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Determinants of the G protein-dependent opioid modulation of neuronal calcium channels

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ABSTRACT **The modulation of a family of cloned neuronal calcium channels by stimulation of a coexpressed μ opioid receptor was studied by transient expression in *Xenopus* oocytes. Activation of the morphine receptor with the synthetic enkephalin [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO) resulted in a rapid inhibition of α_{1A} (by ≈20%) and α_{1B} (by ≈55%) currents while α_{1C} and α_{1E} currents were not significantly affected. The opioid-induced effects on α_{1A} and α_{1B} currents were blocked by pertussis toxin and the GTP analogue guanosine 5′-[β-thio]diphosphate. Similar to modulation of native calcium currents, DAMGO induced a slowing of the activation kinetics and exhibited a voltage-dependent inhibition that was partially relieved by application of strong depolarizing pulses. α_{1A} currents were still inhibited in the absence of coexpressed Ca channel α_2 and β subunits, suggesting that the response is mediated by the α_1 subunit. Furthermore, the sensitivity of α_{1A} currents to DAMGO-induced inhibition was increased ≈3-fold in the absence of a β subunit. Overall, the results show that the α_{1A} (P/Q type) and the α_{1B} (N type) calcium channels are selectively modulated by a GTP-binding protein (G protein). The results raise the possibility of competitive interactions between β subunit and G protein binding to the α_1 subunit, shifting gating in opposite directions. At presynaptic terminals, the G protein-dependent inhibition may result in decreased synaptic transmission and play a key role in the analgesic effect of opioids and morphine.**

Voltage-dependent calcium (Ca) channels serve as crucial mediators between membrane excitability and the regulation of Ca-dependent functions such as neurotransmitter release, gene expression, and pacemaker activity (1, 2). Stimulation of many types of neurotransmitter receptors activates heterotrimeric GTP-binding proteins (G proteins), which subsequently influence Ca channel activity by multiple pathways (3, 4). The inhibition of Ca channels mediated by G proteins has been demonstrated for a variety of seven-helix receptors, including those for noradrenergic, muscarinic, GABAergic (GABA = γ-aminobutyric acid) serotoninergic, dopaminergic, and opiate agonists (3, 4). The opioids have been implicated in relieving pain via a mechanism involving a decrease in presynaptic neurotransmitter release (for review, see ref. 5). However, attempts to correlate the nature of Ca channel subtypes affected by stimulation of various opioid receptors (μ, δ, and κ) have been hampered by the fact that multiple types of Ca channels are coexpressed in opioid-sensitive neurons and that some Ca channels are located along distal dendrites and at synaptic terminals (6, 7).

Molecular cloning and exogenous expression techniques have allowed the determination of the electrophysiological and pharmacological characteristics of individual subtypes of Ca channels. Neuronal Ca channels are composed of at least three subunits including a pore-forming α_1 subunit that determines the major biophysical and pharmacological properties of the channel complex and ancillary α_2 and β subunits that modulate the physiological properties of the α_1 subunits (reviewed in ref. 8). In the mammalian central nervous system, five α_1 subunits that exhibit distinct cellular and subcellular distributions have been described (α_{1A}–α_{1E}). Functional studies indicate that α_{1C} and α_{1D} subunits encode 1,4-dihydropyridine-sensitive L-type Ca channels (9, 10), the α_{1B} subunit encodes an ω-conotoxin GVIA-sensitive N-type channel (11, 12), and the α_{1A} subunit encodes a Ca channel with some properties similar to both P- and Q-type currents (13, 14). The exact native Ca current encoded by the α_{1E} subunit remains to be precisely described (15).

In the present study, we used transient expression in *Xenopus* oocytes to examine the molecular basis for the G protein-dependent modulation of cloned neuronal Ca channels by stimulation of a coexpressed μ-opioid receptor. The results demonstrate the selective μ-opioid-dependent inhibition of α_{1A} (P/Q type) and α_{1B} (N type) Ca channels and are consistent with the properties of modulation seen for native channels. The results also demonstrate that the Ca channel β subunit plays a crucial role in regulating the inhibitory effects of G protein activation.

EXPERIMENTAL PROCEDURES

Transient Expression in *Xenopus* Oocytes. Stage V and VI *Xenopus laevis* oocytes were prepared as described (13, 16), and nuclear injection was performed with 10 nl of a mixture of rat brain cDNAs encoding Ca channel α_1, α_2, and β subunits and the μ opioid receptor (≈2 ng of each in the vertebrate expression vectors pMT2 or pRcCMV). Except for α_{1B}, the expression plasmids have been described [α_{1A}, β_{1b}, β_{2a}, β_3, and β_4 (13); α_{1C} and α_2 (9); α_{1E} (14); μ-opioid receptor MOR1 (a kind gift of Lei Yu, ref. 17)]. The α_{1B} cDNA (rbB-II) differs from the rbB-I isoform (12) at several amino positions and results in Ba currents that activate and inactivate with faster kinetics (18). In preliminary experiments, the magnitude of the μ-opioid-dependent inhibition was found to be more consistent between batches of oocytes if they were injected with synthetic $G\alpha_o$ RNA (results not shown). Consequently, in all of the reported experiments, 12–24 hr after the nuclear cDNA injections, oocytes were injected cytoplasmically with ≈15 nl of *in vitro*-transcribed RNA (0.1 μg/μl) from a rat G protein $G\alpha_o$ subunit cDNA (a kind gift of Mel Simon and Norman Davidson, California Institute of Technology).

Electrophysiological Recording. After incubation for 2–6 days, macroscopic currents were recorded as described (16) in a solution containing 40 mM $Ba(OH)_2$, 25 mM tetraethylammonium hydroxide, 25 mM NaOH, 2 mM CsOH, and 5 mM Hepes (titrated to pH 7.3 with methanesulfonic acid). The endogenous oocyte Ca-activated Cl current was completely

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Abbreviations: PTX, pertussis toxin; G protein, GTP-binding protein; PKC, protein kinase C; GABA, γ-aminobutyric acid; GDP[βS], guanosine 5′-[β-thio]diphosphate; GTP[γS], guanosine 5′-[γ-thio]triphosphate; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin.

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suppressed by injection of a solution containing bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (2–5 mM estimated final concentration; see ref. 19). Activation of the μ-opioid receptor was achieved by rapidly switching from the control superfusion solution to a solution containing the selective μ-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]enkephalin (DAMGO; Research Biochemicals, Natick, MA) at 1 μM. An effective exchange of the bath was achieved within 1–2 sec as indicated by monitoring Ca channel block with 100 μM Cd. For intraoocyte injections, cells were impaled with a fourth pipette and injected with 15–30 nl of the compound solution (dilution factor from the pipette ≈ 1:100; see ref. 16). The effects of DAMGO 10–15 min after injection of guanosine 5′-[β-thio]diphosphate (GDP[βS]) or guanosine 5′-[γ-thio]triphosphate (GTP[γS]) were compared to the initial effects. The effects of pertussis toxin (PTX; 2 μg/ml, overnight incubation) were assayed by comparing paired control and treated batches of oocytes. Current–voltage curves were fitted using a modified Boltzmann equation (16). Exponential functions were used to quantify the changes in current activation kinetics as well as the onset of the DAMGO-induced inhibition of Ca channel activity. Unless otherwise stated, results are presented as the mean ± SEM.

RESULTS

μ-Opioid Receptor Stimulation Selectively Inhibits α_{1A} (P/Q Type) and α_{1B} (N Type) Currents. In agreement with previous studies, the nuclear injection of expression plasmids containing rat brain α_{1A}, α_{1B}, α_{1C}, and α_{1E} Ca channel cDNAs (coexpressed with α_2 and β_4 subunits) produced robust Ba currents with distinct pharmacological and physiological properties (9, 12, 13, 15, 16, 18, 19). Perfusion with the μ-opioid receptor agonist DAMGO (1 μM) resulted in significant reduction ($P < 0.01$) of the α_{1A} and α_{1B} Ba currents by ≈20% and ≈55%, respectively (Fig. 1*A*; α_{1A} = 19.8% ± 8%, n = 38; α_{1B} = 55.3% ± 9%, n = 38). The effect of DAMGO was reversible upon washout and was prevented by addition of the opioid receptor antagonist naloxone (10 μM; Fig. 1*A*). Increasing the concentration of DAMGO (up to 10 μM) did not produce any further inhibition (Fig. 1*A*). In contrast to the α_{1A} and α_{1B} currents, the α_{1E} and α_{1C} Ba currents were not significantly affected by DAMGO application (Fig. 1*B*; α_{1E} = 97% ± 3% of control, n = 10; α_{1C} = 101% ± 1% of control; n = 7). In the absence of the coexpressed μ-opioid receptor, DAMGO application had no direct effect on Ca channel properties (data not shown). Similarly, in the absence of DAMGO, coexpression of the μ-opioid receptor resulted in no detectable changes in current properties, indicating a lack of receptor activity without agonist application (data not shown).

Consistent with the membrane-delimited G protein modulation of native Ca channels (3), a further effect of μ-opioid receptor stimulation was the significant slowing of the α_{1B} and α_{1A} activation kinetics (Fig. 1*B*). Another representative feature of the G protein-dependent inhibition of high threshold neuronal Ca channels is fast onset kinetics of the response (20). As shown in Fig. 2*A*, the time course of inhibition of α_{1A} and α_{1B} by 1 μM DAMGO was rapid, with the onset of the modulation described by a single exponential with time constants of 3.22 ± 0.22 sec (α_{1B}, n = 27) and 4.11 ± 0.28 sec (α_{1A}, n = 29).

Further evidence for the involvement of G protein interactions was indicated by experiments whereby preincubation of oocytes with PTX prevented the DAMGO-induced inhibition of both α_{1B} (by 82% ± 5%, n = 6; Fig. 2*B*) and α_{1A} (by 75% ± 7%, n = 7; Fig. 2*B*). Similarly, injection of GDP[βS] (estimated final concentration ≈ 1 mM) also significantly reduced the DAMGO-induced effects (α_{1B} by 87% ± 3%, n = 6; α_{1A} by 78% ± 11%, n = 9; Fig. 2*B*). The effects of stimulation of the μ-opioid receptor were qualitatively mimicked by intraoocyte injection of the nonhydrolyzable GTP analog, GTP[γS] (≈0.3 mM intraoocyte estimated concentration) in oocytes not coexpressing the receptor (α_{1B}, n = 5; α_{1A}, n = 3; data not shown).

μ-Opioid-Induced Current Inhibition Exhibits Voltage Dependence. The rapid inhibition of Ca currents mediated by G

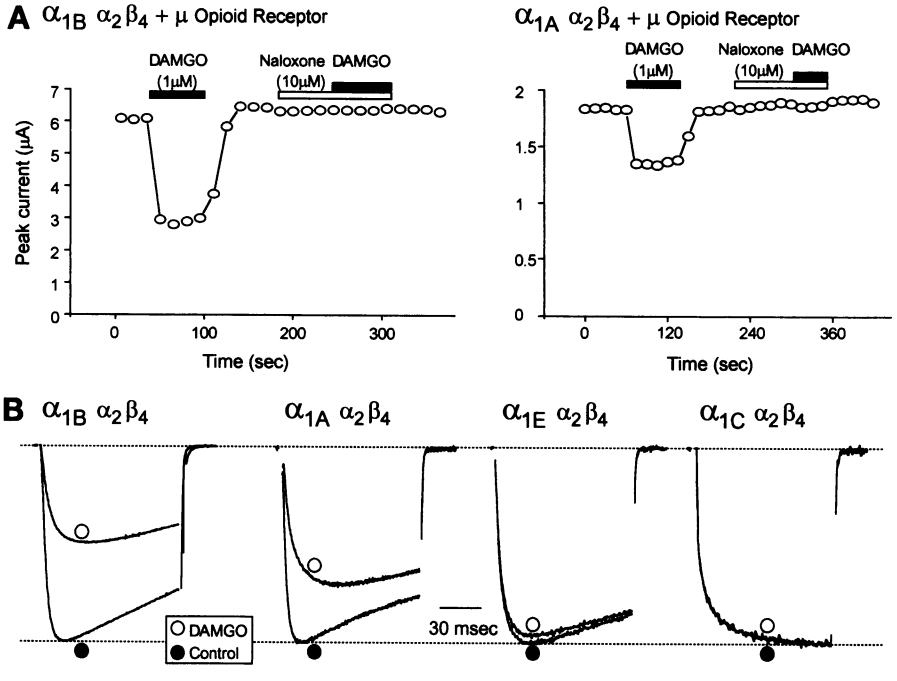

FIG. 1. Activation of the μ-opioid receptor inhibits the α_{1B} and α_{1A} currents in *Xenopus* oocytes. (*A*) Plot of DAMGO-induced inhibition of α_{1B} (*Left*) and α_{1A} (*Right*) peak currents as a function of time. A 100-msec test pulse was applied every 10 or 15 sec from a holding potential of −100 mV to a test potential of +30 mV (α_{1B}) or +10 mV (α_{1A}). Application of DAMGO (1 μM) induced a rapidly developing inhibition of both channels that was suppressed by the opioid receptor antagonist naloxone (10 μM). (*B*) Comparison of the effect of 1 μM DAMGO on the α_{1A}, α_{1B}, α_{1C}, and α_{1E} Ca channels (each in combination with α_2 and β_4). Control peak currents were normalized (dotted line).

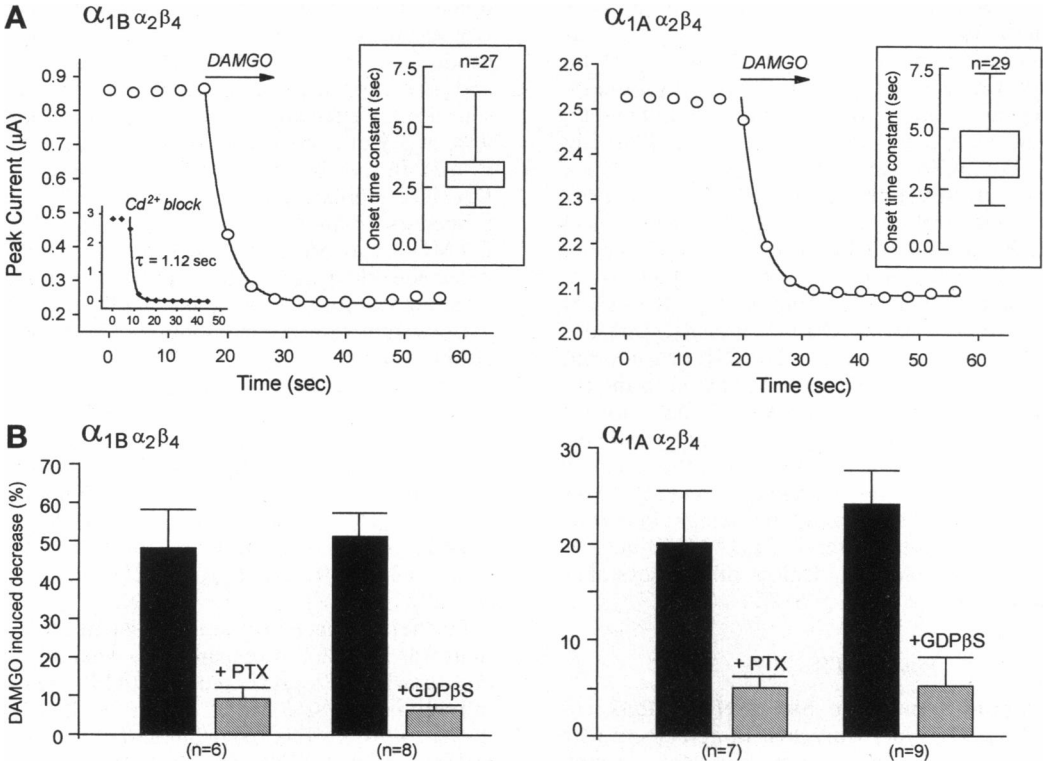

FIG. 2. The μ-opioid receptor affects the α_{1B} and α_{1A} Ca channels through a G protein-dependent mechanism. (*A*) Peak currents were elicited by 100-msec depolarizations every 4 sec from a holding potential of −100 mV. The onset of the DAMGO (1 μM)-induced inhibition was fitted with a single-exponential curve. Examples of these fits are presented with the distribution of the time constants plotted in the *Inset*. The lower and upper limits of the boxes represent the 25th and the 75th percentiles. The central horizontal bar represents the median, and the vertical lines indicate the minimum and maximum values. The 2- to 4-sec time constant of the DAMGO effect was slightly slower than the speed with which bath solutions are exchanged as judged by the fit of the Cd^{2+} blockade (≈1 sec; *Inset*). (*B*) Histograms show the effect of overnight treatment of the oocytes with PTX (2 μg/ml) or injection of GDP[βS] (final estimated concentration ≈ 1 mM).

proteins has been shown to result from a positive shift in the voltage dependence of channel gating (21). A consequence of the shift in voltage dependence is that inhibited channels require a stronger depolarization to open. Fig. 3 shows that the DAMGO-induced inhibition of the α_{1B} and α_{1A} currents exhibited this type of voltage dependence. Typical records of α_{1B} and α_{1A} current–voltage relations show that DAMGO reduced the inward currents elicited by moderate depolarizations more effectively than the outward currents evoked by larger voltage pulses (Fig. 3*A*). A summary of the current blockage at different voltages further exemplifies the voltage-dependent aspect of the inhibition (Fig. 3*A Insets*). Fit of the current–voltage relation with a modified Boltzmann function (see ref. 16) also revealed a slight positive shift of the voltage for half-activation $V_{0.5}$ (by 2 to 5 mV) and an apparent reduction of the number of gating charges reflected by a change of the slope factor K (by 15–35%). Application of positive conditioning prepulses (to +100 mV) prior to the test potential partially relieved both the DAMGO-induced α_{1B} and α_{1A} current inhibitions and slowing of the activation (α_{1B}, n = 10; α_{1A}, n = 14; Fig. 3*B*). The relief of the inhibition initially developed at prepulse potentials near 0–20 mV and was maximal at potentials equal to or greater than +100 mV (data not shown). A similar prepulse effect was also observed in oocytes injected with GTP[γS] without a coexpressed μ-opioid receptor and is consistent with the notion that the μ-opioid receptor inhibition involves a direct G protein interaction (α_{1B}, n = 2; α_{1A}, n = 3; data not shown).

μ-Opioid Receptor-Induced Inhibition Is Antagonized by β-Subunit Coexpression. A number of studies have shown that Ca channel β subunit coexpression modulates a variety of α_1 subunit functional properties including the magnitudes of whole-cell currents and several voltage-dependent and kinetic characteristics (8). Of particular note, most of the observed β-subunit effects were opposite to those induced by stimulation of the μ-opioid receptor. For example, while β subunits increase current amplitudes, speed activation kinetics, and shift the activation-gating toward negative potentials (e.g., refs. 12, 13, and 22), G protein-mediated responses result in an inhibition of whole-cell currents, a slowing of activation kinetics, and a positive shift of the voltage dependence of the activation (refs. 20, 21, 23, and 24; this study, Figs. 1–3). To determine whether β subunits differentially affected the μ-opioid-induced effects, we examined the effects of DAMGO on α_{1B} and α_{1A} coexpressed with four different β subunits. The degree of whole-cell current inhibition of the α_{1A} and α_{1B} currents by DAMGO was not significantly different among the four β subunits (Fig. 4*A*). In contrast, the DAMGO-induced slowing of activation kinetics was significantly more pronounced with β_{2a} compared to the other β subunits [e.g., Fig. 4*B*; mean time to peak with DAMGO for α_{1B} = 29.8 ± 14, 90.7 ± 8, 15.1 ± 6, and 21.7 ± 12 msec with β_{1b}, β_{2a}, β_3, and β_4, respectively; n = 9–30]. For each β subunit, the activation kinetics could be fit by a sum of two exponentials with a fast and slow time constant. With the β_{2a} subunit, the contribution of the slower phase to the overall activation was increased and the time constant was twice that of the other three β subunits (data not shown). Overall, the result is a slower time to peak of the DAMGO-inhibited current with the β_{2a} (Fig. 4*B*).

One possibility to account for the differential effect of the β_{2a} subunit was that G proteins interact directly with the β subunit in the channel complex. However, Fig. 5*A* shows that in the absence of a β subunit, μ-opioid receptor stimulation actually induced an ≈3-fold increase in the inhibition of α_{1A} currents (α_{1A}, n = 12; α_{1A} + α_2 + β_4, n = 16). There was no effect of omitting the α_2 subunit on μ-opioid receptor-induced

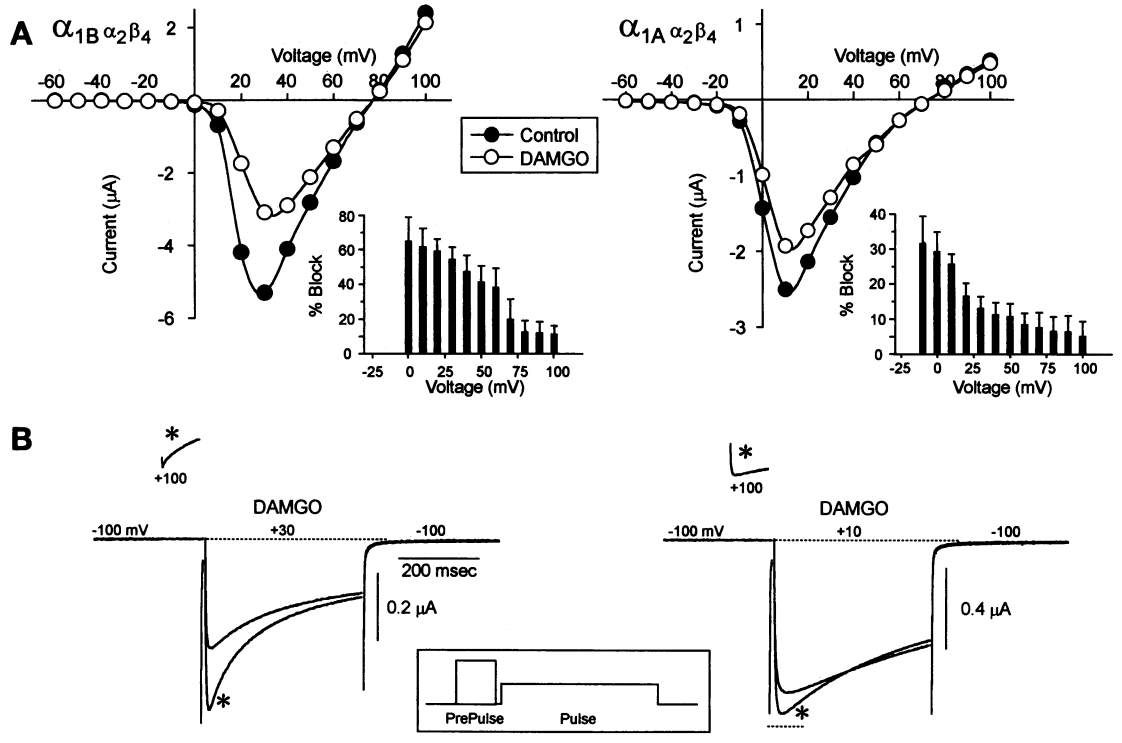

FIG. 3. Voltage dependence of α_{1A} (*Right*) and α_{1B} (*Left*) current inhibition by DAMGO. (*A*) Typical current–voltage relationships for control and after DAMGO application. The histograms (*Insets*) show the fraction of current blocked at the various potentials (α_{1B}, $n = 11$; α_{1A}, $n = 7$). Note the more pronounced block at smaller depolarizations. (*B*) Facilitation of DAMGO-inhibited α_{1A} and α_{1B} currents. The superimposed traces are pairs of currents (400-msec depolarizations to +30 or +10 mV for α_{1B} and α_{1A}, respectively) either preceded (*) or not by a strong 100-msec predepolarization to +100 mV (stimulation protocol in the *Inset*). The dotted line below the peak inward currents represents the control current amplitude before DAMGO application.

effects, indicating that in *Xenopus* oocytes this subunit does not contribute to the G protein-dependent modulation of Ca channels (data not shown). These results indicate that the inhibitory effects of G protein activation occur via the Ca

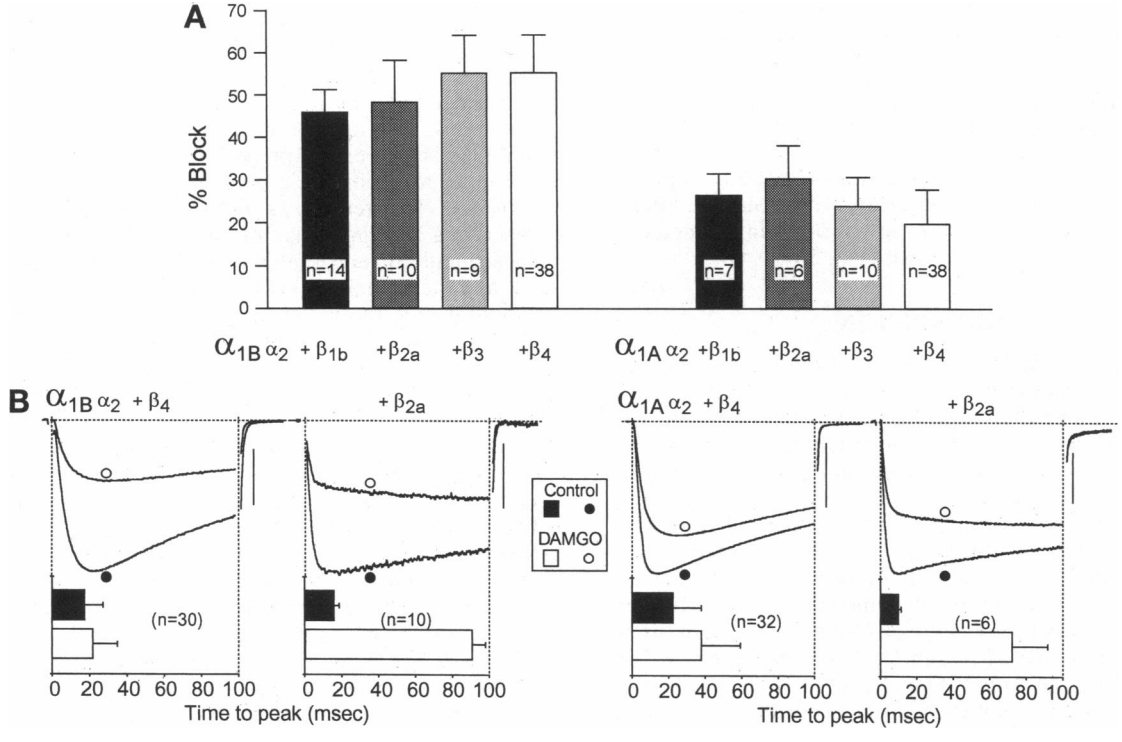

FIG. 4. Effect of β subunit coexpression on α_{1A} and α_{1B} channels modulation by DAMGO. (*A*) Summary of blocking effects of DAMGO on α_{1B} + α_2 and α_{1A} + α_2 channels expressed with β_{1b}, β_{2a}, β_3, or β_4. (*B*) In comparison with the other β subunits, α_{1A} and α_{1B} activation kinetics are differentially affected by β_{2a}. The plot of control and DAMGO-inhibited currents shows an increased time to peak with β_{2a} compared to β_4.

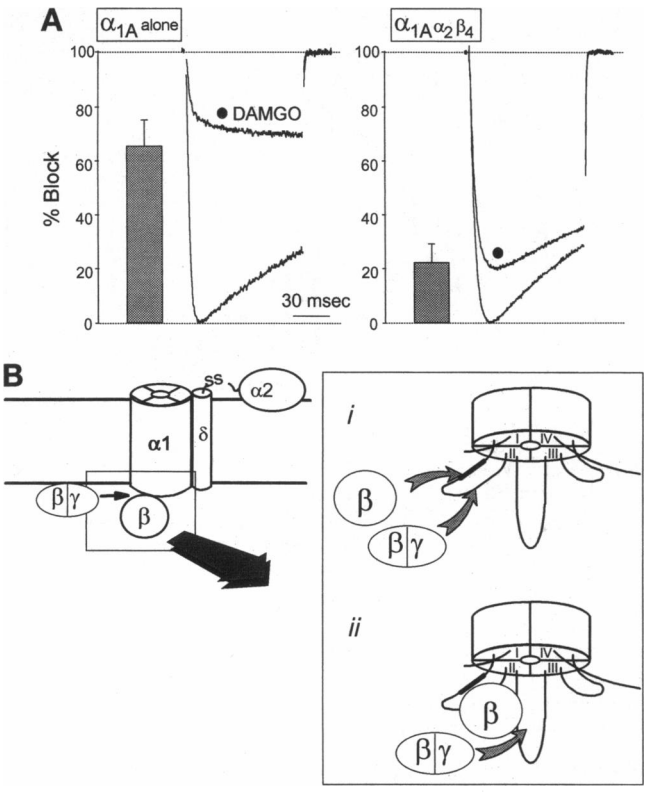

FIG. 5. DAMGO-induced inhibition of α_{1A} Ca channels is antagonized by the β subunit. (*A*) Comparison of the DAMGO block on α_{1A} channels expressed alone or with α_2 and β_4. Note the much more pronounced current inhibition and slowing of the activation with α_{1A} alone. (*B*) Proposed model of the putative direct G protein action on Ca channel underlying the observed DAMGO effects. The cartoon on the left represents the channel complex containing α_1, α_2, and β subunits and an activated G protein in close proximity. Two possible mechanisms are proposed to account for the observed β subunit effects: (*i*) a direct competition between the β subunit and the activated G protein in the domain I-II β binding site, and (*ii*) the activated G protein sterically interferes with normal β subunit interactions elsewhere on the α_1 subunit. For simplicity, the $\beta\gamma$ subunits are shown, although it remains to be determined whether activated Gα subunits or $\beta\gamma$ subunits are involved.

channel α_1 subunit itself. Further, they suggest that G protein binding may either sterically interfere with β subunit binding in the domain I-II linker (25) or affect β-subunit interactions in other regions of the α_1 subunit (Fig. 5*B*).

DISCUSSION

Many central and peripheral neurons exhibit neurotransmitter-induced G protein-dependent inhibition of high-threshold Ca currents. Using the exogenous expression of cloned neuronal Ca channels, our results demonstrate that the stimulation of the μ-opioid receptor selectively inhibits α_{1A} (P/A type) and α_{1B} (N type) currents. The inhibition of α_{1B} currents by DAMGO is consistent with the G protein-dependent block of N-type channels by opioids (21, 26, 27) and other neurotransmitters (e.g., refs. 3, 20, and 28). Similarly, the effects on α_{1A} are consistent with recent studies demonstrating the G protein-dependent inhibition of P- and/or Q-type currents by stimulation of μ-opioid (26, 27) and $GABA_B$ (29) receptors. Immunohistochemical studies show that both of the α_{1A} and α_{1B} channels are widely expressed postsynaptically on dendrites and also presynaptically at a subset of central synapses (6, 7). At synaptic terminals the G protein-dependent inhibition of α_{1A} and α_{1B} Ca currents would likely have dramatic effects on synaptic transmission. Furthermore, while both α_{1A} and α_{1B} are inhibited by the opioid induced G protein activation, only α_{1B} is significantly up-regulated by stimulation of protein kinase C (PKC) (30). This suggests that one mechanism to control levels of presynaptic Ca concentration and neurotransmitter release may be to differentially modulate individual subtypes of Ca channels via distinct second messenger pathways.

There are relatively few reports of the G protein-dependent modulation of neuronal L-type channels, and the insensitivity of the α_{1C} Ca channel to μ-opioid receptor stimulation may be reflective of native L-type channels (for example, see refs. 3, 26, 27, and 31). It is also possible that *Xenopus* oocytes do not express additional factors required for the specific G protein-dependent regulation of α_{1C} channels, and we cannot rule out that these channels may be regulated in neurons. A further possibility is that reports concerning the G protein-dependent modulation of some neuronal L-type Ca channels (e.g., ref. 32) may reflect regulation of the α_{1D} L-type channel.

Mechanism of μ-Opioid Receptor-Induced Modulation. The μ-opioid receptor-induced inhibition of α_{1A} and α_{1B} current closely resembles the G protein-dependent inhibition of N- and P-type currents in native cells (e.g., refs. 20, 21, and 29). While additional experiments are required to confirm that no cytoplasmic messengers are involved, our results are consistent with a direct G protein interaction (membrane-delimited). For both α_{1A} and α_{1B}, current inhibition develops rapidly, is blocked by both PTX and GDP[βS] and can be mimicked by the direct intraoocyte injection of GTP[γS]. There is no evidence that activation of endogenous oocyte second messengers can mimick the observed G protein-dependent inhibition. For example, microinjection of cAMP or the catalytic subunit of protein kinase A do not result in the inhibition of any of the cloned neuronal Ca channels (ref. 16; E.B. and T.P.S., unpublished results). Also, while PKC activation has been implicated in the inhibition of some Ca channels (33), it is unlikely that PKC mediates the opioid-induced inhibition since the oocytes were injected with BAPTA to chelate intracellular Ca, and the majority of endogenous oocyte PKC activity is Ca-dependent and inhibited by this treatment (30).

The opioid-induced inhibition of α_{1A} and α_{1B} appears to influence the gating of the channel as reflected by slower activation kinetics, a more pronounced block at moderate potentials, and a shift in the current–voltage relations. In addition, short positive prepulses appear to reverse the μ-opioid-induced inhibition, a phenomenon also observed in neurons and referred to as facilitation (34). Several models describing the molecular mechanism of facilitation and the subsequent current reinhibition have been proposed (20, 23, 24) and the exogenous expression of the α_{1A} and α_{1B} channels should permit the direct testing of these possibilities.

The exact nature of the G protein(s) involved in the opioid-induced response remains to be determined, although the sensitivity to PTX suggests an involvement of Gα_i and/or Gα_o. It also remains to be determined whether the observed effects are due to activated Gα subunits or to $\beta\gamma$ subunits.

β Subunit Binding and G Protein Activation. A heterogeneous family of Ca channel β subunit genes has been identified, and it has generally been assumed that the major functional role of β subunits is to differentially regulate the electrophysiological properties of the pore-forming α_1 subunits. However, the results presented here, together with two recent studies indicating crucial roles for β subunits in the PKC- and voltage-dependent regulation of neuronal Ca channels (16, 30), suggest that β subunits play a predominant function in controlling neurotransmitter- and activity-dependent modulation of Ca channels. Although the opioid-induced inhibition was of similar magnitude for the four β subunits tested, we observed a significantly increased slowing of the activation kinetics with β_{2a}, and this suggests that distinct

β subunits may make unique contributions to the G protein-dependent modulation of Ca channel activity.

That the opioid-induced effect was present in the absence of a coexpressed α_2 and β subunits suggests that the direct target of the activated G protein is the α_1 subunit itself. Furthermore, the observed ≈3-fold increase in inhibition in the absence of a β subunit also suggests that the activated G protein plays a negative regulatory role with regard to the normal β subunit-dependent modulation of Ca channel properties. This result is in agreement with those of Dolphin and coworkers (35) demonstrating that the magnitude of $GABA_B$-dependent inhibition of N-type currents is enhanced by decreasing β subunit expression using antisense oligonucleotides. The functional consequences of G protein activation are opposite to those of β subunit binding, and we propose a model whereby the activated G protein may either interfere directly with β subunit binding to the α_1 subunit in the domain I-II linker (25) or sterically interrupt β subunit interactions elsewhere on the α_1 subunit (Fig. 5*B*). The binding of the β subunit to the α_1 subunit has been demonstrated to be essentially irreversible after assembly of the channel complex (36), so it seems unlikely that G protein binding results in removal of the β subunit from the channel complex. A more likely scenario is that the activated G protein has access to sites on the α_1 subunit that normally interact with some portion of the β subunit and that these sites directly influence channel gating (Fig. 5*B*). The G protein and β subunit would interact competitively with some of the same sites of the α_1 subunit and would produce opposite effects; the β subunit shifts gating in the hyperpolarizing direction and the G protein shifts gating in the depolarizing direction.

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