Abolition of G protein inhibition of α_{1A} and α_{1B} calcium channels by co-expression of the β_3 subunit

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Abstract **Three different classes of α_1 Ca^{2+} channel (α_{1A}, α_{1B}, α_{1C}) were expressed in *Xenopus* oocytes to determine whether G protein-mediated inhibition is an inherent property of the α_1 subunit itself, and if so, whether co-expression of auxiliary subunits modulates the inhibition seen. From our data it is apparent that either α_{1A} or α_{1B} Ca^{2+} channels expressed alone are sufficient for voltage-dependent G protein inhibition. α_{1C} Ca^{2+} channels expressed alone do not exhibit the G protein inhibition seen in α_{1A} and α_{1B} channels. Additionally, co-expression of the β_3 subunit abolishes the ability of G proteins to inhibit currents through α_{1A} and α_{1B} Ca^{2+} channels. Differential sensitivity of α_1 as well as modulation of properties by β_3 provide a potential mechanism for the regulation of G protein-mediated inhibition in neurons.**

Key words: Calcium channel; G protein; α_{1A} subunit; α_{1B} subunit; α_{1C} subunit; β_3 subunit

1. Introduction

Recently, a variety of Ca^{2+} channel α_1 subunits, as well as several auxiliary subunits have been cloned [1–12]. Expression of the α_1 subunit is sufficient for the formation of functional Ca^{2+} channels [8]. Current through α_1 channels can be modulated by co-expression of the auxiliary subunits. The α_2 subunit is thought to reside mostly extracellularly, and has been shown to cause a modest increase in current amplitude when compared with current through α_1 alone [7,11]. Co-expression of the β subunit with a variety of α_1 clones results in currents with drastically altered characteristics, including a shift in both activation of current and peak current amplitudes to more hyperpolarized levels, an increase in the rate of current activation/inactivation, and an increase in current amplitude [3,7,11,12].

Modulation of calcium channels by G proteins has been demonstrated in a variety of cell preparations [13,14]. N-type Ca^{2+} channel currents frequently undergo G protein-mediated inhibition. However, in some cells N-type currents are resistant to G protein inhibition, even when L-type currents within the same cell are capable of undergoing G protein inhibition [15]. Conversely, L-type currents are frequently resistant to G protein-mediated inhibition within cells in which N-type currents undergo inhibition [16–19]. Differential response of the Ca^{2+} channels to the activated G proteins could arise from differences within a particular α_1, such as would occur by alternate splicing, or from the presence of altogether different α_1 subunits, which have superficially similar pharmacological characteristics. Alternatively, differential modulation of Ca^{2+} channels could arise from differences in the populations of auxiliary subunits associated with the α_1's present. To address the possibility that distinct α_1's may differ in their abilities to undergo G protein-mediated inhibition, we tested the ability of three different classes of α_1 subunit (α_{1A}, α_{1B}, and α_{1C}) to undergo G protein-mediated modulation when expressed alone in *Xenopus* oocytes. Furthermore, the effect of auxiliary subunits on the sensitivity of the channels to G protein-mediated inhibition, was examined by co-expression of α_2 and the $\alpha_2\beta_3$ combination with each of the three α_1's.

2. Materials and methods

2.1. In vitro transcription and oocyte preparation

Capped RNA transcripts encoding full-length rabbit brain α_{1A} (*Xba*I linearized/SP6 RNA polymerase, gift of Dr. Y. Mori; α_{1B} (*Sal*I/SP6, gift of Dr. Y. Fujita); human heart α_{1C} (*Xba*I/T7, gift of Drs. G. Mikala and A. Schwartz); rat brain α_{2b} (*Not*I/T7, gift of Dr. H. Chin) and β_3 (*Not*I/T7, gift of Dr. Edward Perez-Reyes) calcium channel subunits were synthesized using Ambion's mMESSAGE mMACHINE in vitro transcription kit (Austin, Texas). *Xenopus laevis* stage V–VI oocytes were removed and treated with collagenase (Sigma type IV) to remove the follicular layer. The oocytes were then injected with cRNA encoding α_{1A}, α_{1B}, or α_{1C} alone, or in combination with α_{2b} (1:1), or in combination with both α_{2b} and β_3 (1:1:1). The concentrations of all individual RNAs before injection was 0.1 $\mu g/\mu l$, and 20–60 nl of RNA mixed at the above ratios was injected. The oocytes were maintained in culture at 18°C for at least two days in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 2 mg/ml gentamycin.

2.2. Electrophysiological recording and experimental treatments

Two-electrode voltage clamp currents were recorded using a Dagan CA-1 amplifier. Oocytes were clamped at a holding potential of −80 mV and a series of twelve voltage steps ranging from −30 mV to +80 mV in 10 mV increments was imposed. Successive voltage steps were separated by 20 s. Currents were filtered at 1 kHz. Analysis was done off-line, using pClamp software versions 5.51 and 6.0 (Axon Instruments) and a p/2 or p/4 leak subtraction technique. Electrodes contained 3 M KCl and had resistances of 0.5–2 MΩ. Oocytes were placed in a 1 ml chamber and perfused at a rate of 0.5 ml/min. All recordings were made at room temperature using bath solutions containing (in mM): BaOH 10, NaOH 50, CsOH 2, TEA-OH 20, *N*-methyl-D-glucamine 20, HEPES 5, titrated to pH 7.5 with methane sulfonic acid. For the guanine nucleotide experiments, 4.6 nl of a 50 mM stock solution of GDPβS (Calbiochem), or water, for control experiments, was injected using a Drummond microinjector, and the Ba^{2+} current was monitored at 5 min intervals for 30 min. The final concentration of GDPβS inside the oocyte was estimated to be between 200–500 μM using an estimated oocyte volume of 1000 nl. In all experiments, 40 nl of a 50 mM stock solution of BAPTA (Sigma) was injected at least 2 h before the experiment. Final concentration of BAPTA inside the oocyte was estimated to be between 2–5 mM. For experiments using n-ethylmaleimide (NEM) (Aldrich), the NEM was dissolved in the

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Abbreviations: BAPTA, K_3-1,2-bis(aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; NEM, n-ethylmaleimide; GDPβS, guanosine-5′-*o*-(2-thiodiphosphate); DHP, 1,4-dihydropyridine.

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external solution at a concentration of 200 μM, and applied to the oocyte for 2 min.

3. Results and discussion

To determine if the Ca^{2+} channel clones used in our experiments were inhibited by G proteins under basal conditions we injected GDPβS into oocytes expressing individually the α_{1A}, α_{1B}, or α_{1C} calcium channels [9]. GDPβS binds G proteins and blocks the exchange of GDP for GTP, preventing activation of the G protein, since association with GTP is necessary for the active form of the protein. Injection of GDPβS into oocytes expressing the α_{1A} Ca^{2+} channel subunit without auxiliary subunits caused an approximate 2-fold increase in current amplitude and an increase in the rate of current inactivation (Fig. 1). The α_{1B} channel also showed an approximate two-fold increase in current amplitude after GDPβS treatment and a similar increase in the rate of current inactivation (Fig. 1). In contrast to the results obtained with α_{1A} and α_{1B}, no increase in current amplitude or change in current kinetics was seen after treatment with GDPβS in the α_{1C} calcium channel (Fig. 1).

To confirm whether the effect of GDPβS was indeed due to relief of G-protein mediated inhibition we used the sulfhydryl alkylating agent NEM. NEM at low doses (0.1–0.2 mM) has been shown to uncouple certain classes of G proteins from their receptors [20–22]. A two minute application of NEM caused a two-fold increase in current amplitude and an increase in the rate of decay of the inactivating current in both α_{1A} and α_{1B} (Fig. 1C,D,E). To determine whether these two agents were both acting on the same G protein pathway, we tested whether the effects of NEM and GDPβS were additive. After the oocytes were treated with GDPβS and the maximal current was reached, NEM was added. NEM caused no further increase in current amplitude in α_{1A} or α_{1B} (Fig.1D, Table 1), consistent with the treatments acting on the same target. We conclude from this data that there is a tonically active G protein population in *Xenopus* oocytes that inhibits the α_{1A} and α_{1B} but not the α_{1C} calcium channels.

Recently, the α_{1B} calcium channel was found to co-purify with the α_{2b} and β_3 subunits [23]. Using this subset of Ca^{2+} channel subunits to test for tonic G protein inhibition, we co-expressed either α_{1A} or α_{1B} with either α_2 or $\alpha_2\beta_3$. Co-expression of α_{2b} with α_{1A} or α_{1B} had no discernable effect on current amplitude, kinetics, or voltage dependence of the baseline currents when compared to α_{1A} or α_{1B} alone (data not shown). Co-expression of the $\alpha_2\beta_3$ combination with α_{1A} caused an approximately four-fold increase in current amplitude, a shift in the voltage dependency of activation to more hyperpolarized levels, and an apparent increase in the rate of current inactivation when compared to currents through α_{1A} expressed alone. Co-expression of $\alpha_2\beta_3$ with α_{1B} caused effects nearly identical to co-expression with α_{1A}, with the exception that the time constant for the inactivating current was not significantly different after co-expression of the β subunit (Figs. 1E and 2E).

The $\alpha_{1A}\alpha_{2b}$ combination showed a significant increase in cur-

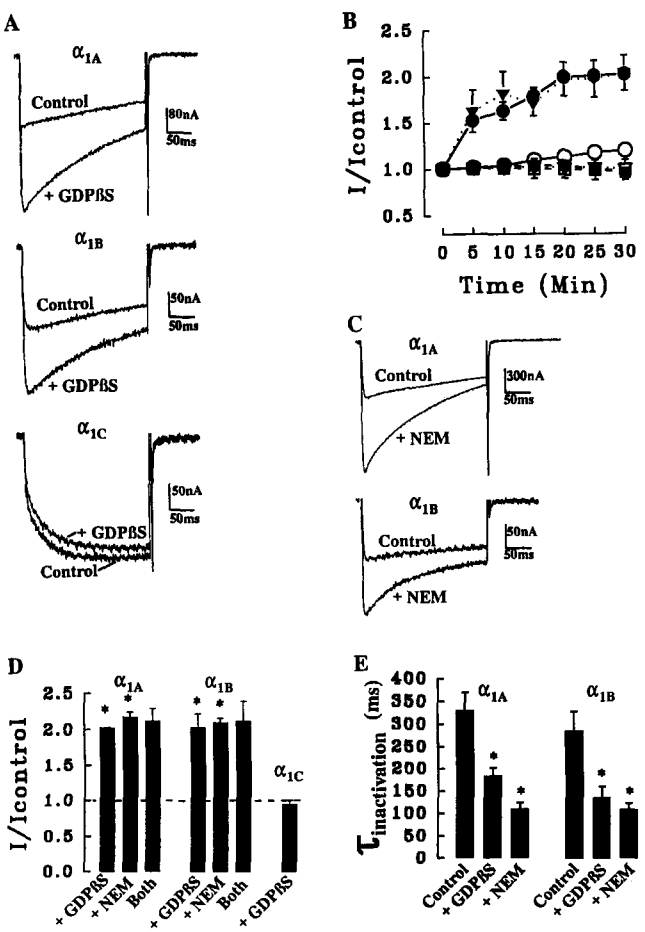

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Fig. 1. Effect of G protein inactivation on three different classes of α_1 subunit expressed individually in *Xenopus* oocytes. Control measurement of peak current was obtained during a series of twelve steps from −30 mV to +80 mV in 10 mV increments. Oocytes were then injected with 5 nl of a 50 mM stock solution of GDPβS or 5 nl of the water vehicle. Peak current amplitude was monitored at 5 min intervals for the next 30 min, normalized to control values, and is expressed as the mean of the normalized current amplitude ± S.E.M. (A) Ba^{2+} currents recorded before and after injection of GDPβS in oocytes expressing either α_{1A}, α_{1B}, or α_{1C}. (B) Peak current amplitude over the 30 min time course of the experiment after injection of GDPβS into the oocyte (α_{1A}, ●; α_{1B}, ▼; α_{1C}, ■), or after injection of the water vehicle (α_{1A}, ○; α_{1B}, ▽; α_{1C}, □). Current amplitudes are normalized to control values and represented as mean ± S.E.M. (C) Ba^{2+} currents recorded before and after treatment with 200 μM NEM. (D) The change in Ba^{2+} currents after administration of GDPβS, NEM, or NEM after GDPβS treatment. In experiments with combined GDPβS and NEM, NEM was applied 30 min after GDPβS injection, by which time current amplitudes had reached maximal levels. Current amplitudes are normalized to control values and expressed as mean ± S.E.M. In experiments in which both NEM and GDPβS were applied, the resulting current amplitudes were not significantly different from either NEM or GDPβS applied alone (* denotes significant difference, Student's independent *t*-test $P < 0.05$). (E) Time constants ($\tau_{inact.}$) of the decaying portion of α_{1A} and α_{1B} currents before and after NEM and GDPβS treatments. Currents were fitted with a single exponential, averaged and expressed, as the mean ± S.E.M. (* denotes significant difference, Student's independent *t*-test, $P < 0.05$). Time constants for α_{1A} could not be fit in many cases because the rate of inactivation was very slow under the conditions of our experiments (10 mM Ba^{2+} as the charge carrier and ~5 mM internal BAPTA). We have selected only α_{1A} currents which inactivate quickly enough to allow for confident fitting. The time constant obtained is therefore much faster than what would be obtained if all the data were included. Thus, the control α_{1A} time constant is valid only for comparison with the α_{1A} values obtained after NEM and GDPβS treatments.

rent amplitude (Table 1) and in decay rate of the inactivating current (data not shown) after both GDPβS and NEM treatments, similar to the effects of GDPβS treatment on α_{1A} alone. However, when the β_3 subunit was added to this combination, the effects of GDPβS or NEM on both current amplitude and kinetics was abolished (Fig. 2). The results were similar when β_3 was expressed with α_{1B} (Fig. 2). The lack of effect of GDPβS or NEM on the $\alpha_{1A}\alpha_{2b}\beta_3$ or $\alpha_{1B}\beta_{2b}\beta_3$ calcium channels does not appear to be due strictly to the increased size of these currents, as we have also observed no potentiation of currents which happened to be small; i.e. within the amplitude range of currents through α_1 by itself. Thus, co-expression of the β_3 subunit can block inhibition mediated by the tonically active G proteins on α_{1A} and α_{1B} calcium channels. In addition, it appears that the α_2 subunit has no effect on this G protein-mediated inhibition.

One type of G protein inhibition which is commonly seen in neurons is dependent on the membrane potential of the cell. Strong depolarizations are thought to temporarily uncouple the activated G protein from the Ca^{2+} channel, producing larger currents after depolarizing prepulses, by relieving the inhibitory effect of the G proteins [24,25]. To test whether the tonic inhibition of α_{1A} and α_{1B} seen in *Xenopus* oocytes displayed similar voltage-dependence, we used a voltage protocol in which two test pulses were separated by a period in which the channels were allowed to recover from inactivation. Shortly before the second test pulse, a strong depolarizing prepulse was given. The currents elicited by the two test pulses were then compared (Fig. 3). Currents through both α_{1A} and α_{1B} channels could be facilitated with a strong depolarizing prepulse using the prepulse voltage protocol illustrated in Fig. 3A. Facilitation of the α_{1B} currents was abolished after GDPβS or NEM treatments (Fig. 3B,C). In addition, no current facilitation was observed when the β_3 subunit was co-expressed with the α_{1B} subunit (Fig. 3C). Similar results were obtained for the α_{1A} (data not shown). We conclude from these data that the inhibition of α_{1A} and α_{1B} currents by the tonically active G protein population is voltage

Table 1
Response of oocytes expressing various subunit combinations to treatments designed to inactivate G proteins

	GDPβS	NEM	NEM after GDPβS	Water
α_{1A}	2.03 ± .01 (n = 4)	2.18 ± .06 (n = 8)	2.13 ± .27 (n = 4)	1.19 ± .08 (n = 6)
$\alpha_{1A}\alpha_2$	1.70 ± .15 (n = 5)	2.16 ± .05 (n = 3)	N.D.	1.02 ± .003 (n = 3)
$\alpha_{1A}\alpha_2\beta_3$	0.93 ± .03 (n = 3)	1.07 ± .01 (n = 5)	N.D.	1.03 ± .02 (n = 2)
α_{1B}	2.04 ± .19 (n = 5)	2.10 ± .06 (n = 7)	2.21 ± .17 (n = 4)	1.00 ± .02 (n = 2)
$\alpha_{1B}\alpha_2$	2.15 ± .07 (n = 2)	2.21 ± .28 (n = 2)	N.D.	N.D.
$\alpha_{1B}\alpha_2\beta_3$	1.12 ± .06 (n = 2)	1.30 ± .07 (n = 4)	N.D.	1.21 ± .05 (n = 3)
α_{1C}	0.95 ± .04 (n = 7)	N.D.	N.D.	0.99 ± .11 (n = 3)
$\alpha_{1C}\alpha_2$	1.01 ± .07 (n = 2)	N.D.	N.D.	N.D.
$\alpha_{1C}\alpha_2\beta_3$	0.85 ± .05 (n = 4)	N.D.	N.D.	0.86 ± .08 (n = 3)

The column labeled water is the vehicle control for the GDPβS experiments. Current amplitudes are represented as $I_{Treat-ment}/I_{Control}$. N.D. = not determined.

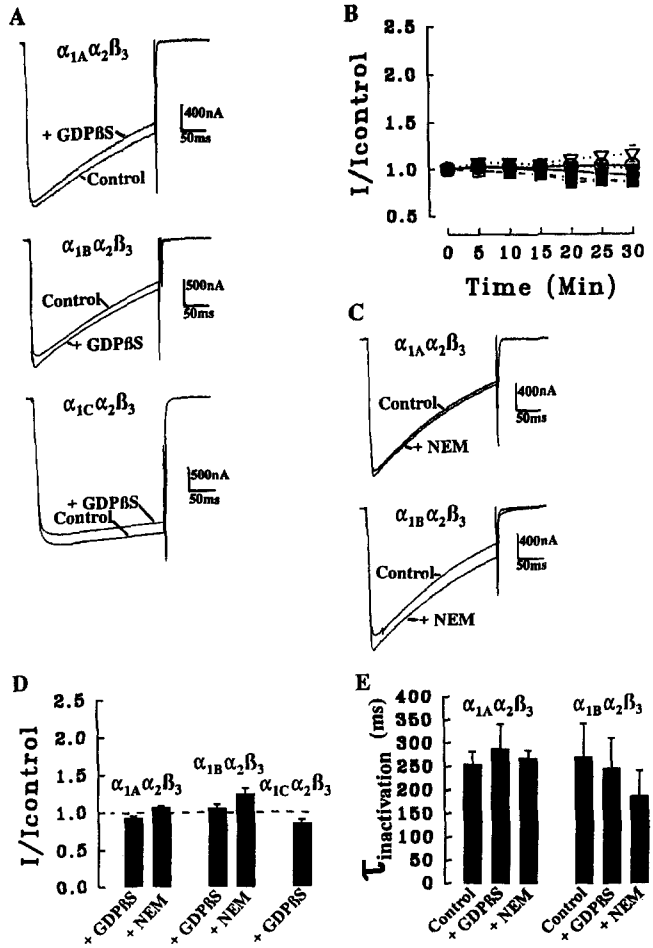

Fig. 2. The effects of co-expression of the β_3 subunit on inhibition of Ba^{2+} currents by the basally-active G protein. α_{1A}, α_{1B}, or α_{1C} were co-expressed with the α_2 and β_3 subunits and tested for G protein inhibition using the same protocol used in Fig. 1. (A) Ba^{2+} currents elicited with a voltage step to +20 mV before and after GDPβS injection into oocytes expressing $\alpha_{1A}\alpha_2\beta_3$, $\alpha_{1B}\alpha_2\beta_3$, or $\alpha_{1C}\alpha_2\beta_3$ combinations. (B) Peak amplitude of currents after GDPβS ($\alpha_{1A}\alpha_{2b}\beta_3$, ●; $\alpha_{1B}\alpha_{2b}\beta_3$, ▼; $\alpha_{1C}\alpha_{2b}\beta_3$, ■) or vehicle ($\alpha_{1A}\alpha_{2b}\beta_3$, ○; $\alpha_{1B}\alpha_{2b}\beta_3$, ▽; $\alpha_{1C}\alpha_{2b}\beta_3$, □) injection over the 30 min time course of the experiment. Amplitude values are normalized to control levels. (C) Ba^{2+} currents recorded before and after treatment with 200 μM NEM. (D) Change in the peak Ba^{2+} current after administration of either GDPβS or NEM. (E) Time constants for the decaying portion of the current both before and after treatments with either GDPβS or NEM. All time constants were fit with a single exponential and expressed as the mean ± S.E.M.

dependent, similar to G protein-mediated inhibition described previously in a variety of neurons [16,17,19,22,24–28]. Additionally, lack of facilitation after co-expression of the β_3 subunit is consistent with our conclusion that co-expression of the β_3 subunit abolishes tonic inhibition of these channels.

The voltage-dependency of inhibition which we observed for α_{1A} and α_{1B} currents, coupled with the persistance of G protein inhibition in the presence of the Ca^{2+} chelator, BAPTA, makes it likely that the G protein inhibition occurring is of the membrane-delimited type [27,28]. In this model, the G protein is believed to interact directly with the channel [29]. Since the α_1 subunit is sufficient for G protein-mediated inhibition of α_{1A} and α_{1B} currents, the G protein presumably interacts directly with this subunit.

Figure 3

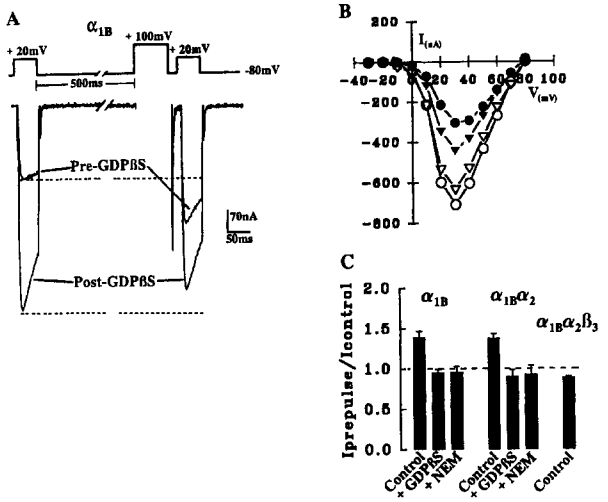

Fig. 3. Voltage-dependence of tonic G protein-mediated inhibition. (A) Facilitation of current recorded from α_{1B} by a depolarizing prepulse is obvious by comparison of the pre-GDPβS trace on the left with the pre-GDPβS trace on the right (elicited after a prepulse). The voltage protocol used is shown above the current traces. Comparison of the post-GDPβS traces show that after this treatment, prepulse facilitation no longer occurs, presumably because of the removal of tonic G protein inhibition by GDPβS. (B) Current vs. voltage plots of peak currents recorded from α_{1B} before (closed symbols), and after (open symbols) GDPβS injection. Currents recorded without a depolarizing prepulse are represented by circles, while currents recorded after a depolarizing prepulse are represented by triangles. (C) Facilitation of current amplitude of the various Ca^{2+} channels using the depolarizing prepulse protocol illustrated in Fig. 3A.

We have demonstrated that co-expression of the β_3 subunit renders α_{1A} and α_{1B} calcium channels incapable of undergoing G protein-mediated inhibition. Presumably, the interference by the β_3 subunit of the tonic G protein-mediated inhibition must take place either by direct competition for the same site, steric masking of a site nearby, or alteration in the conformation of the α_1 by the binding of the β subunit, which renders the G protein interaction impossible.

The α_{1A} and α_{1B} subunits, which are not DHP sensitive, share greater homology to each other than to the α_{1C} which is closer in homology to other DHP sensitive α_1's [30]. The two main areas of divergence are the TM2-TM3 linker and the C-terminus. It will be interesting to determine whether it is one of these areas which is responsible for the difference in G protein action or whether it is a site closer to the β subunit binding site, or possibly the β subunit binding site itself.

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