris base per well. An appropriate dose of Tris base was added to the remaining wells to keep this constant. The concentration of Tris base never exceeded 0.1%. The concentration of prolactin used in these experiments was 0.1 ng/ml. The appropriate vehicle was run to determine that Tris base had no effect on its own. Each dose was done in triplicate and the experiments were repeated three times.

Membrane Preparation

Cells were collected from the cell splittings and stored at -80°C until they were used. All subsequent procedures were conducted at 4°C. Cells (9x10^9 cells in 30 mls of 50 mM Na/K Phosphate buffer, pH 7.4 at 4°C) were homogenized using a Brinkman Polytron (setting 10 for 60 sec). This homogenate was centrifuged at 600 x G (10 min) and the resulting supernatant was centrifuged at 49000 x G (20 min). The pellet obtained from this spin was resuspended in buffer and stored at -80°C until used. Protein concentrations were determined by the method of Lowry et al., (1951).
Binding Assays

Binding of \(^3\text{H}\) Ro5-4864 to crude membranes was performed in 50 mM Na\(^{+}/K^{+}\) phosphate buffer. Total and non-specific binding was determined in the presence and absence of 1 µM Ro5-4864. Final incubation volume was 500 µl, this consisted of 400 µl membrane preparation, 50 µl \(^3\text{H}\) Ro5-4864 to yield the desired final concentration and 50 µl of the solution used to determine total or non-specific binding. Incubations were maintained for 120 min at 4°C. Protein concentrations of 50 to 75 µg/assay were used unless otherwise specified. After incubation, membranes were collected on glass fiber filters (Schleicher and Schuell, no. 32) using an M-24R Brandel Cell Harvester. Filters were washed twice with 5 ml ice cold 50 mM phosphate-buffered saline (PBS, pH 7.4 at 4°C) before harvesting. Incubations were terminated by the addition of 2.5 ml ice cold PBS and membranes were washed three times with 5 ml PBS. Filters were placed in vials, air dried at 37°C, and 10 ml scintillation solution was added to assess radioactivity by scintillation spectroscopy at 51% counting efficiency.

Each experiment was performed three times and individual determinations were in triplicates. To assess the effect of protein concentrations on specific \(^3\text{H}\) Ro5-4864 binding to the crude membrane preparation at a radioligand concentration of 1 nM, 20 to 300 µg protein/assay was used. The association rate of the crude membrane preparation was determined by harvesting membranes at time points ranging from 0 to 180 minutes using a concentration of 1 nM \(^3\text{H}\) Ro5-4864 and 1 µM Ro5-4864 to determine non-specific binding. Competition experiments were performed at radioligand concentrations of 1 nM.
Determination of the IC₅₀ values for inhibition of [³H]- Ro5-4864 binding was performed using the PCNONLIN program for analysis of binding data.

**Statistical Analysis**

The data presented was analyzed by an ANOVA test. Statistical differences between the means were determined by the Newman-Keuls range for multiple comparisons. Differences were considered to be significant at the 95% or above confidence level.
RESULTS

Preliminary Binding Experiments

Before performing the ligand competition studies it was necessary to assess the effect of protein concentration on specific $[^3H]$-Ro5-4864 binding to the crude membrane preparation at a radioligand concentration of 1 nM. For these experiments 20 to 300 ug protein/assay were used. Each protein concentration was incubated in the presence of 1 nM $[^3H]$-Ro5-4864 for 120 minutes. Total and non-specific binding of $[^3H]$-Ro5-4864 was determined in the absence and presence of 1 uM Ro5-4864.

As can be seen from Figure 1 specific binding of $[^3H]$- Ro5-4864 is linear from 39 ug to 237 ug per assay. Since protein was a limiting factor due to the large number of cells required to obtain the desired amount of protein, it was decided to use 50-75 ug/assay in future experiments. Specific binding at these protein concentrations was determined to be 95%.

The next experiment performed was to determine the association time for the crude membrane preparation to come to equilibrium. This was determined by incubating 50-75 ug protein/assay with 1 nM $[^3H]$-Ro5-4864. The membranes were harvested at time points ranging from 0 to 180 minutes. Total and non-specific binding of $[^3H]$-Ro5-4864 was determined in the absence and presence of 1 uM Ro5-4864.
Figure 2 illustrates the association time curve determined from these experiments. The binding of [3H]-Ro5-4864 to the membrane preparation reached equilibrium at 180 minutes. In future experiments an incubation time of 210 minutes was used in order to further ensure that equilibrium would be reached.

**Ligand Competition Curves**

The pharmacological specificity of the [3H] Ro5-4864 binding site on Nb2 cell fragments was evaluated using ligands with differing affinities for the peripheral and central benzodiazepine receptor. [3H] Ro5-4864 (1nM) was incubated for 2.5 hours in the presence or absence of various concentrations of unlabelled Ro5-4864, PK 11195, diazepam, Protoporphyrin IX or clonazepam. The data presented are representative of 2 separate experiments run in triplicate except for Protoporphyrin IX (n=3). Concentrations of unlabelled ligands were 0.3 to 300 nM (Ro5-4864 and PK 11195), 0.3 to 1000 nM (Diazepam), 1 to 1000 nM (Protoporphyrin IX), and 0.1 to 100 uM (Clonazepam). The representative competition curves for the ligands used are presented in Figure 3. The IC50 values found in Table 1 were determine using the PCNONLIN binding program.

Ro5-4864 represented by open squares, and PK 11195 represented by open circles are ligands which bind with high affinity to the peripheral benzodiazepine receptor. Both Ro5-4864 (IC50=6.6 nM) and PK 11195 (IC50=6.8 nM) compete with [3H]-Ro5-4864 binding very effectively as would be expected with ligands that bind with high affinity to the peripheral benzodiazepine receptor. Diazepam
represented by the closed inverse triangles competes moderately well with $[^3\text{H}]$-Ro5-4864 binding ($IC_{50}=73$ nM). Diazepam possesses a moderate affinity for the peripheral benzodiazepine receptor and since it has a lower affinity for this receptor it has a higher $IC_{50}$ value. Clonazepam, a central benzodiazepine receptor agonist is represented by the open triangles. This ligand has little ability to compete with $[^3\text{H}]$-Ro5-4864 binding ($IC_{50}=23$ uM) which would be expected with a ligand that possesses little or no affinity for the peripheral benzodiazepine receptor. Protoporphyrin IX has been suggested to be the endogenous ligand for the peripheral benzodiazepine receptor (Verma et al., 1987). If this were the case in the Nb2 cell it would be expected to compete with $[^3\text{H}]$-Ro5-4864 binding as effectively as Ro5-4864 and PK 11195. Protoporphyrin IX is represented by the open diamonds, it competes rather poorly with $[^3\text{H}]$-Ro5-4864 binding ($IC_{50}=177$ nM). The rank order of potency for inhibition of $[^3\text{H}]$-Ro5-4864 binding by the ligands tested was Ro5-4864=PK11195>Diazepam>Protoporphyrin IX>>Clonazepam. The peripheral benzodiazepine receptor found in the Nb2 cells possesses the same rank order of potency as reported by Wang et al. (1984c).

**Prolactin Dose Response in the Nb2 Cells**

Nb2 cells were aliquoted ($10^4$/ml) into 24-well flat bottom culture plates. Concentrations of prolactin ranging from 0.001 to 10 ng/ml were added to the cell suspensions. The cells were incubated for 96 hours and counted visually using a hemocytometer. The data presented in Figure 4 is the mean ± SEM of three separate experiments run in triplicate. The data is presented as a percent response of cell growth to prolactin. Since the response to prolactin at 1 ng/ml
prolactin is maximal this was considered to be a 100% response. All other values in the figure are expressed as a percentage of the cell number that was counted from the wells containing 1 ng/ml prolactin. Figure 4 illustrates that Nb2 cells respond to prolactin in a dose related manner, with maximal stimulation occurring at 1 ng/ml. The EC50 was determined graphically to be 0.45 ng/ml prolactin. Those cells that received no prolactin (cells only), were arrested in the G0/G1 phase but remained viable. Viability was determined by using the Trypan blue exclusion method, a widely used method for determining cell viability. An important observation from these experiments was the determination of a sub-optimal dose of prolactin to use in further studies. This dose (0.1 ng/ml) is below the EC50 value for prolactin. This concentration of prolactin was used to permit us to evaluate both positive and negative components of the modulatory actions of ligands acting on the peripheral benzodiazepine receptor. Viability was determined to be >95% in all populations tested.

Peripheral Benzodiazepine Ligands Effects on Prolactin Stimulated Mitogenesis

Nb2 cells were aliquoted (10^4) into 24-well flat bottom culture plates. A sub-optimal concentration of prolactin (0.1 ng/ml) was added to each of the wells. In addition, ligand concentrations ranging from 10^-15 to 10^-6 M were added to each of the wells. The cells were incubated for 96 hours and counted visually using a hemocytometer. The data is expressed as a percentage of mitogenesis stimulated by prolactin alone which was considered to be 100%. Each bar represents the mean ± SEM of three separate experiments run in triplicate. Figure 5 shows the results of the study done with Ro5-4864. At a concentration
of 10⁻⁶ M, Ro5-4864 inhibited prolactin stimulated mitogenesis (73% of prolactin alone) while and 10⁻⁹ M enhanced it. Maximal enhancement (76% over prolactin) occurred in the presence of 10⁻⁹ M Ro5-4864. All other concentrations did not significantly alter prolactin-stimulated mitogenesis. Furthermore, when Ro5-4864 was administered alone at any concentration (10⁻⁶ M to 10⁻¹⁵ M) it had no effect on mitogenesis.

Figure 6 shows the results of the same type of experiment using PK 11195. In contrast to the effects of Ro5-4864, 10⁻⁶ M PK 11195 had no effect on prolactin-stimulated mitogenesis. However, concentrations of PK 11195 ranging from 10⁻⁷ M to 10⁻⁹ M enhanced prolactin stimulated mitogenesis. Maximal enhancement occurred in the presence of 10⁻⁹ M PK 11195 (116% over prolactin). Again, when any of the concentrations of PK 11195 (10⁻⁶ M to 10⁻¹⁵ M) were administered alone it had no effect on mitogenesis. The differences in maximal enhancement of mitogenesis at 10⁻⁹ M (PK 11195 1116% vs Ro5-4864 76%) and the ability of PK 11195 to enhance mitogenesis over a wider concentration range may be partially explained by the work of LeFur et al. (1983). They determined that Ro5-4864 binding was temperature dependent whereas PK 11195 was not, since these experiments were performed at 37°C the difference may be due to this.

Identical dose response studies were also performed with the central benzodiazepine ligand, clonazepam. The results of this study can be observed in Figure 7. As can be seen, no inhibition or enhancement occurs at any dose (10⁻⁶ M to 10⁻¹⁵ M) suggests that the lack of effect observed are results from the low
affinity this compound has for the peripheral benzodiazepine receptor. Table 2 summarizes the effects observed with each of the three ligands.

Peripheral Benzodiazepine Ligand Interaction Studies

These studies were carried out in order to determine if these ligands were working at the same binding site to elicit their actions. The concentrations of the ligands used for these studies were 10^{-6} M, 10^{-9} M, and 10^{-15} M. Nb2 cells were aliquoted (10^4) into 24-well flat bottom culture plates. A sub-optimal concentration of prolactin (0.1 ng/ml) was added to each well. In addition, ligand combinations were added to each of the wells. In each experiment the PK 11195 concentration was held constant while the Ro5-4864 concentration was varied; i.e., 10^{-6} M PK 11195 was incubated with 10^{-6} M Ro5-4864, 10^{-9} M Ro5-4864 and 10^{-15} M Ro5-4864 in separate wells. This sequence was repeated until each concentration of one ligand was tested across the three concentrations of the other. The cells were incubated for 96 hours and counted visually using a hemocytometer. These data are expressed as a percentage of mitogenesis stimulated by prolactin alone, which was considered to be 100%. Each value represents the mean ± SEM of three separate experiments run in triplicate.

At a concentration of 10^{-6} M, Ro5-4864 blocked the enhancement of mitogenesis by 10^{-9} M PK 11195. The same held true when 10^{-6} M PK 11195 was added in the presence of 10^{-9} Ro5-4864; i.e., the enhancement of mitogenesis was blocked. The inhibition of prolactin-stimulated mitogenesis by 10^{-6} M Ro5-4864 was also blocked by 10^{-6} M PK 11195. Perhaps the most striking result was that
obtained when 10^{-9} M of both compounds were added together, the response was no different from when either drug was tested alone. This suggests that these ligands interact at the same site since one would expect a synergistic or additive increase if they were acting at different sites to elicit these actions. Curiously, when 10^{-15} M PK 11195 was added in the presence of 10^{-6} M Ro5-4864 the inhibition of mitogenesis was blocked. This effect was unexpected since 10^{-15} M is a very low dose as compared to 10^{-6} M. The results from these studies are shown in Table 3.

**Protoporphyrin IX Studies**

Nb2 cells were aliquoted (10^4) into 24-well flat bottom culture plates. A sub-optimal concentration of prolactin (0.1 ng/ml) was added to each of the wells. In addition, concentrations of Protoporphyrin IX ranging from 10^{-15} to 10^{-6} M were added to each of the wells. Protoporphyrin IX was chosen since Verma et al. (1987) had reported that it was the most potent porphyrin in competing with [^3H]-Ro5-4864 for binding to the peripheral benzodiazepine receptor. The cells were incubated for 96 hours and counted visually using a hemocytometer. These data are expressed as a percentage of mitogenesis stimulated by prolactin alone which was considered to be 100%. Each bar represents the mean ± SEM of three separate experiments run in triplicate.

Figure 8 shows the results obtained from the dose response studies using Protoporphyrin IX. Concentrations ranging from 10^{-6} M to 10^{-15} M had no effect on prolactin stimulated mitogenesis. The porphyrin concentration remaining in
the medium after 96 hours of incubation was determined for each experiment according to the method described by Granick et al. (1972). Approximately 40 percent of the highest dose (10⁻⁶ M) remained, this concentration (4×10⁻⁷ M) is approximately equal to the EC₅₀ found in competition studies. If Protoporphyrin is truly an endogenous ligand for the peripheral benzodiazepine receptor, it would be likely to cause an effect at the same concentrations as Ro5-4864 and PK 11195. This lack of effect even at high doses does not agree with the hypothesis that porphyrins are an endogenous ligand for the peripheral benzodiazepine receptor. Table 4 illustrates the comparison of Protoporphyrin IX with ligands for the peripheral benzodiazepine receptor effects.
FIGURE 1. Protein dependence of specific [³H]Ro5-4864 binding with Nb2 cell membrane fragments. Total (filled circles), non-specific (open triangles) and specific (open circles) were determined using protein concentrations ranging from 39 ug to 239 ug/assay. Non-specific binding was determined in the presence of 1 uM unlabelled Ro5-4864. Samples were harvested after incubating for 120 minutes. Each point is the average of two experiments performed in triplicate and normalized with respect to protein concentration.
FIGURE 2. Association of specific $[^3H]$ Ro5-4864 binding with Nb2 cell membrane fragments. Association (closed circles) of 1 nM $[^3H]$ Ro5-4864 with the peripheral benzodiazepine receptor in the absence and presence of 1 uM unlabelled Ro5-4864 to determine total and non-specific binding, respectively. Samples were harvested after incubating for 0, 15, 30, 45, 60, 90, 120, and 180 minutes. $B_e$ is the specific bound radioligand at equilibrium. Each point is the average of two experiments performed in triplicate and normalized with respect to protein.
FIGURE 3. Competition for specific $[^3]$H Ro5-4864 binding to the Nb2 cell peripheral benzodiazepine receptor. Representative data from competition analysis from separate experiments run in triplicate. Non-specific binding was determined in the presence of 1 μM unlabelled Ro5-4864. Concentrations of competing ligands were 0.1 nM to 300 nM (Ro5-4864 and PK 11195), 3nM to 1000 nM (Diazepam), 1 nM to 10000 nM (Protoporphyrin IX) and 100 nM to 100000 nM (Clonazepam). Concentration of radioligand was 1 nM in all experiments. Ro5-4864 (open square), PK 11195 (open circle), Diazepam (closed inverse triangle), Protoporphyrin IX (open diamond) and Clonazepam (open triangle). IC$_{50}$ values for each ligand can be found in Table 1.
TABLE 1. Estimated IC₅₀ values for ligands competing with [³H] Ro5-4864 for specific binding to the Nb2 cell peripheral benzodiazepine receptor.

<table>
<thead>
<tr>
<th>LIGAND</th>
<th>IC₅₀ VALUE</th>
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<tbody>
<tr>
<td>Ro5-4864</td>
<td>6.6 (2)</td>
</tr>
<tr>
<td>PK 11195</td>
<td>6.8 (2)</td>
</tr>
<tr>
<td>Diazepam</td>
<td>73 (2)</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>177 (3)</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>23000 (2)</td>
</tr>
</tbody>
</table>

* Average estimated IC₅₀ values calculated using the PCNONLIN computer binding program.

* Number of separate experimental determinations.
FIGURE 4. Response of Nb2 cells to prolactin. Quiescent cells resuspended in assay medium were aliquoted (10^4/ml) into 24-well flat bottom culture plates. Prolactin concentrations ranging from 0.001 to 10 ng/ml were added to the wells. The cells were incubated for 96 hours and counted visually using a hemocytometer. All data are expressed as percent response to prolactin. Since maximal cell growth was observed at 1 ng/ml prolactin the cell number counted in that well was considered to be a 100% response. All other responses are expressed as a percentage of this cell number. Each point represents the mean±S.E.M. of three separate experiments run in triplicate.
FIGURE 5. The effects of Ro5-4864 on prolactin-stimulated mitogenesis in the Nb2 cells. Quiescent cells resuspended in assay medium were aliquoted (10⁴/ml) into 24-well flat bottom culture plates. To each well was added a medium containing either no prolactin or 0.1 ng/ml prolactin, a sub-optimal mitogenic concentration. In addition, concentrations of Ro5-4864 (10⁻¹⁵ M to 10⁻⁶ M or none) were added to each well. The cells were incubated for 96 hours and counted visually using a hemocytometer. All data are expressed as percentage of prolactin-stimulated mitogenesis which was considered to be 100%. Each bar represents the mean± S.E.M. of three separate experiments run in triplicate.

* Significantly different than prolactin-stimulated mitogenesis P<0.05.
FIGURE 6. The effects of PK 11195 on prolactin-stimulated mitogenesis in the Nb2 cells. Quiescent cells resuspended in assay medium were aliquoted (10^4/ml) into 24-well flat bottom culture plates. To each well was added a medium containing either no prolactin or 0.1 ng/ml prolactin, a sub-optimal mitogenic concentration. In addition, concentrations of PK 11195 (10^{-15} M to 10^{-6} M or none) were added to each well. The cells were incubated for 96 hours and counted visually using a hemocytometer. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. Each bar represents the mean± S.E.M. from three separate experiments run in triplicate.

* Significantly different than prolactin-stimulated mitogenesis P<0.05.
FIGURE 7. The effects of clonazepam on prolactin-stimulated mitogenesis in the Nb2 cells. Quiescent cells resuspended in assay medium were aliquoted (10^4/ml) into 24-well flat bottom culture plates. To each well was added a medium containing either no prolactin or 0.1 ng/ml prolactin, a sub-optimal mitogenic concentration. In addition, concentrations of clonazepam (10^-15 M to 10^-6 M or none) were added to each well. The cells were incubated for 96 hours and counted visually using a hemocytometer. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. Each bar represents the mean± S.E.M. of three separate experiments run in triplicate.
TABLE 2. Comparison of effects of Ro5-4864, PK 11195 and Clonazepam on prolactin-stimulated mitogenesis in the Nb2 cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>PERCENT OF PROLACTIN RESPONSE</th>
</tr>
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<tbody>
<tr>
<td>10^-6 M Clon+PRL</td>
<td>99±1</td>
</tr>
<tr>
<td>10^-9 M Clon+PRL</td>
<td>100±1</td>
</tr>
<tr>
<td>10^-6 M Ro+PRL</td>
<td>76±3^b</td>
</tr>
<tr>
<td>10^-9 M Ro+PRL</td>
<td>159±5^b</td>
</tr>
<tr>
<td>10^-9 M PK+PRL</td>
<td>169±8^b</td>
</tr>
</tbody>
</table>

a Cells were incubated for 96 hours in the presence of 0.1 ng/ml prolactin, in addition, varying concentrations of the three ligands (10^-15 M to 10^-6 M or none) were added to each of the wells. The cells were counted visually using a hemocytometer. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. These data represent the mean ± S.E.M. of three separate experiments (n=3) run in triplicate.

b Significantly different than prolactin-stimulated mitogenesis P<0.05.

Ro=Ro5-4864 PK=PK 11195 Clon=Clonazepam PRL=Prolactin
TABLE 3. The competition between Ro5-4864 and PK 11195 to modulate prolactin-stimulated mitogenesis in the Nb2 cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>PERCENT OF PROLACTIN RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-6 M Ro+PRL</td>
<td>76±3b</td>
</tr>
<tr>
<td>10-9 M Ro+PRL</td>
<td>159±5b</td>
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<tr>
<td>10-9 M PK+PRL</td>
<td>169±8b</td>
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<tr>
<td>10-6 M Ro+10-6 M PK+PRL</td>
<td>81±7c</td>
</tr>
<tr>
<td>10-6 M Ro+10-9 M PK+PRL</td>
<td>87±8c</td>
</tr>
<tr>
<td>10-9 M PK+10-9 M Ro+PRL</td>
<td>96±3c</td>
</tr>
<tr>
<td>10-9 M Ro+10-9 M PK+PRL</td>
<td>141±19b</td>
</tr>
</tbody>
</table>

The experimental paradigm was: a single concentration (10-6, 10-9, 10-15 M) of PK 11195 was mixed with one of the three concentrations of Ro5-4864. This sequence was repeated until each concentration of one ligand had been tested across the three concentrations of the other. Prolactin was used at a sub-optimal mitogenic level, 0.1 ng/ml. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. These data represent the mean± S.E.M. of three separate experiments (n=3) run in triplicate.

- Significantly different than prolactin-stimulated mitogenesis p<0.05.
- Significantly different than response to the individual ligand p<0.05.

PK=PK 11195 Ro=Ro5-4864 PRL=Prolactin
FIGURE 8. The effects of Protoporphyrin IX on prolactin-stimulated in the Nb2 cells. Quiescent cells resuspended in assay medium were aliquoted (10^4/ml) into 24-well flat bottom culture plates. To each well was added either no prolactin or 0.1 ng/ml prolactin, a sub-optimal mitogenic concentration. In addition, concentrations of Protoporphyrin IX (10^-15 M to 10^-6 M or none) were added to each of the wells. The cells were incubated for 96 hours and counted visually using a hemocytometer. At the end of the incubation period the porphyrin content remaining in the medium was measured. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. Each bar represents the mean ± S.E.M. of three separate experiments run in triplicate.
TABLE 4. Comparison of effects of Ro5-4864, PK 11195 and Protoporphyrin IX on prolactin-stimulated mitogenesis in the Nb2 cells.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>PERCENT OF PROLACTIN RESPONSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁶ Proto IX+PRL</td>
<td>96 ± 0.3</td>
</tr>
<tr>
<td>10⁻⁹ Proto IX+PRL</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>10⁻⁶ Ro+PRL</td>
<td>76 ± 3ᵇ</td>
</tr>
<tr>
<td>10⁻⁹ Ro+PRL</td>
<td>159 ± 5ᵇ</td>
</tr>
<tr>
<td>10⁻⁹ PK+PRL</td>
<td>169 ± 8ᵇ</td>
</tr>
</tbody>
</table>

a Cells were incubated for 96 hours in the presence of 0.1 ng/ml prolactin, in addition, varying concentrations of the three ligands (10⁻¹⁵ to 10⁻⁶ M or none) were added to each of the wells. The cells were counted visually using a hemocytometer. All data are expressed as a percentage of prolactin-stimulated mitogenesis which was considered to be 100%. These data represent the mean ± S.E.M. of three separate experiments (n=3) run in triplicate.

b Significantly different than prolactin-stimulated mitogenesis P<0.05.

Proto IX=Protoporphyrin IX Ro=Ro5-4864 PK=PK 11195
PRL=Prolactin
DISCUSSION

Determination of Hormonal Dependency: Prolactin Dose Response

Nb2 node lymphoma cells can be maintained without loss of hormonal dependency in medium containing 10% fetal calf serum (FCS) as the only source of lactogen; the FCS provides the equivalent of approximately 5 ng prolactin/ml, at which concentration the cells replicate at their maximum rate (Noble et al., 1985). Representative dose response data demonstrates that these cells respond to prolactin in a dose dependent manner and the response reaches a plateau at 1 ng/ml, this parallels the dose response observed by Noble et al. (1985). However the prolactin response seems to increase after multiple passages of these cells and the dose response is shifted to the left as is illustrated by Noble et al. (1985). So although the Nb2 cells are dependent on prolactin for growth, their dependence may be transient.

Studies by Noble and Hoover (1975) have emphasized that progression of hormone-dependent tumors is a continuing process from dependency to autonomy. The mechanism by which this process occurs is poorly understood but it is known that even when tumors start from a homogeneous cell population they can, with the passage of time develop a diversity of phenotypic properties (Fidler and Hart, 1982; Poste, 1982). Nowell proposed that heterogeneity underlies tumor progression to autonomy and that environmental changes can result in a selection of a subpopulation which can proliferate more readily under the changed conditions. This concept could explain the progression of cell cultures towards
autonomy (prolactin independence) which follows a reduction of prolactin levels in the medium. The original dependent culture may be comprised of a number of subpopulations, those that require high levels of prolactin and a minor population with little or no prolactin requirement. When levels of prolactin are high, growth of dependent cells is high enough to ensure that they continue to predominate. However when levels fall the hormone-dependent cells cease to grow and eventually lyse while the autonomous cells become predominant (Noble et al., 1985). Therefore it is important to follow the protocol described in the Materials and Methods. All cells used in these experiments were used between passage number three and fifteen to ensure that a homogeneous population of prolactin-dependent cells were used. Cells were frozen in order to maintain "stocks" of the parent prolactin-dependent cells, these frozen cells were thawed to begin "new" cultures. The freezing process is undertaken every time a "new" population is started in order to maintain a large store of prolactin dependent cells. When tested for prolactin dependency these "new" cells respond in a manner similar to the "parent" cultures. Hence, the prolactin dose response experiment is necessary to assure the prolactin dependence of the cells used and is thus a quality control method which should be used routinely for this purpose.

One may ask, "Why is a growth factor-dependent cell line important?" The importance of such a line is their use as a model system to study factors which may influence or regulate cellular mitogenesis. Thus it is important to maintain the same characteristics of the cell line from experiment to experiment. These cells can be "synchronized" so that the researcher can study the effects of various agents (eg. drugs, growth factors, etc.) on cellular mitogenesis. The
concentration of these factors as well as the time that the cells are exposed to them can be controlled. Complicating factors can be reduced since the researcher, in essence, controls many of the variables. Without a growth-dependent cell line the model is less useful, since the cell growth can no longer be controlled by the removal of this factor. If these cells are maintained as prolactin-dependent the events associated with mitogenesis can be explored in greater detail.

**Study of Peripheral Benzodiazepine Ligands: Effects on Mitogenesis**

There were three lines of evidence that suggested the presence of a specific and functionally active benzodiazepine binding site on the Nb2 node lymphoma cell. First, diazepam at a concentration of $10^{-6}$ M inhibited prolactin-dependent $[^{3}\text{H}]$ thymidine incorporation in these cells (Laird et al., 1987). This observation suggested that diazepam acted through a specific binding site present on these cells to inhibit prolactin-stimulated mitogenesis. Second, Nb2 cells possess immunocytochemical and enzymatic markers characteristic of a thymocyte at an intermediate stage of differentiation (Fleming et al., 1982). These cells are of the same lineage as a mouse thymoma cell line which possess peripheral benzodiazepine receptors (Wang et al., 1984a). These observations suggest that diazepam may also interact with a peripheral benzodiazepine binding site in the Nb2 cell. However, diazepam can bind with equal affinity to both central and peripheral sites (Squires and Braestrup, 1977). Therefore, it was necessary to characterize the effects using ligands that bind specifically to the peripheral benzodiazepine binding site, such as Ro5-4864 (Wang et al., 1984a) ands PK 11195
(Benavides et al., 1985; Dubroeucq et al., 1986). Finally, a high affinity binding site for [3H]Ro5-4864, a specific ligand for the benzodiazepine binding site was found on whole Nb2 cells. This binding site was saturable, homogeneous and pharmacologically similar to peripheral benzodiazepine sites found in other cells (Laird et al., 1989).

This study was undertaken to explore further the role of the peripheral benzodiazepine receptor in prolactin-dependent mitogenesis of the Nb2 cell. Studies had been done previously to determine the peripheral benzodiazepine binding sites effects on cell growth using micromolar concentrations of benzodiazepines (Wang et al. 1984a; Wang et al., 1984b). Wang et al. (1984a) demonstrated a strong positive correlation (r=0.85) between binding constants for peripheral benzodiazepine binding sites and their ED50 in inhibiting [3H]Thymidine incorporation. Laird et al. (1987) found that Ro5-4864 could enhance prolactin-stimulated [3H] thymidine incorporation at low concentrations (10^-9 M) and that high concentrations (10^-6 M) inhibited it.

In order to further characterize the role of the peripheral benzodiazepine binding site, dose responses with the putative agonist, Ro5-4864 and the putative antagonist, PK 11195 (LeFur et al., 1983) as well as the central agonist, clonazepam were studied. High concentrations (10^-6 M) of Ro5-4864 inhibited prolactin-stimulated mitogenesis while a concentration of 10^-9 M it enhanced mitogenesis. Surprisingly, the putative antagonist, PK 11195 enhanced prolactin-stimulated mitogenesis (10^-7 through 10^-9 M) but had no effect at 10^-6 M. Clonazepam had no effect on prolactin stimulated mitogenesis over a
concentration range from $10^{-15}$ M to $10^{-3}$ M. The lack of effect with clonazepam suggests that the effects of Ro5-4864 and PK 11195 on prolactin-stimulated mitogenesis are due to interactions at a peripheral benzodiazepine binding site.

Further studies were carried out in order to determine if the two peripheral ligands, Ro5-4864 and PK 11195, were interacting at the same site. Three concentrations of each drug were used; $10^{-6}$ M (the inhibitory dose of Ro5-4864), $10^{-9}$ M (a stimulatory dose or either ligand), and $10^{-15}$ M (a no response dose for either ligand). At a concentration of $10^{-6}$ M PK 11195 blocked the enhancement of mitogenesis by $10^{-9}$ M Ro5-4864, while $10^{-6}$ M Ro5-4864 blocked the enhancement of mitogenesis by $10^{-9}$ PK 11195. A concentration of $10^{-6}$ M PK 11195 also blocked the inhibition of prolactin-stimulated mitogenesis by $10^{-6}$ M Ro5-4864. When both drugs were added at a concentration of $10^{-9}$ M the response was essentially the same as when both were tested alone.

These data suggest that the two ligands interact at the same site for two reasons: 1) Each ligand can block the other's effect on mitogenesis and 2) No additive or synergistic effect was observed when both drugs were added at $10^{-9}$ M. It is possible that the cells can not be stimulated to grow any further at this concentration of drug and prolactin. Data from the prolactin dose response curve indicates that the dose of prolactin used in these studies (0.1 ng/ml) is a sub-optimal dose. So it is possible for the cells to be stimulated to a higher degree with prolactin, but the ability of the peripheral ligands to enhance mitogenesis may be limited.
The most logical question to any pharmacologist is, how can an agonist and antagonist share the same properties. The definition of an agonist is a compound which interacts at a receptor and initiates a reaction, whereas an antagonist is defined as a compound that blocks the action of an agonist by blocking the receptor. A very intriguing phenomenon is occurring in the Nb2 cells since it is evident that the agonist and the antagonist share a common function i.e. the enhancement of mitogenesis. Two independent groups have observed a similar occurrence when studying O₂ consumption in neuroblastoma cells (Larcher et al., 1989) or respiratory control in rat mitochondria (Hirsch et al., 1988). Both groups found that R05-4864 and PK 11195 produced similar effects in their systems. Hirsch et al. (1988) suggested that since PK 11195 has full intrinsic activity that it is not a peripheral receptor antagonist in their system. In their study they also found that PK 11195 could partially block the ability of Ro5-4864 to decrease respiratory control, and vice versa, in a manner consistent with the ability of these drugs to competitively inhibit the binding of each other to the peripheral benzodiazepine receptor (Hirsch et al., 1988b). Since the actions of Ro5-4864 and PK 11195 are similar but not identical it is not possible to classify either compound to a general pharmacological class. The original differentiation between Ro5-4864 (putative agonist) and PK 11195 (putative antagonist) was determined by thermodynamic studies. A recent report by Raffa and Porreca (1989) suggested that some inconsistencies may result from interpretation of thermodynamic data for classification of agents as agonists and antagonists. Further studies are required before an accurate pharmacological classification of Ro5-4864 and PK 11195 can be made.
Possible Mechanisms of Actions for Peripheral Benzodiazepine Ligands

The fact that the Ro5-4864 and PK 11195 enhance prolactin-stimulated mitogenesis (10^{-9} M) suggests a common mechanism of action for these compounds at this concentration. This mechanism may be the ability of the compounds to displace an endogenous modulator of mitogenesis in the Nb2 cell. One candidate for this modulator is an antizyme-like, polypeptide factor described by Richards et al. (1986). This factor which was extracted from lactogen deprived, quiescent cells, markedly reduced ornithine decarboxylase (ODC) activity in log phase cells. This inhibition was dose dependent. Since ongoing polyamine synthesis regulated by ODC is necessary for continuous Nb2 cell proliferation (Offenstein et al., 1985; Elsholtz et al., 1986), this factor may prove to be the endogenous modulator that is displaced. The peripheral benzodiazepine ligands may be working by displacing this factor since Ro5-4864+prolactin has been shown to enhance ODC activity above prolactin alone (Laird et al., 1989).

Hirsch et al. (1988) performed studies to characterize ligand binding to a peripheral benzodiazepine receptor located on mitochondria (MBR). Various drugs were analyzed for their ability to inhibit [^{3}H]Ro5-4864 or [^{3}H]PK 11195 binding and the nature of the inhibition (competitive or noncompetitive) was also studied. These studies revealed several new classes of MBR ligands and suggested that the relationship between ligand structure and binding affinity is highly complex. This type of work suggests the possibility of a previously undescribed endogenous ligand which may exist in the Nb2 cell. The peripheral benzodiazepine ligands
studied here may displace this ligand in order to enhance prolactin-stimulated mitogenesis. Essentially, the ligands studied may act as pure antagonists at $10^{-9}$ M and displace the endogenous ligand from the receptor, which allows for enhancement of mitogenesis. Only peripheral ligands mediate this event since clonazepam, a central benzodiazepine ligand has no effect.

Ro5-4864 at a concentration of $10^{-6}$ M inhibits prolactin-stimulated mitogenesis, an effect unique to Ro5-4864. This may be due to the fact that Ro5-4864 may also possess agonistic properties in the Nb2 cell. Since such a high concentration is needed to cause an effect it is more likely a partial-agonist and not a full agonist. It may interact with the receptor to attenuate mitogenesis in a manner similar to the endogenous ligand. This may explain the ability of Ro5-4864 to inhibit prolactin-stimulated mitogenesis in the Nb2 cells.

Endogenous Ligand Studies

Verma and Snyder reported (1987) and characterized (1988) the interaction of porphyrins with the peripheral benzodiazepine receptor. This work was done using radioligand binding techniques measuring the ability of various porphyrins to compete with $[^{3}H]$Ro5-4864 for binding to the peripheral benzodiazepine receptor. Verma and Snyder (1988) provided suggestive evidence that the porphyrins were the endogenous ligand for the peripheral benzodiazepine receptor. However, to date no one has undertaken studies to determine if porphyrins interact in a manner similar to the ligands for the peripheral benzodiazepine receptor in a physiological situation. Since Ro5-4864 and PK 11195 modulate prolactin-
stimulated mitogenesis it is reasonable to expect that the porphyrins would elicit a similar response if there is indeed a physiological interaction between the two.

Protoporphyrin IX was chosen for these studies since Verma and Snyder (1988) suggested it was the porphyrin with the highest affinity for the peripheral benzodiazepine receptor. In the Nb2 cell system Protoporphyrin IX had no effect on mitogenesis which suggests that the porphyrins may not be the endogenous ligand in this system. There are several possibilities which may explain this apparent discrepancy between the binding data of Verma and Snyder and the lack of effect under physiological conditions. The first possibility is that another endogenous ligand exists and is present in the Nb2 cells. The possibility of other possible endogenous ligands was raised by Hirsch et al. (1988). A second possibility also raised by Hirsch's study is that Protoporphyrin IX may not be the most potent porphyrin at all. Protoporphyrin IX has an EC50 of approximately 177 nM in competition studies. The porphyrin concentration remaining in the medium after four days of incubation was determined for each experiment according to the method described by Granick et al. (1972). Approximately forty percent of the highest dose (10^-6 M) remained, this concentration (4x10^-7 M) is approximately equal to the EC50 found in the competition studies. If Protoporphyrin IX was the endogenous ligand the concentration remaining after four days should have been high enough to modulate mitogenesis. Their work suggests that other porphyrin's may have a higher affinity for the peripheral benzodiazepine receptor. Therefore, Protoporphyrin IX may not have been the best choice for this type of study. This possibility can be addressed by using other porphyrins such as hemin and deuteroporphyrin in similar studies. The possibility
also exists that porphyrins may not be stable in the cell culture medium or under the conditions under which the cells are grown. Other investigators (Verma and Snyder, 1988; Hirsch et al., 1988) have suggested that porphyrins are unstable in solution and should be made up fresh on the day of the experiment.

Other Possible Roles for the Peripheral Benzodiazepine Receptor in Mitogenesis

Verma and Snyder (1989) suggested that the peripheral benzodiazepine receptor may act as a modulator similar to the effects of the central benzodiazepine receptor at neuronal GABA-A receptors. They may modify a process that is already occurring. This may involve transcriptional modifications, energy processes or any other process critical for cell growth. This seems likely since neither of these ligands elicits an action on their own. The next sections are speculations on possible areas that the peripheral benzodiazepine receptor may affect.

Possible Role for Oncogenes

Another possible mechanism may be the ability of peripheral benzodiazepine ligands to regulate oncogene expression. In 1985, Curran and Morgan observed super induction of the c-fos proto-oncogene in rat pheochromocytoma cells (PC-12) by peripheral benzodiazepine ligands after exposure of these cells to nerve growth factor (NGF). Curran and Morgan suggested that the differentiated or physiological state of the stimulated cell may determine the nature of long term events to which c-fos action is coupled. This may prove to be the case in the Nb2
cells since c-fos expression was not sufficient to elicit cell division but the cells were competent to respond to prolactin (Andrews et al. 1987). If super induction of c-fos occurs after exposure to peripheral ligands those cells may be highly competent to respond to prolactin which would explain the enhance enhancement of mitogenesis. If these cells are "super-competent" they would grow at a faster rate and would be able to do so even at a sub-optimal dose of prolactin.

**Possible Interaction with Second Messenger System of Prolactin-Stimulated Mitogenesis**

In 1986 Anholt proposed that the peripheral benzodiazepine receptor may function as a modulator of cellular energy metabolism. Hirsch et al. (1988a) examined the effects of peripheral benzodiazepine ligands on mitochondrial respiratory control based on Anholt's proposal. They observed that micromolar concentrations of Ro5-4864 inhibited the respiratory control ratio (RCR) 100% over control. This observation may provide an explanation for the inhibition of prolactin-stimulated mitogenesis by $10^{-6}$ M Ro5-4864.

Two groups have suggested that ongoing phospholipase C activity is necessary for Nb2 cell proliferation and that this may be responsible for at least part of the signal transduction cascade (Offenstein and Rillema, 1987; Russell et al., 1987). Phospholipase C is responsible for phosphoinositide turnover. The immediate substrate for phosphoinositide breakdown is phosphatidylinositol 4,5 bisphosphate which may be replenished by phosphorylation of phosphatidylinositol (Berridge, 1984). Since the formation of phosphoinositides is ATP dependent, the
receptor-mediated breakdown of these should be sensitive to changes in energy metabolism. Thus Ro5-4864 at a concentration of 10⁻⁶ may inhibit the cellular energy required for the second messenger system in the Nb2 cells. Without this stimulus these cells will not proliferate as readily as the control (prolactin alone) which may explain the inhibition.

Another possible mechanism through which the inhibition may take place is the inhibition of a calcium-calmodulin (Ca-CaM) kinase system. The hydrolysis of phosphoinositides represents a bifurcation in the signal pathway, in that the formation of two separate second messengers occurs (Berridge, 1984). One limb depends upon diacylglycerol (DAG) activating protein kinase C (PKC) while the other depends on calcium which interacts with calmodulin to elicit a response. This type of bifurcating pathway may provide the versatility necessary to introduce subtle variations in the control of cellular mechanisms. These two pathways may contribute to a final response by acting co-operatively or synergistically. The relative importance of each pathway may also vary with time, in that calcium may be responsible for initiation whereas diacylglycerol may be more important in maintaining the response (Kojima et al., 1983; Zwalich et al., 1983). DeLorenzo et al. (1981) found that micromolar concentrations of diazepam and Ro5-4864, ligands which interact with the peripheral benzodiazepine receptor, inhibit a calcium-calmodulin protein kinase system in brain membranes. The inhibition of the Ca-CaM kinase may not allow for the initiation of events necessary for immediate Nb2 cell proliferation and the protein kinase C pathway may not be as powerful a stimulus to allow for normal proliferation. This pathway does allow for subtle variations which may explain
why PK 11195 at a concentration of 10^{-6} M does not inhibit mitogenesis. It may also provide an explanation for the ability of 10^{-15} M PK 11195 to block the effect of 10^{-6} M Ro5-4864. The Ca-CaM pathway may be activated enough so that normal proliferation may occur thus allowing for the normal growth pattern.
SUMMARY and CONCLUSIONS

The peripheral benzodiazepine ligands, Ro5-4864 and PK 11195 can modulate prolactin-stimulated mitogenesis in the Nb2 cell. Both can enhance mitogenesis at a concentration of $10^{-9}$ M while only Ro5-4864 inhibits mitogenesis at a concentration of $10^{-6}$ M. This observation suggests that Ro5-4864 may be acting as a partial agonist at this receptor. Therefore, the previous classification of these drugs as agonist (Ro5-4864) and antagonist (PK 11195) may have to be reinterpreted in light of this information.

Both Ro5-4864 and PK 11195 seem to be acting upon a common receptor site, the peripheral benzodiazepine receptor site. Both drugs can antagonize the other's effects and when the drugs are added simultaneously at $10^{-9}$ M no additive or synergistic effect was observed. If these drugs acted at different sites one of these effects might be expected. The effect observed in the Nb2 cells is specific for the peripheral benzodiazepine receptor, since clonazepam a central benzodiazepine receptor agonist had no effect on prolactin-stimulated mitogenesis in the Nb2 cells.

The Nb2 node lymphoma cell provides an excellent model with which to study cellular mitogenesis and the factors that affect it. This study has shown that peripheral benzodiazepine ligands modulate prolactin-stimulated mitogenesis in these cells. The mechanism remains unknown. All the possibilities reviewed seem to suggest a modulatory role of events already occurring in response to prolactin stimulation. This role may be compared to that of benzodiazepines
working at the GABA-A receptor. This system can be used to elucidate mechanisms of action such as interactions with second messenger systems, endogenous modulators, and oncogenes.
REFERENCES


