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The β_{1a} subunit regulates the functional properties of adult frog and mouse L-type Ca^{2+} channels of skeletal muscle

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The β_{1a} subunit, one of the auxiliary subunits of $Ca_V1.1$ channels, was expressed in COS-1 cells, purified by electroelution and electrodialysis techniques and identified by Western blot using monoclonal antibodies. The purified β_{1a} subunit strongly interacted *in vitro* with the alpha interaction domain (AID) of $Ca_V1.1$ channels. The actions of the purified β_{1a} subunit on $Ca_V1.1$ channel currents were assessed in whole cell voltage clamp experiments performed in vesicles derived from frog and mouse adult skeletal muscle plasma membranes. L-type inward currents were recorded in solutions containing Ba^{2+} (I_{Ba}). Values of peak I_{Ba} were doubled by the β_{1a} subunit in frog and mouse muscle vesicles and the amplitude of the slow component of tail currents was greatly increased. The actions of the β_{1a} subunit on $Ca_V1.1$ channel currents reached a steady state within 20 min. The β_{1a} subunit had no effect on the time courses of activation or inactivation of I_{Ba} or shifted the current–voltage relation. Non-linear capacitive currents were recorded in solutions that contained mostly impermeant ions. Charge movement depended on voltage with average Boltzmann parameters: $Q_{max} = 28.0 \pm 6.6$ nC μF^{-1}, $V = -58.0 \pm 2.0$ mV and $k = 15.3 \pm 1.1$ mV ($n = 24$). In the presence of the β_{1a} subunit, these parameters remained unchanged: $Q_{max} = 29.8 \pm 3.5$ nC μF^{-1}, $V = -54.5 \pm 2.2$ mV and $k = 16.4 \pm 1.3$ mV ($n = 21$). Overall, the work describes a novel preparation to explore *in situ* the role of the β_{1a} subunit on the function of adult $Ca_V1.1$ channels.

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In skeletal muscle, L-type, voltage-activated Ca^{2+} channels play an essential role in excitation–contraction coupling as the voltage sensors that link the depolarization of the transverse tubular system (T-system) to Ca^{2+} release by the sarcoplasmic reticulum (for reviews see Rios & Pizarro, 1991; Lamb, 1992; Huang, 1993; Melzer *et al.* 1995). In addition to their role as voltage sensors, muscle Ca^{2+} channels give rise to very slowly activated L-type Ca^{2+} currents (Sanchez & Stefani, 1983; for a review, see Melzer *et al.* 1995). Ca^{2+} channels of skeletal muscle are complex molecules composed of α_{1s}, α_2–δ, β_1 and γ subunits. The α_{1s} subunit (now referred to as the $Ca_V1.1$ channel, according to Ertel *et al.* 2000), is the channel-forming subunit (Perez-Reyes *et al.* 1989) that contains the voltage sensor of excitation–contraction coupling and the dihydropyridine binding sites (for reviews see Rios & Pizarro, 1991; Hofmann *et al.* 1994; Isom *et al.* 1994; Catterall, 2000). The β_{1a} subunit is one of the auxiliary subunits of $Ca_V1.1$ channels (Isom *et al.* 1994) and the main isoform among the β_1 subunits present in muscle (Ren & Hall, 1997). The β_{1a} subunit has important effects on the surface expression of α_{1s} but little information is available on its functional effects on $Ca_V1.1$ channels. In these experiments we describe, for the first time, the actions of β_{1a} on the electrophysiological properties of $Ca_V1.1$ channels in adult tissue. We used spherical vesicles derived from skeletal muscle plasma membranes and the whole cell voltage clamp technique. This preparation allows the control of the composition of the internal medium, to which we added the β_{1a} subunit. In addition, it enables proper control of the membrane potential without the complications due to the presence of the T-tubular system in muscle fibres (Camacho *et al.* 1999). We found that the β_{1a} subunit produces major changes in the amplitude of L-type currents without any effect on charge movement. Part of this study has been published in abstract form (Rebolledo *et al.* 2002).

METHODS

Preparation

We used spherical vesicles derived from the plasma membrane of frog and mouse skeletal muscle. Adult frogs were anaesthetized in 15 % ethanol prior to decapitation. Mice were killed by cervical dislocation, performed according to the authorized procedures of our institution. All procedures used conformed with the principles of the UK Animals (Scientific Procedures) Act 1986. The procedure of vesicle formation by enzymatic treatment was originally described for single channel experiments by Standen *et*

al. (1984), and was modified for ‘whole vesicle’ recordings by Camacho *et al.* (1996, 1999) to record muscle K^+ and Ca^{2+} channel currents. In brief, semitendinosus muscles from *Rana montezumae* or extensor digitorum longus (EDL) muscles from mouse (BALB/c) were incubated in 120 mM KCl, buffered at pH 7.2 with 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) with added collagenase (Sigma, type IA, 50 U ml^{-1}). This enzymatic treatment does not produce major changes in the single channel behaviour of Na^+ or K^+ channels present in the vesicles (Standen *et al.* 1984) and collagenase, by itself, does not significantly affect the electrophysiological properties of dihydropyridine receptors in enzymatically dissociated skeletal muscle fibres (Szentesi *et al.* 1997). Vesicles formed spontaneously after a period of about 60 min at 20–22 °C and were selected with diameters ranging from 40 to 50 μm. Recordings were made at 15–17 °C.

Purification, expression and identification of the β_{1a} subunit
COS-1 cells (American Type Culture Collection) were used for expression of the β_{1a} subunit because of the high copy number achieved by SV40 origin-containing plasmids, such as the one used in the present study. Cells were routinely grown in Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 10 % heat-inactivated fetal bovine serum (FBS). Six 100 mm dishes were used for tissue culture. Cells were incubated at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were transiently transfected with a plasmid (a gift from Dr Patricia Powers, University of Wisconsin, USA) containing the cDNA of the β_{1a} subunit (pSG5-Mb1-A) under the control of the SV40 promoter. The plasmid also contained a T7-tag in the amino terminus region that was used to identify the β_{1a} subunit by Western blot (see below). Transfections were performed with Lipofectamine Plus (Life Technologies, Inc.) according to the manufacturers’ protocol. The culture medium was changed 24 h after transfection and cells were harvested 48 h after transfection.

To isolate the β_{1a} subunit, the cells were resuspended in lysis buffer containing (mM): 50 Tris-HCl, 2 EDTA, 2 EGTA at pH 7.4 and protease inhibitors (0.2 mM phenylmethysulfonyl fluoride (PMSF), 100 nM aprotinin, 1 μM pepstatin A, 1 μM leupeptin and 1 mM soybean trypsin inhibitor). Cells were frozen in dry ice–acetone for 5 min and thawed at 37 °C for 5 min. This freeze–thaw protocol was carried out three times. The preparation was centrifuged at 10 080 *g* (12 000 r.p.m.) for 5 min and the pellet was recovered and homogenized in 1 vol. of 0.1 M potassium phosphate buffer (pH 7.4) and 2 vol. of 20 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 20 % (v/v) glycerol, 0.2 % Nonidet P 40 (NP-40), 1 % sodium cholate and 0.15 M NaCl (20 strokes by hand using a Teflon–glass homogenizer as described elsewhere (Newman *et al.* 1992). The use of detergents allowed the separation of the β_{1a} subunit from the membrane fraction (this is a standard procedure in those cases in which proteins are closely associated with membranes; for example, cytochrome P450, present in the endoplasmic reticulum, is efficiently isolated from membranes following a similar procedure, see Newman *et al.* 1992). The homogenate was centrifuged at 165 000 *g* (42 000 r.p.m.) for 60 min in a Type 51.1 rotor (Beckman). The pellet was discarded and the supernatant concentrated using a VIVASPIN 2 concentrator (Vivascience Inc., USA) according to the manufacturers’ protocol and the protein content was estimated by Lowry’s method (Lowry *et al.* 1951).

To identify the β_{1a} subunit, samples from the concentrate were diluted with an equal volume of 2 × sodium dodecyl sulfate (SDS) sample buffer (containing: 50 mM TrisHCl, pH 6.8, 4 % SDS, 20 % β-mercaptoethanol, 0.04 % bromophenol blue) before loading onto 10 % polyacrylamide gels. The gels were stained with Coomassie blue. The electrophoretic pattern was compared between samples from transfected and non-transfected cells and the β_{1a} subunit was identified by its molecular mass. The identification of the β_{1a} subunit was further confirmed by Western blot from the concentrate. Immunoblots were done following standard procedures (Lane, 1990). Protein (100 μg) was electrophoresed in an SDS–polyacrylamide gel (10 % polyacrylamide) and then transferred onto a nitrocellulose membrane. The blots were blocked with 5 % non-fat dried milk in phosphate buffered saline (PBS). A monoclonal antibody directed at the T7-tag (MASMTGGQQMG) amino acids (Novagen Inc., USA) was used to identify the β_{1a} subunit. Alternatively, an antibody that recognizes the skeletal muscle DHPR β subunit as a 52 kDa band on Western blots (Upstate Inc., USA) was also used. Both were used at a 1:1000 dilution. After washing, the membrane was incubated with a rabbit anti-mouse horseradish peroxidase conjugate (Amersham Life Sciences, USA) as secondary antibody.

Once the identity of the β_{1a} subunit was assessed, the corresponding band in the gels stained with Coomassie blue was cut out and purified by electroelution methods, according to Smith (1992). After the electroelution procedure, SDS was removed by electrodialysis with a buffer solution with a low SDS content (0.01 M NH_4CO_3, 0.02 % SDS) for 24 h. During this period the solution was exchanged several times. To further remove all traces of SDS, proteins were precipitated overnight after electrodialysis with acetone (80 %, HPLC grade) at −20 °C. The precipitate was then centrifuged at 10 080 *g* (12 000 r.p.m.) for 10 min at 4 °C and the supernatant was removed. The pellet was gently washed with 100 % acetone, centrifuged again and dried under vacuum to remove the solvent. The dried protein was stored at −20 °C until used. Although we do not have the precise yield numbers, we roughly estimate that about 60 % of the β_{1a} subunit present in the COS-1 cells could be recovered after the purification procedure. Silver staining in a polyacrylamide gel following the method described in detail by Sasse & Gallagher (1992) was done to assess whether a single or multiple bands were present after the purification procedure.

Probing the interaction of the purified β_{1a} subunit with α_{1s}
The fusion protein glutathione-S-transferase (GST)–AIDα_{1s} was used to probe the integrity of purified β_{1a} subunit. To this end, the nucleotide fragment 1251–1401 of rabbit α_{1s} cDNA was amplified by PCR. This fragment codes the 342–392 amino acid region that contains the interaction site with the β subunit (AID) (Pragnell *et al.* 1994). The primers used were:

5′ CGGGATCCAAGGCCAAGTCCAGG 3′

and

5′ CGGAATTCGCCTCCCTCTTCCAA 3′.

The amplified fragment was digested with *Bam*HI and *Eco*RI and cloned into the pGEX2T vector (Pharmacia). The resulting clones were sequenced (ABI PRISM Model 310, Perkin Elmer, USA) prior to being used to transform BL21 (DE3) bacteria (Novagen, Inc., USA). A 5 ml sample of the overnight culture was added to 50 ml medium. The culture was grown until it reached an OD value of 0.6 at 600 nm and then induced with 1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The culture was centrifuged and resuspended in 5 ml NETN buffer, containing (mM): 100 NaCl, 20 Tris-HCl, 1 EDTA, 1 PMSF and 0.5 % NP-40 at pH 8.0. It was then sonicated three times for 30 s each and centrifuged at 10 080 *g* (12 000 r.p.m.) for 10 min. Both GST and GST-AIDα_{1s}

remained in the soluble fraction. An aliquot (500 μl) of this solution was incubated with 100 μl of Glutathione–Sepharose 4B beads (Pharmacia) in NETN plus 0.5 % non-fat dried milk at a temperature of 4 °C for 2 h. The sample was washed three times with NETN and resuspended in an equal volume of NETN buffer with 1 mM of the protease inhibitor PMSF and 0.02 % NaN_3 added. The purification of GST and GST-AIDα_{1s} was carried out using a high-affinity resin and checked with a polyacrylamide gel and Coomassie blue. Identical amounts of GST and GST-AIDα_{1s}, coupled to Glutathione–Sepharose 4B beads were equilibrated in 200 μl of binding buffer containing (mM): 50 Tris-HCl, 100 NaCl, 0.1 DTT at pH 7.4. Purified β_{1a} subunit (5 μl) was added separately to GST and GST-AIDα_{1s} proteins and incubated at 4 °C for 2 h under continuous agitation. Then, the samples were washed five times with a buffer similar to the binding buffer, except that it contained 150 mM NaCl, and the interaction of β_{1a} subunit with GST and GST-AIDα_{1s} proteins was assessed on SDS-PAGE gels and Western blots using the anti-T7-tag monoclonal antibody.

Solutions

The external solution employed to record Ba^{2+} currents contained (mM): 30 Ba^{2+}, 110 TEA^+ and methanesulphonate ($CH_3SO_3^-$) as anion. The composition of the external solution used to record charge movement was (mM): 110 TEA-CH_3SO_3, 10 $CaCl_2$. In the experiments involving the Ca^{2+} channel agonist Bay K 8644 (Ma & Coronado, 1988) a concentration of 1 μM was used. The pipette solution contained (mM): 125 $CsCH_3SO_3$, 2 $MgCl_2$ and 1 EGTA. The presence of Ca^{2+} in the extracellular solution in charge movement experiments was required to maintain the stability of our recordings. Movements of 'on' charge due to non-linear capacitive currents are properly recorded with these saline solutions because, as we have shown previously (Camacho *et al.* 1999), charge moves at potentials more negative than L-type currents. Furthermore, movement of 'on' charge ends within 10 ms after the onset of the depolarizing pulse, during which no significant activation of ionic currents takes place. Finally, the current density values of L-type currents recorded under these conditions are quite small. In fact, currents only became visible as an apparent increase in 'off' charge relative to 'on' charge at potentials more positive than – 30 mV when this $[Ca^{2+}]_O$ was used (Camacho *et al.* 1999). Inward currents were clearly seen during depolarizing pulses when the more permeant Ba^{2+} ions, at a higher concentration, were used as charge carriers (Camacho *et al.* 1999 and this paper).

Extra- and intracellular solutions were buffered with Hepes (5 mM) at pH 7.2 and 7.1, respectively. All chemicals were obtained from either the Sigma Chemical Co. or the Aldrich Chemical Co., USA.

Electrophysiological methods

We followed the recording techniques described in detail by Camacho *et al.* (1996, 1999). In brief, the patch clamp technique was used in the whole cell configuration (Hamill *et al.* 1981). Pipettes were double-pulled from hard glass (KIMAX-51; Kimble Glass, Toledo, OH, USA) using a David Kopf (Tujunga, CA, USA) 700D vertical puller. The tips had resistances of about 2–3 MΩ.

To test the actions of the β_{1a} subunit on Ca_V1.1 channel currents, we first recorded control currents from a preselected muscle vesicle with a pipette that contained the internal solution. Then this pipette was replaced by one filled with 5 μl of the same solution to which the β_{1a} subunit was added at a concentration of 0.30–0.35 μg μl^{-1}. Finally, the currents were again recorded from the same vesicle with a similar pulse protocol. Control experiments were done following this experimental protocol, except that a protease-pretreated β_{1a} subunit was used. In these experiments, 50 μl of the internal solution that contained the β_{1a} subunit were mixed with proteinase K (Merck, Germany), an enzyme with a high protease activity ($\geqslant$ 40 U ml^{-1}). The protease-treated β_{1a} subunit was retrieved following the manufacturers' protocol. Other control experiments involved the use of a second pipette filled with the internal solution with no β_{1a} subunit added.

The diffusion of the β_{1a} subunit through the pipette was estimated in separate experiments in vesicles with similar diameters, ranging from 40 to 50 μm, by measuring the diffusion of fluorescein isothiocyanate labelled-peroxidase (FITC-peroxidase, Sigma), which has a similar molecular mass to that of the β_{1a} subunit. In addition to vesicle size, diffusion also depends on the access resistance (Pusch & Neher, 1988), therefore we used pipettes with similar access resistances to the ones used in electrophysiological experiments. Vesicles were patch clamped with pipettes containing 5 μl of FITC-peroxidase at a similar concentration to that used when the β_{1a} subunit was tested and were illuminated with monochromatic light at a wavelength of 485 nm. Diffusion was measured as the increase in fluorescence emitted by the vesicles at a wavelength of 535 nm. To measure fluorescence, vesicles were photographed with film (Delta 3200, Ilford, UK) at different times after the formation of the seal and the emitted fluorescence was estimated as the density change under the image of the vesicle on film.

Data collection and pulse protocol

Membrane currents (I_m) in response to voltage step depolarizations applied from the holding potential (E_h), were measured with an Axopatch amplifier (Model 200A, Axon Instruments, USA) and sampled by an IBM-PC/AT compatible Pentium-based microcomputer. Analog signals were digitized to a resolution of 12 bits through a LabMaster interface (TL–1 DMA interface, Axon Instruments) that also generated the command pulses. Data were analysed with a combination of pCLAMP (version 6.0, Axon Instruments) and in-house software. I_m was amplified and filtered with an active four-pole, low-pass Bessel filter set at a corner frequency of no more than half the sampling frequency. To measure activation of Ba^{2+} currents, command pulses of 500 ms duration and variable amplitude were delivered. The interval between pulses was at least 1 s to avoid changes in channel kinetics by a previous depolarization, as described by Feldmeyer *et al.* (1990). The pulse sequence was bracketed by five consecutive hyperpolarizing control pulses, −20 mV from E_h that ranged between −80 and −100 mV. The currents generated during the hyperpolarizing pulses were used to calculate the linear membrane capacitance and to measure the leakage current during the experiment.

In double-pulse experiments, done to record the kinetic changes produced by a conditioning depolarization, two consecutive 500 ms depolarizing pulses to +20 mV were delivered. The interval between pulses was 100 ms, during which the membrane potential was clamped to the holding potential (−80 mV).

Facilitation experiments were done with the following pulse protocol: first, a control pulse to +10 mV was applied, then a 2 min pause was allowed for complete recovery from inactivation, after which a conditioning pulse to +60 mV was delivered. This was followed by a brief repolarization pulse to the holding potential of −90 mV, and then by a test pulse to +10 mV.

Steady-state inactivation was investigated by delivering 4000 ms prepulses to several potentials followed by 500 ms test pulses to

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+20 mV. The voltage dependence of inactivation of L-type currents was fitted to a function of the same form as eqn (1) but with ($V_m - V$) in the exponential (see below).

Charge movement was measured in polarized vesicles by delivering 100 ms pulses to preselected depolarizing potentials. We measured the area under the unsubtracted currents at the beginning of the depolarizing pulses. Camacho *et al.* (1999) described that the total charge is mostly linear for hyperpolarizing pulses, whereas depolarizing pulses generate an excess charge that is due to non-linear capacitive currents. Therefore, a straight line was fitted to charge moved by 'on' currents elicited by pulses from −180 to −110 mV from the holding potential of −100 mV. The line was extrapolated to depolarizing values to calculate the non-linear charge moved at each potential. The voltage dependence of activation of non-linear charge movement was fitted to the Boltzmann function:

$$Q = Q_{max}/(1 + \exp((V - V_m)/k)), \quad (1)$$

where Q_{max} is the maximum value of charge, V_m is the membrane potential, V is the potential where $Q = 0.5\,Q_{max}$, and k is a measure of the steepness of the curve.

Unless otherwise indicated, the dialysis time for data presented with the β_{1a} subunit was 15 min.

The fitting of numerical formulae to experimental data employed a non-linear least squares algorithm. Parameter values given in the text and in the table are expressed as means ± S.E.M. To calculate statistical significance, Student's t test was used at the level $P < 0.05$.

RESULTS

Transiently transfected COS-1 cells express the β_{1a} subunit

We found that the β_{1a} subunit can be expressed in the membrane fraction of COS-1 cells using the mammalian expression vector pSG5-Mb1-A. Figure 1*A* illustrates a representative polyacrylamide gel stained with Coomassie blue that shows a distinct band of about 60 kDa. The band was present in transfected cells only. This is consistent with the reported molecular mass of 56 kDa of the β subunit of skeletal muscle (Flockerzi *et al.* 1986), since a slightly higher molecular mass is expected due to the presence of T7-tag amino acids. Two different antibodies, both specific for the muscle β subunit, were used to visualize the expressed protein on immunoblots. The β_{1a} subunit was detected as a single molecular species of about 60 kDa. Figure 1*B* shows a representative immunoblot that illustrates the reactivity of the protein with the antibody directed at the tag amino acids in the N-terminus. Similar results were observed with the antibody directed at the β_{1a} subunit (data not shown), thus providing further evidence that the expressed β_{1a} subunit is an intact full length protein. Figure 1*C* shows a silver-stained polyacrylamide gel. Silver staining of purified β_{1a} subunit from COS-1 cells revealed a single and distinct band with an approximate molecular mass of 60 kDa. No other bands were detected

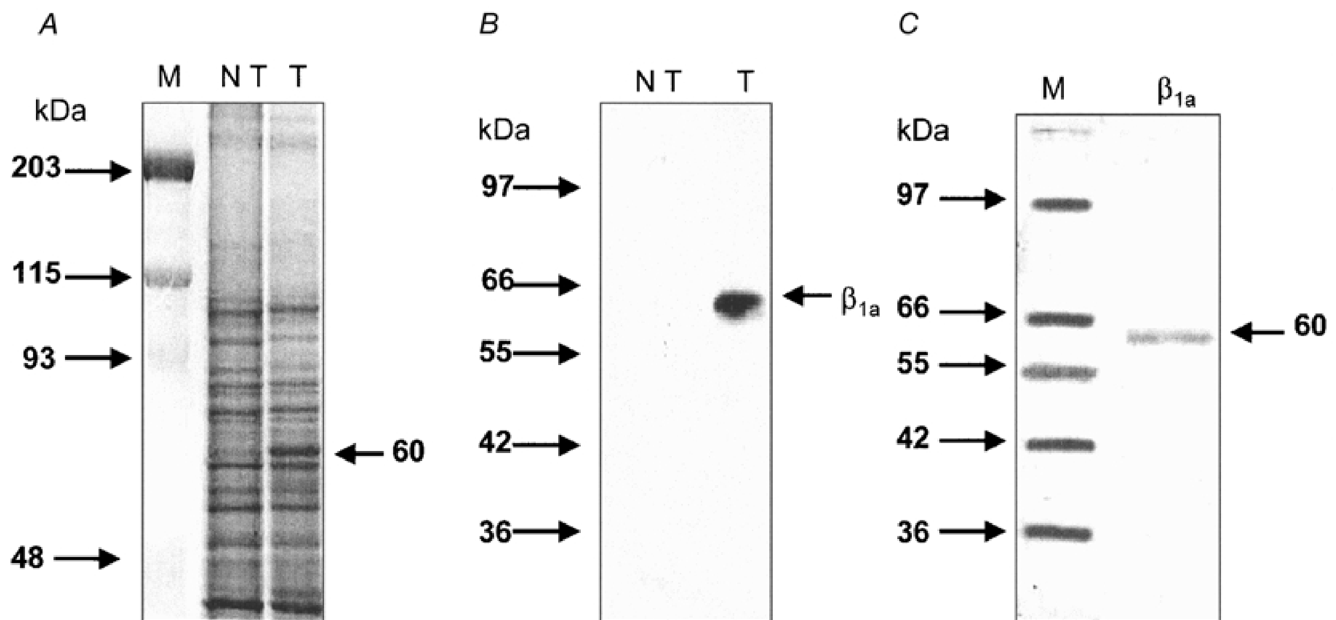

Figure 1. Identification and purification of the β_{1a} subunit expressed in COS-1 cells

A, Coomassie blue-stained SDS–polyacrylamide (10 %) gel of concentrate prepared from non-transfected (NT) and transfected (T) cells. The arrow indicates a protein with an apparent molecular mass of 60 kDa. Notice that this band is only expressed in transfected cells. *B*, an immunoblot from gel-separated concentrate prepared from non-transfected (NT) and transfected (T) cells. A monoclonal antibody directed at the T7-tag was used. Notice that the antibody recognizes a band in transfected cells only. *C*, a silver-stained polyacrylamide gel showing the β_{1a} subunit, purified as indicated in Methods. Markers (M) were used in panels *A* and *C*. The molecular masses of the markers (kDa) are given on the left side of each panel.

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after the purification procedure with this sensitive staining method, suggesting that the purification procedure yielded an essentially free β_{1a} subunit without contamination from other proteins. Although unlikely, the presence in the gel, in addition to the β_{1a} subunit, of a different protein with exactly the same molecular mass cannot be entirely ruled out.

Before testing the actions of the β_{1a} subunit on $Ca_V1.1$ channel currents, it was important to determine whether the integrity of the β_{1a} subunit was preserved after the purification procedure. If this was indeed the case, it would be expected that the high interaction capability of the β_{1a} subunit with α_{1s} would have been maintained. Therefore, we examined *in vitro* the interaction of purified β_{1a} subunit with the conserved motif present on all α_1 subunits and essential for the binding of β subunits (AID). AID was expressed as a 50-amino-acid GST fusion protein. The GST-AID fusion protein was coupled to glutathione–Sepharose beads and used as the substrate for the binding of purified β_{1a} subunit (see Methods). Figure 2*A* shows a representative polyacrylamide gel stained with Coomassie blue that shows a distinct band of about 26 kDa that corresponds to GST. The band that migrated more slowly was associated with GST–AID, as expected, and it corresponded to a molecular mass of 32 kDa. Figure 2*B* shows a representative immunoblot that illustrates binding of GST-AID protein with purified β_{1a} subunit. The β_{1a} subunit reacted with the antibody directed at the T7-tag amino acids in the N-terminus, consistent with the membrane extract immunoblot shown previously (Fig. 1*B*). GST alone did not bind any β subunits (Fig. 2*B*, central lane). Finally, GST–AID Sepharose beads were able to bind purified β_{1a} subunit, indicating that this specific form of interaction was preserved.

Actions of β_{1a} on L-type currents in vesicles

We found that the β_{1a} subunit increased the amplitude of currents flowing through $Ca_V1.1$ channels. This is shown in the experiments illustrated in Fig. 3*A–F*. Figure 3*A* illustrates control membrane currents recorded from a frog muscle vesicle to the potentials indicated (in mV). Inward Ba^{2+} currents, the amplitude and time course of which depended upon the membrane depolarization level during the pulse, were recorded. Currents were not sustained but declined during the pulse by a voltage-dependent inactivation process. These currents closely resembled those we described previously as L-type, dihydropyridine-sensitive currents (Camacho *et al.* 1999). Figure 3*B* illustrates currents recorded from the same vesicle, 15 min after the pipette containing the control solution was replaced by one containing the β_{1a} subunit. The amplitude of the currents was distinctly larger at all potentials (for example, during the pulse to +20 mV) though their time course was similar. To illustrate the fact that the β_{1a} subunit does not produce major kinetic effects, the currents recorded at +20 mV, illustrated in Fig. 3*A* and *B*, were normalized relative to their peak amplitude during

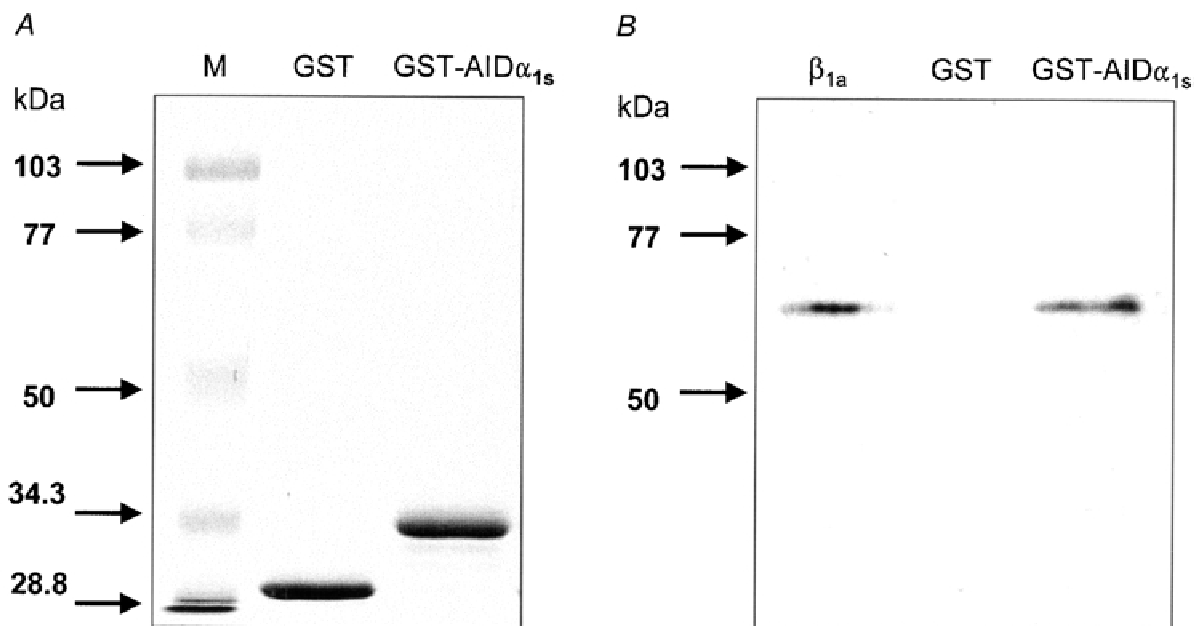

Figure 2. AIDα_{1s} and β_{1a} interactions

A, Coomassie blue-stained SDS–polyacrylamide gel containing 10 μl GST–Sepharose beads (GST) or GST–AIDα_{1s} fusion protein–Sepharose beads. *B*, immunoblots probed with a monoclonal antibody directed at the T7-tag of the β_{1a} subunit. GST and GST–AIDα_{1s} fusion protein were allowed to interact *in vitro* with purified β_{1a} subunit prior to immunoblots. Markers (M) were used in panel *A*. The molecular masses (kDa) of the markers are given on the left side of the panel.

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the pulse. Figure 3*C* shows superimposed records of the currents after the normalization procedure. It is clear that the time course of activation and inactivation of both currents was quite similar, indicating no major effects of β_{1a} subunit on channel kinetics. This conclusion was further supported by results obtained with double-pulse experiments of the type described by Feldmeyer *et al.* (1990) in frog muscle fibres. We found in frog muscle vesicles that the current during a 500 ms test pulse to +20 mV activated faster when compared to the current during the conditioning pulse. The time to peak of I_{Ba} during the conditioning pulse averaged 130.8 ± 3.8 ms ($n = 5$) and it decreased to 98.0 ± 6.4 ms ($n = 5$) during the test pulse, confirming the observations of Feldmeyer *et al.* (1990). In the presence of the β_{1a} subunit, the time to peak of I_{Ba} during the prepulse was 124.8 ± 6.4 ms ($n = 6$) and it decreased to 91.7 ± 3.4 ms ($n = 6$) during the test pulse. These numbers were similar to those obtained under control conditions, further confirming that the β_{1a} subunit has negligible effects on Ca^{2+} channel current kinetics.

The β_{1a} subunit also increased the magnitude of Ca^{2+} channel currents in vesicles derived from mouse skeletal muscle. Figure 3*E* shows superimposed membrane current records under control conditions from a mammalian muscle vesicle and Fig. 3*F* shows the corresponding records, taken 20 min after the control pipette was replaced by one containing the β_{1a} subunit in the same experiment. The β_{1a} subunit increased the amplitude of mammalian L-type currents with minor changes in their time course.

Unlike the effects of β_{1a} on Ba^{2+} current amplitude, charge movement remained unchanged by the addition of the β_{1a} subunit. Charge movement can be seen in the experiment illustrated in Fig. 3*A* and *B* as brief outward deflections at the beginning of the pulses that represented non-linear outward capacitive currents or 'on' currents (Camacho *et al.* 1999). These currents preceded the inward Ba^{2+} currents. Immediately after the pulses, large inward non-linear currents were also recorded. As we previously described in detail (Camacho *et al.* 1999), two distinct components contributed to these currents: tail inward Ba^{2+} currents, arising from deactivation of the channels and non-linear capacitive currents. To determine whether the β_{1a} subunit altered charge movement, we compared the time course and amplitude of 'on' currents before and after the application of the β_{1a} subunit. Figure 3*D* illustrates superimposed traces of the membrane currents generated by a command pulse to +20 mV, from the same experiment illustrated in Fig. 3*A* and *B* but displayed on a much faster time scale. It is apparent that, while the amplitude of the inward Ba^{2+}

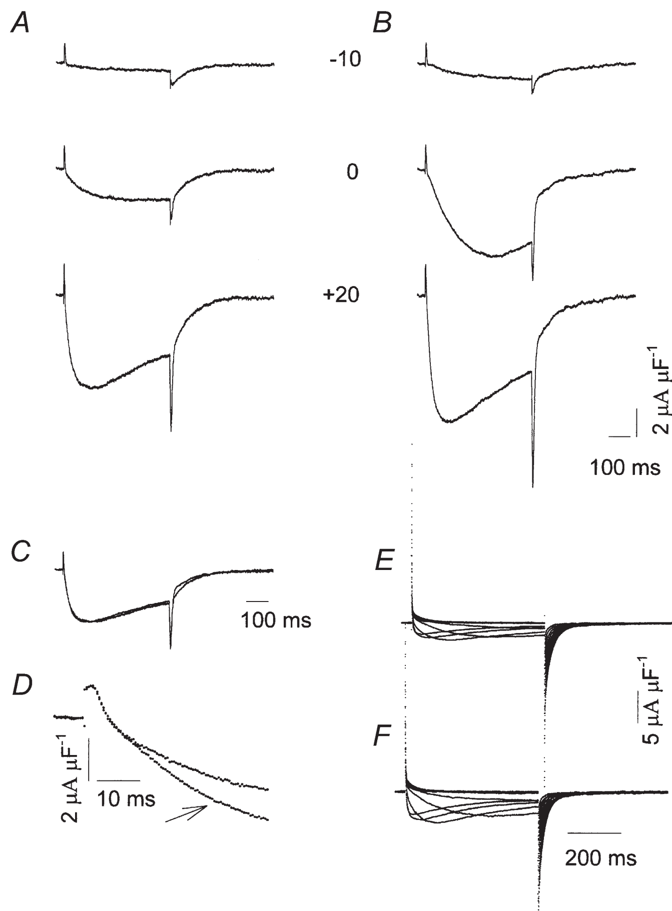

Figure 3. Macroscopic Ba^{2+} currents in membrane vesicles

A, currents during voltage steps to the potentials indicated (mV) after subtraction of linear membrane current components. $E_h = -80$ mV. *B*, the effect of the β_{1a} subunit on membrane currents recorded in the same experiment as in *A*. *C*, superimposed recordings at +20 mV from *A* and *B*; recordings were normalized to their peak amplitude. *D*, superimposed recordings at +20 mV from *A* and *B*. Note that recordings are shown on an expanded time scale. The arrow indicates the recording taken in the presence of the β_{1a} subunit. *E*, superimposed leak-subtracted recordings from a separate experiment taken from a mouse muscle vesicle. Currents were elicited by stepping the membrane potential from −60 mV to +40 mV in 10 mV steps from $E_h = -80$ mV. *F*, same experiment as in *E*, in the presence of the β_{1a} subunit.

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current became larger when the β_{1a} subunit was present in the patch pipette, as shown in Fig. 3 *A* and *B*, 'on' currents were identical, indicating that this subunit had negligible effects on charge movement. This observation was confirmed by performing charge movement experiments in solutions containing mostly impermeant ions. We found that the average values of the Boltzmann parameters fitted to the experimental data were unaffected by the β_{1a} subunit. Table 1 summarizes results from several experiments under control conditions and in the presence of the β_{1a} subunit. In the latter case, data correspond to recordings taken 15 min after the whole cell configuration with the test pipette was achieved.

Figure 4*A* and *B* shows the current–voltage relation from the experiments illustrated in Fig. 3*A*–*D* and Fig. 3*E*–*F*, respectively. Open symbols represent values of peak currents recorded with a control solution and filled symbols, results with the β_{1a} subunit added. In the control solution, activation of both frog (Fig. 4*A*) and mouse (Fig. 4*B*) muscle Ba^{2+} currents began at about −10 mV; the peak Ba^{2+} current was largest at a membrane potential of +20 mV; it declined for larger depolarizations and it was still inward at +60 mV, indicating a very positive reversal potential. Compared to records taken in control conditions, the current-voltage relation in the presence of the β_{1a} subunit, both in frog and mouse muscle vesicles, was similar except for the larger values of peak currents recorded at all potentials, but no major shifts along the voltage axis were observed. To measure possible shifts, the voltage which produced the largest current was determined, as this approach produced the most consistent results. In nine experiments performed in frog muscle vesicles, I_{Ba} peaked at +13.3 ± 2.0 mV. Similarly, it peaked at +11.1 ± 2.6 mV when the β_{1a} subunit was present in the patch pipette. In the same experiments, the value of peak I_{Ba} averaged −3.1 ± 0.4 μA μF^{-1} under control conditions and it increased to −5.6 ± 0.3 μA μF^{-1} in the presence of the β_{1a} subunit. The ratio between peak I_{Ba} in the presence of the β_{1a} subunit relative to its value under control conditions averaged 2.0 ± 0.3 ($n = 9$). In contrast, the protease-inactivated β_{1a} subunit had no effect. The corresponding ratio of peak currents was: 1.05 ± 0.03

Table 1. Actions of the β_{1a} subunit on charge movement in vesicles

	Control *	β_{1a}**
Q_{max} (nC μF^{-1})	28.0 ± 6.6	29.8 ± 3.5
V (mV)	-58.0 ± 2.0	-54.5 ± 2.2
k (mV)	15.3 ± 1.1	16.1 ± 1.3

* $n = 24$, ** $n = 21$.

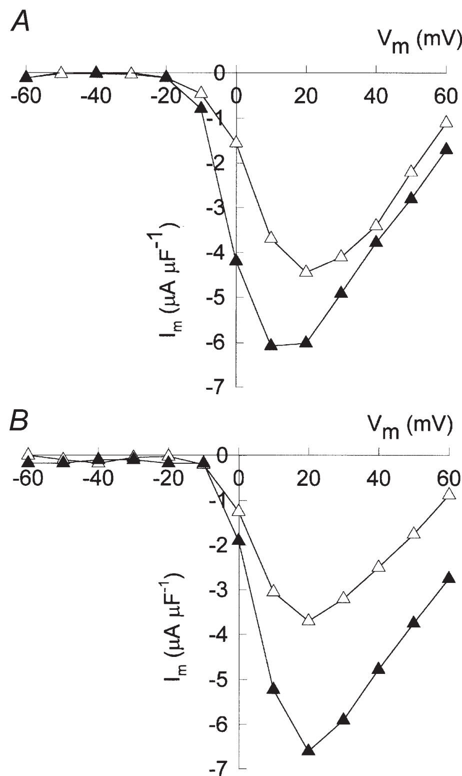

Figure 4. The action of the β_{1a} subunit on the current–voltage relation

A, peak current values taken from the experiment illustrated in Fig. 3*A* (△) and *B* (▲). *B*, the corresponding peak values from the experiment illustrated in Fig. 3*E* (△) and *F* (▲).

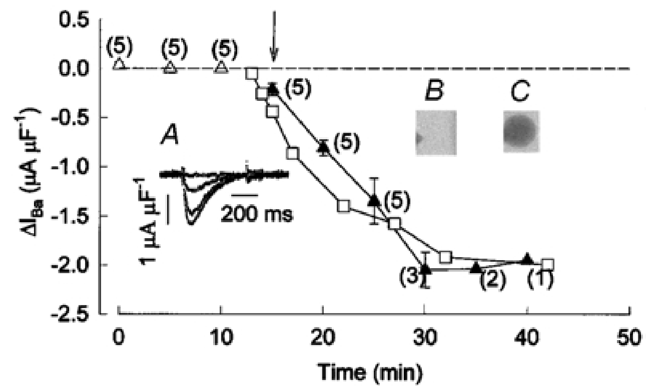

Figure 5. The time course of action of the β_{1a} subunit

Triangles represent peak ΔI_{Ba} values ($I_{Ba,i} - I_{Ba,a}$, where i is every individual current recording and a is the average of the currents recorded under control conditions in every experiment) as a function of time. Currents were recorded at +20 mV. △, the average values under control conditions ($n = 5$). ▲, the average values in the presence of the β_{1a} subunit. Numbers beside each point indicate the number of experiments. The arrow indicates the beginning of the recordings with the pipette containing the β_{1a} subunit. Inset *A*, ΔI_{Ba} recordings from a representative experiment taken at 0, 5, 10 and 15 min after breaking the patch membrane with a pipette containing the β_{1a} subunit. Inset *B*, photograph taken of a vesicle previous to the recording with a patch pipette that contained FITC-peroxidase. Inset *C*, the same vesicle 29 min after the initiation of the 'whole-cell' recording. □, values of optical density (in arbitrary units) from the experiment shown in *B* and *C*.

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($n = 9$). The β_{1a} subunit also increased the amplitude of L-type currents in the presence of Bay K 8644 (1 μM), although the effects were less pronounced. In three experiments, peak I_{Ba} averaged -4.2 ± 0.2 μA μF^{-1} ($n = 3$) under control conditions and -7.1 ± 0.3 μA μF^{-1} ($n = 3$) in the presence of the β_{1a} subunit. Similarly, the corresponding ratio of peak currents was: 1.6 ± 0.2 ($n = 3$). Since Bay K increases the open probability of the channels (Ma & Coronado, 1988), this finding is consistent with the possibility that the β_{1a} subunit also increases their open probability.

In agreement with the results obtained in frog muscle vesicles, the β_{1a} subunit greatly increased the peak current amplitude of mammalian muscle vesicles in Bay K-untreated preparations. In three experiments, peak I_{Ba} averaged -2.2 ± 0.1 μA μF^{-1} ($n = 3$) under control conditions and -4.6 ± 0.1 μA μF^{-1} ($n = 3$) in the presence of the β_{1a} subunit. The mean value of the ratio between peak current amplitude values was: 2.2 ± 0.1 ($n = 3$). These results indicate that the β_{1a} subunit doubles the amplitude of L-type currents flowing through $Ca_V1.1$ channels.

L-type currents are not sustained but decline during the depolarizing pulses by a voltage-dependent inactivation process. Double-pulse experiments were performed to assess the effect of the β_{1a} subunit on steady state inactivation. We found that the main effect of the β_{1a} subunit is a shift in the mid point of inactivation towards more negative potentials. In control experiments, the average Boltzmann parameters were (mV): $V_m = -37.6 \pm 3.2$ ($n = 4$) and $k = 9.1 \pm 0.6$ ($n = 4$). In the same experiments, the addition of the β_{1a} subunit changed these parameters to: $V_m = -53.4 \pm 2.2$ ($n = 4$) and $k = 16.3 \pm 2.2$ ($n = 4$).

Time course of β_{1a} action on L-type currents

To measure the time course of the effect of the β_{1a} subunit on $Ca_V1.1$ channel current amplitude, the membrane potential of frog muscle vesicles was pulsed to +20 mV from the $E_h = -80$ mV at 5 min intervals, under control conditions and after the control pipette was replaced by one containing the β_{1a} subunit. Control currents were averaged and each individual current record, under control conditions and in the presence of the β_{1a} subunit, was subtracted from this average current in every experiment. This procedure provided an estimate of the increase in current amplitude by β_{1a} subunit. Figure 5 summarizes results from several experiments; the plot shows mean values of the difference in peak current as a function of time. As expected, the subtracted currents under control conditions were very close to zero, indicating only minor changes in current amplitude (△). However, when the control pipette was replaced by one containing the β_{1a} subunit (indicated by the arrow), the amplitude of the subtracted current progressively increased until it reached a steady value 15 min after the 'whole cell' configuration with the test pipette was achieved (▲). Inset *A* shows traces from a representative experiment. To determine whether the time course of

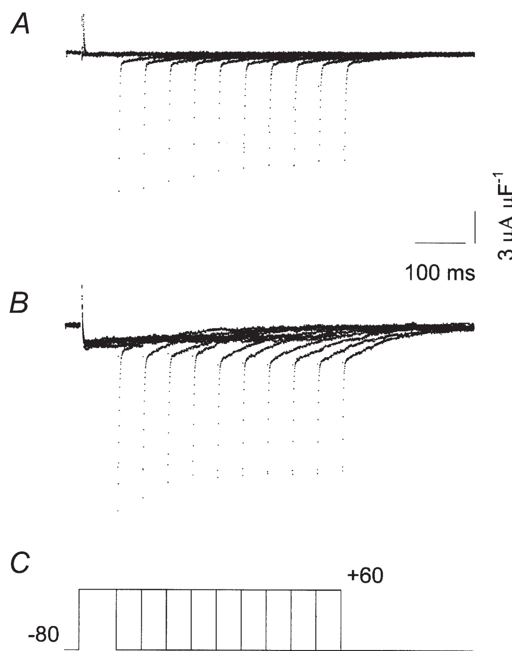

Figure 6. The influence of the β_{1a} subunit on tail currents

A, recordings showing non-linear control currents generated with the pulse protocol shown in *C*. *B*, currents recorded with the same pulse protocol in the presence of the β_{1a} subunit. Pulse durations were (ms): 75, 125, 175, 225, 275, 325, 375, 425, 475 and 525. Same experiment throughout.

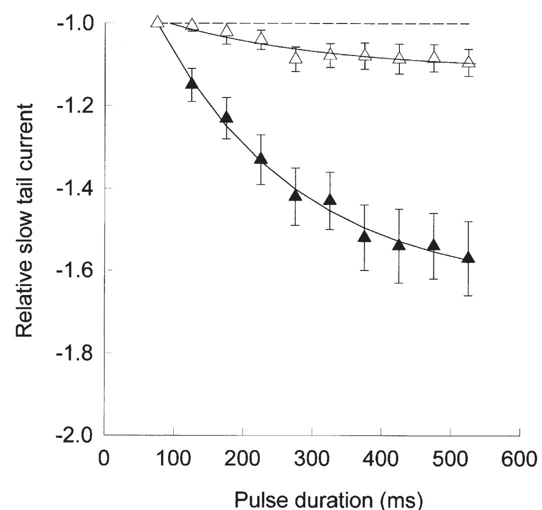

Figure 7. The action of the β_{1a} subunit on the slow component of tail currents

The peak amplitude of 'off' currents (ordinate), relative to its value after a 75 ms pulse, as a function of pulse duration (abscissa). △, average values (± S.E.M., $n = 31$) under control conditions. ▲, average values (± S.E.M., $n = 19$) in the presence of the β_{1a} subunit. The continuous lines represent the single exponential best fit, with τ = 228 ms (△) and τ = 206 ms (▲).

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action of the β_{1a} subunit could be accounted for by its diffusion through the tip of the pipette, we measured the increase in fluorescence emitted by vesicles containing FITC-peroxidase and found that it followed a quite similar time course, as indicated by a representative experiment in Fig. 5 (□). Inset *B* shows a photo image from the same experiment taken just before the formation of the gigaseal. The tip of the patch pipette can be seen on the left hand side. Inset *C* shows an image from the same experiment taken 29 min after the whole cell configuration was achieved. The increase in the fluorescence of the vesicle is apparent. By comparing the density of the pipette tip to that of the vesicle in two separate experiments, we estimated that at 20 min, the concentration of FITC-peroxidase was 0.65 times that of the pipette tip. A similar conclusion was reached when the kinetic equations of diffusional exchange between small cells and a patch pipette were used (Pusch & Neher, 1988). Thus, for a vesicle with a 20 μm radius, we calculated that under experimental conditions, diffusion reached 68 % of its final value at a dialysis time of 20 min. This indicated that the concentration of the β_{1a} subunit inside the vesicles reached a value of 0.4 $\mu g\ \mu l^{-1}$ or 5 nM in our electrophysiological experiments. Based on this estimate, we calculated that 2×10^{15} β molecules per ml were present inside the vesicles. For a vesicle with a 20 μm radius, we estimated that about 66 000 β molecules were added.

Tail Ba^{2+} currents and facilitation in vesicles

Excessive tail currents and facilitation of Ca^{2+} channels of mammalian myotubes have previously been described (Fleig & Penner, 1996). Excessive tail currents, albeit quite small, were also recorded in frog muscle vesicles as an increase in the amplitude of the tail currents generated after pulses of increasing duration (Camacho *et al.* 1999). Figure 6*A* shows membrane currents produced by the pulse protocol shown in panel *C*. Membrane currents were elicited by depolarizing pulses to +60 mV, the duration of which was progressively increased. There was a small and brief outward current at the beginning of each pulse that was similar to the ones illustrated in Fig. 3*A* and *B*. These currents were followed by a small inward Ba^{2+} current that inactivated, in part, during the pulses. Immediately after the depolarizing steps two distinct tail current components were observed: a fast component and a slow one, the magnitude of which increased as the pulse duration increased, confirming previous results (Camacho *et al.* 1999). Panel *B* illustrates the currents from the same experiment after the control pipette was replaced by one containing the β_{1a} subunit. Currents were recorded from the same vesicle with a similar pulse protocol as in Fig. 6*A*. There was a distinct increase in the amplitude of the inward current during the pulse, consistent with the results described in Figs 3 and 4. In addition, the amplitude of the slow tail current component was greatly increased by the β_{1a} subunit.

Figure 7 summarizes results from several experiments. The magnitude of the slow component, measured as the amplitude of the tail current 13 ms after the end of each depolarizing pulse, was normalized relative to its value after the 75 ms pulse, the shortest pulse duration tested. In control experiments (△), the magnitude of the slow component was quite small, reaching an asymptotic value of about 1.1. In contrast, it increased up to 1.6 in the presence of the β_{1a} subunit (▲).

Facilitation has been observed in mammalian myotubes as an increase in current amplitude during test pulses that were preceded by a conditioning depolarization and it has been proposed that excessive tail currents and facilitation have the same origin (Fleig & Penner, 1996). It was, therefore, relevant to test whether facilitation was present in the vesicles and whether the β_{1a} subunit had an influence on it. Figure 8 shows results from a representative facilitation experiment. The membrane potential was clamped according to the pulse protocol shown in panel *C*. In the absence of the β_{1a} subunit (Fig. 8*A*), the control pulse produced an inward current that inactivated almost completely during the pulse. In contrast, the current during the test pulse was virtually absent, indicating that the conditioning prepulse produced a protracted inactivation state. In the presence of the β_{1a} subunit (Fig. 8*B*), the amplitude of the L-type current during the control pulse increased, as shown previously (Figs 3–5), but the amplitude of the current during the test pulse was quite small, suggesting that the channels were similarly inactivated by the conditioning depolarization. Similar results were obtained in another four experiments.

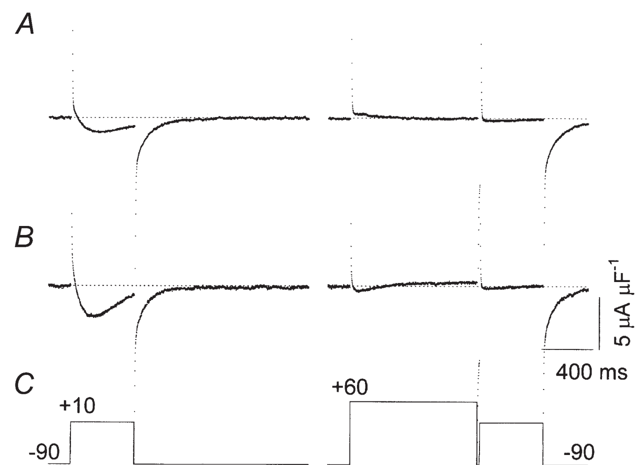

Figure 8. L-type currents after a conditioning depolarization

A, leak-subtracted control currents recorded with the pulse protocol shown in *C*. *B*, currents recorded with the same pulse protocol in the presence of the β_{1a} subunit. Data were taken from the same experiment. The dotted lines in *A* and *B* represent the zero current level. *C*, the pulse protocol; the pulse to +10 mV is the test pulse. The pulse to +60 mV is a conditioning prepulse preceding the test pulse.

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DISCUSSION

A new role of the β subunit in muscle

We here describe for the first time that the auxiliary β_{1a} subunit modulates the activity of pre-existing α_{1s} channels of adult skeletal muscle. Modulation was seen as a rapid increase in the magnitude of L-type currents. Although the underlying mechanism of this form of regulation remains to be established, our present results suggest a direct action of the β_{1a} subunit on α_{1s} without involving an increase in the number of α_{1s} molecules present in the membrane. Several lines of evidence provide support for this conclusion: firstly, we tested the effects of the β_{1a} subunit itself on channel activity in muscle vesicles, a system devoid of internal organelles (Camacho *et al.* 1996) and, therefore, without all the machinery that is required for the synthesis and transport of channels to the membrane. Secondly, addition of the β_{1a} subunit to the vesicle did not increase charge movement, as would be expected if more α_{1s} molecules were incorporated into the membrane. Thirdly, the actions of the β_{1a} subunit on α_{1s} currents developed as soon as it diffused through the patch pipette, suggesting a modulatory effect of this subunit on preexisting channels. Finally, our results show that the purified β_{1a} subunit strongly bound *in vitro* to the AID region of the α_{1s} subunit.

A direct effect of β subunits on Ca^{2+} channel function has been difficult to establish in the past because in many cases the actions of β subunits also involve changes on trafficking of channels (Kamp *et al.* 1996; Josephson & Varadi, 1996; Wei *et al.* 2000). According to Gerster *et al.* (1999), these may be independent functions of the β subunit. In one example, however, functional modifications of the cardiac Ca^{2+} channel currents by β subunits, without changes in the number of channels, have been observed. When α_{1c} was coexpressed in *Xenopus* oocytes with the cardiac β_{2a} subunit, an increase in the magnitude of Ca^{2+} channel currents without changes in charge movement was observed (Neely *et al.* 1993), consistent with the present results in skeletal muscle. However, a significant difference between modulation of the skeletal muscle and the cardiac Ca^{2+} channel by their β subunits is apparent. Unlike the shift produced by β_{2a} on α_{1c} activation parameters (Neely *et al.* 1993), we observed no shifts in the current–voltage relation of skeletal muscle Ca^{2+} channels by the β_{1a} subunit. This suggests that, unlike the effects of the β_{2a} subunit on the cardiac channel, the coupling between charge and the opening of the channel pore remains unchanged when the β_{1a} subunit interacts with the skeletal muscle $Ca_V1.1$ channel.

The dual role of the β subunit in muscle

We found no major effects of the β_{1a} subunit on the time course of activation or inactivation of L-type currents. Early expression experiments on mammalian cell lines had shown acceleration of channel kinetics by coexpression of α_{1s} and β_{1a} subunits (Lacerda *et al.* 1991; Varadi *et al.* 1991). Differences in the phosphorylation level of the β_{1a} subunit, which contains several potential phosphorylation sites (Catterall, 2000), might help to explain the discrepancies between these results and ours. However, channel kinetics in heterologous systems were abnormally slow when α_{1s} was expressed alone. Therefore, it is possible that $Ca_V1.1$ channels are not assembled or regulated properly in cell lines, which may explain the differences from the present findings.

Previous work has shown that the β_{1a} subunit has profound effects on the surface expression levels of the α_{1s} subunit. Coexpression of both α_{1s} and β_1 subunits resulted in an increase in the number of dihydropyridine binding sites (Lacerda *et al.* 1991; Varadi *et al.* 1991) and in an increased amount of α_1 in the membrane (Krizanova *et al.* 1995). In agreement with a role for the β_1 subunit in the surface expression of Ca^{2+} channels, Gregg *et al.* (1996) have shown that the β_{1s} subunit is absent in the membrane of β-null, cultured myotubes (Gregg *et al.* 1996). Likewise, Beurg *et al.* (1997, 1999) reported an increase in charge movement by expression of β_{1a} in β-null myotubes. These results can be easily reconciled with our present findings if the β_{1a} subunit has a dual effect on α_{1s} channels, on the one hand, targeting α_{1s} to the tubular membrane and, on the other, modulating channel function of pre-existing channels (this paper). In this regard, it is interesting to note that Witcher *et al.* (1995) described a pool of β subunits in skeletal muscle that is not tightly associated with α_1 subunits and proposed that they may be shuttled to the membrane to bind with α_1 subunits and modulate channel function. The suggestion that the β_3 subunit directly modulates human α_{1c} currents recorded is also consistent with our view (Yamaguchi *et al.* 1998). Thus, in oocytes pretreated with the V-ATPase inhibitor Bafilomycin A_1, no increase in the levels of α_{1c} were present and yet a 1.8-fold increase in current amplitude was observed, which was interpreted as an allosteric modulation of α_{1c} subunits by β_3 subunits (Yamaguchi *et al.* 1998).

Low-affinity α_{1s} and β_{1a} interactions

Among other possibilities, direct α_{1s}–β_{1a} interactions may play a role in the modulatory action of the β_{1a} subunit on channel function described in the present paper. If indeed these interactions take place, our data do not provide evidence as to whether this occurs by binding to high- or to low-affinity sites on the α_{1s} subunit. High-affinity interaction sites in the α (AID) and in β (BID) subunits of Ca^{2+} channels have been described (Pragnell *et al.* 1994; Walker & De Waard, 1998). AID is a highly conserved region in the loop between domains I and II (Pragnell *et al.* 1994). BID is a 30-amino-acid N-terminal region of the second conserved domain of the β subunit (De Waard *et al.*1994). The BID region of β_{1a} binds strongly to the I–II loop of α_{1s} (Cheng *et al.* 2002). In addition to these high-

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affinity regions, low-affinity sites have been recently described (Cheng *et al.* 2002) and perhaps they interact with the pool of non-bound β_{1a} subunits described by Witcher *et al.* (1995). This would imply that more than one β_{1a} subunit could bind to a single α_{1s} subunit. The association of multiple subunits with a single α_1 subunit has been proposed, based on experiments in which α_{1c} and α_{1E} were expressed in *Xenopus* oocytes, and a new interaction domain in α_{1E} was described (Tareilus *et al.* 1997). Alternatively, it is possible that at least some α_{1s} subunits are not associated with β subunits. This is because a significant fraction of all dihydropyridine binding sites (and therefore α_{1s} subunits) in muscle are not functional channels (Lamb, 1992; Schwartz *et al.* 1985). If the reason why some α_{1s} subunits are not functional is because of the lack of bound β_{1a} subunits, then our data may be explained by binding of β_{1a} subunits to these α_{1s} subunits. The presence of unoccupied channels would be expected if α_{1s}–β_{1a} interactions are reversible. Recently, the main interactions between α_{1c} and β subunits have been shown to be reversible *in vitro* (Bichet *et al.* 2000) and in inside-out patches of membranes containing the α_{1c} subunit (Hohaus *et al.* 2000).

Our data indicate that, when extrapolated *in vivo*, the interaction of the β_{1a} subunit with α_{1s} increases the Ca^{2+} influx during muscle activity. If this interaction is mediated by low-affinity sites, it would be expected to be more easily switched 'on' and 'off', according to the requirements of the muscle fibre for Ca^{2+} ions. Also, the steady-state inactivation changes of α_{1s} currents induced by the β_{1a} subunit may plausibly be explained by low-affinity interactions between these subunits. This type of interaction has been hypothesized to cause the shifts in steady-state inactivation of α_{1B} currents in *Xenopus* oocytes observed after injection of high concentrations of β_3 subunits (Canti *et al.* 2001). These shifts are similar to those described in the present paper.

Role of the β_{1a} subunit on facilitation of muscle Ca^{2+} channels

We found that long and strong depolarizations increased the amplitude of a slow α_{1s} tail current component. The origin of this component is presently unknown, although previous work in heart cells has shown that L-type channels are driven by long depolarizations into a slow gating mode characterized by long openings and high open probability (Pietrobon & Hess, 1990). Our experiments indicate that the β_{1a} subunit promotes an increase in the amplitude of a slow component of tail currents that depends on the duration of the preceding depolarization. This component has been previously described in rat myoballs, where it is quite prominent (Fleig & Penner, 1995), and it has been associated with facilitation of muscle L-type channels (Fleig & Penner, 1996). Facilitation is a use-dependent regulation of channel activity that results in an increased Ca^{2+} influx by a preceding depolarization (Dolphin, 1996). Facilitation in rat myoballs was observed as an increase in current amplitude during a test pulse by a prepulse depolarization (Fleig & Penner, 1996). In agreement with these observations, an additional Ca^{2+} entry through $Ca_V1.1$ channels occurred during tetanic activity in rat myoballs (Sculptoreanu *et al.* 1993). Both phenomena, the slow tail current component and current facilitation during test pulses, have been proposed to depend on a common mechanism in rat myoballs (Fleig & Penner, 1996). In our experiments, no facilitation during test pulses was observed, which gives no support to this proposal. Furthermore, in the presence of the β_{1a} subunit, $Ca_V1.1$ channel currents during the test pulse were still distinctly smaller when compared with those during the control pulse, suggesting that the slow component of tail currents and current facilitation are regulated differently by this auxiliary subunit.

Facilitation of the cardiac and the neuronal L-type channels has been observed when α_{1c} is expressed in heterologous systems and it greatly depends on the coexpression of a β auxiliary subunit (Bourinet *et al.* 1994; Dai *et al.* 1999; Kamp *et al.* 2000). However, the role of the β subunit is less clear in other cases. Thus, facilitation is present even when α_1 of smooth muscle is expressed alone in Chinese hamster ovary (CHO) cells (Kleppisch *et al.* 1994). The fact that the β_{1a} subunit did not promote facilitation of $Ca_V1.1$ channels (this paper) may suggest that facilitation does not depend on the β_{1a} subunit in skeletal muscle. Alternatively, it is also possible that facilitation requires phosphorylation of the β_{1a} subunit. It has been previously shown that an increase in Ca^{2+} influx during repetitive activity depends on phosphorylation by cAMP-dependent protein kinase (Sculptoreanu *et al.* 1993). Finally, it is also possible that, under our experimental conditions, voltage-dependent inactivation of $Ca_V1.1$ channels overcame a relatively small facilitation that would otherwise have be seen.

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