Identification of PK-A Phosphorylation Sites in the Carboxyl Terminus of L-Type Calcium Channel α_1 Subunits†

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Received March 20, 1996; Revised Manuscript Received May 20, 1996⊗

ABSTRACT: Full length L-type calcium channel α_1 subunits are rapidly phosphorylated by protein kinase A (PK-A) in vitro and in vivo at sites located in their long carboxyl terminal tails. In skeletal muscle, heart, and brain the majority of biochemically isolated α_1 subunits lacks these phosphorylation sites due to posttranslational proteolytic processing. Truncation may therefore modify the regulation of channel activity by PK-A. We combined site-directed mutagenesis and heterologous expression to investigate the extent to which putative cAMP-dependent phosphorylation sites in the C-terminus of α_1 subunits from skeletal muscle, heart, and brain are phosphorylated in vitro. The full length size form of wild-type and mutant calcium channel α_1 subunits was obtained at high yield after heterologous expression in *Saccharomyces cerevisiae*. Like in fetal rabbit myotubes [Rotman, E. I., et al. (1995) *J. Biol. Chem. 270*, 16371−16377], the rabbit skeletal muscle α_1 C-terminus was phosphorylated at serine residues 1757 and 1854. In the carboxyl terminus of α_{1S} from carp skeletal muscle and α_{1C} from rabbit heart a single serine residue was phosphorylated by PK-A in vitro. The C-terminus of α_{1D} was phosphorylated at more than one site. Employing deletion mutants, most of the phosphorylation (>70%) was found to occur between amino acid residues 1805 and 2072. Serine 1743 was identified as additional phosphorylation site in α_{1D}. We conclude that in class S and C calcium channels the most C-terminal phosphorylation sites are substrate for PK-A in vitro, whereas in class D calcium channels phosphorylation also occurs at a site which is likely to be retained even after posttranslational truncation.

Voltage-gated L-type calcium channels mediate the depolarization-induced calcium influx into muscle, neuronal, and endocrine cells [for review, see Catterall (1995)]. Their activity is modulated by neurotransmitters, drugs (calcium antagonists), and enzymes. The phosphorylation of L-type channels in skeletal muscle (Sculptoreanu et al., 1993a; Johnson et al., 1994), heart muscle (Sculptoreanu et al., 1993b), or neuroendocrine cells, e.g., pancreatic β-cells (Henquin et al., 1984) or chromaffin cells (Artalejo et al., 1992; Doupnik et al., 1992), leads to channel activation resulting in increased calcium influx [for review, see Dolphin (1995)]. Functional and biochemical studies suggest that, at least in the case of cardiac and skeletal muscle L-type channels, direct phosphorylation of the α_1 subunit is involved in modulation of channel function by PK-A[1] (Lai et al., 1990; Rotman et al., 1992, 1995; Yoshida et al., 1992). However, the contribution of other channel subunits (e.g., the β subunit) or other proteins cannot be ruled out (Flockerzi et al., 1983; De Jongh et al., 1989; Klöckner et al., 1992; Haase et al., 1993).

The majority of calcium channel α_1 subunits biochemically isolated from rabbit skeletal muscle (α_{1Sr}) or heart (α_{1C}) are truncated at their long C-terminal tails (Lai et al., 1990; De Jongh et al., 1991; Rotman et al., 1992; Yoshida et al., 1992). In contrast, truncation was only observed to a very minor extent in fetal rabbit myotubes (Rotman et al., 1995) and is assumed to be the consequence of a site-specific posttranslational proteolytic clip (De Jongh et al., 1991). Only the full length forms were rapidly phosphorylated by PK-A in vitro because truncation eliminates sites of cAMP-dependent phosphorylation located on the C-terminal tail. This finding suggested that proteolytic truncation regulates the extent to which calcium channel function is modulated by cAMP-dependent phosphorylation in adult and embryonic tissue.

Efforts have been made to exactly identify the amino acid residues phosphorylated by PK-A in vitro. By phosphopeptide mapping and sequence-directed antibodies, two phosphorylation sites within the C-terminal tail of α_{1Sr} were identified (Rotman et al., 1992, 1995). In the transverse tubule membrane of skeletal muscle, α_{1Sr} is present in high densities. However, no information is available about the phosphorylation sites of other L-type α_1 subunits. The application of phosphopeptide mapping to identify C-terminal residues phosphorylated by PK-A is limited, owing to the minute amounts of full length α_1 subunits present in, e.g., heart, brain, or neuroendocrine cell membrane fractions, in contrast to skeletal muscle T-tubule preparations. We therefore took advantage of the distinctive features offered by the *Saccharomyces cerevisiae* expression system. Sufficient amounts of the full length size form of α_1 subunits containing all putative C-terminal PK-A phosphorylation sites

† Supported by research grants from the Fonds zur Förderung der Wissenschaftlichen Forschung (S6601-med to H.G.; S6602-med to J.S.).

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations: PK-A, cAMP-dependent protein kinase; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); PAS, protein A-Sepharose.

S0006-2960(96)00683-6 CCC: $12.00

can be easily obtained. The cDNA for mutant α_1 subunits lacking one or more putative phosphorylation sites can be engineered by site-directed mutagenesis and readily introduced into *S. cerevisiae* for constituent expression.

MATERIALS AND METHODS

Construction of chimeric α_1 Subunit cDNAs and Site-Directed Mutagenesis. Amino acid numbering, given in parentheses, is according to rabbit skeletal muscle α_{1S} (α_{1Sr}; Tanabe et al., 1987), carp skeletal muscle α_{1S} (α_{1Sc}; Grabner et al., 1991), α_{1C-a} from rabbit heart (Mikami et al., 1989), and α_{1D}, clone rCACN4A, from rat insuloma cell line RINm5F (Ihara et al., 1995) throughout the paper. Nucleotide (nt) numbering is according to the respective chimeric cDNA clones. Chimeras of different α_1 subunits (see Figure 1A) were constructed as follows:

Chimera TM-Sc. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1852) was constructed as described (Wang et al., 1995) by replacing domains I and II of α_{1Sc} with the corresponding sequence of α_{1C-a}.

Chimera TM. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1426, 1783−1852). A *Bgl*II−*Xba*I fragment (nt 4551−5623) of the chimera TM-Sc was deleted, and recessed 3′-ends were filled using Klenow fragment of *Escherichia coli* DNA polymerase I (Promega) and religated.

For the construction of the following α_1 chimeras, polymerase chain reaction (PCR) was used to create the restriction site *Nru*I by introducing silent mutations (mutational sites indicated by an asterisk). cDNA amplification by PCR (Biomed, Thermocycler 60) was performed with 35 cycles at moderate stringency (1 min 94 °C, 30 s at 50 °C, 1.5 min 72 °C) using Taq polymerase (Promega) or proofreading Pfu polymerase (Stratagene). The integrity of Taq polymerase-generated fragments was confirmed by cDNA sequencing using the dideoxy chain termination method (Sanger et al., 1977).

Chimera TM-Sr. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1Sr} (1465−1948). This chimera was constructed by ligating the *Nru*I*−*Not*I fragment of α_{1Sr} into the *Nru*I* site of TM and the *Not*I site in the polylinker of the plasmid.

Chimera TM-C. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1C-a} (1515−2171). The *Nru*I*−*Not*I fragment of α_{1C-a} was ligated as described for TM-Sr.

Chimera TM-D. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1D} (1517−2203). *Nru*I*−*Not*I fragment of rat neuroendocrine α_{1D} (clone rCACN4A) was ligated as described for TM-Sr.

C-terminal deletion mutants of chimera TM-D were constructed as follows:

Chimera TM-D1. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1D} (1517−1805). A mutation primer (5′-AGCTTTGGACTAGTTGGCGTTATT-3′, nt 5758−5790) was used to introduce a stop codon and a *Spe*I site after position 1805. The PCR-amplified *Nru*I*−*Spe*I* fragment was subcloned into the *Nru*I* site of TM and the *Spe*I site into the polylinker of the plasmid.

Chimera TM-D2. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1D} (1517−1994). A mutation primer (5′-CTGCTGCTGCAACTAGTGCAGGGGC-3′, nt 6325−6351) was used to introduce a stop-codon and a *Spe*I site after position 1994. Subcloning was carried out as described for TM-D1.

Chimera TM-D3. α_{1Sc} (1−60), α_{1C-a} (145−920), α_{1Sc} (806−1410), α_{1D} (1517−2072). A mutation primer (5′-TCCTATAGGACTAGTCGGGTTCAT-3′, nt 6559−6583) was used to introduce a stop codon and a *Spe*I site after position 2072. Subcloning was carried out as described for TM-D1.

Site-directed mutagenesis was performed on chimeras TM-Sc, TM-Sr, TM-C, and TM-D using the Sculptor IVM System (Amersham). Serine residues were replaced by alanine residues in all chimeras, except in TM-C, where serine residues were replaced by glycine residues. To identify desired mutations, mutagenic primers also contained silent nucleotide exchanges creating additional restriction sites.

The following mutations were introduced by mutagenic sense primers: S1738A (TM-Sc1), S1853/1854A (TM-Sr1), S1757A plus S1853/1854A (TM-Sr2), S1848G (TM-C1), S1928G (TM-C2), S1848G plus S1928G (TM-C3), and S1743A (TM-D1.2)

Expression of α_1 Chimeras in S. cerevisiae and Membrane Preparation. All chimeras were subcloned into the yeast episomal plasmid Yep351ADC1 (Kaiser et al., 1994). Transformation of *S. cerevisiae* JB811 (Gal^+, ura3, trp1, len2, ade2-1, pep4-3, prb, prc) was performed as described by Hill et al. (1991), and clones were selected by growth on solid medium lacking leucine. Transformants were grown overnight at 30 °C in yeast minimal medium lacking leucine, harvested at an OD_{600} of 1.2, washed with 50 mM Tris-HCl (pH 7.4)/0.1 mM PMSF, and frozen in liquid N_2. For membrane preparation cells were lysed with glass beads in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.1 mM PMSF. The lysate was spun for 5 min at 500*g* (4 °C), and the supernatant was pelleted for 60 min at 100000*g* (4 °C). This microsomal pellet was resuspended in 50 mM Tris-HCl (pH 7.4)/0.1 mM PMSF at a protein concentration of 2−3 mg/mL and frozen in liquid N_2.

Phosphorylation and Immunoprecipitation. Membrane proteins (1 mg) were solubilized by incubation in RIA buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100] for 30 min on ice. After centrifugation in an Eppendorf centrifuge (5 min, 4 °C), the supernatant was added to 18.5 mg of protein A-Sepharose (PAS) beads (Sigma) for preadsorption of nonspecifically bound proteins. After 30 min of incubation, the resin and insoluble proteins were pelleted, and the supernatant was either immediately used for immunoprecipitation or aliquots (50 μg) were quickly frozen in N_2 and stored until use. Phosphorylation was carried out after purification of samples by immunoprecipitation. For immunoprecipitation 5−50 μg of solubilized protein, corresponding to 100−500 fmol of α_1 subunit, was incubated with either 10 μL of affinity-purified anti-α_1 (directed against residues 1382−1400 of α_{1Sr}; Striessnig et al., 1990) or 10 μL of affinity-purified anti-α_{1C} (directed against residues 848−865 of the α_{1C-a}) in RIA buffer containing 0.5 mg/mL BSA (essentially fatty acid free, Sigma) (RIA/B) at 4 °C overnight. After the sample was rotated with 15 μL of PAS at 4 °C for 30 min, the antigen/antibody/PAS conjugates were pelleted, washed three times with RIA/B and once with phosphorylation buffer [50 mM HEPES-NaOH (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100, 0.05% BSA], and incubated at 30 °C for 20 min in phosphorylation buffer containing 3 μM [γ-^{32}P]ATP at a specific activity of 100 dpm/fmol and 13 units of catalytic subunit of PK-A in a final volume of 25 μL. PAS

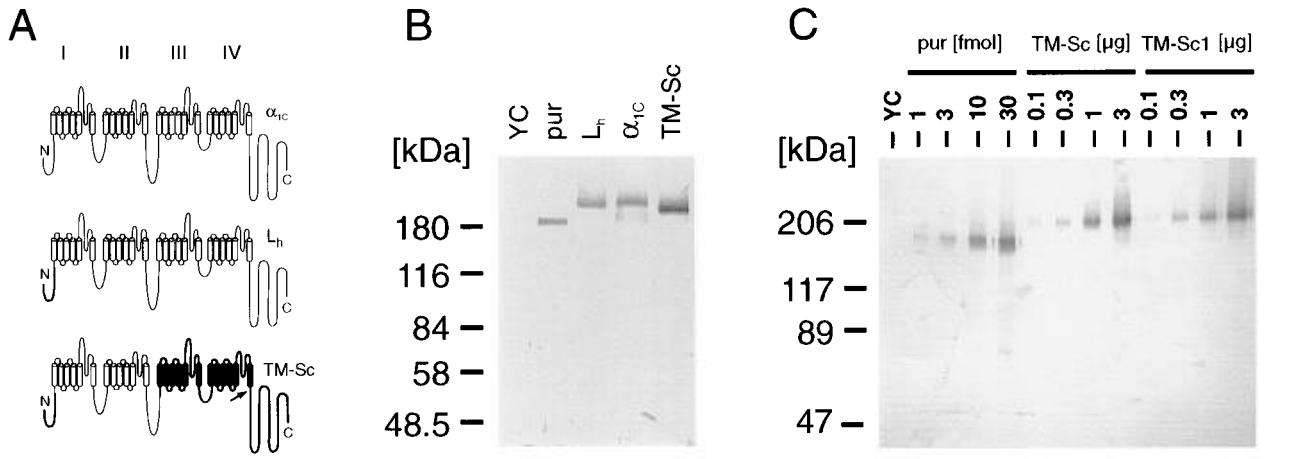

FIGURE 1: Heterologous expression of calcium channel α_1 subunits in *S. cerevisiae*. (A) Schematic representation of calcium channel α_1 subunit chimeras. Sequence stretches from α_{1C} (rabbit heart) are represented by thin lines and white segments. Bold lines and black segments represent sequence stretches from α_{1Sc} (carp skeletal muscle). The arrow indicates the position, where the C-terminal tails of α_{1Sc}, α_{1Sr}, α_{1C}, and α_{1D} were introduced into the transmembrane core chimera TM to yield chimeras TM-Sc, TM-Sr, TM-C, and TM-D, respectively. (B) Immunoblot analysis of wild-type and chimeric α_1 subunits expressed in *S. cerevisiae*. Equal amounts of membrane preparations obtained after heterologous expression of Lh, α_{1C}, and TM-Sc were separated by SDS−PAGE and detected with sequence-directed antibody anti-α_1 (residues 1382−1400 of α_{1Sr}). YC: JB811 transformed with vector alone. pur: partially purified calcium channel preparation from rabbit skeletal muscle. One of three immunoblots yielding essentially the same result is shown. (C) Semiquantitative immunoblotting of chimera TM-Sc and mutant TM-Sc1. Serial dilutions of membrane preparations (TM-Sc, TM-Sc1) and partially purified calcium channel preparations (pur) were separated on the same gel. Immunoblotting was carried out as described (Mitterdorfer et al., 1994), and the intensity of antibody staining was compared by densitometric scanning.

pellets were washed three times with RIA/B including 0.1 mM PMSF, 12.5 mM NaF, and 20 mM β-glycerolphosphate (RIA/B-WB) and either analyzed by SDS−PAGE after one wash with TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) and incubation in sample loading buffer (Striessnig et al., 1990) at 56 °C for 10 min or subjected to a second immunoprecipitation. For a second immunoprecipitation PAS pellets were extracted with 30 μL of 50 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, 20 mM β-glycerolphosphate, 5 mM DTT, and 1% SDS at 56 °C for 30 min. After dilution by adding 300 μL of RIA/B-WB and centrifugation for 5 min, the supernatant was incubated with 10 μL of anti-α_1 or anti-α_{1C} (848−865) at 4 °C for 2 h. The antigen/antibody complex was then collected with 15 μL of PAS for 30 min at 4°C and the PAS washed three times with RIA/B-WB and once with TBS. Sample preparation for SDS−PAGE was carried out as outlined above. Dried SDS−polyacrylamide gels were exposed to film (Kodak X-Omat AR) for 2−20 min.

Semiquantitative Estimation of Phosphorylation Stoichiometry. The amount of incorporated γ-^{32}P was determined by liquid scintillation counting of α_1 subunits excised from SDS gels. Using semiquantitative estimates of α_1 subunit concentrations from immunoblots (see Figure 1C) the incorporation of γ-^{32}P was 0.29 ± 0.03 (TM-Sr, $n = 5$), 0.38 ± 0.12 (TM-Sc, $n = 3$), and 0.42 ± 0.09 (TM-D, $n = 9$) mol/mol α_1 subunit. The stoichiometry for TM-C (34 ± 21, $n = 3$) was difficult to assess as a consequence of a second immunoprecipitation step and the associated variability in resolubilization between separate experiments. Data are given as mean ± SE and assume quantitative immunoprecipitation in a single immunoprecipitation step.

Immunoblot analysis was carried out as described (Mitterdorfer et al., 1994). Prestained molecular weight markers were from Bio-Rad.

RESULTS

Expression of L-Type Calcium Channel α_1 Subunits in S. cerevisiae. Among the expression systems commonly used to biochemically and functionally analyze recombinant proteins, the *S. cerevisiae* system offers the advantages of high expression efficiency and proper posttranslational processing of the recombinant protein. We therefore tested if calcium channel α_1 subunits can also be expressed in *S. cerevisiae* at high yield. α_1 subunit cDNAs were cloned into the yeast/*E. coli* shuttle plasmid YEp351-ADC1 (Kaiser et al., 1994). These constructs were then used to transform *S. cerevisiae* strain JB811. YEp351-ADC1 carries the LEU2 gene, which encodes β-isopropylmalate dehydrogenase from *S. cerevisiae*, conferring leucine prototrophy. This genetic marker allows for rapid and reliable selection of successfully transformed JB811 clones by omitting leucine from the nutrient mix.

Transformation of *S. cerevisiae* with the cardiac α_1 subunit (α_{1C}) resulted in the appearance of a membrane-associated 240 kDa polypepetide identified with antibody anti-α_1. This band was absent in cells transformed with vector alone (Figure 1B,C). The N-terminus of α_{1C} (residues 1−144) was replaced by the corresponding sequence of carp skeletal muscle (residues 1−60) yielding chimera L_h (Figure 1A) to eliminate a putative weak PK-A phosphorylation site. The highest expression density among different chimeras was found for chimera TM-Sc. TM-Sc corresponds to L_h but with domain III, IV, and the C-terminal tail replaced by carp skeletal muscle sequence. Both chimeric constructs form functional calcium channels after expression in *Xenopus* oocytes (Wang et al., 1995). Using purified skeletal muscle calcium channels as a standard in immunoblot experiments, we estimated an expression density of 10 pmol of TM-Sc per mg of membrane protein (Figure 1C). This is about 20−120-fold higher than the density of α_1 subunits observed after transient or stable transfection of mammalian cells (Kim et al., 1990; Lacerda et al., 1991; Varadi et al., 1991; Bosse et al., 1992; Mitterdorfer et al., 1994) or the density of L-type calcium channels in microsomal membrane fractions from brain or heart (Glossmann et al., 1985). *S. cerevisiae* therefore represents a powerful expression system for obtaining high quantities of recombinant calcium channel α_1

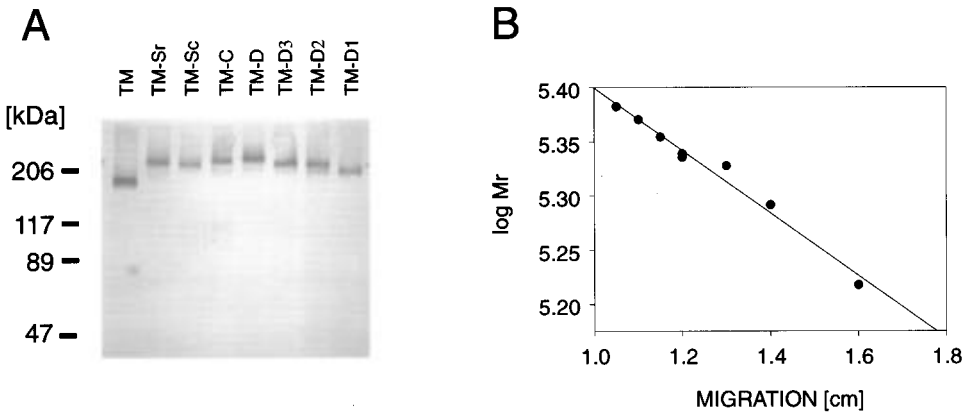

FIGURE 2: Absence of proteolytic processing of α_1 subunit C-termini in *S. cerevisiae*. (A) Immunoblot analysis of α_1 subunit chimeras expressed in *S. cerevisiae*. Comparable amounts of immunoreactivity (about 10 fmol/lane) were separated on the same gel. Immunoblotting was carried out as described (Mitterdorfer et al., 1994). The difference in the electrophoretic mobility of the α_1 chimeras and the C-terminal deletion mutants corresponds to the difference in the their predicted molecular mass. One of four essentially identical blots is shown. (B) Migration distances of α_1 subunit chimeras were plotted against the logarithm of their predicted molecular mass. The correlation coefficient was 0.98 ± 0.01 ($n = 4$). Data are from the immunoblot shown in panel A.

subunits. Consequently, it is highly suitable to study C-terminal phosphorylation of α_1 subunits, provided that no proteolytic translational processing leads to truncation of their C-termini as observed in native tissue (Lai et al., 1990; De Jongh et al., 1991; Rotman et al., 1992, 1995; Hell et al., 1993).

To examine the C-terminal phosphorylation of various L-type α_1 subunit C-termini, the C-terminal tail of chimera TM-Sc was replaced by the corresponding sequence of class S, C, or D α_1 subunits. The hydrophobic transmembrane core (TM) of TM-Sc was used for the generation of these chimeric α_1 subunits, since TM-Sc yielded the highest densities upon heterologous expression in *S. cerevisiae* (Figure 1). In addition, TM does not contain consensus sites for cAMP-dependent phosphorylation. The chimeras were constructed by introducing C-terminal tails of α_1 subunits from rabbit skeletal muscle (α_{1Sr}; Tanabe et al., 1987), rabbit heart (α_{1C-a}; Mikami et al., 1989), and rat neuroendocrine tissue (α_{1D}, clone rCACN4A; Ihara et al., 1995). In addition to TM-Sc (C-terminal tail from carp skeletal muscle, α_{1Sc}; Grabner et al., 1991) this yielded chimeras TM-Sr, TM-C, and TM-D, respectively (for a detailed description, see Materials and Methods). To obtain α_1 species with defined lengths of their C-termini, deletion mutants of TM-D (TM-D1, TM-D2, TM-D3) were also constructed.

Absence of Proteolytic Processing of α_1 Subunit C-Termini in S. cerevisiae. The expression density and apparent molecular mass of the resulting chimeric calcium channel α_1 subunits were assessed by semi-quantitative immunoblots. In Figure 2A, comparable amounts of α_1 immunoreactivity (about 10 fmol/lane) of TM, TM-Sr, TM-Sc, TM-C, TM-D, and TM-D deletion mutants TM-D3, TM-D2, and TM-D1 were separated on the same gel. The difference in their predicted molecular mass is exclusively due to the different length of their C-termini. Several lines of evidence indicate that proteolytic truncation of α_1 subunits does not occur after expression in *S. cerevisiae*: First, we found an excellent correlation ($r = 0.98 \pm 0.01$, $n = 4$) between the theoretical molecular mass of the α_1 chimeras and their electrophoretic mobility (Figure 2B). Second, skeletal muscle calcium channel α_1 subunits purified from native tissue migrated faster than TM-Sc (Figure 1), although their predicted molecular mass is almost identical (212 vs 214 kDa). This is expected because purified skeletal muscle α_1 subunits (but not TM-Sc) are truncated around position 1685−1699, reducing their molecular mass by about 22 kDa (De Jongh et al., 1991). Only very rarely a faint band (less than 10% of α_1 immunoreactivity) was detected in our immunoblots, which migrated with a molecular weight, that could correspond to a truncated form of α_1 (not shown).

Identification of C-Terminal PK-A Phosphorylation Sites by Site-Directed Mutagenesis. The absence of proteolytic processing allowed us to exploit the *S. cerevisiae* expression system for the identification of PK-A phosphorylation sites in the C-terminus of our chimeras. Membranes were solubilized in Triton-X100, and solubilized α_1 subunits were immunopurified with anti-α_1 and phosphorylated with PK-A in the presence of [γ-^{32}P]ATP. Phosphorylated antibody-bound proteins were either directly separated by SDS−PAGE (TM, TM-Sc, TM-Sr, TM-D) or after a second immunoprecipitation step with anti-α_{1C} (TM, TM-C). Figure 3 shows that TM-Sr, TM-Sc, TM-C, and TM-D are substrates for PK-A in vitro. No phosphorylation occurred in membranes of nontransformed *S. cerevisiae* and cells transformed with chimera TM, which lacks sites for cAMP-dependent phosphorylation. Therefore, TM-Sr, TM-Sc, TM-C, and TM-D are phosphorylated at their C-terminal tail.

Next we tested if in α_{1Sr} phosphorylation occurs at the same sites as in the corresponding wild-type α_1 subunit in native membranes. The phosphorylation of the C-terminal tail of α_{1Sr} has been investigated in detail previously (Rotman et al., 1992, 1995) and found at consensus sites corresponding to serines in position 1853/1854 and in position 1757. The same residues were phosphorylated by PK-A in chimera TM-Sr, carrying the C-terminus of α_{1Sr} (Figure 3A). Mutation of serines 1853/1854 to alanine (S1853/1854A) reduced phosphorylation to $69 \pm 4\%$ ($n = 3$) of control values as determined by scintillation counting of α_1-associated ^{32}P. Additional mutation of serine 1757 resulted in a complete loss of phosphorylation of the double mutant suggesting that this residue is also phosphorylated. No detectable change in expression density was found for these mutants in immunoblots ($n = 3$). Thus our results agree with findings from intact fetal rabbit myotubes, where serine residues 1757 and 1853/1854 have been identified as the only targets for cAMP-dependent phosphorylation in vivo (Rotman et al., 1995). The fact that serine 1854 is located only 19 amino acids from the C-terminal end further supports the absence

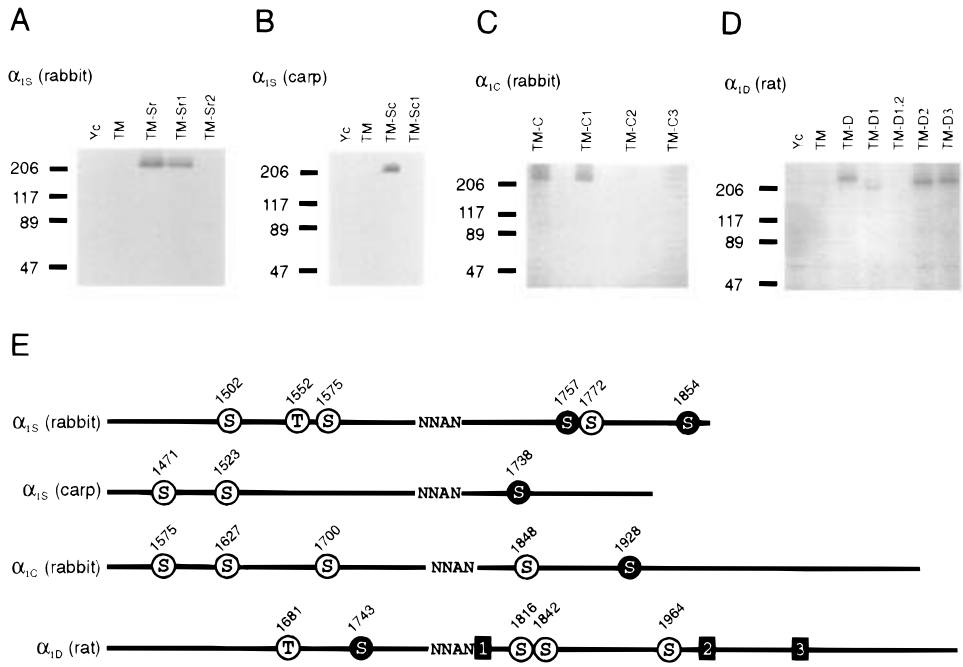

FIGURE 3: Phosphorylation of class S, C, and D calcium channel α_1 subunits by PK-A. Membrane preparations of α_1 chimeras expressed in *S. cerevisiae* were solubilized, immunopurified, and phosphorylated by PK-A as described in Materials and Methods. Phosphorylated antibody-bound proteins were either directly separated by SDS−PAGE (A, B, D) or after a second immunoprecipitation step using antibody anti-α_{1C} (C). Dried gels were subjected to autoradiography, and incorporation of γ-^{32}P into wild-type and mutant chimeric constructs was quantitated by scintillation counting after cutting the respective bands from the gel. All chimeric α_1 subunit constructs were substrates for PK-A in vitro. No phosphorylation occurred in membranes of nontransformed *S. cerevisiae* (Yc) or cells transformed with transmembrane core chimera (TM), which lacks sites for cAMP-dependent phosphorylation. One of three experiments yielding the same result is shown. (A) PK-A phosphorylation sites in the C-terminus of α_{1Sr}. Mutation of serine residues 1853/1854 to alanine (TM-Sr1) reduced phosphorylation to 69 ± 4% ($n = 3$) of control values obtained with wild-type (TM-Sr). Additional mutation of serine 1757 (TM-Sr2) resulted in a complete loss of phosphorylation, suggesting that this residue is also phosphorylated by PK-A. (B) PK-A phosphorylation sites in the C-terminus of α_{1Sc}. The C-terminus of α_{1Sc} is phosphorylated by PK-A in vitro (TM-SC). Mutation of serine 1783 to alanine completely abolished phosphorylation (TM-Sc1). (C) PK-A phosphorylation sites in the C-terminus of α_{1C}. Phosphorylation after mutation of serine 1848 to glycine (TM-C1) was 98 ± 6% ($n = 3$) of control (TM-C). Mutation of serine 1928 to glycine was sufficient to reduce phosphorylation to background values. The same result was obtained with a double mutant, where serine residues 1848 and 1928 were replaced by glycine (TM-C3). (D) PK-A phosphorylation sites in the C-terminus of α_{1D}. C-terminal deletion mutants were created by introducing stop codons at amino acid residues 1805 (TM-D1), 1994 (TM-D2), and 2071 (TM-D3). In vitro phosphorylation of TM-D2 and TM-D3 was 93 ± 17% ($n = 3$) and 101 ± 27% ($n = 3$) of control values obtained with wild-type (TM-D). A substantial amount of phosphorylation (25 ± 5% of TM-D; $n = 3$) was detected in TM-D1. Mutation of serine 1743 to alanine in TM-D1 (TM-D1.2) was sufficient to completely abolish phosphorylation. (E) Schematic representation of PK-A phosphorylation sites in the C-terminus of class S, C, and D α_1 subunits. Serine and threonine residues, which are putative targets for phosphorylation by PK-A, are shown. Their position is approximately drawn to scale. Numbering of residues is according to their respective position in α_{1Sr}, α_{1Sc}, α_{1C}, and α_{1D} (clone rCACN4). Residues which were identified to be phosphorylated by PK-A in our study are represented by filled circles. The approximate position of the putative site for posttranslational proteolytic truncation in α_{1Sr} (De Jongh et al., 1991) is indicated (NNAN). Boxed numbers represent the positions where stop codons were introduced into the α_{1D} chimera (TM-D) to create the deletion mutants TM-D1 (1), TM-D2 (2), and TM-D3 (3).

of substantial proteolytic processing after expression of calcium channel α_1 subunits in *S. cerevisiae*.

Subsequently, we localized the sites of cAMP-dependent phoshorylation in α_1 C-termini that have not been previously investigated. Our main targets were consensus phosphorylation sites located on the C-terminal side of the putative truncation site (indicated by the conserved sequence stretch NNAN in Figure 3) identified in rabbit α_{1S} (De Jongh et al., 1991). The α_{1S} subunit from carp skeletal muscle contains four sites for cAMP-dependent phosphorylation. Three of these sites are located in the C-terminus. We have previously reported that α_1 subunits biochemically isolated from carp skeletal muscle cannot be phosphorylated by PK-A in vitro (Grabner et al., 1991). This finding could be due to removal of a C-terminal phosphorylation site by proteolytic processing. As shown in Figure 3B the C-terminus of α_{1Sc} can indeed serve as a substrate for cAMP-dependent phosphorylation in vitro, supporting this assumption. Of the three potential phosphorylation sites only the most C-terminal one is phosphorylated by PK-A in chimera TM-Sc. Mutation of serine 1738 to alanine (mutant TM-Sc1), located C-terminal of the proposed truncation site (see Figure 3E), was sufficient to completely abolish phosphorylation. Our finding demonstrates that, similar to rabbit skeletal muscle, full length calcium channel α_1 subunits in carp skeletal muscle are also a substrate for PK-A.

In vitro phosphorylation of full length α_1 subunits from rabbit heart by PK-A has previously been shown (Yoshida et al., 1992; De Jongh et al., 1994), but a detailed analysis of the phosphorylated residues has not yet become available. We focused on the most C-terminal consensus phosphorylation sites, serine 1848 and 1928. Mutation of serine 1848 to glycine (mutant TM-C1) did not cause a detectable reduction of phosphorylation (98 ± 6%; $n = 3$). In contrast, phosphorylation of mutant S1928G (TM-C2) was reduced to background values, as was the double mutant S1848G plus S1928G (TM-C3). This indicates, that in the C-terminal tail of the cardiac calcium channel α_1 subunit serine 1928 represents the major substrate for phosphorylation by PK-A in vitro.

α_{1D} subunits have been found to participate in the formation of L-type calcium channels in neuroendocrine

tissues and brain. Phosphorylation of α_{1D} has not yet been studied. Two isoforms of calcium channel α_{1D} subunits have been described (rCACN4A and rCACN4B; Ihara et al., 1995), which result from alternative splicing of a single α_{1D} gene (Seino et al., 1992; Williams et al., 1992; Yaney et al., 1992). The predicted amino acid sequence of these two transcripts differs within a C-terminal domain of 535 amino acids, which contains a cluster of several potential PK-A phosphorylation sites, that are found in only one of the splice variants, rCACN4A.

The deduced amino acid sequence of rCACN4A predicts five potential cAMP-dependent phosphorylation sites (T1681, S1743, S1816, S1842, and S1964). To delineate the localization of sites, which are phosphorylated by PK-A in vitro, phosphorylation studies were carried out with the deletion mutants created by introducing stop codons at amino acid residues 1805 (TM-D1), 1994 (TM-D2), and 2072 (TM-D3). TM-D2 and TM-D3 were constructed to exclude weak phosphorylation sites in positions 2015, 2050, 2065, 2094, and 2099. A stop codon was introduced at the corresponding putative site for posttranslational truncation (position 1805), yielding TM-D1, to determine if the class D C-terminus is also phosphorylated exclusively after this site. As expected, phosphorylation was not reduced in TM-D2 and TM-D3, compared to TM-D. A substantial amount of phosphorylation ($25 \pm 5\%$, $n = 3$) was detected in deletion mutant TM-D1. This finding was surprising, because, in contrast to the other α_1 subunits investigated, this site is on the N-terminal half of the C-terminal tail and would therefore most likely not be removed in case of proteolytic truncation. Site-directed mutagenesis revealed that phosphorylation of deletion mutant TM-D1 occured at serine 1743, as its replacement by an alanine residue completely prevented phosphorylation of TM-D1 ($n = 3$). In contrast, cAMP-dependent phosphorylation was not reduced in TM-D2 and TM-D3. Our data restrict the major sites of phosphorylation of the α_{1D} to the consensus phoshorylation sites at serine residues in position 1816, 1842, and 1964. Splice variant rCACN4B lacks these consensus phosphorylation sites (i.e., serine 1743, serine 1816, serine 1842, and serine 1964).

DISCUSSION

We demonstrate for the first time that L-type calcium channel α_1 subunits can be expressed at high yields in *S. cerevisiae*. The *S. cerevisiae* system offers the advantages of high expression efficiency, proper posttranslational processing, and simple handling. *S. cerevisiae* can be readily transformed with cDNA inserted into appropriate vectors following established procedures (Hill, 1991). Different ion channels and membrane receptors, including nicotinic acetylcholine receptors, muscarinic acetylcholine receptors, benzodiazepine receptor, dopamine receptors, human *mdr1*, and a transmembrane phenylalkylamine calcium antagonist binding protein, were successfully expressed in *S. cerevisiae* (Fujita et al., 1986; Payette et al., 1990; Riond et al., 1991; Huang et al., 1992; Sander et al., 1994; Kuchler et al., 1992; Hanner et al., 1995). As observed in *Xenopus* oocytes and mammalian cells, the expression density varied for different α_1 cDNAs, depending on N- and C-terminal modifications (Wei et al., 1994a,b; Klöckner et al., 1995). The highest densities, observed for TM-Sc and TM-D1, were about 20-fold higher than the densities measured for α_1 subunits in microsomal membrane fractions of mammalian heart or brain (Glossmann et al., 1985).

We exploited this system for a detailed analysis of the C-terminal PK-A phosphorylation sites of L-type calcium channel α_1 subunits. Recent studies have shown that PK-A phosphorylation of α_1 subunits is responsible for L-type calcium channel modulation in skeletal muscle (Sculptoreanu et al., 1993a; Johnson et al., 1994) and heart (Sculptoreanu et al., 1993b). In heart this seems to be due to phosphorylation of the α_1 subunit (Yoshida et al., 1992; Sculptoreanu et al., 1993b). This was in contrast to earlier studies describing that biochemically isolated α_1 subunits from skeletal muscle and heart are either weak (Roehrkasten et al., 1988) or no substrates (Hosey et al., 1989; Grabner et al., 1991; Haase et al., 1993) for PK-A. This discrepancy was resolved by the finding that the majority of biochemically isolated α_1 subunits undergoes a proteolytic cleavage removing a substantial portion of their C-terminal tails, including sites phosphorylated by PK-A (Lai et al., 1990; De Jongh et al., 1991; Rotman et al., 1992; Yoshida et al., 1992; Hell et al., 1993). As only a small fraction of α_1 subunits exists as the full length form that can be phosphorylated in adult skeletal muscle, heart, and brain, this complicates studies designed to identify these phosphorylation sites. So far a detailed analysis was only accomplished for α_{1Sr} subunits (Rotman et al., 1995) which are present at high densities in transverse-tubule membranes and can be easily purified. In this tissue, of five consensus sites only serine residues 1757 and 1854 were found to be phosphorylated by PK-A in vivo and in vitro (Rotman et al., 1995). The same sites were also exclusively phosphorylated by PK-A in *S. cerevisiae*-expressed chimera TM-Sr which contains the C-terminal tail from the rabbit skeletal muscle α_1.

Therefore, we were prompted to use an identical approach to identify regions of phosphorylation for all other classes of DHP-sensitive L-type channel α_1 subunits cloned so far. In the C-terminus of carp skeletal muscle and rat heart α_{1C} only a single serine served as a substrate for PK-A. In both cases this serine residue corresponded to the most C-terminal consensus site for cAMP-dependent phosphorylation. In contrast, the C-terminus of the class D α_1 subunits rCACN4A was phosphorylated at more than one site. Most of the phosphorylation (>70%) occurred within a 267 amino acid sequence stretch that is absent in another α_{1D} splice form (rCACN4B). This suggests that channel modulation by PK-A may not be modified only by posttranslational truncation but also by alternative splicing. Within the α_{1D} C-terminus of rCACN4A we also found evidence for phosphorylation of a residue (serine 1743) located at the N-terminal end of the putative truncation region (see Figure 3E) that is likely to be retained even after posttranslational proteolytic processing. This sets the neurosecretory α_{1D} subunits apart from class C and S α_1 subunits.

Taken together, our study identifies serine 1928 of the α_{1C} subunit as the major site for in vitro phosphorylation by PK-A. Evidence that phosphorylation of this residue is important for the modulation of calcium channel function was recently obtained by Perets et al. (1996). In addition, we demonstrate that the C-termini of carp α_{1S} and rat neuroendocrine α_{1D} (rCACN4A) contain residues that are phosphorylated by PK-A in vitro. These phosphorylation sites are only present when L-type α_1 subunits are not truncated. This finding is in line with the hypothesis that posttranslational processing may serve as an important modulatory mechanism in L-type calcium channels. In addition, our findings pave the way

for antibody mapping of these phosphorylation sites in native cardiac and neuronal tissues as well as intact cells.

Reintroducing the mutant carboxyl terminal tails into the respective parent α_1 subunit also generates the possibility to test for functional consequences of cAMP-dependent phosphorylation after heterologous expression in *Xenopus* oocytes or mammalian cells. Further, extended application of the *S. cerevisiae* expression system for radioligand binding studies will require the coexpression of accessory subunits, especially the β subunit, which is necessary for high affinity 1,4-dihydropyridine binding (Mitterdorfer et al., 1994). In the absence of coexpressed β subunit, we therefore observed no saturable high affinity binding with tritiated calcium antagonists in membrane preparations of TM-Sc. However, the feasibility of the *S. cerevisiae* expression system in obtaining large quantities of full length calcium channel α_1 subunits, which are readily phosphorylated in vitro, can be exploited for a screening assay to identify the protease responsible for posttranslational truncation of L-type calcium channel α_1 subunits in various mammalian tissues.

ACKNOWLEDGMENT

We are grateful to A. Schwartz for generously providing α_{1C-a} and to S. Seino for α_{1D}. We thank P. Kaiser, Institut für Biochemie, Universität Innsbruck for providing JB811, Yep351ADC1, and help in setting up the yeast expression system.

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BI960683O