Identification and Affinity Labeling of Very High Affinity Binding Sites for the Phenylalkylamine Series of Ca^+ Channel Blockers in the *Drosophila* Nervous System†

David Pauron,‡ Janti Qar,‡,§ Jacques Barhanin,‡ Didier Fournier,‖ Andrē Cuany,‖ Madeleine Pralavorio,‖ Jean-Baptiste Berge,‖ and Michel Lazdunski*,‡

Centre de Biochimie du CNRS, Parc Valrose, 06034 Nice Cedex, France, and Station de Nēmatologie et de Gēnētique Molēculaire des Invertēbrēs de l'INRA, BP 78, 06602 Antibes Cedex, France

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ABSTRACT: The interaction of putative Ca^{2+} channels of *Drosophila* head membranes with molecules of the phenylalkylamine series was studied from binding experiments using (−)-[^{3}H]D888 and (±)-[^{3}H]verapamil. These ligands recognize a single class (K_d = 0.1–0.4 nM; B_{max} = 1600–1800 fmol/mg of protein) of very high affinity binding sites. The most potent molecule in the phenylalkylamine series was (−)-verapamil with a K_d value as exceptionally low as 4.7 pM. Molecules in the benzothiazepine and diphenylbutylpiperidine series of Ca^{2+} channel blockers as well as bepridil inhibited (−)-[^{3}H]D888 binding in a competitive way with K_d values between 12 and 190 nM, suggesting a close correlation, as in the mammalian system, between these receptor sites and those recognizing phenylalkylamines. A tritiated (arylazido)phenylalkylamine with high affinity for the *Drosophila* head membranes, phenylalkylamine receptor (K_d = 0.24 nM), was used in photoaffinity experiments. A protein of M_r 135 000 ± 5000 was specifically labeled after ultraviolet irradiation.

Voltage-dependent Ca^{2+} channels are essential for coupling excitation to contraction in cardiac and smooth muscle cells and for coupling excitation to secretion of hormones and neurotransmitters in secretory cells. Different types of Ca^{2+} channels have been recently identified that have different biophysical and pharmacological properties (Carbone & Lux, 1984; Nilius et al., 1985; Nowycky et al., 1985; Cognard et al., 1986; Reynolds et al., 1986a). The best known of these channels is the slow, high-threshold Ca^{2+} channel or L-type Ca^{2+} channel (Nowycky et al., 1985). This particular channel type has a rich pharmacology (Baker & Knight, 1984; Janis & Triggle, 1984; Miller & Freedman, 1984; Ptasienski et al., 1985; Galizzi et al., 1986b). It is blocked by molecules in the 1,4-dihydropyridine series such as nitrendipine, nifedipine, or (+)-PN200-110 and by Ca^{2+} channel blockers belonging to the phenylalkylamine series such as verapamil, D600, and D888.[1] Verapamil receptors have not only been identified in excitable cells, but they are also present in high amount in spermatozoids (Kazazoglou et al., 1985) as well as in plant membranes (Andrejauskas et al., 1985).

Ionic channels that are involved in electrical excitability in mammalian tissues are present in insect tissues (Salkoff, 1986), and it has been shown recently that the biochemical pharmacology of voltage-dependent Na^+ channels in housefly brain is similar to, though not identical with, that observed in mammalian brain (Pauron et al., 1985). The interest of studying ionic channels in *Drosophila* is linked to the nearly unique property of this animal system to lend itself to the genetic approach (Ganetzky & Wu, 1985; Ganetzky, 1986; Salkoff, 1986; Tanouye et al., 1986). Spectacular results have been recently obtained in the genetic study of a variety of *Drosophila* ionic channels including the production of mutants with an altered behavior (Salkoff & Wyman, 1981; Jakson et al., 1985).

The main purpose of this paper is to report the exceptional properties of the ligand–receptor interaction of the phenylalkylamine receptor in the *Drosophila* nervous system and the identification of the polypeptide chain bearing phenylalkylamine binding sites using a photoactivable azido derivative.

Experimental Procedures

Materials. (−)-[^{3}H]Demethoxyverapamil (D888, 2.22 TBq/mmol) was purchased from Amersham, France. (±)-[^{3}H]Verapamil (2.96 TBq/mmol) was obtained from E. I. du Pont de Nemours, France. Unlabeled (+)-PN200-110 was a generous gift from Dr. R. P. Hof (Sandoz, Basel, Switzerland). Nitrendipine was from Bayer AG, FRG. (+)- and (−)-verapamil, (+)- and (−)-D888, (+)- and (−)-gallopamil (D600), and [^{3}H]LU47781 (3.15 TBq/mmol) were from Knoll AG, FRG. *d-cis*-Diltiazem and *l-cis*-diltiazem were from Synthelabo, Paris, France. (+)-Bepridil and (−)-bepridil were from CERM, Riom, France. Fluspirilene was a gift from Janssen Pharmaceutica, Beerse, Belgium.

Preparations of Membranes from Drosophila Heads. A collection of heads was obtained by sieving *Drosophila melanogaster* flies that had been stored at −70 °C. Membrane preparations were performed as described earlier from housefly heads (Pauron et al., 1985) and were kept frozen at −70 °C until use. No loss of binding activity was observed after 1

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* Author to whom correspondence should be addressed.

‡ Centre de Biochimie du CNRS.

§ On leave from the Department of Biological Sciences, Yarmouk University, Irbid, Jordan.

‖ Station de Nēmatologie et de Gēnētique Molēculaire des Invertēbrēs de l'INRA.

[1] Abbreviations: D600, methoxyverapamil; D888, demethoxyverapamil; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [^{3}H]LU47781, (*S*)-1-(3-azidophenyl)-3-[^{3}H$_3$]methyl-7-cyano-7-(3,4,5-trimethoxyphenyl)-8-methyl-3-azanonane hydrochloride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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month of storage. This work has necessitated 20 g of *Drosophila* heads.

Binding Assays. Experiments were done at 22 ± 1 °C except when stated otherwise. Equilibrium binding assays were performed as described previously for (−)-[^{3}H]demethoxyverapamil (Galizzi et al., 1986a) and (±)-[^{3}H]verapamil (Galizzi et al., 1984).

Protein concentrations were determined according to the method of Bradford (1976) using bovine serum albumin as a standard.

Photoaffinity Labeling Experiments. Drosophila nervous membranes were incubated at a final concentration of 2.5 mg of protein/mL with 2.2 nM [^{3}H]LU47781 in 20 mM Hepes–NaOH, pH 7.4, under dim light at 22 °C. Nonspecific incorporation was measured in a parallel incubation in the presence of 100 nM unlabeled (±)-verapamil. After 40 min of incubation, half of each incubation sample was kept in the dark and half was irradiated in a 18 mm diameter Petri dish with a Rayonet photochemical reactor lamp (The SONE Ultraviolet Co., Middletown, CT, 40 W) for 2 min at a distance of 12 cm. Specific binding on irradiated and nonirradiated preparations was determined by filtration of 2 × 20 μL aliquots on GF/C filters (Galizzi et al., 1986a) before addition of 100 nM unlabeled (±)-verapamil to initiate the dissociation of the noncovalently bound [^{3}H]LU47781. Dissociation was followed by filtration of 20-μL aliquots at different times. Finally, 100 μL of each incubation was centrifuged at 12000*g* for 10 min, and the pellets were washed with 1 mL of 20 mM Hepes–NaOH, pH 7.4, buffer before denaturation in 2% SDS, 9% glycerol, 75 mM Tris-HCl, pH 6.8, and 2.5% β-mercaptoethanol (disulfide reducing conditions) or 8 mM iodoacetamide (nonreducing conditions). Denatured samples were loaded onto a 4–14% linear polyacrylamide gradient according to the method of Laemmli (1970). Following electrophoresis slab gels were stained with Coomassie blue and impregnated with Amplify (Amersham) for 30 min for flurography. Gels were dried and exposed to Kodak XAR5 film with an intensifying screen, Du Pont Cronex Hi-plus, for 14 days at −70 °C.

Results

Equilibrium Binding of (−)-[^{3}*H*]*D888 and* (±)-[^{3}*H*]*Verapamil.* (−)-[^{3}H]D888 (Figure 1A) and (±)-[^{3}H]verapamil (Figure 1B) bind specifically to *Drosophila* head membranes in a saturable way. Specific binding represents more than 98% of the total binding at a concentration corresponding to the K_d value. Scatchard plots (Figure 1, insets) clearly demonstrate that (−)-[^{3}H]D888 and the racemic mixture of (±)-[^{3}H]verapamil bind to a single class of receptor sites. Parameters for the interaction between (−)-[^{3}H]D888 and its receptor site are K_d = 0.13 ± 0.06 nM and B_{max} = 1.8 ± 0.2 pmol/mg of protein. K_d and B_{max} values for (±)-[^{3}H]verapamil are 0.36 ± 0.05 nM and 1.6 ± 0.2 pmol/mg of protein, respectively.

Effects of Different Ca^{2+} Channel Inhibitors on (−)-[^{3}*H*]-*D888 Binding.* Competition experiments have been done in order to measure the effects of molecules which belong to different classes of Ca^{2+} channel inhibitors on (−)-[^{3}H]D888 binding. These molecules were phenylalkylamines such as (−)- and (+)-D888, (−)- and (+)-verapamil, and (−)- and (+)-D600, dihydropyridines such as (+)-PN200-110 and nitrendipine, benzothiazepines such as *d-cis* and *l-cis*-diltiazem, and (−)- and (+)-bepridil and fluspirilene, a neuroleptic of the diphenylbutylpiperidine series.

Complete inhibition of (−)-[^{3}H]D888 binding was observed for all drugs except for (+)-PN200-110 and nitrendipine,

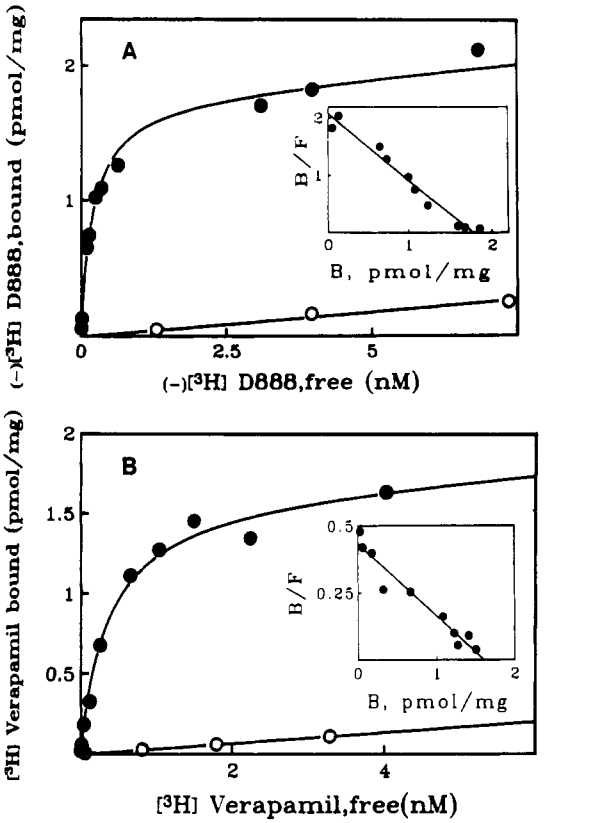

FIGURE 1: Equilibrium binding of (−)-[^{3}H]D888 and (±)-[^{3}H]verapamil to *Drosophila* head membranes. (A) Increasing concentrations of (−)-[^{3}H]D888 were incubated with membranes (0.15 mg of protein/mL) for 90 min at 22–24 °C without (●) or with (○) unlabeled (−)-D888 (1 μM). (Inset) Scatchard plot of the data. (B) Same experiment as in (A) but performed with (±)-[^{3}H]verapamil and 0.098 mg/mL membranes. (Inset) Scatchard plot of the (±)-[^{3}H]verapamil specific binding.

which had an incomplete effect on (−)-[^{3}H]D888 binding at concentrations as high as 10 μM (Figure 2). Inhibition constants K_I for all the different drugs were calculated from the data of Figure 2 according to the relationship $K_{0.5} = K_I(1 + L^*/K_d)$, where $K_{0.5}$ is the concentration of drug that inhibits 50% of the specific binding of (−)-[^{3}H]D888, L^* is the concentration of free radioligand at half-dissociation, and K_d is the dissociation constant of the (−)-[^{3}H]D888–receptor complex. $K_{0.5}$ values and corresponding K_I values are listed in Table I. The order of inhibition potency in the phenylalkylamine series is (−)-verapamil > (−)-D888 = (−)-D600 (Figure 2A, Table I). The (−) and (+) enantiomers present a stereoselectivity for their inhibition potencies. This stereoselectivity is modest for D888 [(−)-D888 is about 3.6-fold more active than (+)-D888], higher for D600 (about 90-fold), and very high for verapamil (about 500-fold).

Results obtained with other calcium channel antagonists are illustrated in Figure 2B and summarized in Table I. Their inhibition constants, K_I, ranged from about 12 to about 190 nM. No stereoselectivity effect was observed for diltiazem and bepridil; *d-cis*-diltiazem is as potent as *l-cis*-diltiazem and the same situation is encountered for (+)- and (−)-bepridil. Competition experiments performed at 37 °C instead of 22 °C gave exactly the same results (data not shown).

Competition of d-cis-Diltiazem, Fluspirilene, and (+)-*Bepridil Binding with* (−)-[^{3}*H*]*D888 Binding.* In order to further characterize the properties of inhibition of tritiated phenylalkylamine binding by *d-cis*-diltiazem, fluspirilene, and (+)-bepridil, direct binding assays were carried out with (−)-[^{3}H]D888 in the presence of increasing concentrations of

Table I: Effects of Calcium Channel Antagonists on (−)-[^{3}H]D888 Binding to Insect Nervous System and Mammalian Skeletal Muscle

	insect nervous system[a]		mammalian skeletal muscle,[b] K_I (nM)
	$K_{0.5}$ (nM)	K_I (nM)	
(−)-D888	0.11	0.052[c]	2
(+)-D888	0.40	0.190[c]	
(−)-verapamil	0.01	0.005[c]	20[d]
(+)-verapamil	4.8	2.3[c]	
(−)-D600	0.11	0.052[c]	40[d]
(+)-D600	10	4.7[c]	
fluspirilene	26	12[e]–14[e]	0.4
(−)-bepridil	35	17[e]–26[e]	20[d]
(+)-bepridil	35	17[e]	
d-cis-diltiazem	410	190[e]–270[e]	60
l-cis-diltiazem	410	190[e]	900

[a] Insect nervous system (*Drosophila*): $K_{0.5}$ values are concentrations of drugs needed for half-maximal binding inhibition under the conditions of Figure 3. [b] Mammalian skeletal muscle (rabbit): K_I values are from Galizzi et al. (1986b). [c] K_I values were determined from competition experiments presented in Figure 2. [d] $K_{0.5}$ and K_I values were obtained from the racemic compound instead of pure enantiomers. [e] K_I values were determined from direct binding experiments presented in Figure 3. Equations to calculate K_I values are given in the text.

these other drugs. Figure 3 presents the family of Scatchard plots corresponding to specific binding of (−)-[^{3}H]D888 in the three conditions. The B_{max} value determined in the absence of inhibitor was not modified by the three other drugs, while the K_d value was increased. This is a typical result for competitive inhibition. The inhibition constant K_I for each drug was calculated from the relationship $K_{app} = K_d(1 + [\text{inhibitor}]/K_I)$, where K_{app} is the apparent dissociation constant of the (−)-[^{3}H]D888–receptor complex measured in the presence of different concentrations of inhibitor, K_d is the true dissociation constant of this complex, and K_I is the inhibition constant. Plots of K_{app}/K_d versus [inhibitor] were linear (not shown), and their slope provided K_I values. These K_I values were 14 nM for fluspirilene, 26 nM for (+)-bepridil, and 270 nM for *d-cis*-diltiazem. These values have been included in Table I.

Photoaffinity Labeling of the Phenylalkylamine Receptor in Drosophila Nervous Membranes. In order to identify which of the polypeptides of *Drosophila* nervous membranes carry the phenylalkylamine receptor, the (arylazido)phenylalkylamine LU47781 was used in photoaffinity labeling experiments. Under dim light, this molecule reversibly binds to the membrane preparation with a K_d value of 0.24 ± 0.05 nM and a B_{max} value of 1.9 ± 0.2 pmol/mg of protein (Figure 4A). After UV irradiation, 50% of the specifically bound tritiated probe had lost the ability to dissociate from its receptor site in the presence of an excess of unlabeled (±)-verapamil (Figure 4B).

Figure 5 shows that [^{3}H]LU47781 was specifically incorporated in a (135 000 ± 5000)-Da band that had the same mobility both under nonreducing conditions and under disulfide reducing conditions. Photolabeling was completely inhibited by 100 nM (±)-verapamil.

Discussion

The most characteristic feature of the phenylalkylamine receptor in *Drosophila* membranes is its very high affinity for some of the phenylalkylamines. Dissociation constants as low as 4.7 and 52 pM are found for (−)-verapamil and for (−)-D600 and (−)-D888, respectively (Table I). These are exceptionally low dissociation constants for ligand–receptor interactions in which the ligand is not itself a protein. Very rare cases of this sort have been described before. The phenylalkylamine receptor is also characterized by a very high stereoselectivity, (−) enantiomers being much more potent than (+) enantiomers (Table I). For verapamil, the affinity ratio is 480 times higher for the (−) enantiomer. This ratio is lower for D888 and D600 enantiomers. It is not very surprising to observe that the largest difference in affinity for the enantiomers is seen with the phenylalkylamine structure, verapamil, that has the highest affinity for its receptor.

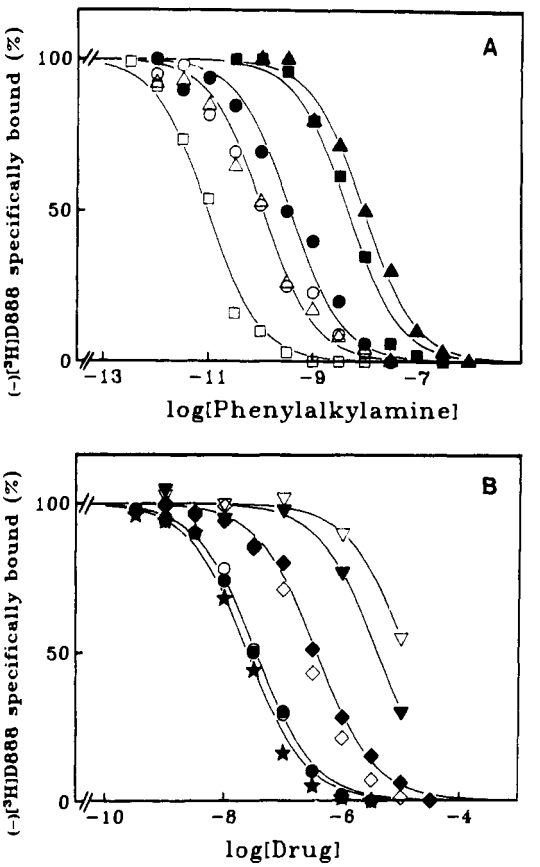

FIGURE 2: Effects of calcium channel antagonists on (−)-[^{3}H]D888 binding to *Drosophila* head membrane preparation. (A) Inhibition of (−)-[^{3}H]D888 binding by phenylalkylamines. Specific binding of (−)-[^{3}H]D888 (0.28 nM) was measured after a 2-h incubation with membranes (48 μg of protein/mL) at 22–24 °C in the presence of increasing concentrations of (−)-verapamil (□), (+)-verapamil (■), (−)-D888 (○), (+)-D888 (●), (−)-D600 (△), and (+)-D600 (▲). Nonspecific binding was measured in a parallel incubation performed with 1 μM (−)-D888 and subtracted from the data. 100% of (−)-[^{3}H]D888 specific binding represents 0.88 pmol/mg of protein. (B) Inhibition of (−)-[^{3}H]D888 binding by other calcium channel antagonists. Competition experiments were done exactly as in (A) but in the presence of fluspirilene (★), (−)-bepridil (○), (+)-bepridil (●), *d-cis*-diltiazem (◇), *l-cis*-diltiazem (◆), nitrendipine (▽), and (+)-PN200-110 (▼).

The best characterized phenylalkylamine receptor is the one associated with Ca^{2+} channels in T-tubule membranes of mammalian skeletal muscle. Affinities in this mammalian system are up to 2000-fold lower than those found in the *Drosophila* nervous system (Table I).

Receptors of relatively high affinity for phenylalkylamines (K_d = 0.5–1 nM) for (−)-[^{3}H]D888 have also been identified in mammalian brain (Reynolds et al., 1986b; Qar et al., 1987) as well as in cardiac (Qar et al., 1987) and smooth muscle (Qar et al., 1987). In all these tissues they coexist with low-affinity sites (K_d = 30–100 nM), and their properties are difficult to analyze because high-affinity sites are in low amounts as compared to low-affinity sites (a difference of at least 1 order of magnitude). In insect neuronal membranes only very high affinity sites have been detected, and they are in a relatively

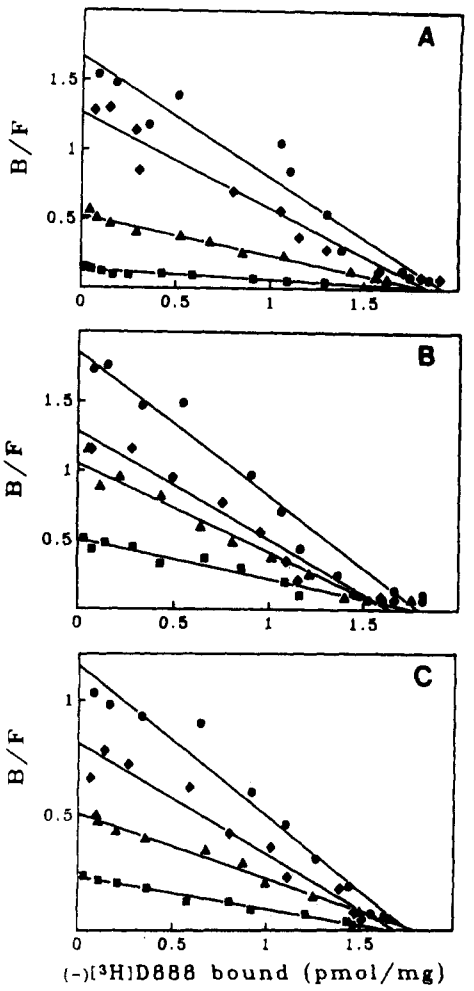

FIGURE 3: Scatchard plots of specific binding of (−)-[^{3}H]D888 to *Drosophila* head membranes in the presence of fluspirilene, (+)-bepridil, and *d-cis*-diltiazem. Membranes (0.14 mg of protein/mL) were incubated at 22–24 °C with increasing concentrations of (−)-[^{3}H]D888 plus fluspirilene (A), (+)-bepridil (B), and *d-cis*-diltiazem (C). Concentrations of the inhibitors: (A) 0 (●), 10 (◆), 30 (▲), and 150 nM (■) fluspirilene; (B) 0 (●), 6 (◆), 15 (▲), and 70 nM (■) (+)-bepridil; (C) 0 nM (●), 80 nM (◆), 300 nM (▲), and 1 μM (■) *d-cis*-diltiazem. The true inhibition constant for each competitor, K_I, was determined as described in the text (see Results).

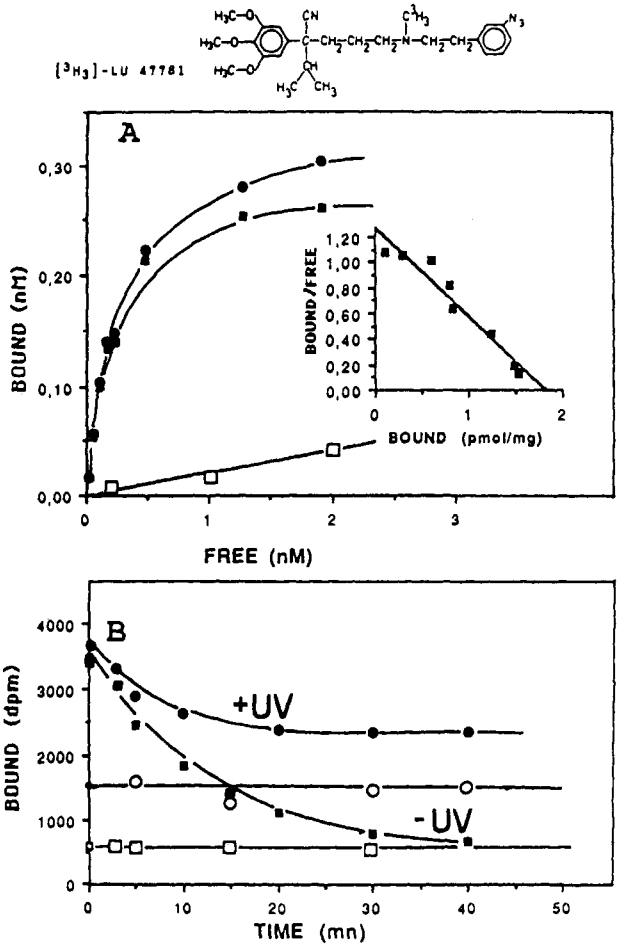

FIGURE 4: Equilibrium binding of [^{3}H]LU47781 to *Drosophila* head membranes. (A) Membranes (0.17 mg of protein/mL) were incubated under the conditions described in Figure 1 with [^{3}H]LU47781 at various concentrations in the absence (●, ■) or in the presence (□) of unlabeled (±)-verapamil (100 nM). (●) Total binding; (■) specific binding. (Inset) Scatchard plot of the specific binding. (B) Dissociation of bound [^{3}H]LU47781 in the presence of 100 nM (±)-verapamil after ultraviolet irradiation (●, ○) or without irradiation (■, □). Open symbols: nonspecific binding measured in the presence of 100 nM (±)-verapamil (see Experimental Procedures). Dpm bound are for 20-μL aliquots of the incubation mixtures.

large amount (2 pmol/mg of protein). Therefore, these membranes appear at present as the best source for the analysis of the properties of very high affinity phenylalkylamine binding sites. Phenylalkylamine binding to skeletal muscle T-tubules was shown to be competitively antagonized by other Ca^{2+} channel blockers such as bepridil and diltiazem (Galizzi et al., 1986a). The same observation had been made in this work for the very high affinity phenylalkylamine binding site in *Drosophila* membranes (Figure 3). Although high-affintiy phenylalkylamine binding sites in mammalian skeletal muscle have much less affinity [a factor of 38 for (−)-D888] for phenylalkylamines than very high affinity sites in *Drosophila* membranes, affinities for diltiazem and bepridil are not too different in *Drosophila* nervous membranes and in mammalian skeletal muscle (Table I). *d-cis*-Diltiazem binds with a similar affinity to insect and mammalian nervous system membranes [K_d = 280 nM (Reynolds et al., 1986b)].

Fluspirilene is also a Ca^{2+} channel blocker (Galizzi et al., 1986b). This molecule inhibits (−)-[^{3}H]D888 binding in a noncompetitive way in mammalian skeletal muscle; this inhibition is of the competitive type at the very high affinity phenylalkylamine binding site in *Drosophila* membranes. The noncompetitive behavior observed in mammalian skeletal muscle suggests that the competitive behavior observed in *Drosophila* membranes may not mean that flusipirilene binds at the phenylalkylamine binding site itself. As in mammalian membranes, fluspirilene may have its own binding site distinct from the phenylalkylamine binding site. When the fluspirilene binding site is occupied, no binding is possible at the phenylalkylamine binding site. The affinity of fluspirilene for its binding site is similar in mammalian brain [K_d = 20 nM (Qar et al., 1987)] and in *Drosophila* nervous membranes.

All these results taken together indicate that the protein (or the protein assembly) that contains very high affinity binding sites for phenylalkylamines also contains sites for other Ca^{2+} channel blockers such as diltiazem, bepridil, and fluspirilene but is devoid of sites with a reasonable affinity for dihydropyridines. Dihydropyridine binding sites have been identified with (+)-[^{3}H]PN200-110 (not shown). However, they were 45 times less abundant (B_{max} = 36 ± 4 fmol/mg of protein, K_d = 1.2 ± 0.2 nM) than very high affinity phenylalkylamine binding sites, and (+)-[^{3}H]PN200-110 binding at these sites were unaffected by phenylalkylkamines at concentrations as high as 1 μM. The present data suggest that, in *Drosophila* brain membranes, very high affinity phenylalkylamine binding sites are situated on a protein component diffeent from (and more abundant than) the protein component that contains the

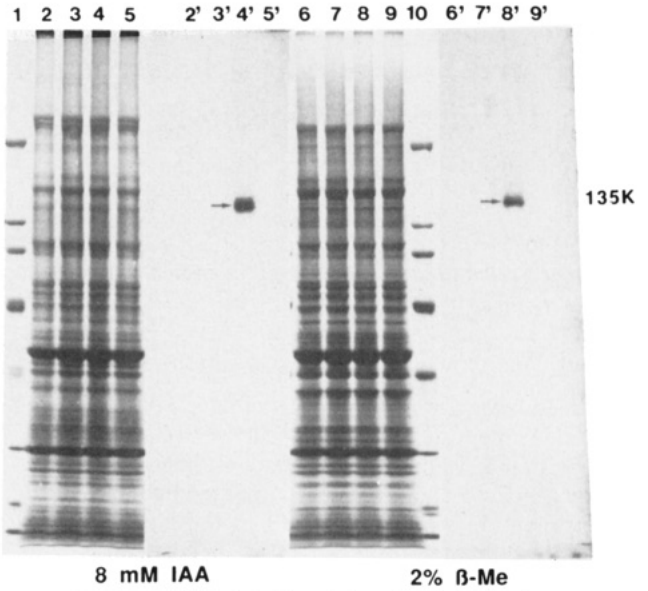

FIGURE 5: Photoaffinity labeling of the phenylalkylamine receptor with [^{3}H]LU47781. Labeling experiments were performed as described under Experimental Procedures. Lanes 1–5 and 6–10, Coomassie blue staining of the gel; lanes 2′–5′ and 6′–9′, autoradiography of the gel. Lanes 2, 4, 6, and 8 and lanes 2′, 4′, 6′, and 8′ show incubation in the absence of unlabeled (±)-verapamil, and lanes 3, 5, 7, and 9 and lanes 3′, 5′, 7′, and 9′ show incubation in the presence of unlabeled (±)-verapamil. Lanes 2, 3, 2′, and 3′ and lanes 6, 7, 6′, and 7′ are from incubations that were not irradiated. Lanes 1 and 10 are molecular weight markers from Bio-Rad: (from the top to the front) myosin (200 000), β-galactosidase (116 500), phosphorylase *b* (96 500), bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (24 500), and lysozyme (14 400). β-Me = β-mercaptoethanol (disulfide reducing conditions); IAA = iodoacetamide (nonreducing conditions). Despite the relatively large nonspecific binding of [^{3}H]LU47781 after UV illumination (Figure 4B), no nonspecifically labeled bands appeared in the autoradiography because all the nonspecific incorporation occurred in phospholipids and migrated ahead of the dye front of the gel.

relatively high affinity dihydropyridine binding sites.

Photoaffinity labeling with [^{3}H]LU47781, an (arylazido)-phenylalkylamine that binds with a high affinity (K_d = 0.24 nM) to the insect putative Ca^{2+} channels, revealed a receptor structure made of a single protein of M_r 135 000. The phenylalkylamine receptor in skeletal muscle T-tubule membranes is situated on a protein of M_r 170 000 (Galizzi et al., 1986a; Striessnig et al., 1987; Vandaele et al., 1987).

It is of interest to note that differences in pharmacological properties of phenylalkylamine receptors in *Drosophila* nerve membranes and rabbit skeletal muscle membranes, respectively, are also associated with differences of size of the polypeptides on which these receptor sites are situated.

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Registry No. (−)-D888, 93468-87-2; (−)-D600, 36622-40-9; LU47781, 110143-76-5; (±)-verapamil, 56949-77-0; (−)-verapamil, 36622-29-4; *d-cis*-diltiazem, 42399-41-7; *l-cis*-diltiazem, 75472-92-3; (+)-bepridil, 110143-74-3; (−)-bepridil, 110143-75-4; fluspirilene, 1841-19-6.

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