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Structures and Functions of Calcium Channel β Subunits

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Calcium channel β subunits have profound effects on how α_1 subunits perform. In this article we summarize our present knowledge of the primary structures of β subunits as deduced from cDNAs and illustrate their different properties. Upon co-expression with α_1 subunits, the effects of β subunits vary somewhat between L-type and non-L-type channels mostly because the two types of channels have different responses to voltage which are affected by β subunits, such as long-lasting prepulse facilitation of α_{1C} (absent in α_{1E}) and inhibition by G protein βγ dimer of α_{1E}, absent in α_{1C}. One β subunit, a brain β2a splice variant that is palmitoylated, has several effects not seen with any of the others, and these are due to palmitoylation. We also illustrate the finding that functional expression of α_1 in oocytes requires a β subunit even if the final channel shows no evidence for its presence. We propose two structural models for Ca^{2+} channels to account for "α_1 alone" channels seen in cells with limited β subunit expression. In one model, β dissociates from the mature α_1 after proper folding and membrane insertion. Regulated channels seen upon co-expression of high levels of β would then have subunit composition $\alpha_1\beta$. In the other model, the "chaperoning" β remains associated with the mature channel and "α_1 alone" channels would in fact be $\alpha_1\beta$ channels. Upon co-expression of high levels of β the regulated channels would have composition $[\alpha_1\beta]\beta$.

INTRODUCTION

Analysis of the subunit composition of voltage-gated calcium channels has shown that they invariably are formed of four subunits: α_1, β, α_2 and δ, of which the last two are a proteolytically processed disulfide-linked dimer that originates from a single precursor mRNA. Six non-allelic α_1, four non-allelic β, and one $\alpha_2\delta$ genes have thus far been discovered (reviewed in Perez-Reyes and Schneider, 1996) and the transcripts of most if not all genes are subject to alternative splicing. This gives rise to a large degree of molecular diversity, based on both the genes that originate the components that make up the functional channel, and the processing to which the individual subunits are subject.

Pore formation, voltage sensing, and drug/toxin binding are properties conferred by the α_1 subunits (Perez-Reyes *et al.*, 1989). Correlative studies based on both, the functional properties of cloned α_1 subunits expressed together with β and $\alpha_2\delta$ subunits in eukaryotic cells and the determination of the sites of expression of the cloned α_1 subunits, have given a picture in which α_{1S} forms the skeletal muscle Ca^{2+} channel, α_{1C} forms the cardiac, the smooth muscle, and a DHP-sensitive form of neuronal Ca^{2+} channels, α_{1D} is responsible primarily for the DHP-sensitive receptor/$G_{i/o}$-regulated Ca^{2+} channel found in endocrine cells, and α_{1B} forms the DHP-insensitive receptor/$G_{i/o}$-regulated Ca^{2+} channel found in neurons. Subcellular distri-

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bution places both α_{1B} and α_{1A} in presynaptic terminals, and functional studies indicate that classical N-type channels are formed mostly by α_{1B}. Although subcellular distribution suggests α_{1A} to be responsible also for P-type channels in cerebellar Purkinje cells, the pharmacological properties of expressed α_{1A} have thus far not mimicked P-type currents sufficiently to allow an unequivocal assignment. This could be because the actual α_1 forming P-type channels has not been cloned, or that P-type channels are formed by particular splice variants of α_{1A} that have not yet been properly assembled, or that proper pharmacology depends not only on the identity of the α_{1A} but also of the combination of both the particular β and $\alpha_2\delta$ subunits that accompany α_{1A} and some as yet undefined factor(s) contributed by the neuronal cells in general or the Purkinje cell in particular. [Noteworthy in this regard is to mention that it has not been possible to observe in *Xenopus* oocytes stimulation of the cardiac α_{1C} by cAMP dependent protein kinase-mediated phosphorylation, i.e., in response to activation of the β adrenergic receptor system or to addition of cAMP, even though when expressed in β adrenergic receptor expressing baby hamster kidney (BHK) cells the clone shows a robust response (Yatani *et al.*, 1995)]. The most elusive molecular identity is that of the T-type channels. Of the cloned α_1 subunits, the one that comes closest to exhibiting T-type properties is α_{1E}. It has permeation properties expected for T-type currents (Bourinet *et al.*, 1996) and activates at lower voltage than all other α_1 subunits. However, the actual voltages at which it begins activating and the kinetic profile of block by Ni differ from what is found upon expressing α_{1E} in model cells. Whether indeed α_{1E} subunits are responsible for T-type currents is an unresolved matter.

Below we provide an overview of the structural and functional features of regulatory β subunits. Except for the results of Dolphin and collaborators, who suppressed β subunit synthesis in natural neuronal cells by microinjection of antisense oligonucleotides (Campbell *et al.* 1995; Berrow *et al.*, 1995), most of the knowledge about roles of β subunits in regulating the functions of α_1 subunits stems from "reconstituting" Ca^{2+} channel activity by coexpression in *Xenopus* oocytes, fibroblast L cells, and human embryonic kidney (HEK) cells. This has been complemented by direct protein–protein interaction analysis (Pragnell *et al.*, 1994; De Waard *et al.*, 1994; Witcher *et al.*, 1995; Qin *et al.*, 1997).

PRIMARY STRUCTURE OF β SUBUNITS

As has been the case for all Ca^{2+} channel subunits, the first β subunit cloned was that of the skeletal muscle and was based on partial amino acid sequence analysis of the β subunit that forms part of the T-tubule dihydropyridine receptor/Ca^{2+} channel complex. (β1, Ruth *et al.*, 1989). In contrast to the then known α_1 subunit, which was predicted on the basis of Kyte–Doolittle analysis to be formed of four homologous repeats, each being homologous to a Shaker voltage-gated K^+ channel with six transmembrane segments of which the fourth (S4) was the positively charged voltage sensor, the cloned skeletal muscle β subunit did not contain hydrophobic segments that would suggest a transmembrane topology. This leads to the conclusion that it had to be cytoplasmic and associated with the channel proper through protein–protein interaction forces.

Cloning on the basis of sequence similarity by F. Hofmann's laboratory in Germany and by several other laboratories, including ours, led in a relatively short time to the identification of alternative splice variants of β1: rat β1b and β1c expressed in brain (Pragnell *et al.*, 1991; Powers *et al.*, 1992), and of other β subunits: β2a, β2b, and β3 expressed in rabbit heart, lung, and aorta (Hullin *et al.*, 1992) and β2a, β3, and β4 expressed in rat brain (Castellano *et al.*, 1992, 1993a,b). More recently, two homologues of the mammalian β_3 subunit were cloned from *Xenopus* oocyte RNA (Tareilus *et al.*, 1997). Table I presents a summary of successful cloning efforts. Table II lists the chromosomal location of β subunit genes in man and mouse. While each of the β genes is found on different chromosomes in man, in the mouse, β_2 and β_4 are both on chromosome 2 proximal to the its centromere.

Figure 1 shows an alignment of the deduced primary amino acid sequences, including the exon–exon boundaries as inferred from the intron–exon structure determined by Flockerzi's laboratory for β3. The alignment clearly highlights the existence of five amino acid sequence similarity domains: two highly variable N-terminal D1 and C-terminal D5 domains, one of two middle D3 domains, and two highly similar "connecting" D2 and D4 domains of ca. 130 and 150 amino acids, respectively. β1 and β2, but apparently not β3 and β4, come in several flavors through the use of alternatively spliced exons to code for sequence similarity domains 1, 3, and 5, which also gives a structural basis for the evolutionary organization, relatedness, and differences among the five similarity domains. As

Table I. GenBank Accession Numbers of Ca^{2+} Channel β Subunit Clones

Type	Subtype	Name in report	Source	GenBank #	Reference
β1	β1a	β	Rabbit skeletal muscle	M25871	Ruth *et al.*, 1989
		β1M	Human skeletal muscle	M92301	Powers *et al.*, 1992
		βc	Human heart	L06112	Collin *et al.*, 1993
		β1c	Human genomic DNA	U86960	Powers *et al.*, direct submission
	β1b	brain β	Rat brain	X61394	Pragnell *et al.*, 1991
		β1B2	Human hippocampus	M92303	Powers *et al.*, 1992
		βa	Human heart	L06110	Collin *et al.*, 1993
	β1c	—	Human genomic DNA	U86961	Powers, *et al.*, direct submission
		brain β2	Human brain	M76560	Williams *et al.*, 1992
		β1B1	Human hippocampus	M92302	Powers *et al.*, 1992
		βb	Human heart	L06111	Collin *et al.*, 1993
		β1c	Human genomic DNA	U86960	Powers *et al.*, direct submission
β2	β2a	β2	Rat brain	M80545	Perez-Reyes *et al.*, 1992
		β2c	Human brain	U95019	Williams, 1997, direct submission
			Rabbit brain	—	Qin and Birnbaumer, unpublished
	β2a′	CaB2a	Rabbit heart	X64297	Hullin *et al.*, 1992
	β2a″	β2a	Mouse brain	L20343	Massa *et al.*, direct submission
	β2a‴	CaB2b β2b	Rabbit heart	X64298	Hullin *et al.*, 1992
	β2c	CaB2c	Rabbit heart	X64299	Hullin *et al.*, 1992
β3	β3a	β3	Rat brain	M88751	Castellano *et al.*, 1993a
		CaB3	Rabbit heart	X64300	Hullin *et al.*, 1992
		β3	Mouse genomic DNA	U20372	Chin *et al.*, direct submission
		β3a	Mouse genomic DNA	X76555	Murakami *et al.*, 1996
		β3	Human embryo	L27584	Collin *et al.*, 1994
		xo28	*Xenopus* oocyte	U33217	Tareilus *et al.*, 1997
		xo32	*Xenopus* oocyte	U33218	Tareilus *et al.*, 1997
	β3b	β3b	Human genomic DNA	X76556	Murakami *et al.*, direct submission
β4	β4	β4	Rat brain	L02315	Castellano *et al.*, 1993b
		β4	Human brain	U95020	Williams, direct submission
β	β		Housefly	X78561	Grabner *et al.*, 1994

deduced from the intron–exon boundaries determined for the β1 and β3 genes by Powers *et al.* (1992) and Murakami *et al.* (1996), respectively, the D1 and D3 domains are encoded in single exons and the D5 domain is formed of the 3′ half of one exon (exon 12 of β3) plus a last coding exon (exon 13 of β3), while the two highly homologous D2 and D4 are encoded in four and six exons respectively. For β1 and β2 there are alternative exons encoding for alterative D1, D3, and D5 domains. The N-terminal D1 domains are

Table II. Chromosomal Location of Ca^{2+} Channel Beta Subunits

Calcium channel β subunit		Species			
Gene	Protein(s)	Human chromosome	Reference	Mouse chromosome	Reference
CACNLB1	β1	17q21–q22	Gregg *et al.*, 1993; Iles *et al.*, 1993	—	—
CACNLB2	β2	10p12	Taviaux *et al.*, 1997	prox. 2	Chin *et al.*, 1995
CACNLB3	β3	12q13	Park *et al.*, 1997	15	Chin *et al.*, 1995
CACNLB4	β4	2q22–q23	Taviaux *et al.*, 1997	prox. 2	Burgess *et al.*, 1997; Chin *et al.*, 1995

Table III. Summary of Principal Roles of Ca^{2+} Channel β Subunits in Ca^{2+} Channel Function

1. Obligatory for surface expression of mature channels
β1 knockout (CACNLB1) is lethal due to lack of skeletral EC[a] coupling
β4 most active or active only with α_{1A}
the "chaperoning" β may remain associated with or dissociate from the mature channel
2. Setting of proper kinetics of activation and voltage dependence of activation
improved (faster) rates of activation
improved coupling of voltage-sensing to pore opening
3. As seen with α_{1C}
stabilization of gating modes 1 and 2
suppression of null's (mode 0)
required for establishment of long-lasting (τ = 20 sec) prepulse facilitation; β2a inactive
4 As seen with α_{1E}
acceleration of inactivation and left shift of the midpotential of steady-state inactivation; β2a has opposite effect
inhibition of inhibition by G_i/G_o-coupled receptors acting via Gβγ dimer through competitive in interaction at a common C-terminal site

[a] EC, excitation contraction coupling: absence of properly assembled and targeted skeletal α_{1S}.

either "long" (45–57 amino acids) or "short" (15–17 amino acids) with short domains being structurally unrelated but long domains showing evolutionary relatedness as evidenced by the presence of a cluster of basic amino acids (bold in Fig. 1) followed by a serine-rich region that begins with an invariant SDGST sequence.

Structurally, and as it turned out also biochemically and functionally, one of the most interesting of the domains is that of the short D1 domain of the type 2 β subunit expressed in brain: although short (16 amino acids), it is homologous to long domain D1 in that it has an incomplete basic amino acid cluster, lacks the GDST-initiated serine-rich region, and displays a unique Cys-Cys doublet (CC) in positions 3 and 4, found in no other β subunit N-terminus. This doublet was recently shown by Hosey and coworkers to be palmitoylated (Chien *et al.*, 1996).

Figure 2 compares β subunit variants in a schematic form based on their sequence similarity domains, along with the phylogenetic relationships of the D2 and D4 domains. It should be noted that since the short AKQKQKX D3 domain is highly conserved among the four β subunits, β subunits with this, rather than the longer forms of D3 found in β1 and β2, are best described as highly homologous except in their N- and C-termini.

A visual inspection of the amino acid sequence differences among the various β subunits (Fig. 1) reveals, however, that the subdivision into D1 through D5 sequence similarity domains is simplistic and likely to be naive. There are pockets of marked differences that in all likelihood could have functional consequences of fundamental nature. Using the exon structure of β3 as a reference, areas of this type are the C-termini, sequences located toward the end of exon 2, the middle of exon 3, the stretch encoded in the second half of exon 4 plus the rather variable exon 5 plus the "extension" in the β1 and β2 subunits that include exon 6A. A limited analysis in which we replaced the C-terminus of β1a with the C-terminus of β1B (118 amino acids longer) gave a β subunit that regulated α_{1E} activation and inactivation in a manner that was indistinguishable from that of β1a (Qin *et al.*, 1996; Olcese, Qin, Stefani, and Birnbaumer, unpublished).

FUNCTIONS OF CALCIUM CHANNEL β SUBUNITS

Functional expression of the skeletal muscle α_1 cDNA in murine L cells, in which β subunit or $\alpha_2\delta$ mRNA levels are undetectable (*Kim, H.S., Perez-Reyes, E., and Birnbaumer, L., unpublished*), showed that the α_1 subunit is a self-contained pore-forming and voltage-sensing Ca^{2+} channel (Perez-Reyes *et al.*, 1989). However, the voltage-sensitive Ca^{2+} channel formed under these conditions was highly anomalous in several aspects. The most notable was that the half-time for activation at 0–10 mV was on the order of 5–10 sec, instead of being in the millisecond range (Lacerda *et al.*, 1991). This indicated that, most likely, proper responsiveness to voltage was dependent on additional factors. The immediate attention fell on the proteins that co-purified with α_{1S} in the dihydropyridine receptor β, $\alpha_2\delta$ and γ. γ appears to be restricted to the skeletal muscle Ca^{2+} channel and has not received much attention. We shall concentrate here on β subunits, interjecting a few recent results with $\alpha_2\delta$ as they relate to its ability to modulate effects of β subunits.

Indeed expression of β1a in α_{1S} expressing L cells normalized the activation kinetics of the Ca^{2+} channel. The importance of β subunits became apparent not only in these studies but independently also through the studies of Singer *at al.* (1991) who injected α_{1C} into *Xenopus* oocytes alone and in combination with β, $\alpha_2\delta$, and γ and observed marked effects of all regulatory subunits. Subsequently, as the remaining

```
               ---------------------------- exon #1 ------------------------------ | -----
rtβ4:          MSSSYAKNGAADGPHSPSSQVARGTTTRRSRLKRSDGST_____TSTSFILRQ | -----      53
rtβ3:                                                  MYDDSYVPGFEDSEA | GSADS      20
xoβ3:                                                  MYDDLYLHGFEDSEV | -----      20
 mβ2a heart                                   MKATWIRLLKRAKGGRLKSSDIC | -----      28
rbβ2a heart                                        MLDRHLAAPHTQGLVLEG | -----      22
rt/hum β2a                    MQCCGLVHRRRVR_______________________VSY | -----      21
rbβ2b heart         MNQASGLDLLKISYGKGARRKNRFKGSDGSTSSSDTTSNSNFVRQ | -----      50
rbβ1:  MVQKTSMSRGPYPPSQEIPMEVFDPSPQGKYSKRKGRFKRSDGSTS__DTTSNSNFVRQ | ---E-      62
rtβ1:     ----SG----------------------------------------------------- | -----      62
                                                                   | *** *

          ------------- exon #2 --------------- | ---------------- exon #3 -
rtβ4:     ------_-----------AI-Q-R-Q--AI------ | S-------K-------A---DV--PS   114
rtβ3:     YTSRPSLDSDVSLEEDRESARREVESQAQQQLERAK | HKPVAFAVRTNVSYCGVLDEECPVQG    82
xoβ3:     -----------------T------R---------R | L---------------A---------    82
rtβ2:     ------_-----------AV---A-R---A---K-- | T-----------R-SAAQEDDV--P-    82
rbβ1:     ------_-----------AL-K-A-R--LA---K-- | T-----------G-NPSPGD-V--E-   123
rtβ1:     ------_----------------------------- | -------------------------Q-   123
          ****** ***********  * * * **   *** * | ******* *** *         **

          -------------- | ------------- exon #4 --------------- | -------
rtβ4:     TAIS-D-------- | --N-----------CE-G-----L---N--IQ----RG | __RFHGG  173
rtβ3:     SGVNFEAKDFLHIKE | KYSNDWWIGRLVKEGGDIAFIPSPQRLESIRLKQEQKAR | RS_____  138
xoβ3:     AAI------------ | --N-----------A--T---------A---------- | --_____  138
rtβ2:     MAIS--------V-- | -FN-----------CE-G-----VK--NM--QH--R-K | QGKFYSS  143
rbβ1:     VAIT--P-------- | --N-----------CEVG-----VK-D-L--L----L- | Q-RLSSS  184
rtβ1:     --------------- | ------------------------------------T-- | -N-----  184
             *  ***** ** | *  ************     *****  *    **  ** |

          exon #5 ------------------ | ------------- exon # 6A --------------
rtβ4:        KSS--S--SLGEMVS-TF-AT-TTT | ___________________________________   198
rtβ3:        ___GNP--LS_DI__GNRRSPPPSL | ___________________________________   157
xoβ3:        ____________GG-MVS---------I | ________________________________   154
rtβ2a/b      KSG--S--SLG-IVPSS-K-T---S | ________AIDIDATGLDAEENDIPANHRSPKPSANSV   198
rb/rt β2c brain          ------------ | ___________________________________
rbβ1:        KSGD-S--SLG-VVT-TRRPTP-AS | GNEMTNLAFELEPLDLEEDEAELGEQSGSAKTSVSSV   247
rtβ1a        ------------------------- | -------------------------------------   239
    b/c      ------------------------- | ___________________________________   209
                   **                 |                            ^ ^ ^  ^^

          ---------------- | - #6B - | -------- exon # 7 -------- | ------ exon
rtβ4:     ________________ | ------V | T--I---------------------- | -----------   243
rtβ3:     ________________ | AKQKQKQ | AEHVPPYDVVPSMRPVVLVGPSLKGYE | VTDMMQKALFD   202
xoβ3:     ________________ | ------- | ---TA---------------------- | -----------   199
rtβ2a/bTSPHSKEKRMPFFKK    | _______ | T--T----------------------- | -----------   251
rbβ2c     ________________ | AKQKQKS | T--T----------------------- | ---------
rbβ1:     TTPPPHGTRIPFFKK  | _______ | T---------------II---------- | -----------   299
rtβ1a     ---------------  | _______ | --------------------------- | -----------   299
    b/c___________________ | AKQKQKS | --------------------------- | -----------   254
          ^  ^       ^^^^^ | ^^^^^^ |  **  **********  ********** | ***********
minimum α1-regulatory
    fragment of β1b          ++++++ | ++++++++++++++++++++++++++++ | ++  211-245

          # 8 ----- | ------------- exon # 9 -------------- | ---------------
rtβ4:     ------D-- | ----------I-----------S--A-----N----L | ---------------R     303
rtβ3:     FLKHRFDGR | ISITRVTADLSLAKRSVLNNPGKRTIIERSSARSSI | AEVQSEIERIFELAK      262
xoβ3:     --------- | -----------------------A------T---- | --------------S-     259
rtβ2:     ------E-- | ----------I-----------S-HA-----NT---L | ---------------R     311
rbβ1:     --------- | ----------I-----------S-HI-----NT---L | ---------------R     359
rtβ1:     --------- | ------------------------------------ | ---------------359/314
          ****** ** | ********* *********** *  *****  *** | **************
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Fig. 1. Amino acid sequence alignment of Ca^{2+} channel beta subunits. β1 through β4 are products of non-allelic genes; a, b, . . . are splice variants. Vertical lines represent exon–exon boundaries of β1 (Powers *et al.*, 1994) and β3 (Murakami *et al.*, 1996) and are extrapolated for all β subunits. Exon 1 amino acids are listed for all β subunits; beginning with Exon 2, sequences are compared to that of rat β3 (reference sequence). -, identical to reference sequence; ___, gap, @, stop. Basic amino acid patterns in the N-termini are highlighted in bold; **RRPTP**, β1a motif phosphorylated by cAMP-dependent protein kinase (DeJongh *et al.*, 1989); *****, minimum β1b sequence that when expressed in *Xenopus* oocytes alters kinetics of α_1 activation (De Waard *et al.*, 1994). Reference sequences are as follows: master sequence (bold) is rat b3, (rtβ_3); non-rat β1's are compared to rbβ_1a; β_2b and β_2c are compared to β_2a. rt, rat; rb, rabbit, hum, human; m, mouse; xo, *Xenopus laevis* oocyte. rtβ_1a = ratβ_1M; rtβ_1c = rtβ_1B1; rtβ_1b = rtβ_1B2. N-termini of β1b, β2b, and β4 show evolutionary relatedness; those of the rat β2a and the human β2a are also related and carry a palmitoylation motif; N-termini of β3, mouse β2a, rat β2a, rabbit β2a, and rabbit β2b appear to have unrelated evolutionary origins.

```
           ----------- exon # 10 -----------------  | ------------ exon # 11 -
rtβ4:      ------------------I---------H-------     | ------K------S---N--LV-A        363
rtβ3:      SLQLVVLDADTINHPAQLAKTSLAPIIVFVKVSSPK     | VLQRLIRSRGKSQMKHLTVQMMAY        322
xoβ3:      T----I---------T--S-----------------     | -------------V---N---L-A        319
rtβ2:      T-----------------S----------Y--I---     | ------K------A---N---V-A        371
rbβ1:      T----A------------S----------YI-IT--     | ------K------S---N--IA-S        419
rtβ1:      ------------------------------------     | ------------------------    419/374
            **** ********* ** *********  *  ***     |****** ****** *** **  *

           --------  | ------------------------------ exon # 12 ------------
rtβ4:      ---A----  | _-M----------E------G---A------TSSSTPMTP_LLGRNVGSTAL        422
rtβ3:      DKLVQCPP  | _ESFDVILDENQLDDACEHLAEYLEVYWRATHHPAPGPGM____________        379
xoβ3:      ---I----  | _-M----------E-A-------M---K-----LHPQNSQNL__________         379
rtβ2:      ---A----  | Q------------E-------D---A--K---P-SSNLPNP_LLSRTLATSTL        431
rbβ1:      E--A----  | _-M--I-------E-----------A--K---P-SSTPPNP_LLNRTMATAAL        478
rtβ1:      --------  | _-------------------------------R-----_----------- 478/433
            ** ****  |  * ** ******* * ****  * * ** ***

           ----------  | ------------------------- exon # 13 ---------- to end
rtβ4:      SPY----S--- | SQR__MRHSNHSTENSPIERRSLMTS__DENYHNERARKSRNRLSS___              475
rtβ3:      LGPPSAIPGLQ | __________NQQLLGERGEEHSPLE__RDSLMPSDEASESSRQAWTG_              416
xoβ3:      IT-----P--- | __________--E--SDAN-------__-----Q------T--KT--A_              416
rtβ2:      PLSPTLASNS- | GSQGDQRTDRSAPRSASQA--EPC--PVKK-QHR-SS-THQNHRSG--R              491
rbβ1:      AASPAPVSN-- | VQVLTSLRRNLSFWGGLETSQRGGGAVPQQQEHAM@                         524
rtβ1a/c-V--------- | -----------------------------------@ 1a(M)/1c(B1)        524/478
       b   -V--------- | GPYLVSGDQPLDRATGEHASVHEYPGELGQPPGLYPSNHPPGRAGTLWA              493
                     * |

rtβ4:      __---H-RD-YPL____________V----PDS-----K---NRG__-PGGCSHDSRHRL@                519
rtβ3:      __SSQRSSRH_______________LEEDYADAYQDLYQPHRQHT__SGLPSANGH___DPQD              457
xoβ3:      __------H-_______________---E-S-T-T-------H-N__---N-T-G-___-SQ-              457
rtβ2:      GL-R-ETFDSETQESRDSAYVEPK---SHEHV-R-V---E-NHREESH-S---RHRE-RH                 551
rtβ1b      LSRQDTFDADTPGSRNSVYTEPGDSCVDMETDPSEGPGPGDPAGGGTPPARQGSWEEEED                 553

rtβ3:      RLLAQDSEHDHNDRNWQRNRP_____________________________WPKDSY@                     484
xoβ3:      ---ER---QN-----------_____________________________------@                     484
rtβ2:      -TRDMGRDQ---ECSK--S-HKSKDRYCDKEGEVISKRRSEAGEWNRDVYIRQ@  2b/2a  628/604
rtβ1b      YEEEMTDNRNRGRNKARYCAEGGGPVLGRNKNELEGWGQGVYIR@                 1b(B2)  597
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Fig. 1. Continued.

types of α_1 were cloned, it became apparent that often co-expression of β augmented macroscopic currents (Wei *et al.*, 1996), to the extent that in some instances it proved to be an intrinsic requirement for development of mature Ca^{2+} channel currents. The extreme case of the latter appears to be α_{1A} (also brain-I or BI; see Mori *et al.*, 1991, and Stea *et al.*, 1994). Many initial studies of newly cloned α_1 subunits were therefore carried out almost exclusively in the presence of co-expressed β (e.g., Ellinor *et al.*, 1993). To some extent this delayed studies of their qualitative effects.

As observed in our laboratory, the functional importance of β subunits became apparent in studies with both α_{1C} and α_{1E}, which in contrast to α_{1A}, α_{1C}, and α_{1E} express readily in *Xenopus* oocytes. This allowed us to compare channel properties expressed in the absence of exogenous β to those obtained in the presence of exogenous β, and hence a comparison of different β subunits.

EFFECTS OF β SUBUNITS ON CHANNEL ACTIVATION AND INACTIVATION

The first evidence that β subunits play important roles in Ca^{2+} channel function came from the studies of Singer *et al.* (1991) who noted major effects on expression and kinetics of the cardiac α_{1C} in *Xenopus* oocytes, and those of Lacerda *et al.* (1991) who showed that grossly abnormal kinetics of the skeletal α_{1S} expressed in L cells could be normalized by co-expression of the β subunits. Numerous subsequent studies, increasingly more sophisticated in their analysis of Ca^{2+} channel currents and carried out primarily but not exclusively in *Xenopus* oocytes, have shown that β subunits affect all aspects of α_1 function. As seen with neuronal non-L-type α_1 subunits, β subunits affect the rates of activation and deactivation by voltage, the rate of voltage-induced inactivation, inhibition by G protein βγ dimers, and coupling of voltage sensing to pore opening. Likewise, when tested using an L-type α_1 subunit, α_{1C}, β subunits markedly improve the coupling of voltage sensing to pore opening and are an obligatory requirement for development of the prepulse facilitation phenomenon.

α_{1C} Regulation by β Subunits

As seen with α_{1C} in *Xenopus* oocytes, β subunits promote a left shift in the current–voltage (*I–V*) and conductance–voltage (*G–V*) relationships (Fig. 3A and

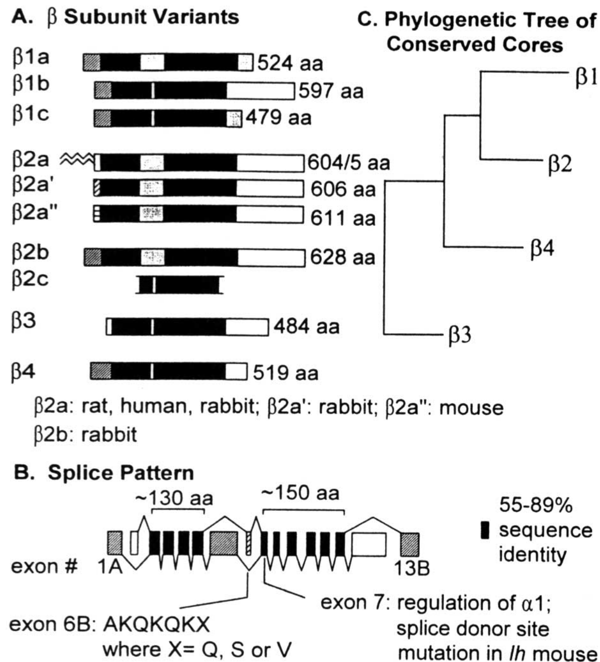


Fig. 2. Ideograms of Ca^{2+} channel β subunit gene and splice variants (panel A) and evolutionary relatedness (growtree phylogram of the Wisconsin GCG Computer Package; panel C). Panel B depicts the splice pattern of β subunits as inferred from Powers *et al.* (1994) and Murakami *et al.* (1996) for β1 and β3 respectively. It also shows the location of the splice donor site mutation in β4 that causes ataxia and seizures in the lethargic (*lh*) mouse (Burgess *et al.* 1997).

3B) and at the same time accelerate the rate at which the channel activates at any given test potential (Fig. 4). By employing the "cut open oocyte" vaseline-gap voltage clamp method it is possible to record not only ionic currents of α_{1C} channels expressed in *Xenopus* oocytes, but also gating currents and their development as a function of the test potential. This revealed that in contrast to ionic currents, the movement of the voltage sensor as a function of voltage (*Q–V* relationship) is not affected by the presence of the β subunit. Moreover, for the particular combination of α_{1C} and β2a, the total charge moved at fully activating potentials was unaffected by the presence of the exogenously expressed β subunit. Since the charge moved is proportional to the number of voltage-sensing molecules under clamp conditions, this indicated that increases in macroscopic current observed upon co-expressing β with α_{1C} are fully accounted for by the shift in the *G–V* relationship. By extension this is proof that the β improves the coupling of charge movement to pore opening (Neely *et al.*, 1993; Noceti *et al.*, 1996). In contrast to β2a, other types of β subunits not only improve intramolecular coupling between the voltage sensing and pore opening, but also the expression/ maturation of the channel proper. As a result, with β1b, for example, both gating currents and coupling of voltage sensing to pore opening are improved, and the increase in macroscopic currents is the result of the sum of more channels each "working" more efficiently.

α_{1E} Regulation by β Subunits

In contrast to L-type channels, non-L-type Ca^{2+} channels not only activate rapidly in response to a test potential but also inactivate. Both responses are affected by β subunits. As seen with α_{1E}, activation of non-L-type α_1 channels is qualitatively similar to that seen with the L-type α_{1C}: co-expression of β subunits improves coupling of voltage sensing to pore opening and, depending on the type of α_1, facilitates (α_{1A}) or does not facilitates (α_{1E}) assembly, maturation, and surface expression of the channel complex.

Coupling of charge movement to pore opening is much tighter in α_{1E} than in α_{1C} (Olcese *et al.*, 1996). The ability of β subunits to facilitate pore opening is thus not seen as one of left-shifting the overall voltage–conductance relationship, but rather one of increasing the "steepness" of the *G–V* curve, which of course is also seen with α_{1C}. The "steepening" of the α_{1E} *G–V* curve (Fig. 5) is well explained by a switching in gating modes with distinct midpotentials ($V_{1/2}$) of activation: one activating with $V_{1/2}$ around 0 mV, the other with $V_{1/2}$ at approximately 40 mV. For α_{1E} alone the *G–V* is well described by a simple sum of 50% of the channels gating in one mode and 50% in the other. Upon co-expression of a β subunit the distribution of channels between these gating modes is changed so that now 75–80% of the channels are activated with $V_{1/2}$ around 0 mV. This is accompanied by a reduction in the proportion of channels that are activated with mid-activation potentials around 40 mV. In addition, for α_{1E} this is also accompanied by an increase in the absolute Po_{max}, presumably through reduction of channels trapped in a non-opening gating mode (nulls, e.g., Noceti *et al.*, 1996). Important for the present discussion is that all β subunits had the same qualitative effect on α_{1E} activation, regardless of the gene of origin or the type of splice variant (Fig. 5).

β4 and α_{1A}

Despite the initial successes in observing an effect of the newly cloned β4 subunit on α_{1C}, this has been

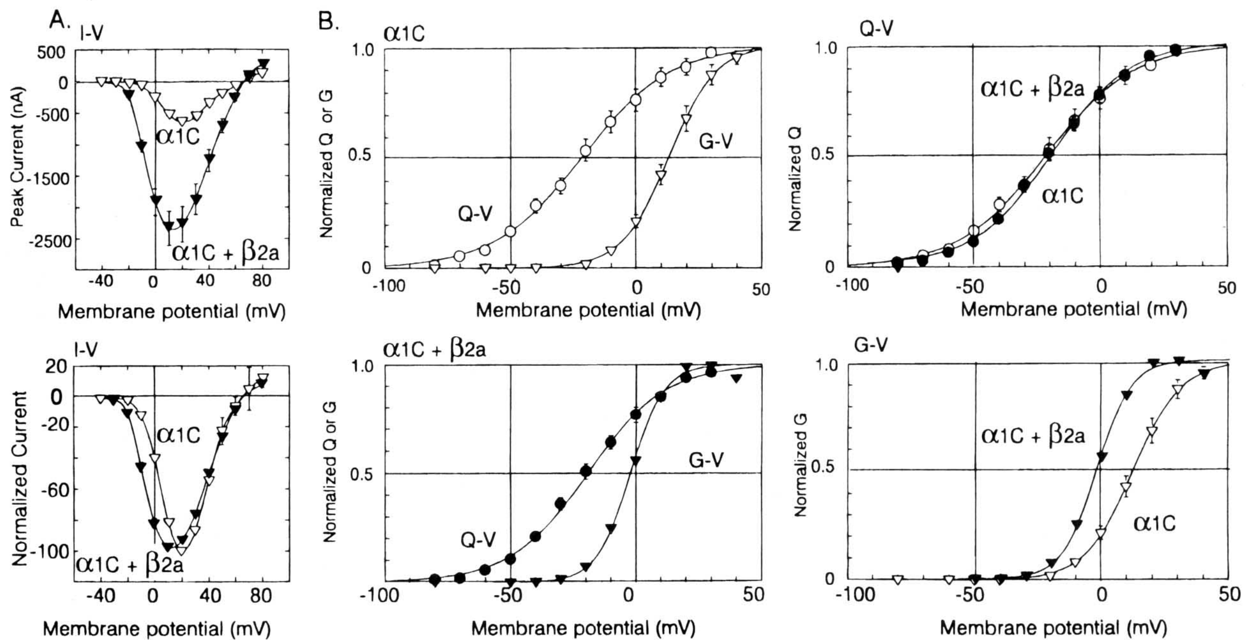


Fig. 3. *I–V, G–V,* and *Q–V* relationships of α_{1C} and $\alpha_{1C}\beta2a$ in *Xenopus* oocytes. Panel A. IV relations obtained using a standard two-electrode voltage clamp. Adapted from Perez-Reyes *et al.* (1991) Panel B, *G–V* and *Q–V* relations replotted from Neely *et al.* (1994). Note that β2a did not affect the *Q–V* relationship but left-shifted the *G–V* relationship. Since the change in conductance (pore opening) is the readout of the effect of a change in voltage on the voltage sensor, the data show that β subunits improve coupling between voltage sensing and pore opening.

an observation that has been difficult to repeat consistently, with most attempts at determining an effect of β4 on either α_{1E} or α_{1C} being unsuccessful. Likewise, functional expression of α_{1A} (either the type I or the type II splice variant, generously provided by T. Tanabe) has also been a low-frequency event, so that we have very few sets of experiments in which the batch of oocytes injected with α_{1A} alone developed measurable Ca^{2+} channel currents (i.e., peak currents greater than 5 nA at 0 mV). Results from one of these sets of experiments are shown in Fig. 4B. Yet in spite of these failures (no effect of β4 and lack of consistent expression of α_{1A} alone), the combination of α_{1A} and β4 leads consistently to expression of $\alpha_{1A}\beta4$ channels (Fig. 4B). The molecular determinants responsible for this selectivity have not been investigated.

Inactivation

α_{1E} channels display a rather rapid voltage-induced inactivation and this inactivation is strongly modulated by β subunits. The first report on the ability of different β's to confer differential inactivation properties to an α_1 came from a study in which inactivation of Doe-1 was studied (Ellinor *et al.*, 1993). This was expanded to other α_1 subunits, particularly α_{1A} which was shown by Stea *et al.* (1994) to inactivate much more slowly when co-expressed with β2a than with other β subunits. The use of α_{1E}, which, in contrast to Doe-1 and α_{1A}, expresses well in *Xenopus* oocytes without requiring the simultaneous co-expression of an exogenous β, uncovered that the effects of β_{2a} and β_{1b} differed not in the extent to which they accelerated inactivation, with β_{2a} being the least effective of the two, but that the two β's had opposing effects: β_{1b} accelerated inactivation and left-shifted the potential at which a given protocol promotes 50% inactivation; β_{2a}, on the other hand, caused the opposite to happen: inactivation was slower in being established and 50% inactivation occurred at higher potentials (Fig. 6).

The primary region of β2a responsible for the opposing effect on α_{1E} inactivation as compared to the effects of other β subunits was localized to its N-terminus (Olcese *et al.*, 1994). In addition, a variable region in the "middle" of the β1 and β2 molecules, inserted between two highly conserved domains, was subsequently found to be a second region able to affect

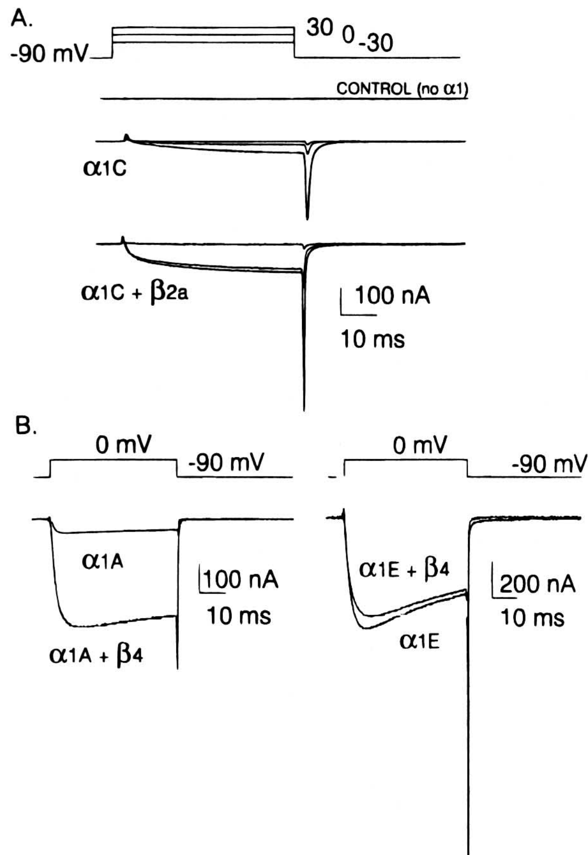


Fig. 4. Representative effects of β subunits on Ca^{2+} channel currents in oocytes injected with different types of α_1 cRNA's. Data in Panel A are adapted from Neely *et al.* (1994). Except when stated otherwise, Ca^{2+} channel currents presented in this and subsequent figures were recorded using the "cut open oocyte" method of Taglialatela *et al.* (1992) using Ba^{2+} as the charger carrier.

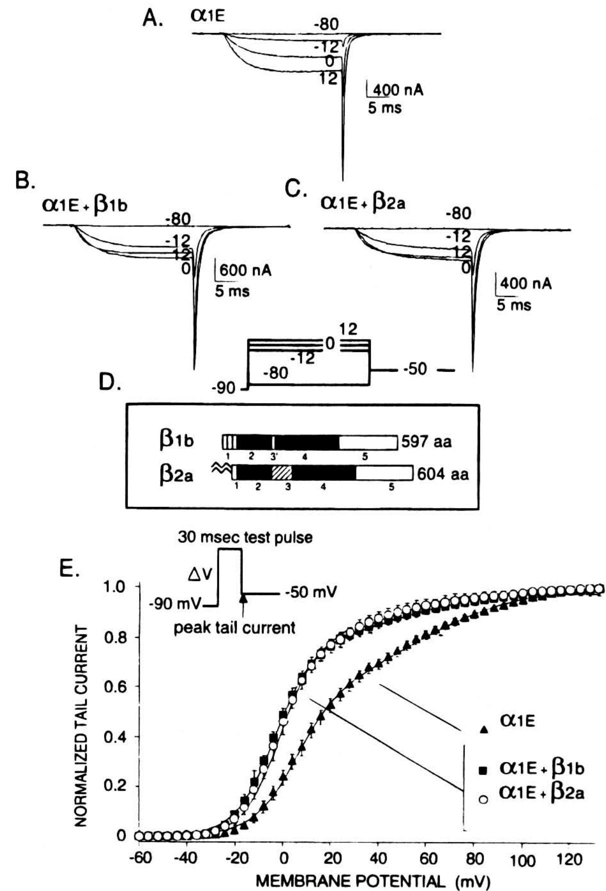


Fig. 5. Facilitation of α_{1E} activation by β_{1b} and β_{2a} subunits. Note that β subunits accelerate activation (top panels) and promote a left-shift in the *G–V* curve. Fits using two Boltzmann distributions showed the effect to be due mainly to an increase in the amplitude of a component that is activated between 0 and 5 mV. Note also that the effects of the two β subunits are indistinguishable. Other experiments (Olcese *et al.*, 1996) showed that at low voltages (−20 to 0 mV) *G–V* and *Q–V* relations are essentially superimposable, indicating a much tighter intrinsic coupling between voltage sensor and pore opening in α_{1E} than α_{1C} (adapted from Olcese *et al.*, 1995).

α_1 inactivation. This region is encoded in the two alternatively used exons #6 (cf. Figs. 1 and 2). When compared to the short counterpart, long D3 domains of β subunits slow inactivation and cause a right shift in the potentials causing 50% steady-state inactivation. In contrast to the N-termini, the effect of the long/short middle domains is cryptic, i.e., only seen when the inactivation-setting N-terminal domains are mutationally removed. Further studies are thus necessary to determine the importance, if any, of this middle domain in regulating α_{1E} inactivation (Qin *et al.*, 1996).

β Subunits and the Phenomena of Prepulse Facilitation

Prepulse facilitation is a phenomenon in which a train of depolarizations or, in its stead, a long and strong depolarizing pulse, induces a form of the Ca^{2+} channel that in response to a given test potential exhibits an increased opening probability that persists for several seconds after repolarization (reviewed in Dolphin, 1996). There are two major classes of prepulse-induced facilitation of Ca^{2+} currents and both are affected by Ca^{2+} channel β subunits, but in opposite ways.

One type of prepulse facilitation is seen with L-type Ca^{2+} channels and is highly variable depending on the tissue or cell as well as the species in which it is studied. Thus, it has been recorded in several neuronal cells (Ikeda, 1991; Artalejo *et al.*, 1991; Kavalali

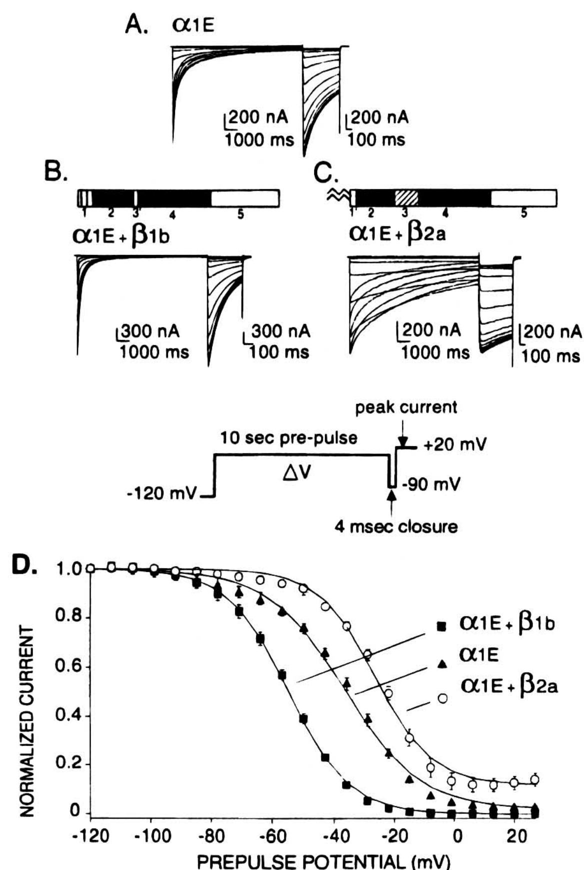


Fig. 6. Differential modulation of α_{1E} inactivation by β_{1b} and β_{2a} subunits. The figure shows voltage-induced inactivation of α_{1E} in oocytes injected with α_{1E} alone, and with β1b or β2a. Voltage change protocols are shown on the figure (adapted from Olcese *et al.*, 1994). Note that in contrast to activation, the two β subunits have opposing effects on inactivation. Other experiments (Qin *et al.*, 1996) showed that the opposing effect is conferred to the β subunit by the N-terminus and that in the absence of an N-terminus, the long form of the middle section of β_{2a} and β_{1b} can also confer the ability to a β subunit to delay channel inactivation and that different C-termini associated with β1 and β2 do not influence activation, deactivation, and inactivation of α_{1E}.

and Plummer, 1996), in skeletal muscle (Johnson *et al.*, 1997), and in guinea pig and amphibian cardiac cells (Brown *et al.*, 1984a, b; Lee, 1987; Fedida *et al.*, 1988; Zygmunt and Maylie, 1990; Noble and Shimoni, 1981a, b), but, for example, not in rodent cardiac cells (Cens *et al.*, 1996). Prepulse facilitation of an L-type Ca^{2+} channel was recapitulated with cloned α_{1C} by Kleppisch *et al.* (1994) in CHO cells that had been transfected with α_{1C} cDNA alone and also by Bourinet *et al.* (1994) in *Xenopus* oocytes. However, in this latter expression system, prepulse facilitation was dependent on co-expression of a β subunit (β1b).

Another type of prepulse facilitation, also referred to as prepulse potentiation, is seen primarily with non-L-type Ca^{2+} channels of neurons and is in fact due to a reversal or attenuation of the inhibition of channel activity imposed by agonists known to act via G_i/G_o-coupled receptors and formation of free Gβγ dimers (Dunlap and Fischbach, 1981; Tsunoo *et al.*, 1986; Bean, 1989; Ikeda, 1996; Herlitze *et al.*, 1996).

Figure 7 illustrates the dependence of prepulse facilitation of α_{1C} on a β subunit and the suppression of "prepulse facilitation" of α_{1E} under negative control by the G_i/G_o-coupled M2 muscarinic receptor.

POSTTRANSLATIONAL MODIFICATIONS OF β SUBUNITS AND ROLE OF N-TERMINAL PALMITOYLATION OF β2A

Phosphorylation

In both the heart and the skeletal muscle, stimulation of the cAMP-dependent protein kinase leads to augmented Ca^{2+} channel currents (cf. Osterrieder *et al.*, 1982). Studies on the susceptibility to phosphorylation and state of phosphorylation of the Ca^{2+} channel components have shown that both the pore-forming α_1 and the regulatory β subunits incorporate phosphate *in vitro* and *in vivo* (Flockerzi *et al.*, 1986; Chang *et al.*, 1991; Haase *et al.*,, 1996), and DeJongh *et al.* (1989) identified the phosphorylated site of the skeletal muscle β subunit as Thr-205 in a RRPTP consensus protein kinase A consensus site located just prior to the D3 domain. Analysis of the amino acid sequences deduced from the cloned cDNA's shows consensus sites for not only cAMP-dependent protein kinase but also for protein kinase C in all β subunits. However, in spite of finding that β subunits are phosphorylated and that, for example, the state of phosphorylation in cardiac cells correlates with the state of activation of protein kinases (Haase *et al.*, 1996), it has not been possible as yet to establish whether β subunit phosphorylation is functionally relevant (see, for example, Gutierrez *et al.*, 1994).

Palmitoylation

Hosey and collaborators made the interesting observation that β2a is unique in that it is palmitoylated at cysteines 3 and 4 of its N-terminus (Chien *et al.*, 1997). Replacement of either Cys-3 or Cys-4 with Ala led to loss of palmitoylation, as did of course double

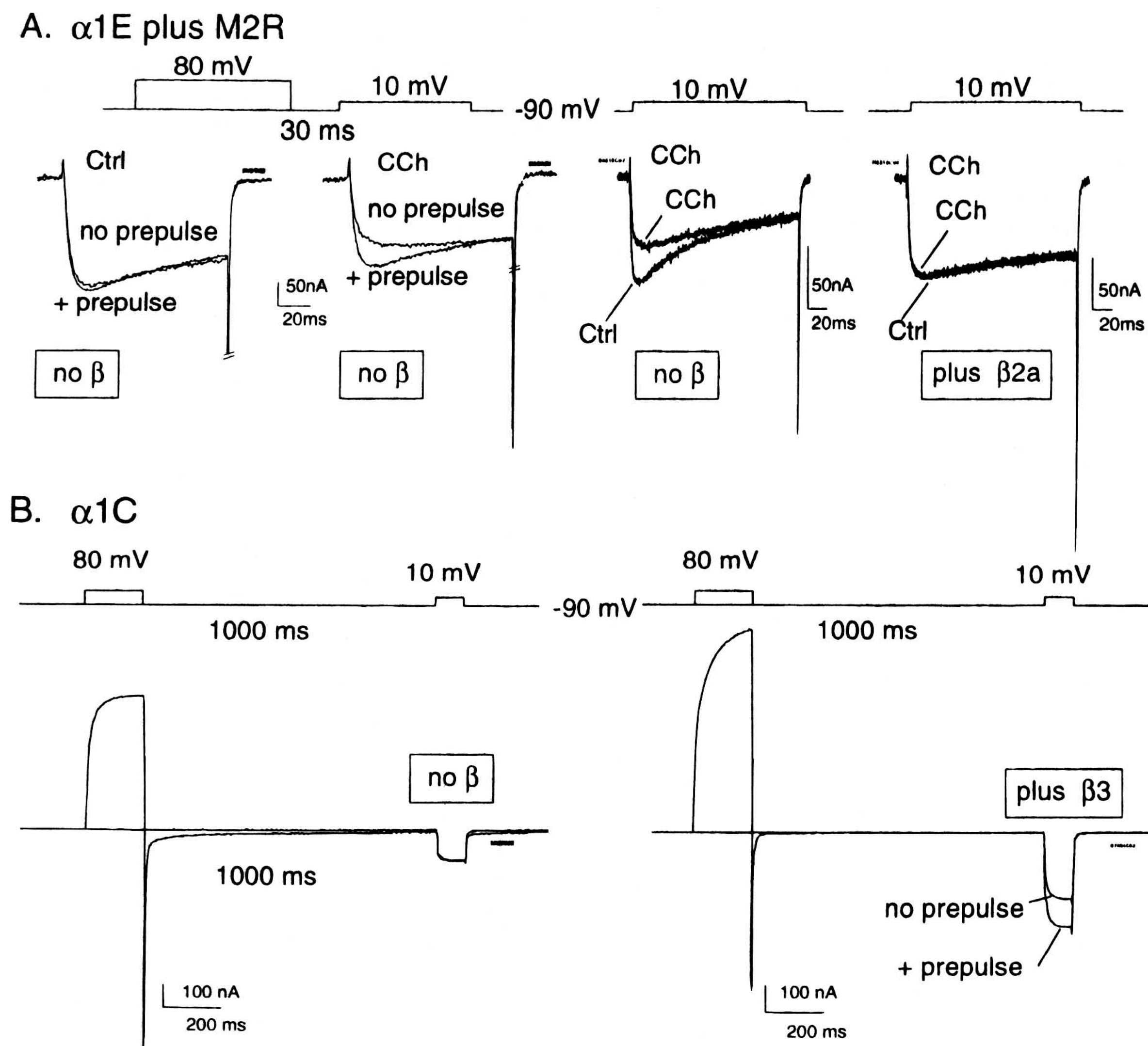


Fig. 7. Distinct forms of prepulse facilitation or potentiation in α_{1C} and α_{1E}. Panel A, dependence of prepulse facilitation of α_{1C} activation on a β subunit. Panel B, dependence of prepulse facilitation of α_{1E} on prior inhibition by a G protein-coupled receptor, in this case the carbachol (CCh)-activated M2-muscarinic receptor and suppression of the G protein-mediated effect by β2a (adapted from Qin *et al.*, 1997). Although somewhat less effective, β1b and β3 affect the G protein-coupled regulation in the same way as β2a (Bourinet *et al.*, 1995; Qin *et al.*, 1997).

replacement of Cys 3 and 4. Although palmitoylation could be assumed to contribute or even be a determinant for membrane localization of this β subunit, Hosey's group also found that $Cys^{3,4}Ala$ still localizes to the membrane fraction of cells in which it is expressed (Chien *et al.*, 1997). As is the case for the B-type splice variant of Kirsten *ras*, a non-isoprenylated form of *ras* (Hancock *et al.*, 1989), membrane localization of the $Cys^{3,4}Ser$ β2a may be determined by its cluster of positive amino acids. In contrast to phosphorylation, palmitoylation has been shown to be an important functional parameter and responsible for many of the properties that distinguish β2a from other β subunits.

PALMITOYLATION OF β2A INTERFERES WITH ITS ABILITY TO SUPPORT PREPULSE FACILITATION OF α_{1C} AND IS RESPONSIBLE FOR RIGHT-SHIFTING STEADY-STATE INACTIVATION CURVES OF α_{1E}

β subunit-dependent long-lasting prepulse facilitation of α_{1C} channels does not develop in oocytes

injected with α_{1C} plus β2a (Cens *et al.*, 1996). The structural determinant responsible for the difference between β2a and other β subunits resides in the nature of its N-terminus, and within it, in the fact that it is palmitoylated. As shown in Fig. 8A, preventing palmitoylation of β2a by mutating the acceptor cysteines to serines (β2a[$Cys^{3,4}$Ser]) "unblocks" its inability to support prepulse facilitation of α_{1C}. In agreement with this interpretation, replacing the N-terminus of β3 with that of the wild type β2a blocked the ability of β3 to support prepulse facilitation. Other experiments showed concurrent loss of palmitoylation of β2a upon introducing the Cys to Ser. As expected β3 carrying the N-terminus of β2a incorporates polmitate (Qin and Birnbaumer, unpublished). We tested whether other "unique" properties of β2a, notably its distinctive ability to affect inactivation in a manner that is opposite to the way other β subunits do (cf. Fig. 6, Olcese *et al.*, 1994), is also related to its N-terminal palmitoylation. As shown in Fig. 8B, this is indeed the case.

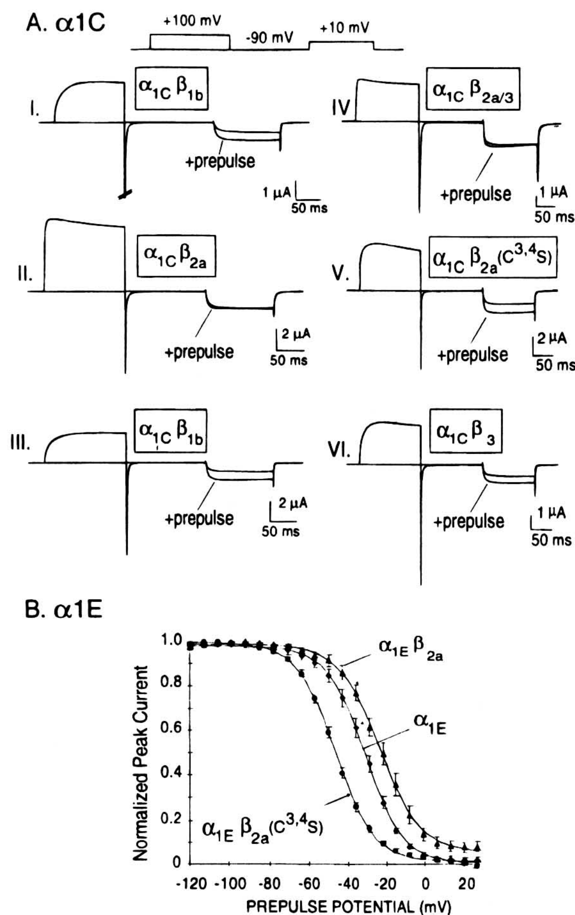


Fig. 8. Palmitoylation confers special properties to β2a. A. Prepulse facilitation of α_{1C}. β1b, β3, and a palmitoylation mutant of β2a support development of prepulse facilitation in α_{1C} while the palmitoylated β2a, or the also palmitoylated (β2a/β3 chimera (β2a[1–16]/β3[16–484]), fail to do so. Voltage protocols are shown on top of the traces; A, B, C, and D show representative records of prepulse facilitation obtained in oocytes injected with α_{1C} and the indicated β subunit cRNAs. B. Steady-state inhibition of α_{1E}. While β2a is unique in right-shifting the voltage inhibition curve of α_{1E}, the non-palmitoylated β2a[$Cys^{3,4}$Ser] causes a left shift as do other β subunits (Fig. 6, Olcese *et al.*, 1994).

β-SUBUNITS ARE ESSENTIAL FOR PROPER CHANNEL ASSEMBLY AND TARGETING

Three lines of evidence indicate that β subunits are essential for proper channel assembly and delivery to the cell surface: *in vitro* expression of α_{1C}, gene targeting of β1, and effects of antisense oligonucleotides in *Xenopus* oocytes. Thus, Hosey and collaborators investigated the subcellular localization of α_{1C} in cells transfected with α_{1C} in the absence and presence of β2a, and found that while the total amount of α_{1C} accumulated in cells in the absence of b2a was not much affected by β2a, β2a markedly increased the amount of α_{1C} in the plasma membrane, where it colocalized with α_{1C}. Expression of β2a alone showed it to localize to the plasma membrane, indicating that while α_{1C} was dependent on β, β was not dependent on α_1 to reach the plasma membrane (Chien *et al.*, 1996). Gregg *et al.* (1993) inactivated the murine CACNLB1 gene encoding the Ca^{2+} channel β1 subunits. This led to death at birth due to asphyxia, secondary to loss of excitation–contraction coupling. Studies of myotubes derived from β1-/-mouse embryos showed that myotubes not only lacked excitation contraction coupling but also that their Ca^{2+} currents were reduced by 10–20-fold and that this was accompanied by absence of detectable α_{1S} in the cells (Strube *et al.*, 1996). These authors concluded that not only is the β subunit important for assembly of functional Ca^{2+} channels but also for the targeting of the α_1 subunits, α_{1S} in their case, to its normal site of expression. In agreement with this conclusion, expression of β1 in β1-/- myotubes rescued Ca^{2+} currents and excitation–contraction coupling (Beurg *et al.*, 1997). The third line of evidence pointing to an obligatory role of β subunit in channel assembly and targeting, in addition to their regulatory roles, was the finding that *Xenopus* oocytes express an endogenous β subunit and that

suppression of this β subunit blocked Ca^{2+} channel expression.

A β SUBUNIT IN *XENOPUS* OOCYTES

Lacerda and colleagues reported the occurrence of endogenous Ca^{2+} channel currents in *Xenopus* oocytes (Lacerda *et al.*, 1994). With a much lower frequency, approximately once every two to three months, we also encountered batches of oocytes that have endogenous Ca^{2+} channel currents. We therefore wondered whether these were α_1 or $\alpha_1\beta$ type channels. This was investigated by searching for presence in the oocytes of mRNA encoding a β subunit. We indeed found such sequences (Tareilus *et al.*, 1997). Using a RACE-PCR approach we cloned from *Xenopus* oocyte mRNA two full-length cDNAs differing in their coding region in only 74 randomly distributed nucleotides and encoding two β subunits of 484 amino acids that differ in 22 amino acids (94.5% sequence identity). *Xenopus* oocytes are tetraploid and the two subunits may either be closely related non-allelic isoforms or two alleles of a highly polymorphic gene. Comparison of the amino acid sequences of the *Xenopus* β subunits to those of the four known mammalian β subunits places them into the type 3 category (Fig. 1). Xoβ3 subunits play an essential role in the expression of endogenous and exogenous α_1 subunits as deduced from the following results: (1) co-injection of α_{1E} or α_{1C} cRNA with Xoβ3 cRNA led to Ca^{2+} channel currents that resembled those obtained by co-expression of these α_1 subunits with rat β3. (2) Injection of antisense oligonucleotides to *Xenopus* β3 resulted in suppression of ionic Ca^{2+} channel currents as well as of the gating currents associated with the expression of α_1 subunits. (3) Injection of oocytes with concentrations of anti-Xoβ3 oligonucleotides that resulted in >95% loss of Ca^{2+} channel currents together with mammalian β1a cRNA "rescued" expression of α_1. This last experiment (illustrated in Fig. 9), together with other experiments in which sense oligonucleotides had no effect on expression of "α_{1E} alone" currents, and Xoβ3 antisense oligonucleotides that suppressed Ca^{2+} channel currents had no effect on the expression of Shaker K^+ channels, indicated that the effect of antisense was not a nonspecific inhibition of the translation machinery of the oocyte. We concluded that the so-called "α_1-alone" currents recorded by us and others from oocytes that had been injected only with an α_1 cRNA, could be due to channels that had at one point or another inter-

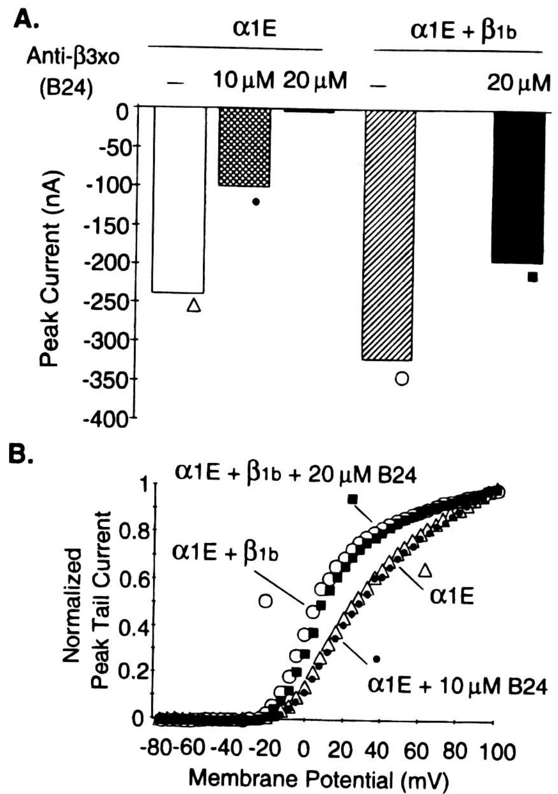


Fig. 9. Evidence for dependence of Ca^{2+} channel currents in *Xenopus* oocytes on an endogenous β subunit: suppression by antisense oligonucleotide and reversal of inhibition by excess mammalian β subunit cRNA. Panel A: Dose-dependent inhibition of α_{1E} by antisense oligonucleotide B24 and rescue by co-injected β1b cRNA. Panel B: G–V relationships of α_{1E} channels expressed in oocytes injected with the indicated cRNA's and 10 μM B24. Note that α_{1E} currents of oocytes in which α_{1E} expression was inhibited 60% by anti-β3xo (10 μM) expressing did not differ from that recorded from oocytes that had received α_{1E} cRNA alone. We interpret this to mean that the dual gating mode inferred from the two-Boltzmann fit is not due to expression of a mixture of α_{1E} alone plus α_{1E}.β channels. Note further that the better than 98% inhibition of expression by 20 μM anti-β3xo is not due to a nonspecific or toxic effect of the oligonucleotide.

acted with the endogenous *Xenopus* β3 (β3xo). The most plausible role of β in this case would be that of aiding in folding, subunit assembly, and targeting, as inferred also from gene knockout experiments and the effect of β to redirect α_1 in tissue culture cells.

Burgess *et al.* (1997) mapped the genomic locus causing ataxia and lethargy in the mouse. They identified the genomic locus as that of the Ca^{2+} channel type-4 β subunit (CACNLB4 or CCB4), which in the lethargic (*lh*) mouse has a four-nucleotide insertion (Fig. 2C) that leads to loss of a splice donor site, exon skipping, and a translational frameshift. As a result

the translated protein is truncated and lacks sequence similarity domains D4 and D5, which include the α_1 binding site of β subunits (De Waard *et al.*, 1994). This is further evidence for the essential role of β subunits in Ca^{2+} channel structure and function.

TWO SITES FOR INTERACTION WITH β SUBUNITS ON NON-NEURONAL α_1 SUBUNITS (TYPE E, A AND B)

The existence in *Xenopus* oocytes of an endogenous β subunit and its obligatory role in the assembly and targeting of channels to the plasma membrane raised the question as to the molecular makeup of Ca^{2+} channels in oocytes injected with α_1 alone. Two possibilities needed to be considered. The first assumes that the endogenous β does not stay with the channel once it is in the plasma membrane and that channels in α_1 alone injected oocytes are indeed α_1 alone channels as assumed. As such they present a typical functional profile that is then modulated by expression of high levels of an exogenous β subunit. The second possibility is that the endogenous β subunit does not dissociate from the channel after it reaches the plasma membrane and that channels in α_1-injected oocytes instead of being formed of α_1 alone are instead $\alpha_1\beta$xo complexes. By extension the functional profile of channels expressed in oocytes not receiving β subunit cRNA's are those of $\alpha_1\beta$xo, and not as commonly assumed "α_1 alone" channels. By extension the modulated functional profile of channels formed in oocytes receiving both the α_1 and the β subunit cRNA's are given by $[\alpha_1\beta]\beta$ complexes. By further extension of this reasoning, α_1 subunits should have a second β subunit binding site: one used by the β subunit aiding in folding, assembly, and targeting and another changing its channel characteristics: facilitating activation, supporting prepulse facilitation, modulating voltage-induced inhibition. Campbell and collaborators had shown that loop 1 (L1), the segment connecting repeat domain I to repeat domain II of α_1 subunits, has the capacity to bind β with high affinity (Pragnell *et al.*, 1994; Witcher *et al.*, 1995). We thus scanned α_{1E} for additional β subunit binding site(s). As illustrated in Fig. 10, an additional site was found in the C-terminus (Qin *et al.*, 1997). The approach used was similar to that used by Pragnell *et al.* (1994), in that we made ^{35}S-labeled β2a by *in vitro* translation of cRNA and asked it to bind to GST fusion proteins formed of glutathione-S-transferase joined to segments of α_1: N-terminus, loop 1 (between repeats I and II), loop 2 (between repeats II and III), loop 3 (between repeats III and IV), and the C-terminus.

We confirmed the finding of Campbell and collaborators that loop 1 interacts wit β. In addition we found that β interacts with the C-terminus (of α_{1E}, Fig. 10). Fine-mapping of this site identified it to reside in a very short 38-amino acid long stretch in the middle of the α_{1E} C-terminus (Fig. 11). A homologous site was also found in α_{1A} and α_{1B}, but not in α_{1C} (not shown). The experiments in Fig. 12 show that the region of the β subunit that interacts with the C-terminal α_{1E} sequence, D4, is the same as that previously determined by Campbell's group to interact with the Loop I–II L1 linker (De Waard *et al.*, 1994).

THE C-TERMINAL β SUBUNIT BINDING SITE IS ALSO A Gβγ BINDING SITE

The fact that no second β binding site was seen for α_{1C} led to two corollaries for the C-terminal β subunit binding site: (1) it could not be responsible for the modulation of our hypothetical $\alpha_1\beta$ complexes, because both non-L and L-type α_1 subunits require β subunits for expression in the oocyte, and (2) its existence raised the question whether it is of functional relevance. Independent studies that we were carrying out in parallel on the mode of action of G protein βγ subunits provided the answer to the question of functional relevance, for we determined that it is the site to which β subunits bind to inhibit the effect of Gβγ dimers (Qin *et al.*, 1997).

CONCLUDING REMARKS

In this article we have illustrated the importance of β subunits in the moment-to-moment regulation of voltage-gated Ca^{2+} channels. Isolated as part of a dihydropyridine binding complex from skeletal muscle T-tubules, β subunits have now been shown to be central players in most if not all functional aspects of Ca^{2+} channel function: facilitation of activation and deactivation in response to voltage, modulation of inactivation by voltage, and support for or inhibition of special α_1-specific phenomena such as long-lasting prepulse facilitation (τ = ca. 20 sec!) and Gβγ-mediated inhibition of non-L-type Ca^{2+} channels (formed by α_{1E}, α_{1B}, or α_{1A}). Some of the immediate questions that need to be addressed are the molecular makeup

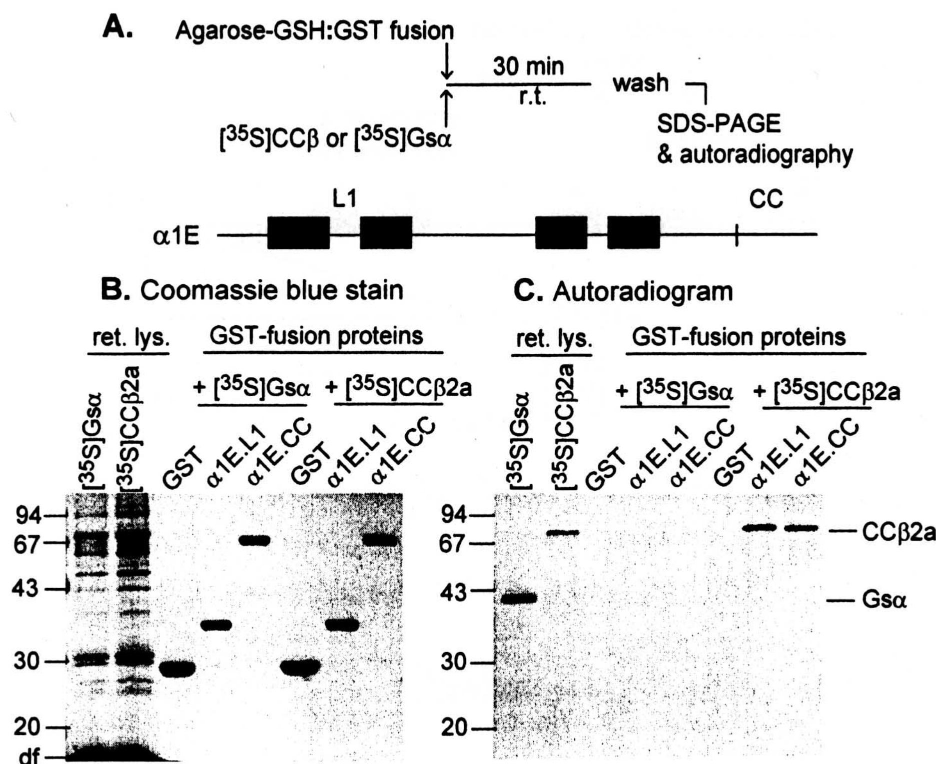


Fig. 10. Identification of two sites on α_{1E} that interact with β subunits. The figure shows binding of *in vitro*-translated, ^{35}S-labeled β_{2a} to the recombinant L1 and the CC regions of α_{1E} synthesized in *E. coli* as fusion proteins fused to bacterial glutathione-S-transferase (GST). Note that β2a interacts not only with the loop connecting α_1 repeat domains I and II (L1), originally identified by Pragnell *et al.* (1994), but also with the carboxyl-half of the C-terminal tail of α_{1E} (last 275 amino acids of the 2312 amino acid α_{1E}, referred to as CC or, elsewhere, as ECC). As outlined in the figure, GST:α_{1E} fusions were adsorbed to glutathione-agarose and incubated with ^{35}S-labeled calcium channel β_{2a} (CCβ2a) or $G_s\alpha$ (control). The beads were then collected by centrifugation, washed several times, and resuspended in Laemmli's sample buffer containing 1% SDS. The mixtures were directly electrophoresed in 9% SDS-PAGE gels, stained with Coomassie blue (Panel A), dried, and autoradiographed (Panel B) to determine whether [^{35}S]$G_s\alpha$ or [^{35}S]CCβ_{2a} had bound to GST or any of the GST fusion proteins. Note also that α_{1E} L1 and α_{1E} CC bound [^{35}S]CCβ_{2a} but not an unrelated protein, [^{35}S]$G_s\alpha$.

of the so-called "α_1 alone" and "$\alpha_1\beta$" channels in *Xenopus* oocytes and the fine mapping of α_1–β interactions.

Truncation of the C-terminus of α_{1E} up to and including the β binding site showed that this loss does not affect regulation of channel kinetics by β subunits and indicated that even though β binds tightly to two distinct sites, only one of them, the binding to the L1 loop between repeat domains I and II, appears to be relevant to regulation of the channel's responses to voltage (Qin *et al.*, 1997).

The existence of two β subunit interaction domains raises the immediate question as to the three-dimensional structures of the binding complexes. A sequence comparison of the region in loop 1 involved in binding to β (De Waard *et al.*, 1994) to those of the C-termini in α_{1A}, α_{1B}, and α_{1E} shows no obvious similarities. Also among the C-terminal binding sequences there is significant variation. A study of the three-dimensional configuration of these complexes should therefore be revealing in terms of further understanding the mechanisms by which β subunits regulate α_1 subunits. Although the L- and C-terminal binding sites of α_1 have the capacity to bind β independent of each other, it is conceivable that in the context of full α_1 the two sites cooperate in interacting with a single β.

The kinetic mechanism by which a β subunit reduces the frequency of nulls is also of interest. Analysis of the effect of β on α_{1C} gating at the single-channel level revealed, surprisingly, that it had little effect on

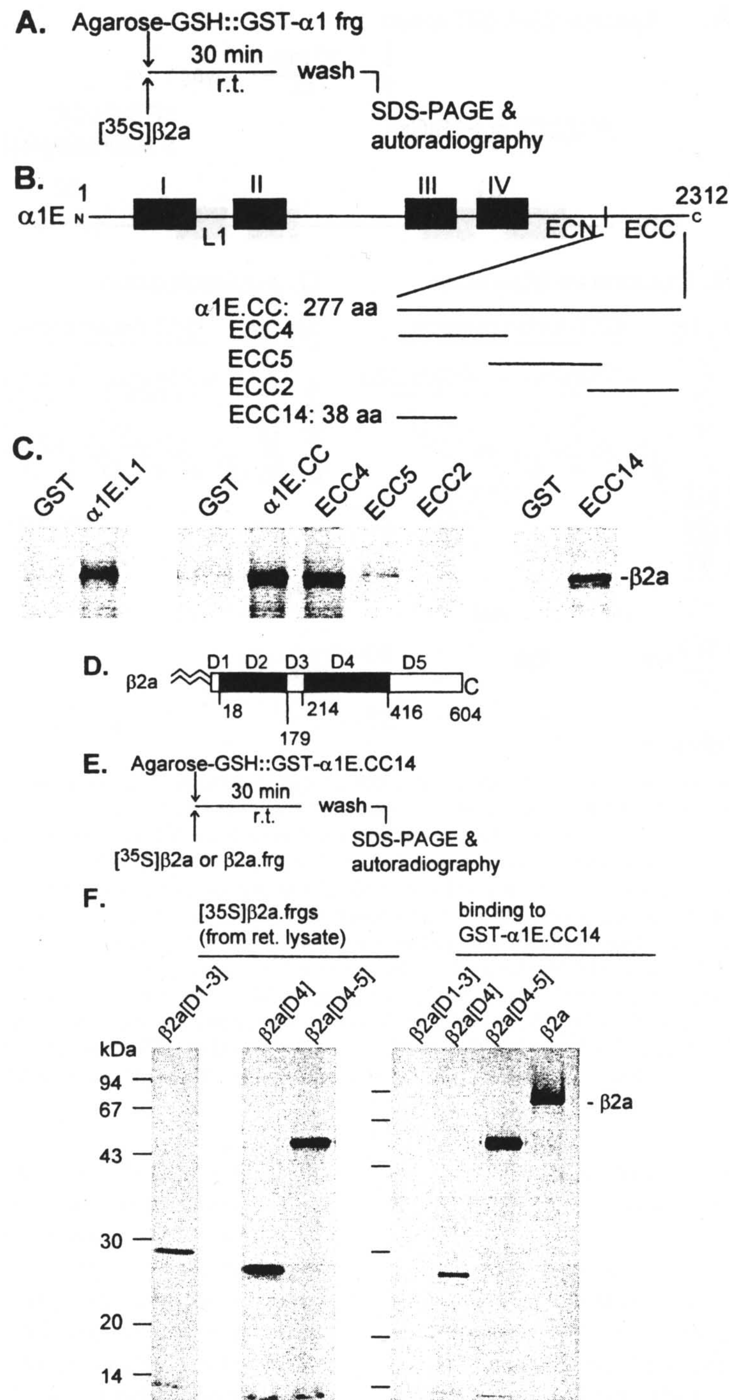
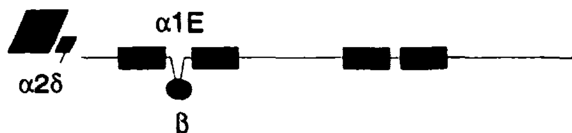


Fig. 11. Binding of β2a to subfragments of the C-terminus of α_{1E} reveals a 38 amino acid β subunit binding domain of α_{1E} (Panels A–C) that binds to the sequence similarity domain 4 (D4) of the β subunit (D–F). A homologue of the α_{1E} β subunit binding site was also found in α_{1B} and α_{1A} but not in α_{1C}. For further details see Qin *et al.* (1997).

Model 1. The mature regulated channel is formed of α1E.β.α2δ



Model 2. The mature regulated channel is formed of [α1E.β]β.α2δ

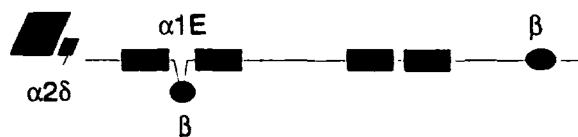


Fig. 12. Two Models of subunit composition of an α_{1E} Ca^{2+} channel under the regulatory effect of β and $\alpha_2\delta$ subunits.

the relative frequency with which an opening channel entered into either type 1 (unwilling) or type 2 (willing) gating modes; rather, it decreased mode switching.

While undoubtedly α_1 subunits are the business end of Ca^{2+} channels and their structure–function study is a fruitful avenue for new findings, it is clear that studies on the interaction and effects of β subunits with α_1 subunits is an equally fruitful road to new discoveries.

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