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[^{3}H]HOE166 defines a novel calcium antagonist drug receptor – distinct from the 1,4 dihydropyridine binding domain

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Summary. Benzothiazinones represent a novel class of drugs which block voltage-dependent L-type calcium channels in different tissues. [^{3}H]HOE166 (R-(±)-3,4-dihydro-2-isopropyl-4-methyl-2-[2-[4-[4-[2-(3,4,5-trimethoxyphenyl)ethyl]-piperazinyl]butoxy]phenyl]-2H-1,4-benzothiazin-3-on-dihydrochloride; ≈ 57 Ci/mmol) a potent optically pure benzothiazinone was employed to characterize receptors associated with skeletal muscle transverse tubule calcium channels. [^{3}H]HOE166 reversibly labels the membrane-bound calcium channels with high affinity (K_d = 0.36 ± 0.05 nM; B_{max} = 18.2 ± 3.3 pmol/mg of membrane protein; means ± SD, n = 13), HOE166 (K_i = 0.76 nM) is 29-fold more potent than the respective (S)-enantiomer (K_i = 22.1 nM). Binding is inhibited by divalent and trivalent cations (Cd^{2+} and La^{3+} being most potent) and other calcium channel drugs (1,4 dihydropyridines, phenylalkylamines, benzothiazepines). High affinity [^{3}H]HOE166 binding activity is maintained (K_d = 4.5–9.0 nM) after solubilization and purification (554–1350 pmoles/mg of protein) of the calcium channel complex from transverse-tubule membranes. The following data support our recent claim (Striessnig et al. 1985, 1988) that HOE166 labels a domain on L-type calcium channels which is distinct from that defined by 1,4 dihydropyridines, phenylalkylamines or benzothiazepines: (1) All 1,4 dihydropyridine-, phenylalkylamine- and benzothiazepine-receptor-selective drugs tested are only very weak inhibitors of [^{3}H]HOE166 binding. (2) (+)-PN200-110 only partially inhibits [^{3}H]HOE166 binding to the purified calcium channel complex. (3) The decay of the [^{3}H]HOE166-receptor complex is monoexponential but the dissociation rate constants depend on the ligand concentration; (+)-PN200-100 accelerates the dissociation in the presence of unlabelled HOE166. (4) Nanomolar concentrations of HOE166 and HOE167 completely inhibit (–)-[^{3}H]desmethoxyverapamil binding to a *Drosophila* phenylalkylamine receptor (which lacks a 1,4 dihydropyridine binding domain). Taken together, these results are incompatible with the view that [^{3}H]HOE166 binds competitively to the calcium channel linked 1,4 dihydropyridine drug receptors.

Key words: Benzothiazinones – Purified calcium channels – *Drosophila* – Calcium channel blockers – Skeletal muscle

Abbreviations. K_d, dissociation constant; K_i, inhibition constant; k_{-1}, k_{+1}, dissociation, association rate constant; SDS, sodium dodecyl sulfate; T-tubule, transverse tubule; $s_{20,w}$, sedimentation coefficient

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Introduction

From the three different types of voltage-dependent calcium channels the so-called L-type is modulated by a variety of organic calcium channel drugs (Janis et al. 1987; Triggle and Janis 1987; Glossmann and Striessnig 1988). These drugs, which belong to different chemical classes bind to distinct, high affinity channel-linked receptor domains. The domains are reciprocally allosterically coupled and reside on a 170–200 kDa polypeptide, termed the alpha$_1$ subunit (Striessnig et al. 1987; Takahashi et al. 1987; Leung et al. 1987; Striessnig 1989; Glossmann et al. 1989). Benzothiazinones (HOE166 and analogues) represent a novel chemical class of calcium antagonists. The most potent derivative of this series is HOE166 (R-(±)-3,4-dihydro-2-isopropyl-4-methyl-2-[2-[4-[4-[2-(3,4,5-trimethoxyphenyl)ethyl]piperazinyl]butoxy]phenyl]-2H-1,4-benzothiazin-3-on-dihydrochloride). HOE166 and its less potent (–)-(S)-enantiomer, HOE167, inhibit KCl-induced smooth muscle contractions, e.g. of guinea-pig pulmonary arteries, rabbit aortic strips, or rat mesenteric artery (Striessnig et al. 1988) and block 1,4 dihydropyridine-sensitive calcium channels in skeletal muscle (Romey et al. 1988), in A7r5 cells (rat aortic cell line) and the insulin secreting RINm5F cells (Qar et al. 1988). It is controversial whether benzothiazinones define a novel calcium channel drug receptor (as proposed by Striessnig et al. 1988) or bind in a simple, competitive manner to one of the well characterized domains, e.g. that defined by 1,4 dihydropyridines (as proposed by Qar et al. 1988). In this paper we report that [^{3}H]HOE166 defines a binding domain for benzothiazinones on skeletal muscle calcium channels definitely distinct from the 1,4 dihydropyridine, phenylalkylamine and benzothiazepine receptor. Our data obtained in T-tubule membranes are complemented by studies employing purified calcium channels and the high affinity phenylalkylamine receptors in *Drosophila* head membranes which lack the 1,4 dihydropyridine binding domain (Pauron et al. 1988; Greenberg et al. 1989).

Materials and methods

Materials. HOE166 (R-(±)-3,4-dihydro-2-isopropyl-4-methyl-2-[2-[4-[4-[2-(3,4,5-trimethoxyphenyl)ethyl]piperazinyl]butoxy]phenyl]-2H-1,4-benzothiazin-3-on-dihydrochloride), HOE167 (the S-enantiomer of HOE166) and [~^{3}H]HOE166 (~57 Ci/mmol) were a gift from Hoechst AG, Frankfurt, FRG. Radiochemical purity was > 95% by thin-layer chromatography and HPLC.

(−)-[^{3}H]desmethoxyverapamil (~ 80 Ci/mmol) and [^{3}H](+)-cis-diltiazem (~ 150 Ci/mmol) were from Amersham (UK). The sources of the other unlabelled calcium antagonists are given elseswhere (Striessnig et al. 1986a, b; Glossmann and Ferry 1985).

Preparation of membranes. Partially purified skeletal muscle T-tubule membranes were prepared from guinea-pig skeletal muscle as described (Glossmann and Ferry 1985). Membranes were finally resuspended (4–8 mg of membrane protein/ml) in 50 mM Tris-HCl, pH 7.4, 0.1 mM PMSF and stored in liquid nitrogen until use. *Drosophila* head membranes were prepared as described previously from fly heads (Greenberg et al. 1989), finally resuspended (3–6 mg of membrane protein/ml) in 50 mM Na^+-HEPES buffer, pH 7.0, and stored in liquid nitrogen until use.

Preparation of purified skeletal muscle calcium channels. All steps were carried out at 4° C. 200 mg of rabbit skeletal muscle T-tubule membranes were solubilized for 30 min in solubilization buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.1 mM benzamidine, 1.0 mM iodoacetamide, 1 μM pepstatin A, 0.1 mM PMSF) containing 1% digitonin (w/v) at a detergent to protein ratio of 5. After centrifugation for 60 min at $40000 \times g$ the resulting supernatant was first purified on a 20 ml wheat germ lectin sepharose 4B column equilibrated in solubilization buffer containing 0.1% (w/v) digitonin (column buffer). The 1,4 dihydropyridine binding activity was eluted from the column with column buffer containing 6% (w/v) N-acetyl-D-glucosamine and loaded onto four 5–20% sucrose density gradients. After centrifugation ($210000 \times g$, 90 min) 2 ml fractions of the gradients were collected, assayed for 1,4 dihydropyridine and phenylalkylamine binding activity, pooled and quickly frozen in liquid nitrogen until use (0.01–0.02 mg of purified protein/ml). To confirm the purity of the calcium channel polypeptides aliquots of the pooled fractions were subjected to SDS-PAGE followed by Coomassie or silver staining and densitometric scanning (Striessnig et al. 1987). Alpha$_1$, alpha$_2$-delta, beta and gamma subunit protein together comprised more than 95% of the total staining intensity.

Binding assays. All binding assays were carried out according to Glossmann and Ferry (1985) in 50 mM Tris-HCl, pH 7.4 or pH 8.0 (in the case of [^{3}H]HOE166 binding) in a final assay volume of 0.5 ml. Drug stock solutions were made 10 mM in DMSO. In order to prevent adsorption of the unlabelled drugs to plastic material, serial drug dilutions made in DMSO were directly added to the incubation mixture as recently described by Boer et al. (1989). Final DMSO concentrations never exceeded 1% (v/v), which did not affect the binding of the radioligands investigated. Using this protocol binding of HOE166 and HOE167 to plastic tubes was minimized and 4–5-fold lower IC_{50} values were obtained as compared to the previously (Striessnig et al. 1988) employed protocol of Glossmann and Ferry (1985).

[^{3}H]HOE166 was incubated for 60 min at 37° C with partially purified skeletal muscle T-tubule membranes or 60 min at 25° C with purified skeletal muscle calcium channels in the absence (total binding) or presence of 1–3 μM unlabelled HOE166 (nonspecific binding). Nonspecific binding was subtracted from total binding to yield specific (i.e. saturable) binding. Bound and free ligand were separated by rapid filtration of the incubation mixture through GF/C Whatman filters which were then washed two times with ice-cold filtration buffer [10% (w/v) polyethylenglycol 6000, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4; see Glossmann and Ferry 1985]. Retained radioactivity was determined by liquid scintillation counting. Detergent-solubilized receptor was precipitated with filtration buffer for 2 min on ice in the presence of carrier protein (1.5 mg/ml bovine serum albumin and 1.5 mg/ml gamma-globulin).

Saturation analysis at equilibrium was performed by incubating increasing concentrations of [^{3}H]HOE166 (up to 6.0 nM) with the indicated concentrations of partially purified T-tubule membrane protein. With purified calcium channels the specific activity of [^{3}H]HOE166 was decreased by addition of increasing concentrations of unlabelled ligand. K_d and B_{max} were determined by Scatchard or Eadie Hofstee-transformation of the specific binding data and linear regression analysis. Binding-inhibition data from 2 experiments (except if stated otherwise) were computer-fitted to the general-dose-response equation (De Lean et al. 1978) by nonlinear methods and constants are given ± asymptotic SD.

The dissociation rate constant was determined from kinetic studies. Dissociation of the [^{3}H]HOE166-receptor-complex at equilibrium (B_e) was initiated by blocking the association reaction by 50-fold dilution of the incubation mixture or by the addition of the indicated concentrations of unlabelled HOE166. At various times, t, after initiation of dissociation, remaining complex, B_t, was determined. The k_{-1} was the negative slope of the line obtained by linear regression analysis of a plot of $\ln(B_t/B_e)$ vs. time.

Protein assays. Protein concentrations were determined according to Lowry (Lowry et al. 1951) or Bradford (1976). Bovine serum albumin was used as a standard.

Results

1. Characteristics of the [^{3}H]HOE166 labeled membrane-bound calcium channel

At 37° C saturable binding of [^{3}H]HOE166 to partially purified skeletal muscle T-tubule membranes is pH dependent (pH optimum at pH 8.0), linearly related to the membrane protein concentration (up to 0.04 mg/ml) and reached a steady-state at 0.1 nM within 35 min (results not shown). Saturation experiments revealed high affinity interaction of [^{3}H]HOE166 with a single, saturable class of sites. A K_d of 0.36 ± 0.05 nM and a B_{max} of 18.2 ± 3.3 pmol/mg of protein (means ± SD) was calculated from 13 experiments. Hill analysis demonstrated that the slope (0.96 ± 0.05) was not significantly different from unity. A representative saturation analysis is shown in Fig. 1. At K_d concentrations of free [^{3}H]HOE166 nonspecific binding was 25% of total binding. Cations (employed as the chloride salts) concentration-dependently inhibited the binding of [^{3}H]HOE166 with the following order of potency (% inhibition at 10 mM, mean ± SD): Na^+ (0) = K^+ (0) < Sr^{2+} (21 ± 7) < Ba^{2+} (26 ± 8) < Mg^{2+} (29 ± 6) < Ca^{2+} (35 ± 15) < Ni^{2+} (41 ± 6) < Co^{2+} (52 ± 4). The most potent divalent and trivalent cations were Cd^{2+} (74 ± 7) and La^{3+} (96 ± 4) with IC_{50} values of 2.9 ± 1.4 μM and 140 ± 20 μM, respectively. Chelation of divalent cations by pretreatment of the membranes with 10 mM EDTA (37° C, 20 min) reduced [^{3}H]HOE166 binding only to 86% of control binding. Re-

Table 1. Inhibition of reversible [^{3}H]HOE166 binding to partially purified guinea-pig skeletal muscle T-tubule membranes by calcium channel drugs.

Drug (s)	[^{3}H]HOE166		Control ligand IC_{50} or K_i(*) [nM]
	IC_{50} or K_i(*) [nM]	slope factor	
Benzothiazinones			
HOE166	0.76± 0.11*	1.00±0.14	–
HOE167	22.1 ± 6.6*	1.08±0.33	–
1,4-Dihydropyridines			
(+)-PN200-110	10.3 ± 2.2	0.80±0.11	0.68±0.05*
(−)-PN200-110	428 ± 96	0.96±0.17	–
(−)-Nitrendipine	> 100	–	–
(+)-Nitrendipine	> 100	–	–
R-202-791	78.9 ± 22.,5	0.61±0.11	11.1 ±1.39*
S-202-791	> 1 μM	–	–
Phenylalkylamines			
(−)-Desmethoxy-verapamil	243 ± 73	0.82±0.18	5.08±1.04*
(+)-Desmethoxy-verapamil	193 ± 3	1.69±0.27	–
(−)-Verapamil	1076 ±242	0.98±0.18	–
(+)-Verapamil	1241 ±261	0.88±0.14	–
(−)-Gallopamil	127 ± 52	0.57±0.12	–
(±)-D-619	no inhibition up to 1 μM		
Benzothiazepines			
(+)-cis-Diltiazem	2785 ±862	0.61±0.12	43.2 ±4.6
(−)-cis-Diltiazem	> 10 μM	–	–
Others			
Trans-Diclofurime	322 ± 53	1.05±0.16	16.7 ±0.9
Fluspirilene	5.6 ± 2.8	0.73±0.24	–
Pimozide	27.8 ± 3.8	0.80±0.15	–

0.008 – 0.04 mg/ml of protein were incubated with 0.1 – 0.6 nM [^{3}H]HOE166 for 60 min at 25° C in the absence (control binding) and presence of increasing concentrations of unlabeled drugs. Data from 2 – 4 experiments employing different membrane preparations were pooled and computer-fitted to the general-dose response equation. The binding parameters obtained (IC_{50}, slope factor, maximal inhibition) are given ± asymptotic SD. Control ligands were (+)-[^{3}H]PN200-110 (0.4 nM) for 1,4 dihydropyridines, (−)-[^{3}H]desmethoxyverapamil (1.5 nM) for phenylalkylamines and [^{3}H](+)-cis-diltiazem (3 nM) for benzothiazepines. K_i values were calculated according to Linden (1981) taking into account ligand and receptor concentration. Maximal inhibition is given as % of control. All drugs, for which IC_{50} or K_i values were calculated, inhibited [^{3}H]HOE166 binding to 100% at the highest concentration tested

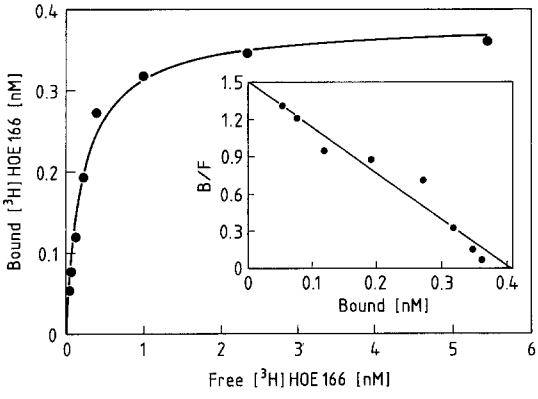

Fig. 1. Saturation analysis with [^{3}H]HOE166 and partially purified guinea-pig skeletal muscle T-tubule membranes. A representative experiment is shown. 0.019 mg/ml of membrane protein were incubated for 60 min at 37° C with increasing concentrations of [^{3}H]HOE166 in the absence and presence of 1 μM HOE166 to define nonspecific binding. Binding parameters were calculated by linear regression analysis after Scatchard transformation (*inset*) of the specific binding data: K_d = 0.267 nM, B_{max} = 404 pM (21 pmol/mg of protein), r = 0.97. Hill slope = 0.97 (r = 0.99)

duction of disulfide bonds or modification of SH-groups by pretreatment with 10 mM concentrations (37° C, 20 min) of dithiothreitol, N-ethylmaleimide or p-chloromercuriphenylsulfonic acid also inhibited [^{3}H]HOE166 binding (to 67%, 51% and 6% of control binding, respectively).

To investigate whether [^{3}H]HOE166 interacts in a simple competitive fashion with the 1,4 dihydropyridine, phenylalkylamine or benzothiazepine domain, inhibition studies with selective drugs were performed. If [^{3}H]HOE166 binds directly to one of the above receptors it must be completely displaced by the respective receptor-selective drug with a pseudo Hill-slope around unity and an inhibition constant close to its own K_d. The results are summarized in Table 1. All compounds tested inhibited [^{3}H]HOE166 binding to 100%. HOE166 and HOE167 inhibited with pseudo Hill slopes close to unity and K_i values of 0.76 and 22.1 nM, respectively. Thus the site defined by HOE166 discriminates the benzothiazinone enantiomers with an eudismic ratio of 29. Benzoxadiazol 1,4 dihydropyridines inhibited [^{3}H]HOE166 in a stereoselective fashion but with high IC_{50} values and/or low pseudo Hill slopes. Nitrendipine enantiomers had IC_{50} values > 100 nM. In control experiments (+)-PN200-110 and (−)-202-791 inhibited (+)-[^{3}H]PN200-110 binding with IC_{50} values of 2.5 ± 0.2 nM (slope factor = 1.03 ± 0.08, K_i = 0.68 nM) and 40.6 ± 5.1 nM (slope factor = 0.90 ± 0.08, K_i = 11.1 nM), respectively. Inhibition of [^{3}H]HOE166 binding by the enantiomers of the phenylalkylamines, verapamil and desmethoxyverapamil, was not stereoselective and of low affinity. Whereas (−)-desmethoxyverapamil has 20-fold higher affinity for the skeletal muscle calcium channel-phenylalkylamine receptor than (−)-verapamil and 6-fold higher affinity than (−)-methoxyverapamil (Striessnig et al. 1987; Goll et al. 1984a, b), (−)-methoxyverapamil is equally (or

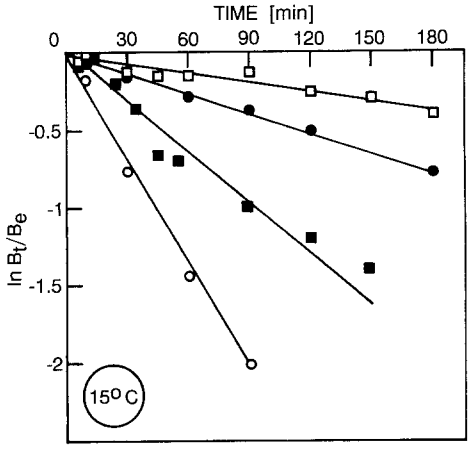

Fig. 2. Dissociation kinetics of [^{3}H]HOE166 in guinea-pig skeletal muscle T-tubule membranes. 0.013–0.03 mg/ml of membrane protein were incubated with 0.2–1.0 nM of [^{3}H]HOE166 for 60 min at 37° C. The incubation mixture was cooled down to 15° C. Dissociation of [^{3}H]HOE166 was induced by 50-fold dilution of the incubation mixture with assay buffer or by addition of unlabeled HOE166 to the indicated final concentrations. The following dissociation-rate constants were determined: Dilution (□): k_{-1} = 0.0018 min^{-1}, $r = 0.95$; 1 μM HOE166 (●): k_{-1} = 0.0039 min^{-1}, $r = 0.99$; 10 μM HOE166 (■): k_{-1} = 0.0099 min^{-1}, $r = 0.98$; 30 μM HOE166 (○): k_{-1} = 0.030 min^{-1}, $r = 0.99$

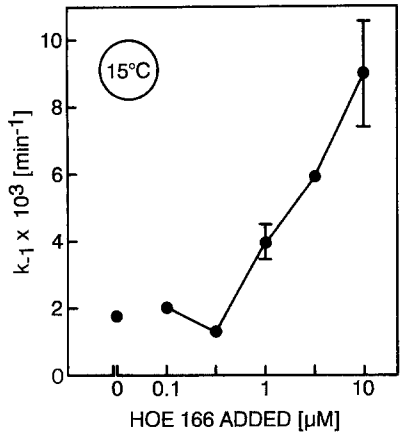

Fig. 3. Dependence of the dissociation rate constants on the concentration of HOE166. The experimental conditions were as described in the legend to Fig. 2. SD is given for three experiments employing different membrane preparations

even more) potent to inhibit [^{3}H]HOE166 binding to its domain compared with (−)-desmethoxyverapamil. A low potency was also found for drugs binding to the benzothiazepine receptor, namely (+)-cis-diltiazem (Glossmann et al. 1983) and trans-diclofurim (Spedding et al. 1987). In control experiments these drugs inhibited [^{3}H](±)-cis-diltiazem binding with IC_{50} values of 43.2 ± 4.6 nM and 16.7 ± 0.9 nM, respectively. These data argue against a high affinity interaction of [^{3}H]HOE166 with either one of the well defined drug receptors, supporting our previously published data obtained with unlabelled HOE166 (Striessnig et al. 1988).

The kinetic experiments shown in Figs. 2 and 3 unmask a so far not described, complex dissociation behaviour of [^{3}H]HOE166. Dissociation of the receptor ligand complex at equilibrium was induced through blockade of the forward

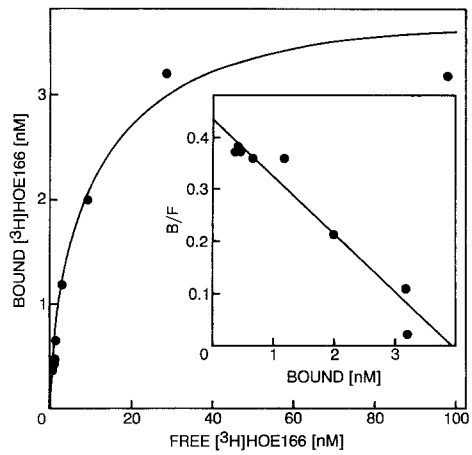

Fig. 4. Saturation analysis of [^{3}H]HOE166 binding to purified skeletal muscle T-tubule calcium channels. Scatchard transformation of the specific binding data is shown. 0.0029 mg/ml of purified protein were incubated with increasing concentrations of [^{3}H]HOE166. Binding parameters were calculated by linear regression analysis: K_d = 9.0 nM, B_{max} = 1356 pmol/mg of protein, $r = 0.98$; Hill slope = 0.90 ($r = 0.97$)

reaction by 50-fold dilution of the incubation mixture into assay buffer or by addition of unlabeled HOE166. Figures 2 and 3 show that the dissociation rate constant was highly dependent on the HOE166 concentration added. At 15° C concentrations of > 1 μM caused a concentration-dependent increase in the dissociation rate constant. As kinetics were slower at 15° C than at 37° C effects of higher concentrations of unlabeled HOE166 (1–10 μM) could be more reliably tested at the lower temperature. Addition of 1 μM (+)-PN200-110 together with 1 μM of HOE166 caused a further increase in the dissociation rate (control: k_{-1} = 0.004 min^{-1}; with (+)-PN200-110: k_{-1} = 0.011 min^{-1}). At 37° C the k_{-1} obtained by the dilution method was smaller (k_{-1} = 0.021 min^{-1}) than by adding saturating concentrations HOE166 (200 nM, k_{-1} = 0.049 min^{-1}; 1800 nM, k_{-1} = 0.21 min^{-1}). Due to the dependency of k_{-1} on unlabelled ligand concentration only dissociation experiments performed by dilution (i.e. in the absence of unlabelled ligand) were used to calculate (a kinetically derived) K_d. From the association rate constant, determined at 37° C (k_{+1} = 0.027 ± 0.016 $nM^{-1} \times min^{-1}$) and the k_{-1} (obtained by dilution) a dissociation constant of 0.77 nM was derived. This value is in fair agreement with the K_d or K_i (Table 1) measured by equilibrium saturation or competition experiments. At 37° C (not shown) as well as 15° C (Fig. 2) dissociation was always monophasic.

2. Co-purification of [^{3}H]HOE166 binding activity with the calcium channel complex

In order to study the interaction of [^{3}H]HOE166 with purified skeletal muscle calcium channels [^{3}H]HOE166 binding activity was extracted from skeletal muscle T-tubules with digitonin and purified by WGA-sepharose affinity chromatography and sucrose-density gradient centrifugation as described previously (Striessnig et al. 1986b; Vaghy et al. 1987). A protein peak ($S_{20,w}$ = 19) contained the calcium channel polypeptides (alpha$_1$, alpha$_2$-delta, beta and gamma as verified by SDS-gel electrophoresis; see Glossmann and Striessnig 1988) as well as (+)-[^{3}H]PN200-

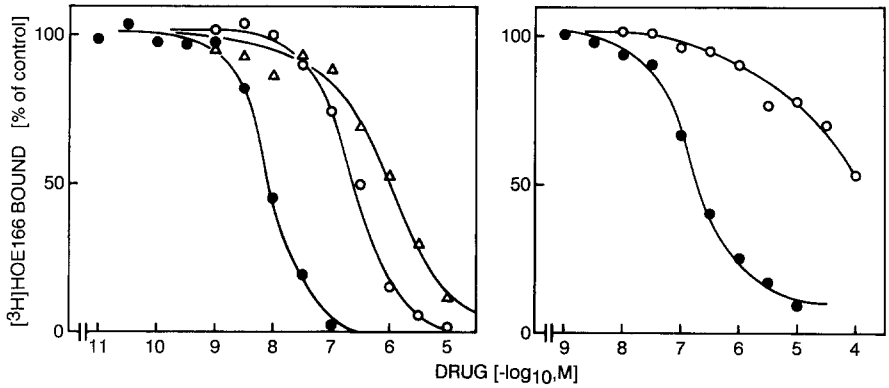

Fig. 5. Pharmacological profile of [^{3}H]HOE166 binding sites on purified skeletal muscle T-tubule calcium channels. A representative experiment is shown. 1.3 nM [^{3}H]HOE166 were incubated with 0.0029–0.0039 mg/ml of purified calcium channel protein for 60 min at 25°C. The following binding parameters (given as mean ± asymptotic SD) were obtained by nonlinear curve fitting for the experiments shown: *Left panel:* HOE166 (●): IC_{50} = 9.0 ± 1.1 nM, K_i = 4.1 nM, slope factor = 1.37 ± 0.19; HOE167 (○): IC_{50} = 280 ± 36 nM, K_i = 181 nM, slope factor = 1.01 ± 0.1; (−)-desmethoxyverapamil (△): IC_{50} = 1.2 ± 0.2 μM, slope factor = 0.84 ± 0.1. *Right panel:* (+)-PN200-110 (●): IC_{50} = 165 ± 22 nM, maximal inhibition to 11 ± 3% of control binding, slope factor = 1.04 ± 0.13; (+)cis-diltiazem (○): ⩾ 10 μM

110, (−)-[^{3}H]desmethoxyverapamil and [^{3}H]HOE166 binding activity. Protein peaks sedimenting at higher and lower sucrose densities (Striessnig et al. 1986a) did not contain binding activity for these ligands (not shown). The purified calcium channel complex was analysed for [^{3}H]HOE166 interaction in more detail. Figure 4 shows a saturation analysis with [^{3}H]HOE166 and the purified calcium channel complex. The maximal density of binding sites (range 554–1350 pmol/mg of protein) is close to the densities measured for 1,4 dihydropyridines (970–1500 pmol/mg of protein; Striessnig 1986a, b) and phenylalkylamines (694 pmol/mg of protein; Glossmann et al. 1988). As reported for 1,4 dihydropyridines and phenylalkylamines (Striessnig et al. 1986a, b, 1987) the affinity of HOE166 for the purified complex was lower (range: K_d = 4.5–9.0 nM; Fig. 4) than for the membrane bound state (K_d = 0.36 nM). Similar to the membrane-bound channel the interaction of the benzothiazinones with the purified preparation was stereoselective and modulated by other calcium channel drugs (Fig. 5). The following binding parameters were calculated from three independent experiments by fitting the data to the general-dose-response equation (means ± asymptotic SD): HOE166: IC_{50} = 10.4 ± 1.4 nM, slope factor = 1.24 ± 0.18; HOE167: IC_{50} = 175 ± 40.4 nM, slope factor = 0.85 ± 0.14; (+)-PN200-110: IC_{50} = 87.9 ± 16.4, slope factor = 1.00 ± 0.17; (−)-desmethoxyverapamil: IC_{50} = 1.56 ± 0.51 μM, slope factor = 0.70 ± 0.13. (+)-PN200-110 only inhibited partially (to 20 ± 3.7% of control binding).

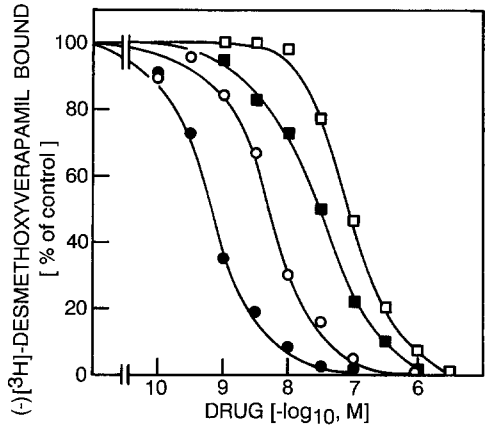

Fig. 6. Inhibition of reversible (−)-[^{3}H]desmethoxyverapamil binding to *Drosophila melanogaster* head membranes by the enantiomers of HOE and desmethoxyverapamil. 0.08 mg/ml of membrane protein were incubated with 0.42–0.53 nM of tritiated ligand for 60 min. Non-specific binding was determined in the presence of 1 μM (±)-desmethoxyverapamil and was 6% of total binding. The following binding parameters (given as mean ± asymptotic SD) were obtained by nonlinear curve fitting for the experiments shown: HOE167 (■): IC_{50} = 30.1 ± 2.5 nM, slope factor = 0.89 ± 0.06; HOE166 (□): IC_{50} = 88.4 ± 5.9 nM, slope factor = 1.14 ± 0.08; (−)-desmethoxyverapamil (●): IC_{50} = 0.69 ± 0.08 nM, K_i = 0.308 ± 0.04 nM, slope factor; (+)-desmethoxyverapamil (○): IC_{50} = 5.7 ± 0.7 nM, K_i = 2.63 ± 0.32 nM, slope factor = 1.21 ± 0.15. K_i values were calculated according to Cheng and Prusoff (1973)

3. Interaction of benzothiazinones with the high affinity phenylalkylamine receptor on putative voltage-dependent calcium channels in the Drosophila nervous system

High affinity phenylalkylamine receptors exist in *Drosophila* head membranes which bind phenylalkylamines (but not 1,4 dihydropyridines) with subnanomolar K_d values (Pauron et al. 1988; Greenberg et al. 1989; Zech et al. 1989). Figure 6 shows that HOE166 and HOE167 inhibit the phenylalkylamine receptor in *Drosophila* head membranes with IC_{50} values in the nanomolar range. The stereoselectivity of the two enantiomers is inverse as compared to mammalian calcium channels – HOE167 was about 3 times more potent than HOE166. (−)-[^{3}H]desmethoxyverapamil binding was completely inhibited by unlabelled (−)-desmethoxyverapamil with a subnanomolar IC_{50} (Fig. 6). Control experiments performed in parallel employing the same serial dilutions of the HOE enantiomers confirmed that HOE166 (IC_{50} = 1.04 ± 0.11 nM; n = 3) is more potent than (−)-desmethoxyverapamil (IC_{50} = 5.08 ± 1.04 nM) or HOE167

(IC_{50} = 5.94 ± 1.2 nM) to inhibit (−)-[^{3}H]desmethoxyverapamil binding to receptors in skeletal muscle T-tubule membranes. As mentioned in the "method" section these values for HOE166 and HOE167 are lower than previously reported, albeit the eudismic ratio is identical.

Discussion

We recently postulated a novel drug receptor domain, distinct for benzothiazinones on "L-type" calcium channels (Striessnig et al. 1988). In particular it was concluded that the domain defined by HOE166 is allosterically coupled to the 1,4 dihydropyridine, phenylalkylamine and benzothiazepine receptors. Our reasoning was based on the effects of unlabelled HOE166 and HOE167 on the kinetic and equilibrium binding parameters of 1,4 dihydropyridine, phenylalkylamine and benzothiazepine receptor-selective radioligands. A recent study in which skeletal muscle T-tubule membranes were directly labelled with [^{3}H]-HOE166 claimed to the contrary that benzothiazinones bind to sites which have the properties of the 1,4 dihydropyridine receptor (Qar et al. 1988). This conclusion was mainly based on the finding that HOE166 concentration-dependently decreased the apparent affinity of (+)-[^{3}H]PN200-110 binding without changes in the B_{max}. However, if the negative cooperativity between an allosteric effector and the radioligand is very great the decrease in affinity for the radioligand induced by the allosteric effector will resemble competitive inhibition in equilibrium binding studies (Ehlert 1988). Therefore kinetic studies are more suitable to discriminate competitive from non-competitive binding.

The equilibrium binding parameters for [^{3}H]HOE166 in partially purified skeletal muscle T-tubule membranes determined by us agree with those reported by Qar et al. (1988): The density of benzothiazinone receptors is equivalent to the domain labelled by 1,4 dihydropyridines (cf. Striessnig et al. 1988) and the K_d is in the subnanomolar range (K_d = 0.36 nM vs. K_d = 0.27 nM determined by Qar et al. (1988). The relative affinities of HOE166 and its (S)-enantiomer, HOE167, were also very similar with eudismic ratios of 29 (Table 1) vs. 24 (Qar et al. 1988) suggesting that the same receptor was labelled. However, our detailed studies of [^{3}H]HOE166 binding modulation by other classes of calcium channel drugs as well as kinetic studies make it very unlikely that [^{3}H]HOE166 competitively binds to the 1,4 dihydropyridine receptor in skeletal muscle. The dissociation rate of the HOE166-receptor-complex is dependent on the concentration of unlabelled HOE166 added to initiate dissociation (Figs. 2 and 3). Similar complex dissociation kinetics have also been described for phenylalkylamines (Goll et al. 1984a; Reynolds et al. 1986; Glossmann et al. 1985) but never for 1,4 dihydropyridines. At 37°C low concentrations (< 1 μM) unlabelled HOE166 induced a dramatic increase in the dissociation rate compared to the dilution method. This phenomenon has to be considered when k_{-1} data are used to derive the dissociation equilibrium constant (see e.g. Qar et al. 1988). How can we explain the complex dissociation behaviour? A distinct possibility is destabilizing site-site interaction of [^{3}H]HOE166 binding. Such negative cooperative effects have been described e.g. for the binding of insulin to its receptor on mononuclear cells (De Meyts et al. 1973; De Meyts and Roth 1975) and were best explained by models assuming K_d to be a function of receptor occupancy (Y). In case of negative cooperativity the apparent K_d increases from $K_{d,low}$ at infinite low receptor occupancy ($Y \rightarrow 0$) to $K_{d,\ high}$ at full ($Y \rightarrow 1$) receptor occupancy. As negative cooperativity does not affect association kinetics (De Lean and Rodbard 1979) the changes in K_d must be due to changes of k_{-1} (decrease in the half-life of the complex) increasing from $K_{-1,low}$ ($Y \rightarrow 0$) to $k_{-1,high}$ ($Y \rightarrow 1$), respectively (De Lean and Rodbard 1979). The cooperativity can be described by the interaction factor $d = k_{-1,high}/k_{-1,low} = K_{d,high}/K_{d,low}$. To test if our data are compatible with this model we calculated d assuming that the dilution-induced k_{-1} is the best estimate for $k_{-1,low}$ whereas k_{-1} at the highest HOE166 concentration added is the best estimate for k_{-1}, high. Minimal d values of 10.5 (0.22/0.021) and 4.4 (0.009/0.0018) were calculated for 37°C and 15°C, respectively. However, with these interaction factors the Scatchard plot of the saturation binding isotherm must be clearly nonlinear and concave up (De Lean and Rodbard 1979). As we did not detect such nonlinearity for the membrane-bound (37°C, Fig. 1) or purified (25°C, Fig. 4) benzothiazinone domain negative, homotropic cooperativity can be ruled out. A second explanation for the observed monophasic-ligand-dependent dissociation could be a negative heterotropic allosteric mechanism between the high affinity benzothiazinone receptor and a second (low affinity) HEO166 binding site. This site could be occupied upon addition of concentrations of unlabelled HOE166 which are in excess of those needed to fully occupy the low (i.e. 0.36 nM) K_d site. It is reasonable to assume that the negative allosteric destabilization of the [^{3}H]HOE166-receptor-complex is linearly correlated to the occupancy of the second, low affinity site. We estimate that the K_d is ≥ 3 μM (Fig. 3). Thus the affinity of HOE166 for this site is > 10000 times lower than for the high affinity domain. It is unlikely that these sites can be detected with [^{3}H]HOE166 either in saturation or in displacement studies. (+)-PN200-110, in the presence of unlabelled HOE166 further accelerated the dissociation.

Our displacement studies with 1,4 dihydropyridine, phenylalkylamine and benzothiazepine-receptor-selective drugs are also compatible with the hypothesis that [^{3}H]HOE166 labels a distinct domain. All drugs tested (Table 1) are only weak inhibitors of [^{3}H]HOE166 binding in skeletal muscle as compared to their directly measured affinities for their respective drug binding domain. Most interesting is that benzoxadiazol-1,4-dihydropyridines are inhibitory whereas nitrendipine is almost ineffective. The non-competitive type of interaction for 1,4 dihydropyridines is underlined by our dissociation studies (see above) and by experiments employing the purified skeletal muscle calcium channel complex. (+)-PN200-110 at the highest concentrations employed, inhibited [^{3}H]HOE166 binding only partially (Fig. 5). Interestingly, the allosteric coupling between the receptor domains is not fully reciprocal: Whereas HOE166 is quite potent to inhibit 1,4 dihydropyridine or phenylalkylamine binding (Striessnig et al. 1988) the reverse is not true (Table 1).

The 1,4 dihydropyridine, phenylalkylamine and benzothiazepine binding domains have been localized on the $alpha_1$ subunit of the skeletal muscle calcium channel complex by photoaffinity labelling (Striessnig et al. 1987; Takahashi et al. 1987; Leung et al. 1987; Vaghy et al. 1987; Glossmann et al. 1989). A prerequisite for photoaffinity labelling of the benzothiazepine domain in the purified calcium channel preparation is the maintainance of binding

activity. Our data show that the [^{3}H]HOE166 site is recovered in the purified calcium channel preparation with high affinity and nearly equal density as is 1,4 dihydropyridine and phenylalkylamine receptor activity. The copurification with the other drug receptors proves that the benzothiazinone receptor is located on one of the polypeptides (most likely alpha$_1$) found in the oligomeric complex rather than on a regulatory protein associated with the calcium channel exclusively in the membrane-bound state. Interaction of HOE166 with the benzothiazinone receptor seems to confer its inhibitory effects on the calcium channel activity as well as excitation-contraction coupling (i.e. the proposed voltage-sensor-function of the alpha$_1$ subunit; Romey et al. 1988).

A unique drug receptor with very high affinity for phenylalkylamines has been recently discovered and biochemically characterized in *Drosophila* head membranes (Pauron et al. 1988; Greenberg et al. 1989; Zech et al. 1989). The binding domain is localized on a 135 kDa polypeptide which has been photoaffinity labelled and partially purified after solubilization (Greenberg et al. 1989). The peculiar feature of this phenylalkylamine receptor is the complete absence of phenylalkylamine binding regulation by 1,4 dihydropyridines or of high-affinity interaction with labelled 1,4 dihydropyridines. In addition there is only very weak interaction with benzothiazepines, interestingly with inverse stereoselectivity (Zech et al. 1989). Thus, the *Drosophila* receptor (shown to be a calcium channel; Pelzer et al. 1989) may represent an ancestor of the mammalian L-type calcium channel which acquired the 1,4 dihydropyridine binding domain later in evolution. A complete lack of benzothiazinone modulation in the *Drosophila* phenylalkylamine receptor system could support the view that benzothiazinones indeed bind directly to the 1,4 dihydropyridine domain. However, our data show that, as in skeletal muscle T-tubule membranes, HOE166 and HOE167 inhibit phenylalkylamine binding in *Drosophila* with IC_{50} values in the nanomolar range-albeit with inversed stereoselectivity. This clearly distinguishes the benzothiazinones from the phenylalkylamines which bind to the *Drosophila* receptor with similar stereoselectivity as to skeletal muscle calcium channels (Goll et al. 1984a, b; Striessnig et al. 1987; Greenberg et al. 1989). Besides piperazinyl-indoles (Zech et al. 1989) and diphenylbutylpiperidines (Pauron et al. 1988) the benzothiazinones are now the third chemical class of compounds known to display high affinity interaction with the *Drosophila* phenylalkylamine receptor.

Comparison of the primary structures of the receptor carrying subunits (e.g. alpha$_1$ of the mammalian L-type channel and the 135 kDa calcium channel polypeptide from *Drosophila*) and photoaffinity labelling studies-combined with site-directed antibodies (see e.g. Tejdor and Catterall 1988; Dolman et al. 1987) should help to elucidate the amino acid sequences which participate in the formation of the respective drug binding domains. It is hoped that such unequivocal structural data will come forward to support the existence of distinct domains for 1,4 dihydropyridines and benzothiazinones.

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References

Boer R, Grassegger A, Schudt C, Glossmann H (1989) (+)-Niguldipine binds with very high affinity to calcium channels and to a subtype of alphal-adrenoceptors. Eur J Pharmacol 172:131–146

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Cheng Y-C, Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108

De Lean A, Munson PJ, Rodbard D (1978) Simultaneous analysis of families of sigmoid curves: Application for bioassays, radioligand assays and physiological dose-response curves. Am J Physiol 4:E97–E102

De Lean A, Rodbard D (1979) Kinetics of cooperative binding. In: O'Brien RD (ed) The receptors. A comprehensive treatise. Plenum Press, New York and London, pp 143–192

De Meyts P, Bianco AR, Roth J (1976) Site-site interactions among insulin receptors: Characterization of the negative cooperactivity. J Biol Chem 251:1877–1881

De Meyts P, Roth J (1975) Cooperativity in ligand binding: A new graphic analysis. Biochem Biophys Res Commun 66:1118

Dohlman HG, Bouvier M, Benovic JL, Caron MG, Lefkowitz RJ (1987) The multiple membrane spanning topography of the beta2-adrenergic receptor. J Biol Chem 262:14282–14288

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. Mol Pharmacol 33:187–194

Glossmann H, Ferry DR (1985) Assay for calcium channels. Meth Enzymol 109:513–550

Glossmann H, Ferry DH, Goll A, Striessnig J, Schober M (1985) Calcium channels: basic properties as revealed by radioligand binding studies. J Cardiovasc Pharmacol 7:20–30

Glossmann H, Linn T, Rombusch M, Ferry DR (1983) Temperature-dependent regulation of d-cis-[^{3}H]diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. FEBS Lett 160:226–232

Glossmann H, Striessnig J (1988) Calcium channels. Vitam Horm 44:155–328

Glossmann H, Striessnig J, Hymel L, Schindler H (1988) Purification and reconstitution of calcium channel drug-receptor sites. Ann NY Acad Sci 522:150–161

Glossmann H, Striessnig J, Knaus HG, Müller J, Grassegger F, Höltje H-D, Marrer S, Hymel L, Schindler H (1989) Structure of calcium channels. Ann NY Acad Sci 560:198–214

Goll A, Ferry DR, Glossmann H (1984a) Target size analysis and molecular properties of Ca^{2+} channels labelled with [^{3}H]verapamil. Eur J Biochem 141:177–186

Goll A, Ferry DR, Striessnig J, Schober M, Glossmann H (1984b) (−)-[^{3}H]Desmethoxyverapamil, a novel Ca^{2+} channel probe. Binding characteristics and target size analysis of its receptor in skeletal muscle. FEBS Lett 176:371–377

Greenberg RM, Striessnig J, Koza A, Devay P, Glossmann H, Hall LM (1989) Native and detergent-solubilized membrane extracts from Drosophila heads contain binding sites for phenylalkylamine calcium channel blockers. Insect Biochem 19:309–322

Janis RA, Silver PJ, Triggle DJ (1987) Drug action and cellular calcium regulation. Adv Drug Res 16:309–591

Leung AT, Imagawa T, Campbell KP (1987) Structural characterization of the 1,4-dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from rabbit skeletal muscle. Evidence for two distinct high molecular weight subunits. J Biol Chem 262:7943–7946

Linden J (1982) Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. J Cyclic Nucleotide Res 8:163–172

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin Phenol Reagent. J Biol Chem 193:265–275

Mir AK, Spedding M (1987) Calcium antagonist properties of diclofurime isomers. II. Molecular aspects: allosteric interactions with dihydropyridine recognition sites. J Cardiovasc Pharmacol 9:469–477

Pauron D, Qar J, Barhanin J, Fournier D, Cuany A, Pralavorio M, Berge JB, Lazdunski M (1987) Identification and affinity labeling of very high affinity binding sites for the phenylalkylamine series of Ca^{+} channel blockers in the Drosophila nervous system. Biochemistry 26:6311–6315

Pelzer S, Barhanin J, Pauron D, Trautwein W, Lazdunski M, Pelzer D (1989) Diversity and novel pharmacological properties of Ca^{2+} channels in *Drosophila* brain membranes. EMBO J 8: 2365–2371

Qar J, Barhanin J, Romey G, Henning R, Lerch U, Oekonomopulos R, Urbach H, Lazdunski M (1988) A novel high affinity class of Ca^{2+} channel blockers. Mol Pharmacol 33:363–369

Reynolds IA, Snowman AM, Snyder SH (1986) (–)-[^{3}H]Desmethoxyverapamil labels multiple calcium channel modulator receptors in brain and skeletal muscle membranes: differentiation by temperatures and dihydropyridines. J Pharmacol Exp Ther 237:731–738

Romey G, Garcia L, Rieger F, Lazdunski M (1988) Targets for calcium channel blockers in mammalian skeletal muscle and their respective functions in excitation-contraction coupling. Biochem Biophys Res Commun 156:1324–1332

Striessnig J (1989) Identification of the purified calcium channel benzothiazepine receptor by photoaffinity labelling and anti-benzothiazepine antibodies. Naunyn-Schmiedeberg's Arch Pharmacol 339:R44

Striessnig J, Goll A, Moosburger K, Glossmann H (1986a) Purified calcium channels have three allosterically coupled drug receptors. FEBS Lett 197:204–210

Striessnig J, Moosburger K, Goll A, Ferry DR, Glossmann H (1986b) Stereoselective photoaffinity labelling of the purified 1,4-dihydropyridine receptor of the voltage-dependent calcium channel. Eur J Biochem 161:603–609

Striessnig J, Knaus HG, Grabner M, Moosburger K, Seitz W, Lietz H, Glossmann H (1987) Photoaffinity labelling of the phenylalkylamine receptor of the skeletal muscle transverse-tubule calcium channel. FEBS Lett 212:247–253

Striessnig J, Meusburger E, Grabner M, Knaus H-G, Glossmann H, Kaiser J, Schölkens B, Becker R, Linz W, Henning R (1988) Evidence for a distinct calcium antagonist receptor for the benzothiazinone compound HOE166. Naunyn-Schmiedeberg's Arch Pharmacol 337:331–340

Takahashi M, Seagar MJ, Jones JF, Reber BF, Catterall WA (1987) Subunit structure of dihydropyridine-sensitive calcium channels from skeletal muscle. Proc Natl Acad Sci [USA] 84:5478–5482

Tejdor FJ, Catteral WA (1988) Site of covalent attachment of alpha-scorpion toxin derivatives in domain I of the sodium channel alpha subunit. Proc Natl Acad Sci [USA] 85:8742–8746

Triggle DJ, Janis RA (1987) Calcium channel ligands. Annu Rev Pharmacol Toxicol 27:347–369

Vaghy PL, Striessnig J, Miwa K, Knaus HG, Itagaki K, McKenna E, Glossmann H, Schwartz A (1987) Identification of a novel 1,4-dihydropyridine- and phenylalkylamine-binding polypeptide in calcium channel preparations. J Biol Chem 262:14337–14342

Zech C, Greenberg RM, Hall L (1989) Very high affinity interaction of sodium channel ligans with drosophila head membranes. Naunyn-Schmiedeberg's Arch Pharmacol 339:R45

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