Dihydropyridine binding and Ca^{2+} -channel characterization in clonal calcitonin-secreting cells

Dietmar KRAUTWURST, Hans SCHERÜBL,* Thomas KLEPPISCH,† Jürgen HESCHELER and Günter SCHULTZ‡

Pharmakologisches Institut der Freien Universität Berlin, Thielallee 69/73, D-1000 Berlin 33, Federal Republic of Germany

1,4-Dihydropyridine-sensitive voltage-dependent Ca^{2+} channels play a crucial role in the extracellular Ca^{2+} -sensing of calcitonin-secreting parafollicular cells of the thyroid (C-cells). To characterize the Ca^{2+} channels in C-cells, we studied 1,4-dihydropyridine binding and performed electrophysiological experiments with Ca^{2+} -sensitive C-cells (rat C-cell line rMTC 44-2) in comparison with 'defective' Ca^{2+} -insensitive C-cells (human C-cell line TT). In membranes of rMTC cells, we detected a high-affinity, stereoselective and Ca^{2+} -dependent binding site for the Ca^{2+} -channel-blocking 1,4-dihydropyridine, (+)-[^3H]PN 200-110. Radioligand binding was saturable ($B_{\text{max}} = 18 \pm 2$ fmol/mg of protein), reversible [K_i for (+)-PN 200-110 = 37 ± 1 pM] and allosterically modulated by the phenylalkylamine (–)-desmethoxyverapamil [(–)-D888] as well as the bis-benzylisoquinoline alkaloid (+)-tetrandrine. Thus the 1,4-dihydropyridine binding in rMTC cells featured all

characteristics of binding to the α_1 -subunit of L-type Ca^{2+} channels. In contrast, in membranes of TT cells, which are known to lack Ca^{2+} -sensitivity, no Ca^{2+} -channel-specific (+)-[^3H]PN 200-110 binding was detected. In voltage-clamp experiments, rMTC cells exhibited slowly inactivating Ca^{2+} currents which proved sensitive to (+)-PN 200-110, (–)-D888 and (+)-tetrandrine. These L-type Ca^{2+} -channel blockers did not affect the Ca^{2+} currents in TT cells. The numbers of 1,4-dihydropyridine-sensitive Ca^{2+} channels in rMTC cells as calculated from both the binding studies and the whole-cell/single-channel recordings were 2000 and 7000/cell respectively. Thus qualitative and quantitative detection of 1,4-dihydropyridine-sensitive Ca^{2+} channels by radioligand-binding in Ca^{2+} -sensitive rMTC cells, but not in Ca^{2+} -insensitive TT cells, reflects the electrophysiological detection of functional Ca^{2+} channels in rMTC cells, but not in TT cells.

INTRODUCTION

Calcitonin is known to lower the Ca^{2+} concentration in the blood, and conversely calcitonin secretion from the parafollicular cells of the thyroid (C-cells) is tightly regulated by the extracellular Ca^{2+} concentration (Gagel et al., 1980; Zeytinoglu & DeLellis, 1987). The extracellular Ca^{2+} sensing is mediated by the coupling of changes of the extracellular Ca^{2+} to corresponding changes in the intracellular Ca^{2+} and, thereby, to calcitonin secretion (Fried & Tashjian, 1986). The existence of two C-cell lines with different Ca^{2+} -sensitivity, i.e. the Ca^{2+} -sensitive C-cell line rMTC 44-2 (Gagel et al., 1980) from a rat medullary thyroid carcinoma and the 'defective' Ca^{2+} -insensitive C-cell line TT (Leong et al., 1981; Haller-Brem et al., 1987) from a human medullary thyroid carcinoma, provide an excellent tool to study the crucial role of 1,4-dihydropyridine-sensitive voltage-operated Ca^{2+} channels in the electrosecretory coupling (Cooper et al., 1986; Muff et al., 1988; Raue et al., 1989; Scherübl et al., 1990).

Within the α_1 -subunit of the voltage-dependent L-type Ca^{2+} channel, distinct binding domains for Ca^{2+} -channel blockers from different chemical classes, e.g. 1,4-dihydropyridines, phenylalkylamines and benzothiazepines, are located (for reviews, see Triggle & Janis, 1987; Hosey & Lazdunski, 1988; Glossmann & Striessnig, 1990). These binding domains interact allosterically via a Ca^{2+} -binding site (Glossmann et al., 1985; Staudinger et al., 1991). In endocrine cells, few characterizations of the 1,4-dihydropyridine-binding site of voltage-dependent

Ca^{2+} channels have been reported (Greenberg et al., 1986; Bression et al., 1987; Kunze et al., 1987; Yaney et al., 1991). Comparing the two calcitonin-secreting cells rMTC and TT, we correlated the binding of the dihydropyridine (+)-PN 200-110 and other organic L-type Ca^{2+} -channel blockers with their actions on voltage-dependent Ca^{2+} channels in these cells. In membranes of the Ca^{2+} -sensitive rMTC cells, we characterized voltage-dependent Ca^{2+} channels by their binding properties for the 1,4-dihydropyridine (+)-[^3H]PN 200-110 and by measuring whole cell and unitary currents through 1,4-dihydropyridine-sensitive Ca^{2+} channels; the combination of binding studies and electrophysiological experiments allowed us to estimate the total number of 1,4-dihydropyridine-sensitive Ca^{2+} channels per rMTC cell. In contrast, the Ca^{2+} -insensitive TT cells lacked high-affinity 1,4-dihydropyridine-binding sites as well as 1,4-dihydropyridine-sensitive voltage-dependent Ca^{2+} channels.

MATERIALS AND METHODS

Materials

Dulbecco's modified essential medium (DMEM) was purchased from Biochrom (Berlin, Germany); horse and fetal-calf sera as well as RPMI-1640 medium were from Gibco (Paisley, U.K.). Tetrodotoxin was obtained from Sigma (Deisenhofen, Germany) and ω -conotoxin (GVIA) from Bissendorf (Hannover, Germany). PN 200-110 was a gift from Sandoz (Basel, Switzerland) and (+)-[^3H]PN 200-110 was purchased from NEN/DuPont (Bad

Abbreviations used: (+)-PN 200-110 or (+)-isradipine, (+)-isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; (–)-D888 or (–)-desmethoxyverapamil, 2,7-dimethyl-3-(3,4-dimethoxyphenyl)-3-cyan-7-aza-9-(3-methoxyphenyl)nonane; nimodipine, isopropyl-(2-methoxyethyl)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate; Bay K 8644, methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate; (+)-tetrandrine, (+)-6,6',7,12-tetramethoxy-2,2'-dimethylberbaman.

* Present address: Universitäts-Klinikum Steglitz, Abt. für Gastroenterologie, Hindenburgdamm 30, D-1000 Berlin-45, Federal Republic of Germany.

† Present address: Physiologisches Institut der Humboldt-Universität Berlin, Hessische Strasse 3–4, D-1040 Berlin, Federal Republic of Germany.

‡ To whom correspondence should be addressed.

Homburg, Germany). (+)-Tetrandrine was kindly provided to us by Dr. H.-G. Knaus (Institut für Biochemische Pharmakologie, Universität Innsbruck, Austria), and (–)-D888 was a gift from Dr. T. Schneider, Physiologisch-Chemisches Institut, Universität des Saarlands, Homburg/Saar, Germany.

Cell culture

Calcitonin-secreting cells of the rat cell line rMTC 44-2 (rMTC cells), subcultured to passages 48–59, were grown as monolayers. The culture medium consisted of DMEM supplemented with 15% (v/v) horse serum and 2.5% (v/v) fetal-calf serum. The medium was changed every second day, and cells were split every week. Calcitonin-secreting cells of the human cell line TT (TT cells) were grown in the same way, except for the use of RPMI-1640 medium supplemented with 16% fetal-calf serum.

Preparation of membranes

Pre-confluent rMTC and TT cells were harvested with a cell scraper, collected by centrifugation at 1600 *g* (4 °C, 20 min) and disrupted by nitrogen cavitation in a buffer consisting of (mM) NaCl 100, EDTA 0.5, KH_2PO_4 50 (pH 7.0). Immediately after cell disruption, the EDTA concentration was increased to 3 mM, and 2-mercaptoethanol was added to a final concentration of 15 mM. Nuclei were removed by short centrifugation at 1000 *g* (4 °C), and membranes were sedimented by centrifugation at 30000 *g* (4 °C, 15 min). Total protein was determined as described by Peterson (1983). Membranes were stored at –70 °C in 10 mM triethanolamine/HCl buffer (pH 7.4).

Binding studies on membrane-bound Ca^{2+} channels

Dihydropyridine-binding assays were performed as described by Glossmann and Ferry (1985) with the following modifications. In all experiments membranes were incubated at 37 °C (triplicates) in 0.2 ml of Tris/HCl buffer (50 mM, pH 7.4) containing CaCl_2 (1 mM) and MgCl_2 (1 mM). When using the allosteric regulators (+)-tetrandrine and (–)-D888 the free Ca^{2+} concentration of the incubation buffer was adjusted to 100 nM with EGTA. In competition experiments, the binding of 0.1 nM (+)-[^3H]PN 200-110 to rMTC cell membranes was performed in the presence of increasing concentrations of non-radioactive 1,4-dihydropyridines. All drugs added were diluted in ethanol, and the final concentration of ethanol never exceeded 0.2% (v/v). Non-specific binding was determined in the presence of 2 μM (+)-PN 200-110. When binding equilibrium was reached after 30 min, 2 ml of ice-cold buffer [20 mM Tris/HCl, 10% (w/v) poly(ethylene glycol) 6000, 10 mM MgCl_2] was added to each tube, and samples were rapidly filtered under vacuum through Whatman GF/B glass-fibre filters. Filters were washed twice, dried under vacuum, and the radioactivity was measured by liquid-scintillation counting. The specific binding was at least 80% of the total binding.

Kinetic studies

In association experiments, membranes were incubated in the presence of 0.1 nM (+)-[^3H]PN 200-110 for various periods of time. In dissociation experiments, rMTC membranes were incubated in the presence of 0.1 nM (+)-[^3H]PN 200-110 until equilibrium was achieved. The dissociation of the radioligand was then initiated by addition of 2 μM (+)-PN 200-110 and stopped by rapid filtration at the indicated times.

Electrophysiology

For electrophysiological experiments, C-cells were cultured on small glass slides (density about 10000/cm²) for 2–5 days. Glass slides with adherent cells were transferred into a perfusion chamber, which was mounted on an inverted microscope (Zeiss, Oberkochen, Germany). C-cells had a capacity of 8 ± 2 pF ($n = 30$). Ba^{2+} currents through voltage-dependent Ca^{2+} channels were measured by the patch-clamp technique in the cell-attached and whole-cell configurations (Hamill et al., 1981). For both single-channel and whole-cell experiments, patch electrodes were prepared from glass capillaries (Jencons, Leighton Buzzard, Beds., U.K.) and had an average resistance of 2–5 M Ω (for single-channel recordings, pipettes were fire-polished and coated).

Whole-cell recordings

Whole-cell currents were recorded in a small (0.2 ml) perfusion chamber (5 ml/min, at 36–37 °C). For determination of inward currents, Ba^{2+} was used as charge carrier in a solution (E1) composed of (mM): choline chloride 125, BaCl_2 10, MgCl_2 1, CsCl 5.4, glucose 10, Hepes 10, pH 7.4 with tetraethylammonium hydroxide at 36 °C. The whole-cell configuration was achieved by suction. The experiments under 80 mM Ba^{2+} conditions (compare Figure 7) were carried out at the same temperature as the single-channel recordings (24 ± 1 °C) in a solution (E2) containing (mM): choline chloride 55, BaCl_2 80, MgCl_2 1, CsCl 5.4, glucose 10, Hepes 10, pH 7.4, with tetraethylammonium hydroxide at 24 °C. The pipette solution for all whole-cell recordings (I1) contained (mM): CsCl 120, CsOH 40, MgCl_2 4, Hepes 10 (pH 7.4 with CsOH, 24 °C), Na_2ATP 3. In rMTC cells, an ω -conotoxin-sensitive current may contribute to the observed voltage-activated whole-cell Ca^{2+} -channel current (Scherübl et al., 1991), but neither is a prerequisite for the electrical activity of rMTC cells nor for the activation of calcitonin secretion (H. Scherübl, unpublished work). Therefore, we added 1 μM ω -conotoxin to the pipette solution when measuring whole-cell currents in E2. This pipette solution (I2) additionally contained 3 μM tetrodotoxin in order to block Na^{2+} channels. Access resistance in all experiments was below 50 M Ω , and the Ohmic background conductance was determined by hyperpolarizing pulses (from –60 mV).

Single-channel recordings

Single-channel currents were recorded at room temperature (24 ± 1 °C). The pipette solution (I3) contained (mM): BaCl_2 80, NaCl 50, Na/Hepes 10 (pH 7.4 at 24 °C), 3 μM tetrodotoxin, 1 μM ω -conotoxin and 5 μM Bay K 8644. Cells were incubated in a solution (E3) containing (mM) potassium aspartate 130, MgCl_2 1, glucose 20, EGTA 5 and Hepes 5 (pH 7.4 with KOH) in order to zero the membrane potential.

Data analysis

Binding studies

All concentration–response curves represent computer-derived best fits as calculated by the logistic equation (DeLean et al., 1978). From non-linear regression analysis, the following binding parameters were obtained: the dissociation constant (K_D), the maximum binding (B_{max}), the apparent Hill slope (h) of the inhibition curves, and the IC_{50} and EC_{50} values as the drug concentrations causing 50% inhibition or 50% stimulation respectively. The K_i values were calculated from the Cheng and Prusoff (1973) equation, by using the free concentrations of

radioligand and unlabelled drug. The co-operativity factor a , as well as the value of $\alpha \times K_A$, i.e. the dissociation constant of the allosteric stimulator for the complex of ligand and receptor, were calculated as described by Ehlert (1987). The association constant (k_{+1}) was obtained from linear regression of the transformed data, which were normalized in accordance with the integrated second-order rate equation:

$$[1/(L_0 - R_0)] \times \ln[(L_0 - RL)/(R_0 - RL)] = k_{+1}t + A \quad (1)$$

where L_0 and R_0 are the total concentrations of radioligand and receptor respectively, RL is the receptor-ligand complex at the indicated times, t , and A is

$$[1/(L_0 - R_0)] \times \ln(L_0/R_0) \quad (2)$$

The data from the dissociation kinetics were transformed in accordance with the equation:

$$\ln(B/B_0) = -k_{-1}t \quad (3)$$

where B is the concentration of specifically bound radioligand at the indicated times, t , and B_0 is specific binding at equilibrium (time 0).

If the binding of (+)-[^3H]PN 200-110 to the 1,4-dihydropyridine-binding site follows a 1:1 stoichiometry, the number of binding sites/cell, S_c , can be calculated from the following equation:

$$S_c = (B_{\max.} \times N)/n_c \quad (4)$$

with $B_{\max.}$ as the maximum specific binding, N as Avogadro's number and $n_c = 5 \times 10^6$ as the number of cells/mg of protein (rMTC cells).

All determinations were performed in triplicate in three independent experiments; data are given as means $\pm$ S.D.

Electrophysiology

Single-channel currents were digitized with a sampling rate of 10 kHz. For analysis, the data were filtered at a cut-off frequency of 800 Hz (8-pole low-pass Bessel filter; Frequency Devices, Haverhill, MA, U.S.A.). The open-state probability (p_o) was determined from the mean open time, $p_o = t_o/t$, with t_o as the time of the channel being in the open state and t as the total recording time.

For the demonstration of the current-voltage relations measured under whole-cell conditions, the currents were leakage-compensated and fitted to voltage according to the Boltzmann equation:

$$f_{\text{(current)}} = g \times (V_{\text{test}} - V_{\text{rev}}) / \{1 + \exp[(V_h - V_{\text{test}})/k]\} \quad (5)$$

where g is the relative conductance, V_{test} is the indicated test potential, V_{rev} is the reversal potential, V_h is the potential of half-maximal activation and k determines the slope.

The number, n , of Ca^{2+} channels/cell was estimated from the equation (Kunze et al., 1987):

$$n = I_{\text{wc}} / (p_o \times I_{\text{sc}}) \quad (6)$$

where I_{wc} is the whole-cell current amplitude, p_o is the open-state probability of the single channel and I_{sc} is the single-channel current amplitude.

RESULTS

Saturation experiments

In rMTC cell-membranes, equilibrium binding studies with increasing concentrations of (+)-[^3H]PN 200-110 revealed a high-affinity binding site ($K_D = 38 \pm 9$ pM, $B_{\max.} = 17 \pm 3$ fmol/mg of protein; $n = 3$) and a low-affinity binding site ($K_D = 8 \pm 6$ nM, $B_{\max.} = 135 \pm 36$ fmol/mg of protein) (Figure 1). According to eqn. (4), the number of high-affinity (+)-[^3H]PN 200-110-binding sites/cell was calculated to 2160. In contrast, membranes of TT cells exhibited only a low-affinity binding site ($K_D = 6 \pm 4$ nM, $B_{\max.} = 160 \pm 35$ fmol/mg of protein) (see Figure 1). High-affinity 1,4-dihydropyridine binding to the α_1 -subunit of voltage-dependent Ca^{2+} channels is known to depend

Figure 1 Equilibrium binding of (+)-[^3H]PN 200-110 to C-cell membranes

Saturation binding experiments were carried out on rMTC (●) and TT (○) membranes with increasing concentrations of (+)-[^3H]PN 200-110. Curves represent best fits assuming two classes of binding sites, or a single class of binding sites, for rMTC and TT cell membranes respectively. The inset magnifies the range of the specific (+)-[^3H]PN 200-110 binding to the high-affinity site of rMTC membranes, with $K_D = 0.041$ nM and $B_{\max.} = 13$ fmol/mg protein. Low-affinity binding parameters for rMTC: $K_D = 8$ nM, $B_{\max.} = 140$ fmol/mg of protein; for TT: $K_D = 8$ nM, $B_{\max.} = 160$ fmol/mg of protein. Similar results were obtained with two independent experiments, whose mean values are given in the Results section.

Table 1 (+)-[^3H]PN 200-110 binding parameters in C-cell membranes

All values are given as means $\pm$ S.D. of three experiments. In TT membranes, no significant (+)-[^3H]PN 200-110 high-affinity binding could be detected.

Cells	High affinity					Low affinity	
	k_{+1} ($\text{pM}^{-1} \times \text{min}^{-1}$)	k_{-1} (min^{-1})	K_D^* (pM)	$K_D^\dagger$ (pM)	$B_{\max.}^\dagger$ (fmol/mg)	$K_D^\dagger$ (nM)	$B_{\max.}^\dagger$ (fmol/mg)
rMTC	0.0019 ± 0.0006	0.126 ± 0.006	66 ± 10	38 ± 9	17 ± 3	8 ± 6	135 ± 36
TT	—	—	—	—	—	6 ± 4	160 ± 35

* The kinetic K_D value was calculated from k_{-1}/k_{+1} .

† Values were obtained from non-linear regression analysis of saturation experiments.

Figure 2 Time-dependent association of (+)-PN 200-110 to C-cell membranes

TT (○) and rMTC (●) membranes were incubated in the presence of 0.1 nM (+)-[³H]PN 200-110 for various periods of time. The inset shows the normalized rMTC data according to the second-order rate equation as described in the Materials and methods section. The slope of the line as an estimate for the association constant was 0.0023 pM⁻¹·min⁻¹ (*r* = 0.98). Similar results were obtained with two independent experiments.

Figure 3 Time-dependent dissociation of 0.1 nM (+)-[³H]PN 200-110 from rMTC cell membranes

Dissociation experiments were performed in the absence (●) or presence of 1 μM (+)-tetrandrine (▲) or 1 μM (-)-D888 (■). Data are reported as the natural logarithm of the ratio of ligand bound at the indicated times (*B*) compared with ligand bound at zero time (*B*₀) versus time of ligand dissociation. The dissociation rate constants were: control, *k*₋₁ = 0.133 min⁻¹, *r* = 0.98; (+)-tetrandrine, *k*₋₁ = 0.07 min⁻¹, *r* = 0.98; (-)-D888, *k*₋₁ = 0.373 min⁻¹, *r* = 0.99. Similar results were obtained with two independent experiments.

on the presence of Ca²⁺ (Glossmann & Ferry, 1985). Whereas the *B*_{max} of 17 fmol/mg of protein of the high-affinity binding of (+)-[³H]PN 200-110 to rMTC cell membranes was strongly diminished in a Ca²⁺-free incubation buffer, the low-affinity

Figure 4 Effect of (+)-tetrandrine on specific (+)-PN 200-110 binding

r-MTC cell membranes were equilibrated with 0.1 nM (●) and 1 nM (○) (+)-[³H]PN 200-110 in low-Ca²⁺ incubation buffer in the presence of (+)-tetrandrine at increasing concentrations. Binding (*B*) was assessed relative to untreated controls (*B*₀). The maximal stimulation of specific (+)-[³H]PN 200-110 high-affinity binding by (+)-tetrandrine was 170%, with an EC₅₀ value of 123 nM. Note the absence of a significant stimulation of (+)-[³H]PN 200-110 binding in the low-affinity range. Similar results were obtained with two independent experiments.

binding in both rMTC and TT cell membranes remained unaffected (results not shown; *n* = 3). This suggests that TT cells do not express the 1,4-dihydropyridine-binding site associated with the α₁-subunit of voltage-dependent Ca²⁺ channels (Zernig, 1990). The binding parameters of (+)-[³H]PN 200-110 are summarized in Table 1.

Association and dissociation kinetics

The time course of (+)-[³H]PN 200-110 association to rMTC and TT cell membranes is shown in Figure 2. In rMTC cell membranes, equilibrium binding of 0.1 nM (+)-[³H]PN 200-110 was reached after approx. 20 min with a mean association constant of 1.9 (±0.6) × 10⁻³ pM⁻¹·min⁻¹. No significant association of (+)-[³H]PN 200-110 to TT cell membranes was observed. The dissociation constant (*k*₋₁) was 0.126 ± 0.006 min⁻¹ (Figure 3; see Table 1). It is well known that the dissociation kinetics of 1,4-dihydropyridines are allosterically modulated by phenylalkylamines and benzothiazepines via distinct binding sites on the α₁-subunit of the L-type Ca²⁺ channel. To verify that the high-affinity binding of (+)-[³H]PN 200-110 in rMTC cell membranes reflects the binding to the Ca²⁺-channel protein, we examined the effects of a positive allosteric regulator, the bis-benzylisoquinoline alkaloid (+)-tetrandrine (King et al., 1988), and of a negative allosteric regulator, the phenylalkylamine (-)-D888 (Glossmann et al., 1985; Ruth et al., 1985) on the dissociation kinetics of (+)-[³H]PN 200-110 (see Figure 3). (+)-Tetrandrine is known to interact directly at the benzothiazepine-binding site within the α₁-subunit of the L-type Ca²⁺ channel, increasing the affinity for 1,4-dihydropyridines to their binding site without changing their maximum number (King et al., 1988). In the presence of (+)-tetrandrine (1 μM), we observed a decreased dissociation rate of (+)-[³H]PN 200-110 with *k*₋₁ = 0.066 ± 0.007 min⁻¹, whereas (-)-D888 (1 μM) increased it to 0.33 ± 0.06 min⁻¹ (see Figure 3 and Table 1).

Equilibrium binding studies

As shown in Figure 4, (+)-tetrandrine exclusively stimulated the high-affinity binding of (+)-[³H]PN 200-110 to rMTC cell

Figure 5 Inhibition of specific (+)-[^3H]PN 200-110 binding to rMTC membranes by various 1,4-dihydropyridines

Membranes were equilibrated with 0.1 nM (+)-[^3H]PN 200-110 in the absence or presence of increasing concentrations of (+)-PN 200-110 (●), (-)-PN 200-110 (○) and nimodipine (□). Results are expressed as percentage of specific binding obtained in the absence of unlabelled drugs. The following binding parameters were obtained: (+)-PN 200-110: $\text{IC}_{50} = 0.075$ nM, $K_i = 0.030$ nM, $h = 1.3$; (-)-PN 200-110, $\text{IC}_{50} = 18.49$ nM, $K_i = 7.44$ nM, $h = 0.8$; nimodipine, $\text{IC}_{50} = 0.419$ nM, $K_i = 0.168$ nM, $h = 1.0$. Similar results were obtained with two independent experiments.

membranes in a concentration-dependent manner while leaving the low-affinity binding site unaffected. In three experiments, (+)-tetrandrine stimulated (+)-[^3H]PN 200-110 high-affinity binding by about 170%, with an EC_{50} of 0.1 ± 0.02 μM . K_A was calculated as 0.125 ± 0.1 μM , and α was 0.6 ± 0.01 . We obtained

Table 2 Effects of various 1,4-dihydropyridines on the specific (+)-[^3H]PN 200-110 binding in rMTC membranes

The values are expressed as means $\pm$ S.D. of three independent experiments with 0.1 nM (+)-[^3H]PN 200-110.

	IC_{50} (nM)	K_i (nM)	h
(+)-PN 200-110	0.086 ± 0.015	0.037 ± 0.01	1.2 ± 0.3
(-)-PN 200-110	17.5 ± 1.4	7.5 ± 0.1	1.0 ± 0.3
Nimodipine	0.34 ± 0.1	0.144 ± 0.034	1.2 ± 0.4

similar results (not shown) with the benzothiazepine diltiazem in the presence of 100 nM Ca^{2+} . Neither (+)-tetrandrine nor diltiazem stimulated the (+)-[^3H]PN 200-110 high-affinity binding in the presence of 1 mM Ca^{2+} (results not shown).

The specificity of the (+)-[^3H]PN 200-110 high-affinity binding was further investigated by testing the ability of various 1,4-dihydropyridines to compete with the radioligand for the binding site (Figure 5). Table 2 summarizes the binding parameters for the stereo-isoforms of PN 200-110 and for nimodipine. We calculated the affinity ratio of nimodipine versus (+)-PN 200-110 as 4 and (-)-PN 200-110 versus (+)-PN 200-110 as 203.

Electrophysiological experiments

In rMTC and TT cells, whole-cell voltage-clamp currents through Ca^{2+} channels were recorded during depolarizing voltage-clamp pulses from a holding potential of -80 mV to several test potentials. At 0 mV test potential, we compared the effects of

Figure 6 Current-voltage relations of whole-cell barium currents through Ca^{2+} channels

In TT (a) and rMTC (b-d) cells, currents were measured during sequences of depolarizing voltage-clamp pulses from a holding potential of -80 mV to various test potentials by using the solutions I1 and E1. The current-voltage relations were determined in the absence (●) or presence of 10 μM nickel (○), 1 μM (+)-PN 200-110 (◇), 10 μM (-)-D888 (□) or 10 μM (+)-tetrandrine (△). Curves are best fits according to the Boltzmann function described in the Materials and methods section. The insets show original current recordings at 0 mV test potential with the horizontal bars indicating time (10 ms for a or 50 ms for b-d) and the vertical bars showing the amplitude (50 pA for a or 100 pA for b-d). The broken lines represent the zero-current level. Each experiment shown is representative of six such determinations.

Figure 7 Ca^{2+} -channel currents in rMTC cells with 80 mM Ba^{2+} as charge carrier

(a) Consecutive recordings of single-channel Ca^{2+} currents in a cell-attached patch of a rMTC cell, by using the bath solution E3 and the pipette solution I3 containing 1 μM Bay K 8644, 3 μM tetrodotoxin and 1 μM ω -conotoxin. The currents were evoked by 200 ms pulses from -80 to 0 mV applied at 0.3 Hz. Individual current recordings were leak-subtracted and filtered at 800 Hz. (b) The average current was calculated from 35 sweeps. Similar results were obtained with four cells. (c) Whole-cell currents measured under the same conditions as in (a), by using solutions E2 with 80 mM Ba^{2+} as charge carrier and I2. In the presence of 1 μM Bay K 8644 (BayK) the current amplitude is increased about 3-fold compared with control currents (con). Similar currents were recorded in 10 different cells. The zero-current level is indicated by broken lines.

Ni^{2+} , (+)-PN 200-110, (-)-D888 and (+)-tetrandrine on Ba^{2+} currents through Ca^{2+} channels (Figure 6). rMTC cells displayed slowly inactivating Ba^{2+} currents with an amplitude of 46 ± 7 pA/pF, which were insensitive to 10 μM Ni^{2+} but were blocked by $33 \pm 8\%$ with (+)-PN 200-110 (1 μM , $n = 6$). (+)-Tetrandrine (10 μM) and (-)-D888 (10 μM) blocked the voltage-activated Ba^{2+} current in rMTC cells by $40 \pm 10\%$ and $36 \pm 7\%$ respectively ($n = 6$; see Figure 6), but failed to affect the Ba^{2+} current in TT cells (results not shown). In contrast, TT cells exhibited fast-inactivating Ba^{2+} currents with an amplitude of 12 ± 1 pA/pF, which were dihydropyridine-insensitive but could be blocked by $58 \pm 7\%$ with Ni^{2+} (10 μM , $n = 6$).

In order to determine the number of functional 1,4-dihydropyridine-sensitive Ca^{2+} channels in rMTC cells, we recorded single Ca^{2+} channels in the cell-attached configuration. Under conditions where Na^{+} channels and ω -conotoxin-sensitive Ca^{2+} channels were blocked (see the Materials and methods section), single Ca^{2+} -channel openings were seen in response to depolarizing pulses from -80 to 0 mV (Figure 7). The amplitude of the currents in the presence of 80 mM Ba^{2+} amounted to 1.3 ± 0.1 pA, and the open-probability of an observed single channel was determined as 0.04 ± 0.01 ($n = 4$). The slow activation of the whole-cell current measured in the presence of high Ba^{2+} is supposed to be due to the right-shifted activation potential relationship with maximum currents at about +30 mV (results not shown; see also McDonald et al., 1986; Hille, 1992). Whole-cell recordings performed under similar conditions to

those for single-channel recordings revealed a current density of 14 ± 8 pA/pF in control cells, which amounted to 47 ± 18 pA/pF with 1 μM Bay K 8644 ($n = 10$). From eqn. (6), with $I_{\text{wc}} = 376$ pA, $p_o = 0.04$ and $I_{\text{sc}} = 1.3$ pA, we obtained a total number of channels/cell, $n = 7230$.

DISCUSSION

In the present study, we characterized voltage-dependent Ca^{2+} channels in the calcitonin-secreting cells rMTC 44-2 and TT by 1,4-dihydropyridine binding as well as by whole-cell and single-channel patch-clamp experiments. In rMTC, but not in TT, cell membranes we detected high-affinity, low-capacity and stereoselective (+)-[^3H]PN 200-110 binding which was allosterically modulated and Ca^{2+} -dependent. In rMTC cell membranes, the B_{max} of the high-affinity site amounted to 17 fmol/mg of protein, and the kinetically determined dissociation constant K_D was 66 pM, which is in good agreement with the measured equilibrium dissociation constant $K_D = 38$ pM. Our data are corroborated by results from (+)-[^3H]PN 200-110 binding to membranes of other endocrine cells such as rat and human pituitaries, PC-12 and RINm5F cells with K_D values of 38–410 pM and B_{max} of 30–110 fmol/mg of protein (Greenberg et al., 1986; Bression et al., 1987; Kunze et al., 1987; Yaney et al., 1991). In our hands, the high-affinity (+)-[^3H]PN 200-110 binding site in rMTC cell membranes exclusively displayed sensitivity to the heterotropic allosteric modulators (-)-D888 and (+)-tetrandrine as well as to Ca^{2+} . Thus the (+)-[^3H]PN 200-110 high-affinity binding site in rMTC membranes features all characteristics of the binding site on the α_1 -subunit of the L-type Ca^{2+} channel (for reviews see Triggle & Janis, 1987; Hosey & Lazdunski, 1988; Glossmann & Striessnig, 1990). The 'endocrine type' (+)-[^3H]PN 200-110 binding sites displayed lower capacity and higher affinity than those in non-endocrine tissues such as smooth, skeletal and cardiac muscle and brain (Schwartz et al., 1985; Hamilton et al., 1986; Glossmann & Striessnig, 1988; Dacquet et al., 1989; Porzig, 1990; Dunn & Bladen, 1991; Hescheler et al., 1991).

Additionally, membranes of both rMTC and TT cells displayed low-affinity (+)-[^3H]PN 200-110 binding sites with similar K_D values, around 160 nM. Although several groups reported on functionally relevant low-affinity (0.01–1 μM) dihydropyridine-binding sites in membranes of skeletal and cardiac muscle cells (Vaghy et al., 1985; Brown et al., 1986; Rogart et al., 1986; Dunn & Bladen, 1991), in our experiments this low-affinity binding was Ca^{2+} -independent and not affected by (+)-tetrandrine. Therefore we do not consider it as binding to the α_1 -subunit of the L-type Ca^{2+} channel, but rather to other structures such as nucleoside transporters in the plasma membrane, as described by Glossman et al. (1989) and Zernig (1990). Furthermore, low-affinity 1,4-dihydropyridine-binding sites may include Na^{+} or K^{+} channels (Yatani and Brown, 1985; Hume, 1985).

To confirm the differential expression of voltage-dependent Ca^{2+} channels in rMTC and TT cells as revealed by our binding studies, we used the same organic Ca^{2+} -channel blockers to characterize whole-cell Ca^{2+} currents in these cells. Under voltage-clamp conditions, the fast-inactivating Ca^{2+} -channel current displayed in TT cells (Scherübl et al., 1990) was insensitive to (+)-PN 200-110, (-)-D888 and (+)-tetrandrine. In contrast, the slowly inactivating Ca^{2+} -channel current in rMTC cells was partially blocked by the organic L-type Ca^{2+} -channel blockers. However, the concentration of (+)-PN 200-110 (1 μM) which we needed to block currents by about 40% in rMTC cells is several orders of magnitude higher than K_D and K_i values for this drug observed in the binding studies. This discrepancy is commonly

explained by the voltage-dependence of the Ca^{2+} -channel block by dihydropyridines (Sanguinetti & Kass, 1984; Hamilton et al., 1986), resulting in binding with higher affinity to the inactivated Ca^{2+} channel than to rested channels (Bean, 1984). In addition, in rMTC cells a 1,4-dihydropyridine-insensitive but ω -conotoxin-insensitive current contributes to the Ca^{2+} -channel current observed in whole-cell voltage-clamp experiments (Scherübl et al., 1991).

The combination of radioligand binding and electrophysiological measurements allowed us to compare the numbers of Ca^{2+} -channel-related 1,4-dihydropyridine-binding sites and functional channels. To estimate the total number of functional 1,4-dihydropyridine-sensitive Ca^{2+} channels, we performed both whole-cell and single-channel voltage-clamp experiments in rMTC cells under similar conditions. We calculated the total number of 1,4-dihydropyridine-sensitive Ca^{2+} channels to about 7000/cell, which number is in reasonable agreement with about 2000 (+)-[^3H]PN 200-110 binding sites/cell. These results provide strong evidence for a close relation of high-affinity (+)-[^3H]PN 200-110 binding sites and functional Ca^{2+} channels, and are in line with calculated values of 1200–6000 channels derived from similar experiments with PC-12 cells (Greenberg et al., 1986; Messing et al., 1986; Kunze et al., 1987; Porzig, 1990). From the binding data and the electrophysiological measurements, we calculated the mean density of 1,4-dihydropyridine-sensitive Ca^{2+} channels in rMTC cells as 2.5 and 8.5 channels per μm^2 membrane area, respectively. The Ca^{2+} -channel density in rMTC cells therefore equals that in ventricular cells of adult guinea pigs with 0.5–5 channels per μm^2 membrane area. However, a difference should be pointed out when comparing rMTC cells with heart or skeletal-muscle cells, where Ca^{2+} -channel-related 1,4-dihydropyridine binding largely exceeds the number of functional channels (Schwartz et al., 1985; Hamilton et al., 1986); more than one 1,4-dihydropyridine binding site associated with one Ca^{2+} -channel molecule has been postulated by several groups (Green et al., 1985; Rogart et al., 1986; Schilling & Drewe, 1986; Lee et al., 1987; Dunn & Bladen, 1991), although this assumption may not hold true.

The expression of completely different voltage-operated Ca^{2+} channels in rMTC and TT cells is reflected by the differing Ca^{2+} -sensitivity of calcitonin secretion from both cell types. Whereas the 1,4-dihydropyridine-sensitive Ca^{2+} channel of rMTC cells works as a Ca^{2+} sensor regulating Ca^{2+} -induced calcitonin secretion (Raue et al., 1989; Scherübl et al., 1990), the fast inactivating Ca^{2+} current observed in TT cells appears to be insufficient to regulate calcitonin secretion in response to Ca^{2+} (Haller-Brem et al., 1987). The exclusive expression of slowly rather than fast-inactivating voltage-dependent Ca^{2+} channels in these two C-cell lines offers a fascinating opportunity to clarify the molecular diversity of voltage-dependent Ca^{2+} channels and their roles in the regulation of cellular functions.

We are grateful to Mrs. Inge Reinsch and Mrs. Monika Bigalke for help with the cell culture, to Wolfgang Stamm for expert technical assistance and to Dr. Rudolf Schubert for helpful discussion. This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

- Bean, B. P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6388–6392
- Bression, D., Chaumet-Riffaud, P., Brandi, A. M., Comte, A., Peillon, F. and Kiechel, J. R. (1987) *Mol. Cell. Endocrinol.* **50**, 255–261
- Brown, A. M., Kunze, D. L. and Yatani, A. (1986) *J. Physiol. (London)* **379**, 459–514
- Cheng, Y.-C. and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108
- Cooper, C. W., Borosky, S. A., Farrell, P. E. and Steinsland, O. S. (1986) *Endocrinology (Baltimore)* **118**, 545–549
- Dacquet, C., Loirand, G., Rakotoarisoa, L., Mironneau, C. and Mironneau, J. (1989) *Br. J. Pharmacol.* **97**, 256–262
- DeLean, A., Munson, P. J. and Rodbard, D. (1978) *Am. J. Physiol.* **235**, E97–E102
- Dunn, S. M. J. and Bladen, C. (1991) *Biochemistry* **30**, 5716–5721
- Ehlert, F. J. (1987) *Mol. Pharmacol.* **33**, 187–194
- Fried, R. M. and Tashjian, A. H., Jr. (1986) *J. Biol. Chem.* **261**, 7669–7674
- Gagel, R. F., Zeytinoglu, F. N., Voelkel, E. F. and Tashjian, A. H., Jr. (1980) *Endocrinology (Baltimore)* **107**, 516–523
- Glossmann, H. and Ferry, D. R. (1985) *Methods Enzymol.* **109**, 513–550
- Glossmann, H. and Striessnig, J. (1988) *ISI Atlas Sci. Pharmacol.* **2**, 202–210
- Glossmann, H. and Striessnig, J. (1990) *Rev. Physiol. Biochem. Pharmacol.* **114**, 1–106
- Glossmann, H., Ferry, D. R., Goll, A., Striessnig, J. and Schober, M. (1985) *J. Cardiovasc. Pharmacol.* **7** (Suppl. 6), S20–S30
- Glossmann, H., Zernig, G., Graziadei, I. and Moshhammer, T. (1989) in *Nimodipine and Central Nervous System Function* (Traber, J. and Gispén, W. H., eds.), pp. 51–67, Schattauer-Verlag, Stuttgart and New York
- Green, F. J., Farmer, B. B., Wiseman, G. L., Jose, M. J. and Watanabe, A. M. (1985) *Circ. Res.* **56**, 576–585
- Greenberg, D. A., Carpenter, C. L. and Messing, R. O. (1986) *J. Pharmacol. Exp. Ther.* **238**, 1021–1027
- Haller-Brem, S., Muff, R., Petermann, J. B., Born, W., Roos, B. A. and Fischer, J. A. (1987) *Endocrinology (Baltimore)* **121**, 1272–1277
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. and Sigworth, J. (1981) *Pflügers Arch.* **391**, 85–100
- Hamilton, S. L., Yatani, A., Brush, K., Schwartz, A. and Brown, A. M. (1986) *Mol. Pharmacol.* **31**, 221–231
- Hescheler, J., Meyer, R., Plant, S., Krautwurst, D., Rosenthal, W. and Schultz, G. (1991) *Circ. Res.* **69**, 1476–1486
- Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd edn., pp. 457–470, Sinauer Press, Sunderland, MA
- Hosey, M. M. and Lazdunski, M. (1988) *J. Membr. Biol.* **104**, 81–105
- Hume, J. R. (1985) *J. Pharmacol. Exp. Ther.* **234**, 134–140
- King, F. V., Garcia, M. L., Himmel, D., Reuben, J. P., Lam, Y. T., Pan, J., Han, G. and Kaczorowski, J. (1988) *J. Biol. Chem.* **263**, 2238–2244
- Kunze, D. L., Hamilton, S. L., Hawkes, M. J. and Brown, A. M. (1987) *Am. J. Physiol.* **31**, 401–409
- Lee, T. R., Smith, T. W. and Marsh, J. D. (1987) *Circ. Res.* **60**, 683–691
- Leong, S. S., Horoszewicz, J. S., Shimaoka, K., Friedmann, M., Kawinski, E., Song, M. J., Zeigel, R., Chu, T. M., Baylin, S. and Mirand, E. A. (1981) in *Advances in Thyroid Neoplasia* (Andreoli, M., Monaco, F. and Robbins, J., eds.), pp. 95–108, Field Educational Italia, Rome
- McDonald, T. F., Cavallie, A., Trautwein, W. and Pelzer, D. (1986) *Pflügers Arch.* **406**, 437–448
- Messing, R. O., Carpenter, C. L. and Greenberg, D. A. (1986) *Biochem. Biophys. Res. Commun.* **136**, 1049–1056
- Muff, R., Nemeth, E. F., Haller-Brem, S. and Fischer, J. A. (1988) *Arch. Biochem. Biophys.* **265**, 128–135
- Peterson, G. L. (1983) *Methods Enzymol.* **91**, 95–119
- Porzig, H. (1990) *Rev. Physiol. Biochem. Pharmacol.* **114**, 209–262
- Raue, F., Serve, H., Grauer, A., Rix, E., Scherübl, H., Schneider, H. G. and Ziegler, R. (1989) *Klin. Wochenschr.* **67**, 635–639
- Rogart, R. B., DeBruyn Kops, A. and Dzau, V. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7452–7456
- Ruth, P., Flockerzi, V., von Nettelbladt, E., Oeken, J. and Hofmann, F. (1985) *Eur. J. Biochem.* **150**, 313–322
- Sanguinetti, M. C. and Kass, R. S. (1984) *Circ. Res.* **55**, 336–348
- Scherübl, H., Schultz, G. and Hescheler, J. (1990) *FEBS Lett.* **273**, 51–54
- Scherübl, H., Schultz, G. and Hescheler, J. (1991) *Mol. Cell. Endocrinol.* **82**, 293–301
- Schilling, W. P. and Drewe, J. R. (1986) *J. Biol. Chem.* **261**, 2750–2758
- Schwartz, L. M., McCleskey, E. W. and Almers, W. (1985) *Nature (London)* **314**, 747–751
- Staudinger, R., Knaus, H.-G. and Glossmann, H. (1991) *J. Biol. Chem.* **266**, 10787–10795
- Triggie, D. J. and Janis, R. A. (1987) *Annu. Rev. Pharmacol. Toxicol.* **27**, 347–369
- Vaghy, P. L., Galube, I., Grupp, G., Grupp, J. and Williams, J. (1985) *Bayer-Symp. 9: Cardiovascular Effects of Dihydropyridine-Type Calcium Antagonist and Agonists*, pp. 156–184, Springer-Verlag, Berlin
- Yaney, G. C., Stafford, G. A., Henstenberg, J. D., Sharp, G. W. G. and Weiland, G. A. (1991) *J. Pharmacol. Exp. Ther.* **258**, 652–662
- Yatani, A. and Brown, A. M. (1985) *Circ. Res.* **56**, 868–875
- Zernig, G. (1990) *Trends Pharm. Sci.* **11**, 38–44
- Zeytinoglu, F. N. and DeLellis, R. (1987) *Endocrinology (Baltimore)* **121**, 352–360