Ca^{2+} Channel Inactivation Heterogeneity Reveals Physiological Unbinding of Auxiliary β Subunits

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ABSTRACT Voltage gated Ca^{2+} channel (VGCC) auxiliary β subunits increase membrane expression of the main pore-forming α_1 subunits and finely tune channel activation and inactivation properties. In expression studies, co-expression of β subunits also reduced neuronal Ca^{2+} channel regulation by heterotrimeric G protein. Biochemical studies suggest that VGCC β subunits and G protein $\beta\gamma$ can compete for overlapping interaction sites on VGCC α_1 subunits, suggesting a dynamic association of these subunits with α_1. In this work we have analyzed the stability of the α_1/β association under physiological conditions. Regulation of the α_{1A} Ca^{2+} channel inactivation properties by β_{1b} and β_{2a} subunits had two major effects: a shift in voltage-dependent inactivation (E_{in}), and an increase of the non-inactivating current (R_{in}). Unexpectedly, large variations in magnitude of the effects were recorded on E_{in}, when β_{1b} was expressed, and R_{in}, when β_{2a} was expressed. These variations were not proportional to the current amplitude, and occurred at similar levels of β subunit expression. β_{2a}-induced variations of R_{in} were, however, inversely proportional to the magnitude of G protein block. These data underline the two different mechanisms used by β_{1b} and β_{2a} to regulate channel inactivation, and suggest that the VGCC β subunit can unbind the α1 subunit in physiological situations.

INTRODUCTION

Different Ca^{2+} channel auxiliary β subunits have been isolated from mammalian brain and heart. They can potentially be associated with any of the 6 α_1 pore-forming subunits of high-voltage activated Ca^{2+} channel (α_{1A}, α_{1B}, α_{1C}, α_{1D}, α_{1E}, α_{1F}, and α_{1S}) via conserved interaction domains: α-interaction domain (AID) on α_1 and β-interaction domain (BID) on β (De Waard et al., 1995, 1996; Pragnell et al., 1994; Walker and De Waard, 1998). These β subunits induced generic as well as specific modifications in the expression, electrophysiological properties, and regulation of any of the α_1 subunits. Increased channel expression, activity, and membrane targeting of the α_1 subunit are common modifications induced by all β subunits, whereas changes in the voltage-dependence and kinetics of activation and inactivation are more specific of a given pair of α_1-β subunits (Mangoni et al., 1997; Sather et al., 1993; De Waard and Campbell, 1995; Jones et al., 1998; Stea et al., 1994). These effects appeared to be mediated by high affinity interactions between the AID (located on the loop connecting domains I and II of each α_1 subunits) (Pragnell et al., 1994) and the BID (located on the beginning of the second conserved region of the β subunit) (De Waard et al., 1994). Modification of these sites, using site-directed mutagenesis, produced a complete loss of the α_1/β subunits co-localization and co-immunoprecipitation, but only partially reverted the increase in current amplitude and change in electrophysiological properties of α_1 (Gerster et al., 1999; De Waard et al., 1994). These latter results suggest that the β subunit modifies channel targeting and/or current amplitude using molecular determinants distinct from those necessary for the modification of the electrophysiological properties (Gerster et al., 1999). Indeed, functional analysis of mutated and truncated α_1 and β subunits revealed that additional interaction sites of lower affinity (on the N- and C-terminal tails of the α_1 and β subunits) may also participate to these regulations (Birnbaumer et al., 1998; Cens et al., 1998; Olcese et al., 1994; Qin et al., 1996; Walker et al., 1998).

It should be noted that although the AID-BID interaction occurs through high-affinity sites, the recent identification of a G protein $\beta\gamma$ binding site overlapping the AID suggests that this interaction can be disrupted in certain circumstances, such as when G proteins are activated (De Waard et al., 1997; Zamponi et al., 1997). Indeed, in an heterologous expression system, the Ca^{2+} channel block by G protein is decreased by expression of an auxiliary β subunit (Bourinet et al., 1996) suggesting competition between overlapping sites and possible disruption of the AID-BID interaction. In normal conditions however, such unbinding has never been observed.

Slowing of inactivation has been reported when the β_{2a} subunit is co-expressed with the neuronal α_{1A}, α_{1B}, α_{1C}, and the α_{1E} subunits (Sather et al., 1993; Mangoni et al., 1997, De Waard and Campbell, 1995; Stea et al., 1994; Jones et al., 1998; Cens et al., 1999). The mechanism underlying this effect has recently been proposed to be due to immobilization of the channel inactivation gate by a membrane-anchoring site (Restituito et al., 2000) constituted of two palmitic acid bound to cysteines 3 and 4 of the amino-terminal tail of the β_{2a} subunit (Chien et al., 1996, 1998; Qin et al., 1998).

Here we report a significant heterogeneity in the voltage-dependence and kinetics of inactivation of the α_{1A} P/Q-type

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Ca^{2+} channel expressed with the β_{2a} or β_{1b} subunits. Variable parameters are the fraction of non-inactivating current (R_{in}) for β_{2a} and the voltage for half-inactivation (E_{in}) for β_{1b}. However, expression of α_{1A} and $\alpha_2 - \delta$ without β results in more homogeneous inactivation properties. A more complete analysis of voltage-dependent activation and inactivation and G protein regulation was performed using different mutated β subunits at different α_{1A}/β_{2a} cDNA ratios and suggested unbinding of the β subunit from the α_1 subunit. Altogether these results provide evidences for a dynamic association between these two subunits.

MATERIALS AND METHODS

Preparation of mutated β subunits

The following calcium channel subunits were used: α_{1A} (Starr et al., 1991), β_{1b} (Pragnell et al., 1991); β_{2a} (Perez-Reyes et al., 1992), and $\alpha_2\delta$. All these cDNAs were inserted into the pMT2 expression vector (Stea et al., 1994). The chimera CD8-β_{2a}C3,4S construction was produced as described previously (Restituito et al., 2000).

Xenopus oocyte preparation and injection

Xenopus oocyte preparation and injection (5–10 nl of a mixture of α_1, $\alpha_{2\text{-}\delta}$, or β subunits cDNAs at ≈0.3 ng/nl each) were performed as described elsewhere (Cens et al., 1996). For Fig. 4, starting cDNA concentration was either 1 or 0.1 ng/nl for α_{1A} and β_{2a} subunits, giving a final concentration of 0.3 or 0.03 ng/nl after dilution. Oocytes were selected as expressing slow- or fast-inactivating currents after individual recordings in voltage-clamp. Oocytes from each pool (fast, slow, non-expressing, and non-injected) were then frozen in liquid nitrogen and kept for Western blotting. Each line was loaded with the equivalent of three oocytes expressing the same quantity of currents. μ opioid receptor and α_O G protein RNA (1 μg/μl) were injected 1 day after cDNA injection. Oocytes were then incubated for 2 to 7 days at 19° C under gentle agitation before recording.

Western blot

Frozen oocytes were homogenized in 5 μl/oocytes of the following lysis buffer [20 mM Tris (pH 7.5), 50 mM NaCl, 50 mM NaF, 10 mM βglycerophosphate, 5 mM $Na_4P_2O_7$, 5 mM EDTA; 5 mM EGTA, 10 mg/ml PMSF, 0.2 mg/ml pepstatin, 0.2 mg/ml leupeptin, and 0.2 mg/ml aprotinin] and centrifuged for 3 min at 20,000 × *g* at 4°C. The supernatant was boiled in sodium dodecyl sulfate (SDS) gel loading buffer (Laemmli), electrophoresed on 10% SDS-polyacrylamide gel, and transferred to nitrocellulose filter. Filters were blocked 1 h at room temperature (8% skim milk powder), rinsed in distilled water, incubated overnight at 4°C with the β-com rabbit polyclonal primary antibody in 0.1% bovine serum albumin (CW24; Vance et al., 1998), washed 3 times, incubated 1 h with an anti-rabbit antibody, and detected by chemiluminescence (Renaissance NEN, Boston, MA).

Electrophysiological recordings

Whole-cell Ba^{2+} currents (<5 μA) were recorded under two electrodes voltage-clamp using the GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA). Current and voltage electrodes (<1 MΩ) were filled with 2.8 M CsCl and 10 mM BAPTA, pH = 7.2, with CsOH. Ba^{2+} current recordings were performed after injection of BAPTA (around 50 nl of (mM): BAPTA-free acid (Sigma), 100; CsOH, 10; HEPES, 10; pH 7.2 CsOH) using the following solution (in mM): BaOH, 10; TEAOH, 20; NMDG, 50; CsOH, 2; HEPES, 10; pH 7.2 with methanesulfonic acid. Currents were filtered and digitized using a Digidata 1200 interface (Axon Instruments), and subsequently stored on a Pentium II-based personal computer by using the version 6.02 of the pClamp software (Axon Instrument). Ba^{2+} currents were recorded during a test pulse from −80 mV to +10 mV of 2.5 sec duration. Current amplitudes were measured at the peak of the current. Pseudo steady-state inactivation (2.5 s of conditioning depolarization followed by a 400 ms test pulse to +10 mV) was fitted using the following equations

$$I/I_{max} = R_{in} + (1 - R_{in})/(1 + \exp((V - E_{in})/k)) \quad (1)$$

where I is the current amplitude measured during the test pulse at +10 mV for conditioning depolarization varying from −80 to +50 mV; I_{max}, the current amplitude measured during the test-pulse for a conditioning depolarization of −80 mV; E_{in}, the potential for half-inactivation; V, the conditioning depolarization; k, the slope factor; and R_{in}, the proportion of non-inactivating current. Current to voltage curves were fitted using the following equation

$$I/I_{max} = G^*(V - E_{rev})/(1 + \exp((V - V_{act})/k)) \quad (2)$$

where I is the current amplitude measured during depolarizations varying from −80 to +50 mV; Imax, the peak current amplitude measured of the current-voltage curve; G, is the normalized macroscopic conductance; E_{rev}, is the apparent reversal potential, V_{act}: the potential for half-activation; V, the value of the depolarization; and k, a slope factor.

Activation of G protein-coupled μ opioid receptor was performed by perfusion of 10 μmol of the specific agonist Tyr-D-Ala-Gly-N-Methyl-Phe-Gly-ol (DAMGO; Sigma-Aldrich, Saint Quentin Fallavier, France). % block induced by DAMGO was calculated by dividing the current amplitude in control conditions by the current amplitude recorded at the steady state effect of 10 μM DAMGO during train of 50-ms depolarizations at 0 mV. All values are presented as mean ± SEM.

RESULTS AND DISCUSSION

Xenopus oocytes were injected with cDNA encoding the $\alpha_{1A} + \alpha_2 - \delta$ and either the β_{1b} or β_{2a} subunit, and Ba^{2+} currents were recorded 2–5 days later. As seen in Fig. 1, *A* and *B* (*left*), and as already shown by others (De Waard and Campbell, 1995; Stea et al., 1994), these two combinations of subunits induced the expression of P/Q type Ba^{2+} currents with fast and slow inactivation, respectively. The voltage protocol applied to the oocytes (shown on top of Fig. 1) allows for the same recording to calculate current-voltage and inactivation curves as well as inactivation kinetics. The slowing of inactivation by the β_{2a} subunit was clear for depolarized (>−20 mV) potentials, and the inactivation curves allow to calculate the potential for half-inactivation (E_{in}) and the proportion of non-inactivating current (R_{in}) for each combination of subunits. Averaged values gave an E_{in} of −28.2 ± 9.8 mV and −12.8 ± 5 mV and an R_{in} of 0.098 ± 0.002 and 0.48 ± 0.004 for β_{1b} (n = 28) and β_{2a} (n = 56). Comparison with the values obtained in the absence of expressed β subunit ($\alpha_{1A} + \alpha_2 - \delta$; E_{in} = −12.2 ± 2.2mV and R_{in} = 0.08 ± 0.002, n = 16) demonstrate that the major effect of β_{1b} subunit was to decrease the availability of the channel by hyperpolarizing E_{in}, whereas the major effect of β_{2a} was to prevent inactivation

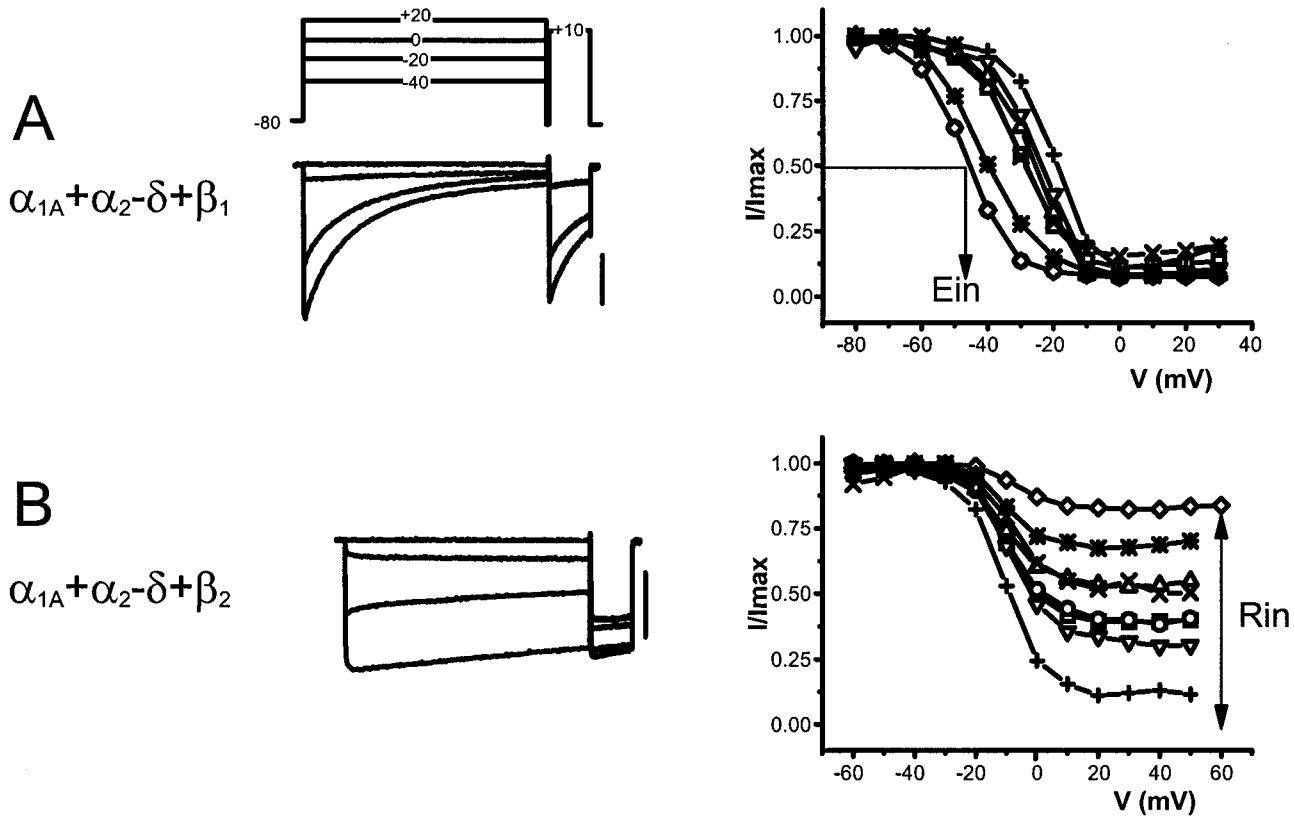

FIGURE 1 Heterogeneity of voltage-dependent inactivation. Voltage-dependent inactivation curves recorded from oocytes expressing the α_{1A} and $\alpha_2 - \delta$ Ca^{2+} channel subunits with either the β_{1b} (*A*) or β_{2a} (*B*) auxiliary subunits. (*Left*) Typical current traces recorded on oocytes expressing $\alpha_{1A} + \alpha_2 - \delta + \beta_{1b}$ or $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ subunit during the voltage protocol shown on top. The conditioning depolarisation had a duration of 2.5s, test-pulse (+10 mV) duration was 0.4 s. Scale bar, 0.5 μA. (*Right*) Example of 8 typical steady-state inactivation curves recorded on different oocytes for each of the two combinations of subunits. Note the variability in the voltage for half-inactivation (E_{in}) and in the proportion of the non-inactivating current (R_{in}).

by increasing R_{in}. However, when typical individual inactivation curves recorded for each combination were plotted on the same graph (Fig. 1, *right*), a large variation in the E_{in} and R_{in} values can be seen for each subunit combination. This variation was better seen in Fig. 2 *A*, in which individual R_{in} values are plotted against their corresponding E_{in} value for all combinations of subunits tested ($n > 200$ oocytes). From such a plot, 3 groups can be identified. The control α_{1A} group (open square), corresponds to oocytes expressing the $\alpha_{1A} + \alpha_2 - \delta$ subunits without β subunit. For this group, the inactivation parameters (E_{in} and R_{in}) are well centered around their average values, as expected for an homogeneous population. The β_2 group (*triangle*) represents oocytes injected with $\alpha_{1A} + \alpha_2 - \delta$ and the β_{2a} subunit cDNAs. This group displays low variability in their E_{in} values (which are almost similar to E_{in} of the α_{1A} group), but large variations in R_{in} ranging from 0.1 to 0.9. In this group, smaller values of R_{in} are close to the average R_{in} value of the α_{1A} group. In the case of the β_1 group (oocytes injected with $\alpha_{1A} + \alpha_2 - \delta + \beta_{1b}$ cDNA; Fig. 2 *A*, *open circle*) the variations are more pronounced for E_{in} (starting from -10 mV, the average value of the control α_{1A} group, and decreasing up to -55 mV), whereas R_{in} appeared almost unchanged when compared to α_{1A} group. Hyperpolarization of the inactivation curve and reduction of inactivation, the two fundamental effects of the β_{1b} and β_{2a} subunits, respectively, are therefore subject to large variations in vivo despite the known high-affinity interaction between the α_{1A} and the β subunits. Such variations has also been found in chromaffin cells and tsA201 cells transfected with the α_{1B}, or α_{1A} and β_{2a} subunits (Cahill et al., 2000; Hurley et al., 2000).

The slowing of inactivation by β_{2a} has been attributed to palmitoylation of cysteines at position 3 and 4 in the β_{2a} subunit (Qin et al., 1998). Modulation of this post-translational modification can thus be a reason for variations in the voltage-dependence and kinetics of inactivation (Chien et al., 1998; Hurley et al., 2000; Qin et al., 1998). To assess this, we have injected cDNA of the β_{2a} subunits mutated at cysteines 3,4 (β_2C3, 4S subunit) with the α_{1A} and $\alpha_2 - \delta$ subunits and analyzed the inactivation parameters of the expressed channels. Mutation of Cys3,4 to Ser decreases R_{in} to values normally recorded with the $\alpha_{1A} + \alpha_2 - \delta$ subunits expressed alone or with β_{1b} (Fig. 2 *B*, *diamond*). The

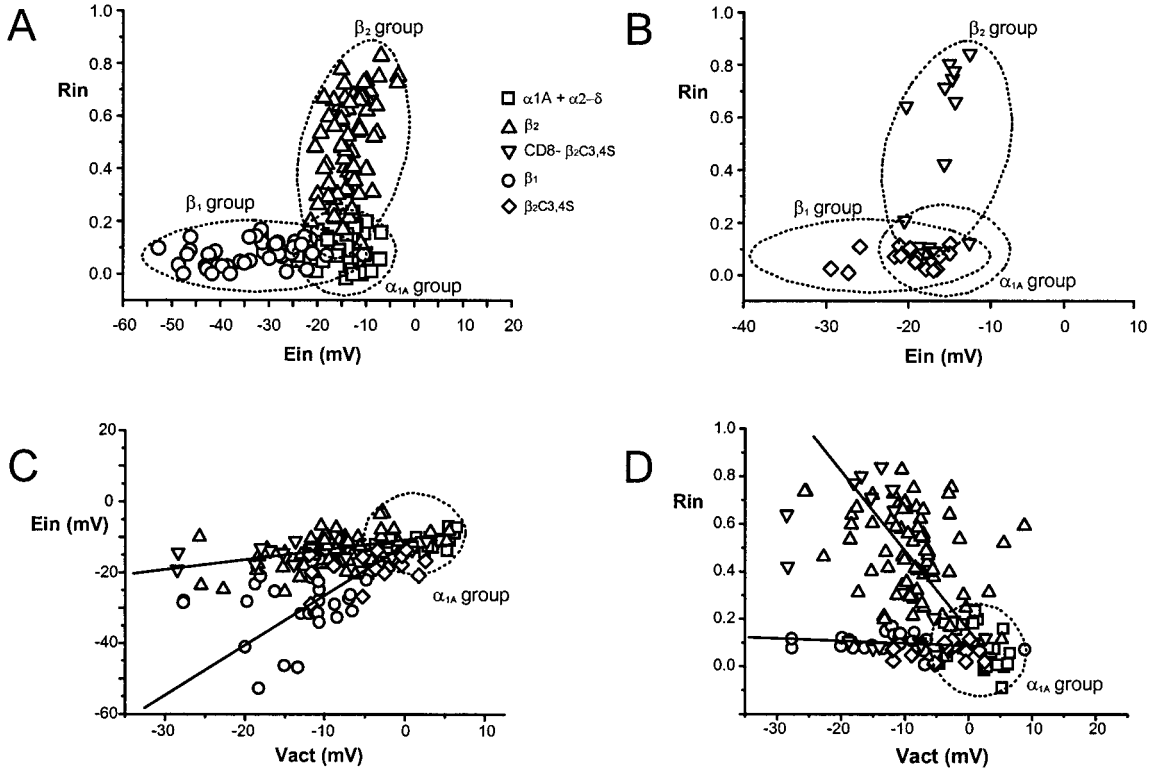

FIGURE 2 β_1 and β_2-induced regulation of inactivation. (*A* and *B*) Scatter plot of R_{in} versus E_{in} for different batches of oocytes (n>200) injected with the α_{1A} and $\alpha_2 - \delta$ subunits alone or in combination with β_{1b}, β_{2a} (*A*), CD8-β_2C3,4S or β_2C3,4S (*B*; see Results and Discussion). Note that these oocytes can be divided into 3 groups. The control α_{1A} group included oocytes expressing the $\alpha_{1A} + \alpha_2 - \delta$ subunits and show limited variability in either E_{in} and R_{in}. The β_2 group, displays the same E_{in} than $\alpha_{1A} + \alpha_2 - \delta$ but with higher R_{in} (includes oocytes expressing $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$, or CD8-$\beta_2$C3, 4S). Currents recorded from oocyte belonging to the β_1 group have the same R_{in} than $\alpha_{1A} + \alpha_2 - \delta$, but more hyperpolarized Ein (group of oocytes expressing $\alpha_{1A} + \alpha_2 - \delta + \beta_{1b}$ or β_2C3, 4S). For the β_{1b} group, variability is more pronounced for E_{in}, while for the β_2 group, R_{in} was the most variable parameter. (*C*) Scatter plot of E_{in} versus V_{act}, the potential of half activation for the oocytes shown on *A*. The two groups of oocytes induced an hyperpolarization of the IV curves. This hyperpolarization was correlated with a negative shift of E_{in} for the β_1 group that was less pronounced for the β_2 group. (*D*) Scatter plot of R_{in} *versus* V_{act}, the potential of half activation, for the currents recorded from the same oocytes shown on A. Hyperpolarizing shift of the IV curve (V_{act}) was correlated with an increase in R_{in} for the β_2, but not the β_1 group. Lines are drawn to show tendencies. Symbols in *C* and *D* are the same as in *A* and *B*.

mutation also shifts E_{in} toward hyperpolarized values to varying degrees. Thus, the effect of the mutation on the β_{2a} subunits seems to transfer the variability in R_{in} associated to β_{2a} subunit to variability in E_{in} normally recorded with the β_{1b} subunit, suggesting that variations of these two parameters arise from a common mechanism. Further analysis of the role of Cys3,4 was done by expressing a chimera where a transmembrane CD8 domain was added at the N terminus of this β_2C3,4S mutated subunit (CD8-β_2C3, 4S subunit). This subunit is able to slow α_{1A} channel inactivation without being palmitoylated (Restituito et al., 2000). Interestingly, this CD8-β_2C3,4S subunit expressed with the $\alpha_{1A} + \alpha_2 - \delta$ subunits gave slowly inactivating channels that behaved exactly like the β_{2a} subunit, i.e., displaying large variations in their R_{in} values but low variability in E_{in} (Fig. 2 *B*, *inverted triangle*). Such results clearly argue against any participation of the palmitoylation of the β_{2a} subunit in the variability recorded with R_{in} as can be seen in other systems (Hurley et al., 2000).

Inspection of the ability of these subunits to modulate the activation properties of the α_{1A} subunit (Fig. 2 *C*) has been done by plotting E_{in} versus the potential for half-activation (V_{act}) for each oocyte. As seen in Fig. 2 *C*, all subunit combinations are capable of hyperpolarizing the voltage-dependence of activation of the channel. In the case of the β_{1b} group, however, this shift was graded with the hyperpolarizing shift in E_{in}. In the case of the β_2 group, almost no change in E_{in} could be recorded. Similarly, when R_{in} was plotted *versus* V_{act} (Fig. 2 *D*), the same type of relation appeared, i.e., the hyperpolarizing shift in V_{act} was graded with the increase in R_{in} value for the β_2 group, but not for the β_1 group. The α_{1A} group (*open square*) still displays a low degree of variability.

One obvious explanation for these large variations in E_{in} and R_{in} is the variable degree of expression of auxiliary β subunit between different oocytes. In this case, since the primary effect of β subunits is to increase channel expression, the magnitude of the effect (shift in E_{in} or increase in

R_{in}) is expected to be correlated to the amplitude of the current, because this latter parameter is proportional to the number of functional channels expressed. Plots of E_{in} and R_{in} values against current amplitude (Fig. 3, *A* and *B*) for these different mutations clearly show that this was not the case, and the lack of clear relation between changes in E_{in} and R_{in} and current amplitude therefore argues against a deficit in β subunit expression as a possible mechanisms to account for the observed effects. For the same reasons, any technical artifacts due to a poor voltage-control also appears very unlikely in these oocytes. Moreover, the fact that small R_{in} values in the β_2 group have E_{in} comparable to the α_{1A} group (Fig. 2 *A*), suggest that these variations are not due to over-expression of an endogenous β_3 subunits (Tareilus et al., 1997) which one would expect to also hyperpolarize E_{in}.

Indeed, the slow inactivation induced by the β_{2a} subunit was never observed when β_{2a} cDNA was not injected. In such conditions, expression was low (0.2–1 μA, see $\alpha_1/\beta_{=}$ 0.1/0 and 1/0, Fig. 3 *C*) and inactivation was fast (low R_{in} values, Fig. 3 *D*). Similar values are obtained for two concentrations of α_{1A} subunit cDNA injected (0.1 and 1ng/nl, $p > 0.05$), suggesting that functional expression of this subunit was already saturating at the lowest cDNA concentration. Co-injection of β_{2a} subunit cDNA, either at low (0.1) or high (1) concentration induced a significant increase in current amplitude (Fig. 3 *C*) and a slowing of inactivation ($p < 0.05$; Fig. 3 *D*). Both effects were recorded for the two α_{1A} concentrations ($\alpha_1/\beta_{=}$ 0.1/1 and 1/1, Fig. 3 *C*). However, whereas variability in current amplitude was recorded at all the concentrations of the α_{1A} and β_{2a} cDNA tested (maximum and minimum amplitude values were almost identical in all cases, $\alpha_1/\beta_{=}$ 0.1/1; 1/1 and 1/0.1), variability in inactivation (R_{in}) was more prominent for the highest α_{1A}/β_{2a} subunit ratio (1/0.1), and only slow-inactivating currents were recorded at $\alpha_1/\beta_{=}$ 0.1/1. The fact that different degree of regulation of inactivation by the β_{2a} subunit can be recorded at two concentrations of $\alpha_{1A,}$ (0.1 and 1, both saturating for expression of functional channels), and that this regulation did not correlate with the expressed current amplitude using two different β subunit concentrations (see 1/0.1, *inverted triangle*; and 1/1, *diamond*; $n = 67$ and 119, respectively, Fig. 3 *E*) suggested that the individual variation was not due to inter-oocyte variability in the level of expression of the β_{2a} subunit.

This hypothesis was tested directly by coupling the electrophysiological measurement with the immunochemical

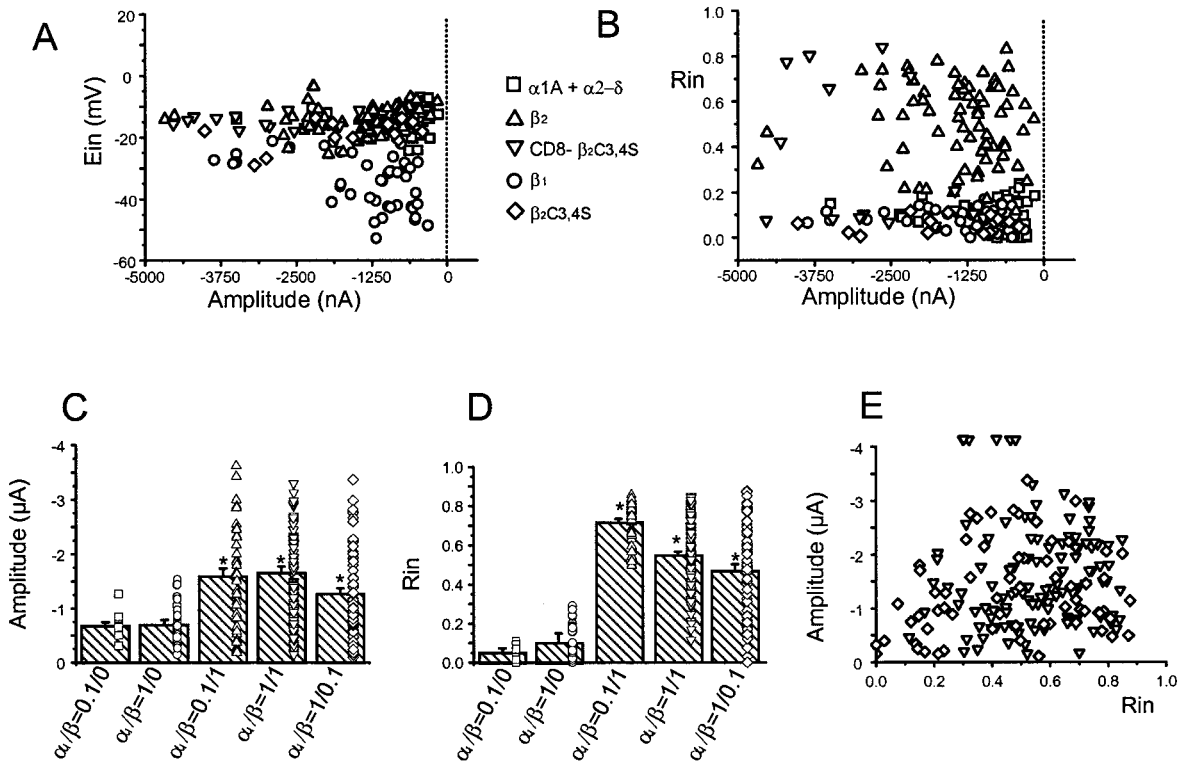

FIGURE 3 (*A* and *B*) Scatter plots of E_{in} (*A*) and R_{in} (*B*) *versus* the maximum amplitude of the corresponding current. In each case variations in E_{in} (for the β_1 group, *A*), or R_{in} (for the β_2 group, *B*) were not related to the amplitude of the current suggesting that deficient expression of the channel auxiliary subunits was not the cause of the variability. (*C* and *D*) Individual and averaged Ba^{2+} current amplitude (*C*) and inactivation (R_{in}, *D*) recorded on oocytes expressing different ratios of α_{1A}/β_2 subunits cDNA. Ten nl of a v/v mixture of α_{1A}, β_2 (at a concentration of 0, 0.1, or 1 μg/μl, as indicated) and $\alpha_2 - \delta$ (1 μg/μl) subunit cDNAs were injected into oocytes, and currents were recorded 3–4 days later. (*E*) Lack of correlation between current amplitude and R_{in} for oocytes injected with ratios of α_{1A}/β_{2a} cDNA giving the largest variability in inactivation (1/1, *inverted triangle*, and 1/0.1, *diamond*).

evaluation of the β_{2a} subunit expression. Oocytes were injected with a α_{1A}/β_{2a} subunit:cDNA ratio of 1:1, and Ba^{2+} currents were recorded 2–3 days later. Current amplitude and inactivation (R_{in}) were then estimated and allowed the selection of the oocytes into two groups (fast and slowly inactivating). Oocytes were then homogenized, and analyzed by SDS-page electrophoresis and Western blot using a anti-β antibody. Bottom of Fig. 4 *A* shows averaged current amplitude and inactivation of these two groups (n = 5 for each group). Despite differences in inactivation, the amplitude of each group was very similar. Moreover Western blot analysis (3 oocytes/line, representing ~5 μA of current) showed that the level of expression of the β_{2a} subunit was almost identical between the fast and slowly inactivating oocytes, and clearly distinct from the non-expressing or non-injected oocytes. Therefore, neither defect in palmitoylation, nor deficient expression of the β_{2a} subunit, could explained the observed changes in inactivation.

Dissociation of a variable proportion of the auxiliary β from α_{1A} subunits, after targeting of the pore-forming subunit to the membrane, may also cause these effects. Indeed, these two different functions of the β subunits have recently been shown to be due to different and independent interaction sites (Gerster et al., 1999) and reversible $\alpha 1/\beta$ interaction has been proposed (Bichet et al., 2000; Gerster et al., 1999). This "unbinding" hypothesis can be tested by analyzing the regulation of the Ba^{2+} currents by G proteins. G protein-induced inhibition of the α_{1A} Ca^{2+} channel has been shown to be greatly reduced by co-expression of β subunits (Bourinet et al., 1996), and can therefore be used as an index of association between the α_{1A} and the β subunits. Oocytes were co-injected with RNA encoding the μ opioid receptor and cDNA of α_{1A} and $\alpha_2 - \delta$ subunits alone or with the β_{2a} subunit. G protein activation was achieved by superfusion of 10 μM DAMGO, a specific μ opioid agonist, and both the level of inactivation of the Ba^{2+} current (determined as R_{in}) and the extend of G protein block of the current were calculated. Fig. 4 *B* shows that perfusion of DAMGO on oocytes expressing $\alpha_{1A}+\alpha_2-\delta$ subunits induced a pronounced inhibition (≈70%) of the rapidly inactivating Ba^{2+} current. This inhibition was voltage-depen-

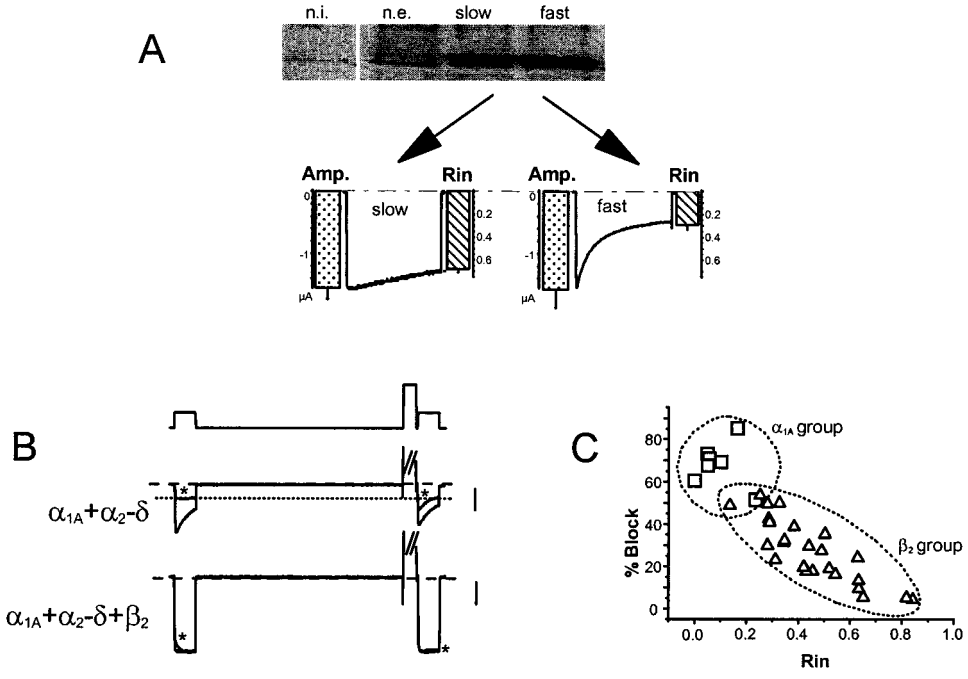

FIGURE 4 (*A*) Oocytes with currents showing fast or slow inactivation expressed the same level of β_{2a} subunit. Oocytes were injected with 10 nl of cDNA with a α_{1A}/β_{2a} ratio of 1/0.1. Three days later, oocytes were selected in 3 batches according to the properties of their Ba^{2+} currents (n.e., no expression; slow, current with an $R_{in} > 0.5$; fast, current with $R_{in} < 0.5$). For each batch, averaged current amplitude and inactivation were recorded (see histogram). Oocytes were then homogenized, centrifuged and processed for Western blotting using an anti-βcom antibody (3 oocytes/line corresponding to ~5 μA; n.i, non-injected). The mean current amplitude and Western blot suggest similar levels of expression of the α_{1A} and β_{2a} subunit in the fast and slow oocytes. (*B*) Typical current traces recorded from oocytes expressing $\alpha_{1A} + \alpha_2 - \delta$ or $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ subunits before and at the steady state effects of DAMGO (*, 10 μM). Holding potential was −80 mV. The second test pulse (0 mV, see voltage protocol at top) is preceded by a prepulse at +100 mV that allow partial relieve of the inhibition induced by DAMGO. Note the differences in inactivation kinetics and block by DAMGO between $\alpha_{1A} + \alpha_2 - \delta$ and $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ oocytes. Scale bar, 0.5 μA. (*C*) Scatter plots of the percentage of inhibition of the Ba^{2+} currents recorded during the perfusion of the μ opioid agonist DAMGO (10 μM), and the inactivation properties of the corresponding current. Inactivation was quantified by calculating R_{in} as above. Two different combinations of subunits were studied $\alpha_{1A} + \alpha_2 - \delta$ and $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$. In the case of $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$, note the correlation between R_{in} and the magnitude of the inhibition by DAMGO.

dent, as it could be partially reversed by applying a 50-ms prepulse to positive voltages (+100 mV). When the same experiment was performed on oocytes expressing the $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ subunits, current inhibition induced by DAMGO was greatly reduced, especially on those oocytes that have the slowest inactivation kinetics (see bottom of Fig. 4 *B*). A scatter plot of this inhibition *versus* the inactivation parameter R_{in} (see Materials and Methods) for different oocytes is shown on Fig. 4 *C*. Both, the fast inactivation kinetics and the marked inhibition by DAMGO recorded with $\alpha_{1A} + \alpha_2 - \delta$ were systematically recorded on these batches of oocytes, and therefore values for R_{in} and percentage of inhibition by DAMGO display a low dispersion (α_{1A} group, Fig. 4 *C*). However, on oocytes injected with $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ cDNA, values for both inactivation (Fig. 1) and percentage of inhibition by DAMGO were more dispersed (β_2 group, Fig. 4 *C*). In this group, the decrease in the inhibition by DAMGO was correlated to a slowing of inactivation. Again, these effects were not related to the absolute amplitude of the current, since G protein-sensitive or G protein-resistant currents of similar amplitude could be recorded from oocytes expressing the $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$ subunits (data not shown).

The simplest explanation for these results is to propose an unbinding of the auxiliary β subunit from the α_{1A}. Such a dissociation removes the modulatory role of the β subunit on inactivation, as well as blockade of G protein modulation. Thus, for each oocyte, a mixed population of channels with or wihtout β subunit in variable proportions, could co-exist on the oocyte membrane. The magnitude of the β subunit effect may therefore result from the proportion of bound β subunits on the AID of channel. Because no clear relation can be found between the magnitude of this effect and current amplitude, we propose that the β subunit can unbind the channel after membrane targeting of the α1 subunits. Moreover, considering that fast or slow inactivating kinetics could be recorded for current of similar amplitudes in the same batch of $\alpha_{1A} + \alpha_2 - \delta + \beta_{2a}$-injected oocytes, lead us to suggest that this unbinding of auxiliary subunit does not affect the probability of opening of the channel, but only the transition to the inactivated state. Unbinding can occur for the β_1 or the β_2 subunits (as well as the different mutants presented) and is best revealed by the modulation of the inhibition by G proteins. Because the presence of a β subunit is an important determinant for channel regulation by G proteins, dissociation occurring in physiological conditions, therefore, represents a new pathway for modulating channel properties and regulation. Preliminary experiments using cAMP derivative and kinase inhibitors (W7, H89) suggest that PKA phosphorylation is not directly involved in this pathway. Recently, based on the different membrane distribution of the α_1 subunit when expressed alone or with the β subunit, it has been hypothesized that α_1/β_2 interaction may results in secondary interactions with other cellular proteins (such as PDZ-domain-containing proteins; Gao et al., 1999) and stabilization of the complex at specific membrane location. Differential expression of such proteins between oocytes of a same batch may therefore constitute an interesting direction for future experiments. Whether β subunits remain bound to the α_1 subunit, using secondary interaction sites of minor importance regarding inactivation and G protein regulations, remains also to be determined.

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