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INTRACELLULAR [^{3}H]DOPAMINE BINDING SITES IN NORMAL AND MALIGNANT CELLS : RELATIONSHIPS TO CELL PROLIFERATION

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SUMMARY: **Dopamine interaction with target cells undoubtably involves binding to plasma membrane receptors. However, the well documented cell growth inhibitory activity of this catecholamine suggests nuclear regulation. To evaluate this possibility, we determined the intracellular localization and binding of [^{3}H]dopamine in human retinoblastoma (Y-79 cells), normal mouse fibroblasts (LM-cells), and in the rat uterus. Cytosol and purified nuclear preparations devoid of plasma membrane components contained specific, saturable, high affinity (Kd~20 nM) binding sites for [^{3}H]dopamine. The nuclear binding affinity for dopamine, L-dopa, and L-dopa methyl ester correlated with the inhibitory effects of these compounds on cell proliferation, suggesting that intracellular dopamine binding sites may also be involved in cellular response to catecholamines.**

Dopamine and related compounds inhibit malignant cell proliferation in vitro (1-4) and mammary tumor and melanoma growth in vivo (5-16). The in vivo effects on mammary tumors have usually been attributed to dopamine inhibition of prolactin release from the pituitary (5-11), however, the in vitro studies demonstrate direct inhibition of tumor cell proliferation by these compounds. Although dopamine regulation of nuclear biosynthetic events is not considered a typical response of target cells to this catecholamine, studies in our laboratory demonstrated that a similar compound, methyl p-hydroxyphenyllactate (MeHPLA), may regulate normal and malignant cell proliferation through interaction with a specific nuclear binding site (17). MeHPLA and dopamine are tyrosine metabolites (18-20), and therefore, parallel mechanisms may exist. For these reasons we examined the accumulation and compartmentallization of [3H]dopamine in Y-79 retinoblastoma cells which were previously shown to synthesize dopamine from tyrosine (21). The results of these studies demonstrate that retinoblastoma cells contain high affinity binding sites for dopamine in the cytosol and nuclear fractions and the binding affinity of dopamine, L-dopa, and methyl l-dopa correlated with inhibitory effects of these compounds on cell

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proliferation. The presence of these binding sites was not restricted to cultured malignant cells as nuclear preparations from the rat uterus also contained high affinity [^{3}H]dopamine binding sites. The direct stimulatory effects of estradiol on the nuclear levels of [^{3}H]dopamine binding sites correlated with the mitogenic activity of this steroid hormone on uterine cellular proliferation and the well documented effects of catecholamines on the uterus. These finding suggest that in addition to mediating target cell response through well documented cell membrane receptor systems , dopamine and related catecholamines may regulate cellular proliferation through intracellular cytoplasmic and nuclear binding mechanisms.

MATERIALS AND METHODS

Y-79 Retino-blastoma cells were grown in our laboratory under standard conditions in RPMI(Y-79) containing 10% fetal bovine serum, insulin and penicillin/streptomycin (21). For cell fractionation experiments and the preparation of cytosol and nuclear fractions for [^{3}H]dopamine binding assays, the cell pellets from 2-3 T-150 flasks (Corning) were collected by centrifugation, washed in TES buffer (10 mM tris, 1.5 mM EDTA, 30% sucrose, pH 7.4 at 22°C), and homogenized for binding analysis as described below. For cell growth inhibition assays, Y-79 were plated in 24-well Co-Star multiplates (seeding density = 20,000 cells/well), and 24-48 hours following plating (Day 0), the cells were treated with the indicated concentrations of dopamine and or related analogues (figure 4) by direct addition of the compounds to the culture medium. Cell number was determined by hemocytometer counts (17) 48 hours following treatment. The compounds were dissolved in 10 µL 100% ethanol and controls were also treated with this vehicle. Minimal suppression (<10%) of cell proliferation was observed in the alcohol treated controls.

For direct labeling experiments, exponentially growing Y-79 cells were cultured in the presence of 40 nM [^{3}H]dopamine (total binding) or 40 nM [^{3}H]dopamine plus 4.0 µM dopamine (non-specific binding) for 24 hours. At this time, the cell suspensions were cooled to 4°C for 30 minutes and washed by resuspension and centrifugation (800 x g) in TES buffer. The final washed cell pellet was homogenized in Kontes glass-glass homogenizers, and the cytosol, mitochondrial, microsomal and nuclear fractions were prepared by differential centrifugation (22,23). Aliquots of these cell fractions were solubilized in NCS (Amersham Radiochemicals) and counted in a liquid scintillation spectrometer to determine specific [^{3}H]dopamine binding (total binding minus non-specific binding).

To determine whether the [^{3}H]dopamine binding in the cytosol and nuclear preparations resulted from contamination by plasma membrane dopamine receptors, we measured alkaline phosphatase activity and [^{3}H]dopamine binding in the cytosol, crude and purified nuclear fractions from Y-70 cells. Alkaline phosphatase is a specific marker enzyme for plasma membrane (22,24). For these experiments, exponentially growing Y-79 retinoblastoma cells (3 T-150 flasks/experiment) were harvested by centrifugation, homogenized in TES buffer, and the cell fractions were prepared by well documented procedures for the preparation of highly purified cytosol, mitochondrial, microsomal and nuclear fractions (22). The cytosol (105,000 x g supernatant), crude nuclear pellet (800 x g pellet), and purified nuclei (obtained by centrifugation through a discontinuous sucrose gradient) were assayed for [^{3}H]dopamine binding as described below. The purified nuclei were essentially devoid of cytoplasmic tags as judged by phase contrast microscopy. The alkaline phosphatase activity in the cytosol and nuclear (crude and purified) fractions was determined by utilizing p-nitrophenylphospate as substrate (22). This assay was validated utilizing highly purified alkaline phosphatase purchased from Worthington Biochemical Corporation (Freehold, NJ).

The methodology used for the measurement of [^{3}H]dopamine binding in cytosol and nuclear fractions was adopted from procedures developed in our laboratory for the measurement of estrogen binding sites by [^{3}H]estradiol exchange (26). Briefly, the various tissue (uterus), and cultured cell preparations (Y-79 and MCF-7 cells) were homogenized in TES buffer, and the cytosol (40,000 x g) and nuclear pellet (800 x g) fractions were obtained by centrifugation. An aliquot of the total homogenate was removed for the determination of DNA by the method of Burton (31). Following centrifugation, the cytosol and nuclear fractions were diluted in TES buffer , and incubated (4°C x 60 minutes) in the presence of [^{3}H]dopamine (2-40 nM) ± 0.2-4.0 µM dopamine. Following the incubation, the cytosol fractions were incubated (4 °C x 15 minutes) in the presence of 500 µL of a hydroxylapetite suspension (60 % in TES buffer) to separate protein bound [^{3}H]dopamine from free ligand, and the HAP pellet was

washed by resuspension and re-centrifugation in TES buffer (26). The final washed HAP pellet was extracted with ethanol and counted in a liquid scintillation counter, and the specific binding was determined. Similarly, free [^{3}H]dopamine was removed from nuclear pellets by washing and recentrifugation (3 times at 800 x g x 10 minutes in a Beckman JS-7.5 rotor), and the [^{3}H]dopamine bound to the nuclear pellet was extracted in ethanol and counted in 4.0 mL of ACS (Amersham Radiochemicals). Specific binding was expressed as pmoles [^{3}H]dopamine bound per cell assuming each nucleus contained 8 pg DNA.

RESULTS AND DISCUSSION

To determine whether mammalian cells contain intracellular [^{3}H]dopamine binding sites, we studied the intracellular localization of this compound in human retinoblastoma (Y-79) cells which have been shown to synthesize dopamine from tyrosine (21). Exponentially growing Y-79 cells were cultured in the presence of [^{3}H]dopamine ±100 fold excess unlabeled dopamine (non-specific binding) and specific binding was determined in the cytosol, mitochondrial, microsomal and nuclear fractions (17, 22) prepared from these cells. Although dopamine receptors have been classically studied is crude cell membrane fractions (23), the present studies demonstrated that the cytosol and nuclear fractions from Y-79 cells contained ~90% of the total cellular [^{3}H]dopamine binding (figure 1A) and only low levels (~10%) were measured in the mitochondrial and microsomal pellet fractions.

Since it was possible that the cytosol and nuclear binding in Y-79 cells represented plasma membrane dopamine receptor (23) contamination, we measured [^{3}H]dopamine binding sites and alkaline phosphatase activity in the total cellular homogenate, cytosol, crude nuclear-myofibrilar pellet, and highly purified nuclear fractions prepared from Y-79 retinoblastoma cells. Alkaline phosphatase is a marker for plasma membrane contamination of nuclear preparations (22, 24). The data (figure 1B) demonstrated that the specific [^{3}H]dopamine binding was equally distributed and quantitatively recovered in the cytosol and nuclear fractions as compared to the total cellular homogenate, and the distribution of alkaline phosphatase activity in these fractions did not correlate with the dopamine binding. Likewise, highly purified nuclei contained ~85% of the [^{3}H]dopamine binding (as compared to crude nuclei), and only 0.02% of the total homogenate alkaline phosphatase activity (figure 1 B). Therefore, the [^{3}H]dopamine binding measured in cytosol and nuclei was not derived from plasma membrane contaminants. Autoradiographic studies demonstrated that [^{3}H]dopamine localizes to specific nuclear regions in rat pituitary cells (25). Very little labeling was observed in the endoplasmic reticulum or the mitochondria (25) which supports our results for the microsomal and mitochondrial pellet fractions (figure 1A).

To characterize the [^{3}H]dopamine binding sites, nuclear fractions were prepared from Y-79 cells (17, 26) and incubated (4°C x 60 minutes) in the presence of [^{3}H]dopamine (4-40 nM) or [^{3}H]dopamine + unlabeled dopamine (0.4-4.0 μM). Following incubation, the nuclear pellets were washed to remove free ligand, and the bound ligand (27-30) was determined (see figure 2 for details). A single saturable specific [^{3}H]dopamine binding site was measured and non-specific binding (not competed with 100-fold excess unlabeled dopamine) was linear, and represented ~50 % of the total binding. On the basis of the nuclear DNA content (31), retinoblastoma cells contained ~30,000 sites/cell assuming one [^{3}H]dopamine binding site/ molecule (figure 1 B). The curvilinearity of the Scatchard plot (figure 2 C) and the

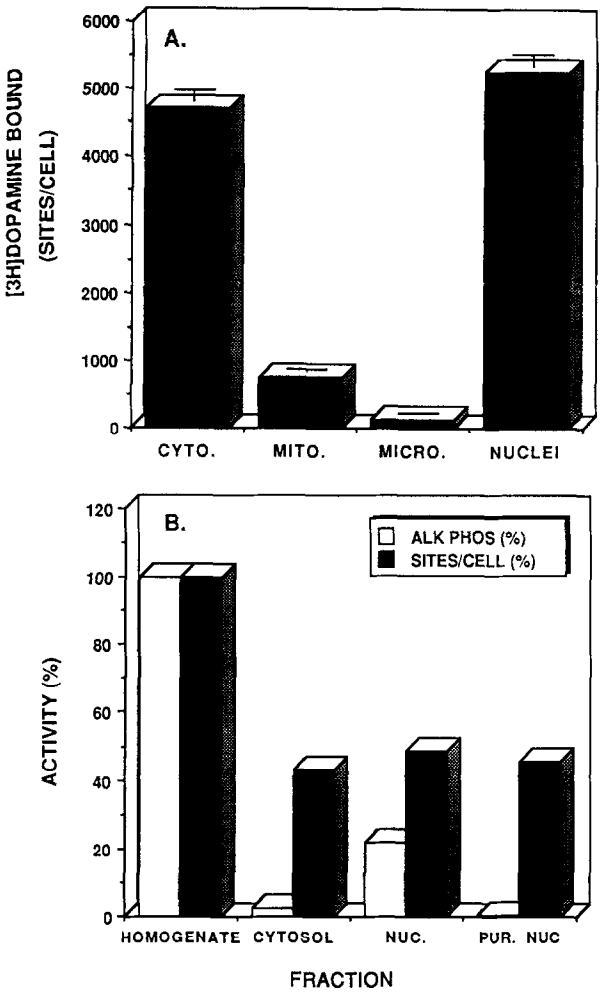

Figure 1. Intracellular Distribution of [^{3}H]Dopamine Binding Sites and Alkaline Phosphatase Activity in Y-79 Retinoblastoma Cells. (A) Exponentially growing Y-79 cells (21) were grown in the presence of 40 nM [^{3}H]dopamine (or 40 nM [^{3}H]dopamine plus 4 μM dopamine) for 24 hours. At this time, the cytosol (Cyto.), mitochondrial (Mito.), microsomal (Micro.) and crude nuclear-myofibrilliar (nuclei) fractions were prepared (22). Aliquots of these cell fractions were counted to determine specific binding (total [^{3}H]dopamine binding minus non-specific binding) and results were expressed on the basis of DNA content (sites/cell) assuming 8 pg DNA per cell nucleus. (B) Exponentially growing Y-79 cells (2-3 T-75 flasks) were harvested, fractionated, and the cytosol, crude nuclear pellet (Nuc.) and highly purified nuclei (Pur. Nuc) were incubated with 40 nM [^{3}H]dopamine ± 4.0 μM dopamine (non-specific binding) at 4° x 60 minutes to determine specific binding. Results (specific [^{3}H]dopamine binding or alkaline phosphatase activity) were expressed as a per cent of the total activity measured in the whole cell homogenate.

determination of a Hill coefficient (~2) for these data (not shown) suggested positive cooperativity. On this basis, we estimated approximately 15,000 [^{3}H]dopamine binding sites per retinoblastoma cell.

Similar binding curves (not shown) were also obtained with purified nuclei from Y-79 cells, mouse fibroblasts (LM-cells) and in cytosol preparations from Y-79 cells (figure 2 D). Therefore, this binding was not a phenomenom observed only in preparations from cultured malignant cells. Our laboratory has obtained similar sigmoidal saturation curves for [^{3}H]estradiol binding to nuclear type *II*

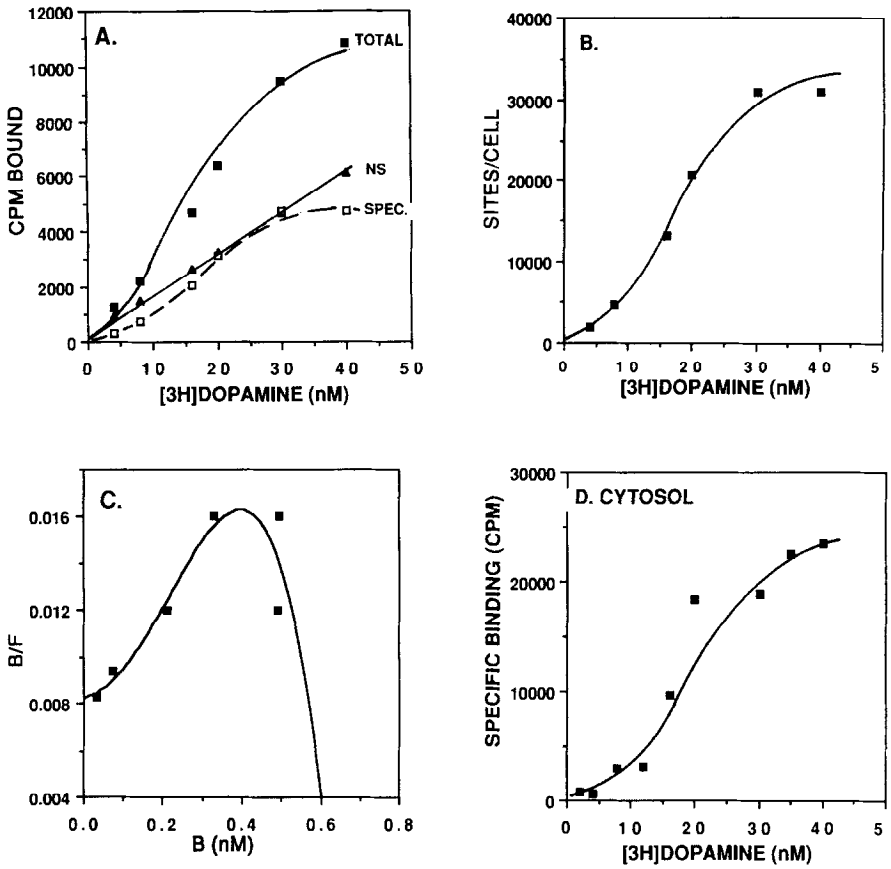

Figure 2. Measurement of [^{3}H]Dopamine Binding Sites in Y-79 Retinoblastoma Cell Cytosol and Nuclear Fractions. (A-C) Nuclear fractions from Y-79 cells were prepared (22, 27-30), resuspended in TES buffer, and aliquots (250 µl) incubated (4°C) with [^{3}H]dopamine (2-40 nM) ± 0.2-4.0 µM unlabeled dopamine (non-specific binding). Specific [^{3}H]dopamine binding in final washed pellets was determined as previously described (27-30) for steroid binding assays. (A) Actual binding measured in the nuclear assay. (B) Specific nuclear binding expressed as sites/cell (see figure 1 for details). (C) Scatchard plot (39) of specific binding data in A. (D) Specific binding determined in cytosol fraction by hydroxylapetite adsorption as described in figure 1.

sites in the rat uterus and a variety of tissues (27-30,33), and MeHPLA is the endogenous ligand for this binding site (17). Since dopamine is structurally similar to MeHPLA, we suspected that the [^{3}H]dopamine might be binding to type II sites. To evaluate this possibility, rat uterine nuclear preparations were assayed for [^{3}H]dopamine binding sites, and binding specificity was also determined. These experiments demonstrated that rat uterine nuclei contained [^{3}H]dopamine binding sites and the level of this site was dramatically increased 2-3-fold following in vivo treatment with estradiol (figure 3 A). However, neither estradiol (E_2) or MeHPLA competed for [^{3}H]dopamine binding in rat uterine nuclei whereas both compounds interact with nuclear type II binding sites with a very high affinity (17, 27, 28). Therefore, nuclear [^{3}H]dopamine binding sites are not type II estradiol binding sites.

The function of [^{3}H]dopamine binding sites in the rat uterus remains to be resolved. These sites were increased by estrogen (figure 3A) under conditions where cellular hypertrophy, hyperplasia and

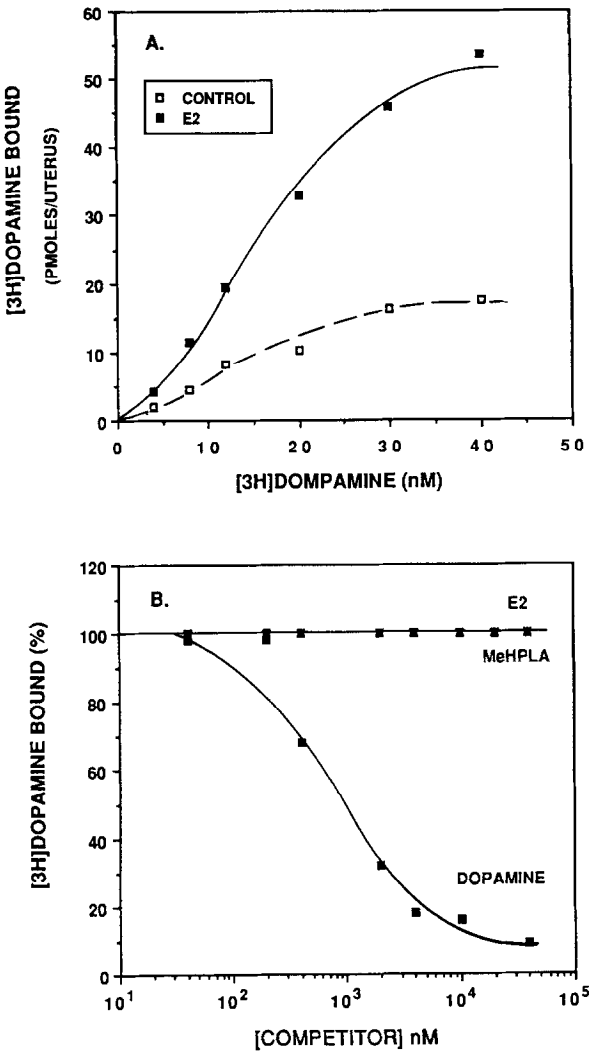

Figure 3. [^{3}H]Dopamine Binding Sites in the Rat Uterus. (A) Adult-ovariectomized female rats were implanted with beeswax pellets (controls) containing estradiol (E2; 2mg) for 96 hours (30-32). The uteri were removed, homogenized in TES buffer, and the nuclear pellet fractions were assayed for [^{3}H]dopamine binding sites as described in figure 2. (B) Rat uterine nuclei were incubated with 40 nM [^{3}H]dopamine in the presence of the indicated concentration of competitor. Results were expressed as % bound in the absence of competing ligand where 100% was ~5500 cpm.

DNA synthesis were observed suggesting a direct correlation with uterine cellular proliferation (27-29). Dopamine, nor-epinephrine, and epinephrine levels vary in the rat uterus with the estrus cycle (34), as does the uptake of circulating [^{3}H]epinephrine (35). Furthermore, we found that competable binding was detectable in the cytosol and nuclear fractions prepared from the mouse uterus 2 hours following a subcutaneous injection of [^{3}H]dopamine (382 ng; 100 μCi). In fact, the specific cpm measured in total uterine tissue (121 ± 13 fmoles/uterus), was quantitatively accounted for in the cytosol (106 ± 3 fmoles/uterus) and nuclear (15 ± 1.9 fmoles/uterus) fractions. Therefore, [^{3}H]dopamine and/or a metabolite(s) accumulated in the cytoplasm and nucleus of uterine cells and we suspect this was due to a

Table I. Relative Binding Affinity of Phenols and Catechols for [^{3}H]Dopamine Binding in Retinoblastoma Cell Nuclei

Compound	*Relative Binding Activity (%)
Dopamine	100.0
D-Dopa	2.6
L-Dopa	2.6
L-Dopa Methyl Ester	40.0
5-hydroxydopamine	80.0
6-hydroxydopamine	2.6
nor-epinephrine	100.0
epinephrine	100.0
5,6-hydroxytyramine	80.0
5,7-hydroxytyramine	0.0
Serotonin	0.0
tyrosine	0.0
phenylalanine	0.0

* Relative Binding Activity (RBA) = $\frac{\text{[dopamine] at 50\% inhibition} \times 100}{\text{[competitor] at 50 \% inhibition}}$.

binding interaction with the cytosol and nuclear dopamine binding sites. We are currently studying the relationships between nuclear dopamine binding and uterine responses to estrogenic hormone stimulation.

To assess the specificity of the dopamine binding sites in Y-79 cell nuclei, competition analysis was performed with a number of dopamine related compounds. Dopamine, nor-epinephrine, and epinephrine possessed the highest binding affinities (Table I) for the nuclear [^{3}H]dopamine binding site. Apparently, some stereospecificity is associated with the catechol ring since 5-hydroxydopamine competed very well for dopamine binding to this site (RBA 80%), as compared to 6-hydroxydopamine (RBA 2.6%). Equivalent relationships were observed for 5,6- and 5,7-dihydroxytyramine, respectively. Conversely, serotonin, L-tyrosine, and L-phenylalanine did not compete for [^{3}H]dopamine binding demonstrating that the catechol ring is essential for this binding interaction.

Since L-dopa methyl ester has been shown to inhibit human melanoma cell proliferation in vivo and in vitro (13-16), we evaluated the ability of this compound, as well as L-dopa, to compete for [^{3}H]dopamine binding in Y-79 cell nuclei. L-dopa-methyl ester possessed approximately a 15-fold higher binding affinity for the nuclear [^{3}H]dopamine binding site than L-dopa (figure 4A and Table I) and in fact was equivalent to dopamine. These finding are analagous to our studies with MeHPLA (17) demonstrating that esterification of the carboxylic acid imparts high binding affinity on this type of molecule. MeHPLA binds in nuclei with a 20-fold higher affinity than HPLA, and this directly correlates with MeHPLA, but not HPLA, inhibition of normal and malignant cell proliferation (17). To determine whether a similar phenomenom exists with L-dopa and L-dopa methyl ester, we assessed the effects of these compounds, as well as dopamine, on the proliferation of Y-79 cells. Both dopamine and L-dopa methyl ester inhibited Y-79 cell proliferation (figure 4 B) at much lower concentrations (50% inhibition at ~ 20 μM) than L-dopa (50% inhibition at ~200 μM). Since L-dopa interacts with nuclear [^{3}H]dopamine binding sites with ~15-fold lower affinity than L-dopa methyl ester or dopamine (figure 4A), a correlation exists between the binding affinity and cell growth inhibition. That ~1.0% of this type of compound is

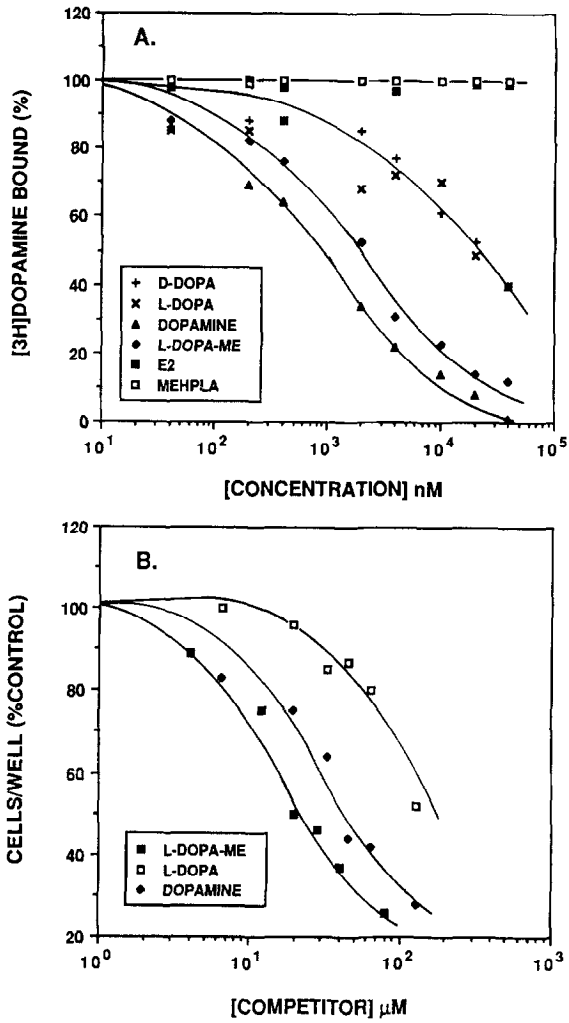

Figure 4. Effects of Dopamine and Related Analogs on [^{3}H]Dopamine Binding in Y-79 Cell Nuclei and on Cell Proliferation. (A) Nuclear fractions from retinoblastoma cells were incubated (4°C x 60 minutes) in the presence of 40 nM [^{3}H]dopamine ± the indicated concentration of the competitor (0.04-40 μM) and results were expressed as per cent bound (100 % ~5000 cpm) measured in the absence of competitor. (B) Exponentially growing Y-79 cells were treated with the indicated compound (2-200μM) and cell number (cells/well; 12 determinations per concentration) was quantitated 24 hours following treatment (17). Abbreviations were: L-dopa (L-DOPA), L-dopa methyl ester (L-DOPA-ME), estradiol (E2), methyl p-hydroxyphenyllactate (MEHPLA), dopamine (DOPAM.).

actually taken up by Y-79 cells under these experimental conditions (not shown) suggests that the effective concentration of these compounds is ~200 nM (1.0% of 20 μM). Perhaps L-dopa methyl ester or a similar methylated dopamine metabolite is the endogenous ligand for the [^{3}H]dopamine binding sites. Dihydroxyphenylacetic acid (DOPAC) is a dopamine metabolite (36) and it is possible that this compound may be methylated in vivo to MeDOPAC. Although the ability of dopamine and other catechols to inhibit cellular proliferation has usually been attributed to the auto-oxidation of these compounds to quinones which covalently bind to protein (1-4, 37), our data suggest that perhaps more specific events are involved. If quinone formation were the only factor involved in cell growth inhibition, similar dose

response curves would have been obtained with L-dopa, L-dopa methyl ester and dopamine (figure 4 B), and this was not the case.

Whether the [^{3}H]dopamine binding sites represent intracellular catecholamine receptors remains to be established. [^{3}H]Dopamine localizes in specific nuclear regions of pituitary cells in vitro (25), and we have observed nuclear binding following exposure to [^{3}H]dopamine in vitro (figure 1) or in vivo (mouse uterus). The nuclear [^{3}H]dopamine binding site resists high salt extraction (not shown), suggesting association with the nuclear matrix where DNA replication occurs (38). The localization of [^{3}H]dopamine binding sites on the nuclear matrix is consistent with the observed direct regulatory effects of dopamine and related compounds on normal and malignant cell proliferation (figure 4 B and 13-16). Inhibition of mammary tumor growth in vivo has classically been attributed to indirect effects of dopamine on pituitary prolactin secretion (5-11), however, our data support direct intracellular effects of dopamine and related compounds on malignant cells. If this is the case, the regulation of normal and malignant cell function by dopamine may not only be mediated by binding to cell membrane receptors (23), but may also involve direct interactions with intracellular cytoplasmic and nuclear catecholamine binding sites. The regulation of nuclear biosynthetic events by dopamine and related compounds through a nuclear binding interaction may lead to new inroads regarding the mechanism of action of catecholamines in target cells.

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