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Endothelin-1 Significantly Increased Number of Specific High-Affinity 1,4-Dihydropyridine (DHP) Binding Sites Photolabelled on Vascular Smooth Muscle Cells with (−)-[^{3}H]-Azidopine

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Summary

The effects of endothelin-1 (ET-1) on surface membrane Ca^{2+} channels were studied on cultured human embryonal vascular smooth muscle cells (VSMC) and on isolated rat aorta using photoaffinity labelling with DHP Ca^{2+} channel antagonist (−)-[^{3}H]-azidopine (AZI). The AZI-labelled saturable population of sites on VSMC with B_{max} = 1.59±0.10 pmol/mg of protein and K_D = 5.40±1.70 nmol/l; and 1.32±0.11 pmol/mg w.w. and K_D = 1.09±0.20 nmol/l in isolated rings of the rat aorta. Preincubation with ET-1 (0.1, 1.0 and 10 nmol/l) increased (in a concentration-dependent manner) the total number of sites specifically photolabelled on VSMC. The number of sites labelled with AZI on ET-1 preincubated VSMC increased markedly when divalent cations (Ca^{2+} or Mg^{2+} in other experiments) were present in the incubation medium. Specific photolabelling also significantly increased in VSMC pretreated with intrinsically photoreactive nifedipine. A protein kinase C inhibitor staurosporine, added to the incubation medium, significantly reduced the enhanced specific photolabelling after ET-1. The increase in specific photolabelling after ET-1 preincubation (+ 197±46 %; P<0.05) was also observed in rings of the rat aorta and it was significantly reduced after preincubation with S-(+)-niguldipine.

Key words

Vascular smooth muscle – Dihydropyridine receptor – Photoaffinity labelling – (−)-[^{3}H]-azidopine – Ligand binding – Endothelin-1 – Staurosporine – S-(+)-niguldipine

Introduction

The product of vascular endothelial cells, endothelin-1 (ET-1), is the most potent endogenous vasoconstrictor and comitogen for vascular smooth muscle cells (Yanagisawa *et al.* 1988). In whole cell patch clamp experiments with mesangial cells the coupling between presumably endothelin receptors and L-type ion channels was reported (Hu *et al.* 1993). It is, as yet, not clear whether this is the result of direct, or casually coupled indirect effects. Increased plasma endothelin levels in coronary venous blood of patients with vasospastic angina pectoris (Toyo-Oka *et al.* 1991) suggested that endothelin could be one of the factors causing vasospasms in these patients. Recent development in physiology of cell signalling processes in vascular smooth muscle (VSM) appeared to confirm a possible dual function of a voltage sensor and a Ca^{2+} channel (Beam *et al.* 1992, Marks 1992). However, the importance of ET-1, as an intracellular mediator and activator of Ca^{2+} channels in VSM, is not clear.

When added to the medium in the physiological range of concentrations (from 10 fmol/ml to 10^2 pmol/ml), ET-1 failed to change the membrane potential in canine isolated coronary artery preparations and induced sustained depolarization at higher concentrations (Nakashima and Vanhoutte 1993). In whole cell vascular smooth muscle preparations ET-1 induced a complex response: it significantly increased $(Ca^{2+})_i$ and activated specific phospholipase C, stimulated Na^+/H^+ exchange (Simonson *et al.* 1989, Hubel and Highsmith 1995) and also increased mitogen-activated protein kinase (Wang *et al.* 1993). Our recent studies with low concentrations

of human and porcine ET-1 showed the prevalence of inactive Ca^{2+} channels in primate renal cells (Dřímal 1994, Dřímal and Boháčik 1994).

In the present study, we studied the characteristics of specific 1,4-dihydropyridine binding identified on cultured human embryonal vascular smooth muscle cells (VSMC) and on rings of the rat aorta with photoaffinity labelling and ligand binding technique both before and after preincubation with endothelin-1.

Because the presence of damaged cells, which are presumably depolarized, may contribute substantially to DHP binding (Wei *et al.* 1989), and binding of DHPs in the whole cell preparation is strongly modulated by the existing membrane potential (Sanquinetti and Kass 1984). Furthermore, the affinity of a given DHP for a DHP binding sites on a Ca^{2+} channel in VSMC may increase with the Ca^{2+} inflow and with the proportion of inactivated channels (Morel and Godfraind 1987, 1994), so that photoaffinity labelling studies were also carried out after preincubation of VSMC with pmol/l concentrations of DHP Ca^{2+} channel blockers.

Methods

Chemicals were either gifts, or were purchased from the following producers: BAY K 8644, endothelin-1 (both Calbiochem); nifedipine (Drug Research, Modra); nitrendipine (Bayer A.G.); S-(+)-niguldipine (RBI); (+)-PN200-110 (Sandoz); staurosporine (RBI).

The following radiochemicals were used: [^{3}H]-azidopine (2,6-dimethyl-4-(2'-trifluoro-methyl-phenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid-ethyl, (N-4"-azido-[3",5"-^{3}H]benzoylaminoethyl) diester, specific activity 50 Ci/mmol (Amersham, UK), and (−)-[^{3}H]-azidopine, an active enantiomer of the photoaffinity probe, prepared as described by Ferry *et al.* (1984). Serum for cell cultures and other cell culture reagents were from Gibco (Grand Island, NY, USA). All other materials used were from sources as listed in our previous papers (Dřímal 1988, 1994). Twenty Wistar Hannover/Rosice/Dobrá Voda male rats (weighing 220−250 g) were used. They were anaesthetized with sodium pentobarbital (45 mg/kg i.p.) and the thoracic aorta was immediately removed and placed in HEPES-buffered physiological salt solution (see Cell Culture), cleaned of adherent connective and fatty tissue, denuded with a thin Eschmann embolectomy catheter (No.1.1) and cut in rings.

In three rats after preparation of the right carotid artery, a bipolar stimulation electrode was introduced into the aortic arch and programmable periarterial stimulation was applied with pulses of submaximal intensity usually 1−3 V (duration of impulse 1 ms and frequency 10 Hz) for 6 x 60 s by a Disa Multistim. Immediately after the stimulation preparation of rat aorta continued as described above.

Cell culture

Approved by the local Ethics Committee, our experiments were performed with cell lines of human embryonal vascular smooth muscle (VSMC) (embryos 4−8 weeks of gestation). Only grown cultures of VSM cells (mostly from the third to the eleventh passage, containing approximately 10^5-10^6 cells/cm^2) were used. The stock cultures were grown at 37 °C in complete media consisting of 90 % of Dulbeco's minimum essential medium (DMEM), glucose and 10 % of heat inactivated foetal bovine serum, penicillin G (50 IU/ml), streptomycin sulfate (50 μg/ml) in an atmosphere of 5 % CO_2 and 95 % of air. VSMC were harvested by short incubation (2 min) in fresh DMEM without serum containing 0.05 % of trypsin and 0.02 % of EDTA. The suspension was then quickly centrifuged, the supernatant aspirated and packed VSMC were resuspended for 60 min in DMEM. VSMC were then centrifuged, the medium was discarded and cells were resuspended in HEPES-buffered physiological salt solution (PSS, in mmol/l): NaCl 135, $MgCl_2$ 1.0, KH_2PO_4 0.44, $NaHPO_4$ 0.34, $NaHCO_3$ 2.6, HEPES 20.0, glucose 5.56, pH = 7.4. VSMC viability was judged by optical inspection of the preparation and by using the colourimetric NBT (tetrazolium) assays. The cell line, the cell type, the morphology of the whole cell VSM preparation and the total numbers of surface membrane bound receptors present on VSMC have been described elsewhere (Dřímal 1989, 1992).

Whole cell binding assays − reversible binding

The saturation binding assays were performed in the dark on VSMC in HEPES-buffered PSS. Aliquots of VSMC (usually 1.0−1.25 x 10^6 cells) were incubated (at 25 °C; 60 min) in darkness with 12 gradually increasing concentrations of (±)-[^{3}H]-azidopine (0.5−20.0 nmol/l). Non-specific binding was defined with 1.0 μmol/l of PN200-110.

Photoaffinity labelling experiments

Rat aorta

Rings of the denuded rat aorta (1−2 mg w.w.) were incubated for 60 min on microtitration wells (Koh-i-noor Hardtmuth, České Budějovice) in physiological salt solution (PSS), maintained at 25 °C and aerated with a mixture of 95 % of O_2 and 5 % of CO_2 containing (−)-[^{3}H]-azidopine (0.1 to 7.0 nmol/l).

At the beginning of incubation, preparations were briefly irradiated (2.5 min at a distance of 10 cm) with a UV lamp (Phillips 40 W). At the end of the incubation time, each ring was dried on filter paper and weighed. The radioactivity of the tissue was counted by a liquid scintillation counter (LKB Wallace, Turku,

Finland) at efficiency of 48 %. Non-specific binding was determined in solutions containing 1.0 μmol/l of PN200-110 and subtracted from the total binding values.

Cultured human vascular smooth muscle cells

Aliquots of VSMC (0.1–0.2 mg of protein) were incubated for 60 min in total darkness with (−)-[^{3}H]-azidopine (0.5–10 nmol/l) in the absence (total labelling) and in the presence of PN200-110 (1 μmol/l), (non-specific labelling). At the beginning of incubation, the mixtures were briefly (2.5 min) photoirradiated. After the end of incubation (60 min totally), VSMC were separated by rapid filtration on Wathman GF/C glass fibre filter and washed with 3.0 ml of ice cold PBS. The radioactivity retained on the filters was collected in 10 ml of Bray scintillation cocktail and counted in a 2500 TR Tricarb Liquid Scintillation Analyzer (Packard) at efficiency of 65 %.

Protein concentrations were measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical analysis

Data are presented as arithmetic means ± S.E.M. Differences were tested for statistical significance using Student's t-test. P = 0.05 was considered to be significant. A statgraphic program packet with non-linear regression analysis was used.

Results

The trypsin/EDTA and low speed centrifugation procedures used in our study with VSMC slightly increased the total number of sites labelled with DHP (10–15 %). A similar range of changes in the characteristics of DHP binding were also observed after one or two consecutive freezing and thawing of VSMC preparation in our experiments (Dřímal, unpublished data).

Kinetics and reversibility of [^{3}H]-azidopine binding

The association rate constant determined with 1.0 nmol/l of [^{3}H]AZI for six VSMC preparations at 25 °C (the temperature employed in photoaffinity labelling experiments) was k_{+1} = 0.044±0.016 nmol.l^{-1}.s^{-1}. Preincubation of VSMC with ET-1 in concentration of 1.8 nmol significantly increased k_{+1} = 0.134 nmol.l^{-1}.s^{-1} ($P<0.05$). In other experiments, the dissociation of the AZI-receptor complex was induced by addition of a final concentration of 1.0 μmol/l PN200-110 to the incubation medium at zero time. When VSMC were preincubated shortly with nifedipine or nisoldipine (both DHPs with 2'-NO_2 substitution on DHP rings are intrinsically photoreactive and form free radicals after photoirradiation) and irradiated with UV light, shortly before the kinetic experiments, the dissociation rate constant was k_{-1} = 0.014/min.

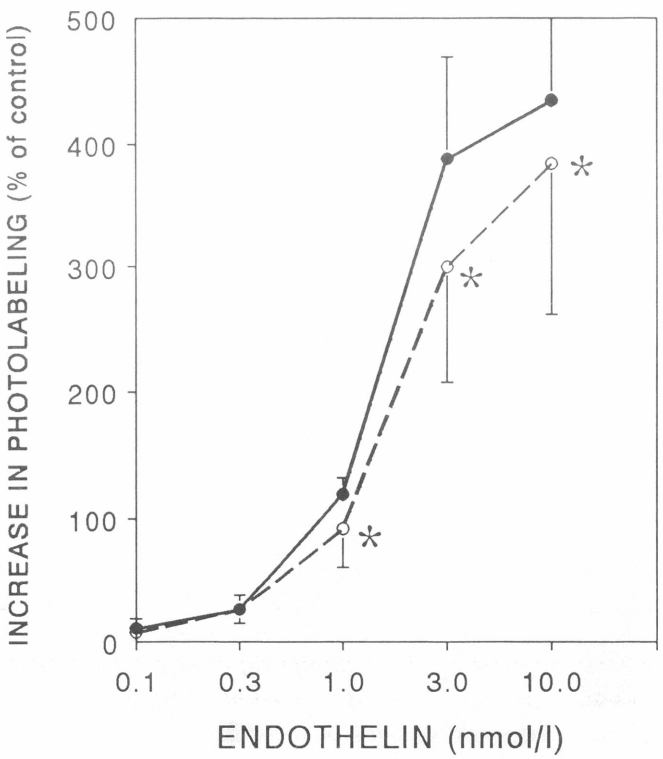

Fig. 1
*Increase in specific photolabelling (in % of control B_{max}) after preincubation of cultured human vascular smooth muscle cell (VSMC) preparations with 0.1–10 nmol/l of endothelin (full line) and after pretreatment of VSMC with 10.0 pmol/l S-(+)-niguldipine (broken line), (n = 12). Values are means ± S.E.M. *Significant increase ($P<0.05$).*

Specific binding of (±)-[^{3}H]AZI to VSMC was saturable and reversible. The Scatchard analysis of the specific binding fraction was consistent with the interaction of the arylazide ligand with a single population of high affinity sites. The six preparations of VSMC had an average of K_D = 620±50 pmol and the total number of specific binding B_{max} = 0.846±0.120 pmol/mg of protein, indicating an approximately 10 DHP binding sites per cell.

A significant decrease in the density of specific (±)-[^{3}H]AZI binding sites was observed after preincubation of VSMC with 0.1–1.0 pmol/l of BAY K 8644 (−46.5±11 %, $P<0.05$). The total number of specific binding sites significantly increased after preincubation of VSMC with 10.0 pmol/l of nifedipine, nitrendipine or with S-(+)-niguldipine by 52±16, 43±19, and 25±9 %, respectively ($P<0.05$).

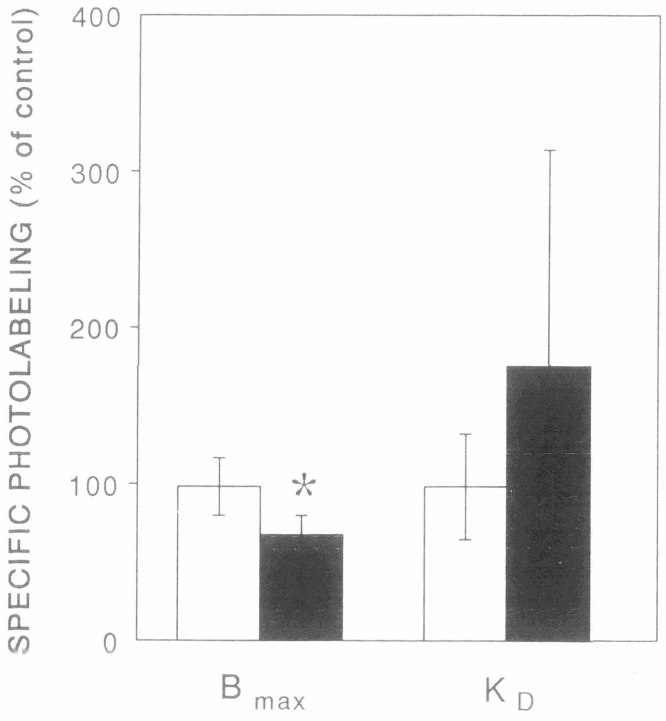

Fig. 2
*Characteristics of specific photolabelling (B_{max} and K_D, both in % of control) after preincubation of VSMC preparation with endothelin (10.0 nmol/l) (open columns) and after pretreatment of VSMC with protein kinase C inhibitor staurosporine (PKI, 1.0 μmol/l), (hatched columns), (n=12). *Significant change when compared to the control ($P<0.05$).*

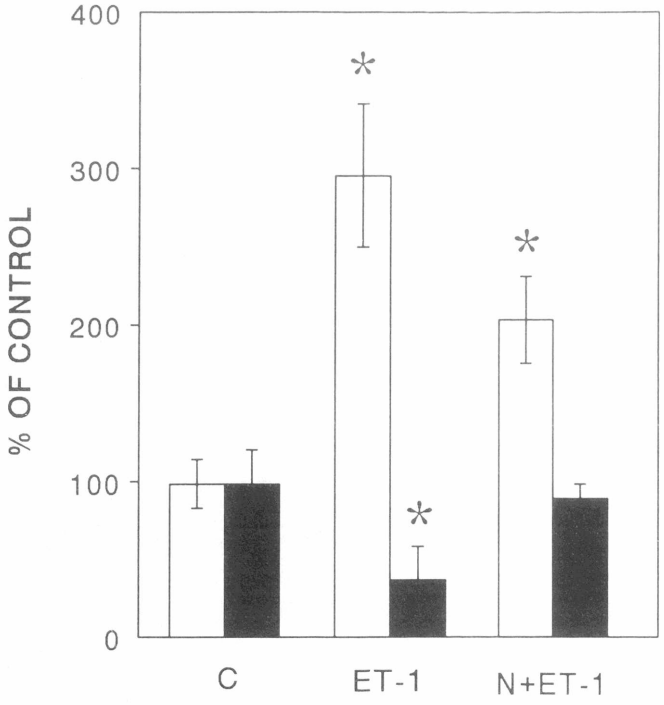

Fig. 3
*The specific labelling with (−)-[^{3}H]-azidopine in ring preparations of denuded rat aorta preincubated with endothelin and DHP Ca^{2+} channel antagonist S-(+)-niguldipine: open columns – number of sites, full columns – K_D. For details see Method. C = control, ET-1= endothelin-1, N+ET-1 = effect of endothelin in preparations preincubated with S-(+)-niguldipine. At the bottom are control values of B_{max} and K_D. *Significant change when compared to the control ($P<0.05$).*

Photoaffinity labelling with (−)-[^{3}H]-azidopine – photolabelling of vascular smooth muscle cells

(−)-[^{3}H]AZI specifically photolabelled a saturable population of high affinity binding sites on VSMC with K_D = 5.4±1.2 nmol/l and with a density B_{max} = 1.59±0.09 pmol/mg of protein. The specific photolabelling increased significantly in cells preincubated with 1.0, 3.0 and 10.0 nmol of ET-1. The increased specific photolabelling was concentration-dependent (Fig. 1). Verapamil (1.0 μmol/l) and the trisodium salt of EDTA (1.0 mmol/l) slightly but significantly increased photolabelling in control VSMC (+26±8 and +21±9 %, respectively). Both agents, however, were without any significant effect on the increased photolabelling induced in VSMC by 1.85 nmol/l endothelin (EC_{50} of ET-1). The increase of photoaffinity labelling induced by ET-1 was reduced in VSM cells preincubated with 1.0 μmol/l of staurosporine (Fig. 2). When Ca^{2+} or Mg^{2+} (5.0−7.5 mmol/l) were added in other experiments to the incubation medium in staurosporine-preincubated VSMC (n=6), the increase in photolabelling induced by 1.85 nmol/l ET-1 was much larger than that in untreated VSMC.

Photolabelling of rat aortic rings

The effects of ET-1 on specific photolabelling was also measured in 72 isolated, denuded ring preparations of the rat aorta. Fig. 3 shows the characteristics of specific photolabelling in control (B_{max} = 1.30±0.10 pmol/l and K_D = 1.1±0.2 nmol/l) and increased specific photolabelling (+197±46 %; $P<0.05$) after preincubation with 1.8 nmol/l ET-1. Previous preincubation of the rat aorta with S-(+)-niguldipine partially reduced (−32±8 %; $P<0.05$) the increase in photolabelling induced by ET-1. Photolabelling measured on aortic rings subjected to the protocol of repeated periarterial stimulations significantly decreased B_{max} (0.86±0.19 pmol/mg w.w. and K_D = 9.3±2.3 nmol/l, $P<0.05$).

Discussion

We have demonstrated, for the first time, that the exposure of human vascular smooth muscle cells to nifedipine (or nisolpidine, both DHPs are intrinsically photoreactive and capable of derivatization when exposed to UV-light) and subsequent photoirradiation, significantly increased the total number of specific, high affinity binding sites, photolabelled on VSM cells with (−)-[^{3}H]-azidopine. Furthermore it was also shown that preincubation of VSM cells with the vasoconstrictor peptide endothelin-1 produced similar changes, it increased significantly the number of high affinity binding sites, covalently labelled on cells. Evidence was provided by using of ligand binding and photoaffinity labelling studies.

Photogenerated and subsequently irreversibly trapped near or at the DHP binding site, the photoaffinity probe (−)-[^{3}H]-azidopine helped in identifying specific sites on presumably inactivated Ca^{2+} channels in VSMC.

As the photoreactive analog of Ca^{2+} channel blocker AZI has its photoreactive group on the DHP side chain that (in extended form) protrude 10−15 Å from the core of the molecule, the probe labels covalently the region of the DHP binding site that is relatively at the periphery, i.e. distant from the functional DHP receptor (Varadi *et al.* 1995). As tools for "locally photogenerated free radicals on Ca^{2+} channels" we used three 1,4-DHP Ca^{2+} channel antagonists in the present study: intrinsically photoreactive nifedipine, blocking access to the orthosteric DHP binding site, intermediary nitrendipine, the DHP without intrinsic photoreactivity, capable of derivatization, and niguldipine presumably labelling the core of the DHP region and having a "voluminous side chain substituent" on the DHP ring. Three DHPs that differed in their intrinsic photoreactivity as well as in their capability to derivatize and form free radicals under photoirradiation, as matter of fact, produced different effects: nifedipine significantly increased, nitrendipine was without effect and S-(+)-niguldipine significantly reduced specific photolabelling on VSMC.

Our present results and interpretations agree well with those reported in our previous studies (Dřímal 1994, Dřímal and Boháčik 1994) in which preincubation with endothelin increased the total number of binding sites identified on renal cells. However, the total number of high affinity binding sites specifically photolabelled on VSMC was higher. In our previous studies we speculated that as the photoaffinity probe, (−)-[^{3}H]-azidopine with the inert side chain substituent in position-2' of the phenyl ring (-CF_3 group), may covalently label the periphery of the DHP receptor site on Ca^{2+} channels without production of undesirable effects in the core of DHP binding site. More suitable in this respect are 1,4-dihydropyridines with the small alkyl substitutions situated on the port side of the DHP ring, mostly compounds that derivatize when exposed to UV irradiation. They may presumably label and also affect the centre of the DHP binding site. The significant reduction in specific photolabelling observed in the present study on VSMC preincubated with niguldipine (DHP with the voluminous substituent on the port side of the ring) supports the view that possibly both, the formation of a covalent bond between the ligand and target in the centre of the DHP binding site, and a voluminous substitution situated on the port side of the DHP ring, may seriously interfere with the proper distinguishing and further interaction of the DHP ligand with its specific binding site on the Ca^{2+} channel. Free radicals

that are generated after photoirradiation and subsequent aromatization of the 1,4-DHP ring (under photolysis of bound DHP) with covalent bonding presumably in the centre (selected 1,4-DHP) and also on the periphery of the DHP binding site (photolabelled with (−)-[^{3}H]AZI) are of prime importance for the inactivation of the Ca^{2+} channel.

It is well established that ET-1, with a pattern similar to other calcium mobilizing agonists, induces a series of repetitive free calcium transients in certain type of cells (Serradeil-Le Gal *et al.* 1991) and that ET-1 inhibits the plasmalemmal Ca^{2+} pump (Jouneaux *et al.* 1993). Correspondingly, decreased specific photolabelling observed in our study on VSMC preincubated with Ca^{2+} channel agonist BAY K 8644 and reduced B_{max} for (−)-[^{3}H]AZI in aortic rings subjected to periarterial sympathetic stimulation represent the type of preparation with a preference for "an active Ca^{2+} channel".

Increased photolabelling observed in VSMC following preincubation with the Ca^{2+} channel antagonist niguldipine, endothelin-1 and photoirradiation in DHP-pretreated VSMC indicate that the vasoactive peptide, by acting through mobilization of Ca^{2+} and stimulation of protein kinase C, may lead to inactivation of membrane Ca^{2+} channels by a similar mechanism. As revealed by photoaffinity labelling, the DHP type Ca^{2+} channel ligands bind with high affinity to distinct receptor sites on Ca^{2+} channels. It was suggested that in skeletal muscle DHP type Ca^{2+} channel antagonists approach their receptor sites on Ca^{2+} channels from the extracellular portion of the Ca^{2+} channel and presumably label the alpha(1) subunit (Catterall and Striessing 1992). Recent study of molecular determinants of calcium-dependent inactivation in cardiac L-type Ca^{2+} channels suggested a close link between the site(s) regulated by Ca^{2+}, residing presumably in the cytoplasmic region of the cardiac alpha(1) subunit and Ca^{2+} induced mode shifts, leaving direct binding of Ca^{2+} as a likely event, initiating processes of inactivation of the Ca^{2+} channel (Zong *et al.* 1994, Inredy and Yue 1994).

It is interesting to note, in this respect, that protein kinase activators and also some peptides that stimulate phospohoinositide breakdown are capable of producing both activation of Ca^{2+} release from internal stores and also a partial shut-off of Ca^{2+} entry *via* membrane bound Ca^{2+} channels in vascular smooth muscle (Galizzi *et al.* 1987). It was previously reported that ET-1 partially inhibited the decrease in DHP Ca^{2+} channel antagonist binding sites induced by BAY K 8644 in rat hippocampal slices (Huguet *et al.* 1993) and that ET-1 stimulated mitogen-activated protein kinase activity by phosphorylation of the enzyme (Wang *et al.* 1993). The Ca^{2+} binding site responsible for the inactivation of the Ca^{2+} channel is presumably encoded in the alpha(1) subunit and it was proposed that it is located near the inner channel mouth but outside the membrane electric field (Neely *et al.* 1994).

In agreement with the proposal of an independent Ca^{2+} channel associated binding site, presumably located on the surface cell membrane, within the vicinity of the channel protein found, also in our experiments, the preincubation of VSMC with niguldipine (the DHP with a voluminous substituent on the 1,4 dihydropyridine ring) and the pretreatment with protein kinase inhibitor staurosporine, partially but significantly reduced the photolabelling in ET-1 preincubated VSMC.

The number of channels inactivated in rings of the rat aorta at rest could be related generally to the level of the resting potential and it could also be the result of the depolarization induced in the edges of the aortic ring by cutting and other experimental manipulations. The significant reduction in the specific photolabelling in the group of rat aortas subjected to massive sympathetic stimulation and the markedly increased proportion of sites photolabelled with (−)-[^{3}H]AZI in ET-1 preincubated rat aortic rings indicate that the influence of the marginal zone on the total number of specific sites labelled with (−)-[^{3}H]AZI is negligible in this type of preparation.

Our results suggest that increased photoincorporation of the high affinity Ca^{2+} channel ligand (−)-[^{3}H]-azidopine, induced by endothelin in cultured human vascular smooth muscle, i.e. an increase identified with the photoaffinity probe that showed significant preference for inactive Ca^{2+} channels, is complex and it is partially dependent on the activity of protein kinase.

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