Target size analysis of skeletal muscle Ca^{2+}channels

Positive allosteric heterotropic regulation by d-*cis*-diltiazem is associated with apparent channel oligomer dissociation

A. Goll+, D.R. Ferry+ and H. Glossmann*

Rudolf Buchheim-Institut für Pharmakologie, Frankfurter Straße 107, D-6300 Giessen, FRG

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[^{3}H]PN 200-110, a potent chiral benzoxadiazol 1,4-dihydropyridine Ca^{2+}antagonist was used to label guinea pig skeletal muscle Ca^{2+} channels. [^{3}H]PN 200-110 binds with a K_d of ~1 nM to a homogeneous population of non-interacting binding sites; d-*cis*-diltiazem, but not l-*cis*-diltiazem increases the B_{max} of [^{3}H]PN 200-110 by 25% and slows the dissociation rate 3-fold at 37°C. Target size analysis of the [^{3}H]PN 200-110-labelled Ca^{2+} channels with 10 MeV electrons gave an M_r of 136000 which was reduced to 75000 by d-*cis*-diltiazem treatment of membranes. It is concluded that positive heterotropic allosteric regulation by d-*cis*-diltiazem is accompanied by channel oligomer dissociation.

[^{3}H]PN 200-110 *d-cis-Diltiazem* *Skeletal muscle* *Target size* *Ca^{2+} channel* M_r

1. INTRODUCTION

The Ca^{2+} channels of skeletal muscle have been directly identified with the Ca^{2+} antagonist [^{3}H]nimodipine. [^{3}H]Nimodipine binds to a homogeneous population of saturable, stereospecific, non-interacting binding sites in a fully reversible manner [1]. Using [^{3}H]nimodipine to label Ca^{2+} channels, subcellular fractionation of skeletal muscle has led to the conclusion that the [^{3}H]nimodipine-labelled Ca^{2+} channel is located almost exclusively in t-tubules [2]. This result is in agreement with findings from electrophysiological studies [3].

We have labelled skeletal muscle Ca^{2+} channels with the potent Ca^{2+} antagonist [^{3}H]PN 200-110 (isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-pyridine-3-carboxylate) [4] which labels more channels than [^{3}H]nimodipine, [^{3}H]nitrendipine or [^{3}H]nifedipine [5]. Here, we report on the positive heterotropic allosteric regulation of [^{3}H]PN 200-110 binding by d-*cis*-diltiazem and the associated parallel reduction of the Ca^{2+} channel molecular size as assessed by in situ target size analysis.

2. MATERIALS AND METHODS

2.1. *Radiation-inactivation of Ca^{2+} channels*

Male or female guinea pigs were stunned and bled and skeletal muscle membranes prepared as in [1]. The membranes were then pre-incubated at 37°C for 30 min under 3 conditions:

(i) In 50 mM Tris–HCl supplemented with 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4 (buffer A);
(ii) In buffer A supplemented with 10 μM d-*cis*-diltiazem;
(iii) With 10 μM l-*cis*-diltiazem.

The membranes were then flash-frozen in liquid nitrogen in 2-ml aliquots. Pretreated membranes were irradiated with 10 MeV electrons from a linear accelerator (CSF, Thompson, Paris) using a

* To whom all correspondence should be addressed

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focused electron beam. The temperature was automatically maintained at −110°C as in [6]. Dosimetry was performed with radiochromic dye films [7]. The dose of radiation reducing the starting biological activity to 37% (D_{37}) was used to compute the M_r-value from the empirical formula [8]:

$$M_r = f\frac{6.4 \times 10^5}{D_{37}\ [\text{Mrad}]}$$

where:

f = a temperature correction factor of 2.8 at −110°C [7].

2.2. *Ligand-binding assays*

Ligand-binding assays were performed under sodium vapour lighting in 1.0 ml in buffer A, with 20–100 μg membrane protein/ml and unlabelled drugs as indicated. Non-saturable [^{3}H]PN 200-110 (2.6 Ci/mmol, ⩾95% radiochemical purity)-binding was defined either by 1 or 10 μM unlabelled PN 200-110 or by 1 μM unlabelled nimodipine, all of which gave identical results. Non-saturable binding was 3–8% of bound [^{3}H]PN 200-110 at the K_d of [^{3}H]PN 200-110. After 15 min incubation, bound and free [^{3}H]ligand were separated by rapid filtration as in [1].

Acetylcholine esterase (EC 3.1.1.7) was assayed as in [9] with a Beckman DU 8 spectrophotometer.

2.3. *Experimental design and statistics*

Data are presented from 3 complete and independent radiation-inactivation experiments in which the differentially preincubated membranes were irradiated simultaneously. The acetylcholine esterase activity determination and binding assays were performed in the same samples. All means are arithmetic means, all p-values were computed with the two-tailed Student's t-test.

2.4. *Materials*

Radiochromic dye films were purchased from Far-West Technology (Goleta CA). Sources for other chemicals are given in [1,5].

3. RESULTS

3.1. *Saturability of [^{3}H]PN 200-110-binding*

[^{3}H]PN 200-110 binds to a saturable, non-interacting set of binding sites in skeletal muscle membranes. The K_d of [^{3}H]PN 200-110 at 37°C is 1.4 ± 0.5 nM (n = 3) and the B_{max} is 21 ± 1.5 pmol/mg protein. Fig.1a shows a saturation experiment performed over a 2200-fold range of free [^{3}H]ligand concentrations. As can be seen,

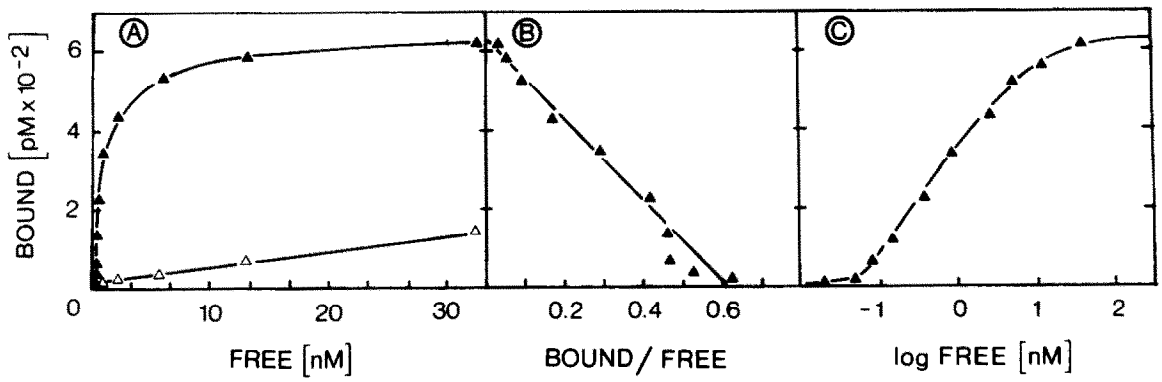

Fig.2. (A) Stereoselective inhibition of [^{3}H]PN 200-110-binding to skeletal muscle membranes by the optical enantiomers of PN 200-110. B is the concentration of the specifically bound [^{3}H]ligand in the absence and B_0 in the PN 200-110 is subtracted from total binding to yield saturable (▲) binding. The range of the free (+)enantiomer of [^{3}H]PN 200-110 employed, is from 0.016–34 nM. As can be seen at the K_d, the binding to saturable binding sites is >90% of filter-retained radioactivity. The assay is performed in 1.0 ml at 37°C at 32 μg protein/ml. (B) Linear transformation of the data (shown in A) presented as a Hofstee plot. Linear regression analysis gives a B_{max} of 0.61 nM, which is equivalent to 19.3 pmol/mg protein. (C) The concentration of bound ligand is plotted as a function of the log free-ligand. Non-linear curve fitting to the general dose–response equation yields a K_d (± asymptotic standard deviation) of 1.1 ± 0.1 nM and a B_{max} of 18.1 ± 0.2 pmol/mg protein.

blank values are extremely low and the signal to noise ratio is remarkably good for this labelled Ca^{2+} antagonist in skeletal muscle membranes. The transformations of the equilibrium-binding data show linearity in the Hofstee plot and the symmetry of the bound *vs* log free-ligand plot.

3.2. *Characteristics of [^{3}H]PN 200-110-binding*

The Ca^{2+} channel-binding of [^{3}H]PN 200-110 is stereoselectively inhibited by the optically pure enantiomers of PN 200-110, (+)205-033 and (−)205-034 (fig.2). The binding-inhibition data of both enantiomers are characterized by a slope factor of 1.0 ± 0.1, with the (+)enantiomer being about 300-times more potent than the (−)enantiomer. This stereoselective inhibition is similar to that reported for the binding of these enantiomers at the [^{3}H]nimodipine-labelled skeletal muscle Ca^{2+} channel [1]. As the (+)enantiomer is about 300-times more potent than the (−)enantiomer we have used the concentration of the free (+)enantiomer of [^{3}H]PN 200-110 in all calculations neglecting the (−)enantiomer.

3.3. *Regulation of [^{3}H]PN 200-110-binding by diltiazem diastereoisomers*

d-*cis*-Diltiazem, but not l-*cis*-diltiazem, increases

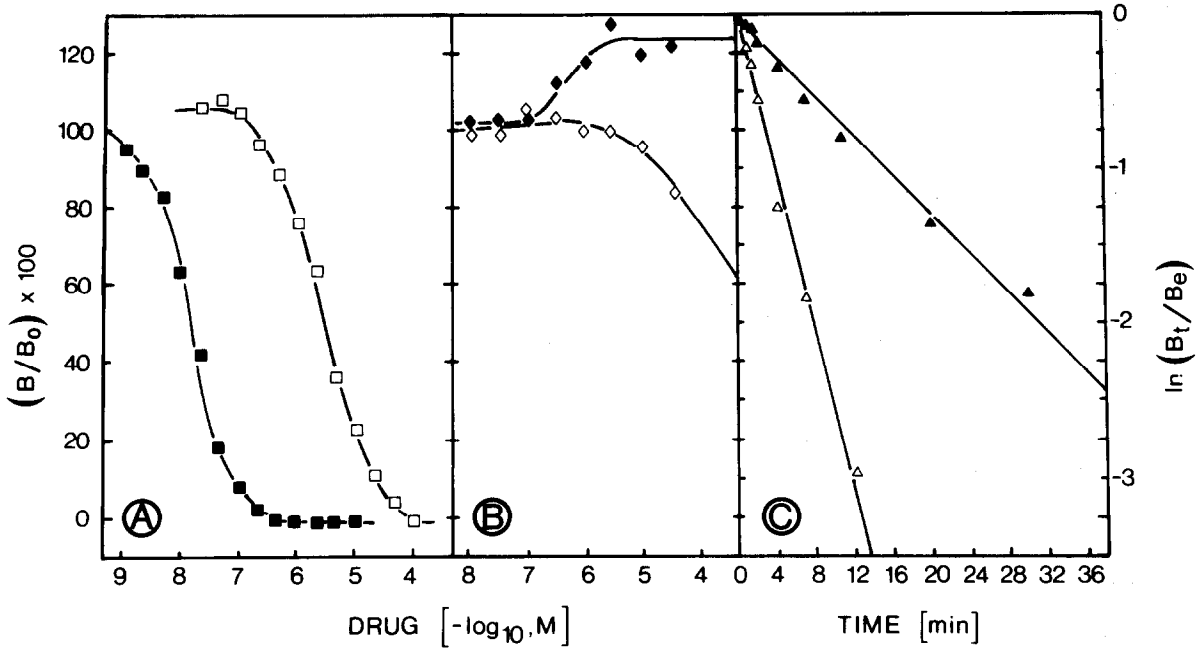

Fig.2. (A) Stereoselective inhibition of [^{3}H]PN 200-110-binding to skeletal muscle membranes by the optical enantiomers of PN 200-110. *B* is the concentration of the specifically bound [^{3}H]ligand in the absence and B_o in the presence of the competitor, respectively. Receptor is 1.5 nM, bound ligand (B_o), 1.2 nM and the [^{3}H]ligand, 6.7 nM. The (+)enantiomer of PN 200-110, (+)205-033 (■) is about 300-times more potent than the (−)enantiomer of PN 200-110, (−)205-034 (□). The K_d-values are calculated by eq. (22) and eq. (23) of [12]: (+)205-033, 1.3 nM; (−)205-034, 375 nM. (B) Stereospecific interaction of d-*cis*-diltiazem (◆) and l-*cis*-diltiazem (◇) with [^{3}H]PN 200-110-labelled Ca^{2+} channels. d-*cis*-Diltiazem causes a small (~25%) stimulation of Ca^{2+} channel-labelling by [^{3}H]PN 200-110; l-*cis*-diltiazem causes only a small inhibition (15% at 50 μM). Protein is 19.2 μg/ml and bindable [^{3}H]PN 200-110, 5.0 nM. (C) Dissociation of ([^{3}H]PN 200-110 Ca^{2+}–channel) complexes induced by blockade of the association reaction with 1 μM unlabelled PN 200-110. B_t is the saturable binding at the times indicated, and B_e saturable binding at equilibrium (time = zero). B_e is 0.55 nM in the absence of d-*cis*-diltiazem and 0.70 nM in the presence of 10 μM d-*cis*-diltiazem; the concentration of bindable [^{3}H]PN 200-110 is 4.2 nM and protein, 43.9 μg/ml. In this experiment, the k_{-1} in the absence of d-*cis*-diltiazem (△) was 0.25 min^{-1} and in the presence of d-*cis*-diltiazem (▲) 0.07 min^{-1}.

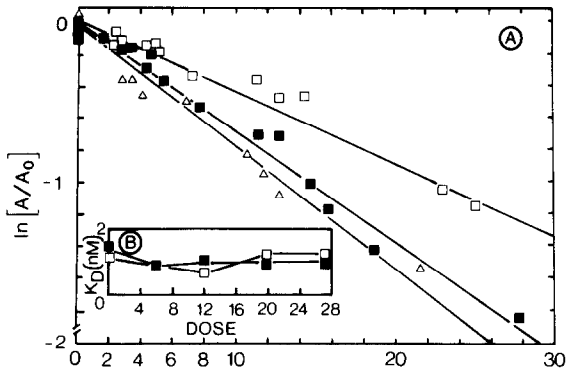

Fig.3.

Fig.3. Radiation inactivation of [^{3}H]PN 200-110-labelled Ca^{2+} channels. (A) A is the specific binding at a given dose of radiation, and A_o the saturable binding in non-irradiated controls. The skeletal muscle membranes are either pre-incubated and assayed in buffer (■), 10 μM d-*cis*-diltiazem (□) or 10 μM l-*cis*-diltiazem (△). In this experiment, [^{3}H]PN 200-110 is 5.3 nM and protein, 60 μg/ml. In the non-irradiated controls the concentrations of specifically bound [^{3}H]PN 200-110 are as follows: for buffer pre-incubation, 1.59 ± 0.04 nM; for 10 μM d-*cis*-diltiazem pre-incubation, 2.02 ± 0.05 nM; for l-*cis*-diltiazem pre-incubation, 1.23 ± 0.06 nM (n = 3). The D_{37}-values of the [^{3}H]PN 200-110-labelled Ca^{2+} channels under the pre-incubation conditions are, buffer D_{37} = 14.01 Mrad (M_r 128000), 10 μM d-*cis*-diltiazem, D_{37} = 23.3 Mrad (M_r 77000), 10 μM l-*cis*-diltiazem D_{37} = 12.9 Mrad (M_r 139000). (B) The K_d of [^{3}H]PN 200-110 for residual Ca^{2+} channels is not changed by the radiation for doses up to 27 Mrad.

in a concentration-dependent manner [^{3}H]PN 200-110-labelling of Ca^{2+} channels. The ED_{50} of d-*cis*-diltiazem stimulation is 1–2 μM (fig.2) which is in the same range as the ED_{50} of d-*cis*-diltiazem to stimulate [^{3}H]nimodipine-binding [1]. The stimulation of [^{3}H]PN 200-110-labelling of calcium channels by d-*cis*-diltiazem is due to an increase in B_{max} from 21 ± 1.5 pmol/mg protein (n = 3) to 26 ± 1 pmol/mg protein (n = 3; $p < 0.05$).

Table 1

M_r-values of Ca^{2+} channels and an enzyme standard as determined by radiation inactivation

	Pre-incubation condition		
	Buffer	10 μM d-*cis*-diltiazem	10 μM l-*cis*-diltiazem
Skeletal muscle Ca^{2+} channels labelled with [^{3}H]PN 200-110	(c) 136000 ± 12000	(a,b) 75000 ± 4800	(d) 123000 ± 6700
Skeletal muscle Ca^{2+} channels labelled with [^{3}H]nimodipine[a]	178000 ± 7000	111000 ± 7000	158000 ± 7100
Skeletal muscle acetylcholine esterase	66000	58000	69000
Skeletal muscle Ca^{2+} channels labelled with [^{3}H]nitrendipine[b]	210000 ± 20000	n.d.	n.d.

[a] Data are from Ferry et al., submitted

[b] Data are from [11]. Data for the M_r of the membrane-bound acetylcholine esterase, which is the internal control, are included

n.d., not determined

M_r-values are calculated from monophasic decay curves of the given biological activity according to the empirical formula in [8]. Means with standard error for n = 3 experiments are shown: (a) $p < 0.01$ for [^{3}H]PN 200-110-labelled calcium channels pre-incubated in 10 μM d-*cis*-diltiazem *vs* pre-incubation in buffer; (b) $p < 0.013$ for [^{3}H]PN 200-110-labelled calcium channels *vs* [^{3}H]nimodipine-labelled calcium channels, both pre-incubated with 10 μM d-*cis*-diltiazem; (c) $p < 0.04$ for [^{3}H]PN 200-110-labelled *vs* [^{3}H]nimodipine-labelled, both pre-incubated in buffer; (d) $p < 0.06$ for both pre-incubated in l-*cis*-diltiazem

3.4. *Reversibility and dissociation kinetics*

The binding of [^{3}H]PN 200-110 to Ca^{2+} channels is fully reversible. When an equilibrium population of (channel–[^{3}H]PN 200-100) complexes is perturbed by addition of 1 μM unlabelled PN 200-110, the complexes decay monoexponentially with respect to time. At 37°C the k_{-1} is 0.3 min^{-1}. In the presence of a saturating concentration of d-*cis*-diltiazem the k_{-1} of (channel–[^{3}H]PN 200-110) complexes is 0.07 min^{-1} which is 4-times smaller than in the absence of d-*cis*-diltiazem (fig.2).

3.5. *Target size analysis*

Irradiation of skeletal muscle microsomes decreases [^{3}H]PN 200-110-labelling of Ca^{2+} channels, due to a decrease in B_{max}. The K_d of [^{3}H]PN 200-110 for the remaining Ca^{2+} channels remains unchanged (fig.3a). When the decline of [^{3}H]PN 200-110 Ca^{2+} channel-labelling is plotted against the radiation dose, a monoexponential decay curve is found for membranes which were pre-incubated in buffer A or in buffer A supplemented with 10 μM d- or l-*cis*-diltiazem, respectively (fig.3b). These monoexponential decay curves are shown as linear transformations in fig.3c.

When membranes are pre-incubated in buffer A, the D_{37} of the [^{3}H]PN 200-110-labelled Ca^{2+} channel is 13.2 ± 0.2 Mrad (n = 3), which corresponds to M_r 136000. Pre-incubation with d-*cis*-diltiazem increased the D_{37} to 24.3 ± 2 Mrad (n = 3), corresponding to a decrease in M_r of 61000 mass units. Pre-incubation with l-*cis*-diltiazem does not significantly decrease the M_r (table 1). The M_r of the membrane-bound acetylcholine esterase, which is measured as an internal standard, is identical for all 3 pre-incubation conditions (table 1).

4. DISCUSSION

Here we found that the apparent molecular size of the [^{3}H]PN 200-110-labelled Ca^{2+} channel is 136000. Pre-incubation with d-*cis*-diltiazem, but not l-*cis*-diltiazem, reduced the M_r of the [^{3}H]PN 200-110-labelled channel. The specificity of the d-*cis*-diltiazem regulation of the Ca^{2+} channel M_r is underlined by the ineffectiveness of l-*cis*-diltiazem to induce a decrement of the M_r, and the fact that acetylcholine esterase has an identical M_r under all 3 pre-incubation conditions. This rules out the possibility that non-specific effects such as 'radical capture' are responsible for the observed M_r regulation. In this respect, the Ca^{2+} channel is analogous to the benzodiazepine receptor/chloride ionophore, where γ-aminobutyric acid acts a positive heterotropic allosteric regulator and thereby induces a decrease in molecular size of the [^{3}H]flunitrazepam-labelled complex [10].

Our value for the M_r of the skeletal muscle Ca^{2+} channel labelled with PN 200-110 is smaller than that in [11]. We can rule out methodological or species differences to explain this apparent discrepancy, because we have measured the M_r of the Ca^{2+} channel with different labelled 1,4-dihydropyridine Ca^{2+} antagonists and our data (e.g., with [^{3}H]nimodipine) are statistically indistinguishable from the M_r-value reported in [11] (see table 1). It should be noted that in skeletal muscle the 3-nitrophenyl 1,4-dihydropyridines, [^{3}H]nitrendipine and [^{3}H]nimodipine label an identical number of high-affinity binding sites, whereas the benzoxadiazol 1,4-dihydropyridine derivative [^{3}H]PN 200-110 labels significantly more sites [5]. In addition, the d-*cis*-diltiazem stimulation of [^{3}H]PN 200-110 binding capacity is only 20% compared to the 200–300% increase seen with [^{3}H]nitrendipine or [^{3}H]nimodipine. We have suggested that this may be due to an 'efficacy factor' whereby the given 1,4-dihydropyridine stabilizes a conformer of the channel with high affinity for this class of drugs [5]. Because [^{3}H]PN 200-110 (in comparison to nifedipine, nitrendipine and nimodipine) can stabilize more Ca^{2+} channels in a high affinity state, the action of d-*cis*-diltiazem (which allows channels at 37°C to have high affinity for 1,4-dihydropyridines) is much less pronounced.

As can be seen from table 1, [^{3}H]PN 200-110-labelling of Ca^{2+} channels can be also differentiated from [^{3}H]nimodipine (or [^{3}H]nitrendipine)-labelling by in situ target size analysis. Thus [^{3}H]PN 200-110-labelling of Ca^{2+} channels yields M_r-values which are 37000–42000 mass units smaller than the corresponding M_r-values of [^{3}H]nimodipine-labelled channels.

How can we explain the difference in M_r measured with [^{3}H]PN 200-110 as opposed to nimodipine or nitrendipine and the decrement in functional size induced by d-*cis*-diltiazem? In the

Table 2

Average target sizes of putative elements of the skeletal muscle Ca^{2+} channel in situ

Element	M_r	Pre-incubation condition	Ca^{2+} channel label	Proposed function
α	<75000	derived		Binds 1,4-dihydropyridines
β	75000	d-*cis*-Diltiazem	[^{3}H]PN 200-110	Facilitates PN 200-110-binding
γ	111000	d-*cis*-Diltiazem	[^{3}H]Nimodipine	Facilitates nimodipine-binding
δ	60000	derived		Dissociated by d-*cis*-diltiazem
$(\beta \cdot \delta)$	138000	buffer	[^{3}H]PN 200-110	–
$(\gamma \cdot \delta)$	178000	buffer	[^{3}H]Nimodipine	–

Elements of the model are the limiting largest structures which allow the data of the target size analysis (table 1) to be unambiguously interpreted. In frozen membranes α always behaves as an independent target, $(\beta \cdot \delta)$ and $(\gamma \cdot \delta)$ are assembled targets which are dissociated upon pre-treatment with d-*cis*-diltiazem. The M_r of δ is derived by subtracting the M_r of either [^{3}H]nimodipine or [^{3}H]PN 200-110-labelled Ca^{2+} channels pre-incubated with d-*cis*-diltiazem from the M_r of buffer pre-incubated channels. The M_r of α, as a condition of the model, must be smaller than any measured M_r

absence of more biochemical information on the structural composition of the Ca^{2+} channel we would like to propose the following model. This model uses the assumption that a single hit destroys the total structure of a given 'target' [8] whether that target is composed of a single protomer or assembled from heterologous or homologous protomers. Assume that the Ca^{2+} channel consists of 4 elements (α, β, γ, δ) which may or may not correspond to the true protomers of the oligomeric channel. These elements are defined as the largest components required for the measured biological activity; e.g., [^{3}H]PN 200-110, [^{3}H]nimodipine or [^{3}H]nitrendipine-labelling after irradiation. Table 2 lists the elements of the model and their measured or deduced M_r-values under the different experimental conditions. The element α (carrying the 1,4-dihydropyridine Ca^{2+} antagonist binding site) has to associate with the (facilitatory) element β when PN 200-110 labels the channel, but with the (facilitatory) element γ when [^{3}H]nimodipine or [^{3}H]nitrendipine labels.

The M_r difference $(\gamma - \beta)$ is 40000, because under all preincubation conditions (table 1) the channel assayed by [^{3}H]nimodipine or [^{3}H]nitrendipine is larger by this increment than when assayed with PN 200-110. The conclusion is, that we have not determined the M_r of α, but that of the facilitatory elements (β or γ). The following rank order of M_r-values must then apply: $\alpha < \beta < \gamma$. Because preincubation with d-*cis*-diltiazem decreases the M_r by about 60000 we have to postulate that β and γ are functionally coupled to the element δ; δ is the element which d-*cis*-diltiazem uncouples from either β or γ. Therefore, according to the model, in buffer-preincubated membranes, frozen at -110°C, the elements exist as α, $(\beta \cdot \delta)$ and $(\gamma \cdot \delta)$ where $(\beta \cdot \delta)$ and $\gamma \cdot \delta)$ are assembled (coupled) elements. In d-*cis*-diltiazem-preincubated membranes the elements exist as α, β, γ, δ.

Admittedly this model is speculative but it can explain the experimental results and gives an estimate of the functional size of the 1,4-dihydropyridine binding site. In any event our data demonstrate that 1,4-dihydropyridines interact differently with the Ca^{2+} channels as shown by target size analysis in this study and by comparative ligand-binding experiments elsewhere [5]. Our experiments have yielded the important result that the allosteric regulator d-*cis*-diltiazem decreases the in situ M_r of the Ca^{2+} channel as labelled by 1,4-dihydropyridines, and that slow channel blocker–channel interactions are of a complex nature at the molecular level.

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