

β -subunit sequestration differently blocks Ca^{2+} channel current and exocytosis in chromaffin cells.

*Marcos Aldea^a, Inmaculada Cuchillo-Ibáñez^a, Jacques Brocard^b,
Almudena Albillos^a, Michel Villaz^b, Antonio G. García^a and
Michel De Waard^b*

*a. Instituto Teófilo Hernando, Departamento de Farmacología y Terapéutica,
Facultad de Medicina, Universidad Autónoma de Madrid, Spain.*

*b. INSERM EMI 99-31, CEA, UJF, Laboratoire Canaux Ioniques et
Signalisation, Grenoble, France.*

Correspondence: Dr. Marcos Aldea, Departamento de Farmacología,
Facultad de Medicina, Arzobispo Morcillo 4, Universidad Autónoma de Madrid.
28029 Madrid. Spain.

Phone: 34-91-4975384. **Fax:** 34-91-4975380. **Email:** marcos.aldea@uam.es

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Chromaffin cells represent a good neuron-like model for studying calcium channels and secretion. The identity of the calcium channels involved in chromaffin cell secretion has been investigated thoroughly¹⁻³. Pharmacological studies suggest that all calcium channel subtypes are involved in the secretory process, but depending on the experimental conditions used, these channels behave at different ways. So far, it remains unclear why chromaffin cells require so many channel types to fulfil a single and fundamental process. The question is partly answered by the observation that, following inactivation by membrane depolarization, secretion can be induced by channel types less sensitive to inactivation⁴.

High voltage activated calcium channels (HVA) are heteromeric complexes formed by at least three different subunits: α_1 , α_2 , and β . The α_1 subunit forms the pore of the channel and confers the pharmacological properties, while the others ones are auxiliary subunits. The auxiliary β subunit is essential for functional expression of high-voltage activated Ca^{2+} channels. The α_2 subunit binds to the α_1 subunit through the I-II loop of the α_1 subunit. All the I-II loops from the different α_1 subunits can bind all the α_2 subtypes, with high affinity but with different selectivity between different α_1 subtypes. In addition the I-II loop contains a reticular retention signal that restricts its incorporation to the plasma membrane, and this sequence is antagonized by the β subunit, releasing the α_1 subunit from the endoplasmic reticulum and facilitating the incorporation of this subunit to the plasma membrane. This property suggests that an overexpression of a construct containing the loop I-II from an α_1 subunit would act as a lure sequestering the endogenous β subunit and altering its intracellular function. This is the case in heterologous expression systems like *Xenopus* oocytes, where the β subunit regulates the correct assembling and the localization of calcium channels in the membrane⁵⁻⁸. We describe here a lure sequence designed to sequester the β subunits in transfected bovine adrenal medulla chromaffin cells, and the functional consequences for the whole-cell inward Ca^{2+} channel current (I_{Ca}) and exocytosis (I_{Cm}) using the perforated configuration of the patch-clamp technique.

RESULTS AND DISCUSSION

We used the I-II loop of the β_1 subunit that forms the pore of the P/Q-type Ca^{2+} channels to design the -lure sequence. The resulting chimeric protein contains extracellular and transmembrane region of the β_1 chain of the human CD8, the I-II loop of β_{1A} subunit and the enhanced green fluorescent protein (EGFP). This construct has several advantages which are to be coupled to the membrane fraction of the cell and its detection is greatly facilitated by EGFP fluorescence. The presence of CD8 allows immunocytochemical detection using an anti-CD8 antibody.

When we evaluated the transfection efficiency of this construct in chromaffin cells maintained in culture, we saw that only around 2-5% of the cells expressed EGFP. The distribution of CD8-I-II-EGFP appeared often patchy and mostly intracellular.

We next wanted to evaluate the functional effects of the expression of the -lure sequence onto native calcium channel currents in bovine chromaffin cells. Since expression of CD8-I-II-EGFP appeared reliable for only a few days, we evaluated its effect on current density 2 days after transfection. Figure 1A shows the protocol used; 200 ms duration depolarizing pulses were applied at 2 min intervals under the perforated-patch configuration of the patch-clamp technique, and the capacitance increase evoked by this pulse was recorded. The extracellular Ca^{2+} concentration to evoke the Ca^{2+} current and the capacitance increase was 2 mM. The peak of the current traces obtained in control untransfected cells was 441.5 ± 47.9 pA, while in CD8-I-II-EGFP transfected cells averaged 271 ± 69.1 pA, which correspond to a significant 62% reduction in calcium influx through calcium channels ($p < 0.05$). When the current was normalized using the cell size, estimated by the capacitance of the resting cell (current density measured in pA/pF) before the pulse application, we observed a 54% reduction ($p < 0.05$). These results show a direct effect of the CD8-I-II-EGFP protein to promote a reduction in the β subunits availability that leads to a reduction in the Ca^{2+} entry to the cell.

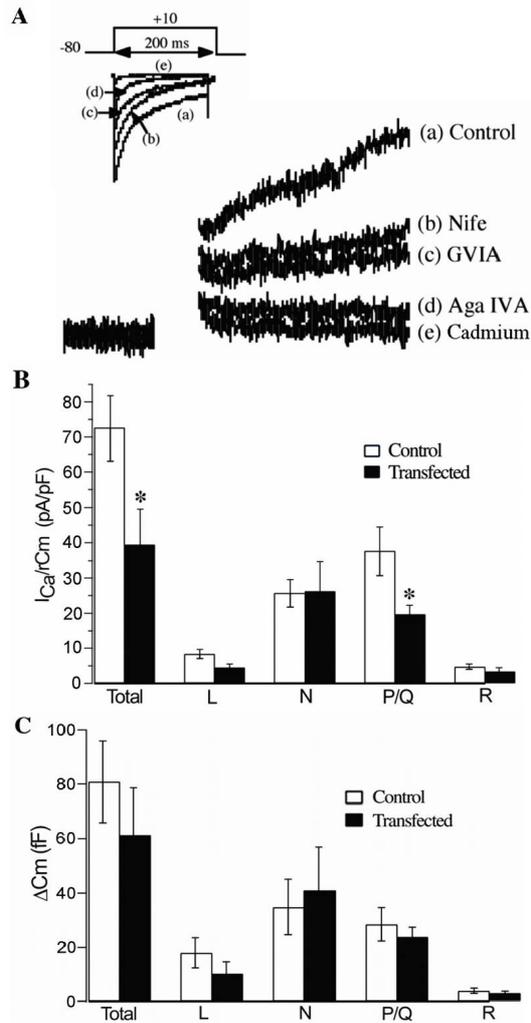


Figure 1. Effects of the CD8-I-II-EGFP construct on the Ca^{2+} current density (I_{Ca}/rCm) and the capacitance increase (ΔCm) in bovine chromaffin cells. **A) The Protocol used for the estimation of the Ca^{2+} current density and the capacitance increase consisted in 200 ms duration depolarizing pulses that evoked an initial capacitance increase reduced by the accumulative application of the Ca^{2+} channel blockers (nifedipine, ω -conotoxin GVIA, ω -agatoxin IVA and Cd^{2+}). **B**) Average data of total calcium entry and contribution for the different Ca^{2+} channel subtypes to this entry in control and transfected cells. **C**) Total secretory response and contribution of the different Ca^{2+} channel subtypes to the exocytosis in control and transfected cells. * $p < 0.05$.**

Since the sequence of the I-II loop used in these studies derived from the β_{1A} subunit, that forms the pore of the P/Q Ca^{2+} channels, we wanted to verify if this sequence interfere with the formation of all calcium channel subtypes or if this inhibition affects preferentially to a specific calcium channel subtype (i.e. P/Q calcium channels). Pharmacological dissection of the whole-cell I_{Ca} , and its associated C_m response in the same cell, was achieved through the use of 3 μ M nifedipine (L-type channel blocker), 1 μ M ω -conotoxin GVIA (N-type channel blocker), 1 μ M ω -agatoxin IVA (P/Q-type Ca^{2+} channel blocker) and 200 μ M Cd^{2+} (blockade of residual current). These calcium channel blockers were applied sequentially and accumulatively to each cell, to evaluate the residual calcium current and the associate secretory response (R-type calcium channels).

Figure 1B shows the different blockade of the current density (I_{Ca}/rC_m expressed in pA/pF) obtained for the different Ca^{2+} channel blockers. When comparing the pairs of bars we only observed significant difference in the case of the P/Q-type calcium channels that are reduced in CD8-I-II-EGFP transfected cells. The current density through P/Q-type calcium channels decrease from 37.7 ± 8.9 pA/pF in control cells to 19.7 ± 2.6 pA/pF in transfected cells ($p < 0.05$). This result suggests a specific association between the I-II loop of β_{1A} subunit and a determinate isoform of α subunit, perhaps the α_4 -type subunit. This inhibition is specific of the P/Q-type calcium channels and no compensation of any other calcium channel subtypes occurred (L, N, R). The sequestration of the β subunit preferentially associated to the β_{1A} subunit favoured the endoplasmic reticulum retention of this protein⁹. The L-type Ca^{2+} channels contributed to the total calcium entry with 8.4 ± 1.3 pA/pF, the N-type Ca^{2+} channels 25.8 ± 3.9 pA/pF and the R-type channels contribution were 4.8 ± 0.7 pA/pF, in control cells. In transfected cells the contribution to the total current density for each channel was: L-type 4.5 ± 1 pA/pF, N-type 26.3 ± 8.5 pA/pF and R-type 3.5 ± 1 pA/pF.

Then we wanted to evaluate the secretory response in control and CD8-I-II-EGFP transfected cells. Figure 1C shows the total capacitance increase (ΔC_m) and the different blockade obtained using the indicated calcium channel blockers in control and transfected cells.

The total secretory response amounted to 80.8 ± 15.1 fF and 61.24 ± 17.27 fF, in control and transfected cells, respectively. We didn't find any difference in the total secretory response when comparing control and transfected cells. The L-type Ca^{2+} channels were responsible for 17.8 ± 5.6 fF, N-type for 34.8 ± 10.2 fF, P/Q type for 28.5 ± 6.2 fF and R-type 3.9 ± 0.9 fF, in control cells. In transfected cells the L-type Ca^{2+} channels contribution were 10.2 ± 4.4 fF, N-type Ca^{2+} channels were $41.1 \pm 15.8.4$ fF, P/Q-type 23.8 ± 3.6 fF and R-type Ca^{2+} channels contribution to the total secretory response were 3.1 ± 0.7 fF. We didn't find any significant difference when comparing control and transfected cells.

CONCLUSION

Our data suggest that CD8-I-II-EGFP acts as a specific repressor of the expression of P/Q-type calcium channels and this effect is probably due to the ability of the I-II loop from the β_{1A} subunit to sequester a certain subtype which is preferentially associated with the β_{1A} subunit, favouring its release from the endoplasmic reticulum and its incorporation to the plasma membrane.

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