Purified calcium channels have three allosterically coupled drug receptors

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(−)-[^{3}H]Desmethoxyverapamil and (+)-[^{3}H]PN 200-110 were employed to characterize phenylalkylamine-selective and 1,4-dihydropyridine-selective receptors on purified Ca^{2+} channels from guinea-pig skeletal muscle t-tubules. In contrast to the membrane-bound Ca^{2+} channel, d-*cis*-diltiazem ($EC_{50} = 4.5 \pm 1.7$ μM) markedly stimulated the binding of (+)-[^{3}H]PN 200-110 to the purified ionic pore. In the presence of 100 μM d-*cis*-diltiazem (which binds to the benzothiazepine-selective receptors) the B_{max} for (+)-[^{3}H]PN 200-110 increased from 497 ± 81 to 1557 ± 43 pmol per mg protein, whereas the K_d decreased from 8.8 ± 1.7 to 4.7 ± 1.8 nM at 25°C. P-*cis*-Diltiazem was inactive. (−)-Desmethoxyverapamil, which is a negative heterotropic allosteric inhibitor of (+)-[^{3}H]IN 200-110 binding to membrane-bound channels, stimulated 1,4-dihydropyridine binding to the isolated channel. (−)-[^{3}H]Desmethoxyverapamil binding was stimulated by antagonistic 1,4-dihydropyridines [(+)-PN 200-110 > (−)(*R*)-202-791 > (+)(4*R*)-Bay K 8644] whereas the agonistic enantiomers (+)(*S*)-202-791 and (−)(4*S*)-Bay K 8644 were inhibitory and (−)-PN 200-110 was inactive. The results indicate that three distinct drug-receptor sites exist on the purified Ca^{2+} channel, two of which are shown by direct labelling to be reciprocally allosterically coupled.

Ca^{2+} channel *(Skeletal muscle)* *Allosterism* *1,4-Dihydropyridine* *Phenylalkylamine*

1. INTRODUCTION

Skeletal muscle Ca^{2+} channels, first labelled directly with the 1,4-dihydropyridine [^{3}H]nimodipine [1], have been shown to be nearly exclusively localized in t-tubule membranes [2,3]. As the density of these channels compared to, e.g. heart, brain or smooth muscle is high [4], they have become the most favoured for solubilization and purification of the channel proteins [5–8], photoaffinity labelling [9,10], reconstitution [11] and detailed characterisation of distinct drug-receptor sites, including target size analysis [12]. There is strong evidence that the Ca^{2+} channel has 3 drug-receptor domains (one selective for 1,4-dihydropyridines, one for phenylalkylamines and one for d-*cis*-diltiazem) which are functionally linked to each other by reciprocal allosteric coupling mechanisms and to Ca^{2+}-binding sites [13,14]. Binding of the phenylalkylamine verapamil to its site lowers the affinity of the 1,4-dihydropyridine-selective site [1], whereas the potent 1,4-dihydropyridine (+)-PN 200-110 inhibits, via a similar allosteric mechanism, binding of the potent phenylalkylamine (−)-[^{3}H]desmethoxyverapamil to its receptor [15]. So far all of these studies have been performed with particulate or solubilized Ca^{2+} channels [4]. Here, we describe that the purified channel also shows reciprocal allosteric regulation between (+)-[^{3}H]PN 200-110-labelled 1,4-dihydropyridine receptors and the (−)-[^{3}H]-desmethoxyverapamil-labelled phenylalkylamine site. In addition, we find that the purified preparation has stereospecific sites for the diltiazem diastereoisomers, indicating that the 3 drug-receptor sites copurify.

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2. MATERIALS AND METHODS

2.1. *Materials*

(−)-[^{3}H]Desmethoxyverapamil (83 Ci/mmol) and the unlabelled optically pure enantiomers of desmethoxyverapamil were gifts from Knoll; (+)-[^{3}H]PN 200-110 (binding ability > 85%; 75 Ci/mmol) was from Amersham. The PN 200-110 enantiomers, (−)(*R*)- and (+)(*S*)-202-791 [isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridine carboxylate], were from Sandoz; the optically pure enantiomers (+)(4*R*)-Bay K 8644 and (−)(4*S*)-Bay K 8644 and (±)-[^{3}H]Bay K 8644 (78 Ci/mmol) were from Bayer. Benzamidine and pepstatin A were from Sigma.

2.2. *Membrane preparation and channel purification*

Partially purified skeletal muscle t-tubule membranes from guinea-pig hind limbs were prepared as described in [1] and kept in liquid nitrogen for up to 1 month untill use. The purification procedure followed essentially the strategies outlined in [4,5] with the following modifications: 0.1 mM benzamidine, 1.0 mM iodoacetamide, 1 μM pepstatin A were present in all buffers and sucrose gradient centrifugation after affinity chromatography was performed in a Beckman VT I 50 rotor as described [7]. Purity of the channel was assessed by SDS-PAGE on 5–15% polyacrylamide gradient gels and silver staining [16], photoaffinity labelling [9,10] and post-labelling with (+)-[^{3}H]PN 200-110. With the latter method the specific activity of the channel was ⩾ 1500 pmol per mg protein, which is equal to the calculated value of the channel purified from rabbit skeletal muscle t-tubule membranes [7,8].

2.3. *Binding assays*

The conditions for the binding assay with purified channels were essentially as in [4], except that all incubations were performed in a volume of 0.25 ml for 60 min at 25°C. (+)-[^{3}H]PN 200-110 (for experiments to evaluate the effects of unlabelled drugs) was 2–3.5 nM. For saturation studies the free ligand concentration was varied between 0.4 and 30 nM. The (−)-[^{3}H]desmethoxyverapamil concentration was 6–10 nM; for saturation studies the free concentration was varied between 3 and 350 nM by decreasing the specific activity with unlabelled (−)-desmethoxyverapamil. The non-specific binding for (+)-[^{3}H]PN 200-110 assays was defined using 3 μM (+)-PN 200-110 and 30 μM (−)-desmethoxyverapamil for (−)-[^{3}H]desmethoxyverapamil assays, respectively. Bound and free ligand were separated after addition of carrier protein with the polyethylene glycol method [4]. For membrane-bound channels conditions were as described [4]. The non-specific binding for (±)-[^{3}H]Bay K 8644 and (+)-[^{3}H]PN 200-110 experiments with particulate membranes was defined using 1 μM PN 200-110. Data are given as means ± SE for 3 independent experiments except for binding inhibition or stimulation data where the asymptotic standard deviation obtained by parameter fitting of pooled data with non-linear methods is presented [4,5]. IC$_{50}$ and EC$_{50}$ values indicate the drug concentration causing 50% inhibition or 50% stimulation, respectively. Inhibition is defined as $100 \times (B_0 - B)/B$ where B is the specific binding of ^{3}H-labelled ligand in the presence and B_0 in the absence of added drug. Stimulation is defined as $100 \times (B - B_0)/B_0$.

3. RESULTS

3.1. *Characteristics of the 1,4-dihydropyridine receptors*

Previously is was shown that binding of [^{3}H]-nifedipine > [^{3}H]nimodipine = [^{3}H]nitrendipine ⩾ [^{3}H]PN200-110 to particulate calcium channels in skeletal muscle was stimulated by d-*cis*-diltiazem at 37°C, mainly by increasing the apparent density of receptors which were stabilised in the high-affinity state [17]. Fig.1A shows that the binding of (+)-[^{3}H]PN 200-110 to purified channels is stimulated by d-*cis*-diltiazem in a concentration-dependent manner. The IC$_{50}$ value ($n = 3$) was 4.5 ± 1.7 μM, the maximum stimulation being 369 ± 40%. Saturation analysis gave a K_d value for (+)-[^{3}H]PN 200-110 of 8.8 ± 1.7 nM ($n = 3$) in the absence of d-*cis*-diltiazem whereas with 100 μM d-*cis*-diltiazem the dissociation constant decreased to 4.7 ± 1.8 nM. A concomitant increase in B_{max} was observed (497 ± 81 vs 1557 ± 43 pmol per mg protein). An example is shown in fig.1B. The diastereoisomer l-*cis*-diltiazem, which is not a Ca^{2+}-

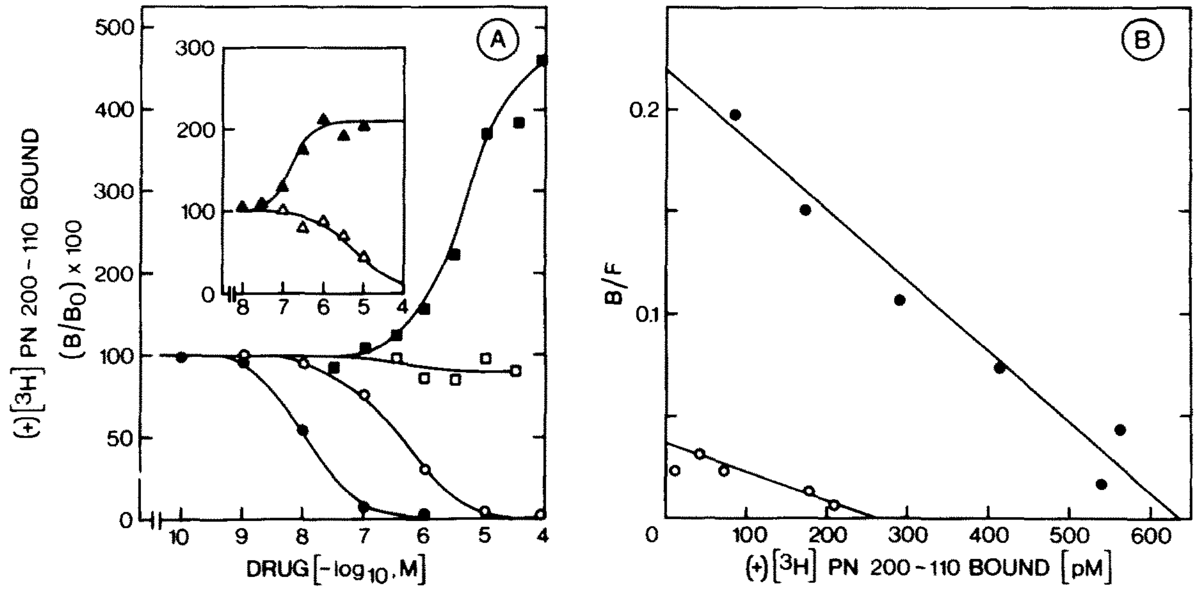

Fig.1. Equilibrium binding properties of the 1,4-dihydropyridine-selective receptor, labelled by (+)-[^{3}H]PN 200-110. (A) Stereospecific regulation of (+)-[^{3}H]PN 200-110 binding by the diastereoisomers of diltiazem, and the enantiomers of PN 200-110 and desmethoxyverapamil (inset). (+)-[^{3}H]PN 200-110 (2.0-3.5 nM) was incubated with 0.08-0.17 μg channel protein for 60 min at 25°C. B_0 is the specifically bound ligand in the absence, and B in the presence of unlabelled drug. Data were obtained from at least 2-3 independent experiments. IC_{50} (or EC_{50}) values and apparent Hill slopes (n_H) are given ± asymptotic SD. (●) (+)-PN 200-110 - $IC_{50} = 12.3 \pm 3.5$ nM, $n_H = 1.05 \pm 0.1$, maximal inhibition = 100%; (○) (−)-PN 200-110 - $IC_{50} = 434 \pm 140$ nM, $n_H = 0.83 \pm 0.20$, maximal inhibition = 100%; (■) d-*cis*-diltiazem - $EC_{50} = 4.5 \pm 1.7$ μM, $n_H = 1.0 \pm 0.34$, maximal stimulation = 269 ± 40%; (□) l-*cis*-diltiazem - < 10% inhibition at 10 μM; (▲) (−)-desmethoxyverapamil - $EC_{50} = 187 \pm 113$ nM, $n_H = 2.2 \pm 0.21$, maximal stimulation = 110 ± 16%;.(△) (+)-desmethoxyverapamil - 56% inhibition at 10 μM. (B) Saturation analysis of equilibrium binding in the absence or presence of d-*cis*-diltiazem. (+)-[^{3}H]PN 200-110 (0.4-30 nM) was incubated with 0.105 μg channel protein for 60 min at 25°C with (●) and without (○) 100 μM d-*cis*-diltiazem in the incubation mixture. Scatchard transformation of the data is shown. The following parameter estimates represent the best fit of the specific binding data to a monophasic saturation isotherm: d-*cis*-diltiazem absent - $K_d = 7.2 \pm 1.1$ nM, $B_{max} = 264.7 \pm 14$ pM (equivalent to 627 ± 33 pmol/mg protein); d-*cis*-diltiazem present - $K_d = 2.87 \pm 0.5$ nM, $B_{max} = 630 \pm 35$ pM (equivalent to 1500 ± 83 pmol/mg protein).

channel antagonist [18], was inactive. The phenylalkylamine (−)-desmethoxyverapamil inhibits 1,4-dihydropyridine binding to particulate Ca^{2+} channels (fig.2). (−)-Desmethoxyverapamil is approx. 3 orders of magnitude more potent at inhibiting the binding of the overt agonist (±)-[^{3}H]Bay K 8644 than the potent antagonist (+)-[^{3}H]PN 200-110. The mechanism of inhibition was evaluated for (+)-[^{3}H]PN 200-110 and is of a mixed type (B_{max} decrease, K_d increase). In contrast, (−)-desmethoxyverapamil did not inhibit (+)-[^{3}H]PN 200-110 binding to purified channels but stimulated it. The EC_{50} value was 187 ± 113 nM ($n = 3$), the maximum stimulation being 110 ± 16%. Interestingly, the (+) enantiomer of desmethoxyverapamil was inhibitory for both the membrane-bound and purified channel (not shown). As reported for the particulate channel [4], the 1,4-dihydropyridines (+)-PN 200-110 and (−)-PN 200-110 exhibited stereospecific competition.

3.2. *Characteristics of the (−)-[^{3}H]desmethoxyverapamil-labelled phenylalkylamine receptors*

The interaction of the high-affinity probe (−)-[^{3}H]desmethoxyverapamil with the phenylalkylamine-selective receptors of the particulate Ca^{2+}

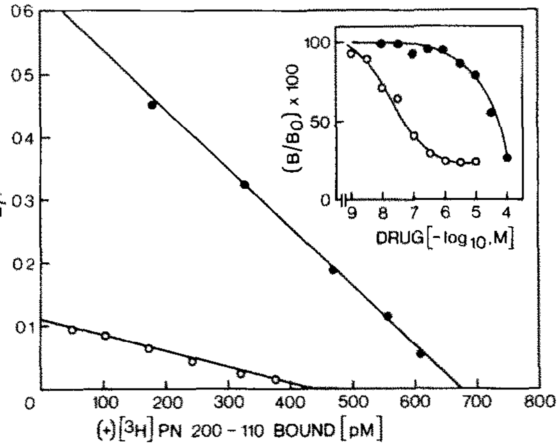

Fig.2. Mechanism of inhibition of (+)-[^{3}H]PN 200-110 binding to partially purified skeletal muscle t-tubule membranes by (−)-desmethoxyverapamil. (+)-[^{3}H]PN 200-110 (0.5-24 nM) was incubated with 7.8 μg membrane protein for 60 min at 25°C in the absence and presence of 100 μM (−)-desmethoxyverapamil. Scatchard transformation of the specific binding data is shown. Control (●) - $K_d = 1.0 \pm 0.3$ nM, $B_{max} = 672 \pm 5.8$ pM (equivalent to 21.5 ± 0.2 pmol/mg protein); 100 μM (−)-desmethoxyverapamil present (○) - $K_d = 3.93 \pm 0.12$ nM, $B_{max} = 432.7 \pm 4.7$ pM (equivalent to 13.8 ± 0.1 pmol/mg protein). (Inset) Comparison of inhibition of (+)-[^{3}H]PN 200-110 (●) and (±)-[^{3}H]Bay K 8644 (○) binding to particulate skeletal muscle t-tubule membranes by (−)-desmethoxyverapamil. 0.4-0.6 nM (+)-[^{3}H]PN 200-110 or 1.5-3.0 nM (±)-[^{3}H]Bay K 8644 were incubated with 7-10 or 20-30 μg membrane protein, respectively. Ligand bound in the presence of increasing concentrations of (−)-desmethoxyverapamil (*B*) was normalized with respect to control binding (*B*$_0$). Points are means from at least 2-3 experiments. IC$_{50}$ values (± asymptotic SD) for binding inhibition were 38.9 ± 10 nM (76% ± 4% maximal inhibition) with (±)-[^{3}H]Bay K 8644 and 46 ± 11 μM (assuming a maximal inhibition of 100%) when (+)-[^{3}H]PN 200-110 was the ligand.

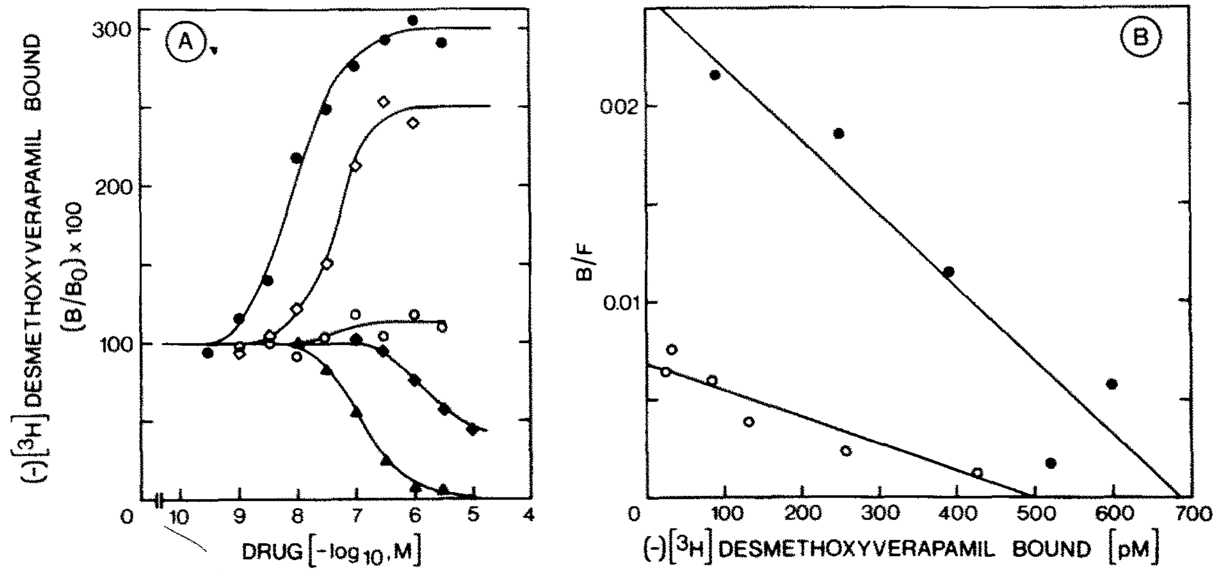

Fig.3. Equilibrium binding properties of the phenylalkylamine-selective receptor, labelled by (−)-[^{3}H]desmethoxyverapamil. (A) Stereospecific regulation of (−)-[^{3}H]desmethoxyverapamil binding by the enantiomers of PN 200-110 and 202-791. The competitive inhibition by unlabelled (−)-desmethoxyverapamil is also illustrated. (−)-[^{3}H]Desmethoxyverapamil (6-10 nM) was incubated with 0.3-0.6 μg channel protein for 60 min at 25°C. *B*$_0$ and *B* are defined as in fig.1. Points are means from at least 2-3 experiments. IC$_{50}$ (or EC$_{50}$) values and apparent Hill slopes (n_H) are given ± asymptotic SD: (●) (+)-PN 200-110 - EC$_{50}$ = 7.0 ± 1.9 nM, n_H = 0.95 ± 0.23, maximal stimulation = 201 ± 9%; (○) (-)-PN 200-110 - 10% stimulation at 3 μM; (◇) (*R*)-202-791 - EC$_{50}$ = 44 ± 8 nM, n_H = 1.4 ± 0.3, maximal stimulation = 151 ± 8%; (◆) (*S*) − 202-791 - EC$_{50}$ = 1.46 ± 0.48 μM, n_H = 1.5 ± 0.6, 58.1% maximal inhibition at 10 μM; (▲) (-)-desmethoxyverapamil - EC$_{50}$ = 99 ± 18 nM, n_H = 1.01 ± 0.17, maximal inhibition 100%. (B) Mechanism of stimulation of (-)-[^{3}H]desmethoxyverapamil binding by (+)-PN 200-110. Saturation experiments were performed as described in section 2, with and without 1 μM (+)-PN 200-110 in the incubation mixture. Scatchard transformation of the data of one typical experiment is shown. Best fits were obtained with the following parameter estimates. Control (○): K_d = 73.7 ± 10 nM, B_{max} = 490.7 ± 42 pM. 1 μM (+)-PN 200-110 present (●): K_d = 26.4 ± 5.6 nM, B_{max} = 682 ± 51 pM.

channel in skeletal muscle was evaluated previously in detail [15]. Fig.3A shows that (+)-PN 200-110 stimulates (−)-[^{3}H]desmethoxyverapamil binding (EC_{50} = 7.0 ± 1.9 nM) to the purified channel whereas it was an allosteric inhibitor for particulate preparations [15]. The average maximum stimulation was 201 ± 9% ($n = 3$). In saturation studies the K_d for (−)-[^{3}H]desmethoxyverapamil was 91 ± 17 nM; with 1 μM (+)-PN 200-110 present the K_d decreased to 48 ± 11 nM ($n = 3$) (fig.3B), whereas B_{max} increased by 64 ± 24%. (−)-PN 200-110 was without effect. We have also investigated the enantiomers of so-called agonistic 1,4-dihydropyridines. As can be seen in fig.3 (−)(*R*)-202-791 stimulated whereas (+)(*S*)-202-791 was inhibitory. The (*R*) enantiomer is a potent Ca^{2+}-channel blocker whereas the (*S*) enantiomer is a potent agonist [19]. Likewise (+)(4*R*)-Bay K 8644 (a channel blocker) stimulated (EC_{50} = 100 ± 58 nM, maximal stimulation = 33 ± 9%) whereas (−)(4*S*)-Bay K 8644, a channel agonist [20], was inhibitory (not shown). The benzothiazepine diastereoisomers blocked (−)-[^{3}H]desmethoxyverapamil binding in a stereospecific manner. The IC_{50} value for d-*cis*-diltiazem was 3.8 ± 2 μM vs 19.0 ± 3 μM for l-*cis*-diltiazem.

4. DISCUSSION

In this report we show that 2 of the postulated 3 drug-receptor sites of the Ca^{2+} channel can be directly identified in purified preparations with a post-labelling procedure, namely the 1,4-dihydropyridine-selective and the phenylalkylamine-selective receptor. The presence of a third receptor (the benzothiazepine-selective site) is inferred from the stimulation of 1,4-dihydropyridine (e.g. (+)-[^{3}H]PN 200-110) binding by the Ca^{2+}-channel blocker d-*cis*-diltiazem, whereas the diastereoisomer l-*cis*-diltiazem was inactive. The 1,4-dihydropyridine receptor recognized the optically pure enantiomers of the benzoxadiazole 1,4-dihydropyridine PN 200-110 with a eudismic ratio of > 30. This strongly supports our claim that we have characterized the 1,4-dihydropyridine receptor of the Ca^{2+} channel and not an unrelated 1,4-dihydropyridine site. Such sites reside, e.g. on the nucleoside transporter [21] which has the same molecular mass (50 kDa) as the β-subunit of the Ca^{2+} channel [7,8].

Some features clearly distinguish purified and membrane-bound channels. It has been reported that membrane-bound and solubilized channels have a K_d of 0.7 nM for (+)-[^{3}H]PN 200-110 [4]. The affinity for this benzoxadiazole 1,4-dihydropyridine is decreased upon purification by a factor of 10, the K_d now amounting to 8.8 nM. Whereas with respect to B_{max} and K_d there are only small effects of d-*cis*-diltiazem on (+)-[^{3}H]PN 200-110 binding to the membrane-bound structure [4,17] there is a dramatic increase of B_{max} in the purified preparation, accompanied by a 2-fold increase in affinity. Apparently, (+)-[^{3}H]PN 200-110 is not able to stabilise all of the 1,4-dihydropyridine receptors in the high-affinity state unless d-*cis*-diltiazem (acting through its own site) shifts the equilibrium towards the high-affinity state. There is compelling evidence that in intact cells this high-affinity state corresponds to the inactivated channel [22]. In particulate preparations high-affinity stabilisation is dependent on temperature and on the structure of the 1,4-dihydropyridine. Overt agonists (as is (±)-[^{3}H]Bay K 8644) stabilise (in contrast to the potent antagonist (+)-[^{3}H]PN 200-110) only a minor fraction of channels in the high-affinity state [12]. The partial agonists [^{3}H]nifedipine, [^{3}H]nimodipine or [^{3}H]nitrendipine are intermediate in behaviour and d-*cis*-diltiazem is required to label maximally the 1,4-dihydropyridine receptors with these ligands. Thus, the purified channel has acquired properties with respect to (+)-[^{3}H]PN 200-110 binding which the membrane-bound structure shows only for partial or overt agonists. (−)-Desmethoxyverapamil is not a negative allosteric heterotropic inhibitor of 1,4-dihydropyridine binding as it is for the particulate preparation but stimulates (as does d-*cis*-diltiazem). The stimulatory effect of (−)-desmethoxyverapamil on (+)-[^{3}H]PN 200-110 binding does not indicate that this phenylalkylamine binds to the benzothiazepine-selective receptor. Instead, stimulatory or inhibitory effects of a Ca^{2+} channel drug specific for the phenylalkylamine receptor on the kinetic paraeters of a ligand binding to the 1,4-dihydropyridine receptor are best explained by an allosteric model [12,23]. In this model the equilibrium of two states (e.g. inactivated and resting) is governed by the allosteric constant in the absence of drug. If the labelled ligand binds better to the inactivated state the second drug may or may not affect the equilibrium by changing the allosteric constant. This can result in either an increase or decrease of the fraction of channels labelled in the high-affinity state. Interesting examples in this context are the stimulation of [^{3}H]-nimodipine binding to membrane-bound channels by the potent phenylalkylamine (–)-D-600 (methoxyverapamil) [13] and the 1000-fold higher potency of (–)-desmethoxyverapamil in inhibiting binding of the overt agonist (±)-[^{3}H]Bay K 8644 compared to the overt antagonist (+)-[^{3}H]PN 200-110. Clearly, these effects can only be rationalized within an allosteric model. The question arises as to whether the conformational changes in the two directly labelled receptor domains of the purified channel are coupled reciprocally as shown by us for the membrane-bound structure. We can convincingly demonstrate that this is indeed the case. The potent Ca^{2+}-channel antagonist (+)-PN 200-110 is a negative heterotropic allosteric regulator of (–)-[^{3}H]desmethoxyverapamil binding to the membrane-bound channel. In the case of the purified channel it stimulated the binding of the phenylalkylamine as the phenylalkylamine stimulated (+)-[^{3}H]PN 200-110 binding. The antagonistic enantiomers (–)(*R*)-202-791 and (+)(4*R*)-Bay K 8644 were also stimulatory whereas the agonistic enantiomers (–)(4*S*)-Bay K 8644 and (+)(*S*)-202-791 were inhibitory on (–)-[^{3}H]desmethoxyverapamil binding. This finding suggests that the high- and low-affinity states of the isolated channel are in analogy to the respective inactivated and resting open conformations of the channel in situ and that the drugs act as agonists and antagonists by changing the ratio of these states. Clearly, the ratio of states (i.e. the allosteric constant) is changed when the channel is purified. This is accompanied by an increase in the dissociation constants by one or even two orders of magnitude for our labels (+)-[^{3}H]PN 200-110 and (–)-[^{3}H]-desmethoxyverapamil. Others have tried to maintain the Ca^{2+} channel in the high-affinity state (with respect to 1,4-dihydropyridines) by prelabelling with [^{3}H]nitrendipine in the presence of d-*cis*-diltiazem [7,8]. However, we have shown by radiation-inactivation that d-*cis*-diltiazem induces a drastic decrease in the size of the 1,4-dihydropyridine-selective site, namely by 60 kDa [24,25]. It is yet not known whether this indicates the dissociation of a subunit or reflects only a change in the radiation-sensitive domain. Nevertheless, prelabelling in the presence of an allosteric regulator may cause the loss of a channel component unless proven otherwise. Alterations of the allosteric constant upon purification have important consequences for monitoring the drug receptors of the Ca^{2+} channel with the currently available labels. From the above considerations radioactive ligands with very high affinity for the inactivated state should be helpful. Alternatively, probes which bind to the channel in a voltage-independent manner may be used. The reasons for the observed differences between the membrane-bound and purified channel have not yet been clarified. The loss of an endogenous regulator, altered composition of associated lipids, loss or dephosphorylation of a subunit (e.g. the β-subunit) must be considered as possibilities. Finally, although we have demonstrated by direct labelling that two drug-receptor sites are reciprocally allosterically coupled on purified channels, the presence (and coupling) of the third (the benzothiazepine-selective site) has only been indirectly proven. The quantitation and characterisation of this site require probes of higher affinity than the currently available d-*cis*-[^{3}H]diltiazem.

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