0026-895X/88/040363-07$02.00/0

MOLECULAR PHARMACOLOGY, 33:363-369

ACCELERATED COMMUNICATION

A Novel High Affinity Class of Ca^{2+} Channel Blockers

JANTI QAR,[1] JACQUES BARHANIN, GEORGES ROMEY, RAINER HENNING, ULRICH LERCH, RAYMOND OEKONOMOPULOS, HANSJORG URBACH, and MICHEL LAZDUNSKI

Centre de Biochimie du Centre National de la Recherche Scientifique, Parc Valrose, 06034 Nice Cedex, France (J.Q., J.B., G.R., M.L.) and Hoechst AG, Postfach 80 03 20, D-6230 Frankfurt/Main 80, Federal Republic of Germany (R.H., U.L., R.O., H.U.)

Received December 18, 1987; Accepted February 2, 1988

SUMMARY

Benzolactams (HOE 166 and analogs) form a new class of molecules acting on the 1,4-dihydropyridine-sensitive L-type Ca^{2+} channels. The main binding properties of HOE 166 and analogs to rabbit skeletal muscle membranes are as follows. (i) The compounds have a specific binding site to which they associate with a high affinity (0.25 nM for HOE 166). (ii) Unlabeled HOE 166 and analogs completely inhibit 1,4-dihydropyridine binding [(+)-[^{3}H]PN 200-110] in a competitive way. (iii) Affinity values measured for HOE 166 inhibition of (+)-[^{3}H]PN 200-110 ($K_{0.5}$ = 0.25 nM and K_i = 0.55 nM) and of [^{3}H]HOE 166 binding ($K_{0.5}$ = 0.5 nM) are in good agreement. They also fit with results from direct binding experiments with tritiated HOE 166 (K_d = 0.27 nM) and from kinetic experiments (K_d = 0.39 nM). (iv) HOE 166 completely inhibits the specific binding of other classes of Ca^{2+} channel antagonists such as phenylalkylamines [(−)[^{3}H] desmethoxyverapamil], benzothiazepines (*d*-cis-[^{3}H]diltiazem), diphenylbutylpiperidines ([^{3}H]fluspirilene), and [^{3}H]bepridil. In all these cases the binding inhibition is of a noncompetitive nature. (v) The maximum binding capacity for [^{3}H]HOE 166 binding to transverse tubule membranes, 65 pmol/mg of protein, is the same as that found for other classes of Ca^{2+} channel antagonists. $^{45}Ca^{2+}$ uptake experiments performed with the rat aortic cell line A7r5 and the insulin-secreting cell line RINm5F demonstrate that HOE 166 and analogs fully inhibit the 1,4-dihydropyridine-sensitive $^{45}Ca^{2+}$ influx elicited by depolarization. There is a good correlation between inhibitory potencies of compounds in the HOE 166 series measured on (+)-[^{3}H]PN 200-110 binding to A7r5 membranes and on the activity of Ca^{2+} channels followed by $^{45}Ca^{2+}$ fluxes with the same cells. Structure-function relationships of HOE 166 and analogs for Ca^{2+} channel blockade in A7r5 and RINm5F cells were also in good correlation. Finally, voltage-clamp experiments confirmed that voltage-dependent L-type Ca^{2+} channels are completely blocked by 100 nM HOE 166 even at a membrane potential held at −80 mV.

Voltage-dependent Ca^{2+} channels play an essential role in excitation-contraction coupling in cardiac, skeletal, and smooth muscle as well as in excitation-secretion coupling leading to hormone or neurotransmitter release.

Ca^{2+} channel inhibitors form an important class of therapeutic agents used in cardiovascular diseases (1, 2) with important hopes that they might also be used for other types of pathologies (3, 4).

Different types of voltage-dependent Ca^{2+} channels have been identified (5–12). However only the L type (8), *i.e.*, the slow and high threshold type of Ca^{2+} channel, has a rich pharmacology (13, 14). It is blocked by DHPs such as nitrendipine or (+)-PN 200-110, by phenylalkylamines such as verapamil and D888, by bepridil, by benzothiazepines such as diltiazem, or by diphenylbutylpiperidines such as fluspirilene (15, 16).

This paper demonstrates that new chemical series having the properties to block L-type Ca^{2+} channels can still be found. It characterizes binding sites for a new class of molecules (HOE 166 and analogs), examines the interactions between these binding sites and those previously identified for classical Ca^{2+} channel blockers, and demonstrates Ca^{2+} channel blockade by this new series of molecules in smooth muscle and insulin-secreting cells.

Materials and Methods

Cell cultures. The A7r5 embryonic rat aortic smooth muscle cell line was obtained from the American Type culture collection, Rockville, MD. Cells were plated at a density of 7000 cells/well (Falcon 24-well

This work was supported by the Centre National de la Recherche Scientifique.
[1] On leave from the Department of Biological Sciences, Yarmouk University, Irbid, Jordan.

ABBREVIATIONS: DHP, 1,4-dihydropyridine; T-tubule membranes, transverse-tubule membranes; EDTA, ethylenediaminetetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HOE 166, *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; D888, desmethoxyverapamil; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*^{1},*N*^{1}-tetraacetic acid; PN 200-110, isopropyl 4-(2,1,3,-benzoxadiazol-4 yl) 1-4-dihydro-2,6-dimethyl-5-(methoxycarbonyl)-pyridine-3-carboxylate.

tissue culture plates) or at a density of 3.5×10^6/350 ml of culture medium (Falcon roller bottles, 850-cm² style) and grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum. RINm5F is an insulin-secreting cell line derived from a rat islet cell tumor. Cells were plated at a density of 2×10^5 cells/well (Falcon 24-well tissue culture plates) and grown as previously described (17, 18).

Preparation of membranes. Transverse tubule (T-tubule) membranes from rabbit skeletal muscle were prepared according to the method of Galizzi *et al.* (19) in the presence of 0.1 mM phenylmethylsulfonylfluoride, 1 mM iodoacetamide, and 10 mM EDTA. Microsomal preparations from rabbit skeletal muscle consisted in a simplified preparation in which the sucrose gradient step was omitted (20). Microsomes from A7r5 cells were obtained as follows. After 10 days of culture, cells were rinsed four times with ice-cold 20 mM Hepes-NaOH buffer, pH 7.5, containing 0.2 M sucrose and 1 mM EDTA, scraped in the same buffer, and pelleted by centrifugation at $2000 \times g$ for 10 min. Cells were homogenized with a Potter-Elvejhem apparatus in 50 ml of the above buffer by 10 strokes and sonicated for 10 sec. The homogenate was centrifuged for 30 min at $60000 \times g$ and the pellet was resuspended in 20 mM Hepes-NaOH, pH 7.5, 1 mM EDTA. After a second centrifugation for 30 min at 60,000 *g*, the pellet was resuspended in 40 mM Hepes-NaOH, pH 7.5, at a concentration of 5 mg of protein/ml and stored in liquid nitrogen. Ten ml of microsomal preparation were obtained from six Falcon roller bottles.

Binding assays. Incubation of skeletal muscle membrane preparations with the different ligands was performed in a 1-ml solution of 20 mM Hepes-NaOH, pH 7.5, 0.01% bovine serum albumin, 2 mM $CaCl_2$ for 45 min at 20° (equilibrium binding experiments). Then two 400-μl aliquots of incubation were filtered on GF/C glass fiber filters equilibrated in 0.05% polyethyleneimine in 100 mM Tris-HCl at pH 7.5 as previously described by Cognard *et al.* (21) for (+)-[^{3}H]PN 200-110 binding, by Galizzi *et al.* (22) for (−)-[^{3}H]D888, *d*-cis-[^{3}H]diltiazem and (±)-[^{3}H]bepridil binding, and by Galizzi *et al.* (15) for [^{3}H]fluspirilene. For experiments involving (−)D888, *d*-cis-diltiazem, (±)bepridil and fluspirilene, no calcium was present in the incubation mixture. Competition of (+)-[^{3}H]PN 200-110 binding to A7r5 microsomal preparation (0.2 mg of protein/ml) by different unlabeled drugs were analyzed at 20° in the same buffer as the one used in flux experiments and in the presence of 0.1 nM (+)-[^{3}H]PN 200-110. Under these conditions the measured $K_{0.5}$ values are very close to the true K_d values of the different drugs.

$^{45}Ca^{2+}$ uptake experiments. Ca^{2+} uptake studies on A7r5 and RINm5F cells were carried out in 24-well culture plates at 37° as described for A7r5 cells (16, 23). Cells were washed in 20 mM Hepes-NaOH, pH 7.4, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.01% bovine serum albumin and preincubated for 10 min with 200 μl of this solution (polarized conditions) in the presence of the molecule to be assayed. For depolarized conditions, the salt solution contained 55 mM KCl instead of 5 mM K^+ and 85 mM NaCl instead of 135 mM NaCl. $^{45}Ca^{2+}$ uptake measurements were made after 3-min incubations in the buffer containing 0.6 μCi/ml $^{45}CaCl_2$ and 0.1 mM $CaCl_2$ instead of 0.1 mM EGTA.

Electrophysiology. Voltage-clamp experiments were done at 32 ± 2° on the A7r5 cell line by using the whole cell configuration of the patch-clamp method (24). The external solution contained 40 mM tetraethylammonium chloride, 10 mM $CaCl_2$, and 1 mM $MgCl_2$. This solution was buffered at pH 7.4 with 10 mM Hepes/KOH. The pipette solution contained 140 mM CsCl, 5 mM EGTA, 4 mM $MgCl_2$, 3 mM ATP, and was buffered at pH 7.2 with 10 mM Hepes/CsOH. Patch pipette (2–6 MΩ) were connected to the head stage of the recording apparatus (RK 300, Bio-Logic, Grenoble, France).

Chemicals. [^{3}H]HOE 166 (0.81 TBq/mmol), unlabeled HOE 166, and its analogs (see Table 1 under Results) were from Hoechst (Frankfurt am/Main, FRG). Tritiated (0.33 TBq/mmol) and unlabeled fluspirilene were from Janssen Pharmaceutica (Beerse, Belgium). (±)-[^{3}H]Bepridil (1.4 TBq/mmol) was from Commissariat à l'Energie Atomique (Saclay, France) and unlabeled bepridil was from CERM (Riom, France). Unlabeled (+)-PN 200-110 was from Sandoz (Basel, Switzerland), (−)D888 was from Knoll AG (FRG) and *d*-cis-diltiazem was from Synthelabo (Paris, France). (+)-[^{3}H]PN 200-110 (2.96 TBq/nmmol), (−)-[^{3}H]D888 (3 TBq/mmol), and *d*-cis-[^{3}H]diltiazem (5.9 TBq/mmol) were from Amersham.

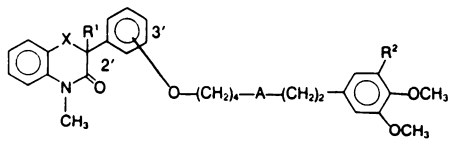
Results

Inhibition by HOE 166 of the binding of different types of labeled calcium channel antagonists to rabbit skeletal muscle membranes. Binding sites for the different types of Ca^{2+} channel blockers have been characterized in rabbit skeletal muscle T-tubule membranes. They include receptors for DHP, for phenylalkylamines, for benzothiazepines, and for bepridil, and receptors for one neuroleptic (fluspirilene) of the diphenylbutylpiperidine series (15, 22, 25, 26).

Protection experiments demonstrate that unlabeled HOE 166 or its *S*-(−)-enantiomer, analog **2** (Table 1), completely inhibits the binding of (+)-[^{3}H]PN 200-110 (DHP) (Fig. 1A, *inset*), (−)-[^{3}H]D888 (phenylalkylamine), *d*-cis-[^{3}H]diltiazem (benzothiazepine), [^{3}H]fluspirilene (diphenylbutylpiperidine), and (±)-[^{3}H]bepridil to T-tubule membranes (Fig. 1B, *inset*). The $K_{0.5}$ value for the action of the most active compound HOE 166 on (+)-[^{3}H]PN 200-110 binding was in the subnanomolar range (0.25 nM) and the Hill coefficient was close to 1. Inhibition of binding by other radioligands occurred at higher drug concentrations which ranged from 6 to 25 nM. The stereospecificity was more pronounced for inhibition of (+)-[^{3}H]PN 200-110 binding, the active enantiomer HOE 166 being 52 times more potent than the *S*-(−)-enantiomer (Fig. 1A, *inset*).

Fig. 1 shows families of Scatchard plots describing (+)-[^{3}H]PN 200-110 (Fig. 1A) and (−)-[^{3}H]D888 (Fig. 1B) binding to a microsomal preparation in the presence of increasing concentrations of HOE 166. The maximal binding capacity for (+)-[^{3}H]PN 200-110 was not modified in the presence of HOE 166, whereas apparent affinity was decreased from $K_d = 0.08$ nM in the absence of HOE 166 to 0.16, 0.39, and 0.72 nM in the presence of 0.5, 2, and 5 nM HOE 166, respectively. This is a typical result for a competitive inhibition, and the inhibition constant, K_I, calculated from these values is 0.55 nM, in good agreement with the $K_{0.5}$ value of 0.25 nM found in competition

Table 1
Benzolactams used in this investigation



No.	R¹	R²	A	X	Position of side chain	Absolute configuration
1 (HOE 166)	$(CH_3)_2CH$	OCH_3		S	2′	*R*
2	$(CH_3)_2CH$	OCH_3		S	2′	*S*
3	*n*-C_4H_9	OCH_3		O	2′	*R,S*
4	$(CH_3)_2CH$	H		S	3′	*R,S*
5	$(CH_3)_2CH$	OCH_3		CH_2	2′	*R,S*
6	$(CH_3)_2CH$	OCH_3		SO_2	2′	*R*

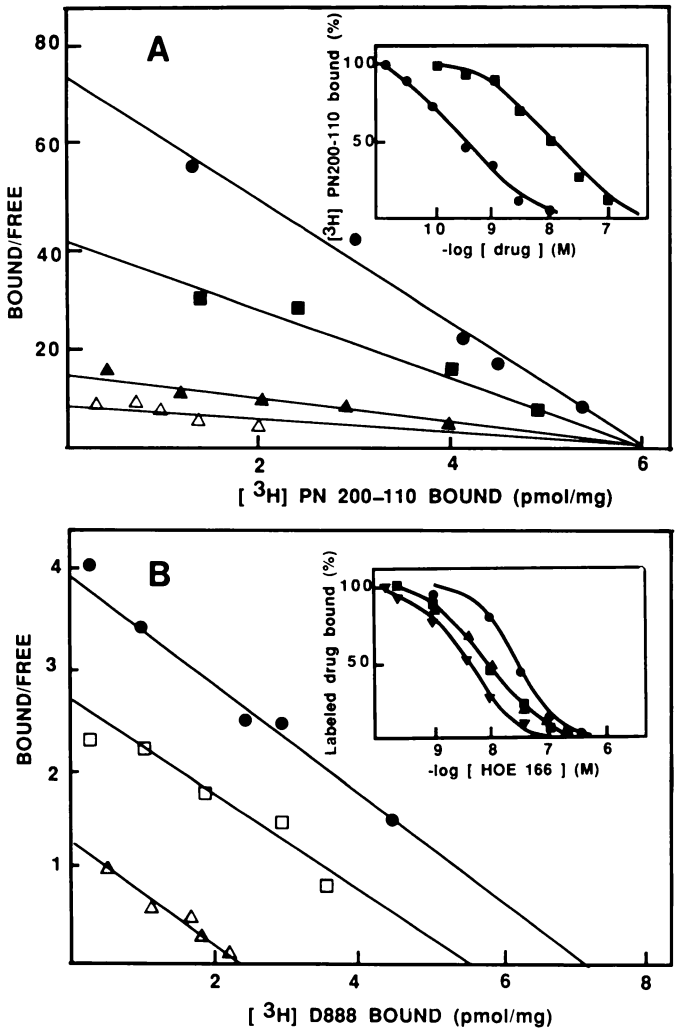


Fig. 1. Scatchard plots of the specific binding of (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]D888 to rabbit skeletal muscle microsomes in the presence of HOE 166. A. Membranes (0.01 mg of protein/ml) were incubated at 20° with increasing concentrations of (+)-[^{3}H]PN 200-110 in the absence of (●) or presence of 0.5 nM (■), 2 nM (▲), and 5nM (△) unlabeled HOE 166. *Inset:* Dose response curves for inhibition of (+)-[^{3}H]PN 200-110 binding by unlabeled HOE 166 (●) and its *S*-(−)-enantiomer (analog **2**) (■). $K_{0.5}$ values are 0.25 nM and 13 nM for HOE 166 and analog **2**, respectively. B. Equilibrium binding of (−)-[^{3}H]D888 at 20° (0.05 mg of protein/ml) in the absence (●) or presence of 6 nM (□) and 30 nM (△) HOE 166. *Inset:* Dose response curves for inhibition of (−)-[^{3}H]D888 (●), (±)-[^{3}H]bepridil (■), *d*-cis-[^{3}H]diltiazem (▲), and [^{3}H]fluspirilene (▼) by unlabeled HOE 166. $K_{0.5}$ values are 25 nM, 9 nM, 10 nM, and 6 nM for inhibition of (−)-[^{3}H]D888, (±)-[^{3}H]bepridil, *d*-cis-[^{3}H]diltiazem, and [^{3}H] fluspirilene binding, respectively. The corresponding $K_{0.5}$ values for the *S*-(−) compound are (nM): 30, 16, 9, and 40.

experiments. The presence of 30 nM HOE 166 did not modify dissociation kinetics of bound (+)-[^{3}H]PN 200-110 from its receptor (not shown). This result is another indication of the possible competitive interaction of benzolactams at the DHP binding site. The situation was different for (−)-[^{3}H]D888 binding. The affinity of the phenylalkylamine marker was not modified in the presence of HOE 166 (K_d = 1.8 nM). Conversely, the B_{max} value was decreased in the presence of unlabeled HOE 166. The same type of result was also observed for the inhibition of *d*-cis-[^{3}H]diltiazem, [^{3}H]fluspirilene, and (±)-bepridil (not shown). This behavior corresponds to a noncompetitive inhibition.

Specific binding of [^{3}H]HOE 166 to rabbit skeletal muscle T-tubule membranes. Fig. 2A shows a typical equilibrium binding experiment of [^{3}H]HOE 166 to T-tubule membranes. The nonspecific binding component measured in the presence of 1 μM unlabeled HOE 166 was low, even at saturating concentrations of [^{3}H]HOE 166. A Scatchard plot for the specific binding component (Fig. 2A, *inset*) shows that [^{3}H] HOE 166 specifically associates with a single class of sites with a K_d value of 0.27 nM and a B_{max} value of 65 pmol/mg of protein.

Fig. 2, B and C, shows association and dissociation kinetics relative to the interaction of [^{3}H]HOE 166 with T-tubule membranes. Under the chosen experimental conditions the association reaction is of pseudo-first order with a rate constant value, k_1, of 2.3×10^6 M^{-1} sec^{-1}. The first order rate constant of dissociation of the complex (k_{-1}) is 8.94×10^{-4} sec^{-1}, corre-

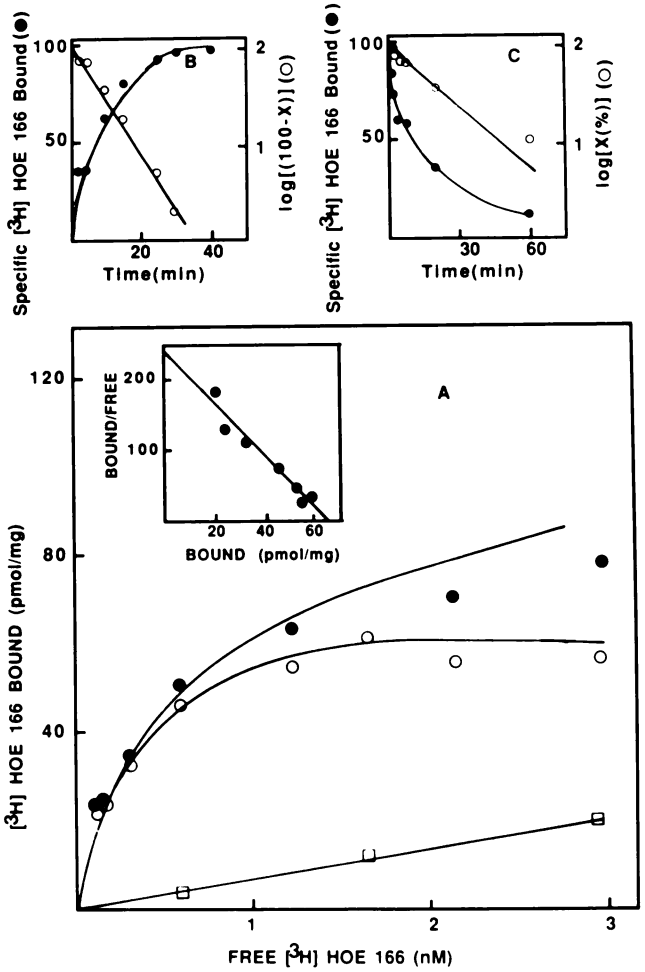


Fig. 2. A. Direct binding of [^{3}H]HOE 166 to T-tubule membranes at 20° (0.008 mg of protein/ml). ●, total binding; □, nonspecific binding measured in the presence of 1 μM unlabeled HOE 166; ○, specific binding. *Inset:* Scatchard plot of the specific binding. B and C. Association (B) and dissociation (C) kinetics for the binding of [^{3}H]HOE 166 to T-tubule membranes at 20°. B. Association kinetics were started by addition of 0.5 nM [^{3}H]HOE 166 to T-tubule membranes (0.003 mg of protein/ml corresponding to a receptor concentration of 0.195 nM). The maximum concentration of specifically bound [^{3}H]HOE 166 which corresponded to 100% was 0.108 nM. The concentration of free [^{3}H]HOE 166 only varied by 21% and the reaction is of pseudo-first order. C. Dissociation was initiated after 45 min of association by addition of 1 μM unlabeled HOE 166. ○, semilogarithmic representation of association (B) and dissociation (C) data. *X*, percentage of the maximal [^{3}H]HOE 166 specifically bound at time *t*.

sponding to a half-life of dissociation of 13 min at 20°. The equilibrium dissociation constant (K_d) from the kinetic data (k_{-1}/k_1 = 0.39 nM) is in good agreement with the K_d value found in Fig. 2A and with the K_I value calculated from Fig. 1A.

Effects of classical calcium channel antagonists and of HOE 166 analogs on the specific binding of [^{3}H]HOE 166 to T-tubule membranes. Fig. 3A shows that various calcium channel antagonists completely inhibit the specific binding of [^{3}H]HOE 166 to T-tubule membranes with $K_{0.5}$ values between 0.4 and 1800 nM. The rank order of potency was as follows : (+)-PN 200-110 ($K_{0.5}$ = 0.4 nM) > fluspirilene ($K_{0.5}$ = 10 nM) > (−)-D888 ($K_{0.5}$ = 50 nM) > (±)-bepridil ($K_{0.5}$ = 100 nM) > *d*-cis-diltiazem ($K_{0.5}$ = 1800 nM). *d*-cis-Diltiazem also inhibited (100%) [^{3}H]HOE 166 binding at 37° (not shown). Fig. 3B shows the inhibition of the specific binding of [^{3}H] HOE 166 to T-tubule membranes by unlabeled HOE 166 and its analogs (Table 1). The $K_{0.5}$ value found in these experiments for unlabeled HOE 166 is 0.5 nM, which is similar to the K_d value of 0.27 nM found from equilibrium data and to the K_d value of 0.39 nM calculated from kinetic data. $K_{0.5}$ values for the different HOE 166 analogs are comprised between 12 and 50 nM and are given in the legend of Fig. 3.

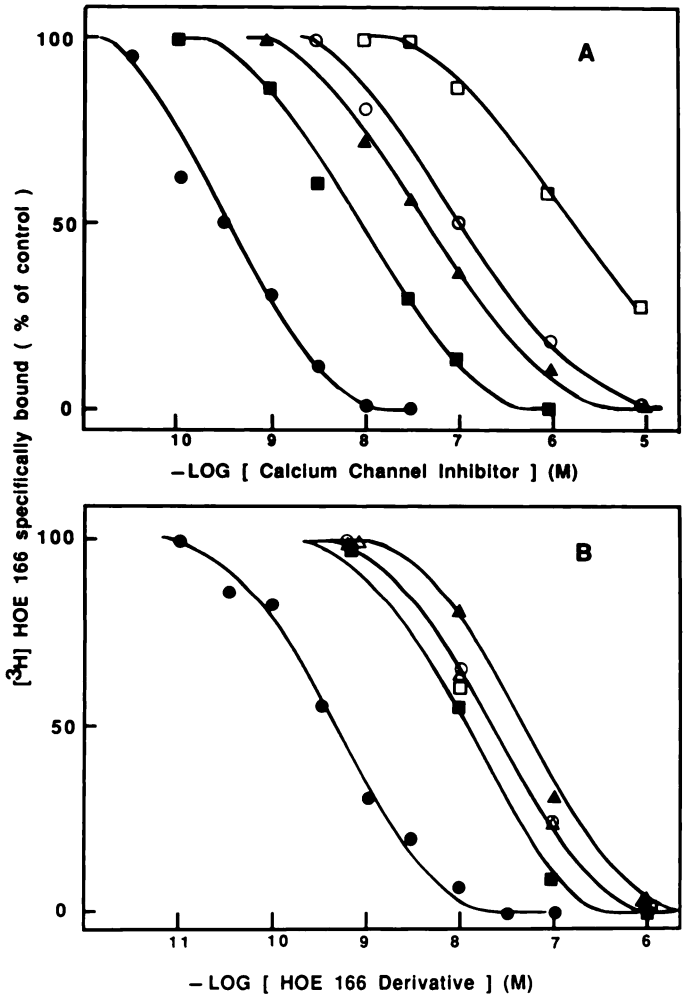


Fig. 3. Inhibition of [^{3}H]HOE 166 binding to T-tubule membranes by different calcium channel antagonists (A) and HOE 166 derivatives (B). Specific binding of [^{3}H]HOE 166 (0.4 nM) was measured after 45 min incubation at 20° with T-tubule membranes (0.003 mg of protein/ml) in the presence of increasing concentrations of (A): (+)PN 200-110 (●), (−)-D888 (▲), fluspirilene (■), (±)-bepridil (○), and *d*-cis-diltiazem (□); or (B): HOE 166 (●) and analogs **2** (■), **3** (▲), **4** (□), **5** (△), and **6** (○). Nonspecific binding was measured in the presence of 1 μM HOE 166 and was lower than 10% of the total binding. The 100% value corresponded to 0.1 nM specifically bound [^{3}H]HOE 166. $K_{0.5}$ values are (nM): (+)-PN 200-110, 0.4; fluspirilene, 10; (−)-D888, 50; (+)-bepridil, 100; *d*-cis-diltiazem, 1800; HOE 166, 0.5; analog **2**, 12; analog **4**, 13; analog **6**, 18; analog **5**, 20; and analog **3**, 50.

Effect of HOE 166 and analogs on DHP-sensitive $^{45}Ca^{2+}$ uptake in vascular A7r5 cells and RINm5F cells in culture. The effect of HOE 166 and derivatives on the activity of voltage-dependent Ca^{2+} channels was investigated by $^{45}Ca^{2+}$ flux experiments performed on two different cell lines: the aortic cell line A7r5 and the insulin-secreting cell line RINm5F.

In a medium containing 5mM K^+, the different drugs assayed were without effect on the basal $^{45}Ca^{2+}$ uptake in both cell types. Conversely, the $^{45}Ca^{2+}$ uptake component due to Ca^{2+} channel activity elicited by depolarization in 55 mM K^+ was completely inhibited by molecules of the HOE 166 family. Concentration dependences for inhibition by the different benzolactams of the HOE 166 family are presented in Fig. 4A for A7r5 cells and in Fig. 4B for RINm5F cells. Although Ca^{2+} channel activity measured by $^{45}Ca^{2+}$ uptake was 5–25 times more sensitive to the different inhibitors assayed [benzolactams, (+)-PN 200-110, (−)-D888, *d*-cis-diltiazem] in A7r5 than in RINm5F cells, a very good correlation was found for the relative inhibitory potencies of the different drugs in both cell lines (Fig. 5, *right*).

It would have been interesting to compare inhibitory potencies of HOE 166 and derivatives measured from Ca^{2+} uptake experiments on the one hand and from [^{3}H]HOE 166 binding on the other hand. Unfortunately, the nonspecific binding component of [^{3}H]HOE 166 binding to A7r5 and RINm5F cells was too large to allow good enough determinations of $K_{0.5}$ values for the different drugs using [^{3}H]HOE 166 as a marker. Therefore $K_{0.5}$ values for each of the unlabeled drugs of interest were determined from their inhibition of (+)-[^{3}H]PN 200-110 binding. A very good correlation was found between IC_{50} values determined by Ca^{2+} flux studies and $K_{0.5}$ values measured by binding studies (Fig. 5, *left*).

Voltage-clamp analysis of the effects of HOE 166 on A7r5 cells. Two experimental protocols have been used to investigate the blocking effect of HOE 166 on the Ca^{2+} current of A7r5 cells. In the first protocol (I), the effect of the drug was tested under polarized conditions i.e., at a holding potential, V_H = −80 mV. The voltage dependence of the drug effect was investigated by exposing the cell first to a fixed concentration of the drug under the polarized conditions (protocol I) until a steady state effect on the current was reached. Next, V_H was held for 3 min at −20 mV in order to set the Ca^{2+} channel in the inactivated state; then, V_H was returned to −80 mV for 1 min before the test pulse (protocol II).

The blocking effect of HOE 166 on the Ca^{2+} channels of A7r5 cells was compared to that of (+)-PN 200-110. Fig. 6 (A and B) shows that the more potent blocker of the Ca^{2+} channels of A7r5 cells at −80 mV is HOE 166 since 10 nM HOE 166 blocked 47% of the current under polarized conditions (protocol I), whereas the same concentration of (+)-PN 200-110 blocked only 34% of the peak current under protocol I. Under protocol II, blockade by HOE 166 passed from 47 to 66%, whereas blockade by (+)-PN 200-110 passed from 34% to 72%, indicat-

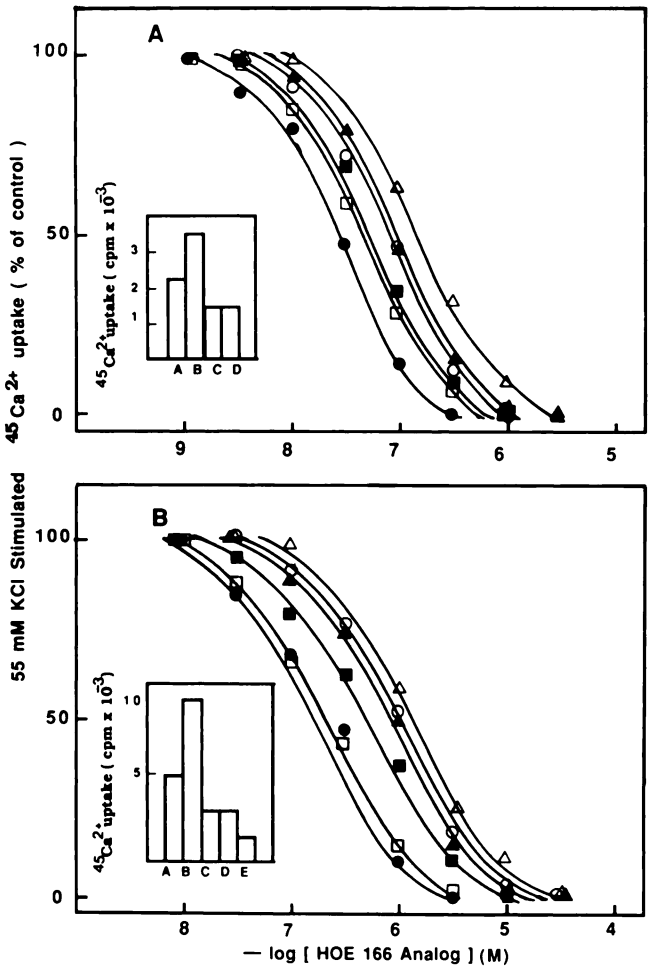


Fig. 4. Inhibition of DHP-sensitive $^{45}Ca^{2+}$ uptake into A7r5 and RINm5F cells by HOE 166 analogs. Times of uptake were 3 min. A. Inhibition of K^+-induced $^{45}Ca^{2+}$ uptake into A7r5 cells by increasing concentrations of HOE 166 (●) and analogs **2** (△), **3** (▲), **4** (○), **5** (■), and **6** (□). *Inset:* basal uptake in 5 mM K^+ buffer in the absence (A) or presence (C) of 0.1 μM (+)-PN 200-110. Activated uptake in 55 mM K^+ buffer in the absence (B) and presence (D) of 0.1 μM (+)-PN 200-110. Results are expressed as $^{45}Ca^{2+}$ cpm/12-mm well. B. Inhibition of K^+-induced $^{45}Ca^{2+}$ uptake into RINm5F cells by the same drugs as in A. *Inset:* basal uptake in 5 mM K^+ buffer (A) and activated uptake in 55 mM K^+ in the absence of blocker (B) or in the presence of 10 μM (+)-PN 200-110 (C), 100 μM *d*-cis-diltiazem (D), and 10 μM D888 (E).

ing a higher voltage dependence for the 1,4-dihydropyridine. Complete Ca^{2+} channel blockade was observed at 100 nM HOE 166. This result is consistent with $^{45}Ca^{2+}$ flux data (Fig. 6).

Discussion

Voltage-dependent ionic channels are also drug receptors. For example, voltage-dependent Na^+ channels have binding sites for multiple classes of neurotoxins (27–29). More recent data also indicate that different types of toxin receptors are present on voltage-dependent K^+ channels (30, 31). Functional and structural knowledge of these channels was greatly assisted by the availability of the specific toxins (32). Most of the important new findings concerning voltage-dependent Ca^{2+} channel have also necessitated the use of specific pharmacological tools. In that case they have mainly been drugs coming from the cardiovascular field, such as DHP, phenylalkylamines, bepridil, or diltiazem.

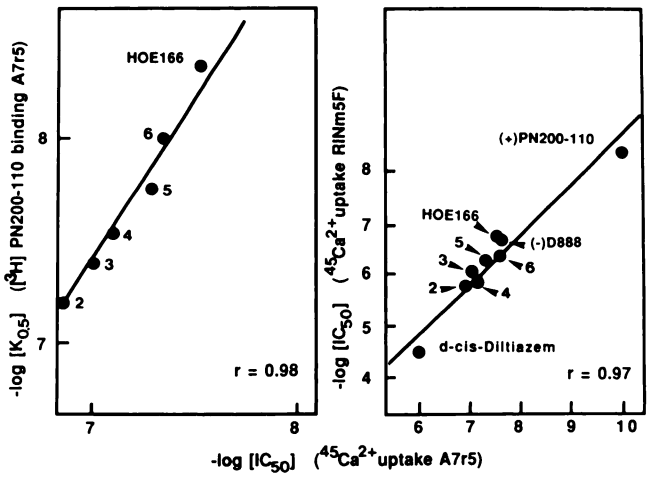


Fig. 5. *Left:* Correlation of the inhibitory potencies of HOE 166 and derivatives on $^{45}Ca^{2+}$ uptake by A7r5 cells and (+)-[^{3}H]PN 200-110 binding to A7r5 microsomes. *Right:* Correlation of the inhibitory potencies of HOE 166, derivatives, and (+)-PN 200-110, *d*-cis-diltiazem, and (−)-D888 for Ca^{2+} channel blockade in A7r5 and RINm5F cells.

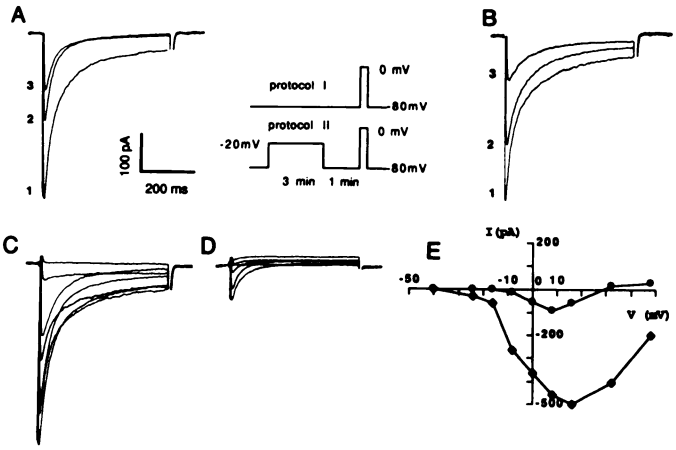


Fig. 6. Blocking effects of HOE 166 and (+)-PN 200-110 on Ca^{2+} channels of A7r5 cells at 32°. The external solution contained 10 mM Ca^{2+} (A and B). Ca^{2+} currents associated with a step depolarization to 0 mV from a holding potential (V_H) = −80 mV. A. *1:* Control current; *2:* steady state effect of 10 nM HOE 166 (protocol I); *3:* additional blockade of the Ca^{2+} current after V_H was held at −20 mV for 3 min and returned to −80 mV for 1 min (protocol II). B. *1:* Control current; 2. steady state effect of 10 nM (+)-PN 200-110 (protocol I), *3:* additional blockade of the Ca^{2+} current (protocol II). C and D. Superimposed Ca^{2+} current traces associated with depolarizing steps to −40, −24, −16, −8, 0, +8, +16, +32, and +48 mV from V_H = − 80 mV. C. Control currents. D. After a 10-min exposure to HOE 166 (100 nM) E. Peak Ca^{2+} current-membrane potential relationship for the experiments illustrated in C (♦) and D (●).

The discovery of new classes of Ca^{2+} channel ligands is potentially important both for therapeutic purposes and for fundamental research concerning channel structure and function.

Three different approaches have been used in this report to define interactions of a new molecule, HOE 166, and some of its analogs, with Ca^{2+} channel structures present in rabbit skeletal muscle, rat aortic cells, and insulin-secreting cells. These approaches included binding studies, $^{45}Ca^{2+}$ flux experiments, and electrophysiological analysis on cultured cells.

T-Tubule membranes have been found to be the richest source of receptors for all kinds of Ca^{2+} channel blockers (25). They have also served as the essential source of material for

the purification of the channel (20, 33–38), and skeletal muscle has been used for the cDNA cloning of one of the constituting subunits of this channel (39). For all these reasons binding studies on T-tubule skeletal muscle membranes were first used for the identification of receptors of molecules of the HOE 166 series. The two salient properties of [^{3}H]HOE 166 binding to T-tubule membranes are a high affinity, with a K_d of 0.27 nM, and a large number of receptors, corresponding to a B_{max} value of 65 pmol/mg of protein. Since HOE 166 and its analogs completely prevent the binding of all known classes of Ca^{2+} channel antagonists including (+)-[^{3}H]PN 200-110, (−)-[^{3}H] D888, *d*-cis[^{3}H]diltiazem, [^{3}H]bepridil, and [^{3}H]fluspirilene, and since its binding stoichiometry to T-tubules is nearly identical to that of the molecule of other Ca^{2+} channel blockers, it is then clear that the HOE 166 receptor appears to be associated with the same kind of voltage-dependent Ca^{2+} channel structure as receptors for the other drugs.

A more detailed analysis of the inhibitory effect of HOE 166 and analogs on (+)-[^{3}H]PN 200-110 and (−)-[^{3}H]D888 binding revealed that the inhibition was competitive for the former tritiated ligand and noncompetitive for the latter one. Moreover, as previously described for DHP (13), inhibition of [^{3}H] DHP binding by HOE 166 occurs with higher affinity than inhibition of other types of molecules (Fig. 1). A similar type of situation was found for analogs of PN 200-110 in the DHP series. However, there are two marked differences : (i) molecules of the DHP series do not completely abolish the binding of other classes of tritiated Ca^{2+} channel antagonists such as phenylalkylamine, diltiazem or bepridil, whereas HOE 166 does; and (ii) *d*-cis-diltiazem enhances the binding of (+)-[^{3}H] PN 200-110, whereas it inhibits [^{3}H]HOE 166 binding at 20° as well as at 37°.

For all these reasons, it seems that the HOE 166-binding site on skeletal muscle membranes has only part of the properties of the DHP-binding sites and is clearly different from the fluspirilene-binding site (15, 16) or from binding sites for phenylalkylamine, benzothiazepine or bepridil (22). Such binding behavior is interesting since HOE 166 includes some structural elements from diltiazem and verapamil families of compounds bridged by a piperazine linkage (Table 1).

It is, of course, important to show that sites identified by binding experiments correspond to sites modulating Ca^{2+} channel activity. This is demonstrated using two different cell lines in culture: the aortic cell line A7r5 and the insulin-secreting cell line RINm5F. The A7r5 cells have previously been shown to be excellent for the analysis of the pharmacology of Ca^{2+} channels by Ca^{2+} flux and of the regulation of the channel by peptides and protein kinase C activators (23).

A striking parallelism does exist between the occupancy of the HOE 166 receptor as shown by inhibition of (+)-[^{3}H]PN 200-110 binding to A7r5 membranes and the Ca^{2+} channel blockade in the A7r5 cells measured by $^{45}Ca^{2+}$ flux experiments (Fig. 5, *left*, $r = 0.98$).

Electrophysiological analysis of A7r5 cells provides an independent demonstration that the voltage-dependent Ca^{2+} channel of the L type (i.e., blocked by DHP) is blocked by low concentrations of HOE 166. One difference for the blockade of the Ca^{2+} channel by HOE 166 and (+)-PN 200-110, respectively, is the voltage dependence of the drug effect. DHP molecules are known to bind more tightly to the L-type Ca^{2+} channel structure in a variety of excitable cells at more depolarized membrane potentials (21, 40). This is due to their higher affinity for the inactivated state of the channel. It has been shown in this paper (Fig. 6) that HOE 166 efficacy is also sensitive to membrane potential but less than (+)-PN 200-110 efficacy.

Ca^{2+} channels are also essential for coupling excitation to secretion in insulin-secreting cells. This report shows that DHP-sensitive Ca^{2+} channels can be studied by $^{45}Ca^{2+}$ uptake measurements on RINm5F insulinoma cells. The pharmacological profile of Ca^{2+} channel blockade in insulinoma cells, including blockade by substances in the HOE 166 series, is similar to that found in A7r5 smooth muscle cells. A good correlation ($r = 0.97$) was found between the $K_{0.5}$ values determined for the different Ca^{2+} channel blockers on the two cell lines, showing that compounds that are less potent on A7r5 cells are also less potent on RINm5F. A less strict correlation was observed between the drug potencies found for benzolactam-induced inhibition of (+)-[^{3}H]PN 200-110 binding to skeletal muscle and to the two cell lines (Figs. 3 and 5). These differences probably reflect differences in properties of L-type Ca^{2+} channel properties in these different tissues.

A number of toxins acting on Na^+ and K^+ channels have now been shown to have endogenous equivalents in the mammalian brain (41, 42). Therefore, it is not unreasonable to believe that endogenous equivalents of Ca^{2+} channel blockers also exist. If this assumption is correct, then it is to be expected that the more numerous the chemical families of Ca^{2+} channel blockers, the more numerous the families of endogenous equivalents of Ca^{2+} channel blockers.

Note added in proof.

HOE 166 also blocks K^+-evoked contractions of aortic smooth muscle preparations and [^{3}H]noradrenaline release from PC12 cells (Usinger, P., U. Albus, W. Linz, and R. Henning. HOE 166, a new calcium antagonist, has a binding site different from 1,4-DHP. *Eur. J. Pharmacol.* in press (1988).

Acknowledgments

We thank Dr. R. P. Hof (Sandoz, Basel, Switzerland) for the gift of unlabeled (+)-PN 200-110; Dr. P. Traut, and Dr. M. Hollman (Knoll AG, FRG) for the gift of (+)- and (−)-D888; Dr. Busch (CERM, Riom, France) for the gift of (+)- and (−)-bepridil; Dr. N. Laduron (Janssen Pharmaceutica, Beerse, Belgium) for the gift of tritiated and unlabeled fluspirilene; Synthelabo (Paris, France) for the gift of *d*-cis and *l*-cis-diltiazem; and Dr. E. Van Obberghen (Unité INSERM 145, Nice, France) for the gift of RINm5F cells. The excellent technical assistance of Marie-Madeleine Larroque and the expert secretarial assistance of C. Roulinat-Bettelheim is greatly appreciated and acknowledged.

References

1. Fleckenstein, A., C. Van Breemen, R. Gross, and F. Hoffmeister. *Cardiovascular Effects of Dihydropyridine-type Calcium Antagonists and Agonists.* Springer-Verlag, Berlin, 511 (1985).
2. Urthaler, F. Review: role of calcium channel blockers in clinical medicine. *Am. J. Med. Sci.* **292**:217–230 (1986).
3. Janis, R. A., and D. J. Triggle. New developments in Ca^{2+} channel antagonists. *J. Med. Chem.* **26**:775–785 (1983).
4. Godfraind, T., R. Miller, and M. Wibo. Calcium antagonism and calcium entry blockade. *Pharmacol. Rev.* **38**:321–416 (1986).
5. Carbone, E., and H. D. Lux. A low voltage-activated, fully inactivating Ca channel in vertebrate sensory neurons. *Nature (Lond.)* **310**:501–502 (1984).
6. Bossu, J. L., A. Feltz, and J. M. Thomann. Depolarization elicits two distinct calcium currents in vertebrate sensory neurons. *Pfluegers Arch.* **403**:360–366 (1985).
7. Nilius, B., P. Hess, J. B. Lansman, and R. W. Tsein. A novel type of cardiac calcium channel in ventricular cells. *Nature (Lond.)* **316**:443–446 (1985).
8. Nowycky, M. C., A. P. Fox, and R. W. Tsien. Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature (Lond.)* **315**:440–442 (1985).
9. Cognard, C., M. Lazdunski, and G. Romey. Different types of Ca^{2+} channels in mammalian skeletal muscle cells in culture. *Proc. Natl. Acad. Sci. USA* **83**:517–521 (1986).
10. Reynolds, I. J., J. A. Wagner, S. H. Snyder, S. A. Thayer, B. M. Olivera, and

R. J. Miller. Brain voltage-sensitive calcium channel subtypes differentiated by α-conotoxin fraction GVIA. *Proc. Natl. Acad. Sci. USA* **83**:8804–8807 (1986).
11. McCleskey, W. W., A. P. Fox, D. H. Feldman, L. J. Cruz, B. M. Olivera, R. W. Tsien, and D. Yoshikami. ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not in muscle. *Proc. Natl. Acad. Sci. USA* **84**:4327–4331 (1987).
12. Miller, R. J. Multiple calcium channels and neuronal function. *Science (Wash. D.C.)* **235**:46–52 (1987).
13. Fosset, M., and M. Lazdunski. Biochemical characterization of the skeletal muscle Ca^{2+} channel, in *Structure and Physiology of the Slow Inward Calcium Channel. Receptor, Biochemistry and Methodology.* (J. C. Venter D. J. Triggle, eds.), Vol. 9. Alan R. Liss, Inc., New York, 141–159 (1987).
14. Triggle, D. J., and A. R. Janis. Calcium channel ligands. *Annu. Rev. Pharmacol. Toxicol.* **27**:347–369 (1987).
15. Galizzi, J. P., M. Fosset, G. Romey, P. Laduron, and M. Lazdunski. Neuroleptics of the diphenylbutylpiperidine series are potent calcium channel inhibitors. *Proc. Natl. Acad. Sci. USA* **83**:7513–7517 (1986).
16. Qar, J., J. P. Galizzi, M. Fosset, and M. Lazdunski. Receptors for diphenylbutylpiperidine neuroleptics in brain, cardiac, and smooth muscle membranes. Relationship with receptors for 1,4-dihydropyridines and phenylalkylamines and with Ca^{2+} channel blockade. *Eur. J. Pharmacol.* **141**:261–268 (1987).
17. Chick, W. L., S. Warren, W. Shields, R. N. Chute, A. A. Like, V. Lauris, and K. C. Kitchen. A transplantable insulinoma in the rat. *Proc. Natl. Acad. Sci. USA* **74**:628–632 (1977).
18. Praz, G. A., P. A. Halban, C. B. Wollheim, B. Blondel, A. J. Strauss, and A. E. Renold. Regulation of immunoreactive-insulin release from a rat cell line (RINm5F). *Biochem. J.* **210**:345–352 (1983).
19. Galizzi, J. P., M. Fosset, and M. Lazdunski. Properties of receptors for the Ca^{2+} channel blocker verapamil in transverse-tubule membranes of skeletal muscle. Stereospecificity, effect of Ca^{2+} and other inorganic cations, evidence for two categories of sites and effect of nucleoside triphosphates. *Eur. J. Biochem.* **144**:211–215 (1984).
20. Borsotto, M., J. Barhanin, M. Fosset, and M. Lazdunski. The 1,4-dihydropyridine receptor associated with the skeletal muscle voltage-dependent Ca^{2+} channel. Purification and subunit composition. *J. Biol. Chem.* **260**:14255–14263 (1985).
21. Cognard, C., G. Romey, J. P. Galizzi, M. Fosset, and M. Lazdunski. Dihydropyridine-sensitive Ca^{2+} channels in mammalian skeletal muscle cells in culture: electrophysiological properties and interactions with Ca^{2+} channel activator (Bay K8644) and inhibitor (PN 200-110). *Proc. Natl. Acad. Sci. USA* **83**:1518–1522 (1986).
22. Galizzi, J. P., M. Borsotto, J. Barhanin, M. Fosset, and M. Lazdunski. Characterization and photoaffinity labeling of receptor sites for the Ca^{2+} channel inhibitors *d*-cis-diltiazem, (±)bepridil, desmethoxyverapamil, and (+)PN 200-110 in skeletal muscle transverse tubule membranes. *J. Biol. Chem.* **261**:1393–1397 (1986).
23. Galizzi, J. P., J. Qar, M. Fosset, C. Van Renterghem, and M. Lazdunski. Regulation of calcium channels in aortic muscle cells by protein kinase C activators (diacylglycerol and phorbol esters) and by peptides (vasopressin bombesin) that stimulate phosphoinositide breakdown. *J. Biol. Chem.* **262**:6947–6950 (1987).
24. Hamill, O. P., A. Marty, E. Neher, B. Sackmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
25. Fosset, M., E. Jaimovich, E. Delpont, and M. Lazdunski. [^{3}H]Nitrendipine receptors in skeletal muscle. Properties and preferential localization in transverse tubules. *J. Biol. Chem.* **258**:6086–6092 (1983).
26. Glossmann, H., T. Linn, M. Rombusch, and D. R. Ferry. Temperature-dependent regulation of *d*-cis-diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. *FEBS Lett.* **160**:226–232 (1983).
27. Catterall, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxciol.* **20**:15–43 (1980).
28. Lazdunski, M., C. Frelin, J. Barhanin, A. Lombet, H. Meiri, D. Pauron, G. Romey, A. Schmid, H. Schweitz, P. Vigne, and H. P. M. Vijverberg. Polypeptide toxins as tools to study voltage-sensitive Na^+ channels. *Ann. N. Y. Acad. Sci.* **479**:204–220 (1986).
29. Strichartz, G., T. Rando, and G. K. Wang. An integrated view of the molecular toxicology of sodium channel gating in excitable cells. *Annu. Rev. Neurosci.* **10**:237–267 (1987).
30. Bidard, J.-N., C. Mourre, and M. Lazdunski. Two potent central convulsant peptides, a bee venom toxin, the MCD peptide, and a snake venom toxin, dendrotoxin I, known to block K^+ channels, have interacting receptor sites. *Biochem. Biophys. Res. Commun.* **143**:383–389 (1987).
31. Schmidt, R., H. Betz, and H. Rehm. Inhibition of β-bungarotoxin binding by toxin I, MCD-peptide and EGTA. *Biochemistry* **27**:963–967 (1988).
32. Kao, C. Y., and S. R. Levinson (eds.). Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel. *Ann. N. Y. Acad. Sci.* **479**:1–445 (1986).
33. Borsotto, M., J. Barhanin, R. I. Norman, and M. Lazdunski. Purification of the dihydropyridine receptor of the voltage-dependent Ca^{2+} channel from skeletal muscle transverse tubules using (+)[^{3}H]PN 200-110. *Biochem. Biophys. Res. Commun.* **122**:1357–1366 (1984).
34. Curtis, B. M., and W. A. Catterall. Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse-tubules. *Biochemistry* **23**:2113–2118 (1984).
35. Takahashi, M., M. J. Seagar, J. F. Jones, B. F. X. Reber, and W. A. Catterall. Subunit structure of DHP-sensitive calcium channels from skeletal muscle. *Proc. Natl. Acad. Sci. USA* **84**:5478–5482 (1987).
36. Sharp, A. H., T. Imagawa, A. T. Leung, and K. P. Campbell. Identification and characterization of the dihydropyridine-binding subunit of the skeletal muscle dihydropyridine receptor. *J. Biol. Chem.* **262**:12309–12315 (1987).
37. Vaghy, P. L., J. Striessnig, K. Miwa, H. G. Knaus, K. Itagaki, E. McKenna, H. Glossmann, and A. Schwartz. Identification of a novel 1,4-dihydropyridine and phenylalkylamine binding polypeptide in calcium channel preparations. *J. Biol. Chem.* **262**:14337–14342 (1987).
38. Hosey, M. M., J. Barhanin, A. Schmid, S. Vandaele, J. Ptasienski, C. O'Callahan, C. Cooper, and M. Lazdunski. Photoaffinity labelling and phosphorylation of a 165 kilodalton peptide associated with dihydropyridine- and phenylalkylamine-sensitive calcium channels. *Biochem. Biophys. Res. Commun.* **147**:1137–1145.
39. Tanabe, T., H. Takeshima, A. Milkami, V. Flockerzi, H. Takahashi, K. Kangawa, M. Kojima, H. Matsuo, T. Hirose, and S. Numa. Primary structure of the receptor for calcium channel blockers from skeletal muscle. *Nature (Lond.)* **328**:313–318 (1987).
40. Bean, B. P. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA* **81**:6388–6392 (1984).
41. Lombet, A., M. Fosset, G. Romey, Y. Jacomet, and M. Lazdunski. Identification in mammalian brain of an endogeneous substance with Na^+ channel blocking activities similar to those of tetrodotoxin. *Brain Res.* **417**:327–334 (1987).
42. Fosset, M., H. Schmid-Antomarchi, M. Hugues, G. Romey, and M. Lazdunski. The presence in pig brain of an endogeneous equivalent of apamin, the bee venom peptide that specifically blocks Ca^{2+}-dependent K^+ channels. *Proc. Natl. Acad. Sci. USA* **81**:7228–7232 (1984).

Send reprint requests to: Professor Michel Lazdunski, Centre de Biochimie du CNRS, Parc Valrose, 06034 Nice Cedex, France.