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1 **Caloxin-derived peptides for the inhibition of plasma membrane calcium ATPases**

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38 **Abstract:** Plasma membrane calcium ATPases (PMCAs) are a family of transmembrane proteins
39 responsible for the extrusion of cytosolic Ca²⁺ to the extracellular milieu. They are important players
40 of the calcium homeostasis possibly implicated in some important diseases. The reference inhibitors of
41 PMCA extruding activity are on one hand ortho-vanadate (IC₅₀ in the 30 mM range), and on the other
42 a series of 12- to 20-mer peptides named caloxins (IC₅₀ in the 100 μM scale). As for all integral
43 membrane proteins, biochemistry and pharmacology are difficult to study on isolated and/or purified
44 proteins. Using a series of reference blockers, we assessed a pharmacological window with which we
45 could study the functionality of PMCAs in living cells. Using this system, we screened for alternative
46 versions of caloxins, aiming at shortening the peptide backbone, introducing non-natural amino acids,
47 and overall trying to get a glimpse at the structure-activity relationship between those new peptides
48 and the protein in a cellular context. We describe a short series of equipotent 5-residue long analogues
49 with IC₅₀ in the low μM range.

50 **Keywords:** PMCA; peptide inhibitor; caloxins; calcium efflux; cellular test.

51

52 **1. Introduction**

53 Plasma membrane calcium ATPases (PMCA) are key components of the cellular calcium
54 homeostasis [1]. They form a family of four-member family of transmembrane proteins that actively
55 transports calcium throughout the plasma membrane in a highly regulated manner in non-excitable
56 cells [1,2]. They correspond to ATP-driven Ca^{2+} pumps largely expressed in eukaryotic cells and their
57 role is mainly to maintain a low resting cytosolic concentration of calcium. These integral membrane
58 proteins exist in many different isoforms resulting from differential splicing for which individual roles
59 are still largely missing. They are involved in a wide range of pathological conditions in animal
60 models and human diseases [3]. Recent advances strongly suggest that PMCA might have a role in
61 surviving of cancer cells [4], particularly in pancreas cancer cells [5]. Therefore, new actors in the
62 pharmacology of these transporters could present interesting new clinical routes towards anticancer
63 therapies.

64 Methodologies to isolate and address PMCA function have essentially relied on their purification
65 and reconstitution in artificial vesicles [6], and often focused on the detection of the ATPase activity
66 of the pump rather than on the calcium fluxes themselves. For the sake of screening purposes, we
67 reasoned that PMCA function could be specifically addressed in living cells (*i*) by using calcium
68 movements as readout and (*ii*) by designing a “functional isolation” approach instead of using the
69 classical “physical isolation” strategy where the pump works in a completely artificial environment.
70 Indeed, in a living cell, cytosolic calcium concentration at each time point is a snapshot of the dynamic
71 equilibrium established between the activity of the different Ca^{2+} channels/pumps bringing calcium to
72 the cytosol and the concomitant reverse activity of channels and pumps by extrusion to the
73 extracellular space and/or by sequestration into subcellular organelles leading to the global cellular
74 calcium homeostasis (**Scheme 1A**). Pumps removing calcium are particularly active when calcium
75 concentration is high during which time, they take a net lead over the calcium influx mechanisms.
76 Using the available well characterized inhibitors of the contributors of the calcium homeostasis, we
77 defined a combination of pharmacological blockers capable to functionally trigger a net cytosolic
78 calcium clearance condition (*i.e.* “window”) exclusively supported by the PMCA function. This
79 pharmacological window can be easily studied by kinetic fluorescence 96-well plate imaging of living

80 cultured cells. For this purpose, we used HEK 293 cells in which PMCA4 is the main native PMCA
81 isoform expressed [4,7].

82

83 Since the concentration of one of the main reference PMCA inhibitors, ortho-vanadate (oVa),
84 used in the cellular assays is very high (in the 30 mM range) and its specificity towards other cellular
85 systems not guaranteed, several possibilities were previously explored to find alternative reference
86 compounds inhibiting PMCA such as lanthanum salts [8] or carboxyeosine [9]. Lanthanum salts tend
87 to precipitate with many different anions from μM concentrations and inhibit PMCA at mM
88 concentrations, while the use of carboxyeosin is hampered by its membrane impermeability (see Chen
89 et al for discussion [10]). In addition, the group of Grover found by different means several 20-amino-
90 acid long peptides, named caloxins, with interesting inhibitory capacities on these particular proteins
91 [11]. Nevertheless, these peptides were not optimized and thus, the field still suffers of a lack of more
92 easily attainable and trustable tools.

93 In the present study, we designed an assay for PMCA inhibition using HEK 293 cells [7]. Our
94 understanding is that HEK 293 cells express mainly PMCA4 with traces of PMCA1, leading to the
95 possibility that even small amount of PMCA1 could interfere with the whole process. The goal was to
96 discover potent inhibitors of PMCA-dependent calcium movements in living cells in a screening-
97 friendly 96 wells plate format. We used a FLIPR^{TETRA} in a high-throughput assay mode (as opposed to
98 high-throughput screening format). This automatic instrument insured the repetitivity of the assays
99 within a 96-well plate format. The instrument has been previously described for intracellular Ca^{2+}
100 measurement by the manufacturer [12] and by several laboratories [13,14], including ours, in the
101 characterization of newly discovered receptor functionality [15]. We used all the reported caloxins as
102 starting materials [16–19] with the aim of simplifying the sequence as a first step, particularly by
103 shortening these peptides. Their use in an initial step of our assay also validated the assay, as
104 comforting results were obtained, in comparison to the original data obtained with a completely
105 different assay [20]. More than one hundred of analogues were synthesized, characterized, and tested.
106 The final peptides we described are more potent than the parent peptides and potentially more specific
107 than the reference PMCA inhibitor, oVa. Incidentally, the present work is among the first ones

108 screening for finding PMCA inhibitors by measuring its calcium trafficking, as opposed to the often
109 use of PMCA ATPase activity measurement in most of the research for PMCA inhibitor/antagonist
110 [21–23].

111

112 **2. Materials and Methods**

113 **2.1 Reagents:** Standard Fmoc-amino acids and D-counterparts, Oxyma Pure, *N,N'*-
114 diisopropylcarbodiimide (DIC), Fmoc-Rink-amide aminomethyl-polystyrene resin, and preloaded
115 Fmoc-Gly-2-chlorotrityl resin and Fmoc-Arg(Pbf)-Wang resin were purchased from Iris Biotech
116 (Marktredwitz, Germany). Boc-Lys(Fmoc)-OH and other non-standard Fmoc-amino-acid building
117 blocks are from Sigma-Aldrich (Saint-Quentin-Fallavier, France), Iris Biotech, Eurogentec (Seraing,
118 Belgium), ACROS (Illkirch-Graffenstaden, France), Eurogentec (Seraing, Belgique), Bachem
119 (Bubendorf, Switzerland) or custom-synthesized for Servier Laboratories. Acetic anhydride,
120 acetonitrile (HPLC grade), ascorbic acid, dimethylformamide (DMF), dimethylsulfoxide (DMSO),
121 dichloromethane (DCM), hexafluoroisopropanol (HFIP), I₂, methanol, diethylether, trifluoroacetic
122 acid (TFA), piperidine and triisopropyl silane (TIS) were supplied from Sigma-Aldrich.

123
124 **2.2 Peptide synthesis:** All peptides were synthesized by Fmoc solid phase methodology on a
125 SymphonyX instrument (Gyros Protein Technologies AB, Uppsala, Sweden) using the standard
126 manufacturer's procedures at 100 μmol scale. Briefly, The standard deprotection-coupling cycle for
127 each residue consists of six steps: (i) Wash of the resin with 5 mL of DMF (3 x 30 sec); (ii) On-resin
128 Fmoc group deprotection with 5 mL of 20% piperidine in DMF (3 x 3 min); (iii) Wash of the resin
129 with 5 mL of DMF (3 x 30 sec); (iv) Fmoc-AA-OH coupling (2 x 60 min), a) 5 mL of 0.2 M (10 eq.)
130 Fmoc-AA-OH in DMF, b) 2 mL of 0.5 M (10 eq.) Oxyma Pure in DMF, and c) 1 mL of 1 M (10 eq.)
131 DIC in DMF; (v) Capping with 5 mL of 10% Ac₂O in DMF (7 min) and finally (vi) Wash with 5 mL
132 of DMF (3 x 30 sec). After completion of the chain assembly, peptidyl resins were washed thrice with
133 DCM. Peptides were deprotected and cleaved from the resin by adding 10 mL of cleavage cocktail
134 TFA/TIS/H₂O (90:5:5). for 180 min at room temperature. Cleavage solution was filtered from the
135 resin and peptides were precipitated by addition to 40 mL of ice-cold diethylether. After >1 h at -20
136 °C, ethered layer was centrifuged at 3500 RCF and the supernatant was decanted. Pellets were washed
137 twice with diethylether and then dried for >3 h in a vacuum desiccator prior to dissolution for
138 analytical characterization and purification. The peptides were analyzed by UPLC at 214 nm on a 150
139 × 2.1 mm BEH C₁₈ column (Waters, Guyancourt, France) using a linear gradient of acetonitrile/TFA
140 (99.9:0.1) at a flow rate of 0.6 mL/min and by ESI mass spectrometry.

141
142 **2.3 Cal1b332 head-to-tail cyclic peptide synthesis:** The linear precursor of **cal1b332** was
143 synthesized on a preloaded Fmoc-Gly-2-chlorotrityl resin as described above. After completion of the
144 chain assembly, the linear peptide was released from the resin as a fully sidechain protected peptide by
145 adding 20 mL of the mixture HFIP/DCM (2:8, 3 × 30 min, room temperature). The combined organic
146 layers were concentrated under reduced pressure to obtain crude peptide. The linear protected peptide
147 was then dissolved in DCM (1 mM) and 2 eq. of Oxyma Pure and 2 eq. of DIC were added. Reaction
148 mixture was stirred overnight at room temperature for lactamization. Solvent was removed under

149 reduced pressure and the crude head-to-tail cyclic peptide was side-chain deprotected and
150 characterized as described above.

151

152 **2.4 Disulfide bridge formation:** Crude sulfhydryl peptides were dissolved (1 mM) in H₂O/TFA
153 (99.9:0.1) and a 60 mM solution of iodine in MeOH was added dropwise with rapid stirring until the
154 solution had a slight yellow color. Iodine excess was quenched with 1 M ascorbic acid prior to
155 analytical characterization as described above.

156

157 **2.5 Peptide purification:** Crude peptides were purified by reversed-phase HPLC with a Delta
158 Prep 4000 system (Waters) on a 50 × 300 mm Vydac Denali prep C-18 (10 μm, 120 Å) column using
159 an appropriate gradient of acetonitrile/TFA (99.9:0.1) at a flow rate of 60 mL/min. Eluting fractions,
160 detected at 214 nm, are collected, and injected onto analytical column as described above. Fractions
161 containing the pure target peptide were then combined and lyophilized. Qualification data for all
162 synthesized peptides are listed on **Tables 1a** and **1b** together with their respective sequences. Non-
163 standard Fmoc-amino-acid building blocks are listed in **Table 2**.

164

165 **2.6 Peptide sample preparation:** All peptides were classically solubilized in pure DMSO at 100
166 mM (net peptide content). Then, a 10x working concentration was reached by diluting the stock
167 solution in PBS buffer. For each solution, we tested the number of particles in the solution to survey
168 the possible aggregation by light scattering measurements at 400 nm as previously described [24]. No
169 aggregation was detected under our experimental conditions in the 10x solutions.

170

171 **2.7 FLIPR^{TETRA} instrument:** The FLIPR Tetra system (Molecular Devices, Sunnyvale, CA)
172 with whole-plate imaging allows the measurement to be accomplished in a 96-well format with well-
173 by-well analyses, video archiving of data, and automated data analysis. The system has an on-board
174 liquid handling for dispensing simultaneously into the 96 wells of the plate, compound(s) or reagent(s)
175 from dedicated plates or vials. These additions are time controlled. Incidentally, the variation on
176 pipetted volumes was measured to be less than 0.2%. The acquisition of the data in the instrument is
177 made easy by the use of plates in which the entire bottom is illuminated with excitation light and then
178 fluorescence emission is imaged onto a fast CCD camera. The signal from individual wells is
179 discerned by appropriate binning of pixels associated with each well, and the average intensity is
180 recorded as a function of time. This allows kinetic measurements to be performed on a well-by-well
181 basis with superior signal to noise ratio. This allows dosing to be controlled very accurately from both
182 a volume and timing perspective [12]. Each well is considered as a single timely measure.

183

184 **2.8 PMCA-dependent Ca²⁺ traffic measurement:** HEK 293 cells, cultivated as described [25],
185 were seeded on poly-D-Lysine coated 96-well plates (Corning, Sigma Aldrich) 24 hours before the
186 assay at a density of 40,000 cells/well in PBS buffer. Cells were then loaded for 1 hour with 5 μ M
187 Rhod-4-AM (Molecular Devices, San José, CA, USA) as previously described [26] in the presence of
188 20 μ M sulfinpyrazone (Sigma-Aldrich), an anion transport blocker [27]. The design of the
189 experimental protocol is described in the corresponding paragraph (see Results section). The cytosolic
190 calcium-related fluorescence, subtracted from the baseline level, was measured at 555 nm (λ_{ex} = 488
191 nm) for up to 15 minutes at room temperature. All measurements were performed with the FLIPR-
192 TETRA instrument in quadruplicates (with some experimental variations) and independently repeated
193 (n = 3-6). Up to the point where the experimental test parameters were validated, we present the data
194 as direct measurements of the fluorescence due to the Rhod-4/Calcium complex.

195 Once the various parameters were fixed, the data were converted as follows (**Scheme 2**).
196 Measuring the baseline during 10 sec in each well before the addition of the test compounds, the
197 instrument established the baseline control from each well, and subtracted it to the assay measurement,
198 for each individual well. It then transformed this data into an area under the curve (AUC) during the
199 time of the experiment (ending after 15 minutes). This value was taken as representative of the effect
200 of the test compound on the calcium efflux. Each value was thus the mean of the effect of the test
201 compound on 40,000 cells measured at least in triplicate. The effect of PMCA inhibitors was
202 evaluated by subtracting the AUC of the condition without inhibitor from that of with inhibitor (each
203 of them being its own control measured before adding the compound). Delta AUC (Δ AUC) were
204 plotted against concentrations of the inhibitor candidate, the higher the value of the Δ AUC was, the
205 better the inhibitory activity. A PMCA unspecific inhibitor, ortho-vanadate – oVa –, was tested on
206 each plate at concentrations ranging from 10^{-5} to 10^{-2} M as positive control (see Results section for full
207 description).

208

209 **2.9 Statistical analysis:** Δ AUC data, expressed as mean \pm SEM, were analyzed with Prism
210 Software (Graphpad Software, San Diego, CA, USA) and considered significant as follows, * P < 0.05,
211 ** P < 0.01 or *** P < 0.001.

212

213 **3. Results**

214 **3.1 Design of a pharmacological assay to assess the PMCA function.** The assay was based on
215 cytosolic calcium fluorescence recording from HEK 293 cells that express mainly PMCA4 and only
216 traces of PMCA1 [4,7].

217 In a living cell, the cytosolic calcium originates either from intracellular store release or
218 extracellular importation. One can regard the cytosolic calcium concentration as the result of a balance
219 between two opposite processes (**Scheme 1A**). The Ca^{2+} that permeates from the endoplasmic
220 reticulum (ER) through IP_3 receptors (IP_3R) [28] and ryanodine receptors (RyR) [29] as well as from
221 mitochondrial calcium fluxes [30] and Na/Ca-exchanger (NCX) [31] and from the transmembrane
222 channels or transporters tends to increase calcium cytosolic concentration while PMCA and calcium
223 uptake into the ER *via* the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) lead to its reduction.
224 PMCA and SERCA are the major Ca^{2+} clearance pathways in most non-excitable with other Ca^{2+}
225 fluxes such as mitochondrial Ca^{2+} fluxes, NCX and other Ca^{2+} channels/transporters contributing
226 either no or a minimal role in maintaining steady state Ca^{2+} homeostasis [32].

227 Therefore, we aimed at reaching an experimental condition where the cytoplasmic calcium is
228 high, the extracellular calcium influxes are blocked, and their uptake mechanism abolished. When
229 measuring the concentration of the cytosolic calcium *via* a specific intracellular fluorescent probe in
230 this situation, a descending fluorescent signal would reflect mainly the PMCA-dependent calcium
231 extrusion activity which is the core of our study. The Rhod-4 probe was preferred to Fluo-8 indicator
232 because of its higher dynamic response to calcium. This choice did not permit to use the ruthenium red
233 to inhibit mitochondria calcium import so we cannot rule out its intervention in our assay. However,
234 caloxins do not interact with intracellular processes due to their peptide nature that limits their action
235 to the extracellular side. The effects studied in this assay should then rely only to transmembrane
236 effector regulation, *i.e.* theoretically PMCA.

237 Thanks to the benefit of several decades of calcium homeostasis studies, numerous chemicals
238 have been characterized to block cellular calcium fluxes operated by channels and pumps.
239 Experimentally, the initial step which was to empty the internal ER stores of HEK 293 cells was
240 achieved by applying 10 μM of 2-benzyl-3-[hydroxy-(2-phenylacetyl)amino]propanoic acid (*a.k.a.*
241 cyclopiazonic acid, CPA, a blocker of the ER calcium uptake SERCAs [33]) in a calcium-free
242 medium. As a second step, the addition of extracellular calcium (CaCl_2) to a final concentration of 1.2
243 mM, then triggered a rise of the cytosolic calcium carried principally by the opening of ORAI, a
244 transmembrane calcium channel opened by intracellular store depletion (Store-Operated Calcium
245 Entry, SOCE [34]). At this point, due to the SERCA inhibition by CPA the ER remains depleted and
246 the continuous activation of ORAI channels and other minor calcium influxes should be stopped to
247 reveal the PMCA calcium extrusion activity. The efficacy of different calcium entry blockers was

248 studied by challenging the pharmacological sensibility of the rise in cytosolic calcium following
249 external calcium addition after complete internal calcium depletion. For this purpose, the addition of
250 extracellular calcium was accompanied with increasing concentrations of different standard Ca^{2+} entry
251 inhibitors in the presence of CPA (**Figure 1A**). Among the various Ca^{2+} entry blockers tested (oVa,
252 NiCl_2 , CdCl_2 , SKF96365, S66, econazole and 2-APB), Ni^{2+} , S66, econazole and 2-APB efficiently
253 blocked almost entirely the increase in fluorescence induced by the 1.2 mM Ca^{2+} solution, indicating
254 that calcium entry was nearly abolished. Despite 2-APB displaying some variations at μM doses
255 (**Figure 1A**), we selected this inhibitor for safety, availability, and supply cost reasons. As variations
256 might be due to a residual 2-APB-resistant Ca^{2+} entry, the assay was further optimized by testing the
257 remaining calcium inhibitors in combination with 2-APB. As shown (**Figure 1B**), Ni^{2+} concentration-
258 dependently prevented residual cytosolic calcium increase in the presence of 50 μM of 2-APB. The
259 increase in fluorescent signal at high concentrations for SKF 93365 and Cd^{2+} can be attributed to
260 solubility issues and to chelation with Rhod-4 indicator, respectively. In summary, after attempting the
261 use of a series of experimental maneuvers to inhibit the calcium fluxes, the most accurate condition for
262 assessing PMCA function was obtained by a combination of the Ca^{2+} entry blockers, 2-APB and
263 NiCl_2 , in the presence of CPA according to a final protocol summarized in **Scheme 2**.

264 In these conditions, this assay allows to measure mostly the strict PMCA-dependent decrease in
265 the cytosolic calcium in a screening-friendly format under a FLIPR experimental setup. For the
266 evaluation of PMCA inhibitor candidates, we used oVa as an internal and systematic reference
267 compound. We chose oVa because (i) it was much more often used than the other ones, and (ii)
268 carboxyeosin is a potent fluorophore [35] that would interact with Rhod-4 measurements, leading to
269 misinterpretations. oVa was used at concentrations ranging from 10^{-5} to 10^{-2} M. Above 10^{-3} M, its
270 specificity of action is however uncertain particularly regarding phosphorylation and
271 dephosphorylation events. Indeed, vanadate is known for decades to inhibit kinases and phosphatases
272 [36–38]. Prototypical traces of fluorescence recordings are presented in **Figure 2A**. Concentration-
273 response curves are finally built by plotting AUC variation as a function of the concentrations of the
274 test compounds as exemplified for oVa and **cal1b1** (**Figure 2B**).

275

276 **3.2 Practical description and considerations.** Cells were seeded and 24 h later, loaded with 5
277 μM Rhod-4 in the presence of 20 μM sulfipyrazone as described above. Rhod-4 was preferred over
278 other calcium fluorescent indicators essentially because it is a good probe of cytosolic calcium, in
279 various situations [26]. At the end of the loading procedure, cells were resuspended in a Ca^{2+} -free 10
280 μM CPA solution. After 10 min of baseline measurements, the cells were challenged with a caloxin
281 derivative or oVa in the presence of 1.2 mM CaCl_2 , 50 μM of 2-APB and 300 μM NiCl_2 in PBS buffer
282 (**Scheme 1C**). Typical recorded profiles are presented in **Figure 3A** that represents a FLIPR screen-

283 copy of a single plate measurement, in real time. **Figure 3B** shows the first 3 minutes of the recording,
284 during which turbidimetry leads to undershooting traces (below zero). These fluorescence traces
285 appear to be dynamically changing over time, most likely due to variations in Ca^{2+} fluxes and minor
286 turbidity changes due to addition of reagents. However, any difference in baseline control condition
287 (without PMCA inhibitor) compared to following the addition of putative peptides can be assumed to
288 be due to inhibition of PMCA leading to an increase in resting Ca^{2+} concentration. Therefore, any
289 difference in AUC measured during these periods represents the degree of PMCA inhibition that can
290 then be plotted as concentration response curves in all subsequent data.

291 The first layer of transformation was then the subtraction of the AUC of the baseline condition
292 (before the addition of the compound) to the AUC recorded in the presence of the compound. This
293 leads to a representation as shown in **Figure 3C** (right panel). This figure depicted the automatically
294 obtained graph of the kinetical action of any individual compound, as calculated thanks to a FLIPR
295 dedicated function [39]. This data was transformed a second time as follows: The AUC, as it remains
296 after the subtraction of the baseline (to each point), was considered as the value of reference for the
297 effect of the compound. This data was then constructed as a plot as a function of the various
298 concentrations tested, most of the time from 1 to 100 μM , in the routine format. A representative plot
299 is shown in **Figure 3C** for some compounds studied as actual examples of the whole process. Note
300 that for every compound at least 3 curves were recorded per tested concentration and transformed to
301 lead to a final number with its standard variation.

302

303 At that stage, the assay was consolidated and was ran as described in **Scheme 1C**. The
304 compounds were tested in the presence of CPA (10 μM), 2-APB (50 μM) and Ni^{2+} (300 μM) in PBS
305 buffer. From now on, the system is setup in a way that permit to calculate directly the ΔAUC .
306 Although we cannot completely rule out a minor component from additional Ca^{2+} fluxes contributing
307 to the measured AUC, we can assume that these may be the same during the baseline *versus* treatment
308 periods. Therefore, the ΔAUC is the best estimate of PMCA inhibition that combines the convenience
309 and power of a high throughput screening of large numbers of putative PMCA inhibitors

310

311 **3.3 The evaluation of a first series of caloxin1a, -1b, -1c, -2a and -3a derived peptides.** Our
312 first set of experiments was to validate the data published for the reference caloxins -1a, -1b, -1c, -2a
313 and -3a (**Table 3**) in our own system. The **cal1b1**, **cal1b3**, and **cal1c2** variants were originally
314 described with the best activities [20]. In our cellular system, we found **cal1b1** and **cal1b3** to perform
315 modestly at 30 μM , but better than all the other caloxins tested herein (**Figure 4** and data not shown),
316 somehow validating the accuracy of our PMCA-based assay. These peptides were less efficient but

317 600 to 1000 times more potent than oVa (EC₅₀ from 17 to 30 μM versus 30 mM). To the best of our
318 knowledge, these peptides, once discovered by phage display, were not optimized and analogues were
319 not reported in the literature review [20]. Our choice was to start by exploring the most obvious steps
320 in peptide medicinal chemistry for both **cal1b1** (TAWSEVLHLLSRGGG-NH₂) and **cal1b3**
321 (TIPKWISIIQALRGGGSK-NH₂). Indeed, we disregarded **cal1c2** because this caloxin was as active
322 as **cal1b3** in our hands (data not shown) and this peptide is an analogue sequence-wise of the two
323 previous ones. Thus, we reasoned that the structural message borne by the **cal1c2** should be kept in
324 **cal1b1** and **cal1b3**. For the sake of simplicity, we adopted a nomenclature inspired from the previous
325 research published on caloxins: the parent compound name, *e.g.*, **cal1b1**, is followed by its analogue
326 number, following the order of synthesis such as **cal1b12**, **cal1b13**, or **cal1b31**, **cal1b32** and so on. To
327 start implementing a peptide-driven classical approach to analogues of **cal1b1**, we synthesized a series
328 of analogues aiming at finding the shortest active sequence.

329

330 To investigate the contribution of the N-terminal residue, the Trp moiety and the C-terminal region of
331 **cal1b1** in PMCA-mediated Ca²⁺-efflux inhibiting activity, we have generated point-substituted and
332 down-sized analogues of the peptide (**Table 4**, **cal1b1-cal1b16**). Deletion of the three contiguous C-
333 terminal Gly units (**cal1b11**) or their substitution by a 6-aminohexanoic acid (**X6**) moiety (**cal1b12**)
334 did not affect the biological activity of the peptide (**Figure 5A**). Similarly, replacement of the Trp
335 residue in position 3 of **cal1b1** by classical counterparts such as 2-naphthyl-alanine (**X7**) or β-
336 (benzothiazolyl)-alanine (**X16**) led to **cal1b13** and **cal1b14** as potent as the lead peptide. Finally, N-
337 terminal acetylation of the Thr residue or its substitution by azetidine-2-carboxylic acid (**X9**) did not
338 improve the PMCA inhibiting property of **cal1b1** (**cal1b15** and **cal1b16**). Since no significant
339 improvement was obtained in PMCA inhibition with **cal1b1** analogues, we turned to **cal1b3** for a
340 similar approach aiming to find the smallest active sequence (**Table 5**).

341 **Figure 6** shows that successive deletions of 1 to 5 C-terminal residue(s) did not affect the biological
342 activity of the peptides (**cal1b31** to **cal1b35**). However, additional deletions of Arg¹³ (**cal1b311**),
343 Leu¹²-Arg¹³ (**cal1b312**) and Ala¹¹-Leu¹²-Arg¹³ (**cal1b313**) markedly reduced the potency of **cal1b3**
344 analogues. Similarly, N-terminal truncations of 1 to 5 amino acids of either **cal1b3** (**cal1b36** to
345 **cal1b39** and **cal1b310**) or **cal1b35** (**cal1b314** to **cal1b316**) sequences yielded less potent analogues
346 (**Figure 6**). This loss of activity is particularly marked for peptides shortened by at least 4 residues
347 (**cal1b39** and **cal1b310**). Taken as a whole, **cal1b35** represents the iso-active sequence of **cal1b3** and
348 was considered as a template for further optimization.

349 The contribution of Lys⁴, Trp⁵, Ser⁷ and Gln¹⁰ side chains to the biological activity of **cal1b35** and
350 close analogues was evaluated by systematic alanine replacements (Ala-scan) (**Table 6**). **Figure 7**
351 compares the efficacy of all these compounds (30 μM) as PMCA inhibitors to oVa (10 mM). Globally,

352 all these peptide derivatives were less efficient than oVa. Among this series, the concomitant Ala-
353 substitution of Ser⁷ and Gln¹⁰ of **cal1b35** (**cal1b322**), its analog modified in position 1 by a γ -
354 aminobutyric acid (**X1**; **cal1b327**) and its 3 N-terminal residue deleted counterpart (**cal1b340**) were
355 the most active members, scoring in the 15 to 25% range inhibition compared to 100% of inhibition
356 for oVa at a 3 orders of magnitude higher concentration. Interestingly, it appears that the beneficial
357 effect of the double Ala-substitution in compounds **cal1b322** and **cal1b327** exceeded the loss of
358 activity initially detected by the shortening of 3 residues in caloxin **cal1b316** in comparison to
359 **cal1b35** since **cal1b340** was as potent as **cal1b322** and **cal1b327**. In order to design the shortest
360 caloxin possible, we have continued our sequence optimization process on the structure of **cal1b340**
361 (KWIAIIAALR-NH₂) with the proposed nomenclature of **cal1d** derivatives (**Table 1b**). As a first step,
362 it was attempted to modify the N-terminus of this peptide, by entering in place of the Lys¹ residue two
363 non-proteinogenic amino acids, either 2,4-diamino-butyric acid (**X13**) or 2,3-diamino-propionic acid
364 (**X2**). Incorporation of these two building blocks resulted in a slight decrease in the activity of the
365 corresponding peptides **cal1d19** and **cal1d20** at 30 μ M (**Table 7**) in comparison to the starting
366 **cal1b340** peptide. The substitution of the C-terminal arginine of **cal1b340** by an Ala moiety led to an
367 almost inactive peptide (**cal1d21**), strongly enforcing the key nature of the basic charge at the C-
368 terminus as previously suggested. Similarly, the substitution of the same residue by a lysine led to a
369 diminished inhibitory capacity of the corresponding peptide (**cal1d22**) compared to the starting one
370 (**cal1b340**). Finally, we synthesized an all-D version of **cal1b340**. The resulting peptide **cal1d23** was
371 poorly active, barely 20% of the starting peptide activity at 30 μ M.

372

373 **3.4 Evaluation of caloxin1d derived peptides - Central region.** We have shown above that the
374 central residues of **cal1b340** (-IAIIAA-) tolerated particularly well their substitution by an alanine
375 with minor loss of activity suggesting this region acted as a spacer between both extremities where
376 alanine replacement led to a loss of PMCA inhibition. Thus, **cal1b340** can be regarded as a 3-zone
377 delineated sequence: a N-terminal KW motif, a long stretch of alkyl-side-chain-bearing amino acids
378 (isoleucine and alanine) and a C-terminal dipeptide leucine-arginine, the later with a positively
379 charged guanidino group (**Figure 8**).

380

381 We first explored the central zone corresponding to the aliphatic, rather hydrophobic and extended
382 region -IAIIAAL-, by substituting this sequence by different spacers of various length. Description of
383 the linkers inserted within the **cal1b340** sequence leading to **caloxin1d** analogues and their respective
384 PMCA inhibiting activity are reported in **Table 8**. Noticeably, the dipeptides KW-NH₂ (**cal1d69**) and
385 LR-NH₂ (**cal1d68**) as well as an equimolar mixture of the dipeptides (**cal1d70**) did not exhibit any

386 inhibiting activity in our model of PMCA functionality demonstrating the pivotal role of the linker
387 between these two parts. With the aim to understand the optimal structure/length of this extended
388 region in **cal1d**, we selected a series of building blocks to vary the length between the N- and C-
389 terminal extremities from 3 to 24 heavy atoms in the backbone. Most of the attempts failed, leading to
390 **cal1d** derivatives with weak - if any - inhibitory property towards PMCA-mediated calcium
391 movements (**Table 8**). Surprisingly, **cal1d16** and **cal1d17** which encompassed spacers as long as the
392 parent peptide **cal1b340** with a comparable hydrophobicity pattern were totally devoid of activity as
393 well as caloxins with shorter but similar linkers (**cal1d14**, **cal1d15** and **cal1b342**). This feature was
394 also seen in a few previous data, in which Ala-substitution led to many peptides with null activity such
395 as **cal1b39** and **cal1b334**, among many others (**Figure 7**). This feature was somehow contradicted by
396 the finding of the possible replacement of this central core by a single amino acid, in which the
397 succession of N-C α -CO moiety was absent. Indeed, the optimum size of the linker was in the 13-atom
398 range, as seen for **cal1d1**, encompassing a 12-amino-dodecanoic acid moiety (**X5**), which was as
399 potent as **cal1b340** at 30 μ M. Longer or shorter versions failed to enhance the inhibitory capacity of
400 the corresponding peptides, as exemplified by compounds **cal1d47** and **cal1d13** containing 15-amino-
401 pentadecanoic acid (**X20**) and 5-amino-pentanoic acid (**X11**) residues, respectively. We have also
402 introduced the **X5LR** motif on the side-chain amino ϵ group of the KW-NH₂ dipeptide. Surprisingly,
403 the resulting branched peptide **cal1d54** K(**X5LR**)W-NH₂ retained about 50% of the activity of the
404 linear parent peptide (Δ AUC 435 ± 78 vs. 573 ± 94 for **cal1d1**).

405 In this series, we also verified that the leucine moiety was mandatory and could not be directly
406 included in a longer amino-acid linker such as 15-amino-pentadecanoic acid (**X20**) present in **cal1d48**,
407 which encompasses the 12 heavy atoms of 11-amino-undodecanoic acid (**X4**) and 4 extra ones
408 corresponding to almost the 3 heavy atoms of the leucine backbone of **cal1d11**. The same approach
409 was applied by substituting the -IAIIAA- hydrophobic scaffold by 15-amino-4,7,10,13-tetraoxa-
410 pentadecanoic acid (**X19**, amino-(PEG)₄-carboxylic acid) moiety in **cal1d39** for comparison with a
411 “PEG₃”-containing **cal1d37** (**X15**, 12-amino-4,7,10-trioxa-dodecanoic acid). In both cases, this
412 modification had little effect on the potency of the corresponding peptides.

413 The full retro sequence of **cal1d1** was also prepared in which the peptide backbone corresponded to
414 the anti-wise sequence RLX5WK-NH₂ (**cal1d79**). Not surprisingly, **cal1d79** was at least as active as
415 the parent compound (data not shown), strongly suggesting that the scheme of **Figure 8** is confirmed
416 and that the central zone only plays an “addressing” role for both terminal parts. Finally, the retro-
417 inverso analogue, **cal1d80** (rLX5wk-NH₂), displayed a higher inhibiting activity than **cal1d1**.
418 Surprisingly, this result is at the odd of that of obtained with the all-D peptide **cal1d23**, the retro-
419 inverso version of **cal1b340**, that lost more than 90% of the activity (**Table 7**).

420 At this stage, **Figure 9** shows the inhibiting activities of the 2 most interesting caloxins **cal1b3** and
421 **cal1d1** in comparison to the reference inhibitor oVa. The short caloxin **cal1d1** was at least as active as
422 **cal1b3** and exhibited an efficacy at 100 μM that could be useful for a reference PMCA inhibitor.
423 Therefore, we kept the structure of **cal1d1** as a model for further explorations.

424 In further attempts to better understand the structure-activity relationship of these **cal1d1** derivatives,
425 we synthesized other centrally modified caloxins containing cycle or branched alkyl backbones
426 between the KW- and -LR-Nus. These peptides necessitated the synthesis of the Fmoc-protected non-
427 natural amino acids (**Table 2**) and those results came very late in the program, somehow impairing the
428 design of new analogues. With the exception of compound **cal1d67**, these caloxins were better
429 inhibitors at 30 μM than the parent peptide **cal1d1** (**Table 9**). However, considering the difficulties of
430 access to those amino acids), the **X5** linker was chosen for the following steps of optimization.

431
432 **3.5 Evaluation of caloxin1d1 derived peptides – N-terminal extremity.** We continued the
433 optimization of the **cal1d1** sequence by detailing the properties of its N-terminal extremity (region 1),
434 *i.e.*, the lysine-tryptophan dipeptide. **Table 10** summarizes the various substitutions and the results
435 obtained with the corresponding peptides on the PMCA-mediated calcium movement in our model at
436 30 μM . As demonstrated above, we maintained the distance between zones 1 and 3 at 12 heavy atoms
437 by systematically (with the exception of **cal1d41**) using 12-amino-dodecanoic acid (**X5**) as a spacer.
438 Acetylation of the N-terminal extremity of compound **cal1d1** had a strong detrimental effect onto the
439 inhibitory potency of **cal1d51**. An unexpected solubility issue cannot be totally ruled out at the stage,
440 **cal1d51** being significantly more hydrophobic than **cal1d1**. Similarly, replacement of the Trp moiety
441 by a His residue led to peptide **cal1d27** totally devoid of inhibitory capacity, whereas changing into
442 Tyr (**cal1d45**) or Phe (**cal1d44**) had fewer radical effects. Substitution of the Lys residue by Orn or
443 Dab units did not drastically affect the potency of the corresponding analogues **cal1d30** and **cal1d29**,
444 respectively, even though a slight enhancement could be noted for one methylene shortening in the
445 side chain of lysine. Intriguingly, further side-chain methylene shortening yielded the Dap-containing
446 **cal1d28** which presented the best inhibiting activity of this series. When the nature of the amino acid
447 was changed (basic for Lys for neutral Gly or Ala, like in **cal1d76** or **cal1d77**), the respective resulting
448 activities were not drastically changed from **cal1d1**, but when the length of the side chain is
449 maintained (Lys and n-Leu), the activity remains in the 700 Δ AUC range. Finally, replacement of the
450 Ne amine side chain function of lysine by a carboxylic acid function (**X36**) in **cal1d83**, or by a -CH₂-
451 COOH group (**X27**) in **cal1d84** greatly reduced the activity of **cal1d1** in the same way as the loss of
452 N-terminal amine function (**X6**) in **cal1d85**.

453 **3.6 Evaluation of caloxin1d derived peptides – C-terminal extremity.** Because of the non-
454 polar, aliphatic, and bulky nature of the leucine sidechain, the possibilities of incorporating

455 counterparts into the C-terminal LR dipeptide were limited (**Table 11**). The leucine in **cal1d1** can be
456 changed without major differences in the PMCA inhibiting activity by hydrophobic amino acids such
457 as Ile (**cal1d87**), its straight-chain analogue norleucine (**cal1d88**) or, more surprisingly, the shorter and
458 functionalized homo-Cys (**cal1d89**). Finally, substitution of the C-terminal arginine by its
459 homologated version, homo-Arg, led to a significant reduction in the activity (**cal1d31**), whereas
460 dimethylation of the guanidino group (**X25**), its protection by a nitro function (**X26**) or its substitution
461 by an ureido group (**X24**) almost nullified the inhibiting activity of **cal1d58**, **cal1d59** and **cal1d56**,
462 respectively. None of these three compounds were more potent than their parent **cal1d1**. Noticeably,
463 **cal1d56**, was practically inactive pointing out to a key role of the guanidino moiety of arginine as
464 opposed to the ureido group of citrulline (**Table 11**).

465

466 4. Discussion

467 The use of improperly characterized pharmacological tools is a source of confusion and
468 misleading results. The literature is full of use of inhibitors in cellular conditions that have never been
469 characterized for their actions in such a context. Another type of examples is the use of antagonists at
470 G-protein coupled receptors in vivo, while their stability and their uptake have never been studied (see
471 our discussions for example in Boutin & Legros [40] or in Boutin et al. [41]). This is one of the main
472 reasons why we attempted the present research campaign using a living system. Another aspect is the
473 challenging access to purified integral membrane proteins, such as GPCRs [42] and channels .

474 The extraordinary diversity of tools, more or less characterized in terms of stability and/or
475 specificity, led us to the possibility to create a pharmacological model in which only our target protein,
476 PMCA - presumably PMCA4 -, was functionally active. Indeed, our interpretation of this system is as
477 follows: CPA blocks SERCAs leading to a net efflux of an irreversible leak of Ca^{2+} from the RE to the
478 cytosol. The cellular compensatory response leads to the opening of ORAI to promote extracellular
479 calcium entry to refill the RE, system nevertheless blocked due to the inhibition of SERCAs by CPA.
480 Therefore, under those conditions, the only actors left for the Ca^{2+} homeostasis are ORAI and PMCA.
481 Finally, the function of ORAI is blocked by the addition of 50 μM 2-APB. The net result of this
482 pharmacological manipulation of the different actors of the Ca^{2+} led to the measurement of cytosolic
483 Ca^{2+} accumulation depending only on the capacity of PMCA to exclude Ca^{2+} from the
484 overconcentrated cytosol (**Scheme 1**).

485 Small molecules, in particular synthetic compounds, are often coming from the analogizing of
486 natural products [43]. For example, more than a thousand analogues of melatonin as melatonin
487 receptor agonists, were designed in a somewhat rational approach [41]. Alternatively, one can perform
488 HTS campaigns permitting to discover *de novo* structure(s) with potent antagonist or inhibitory
489 capacities with chemical structures unrelated to the natural ligand or substrate/co-substrate of the
490 targeted protein. This situation is slightly different whenever compounds issued from natural sources
491 (particular from plant extracts) are screened and new candidates discovered from this origin. As
492 discussed in Lautié et al. [43], a common feature of natural products is that they statistically recognize
493 a smaller number of protein targets per molecule than synthetic ones (in a roughly 3 to 6 ratio).

494 Finally, peptides form a third category of chemicals that often offer two advantages that are
495 probably inter-dependent: (i) they are built of simple bricks that are similar and/or identical to those
496 making the proteins, offering theoretically a better “guarantee” of specificity because they are slightly
497 larger than the common synthetic compounds (see discussion in Gruber et al [44]). Therefore, one can
498 hypothesize that by enhancing the number of contacts between the chemical and the targets, one can
499 hope a more intimate complementarity and, therefore, less off-target affinity for unrelated proteins. (ii)

500 Peptides offer an almost infinite number of analogues because it exists literally hundreds of amino
501 acids that can be integrated in the starting sequence with the relative simplicity of solid phase
502 synthesis. Several in depth views of the specificity of peptides has been done and computerized (see
503 for example Bhattacharjee and Wallin [45]). They would argue that the complexity of the binding of
504 peptides onto a protein target is dependent on too numerous parameters that only *in silico* simulations
505 [46] and/or co-crystallization of their complex [47] can reveal.

506 The next level of difficulty is then the starting points. Roughly three sources of peptides can be
507 discovered: the combinatorial library approach [48,49], the analogues to the natural ligand [50] and the
508 phage display approach [51]. To these sources, could be added the use of peptides included in massive
509 screening campaigns as part of the global chemical sources of hits in the HTS approach, although
510 quite often, the discovery of peptides as hits in such campaigns is disregarded to the profit of smaller
511 organic chemicals at least in the pharma industry that is the main source of such campaigns.

512 PMCA is a key component of the calcium homeostasis of the cells. PMCA's have been linked to
513 many pathological situations [see [5] for complete review]. The main pharmacological tool used in
514 this area remains *ortho*-vanadate. This compound has been described for ages as a strong inhibitor of
515 PMCA [52]. It is considered an analog of phosphate and has been reported numerous times for its
516 capacity to inhibit kinases and phosphatases rendering its use difficult in a living system, including in
517 cells.

518 Several compounds were reported following HTS campaigns on this PMCA target [21]. For
519 example, aurintricarboxylic acid was discovered during such a campaign [53]. This polymeric dye is
520 typical of HTS campaign findings. Indeed, this compound was also reported over the years as an
521 inhibitor of protein-nucleic acid interactions, a potent inhibitor of ribonuclease and of topoisomerase II
522 [54]. It also stimulates tyrosine phosphorylation processes, probably by inhibiting phosphatase(s) [55].
523 Finally, it inhibits apoptosis [56,57], prevents the downregulation of GluR2 receptors [58] and inhibits
524 calpain, a Ca²⁺-activated protease that is activated during apoptosis [59]. It is important to point out
525 that this compound could not be defined as a specific inhibitor at PMCA [60]. In the 1980s, Grover
526 group applied the technique of phage display to screen for alternate inhibitors with peptide structures
527 and found a series of peptides from 12 to 20 amino acid long (see **Table 1a**) [16]. No report on the
528 optimization of those peptides was reported in the literature.

529 As stated above, small organic chemical could be interestingly challenged by peptides or
530 pseudopeptides [61] or natural compounds [43] because those compounds are potentially more
531 specific of a given target than the former ones. We thus embarked in a long program aiming at finding
532 smaller alternative compounds to the caloxins already described by Grover's group [16]. The main
533 purpose of the study was indeed to find a more practical (*i.e.*, shorter) peptide that could be used in
534 studies on PMCA-dependent Ca²⁺ movement in cellular contexts. One should recall that the longer the

535 peptide (or the heavier the organic compound), the more costly and complicated its synthesis will be.
536 Introducing such a size parameter in pharmacological research is also the first step towards
537 rationalizing the future and possible passage from an experimental compound usable in research to a
538 druggable compound aiming at therapeutical purpose. Furthermore, in more fundamental
539 considerations, peptide structures are uneasy to obtain in crystallization experiments, and the common
540 lack of structural information on active peptide is also a limit to their use [61]. Finally, a discussion
541 still exists on whether a shorter compound would be more as opposed to less specific of its target,
542 because it retains less structural information than their longer counterpart(s).

543 We applied a simple step by step classical strategy by which we (i) defined the best starting point
544 under our experimental conditions; (ii) applied the standard shortening of the peptides; (iii) tried to
545 define the final molecule using simple concepts. Final peptides were already a great step from the
546 initial caloxin (*i.e.*, **cal1b3**).

547 Finally, two other parameters were measured but not considered: the height of the Ca²⁺ peak and
548 the duration of that peak. These parameters would permit to finely evaluate, and these data are
549 gathered in a supplementary table for the main peptides issued from the present sets of experiments.

550 As a conclusive remark, one should first point out to the relative value of data obtained in living
551 cells – or in living animals – where repetitions from experiments to experiments remain often within a
552 large variation interval. To limit this fact, we chose two ways: (i) we only compare results obtained
553 within a set of experiments run the same days, at multiple occasions, often at least three different
554 plates containing each triplicate of a same experiment. These are the numbers one can find in tables. In
555 these cases, the present variation was generally below 10%; and (ii) we express at the end of the
556 experiment the main results in a format considering for the 15 “best” peptides the ratio to both **cal1b3**
557 and **cal1d1** results obtained in the same experiments as well as, for these same peptides, an IC50
558 approximated from 3 concentration values of the set of experiments (**Table 12**). This list is a summary
559 and an indication of the next steps that should be undertaken to further qualify the “best” peptides. For
560 example, the assessment of the stability of the candidate could be predicted – the all-D peptide:
561 **cal1d80** should be a strong candidate – but the actual data is missing. On the other hand, we did not
562 have the opportunity to screen all the candidates in a single set of experiments, where their potencies
563 could be expressed relatively to each other and to the initial **cal1b3**. Finally, one can wonder if the best
564 candidate(s) would be specific enough of a set of relevant off target proteins and channels.

565 Of course, despite a cellular approach – that is, a living system, – these molecules are far from a
566 perfect tool to study PMCA-mediated Ca²⁺ movements in a more complex system and even farer from
567 a candidate to solve therapeutical problems. Nevertheless, there are good and simpler candidates on
568 which further hit-to-lead strategies will be applicable.

569

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572

573

574 **5. Conflicts of interest**

575 The authors declare no competing financial interest.

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580 **7. Author contribution:**

581 JAB. Conceptualization, Peptide design, Pharmacological experiments, Data curation Writing,
582 original, review & editing; LF: Peptide design; SB: Pharmacological experiments, Data curation,
583 Writing, review & editing; MJ: Peptide design, Writing, review & editing; MG: Writing, review &
584 editing, PG: Peptide design; GF: Pharmacological experiments, Data curation Writing, review &
585 editing; KP: Peptide design, Writing, review & editing; JL: Data curation, Writing, original, review &
586 editing.

587

588

589 **8. Legends to the figures**

590

591 **Scheme 1: Calcium homeostasis in a mammalian resting cell and pharmacological window for**
592 **PMCA function assessment.**

593 **A.** Simplified schematic representation of Ca^{2+} dynamics in a mammalian resting cell;

594 Cytosolic Ca^{2+} concentrations are maintained at nM levels against a more than 4 orders of
595 magnitude higher levels in intracellular stores and extracellular space. Orchestrated actions
596 regulate calcium homeostasis summarized by proteins able to (i) either pump calcium ions out
597 from cytosol to the extracellular milieu (PMCA) or into intracellular stores such as ER (SERCA)
598 and (ii) fill out the cytoplasm by reverse action involving ORAI and $\text{IP}_3\text{R/RyR}$, respectively.

599

600 **B.** Schematic representation of the pharmacological treatment for PMCA function assessment. In
601 extracellular Ca^{2+} -free condition, CPA triggers ER store depletion; this calcium release in the
602 cytoplasm induces STIM to couple with the plasma membrane Ca^{2+} channels ORAI for refill
603 (store-operated calcium entry process). Extracellular calcium demand is supplemented by
604 addition of CaCl_2 . Concurrently, treatment with 2-APB blocks ORAI calcium entry.
605 Mitochondrial calcium leak is prevented by addition of Ni^{2+} (not shown).

606

607 **Figure 1: Validation of reference compounds**

608 Design of the pharmacological window for PMCA function assessment in HEK 293 cells. **A.** Effect of
609 calcium entry inhibitors on intracellular calcium concentration of HEK 293 cells obtained in the
610 presence of CPA (10 μM). Representative concentration-response curves for NiCl_2 (orange closed
611 circle), CdCl_2 (red closed square), SKF 96365 (blue closed triangle up), S66 (green closed triangle
612 down), econazole (purple closed diamond) and 2-APB (black open circle). Blue closed star represents
613 the signal in calcium-free condition. Data are mean \pm SEM of triplicate from, at least, three
614 independent experiments. **B.** Effect of calcium entry inhibitors on intracellular calcium concentration
615 of HEK 293 cells obtained in the presence of CPA (10 μM) and 2-APB (50 μM). Representative
616 concentration-response curves for NiCl_2 (orange closed circle), CdCl_2 (red closed square), SKF 96365
617 (blue closed triangle up), S66 (green closed triangle down) and econazole (purple closed diamond).
618 Blue closed star represents the signal in calcium-free condition. Data are mean \pm SEM of triplicate
619 from, at least, three independent experiments.

620

621 **Figure 2: Calcium kinetics in HEK 293 cells.**

622 **A**, Changes in cytosolic calcium content (normalized fluorescent intensity units) of HEK 293
623 cells measured in FLIPR assay over a period of 30 min (15,000 sec). Examples of kinetic
624 profiles of different caloxin analogues obtained in the presence of CPA (10 μ M), 2-APB (50
625 μ M) and Ni²⁺ (300 μ M). The AUC during the control condition (without PMCA inhibitor) can
626 be subtracted from the AUC with the putative PMCA inhibitor to give Δ AUC. This Δ AUC
627 represents the degree of PMCA inhibition and is thus the basis of all subsequent
628 concentration-response curves. Grey curve, solvent (control).

629 **B**, Effect of PMCA inhibitors on intracellular calcium concentration of HEK 293 cells.
630 Representative concentration-response curves for oVa (black open circle) and **cal1b1** (red
631 closed square) obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni²⁺ (300 μ M).
632 Data are mean \pm SEM of triplicate from, at least, three independent experiments.

633

634 **Figure 3: Practical representations of the various steps of PMCA-dependent calcium efflux**
635 **measurements.**

636 **A, Representative FLIPR traces of the PMCA-based measurement in HEK 293 cells.**

637 Half-log diluted concentration-response traces are displayed from row A (10 μ M) to row G
638 (0.01 μ M). 0 concentration replicates are H01, H02 and H03. Columns 1-3 **caloxin 1b3**,
639 columns 4-6 **caloxin 1b34**, columns 7-9 **caloxin 1b35**, columns 10-12: **ortho-vanadate**
640 (oVa).

641 **B, Comparison of kinetic measurements of the inhibition of the PMCA-driven efflux of**
642 **Ca²⁺ in HEK 293 cells by various comopounds at a single concentration.** Typical FLIPR
643 traces recorded during the first four minutes

644 **C, Exemplification of data transformation from kinetic traces to Δ AUC graph.** Left
645 panel: comparison of kinetic measurement of the inhibition of the PMCA-driven efflux of Ca²⁺
646 in HEK 293 cells by 4 differents compounds at 30 μ M. The grey trace displays solvent effect
647 (control). Comparison of concentration-response curves for the same 4 different compounds
648 (same color codes). Right pannel: the areas under the curve (AUC) were calculated and
649 plotted *versus* the concentration of the compound.

650

651 **Scheme 2: Schematic representation of the experimental setup.**

652 HEK 293 cells are seeded in 96-well plates as indicted in the Material and Methods section (C1). After
653 18-24 h, cells are loaded with 5 μ M of the calcium probe Rhod-4-AM in the presence of 20 μ M
654 sulfinpyrazone (C2). One hour later, fluorescence recording started (C3) just before triggering the ER

655 depletion with 10 μ M CPA treatment (C4) for 10 minutes in Ca^{2+} -free condition. Then, cells are
656 challenged by 50 μ M 2-APB, 300 μ M NiCl_2 and 1 mM CaCl_2 in PBS buffer concomitantly with
657 graded concentrations of caloxins or oVa as positive control (C5) for at least 15 minutes of
658 fluorescence measurement (C6).

659
660

661 **Figure 4: Effect of reference caloxins on PMCA intracellular calcium concentration of HEK 293**
662 **cells compared to the classical PMCA inhibitor, ortho-Vanadate.**

663 **A**, Representative concentration-response curves for oVa (black open circle), **cal1b1** (blue
664 closed triangle up) and **cal1b3** (red closed square) obtained in the presence of CPA (10 μ M),
665 2-APB (50 μ M) and Ni^{2+} (300 μ M).

666 **B**, Representative concentration-response curves for oVa (black open circle), **cal2a1** (red
667 closed square), **cal3a1** (blue closed triangle up) and **cal3a2** (green closed triangle down)
668 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni^{2+} (300 μ M).

669 **C**, Representative concentration-response curves for oVa (black open circle), **cal1a1** (red
670 closed square) and **cal1a2** (blue closed triangle up) obtained in the presence of CPA (10 μ M),
671 2-APB (50 μ M) and Ni^{2+} (300 μ M).

672 Data are mean \pm SEM of triplicate from, at least, three independent experiments. * p <0.05, ** p <0.01
673 and *** p <0.001 vs. control condition as assessed by ANOVA and Bonferroni post hoc test. Reference
674 caloxins were described by Grover [20].

675

676 **Figure 5: Effect of caloxin 1b1-derived peptides on PMCA intracellular calcium concentration of**
677 **HEK 293 cells compared to the reference caloxin 1b1.**

678 **A**, Representative concentration-response curves for **cal1b1** (black open circle), **cal1b11** (red
679 closed square), **cal1b12** (blue closed triangle up) and **cal1b13** (green closed triangle down)
680 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni^{2+} (300 μ M).

681 **B**, Representative concentration-response curves for **cal1b1** (black open circle), **cal1b14** (red
682 closed square), **cal1b15** (blue closed triangle up) and **cal1b16** (green closed triangle down)
683 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni^{2+} (300 μ M).

684 Data are mean \pm SEM of triplicate from, at least, three independent experiments.

685

686 **Figure 6: Effect of caloxin 1b3-derived peptides on PMCA intracellular calcium concentration of**
687 **HEK 293 cells compared to the reference caloxin 1b3 and classical PMCA inhibitor, ortho-**
688 **Vanadate.**

689 **A**, Representative concentration-response curves for **cal1b3** (black open circle), **cal1b31** (red
690 closed square), **cal1b32** (blue closed triangle up) and **cal1b33** (green closed triangle down)
691 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni²⁺ (300 μ M).

692 **B**, Representative concentration-response curves for **cal1b3** (black open circle), **cal1b34** (red
693 closed square), **cal1b35** (blue closed triangle up) and **cal1b36** (green closed triangle down)
694 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni²⁺ (300 μ M).

695 **C**, Representative concentration-response curves for **cal1b3** (black open circle), **cal1b37** (red
696 closed square), **cal1b38** (blue closed triangle up) and **cal1b39** (green closed triangle down)
697 obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni²⁺ (300 μ M).

698 **D**, Representative concentration-response curves for **cal1b3** (black open circle) and **cal1b310**
699 (red closed square) obtained in the presence of CPA (10 μ M), 2-APB (50 μ M) and Ni²⁺ (300
700 μ M).

701 Data are mean \pm SEM of triplicate from, at least, three independent experiments. ** p <0.01 and
702 *** p <0.001 vs. control condition as assessed by ANOVA and Bonferroni post hoc test.

703

704 **Figure 7: Percentage of PMCA inhibition induced by oVa, reference caloxins cal1a1, -1b1, -1c2, -**
705 **2a1, -3a1 and cal1b3 derivatives.**

706 All caloxins except oVa were tested at 30 μ M. (*) oVa was tested at 10 mM. Data are mean \pm SEM of
707 triplicate from, at least, three independent experiments.

708

709 **Figure 8: Chemical structures of cal1b340, H-Lys-Trp-Ile-Ala-Ile-Ile-Ala-Ala-Leu-Arg-NH₂.**

710 Caloxin **cal1b340** is organized in 3 successive parts, corresponding to a N-terminal KW motif, a
711 central hydrophobic stretch and a C-terminal LR-NH₂ extremity.

712

713 **Figure 9: Effect of new caloxin derived peptides on PMCA intracellular calcium concentration**
714 **of HEK 293 cells compared to the reference caloxin 1b3 and classical PMCA inhibitor, ortho-**
715 **Vanadate.**

716 **A**, Representative concentration-response curve for oVa (black open circle), and 4
717 concentration-response curves for **cal1b3** obtained in the presence of CPA (10 μ M), 2-APB
718 (50 μ M) and Ni²⁺ (300 μ M).

719 **B**, Representative concentration-response curve for oVa (black open circle), and 4
720 concentration-response curves for **cal1d1** obtained in the presence of CPA (10 μ M), 2-APB
721 (50 μ M) and Ni²⁺ (300 μ M).

722 **C**, Two concentration-response curves for **cal1d53** obtained in the presence of CPA (10 μ M),
723 2-APB (50 μ M) and Ni²⁺ (300 μ M).

724 **D**, Two concentration-response curves for **cal1d43** obtained in the presence of CPA (10 μ M),
725 2-APB (50 μ M) and Ni²⁺ (300 μ M).

726 **E**, Two concentration-response curves for **cal1d28** obtained in the presence of CPA (10 μ M),
727 2-APB (50 μ M) and Ni²⁺ (300 μ M). Data are mean \pm SEM of triplicate.

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740 **9. References**

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983 **Table 1a:** Chemical data for a first series of caloxin-derived peptides.

Code	Sequence			Rt ^a (min)	Purity (%)	MW exp.	MW theo.
calla1	H-	ACPWWSPHACGGG ^b	-NH ₂	3.34	97.90	1325.60	1325.49
calla2	H-	ACPIWQPHYCGGG ^b	-NH ₂	3.16	95.10	1385.50	1385.99
callb1	H-	TAWSEVLHLLSRGGG	-NH ₂	1.64	96.80	1581.20	1581.80
callb11	H-	TAWSEVLHLLSR	-NH ₂	5.71	95.88	1410.08	1410.62
callb12	H-	TAWSEVLHLLSRX6 ^c	-NH ₂	5.81	95.44	1523.64	1523.78
callb13	H-	TAX7SEVLHLLSRGGG	-NH ₂	5.84	95.83	1592.64	1592.80
callb14	H-	TAX16SEVLHLLSRGGG	-NH ₂	5.58	96.34	1599.58	1599.80
callb15	Ac-	TAWSEVLHLLSRGGG	-NH ₂	5.61	95.20	1624.01	1623.80
callb16	H-	X9AWSEVLHLLSRGGG	-NH ₂	5.49	95.44	1563.69	1563.80
callb3	H-	TIPKWISIIQALRGGGSK	-NH ₂	3.75	95.01	1924.38	1924.33
callb31	H-	TIPKWISIIQALRGGGS	-NH ₂	8.54	96.45	1796.07	1796.13
callb310	H-	ISIIQALRGGGSK	-NH ₂	7.61	95.71	1298.63	1298.54
callb311	H-	TIPKWISIIQAL	-NH ₂	2.82	95.21	1381.42	1381.70
callb312	H-	TIPKWISIIQA	-NH ₂	2.39	95.57	1268.90	1268.54
callb313	H-	TIPKWISIIQ	-NH ₂	2.37	95.27	1197.01	1197.47
callb314	H-	IPKWISIIQALR	-NH ₂	2.58	96.04	1436.50	1436.80
callb315	H-	PKWISIIQALR	-NH ₂	2.42	96.94	1323.20	1323.63
callb316	H-	KWISIIQALR	-NH ₂	2.32	95.69	1226.10	1226.51
callb317	H-	TIPKWISIIAALR	-NH ₂	4.20	95.01	1480.82	1480.84
callb318	H-	TIPKWIAIIQALR	-NH ₂	2.64	95.00	1521.10	1521.89
callb319	H-	TIPKAISIIQALR	-NH ₂	2.32	95.5	1422.63	1422.76
callb32	H-	TIPKWISIIQALRGGG	-NH ₂	8.59	96.49	1709.04	1709.06
callb320	H-	TIPAWISIIQALR	-NH ₂	2.96	95.17	1480.40	1480.79
callb321	H-	TIPKWIAIIAALRG	-NH ₂	4.33	97.01	1521.10	1521.90

cal1b322	H-	TIPKWIAIIAALR	-NH ₂	4.38	96.04	1464.50	1464.85
cal1b323	H-	TIPKWIAIIQAL	-NH ₂	4.64	95.71	1365.01	1365.71
cal1b324	H-	TIPKWIAIIQA	-NH ₂	4.04	97.49	1252.40	1252.55
cal1b325	H-	TIPKWIAIIQ	-NH ₂	4.03	95.03	1181.38	1181.48
cal1b326	H-	TIPKWIAIIQALR	-OH	4.24	97.28	1522.08	1522.88
cal1b327	H-	X1IPKWIAIIAALR	-NH ₂	4.30	96.95	1448.93	1448.90
cal1b328	H-	TIPX2WIAIIAALR	-NH ₂	4.16	95.90	1422.75	1422.79
cal1b329	H-	TIPKYIAIIAALR	-NH ₂	3.90	95.01	1441.31	1441.82
cal1b33	H-	TIPKWISIIQALRGG	-NH ₂	8.71	96.35	1651.44	1652.01
cal1b330	H-	TIPKX3IAIIAALR	-NH ₂	3.29	95.53	1426.51	1426.82
cal1b331	H-	CTIPKWIAIIAALRC	-NH ₂	4.41	96.11	1668.98	1669.13
cal1b332		TIPKWIAIIAALRG ^d		4.91	97.05	1505.04	1504.89
cal1b333	H-	TIPKWX4LR	-NH ₂	3.73	97.49	1095.72	1095.50
cal1b334	H-	TIPKWX1X1X1R	-NH ₂	2.66	96.7	1054.90	1054.30
cal1b336	H-	TIpKWIAIIAALR	-NH ₂	4.13	95.85	1464.59	1464.85
cal1b337	H-	TIPKWAIAALR	-NH ₂	3.46	98.03	1238.00	1238.53
cal1b34	H-	TIPKWISIIQALRG	-NH ₂	8.72	95.81	1594.99	1594.95
cal1b340	H-	KWIAIIAALR	-NH ₂	3.77	98.73	1153.20	1153.47
cal1b341	H-	TIPKWIGIIGALR	-NH ₂	3.95	96.23	1436.86	1436.79
cal1b342	H-	KWALR	-NH ₂	2.45	97.33	671.67	671.84
cal1b343/cal1d1 ^e	H-	KWX5LR	-NH ₂	3.51	98.48	798.17	798.09
cal1b35	H-	TIPKWISIIQALR	-NH ₂	8.79	96.38	1537.86	1537.90
cal1b36	H-	IPKWISIIQALRGGGSK	-NH ₂	8.25	97.28	1823.12	1823.20
cal1b37	H-	PKWISIIQALRGGGSK	-NH ₂	8.05	95.3	1710.09	1710.04
cal1b38	H-	KWISIIQALRGGGSK	-NH ₂	7.93	96.63	1612.44	1612.93
cal1b39	H-	WISIIQALRGGGSK	-NH ₂	8.36	95.43	1484.71	1484.75
cal1c2	H-	TAWSEVLDLLRRGGGSK	-NH ₂	3.67	97.02	1843.69	1843.11

cal2a1	H-	VSNSNWPSFPSSGGG	-NH ₂	3.20	95.06	1478.20	1478.54
cal3a1	H-	WSSTSSVSAPLFGGGGSAK	-NH ₂	4.51	95.57	1782.01	1781.95
cal3a2	H-	DSHINNEPSRRKGGGK	-NH ₂	1.56	95.16	1750.33	1750.90

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^a Retention time determined by by HPLC or by UPLC.

985

^b Disulfide-bridged peptides.

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^c **X1**: γ -amino-butyric acid; **X2**: 2,3-diamino-propionic acid; **X3**: 3-(4-pyridyl)-Ala-OH; **X4**: 11-amino-undecanoic acid; **X5**: 12-amino-dodecanoic acid; **X6**: 6-amino-hexanoic acid; **X7**: H-Ala(2-naphtyl)-OH; **X9**: (*S*)-azetidine-2-carboxylic acid; **X16**: β -(2-benzothiazolyl)-Ala-OH. Fmoc-derivatives are reported in Table 2.

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^d Head-to-tail cyclopeptide.

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^e **Cal1b343** was renamed **cal1d1** (see below, **Table 1b**).

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Table 1b: Chemical data for caloxin1d1-derived peptides.

Code	Sequence			Rt ^a (min)	Purity (%)	MW exp.	MW theo.
cal1d1	H-	KWX5LR ^b	-NH ₂	3.51	98.48	798.17	798.09
cal1d11	H-	KWX4LR	-NH ₂	3.47	98.84	784.28	784.06
cal1d12	H-	KWX10LR	-NH ₂	3.03	99.29	742.50	741.98
cal1d13	H-	KWX11LR	-NH ₂	2.62	97.64	700.00	699.90
cal1d14	H-	KWAILR	-NH ₂	3.15	95.43	898.40	898.15
cal1d15	H-	KWAILR	-NH ₂	2.85	96.82	785.20	784.99
cal1d16	H-	KWIIIIILR	-NH ₂	4.55	90.02	1279.40	1279.71
cal1d17	H-	KWAAAAAALR	-NH ₂	2.83	95.97	1027.01	1027.23
cal1d18	H-	X12WIAIIAALR	-NH ₂	3.76	95.18	1139.02	1139.50
cal1d19	H-	X13WIAIIAALR	-NH ₂	3.80	95.73	1125.66	1125.41
cal1d20	H-	X2WIAIIAALR	-NH ₂	3.88	95.38	1111.98	1111.38
cal1d21	H-	KWIAIIAALA	-NH ₂	4.35	97.94	1068.40	1068.36
cal1d22	H-	KWIAIIAALK	-NH ₂	3.84	97.31	1125.74	1125.45
cal1d23	H-	rlaaiiwiwk ^{c,d}	-NH ₂	3.62	95.18	1153.72	1153.47
cal1d25	H-	KX8IAIIAALR	-NH ₂	4.00	97.26	1171.00	1170.50
cal1d26	H-	KX14IAIIAALR	-NH ₂	3.23	98.68	1154.46	1154.40
cal1d27	H-	KHX5LR	-NH ₂	2.82	95.54	750.00	749.10
cal1d28	H-	X2WX5LR	-NH ₂	3.66	98.91	755.79	755.99
cal1d29	H-	X13WX5LR	-NH ₂	3.62	97.55	769.74	770.02
cal1d30	H-	X12WX5LR	-NH ₂	3.62	98.01	783.72	784.10
cal1d31	H-	KWX5LX21	-NH ₂	3.65	98.95	811.86	812.10
cal1d32	H-	KWX11X11LR	-NH ₂	3.49	97.49	798.56	799.02
cal1d33	H-	KWX11X11X11LR	-NH ₂	3.46	96.84	898.08	898.15
cal1d34	H-	KWX11X11X11X11LR	-NH ₂	2.83	96.22	996.62	997.30

cal1d35	H-	KWGGGGGGLR	-NH ₂	2.17	98.02	942.60	943.07
cal1d36	H-	KWX10X10LR	-NH ₂	3.37	99.01	883.10	883.20
cal1d37	H-	KWX15LR	-NH ₂	2.73	98.18	803.40	804.01
cal1d38	H-	KWX19LR	-NH ₂	2.82	97.53	847.40	848.06
cal1d39	H-	KWX19R	-NH ₂	2.31	96.49	734.04	734.90
cal1d40	H-	KWX17X17R	-NH ₂	3.37	98.30	741.32	741.97
cal1d41	H-	X12HIAIIAALR	-NH ₂	2.88	98.94	1089.72	1090.20
cal1d42	H-	kWX5LR ^d	-NH ₂	4.33	98.22	797.58	798.07
cal1d43	H-	KX18X5LR	-NH ₂	3.69	98.14	811.58	812.10
cal1d44	H-	KFX5LR	-NH ₂	4.29	97.38	758.54	759.04
cal1d45	H-	KYX5LR	-NH ₂	3.99	99.79	774.48	775.04
cal1d46	H-	KX7X5LR	-NH ₂	3.87	99.06	808.50	809.10
cal1d47	H-	KWX20LR	-NH ₂	4.08	99.68	839.61	840.17
cal1d48	H-	KWX20R	-NH ₂	3.66	98.96	726.32	727.01
cal1d49	H-	X12WX5LX21	-NH ₂	3.55	99.10	797.18	798.09
cal1d50	H-	KWX22LR	-NH ₂	3.48	96.79	795.55	796.07
cal1d51	Ac-	KWX5LR	-NH ₂	3.94	98.71	839.60	840.13
cal1d52	H-	X9WX5LR	-NH ₂	3.94	96.78	752.80	753.01
cal1d53	H-	X23WX5LR	-NH ₂	4.22	99.62	782.41	783.08
cal1d54	H-	K(X5RL)W ^e	-NH ₂	3.32	97.79	797.90	798.09
cal1d55	H-	KWX5LR	-OH	3.59	98.95	798.60	799.07
cal1d56	H-	KWX5LX24	-NH ₂	3.85	98.17	798.70	799.07
cal1d57	H-	X9KWX5LR	-NH ₂	3.58	95.25	880.80	881.18
cal1d58	H-	KWX5LX25	-NH ₂	3.53	98.93	825.80	826.14
cal1d59	H-	KWX5LX26	-NH ₂	3.93	99.15	842.70	843.09
cal1d60	H-	KX23X5LR	-NH ₂	3.76	99.33	814.50	815.14
cal1d61	H-	KX14X5LR ^f	-NH ₂	2.85	97.05	798.90	799.08

cal1d62	H-	KX14X5LR ^f	-NH ₂	3.02	95.05	799.10	799.08	
cal1d63	H-	KWX27LR	-NH ₂	3.67	95.08	824.26	824.13	
cal1d64	H-	KWX28LR	-NH ₂	3.19	97.74	793.64	794.06	
cal1d65	H-	KWX29LR	-NH ₂	3.29	97.69	815.94	816.12	
cal1d66	H-	KWX30LR	-NH ₂	3.09	95.36	799.62	800.06	
cal1d67	H-	KWX31LR	-NH ₂	2.63	98.83	822.80	823.06	
cal1d68	H-	KW	-NH ₂	1.69	98.79	331.50	331.42	
cal1d69	Ac-	LR	-NH ₂	2.11	97.94	328.80	328.22	
cal1d70	H-	KW + LR ^g	-NH ₂	same as above				
cal1d71a	H-	KWX32LR ^h	-NH ₂	3.79	95.49	837.60	838.16	
cal1d71b	H-	KWX32LR ^h	-NH ₂	3.81	96.69	837.60	838.16	
cal1d72	H-	KWX33LR	-NH ₂	3.45	99.26	832.14	832.11	
cal1d73	H-	KWX34LR	-NH ₂	3.63	99.49	812.57	812.12	
cal1d74	Ac-	TIPKWIX35AALR	-NH ₂	3.72	94.44	1396.88	1397.02	
cal1d76	H-	GWX5LR	-NH ₂	3.98	95.13	726.88	726.97	
cal1d77	H-	AWX5LR	-NH ₂	3.84	96.81	740.84	741.00	
cal1d78	H-	X23WX5LR	-NH ₂	4.12	97.45	782.94	783.08	
cal1d79	H-	RLX5WK ⁱ	-NH ₂	3.58	90.47	797.58	798.10	
cal1d80	H-	rIX5wk ^{dj}	-NH ₂	3.49	98.82	798.10	798.09	
cal1d81 ^k	(SR)	H-	X12X14X5X26	-NH ₂	3.15	95.65	829.90	830.10
	(RS)				3.15	95.65		
cal1d82	H-	EWX5LR	-NH ₂	3.83	97.47	799.00	799.10	
cal1d83	H-	X36WX5LR	-NH ₂	3.90	97.86	826.92	827.08	
cal1d84	H-	X37WX5LR	-NH ₂	3.95	97.49	840.54	840.11	
cal1d85	H-	X6WX5LR	-NH ₂	3.91	98.03	782.70	783.08	
cal1d86	H-	KWX5AR	-NH ₂	3.08	95.15	755.98	756.10	
cal1d87	H-	KWX5IR	-NH ₂	3.44	97.21	798.00	798.10	

cal1d88	H-	KWX5X23R	-NH ₂	3.61	96.91	798.00	798.10
cal1d89	H-	KWX5X38R	-NH ₂	3.23	95.08	802.00	802.10

- 994 ^a Retention time determined by by HPLC or by UPLC.
- 995 ^b **X2**: 2,3-diamino-propionic acid; **X4**: 11-amino-undecanoic acid; **X5**: 12-amino-dodecanoic acid; **X6**: 6-amino-
996 hexanoic acid; **X7**: H-Ala(2-naphtyl)-OH; **X8**: β-(3-benzothieryl)-Ala-OH; **X9**: (*S*)-azetidine-2-carboxylic acid;
997 **X10**: 8-amino-octanoic acid; **X11**: 5-amino-pentanoic acid; **X12**: ornithine; **X13**: 2,4-diamino-butyric acid; **X14**:
998 (DL)-7-azatryptophan; **X15**: 12-amino-4,7,10-trioxa-dodecanoic acid; **X17**: 7-amino-heptanoic acid; **X18**: H-
999 Trp(Me)-OH; **X19**: 15-amino-4,7,10,13-tetraoxa-pentadecanoic acid; **X20**: 15-amino-pentadecanoic acid; **X21**: H-
1000 homoArg-OH; **X22**: 12-amino-dode-7-enoic acid; **X23**: H-Nle-OH; **X24**: H-Cit-OH; **X25**: H-Arg(*N*ω,*N*ω-
1001 dimethyl)-OH; **X26**: H-Arg(NO₂)-OH; **X27**: 14-amino-tetradec-2-enoic acid; **X28**: 11-amino-undec-8-ynoic acid;
1002 **X29**: 9-(aminomethylsulfanyl)nonanoic acid; **X30**: 3-(7-aminoheptoxy)propanoic acid; **X31**: 4-[1-(5-
1003 aminopentyl)triazol-4-yl]butanoic acid; **X32**: 3-[4-(6-aminohexyl)cyclohexyl]propanoic acid; **X33**: 3-[4-(6-
1004 aminoethyl)phenyl]propanoic acid; **X34**: 12-amino-8-methyl-dodecanoic acid; **X35**: 3,4-diamino-benzoic acid;
1005 **X36**: 2-amino-heptane-1,7-dioic acid; **X37**: 2-amino-octane-1,8-dioic acid; **X38**: H-homoCys-OH. Fmoc-
1006 derivatives are reported in Table 2.
- 1007 ^c Retro-inverso version of **cal1b340**.
- 1008 ^d Lowercase letters indicate D-amino acid.
- 1009 ^e The “**X5RL**” moiety is branched onto the ε-NH₂ of the lysine residue.
- 1010 ^f **X14** was used as racemate. Diastereoisomers (**cal1d61** and **cal1d62**) were separated and purified independently.
1011 No enantiomeric identification was performed.
- 1012 ^g Equimolar mixture of the two dipeptides.
- 1013 ^h **X32** was used as racemate. Diastereoisomers (**cal1d71a** and **cal1d71b**) were separated and purified
1014 independently. No enantiomeric identification was performed.
- 1015 ⁱ Retro version of **cal1d1**.
- 1016 ^j Retro-inverso version of **cal1d1**.
- 1017 ^k **X14** was used as racemate, but as opposed to precedingly (^f), the diastereoisomers were not separated and
1018 purified, but the mixture was tested as such.
- 1019
- 1020

1021

1022 **Table 2:** Non-standard Fmoc-amino acid building blocks.

Residue X code	Building block name	Residue name	CAS number
X1	Fmoc- γ -amino-butyric acid	Abu	116821-47-7
X2	<i>N</i> α -Fmoc- <i>N</i> β -alloc-L-2,3-diamino-propionic acid	Dap	188970-92-5
X3	Fmoc-3-(4-pyridyl)-Ala-OH	Pal	169555-93-5
X4	Fmoc-11-amino-undecanoic acid	Aun	88574-07-6
X5	Fmoc-12-amino-dodecanoic acid	Ado	128917-74-8
X6	Fmoc-6-amino-hexanoic acid	Ahx	88574-06-5
X7	Fmoc-3-(2-naphtyl)-Ala-OH	Nal	112883-43-9
X8	Fmoc- β -(3-benzothienyl)-Ala-OH	Bta	177966-60-8
X9	1-Fmoc-(<i>S</i>)-azetidine-2-carboxylic acid	Aze	136552-06-2
X10	Fmoc-8-amino-octanoic acid	Aoc	126631-93-4
X11	Fmoc-5-amino-pentanoic acid	Ava	123622-48-0
X12	<i>N</i> α -Fmoc- <i>N</i> δ -Boc-ornithine	Orn	109425-55-0
X13	<i>N</i> α -Fmoc- <i>N</i> γ -Boc-L-2,4-diamino-butyric acid	Dab	125238-99-5
X14	Fmoc-(DL)-7-azatryptophan	-	7146-37-4 ^a
X15	Fmoc-12-amino-4,7,10-trioxa-dodecanoic acid	-	867062-95-1
X16	Fmoc- β -(2-benzothiazolyl)-Ala-OH	-	959583-56-3
X17	Fmoc-7-amino-heptanoic acid	-	127582-76-7
X18	Fmoc-Trp(Me)-OH	Trp(Me)	1334509-86-2
X19	Fmoc-15-amino-4,7,10,13-tetraoxa-pentadecanoic acid	-	557756-85-1
X20	Fmoc-15-amino-pentadecanoic acid	-	17437-21-7 ^a
X21	Fmoc-homoArg(Pbf)-OH	<i>h</i> Arg	401915-53-5
X22	Fmoc-12-amino-dode-7-enoic acid	-	N/A
X23	Fmoc-Nle-OH	Nle	77284-32-3
X24	Fmoc-Cit-OH	Cit	133174-15-9
X25	<i>N</i> α -Fmoc- <i>N</i> ω , <i>N</i> ω -dimethyl-Arg(Pbf)-OH	ADMA	11858411-84-2
X26	Fmoc-Arg(NO ₂)-OH	Arg(NO ₂)	58111-94-7

X27	Fmoc-14-amino-tetradec-2-enoic acid	-	^b
X28	Fmoc-11-amino-undec-8-ynoic acid	-	N/A
X29	Fmoc-9-(aminomethylsulfanyl)nonanoic acid	-	N/A
X30	Fmoc-3-(7-aminoheptoxy)propanoic acid	-	^b
X31	Fmoc-4-[1-(5-aminopentyl)triazol-4-yl]butanoic acid	-	N/A
X32	Fmoc-3-[4-(6-aminohexyl)cyclohexyl]propanoic acid	-	^b
X33	Fmoc-3-[4-(6-aminohexyl)phenyl]propanoic acid	-	^b
X34	Fmoc-12-amino-8-methyl-dodecanoic acid	-	N/A
X35	Fmoc-3-amino-Boc-4-amino-benzoic acid	Dbz	1823479-63-5
X36	Fmoc-2-amino-heptanedioic acid-7-tert-butyl ester	-	159751-46-9
X37	Fmoc-2-amino-octanedioic acid-8-tert-butyl ester	Asu	276869-41-1
X38	Fmoc-homoCys(Trt)-OH	<i>h</i> Cys	167015-23-8

1023 ^aThe CAS number belongs to the unprotected derivative. Fmoc protecting group was introduced as previously
1024 described [62].

1025 ^bCompounds custom-synthesized for the Institut de Recherches Servier.

1026 N/A, not applicable.

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1029 **Table 3:** Sequences and K_i of reference caloxins.

Code	Sequence			PMCA1	PMCA2
				K_i (μM) ^a	
cal1a1	H-	ACPWWSPHACGGG ^b	-NH ₂		500
cal1a2	H-	ACPIWQPHYCGGG ^b	-NH ₂		190
cal1b1	H-	TAWSEVLHLLSRGGG	-NH ₂	100	46
cal1b3	H-	TIPKWISIIQALRGGGSK	-NH ₂	17	45
cal1c2	H-	TAWSEVLDLLRRGGGSK	-NH ₂	21	2
cal2a1	H-	VSNSNWPSFPSSGGG	-NH ₂		500
cal3a1	H-	WSSTSSVSAPLFGGGGSAK	-NH ₂		700
cal3a2	H-	DSHINNEPSRRKGGGK	-NH ₂		86

1030 ^a Adapted from Grover et al., [20].

1031 ^b Disulfide-bridged peptides.

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1034 **Table 4:** First series of caloxin 1b1 analogues.

Code	Sequence		
cal1b1	H-	TAWSEVLHLLSRGGG	-NH ₂
cal1b11	H-	TAWSEVLHLLSR	-NH ₂
cal1b12	H-	TAWSEVLHLLSR X6 ^a	-NH ₂
cal1b13	H-	T X7 SEVLHLLSRGGG	-NH ₂
cal1b14	H-	T X16 SEVLHLLSRGGG	-NH ₂
cal1b15	Ac-	TAWSEVLHLLSRGGG	-NH ₂
cal1b16	H-	X9 AWSEVLHLLSRGGG	-NH ₂

1035 ^a **X6:** 6-amino-hexanoic acid; **X7:** H-Ala(2-naphtyl)-OH; **X9:** (*S*)-
1036 azetidine-2-carboxylic acid; **X16:** H-Ala(2-benzothiazolyl)-OH.
1037 Fmoc-derivatives are reported in Table 2.

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1042 **Table 5:** Sequence alignments of caloxin 1b3 downsized analogues.

Code	Sequence		
cal1b3	H-	TIPKWISIIQALRGGGSK	-NH ₂
cal1b31	H-	TIPKWISIIQALRGGGS	-NH ₂
cal1b32	H-	TIPKWISIIQALRGGG	-NH ₂
cal1b33	H-	TIPKWISIIQALRGG	-NH ₂
cal1b34	H-	TIPKWISIIQALRG	-NH ₂
cal1b35	H-	TIPKWISIIQALR	-NH ₂
cal1b311	H-	TIPKWISIIQAL	-NH ₂
cal1b312	H-	TIPKWISIIQA	-NH ₂
cal1b313	H-	TIPKWISIIQ	-NH ₂
cal1b36	H-	IPKWISIIQALRGGGSK	-NH ₂
cal1b37	H-	PKWISIIQALRGGGSK	-NH ₂
cal1b38	H-	KWISIIQALRGGGSK	-NH ₂
cal1b39	H-	WISIIQALRGGGSK	-NH ₂
cal1b310	H-	ISIIQALRGGGSK	-NH ₂
cal1b314	H-	IPKWISIIQALR	-NH ₂
cal1b315	H-	PKWISIIQALR	-NH ₂
cal1b316	H-	KWISIIQALR	-NH ₂

1043

1044 **Table 6:** Point-substituted analogues of **cal1b35**.

Code	Sequence		
cal1b35	H-	TIPKWISIIQALR	-NH ₂
cal1b317	H-	TIPKWISIIAALR ^a	-NH ₂
cal1b318	H-	TIPKWIAIIQALR	-NH ₂
cal1b319	H-	TIPKAISIIQALR	-NH ₂
cal1b320	H-	TIPAWISIIQALR	-NH ₂
cal1b321	H-	TIPKWIAIIAALRG	-NH ₂
cal1b322	H-	TIPKWIAIIAALR	-NH ₂
cal1b323	H-	TIPKWIAIIQAL	-NH ₂
cal1b324	H-	TIPKWIAIIQA	-NH ₂
cal1b325	H-	TIPKWIAIIQ	-NH ₂
cal1b326	H-	TIPKWIAIIQALR	-OH
cal1b327	H-	X1 IPKWIAIIAALR ^b	-NH ₂
cal1b328	H-	TIP X2 WIAIIAALR	-NH ₂
cal1b329	H-	TIPKYIAIIAALR	-NH ₂
cal1b330	H-	TIPK X3 IAIIAALR	-NH ₂
cal1b331	H-	CTIPKWIAIIAALRC ^c	-NH ₂
cal1b333	H-	TIPKW X4 LR	-NH ₂
cal1b334	H-	TIPKW X1X1X1R	-NH ₂
cal1b336	H-	TIpKWIAIIAALR ^d	-NH ₂
cal1b337	H-	TIPKWIAAALR	-NH ₂
cal1b340	H-	KWIAIIAALR	-NH ₂
cal1b341	H-	TIPKWIGIIGALR	-NH ₂
cal1b332		TIPKWIAIIAALRG ^e	
cal1b342	H-	KWALR	-NH ₂
cal1d19	H-	X13 WIAIIAALR	-NH ₂

1045 ^a Red letters denote ala-scan position.

1046 ^b **X1**: γ -amino-butyric acid; **X2**: 2,3-diamino-propionic acid; **X3**: H-
 1047 Ala(4-pyridyl)-OH; **X4**: 11-amino-undecanoic acid; **X13**: 2,4-
 1048 diamino-butyric acid. Fmoc-derivatives are reported in Table 2.

1049 ^c Disulfide-bridged peptide.

1050 ^d Lowercase letters indicate D-amino acid.

1051 ^e Head-to-tail cyclopeptide.

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1055 **Table 7:** First series of caloxin **1b340** analogues.

Code	Sequence			Δ AUC (30 μ M) ^a
cal1b340	H-	KWIAIIAALR	-NH ₂	615.7 \pm 49.3
cal1d19	H-	X13 WIAIIAALR ^b	-NH ₂	452.2 \pm 27.1
cal1d20	H-	X2 WIAIIAALR	-NH ₂	339.8 \pm 23.8
cal1d25	H-	KX16 IAIIAALR	-NH ₂	414.7 \pm 24.9
cal1d26	H-	KX14 IAIIAALR	-NH ₂	0.0 \pm 0.0
cal1d21	H-	KWIAIIAALA	-NH ₂	88.3 \pm 7.9
cal1d22	H-	KWIAIIAALK	-NH ₂	377.9 \pm 41.6
cal1d23	H-	kwiaiiialr ^c	-NH ₂	49.0 \pm 1.0

1056 ^a The higher the Δ AUC, the more potent the inhibition.

1057 ^b **X2**: 2,3-diamino-propionic acid; **X13**: 2,4-diamino-butyric acid; **X14**: (DL)-7-azatryptophan;
 1058 **X16**: H-Ala(2-benzothiazolyl)-OH. Fmoc-derivatives are reported in Table 2.

1059 ^c Retro-inverso version of **cal1b340**.

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1063 **Table 8:** Chemical data and PMCA inhibiting activities of centrally modified caloxin1d (**cal1b340**) analogues.

Code	N-ter	Sequence of the heavy atoms in the central region	C-ter	Number of heavy atoms in the central region	Δ AUC (30 μ M) ^a	Chemical name of the spacer
cal1b340	KW-	(N-C-CO) ₆	-LR-NH ₂	18	615.7 \pm 55.4	-Ile-Ala-(Ile) ₂ -(Ala) ₂ -
cal1d68		0	-LR-NH ₂	N/A	0.8 \pm 0.0	N/A
cal1d69	KW-	0	-NH ₂	N/A	5.2 \pm 0.5	N/A
cal1d70	KW-	0	-LR-NH ₂	N/A	4.2 \pm 0.3	N/A
cal1b342	KW-	N-C-CO	-LR-NH ₂	3	0.0 \pm 0.0	-Ala-
cal1d13	KW-	N-C ₄ -CO	-LR-NH ₂	6	0.0 \pm 0.0	- X11 -, 5-amino-pentanoic acid
cal1d15	KW-	(N-C-CO) ₂	-LR-NH ₂	6	0.0 \pm 0.0	-Ala-Ile-
cal1d14	KW-	(N-C-CO) ₃	-LR-NH ₂	9	0.0 \pm 0.0	-Ile-Ala-Ile-
cal1d12	KW-	N-C ₇ -CO	-LR-NH ₂	9	0.0 \pm 0.0	- X10 -, 8-amino-octanoic acid
cal1d11	KW-	N-C ₁₀ -CO	-LR-NH ₂	12	0.0 \pm 0.0	- X4 -, 11-amino-undecanoic acid
cal1d32	KW-	(N-C ₄ -CO) ₂	-LR-NH ₂	12	0.0 \pm 0.0	- X11 -, 5-amino-pentanoic acid
cal1d66	KW-	N-C ₇ -O-C ₂ -CO	-LR-NH ₂	12	0.0 \pm 0.0	- X30 -, 3-(7-aminoheptoxy)propanoic acid
cal1d37	KW-	(O-C ₂) ₃	-LR-NH ₂	12	133.6 \pm 8.0	- X15 -, 12-amino-4,7,10-trioxa-dodecanoic acid
cal1d64	KW-	N-C ₂ -C \equiv C-C ₆ -CO	-LR-NH ₂	12	125.7 \pm 12.6	- X28 -, 11-amino-undec-8-ynoic acid
cal1d39	KW-	(O-C ₂) ₄	-R-NH ₂	12	128.1 \pm 7.7	- X19 -, 15-amino-4,7,10,13-tetraoxa-pentadecanoic acid
cal1d1 (cal1b343)	KW-	N-C ₁₁ -CO	-LR-NH ₂	13	572.7 \pm 40.1	- X5 -, 12-amino-dodecanoic acid
cal1d65	KW-	N-C-S-C ₈ -CO	-LR-NH ₂	12	72.9 \pm 5.1	- X29 -, 9-(aminomethylsulfanyl)nonanoic acid
cal1d50	KW-	N-C ₄ -C=C-C ₅ -CO	-LR-NH ₂	13	239.5 \pm 19.2	- X22 -, 12-amino-dode-7-enoic acid
cal1d63	KW-	N-C ₁₁ -C=C-CO	-LR-NH ₂	15	519.7 \pm 46.8	- X27 -, 14-amino-tetradec-2-enoic acid
cal1d38	KW-	(O-C ₂) ₄	-LR-NH ₂	12	170.1 \pm 8.5	- X19 -, 15-amino-4,7,10,13-tetraoxa-pentadecanoic acid
cal1d40	KW-	(N-C ₆ -CO) ₂	-LR-NH ₂	16	167.4 \pm 6.7	- X17 -, 7-amino-heptanoic acid
cal1d47	KW-	N-C ₁₄ -CO	-LR-NH ₂	16	116.6 \pm 10.5	- X20 -, 15-amino-pentadecanoic acid
cal1d48	KW-	N-C ₁₄ -CO	-R-NH ₂	16	166.3 \pm 18.3	- X20 -, 15-amino-pentadecanoic acid
cal1d16	KW-	(N-C-CO) ₆	-LR-NH ₂	18	0.0 \pm 0.0	-(Ile) ₆ -

cal1d17	KW-	(N-C-CO) ₆	-LR-NH ₂	18	0.0 ± 0.0	-(Ala) ₆ -
cal1d33	KW-	(N-C ₄ -CO) ₃	-LR-NH ₂	18	0.0 ± 0.0	- X11 -, 5-amino-pentanoic acid
cal1d35	KW-	(N-C-CO) ₆	-LR-NH ₂	18	0.0 ± 0.0	-(Gly) ₆ -
cal1d36	KW-	(N-C ₇ -CO) ₂	-LR-NH ₂	18	10.3 ± 0.6	- X10 -, 8-amino-octanoic acid
cal1d34	KW-	(N-C ₄ -CO) ₄	-LR-NH ₂	24	0.0 ± 0.0	- X11 -, 5-amino-pentanoic acid

1064 ^a The higher the ΔAUC, the more potent the inhibition.

1065 N/A: not applicable.

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1068 **Table 9:** Chemical data and PMCA inhibiting activities of other centrally modified caloxin1d (**cal1b340**) analogues.

Code	N-ter	Sequence of the heavy atoms in the central region	C-ter	Number of heavy atoms in the central region	Δ AUC (30 μ M) ^a	Chemical name of the spacer
cal1d67	KW-	N-C ₅ -triazole-C ₃ -CO	-LR-NH ₂	13	0.0 \pm 0.0	- X31 -, 4-[1-(5-aminopentyl)triazol-4-yl]butanoic acid
cal1d72	KW-	N-C ₆ -phenyl-C ₂ -CO	-LR-NH ₂	14	710.1 \pm 63.8	- X33 -, 3-[4-(6-aminohexyl)phenyl]propanoic acid
cal1d71	KW-	N-C ₆ -cyclohexyl-C ₂ -CO	-LR-NH ₂	14	846.3 \pm 59.2	- X32 -, 3-[4-(6-aminohexyl)cyclohexyl]propanoic acid
cal1d73	KW-	N-C ₄ -C(CH ₃)-C ₆ -CO	-LR-NH ₂	13	902.4 \pm 126.4	- X34 -, 12-amino-8-methyl-dodecanoic acid

1069 ^a The higher the Δ AUC, the more potent the inhibition.

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1077 **Table 10:** Chemical data and PMCA inhibiting activities of N-terminal modified **cal1d1** analogues.

Code	Sequence			Δ AUC (30 μ M) ^a
cal1d1	H-	KWX5LR ^b	-NH ₂	572.7 \pm 39.2
cal1d41	H-	X12HIAIIAALR	-NH ₂	169.5 \pm 17.4
cal1d18	H-	X12WIAIIAALR	-NH ₂	443.0 \pm 27.1
cal1d55	H-	KWX5LR	-OH	120.7 \pm 10.0
cal1d51	Ac-	KWX5LR	-NH ₂	4.5 \pm 0.0
cal1d42	H-	kWX5LR	-NH ₂	694.6 \pm 83.3
cal1d76	H-	GWX5LR	-NH ₂	645.5 \pm 45.4
cal1d77	H-	AWX5LR	-NH ₂	524.4 \pm 47.1
cal1d83	H-	X36WX5LR	-NH ₂	331.4 \pm 33.2
cal1d84	H-	X37WX5LR	-NH ₂	309.2 \pm 36.8
cal1d85	H-	X6WX5LR	-NH ₂	286.6 \pm 32.2
cal1d78	H-	X23WX5LR	-NH ₂	775.4 \pm 101.4
cal1d82	H-	EWX5LR	-NH ₂	275.6 \pm 18.9
cal1d28	H-	X2WX5LR	-NH ₂	874.6 \pm 61.2
cal1d29	H-	X13WX5LR	-NH ₂	495.1 \pm 39.7
cal1d30	H-	X12WX5LR	-NH ₂	688.8 \pm 62.1
cal1d49	H-	X12WX5LX21	-NH ₂	701.6 \pm 69.8
cal1d53	H-	X23WX5LR	-NH ₂	635.2 \pm 69.6
cal1d57	H-	X9KWX5LR	-NH ₂	255.7 \pm 33.4
cal1d52	H-	X9WX5LR	-NH ₂	292.9 \pm 22.9
cal1d43	H-	KX18X5LR	-NH ₂	765.2 \pm 60.7
cal1d44	H-	KFX5LR	-NH ₂	362.8 \pm 25.0
cal1d45	H-	KYX5LR	-NH ₂	241.8 \pm 10.3
cal1d27	H-	KHX5LR	-NH ₂	10.0 \pm 0.0
cal1d46	H-	KX7X5LR	-NH ₂	620.7 \pm 62.1
cal1d60	H-	KX23X5LR	-NH ₂	326.7 \pm 22.8
cal1d61	H-	KX14X5LR ^c	-NH ₂	224.2 \pm 20.2
cal1d62	H-	KX14X5LR ^c	-NH ₂	402.6 \pm 35.9

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1080 ^a The higher the Δ AUC, the more potent the inhibition.

1081 ^b **X2**: 2,3-diamino-propionic acid; **X5**: 12-amino-dodecanoic acid; **X6**: 6-amino-hexanoic
1082 acid; **X7**: H-Ala(2-naphtyl)-OH; **X9**: (*S*)-azetidino-2-carboxylic acid; **X12**: ornithine; **X13**:
1083 2,4-diamino-butyric acid; **X14**: (DL)-7-azatryptophan; **X18**: H-Trp(Me)-OH; **X21**: H-
1084 homoArg-OH; **X23**: H-Nle-OH; **X36**: 2-amino-heptane-1,7-dioic acid; **X37**: 2-amino-
1085 octane-1,8-dioic acid. Fmoc-derivatives are reported in Table 2.

1086 ^c **X14** was used as racemate. Diastereoisomers (**cal1d61** and **cal1d62**) were separated and
1087 purified independently. No enantiomeric identification was performed.
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1089 **Table 11:** Chemical data and PMCA inhibiting activities of C-terminal modified **cal1d1** analogues.

Code	Sequence			Δ AUC (30 μ M) ^a
cal1d1	H-	KWX5LR ^b	-NH ₂	572.7 \pm 39.2
cal1d86	H-	KWX5AR	-NH ₂	298.9 \pm 20.8
cal1d87	H-	KWX5IR	-NH ₂	851.5 \pm 76.7
cal1d88	H-	KWX5X23R	-NH ₂	830.7 \pm 91.3
cal1d89	H-	KWX5X38R	-NH ₂	855.5 \pm 111.1
cal1d31	H-	KWX5LX21	-NH ₂	344.1 \pm 10.4
cal1d56	H-	KWX5LX24	-NH ₂	11.7 \pm 1.1
cal1d58	H-	KWX5LX25	-NH ₂	116.6 \pm 5.9

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1091 ^a The higher the Δ AUC, the more potent the inhibition

1092 ^b **X5:** 12-amino-dodecanoic acid; **X21:** H-homoArg-OH; **X23:** H-Nle-OH; **X24:** H-Cit-OH; **X25:** H-
1093 Arg(*N* ω ,*N* ω -dimethyl)-OH; **X38:** H-homoCys-OH. Fmoc-derivatives are reported in Table 2.

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1095 **Table 12:** Summary, apparent potencies of the 12 best peptides.

Code	Sequence			Ratio to cal1d1	Ratio to cal1b3	Potency ^a
cal1b3	H-	TIPKWISIIQALRGGGSK	-NH ₂		1.0	
cal1d1	H-	KWX5LR ^b	-NH ₂	1.0	1.6	
cal1d80	H-	rlX5wk ^{c,d}	-NH ₂	2.1	3.5	
cal1d49	H-	X12WX5X21	-NH ₂	2.1	4.3	
cal1d53	H-	X23WX5LR	-NH ₂	1.9	3.9	
cal1d71a	H-	KWX32LR ^e	-NH ₂	1.9	3.1	
cal1d79	H-	RLX5WK	-NH ₂	1.9	3.1	
cal1d73	H-	KWX34R	-NH ₂	1.9	3.0	
cal1d89	H-	KWX5X38R	-NH ₂	1.8	2.9	
cal1d87	H-	KWX5IR	-NH ₂	1.8	2.9	
cal1d71b	H-	KWX32LR ^e	-NH ₂	1.8	2.8	
cal1d88	H-	KWX5X23R	-NH ₂	1.7	2.8	
cal1d28	H-	X2WX5LR	-NH ₂	1.5	3.9	
cal1d72	H-	KWX33LR	-NH ₂	1.5	2.4	
cal1d43	H-	KX18X5LR	-NH ₂	1.3	3.4	
cal1d42	H-	kWX5LR	-NH ₂	1.2	3.1	
cal1d30	H-	X12WX5LR	-NH ₂	1.2	3.0	
cal1d46	H-	KX7X5LR	-NH ₂	1.1	2.7	

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 1097 ^a Red, apparent IC₅₀ around 30 μM; yellow: apparent IC₅₀ around 10 μM; green: apparent IC₅₀ around
 1098 5 μM.

1099 ^b X2: 2,3-diamino-propionic acid; X5: 12-amino-dodecanoic acid; X7: H-Ala(2-naphtyl)-OH; X12:
 1100 ornithine; X18: H-Trp(Me)-OH; X21: H-homoArg-OH; X23: H-Nle-OH; X32: 3-[4-(6-
 1101 aminoethyl)cyclohexyl]propanoic acid; X33: 3-[4-(6-aminoethyl)phenyl]propanoic acid; X34: 12-
 1102 amino-8-methyl-dodecanoic acid; X38: H-homoCys-OH. Fmoc-derivatives are reported in Table 2.

1103 ^c Lowercase letters indicate D-amino acid.

1104 ^d Retro-inverso version of cal1d1.

1105 ^e X32 was used as racemate. Diastereoisomers (cal1d71a and cal1d71b) were separated and purified
 1106 independently. No enantiomeric identification was performed.

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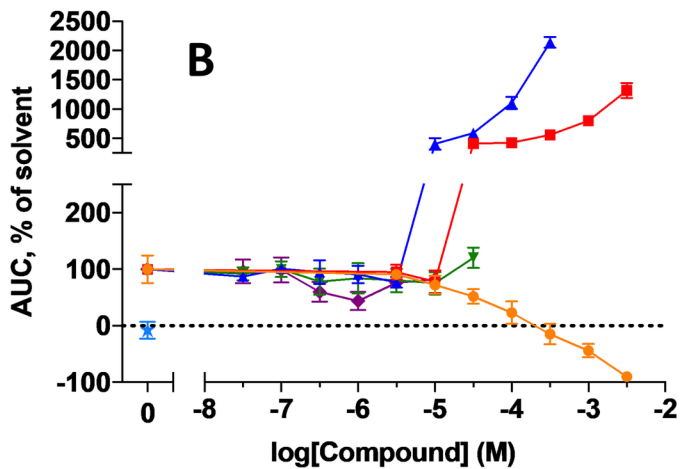
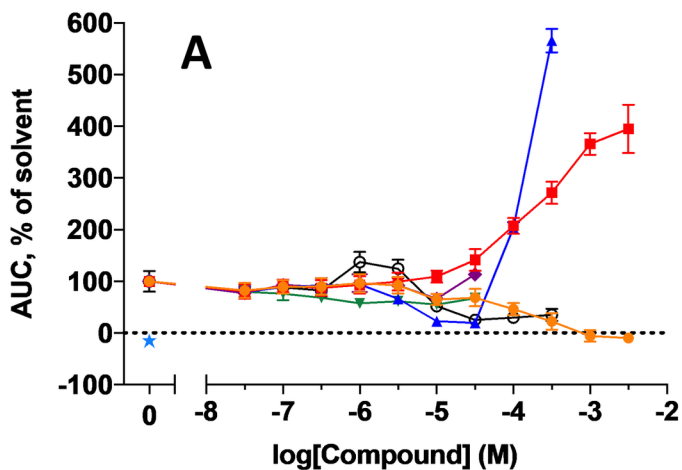


Figure 1

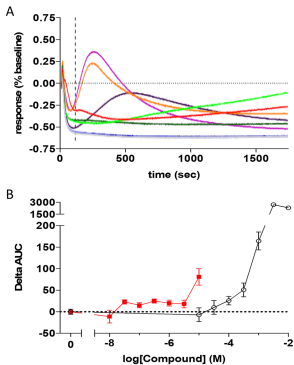


Figure 2

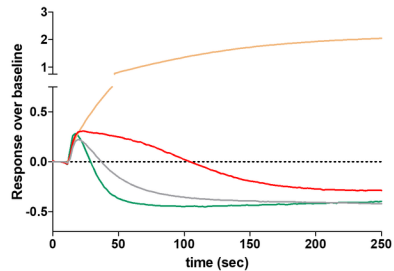
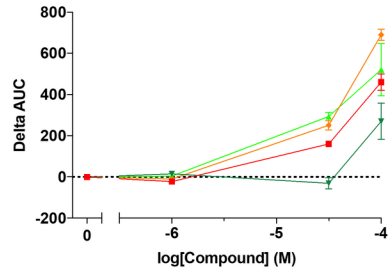
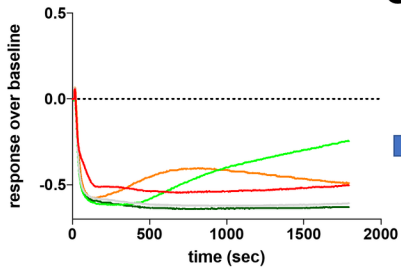
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Figure 3

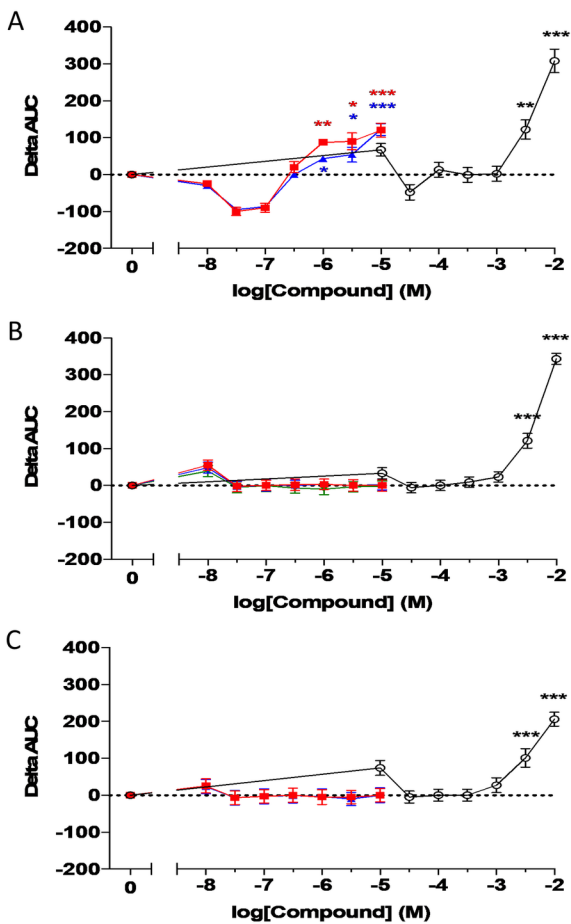


Figure 4

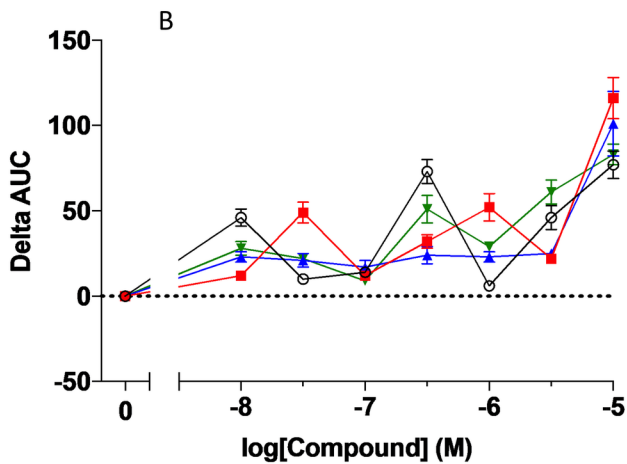
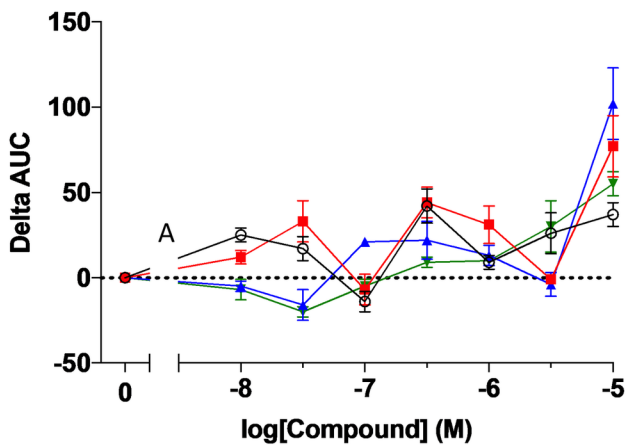


Figure 5

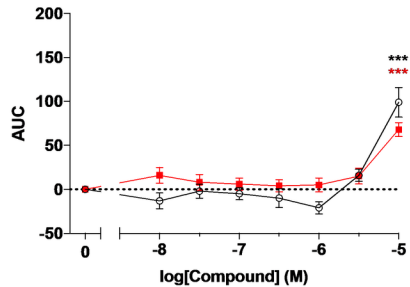
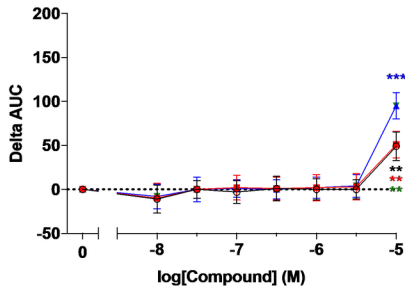
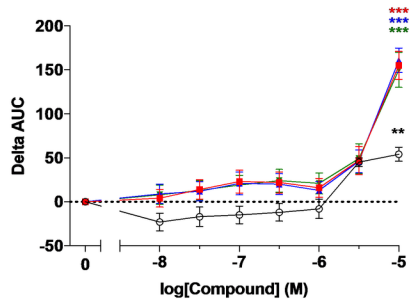
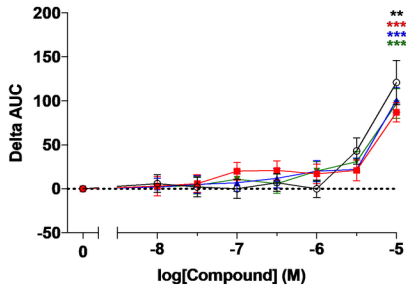


Figure 6

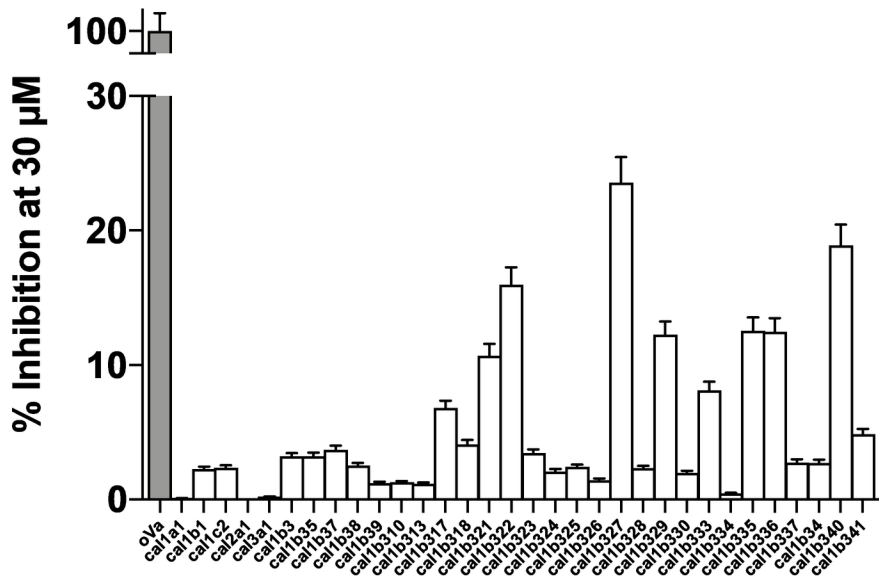


Figure 7

N-terminal
extremity

central region

C-terminal
extremity

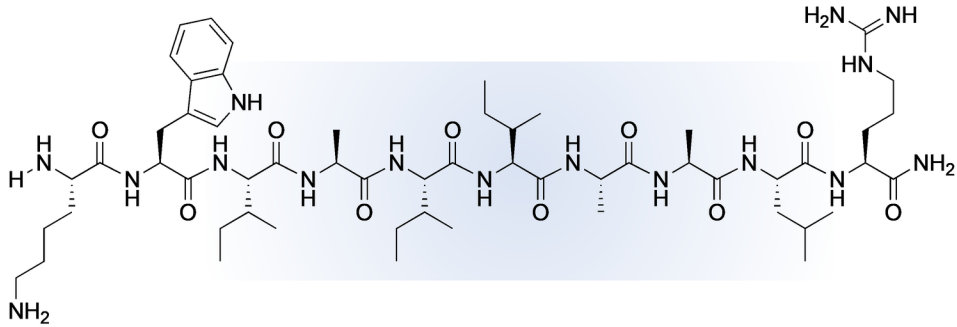


Figure 8

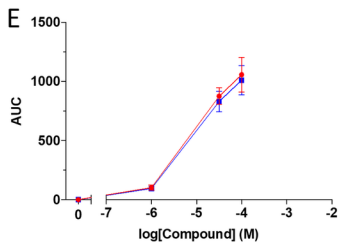
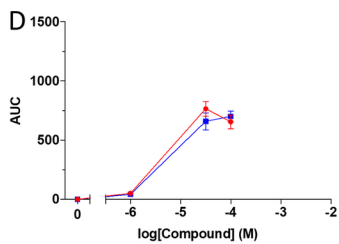
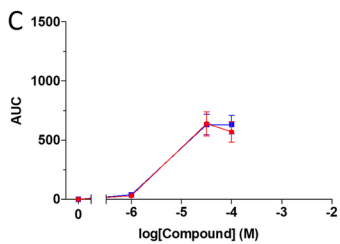
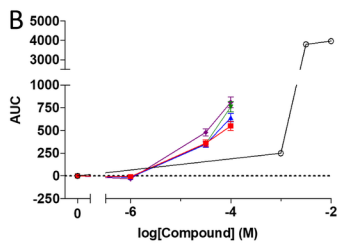
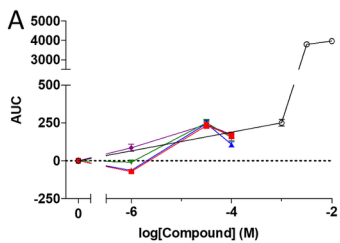


Figure 9