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The influence exerted by the β_3 subunit on MVIIA ω-conotoxin binding to neuronal N-type calcium channels

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Abstract

In the present study, two-electrode voltage-clamp techniques have been used to assess the interaction between the MVIIA ω-conotoxin and an isoform of the N-type Ca^{2+} channel α subunit (α_{1B-d}). Cloned α_{1B-d} Ca^{2+} channels were expressed in *Xenopus laevis* oocytes in the presence and absence of the β_3 subunit. Coexpression of the β_3 subunit significantly shifted the IC_{50} value for MVIIA inhibition of central N-type Ca^{2+} channel current. Analysis of the peak conductance vs. depolarising voltage dependence suggested that the β_3 subunit has no apparent effect on the gating charge which accompanies the closed–open transition of the channels. Instead, coexpression of the β_3 subunit led to an approx. 10 mV shift to more hyperpolarised potentials in the voltage-dependent activation of N-type Ca^{2+} channels. We conclude that MVIIA alters the surface charge on the N-type Ca^{2+} channels and might induce allosteric changes on the structure of the channel, leading to an increase in the dissociation constant of MVIIA binding.

Keywords: Neuronal calcium channel; *Xenopus* oocyte; ω-Conotoxin; Two-electrode voltage clamp

1. Introduction

Voltage-dependent ionic channels are integral proteins with paramount importance for the activity of excitable biological membranes. Most ion channels are membrane proteins with oligomeric architecture consisting of both transmembrane and cytoplasmic subunits [1,2]. Like for other voltage-sensitive cation channels, the main subunit (termed α_1) of voltage-dependent Ca^{2+} channels has 24 transmembrane segments packed into four similar 'pseudosubunits' of six transmembrane segments each and forms the ion permeant pore of the channel [3,4]. Cloning experiments corroborated with intracellular recording have proven that genetic expression of the α_1 subunit suffices for the formation of ion channels with voltage- and time-dependent gating, whose biophysical and pharmacological properties closely resemble the corresponding native channel ones [3,5,6]. Based upon electrophysiological and pharmacological properties, voltage-dependent Ca^{2+} channels can be classified into five major groups (L, N, T, R and P/Q types) [7–9].

N-Type Ca^{2+} channels are essential proteins for both regulating neurotransmitter release in the central and peripheral nervous system and controlling endocrine secretion. Purification experiments have shown that besides the main pore-forming subunit (170–240 kDa), N-type calcium channels contain an additional α_2-δ subunit (140–170 kDa) and a β sub-

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unit (52–78 kDa) [10,11]. The α_2-δ subunit consists of two disulphide-linked peptides, encoded by the same gene, and it is shown to be membrane anchored with a heavily glycosylated extracellular domain; in contrast, the β subunit is an entirely cytoplasmic polypeptide [12]. Protein sequencing techniques and molecular genetic studies have pointed out that there exist at least six α_1 genes (termed A, B, C, D, E and S), each containing multiple splice variants, one α_2-δ gene and four β genes (namely β_1, β_2, β_3, β_4) [13]. Although the main electrophysiological and pharmacological properties of N-type Ca^{2+} channels are conferred by their pore-forming α_1 subunit, overall channel folding and many of their gating properties are greatly modulated by the associated auxiliary subunits. Recent biochemical studies have helped in identifying complementary interaction sites between α_1 and β subunits; that is, the β subunit modulates channel activity by binding to the cytoplasmic linker of the I–II loop of all α_1 subunits. The interaction site for the β subunit is located at the N-terminus and encompasses approx. 30 amino acids [12]. Although electrophysiological analysis of N-type calcium channels resulting from different combinations of their subunits in heterologous expression systems has resulted in accumulation of extensive information about the functional effects of auxiliary subunits, the mechanisms of subunit regulation remain largely speculative. One type from the possible regulation mechanisms suggests that interaction between the α_1 subunit and the β subunit induces conformational alteration in the channel's tertiary structure, causing modification of the open probability or in the efficiency of coupling of gating-charge movement to channel gating [14]. Another mechanism which stems from several studies suggests that the β subunit helps in increasing the number of surface-expressed channels, supported by the observation that β subunits increase both maximal recorded gating currents and total binding to channel ligands [12,15]. Other experiments have shown that coexpression of the β subunit increases the proportion of N-type calcium channels in their inactivated state [16] or that their presence is a prerequisite for the normal activity of N-type channels; when the β_2 subunit was transiently coexpressed along the α_{1B-1} subunit, identified in human neuroblastoma cells, the channel became irreversibly blocked by ω-CgTx but insensitive to dihydropyridines [17]. To the opposite, recent studies show that expression of the β subunit modulates the local surface electrical charge and therefore increases the sensitivity of channel activation to surface potentials [18].

In this study, two-electrode voltage-clamp (TEVC) techniques have been used to assess the interaction between the MVIIA ω-conotoxin and a central isoform of the N-type Ca^{2+} channels (α_{1B-d}). It is shown that coexpression of the β_3 subunit significantly shifted the IC_{50} value for MVIIA inhibition of central N-type Ca^{2+} channel currents. Moreover, it is suggested that coexpression of the β_3 subunit has no apparent effect on the gating charge which accompanies the closed–open transition of the channels. The β_3 subunit was chosen for this set of experiments, due to its ability to coassemble with the α_{1B} subunit from N-type calcium channels [13]. It has already been shown that the β_3 subunit influences the potency of local anaesthetics [19] and spider toxins [20] for the α_{1A} subunit of the P/Q type of calcium channels expressed on oocytes and mammalian cells. Since coexpression of the β_3 subunit led to an approx. 10 mV shift to the left in the voltage-dependent activation of N-type Ca^{2+} channels, a strong ground is provided to assume that the modulatory effects of the β_3 subunit on the biophysical properties of central α_{1B-d} N-type Ca^{2+} channels can be tackled on the frame of the apparent increase in the external surface charge density. As a hypothesis, one is bound to assume a similar mechanism for the pharmacological modifications of these channels by their interaction with the β_3 subunit.

2. Materials and method

Oocytes were surgically removed from mature *Xenopus laevis* frogs anaesthetised by submersion in 0.1% ethyl *m*-aminobenzoate (MS 222). Stage V–VI oocytes were prepared for injection by dissociation in Ca^{2+}-free solution containing (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.4), plus 1 mg/ml collagenase (Sigma type I) for 1 h at room temperature. The oocytes were then maintained in storage solution containing (mM): 96 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 5 pyruvate, plus 50 μg/ml gentamicin (pH 7.4). Each oocyte was injected with 25–50 ng

of total cRNA using a precision injector (Drummond 'Nanojet') and incubated at 19°C in storage solution for 3–5 days. cRNA was synthesised in vitro from linearised template DNA encoding the α_{1B-d} subunit of the N-type VSCC from rat brain using an Ambion mMessage mMachine kit. For the coexpression experiments, the β_3 subunit was coinjected together with the α_{1B-d} subunit at a 1:1 ratio. Recordings were made in a nominally Ca^{2+}-free solution of the following composition (mM): 85 TEAOH, 5 $BaCl_2$, 5 KCl, 5 HEPES, adjusted to pH 7.4 with methanesulphonic acid. Depolarisation-activated Ba^{2+} currents were recorded using two-electrode voltage clamp (GeneClamp 500B amplifier; Axon Instruments, Foster City, CA) at room temperature (21–23°C). Voltage and current electrodes were filled with 3 M KCl and had a final resistance of 0.2–1.0 MΩ. Depolarisation-activated VSCC currents in oocytes were evoked from a holding potential of −80 mV and test voltages generated by a PC Pentium computer, with A/D and D/A converters (Digidata 1200 interface) and pClamp programmes (Axon Instruments). Membrane currents were filtered at 2 kHz, sampled at 10 kHz and stored on a computer hard disk for subsequent detailed analysis. Various concentrations of the MVIIA ω-conotoxin were added cumulatively to the Teflon recording chamber (800 μl volume) and the inhibition of peak Ba^{2+} current amplitude was measured before and at least 5 min after incubation of oocytes with ω-conotoxin. Dose–response curves for MVIIA inhibition of channel activity were fitted by Origin 6.0 software (Microcal Origin, USA), according to the following equation:

$$I_{\text{maxnormalised}} = 1 - \frac{[\text{MVIIA}]^n}{\text{IC}_{50} + [\text{MVIIA}]^n}$$

where $I_{\text{max normalised}}$ represents the peak Ba^{2+} current amplitude at a certain concentration of MVIIA relative to the case when MVII was absent, IC_{50} represents the concentrations of half-maximal block and n represents the Hill coefficient.

3. Results and discussion

Fig. 1A shows the original data traces of Ba^{2+} currents elicited as a result of a voltage step from a −80 mV holding potential to 10 and 0 mV, respectively, through the α_{1B-d} subunit of the central N-type calcium channel expressed alone and coexpressed with the auxiliary β_3 subunit at a 1:1 ratio. One easily notices the very strong influence of the β_3 subunit on the peak magnitude of the recorded Ba^{2+} current. The reason for displaying these original traces recorded at different voltage steps lies in the possibility to compare the activating time constants of the N-type channels in the absence and presence of the β_3 subunit. As is inferred from Fig. 1B, coexpression of the β_3 subunit entails an almost 10 mV shift toward negative values of the depolarising voltage which leads to a maximal peak Ba^{2+} current through the α_{1B-d} subunit of the central N-type calcium channel. Therefore, in order to check whether the coexpression of the auxiliary β_3 subunit exerts any influence on the activating time constants of the N-type channels, it is necessary to exclude from the possible mechanisms those related to the voltage-dependent gating of the channels. In order to do so, the macroscopic kinetic analysis of the N-type channel activation in the absence and presence of the β_3 subunit was performed on original traces recorded at a 10 mV difference with respect to the depolarising voltage step. Detailed evaluation points to a statistically insignificant ($P = 0.25$, $n = 3$–5) difference of the activation time of the channel when the β_3 subunit was either absent ($\tau_{\text{activation}} = 4.3 \pm 0.5$ ms) or present ($\tau_{\text{activation}} = 5.4 \pm 0.7$ ms).

After measuring the peak Ba^{2+} current in response to a certain depolarisation ramp (as shown previously in Fig. 1), the dependence of maximum conductance vs. potential of the expressed Ca^{2+} channels has been evaluated in the presence and absence of the modulatory β_3 subunit (Fig. 1C). These points were inferred by dividing the peak current data to ($V_{\text{depolarisation}} - V_{\text{reversal}}$) values. Upon fitting these data points to a Boltzmann relation (see Table 1), two important parameters were estimated, namely the slope factor ($q \times d$) and the half-activating voltage. The main conclusion arrived at was that the apparent gating charge of the central N-type calcium channels remains unaffected by the interaction with the β_3 subunit; the sole modification observed was that regarding the half-activating voltage of the channels ($V_{1/2}$). A paired two-population t-test at a 95% confidence level was employed to assess the statistical difference between slope factors and half-acti-

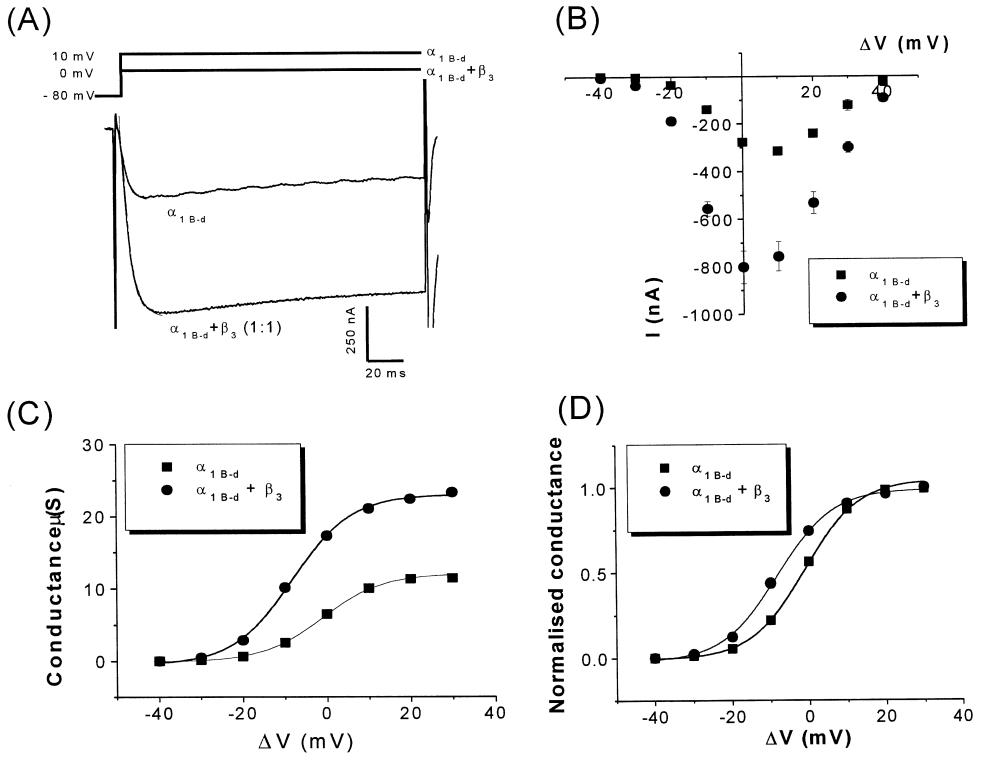

Fig. 1. (A) Original data traces of Ba^{2+} currents elicited as a result of a voltage step from a −80 mV holding potential to 10 and 0 mV respectively, through the α_{1B-d} subunit of the central N-type calcium channels expressed alone and coexpressed with the auxiliary β_3 subunit at a 1:1 ratio. (B) *I–V* curves for the peak Ba^{2+} currents elicited as a result of a voltage step from a −80 mV holding potential to 10 and 0 mV respectively, through the α_{1B-d} subunit of the central N-type calcium channel expressed alone and coexpressed with the auxiliary β_3 subunit at a 1:1 ratio. (C) Dependence of maximum conductance vs. potential of the expressed Ca^{2+} channels evaluated in the presence and absence of the modulatory β_3 subunit. (D) Normalised maximum conductance vs. potential points evaluated in the presence and absence of the modulatory β_3 subunit.

vating voltage values estimated in the absence and presence the β_3 subunit (Table 1). In order to facilitate a better glance of these parameters, the two conductance vs. potential curves have been normalised and superimposed afterwards (Fig. 1D). From these observations it can be speculated that coexpression of the β_3 subunit hardly affects the gating charge which accompanies the closed–open transition of the central N-type calcium channels. In the simplest scenario, the fact that central N-type calcium channels which coassemble with the modulatory β_3 subunit display a significant hyperpolarising shift of their voltage-dependent activation supports could be tacked on the frame of an apparent increase in the external surface charge density induced by the β_3 subunit. In a recent paper [18], it has been proven that expression of any of the β_{1b}, β_{2a}, β_3, β_4 subunits in *Xenopus* oocytes significantly increased the estimated surface charge density of cell membranes which embedded the α_{1A} isoform of P/Q Ca^{2+} channels, from a value of 0.08 e/nm^2 (α_{1A} alone) to 0.11–0.13 e/nm^2 (when a β subunit was expressed). Although such experiments were not specifically performed in the present study, similar changes in the estimated external surface charge density could be supported by the observed increased sensitivity of the central N-type calcium channels to voltage-de-

Table 1
The estimated values of slope and half-activating voltage values from the fit of the maximum conductance vs. potential data points with a Boltzmann relation, as inferred when the α_{1B-d} subunit was expressed alone and coexpressed with the modulatory β_3 subunit ($n = 3$–5)

	$V_{1/2}$ (mean ± S.E.) ($P = 4.03E-6$)	$q \times d$ (mean ± S.E.) ($P = 0.26$)
α_{1B-d}	−1.6 ± 0.2	6.4 ± 0.2
$\alpha_{1B-d}+\beta_3$	−8.2 ± 0.4	7.1 ± 0.5

pendent activation. The explanation of the possible molecular mechanisms through which a putative intracellular modulatory subunit (i.e., the β_3 subunit) could affect the charge density of the external surface from the vicinity of the channel still remains elusive. It should, however, be noted that previous results have demonstrated the ability of intracellular interactions to induce a long-range modification in the structure of other ion channels and thus affect their particular extracellular interaction with various cations [21].

In order to study the influence exerted by the modulatory β_3 subunit on the interaction between N-type calcium channels and the synthetic MVIIA ω-conopeptide, different concentrations from the ω-conopeptide were cumulatively added to the extracellular part of the channel and their effect on the peak Ba^{2+} current was measured. In Fig. 2A an example is shown of depolarisation-activated Ba^{2+} currents elicited in the absence and presence of 10^{-9} M MVIIA.

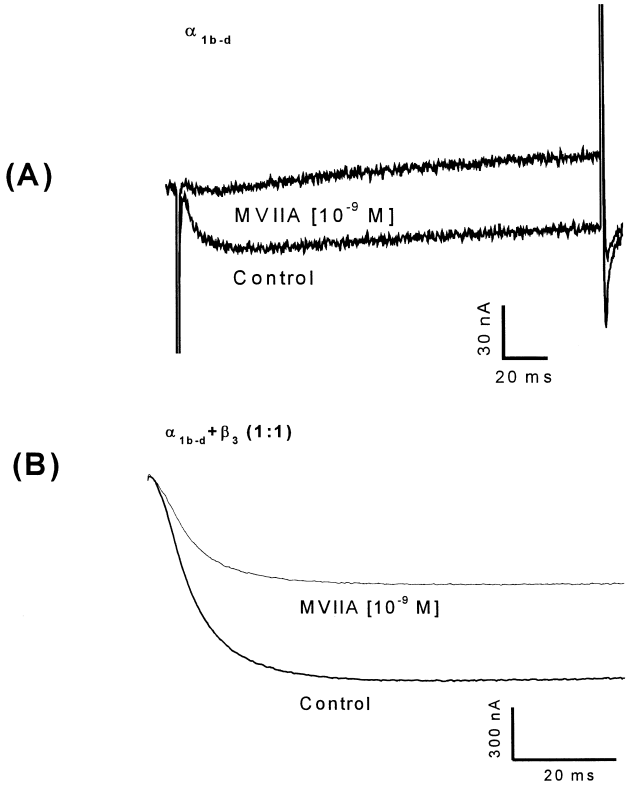

Fig. 2. Original data showing the depolarisation-activated Ba^{2+} currents elicited through the α_{1B-d} subunit of the central N-type calcium channels, in the absence and presence of 10^{-9} M MVIIA, when the main subunit was expressed alone (A) or coexpressed along the modulatory β_3 subunit (B).

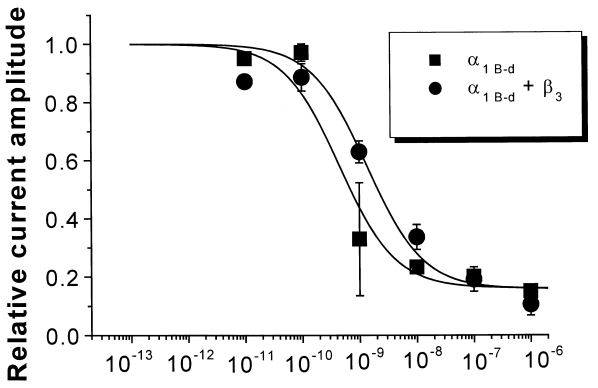

Fig. 3. The dose–response points of the inhibitory effect exerted by the MVIIA ω-conotoxin inferred for the situation when the modulatory β_3 subunit was either absent or present.

From the visual inspection of this figure, it is obvious that the MVIIA ω-conopeptide has a strong inhibitory effect on the macroscopic current through N-type calcium channels expressed in the absence of the modulatory β_3 subunit. When the β_3 subunit was coexpressed alongside the central α_{1B-d} isoform, a similar concentration of the MVIIA ω-conopeptide (10^{-9} M) proves to have a rather significant lower potency in inhibiting peak Ba^{2+} currents through the expressed channels (Fig. 2B).

In order to comprehensively display the aforementioned affinity difference of the MVIIA ω-conopeptide for the studied ion channels, various concentrations of the MVIIA ω-conotoxin were added cumulatively to the Teflon recording chamber (800 μl volume) and the inhibition of peak Ba^{2+} current amplitude was measured. The resulting dose–response points inferred for the two studied situations (i.e., β_3 subunit either absent or present) are shown in Fig. 3. By fitting these experimental points to a Hill equation, the concentrations of half-maximal block (IC_{50} values) were deduced (4.5E-10 ± 2.1E-10 M in the absence and 1.38E-9 ± 5.5E-10 M in the presence of the β_3 subunit, $P = 0.09$, $n = 3$–5).

A structure–activity study performed on the GVIA has pointed to two key amino acids (Lys^2 and Tyr^{13}) important in the interaction between the ω-conopeptide and its binding site on the α subunit on the central N-type calcium channel [22]. Furthermore, taking into account that the solution structures of MVIIA and another close related ω-conopeptide (GVIA) are quite similar, it has been speculated that GVIA and MVIIA bind to the N-type calcium

channel in a similar manner (although in the case of MVIIA Arg^{10}, Leu^{11} and Arg^{21} are equally important for its affinity for the N-type calcium channel) [23,24]. Chimeral studies of the α subunits of N-type calcium channels have suggested that the important region for the GVIA interaction with the channel is located to the putative external loop between the IIIS5 and IIIH5 regions of the channel and key amino acids are glutamate residues [25]. It can therefore be suggested that interaction between the MVIIA ω-conopeptide and N-type calcium channels lies mostly on electrostatic interactions, with a rather large distributed binding surface on the conopeptide. In this case, it will come as no surprise that alterations of the channel external surface potential influence the dissociation constant of the MVIIA ω-conopeptide. What does constitute an unexpected result, is that this alteration appears to be in the sense that more negative charges on the channel external surface (as a result of the β_3 subunit coexpression) decrease the affinity of the MVIIA for the channel (remember that the amino acids from the MVIIA structure with a key role in its interaction with the channel are positively charged). A plausible explanation for this phenomenon would be that an alteration in the surface charge on the N-type calcium channels does induce allosteric conformational changes in the overall three-dimensional structure of the channel and by this, the *on* and *off* reaction rates of MVIIA are altered such that the macroscopic dissociation constant increases. Although more site-directed mutagenesis experiments are needed to clarify the mechanism of MVIIA interaction with central α_{1B-d} N-type Ca^{2+} channels, the results presented in this communication strengthen the important roles played by electrostatic forces for the interaction of central N-type Ca^{2+} channels with synthetic ω-conopeptides, and at the same time draw attention to the non-trivial mechanism of such interactions.

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