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Paradoxical facilitation of exocytosis by inhibition of L-type calcium channels of bovine chromaffin cells

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ABSTRACT

Ca^{2+} entry through the L-subtype (α_{1D}, $Ca_v1,3$) of voltage-dependent calcium channels (VDCCs) seems to selectively regulate the endocytotic response after the application of a single depolarizing pulse to voltage-clamped bovine chromaffin cells. Here we have found that L channel blockade with nifedipine transformed the exocytotic responses elicited by a double-pulse protocol, from depression to facilitation. This apparent paradoxical effect was mimicked by pharmacological interventions that directly block endocytosis namely, dynasore, calmidazolium, GTP-γS and GDP-βS. This reinforces our view that Ca^{2+} entry through PQ channels (α_{1A}; $Ca_v2.1$) regulates fast exocytosis while Ca^{2+} entry through L channels preferentially controls rapid endocytosis.

1. Introduction

The expression of multiple types of voltage-dependent calcium channels (VDCCs) in neurons [1] and chromaffin cells [2] poses the interesting question of their specialisation to regulate different physiological functions within the same cell. Geometry makes easier to understand such specialisation in neurons. For instance N channels (α_{1B}, $Ca_v2.2$) and PQ channels (α_{1A}, $Ca_v2.1$), are preferentially found along apical dendrites and in the axon terminals that synapse with them [3]; this strategic location permits a tight control of neurotransmitter release by Ca^{2+} entering through those channels [4]. On the other hand, VDCCs of the L-subtype (α_{1D}, $Ca_v1.3$) which are located in proximal dendrites and neuronal cell bodies [3,5] have been associated with the regulation of gene expression and enzyme activities in cortical and hippocampal neurons [6,7].

Alike neurons, adrenal medullary chromaffin cells express L, N and PQ VDCCs. However, unlike neurons, these cells adopt a spherical shape while in culture. Thus, studies on the contribution of each channel type to regulate the Ca^{2+}-dependent endocytotic responses to depolarising stimuli have provided controversial results [2]. Concerning Ca^{2+}-dependent endocytosis, we found that L channel blockade with nifedipine suppressed the endocytotic response triggered by a long depolarising pulse applied to bovine chromaffin cells [8]. Later on, Ca^{2+} entry through L channels was found to control endocytosis also at the mouse neuromuscular junction [9] and at *Drosophila* synapse [10]. Recent immunoimaging experiments have discarded a co-localisation of L channels with proteins of the endocytotic machine; rather we favoured the hypothesis that the sustained mode of Ca^{2+} entry through slow-inactivating L channels determines the selective action on endocytosis [11]. Here we used a double-pulse protocol [12] to study the contribution of VDCC subtypes to the regulation of depression or facilitation of exocytotic responses. We surprisingly found that L channel blockade with nifedipine transformed a depressed response into a facilitated exocytotic response. This reinforces our view that PQ and L channels may preferentially regulate exocytosis and endocytosis, respectively, in voltage-clamped bovine chromaffin cells.

2. Materials and methods

2.1. Isolation and culture of bovine chromaffin cells

Bovine adrenal glands were obtained from a local slaughterhouse. Chromaffin cells were isolated by digestion of the adrenal medulla with collagenase as described previously by Livett [13] and maintained in culture for 2–5 days.

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2.2. Recording of Ca^{2+} currents and membrane capacitance of chromaffin cells

All recordings in this study were obtained with the whole-cell configuration of the patch-clamp technique [14,15]. During recording, cells were constantly perifused with a standard external solution containing (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 10 Hepes, pH 7.4 adjusted with NaOH. Cells were internally dialysed with a solution containing (in mM): 10 NaCl, 100 CsCl, 20 TEA-Cl, 0.1 EGTA, 20 Hepes, 5 Mg.ATP, 0.3 Na.GTP, pH 7.2. All experiments were performed at room temperature (24–26 °C). Electrophysiological data were acquired with an EPC-9 amplifier under the control of Pulse software (HEKA Elektronik). Cell membrane capacitance (Cm) changes were estimated by the Lindau–Neher technique [16] applying a sinusoidal wave function (1 kHz, 40 mV peak to peak amplitude) before and after the depolarizing pulses. Cells were held at −80 mV and a double depolarizing pulses protocol to different voltages (−10 and 0 mV) was applied.

2.3. Data analysis

The whole-cell inward Ca^{2+} current peak (I_{Ca}) was analyzed after the initial 10 ms of each depolarizing pulse, to discard the Na^+ current. In this study, exocytosis was measured by monitoring changes in cell capacitance (Cm). Exocytotic response (ΔC1) to the first depolarizing pulse (P1) was measured by subtracting the basal mean Cm obtained 400 ms previous to depolarisation, to that obtained 50 ms after the end of the depolarizing pulse, to avoid a possible Na^+ channel gating artefact [17]. Exocytotic response (ΔC2) to the second depolarising pulse (P2) was calculated subtracting the Cm obtained 50 ms after the end of the P2 to that obtained after P1. Comparisons between means of group data were performed by one-way analysis of variance (ANOVA) followed by Duncan post hoc test when appropriate. A p value equal or smaller than 0.05 was taken as the limit of significance.

2.4. Materials

Collagenase was purchased from Roche (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin fraction V, foetal calf serum and antibiotics were purchased from Gibco (Madrid, Spain). Nifedipine, dynasore, calmidazolium, GTP-γS and GDP-βS were from Sigma (Madrid, Spain). ω-Agatoxin-IVA was from Peptide Institute (Osaka, Japan). ω-Conotoxin GVIA was from Bachem Feinchemikalien (Weil am Rhein, Germany). ω-Conotoxin GVIA and ω-agatoxin-IVA were dissolved in distilled water and stored frozen in aliquots at 0.1 mM. Nifedipine and roscovitine (10^{-2} M) were prepared in dimethylsulphoxide (DMSO) and protected from light. Final concentrations of drugs were obtained by diluting the stock solution directly into the extracellular solution. At these dilutions, solvents had no effect on the parameters studied.

3. Results and discussion

Gillis et al. [12] used a double-pulse protocol to study the effects of protein kinase C on vesicle pool dynamics in chromaffin cells. We used this protocol here consisting of the application of two 100-ms depolarizing pulses, 100 ms apart, from −80 to −10 mV (P1) and to 0 mV (P2) (Fig. 1A). In so doing, we intended to get two I_{Ca} with similar total Ca^{2+} entry [12,18,19]. Despite P2 current being smaller (134 pA) than P1 (152 pA) (Fig. 1A) pooled data from 22 cells showed no statistical differences (190.3 ± 17 pA versus 168 ± 14 pA; $p = 0.14$).

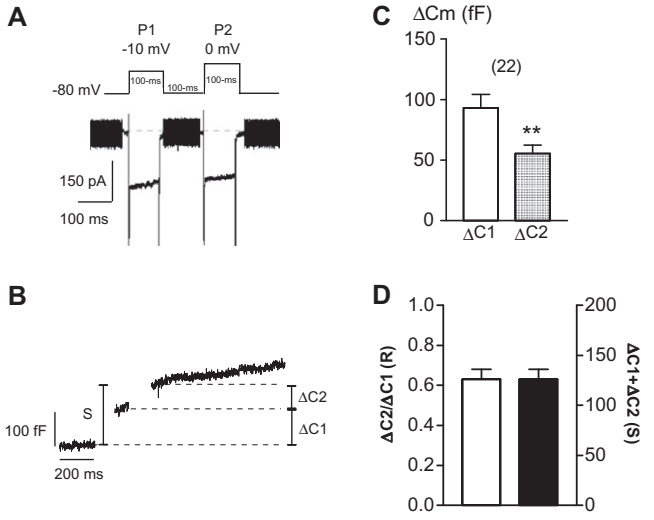

Fig. 1. Calcium currents and the exocytotic responses elicited by stimulation with a double-pulse protocol of bovine chromaffin cells. Example of the Ca^{2+}-currents (A) and capacitance responses (B) to a double-pulse protocol described in A (*top trace*) using the whole-cell configuration of the patch-clamp technique. Two step depolarisations of 100 ms with a 100 ms interval were applied in chromaffin cells held at −80 mV, perifused with a control solution containing 2 mM Ca^{2+}. The intensity of the first pulse (P1; to −10 mV) was adjusted to produce a similar Ca^{2+} current (I_{Ca}) evoked by the second pulse (P2; 0 mV) [12]. (C) ΔCm responses (fF) of 22 cells that exhibited clear depression on the secretory response in P2 (ΔC2). (D) Average values for S (sum of ΔCm elicited by P1 (ΔC1) and P2 (ΔC2)) and R (ratio of ΔCm responses of ΔC2 over ΔC1) calculated from responses to the dual 100 ms protocol. S represents a crude estimate of vesicle pool size; R represents depression or facilitation of the secretory responses. Data in bar graphs C and D are means ± SE of 22 cells from at least 3 different cultures. $^{**}p < 0.01$ compared to control.

Exocytotic responses generated by Ca^{2+} entry during P1 and P2 were measured by means of membrane capacitance increases (ΔCm). Ratios (R) between exocytotic obtained during P2 (ΔC2) and that obtained during P1 (ΔC1) were taken as an indication of facilitation ($R > 1$) or depression of exocytosis ($R < 1$). A crude estimation of RRP (ready-release vesicle pool) was done by adding ΔC1 + ΔC2 (S), assuming that recovery during the 100-ms interpulse period could be neglected [12,18]. Cm traces corresponding to I_{Ca} traces of Fig. 1A, are shown in Fig. 1B where values of ΔCm of 93 and 75 fF for P1 and P2, respectively, were found. Pooled data from 22 cells show a 40% depression of exocytosis (Fig. 1C); this is considered as a form of synaptic depression with a presynaptic origin [20] and assumes pool exhaustion when the ΔC2 response is significantly smaller than ΔC1 [12,18,19,21]. Mean value for R obtained from 22 cells was 0.63 ± 0.05 and RRP value was 163 ± 13 fF corresponding to around 80 vesicles, assuming a mean capacitance of ≈ 2 fF per dense-cored vesicles of chromaffin cells (Fig. 1D) [22–25].

Several studies have approached the question on the extent to which each VDCC subtype contributes to the exocytotic release of catecholamines from chromaffin cells of various mammalian species [2]. However, no studies are available to find out the relative contribution of L, N and PQ channels to the exocytotic responses triggered by a double-pulse protocol. To approach this question, supramaximal concentrations of selective blockers of L channels (3 μM nifedipine), N channels (1 μM ω-conotoxin GVIA; GVIA) or PQ channels (1 μM ω-agatoxin IVA; AgaIVA) were used [2]. A single blocker was tested in a given cell and it was perifused for 5 min before applying the double-pulse protocol. As an example, the left panel of Fig. 2A shows two I_{Ca} traces obtained in the presence of nifedipine; the trace generated by P2 had a slightly smaller amplitude. In spite of this, the ΔC2 was nearly twice higher than ΔC1, as indicated in Fig. 2A (right panel) where the original