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Regulation of cardiac L-type Ca^{2+} channel by coexpression of $G_{\alpha s}$ in *Xenopus* oocytes

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Abstract Activation of $G_{\alpha s}$ via β-adrenergic receptors enhances the activity of cardiac voltage-dependent Ca^{2+} channels of the L-type, mainly via protein kinase A (PKA)-dependent phosphorylation. Contribution of a PKA-independent effect of $G_{\alpha s}$ has been proposed but remains controversial. We demonstrate that, in *Xenopus* oocytes, antisense knockdown of endogenous $G_{\alpha s}$ reduced, whereas coexpression of $G_{\alpha s}$ enhanced, currents via expressed cardiac L-type channels, independently of the presence of the auxiliary subunits α_2/δ or β_{2A}. Coexpression of $G_{\alpha s}$ did not increase the amount of α_{1C} protein in whole oocytes or in the plasma membrane (measured immunochemically). Activation of coexpressed β2 adrenergic receptors did not cause a detectable enhancement of channel activity; rather, a small cAMP-dependent decrease was observed. We conclude that coexpression of $G_{\alpha s}$, but not its acute activation via β-adrenergic receptors, enhances the activity of the cardiac L-type Ca^{2+} channel via a PKA-independent effect on the α_{1C} subunit.

Key words: Calcium channel; G-protein; *Xenopus* oocyte

1. Introduction

Sympathetic (β-adrenergic) augmentation of cardiac contraction force is mediated primarily by the enhancement of activity of the voltage-dependent Ca^{2+} channels of the L-type (see [1]). The molecular mechanism of this classical example of ion channel modulation via a G protein-mediated pathway is still incompletely understood. Clearly, the β-adrenergic effect is mediated via the $G_{\alpha s}$-adenylyl cyclase pathway; a major part of enhancement of the Ca^{2+} current (I_{Ca}) is due to protein kinase A (PKA)-catalyzed phosphorylation [2]. In addition, a membrane-delimited, cAMP-independent potentiation of cardiac and skeletal muscle Ca^{2+} channel activity by purified, GTPγS-activated $G_{\alpha s}$ has been observed [3–6]. It has been proposed that a direct interaction between $G_{\alpha s}$ and the L-type Ca^{2+} channel mediates a minor, fast component of β-adrenergic stimulation of I_{Ca}, whereas the cAMP-dependent phosphorylation mediates a major but slower component [4,7–9]. However, many of the data concerning the membrane-delimited effect of purified $G_{\alpha s}$ were obtained in non-physiological conditions, with channels incorporated into artificial lipid bilayers [3–5]. What happens under physiological conditions remains controversial (reviewed in [10]), with evidence in favor [7,9–11] and against [12] a cAMP-independent effect of $G_{\alpha s}$. There is no biochemical evidence for direct interaction between the cardiac L-type channel and $G_{\alpha s}$; co-immunoprecipitation of the related (but quite dissimilar) skeletal muscle Ca^{2+} channel with $G_{\alpha s}$ has been reported, but only less than 10% of channel protein appeared associated with $G_{\alpha s}$ [6]. In all, it remains unknown whether the membrane-delimited interaction contributes to modulation of I_{Ca} in intact cardiac cells.

The cardiac L-type Ca^{2+} channels are composed of three subunits: the main, pore-forming α_{1C}, the cytosolic β2, and the $\alpha_2\delta$ subunit which is mostly extracellular (for review, see [13,14]. α_{1C} is composed of four homologous membrane domains numbered I–IV and large cytosolic segments: N- and C-terminal domains and the linkers connecting the domains I-II, II-III, and III-IV [13]. Both α_{1C} and β subunits (but not $\alpha_2\delta$) are PKA substrates [15]. Recent studies suggest that α_{1C} plays a major role in mediating the enhancement of channel activity caused by PKA ([16–18]; but see [19], and a single serine residue in the C-terminus (serine 1928) was proposed as the site whose phosphorylation mediates most or all of the PKA effect ([16,20–22]. It is not known how phosphorylation of this residue modulates channel activity; one possibility is that it obstructs an inhibitory action of the C-terminus that has been implicated in channel inactivation and block [23–25].

The aim of this study was to verify and characterize the interaction of $G_{\alpha s}$ with the cardiac Ca^{2+} channel in a heterologous expression system. Antisense knockdown and coexpression experiments in *Xenopus* oocytes demonstrated a positive correlation between $G_{\alpha s}$ expression and the L-type channel function. Coexpression of $G_{\alpha s}$ resulted in an increase in whole-cell current amplitude but not in the number of channels in the plasma membrane. However, we could not find evidence in support of a direct acute regulation of the cardiac L-type channel by G_s upon activation of a β-adrenergic receptor. A possible role for G_s as a part of a protein complex which includes the channel and additional proteins is proposed.

2. Materials and methods

2.1. DNA constructs and mRNA

cDNA of G_α proteins, originally cloned into pGEM2, were generously provided by M. Simon (California Institute of Technology). cDNA of rat $G_{\alpha s}$ [26] was excised with *Nco*I and *Hin*dIII and subcloned into the corresponding sites of a vector derived from pGEM-9zf(−) which contains a 180 bp sequence at the 5′ untranslated region and a 3′ polyadenylation sequence, kindly provided by S.A.N. Goldstein (Brandeis University) [27]. For RNA synthesis, the cDNA was linearized with *Not*I. The coding sequences of rat $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha o}$ [26] were amplified using a standard PCR procedure with Vent polymerase (New England Biolabs) with forward primers containing an *Eco*RI site just 5′ of the initiation codon, and a *Hin*dIII site immediately

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3′ of the termination codon. The PCR products were digested with *Eco*RI and *Hin*dIII and subcloned into the corresponding sites of a high-expression vector, pGEM-HJ, which is a modified version of the pGEMHE [28], to which several additional restriction sites have been added (kindly provided by Z. Selinger, Hebrew University). These DNAs were linearized with *Nhe*I or *Not*I for in vitro RNA synthesis. All PCR products were sequenced at the Tel Aviv University Sequencing Facility. Phosphorothioate antisense oligonucleotides directed against cDNAs of *Xenopus* G protein α subunits were synthesized as described [29]. Capped mRNAs were synthesized in vitro using the suitable RNA polymerases, as described [30]. RNAs specific for the cardiac α_{1C}, β_{2A} and the skeletal muscle α_2/δ subunits were synthesized in vitro using *Asp*718-cleaved pCaH, *Not*I-cleaved pCaB2a and *Sal*I-cleaved pCaA2 as templates [31,32]. RNA of the β_2-adrenergic receptor was synthesized as described [33]. Materials and enzymes for molecular biology were purchased from Boehringer-Mannheim, Promega, or MBI Fermentas.

2.2. Oocyte culture and electrophysiology

Xenopus laevis frogs were anesthetized in a 0.15% solution of procaine methanesulfonate (MS222), portions of ovary were removed through a small incision on the abdomen, the incision was sutured, and the animal was returned to water [30]. The oocytes were collected, defolliculated and injected with RNA as [30,34]. In each experiment, oocytes were injected with equal amounts (by weight) of the mRNAs of the various channel subunits in the desired combinations, and with RNAs of additional proteins or with antisense oligodeoxynucleotides (ODNs) as detailed in the figure legends. Oocytes were incubated at 20–22°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. Whole cell currents were recorded using two-electrode voltage clamp as described [31], in a solution containing 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KOH and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid. All inorganic substances were of analytical or molecular biology grade. Organic materials were from Sigma (Rishon-le-Zion, Israel), unless indicated otherwise. H-89 was from Biolog (Bremen, Germany). H-89 was dissolved in dimethyl sulfoxide (DMSO) at 50 mM and stored in aliquots at −20°C. In the experiments where the effects of H-89 were studied, a control group of oocytes was incubated in H-89-free medium with the addition of DMSO to a final concentration equal to that in the test group.

2.3. Immunochemistry

This was performed as described [27,34]. Oocytes were injected with mRNAs and incubated in NDE solution, containing 0.5 mCi/ml [^{35}S]methionine/cysteine (Amersham) for 3–4 days at 22°C. Plasma membranes together with the vitelline membranes (extracellular collagen-like matrix) were removed manually with fine forceps after a 5–15 min incubation in a low osmolarity solution. The remainder of cell (internal fraction) was processed separately. Ten to 30 plasma membranes and 10 internal fractions were solubilized in 100 μl buffer (4% SDS, 10 mM EDTA, 50 mM Tris (pH 7.5), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM pepstatin, and 1 mM 1,10-phenanthroline) and heated to 100°C for 2 min. Following the addition of 100 μl of H_2O and 800 μl of the immunoprecipitation buffer (190 mM NaCl, 6 mM EDTA, 50 mM Tris (pH 7.5), and 2.5% Triton X-100), homogenates were centrifuged for 10 min at $1000\times g$ at 4°C. The supernatant was incubated for 16 h with the Card-I polyclonal antibody [35], incubated for 1 h at 4°C with protein A Sepharose and pelleted. Immunoprecipitates were washed three times with immunowash buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, and 0.02% SDS). Samples were boiled in SDS-gel loading buffer and electrophoresed on 3–8% polyacrylamide-SDS gradient gel together with standard molecular mass markers (45–205 kDa). Gels were dried and placed in a PhosphorImager (Molecular Dynamics) cassette for up to 3 days. The protein bands of the image were estimated quantitatively using the software ImageQuant, as described [34].

2.4. Data presentation and statistical analysis

The results are always presented as mean ± S.E.M. Multiple-group comparisons have been done by one-way ANOVA test followed by Dunnett's test. Two-group comparisons were done using Student's *t*-test.

3. Results

3.1. Knockdown of $G_{\alpha s}$ by antisense oligodeoxynucleotide reduces Ca^{2+} channel currents

Antisense knockdown methodology has been successfully used in the past to study the coupling of various G_α subunits to phospholipase C and to G protein-activated K^+ channels (GIRK) in *Xenopus* oocytes [29,36,37]. Phosphorothioate ODNs directed against unique parts of the RNAs of *Xenopus* G_α subunits [29] were injected 1–2 days after injection of the RNAs of Ca^{2+} channel subunits (α_1, $\alpha_2\delta$ and β), 2 days be-

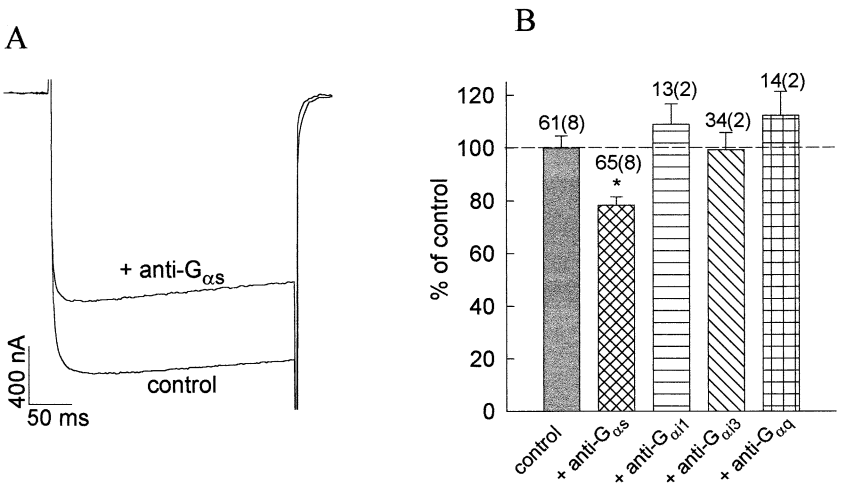

Fig. 1. Effects of antisense ODNs directed against various G_α subunits, on I_{Ba}. Channels were expressed in full-subunit composition, $\alpha_1\alpha_2\delta\beta$ (1 or 2 ng of RNA of each subunit per oocyte was injected). ODNs (30 nl of 1 ng/nl per oocyte) were injected 2 days before testing the Ca^{2+} channel current. The control group was injected with a mismatch ODN. A: Representative Ba^{2+} currents in control and anti-$G_{\alpha s}$ ODN-injected oocytes of one batch, elicited by voltage steps from −80 to +20 mV. B: Summary of the effects of ODNs on the amplitude of I_{Ba}. In each oocyte I_{Ba} was expressed as percent of the mean amplitude of the current in the control group of oocytes from the same donor. These normalized values were averaged across all oocyte batches tested. Data are presented as mean ± S.E.M. Numbers above bars indicate the number of cells assayed, numbers in parentheses indicate the number of donors (oocyte batches). Asterisks indicate statistically significant differences ($P < 0.05$) obtained by one-way ANOVA test followed by Dunnett's test.

fore testing the Ca^{2+} channel currents. A control group of oocytes was injected with a mismatch ODN. The currents were measured using the two-electrode voltage clamp technique with 40 mM Ba^{2+} as charge carrier. Fig. 1A presents an example of the Ba^{2+} current (I_{Ba}) elicited by a voltage step from −80 to 20 mV in two oocytes of the same donor (batch), one injected with a mismatch ODN and the other with anti-$G_{\alpha s}$ ODN. A summary of all experiments is presented in Fig. 1B. Antisense ODN against $G_{\alpha s}$ reduced the amplitude of Ca^{2+} channel currents by 22% on average, whereas ODNs against $G_{\alpha i1}$, $G_{\alpha i3}$, and $G_{\alpha q}$ had no effect. These results imply the possibility of a functional coupling between $G_{\alpha s}$ and the cardiac L-type Ca^{2+} channel expressed in the oocytes.

3.2. Coexpression of $G_{\alpha s}$ increases Ca^{2+} channel currents and modulates inactivation

The possibility of a functional coupling between G_{α} subunits and the Ca^{2+} channel was further examined by the coexpression methodology. RNAs encoding the various G_{α} subunits were coinjected with RNAs of all channel subunits, and the currents were measured 3–4 days later. Expression of the $G_{\alpha s}$ protein was verified by Western blot methodology (data not shown). Coexpression of $G_{\alpha s}$ increased I_{Ba} (an example of an experiment obtained in one oocyte batch is shown in Fig. 2A; see summary of all experiments in Fig. 2E, leftmost pair of columns). The optimal effect was obtained at 0.5–1 ng of $G_{\alpha s}$ RNA per oocyte (Fig. 2B); higher doses often caused oocyte deterioration. The current increase caused by 0.5 ng/oocyte $G_{\alpha s}$ RNA was observed in oocytes of all 10 oocyte batches tested. It averaged 62 ± 6% but varied among batches, ranging between 23% and 145% above control. This may reflect a variability in the amount of endogenous $G_{\alpha s}$ or of another unknown endogenous factor(s). Fig. 2C shows that only coexpression of $G_{\alpha s}$, but not of $G_{\alpha i1}$, $G_{\alpha i2}$, or $G_{\alpha o}$ affected the Ba^{2+} current. The *I-V* curve was not affected by any of the G_{α} subunits tested except $G_{\alpha s}$, which caused a slight (3–5 mV) shift to more negative potentials, suggesting a slightly higher sensitivity to depolarization in the presence of $G_{\alpha s}$ (Fig. 2D).

The importance of the different subunits of Ca^{2+} channel in supporting the effect of $G_{\alpha s}$ on I_{Ba} was examined by expressing the main channel subunit, α_1, with either $\alpha_2\delta$ or β_{2A} (β_2 is the prevalent cardiac β subunit [14,38]). Fig. 2E shows that the enhancement of I_{Ba} occurred in channels of all three sub-

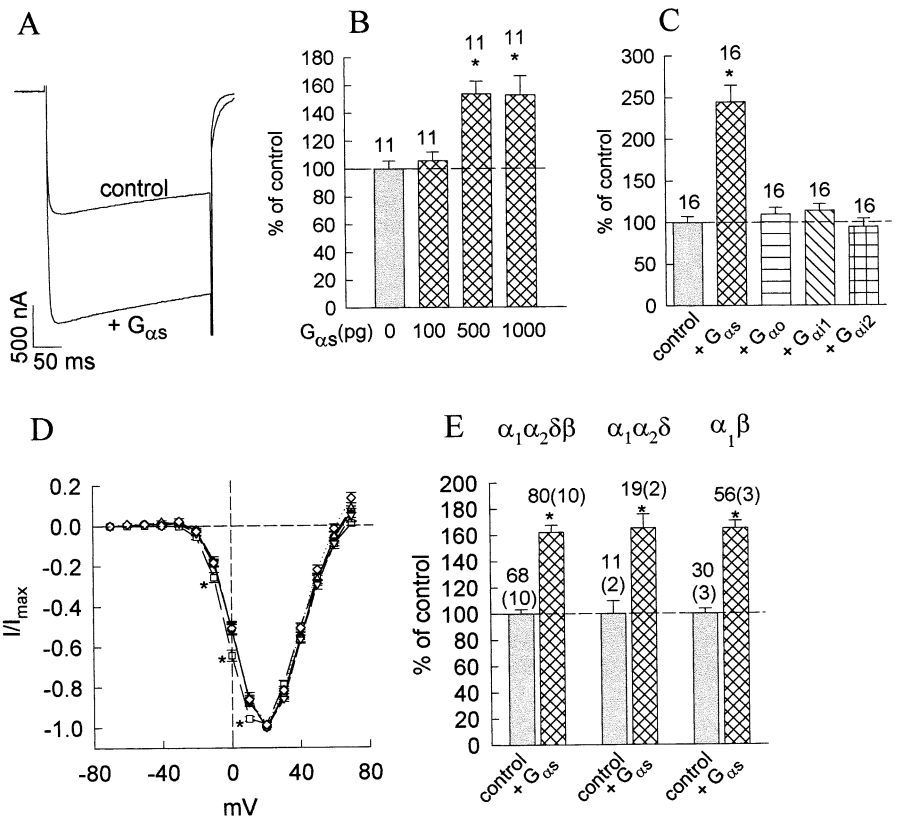

Fig. 2. Effect of coexpression of G_{α} proteins on I_{Ba}. G_{α} subunit RNA (500 pg/oocyte) was injected simultaneously with the RNAs of channel subunits. Asterisks indicate statistically significant differences ($P < 0.05$) obtained by one-way ANOVA and Dunnett's tests (B, C) or by two-tailed *t*-test (D, E). In B–E, data were normalized as described in the legend to Fig. 1B. A: Representative Ba^{2+} currents in control and $G_{\alpha s}$-injected oocytes of one batch, measured by voltage steps from −80 to +20 mV. Channels were expressed in full-subunit composition, $\alpha_1\alpha_2\delta\beta$ (1 ng of each subunit's RNA per oocyte). B: Dependence of $G_{\alpha s}$-induced increase in I_{Ba} on the amount of the injected $G_{\alpha s}$ RNA (indicated on the abscissa). Data from one batch of oocytes. Numbers above bars indicate the number of cells assayed. C: Effects of coexpression of G_{α} proteins (500 pg RNA each) on the amplitude of I_{Ba} in a representative batch expressing channels of full subunit composition. D: *I-V* curves normalized to the peak current from control and G_{α}-injected oocytes of one batch (eight oocytes in each group). ●, control (no G_{α}); □, $G_{\alpha s}$; △, $G_{\alpha o}$; ▽, $G_{\alpha i1}$; ◇, $G_{\alpha i2}$. E: Coexpression of $G_{\alpha s}$ with the L-type Ca^{2+} channels: effects of subunit composition. Combinations of RNAs of channel subunits $\alpha_{1C}+\alpha_2\delta+\beta_{2A}$ (1 or 2 ng of each subunit per oocyte) and $\alpha_{1C}+\alpha_2\delta$, $\alpha_{1C}+\beta_{2A}$ (2.5 ng of each subunit per oocyte) were injected with or without $G_{\alpha s}$ RNA (500 pg/oocyte). Numbers above bars indicate the number of cells assayed, and the number of oocyte batches (in parentheses).

unit combinations tested, suggesting that neither $\alpha_2\delta$ nor β_{2A} was necessary for this effect, and that it was most probably mediated via an interaction with α_1.

3.3. Coexpression of $G_{\alpha s}$ does not increase the amount of α_1 protein

$G_{\alpha s}$ could increase I_{Ba} amplitude by altering the expression, transport to the membrane, degradation, or other processes that regulate the amount of functional channels in the plasma membrane. In *Xenopus* oocytes, the plasma membrane can be mechanically separated from the rest of the cell, called internal fraction, and then the two fractions can be analyzed separately [27]. Newly synthesized proteins are metabolically labeled with ^{35}S by incubating the oocytes in [^{35}S]methionine/cysteine for 3 days following the RNA injection, immunoprecipitated, subjected to SDS-PAGE, and the relative amount of protein is quantified using an imaging procedure. This allows high-precision measurements of changes in the content of protein in whole oocytes and in plasma membrane, caused by coexpression of other proteins [27,34].

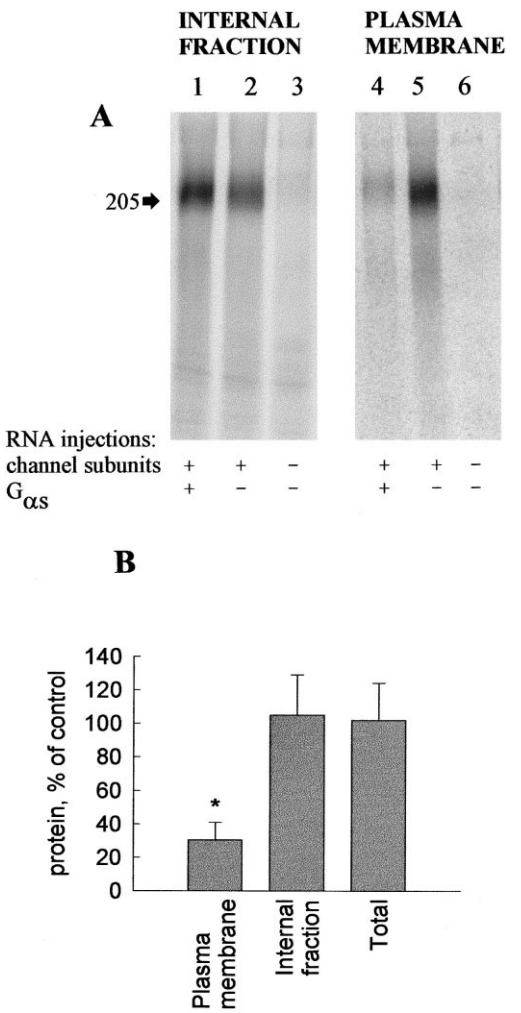

Fig. 3. Effect of coexpression of $G_{\alpha s}$ on the level of α_{1C} protein in *Xenopus* oocytes. Channels were expressed in full-subunit composition, $\alpha_1\alpha_2\delta\beta$ (5 ng of each subunit's RNA per oocyte). $G_{\alpha s}$ RNA (1 ng per oocyte) was injected simultaneously with RNAs of the channel subunits. A: Digitized PhosphorImager scan of SDS-PAGE analysis of the immunoprecipitated α_{1C} protein labeled with [^{35}S]Met/Cys. α_{1C} protein was immunoprecipitated from 10 internal fractions (lanes 1–3) and from 30 plasma membranes (lanes 4–6). Arrow indicates the 205 kDa protein marker. B: Relative amount of the α_{1C} protein in oocytes expressing the channel with $G_{\alpha s}$, expressed as percent of the amount of α_{1C} in oocytes of the same batch expressing the channel alone. In each batch, the amount of α_{1C} per oocyte in the plasma membrane or in the internal fraction was calculated, normalized to control in the same batch as explained above, and the results were averaged across all four batches tested. The total amount of α_{1C} per oocyte was calculated as the sum of the amounts in the plasma membrane and in the internal fraction. Asterisk indicates statistically significant difference ($P < 0.05$; two-tailed *t*-test).

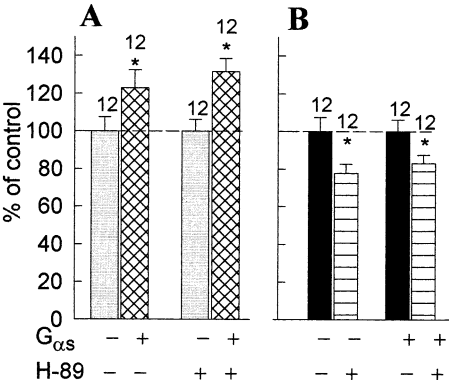

Fig. 4. The PKA inhibitor H-89 (10 μM) does not change the effect of coexpression of $G_{\alpha s}$ on I_{Ba}. Oocytes of one batch were injected with RNAs of all channel subunits (2 ng of each subunit per oocyte) with or without $G_{\alpha s}$ (500 pg per oocyte) and incubated in H-89 for 3 days until the currents were measured. A: Effect of $G_{\alpha s}$ on the amplitude of I_{Ba} with or without H-89. B: Same results as in A are presented to show the effect of H-89 on the amplitude of I_{Ba} with or without $G_{\alpha s}$. Normalization of the data was done as described in Fig. 1B. Numbers above bars indicate the number of cells assayed. Asterisks indicate statistically significant differences ($P < 0.05$; one-tailed *t*-test).

The channels were expressed in the full subunit composition, with or without $G_{\alpha s}$. The α_1 subunit was immunoprecipitated [34] with the Card-I antibody [35] and analyzed as explained above. Fig. 3A shows a representative experiment (one out of four) which demonstrated that coexpression of $G_{\alpha s}$ did not significantly affect the amount of α_1 protein in the internal fraction (left panel, lanes 1 and 2). Strikingly, the amount of α_1 in the plasma membrane was decreased, rather than increased, by the coexpression of $G_{\alpha s}$ (Fig. 3A, right panel, compare lanes 4 and 5). Oocytes uninjected with RNA gave no signal (Fig. 3A, lanes 3 and 6). Fig. 3B summarizes the results of the quantitative analysis of the experiments in all four oocyte batches. It shows that coexpression of $G_{\alpha s}$ significantly reduced (by 70%) the amount of α_1 protein in the plasma membrane. The total cellular content of α_1 protein, as well as its amount in the internal fraction, remained unchanged. (The internal fraction contained $96.9 \pm 1.0\%$ of total α_1 protein in this series of experiments; hence the almost full coincidence between total and internal fraction α_1.) Thus, it is unlikely that the enhancement of I_{Ba} caused by coexpression of $G_{\alpha s}$ is due to an increase in the amount of channels in the plasma membrane.

3.4. The $G_{\alpha s}$-induced increase in I_{Ba} is not mediated by protein kinase A

Theoretically, overexpression of $G_{\alpha s}$ could cause a constitutive activation of adenylyl cyclase and thus of PKA, resulting in increased I_{Ba}. Cardiac Ca^{2+} channels expressed in *Xenopus*

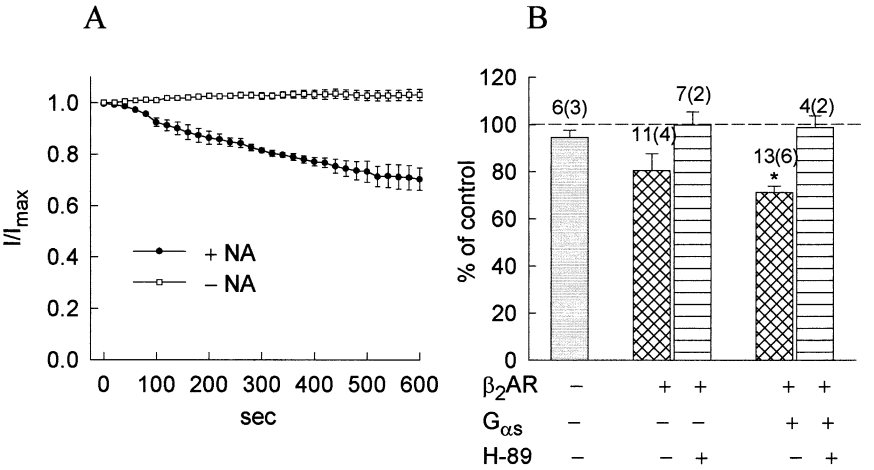

Fig. 5. Effect of noradrenaline (NA) on the L-type Ca^{2+} channel current. Channels were expressed in full-subunit composition, $\alpha_1\alpha_2\delta\beta$ (2 ng of each subunit's RNA per oocyte) and, where indicated, RNAs of β_2AR (50 pg per oocyte) and $G_{\alpha s}$ RNA (100 pg per oocyte) were also injected. After stabilization of the current, NA (10^{-6} M) was added for 10 min. In each oocyte, I_{Ba} was expressed as percent of the current amplitude before application of noradrenaline. These normalized values were averaged across all oocytes in all batches tested. A: Time course of the effect of NA in three oocytes from two batches. Cells bathed in Ba^{2+} solution not containing NA are presented as the control group (five oocytes from two batches). B: Summary of the effects of noradrenaline on the amplitude of I_{Ba}, 10 min after application. Where indicated, the oocytes were incubated for 2 h in H-89 (50 μM). Numbers above bars indicate the number of cells assayed, numbers in parentheses indicate the number of batches. Asterisk indicates statistically significant difference ($P < 0.05$; two-tailed t-test).

oocytes are not up-regulated by acute elevation of cAMP levels [18,39]. However, it was important to rule out the possibility that a persistent increase in intracellular cAMP might underlie the effect of $G_{\alpha s}$. To do this, oocytes were treated with a PKA inhibitor, H-89 (10 μM), during the whole period of incubation (3–4 days) following RNA injection. Fig. 4A shows that this treatment did not alter the effect of $G_{\alpha s}$ coexpression: the increase in I_{Ba} caused by $G_{\alpha s}$ was practically identical in untreated cells (left pair of columns) and in H-89-treated cells (right pair of columns). The efficiency of H-89 treatment in inhibiting PKA was confirmed by comparing, in the same experiments, the amplitudes of I_{Ba} in H-89-treated vs. untreated cells (Fig. 4B). H-89 decreased the amplitude of I_{Ba} by 20–25%, independently of the presence of coexpressed $G_{\alpha s}$. This reduction is very similar to that observed after a 2–4 h incubation in H-89 [20]; it is believed to reflect dephosphorylation of a crucial PKA site by constitutively active phosphatases in the absence of an active PKA [16,18,20].

An additional test of involvement of PKA was done by coexpressing $G_{\alpha s}$ with a mutant of the α_1 subunit (α_{1C}S1928A) lacking a crucial PKA phosphorylation site, Ser-1928 [16,20–22]. Channels containing this mutant subunit do not respond to PKA inhibition [20] or activation [16] in expression systems. In four oocyte batches (22 oocytes), coexpression of $G_{\alpha s}$ with a full-subunit channel containing α_{1C}S1928A increased I_{Ba} by 79 ± 11%, which is not significantly different from the 62 ± 6% increase observed in the wild-type channels. Taken together, these results suggest that $G_{\alpha s}$-induced increase in I_{Ba} is not mediated by the cAMP pathway.

3.5. Acute activation of G_s by β_2-adrenergic receptor (β_2AR) does not enhance I_{Ba}

Despite the bold enhancing effect of coexpression of $G_{\alpha s}$ on the amplitude of I_{Ba}, acute activation of G_s via β_2AR did not enhance the Ca^{2+} channel current. In this series of experiments, the oocytes were injected with RNA of the channel (full composition) and, when desired, RNAs of β_2AR and $G_{\alpha s}$. The latter was injected in a lower amount than in the previous coexpression experiments (100 instead of 500 pg), to avoid an excessively high basal activity of the overexpressed $G_{\alpha s}$ (cf. Fig. 2B), in which case activation of β_2AR might be ineffective. Fig. 5A shows that the adrenergic agonist (−)noradrenaline (NA) reduced (rather than enhanced) I_{Ba}. In control groups of oocytes exposed to Ba^{2+} solution not containing NA for 10 min, there was no decrease in I_{Ba}, demonstrating the absence of rundown. The NA-induced inhibition developed within several minutes, reaching a maximum of 28 ± 3% inhibition after 10 min (see Fig. 5B for a summary). Similar, though weaker, inhibition was observed in cells not injected with $G_{\alpha s}$ RNA, presumably due to the presence of an endogenous $G_{\alpha s}$ in the oocytes. In oocytes that did not express exogenous β_2AR, I_{Ba} was not significantly reduced by NA (Fig. 5B). The slow development of the inhibition suggested the involvement of an intermediate product, such as cAMP. Indeed, incubation in H-89 completely abolished the inhibitory effect of noradrenaline, but did not 'uncover' any enhancing PKA-independent effects (Fig. 5B).

4. Discussion

4.1. $G_{\alpha s}$ increases Ca^{2+} channel currents via an interaction with the α_{1C} subunit

Our results demonstrate that $G_{\alpha s}$ up-regulates Ca^{2+} channels expressed in *Xenopus* oocytes. The positive correlation between the level of $G_{\alpha s}$ and Ca^{2+} channel current amplitude was shown in two ways: (i) decreasing the level of endogenous $G_{\alpha s}$ in the oocytes by antisense knockdown decreased the current; (ii) overexpression of exogenous $G_{\alpha s}$ increased the current. The positive modulation by coexpressed $G_{\alpha s}$ is cAMP-independent, because it is not affected by a PKA inhibitor, and is observed with a phosphorylation-deficient mutant of α_{1C}.

$G_{\alpha s}$ enhances the functioning of the expressed channel rather than its overall expression. The immunochemical meas-

urements actually indicate a more than twofold decrease in the amount of channels in the plasma membrane, although the total cellular α_{1C} content is not altered by $G_{\alpha s}$. This would have caused a decrease in the whole-cell Ca^{2+} channel, unless a counteracting (and overriding) enhancement in channel's gating was present. The small but reproducible leftward shift in the voltage dependence of activation (*I-V* curve) is indicative of a change in gating properties. An alternative (or additional) possibility is that not all of the channels found in the plasma membrane are functional, in which case the change detected by the immunochemical measurements may not reflect the actual situation in the pool of functional channels. In this case, the increase in total Ca^{2+} current would indicate mainly an increased amount of correctly processed or assembled functional Ca^{2+} channels, whereas the concomitant decrease in the amount of α_{1C} protein in plasma membrane may indicate a more efficient elimination of the 'incorrect', non-functional channels. It may be difficult to distinguish between these possibilities at present; development of new methods to distinguish between functional and non-functional channels in the membrane may be necessary.

The main site of interaction of $G_{\alpha s}$ with the channel is the main subunit, α_{1C}. Channels missing either one of the auxiliary subunits ($\alpha_2\delta$ or β) were regulated by coexpression of $G_{\alpha s}$ in the same way as the full-composition channels. At present it is not clear whether the interaction is direct or via intermediate proteins.

Our results suggest high specificity of the L-type Ca^{2+} channel toward $G_{\alpha s}$. Of the four G_α subunits tested, only coexpression of $G_{\alpha s}$ increased I_{Ba}. Correspondingly, knockdown of the endogenous $G_{\alpha s}$ decreased I_{Ba}, while antisense ODNs against several other G_α subunits tested ($G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha q}$) were without effect. The inefficiency of anti-$G_{\alpha i1}$, $G_{\alpha i3}$, $G_{\alpha q}$ ODNs could not be due to a technical problem, since the very same antisense ODNs (except anti-$G_{\alpha i3}$) strongly modulated other G protein-mediated responses in *Xenopus* oocytes: ODN against $G_{\alpha q}$ attenuated receptor-evoked phospholipase C activation [36], whereas anti-$G_{\alpha i1}$ ODN specifically enhanced activation of a G protein-activated K^+ channel by a muscarinic receptor [29]. Similarly, coexpression of $G_{\alpha i2}$ potentiates G protein-activated K^+ channel currents (D. Vorobiov and N. Dascal, unpublished observations).

4.2. *cAMP-dependent and cAMP-independent modulation of the L-type channel by $G_{\alpha s}$*

When this study was initiated, we reasoned that *Xenopus* oocyte may be a perfect system to study cAMP-independent modulation of the cardiac L-type channel, because in this preparation the expressed cardiac L-type channel is not enhanced by cAMP or/and PKA [18,39]; similar behavior is observed in several other cell types such as CHO and HEK [16,40,41]. In HEK cells, PKA up-regulation of cardiac L-type channel can be partially restored by coexpression of AKAP-79 [16]. In the present experiments activation of $G_{\alpha s}$ via the β_2AR failed to increase I_{Ba}. We interpret these data to support the conclusions of Hartzell et al. [12] that an acute cAMP-independent β-adrenergic enhancement of Ca^{2+} current does not normally take place in intact cells.

We propose a hypothesis based on the concept emerging from several recent studies indicating that the L-type Ca^{2+} channel is a part of a multimolecular complex which also includes PKA, PKA-anchoring proteins, phosphatase(s), and possibly adenylyl cyclase ([16,38,42–44]; see [45] for review). We believe that $G_{\alpha s}$ interacts with the channel (directly or via an intermediate) within such a complex, and its presence is important for the correct functioning of the whole complex, including the Ca^{2+} channel.

The small but reproducible decrease in I_{Ba} caused by activation of $G_{\alpha s}$ via the β_2AR is opposite to what is observed in the heart and warrants an explanation. This decrease appears to be PKA-mediated since it is completely blocked by H-89. This effect is not peculiar to *Xenopus* oocytes but has been observed in smooth muscle cells [46,47] where cAMP also does not up-regulate the activity of L-type channels. It is possible that the absence of a PKA-induced enhancement in the oocytes uncovers a smaller but genuine decrease in channel activity caused by phosphorylation of a separate PKA site in one of the channel subunits. Another possibility is that the target of PKA phosphorylation (that caused the decrease in I_{Ba}) is not the channel itself but the β_2AR which uncouples from $G_{\alpha s}$ and couples to a G_i/G_o protein upon PKA phosphorylation [48].

Our results correlate perfectly with the recent report (published while this manuscript was in the final stages of preparation) that cardiac L-type Ca^{2+} channel current in transgenic mice overexpressing $G_{\alpha s}$ is constitutively increased compared with the wild type, and that this effect is cAMP-independent and does not involve an increase in total cellular content of channel protein monitored by dihydropyridine binding [49]. The authors propose that $G_{\alpha s}$ may participate in regulation of the 'baseline' activity of the Ca^{2+} channels in the heart. The fact that overexpression of $G_{\alpha s}$ similarly affects Ca^{2+} channels in the heart and in the expression system used by us emphasizes the physiological relevance of our findings and strongly suggests a similar mechanism for G_s regulation in both preparations. Furthermore, it opens the way for the use of *Xenopus* oocytes for further elucidation of $G_{\alpha s}$ modulation mechanism.

In view of these considerations, the physiological significance of our findings can be viewed in the following context. First, we confirm that the level of expression of $G_{\alpha s}$ is likely to set the background level of Ca^{2+} channel activity in the heart. Secondly, our data help to resolve the existing controversy on the possibility of a direct regulation of L-type Ca^{2+} channel by sympathetic agonists; they support the conclusions of Hartzell et al. [12] suggesting that such an effect does not contribute to an acute modulation of Ca^{2+} channels in the heart. Finally, the cAMP-mediated decrease in L-type Ca^{2+} channel current, normally not observed in the heart, is a well-characterized phenomenon in smooth muscle cells, suggesting that the *Xenopus* oocyte expression system may be used to study the molecular mechanism of this phenomenon. The absence of an enhancing effect of cAMP, already discussed above and in [16,18], a hallmark of this expression system, may actually turn out to be useful in this respect.

5. Conclusions

The main findings of this study may be summarized as follows: $G_{\alpha s}$ specifically modulates the cardiac L-type Ca^{2+} channel by changing its functioning via a functional interaction with the pore-forming subunit, α_{1C}. Our data do not support the hypothesis that a direct interaction of $G_{\alpha s}$ with L-type Ca^{2+} mediates part of the current enhancement caused

by activation of β-adrenergic receptors in the heart, but do suggest a role for $G_{\alpha s}$ in modulation of Ca^{2+} channel function, possibly within a multimolecular complex that serves to localize and compartmentalize the effectors, and to accelerate the signal transduction.

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