

**CRITICAL AMINO ACID RESIDUES OF MAUROCALCINE INVOLVED  
IN PHARMACOLOGY, LIPID INTERACTION AND CELL  
PENETRATION**

Kamel Mabrouk<sup>a</sup>†, Narendra Ram<sup>b</sup>†, Sylvie Boisseau<sup>c</sup>, Flavie Strappazon<sup>c</sup>, Amel Rehaïm<sup>d</sup>, Rémy Sadoul<sup>c</sup>, Hervé Darbon<sup>e</sup>, Michel Ronjat<sup>b</sup> & Michel De Waard<sup>b\*</sup>

<sup>a</sup>Laboratoire d'Ingénierie des Protéines, CNRS FRE 2738, Faculté de Médecine Nord, Boulevard Pierre Dramard, 13916 Marseille, France.

<sup>b</sup>Inserm U607, Canaux Calciques, Fonctions et Pathologies, CEA, Département Réponse et Dynamique Cellulaire, Bâtiment C3, 17 rue des Martyrs, 38054 Grenoble Cedex 09, France.

<sup>c</sup>Equipe Inserm 108 / UJF, Pavillon de Neurologie, CHU de Grenoble, 38043 Grenoble, France.

<sup>d</sup>Unité de Biochimie et Biologie Moléculaire, Faculté des Sciences de Tunis, Campus Universitaire, 2092 El Manar, Tunis, Tunisia.

<sup>e</sup>CNRS, Laboratoire AFMB, 31 chemin Joseph-Aiguier, F-13402 Marseille cedex 20, France.

\* Address correspondence to: Michel De Waard, Inserm U607, CEA, Département Réponse et Dynamique Cellulaire, 38054 Grenoble Cedex 09, France, Tel.: (33) 4 38 78 68 13 ; Fax: (33) 4 38 78 50 41 ; E-mail: [mdewaard@cea.fr](mailto:mdewaard@cea.fr)

† Both authors contributed equally to this work.

**Key words:** maurocalcine, ryanodine receptor, cell penetration, cell penetrating peptide, lipid interaction, cell toxicity

## Summary

Maurocalcine (MCa) is a 33 amino acid residue peptide that was initially identified in the Tunisian scorpion *Scorpio maurus palmatus*. This peptide triggers interest for three main reasons. First, it helps unravelling the mechanistic basis of  $\text{Ca}^{2+}$  mobilization from the sarcoplasmic reticulum because of its sequence homology with a calcium channel domain involved in excitation-contraction coupling. Second, it shows potent pharmacological properties because of its ability to activate the ryanodine receptor. Finally, it is of technological value because of its ability to carry cell-impermeable compounds across the plasma membrane. Herein, we characterized the molecular determinants that underlie the pharmacological and cell-penetrating properties of maurocalcine. We identify several key amino acid residues of the peptide that will help the design of cell-penetrating analogues devoid of pharmacological activity and cell toxicity. Close examination of the determinants underlying cell penetration of maurocalcine reveals that basic amino acid residues are required for an interaction with negatively charged lipids of the plasma membrane. Maurocalcine analogues that penetrate better have also stronger interaction with negatively charged lipids. Conversely, less effective analogues present a diminished ability to interact with these lipids. These findings will also help the design of still more potent cell penetrating analogues of maurocalcine.

## List of abbreviations

BSA, bovine serum albumin;  
CGN, cerebellar granule neurons;  
CHO, Chinese hamster ovary;  
CPP, cell penetrating peptide;  
DHE, dihydroethidium;  
DHP, dihydropyridine;  
DHPR, dihydropyridine receptor;  
DMEM, Dulbecco's modified eagle's medium;  
DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid;  
FACS, fluorescence activated cell sorter;  
Fmoc, N- $\alpha$ -fluorenylmethyloxycarbonyl;  
HEK293, human embryonic kidney 293 cells;  
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;  
HMP, 4-hydroxymethylphenoxy;  
<sup>1</sup>H-NMR, proton nuclear magnetic resonance;  
HPLC, high pressure liquid chromatography;  
M<sub>Ca</sub>, maurocalcine;  
M<sub>Ca<sub>b</sub></sub>, biotinylated maurocalcine;  
MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide;  
PC<sub>50</sub>, half-maximal penetration concentration;  
PBS, phosphate buffered saline; PtdIns, phosphatidylinositol;  
RyR, ryanodine receptor;  
SR, sarcoplasmic reticulum;  
Strep-Cy5 (Cy3), streptavidine-cyanine 5 (cyanine 3);  
TFA, trifluoroacetic acid.

## Introduction

Maurocalcine (MCA) is a 33 amino acid residue peptide that originates from the venom of the chactid scorpion *Scorpio maurus palmatus* [1]. It can be produced by chemical synthesis without structural alteration [1]. The solution structure of MCA, as defined by <sup>1</sup>H-NMR, displays an inhibitor cystine knot motif [2] containing three  $\beta$ -strands (strand 1 from amino acid residues 9 to 11, strand 2 from 20 to 23, and strand 3 from 30 to 33). Both  $\beta$ -strands 2 and 3 form an antiparallel sheet. The folded/oxidized peptide contains three disulfide bridges arranged according to the pattern: Cys<sup>3</sup>-Cys<sup>17</sup>, Cys<sup>10</sup>-Cys<sup>21</sup> and Cys<sup>16</sup>-Cys<sup>32</sup> [1]. MCA now belongs to a family of scorpion toxins since it has strong sequence identity with imperatoxin A from the scorpion *Pandinus imperator* (82% identity; [3]) and with both opicalcine 1 and 2 from the scorpion *Opisththalmus carinatus* (91 and 88% identities, respectively, [4]).

MCA and its structurally related analogues trigger interest for three main reasons. First, MCA is a powerful activator of the ryanodine receptor (RyR), thereby triggering calcium release from intracellular stores [5, 6]. MCA binds with nanomolar affinity onto RyR, a calcium channel from the sarcoplasmic reticulum (SR), and generates greater channel opening probability interspersed with long-lasting openings in a mode of sub-conductance state [1]. Second, MCA has a unique sequence homology with a cytoplasmic domain (termed domain A) of the pore-forming subunit of the skeletal muscle dihydropyridine (DHP)-sensitive voltage-gated calcium channel (DHP receptor, DHPR). This homology implicates a DHPR region that is well known for its involvement in the mechanical coupling between the DHPR and RyR, a process whereby a modification in membrane potential is sensed by the DHPR, transmitted to RyR, and produces internal calcium release followed by muscle contraction. It is therefore expected that a close examination of the cellular effects of MCA on the process of excitation-contraction coupling may reveal intimate details of the mechanistic aspects linking the functioning of the DHPR to that of RyR. In that sense, a role of domain A in the termination of calcium release upon membrane repolarisation has been proposed through the use of MCA [7]. Third, MCA appears unique in the field of scorpion toxins for its ability to cross the plasma membrane. Application of MCA in the extracellular medium of cultured myocytes triggers a transient calcium release from the SR intracellular store within seconds suggesting a very fast passage across the membrane [5]. The identification of MCA's binding site on RyR and the cytoplasmic localization of this site indicate that MCA crosses the plasma membrane to reach the cytoplasm of the cell [8]. To demonstrate the ability of MCA to cross the plasma membrane, a biotinylated derivative of MCA (MCA<sub>b</sub>) was synthesized, coupled to a fluorescent derivative of streptavidine, and the entire complex was shown to reach the cytoplasm of many cell types [9, 10]. This cell penetration does not require metabolic energy but endocytosis cannot be ruled out. It is rapid, reaches saturation within

minutes, is dependent on the transmembrane potential and occurs at concentrations as low as 10 nM [10]. Cell penetration of a large protein such as streptavidine indicates that MCa can be used as a vector for the cell penetration of cell impermeable compounds. This raises considerable technological interest in the peptide.

Considering its characteristics, it makes no doubt that it now belongs to the structurally unrelated family of cell-penetrating peptides (CPPs). Known CPPs include the HIV-encoded transactivator of transcription (Tat) [11], the insect transcription factor Antennapedia (Antp or penetratin) [12], the herpes virus protein VP22, a transcription regulator, the chimeric peptide transportan [13] made in part by the neuropeptide galanin and by the wasp venom peptide mastoparan, and polyarginine peptides [14]. The only structural feature that MCa has in common with these other CPPs is the presence of a large basic domain. Indeed, MCa carries a net positive charge of +8, 12 residues out of 33 are basic, and the electrostatic surface potential of MCa indicates that it presents a basic face involving the first amino terminal Gly residue and all Lys residues of the peptide. CPPs are efficient vectors for the cell penetration of oligonucleotides [15], plasmids [16], antisense peptide nucleic acids [17], peptides [18], proteins [19, 20], liposomes [21] and nanoparticles [22]. As such, CPPs appear invaluable for numerous medical, technological and diagnostic applications.

Because of the potential of MCa to deliver membrane impermeable compounds without signs of cell toxicity, and considering its efficiency at low concentrations, it appears essential to define new analogues that lack the pharmacological effects of wild-type MCa but preserve or enhance its cell penetration efficiencies. Several new mutated analogues were chemically synthesized, and assessed for their effects on RyR and their penetration efficiencies, as well as compared for their toxicity on primary cultures of neurons. Finally, these analogues were also evaluated for their ability to interact with membrane lipids that are presumed to be implicated in the cell penetration of CPPs. The data obtained indicate that monosubstitution of amino acids can improve MCa's ability to penetrate within cells and further decrease the neuronal toxicity of the peptide. They confirm the contribution of the basic amino acid residues in cell penetration and provide new leads for the synthesis of peptide analogues devoid of pharmacological effects.

## Materials and Methods

*Chemical synthesis of biotinylated maurocalcine and point-mutated analogues* - N- $\alpha$ -fluorenylmethyloxycarbonyl (Fmoc) L-amino acids, 4-hydroxymethylphenyloxy (HMP) resin, and reagents used for peptide synthesis were obtained from Perkin-Elmer. N- $\alpha$ -Fmoc-L-Lys(Biotin)-OH was purchased from Neosystem group SNPE. Solvents were analytical grade products from Carlo Erba – SDS (Peypin, France). Biotinylated MCa (MCa<sub>b</sub>) and its biotinylated point-mutated analogues were obtained by the solid-phase method [23] using an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.). Sixteen different analogues were designed such that Ala replaced a native amino acid of MCa (Figure 1). Peptide chains were assembled stepwise on 0.25 mEq of hydroxymethylphenyloxy resin (1% cross-linked; 0.77 mEq of amino group/g) using 1 mmol of N- $\alpha$ -Fmoc amino acid derivatives. The side chain-protecting groups were: trityl for Cys and Asn; *tert*-butyl for Ser, Thr, Glu, and Asp; pentamethylchroman for Arg, and *tert*-butyloxycarbonyl or Biotin for Lys. N- $\alpha$ -amino groups were deprotected by treatment with 18% and 20% (v/v) piperidine/N-methylpyrrolidone for 3 and 8 min, respectively. The Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole active esters in N-methylpyrrolidone (4 fold excess). After peptide chain assembly, the peptide resin was treated between 2 and 3 hrs at room temperature, in constant shaking, with a mixture of trifluoroacetic acid (TFA)/H<sub>2</sub>O/thioanisole/ethanedithiol (88/5/5/2, v/v) in the presence of crystalline phenol (2.25 g). The peptide mixture was then filtered, and the filtrate was precipitated by adding cold *t*-butylmethyl ether. The crude peptide was pelleted by centrifugation (3,000 g; 10 min) and the supernatant was discarded. The reduced peptide was then dissolved in 200 mM Tris-HCl buffer, pH 8.3, at a final concentration of 2.5 mM and stirred under air to allow oxidation/folding (between 50 and 72 hrs, room temperature). The target products, MCa<sub>b</sub> and its analogues, were purified to homogeneity, first by reversed-phase high pressure liquid chromatography, HPLC, (Perkin-Elmer, C<sub>18</sub> Aquapore ODS 20  $\mu$ m, 250x10 mm) by means of a 60-min linear gradient of 0.08% (v/v) TFA/0-30% acetonitrile in 0.1% (v/v) TFA/H<sub>2</sub>O at a flow rate of 6 ml/min ( $\lambda$ =230 nm). The homogeneity and identity of the peptides were assessed by: (i) analytical C<sub>18</sub> reversed-phase HPLC (Merck, C<sub>18</sub> Li-chrospher 5  $\mu$ m, 4 x 200 mm) using a 60-min linear gradient of 0.08% (v/v) TFA/0-60% acetonitrile in 0.1% (v/v) TFA/H<sub>2</sub>O at a flow rate of 1 ml/min; (ii) amino acid analysis after acidolysis (6N HCl / 2% (w/v) phenol, 20 hrs, 118°C, N<sub>2</sub> atmosphere); and (iii) mass determination by matrix assisted laser desorption ionization-time of flight mass spectrometry.

*Conformational analyses of MCa, MCa E12A, MCa K20A and MCa R24A by circular dichroism* –

Circular dichroism (CD) spectra were recorded on a Jasco 810 dichrograph using 1-mm-thick quartz cells.

Spectra were recorded between 180 and 260 nm at 0.2 nm/min and were averaged from three independent acquisitions. The spectra were corrected for water signal and smoothed by using a third-order least squares polynomial fit.

*Preparation of heavy SR vesicles* - Heavy SR vesicles were prepared following the method of Kim *et al.* [24] modified as described previously [25]. Protein concentration was measured by the Biuret method.

*[<sup>3</sup>H]-ryanodine binding assay* - Heavy SR vesicles (1 mg/ml) were incubated at 37°C for 3 hrs in an assay buffer composed of 5 nM [<sup>3</sup>H]-ryanodine, 150 mM NaCl, 2 mM EGTA, 2 mM CaCl<sub>2</sub> (pCa=5), and 20 mM HEPES, pH 7.4. Wild-type or mutant MCa<sub>b</sub> was added to the assay buffer just prior the addition of heavy SR vesicles. [<sup>3</sup>H]-ryanodine bound to heavy SR vesicles was measured by filtration through Whatmann GF/B glass filters followed by three washes with 5 ml of ice-cold washing buffer composed of 150 mM NaCl, 20 mM HEPES, pH 7.4. Filters were then soaked overnight in 10 ml scintillation cocktail (Cybscint, ICN) and bound radioactivity determined by scintillation spectrometry. Non-specific binding was measured in the presence of 20 μM cold ryanodine. Each experiment was performed in triplicate and repeated at least two times. All data are presented as mean ± S.D.

*Cell culture* - Chinese hamster ovary (CHO) cell line (from ATCC) were maintained at 37°C in 5% CO<sub>2</sub> in F-12K nutrient medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Invitrogen) and 10,000 units/ml streptomycin and penicillin (Invitrogen).

*Primary culture of cerebellar granule neurons* - Media used for the culture of cerebellar granule neurons (CGN) was based on Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 10 unit/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine, and 10 mM HEPES (K5 medium). KCl was added to a final concentration of 25 mM (K25 medium) and supplemented with 10% fetal bovine serum (K25+S medium). Primary cultures of CGN were prepared from 6-day-old S/IOPS NMRI mice (Charles River Laboratories), as described previously [26, 27] with some modifications. The cerebella were removed, cleared of their meninges, and cut into 1-mm pieces. They were then incubated at 37°C for 10 min in 0.25% trypsin-EDTA (Invitrogen) in DMEM. An equal volume of K25+S medium and 3,000 units/ml DNase I (Sigma) were added before dissociation by triturating using flame-polished Pasteur pipettes. Next, an equal volume of DMEM containing trypsin inhibitor (Sigma) and 300 units/ml DNase I were added to the cells. Dissociated cells were centrifuged for 5 min at 500 g. The pellet was resuspended in fresh K25+S medium and cells were plated onto poly-D-lysine (10 μg/ml, Sigma) precoated 96-well plates at a density of  $8 \times 10^4$  cells/well. The cerebellar granule neurons were grown in K25+S medium in a

humidified incubator with 5% CO<sub>2</sub> / 95% air at 37°C. Cytosine-β-D-arabinoside (10 μM, Sigma) was added after 1 day *in vitro* to prevent the growth of non-neuronal cells.

*MTT assay* - Primary cultures of CGN were seeded into 96 well micro plates at a density of approximately  $8 \times 10^4$  cells/well. After 4 days of culture, the cells were incubated for 24 hrs at 37°C with MCa<sub>6</sub> or its analogues at a concentration of 1 or 10 μM. Control wells containing cell culture medium alone or with cells, both without peptide addition, were included in each experiment. The cells were then incubated with 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) for 30 min. Conversion of MTT into purple colored MTT formazan by the living cells indicates the extent of cell viability. The crystals were dissolved with dimethyl sulfoxide (DMSO) and the optical density was measured at 540 nm using a microplate reader (Biotek ELx-800, Mandel Scientific Inc.) for quantification of cell viability. All assays were run in triplicates.

*Membrane integrity LDH assay* – A lactate dehydrogenase (LDH) assay was used to probe membrane integrity disturbance by MCa, MCa E12A and MCa K20A according to the protocol provided by the manufacturer (CytoTox-ONE™, Promega). Briefly, CHO cells were incubated with 10 μM peptide for 24 hrs, then 30 min at 22°C, a volume of CytoTox-ONE™ reagent was added, mixed and incubated at 22°C for 10 min, 50 μl of STOP solution added, and the fluorescence was measured (excitation wavelength of 560 nm and emission at 590 nm). Control experiments were also performed to measure background LDH release in the absence of the peptide, and also maximal release of LDH after total cell lysis. Percentage of cell toxicity is measured as follows:  $100 \times (\text{Experimental with peptide} - \text{cultured media background}) / (\text{maximal LDH release} - \text{cultured media background})$ .

*Formation of MCa<sub>6</sub> (wild-type or mutant) /streptavidin-cyanine complex* - Soluble streptavidine–cyanine 5 or streptavidine–cyanine 3 (Strep-Cy5 or Strep-Cy3, Amersham Biosciences) was mixed with four molar equivalents of wild-type or mutant MCa<sub>6</sub> 2 hrs at 37°C in the dark in PBS (phosphate-buffered saline) (in mM): NaCl 136, Na<sub>2</sub>HPO<sub>4</sub> 4.3, KH<sub>2</sub>PO<sub>4</sub> 1.47, KCl 2.6, CaCl<sub>2</sub> 1, MgCl<sub>2</sub> 0.5, pH 7.2.

*Flow cytometry* – Wild-type or mutant MCa<sub>6</sub>–Strep–Cy5/Cy3 complexes were incubated for two hrs with live cells to allow cell penetration to occur. The cells were then washed twice with PBS to remove the excess extracellular complexes. Next the cells were treated with 1 mg/ml trypsin (InVitrogen) for 10 min at 37°C to remove remaining membrane-associated extracellular cell surface-bound complexes. After trypsin incubation, the cell suspension was centrifuged at 500 g and suspended in PBS. Flow cytometry analyses were performed with live cells using a Becton Dickinson FACSCalibur flow cytometer (BD



Biosciences). Data were obtained and analyzed using CellQuest software (BD Biosciences). Live cells were gated by forward/side scattering from a total of 10,000 events.

*Confocal microscopy and immunocytochemistry* - For analysis of the subcellular localization of wild-type or mutant M<sub>Ca</sub><sub>v</sub>-Strep-Cy3/Cy5 complexes in living cells, cells were incubated with the complexes for 2 hrs, and then washed with DMEM alone. Immediately after washing, the nucleus was stained with 1 µg/ml dihydroethidium (DHE, Molecular probes, USA) for 20 min, and then washed again with DMEM. DHE shows a blue fluorescence (absorption/emission: 355/420 nm) in the cytoplasm of cells until oxidization to form ethidium which becomes red fluorescent (absorption/emission: 518/605 nm) upon DNA intercalation. Only the red fluorescence was measured in the nucleus. After this step, the plasma membrane was stained with 5 µg/ml FITC-conjugated Concanavalin A (Molecular Probes) for 3 min. Cells were washed once more, but with PBS. Live cells were then immediately analyzed by confocal laser scanning microscopy using a Leica TCS-SP2 operating system. Alexa-488 (FITC, 488 nm) and Cy3 (543 nm) or Cy5 (642 nm) were sequentially excited and emission fluorescence were collected in z-confocal planes of 10-15 nm steps. Images were merged in Adobe Photoshop 7.0.

*Interaction of M<sub>Ca</sub><sub>v</sub> and its mutants with different lipids* – Strips of nitrocellulose membranes containing spots with different phospholipids and sphingolipids were obtained from Molecular probes. These membranes were first blocked with TBS-T (150 mM NaCl, 10 mM Tris-HCL, pH 8.0, 0.1% (v/v) Tween 20) supplemented with 0.1% free bovine serum albumin (BSA) for about 1 hr at room temperature. Later, these membranes were incubated with 100 nM wild-type or mutant M<sub>Ca</sub><sub>v</sub> along with TBS-T and 0.1% free BSA for 2 hrs at room temperature. Incubation with 100 nM biotin alone was used as a negative control condition. The membranes were then washed a first time with TBS-T 0.1% free BSA using gentle agitation for 10 min. Binding of wild-type or mutant M<sub>Ca</sub><sub>v</sub> onto the lipid strips was detected by incubating 30 min the lipid strips with 1 µg/ml ready to use streptavidine horse radish peroxidase (Vector labs, SA-5704). The membranes were washed a second time with TBS-T 0.1% free BSA. Interaction was detected by incubating the membranes with horse radish peroxidase substrate (Western Lightning, Perkin-Elmer Life Science) for 1 min in each case followed by exposure to Biomax film (Kodak).

## Results

*Synthesis of MCA analogues* – Figure 1 illustrates the primary structure of the various analogues of MCA that were chemically synthesized. Most of the analogues were biotinylated at their amino-terminus to favor their coupling to streptavidine. All substitutions were made by alanine residues. The global net charge of each peptide is also indicated. Biotinylation does not alter the global net charge of MCA. Some of the mutated analogues of MCA were reported previously, but in their non biotinylated version [5].

*Effects of MCA analogues on [<sup>3</sup>H]-ryanodine binding to RyR1* – The purpose of this study is to compare the pharmacological efficacy of various MCA analogues to their efficiency of cell penetration. The ultimate aim is to help the design of new point mutated analogues of MCA that can serve as good cell penetration carriers without displaying an adverse pharmacological effect once penetrated inside cells. The pharmacological potential of MCA analogues can be assessed by their efficacy in stimulating [<sup>3</sup>H]-ryanodine binding onto RyR1 oligomers from SR vesicles, an effect that appears linked to the promotion of opening modes of the channel. Since the analogues synthesized were biotinylated for the purpose of forming a complex with fluorescent derivatives of streptavidine, we first compared the efficacy of [<sup>3</sup>H]-ryanodine binding stimulation between MCA and MCA<sub>b</sub> (Table 1). Adding a biotin group to the N-terminus of MCA did not alter significantly the EC<sub>50</sub> value for [<sup>3</sup>H]-ryanodine binding stimulation or the stimulation factor (between 16- and 18-fold). The properties of all the point mutated analogues of MCA<sub>b</sub> (14 of the 16 mutants) should thus also be comparable to similar point mutated analogues of MCA (2 of 16 the mutants). Next, all the mutants were compared using a similar batch of SR preparation. We noticed that basal binding of [<sup>3</sup>H]-ryanodine binding (in the absence of any analogues) varies greatly with the SR batch although saturation appears more or less similar. Hence, the stimulation efficacy of the binding (ratio between stimulated [<sup>3</sup>H]-ryanodine binding and basal [<sup>3</sup>H]-ryanodine binding) also varies with the SR batch (data not shown). Figure 2 thus compares the effects of various point-mutated analogues of MCA<sub>b</sub> onto the [<sup>3</sup>H]-ryanodine binding to SR vesicles from the same batch preparation. The main results are summarized in Table 1. Six analogues have greater affinity for RyR1 than MCA<sub>b</sub>: MCA<sub>b</sub> D2A, MCA<sub>b</sub> P5A, MCA<sub>b</sub> L9A, MCA<sub>b</sub> E12A, MCA<sub>b</sub> N13A and MCA<sub>b</sub> D15A. It is of interest that a greater affinity for RyR1 can be triggered by mutating negatively charged amino acid residues that increase the net positive of MCA from +8 to +9 (Figure 1). Of note, none of these mutants affected the binding stimulation factor suggesting that these analogues behave similarly than MCA and MCA<sub>b</sub> at saturating concentrations. Alanine substitution of many of the positively charged amino acid residues of MCA that belong to the basic face of the molecule [10] reduce the affinity of MCA for RyR1. These residues comprise Lys<sup>8</sup>, Lys<sup>19</sup>, Lys<sup>20</sup>, and Lys<sup>22</sup>, which confirms previous findings with similar non-biotinylated MCA analogues [5].

Mutation of two other positively charged residues, Arg<sup>23</sup> and Arg<sup>24</sup>, induce similar effects. Besides these six analogues, MCa<sub>b</sub> L7A and MCa<sub>b</sub> T26A also display a significant reduction in the affinity of MCa<sub>b</sub> for RyR1. All MCa<sub>b</sub> analogues that were shown to have reduced affinity for RyR1 also present reduced potencies of [<sup>3</sup>H]-ryanodine binding stimulation (Table 1). A form of correlation appears to occur between the reduction in affinity and stimulation efficacy, with the notable exception of MCa<sub>b</sub> T26A that kept a rather good stimulation efficacy despite a strong reduction in affinity. Finally, among the sixteen mutants that were tested, MCa<sub>b</sub> L4A and MCa<sub>b</sub> H6A did neither change the affinity nor the stimulation efficacy of MCa<sub>b</sub>.

In conclusion, for the purpose of selecting mutated analogues of MCa defective in pharmacological effects, mutations at amino acid positions 7, 23, 24 and 26 appear particularly interesting since they do not contribute to the presentation of the basic face of MCa that is presumably required for cell penetration of MCa [10].

*Effects of point mutations of MCa<sub>b</sub> on its cell penetration efficiency* – To perform a quantitative comparison of the cell penetration efficacy of the various mutated analogues of MCa<sub>b</sub>, FACS analyses were performed with living CHO cells (Figure 3). Cells were incubated for 2 hrs with 1 μM of wild-type or mutant MCa<sub>b</sub> / streptavidine-Cy5 complexes (4 μM of wild-type or mutant MCa<sub>b</sub> for 1 μM of streptavidine-Cy5) before washing, 10 min treatment with 1 mg/ml of trypsin, and FACS analysis. Representative histograms indicating the intensity of cell fluorescence for wild-type and two mutants MCa<sub>b</sub> / streptavidine-Cy5 complexes are shown in figure 3A. MCa<sub>b</sub> K20A induced a mean cell penetration of streptavidine that was 12.5-fold less pronounced than wild-type MCa<sub>b</sub>, whereas MCa<sub>b</sub> E12A increased its cell penetration by a factor of 2.2-fold. A quantitative comparison of the cell penetration of the various MCa<sub>b</sub> analogues was performed and is shown in Figure 3B. Not all point mutated analogues of MCa<sub>b</sub> behave similarly in terms of cell penetration, at least at the concentration tested (1 μM) and for the duration examined (2 hrs). Besides MCa<sub>b</sub> E12A, two other mutants provide a better cell penetration than MCa<sub>b</sub> itself (MCa<sub>b</sub> H6A and MCa<sub>b</sub> K8A). In contrast, two other mutants almost completely inhibit the cell penetration (MCa<sub>b</sub> K19A and MCa<sub>b</sub> K20A). Mild reduction in cell penetration efficacy is observed for MCa<sub>b</sub> L4A, N13A, K22A and R24A (between 1.9- and 3.2-fold). These data thus indicate that the cell penetration properties of MCa<sub>b</sub> can be greatly modulated by selective point mutation of its amino acid sequence. Improved analogues, as well as less potent derivatives, can be produced using simple amino acid substitutions.

To determine the reasons that may underlie these differences in cell penetration efficacies between the various analogues, a dose-response curve on CHO cells was performed for the most and the least penetrating analogues, MCa<sub>b</sub> E12A and MCa<sub>b</sub> K20A, respectively (Figure 4). As shown, the two mutants

differed in their half-effective concentration for cell penetration efficacy over a 2 hrs incubation period. M<sub>Ca<sub>b</sub></sub> E12A penetrates with a half-effective concentration of  $PC_{50} = 113 \pm 16$  nM. In contrast, M<sub>Ca<sub>b</sub></sub> K20A penetrates with an estimated half-effective concentration of  $PC_{50} = 1300-1400$  nM. For the M<sub>Ca<sub>b</sub></sub> K20A mutant, concentrations higher than 2.5  $\mu$ M could not be tested because of cell toxicity of streptavidine. In spite of this toxicity, it was observed that, at saturating concentrations, the same maximal amount of streptavidine-Cy5 could be carried into the cell as with M<sub>Ca<sub>b</sub></sub> E12A. Therefore, it is possible that the differences in penetration efficacies, observed between the various mutated analogues in Figure 3C, is more linked to differences in “cell affinity” for one or several membrane components than to an impairment of the mechanism of cell penetration. The observation that the cell penetration of M<sub>Ca<sub>b</sub></sub> E12A / streptavidine-Cy5 can saturate, is an indication that the process is halted once a form of concentration equilibrium is reached or that the plasma membrane constituents required for cell penetration of the complexes are present in finite amounts.

Finally, we also determined whether differences in pharmacological effects and cell penetration among various M<sub>Ca</sub> analogues could be linked to adverse structural alterations introduced by the point mutations. This was performed by circular dichroism analyses of wild-type M<sub>Ca</sub>, M<sub>Ca</sub> E12A, M<sub>Ca</sub> K20A and M<sub>Ca</sub> R24A since these analogues represented some extreme cases (Figure 5). As shown, the CD spectra of all these peptides are fully superimposed. One can thus deduce that the three analogues possess the same conformation as native M<sub>Ca</sub>.

*Altered cell penetration efficacies of the M<sub>Ca<sub>b</sub></sub> analogues is not associated with differences in cell localization* – To check the cell distribution of M<sub>Ca<sub>b</sub></sub> / streptavidine-Cy5 complexes, it is recommended to work on living cells since cell fixation has been reported to alter the cell distribution of various CPPs [28]. As shown in Figure 6, M<sub>Ca<sub>b</sub></sub> / streptavidine-Cy5 complexes are present as punctuate dots in the cytoplasm of living CHO cells (2 hrs of incubation with the complex, wash, staining and immediate observation by confocal microscopy). The plasma membrane is labeled with concanavaline A, whereas the nucleus is stained with DHE. The distribution of the M<sub>Ca<sub>b</sub></sub> / streptavidine-Cy5 complex is not different than the one observed in fixed HEK293 cells [10], suggesting that, contrary to other CPPs, cell fixation does not alter the distribution of this particular CPP. With mutated M<sub>Ca<sub>b</sub></sub> analogues that penetrate better or less than wild-type M<sub>Ca<sub>b</sub></sub> (M<sub>Ca<sub>b</sub></sub> E12A and M<sub>Ca<sub>b</sub></sub> K20A), a similar subcellular distribution of the complex is observed suggesting that the mechanism of cell penetration is not altered by point mutation of M<sub>Ca<sub>b</sub></sub>. Corroborating the FACS experiments, a significant reduction of complex entry is observed with the M<sub>Ca<sub>b</sub></sub> K20A mutant, whereas a clear increase is observed with the M<sub>Ca<sub>b</sub></sub> E12A mutant. Similar observations were made in HEK293 cells, 3T3 cells, hippocampal and DRG neurons (unpublished observations).

*Differential interaction of M<sub>Ca<sub>b</sub></sub>* and its analogues with negatively charged lipids – For a direct membrane translocation of M<sub>Ca</sub> into cells, a process whereby the peptide would flip from the outer face of the plasma membrane to the inner face, then released free into the cytoplasm, the peptide should interact with various lipids of the membrane, preferably negatively charged ones. These interactions were challenged by incubating 100 nM of M<sub>Ca<sub>b</sub></sub> with lipid strips from Molecular Probes. The immobilized peptide was then revealed with streptavidine-horse radish peroxidase (HRP). M<sub>Ca<sub>b</sub></sub> was found to strongly interact with phosphatidylinositol (PtdIns)(3)P, PtdIns(4)P, PtdIns(5)P, phosphatidic acid and sulfatide, and more weakly with lysophosphatidic acid, PtdIns(3,5)P<sub>2</sub> and phosphatidylserine (Figure 7, Table II). These are all negatively charged lipids. In contrast, at 100 nM, it does not interact with lysophosphatidylcholine, phosphatidylcholine, sphingosine, sphingosine 1-phosphate, phytosphingosine, ceramide, sphingomyelin, sphingosylphosphocholine, myriocin, monosialoganglioside, disialoganglioside, sphingosylgalactoside, cholesterol, lysophosphatidyl choline, or phosphatidylcholine (Figure 7). As control experiment, 100 nM biotin was probed alone and no interaction was found with any of the lipids (data not shown). In comparison with wild-type M<sub>Ca<sub>b</sub></sub> and of all the M<sub>Ca<sub>b</sub></sub> mutants tested, two mutants displayed a new lipid interaction with PtdIns (M<sub>Ca<sub>b</sub></sub> L4A and M<sub>Ca<sub>b</sub></sub> L7A) and four mutants with PtdIns(4,5)P<sub>2</sub> (M<sub>Ca<sub>b</sub></sub> D2A, M<sub>Ca<sub>b</sub></sub> L4A, M<sub>Ca<sub>b</sub></sub> H6A and M<sub>Ca<sub>b</sub></sub> N13A). These new interactions were mostly weak, except for M<sub>Ca<sub>b</sub></sub> L4A with PtdIns(4,5)P<sub>2</sub>. In contrast, many of the weak lipid interactions of M<sub>Ca<sub>b</sub></sub> were lost as a result of various mutations. This is the case for the interactions with lysophosphatidic acid, PtdIns(3,5)P<sub>2</sub> and phosphatidylserine, although it should be mentioned that in the case of PtdIns(3,5)P<sub>2</sub> the interaction could be reinforced. Although it is difficult to correlate specific lipid interactions with the strength of cell penetration, some basic conclusions can be reached. The M<sub>Ca<sub>b</sub></sub> E12A mutant displayed the strongest lipid interactions of all mutants, which is probably at the basis of its better cell penetration properties. In contrast, both M<sub>Ca<sub>b</sub></sub> K19A and M<sub>Ca<sub>b</sub></sub> K20A, that had the weakest cell penetration properties, also displayed the poorest repertoire of lipid interaction and/or the weakest interactions. Further detailed lipid pharmacology combined with FACS analysis will be required to determine the identity of the lipid(s) that are essential for an efficient cell penetration of M<sub>Ca</sub>.

*Cell toxicity of M<sub>Ca<sub>b</sub></sub>* and mutated analogues – M<sub>Ca</sub> was shown to be non toxic to HEK293 cells for incubation periods of 24 hrs and at concentrations up to 5  $\mu$ M [10]. Since HEK293 cells might be more resistant to toxic agents than cells from primary origin, the effect of M<sub>Ca<sub>b</sub></sub> and mutated analogues were also tested on the survival rate of cerebellar granule cells maintained in primary culture (Figure 8). As seen, no cell toxicity could be observed by transmitted light microscopy for a 17 hrs incubation period with 1 or 10  $\mu$ M M<sub>Ca</sub> (Figure 8A). However, using the more sensitive MTT assay, M<sub>Ca</sub> produced  $16.0 \pm 2.8\%$  cell toxicity at 1  $\mu$ M. The biotinylated analogue of M<sub>Ca</sub>, M<sub>Ca<sub>b</sub></sub>, behaved similarly to M<sub>Ca</sub> (not

shown) suggesting that biotinylated and non biotinylated analogues could be compared among each others. However, most M<sub>Ca<sub>b</sub></sub> analogues showed either no neuronal toxicity or limited toxicity at a concentration of 1  $\mu$ M (Figure 8B). Incubation of neuronal cells with a higher concentration of M<sub>Ca</sub> (10  $\mu$ M) produced greater cell death ( $30.4 \pm 17.6\%$ ). Similar neuronal survival percentages were obtained for M<sub>Ca<sub>b</sub></sub> H6A, M<sub>Ca<sub>b</sub></sub> K8A, M<sub>Ca<sub>b</sub></sub> K22A and M<sub>Ca<sub>b</sub></sub> R24A. At 10  $\mu$ M however, most other peptides remained non toxic including many of the best cell-penetrating analogues such as M<sub>Ca<sub>b</sub></sub> D2A, M<sub>Ca<sub>b</sub></sub> P5A, M<sub>Ca<sub>b</sub></sub> L7A, M<sub>Ca<sub>b</sub></sub> L9A, M<sub>Ca<sub>b</sub></sub> E12A and M<sub>Ca<sub>b</sub></sub> D15A. In conclusion, most M<sub>Ca<sub>b</sub></sub> analogues show almost no toxicity on neurons provided that they are used at 1  $\mu$ M, a concentration range that displays a very significant extent of cell penetration for most mutants in complex with streptavidine. Higher concentrations can still be used without adverse effects for several analogues of M<sub>Ca</sub>. Since the peptides interact with the cell membrane lipids, we determined whether some representative peptides could have any effect on cell membrane integrity by assessing LDH release from cells (Figure 8C). As seen, no greater LDH release is produced by a 24 hrs incubation of 10  $\mu$ M M<sub>Ca</sub>, M<sub>Ca</sub> E12A or M<sub>Ca</sub> K20A with CHO cells than expected from limited cell toxicity at this concentration.

## Discussion

### *Cell toxicity and pharmacological profile of MCa*

MCa is a potent activator of RyR. As such, it has the potential to influence  $\text{Ca}^{2+}$  homeostasis in each cell type that expresses a RyR channel. In spite of its effect on  $\text{Ca}^{2+}$  mobilization from intracellular stores, MCa presents limited cell toxicity. No toxicity has been observed on CHO cells or on HEK293 cells, possibly because of the absence of expression of RyR [10]. Cell toxicity can be detected to a very limited extent on primary cultures of cerebellar granule cells, in which the presence of RyR is known, but it is difficult to correlate this toxicity to the pharmacological action of the peptide. Indeed, a similar limited toxicity is apparent at concentrations above 1  $\mu\text{M}$  for the MCa R24A mutant that has not the ability to mobilize  $\text{Ca}^{2+}$  from SR [9], whereas, in contrast, the MCa E12A mutant, that stimulates [ $^3\text{H}$ ]-ryanodine binding onto RyR1 at lower concentrations than MCa, does not display any sign of cell toxicity at 10  $\mu\text{M}$ . It should be emphasized that neurons are more likely to express the RyR2 and RyR3 isoforms than the RyR1 isoform tested herein. Since neither the pharmacological action of MCa onto these two isoforms, nor the key residues responsible for this potential effect, have been determined, it remains difficult to make a precise correlation between a key amino acid residue of MCa and some adverse effect on neuronal survival. Nevertheless, several conclusions are within reach for the design of an efficient drug carrier based on MCa's sequence. First, it is highly desirable to develop a carrier that is devoid of effect on any of the RyR isoforms, particularly with regard to  $\text{Ca}^{2+}$  mobilization. The partial alanine scan we performed on the sequence of MCa reveals that this goal is largely accessible experimentally. Further mutations may be planned, and more extensive tests will be required on the two other RyR isoforms, but it is clear that many key residues already represent starting leads for the rationale design of pharmacologically inert analogues of MCa. The amino acid residues of interest include L7, K8, K19, K20, K22, R23, R24 and T26, which represents a sufficiently diverse array of workable opportunities. Second, developing a cell penetrating analogue of MCa that is devoid of cell toxicity is also clearly within reach. Most analogues were non toxic to neurons, and many other appear as efficient protein carriers when used at concentrations well below the toxic concentration. For instance, the MCa E12A mutant is maximally effective in carrying streptavidine into cells when used at a concentration of 250 nM, whereas it is non toxic even at 10  $\mu\text{M}$ . Among the pharmacologically least active analogues devoid of cell toxicity, we can mention those that are mutated at position L7, K19, K20, R23 and T26. This still represents sufficient diversity for the design of numerous novel cell penetrating analogues lacking both pharmacological activity and signs of cell toxicity. Third, taking into account the two above mentioned criteria, lack of pharmacological activity and lack of cell toxicity, any novel analogue designed should keep potent cell penetration efficiency. Among the residues

of interest in terms of pharmacology (L7, K19, K20, R23 and T26), we tested three of them for their penetration efficiency (L7, K19 and K20). Only the L7 locus appears as an interesting lead position since the M<sub>Ca</sub> L7A mutant displays an efficient cell penetration. The L7A being a rather conservative substitution, the strong impact it displays on the pharmacology of M<sub>Ca</sub> is quite encouraging. It seems very likely that less conservative substitutions at this position should further impact the pharmacology without impairing cell penetration. It should be mentioned also that for the design of novel potent analogues, an alternative strategy to a mono substitution would be to perform several substitution at a time: one that impairs the pharmacological impact of M<sub>Ca</sub> and shows a lack of toxicity, combined with another that has improved cell penetration efficiency. Obviously, this study illustrates that many leading strategies can be developed in the future for the production of highly competent analogues of M<sub>Ca</sub>. Further development of a potent M<sub>Ca</sub> analogue will also be based on the chemical strategies for coupling M<sub>Ca</sub> to cargoes. As such, the recent finding that a M<sub>Ca</sub> analogue devoid of internal cysteine residues, required for the disulfide bridges formation, is of great interest if N-terminal cysteine chemistry is pursued as coupling strategy (data not shown).

*The molecular determinants of M<sub>Ca</sub> implicated in pharmacology and cell penetration overlap partially*

A peptide that (i) has homology with a calcium channel sequence (domain A of the II-III loop of Ca<sub>v</sub>1.1), (ii) acts as a pharmacological activator of RyR, and (iii) penetrates efficiently into cells by crossing the plasma membrane puts considerable stringency on its primary structure. Proof of this fact comes from a sequence comparison with M<sub>Ca</sub> related peptide (IpTx A, opicalcine 1 and opicalcine 2) that demonstrates very little sequence variation. Amino acid residues of M<sub>Ca</sub> analogous to domain A include K19, K20, K22, R23, R24, and T26. Absolutely all these residues are essential for the pharmacological effect of M<sub>Ca</sub>, even though they are not solely implicated in the recognition of RyR. A similar residue profile appears to exist for the interaction of M<sub>Ca</sub> with negatively charged lipids (Table II) and for cell penetration (Figure 3), although R23 and T26 mutants were not tested. Undoubtedly, the most prominent effects were observed for K19 and K20 mutations, with milder effects for K22 and R24 mutations, suggesting that cell penetration was less deeply hampered by single point mutations of M<sub>Ca</sub> residues that are analogous to those of domain A. Proof that structural divergence exists between M<sub>Ca</sub> and domain A is the observation that domain A is unable to penetrate into cells in spite of its homology by the presence of basic residues (data not shown). The structural requirements for the cell penetration efficiency of M<sub>Ca</sub> are less stringent than those involved in the pharmacological effect of the peptide. This is in line with what is known on other CPPs, with the main structural requirement being the presence of a basic surface for an efficient cell penetration. This lower stringency is also in line with the considerations developed above on the fact that it should be feasible to uncouple both properties by developing peptide analogues devoid of



pharmacological effects but keeping considerable cell penetration efficiencies. Nevertheless, a particular mention should be made to the fact that each mutation of M<sub>Ca</sub> that increases the net positive charge of the peptide (D2A, D15A and E12A), all improve the EC<sub>50</sub> effect on [<sup>3</sup>H]-ryanodine binding. This may implicate that the nature of the interaction between M<sub>Ca</sub> and its binding site on RyR is electrostatic. In the RyR sites identified for the binding of domain A or M<sub>Ca</sub>, there is indeed a stretch of negatively charged amino acid residues within site F7.2 that look as promising candidates for the interaction [8]. Further experimental will be required to test out this hypothesis. Only one of the mutations of the negatively charged residues of M<sub>Ca</sub> (E12A and not D2A or D15A) contributes to an improved cell penetration when tested at a micromolar concentration. But since the E12A mutant also displays an improved dose-response curve for cell penetration, similar dose-responses will need to be performed in order to really efficiently compare the cell penetration potency of M<sub>Ca</sub> D2A and M<sub>Ca</sub> D15A. Conversely, the K8A mutation induces a slightly better cell penetration, also indicating that increasing the net global positive charges of the molecule is not a prerequisite for a better penetration. Since this mutation increases the dipole moment of the molecule, this may better explain the greater potential of this analogue.

*A correlation appears to exist between the strength of lipid interaction and the potency of cell penetration of M<sub>Ca</sub>'s analogues*

The mechanism of cell penetration of CPPs is not well dissected. Two modes of cell penetration have been proposed. One involves a direct interaction of CPPs with charged heparan sulfates present at the cell surface, followed by macropinocytosis. Delivery into the cytoplasm would be partial and would occur from late endosomes. A second one implies a direct interaction of the CPPs with negatively charged lipids, followed by a translocation mechanism that delivers the penetrating peptide and its cargo into the cytoplasm. Both modes are not contradictory and may well coexist in a variety of cell types. Also, the concept that one set of interactions directs the mode of penetration (lipids for translocation, and heparin sulfates for a form of endocytosis) is by itself unproven. Herein, we have further examined the interaction of M<sub>Ca</sub> and mutated analogues with several lipids. The observations made are coherent with the presence of a basic surface on M<sub>Ca</sub>. The peptides interact well with many negatively charged lipids which is a prerequisite for mechanism of penetration that is based on membrane translocation. Among all the lipids identified, it is difficult to declare that a particular lipid is responsible for the penetration of M<sub>Ca</sub>. Even for the least efficient M<sub>Ca</sub> analogue, M<sub>Ca</sub> K20A, that interacts only with PtdIns(3)P and sulfatide, there is still some degree of cell penetration. What seems to differ among all analogues is not whether these peptides penetrate or not, it is rather the effective concentration that is required for cell penetration. This was obvious when comparing the dose-response curve for cell penetration of the two extreme mutants, M<sub>Ca</sub> E12A and M<sub>Ca</sub> K20A. It is therefore tempting to conclude that cell penetration requires both a good

affinity for many of the negatively charged lipids and a wide set of interactions. Further experimental work will be required to determine the affinity of MCa for each of the negatively charged lipid identified here in order to determine whether a particular correlation may exist between the half-effective concentration of MCa for penetration and its affinity for some lipids. Also, the evidence that the penetrated streptavidine appears as punctuate dots in the cytoplasm of cells may after all indicate a strong contribution of the interaction of MCa with lipids in a form of endocytosis. Clarification will be needed on this issue.

Our data indicate that many basic residues of MCa are involved in the interaction with lipids. The fact that a simple mutation of some chosen basic residues can so drastically alter the interaction with some lipids is surprising owing to the fact that many other basic residues remain present within MCa. Nevertheless, these data indicate that amino acid substitution may thus represent a very interesting alternative to develop MCa analogues presenting greater affinity for these lipids or even wider selectivity. Ultimately, this should lead to the development of analogues of greater potential for cell penetration. Another surprising finding is that many of the residues that appear important for an interaction with negatively charged lipids are also those that are involved in sequence homology with domain A and in the pharmacological effect of MCa. This leads to the interesting and unexplored possibility that (i) domain A may itself interact with negatively lipids adding complexity to the role of the plasma membrane in the process of excitation-contraction, and (ii) lipids may competitively favor or suppress the interaction of MCa or domain A with its binding site on RyR. Obviously further work will be required to address these challenging issues.

## **Acknowledgements**

We acknowledge financial support of Inserm, Université Joseph Fourier and CEA for this project. Narendra Ram is a fellow of the Région Rhône-Alpes and is supported by an Emergence grant. We acknowledge the technical support of Pascal Mansuelle in peptide analyses.

## References

- [1] Z. Fajloun, R. Kharrat, L. Chen, C. Lecomte, E. di Luccio, D. Bichet, M. El Ayeub, H. Rochat, P.D. Allen, I.N. Pessah, M. De Waard, J.M. Sabatier, Chemical synthesis and characterization of maurocalcine, a scorpion toxin that activates Ca<sup>2+</sup> release channel/ryanodine receptors. *FEBS Lett* 469 (2000) 179-785.
- [2] A. Mosbah, R. Kharrat, Z. Fajloun, J.G. Renisio, E. Blanc, J.M. Sabatier, M. El Ayeub, H. Darbon, A new fold in the scorpion toxin family, associated with an activity on a ryanodine-sensitive calcium channel. *Proteins* 40 (2000) 436-442.
- [3] R. El Hayek, A.J. Lokuta, C. Arevalo, H.H. Valdivia, Peptide probe of ryanodine receptor function. Imperatoxin A, a peptide from the venom of the scorpion *Pandinus imperator*, selectively activates skeletal-type ryanodine receptor isoforms. *J Biol Chem* 270 (1995) 28696-28704.
- [4] S. Zhu, H. Darbon, K. Dyason, F. Verdock, J. Tytgat, Evolutionary origin of inhibitor cystine knot peptides. *FASEB J* 17 (2003) 1765-1767.
- [5] E. Estève, S. Smida-Rezgui, S. Sarkozi, C. Szegedi, I. Regaya, L. Chen, X. Altafaj, H. Rochat, P. Allen, I.N. Pessah, I. Marty, J.M. Sabatier, I. Jona, M. De Waard, M. Ronjat, Critical amino acid residues determine the binding affinity and the Ca<sup>2+</sup> release efficacy of maurocalcine in skeletal muscle cells. *J Biol Chem* 278 (2003) 37822-37831.
- [6] L. Chen, E. Estève, J.M. Sabatier, M. Ronjat, M. De Waard, P.D. Allen, I.N. Isaac, Maurocalcine and peptide A stabilize distinct subconductance states of ryanodine receptor type I, revealing a proportional gating mechanism. *J Biol Chem* 278 (2003) 16095-16106.
- [7] S. Pouvreau, L. Csernoch, B. Allard, J. M. Sabatier, M. De Waard, M. Ronjat, V. Jacquemond, Transient loss of voltage control of Ca<sup>2+</sup> release in the presence of maurocalcine in mouse skeletal muscle. *Biophys J* (in press).
- [8] X. Altafaj, E. Cheng, E. Esteve, J. Urbani, D. Grunwald, J.M. Sabatier, R. Coronado, M. De Waard, M. Ronjat, Maurocalcine and domain A of the II-III loop of the dihydropyridine receptor Ca<sub>v</sub>1.1 subunit share common binding sites on the skeletal ryanodine receptor. *J Biol Chem* 280 (2005) 4013-4016.
- [9] E. Esteve, K. Mabrouk, A. Dupuis, S. Smida-Rezgui, X. Altafaj, D. Grunwald, J.C. Platel, N. Andreotti, I. Marty, J.M. Sabatier, M. Ronjat, M. De Waard M, Transduction of the scorpion toxin maurocalcine into cells. Evidence that the toxin crosses the plasma membrane. *J Biol Chem* 280 (2005) 12833-12839.
- [10] S. Boisseau, K. Mabrouk, N. Ram, N. Garmy, V. Collin, A. Tadmouri, M. Mikati, J.M. Sabatier, M. Ronjat, J. Fantini, M. De Waard, Cell penetration properties of maurocalcine, a natural venom peptide active on the intracellular ryanodine receptor. *Biochim Biophys Acta* 2006;1758:308-319.
- [11] A.D. Frankel, C.O. Pabo, Cellular uptake of the Tat protein from human immunodeficiency virus. *Cell* 55 (1988) 1189-1193.
- [12] D. Derossi, S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing, A. Prochiantz, Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J Biol Chem* 271 (1996) 18188-18193.
- [13] M. Pooga, M. Hällbrink, M. Zorko, Ü Langel, Cell penetration of transportan. *FASEB J* 12 (1998) 67-77.
- [14] S.M. Fuchs, R.T. Raines, Pathway for polyarginine entry into mammalian cells, *Biochemistry* 43 (2004) 2438-2444.
- [15] A. Astriab-Fischer, D. Sergueev, M. Fiscjer, B.R. Shaw, R.L. Juliano, Conjugates of antisense oligonucleotides with the Tat and antennapedia cell-penetrating peptides: effects on cellular uptake, binding to target sequences, and biologic actions. *Pharm Res (N.Y.)* 19 (2002) 744-754.
- [16] I.A. Ignatovich, E.B. Dizhe, A.V. Pavlotskaya, B.N. Akifiev, S.V. Burov, S.V. Orlov, A.P. Perevozchikov, Complexes of plasmid DNA with basic domain 47-57 of the HIV-1 tat protein are

- transferred to mammalian cells by endocytosis-mediated pathways. *J Biol Chem* 278 (2003) 42625-42636.
- [17] M. Pooga, U. Soomets, M. Hällbrink, A. Valkna, K. Saar, K. Rezaei, U. Kahl, J.X. Hao, X.J. Xu, Z. Wiesenfeld-Hallin, T. Hökfelt, T. Bartfai, Ü. Langel, Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat Biotech* 16 (1998) 857-861.
- [18] N. Shibagaki, M.C. Udey, Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity. *J Immunol* 168 (2002) 2393-2401.
- [19] M. Rojas, J.P. Donahue, Z. Tan, Y.Z. Lin, Genetic engineering of proteins with cell membrane permeability. *Nat Biotech* 16 (1998) 370-375.
- [20] M. Pooga, C. Kut, M. Kihlmark, M. Hällbrink, S. Fernaeus, R. Raid, T. Land, E. Hallberg, T. Bartfai, Ü. Langel, Cellular translocation of proteins by transportan. *FASEB J* 15 (2001) 1451-1453.
- [21] V.P. Torchilin, R. Rammohan, V. Weissig, T.S. Levchenko, TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc Natl Acad Sci USA* 98 (2001) 8786-8791.
- [22] M. Lewin, N. Carlesso, C.H. Tung, X.W. Tang, D. Cory, D.T. Scadden, R. Weissleder, Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotech* 18 (2000) 410-414.
- [23] B. Merrifield, Solid phase synthesis. *Science* 232 (1986) 341-347.
- [24] D.H. Kim, S.T. Ohnishi, N. Ikemoto, Kinetic studies of calcium release from sarcoplasmic reticulum in vitro. *J Biol Chem* 258 (1983) 9662-9668.
- [25] I. Marty, D. Thevenon, C. Scotto, S. Groh, S. Sainnier, M. Robert, D. Grunwald, M. Villaz, Cloning and characterization of a new isoform of skeletal muscle triadin. *J Biol Chem* 275 (2000) 8206-8212.
- [26] V. Gallo, A. Kingsbury, R. Balazs, O.S. Jorgensen, The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. *J Neurosci* 7 (1987) 2203-2213.
- [27] T.M. Miller, E.M. Johnson Jr, Metabolic and genetic analyses of apoptosis in potassium/serum-deprived rat cerebellar granule cells. *J Neurosci* 16 (1996) 7487-7495.
- [28] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L.V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 278 (2003) 585-590.

## Figure legends

Fig. 1. Amino acid sequences of M<sub>Ca</sub> and of its biotinylated analogues. Amino acid residues are described by single letter code. K<sub>b</sub> = biotinylated version of lysine residue. The net global charge of the molecule is provided for each M<sub>Ca</sub> analogue.

Fig. 2. Concentration-dependent effect of M<sub>Ca</sub> analogues on [<sup>3</sup>H]-ryanodine binding to heavy SR vesicles. [<sup>3</sup>H]-ryanodine binding was measured at pCa 5 (free Ca<sup>2+</sup> 10 μM) in the presence of 5 nM [<sup>3</sup>H]-ryanodine for 3 hrs at 37°C. Non specific binding was less than 5% of the total binding and was independent of the concentration of each M<sub>Ca</sub> analogue. Data were fitted with a logistic function of the type  $y = y_0 + (a/(1+(x/EC_{50})^b))$  where  $y_0$  is the basal [<sup>3</sup>H]-ryanodine binding in the absence of M<sub>Ca</sub> or its analogues,  $a$  the maximum achievable binding,  $b$  the slope coefficient and  $EC_{50}$  the concentration of half-binding stimulation. Experimental data for the effect of M<sub>Ca</sub> is shown once (top left binding curve, filled circles) and the fit of these data shown each time as dashed line for the purpose of comparison with each M<sub>Ca</sub> analogue.  $EC_{50}$  values and binding stimulation factors are provided in Table 1. Data are triplicates. Representative cases of n=3 experiments.

Fig. 3. Cell penetration of wild-type and mutated M<sub>Ca<sub>b</sub></sub> in complex with streptavidine-Cy5 in CHO cells as assessed quantitatively by FACS. The concentration used for all the analogues is 4 μM combined with 1 μM streptavidine-Cy5. Cells were incubated 2 hrs with the complex before being washed and treated for 10 min with 1 mg/ml trypsin. *A*, Comparison of FACS data on CHO cells for streptavidine-Cy5 alone (control), for M<sub>Ca<sub>b</sub></sub> K20A, the least penetrating mutant, for M<sub>Ca<sub>b</sub></sub>, and for M<sub>Ca<sub>b</sub></sub> E12A, the most penetrating analogue. *B*, Comparison of mean fluorescence intensity for the cell penetration of all M<sub>Ca<sub>b</sub></sub> analogues in CHO cells. The dash represents the mean fluorescence of cells alone. All data performed the same day with the same batch of cells. Representative case of n=3 experiments.

Fig. 4. Mean cell fluorescence intensity as a function of concentration of cell penetrating complexes. The indicated concentrations are for streptavidine-Cy5 (from 10 nM to 2.5 μM). Streptavidine concentrations higher than 2.5 μM are toxic to cells when allowed to penetrate and cannot be tested. M<sub>Ca<sub>b</sub></sub> K20A and M<sub>Ca<sub>b</sub></sub> E12A concentrations are fourfold greater than streptavidine-Cy5 concentrations (from 40 nM to 10 μM). The data for M<sub>Ca<sub>b</sub></sub> E12A could be fit by a sigmoid function of the type  $y = a/(1+\exp(-(x-PC_{50})/b))$  where  $a = 2541 \pm 108$  a.u., the maximal fluorescence intensity,  $PC_{50} = 113 \pm 16$  nM, the half-maximal effective penetrating concentration, and  $b = 20.9 \pm 17.9$ . Experiments were repeated three times.

Fig. 5. Conformational analysis of maurocalcine analogues by circular dichroism. Far-UV CD spectra of MCa and three of its analogues at 50  $\mu$ M in pure water recorded at 20°C. Each spectrum is the mean of three independent acquisitions.

Fig. 6. Confocal immunofluorescence images of living CHO cells incubated for 2 hrs with 1  $\mu$ M streptavidine-Cy5 in complex with 4  $\mu$ M MCa<sub>b</sub>, MCa<sub>b</sub> E12A or MCa<sub>b</sub> K20A. Streptavidine-Cy5 appears in blue, the nucleus in red and the plasma membrane in green. Note the lack of differences in cell distribution of streptavidine-Cy5 with the MCa<sub>b</sub> analogues but the obvious differences in cell penetration efficiencies. Images are from a single confocal plane. Control images were also included for cells incubated with MCa<sub>b</sub> alone to show that the blue fluorescence observed is only due to the presence of streptavidine and is not related to the ability of DHE to fluoresce in blue in the cytoplasm (top panel).

Fig. 7. Interaction of MCa<sub>b</sub> or mutated analogues with membrane lipids. The MCa<sub>b</sub> analogues were used at a concentration of 100 nM and incubated for 2 hrs with the lipid strips. Each dot possesses 100 pmoles of lipid immobilized on the strip. Representative examples are shown for MCa<sub>b</sub>, MCa<sub>b</sub> L7A, MCa<sub>b</sub> E12A, MCa<sub>b</sub> K20A and MCa<sub>b</sub> R24A. *A*, Interaction with various phospholipids. The lipids are identified by their numbered positions on the strip (left panel). Numbers refer to: lysophosphatidic acid (1), lysophosphatidylcholine (2), phosphatidylinositol (PtdIns) (3), PtdIns(3)P (4), PtdIns(4)P (5), PtdIns(5)P (6), phosphatidylethanolamine (7), phosphatidylcholine (8), sphingosine 1-phosphate (9), PtdIns(3,4)P<sub>2</sub> (10), PtdIns(3,5)P<sub>2</sub> (11), PtdIns(4,5)P<sub>2</sub> (12), PtdIns(3,4,5)P<sub>3</sub> (13), phosphatidic acid (14), phosphatidylserine (15), and no lipids (16). *B*, Interactions of MCa<sub>b</sub> and mutated analogues with sphingolipids. Numbers refer to: sphingosine (1), sphingosine 1-phosphate (2), phytosphingosine (3), ceramide (4), sphingomyelin (5), sphingosylphosphocholine (6), lysophosphatidic acid (7), myriocin (8), monosialoganglioside (9), disialoganglioside (10), sulfatide (11), sphingosylgalactoside (12), cholesterol (13), lysophosphatidyl choline (14), phosphatidylcholine (15), and blank (16).

Fig. 8. Neuronal toxicity of MCa and mutated analogues. Peptides were tested alone without coupling to streptavidine. *A*, Transmitted light images (differential interference contrast) illustrating cerebellar granule cells in primary culture before (0 hr) and after (17 hrs) incubation with 1 or 10  $\mu$ M MCa. *B*, Neuronal survival after a 17 hrs incubation period with either 1 or 10  $\mu$ M MCa or one of its mutated biotinylated analogue as assessed by the MTT test. MCa and MCa<sub>b</sub> produced similar results. These experiments were repeated three times, data shown as triplicates. Significance is provided as a deviation of three times the SD value from 100% (denoted as asterisks). *C*, Cell toxicity effect of 10  $\mu$ M MCa, MCa E12A or MCa K20A incubated 24 hrs with CHO cells as measured by LDH release.

Table I. Effect of  $\text{MCA}_b$  and its analogues on the parameters of [ $^3\text{H}$ ]-ryanodine binding onto SR vesicles. The  $\text{EC}_{50}$  values and stimulation factors are obtained from the fit of the data shown in Figure 2.

Table II. Strength of lipid interactions for  $\text{MCA}_b$  and its analogues. Experimental conditions as described in the legend of Figure 6.