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A *Xenopus* oocyte β subunit: Evidence for a role in the assembly/expression of voltage-gated calcium channels that is separate from its role as a regulatory subunit

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ABSTRACT **Two closely related β subunit mRNAs (xo28 and xo32) were identified in *Xenopus* oocytes by molecular cloning. One or both appear to be expressed as active proteins, because: (*i*) injection of *Xenopus* β antisense oligonucleotides, but not of sense or unrelated oligonucleotides, significantly reduced endogenous oocyte voltage-gated Ca^{2+} channel (VGCC) currents and obliterated VGCC currents that arise after injection of mammalian α_1 cRNAs (α_{1C} and α_{1E}); (*ii*) coinjection of a *Xenopus* β antisense oligonucleotide and excess rat β cRNA rescued expression of α_1 Ca^{2+} channel currents; and (*iii*) coinjection of mammalian α_1 cRNA with cRNA encoding either of the two *Xenopus* β subunits facilitated both activation and inactivation of Ca^{2+} channel currents by voltage, as happens with most mammalian β subunits. The *Xenopus* β subunit cDNAs (β3xo cDNAs) predict proteins of 484 aa that differ in only 22 aa and resemble most closely the sequence of the mammalian type 3 β subunit. We propose that "α_1 alone" channels are in fact tightly associated $\alpha_1\beta$3xo channels, and that effects of exogenous β subunits are due to formation of higher-order $[\alpha_1\beta]\beta_n$ complexes with an unknown contribution of β3xo. It is thus possible that functional mammalian VGCCs, rather than having subunit composition $\alpha_1\beta$, are $[\alpha_1\beta]\beta_n$ complexes that associate with $\alpha_2\delta$ and, as appropriate, other tissue-specific accessory proteins. In support of this hypothesis, we discovered that the last 277-aa of α_{1E} have a β subunit binding domain. This β binding domain is distinct from the previously known interaction domain located between repeats I and II of calcium channel α_1 subunits.**

Xenopus oocytes translate exogenously injected mRNAs and cRNAs with relatively high efficiency. This has made them systems of choice for the functional expression and characterization of many cloned molecules, such as neuronal ligand-gated ion channels, G protein-coupled receptors, and many voltage-gated ion channels, including voltage-dependent Ca^{2+} channels. Voltage-dependent Ca^{2+} channels are formed of an α_1 pore-forming and voltage-sensing subunit and β and $\alpha_2\delta$ regulatory subunits. Functional expression in *Xenopus* oocytes has not only been used to define structure–function relations of voltage-gated calcium channels by assessing the effects of specific mutations of the α_1 channel protein, but also to define identity and roles of the regulatory subunits in promoting α_1 expression or modifying the properties of the expressed α_1 subunit. Several nonallelic genes encoding α_1 subunits, termed α_{1S} and α_{1A}–α_{1E}, have been identified by molecular cloning (1–4). Of these, all except α_{1S} have been functionally expressed in the *Xenopus* oocyte. However, while certain variants of α_{1C} and α_{1E} can be expressed without coinjection of other subunit cRNAs (e.g., refs. 5 and 6), others, particularly α_{1A}, yield only minimal currents in the absence of additional subunits, notably a β subunit (7, 8). The reasons for these differences are not understood.

The interpretation of results obtained expressing cloned Ca^{2+} channel subunits to define intrinsic properties of α_1 and the effects of regulatory subunits have, of course, assumed that oocytes do not express equivalent endogenous subunits. Yet this assumption has not been rigorously tested. We report here the detection of β subunit mRNA in defolliculated, stage IV–VI oocytes, such as are used for α_1 subunit expression. We cloned two full-length cDNAs derived from what appear to be two alleles of the same gene and show that the product of this gene is constitutively expressed in oocytes. In addition, we report that α_{1E} has two independent β subunit binding domains.

METHODS

Molecular Cloning of Calcium Channel β Subunit cDNAs from Oocyte mRNA. Oligo(dT)-purified mRNA (0.3 g) of stage IV–VI oocytes that had been defolliculated by treatment with collagenase was prepared using the FastTrack RNA Isolation Kit (Invitrogen). Presence of mRNA encoding *Xenopus* calcium channel β subunit homolog(s) was tested using PCR. Full-length cDNAs were cloned using a strategy that combined PCR and rapid amplification of cDNA ends (RACE) PCR with reagents and protocols supplied by the Marathon Amplification Kit (CLONTECH). The reaction products from each of the PCR and RACE PCR reactions were cloned into the Invitrogen pCRII TA cloning vector. Individual clones with inserts of the expected sizes were picked, expanded, and sequenced with Sequenase II (United States Biochemical) by the dideoxynucleotide chain termination method (9), using double-stranded DNA as template. The true nucleotide sequence of the full-length cDNA was deduced from sequencing five PCR clones, each from an independent PCR amplification. Full-length coding sequences of two β subunit-like sequences were obtained, xo28 and xo32, and spanned nucleotide −60, before the ATG initiator codon (A of ATG = 1), through nucleotide 22, after the TGA stop codon. The open reading frames of clones 2-2 of xo28 and 4-3 of xo32 were subcloned into the transcription competent plasmid pAGA2 (10). *Taq* polymerase purchased from Takara Shuzo (Kyoto) was used throughout. We noted an error rate

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Abbreviations: RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase.

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of 1 nt in 400. Due to the engineering of the *Nco*I site at the ATG initiation codon, the N termini of xo28 and xo32 from pAGA2 cRNAs were Met-Val instead of Met-Tyr.

cRNAs of the β3xo, β1b (11), human α_{1E} (6), and the high expressor ΔN60 mutant of the rabbit cardiac α_{1C}, which lacks amino acids 1–59 (X. Wei and L.B., unpublished work), were synthesized using mMessage mMachine reagents and protocols purchased in kit form from Ambion (Austin, TX).

Xenopus laevis oocytes were isolated and injected as described (12), and electrophysiological recordings from oocytes were made using the cut-open Vaseline gap voltage-clamp method (13) as modified in ref. 12.

Calcium Channel Currents. Except when indicated otherwise, the external solution had the following composition: 10 mM Ba^{2+}, 96 mM Na^+, and 10 mM Hepes, titrated to pH 7.0 with methanesulfonic acid (CH_3SO_3H). The solution in contact with the oocyte interior was 110 mM K-glutamate and 10 mM Hepes, titrated to pH 7.0 with KOH. Low access resistance to the oocyte interior was obtained by permeabilizing the oocyte with 0.1% saponin. Activation of Cl^- current by Ba^{2+} influx through the Ca^{2+} channel was eliminated by injecting 100–150 nl of 50 mM BAPTA-Na_4 (1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetate) before recording (12). The BAPTA solution was adjusted to pH 7.0 with methanesulfonic acid. The holding potential was −90 mV. The linear components of the currents, corresponding to the scaled currents elicited by small negative control pulses of one-fourth the amplitude of the stimulating pulse, from −90 mV subtracting holding potential, were subtracted on-line. The data were sampled at 10 kHz and filtered at 2 kHz.

Glutathione *S*-Transferase (GST) Fusion Proteins, *in Vitro* Translation of Ca^{2+} Channel β2a, and Binding of β2a to GST Fusion Proteins. GST fusion plasmids were based on pGEX-4T-1 (Pharmacia) and were constructed by conventional means using either natural restriction fragments of calcium channel α_1 subunits or defined fragments excised by PCR. After transfection into *Escherichia coli* BL21, synthesis of the fusion proteins was induced with 0.2 mM isopropyl β-D-thiogalactoside (IPTG) in a liquid culture grown to OD of 1.0. After 2–3 hr at 37°C, the cells were collected by centrifugation, resuspended in NETN lysis buffer (0.5% Nonidet P-40/1 mM EDTA/20 mM Tris·HCl, pH 8.0/100 mM NaCl; 1.0 ml per 20 ml of culture) and lysed by sonication. The lysate was cleared by centrifugation at 10,000 × *g* for 10 min at 4°C. The GST fusion proteins in the supernant were adsorbed for 30 min at room temperature to glutathione-agarose beads in NETN [1 volume of lysate:1 volume of 50% (vol/vol) slurry of agarose-GSH beads (Pharmacia) in NETN]. The last wash was with binding buffer [1% (vol/vol) Lubrol-PX/2 mM EDTA/100 mM NaCl/20 mM Tris·HCl, pH 8.0] instead of NETN. Slurries [50% (vol/vol)] slurries of washed agarose-GSH beads with GST fusion proteins adsorbed to them (agarose-GSH::GST fusions) were then incubated for 30 min at room temperature in an equal volume of binding buffer with ^{35}S-labeled β2a (Promega), synthesized by coupled transcription–translation (Promega), as described in Pragnell *et al.* (14). After a 50-fold dilution in binding buffer, the beads were centrifuged and washed three times with binding buffer and resuspended in an equal volume of 4× Laemmli's sample buffer. Proteins released from the beads were analyzed by 10% SDS/PAGE followed by autoradiography to detect retention of β2a by the α_1 fragments fused to GST. *In vitro*-translated [Q212L]$G_s\alpha$, a constitutively activated human 379-aa human $G_s\alpha$ subunit was used in place of calcium channel β2a to test for specificity in the interaction of β2a with the α_{1E} fragments.

RESULTS

A Calcium Channel β Subunit mRNA in *Xenopus* Oocytes: β3xo. We designed mixtures of oligonucleotides based on regions of high amino acid sequence identity among the four known types of mammalian β subunits, and we probed oocyte mRNA for presence of a β subunit-like sequence using reverse transcription-PCR. Two very similar PCR products, xo28 and xo32, were identified. The xo28 and xo32 PCR products were each of 430 nt (excluding the PCR primers) and differed in 25 nt. The sequences in xo28 and xo32 coded for 143 aa of two β subunits that differ in only 6 aa. Full-length cDNA versions of xo28 and xo32 were then cloned by a combination of PCR and RACE PCR techniques and found to be almost identical along their entire length. As determined by sequencing, xo28 has an open reading frame of 1455 nt comprising 484 codons preceded by 89 nt of 5′ untranslated sequence with an in-frame stop codon 42 nt upstream of the putative initiator ATG. The open reading frame is followed by 578 nt of 3′ untranslated sequence. xo32 has an open reading frame of the same length as xo28, differing from xo28 in 74 roughly randomly distributed nucleotides, and encodes a protein that differs from xo28 in 22 aa. The 3′ untranslated sequence of xo32 is of 588 nt and differs from that of xo28 in 15 nt, 14 of which are clustered in the last 50 nt before the poly(A) tail. xo32 lacks nucleotides 1991–1993 of xo28, reads C instead of T at position 2033 of xo28, the last nucleotide before the poly(A) tail, and has an additional 14 nt before the initiation of the poly(A) tract. For xo32, 5′ RACE PCR yielded 126 nt upstream of the initiator ATG, of which the first 89 nt 5′ of the initiator ATG differ from those of xo28 in a single nucleotide at position −11.

Multiple sequence analysis, in which the deduced amino acid sequences of xo28 and xo32 were compared with the amino acid sequences of mammalian β1, β2, β3, and β4, showed that the *Xenopus* sequences are β3-like. We refer to them as β3xo subunits. Existence of two β3xo genes should not be surprising, as *X. laevis* is a tetraploid organism (cf. ref. 15). Fig. 1 compares the β3xo sequences to the mammalian β sequences. The high degree of sequence identity of xo28 and xo32 and their similarity to mammalian β3 subunits should be noted.

Functional Expression of *Xenopus* β3. Coinjection of either of the β3xo cRNAs with mammalian α_{1E} mimicked the previously reported effects of coinjecting mammalian β3 (16) in that both variants facilitated voltage induced activation and inactivation of the α_{1E} channel. As illustrated in Fig. 2 for the xo28 *Xenopus* β subunit, conductance–voltage (*G*–*V*) relations obtained in its presence and absence were fitted well by two Boltzmann distributions, the effect of β3xo being primarily to increase the relative amplitude of the proportions of channels activated at the lower voltage. Likewise, steady-state inactivation curves obtained as a function of voltage were shifted to more negative potentials by ≈20 mV by the coexpression of the β3xo. Essentially the same results were obtained with the xo28 and xo32 *Xenopus* β subunit (data not shown). This showed that xo28 and xo32 each encode a bona fide calcium channel β subunit.

Effects of xo28 and xo32 Antisense Oligonucleotides on Expression of Calcium Channel Currents. Two sets of experiments were performed to test for a possible role of endogenously expressed oocyte β3 subunits. The first probed for effects of antisense oligonucleotides on expression of endogenous oocyte calcium currents. The second tested for the effects of antisense oligonucleotides on expression of exogenously injected mammalian α_1 subunits.

Previous studies by Lacerda *et al.* (17) had shown enhancement of endogenous oocyte T- and L-type currents by expression of a mammalian β subunit. This indicated that *Xenopus* Ca^{2+} channels are susceptible to regulation by β subunits. The finding that oocytes have mRNA encoding a β subunit therefore raised the possibility that endogenous Ca^{2+} channel currents might be dependent on endogenous β subunits, as has been found for expression of the mammalian α_{1A}. We thus tested for an effect of anti-*Xenopus* oocyte oligonucleotides on expression of endogenous Ca^{2+} channel currents. In contrast

A

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rtβ4:          MSSSYAKNGAADGPHSPSSQVARGTTTRRSRLKRSDGSTTSTSFILRQ----   52
rtβ3:                                                ----S-VP------A----   19
xoβ3-32:                                             --E------I---------   19
xoβ3-28:                                             MYDDLYLHGFEDSEVGSAD   19
rtβ2a:                                               MQCCGLVHRRRVRVSY----   20
    b:              MNQASGLDLLKISYGKGARRKNRFKGSDGSTSSSDTTSNSNFVRQ----   49
rbβ1:   MVQKTSMSRGPYPPSQEIPMEVFDPSPQGKYSKRKGRFKRSDGSTS-DTTSNSFVRQ---E   61

rtβ4:   --------_-----------AI-Q-R-Q--AI------S-------K-------A---DV-  111
rtβ3:   --------------------S------S----------KH---------------V------   79
xoβ3-32 ---------------------A----------E-------------------------------   79
xoβ3-28 SYTSRPSLDSDVSLEEDRETARREVERQAQQQLERARLKPVAFAVRTNVSYCGALDEECP   79
rtβ2a:  --------_-----------AV---A-R---A---K--T-----------R-SAAQEDDV-   79
rbβ1:   --------_-----------AL-K-A-R--LA----K--T-----------G-NPSPGD-V-  119

rtβ4:   -PSTAIS-D-----------_N------------CE-G-----L---N--IQ----RG__  168
rtβ3:   ---SGV---------------_S-----------G--A---------S------------  138
xoβ3-32 ---------------------S-D----------G-------------------------  139
xoβ3-28 VQGAAINFEAKDFLHIKEKY_NNDWWIGRLVKEGADITFIPSPQRLEAIRLKQEQKARRS  138
rtβ2a:  -P-MAIS--------V---F_N------------CE-G-----VK--NM--QH--R-KQG  138
rbβ1:   -E-VAIT--P-----------N------------CEVG-----VK-D-L--L----L-Q-  179

rtβ4:   RFHGGKSS--SS---GEMVS-TF-AT-TTT_____________________________  198
rtβ3:   _________GNP_SSLS-I__----------L_____________________________  157
xoβ3-32 ---------------------T-----------------------------------------  154
xoβ3-28 ----------------GGDMVSGNRRSPPPSI_____________________________  154
rtβ2a:  KFYSSKSG--SS---G--VPSS-K-T---S_______AIDIDATGLDAEENDIPANHRSP  191
rbβ1:   RLSSSKSGD-SS---G-VVT-T--PT--ASGNEMTNLAFELEPLDLEEDEAELGEQSGSA  239

rtβ4:   _____________________------VT--I-----------------------------  236
rtβ3:   _____________________----------VP-----------------------------  195
xoβ3-32 _____________________---------I-----------------------------  192
xoβ3-28 _____________________AKQKQKQAEHTAPYDVVPSMRPVVLVGPSLKGYEVTDM  192
rtβ2a:  KPSANSVTSPHSKEKRMPFFKK_______T--T-----------------------------  244
rbβ1:   KTSVSSVTTPPPHGTRIPFFKK_______T--------------II--------------  292

rtβ4:   ------------D----------I----------S--A-----N----L--------  296
rtβ3:   ---------------------------------T------A----A-------  255
xoβ3-32 --------MR-------------------------------------A------  252
xoβ3-28 MQKALFDFLKHRFDGRISITRVTADLSLAKRSVLNNPGKRAIIERSSTRSSIVEVQSEIE  252
rtβ2a:  -------------E----------I----------S-HA-----NT---L--------  304
rbβ1:   -----------------------I----------S-HI-----NT---L--------  352

rtβ4:   ------R-------------------I---------H-------------K------S---  356
rtβ3:   -----A-S----V----------A--A-----------------R------M---  315
xoβ3-32 -----A----------------S---------V-----------------------  312
xoβ3-28 RIFELSKTLQLVILDADTINHPTQLSKTSLAPIIVFVKVSSPKELQRLIKSRGKSQVKHL  312
rtβ2a:  ------RT----------------S---------Y--I----------K------A---  364
rbβ1:   ------RT----A----------S---------YI-IT----------K------S---  412

rtβ4:   N--LV-A---A----_-M----------E------G---A------TSSSTPMTP_L-G  422
rtβ3:   T---M-Y---V----_-S----------D---------L----R----___--APGPGMLG  379
xoβ3-32 ---------------_-------------------------------------P----  379
xoβ3-28 NVQMLAADKLIQCPP_EMFDVILDENQLEDACEHLAEYMEVYWKATHHPLHPQNSQNLIT  379
rtβ2a:  N---V-A---A----Q-----------E------D---A--K---PPSSNL-N-_L-S  431
rbβ1:   N--IA-SE--A----_-M--I-------E----------A--K---PPSSTP-N-_L-N  478

rtβ4:   RNVGSTA-SPYPTAISGLQSQR__MRHSNHSTENSPIERRSLMTS__DENYHNERARKSR  467
rtβ3:   --S--------Q--GE_____-GE------_-----P------S  408
xoβ3-32 ---V-------QPV--_____-------L-_-------------  408
xoβ3-28 PPTAIPGLQNQELLSD_____RANEHSPFE__RDSLMQSDEASET  408
rtβ2a:  RTL-TST-PLSPT-ASNSQGSQDQRTDRSAPRSASQA--EPC--PVKK-QHR-SS-THQ  483
rbβ1:   RTM-TAA-AASPAPVSNLQVQVLTSLRRNLSFWGGLETSQRGGGAVPQQQEHAM@      524

rtβ4:   NRLSS_____---H-RD-YPL_____V----PDS-----K---NRG__-PGGCSHD  514
rtβ3:   --QA--G___------R-_____---D-A-A-Q-------Q-T___---P-A--  449
xoβ3-32 -------___--------_____----------V--------_--------  449
xoβ3-28 SRKTWTA___SSQRSSHH_____LEEEYSDTYTDLYQPHRHHN__SGLNSTNG  449
rtβ2a:  NHRSG--RGL-R-ETFDSETQESRDSAYVEPK---SHEHV-R-V---E-NHREESH-S--  543

rtβ4:   SRHRL@                                                  519
rtβ3:   -___-P-----AQ---HD----------_____-------@  484
xoβ3-32 -----------------------------_____-------@  484
xoβ3-28 H___DSQDRLLERDSEQNHNDRNWQRNRP_____WPKDSY@  484
rtβ2a:  -RHRE-RH-TRDMGRDQ---ECSK--S-HKSKDRYCDKEGEVISKRRSEAGE-NR-V-IRQ@ 604
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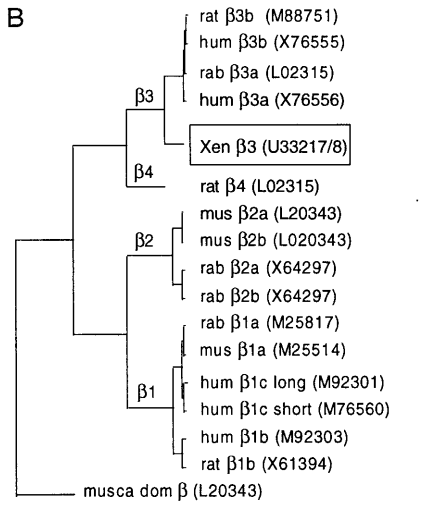

FIG. 1. Amino acid sequence of *Xenopus* β subunits as deduced from the xo28 and xo32 cDNAs and comparison to sequences of mammalian β1b, β2a, β2b, β3, and β4 (*A*) and phylogenetic tree of calcium channel β subunits (*B*). (*A*) xoβ3-28 is the reference. –, Same amino acid as in xoβ3-28; –, gap; @, stop; rt, rat; rb, rabbit; hum, human; and xo, *X. laevis* oocyte. β2a differs from β2b only at the N terminus. β1, β1b splice variant. The phylogram shown in *B* was calculated by the neighbor-joining technique of Kimura, using version 8.0 of the GCG Sequence Analysis Software Package.

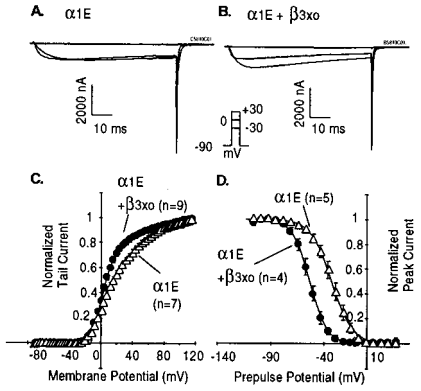

FIG. 2. Effect of β3xo (xo28) on α_{1E}. (*A* and *B*) Time courses of activation of α_{1E} in oocytes injected with α_{1E} cRNA alone or cRNAs encoding both α_{1E} and β3xo (clone xo28). (*C* and *D*) Effect of β3xo on voltage-dependent activation and inactivation of α_{1E}. *G–V* curves were obtained from peak tail currents measured by stepping to −50 mV after depolarizing pulses of 25-msec duration from −88 to 116 mV in 4-mV increments. The data were sampled at 10 kHz and filtered at 2 kHz. The data points were fitted by the sum of two Boltzmann distributions. For α_{1E}, the first component had a $V_{1/2}$ = 2.7 mV, an effective valence ($z\delta$) = 2.9 e, and a relative amplitude of 51%; the second had a $V_{1/2}$ = 41.8 mV and a $z\delta$ = 1.4 e. For α_{1E} + β3xo, the first component had a $V_{1/2}$ = 2.7 mV, a $z\delta$ = 3.4 e and a relative amplitude of 75%; the second had a $V_{1/2}$ = 51.8 mV and a $z\delta$ = 1.3 e. Steady-state inactivation curves were derived from peak currents elicited by a pulse to +20 mV following a conditioning pulse of 10 sec to potentials from −120 to 27 mV in 7 mV increments and a brief (4-msec) pulse to −90 mV. The data were sampled at 500 Hz and filtered at 100 Hz. Sweeps were separated by 20 sec to allow a full recovery from inactivation. Data points were fitted by a Boltzmann distribution. The effective valences were 2.7 ± 0.1 e for α_{1E} and 3.4 ± 0.1 e for α_{1E} + β3xo; the half-inactivation potentials were −32.2 ± 3.4 mV (n = 5) for α_{1E} and −53.5 ± 1.1 mV (n = 4) for α_{1E} + β3xo.

to Lacerda *et al.* (17), who found a high proportion of oocyte batches with endogenous calcium channel currents, we were for the most part unable to find batches of oocytes with currents exceeding 5 nA (tested at +30 mV with 74 mM external Ba^{2+}). In fact, we identified only one batch (out of 15 tested) with oocytes displaying peak inward currents of between 5 and 15 nA. These appeared to be calcium channel currents, based on their characteristic current–voltage relationship and their susceptibility to being reversibly blocked by Co^{2+} (Fig. 3*A*). As shown in Fig. 3*B*, injection of the B11 antisense oligonucleotide (complementary to nucleotides 210–235 of the coding sequences of xo28 and xo32; for composition see Table 1), resulted in a significant reduction in their endogenous calcium channel currents, when compared with currents recovered oocytes of the same batch injected with control oligonucleotide B10.

For the second approach, we coinjected antisense oligonucleotides together with mammalian α_{1C} or α_{1E} subunits, which according to our previous experience do not require coexpression of β subunits for their expression. We found that anti-xo28/xo32 oligonucleotides completely suppressed the functional expression of the type C and E α_1 subunits (Table 1 and Fig. 4). This suppression of expression of α_{1C} and α_{1E} was not due to a poisoning effect of the antisense oligonucleotides because: (*i*) the same effect was obtained with three different antisense oligonucleotides (B8, B11, and B24); (*ii*) sense oligonucleotides synthesized and purified in parallel failed to affect expression of exogenous α_1 subunits; (*iii*) β3xo antisense oligonucleotides that prevented α_{1C} and α_{1E} expression did not interfere with expression of the Shaker K^+ channel—i.e., of an

Numbers in parenthesis next to names of β subunits are the GenBank accession numbers: for xo28, it is U33217, and for xo32, it is U33218.

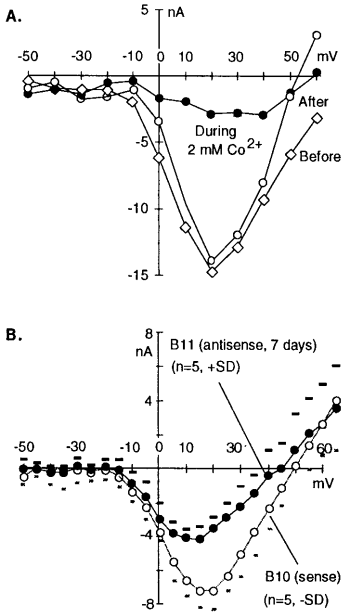

FIG. 3. Inhibition by anti-β3xo oligonucleotide B11 of endogenous Ca^{2+} channel currents in oocytes of one frog presenting such currents. Expression of Ca^{2+} channel currents in *Xenopus* oocytes was determined on the day after isolation and collagenase treatment of oocytes. Oocytes from one frog expressing 5–10 nA at +30 mV with 74 mM Ba^{2+} in the external solution were injected with 50 nl of 100 μM B11 (antisense) or B10 (sense) oligonucleotide. The injected oocytes were then tested 3, 5, and 7 days later for Ca^{2+} channel activity. (*A*) Representative I_{Ba} recorded from one uninjected oocyte. (*B*) *I–V* relations obtained 7 days after injection of sense or antisense oligonucleotides. The external solution was 80 mM Ba^{2+}/10 mM Hepes, titrated to pH 7.0 with methanesulfonic acid (CH_3SO_3H).

unrelated ion channel (data not shown); and (*iv*) α_1 currents could be rescued from suppression by antisense oligonucleotide by coinjection of rat β1b cRNA (Fig. 4). Table 1 summarizes our results on effects of xo28 and xo32 antisense oligonucleotides on the functional expression of mammalian α_1 subunits.

Table 1. Effect of oocyte β subunit antisense oligonucleotide on functional expression of coinjected calcium channel α_1 subunit

Injections		Ion channel activity,† frequency of success
α_1 subunit cRNA	Oligonucleotide,* 20 nl per oocyte	
α_{1C}‡	—	20/22
α_{1C}	B10 sense, 100 μM	6/6
α_{1C}	B11 antisense, 100 μM	0/10
α_{1C}	B11 antisense, 10 μM	0/14
α_{1E}	—	27/29
α_{1E}	B10 sense, 100 μM	10/10
α_{1E}	B11 antisense, 100 μM	0/4
α_{1E}	B8 antisense, 100 μM	3/16§
α_{1E}	B24 antisense, 100 μM	0/12
α_{1E}	B24 antisense, 10 μM	20/20¶
α_{1E}	B11 antisense, 10 μM	1/9‖

*Sense and antisense refer to coding strand of common sequences of *Xenopus* oocyte xo-28 and xo-32 cDNAs. B8, 5′TTGAGCC[A/T]GCCTCTCCACCTC; B10, 5′AGATTTCCTACATATCAAGGAGAAA; B11, 5′GCACTCCTCATCCAGCGCTCCACAG; and B24, 5′TGAACCCACTTCTGAGTCTTCAAA.

†*x* of *y* oocytes from 2 to 3 frogs.

‡All α_{1C} experiments were performed with the ΔN60 mutant of α_{1C} lacking amino acids 1–60 at the N terminus.

§1–5% of control in the three positives.

¶16 ± 8% of control at 30 mV.

‖3% of control for the only positive.

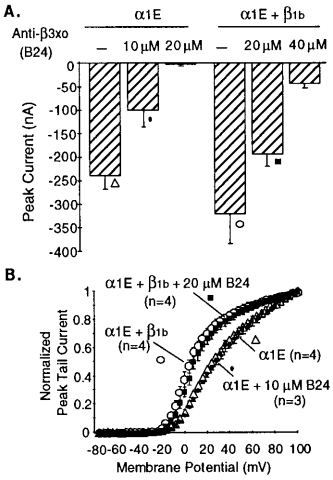

FIG. 4. Inhibition by anti-β3xo oligonucleotide B24 of expression of α_{1E} currents and rescue of α_{1E} currents by coinjection of cRNA encoding the rat β1b subunit. Oocytes were injected with 50 nl of a solution containing 100 μg/ml of α_{1E} cRNA alone or in combination with 100 μg/ml β1b cRNA and the indicated concentrations of B24 oligonucleotide. Ca^{2+} channel currents were recorded 6 days after injection. The results presented on this figure were obtained with oocytes obtained from a single frog. Similar results but varying in the concentration of B24 needed to affect α_{1E} currents, were obtained in two other experiments. (*A*) Peak inward α_{1E} currents recorded from oocytes after a 250-msec test pulse from a holding potential of −90 mV. Injection of cRNAs and B24 oligonucleotide are indicated above the bars, which are means ± SEM of 4–5 oocytes. (*B*) Averaged *G–V* curves (mean ± SEM) of α_{1E} currents in oocytes coinjected or not with B24 and B24 plus β1b cRNA. The composition of B24 is given in Table 1.

Two Sites for Interaction with a β Subunit on α_{1E}. Campbell and coworkers (14) identified the existence of a β subunit binding domain within the L1 loop that connects the homology repeats I and II of α_1 subunits. We used a very similar technique to test for the possible existence of a second β subunit binding domain by incubating fragments of α_{1E} fused to GST (GST fusions) with a Ca^{2+} channel β subunit (CCβ) made and labeled by *in vitro* translation in the presence of [^{35}S]methionine as described by Pragnell *et al.* (14). As shown in Fig. 5, we found that β2a, in addition to binding to L1, binds to a domain contained within the last 277 aa of the >400-aa C terminus of α_{1E} (fragment CC). It follows that, at least *in vitro*, α_{1E} has two interaction domains able to interact with and forming stable complexes with a calcium channel β subunit.

DISCUSSION

Natural Expression of a Calcium Channel β Subunit in Stage IV–VI *Xenopus* Oocytes. Throughout these studies, we gathered four types of evidence indicating that *Xenopus* oocytes express an endogenous β subunit that is active and functional. First, we identified by molecular cloning two mRNAs encoding type-3 β subunits (xo28 and xo32). These sequences were found in mRNA isolated from oocytes that had been thoroughly defolliculated by the collagenase treatment routinely used by us in the preparation of oocytes for injection of cRNAs (see *Methods*). This makes it unlikely that xo28 and xo32 were derived from non-oocyte mRNA. Second, coinjection into oocytes of α_{1E} cRNA and either one of the newly cloned xoβ cRNAs led to expression of Ca^{2+} channel currents that were indistinguishable from those obtained upon coexpression of α_{1E} with mammalian β3, indicating that the cloned sequences encode a β subunit that is functional in an assay that we and others have used previously to identify β subunit function (cf. 8, 18, and 19). Third, injection of the B11

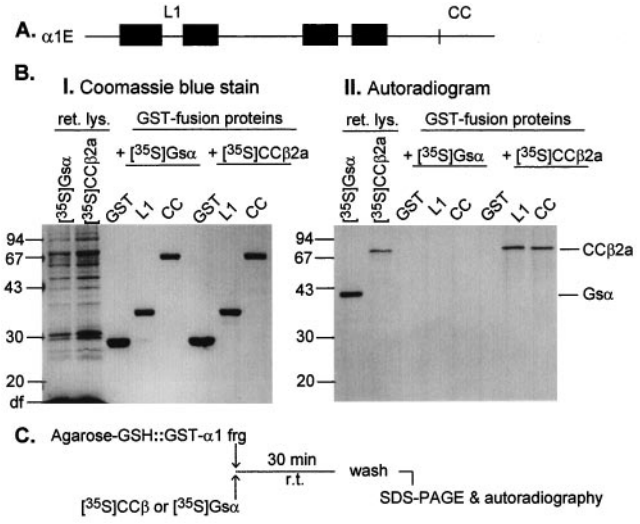

FIG. 5. Identification of two sites on α_{1E} that interact with β subunits. (*A*) Ideogram of a Ca^{2+} channel α_1 subunit with linear N and C termini, four homologous repeats (filled boxes), and connecting loops. (*B*) 12% SDS/PAGE analysis of GST fusion proteins and bound calcium channel β2a or G protein α_s subunits synthesized by reticulocyte lysates in the presence of [^{35}S]methionine. BI, Coomassie blue stain; BII, autoradiogram of the gel shown in BI. (*C*) Experimental design of the test for protein–protein interaction. The figure shows binding of β_{2a} to the L1 and the CC regions of α_{1E}. L1, α_{1E}[356–451]; and CC, α_{1E}[2036–2312]. Note that both α_{1E} L1 and α_{1E} CC bound [^{35}S]CCβ_{2a} but not an unrelated protein, [^{35}S]G$_s\alpha$.

antisense oligonucleotide (Table 1), which was designed to be complementary to a region of the coding sequence of xo28 and xo32 showing no differences in nucleotide composition, resulted in partial but significant suppression of expression of endogenous voltage-dependent Ca^{2+} currents, as would be expected if endogenous Ca^{2+} channel function were dependent on presence of a regulatory β subunit. Fourth, injection of xo28/xo32 antisense oligonucleotides (B11, B8 and B24) resulted in suppression of the functional expression of exogenously injected α_1 subunits. B11, B8, and B24 are complementary to nucleotides 210–235, nucleotides 127–147, and nucleotides 28–51, respectively, of xo28 and xo32. Coinjection of sense type xo28/xo32 oligonucleotides (e.g., B10) did not mimic the effects of B11, B8, and B24.

The role of this endogenous β subunit has not been explored, but is likely to relate to the fact that there are batches of oocytes expressing endogenous voltage-dependent Ca^{2+} currents. Type-3 *Xenopus* β is thus likely to be part of the molecular makeup of endogenous oocyte Ca^{2+} channels.

Implications for Interpretation of Calcium Channel Properties Observed in *Xenopus* Oocytes. The most significant finding of the present work is not that oocytes express a calcium channel β subunit, but rather that β3xo antisense oligonucleotides completely obliterated expression of exogenous calcium channel currents that would otherwise have been expressed, and also the apparent inability of the endogenous xoβ subunits to affect the gating properties of the channel they are helping to express. Thus, there was no expression of α_1 without an endogenous β, but on the other hand, the level of expression of the endogenous β was not sufficient to regulate the activity of the channel. Related to this is the question of whether calcium channel currents measured in oocytes that were injected with α_1 subunits alone are currents mediated by calcium channels formed of α_1 alone (i.e., devoid of β) or whether these currents are mediated by complexes formed of the injected α_1 plus an endogenous *Xenopus* β, an xoβ.

One possibility is that α_1 subunits are expressed only in association with a β (i.e., as $\alpha_1\beta$ complexes) and that differences in properties between α_1·xoβ and α_1·exogenous β reflect differences in the regulatory abilities of xoβ vs. those of the exogenous mammalian β. The result with B11 (the anti-xoβ oligonucleotide) on endogenous calcium channel currents would be consistent with this possibility, as the *I–V* relationships in B11 injected oocytes were the same as those found in oocytes injected with inactive control oligonucleotide. In this case, the effect of the antisense oligonucleotide is well explained by assuming that residual currents reflect currents due to the residual α_1·xoβ complexes expressed on the surface of the oocyte. On the other hand, by the same reasoning, injection of xoβ should not have affected the properties of currents of mammalian α_1 subunits, but merely increased them. Yet, *Xenopus* β3 (i.e., βxo28 or βxo32), rather than merely augmenting α_{1E} currents, modified the properties of α_{1E} currents changing both their *G–V* relations and voltage-induced inactivation. Since in the absence of xoβ (in B11-, B8-, or B24-injected oocytes) there was no expression of a functional α_1 whatsoever as seen from absence of gating currents at the beginning of the time courses of channel activation (data not shown), one would have to argue that the endogenous xoβ must have acted as a "chaperone" aiding in the folding and transit of α_1 to the surface of the oocyte, but that once at the cell surface, formation of stable $\alpha_1\beta$ complexes requires "high" levels of β, higher than naturally supplied by the oocyte. This raises the possibility that currents in α_{1E}-injected oocytes, generally referred to as α_1 alone are in reality the result of mixtures of α_{1E} alone plus α_{1E}·βxo complexes. Analysis of *G–V* curves of α_{1E} alone oocytes could indeed be interpreted in this way: (*i*) the channel behaves as if it existed in two main states, one, ≈50% of the total, responding to voltage with a $V_{1/2}$ of −5 mV, and the other responding with a $V_{1/2}$ of +50 mV; and (*ii*) the effect of exogenous β subunits (mammalian β or β3xo; Figs. 2 and 4) on activation is essentially to increase the proportion of channels responding to voltage with a $V_{1/2}$ of −5 mV. We could presume that the channels responding at low voltage represent $\alpha_1\beta$ complexes, while channels responding at the higher voltage represent α_1 alone, which however required xoβ for their successful targeting to the plasma membrane. Against this reasoning is a consistent failure to observe a decrease in the proportion of the channel molecules responding to low voltage coincident with reduction in the level of residual current. As shown in Fig. 4, the *G–V* curves of α_{1E} alone are indistinguishable from the *G–V* curves of oocytes in which α_{1E} expression was reduced by 50% by injection of 10 μM of B24, an anti-β3xo oligonucleotide.

Taking our findings into account, it would seem that the maturing and regulation of a voltage-gated calcium channel under the influence of a β subunit should be envisaged as depending on two independent actions of β subunits: one, requiring only low levels of β, would be to serve as an organizer of the secondary and tertiary structure of α_1 and to help in one or more of the many steps that intervene between its initial translation and its appearance on the cell surface; the second, requiring higher concentrations of β, would be to regulate the activity of the assembled channel. The steps between translation and localization to the plasma membrane may include simple folding, membrane insertion, aid in co- and posttranslational modification, and targeting of the inserted α_1 to the particular plasma membrane domain where it is to function—e.g., cell body, axon, and synapse. It is possible that once inserted and targeted to the cell surface, the site or sites of α_1–β interaction lose affinity for each other so that further actions of β subunits require higher levels of the β subunit. Such higher concentrations would of course be readily attained upon injection of massive amounts of cRNA into an oocyte.

The thus far known regulatory effects of β subunits include: (*i*) facilitating the coupling between the voltage sensor and pore opening, thus facilitating channel activation (5); (*ii*) modulating switching between gating modes (20); (*iii*) confer-

ring sensitivity to prepulse potentiation to α_{1C}, which is an effect seen with β1b but not with β2a subunits (21, 22); (*iv*) facilitating or decreasing, in an also β subunit-specific manner, voltage-induced inactivation (16); and (*v*) interfering with inhibitory regulation of dihydropyridine-insensitive Ca^{2+} channels by activated G proteins (23, 24). Work by Pragnell *et al.* (14) and Witcher *et al.* (25) has led to the identification of a high-affinity binding site for β within the L1. This site is present on all α_1 subunits and binds to all β subunits. Its existence does not easily explain diverse effects of β subunits on Ca^{2+} channel activity that vary with the type of α_1 and/or the type of β. Moreover, its high affinity does not predict easy dissociation of β from α_1 to allow for appearance of the α_1 alone-type channels seen in *Xenopus* oocytes in the absence of exogenous injection of a β subunit cRNA.

An alternative explanation for the observations at hand is that the interaction between α_1 and β mediated by the loop I–II site is permanent and has a structural role and that the regulatory effects result from interaction of $\alpha_1\beta$ complexes with a second and possibly more β subunits. Our finding that the C-terminal half of α_{1E} is able to form a stable complex with a β subunit *in vitro* without the participation of the L1 loop (Fig. 5), while not proving participation of more than one β subunit in the normal functioning of a channel in the environment of an intact cell, is at least compatible with this possibility. The interaction and regulation of phosphorylase kinase with calmodulin is an example of this type of regulation (26). Phosphorylase kinase has subunit composition $(\alpha\beta\gamma\delta)4$ (M_r, 1,280,000), of which the δ subunit is calmodulin, which remains associated to the complex even in 8 M urea, provided Ca^{2+} is present. The activity of this complex is dependent on Ca^{2+} and is stimulated 5-to 6-fold by addition of exogenous calmodulin, which reaches a maximum at molar ratios of calmodulin to phosphorylase kinase of 20 (26).

In conclusion, our findings are consistent with the hypothesis that β subunits may regulate $\alpha_1\beta$ complexes: (*i*) inhibition of β subunit biosynthesis prevents channel formation (Table 1 and Fig. 4); (*ii*) biosynthesis of "low levels" of β, such as might constitutively be made by *Xenopus* oocytes, allows for assembly of functional channels that functionally appear unaffected by a β subunit (e.g., α_{1E} alone traces in Fig. 2 *A*, *C*, and *D* and Fig. 4*B* may in fact be due to $\alpha_1\beta$); and (*iii*) expression of high(er) levels of β subunits, as obtained upon injection of cRNA encoding a β (e.g., Fig. 2 *B*, *C*, and *D* and Fig. 4*B*) allows for development of Ca^{2+} channels with properties that may vary not only with the type of α_1 but also the type of β subunit. Higher-order regulatory complexes, based on lower affinity interactions, may not withstand prolonged purification procedures and could therefore have been missed in previous biochemical studies. Specific studies analyzing immunoprecipitates obtained under nondenaturing conditions that favor preservation of multicomponent complexes will have to be carried out to substantiate the formation of complexes of $\alpha_1\beta$ with β in a normal cellular environment.

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