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Functional coupling between human E-type Ca^{2+} channels and μ opioid receptors expressed in *Xenopus* oocytes

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Abstract Neuronal α_{1E} Ca^{2+} channels were expressed in *Xenopus laevis* oocytes alone and in combination with the μ opioid receptor. Macroscopic currents were recorded under voltage clamp conditions. The stimulation of the morphine receptor by the synthetic [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5] enkephalin (DAMGO) produced a 20% reduction in the α_{1E} ionic current. This effect was associated with a large change in the decay phase of the Ba^{2+} current. The effect of 1 μM DAMGO was fully antagonized by the universal μ opioid receptor antagonist naloxone and by the selective antagonist β-funaltrexamine. The ionic current inhibition induced by DAMGO was partially recovered by preceding strong depolarizations. The injection of the catalytic subunit of pertussis toxin (A-protomer) abolished the effect of DAMGO, suggesting the involvement of a GTP binding protein in the α_{1E} modulation. The coexpression of the regulatory β_{2a} Ca^{2a} channel subunit, together with the α_{1E} subunit and the μ opioid receptor, prevented the reduction of the ionic current following the receptor stimulation with DAMGO, whereas the coexpression with the β3 subunit reduced by ∼50% the modulatory effect of DAMGO. The effect produced by the stimulation of the opioid receptor could be mimicked by coexpressing the α_{1E} channel with the G-protein βγ subunits.

Key words: α_{1E}; Ca^{2+} channel; Beta subunit; Mu opioid receptor; G-protein

1. Introduction

Opioid peptides modulate nociception by inhibiting Ca^{2+} dependent neurotransmitter release from the synaptic terminals via the inhibition of different classes of voltage dependent ion channels [1]. N and P/Q type Ca^{2+} channels have been shown to be negatively coupled to the opioid receptors in native and cloned channels [2–5]. The stimulation of μ, δ, and κ opioid receptors activate GTP-binding proteins (G-proteins) which can modulate ion channel activity [6]. Ca^{2+} channel modulation by G-proteins [7] has been investigated in neurons and cell lines where different effects have been reported. This variability is likely due to the complexity of the systems used, involving multiple ion channel types, converging pathways, and unknown channel subunit composition. In fact, voltage dependent Ca^{2+} channels are multimeric proteins formed by the association of different subunits [8,9]. The α_1 pore forming subunit is associated to the regulatory α_2/δ and β subunits [10] which profoundly modify some of the biophysical properties of the channel voltage dependence, kinetic properties, coupling efficiency [11–15] and the expression levels [16].

It has been shown that the β subunit interferes with G-protein modulation of Ca^{2+} channels [17,18]. Because Ca^{2+} channel β subunits bind to the intracellular loop between repeats I and II (I-II loop) of the calcium channel α_1 subunits [19], Bourinet and collaborators [5] proposed that G-proteins could also bind to the I-II loop, and different groups [20–22] pointed out the importance of the I-II loop in G-protein modulation. However, Zhang and collaborators [23] have shown that the I-II loop of α_{1B} channel is not the primary responsible for the G-protein sensitivity, and Qin et al. [24] have confirmed this finding also for the α_{1E} clone. Instead, multiple structural elements in the repeat I and the C-terminal region of the channel are responsible for the G protein modulation. Still, the overlapping of binding sites for G-protein βγ dimer and Ca^{2+} channel β subunits, or a steric hindrance, are possible reasons for their competitive actions on the channel (for a review see [25]).

G-protein modulation of α_{1E} has been controversial. Two groups failed to measure significant G-protein modulation of α_{1E} channel [5,26], while other investigators have shown its G-protein sensitivity [24,27,28].

We coexpressed in *Xenopus* oocytes the human neuronal α_{1E} Ca^{2+} channel [29] together with the μ opioid receptor. By stimulating the μ opioid receptor with [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5] enkephalin (DAMGO), we found that the α_{1E} Ba^{2+} current was inhibited via a G-protein mediated mechanism. This effect was reduced by coexpressing the accessory calcium channel β_3 subunit and practically abolished by the β_{2a} subunit.

2. Materials and methods

2.1. RNA synthesis

We used the neuronal pore forming a_{1E} Ca^{2+} channel subunit

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which was expressed alone or in combination with the regulatory b_{2a} subunit and the μ opioid receptor [10,29,30]. Transcription of α_{1E}, β_{2a}, β_3, G-protein β_1 and G-protein γ_2 was done at 37°C in a volume of 25 μl containing 40 mM Tris-HCl (pH 7.2), 6 mM $MgCl_2$, 10 mM dithiothreitol, 4 mM spermidine, 0.4 mM each of adenosine triphosphate, guanosine triphosphate, cytosine triphosphate and uridine trisphosphate, 1 mM 7-methyl guanosine triphosphate, 0.5 mg linearized DNA template and 10 units of T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA).

The murine μ opioid receptor [30] was subcloned into an *Eco*RI site of pcDNA3 vector (Invitrogen, San Diego, CA, USA). The plasmid was linearized by *Not*I digestion and then used as a template for cRNA synthesis using the mMESSAGEmMACHINE kit (Ambion, Austin, TX, USA).

The β_{2a} and the β_3 subunits were subcloned into pAGA2, derived from pGEM-3 (Promega). pAGA2 contains at the 5′ end an alfalfa mosaic virus translational initiation site and at 3′ end a poly A tail to facilitate the expression in *Xenopus* oocytes. Briefly, the full length cDNAs were PCR-amplified from the original clones in pBS with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). Primers containing a *Nco*I site and a *Xba*I site were used. The PCR product was digested with *Nco*I and *Xba*I and subcloned into the pAGA2 vector digested with the same restriction enzymes. Fidelity was confirmed by sequencing.

2.2. Oocyte preparation

Frogs were anesthetized by immersion in water containing 0.15–0.17% tricaine methanesulfonate for about 20 min or until full immobility. The ovaries were removed under sterile conditions by surgical abdominal incision. The animals were then killed by decapitation. The animal protocol was performed with the approval of the Institutional Animal Care Committee of the University of California, Los Angeles.

Before injection, oocytes were defolliculated by collagenase treatment (type I, 2 mg/ml for 40 min at room temperature; Gibco BRL, Gaithersburg, MD, USA). Oocytes were maintained at 19.5°C in Barth solution. Recordings were done 4–12 days after the RNA injection.

2.3. Recording techniques

The cut-open oocyte voltage clamp technique was used to record ionic currents [31]. The external solution (recording chamber and guard compartments) had the following composition: 10 mM Ba^{2+}, 96 mM Na^+, 10 mM HEPES, titrated to pH 7.0 with methanesulfonic acid (CH_3SO_3H) (MES). The lower chamber (facing the interior of the oocyte previously permeabilized with 0.1% saponin) contained 110 mM K-glutamate, 10 mM HEPES titrated to pH 7.0 with NaOH. Because the oocytes endogenous Ca^{2+} dependent Cl^- current is also activated by Ba^{2+} (permeating through the expressed Ca^{2+} channels), all the oocytes were injected prior to recording with 100–150 nl of BAPTA-Na_4 50 mM titrated to pH 7.0 with MES. This procedure prevents the activation of Ca^{2+} and Ba^{2+} activated Cl^- channels [32]. To remove contaminating non-linear charge movement related to the Na/K ATPase [33] 0.1 mM ouabain was added to the solutions. Leakage and linear capacity currents were compensated analogically and subtracted on line using P/-4 subtracting protocol from −90 mV holding potential (SHP).

The μ opioid receptor agonist (DAMGO, Peninsula Lab.) and antagonists (naloxone and β-funaltrexamine (β-FNA), RBI, Natick, MA, USA) were either dissolved in the external solution and perfused, or added concentrated into the recording chamber.

Signals were filtered with an eight pole Bessel filter to 1/5 of the sampling frequency. All the experiments were performed at room temperature (20–22°C). Values are average ± standard error of the mean (S.E.M.).

3. Results

3.1. α_{1E} channels and the μ opioid receptors are functionally coupled

The expression in *Xenopus* oocytes of neuronal α_{1E} Ca^{2+} channels alone gave rise to a large Ba^{2+} current which remained unmodified either by the application of the μ receptor agonist DAMGO (1 μM) or the antagonist naloxone (10 μM) ($n = 5$). Instead, when the α_{1E} subunit was coexpressed with the μ opioid receptor (MOR), the ionic current was inhibited 23.1% ± 1.5% ($n = 24$, six batches of oocytes) by the application of 1 μM DAMGO to the external solution (Fig. 1A). This inhibition was relieved either by the addition of the universal opioid antagonist naloxone (10 μM) to the external solution (Fig. 1A,B) or by the μ receptor specific antagonist β-FNA (data not shown).

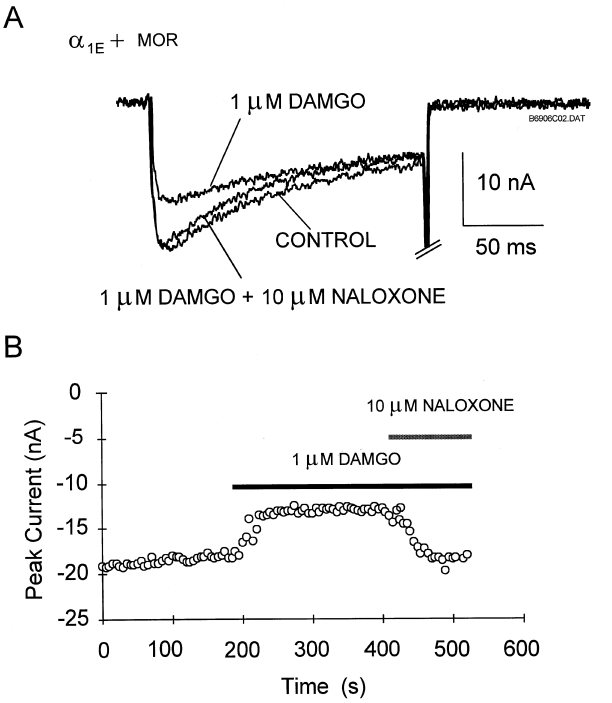

Fig. 1. DAMGO reversibly inhibits α_{1E} Ba^{2+} current. The upper panel shows superimposed current traces (A) elicited by depolarization to 10 mV in an oocyte expressing α_{1E} channels and μ opioid receptors. No auxiliary subunits were coexpressed. The membrane conductance was reduced by the perfusion of 1 μM DAMGO to the external solution and was fully recovered by the addition of 10 μM naloxone. B: The time course plot of the experiment shown in A.

Fig. 2 shows two families of Ba^{2+} currents in control conditions (A) and after the application of 1 μM DAMGO (B). The relative current-voltage relationships (I(V)) are shown in panel (C).

After the inhibition by DAMGO, the channels available for opening maintained a very similar voltage dependence of activation as in control conditions. The conductance-voltage relationships (G(V)) (Fig. 2F) were constructed plotting the tail current during repolarization to −50 mV against the membrane potential during 25 ms test pulses ranging from −88 mV to 132 mV [13]. After the application of DAMGO, a clear reduction of the membrane conductance was present at all tested potentials (Fig. 2F). However, as shown in Fig. 2G, when normalized to the maximum conductance, the two G(V) curves did not show significant changes in their voltage dependence.

3.2. The current inhibition triggered by activation of the μ receptors, is accompanied by a slowdown in the decay phase

The inhibition of the current following the application of

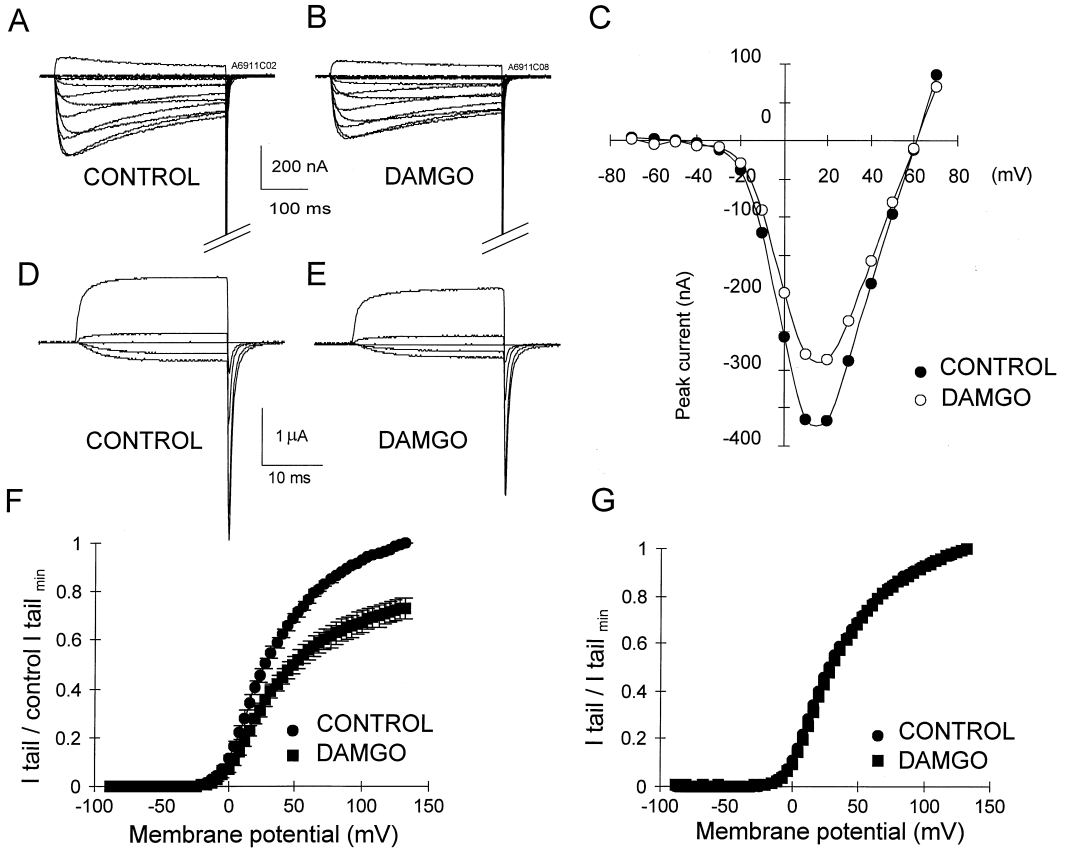

Fig. 2. The inhibited current maintains the voltage dependence of activation of the control. A, B: Two families of Ba^{2+} currents from an oocyte expressing α_{1E}+μ receptors evoked by depolarizing voltage steps from −50 mV to 70 in 10 mM increment, before and after application of 1 μM DAMGO. The relative current-voltage relationship (I(V) curve) in control condition (●) and after perfusion of 1 μM DAMGO (■) is shown in C. D, E: Representative current traces evoked by voltage steps of 25 ms. The returning potential is −50 mV. This pulse protocol was used to construct the activation curves (G(V) curves, F) from the tail currents ($n = 5 \pm$ S.E.M.). The plot in F shows reduction in the membrane conductance following the application of 1 μM DAMGO. No significant changes in the voltage dependence of activation were observed: the normalized G(V) curves for control (●) and 1 μM DAMGO (■) shown in G are practically superimposed.

DAMGO was accompanied by a slowdown of the ionic current decay phase, while the activation kinetics remained substantially unchanged.

In Fig. 3A, the activation kinetics for the control and the DAMGO inhibited current are shown superimposed and in an expanded scale. The first 12–15 ms of current installation during depolarizations to 10 mV were well fitted with a single exponential function. We obtained activation time constants that were practically identical for the control current ($\tau = 4.5 \pm 0.5$ ms) and for the DAMGO inhibited current ($\tau = 4.6 \pm 0.5$ ms ($n = 12$)). The current inhibition in this set of experiments was $23 \pm 2\%$. In the same current records where the activation time constants were measured, we fitted the ionic current inactivation with a single exponential function before and after application of DAMGO 1 μM (Fig. 3B). Comparing the inactivation kinetics before and after the perfusion of DAMGO, we found a change of ∼2 fold in the time constant of inactivation (τ_{inact}). The averaged values were: $\tau_{inact} = 154.9 \pm 16.0$ ms for the control, and $\tau_{inact} = 324.1 \pm 54.1$ ms in 1 μM DAMGO ($P < 0.005$, $n = 12$). For a better estimate of the time constants, we also fitted the current inactivation during 1 s pulses to 10 mV with the sum of two exponential functions (Fig. 3C). Both components of the current inactivation became slower after the application of DAMGO. In the control current the two components of the inactivation had the following time constant: for the fast component $\tau_{fast} = 94.4 \pm 12.2$ ms, and for the slow component $\tau_{slow} = 488.3 \pm 52.5$ ms. Instead, the DAMGO inhibited current had $\tau_{fast} = 197.9 \pm 34.8$ ms, and $\tau_{slow} = 855.9 \pm 163.7$ ms ($n = 9$). The relative amplitudes of τ_{fast} were $33 \pm 1\%$ in control, and $37 \pm 3\%$ in DAMGO. Fig. 3D,E summarizes the changes in activation and inactivation time constants upon DAMGO perfusion.

3.3. Inhibition by DAMGO can be partially relieved by strong depolarization

As first pointed out by Marchetti et al. [34] and Tsunoo et al. [35], the block of voltage gated Ca^{2+} channels mediated by G-protein coupled receptors, shows a marked voltage dependence. We found that in α_{1E} the fraction of current inhibited by DAMGO could be partially recovered by applying strong depolarizations. Before perfusing the opioid agonist (Fig. 4A, control) the current measured during a test pulse to +10 mV did not undergo kinetic and/or major amplitude changes, regardless of the application of a 100 ms depolarizing prepulse to 100 mV. Often, a small reduction in current amplitude ($3.4 \pm 1.7\%$, $n = 8$) was observed at the test potential after the depolarizing prepulse, due to the voltage dependent

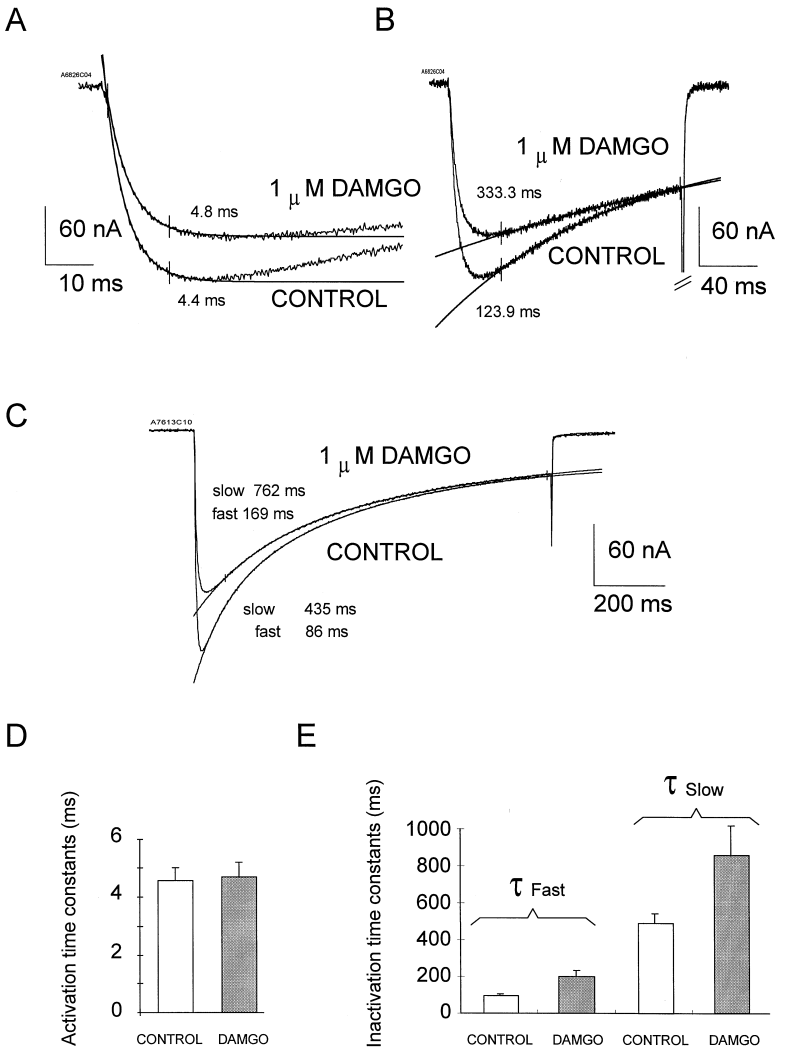

Fig. 3. Kinetic effect of opioid stimulation. In A, the current installation during depolarization to 10 mV in control and DAMGO inhibited currents are shown superimposed in an expanded scale: the continuous lines are the fits to a single exponential function of the initial current installation. No relevant changes in the activation time constant (τ_{act}) were detected comparing control and DAMGO inhibited currents (τ_{act} control = 4.4 ms, τ_{act} DAMGO 4.5 ms). Following perfusion of DAMGO, an evident change in the time course of decay phase occurs: in B, superimposed current traces before and after application of DAMGO are shown (same experiment as in A). The inactivation time constants (τ_{inact}) were calculated by fitting of the current inactivation during 150 ms voltage steps to 10 mV to a single exponential function. The best fits are shown superimposed as continuous lines. C: Representative Ba^{2+} currents evoked by 1 s depolarization to 0 mV before and after the application of 1 μM DAMGO. Current inactivation was fitted with the sum of two exponential functions (fits are superimposed and the relative time constant for fast and slow components are next to the traces). D, E: Bar plots showing respectively the changes in the activation and inactivation time constants occurring after perfusion of DAMGO during depolarization to 10 mV; error bars are the standard errors of the mean (activation, $n = 11$; inactivation, $n = 9$).

inactivation properties of the α_{1E} clone. The application of a strong depolarizing prepulse in the presence of DAMGO (Fig. 4B), partially relieved the block, resulting in a larger current with a faster decay. In average, after the perfusion of 1 μM DAMGO, the positive prepulse recovered 41.9 ± 11% of the blocked current ($n = 8$). The fact that the block was not relieved during large depolarization in the G(V) experiments (Fig. 2E,F), can be explained by the relatively short (25 ms) depolarizing pulses used for the G(V) protocol (Fig. 2C,D). As α_{1E} channels have a marked voltage dependent inactivation [13], longer pulses for the construction of the G(V) curves would have led to an erroneous estimate of the activation curves, by partially inactivating the channels to a different extent depending on the voltage and on the presence of DAMGO.

3.4. Ca^{2+} channel β_{2a} and β_3 compete with the effect of opioid stimulation on α_{1E}

When the regulatory β_{2a} subunit was coexpressed together with α_{1E} and the μ opioid receptor, perfusion of 1 μM DAMGO did not affect the current amplitude or the overall kinetics of the current amplitude. The inhibited current in the presence of β_{2a} was 0.5 ± 1% ($n = 6$). Fig. 5A shows an experiment illustrating this result. We also tested the effect of the Ca^{2+} channel β_3 subunit [36]. While β_{2a} almost completely prevented the opioid effect, in the presence of β_3 the effect of 1 μM DAMGO was only attenuated by ~50%. β_3 reduced the peak current amplitude by 9.0 ± 1.2% ($n = 5$) as shown in Fig. 5B. In the same batch of oocytes, in the absence of the β_3 subunit, the block was 23.2 ± 1.1% ($n = 4$). The proper expression of the regulatory β subunits was monitored by measuring

its effects on the voltage dependence of the activation (G(V) curve) typical of the β subunit modulation on α_{1E} channels [13].

3.5. The modulation of α_{1E} channels by opioid receptor stimulation occurs via a pertussis toxin sensitive G-protein

Mu opioid receptors are typical seven transmembrane domain proteins known to be coupled to GTP-binding proteins [37]. The voltage dependence of Ca^{2+} channel inhibition, and the interference by the Ca^{2+} channel β subunit on that inhibition, have been described as properties characteristic of the modulation of voltage dependent Ca^{2+} channels by G-proteins [38]. To confirm the involvement of a G-protein in the action of the μ opioid receptor in the oocyte, we injected into the oocytes the A-protomer of pertussis toxin (catalytic subunit) ~3 h before current measurements. This maneuver completely eliminated the response evoked by opioid stimulation (DAMGO block 1.3 ± 1.4%, $n = 4$, Fig. 5C).

3.6. Coexpression of α_{1E} with the G-protein βγ subunits mimics the effect of opioid receptor stimulation

We coexpressed α_{1E} channel with the G-protein β_1-γ_2 (G-βγ) subunits in order to confirm the G-protein dependent regulation of α_{1E}. Fig. 6 shows current traces from two oocytes of the same batch expressing α_{1E} alone (A,B) and α_{1E}+G-$\beta_1\gamma_2$ (B,C), obtained in response to depolarizations ranging from −50 to +80 mV. The overexpression of G-βγ produced an evident slowdown of the decay phase of the current (comparing Fig. 6A with Fig. 6C). The application of a 100 ms prepulse to 100 mV slightly diminished the current evoked during the test pulse to 10 mV (−2.4 ± 1.4%, $n = 5$) in the α_{1E} expressing oocytes (Fig. 6B); in contrast, in oocytes expressing G-βγ (same batches of oocytes) the current during the test pulse was augmented by the positive prepulse by 20.8 ± 2.5% ($n = 9$), and the decay phase was accelerated.

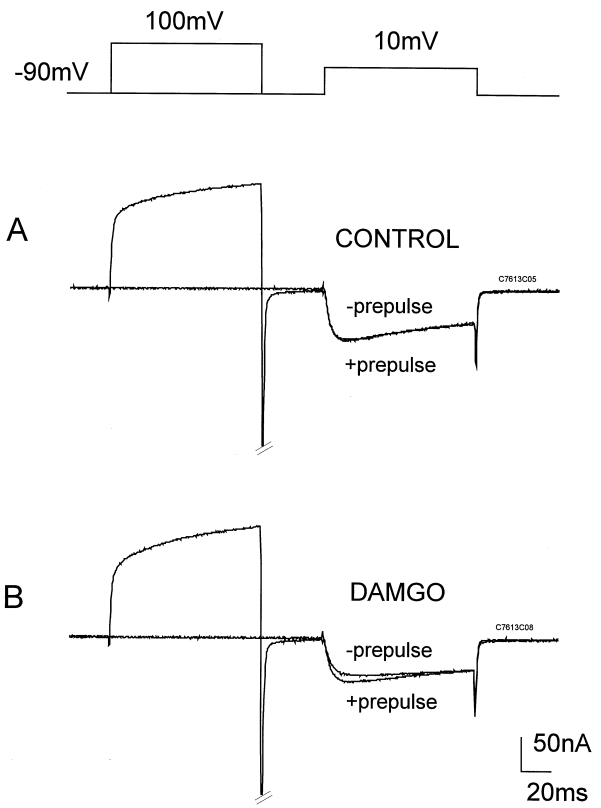

Fig. 4. The block by DAMGO is partially relieved by strong depolarizations. A: Superimposed current traces evoked by depolarization to 10 mV either preceded or not by a 100 ms prepulse to +100 mV in an oocyte expressing α_{1E}+μ opioid receptors. B: Current traces obtained with the same pulse protocol as in A, and from the same oocyte, in the presence of 1 μM DAMGO in the external solution. The block of DAMGO was partially relieved by the positive prepulse. The pulse protocol is shown in the upper panel.

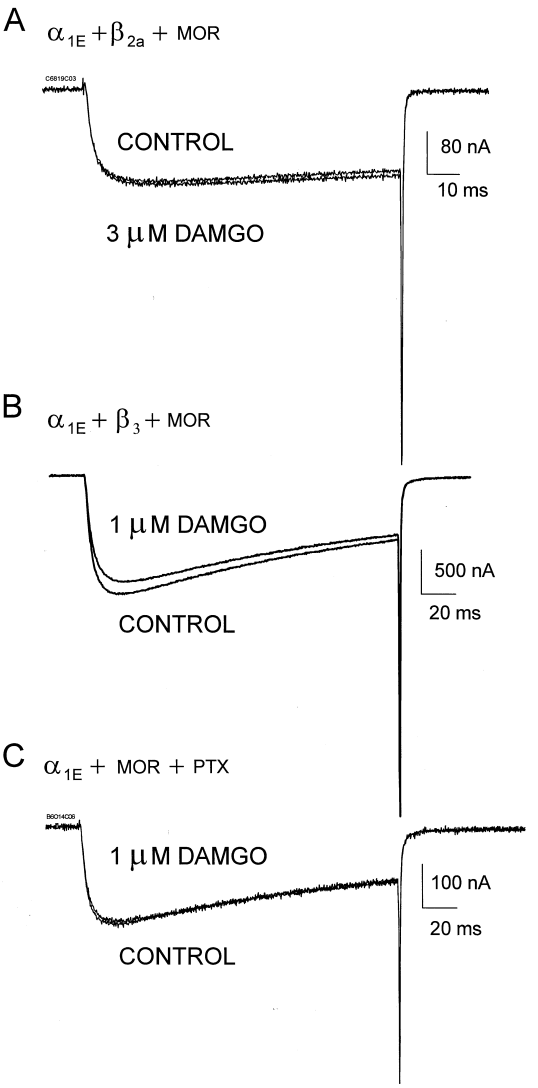

Fig. 5. Ca^{2+} channel β_{2a} subunit and PTX prevent DAMGO effect on α_{1E}. A: Superimposed Ba^{2+} current traces from an oocyte expressing α_{1E}+β_{2a}+μ opioid receptors. Application of 3 μM DAMGO failed to inhibit the current (block 0.5 ± 1.0%, $n = 6$). B: Superimposed current traces from an oocyte expressing of α_{1E}+β_3 calcium channel subunits. The opioid effect (1 μM DAMGO) is reduced (block 9.0 ± 1.2%, $n = 5$). C: Current traces from an oocyte injected with pertussis toxin catalytic subunit (25 nl, 0.1 ng/nl). The effect of 1 μM DAMGO was greatly reduced (block 1.3 ± 1.4%, $n = 4$). All the test pulses were to 10 mV from −90 mV holding potential.

4. Discussion

We have studied the modulation of the neuronal α_{1E} channels by opioids. The results presented in this work show that

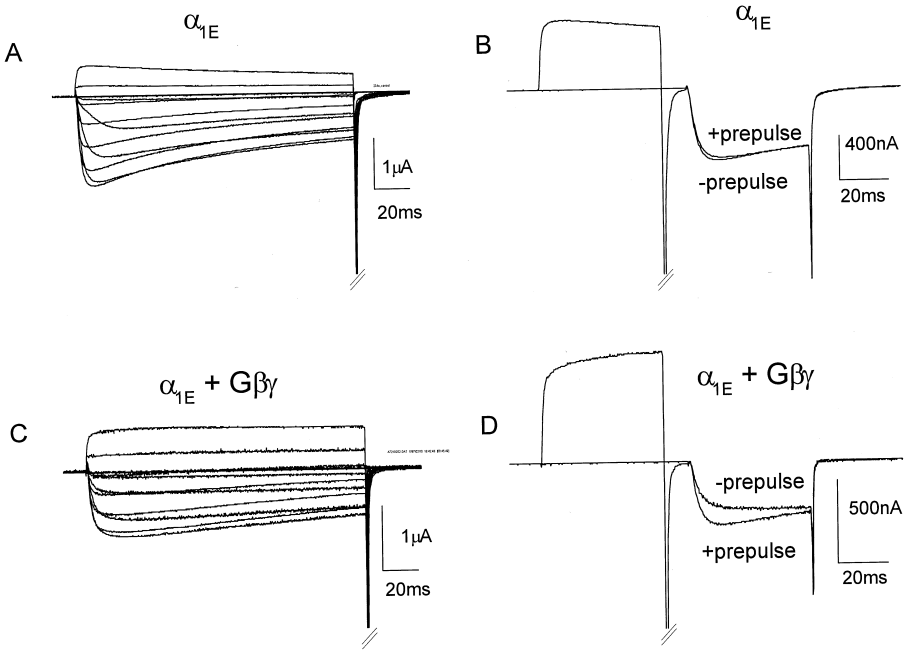

Fig. 6. Coexpression of G-protein βγ subunits mimics the effect of opioid In A, α_{1E} current traces evoked by depolarizations from −50 to +80 mV in 10 mV increments are shown. In B is shown the effect of a prepulse to 100 mV on the current elicited by a test pulse to 10 mV. Coexpression of the G-protein βγ subunits produces mainly a slowdown of the inactivation kinetics as shown in the superimposed current traces shown in C: the pulse protocol is the same as in A. The application of a prepulse to 100 mV increased the current during the test pulse and the inactivation kinetics were accelerated. The expression of G-protein βγ subunits has effects similar to opioid stimulation of α_{1E}. Experiment were done the same day in the same batch of oocytes.

the α_{1E} channel and the μ opioid receptor, when coexpressed in the *Xenopus* oocyte system, are functionally coupled. The current flowing through the recombinant α_{1E} channels was reduced by stimulation of the coexpressed μ opioid receptors with the specific agonist DAMGO via a mechanism involving a PTX sensitive G-protein, probably G_i or G_o, [39]. As demonstrated by Ikeda [40] and Herlitze et al. [41] for the N-type Ca^{2+} channels, α_{1E} Ca^{2+} channels are also modulated by the G-βγ subunit. In fact, coexpression of the Gβγ with α_{1E} Ca^{2+} channels mimicked the modulation occurring via μ opioid receptor activation.

Our results demonstrate that coexpression of the β_{2a} Ca^{2+}channel subunit fully prevents the modulation of α_{1E} channels by the endogenous G-protein, and that the β_3 subunit strongly attenuates this modulation. These findings could explain the results of Toth and collaborators [26] and Bourinet and collaborators [5], showing that α_{1E} Ca^{2+} channels coexpressed with the Ca^{2+} channel $\alpha_2\delta$ and β_{1b} or β_4 subunits, were not significantly modulated by PTX sensitive G-proteins. The fact that in those studies the E-type channels were not modulated by G-protein can be explained by a strong inhibitory effect of the β subunit tested. Because the α_{1E} channel can be expressed without auxiliary subunits, its susceptibility to G proteins modulation in vivo could depend on the amount and type of β subunits available, being the overall sensitivity to modulation by opioid, dependent on the relative concentration of G-protein and Ca^{2+} channel β subunits.

A well documented effect of G-protein inhibition of N-type Ca^{2+} channels is the slowdown of the activation rate of the macroscopic current [34,42,43]. Single channel studies of this phenomenon have shown that this kinetic effect is related to an increase in the delay between the depolarizing voltage step and the first channel opening (first latency) [44,45]. We did not detect significant changes in the activation time constant of current installation during depolarizations. Instead, our data show that, at least at the macroscopic level, the main kinetic effect of G-protein modulation on α_{1E} occurs during the decay phase of the currents. However, it is possible that this change reflects a small modification of the rates in some earlier transitions during the activation pathway, slightly affecting the current activation time constants. If this were the case, the first latency of single α_{1E} channel events would be delayed, consistently with the observation reported for the N-type Ca^{2+} channels [44,45]. Considering a simple sequential kinetic model for α_{1E} channel (e.g. $C \rightleftharpoons C \rightleftharpoons O \rightleftharpoons I$, in which C, O, I are the conformational states closed, open and inactivated), because of the relatively fast development of the current, small changes in the rates of the activation pathway ($C \rightleftharpoons C \rightleftharpoons O$) as well as changes in the rates of inactivation ($I \rightleftharpoons O$) can produce relevant changes in the macroscopic time course of the inactivation. Therefore, the question remains open whether the observed effect on the decay phase of the experimental currents is due to a decrease of the inactivation rates or to a slowdown of the transition rates in the activation pathway. The fact that the current facilitated by the positive prepulse shows a faster development when the G-βγ subunit is overexpressed (i.e. maximal G-protein block) (Fig. 6D), may be suggestive of a change in the first latency. The first latency may become shorter after the removal of the block by the prepulse, affecting consequently the decay phase of the current.

As shown by immunocytochemical staining by Yokoyama et al. [46], α_{1E} channels are widely distributed in the brain both in the cell body and in dendrites. Interestingly, some

of the regions with the most prominent staining for the α_{1E} subunit such as caudate putamen, amygdala, thalamus, hypothalamus, and brainstem are shown to abundantly express mRNA encoding for μ opioid receptors. This evidence implies that the coupling between α_{1E} channels and μ opioid receptors could have a real physiological meaning also, where endogenous opioid peptides modulate the activity of calcium channels.

In summary we found that the neuronal α_{1E} channels are functionally coupled to μ opioid receptors when coexpressed in *Xenopus* oocytes, and prone to PTX sensitive G-protein modulation. This modulation is practically fully prevented by the coexpression of the Ca^{2+} channel regulatory β_{2a} subunit and reduced by $\sim$50% by the β_3 subunit.

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