¹²⁵I-Labelled mapacalcine: a specific tool for a pharmacological approach to a receptor associated with a new calcium channel on mouse intestinal membranes

Patricia VIDALENC, Jean-Luc MOREL, Jean MIRONNEAU and Michel HUGUES¹

Centre National de la Recherche Scientifique, ESA 5017, Physiopathologie et Pharmacologie Vasculaire, Faculté de Pharmacie, Université Victor Segalen (Bordeaux 2), 146 rue Léo Saignat, 33076 Bordeaux Cedex, France

Mapacalcine is a small protein ($M_r = 19041$) composed of two homologous chains purified from the marine sponge *Cliona vastifica*. Recently, we demonstrated that it was able to specifically block a Ca^{2+} channel which could not be related to already described channels on mouse intestinal myocytes. This Ca^{2+} current was insensitive to the known peptidic and organic calcium channel blockers. Mapacalcine was ineffective on T-type and L-type Ca^{2+} currents present on rat portal vein myocytes [Morel, Drobecq, Sautière, Tartar, Mironneau, Qar, Lavie, and Hugues (1997) Mol. Pharmacol. 51, 1042–1052]. We report here the

preparation and purification of a monoiodo-derivative of mapacalcine which retains its biological properties. Binding parameters of mapacalcine to its receptors have been characterized on mouse intestinal membranes. It binds to its receptors with a $K_d = 0.8$ nM, and a maximal binding capacity of 171 fmol/mg of protein on membrane preparations. Our data show that we have prepared a tool that is usable for pharmacological studies of a receptor associated with a new type of calcium channel for which no ligand was available until now.

INTRODUCTION

Calcium channels play an essential role in biological functions such as muscle contraction, neurotransmission or hormone release and cell signalling or spike firing. The recent contribution of molecular biology techniques has dramatically increased the diversity of calcium channels known, but for a great number of them no pharmacological tools are available. Animal toxins have become essential tools, allowing identification of multiple classes of calcium channels [1]. Pharmacological and biochemical studies of the biological target of natural peptides are easier when labelled derivatives of these peptides are available. Knowledge of a wide variety of ionic channels, such as sodium channels [2,3], L- or N-type calcium channels [1,4] and calcium-dependent potassium channels [5–8], has been greatly improved by the use of labelled derivatives of their natural ligands. A specific marker of an important biological target such as calcium channels also represents an invaluable tool for screening molecule banks to discover new compounds usable for therapeutic applications. Recently, we have described the existence of a new type of calcium channel in mouse intestinal myocytes [9]. These channels are low-voltage activated and are insensitive to the pharmacological agents known to specifically block L-, N-, P- or Q-type calcium channels. Moreover, electrophysiological properties of these channels are different from those already described for the known calcium channels. In [9] we also reported the purification and characterization, from marine sponge, of a dimeric ($M_r = 19041$) protein, mapacalcine, which is a specific inhibitor of this new type of calcium channel. Mapacalcine was shown to be ineffective on T- and L-type calcium currents presents in rat portal vein myocytes [9]. In the present paper we report the preparation and purification of a monoiodo derivative of mapacalcine that can be labelled at 2200 Ci/mmol. The biological and pharmacological properties of the iodinated mapacalcine are not affected by the labelling procedure. The ¹²⁵I-labelled mapacalcine has been used to characterize the biochemical properties of its interaction with its receptors on mouse intestinal membranes.

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EXPERIMENTAL PROCEDURES

Materials

Mapacalcine ($M_r = 19041$) was purified from *Cliona vastifica* sponge as previously described [9]. Collagenase (E.C. 2.4.24.3) was from Worthington (Freehold, NJ, U.S.A.). Pronase (E.C. 3.4.24.31), bovine serum albumin, tetrodotoxin and amiloride were from Sigma (St Louis, MO, U.S.A.). Charybdotoxin, scyllatoxin, iberiotoxin, MCD peptide, ω -conotoxin GVIA, ω -agatoxin IVA and ω -conotoxin MVIIC were from Latoxan (Rosan, France). Fenoverine was from Delalande (Paris, France). Desmethoxyverapamil was from Knoll (Ludwigshafen, Germany). Oxodipine and elgodipine were a gift from Dr. A. Galiano (IQB, Madrid, Spain). Diltiazem was from Synthelabo (Paris, France). Iodo Beads (N-chlorobenzene sulphonamide derivatized uniform non-porous polystyrene beads) were purchased from Pierce (Interchim, France).

Iodination of mapacalcine

Mapacalcine was iodinated under two different sets of experimental conditions. Large amounts of toxin were iodinated at low specific radioactivity or with non-radioactive iodine (¹²⁷I)Na

Abbreviations used: $[Ca^{2+}]_i$, intracellular calcium concentration; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed.

to characterize the molecular and physiological properties of the labelled toxin. On the other hand, small amounts of mapacalcine were iodinated with [125 I]Na to obtain a monoiodo derivative of very high specific radioactivity, suitable for binding experiments.

Iodination at low specific radioactivity or with [127 I]Na

Chromatographic properties of the native mapacalcine were first determined by injecting the toxin on an HPLC device (Beckman Biosys 510) using a reverse-phase C8 column (Lichrosorb 100 RP 8, Merck, 4×250 mm) equilibrated with water containing 0.1 % trifluoroacetic acid (TFA). The column was then eluted at 1 ml/min using a gradient of from 10 to 60 % acetonitrile containing 0.1 % TFA during 62.5 min. Iodination was performed by incubating 15.7 nmol of NaI at 1 Ci/mole with two Iodo Beads in 50 μ l of 1 M Tris/HCl, pH 7.4 (final volume of 500 μ l) during 10 min at room temperature. Equivalent molar amounts of mapacalcine were then added and incubated during 20 min. Separation of the iodination mixture was achieved using the chromatographic conditions described above for the native toxin. Fractions were collected at one minute intervals in tubes containing 50 μ l of 1 M Tris/HCl buffer, pH 7.4. The chromatogram was monitored at 280 nm. The concentration of iodine present in each labelled fraction was estimated by measuring the radioactivity contained in these fractions with a Riastar (Packard) gamma counter, knowing its specific activity (1 Ci/mol). Parallel measurements of the mapacalcine concentration in each fraction were achieved by measuring the absorbance contained in the fractions (using ϵ_m at 280 nm = 24200, determined by measurement of the absorbance (A) at 280 nm of a solution of mapacalcine with a known toxin concentration ($\hat{C}$), according to the relation $\epsilon_m = A/(Cl)$ where $l = 1$ cm). The number of iodine per mapacalcine molecules was estimated by calculating the ratio between the concentration of iodine versus the concentration of mapacalcine for each fraction obtained. Mapacalcine was also iodinated using the non-radioactive isotope of iodine (127 I). The non-radioactive iodinated mapacalcine was obtained by the purification procedure described above and used for competition experiments. Biological activity of the iodinated toxin was assayed by electrophysiological experiments in mouse intestinal myocytes.

Iodination at high specific radioactivity

Experimental conditions were similar to those used for low specific activity radiolabelling. Some 0.52 nmol of [125 I]Na (2200 Ci/mmol) were incubated with one Iodo Bead and 20 μ l of 1 M Tris/HCl buffer, pH 7.4, during 10 min (200 μ l, final volume). Subsequently, 10 μ g (0.52 nmol) of mapacalcine were added and incubated for 20 min at room temperature. The reaction mixture was separated using the protocol described above for the low-radioactivity labelled toxin. The fractions containing the iodo derivative of mapacalcine were stored at 4 °C and used for binding experiments.

Intestinal membrane preparation

Swiss mice (20–25 g) were killed by cervical dislocation. Small intestines were removed and rinsed with ice-cold buffer containing 5 mM Tris/HCl at pH 7.4, 140 mM NaCl and 0.1 mM PMSF. The intestines were then opened and scraped to remove the epithelial cell layer. Intestinal smooth muscles were diluted 10-fold (w/v) with the washing buffer, homogenized for 30 s in a Waring Blendor apparatus, then twice for 30 s with a Polytron homogenizer. Homogenate was centrifuged at 4500 *g* for 7 min. The

supernatant was then centrifuged for 45 min at 140000 *g*. All steps were performed at 4 °C. The pellets were resuspended in the washing buffer and frozen at -80 °C until use. Proteins were measured with Bio-Rad proteins assay reagent using lysozyme as standard.

Patch-clamp experiments

The physiological properties of monoiodo-mapacalcine were tested and compared with those of native mapacalcine using the patch-clamp technique [9].

Cell preparation

The longitudinal layer of the duodenal smooth muscle from Swiss mice was cut into several pieces and incubated for 10 min in a low Ca^{2+} (40 μ M) physiological solution. Then, 0.8 mg/ml collagenase, 0.2 mg/ml pronase E and 1 mg/ml bovine serum albumin were added and the mixture was maintained at 37 °C for 20 min. The solution was then removed and the pieces of mouse duodenum incubated under the same conditions for a further 20 min period. Tissues were then placed in enzyme-free solution and dissociated using a fire-polished Pasteur pipette to release cells. Cells were maintained in short-term primary culture in M199 medium containing 10 % fetal calf serum, 1 mM glutamine, 2 mM pyruvate, 20 units/ml penicillin and 20 μ g/ml streptomycin. Cells were maintained in culture at 37 °C in an incubator under a controlled atmosphere (5 % CO_2) and used for electrophysiology within 36 h.

Patch-clamp measurement

Voltage-clamp and membrane current recordings were made with a standard patch-clamp technique using a List EPC-7 patch-clamp amplifier. The whole-cell recording mode was performed with patch pipettes of 1–3 M Ω resistance. Membrane potential and current records were stored and analysed using an IBM-PC computer (P-clamp system).

Solutions

The normal physiological solution contained (mM): 130 NaCl, 5.6 CsCl, 1 MgCl₂, 2 CaCl₂, 11 glucose, 10 Hepes/NaOH at pH 7.4 and 0.05 % bovine serum albumin. The basic pipette solution contained (mM): 130 CsCl, 10 Hepes/CsOH, pH 7.3. For calcium current recordings, CsCl was used instead of KCl in the pipette and external solutions to block outward potassium currents. In addition, 10 mM EGTA, 5 mM Na₂ATP and 1 mM MgCl₂ were added to the basic pipette solution. Iodinated and native mapacalcine were applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the records. In addition, labelled and native mapacalcine were also tested by adding them to the perfusion solution. Experiments were conducted at 26 ± 1 °C.

Binding experiments

The standard incubation buffer (1 ml final volume) consisted of (mM): 50 Tris/HCl, pH 7.4, 130 NaCl, 5.6 KCl, 2 CaCl₂, 0.24 MgCl₂, 11 glucose and 0.05 % bovine serum albumin, this buffer corresponded to the one used for electrophysiological assays. Glass fibre filters (Whatman GF/C) used for the binding experiments were incubated for 1 h in 10 mM Tris/HCl at pH 7.4 (washing buffer) and washed once with 5 ml of the same buffer at 4 °C just before use. Binding experiments were performed by

filtering duplicate aliquots (450 μl) of the incubation medium. Filters were rapidly washed twice with 5 ml of the washing buffer. The radioactivity retained by the filters was then counted. Non-specific binding was determined in parallel experiments in the presence of an excess of unlabelled mapacalcine (1 μM) and subtracted from total binding to obtain the specific binding.

Kinetic binding studies

Association and dissociation kinetics were measured in standard binding buffer at 4 °C. In experiments utilizing a single ligand concentration, incubations contained 0.3 nM [^{125}I]mapacalcine and a protein concentration of 0.5 mg/ml. Under these conditions, the concentration of free ligand available for binding could be assumed to be constant in each assay during the time course of experiments. In a series of experiments, association was measured at 26 °C (temperature used for patch-clamp measurement).

Association kinetics were initiated by addition of membranes and bound mapacalcine was measured at different times by the filtration of 450 μl aliquots according to the filtration technique described above. After equilibrium was achieved, dissociation was initiated by the addition of excess unlabelled mapacalcine (1 μM), preventing re-association of labelled ligand. Part of the binding mixture did not receive unlabelled toxin to assess the stability of binding which remained stable throughout the course of dissociation. Dissociation of labelled toxin was followed by measurement of the decrease in bound [^{125}I]mapacalcine by the filtration technique. Association kinetics utilizing multiple ligand concentrations were performed as described above except that mapacalcine concentration varied from 0.3 to 3.4 nM. Non-specific binding was determined in parallel experiments conducted in the presence of 1 μM native mapacalcine. Specific binding was obtained by subtracting non-specific binding from the total binding.

Equilibrium binding experiments

Intestinal membranes (0.5 mg protein/ml) were incubated with increasing concentrations of [^{125}I]mapacalcine for 120 min at 4 °C. Duplicate aliquots (450 μl) were then filtered and the bound radioactivity was measured as described above.

Competition experiments between [^{125}I]mapacalcine, native mapacalcine and [^{127}I]mapacalcine

Mouse intestinal membranes (0.5 mg protein/ml) were incubated for 120 min at 4 °C with 0.3 nM [^{125}I]mapacalcine and various concentrations of unlabelled mapacalcine or [^{127}I]mapacalcine. The amount of [^{125}I]mapacalcine remaining bound to intestinal membranes in the presence of native or [^{127}I]mapacalcine was estimated as described above. Competition experiments between [^{125}I]mapacalcine and the native toxin have also been performed at 26 °C.

Competition experiments between [^{125}I]mapacalcine and different drugs

Intestinal membranes (0.5 mg protein/ml) were incubated for 120 min at 4 °C in the standard incubation buffer with 0.3 nM [^{125}I]mapacalcine and 1 μM of the different drugs tested (except for iberiotoxin: 0.1 μM). The amount of [^{125}I]mapacalcine that remained bound in the presence of the tested drugs was estimated as described above.

Competition experiments between [^{125}I]mapacalcine, Ca^{2+} , Mg^{2+} , Na^{+} and K^{+}

In these experiments, intestinal membranes (0.5 mg protein/ml) were incubated for 120 min at 0 °C with 0.3 nM [^{125}I]mapacalcine in a buffer containing 50 mM Tris/HCl, pH 7.4, 0.1 % bovine serum albumin and various concentrations of the cation tested. Free calcium concentrations were obtained using a calcium/EGTA buffer [10]. The amount of [^{125}I]mapacalcine that remained bound in the presence of different cations was estimated as described above.

Data analysis

The results are expressed as means $\pm$ S.E.M. Competition and Michaelis curves were analysed by a non-linear least-squares fitting program according to models involving one or two binding sites. Linear plots were analysed by a linear least-squares fitting program.

RESULTS

Iodination of mapacalcine

Mapacalcine is a dimeric protein ($M_r = 19041$) composed of two identical chains. Each chain contains 89 amino acids, including nine cysteines. There are 10 tyrosines in the mapacalcine sequence, which represent potent iodination sites. To characterize the iodinated products obtained after the labelling reaction of mapacalcine, 300 μg of toxin were iodinated with a mixture of [^{127}I]Na and radioactive [^{125}I]Na at low specific radioactivity (1 Ci/mol). The chromatogram obtained after purification of the iodination mixture on reverse-phase HPLC demonstrated a single peak with a retention time of 39.5 min (not shown); all the radioactivity eluted under this peak. The native mapacalcine injected under the same conditions was eluted earlier than the labelled mapacalcine (retention time: 34 min). In some experiments, the low specific radioactivity labelled mapacalcine was evaporated, mixed with 150 μg of native mapacalcine and resubmitted to the purification procedure. The chromatogram obtained demonstrated the presence of two peaks, with retention times of 34 and 39.5 min, corresponding to the retention times of the native mapacalcine and of the labelled material eluted after iodination, respectively (results not shown). These data taken together with the absence of native mapacalcine on the chromatogram obtained after injection of the iodination mixture suggest that all the toxin was iodinated. The concentration ratio [^{125}I]/[toxin] obtained as described in the Experimental procedures section was 1.20 ± 0.23 ($n = 4$), suggesting that mapacalcine was mono-iodinated. The fractions corresponding to the peak of iodinated mapacalcine at low specific radioactivity were used to test the biological properties of the labelled toxin by the patch-clamp technique in mouse intestinal myocytes.

In another series of experiments, mapacalcine was iodinated with [^{125}I]Na at high specific activity (2200 Ci/mmol) as the sole source of iodine. The iodination mixture obtained was purified under the same conditions as used for iodination at low specific activity. The chromatogram obtained demonstrated a labelled peak which eluted at the same retention time as the peak of monoiodo-mapacalcine obtained after low specific radioactivity labelling (results not shown). The radioactive peak was then identified as the monoiodo-mapacalcine and used for binding experiments. The specific radioactivity of monoiodo-mapacalcine obtained after purification on reverse-phase HPLC was equal to

Figure 1 Effect of monoiodo-mapacalcine on the oxodipine-resistant Ca^{2+} current on mouse intestinal myocytes

Ca^{2+} current was induced by depolarization from a holding potential of -80 to 0 mV, at 0.1 Hz, in the presence of $1 \mu\text{M}$ oxodipine; Ca^{2+} currents, expressed as a fraction of their control values (I/I_c), were obtained in the control condition (open columns) and during application of the toxin for 3 min (hatched columns). Numbers in parentheses indicate the number of cells tested. Inset (A) effect of the native mapacalcine; Ca^{2+} currents under control conditions (a) and in the presence of $0.1 \mu\text{M}$ mapacalcine for 3 min (b). (B) Effect of the iodinated mapacalcine under the same conditions as in A.

that of $[^{125}\text{I}]\text{Na}$, i.e. 2200 Ci/mmol . These data demonstrate that it is possible to prepare and purify a monoiodo derivative of mapacalcine that can be radiolabelled at high specific radioactivity (2200 Ci/mmol).

Effects of $[^{125}\text{I}]\text{mapacalcine}$ on calcium currents

The native mapacalcine has been shown to inhibit a non-L-type calcium current in mouse intestinal myocytes. This calcium current was elicited by depolarizations from a holding potential of -80 to 0 mV either at a stimulation frequency of 0.1 Hz (at this stimulation frequency the L-type calcium current was totally inactivated in 6–9 min) or in the presence of $1 \mu\text{M}$ oxodipine, a specific inhibitor of L-type calcium current [11]. The concentration of native mapacalcine required to reduce the non-L-type calcium current by 50% was near $0.2 \mu\text{M}$ [9]. Under the conditions used here, the native mapacalcine ($0.1 \mu\text{M}$) inhibited the non-L-type calcium current by $38 \pm 8\%$ (12 cells tested). Similarly, the iodinated mapacalcine ($0.1 \mu\text{M}$), ejected on the same pool of myocytes, inhibited the calcium current by $42 \pm 7\%$ (14 cells tested) (Figure 1). The effects of iodinated mapacalcine were partially reversed (30–40%) after a 8–10 min return to the physiological solution. Therefore, the inhibitory effects of native and iodinated mapacalcine were not significantly different, demonstrating that the iodination procedure used here led to a monoiodo derivative of mapacalcine which conserved the physiological properties of the native toxin. This derivative could then

Figure 2 Kinetics experiments

(A) Time course of association of 0.3 nM iodo-mapacalcine to mouse intestinal membranes (0.5 mg/ml) at 4°C . Reaction was initiated by the addition of membranes, aliquots were filtered off at various time intervals. Inset: semi-logarithmic transformation of the data; Be and B represent binding signal at equilibrium and at time t , respectively. (B) Time course of dissociation of dissociable labelled mapacalcine from intestinal membranes at 4°C . Dissociation was initiated by adding excess ($1 \mu\text{M}$) unlabelled mapacalcine to a part of the association mixture after equilibrium had been reached. Aliquots were filtered off at time points indicated. Inset: semi-logarithmic plot of the data: y-axis is the $\ln$ of the percentage of binding remaining at time t . Only 40% of specific binding could be dissociated after maximal dissociation was reached.

be used for pharmacological characterization of the biological target of mapacalcine.

Kinetic binding studies

Association kinetics

Specific association of 0.3 nM $[^{125}\text{I}]\text{mapacalcine}$ with mouse intestinal membranes at 4°C reached equilibrium after 90 min (Figure 2A). The concentration of free ligand varied less than 15% during the course of these experiments, fulfilling the criteria for use of the pseudo-first-order plot of the time course to determine the rate constant k_{obs} [12]. A semi-logarithmic plot of these data was linear. The apparent rate constant of association (k_{obs}) was then determined from the slope of the linear representation of the association and found to be $k_{\text{obs}} = 2.7(\pm 0.2)10^{-4} \text{ s}^{-1}$ ($n = 3$). Associations performed at 26°C led to a k_{obs} of $4.0(\pm 0.3) \times 10^{-4} \text{ s}^{-1}$ ($n = 3$).

Figure 3 Ligand concentration dependence of observed rate constant of association of labelled mapacalcine

Association experiments were performed at 4 °C with ligand concentrations which varied from 0.3 to 3.4 nM. For a simple bimolecular association reaction, the slope of the line is equal to the association rate constant (k_a) and the y -intercept is equal to the dissociation rate constant (k_d), according to: $k_{\text{obs}} = k_a[L] + k_d$.

Dissociation kinetics

Dissociation of labelled mapacalcine (0.3 nM) bound to mouse intestinal membranes was examined by adding an excess of unlabelled mapacalcine (1 μM) to incubations after they reached equilibrium (Figure 2B). Binding was only partially reversible and approximately 40% could be dissociated after maximum achievable dissociation. The presence of excess of unlabelled ligand prevented re-association of [^{125}I]mapacalcine, yielding a first order dissociation process with k_d as dissociation rate constant. A semi-logarithmic plot of data obtained was linear and value of k_d was $1.3(\pm 0.2) \times 10^{-4} \text{ s}^{-1}$ ($n = 5$). Once k_{obs} and k_d were defined, the second order rate constant of association (k_a) of labelled mapacalcine could be calculated by the general equation:

$$k_a = (k_{\text{obs}} - k_d)/[L] \quad (1)$$

where k_a and k_d represent the second order rate constant of association and the first order rate constant of dissociation of the mapacalcine/receptor complex, respectively, and $[L]$ the ligand concentration corresponding to k_{obs} . The value of k_a obtained using k_{obs} at $[L] = 0.3 \text{ nM}$ was $4.6(\pm 0.2) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Once k_a and k_d were known, the value of the dissociation constant of the complex mapacalcine/receptor (K_d) at 4 °C was kinetically determined as the ratio of the 'off' to 'on' constants (k_d/k_a). The value obtained in this manner was $K_d = k_d/k_a = 0.3 \text{ nM}$.

Associations using multiple ligand concentrations

Since the complex mapacalcine receptor was partially dissociable, we performed associations of mapacalcine using a range of ligand concentrations to confirm the values of k_d and k_a previously determined in single ligand concentration experiments. The k_{obs} were determined for each set of experiments and plotted versus ligand concentration. Indeed Figure 3 demonstrated that this plot was linear with a slope equal to the second order association rate constant (k_a) and a y -intercept equal to the dissociation rate constant (k_d). According to eqn. (2):

$$k_{\text{obs}} = k_a[L] + k_d \quad (2)$$

Figure 4 Specific binding of [^{125}I]mapacalcine to mouse intestinal membranes

Scatchard plot of direct binding experiment data obtained at 4 °C. The dissociation constant (K_d^*) obtained was $0.5 \pm 0.2 \text{ nM}$ and the maximal binding capacity (B_{max}) $171 \pm 22 \text{ fmol/mg}$ of protein. Inset: the [^{125}I]mapacalcine binding was obtained in the presence (non-specific binding ■) or in the absence (total binding ▲) of a large excess (1 μM) of unlabelled mapacalcine. The specific binding (○) is defined as the difference between total and non-specific binding.

values for k_a and k_d were found to be $2.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $2.3 \times 10^{-4} \text{ s}^{-1}$, respectively. These values are in good agreement with those obtained from single ligand concentration experiments.

Specific binding of [^{125}I]mapacalcine

Figure 4 (inset) shows the results of a binding experiment in which increasing concentrations of [^{125}I]mapacalcine (0.01–5 nM) were added to a fixed concentration of intestinal membranes, either in the presence (non-specific binding) or in the absence (total binding) of a large excess (1 μM) of unlabelled mapacalcine. The specific binding is defined as the difference between total and non-specific binding. Figure 4 represents a typical Scatchard plot of the binding data obtained. The dissociation constant of the complex [^{125}I]mapacalcine/receptor formed in mouse intestinal membrane, $K_d^* = 0.5 \pm 0.2 \text{ nM}$ ($n = 4$), is in good agreement with kinetic data. The maximal binding capacity obtained was $171 \pm 22 \text{ fmol/mg}$ of protein ($n = 4$). The linearity of the Scatchard plot obtained demonstrated that [^{125}I]mapacalcine binds to a single class of non-interacting sites under our experimental conditions. Some experiments were conducted in the presence of a wider range of mapacalcine concentrations (0.01 nM–5 μM) to detect a putative low-affinity binding site. Under these conditions we could only detect the binding sites described above and no specific binding occurred in the presence of the highest mapacalcine concentrations (results not shown).

Competition between [^{125}I]mapacalcine, native mapacalcine and ^{127}I -labelled mapacalcine

Figure 5(A) shows that unlabelled mapacalcine decreases [^{125}I]mapacalcine specific binding to its receptor. The concentration of native toxin inducing 50% inhibition of [^{125}I]mapacalcine specific binding was $K_{0.5} = 1.3 \pm 0.2 \text{ nM}$ ($n = 4$). The expression for $K_{0.5}$ is:

$$K_{0.5} = K_d(1 + [L^*]_{0.5}/K_d^*) \quad (3)$$

Figure 5 Competition between $[^{125}\text{I}]$ mapacalcine, unlabelled mapacalcine and $[^{127}\text{I}]$ mapacalcine

(A) $[^{125}\text{I}]$ Mapacalcine binding was measured in the presence of increasing concentrations of unlabelled mapacalcine at 4 °C. The effect of unlabelled mapacalcine on $[^{125}\text{I}]$ mapacalcine binding was expressed as the % of binding inhibition. (B) Same experiment as in A except that competition was achieved between $[^{125}\text{I}]$ mapacalcine and the non-radioactive labelled mapacalcine ($[^{127}\text{I}]$ mapacalcine).

where $[L^*]$ is the concentration of free $[^{125}\text{I}]$ mapacalcine at half inhibition; K_d^* and K_d are dissociation constants of the complex formed between intestinal membranes and $[^{125}\text{I}]$ mapacalcine or unlabelled mapacalcine, respectively. The value of K_d^* determined in direct binding experiments (Figure 4) is 0.5 ± 0.2 nM and the value of $[L^*]$ was 0.3 nM under the experimental conditions depicted in Figure 5. The value of K_d calculated from eqn. (3) is then 0.8 nM. This value is in good agreement with the affinity constant found for $[^{125}\text{I}]$ mapacalcine. Similar data were obtained from competition experiments performed at 26 °C. Figure 5(B) shows that $[^{127}\text{I}]$ mapacalcine decreases $[^{125}\text{I}]$ mapacalcine specific binding to its receptor. The concentration of $[^{127}\text{I}]$ -labelled toxin inducing 50 % inhibition of $[^{125}\text{I}]$ mapacalcine specific binding was $K_{0.5} = 1.2 \pm 0.1$ nM ($n = 4$). The K_d value calculated as described above was 0.7 nM, which is in good agreement with the values obtained from direct binding and competition experiments. These data, taken together, confirm the electrophysiological observations showing that iodination, under the conditions used, does not alter the biological properties of mapacalcine.

Effects of various drugs specific for ionic channels on $[^{125}\text{I}]$ mapacalcine binding

To check the specificity of the binding of mapacalcine, we tested

Table 1 Competition between $[^{125}\text{I}]$ mapacalcine and different drugs

$[^{125}\text{I}]$ Mapacalcine (0.3 nM) was incubated with intestinal membranes (0.5 mg of protein/ml) and 1 μM of unlabelled molecules for 120 min at 4 °C. These molecules are specific inhibitors of different types of ionic channels. The radioactivity bound to intestinal membranes was determined by the filtration technique described in the 'Experimental procedures' section. Data are expressed as the mean percentage ($\pm$ S.E.M.) of specific binding obtained from three independent experiments conducted in duplicate.

Drug (10^{-6} M)	Drug target channels	$[^{125}\text{I}]$ Mapacalcine binding (%)
Control	(Non-L-type Ca^{2+} channel)	100 $\pm$ 8
Oxodipine	L-type Ca^{2+}	98 $\pm$ 4
Elgodipine	L-type Ca^{2+}	88 $\pm$ 12
Tetrodotoxin	Na^+	95 $\pm$ 4
Amiloride	Na^+	98 $\pm$ 2
ω -Conotoxin MVIIC	Q-type Ca^{2+}	94 $\pm$ 2
ω -Conotoxin GVIA	N-type Ca^{2+}	95 $\pm$ 4
ω -Agatoxin IVA	P-type Ca^{2+}	98 $\pm$ 10
Fenoverine	L-type Ca^{2+}	82 $\pm$ 16
Diltiazem	L-type Ca^{2+}	90 $\pm$ 2
Desmethoxyverapamil	L-type Ca^{2+}	93 $\pm$ 3
Charybdotoxin	Ca^{2+} -dependent K^+ (BK)	90 $\pm$ 4
Scyllatoxin	Ca^{2+} -dependent K^+ (SK)	97 $\pm$ 2
Iberiotoxin*	Ca^{2+} -dependent K^+	95 $\pm$ 6
MCD peptide	Volt.-dependent K^+	91 $\pm$ 5

* 0.1 μM .

a variety of drugs which were previously demonstrated to interact specifically with different ionic channels. Table 1 shows the list of the drugs tested on $[^{125}\text{I}]$ mapacalcine binding. This list includes specific inhibitors of sodium channels, voltage-dependent potassium channels, calcium-dependent potassium channels, various inhibitors of L-type calcium channels and specific inhibitors of N-, P- and Q-type calcium channels. All the drugs, at the concentration tested, had no effect on $[^{125}\text{I}]$ mapacalcine binding. These data are in good agreement with previous functional observations that show that mapacalcine was inactive on potassium, calcium-dependent chloride channels, and L- and T-type calcium channels, and that L-, P-, Q- and N-type calcium antagonists were inactive on the mapacalcine-sensitive calcium current [9].

Influence of cations on $[^{125}\text{I}]$ mapacalcine binding

The effect of several cations was tested on mapacalcine binding. At free calcium concentrations ranging from 10^{-6} to 10^{-2} M no effect on $[^{125}\text{I}]$ mapacalcine binding was observed. In the same way, magnesium, sodium and potassium tested up to 10^{-1} M ($n = 3$) had no significant influence on $[^{125}\text{I}]$ mapacalcine binding (not shown).

DISCUSSION

Data reported herein demonstrate that it is possible to prepare a monoiodo derivative of mapacalcine which retains its biological and pharmacological properties. The binding of $[^{125}\text{I}]$ mapacalcine is specific and saturable. None of the drugs known to interact with a variety of ionic channels, even with calcium channels, are able to compete with $[^{125}\text{I}]$ mapacalcine for its receptor, as expected from previous data using the patch-clamp technique [9]. The $[^{125}\text{I}]$ mapacalcine binding is also insensitive to the presence of cations at their physiological concentration. Kinetic studies show that mapacalcine associates to its receptor with a rate constant (k_a) of $4.6 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ which is an intermediate value

as compared with those found for apamin [13] on low-conductance calcium-dependent potassium channels ($k_a = 2.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) and for snake neurotoxin ($k_a = 4.8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) on the nicotinic receptor [14]. This value is close to that observed for *Escherichia coli* heat-stable enterotoxin for its association to its low-affinity receptor on mouse intestinal membranes ($k_a = 1.46 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) [15]. Dissociation studies demonstrate that mapacalcine binding to its receptor is only partially reversible, which is in good agreement with previous functional observations on mouse intestinal myocytes [9]. About 60 % of the total binding remains associated after maximum dissociation is achieved. This observation was obtained using either excess native mapacalcine, [^{127}I]mapacalcine or dilution to initiate dissociation. Furthermore, we carried out kinetic studies at 26 °C; under these conditions, the plateau level reached after association was comparable to the one obtained at 4 °C. These observations taken together suggest that the non-dissociable binding is probably not owing to an internalization process, which has few chances to occur at 4 °C on membrane preparations. The molecular basis for this partial dissociation of binding remains unknown. However, two possible explanations are as follows. (i) Because mapacalcine is an homodimer containing 18 cysteines it is possible that it forms a mixed disulphide with its receptor. This mechanism has already been described for *E. coli* heat-stable enterotoxin [15–18]. Since [^{125}I]mapacalcine is monoiodinated, only one chain, of the two, contains iodine; after a putative disulphide reaction with the receptor, one chain could remain covalently linked to the receptor while the other could dissociate. In this case, we would observe a maximal theoretical dissociation of 50 % which is close to our observations. (ii) Mapacalcine could behave similarly to endothelin. Endothelin has recently been demonstrated to irreversibly bind to ET_A receptor in a manner that is independent of the temperature at which it is associated. Only 50 % of endothelin can be dissociated by acidic treatment. The authors claim that the irreversibility of endothelin binding, which is not owing to mixed disulphide formation, could be involved in ligand/receptor complex interaction with a G-protein and could account for the refractory nature and slow regeneration of the endothelin receptor [19]. In the same way, mapacalcine could interact with a protein associated with a non-L-type calcium channel.

Previously, we reported that mapacalcine was able to specifically block a non-L-type calcium current in mouse intestinal myocytes, with an IC_{50} of 0.2 μM , using patch-clamp experiments [9]. In this paper we report a dissociation constant in the 0.8 nM range for the complex mapacalcine/receptor. Previous data suggest that this difference cannot be owing to a potential dependence of mapacalcine interaction with the non-L-type calcium channel [9]. This kind of discrepancy is currently encountered when the physiological effect of a drug is compared with biochemical data that only addresses the drug/receptor interactions [20,21]. Electrophysiological measurements are obtained after toxin application periods no longer than 3 min because of technical reasons. Under electrophysiological conditions, knowing the association rate constant (k_{obs}) at 26 °C in the presence of 0.3 nM mapacalcine and since value of k_{obs} varies linearly with mapacalcine concentration, we can estimate that in the presence of 0.8 nM mapacalcine (value of K_a determined in binding studies at 4 and 26 °C) only 17 % of the receptors would be occupied by mapacalcine [22]. Moreover, the two experimental approaches require different sets of experimental conditions: electrophysiology is performed on entire cells on which the toxin is ejected, while binding is performed on membrane preparations incubated in homogeneous solutions of toxin. The impossibility of detecting low-affinity binding sites for mapacalcine in the

0.2 μM range suggests that the biological consequence of receptor occupation by mapacalcine requires other event(s), with their own kinetic requirements. Increasing the toxin concentration to 0.2 μM during functional assays would then help to offset these kinetic constraints and to obtain the expected calcium current blockade within 3 min. These observations taken together would suggest that mapacalcine could interact with a protein associated with the non-L-type calcium channel on mouse intestinal myocytes. Our data support the previous conclusion that mapacalcine is a specific marker for a receptor associated to an interesting type of calcium channel for the following reasons: (i) we show here that different classes of calcium channel antagonists are unable to compete with mapacalcine on its receptor; (ii) the mapacalcine-sensitive calcium current has been shown to be unrelated to L-, N-, P-, Q- or T-type calcium currents [9].

A felodipine-resistant calcium conductance has previously been reported on guinea pig ileum [23]. Consequently, mapacalcine-sensitive calcium channels could, in part, exert control of the smooth muscle contractility. On the other hand, the great diversity of calcium channels explains the failure of dihydropyridines to protect neurons against ischaemia [24], suggesting that mapacalcine-sensitive calcium channels could also play an important role in cell calcium invasion during ischaemia. The use of [^{125}I]mapacalcine would then be helpful to search for the presence of mapacalcine receptors in a variety of tissues, such as brain or heart, and to investigate their physiological implications in these tissues. This derivative should also facilitate the mapping of mapacalcine receptors using autoradiographic techniques. Affinity labelling of the mapacalcine receptor will be performed to elucidate its subunitary composition in order to determine if the subunitary pattern obtained is directly related to a typical calcium channel or if it is different, suggesting that it could be a protein associated with the 'non-L-type' calcium channel previously described on mouse intestinal myocytes [9]. Further experiments will also be performed to elucidate the molecular basis and physiological meaning of the partially reversible mapacalcine binding to its receptor.

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