

Modulation of High Affinity Phenylalkylamine Binding Sites on Cultured Human Embryonal Vascular Smooth Muscle Cells

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Abstract. Two phenylalkylamine Ca^{2+} channel ligands, (\pm) - $[\text{H}]$ verapamil $((\pm)$ - $[\text{H}]$ V) and $(-)$ - $[\text{H}]$ desmethoxyverapamil $((-)$ - $[\text{H}]$ DV), were employed in whole cell binding assays to characterize the specific high affinity binding sites on Ca^{2+} channels, their cooperativity and modulations induced on cultured human embryonal vascular smooth muscle preparation (VSM) by 1) Beta-adrenergic stimulation of the cell, 2) exposure to high K^+ concentration, 3) exposure to high concentration of Mg^{2+} ions, 4) the presence of a benzothiazepine Ca^{2+} channel antagonist and modulator d-cis-diltiazem, and 5) guanylylimidodiphosphate

The total amounts of specific (\pm) - $[\text{H}]$ V and $(-)$ - $[\text{H}]$ DV binding sites present on VSM cells increased significantly after beta-adrenergic receptor activation, following cell membrane depolarization induced by high concentrations of K^+ , in the presence of Ca^{2+} chelator Na_3EDTA , and after incubation of VSM cells with a benzothiazine-type Ca^{2+} channel blocker d-cis-diltiazem. A marked reduction of $(-)$ - $[\text{H}]$ DV binding was observed after permanent G-protein activation by a nonhydrolyzable analog of guanylylimidodiphosphate, after incubation of the cells with norepinephrine, and after incubation of VSM cells with millimolar concentration of Mg^{2+} .

The results suggest the existence of multiple modulations of specific $(-)$ - $[\text{H}]$ DV binding sites on Ca^{2+} channel corresponding to the way of activation of the cell and also to the immediate "state" of the membrane bound Ca^{2+} channels present on VSM cells, the positive heterotropic interaction after beta-adrenergic stimulation, the homotropic positive allosteric interaction induced by d-cis-diltiazem and pure noncompetitive inhibition induced by guanylylimidodiphosphate. The presence of high concentrations of Mg^{2+} inhibited whereas the presence of Ca^{2+} chelator, of ethylenediamine-tetraacetic acid sodium salt, significantly increased the total number of specific high affinity $(-)$ - $[\text{H}]$ DV binding sites on VSM cells.

Key words: Calcium channel — Phenylalkylamine receptor — Calcium channel modulators — Human embryonal vascular smooth muscle cells — (-) — [³H]desmethoxyverapamil — (±) — [³H]verapamil — ligand binding

Introduction

Vascular smooth muscle (VSM) is presumably a therapeutic target of a class of compound known as Ca²⁺ antagonists. The Ca²⁺ channel protein binds Ca²⁺ channel drugs with high or low affinity to the resting, open, or inactivated state of the channel. There is evidence for phenylalkylamine (PAA), 1,4-dihydropyridine (DHP) and benzothiazepine (BTZ) receptors existing on Ca²⁺ channels as three distinct receptor sites that are reciprocally allosterically coupled (Ferry et al. 1984, Galizzi et al. 1984, Goll et al. 1984, Glossmann et al. (1985). Ligand binding studies with skeletal muscle Ca²⁺ channels showed that PAAs may label specific binding sites that are inhibited by Ca²⁺ or other divalent cations (Schneider et al. 1991).

Furthermore it has been shown that purified Ca²⁺ channel receptor protein binds PAAs with significantly lower affinity than does a membrane-bound receptor (Janis et al. 1987, Glossmann and Striessing 1988). Somewhat conflicting findings on PAA binding in purified Ca²⁺ channel proteins were recently explained by the existence of different states of Ca²⁺ channel attributed to the different conformation of the alpha subunit of the Ca²⁺ channel protein (Mikami et al. 1989). It has been proposed that association of PAA type Ca²⁺ channel ligand with the Ca²⁺ channel protein is possible only in physiologically relevant open state of the channel (Striessing et al. 1990). The cyclic version of the modulated Ca²⁺ receptor model assumes closed channels activating upon depolarization or stimulation with agonists and open transiently before passing into inactivated state (Jones 1991). Only the activation of Ca²⁺ channel is presumably directly affected by voltage, with the opening of the channel being faster and the closing slower at the depolarized voltages (Bean 1989).

The proposed allosteric model of modulated Ca²⁺ channel has recently been characterized also in vascular smooth muscle (Dřimal 1989, 1991, Dřimal et al. 1991). Our previous whole cell ligand binding studies have indicated that verapamil class drugs interact in an allosteric fashion with 1,4-DHP receptors on VSM cells to inhibit 1,4-DHP binding. The present study addresses these questions with pharmacologically modulated membrane-bound phenylalkylamine Ca²⁺ channel receptor of human embryonal vascular smooth muscle cell. Using this cell model we describe the general characteristics of specific binding of (-) — [³H]desmethoxyverapamil ((-) — [³H]DV) and [³H]verapamil ((±) — [³H]V) to native and pharmacologically pretreated human embryonal vascular smooth muscle cells.

Materials and Methods

Chemicals

(-)-[³H]desmethoxyverapamil, specific activity 81.1 Ci/mmol (Knoll AG, Ludwigshafen, Germany); N-methyl-[³H]-verapamil-hydrochloride, specific activity 81 Ci/mmol (NEN Res. Prod., DuPont, Boston Ma., USA); BAYK8644 (Calbiochem); d-cis-diltiazem; (Lachema); guanylylimidodiphosphate (RBI); isoprenaline-hydrochloride (Fluka); phorbol 12-myristate-13-acetate (Sigma); noradrenalin (Sigma); Tris(hydroxymethyl)-aminomethane HCl (Serva); trisodium salt of ethylenediamine tetraacetic acid (Sodium Versenate, Riker Labs). Serum for cell cultures and other cell culture reagents were from Gibco (Grand Island, N.Y., USA).

Cell culture

VSM cells were originally obtained from human embryonal aorta (embryos 4–6 wk. of gestation). The VSM cells were grown in Dulbecco's modified Eagle's medium containing glucose and 10% heat inactivated (55 °C for 60 min) fetal bovine serum, penicillin G (50 i.u./ml), streptomycin-sulfate (50 µl/ml) at 37 °C in an atmosphere of 5% CO₂ and 95% air. The cell line was maintained in the exponential growth phase by passaging every 4 days. Vascular smooth muscle cells had a characteristic spindle-like morphology and showed typical growth. The cell type used and the membrane bound receptors present on cells were characterized elsewhere (Dřimal 1989, Dřimal et al.1991). Experiments were performed on confluent cells in the eighth to fifteenth passage. The cells used for experimentation were plated in 35 ml plastic tissue culture dishes at a density 10⁵ cells/mm². After the VSM cells reached confluency they were washed with 5 ml of Tris-HCl — trisodium- ethylenediamine-tetraacetic acid buffer (0.54 mmol.l⁻¹ (sodium versenate) and 0.02 mol.l⁻¹ Tris-HCl, pH 7.4, at 37 °C) and subsequently briefly with collagenase solution (0.1%), and centrifuged (at 250 ×g for 5 min) together with the cultivation medium without serum and glucose to collect detached cells. The cells were washed twice with the cultivation medium and then incubated for 60 min with the medium containing pharmacologically active substances. All experiments were carried out on confluent quiescent cells.

Cell viability

Cell counting was performed with Trypan blue according to Phillips (1973).

Radoligand binding to human VSM cells

At the end of 60 min incubation period both control and pretreated cells were scraped off the plates and harvested onto Whatman GF/C glass fiber filters. The cells were washed twice with 1.5 ml assay buffer (a mixture, containing 80% of Dulbecco's modified Eagle's medium (without glucose and serum) with 20% of 50 mmol.l⁻¹ Tris/HCl, pH 7.51) and incubated for 60 min with (-) - [³H]desmethoxyverapamil or (±) - [³H]-verapamil (0.1 – 12 nmol.l⁻¹). After incubation the cells were washed twice with 3.0 ml of assay buffer and bound drug was separated from free drug by rapid filtration through GF/C glass microfibre filters followed by two consecutive buffer washes. The radioactivity bound on filters was collected in 10 ml scintillation cocktail LSD Spolana Neratovice and counted in a Rack Beta-Liquid Counter LKB Wallace Turku, Finland at an efficiency of 65%.

Analysis of binding data was performed as described previously (Dřimal 1988, 1989). The specific binding was determined by subtracting binding in the presence of $0.1 \mu\text{mol}$ of unlabeled compound from values measured in the absence of the antagonist.

Experiments with guanylylimidodiphosphate were done on electropermeabilized cells. For purposes of permeabilization of the cell membrane, we used the method according to Rols et al. (1992) now widely applicable in studies with mammalian cells. The depolarization conditions of cells were produced by incubating of cells in buffers containing high K^+ concentrations (10, 20, 40 and $60 \text{ mmol.l}^{-1} \text{ K}^+$).

Other determinations

Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as standard. Data reported in the present study are mean values and the corresponding standard error of the mean. Non-linear regression analysis, Student's *t*-test for either paired or unpaired observations, and Statgraphic program package were used. Significance was considered to be present when $P < 0.05$.

Results

Cell viability: Confluent cultures of vascular smooth muscle cells derived from large arteries of human embryos contained a mixture of cells, endothelial cells and mostly adventitial fibroblasts. The cell responses to serum were dependent on the serum concentration in the cultivation medium, with the endothelial cell requirements being relatively large when compared to smooth muscle cells. A light micrograph showed the characteristic appearance of confluent smooth muscle cells: typical outgrowth (2–4 days of logarithmic growth), spindle-like cell form and more or less irregular shape.

The cells incubated in the presence of the pharmacologically active substances (in $\mu\text{mol.l}^{-1}$) guanylylimidodiphosphate (10.0), *d*-cis-diltiazem (0.1–1.0) and isoprenaline (0.1–20.0), or (in mmol.l^{-1}) MgCl_2 (5.0–7.5) and trisodium salt of ethylenediamine tetraacetate (Sodium Versenate), (0.1–1.0) showed no evidence of lysis when compared by light microscopy with control cultures. Cell mortality, determined with the Trypan blue test was $3.2 \pm 1.1\%$ in both groups.

Characterization of (–)– ^3H –desmethoxyverapamil and (±)– ^3H –verapamil binding sites on native, polarized human embryonal vascular smooth muscle cells

Confluent human embryonal VSM cells were frozen for 2–3 days and thawed before use. It was found that VSM preparations can be stored for several days at -20°C without significant loss of growth in culture or loss of binding activity. Saturation analysis with two "open channel ligands" showed saturable binding to a homogenous population of high affinity binding sites. The tritiated Ca^{2+} channel antagonists of phenylalkylamine type used in the present study were (–)– ^3H –DV and the more polyvalent, hydrophobic (±)– ^3H –verapamil, with more promiscuous

receptor binding affinity. As expected, $(-)-[{}^3\text{H}]\text{V}$ labeled in polarized VSM cells significantly more binding sites with comparably lower affinity than did $(-)-[{}^3\text{H}]\text{DV}$, with B_{max} being 745 ± 21 and 501 ± 21 fmol.mg $^{-1}$ and K_D 12.2 ± 1.1 and 2.1 ± 0.18 respectively (Fig. 1).

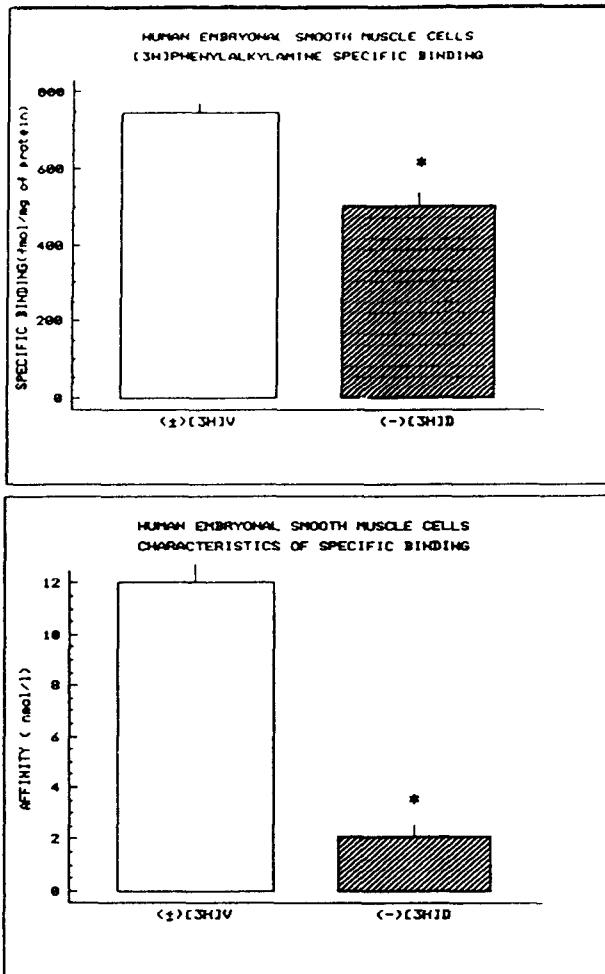


Figure 1. Characteristics of specific $(\pm)-[{}^3\text{H}]\text{verapamil}$ $(\pm)[{}^3\text{H}]\text{V}$ and $(-)-[{}^3\text{H}]\text{desmethoxyverapamil}$ $(-)[{}^3\text{H}]\text{D}$ binding sites identified on native cultured human embryonal vascular smooth muscle cells ($n = 24$). Note the significantly higher capacity and relatively low affinity of $(\pm)[{}^3\text{H}]\text{verapamil}$ binding sites on human embryonal smooth muscle cells. Asterisks indicate significant difference.

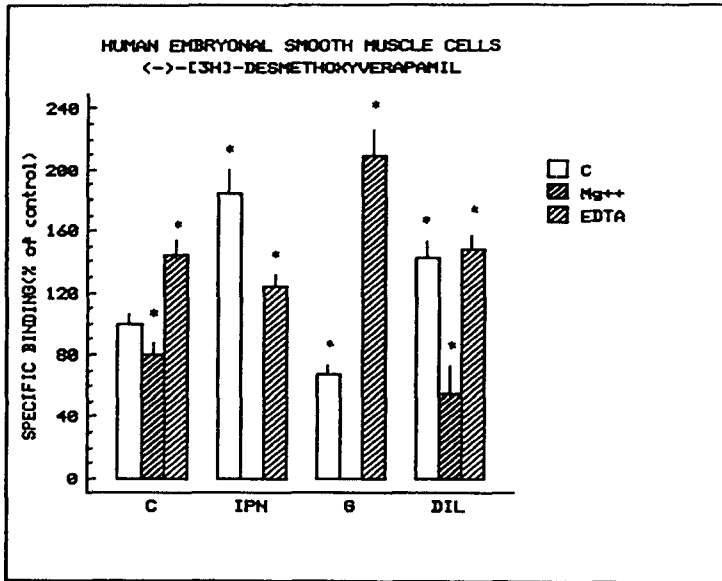


Figure 2. Summary of results; specific binding of (-)-³H]desmethoxyverapamil in human embryonal cell line exposed to concentrations (in $\mu\text{mol.l}^{-1}$) producing half-maximal effects on specific (-)³H]desmethoxyverapamil binding: Isoprenaline (IPN, 10.0), guanylylimidodiphosphate (G, 10.0), d-cis-diltiazem (DIL, 1.0) in control (C) and in the presence of (in mmol.l^{-1}): Mg^{2+} (5.0) and EDTA (0.1), ($n = 36$). Asterisks indicate significant difference vs. control

Characterization of (-)-³H]desmethoxyverapamil binding to depolarized embryonal VSM cells

In depolarized VSM cells, shortly incubated in media containing 10, 20, 40 and 60 mmol.l^{-1} K^+ , there was a marked and concentration dependent increase in specific binding of (-)-³H]DV ($+32 \pm 7$, $+58 \pm 11$ and $+59 \pm 10\%$) with bath concentration of 20 mmol.l^{-1} and a half maximal effect observed at approximately 40 mmol.l^{-1} K^+ in the medium (Fig. 2 and 3). The affinity of (-)-³H]DV for receptor binding sites in these experiments was slightly higher with the numerical values of K_D for the concentration of K^+ producing half maximal increase in B_{max} being $-18.9 \pm 5\%$, ($P < 0.05$). Slight, but significant decrease in K_D ($-31.8 \pm 7\%$) was seen with 60 mmol.l^{-1} K^+ .

Pharmacology of (-)-³H]desmethoxyverapamil specific binding sites in human VSM cells

In order to further explore the mechanisms of allosteric activation or inhibition of Ca^{2+} channel phenylalkylamine receptors in vascular smooth muscle cultured hu-

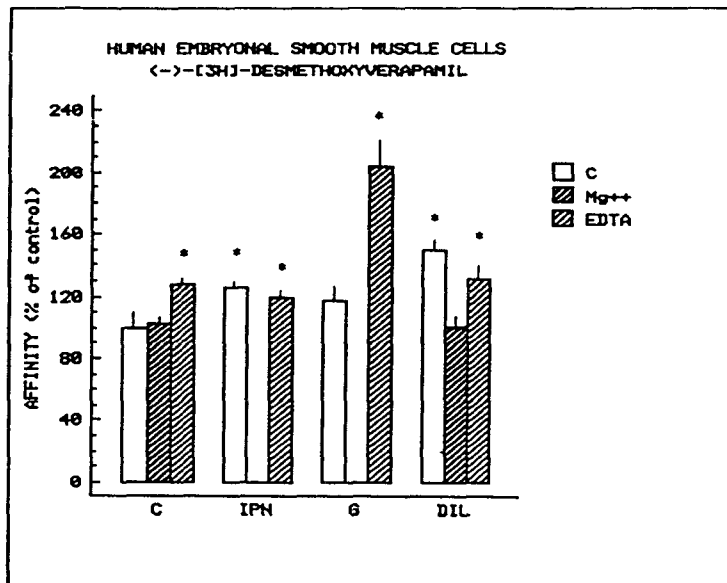


Figure 3. Affinity of (-) - [³H]desmethoxyverapamil binding for specific binding sites identified on native (C) human embryonal cells, or cells exposed to ($\mu\text{mol.l}^{-1}$): isoprenaline, guanylylimidodiphosphate and d-cis-diltiazem. For explanation and symbols see previous figure.

man embryonal cells were incubated with different pharmacological agents. Isoprenaline induced beta- adrenergic activation of VSM resulted in a significant increase in specific (-) - [³H]DV binding ($+6 \pm 2$, $+11 \pm 5$, $+31 \pm 8$, $+64 \pm 11\%$ with a half maximal effect at $10.0 \mu\text{mol.l}^{-1}$ of isoprenaline and a slight but significant reduction in affinity of specific (-) - [³H]DV binding sites on VSM cells (Figs. 2 and 3). The positive modulation of (-) - [³H]DV specific binding induced by beta-adrenergic activation of VSM was significantly attenuated after short incubation of VSM in the presence of Ca^{2+} chelator of sodium-versenate.

On the contrary, incubation of native VSM cells with sodium versenate containing buffer significantly potentiated (-) - [³H]DV specific binding to VSM cells. To characterize the regulatory effect of bivalent cations on (-) - [³H]DV binding in VSM cells the cell line was incubated in the presence of high concentrations of Mg^{2+} . In the presence of 5.0 and 7.5 mmol.l^{-1} Mg^{2+} in the media, specific (-) - [³H]DV binding sites identified on VSM cells were significantly reduced without any significant change in apparent affinity. Direct G-protein activation with guanylylimidodiphosphate significantly reduced the specific binding of the phenylalkylamine Ca^{2+} channel antagonist. Incubation of VSM cells in buffer

with the Ca^{2+} chelator sodium-versenate attenuated the reduction in high affinity $(-)-[{}^3\text{H}]\text{DV}$ specific binding induced by guanylylimidodiphosphate. Phorbol-myristate-acetate did not change the specific binding of $(-)-[{}^3\text{H}]\text{DV}$ in VSM. Incubation of VSM cells with the higher of the two concentrations of benzothiazepine reduced affinity ($K_D = 150 \pm 9\%$) and slightly but significantly increased the maximal number of specific binding sites identified on VSM cells. The presence of Ca^{2+} chelator and d-cis-diltiazem markedly increased affinity (a decrease by $70.9 \pm 5\%$ in numerical values of K_D) without any significant change in B_{max} . A combination of high Mg^{2+} and d-cis-diltiazem in the incubation medium resulted in a significant reduction in both binding parameters ($B_{\text{max}} = -69.8 \pm 7$ and $K_D = -22 \pm 5\%$). Upon increasing concentrations of noradrenaline ($0.1 - 5.0 \mu\text{mol l}^{-1}$) B_{max} was reduced ($+6 \pm 1.2$, $+8 \pm 2$, $+12 \pm 4$ and $+32 \pm 6\%$) and K_D of $(-)-[{}^3\text{H}]\text{DV}$ increased significantly.

Discussion

These results are in agreement with the concept that phenylalkylamines bind selectively to the open channel state in vascular smooth muscle (Sanquinetti and Kass 1984, Bean 1989). Furthermore, our results are in agreement with the view that phenylalkylamines may bind within the intracellular opening of the transmembrane pore of the ion channel (Striessing et al. 1990).

Previous studies have shown that $(-)-[{}^3\text{H}]\text{desmethoxyverapamil}$ labeled multiple calcium channel receptors in brain and skeletal muscle (Reynolds et al. 1986), and that guaninenucleotide binding proteins may modulate calcium channels in cell membranes of equine portal vein smooth muscle (Rakotoarisoa et al. 1991). Our results obtained with the continuous cell line that expresses binding sites with the characteristics of the receptors on Ca^{2+} channels have proven an extremely useful tool in studying the regulation of membrane bound binding sites by events associated with activation or inhibition of heterologic receptors on cells. Our data obtained from experiments with exclusive labeling 1,4-DHP Ca^{2+} channel agonist $(-)-\text{S}-[{}^3\text{H}]\text{-BAYK 8644}$ and 1,4-DHP Ca^{2+} channel antagonist $(\pm)-[{}^3\text{H}]\text{PN 200-110}$ binding sites on cultured vascular smooth muscle cells document the presence of "open, presumably active" and "inactive" Ca^{2+} channels on native VSM cells in a proportion of an approximately 1:1, and modulations of 1,4-DHP binding sites in the presence of phenylalkylamine Ca^{2+} channel blocker (Dřimal unpublished). Consistently with this, the present study shows approximately 60% of phenylalkylamine binding sites being labeled with $(-)[{}^3\text{H}]\text{DV}$ on quiescent VSM cells and a further increase in $(-)[{}^3\text{H}]\text{DV}$ specific binding sites on pharmacologically activated cells. Kostyuk et al. (1983) have postulated high affinity Ca^{2+} , Mg^{2+} ($\text{p}K_{\text{Ca}} = 6.6$ and $\text{p}K_{\text{Mg}} = 4.2$) binding sites on the extracellular side of the Ca^{2+} channels, which control the Ca^{2+} channel function. In the light of these findings documented on

molluscan neurons, results obtained in our study suggest the existence of a regulatory site/s modulating the "open channel ligand" (-) - [³H]desmethoxyverapamil binding on cultured VSM cells, with the [³H]phenylalkylamine binding modulating the bivalent ion regulatory site and *vice versa*.

Taking the recently published concept of "cyclic version of Ca²⁺ channel" as a suitable model system of activation and inactivation response in polarized cells (Jones 1991) and the "open channel ligand" (-) - [³H]desmethoxyverapamil as a suitable tool for the identification of "active" Ca²⁺ channels on VSM cells, one can expect that upon depolarization the closed state of Ca²⁺ channel is destabilized transiently, before passing into inactivated state. This transient opening is the most probable explanation for the increased capacity of specific (-) - [³H]desmethoxyverapamil binding seen in the present study shortly after depolarization of VSM cells.

The findings of the positive modulation of (-) - [³H]desmethoxyverapamil specific binding, induced by beta-adrenergic stimulation of VSM, being markedly reduced in the presence of Ca²⁺ chelator in the incubation medium may mean that the presence of Ca²⁺ is a prerequisite for the appearance of positive modulation. The observation that positive modulation of (-) - [³H]desmethoxyverapamil binding, induced in the present study by benzothiazepine Ca²⁺ channel blocker, was markedly reduced by high Mg²⁺ may support the view that the presence of benzothiazepine, and possibly also bivalent cations in the channel, may produce a steric hindrance for the positive modulation of (-) - [³H]DV specific binding and/or this fact may indicate that the regulatory binding site(s) for Ca²⁺ and for benzothiazepines are presumably situated more proximally (superficially) than the specific binding site for (-) - [³H]desmethoxyverapamil. The negative modulatory effect of guanylylimidodiphosphate can be attributed to the loss of high affinity (-) - [³H]-desmethoxyverapamil binding as the result of permanent G-protein activation.

In conclusion, these findings suggest that (-) - [³H]phenylalkylamine specific binding sites identified on cultured human embryonal smooth muscle cells are modulated according to the state and immediate function of the Ca²⁺ channel receptor complex. The presence of high Mg²⁺ markedly reduces, and Ca²⁺ channel chelator EDTA in the cultivation medium increases, the specific binding. The benzothiazepine Ca²⁺ channel antagonist d-cis-diltiazem increased, and permanent G-protein activation with guanylylimidodiphosphate in electropermeabilized cells significantly reduced high affinity binding sites on VSM cells. Further data are needed to explain external factors imposed by other pharmacologically active agents on the intact cell in negative (inhibitory) modulation of Ca²⁺ channels. Such experiments are our current focus.

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