

Identification of Dihydropyridine (DHP) Binding Sites on Cultured Monkey Renal Cells (GMRC) with a Photoaffinity Probe (-)-[³H]-Azidopine

J. DŘÍMAL

*Institute of Experimental Pharmacology, Slovak Academy of Sciences,
Dúbravská cesta 9, 842 16 Bratislava, Slovak Republic*

Abstract. The low affinity binding sites identified in crude membranes from different excitable tissues with the dihydropyridine (DHP) calcium (Ca²⁺) channel ligands have confused researches in the field of Ca²⁺ channels as they can represent low affinity state(s) of the DHP receptor, or they can be labelled with DHP-type Ca²⁺ channel ligands. The aim of this communication was to provide more evidence for the existence of separate DHP binding sites on the surface of cultured green monkey renal cells (GMRC). The saturation ligand binding experiments with [³H]-nitrendipine (NTP) and photoaffinity labelling studies with (-)-[³H]-azidopine (AZI) were performed in order to identify and further characterize the DHP receptor on cultured GMRC.

Specific high affinity sites identified on GMRC with [³H]-NTP ($B_{\max} = 0.78 \pm 0.03$ pmol/mg protein and $K_D = 0.06 \pm 0.1$ nmol/l in native cells) and photolabelled with AZI represent DHP receptor on L-type Ca²⁺ channels.

The low affinity binding sites photolabelled with AZI on GMRC (9.84 ± 2.4 pmol/mg protein and $K_D = 3.21 \pm 1.25$ nmol/l in native cells) were significantly increased after preincubation of GMRC with low concentrations of DHPs nitrendipine and nisoldipine.

Preincubation of GMRC with Ca²⁺ channel agonist (-)BAYK 8644 significantly reduced specific photolabelling with AZI on GMRC and increased low affinity labelling.

Preincubation of (+)BAYK 8644 was without any effect. Niguldipine (DHP with the voluminous substituent on the port side of the DHP ring) partially inhibited specific photolabelling with AZI on GMRC and also partially reduced the maximal number of low affinity binding sites labelled with AZI.

Our results support the hypothesis of separate subsites in the region of DHP receptor of GMRC and the existence of the "marginal" photolabelling of specific DHP binding sites identified on Ca²⁺ channels.

Key words: Calcium channel — Calcium antagonist — Dihydropyridine receptor — Cultured renal cells — Green monkey — [^3H]-azidopine — [^3H]-nitrendipine

Introduction

Three hypotheses are currently proposed concerning the association of dihydropyridine (DHP) calcium (Ca^{2+}) channel blockers with binding sites on Ca^{2+} channels: The first suggests that DHPs specifically bind with high affinity to a distinct receptor site on L-type of Ca^{2+} channels, predominantly in heart and in vascular smooth muscle (Bean et al. 1986, Kokubun et al. 1986, Wei et al. 1989) and in skeletal muscle, where this site is allosterically regulated (Glossmann et al. 1985), the second suggests that DHP Ca^{2+} channel agonists and antagonists bind to two separate binding sites on Ca^{2+} channels (Triggle et al. 1989), and the third hypothesis suggests distinct subsites on the DHP receptor, the central subsite(s) being labelled by nitrendipine and by other DHPs, while the peripheral subsite is labelled mostly by arylazides (Catterall and Striessing 1992).

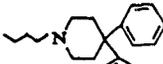
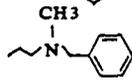
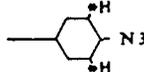
Biochemical studies show that mammalian DHP-sensitive L-type Ca^{2+} channel is composed of five distinct subunits (Catterall 1988) and molecular cloning experiments show that mammalian cells express several classes of the alpha-1 subunit. Some of its isoforms are most closely related to DHP sensitive Ca^{2+} channels. The DHP binding domain is thought to be localized close to the extracellular surface of the cell membrane (Kass et al. 1991; Strubing et al. 1993) or to the carboxyl terminus of the alpha-1 subunit, presumably more closely to the phenylalkylamine binding site, probably in the channel (Regulla et al. 1991).

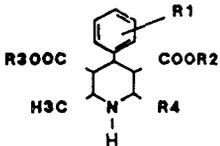
The molecular identification of Ca^{2+} channel has been confined mostly to L-type of Ca^{2+} channels. Mori et al. (1991) have reported the cloning of a brain Ca^{2+} channel and Tanabe et al. (1987) have deduced the primary structure for the DHP receptor of skeletal muscle Ca^{2+} channel. Another group reported cloning and sequencing also of the alpha-1 subunit of the N-type Ca^{2+} channels (Dubel et al. 1992). The L-type Ca^{2+} channels in different cells show however a marked molecular diversity (Tsien et al. 1991). The DHP receptor probably contains four internal repeats with homologous sequences. The proposed model of skeletal muscle DHP receptor presumably contains seven potential cyclic-AMP dependent phosphorylation sites (Numa 1989). It has been demonstrated in support of the second hypothesis that the long duration of action of certain DHPs may be partially related to the formation of a high affinity complex with the inactivated state of the Ca^{2+} channel (Godfraind and Salomone 1991). There is still considerable uncertainty about how DHP interacts with the membrane Ca^{2+} channels and few details of their interaction mechanisms are clearly understood.

The DHPs are sensitive to UV light. In aqueous solution most of DHPs, substituted in position-1 of the phenyl ring like nifedipine (Table 1), are intrinsically

Table 1. Structures of selected dihydropyridines used in the present study. Ligands used for saturation experiments and photoaffinity labelling of DHP receptors on GMRC cells.

STRUCTURAL FORMULAE OF SELECTED DIHYDROPYRIDINES

	R1	R2	R3	R4
NIFEDIPINE	2'NO ₂	Me	Me	Me
NISOLDIPINE	2'NO ₂	iBu	Me	Me
NIGULDIPINE	3'NO ₂		Me	Me
NIMODIPINE	3'NO ₂	iPr	MeOMe	Me
NITRENDIPINE •	3'NO ₂	Et CH3	Me	Me
NICARDIPINE	3'NO ₂		Me	Me
ISRADIPINE	2'3'NON	Me	iPr	Me
AZIDOPINE •	CF ₃	COOCH ₂ CH ₂ NHCO 	Et	Me



photoreactive, that is, they are photoactivable to label covalently their receptor sites, without derivatization (Bayley 1983). The high affinity Ca²⁺ channel antagonist, the photoactivable arylazide azidopine, used frequently as photolabel in photoaffinity studies, also belongs to this category of agents. The ligand conceals a highly reactive intermediate unmasked by UV irradiation. In contrary to nifedipine, azidopine is extrinsically photoreactive (Bayley 1983). We have shown that the whole cell binding assays with the specific Ca²⁺ channel ligands represent a very suitable experimental model for the characterization of membrane-bound Ca²⁺ channels on native polarized cells (Dřimal 1991). The aim of the present paper was to explore the existence of different subsites of DHP binding on Ca²⁺ channels. The hypothesis tested is that the different DHP Ca²⁺ channel antagonists may label distinct receptor sites. For that purpose we identified specific DHP binding sites on surface of GMRC by photoaffinity labelling with two structurally different specific Ca²⁺ channel ligands. The characteristics of covalent photolabelling of DHP binding sites identified on GMRC were analysed in experiments with perturbation of the cell membrane induced by preincubation of GMRC with three different membrane-active compounds with chlorpromazine partitioning in the outer and with vinblastine partitioning in the inner membrane compartment

(Zachowski 1993) and finally with the local cell mediator endothelin (Masaki et al. 1992).

We report here on the interactions of two specific Ca^{2+} channel ligands with the surface DHP receptors on GMRC: [^3H]-nitrendipine, labelling presumably the core of the DHP region and the photoactivable arylazide (-)-[^3H]-azidopine, a covalent label for the periphery of the DHP site.

Materials and Methods

Radiochemicals

[^3H]-azidopine, specific activity 1.85 TBq/mmol (Amersham, Buckinghamshire, England); [^3H]-CGP12177, specific activity 1.26 TBq/mmol (Amersham); [5-methyl- ^3H]-nitrendipine, specific activity 3000.7 GBq/mmol (NEN Research Product Boston, MA, USA). (-)-[^3H]-azidopine was prepared as described by Ferry et al. (1984).

Chemicals were purchased from producers as follows: BAYK 8644 (Calbiochem); nifedipine, nimodipine (Inst. Drug Res. Modra, Slovak Republic); S-(+)-niguldipine (Byk Gulden); nitrendipine (Bayer A.G); Tris(hydroxymethyl)-aminomethane-HCl (Serva). Serum for cell cultures and other cell culture reagents were from Gibco (Grand Island, N.Y., USA).

Cell culture

All experiments were performed on green monkey renal cells (GMRC) obtained from the Institute of Virology (Slovak Academy of Sciences, Bratislava) and USOL (Prague, Czech Republic). Stock cultures were grown at 37°C in a complete medium consisting of Dulbecco's modification of Eagle's minimal essential medium, supplemented with glucose (1000 mg/l) and 10% heat inactivated foetal bovine serum, penicillin G (50 i.u./ml), streptomycin-sulphate (50 µg/ml) in an atmosphere of 5% CO_2 in air.

Whole cell binding assays

The cells were subcultured every 4 days and discarded after twelve passages. The surface cell membrane binding sites and affinity of specific binding of selected Ca^{2+} channel ligands were characterized elsewhere (Dřimal et al. 1991; Dřimal 1992). The GMRC were washed twice with the cultivation medium without glucose and serum. Aliquots of GMRC (0.08–1.11 mg of protein) were incubated for 40 min with buffer I containing (in mmol/l): NaCl 135.0; MgCl_2 1.5; KH_2PO_4 0.44; NaH_2PO_4 0.34; NaHCO_3 2.6; HEPES 20.0; glucose 5.56; pH 7.4, (or with buffer II (Tris-HCl 50.0 and MgCl_2 2.5, supplemented with glucose) in experiments with measurement of the effect of physical factors on GMRC) with tritiated ligands and competing compounds. Binding assays with [^3H]CGP 12177 to intact cells were described in detail elsewhere (Dřimal 1989).

Photoaffinity labelling with (-)-[^3H]-azidopine

Pilot kinetic experiments for photoaffinity studies on GMRC were performed with [^3H]-azidopine. In photoaffinity labelling experiments aliquots of GMRC (10^5 cells per well) were incubated in buffer I with (-)-[^3H]-azidopine (saturation studies). Twelve gradually increasing concentrations (0.5–12 nmol/l) of ligand were incubated in the absence and in the presence of unlabelled azidopine (1.0 µmol/l) or with 10.0 µmol/l (+)-isradipine

in the dark. Incubation mixtures were irradiated for 4 min with UV light (Phillips 20 W/UV lamp) from a distance of 5 cm. After the end of 1 h incubation period the cells were separated by rapid filtration on Whatman GF/C glass microfibre filters, washed with 4.5 ml of cold assay buffer and the radioactivity bound to glass filters was collected in 10 ml scintillation cocktail (Bray, Spolana Neratovice, Czech Republic) and counted in rack beta-liquid counter (LKB Wallace, Turku, Finland) at an efficiency of 65%.

Other determinations

Cell viability: Confluent cultures of GMRC were stored for several days at -20°C and thawed before use without significant loss of growth in culture or without loss of specific binding activity. The cells were incubated for 60 min in the presence of pharmacologically active substances (in $\mu\text{mol/l}$): azidopine (0.1–1.0); (+)-BAYK 8644 (1–10.0); endothelin (0.001–0.1); nicardipine, nifedipine (1.0); (+)-S-niguldipine (1.0); nisoldipine (0.1–1.0); chlorpromazine (0.1–10.0); vinblastine (0.1). At low serum concentrations proliferating cultures enter quiescent state. A light micrographs showed characteristic appearance of confluent GMRC. The GMRC reentered proliferating phase if the serum content was increased, or if the appropriate growth factors were added. The native cells, the GMRC incubated in the presence of picomolar concentrations of selected dihydropyridines (preincubated cells), or the cells pretreated with pharmacologically active substances (mostly at $\mu\text{mol/l}$ concentrations) showed no evidence of lysis. Cell viability was determined with the use of Trypan blue stain exclusion test (0.4%) according to Phillips (1973). Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as standard. The data are mean values and the corresponding standard error of mean. Non-linear regression analysis, Statgraphic Program Package, Student's *t*-test for either paired or unpaired observations were used. Results were considered to be significant at $P < 0.05$.

Results

Vitality of cultured cells

Cell mortality, as determined with the Trypan blue exclusion test was $4.3 \pm 1.5\%$ in all groups.

Saturation of [^3H]-nitrendipine binding sites

Specific binding of [^3H]-nitrendipine on polarized GMRC was saturable ($B_{\text{max}} = 0.780 \pm 0.03$ pmol/mg of protein) and of high affinity ($K_D = 0.06 \pm 0.01$ nmol/l). Preincubation of GMRC with (+)isradipine, S-(+)niguldipine, or with nisoldipine at low nanomolar concentrations (0.01–0.1 nmol/l) significantly reduced specific binding of (^3H)-nitrendipine (to 0.415 ± 0.039 ; 0.565 ± 0.076 and 0.609 ± 0.059 pmol/mg of protein respectively; $P < 0.05$).

Photolabelling of DHP receptor sites with (-)-[^3H]-azidopine

The arylazide azidopine binds specifically and with high affinity to two saturable populations of sites on GMRC with $K_{D1} = 0.06 \pm 0.01$ and $K_{D2} = 3.21 \pm 1.25$ nmol/l ($P < 0.05$) and with the density ($B_{1\text{max}} = 0.846 \pm 0.032$ and $B_{2\text{max}} = 9.84 \pm 2.45$

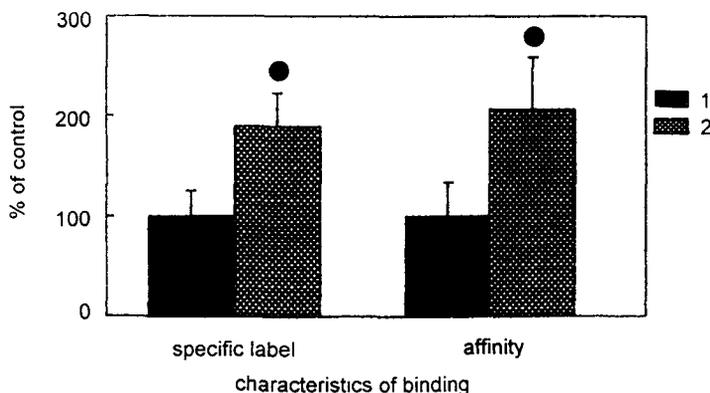


Figure 1. Effects of endothelin (ENDO, 0.1 $\mu\text{mol/l}$) on characteristics of specific high affinity binding sites (B_{max} and K_D) photolabeled on cultured green monkey renal cells (GMRC) with photoaffinity probe (-)-[^3H]-azidopine. Axis x: two parameters of labelling: The maximal number of sites (B_{max}) and affinity (K_D), (both in % of control). Control (GMRC pretreated with endothelin (1)); NIFE (GMRC preincubated with nifedipine (1.0 $\mu\text{mol/l}$) and pretreated with ENDO (2)). Values are: mean + standard error of mean ; • Significant change when compared with control. ($n = 12$).

pmol/mg of protein. The high affinity specific labelling was significantly increased with endothelin ($B_{\text{max}} = 1.54 \pm 0.21$ pmol/mg of protein and $K_D = 4.2 \pm 2.1$ nmol/l; Fig.1).

Preincubation of nifedipine (up to 1.0 $\mu\text{mol/l}$) was without any effect on observed increase in specific labelling of surface DHP binding sites on GMRC induced by endothelin. The high affinity binding sites were blocked selectively with the nifedipine (at low nanomolar concentrations) while the low affinity high capacity (-)-[^3H]-azidopine binding sites identified on GMRC significantly increased after preincubation of nifedipine, nisoldipine, but not with nifedipine (Fig. 2). Preincubation of 0.1–1.0 $\mu\text{mol/l}$ of Ca^{2+} channel agonist (-)-S-BAYK 8644K significantly reduced the maximal number of high affinity sites labelled by (-)-[^3H]-azidopine (0.501 ± 0.07 pmol/mg of protein) and increased the photolabelling of low affinity binding sites with azidopine. Preincubation of (+)BAYK 8644K was without any significant effect.

Photolabelling of DHP receptor sites on perturbed GMRC

The characteristics of covalent photolabelling of DHP binding sites identified on GMRC by (-)-[^3H]azidopine was further analysed in experiments with the perturbations of the cell membrane induced by premedication of GMRC with three membrane-active compounds: chlorpromazine, vinblastine and endothelin. Fig. 3 shows the effects of three selected membrane active drugs. In the presence of

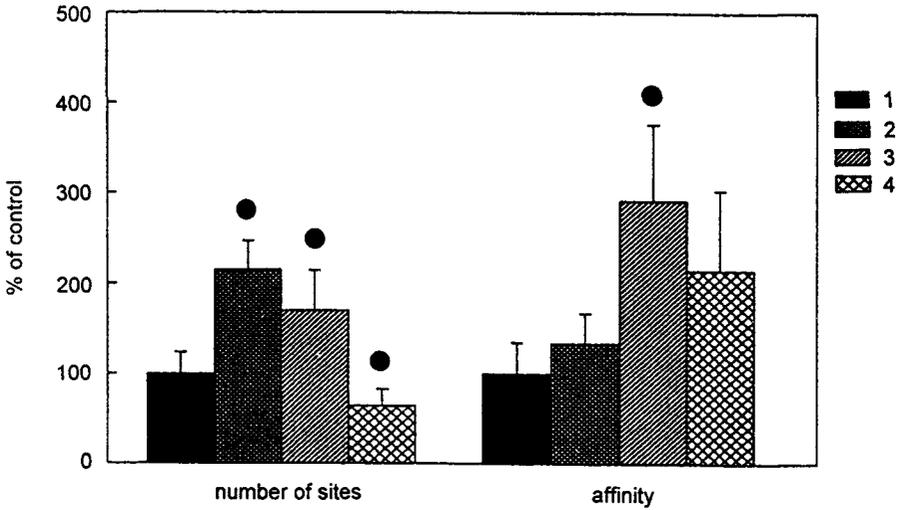


Figure 2. Low affinity labelling with (-)-[³H]-azidopine on GMRC . Axis x : number of sites and affinity (both in % of control) Control: GRMC preincubated with dihydropyridine ($\mu\text{mol/l}$) (1); NIFE (1.0 nifedipine) (2); NISL (1.0 nisoldipine) (3); NGLD (1.0 niguldipine) (4). Explanation and symbols as in Fig 1. ($n = 12$).

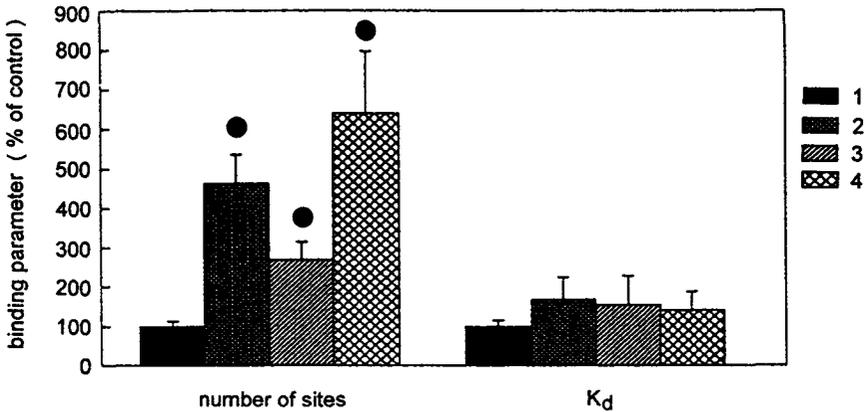


Figure 3. Perturbation of the cell membrane asymmetry in GMRC (Control – native GMRC (1)) and cells exposed to different membrane-active amphiphilic compounds (in $\mu\text{mol/l}$): CHPMZ – chlorpromazine (10.0; affecting the outer leaflet of the membrane (2)), VIBL – vinblastine (0.1; affecting mostly the inner membrane leaflet (3)), and ENDOT = endothelin (0.01; local mediator, directly stimulating Ca^{2+} channels (4)). ($n = 20$).

chlorpromazine the maximal number of sites photolabeled with (-)-[³H]-azidopine significantly increased ($463 \pm 77\%$, $P < 0.05$), similarly vinblastine increased sig-

nificantly photolabelling ($268 \pm 14\%$, $P < 0.05$) and the locally acting cell mediator and Ca^{2+} channel activator endothelin caused profound increase in the total number of sites labelled with azidopine ($639 \pm 157\%$) in these experiments. Sodium salt of butyric acid, a powerful membrane inductor significantly increased the maximal number of sites prelabeled with azidopine.

Equilibrium binding of [^3H]-CGP 12177 to surface beta-adrenergic receptor on UV irradiated GMRC

In this group of experiments we examined the binding of [^3H]-CGP 12177 to the GMRC comparing the native cells with UV irradiated and nifedipine (at low picomolar concentrations) to determine the effects of physical factors (UV phototherapy), pretreatment (low concentrations of nifedipine plus UV phototherapy) on surface beta-adrenergic receptor binding on GMRC. Specific binding of [^3H]CGP 12177 on GMRC was saturable and consistent with an interaction of ligand with a single population of binding sites.

Table 2. Surface beta-adrenergic receptors identified on GMRC with specific beta-adrenergic ligand [^3H]-CGP 12177.

	Number of sites (fmol/mg prot.)	Affinity (nmol/l)
Control	889.3 ± 250	8.8 ± 1.0
Nifedipine	879.9 ± 356	7.9 ± 1.4

Control – cells exposed to UV phototherapy (see Materials and Methods); Nifedipine – cells preincubated with DHP ($10.0 \mu\text{mol/l}$); ($n = 6$).

As shown in Table 2, UV irradiation of GMRC (see Materials and Methods) or pretreatment of GMRC with low picomolar concentrations of the nifedipine (DHP with $2'\text{NO}_2$ substituent on phenyl ring) and subsequent UV phototherapy were without any significant effects either on specific high affinity binding or affinity of binding sites on pretreated GMRC for [^3H]CGP 12177. The use of incubation media with lower ionic strength (Tris-buffered saline, applicable for broken-cell preparations) resulted in comparably lower maximal binding capacity (B_{max}). In quiescent whole cell saturation studies with [^3H]CGP 12177 on GMRC and incubated in buffer II $B_{\text{max}} = 0.409 \pm 0.104 \text{ pmol/mg}$ of protein and $K_D = 9 \pm 4 \text{ nmol/l}$ ($P < 0.05$). Similarly, the lower maximal binding capacity in buffer II was observed also in UV-irradiated cells.

Discussion

The total number of specific, high affinity DHP Ca^{2+} channel antagonist binding sites photolabelled on GMRC with arylazide (-)-[^3H]-azidopine in the present study were comparable to those identified with [^3H]-PN 200-110 on aortic cell (Kuga et al. 1990), RINm5F cultured cells (Yaney et al. 1991), or on human embryonal cells (Dřimal 1992) and higher than the maximal number of sites identified on cultured ventricular myocytes (Wei et al. 1989). The fact that the high affinity DHP binding sites are labelled by two separate DHP Ca^{2+} channel ligands and that the alternative use of DHP-ligand with the voluminous substituent on DHP ring significantly reduced only the high affinity specific photolabelling with arylazide azidopine in the present experiments strongly suggest that there are at least two different subsites present in the high affinity DHP receptor region on GMRC. These subsites were found as subsites regulated according to the actual functional state of Ca channel.

The specific labelling with (-)-[^3H] azidopine on GMRC increased after preincubation of GMRC with pmol concentration of DHPs and decreased after preincubation of cells Ca^{2+} channel agonist BAYK 8644. With the use of two Ca^{2+} channel ligands (radiolabeled agonist and antagonist in saturation studies) we have recently shown that both functional states of Ca^{2+} channels, active and inactive, are equally distributed on polarized human embryonal cells (Dřimal 1992). Similarly also in the present study preincubation of GMRC with Ca^{2+} channel agonist or antagonist modulated negatively or positively the number of sites presumably on Ca^{2+} channels, labeled with arylazide. The low affinity binding sites covalently identified on GMRC were found to be abundant by order of magnitude compared to high affinity Ca^{2+} channel linked DHP receptors.

The second class of sites with typical Ca^{2+} channel specific binding-inhibition profile were earlier reported by Ferry et al. (1987) with guinea-pig heart membranes. The use of a high ionic strength media in the present study has facilitated the unmasking of the high capacity, low affinity binding sites on GMRC with the $B_{\text{max}} = 9.84 \pm 2.45$ pmol/mg of protein and $K_D = 3.21 \pm 1.45$ nmol/l. The existence of low affinity binding sites in purified membrane proteins was described previously by Glossmann et al. (1987) in experiments with purified calcium channel protein photo-labelled by arylazide (-)-[^3H]-azidopine. An important tool to discriminate Ca^{2+} channel-linked receptors from low affinity binding sites is Ca^{2+} channel agonist BAYK 8644 which presumably does not bind to the low affinity binding sites (Glossmann and Striessing 1988). The preincubation of the GMRC with the Ca^{2+} channel agonist in the present study significantly reduced and, on the contrary, preincubation of the cells with the Ca^{2+} channel antagonists nitrendipine, nimodipine and nifedipine significantly increased the B_{max} of low affinity of binding sites. According to a bilayer couple hypothesis (Deuticke 1968) after

pretreatment of cultured cells with amphiphilic membrane active drugs there is a possibility for selective perturbation of the membrane asymmetry by accumulation of compounds in the outer or in the inner leaflet of the membrane. The DHP Ca^{2+} channel ligands approach their receptor sites on membrane-bound Ca^{2+} channels presumably by lateral diffusion after partition with the cell membrane lipid bilayer. To address the question of the pertinence of an increased B_{max} of the low affinity binding sites identified in our study, experiments were carried out in the presence of membrane-active compound: chlorpromazine, accumulating in the outer, and sodium butyrate, a lipophilic membrane inductor, vinblastine, perturbing the inner membrane leaflet (Schrier et al. 1986). The differences between chlorpromazine and vinblastine and butyrate clearly indicate that the perturbing of the membrane asymmetry in the outer or in the inner leaflet was responsible for marked increase in the total number of sites photolabelled on GMRC by azidopine. Moreover, by comparison with chlorpromazine, endothelin in the present study caused very profound increase in B_{max} . It has been recently shown that endothelin, a potent vasoconstrictor peptide either directly, or indirectly activated the DHP-sensitive, voltage dependent Ca^{2+} channel in porcine coronary artery smooth muscle (Goto et al. 1989).

Some authors believe that endothelin and other endogenous activators may exert little effect on L-type Ca^{2+} channels that are sensitive to DHP, they ascribe these effects to the activation of receptor operated Ca^{2+} channels. We therefore used endothelin in our studies with the perturbation of membranes on GMRC and photoaffinity labelling of DHP receptor. In our study, preincubation of nitrendipine at concentration 1/10 of that used in the study of Goto et al. failed to reduce the observed increase in B_{max} of high affinity sites labelled with arylazide azidopine. The affinity of these sites increased significantly after preincubation of nitrendipine. The findings presented here and our previous observations suggest that there are two subtypes of DHP receptor on the Ca^{2+} channels and that at least a fraction of low affinity binding sites labelled on cultured renal cells with arylazide azidopine may represent DHP receptor on presumably open, "active" Ca^{2+} channels:

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