Phosphorylation of the Consensus Sites of Protein Kinase A on α_{1D} L-type Calcium Channel*

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The novel α_{1D} L-type Ca^{2+} channel is expressed in supraventricular tissue and has been implicated in the pacemaker activity of the heart and in atrial fibrillation. We recently demonstrated that PKA activation led to increased α_{1D} Ca^{2+} channel activity in tsA201 cells by phosphorylation of the channel protein. Here we sought to identify the phosphorylated PKA consensus sites on the α_1 subunit of the α_{1D} Ca^{2+} channel by generating GST fusion proteins of the intracellular loops, N terminus, proximal and distal C termini of the α_1 subunit of α_{1D} Ca^{2+} channel. An *in vitro* PKA kinase assay was performed for the GST fusion proteins, and their phosphorylation was assessed by Western blotting using either anti-PKA substrate or anti-phosphoserine antibodies. Western blotting showed that the N terminus and C terminus were phosphorylated. Serines 1743 and 1816, two PKA consensus sites, were phosphorylated by PKA and identified by mass spectrometry. Site directed mutagenesis and patch clamp studies revealed that serines 1743 and 1816 were major functional PKA consensus sites. Altogether, biochemical and functional data revealed that serines 1743 and 1816 are major functional PKA consensus sites on the α_1 subunit of α_{1D} Ca^{2+} channel. These novel findings provide new insights into the autonomic regulation of the α_{1D} Ca^{2+} channel in the heart.

L-type Ca^{2+} channels are essential for the generation of normal cardiac rhythm, for induction of rhythm propagation through the atrioventricular node and for the contraction of the atrial and ventricular muscles (1–5). L-type Ca^{2+} channel is a multisubunit complex including α_1, β and α_2/δ subunits (5–7). The α_1 subunit contains the voltage sensor, the selectivity filter, the ion conduction pore, and the binding sites for all known Ca^{2+} channel blockers (6–9). While α_{1C} Ca^{2+} channel is expressed in the atria and ventricles of the heart (10–13), expression of α_{1D} Ca^{2+} channel is restricted to the sinoatrial

$(SA)^2$ and atrioventricular (AV) nodes, as well as in the atria, but not in the adult ventricles (2, 3, 10).

Only recently it has been realized that α_{1D} along with α_{1C} Ca^{2+} channels contribute to L-type Ca^{2+} current (I_{Ca-L}) and they both play important but unique roles in the physiology/pathophysiology of the heart (6–9). Compared with α_{1C}, α_{1D} L-type Ca^{2+} channel activates at a more negative voltage range and shows slower current inactivation during depolarization (14, 15). These properties may allow α_{1D} Ca^{2+} channel to play critical roles in SA and AV nodes function. Indeed, α_{1D} Ca^{2+} channel knock-out mice exhibit significant SA dysfunction and various degrees of AV block (12, 16–19).

The modulation of α_{1C} Ca^{2+} channel by cAMP-dependent PKA phosphorylation has been extensively studied, and the C terminus of α_1 was identified as the site of the modulation (20–22). Our group was the first to report that 8-bromo-cAMP (8-Br-cAMP), a membrane-permeable cAMP analog, increased α_{1D} Ca^{2+} channel activity using patch clamp studies (2). However, very little is known about potential PKA phosphorylation consensus motifs on the α_{1D} Ca^{2+} channel. We therefore hypothesized that the C terminus of the α_1 subunit of the α_{1D} Ca^{2+} channel mediates its modulation by cAMP-dependent PKA pathway.

EXPERIMENTAL PROCEDURES

Subcloning of Intracellular Loops, N Terminus, Proximal or Distal C Terminus of the α_1 Subunit of the Rat α_{1D} Ca^{2+} Channel into pGEX-6P-1 Vector—pCMV6b/rat α_{1D} plasmid was kindly provided by Dr. Susumu Seino from Kobe University, Japan. Two sets of primers were used to amplify each of the intracellular loops, proximal, or distal C terminus of α_1 subunit of rat α_{1D} Ca^{2+} channel. Forward primer had a BamHI site and a reverse primer had a SalI site. Intracellular loop 1: forward: 5′-cgcggatccggtgtccttagtggagaattc-3′; reverse: 5′-acgcgtcgacg-acagacttcacagctgc-3′. Intracellular loop 2: forward: 5′-cgcggat-ccctgaagctcttcttggccat-3′; reverse: 5′-acgcgtcgacgtggtggttgatg-agtttgtg-3′. Intracellular loop 3: forward: 5′-cgcggatccatgaatat-cttcgtgggcttcg-3′; reverse: 5′-acgcgtcgaccgaggagttcaccacgta-3′. Proximal C terminus: forward: 5′-cgcggatccgacaattttgactat-ctgac-3′ and reverse: 5′-acgcgtcgacaagctgctcgtctcctga-3′. Distal C terminus: forward: 5′-cgcggatccccaaccattttccgtgaag-

[2] The abbreviations used are: SA, sinoatrial; AV, atrioventricular; PKA, cAMP-dependent protein kinase; GST, glutathione *S*-transferase; WT, wild type; SNS, sympathetic nervous system.
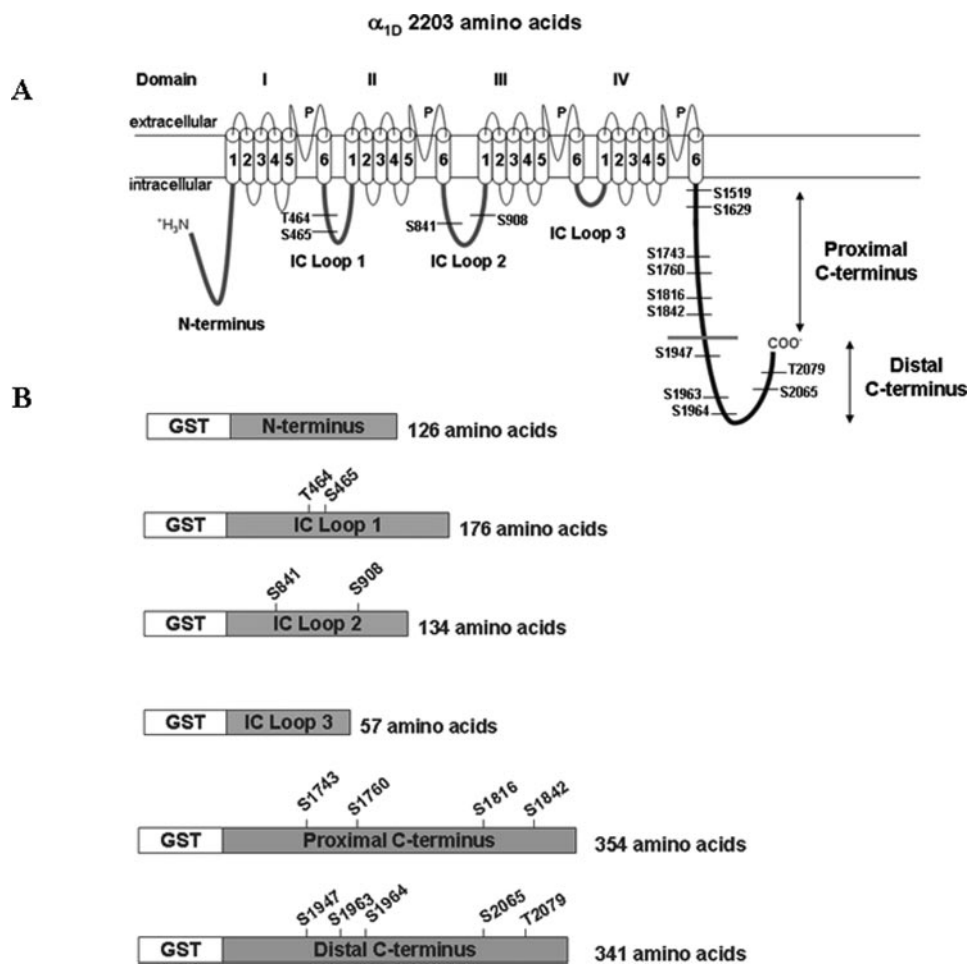
FIGURE 1. **Generation of GST fusion proteins of intracellular loops, N terminus and C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel.** *A*, sketch of the α_1 subunit of α_{1D} Ca^{2+} channel with the four domains, N terminus, proximal and distal C terminus. *B*, PKA consensus sites as determined by scansite on intracellular loops, proximal and distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel. *IC Loop*, intracellular loop.

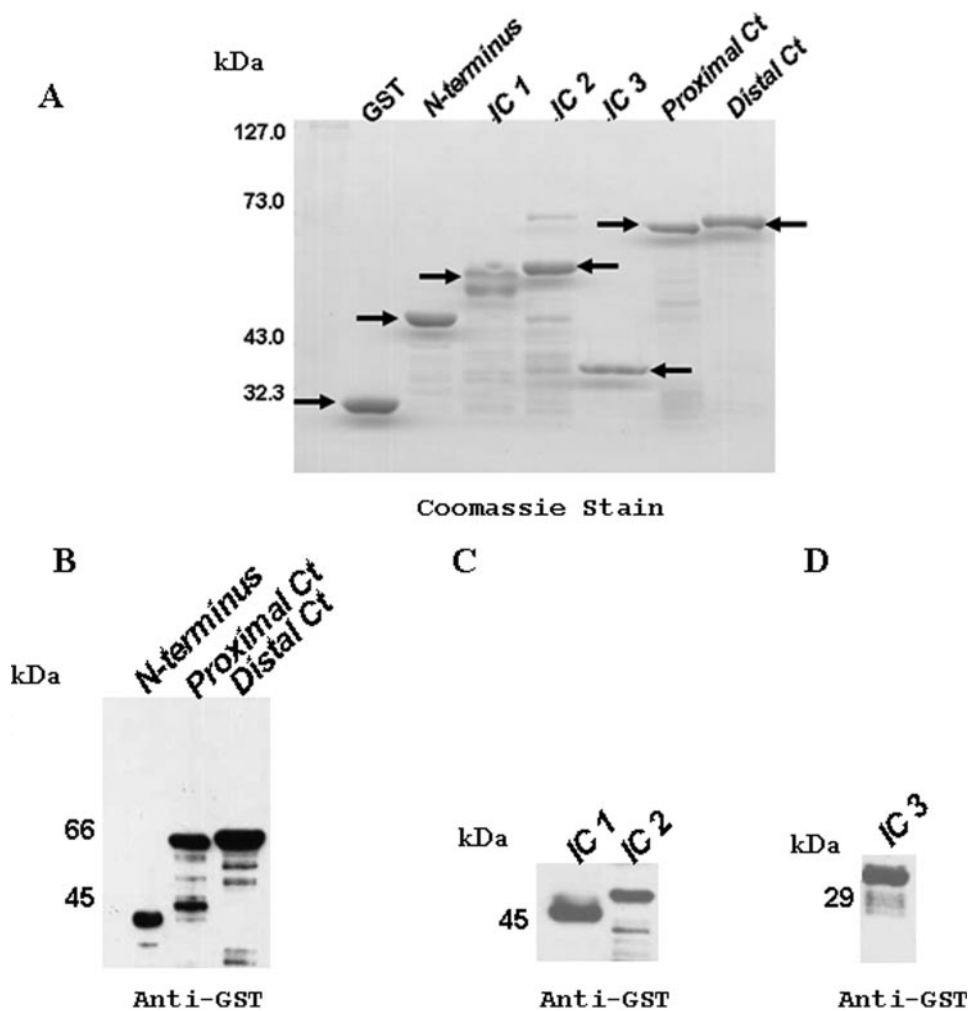positive control. Untransformed DH5α *E. coli* and uninduced cultures of the transformed DH5α *E. coli* served as a negative control.

Western Blot with Anti-GST Antibody—Equal amounts of GST fusion proteins were loaded on 10% SDS-polyacrylamide gel. The samples were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk and 3% bovine serum albumin in 0.1% Tween-20 TBS (TBST). The membrane was washed with 0.1% TBST, and was then probed with mouse monoclonal anti-GST at a dilution of 1:1000. The membrane was washed with 0.1% TBST before being probed with rabbit anti-mouse horseradish peroxidase conjugate antibody at 1:6000. The membrane was washed again with 0.1% TBST and was developed with the ECL Plus Western blotting detection system (Amersham Biosciences) and Kodak developer.

In Vitro Kinase Assay for PKA—The kinase assay was performed on immobilized GST fusion proteins as described by the manufacturer (PKA assay kit, 17-134, Millipore). Briefly, immobilized GST fusion proteins were washed five times with 1$\times$ Tris-buffered saline (TBS). Then 25 μl of kinase assay mixture was added to each tube. The mixture consisted of 5 μl of 5$\times$ reaction buffer, 2 ng of recombinant PKA catalytic subunit, 7.5 μl of double distilled water, and diluted 1:1 magnesium/ATP (PKA assay kit, 17–134, Millipore). The reaction was allowed to occur for 30 min at 30 °C in a water bath with shaking. The reaction was stopped by adding 2$\times$ SDS loading buffer, and boiling for 5 min. Negative controls consisted of GST fusion proteins subjected to an *in vitro* kinase assay without the catalytic subunits of PKA.

Western Blot with Anti-PKA Substrate and Anti-phosphoserine Antibody—Equal amounts of phosphorylated GST fusion proteins or negative controls of GST fusion proteins, as outlined in the *in vitro* kinase assay, were loaded on 10% SDS-polyacrylamide gel. The samples were transferred to a nitrocellulose membrane, and the membrane was blocked with 5% nonfat dry milk and 3% bovine serum albumin in 0.1% TBST. The membrane was probed with mouse monoclonal anti-PKA substrate antibody at 1:200 for negative controls and at 1:500 for the remaining blots (PhosphoDetect™, 700 μg/ml, Calbiochem) or with mouse monoclonal anti-phosphoserine antibody at 1:500 for negative controls and at 1:1000 for the remaining blots (mouse ascites fluid, Sigma). The membrane was washed with 0.1% TBST before being probed with rabbit anti-mouse

3′, reverse: 5′-acgcgtcgacctacaaggtggtgatgcaaa-3′. For the subcloning of the N terminus of α_1 subunit of rat α_{1D} Ca^{2+} channel into pGEX 6P-1 vector, the forward primer had a BamHI site, and the reverse primer had an EcoRI site. Primers: forward: 5′-cgcggatcccagcatcaacggcagcagcaa-3′; reverse: 5′-ccggaattctttccaatccactatactaat-3′.

All amplified fragments were then subcloned into a glutathione *S*-transferase (GST) bacterial expression vector pGEX-6p-1. The DH5α bacterial strain of *Escherichia coli* was transformed with the recombinant pGEX-6P-1 vectors, and sequencing of clones with recombinant vectors were confirmed by Genemed (South San Francisco, CA).

Expression of GST Fusion Proteins of Intracellular Loops, N Terminus, Proximal and Distal C Terminus of α_1 Subunit of Rat α_{1D} Ca^{2+} Channel—GST fusion proteins were expressed under the induction of the lactose analog isopropyl β-D-thiogalactoside as described before (Handbook of GST Gene Fusion System, 18-1157-58, Amersham Biosciences). Purification of the fusion proteins was performed as previously described (23). All samples were run on 12% Bis-acrylamide gel under reducing conditions and the gel was stained with Coomassie Blue. To check the success of the isopropyl-1-thio-β-D-galactopyranoside induction, DH5α transformed with pGEX-6P-1 served as a

FIGURE 2. **Expression of GST fusion proteins.** Representative Coomassie Blue staining showing GST fusion proteins. Two fragments that span the C terminus (proximal (69 kDa) and distal (68 kDa)), together with N terminus (43 kDa) and intracellular loops 1–3 (49, ~49, and 36 kDa) of the α_1 subunit of α_{1D} Ca^{2+} channel were subcloned into the BamHI and SalI sites of pGEX vector. GST fusion proteins were separated by the GST affinity pull-down assay, run on 10% polyacrylamide gel under reducing conditions and stained with Coomassie Blue or probed with anti-GST antibodies. *A*, Coomassie Blue stain of GST fusion proteins of intracellular loops, N terminus, proximal and distal C terminus of α_1 subunit of the α_{1D} Ca^{2+} channel. *B*, Western blot of GST fusion proteins of N terminus, proximal, and distal C terminus probed with anti-GST antibody. *C*, Western blot of GST fusion protein of intracellular loops 1 and 2 probed with anti-GST antibody. *D*, Western blot of GST fusion proteins of intracellular loop 3 probed with anti-GST antibody. *CT*, C terminus; *IC*, intracellular. Note that on Coomassie gel (*panel A*), a 43-kDa marker was used. On the Western blot (*panel B*), a 45-kDa protein marker was used.

horseradish peroxidase conjugate antibody (1:2000 or 1:4000, Sigma). Membrane was washed again with 0.1% TBST and was developed with the ECL Plus Western blotting detection system (Amersham Biosciences) and a Kodak developer.

Mass Spectrometry—Protein bands of GST fusion proteins for intracellular loops, N terminus, proximal and distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel were excised from Coomassie Blue-stained 12% polyacrylamide gels and subjected to in-gel trypsin or chymotrypsin digestion for liquid chromatography-mass spectrometry analysis protocol at the Proteomics & Mass Spectrometry Facility of the University of Massachusetts Medical School (UMMS). Negative controls, which consisted of GST fusion proteins subjected to an *in vitro* kinase assay without the catalytic subunits of PKA were also sent for mass spectrometric analysis.

Site-directed Mutagenesis—Site-directed mutagenesis was performed on pCMV6b/rat α_{1D} using QuikChange TM site-directed mutagenesis kit from Stratagene (La Jolla, CA) according to the manufacturer's instructions. Serine residues were replaced by alanine in all substitution mutants. The following mutations were introduced by mutagenic primers: S1743A, S1816A, and S1964A. The presence of mutations was confirmed by sequencing at the Laval University sequencing facility (Quebec, Canada).

Transfection of the tsA201 Cell Line—Mammalian tsA201 cells were grown in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin G (100 units/ml), and streptomycin (10 mg/ml, Invitrogen) from our laboratory. Cells were incubated in 5% CO_2 humidified atmosphere. The tsA201 cells were transfected using the Ca^{2+} phosphate method with the following modification: to identify transfected cells, 7 μg of EBO/CD8 plasmid was cotransfected with 7 μg of each of the subunits of α_{1D} Ca^{2+} channel: α_1, β, and α_2/δ cDNAs. For patch clamp experiments, 2–3 days post-transfection cells were incubated for 2 min in a medium containing anti-CD8a-coated beads (Dynabeads, M-450 CD8a). The unattached beads were removed by washing with extracellular solution (in mM): 135 choline chloride, 1 $MgCl_2$, 2 $CaCl_2$, and 10 HEPES; adjusted to pH 7.4 with tetraethylammonium hydroxide (TEA-OH). Cells expressing CD8a and therefore binding beads were distinguished from non-transfected cells by light microscopy.

Electrophysiology—Ca^{2+} currents were recorded in whole cell configuration of the patch clamp technique (pClamp 9, Axon Instrument) (2, 24). The internal solution contained (in mM): 135 CsCl, 4 $MgCl_2$, 4 ATP, 10 HEPES, 10 EGTA, and 1 EDTA; adjusted to pH 7.2 with TEA-OH. Data were digitized at 5 kHz with an analog-to-digital converter. The recordings were filtered with a low-pass corner frequency of 2 kHz. For the time course, α_{1D} Ca^{2+} current was continuously recorded at a test potential of 10 mV from a holding potential of 100 mV.

Statistics—Data were expressed as means $\pm$ S.E. Percent of inhibition was calculated as difference of the current amplitude by the intervention over the control value. When indicated,
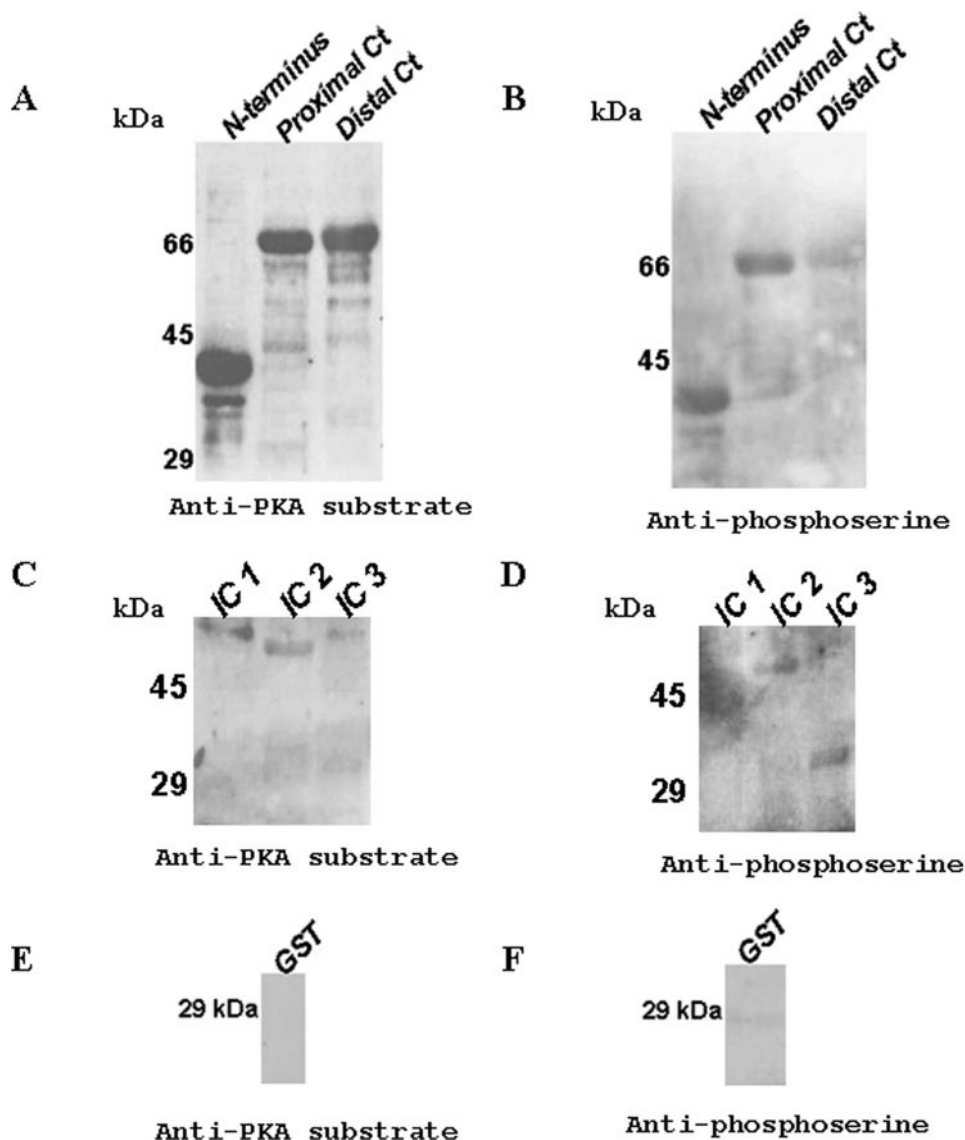
FIGURE 3. **Western blot showing that the N terminus and proximal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel are phosphorylated by PKA.** Immobilized GST fusion proteins of N terminus, proximal and distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel were subjected to an *in vitro* PKA kinase assay. The fusion proteins were then run on 10% polyacrylamide gel and subjected to Western blotting using one of two antibodies: anti-PKA substrate or anti-phosphoserine. GST served as a negative control. *A*, Western blot of GST fusion proteins of N terminus, proximal and distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel probed with anti-PKA substrate antibody. *B*, Western blot of GST fusion proteins of N terminus, proximal and distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel probed with anti-phosphoserine antibodies. Distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel was minimally phosphorylated. *C*, Western blot of GST fusion proteins of intracellular loops of the α_1 subunit of α_{1D} Ca^{2+} channel probed with anti-PKA substrate antibody. The antibodies detected minimal phosphorylation for intracellular loops 1 and 2 and almost none for intracellular loop 3. *D*, Western blot of GST fusion proteins of intracellular loops of the α_1 subunit of α_{1D} Ca^{2+} channel probed with anti-phosphoserine antibodies. The antibodies detected minimal phosphorylation of the three intracellular loops. *E* and *F*, Western blot of GST protein probed with anti-PKA substrate or anti-phosphoserine antibodies, respectively. *CT*, C terminus; *IC*, intracellular.

such a way that the GST tag was on the N terminus of different fragments of the α_1 subunit of the α_{1D} Ca^{2+} channel protein (Fig. 1*B*). GST fusion proteins spanning the intracellular loops, N terminus and C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel were then generated (Fig. 2*A*). The expected size of GST fusion proteins of N terminus, proximal and distal C terminus is 43, 69, and 68 kDa, respectively. The expected size of GST fusion proteins of intracellular loops 1, 2, and 3, is 49, 44, and 36 kDa, respectively. Coomassie Blue staining showed that GST fusion proteins of N terminus, proximal and distal C terminus were migrating at higher than 40 kDa on polyacrylamide gel (Fig. 2*A*). Coomassie Blue stained gels also showed that GST fusion proteins of intracellular loops 1, 2, and 3 were migrating at 49, close to 49 and 36 kDa, respectively (Fig. 2*A*). Western blots showed the expression of GST fusion proteins of N terminus, proximal and distal C terminus, and intracellular loops (Fig. 2, *B–D*, respectively).

GST Fusion Proteins Were Phosphorylated—After successfully generating the GST fusion proteins of intracellular loops and the N terminus and C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel, these fusion proteins were subjected to an *in vitro* PKA kinase assay followed by Western blot to determine which of the PKA consensus sites is phosphorylated by PKA. The phosphorylation status of the GST fusion proteins was tested by two antibodies. The first antibody was an anti-PKA substrate that is capable of detecting phosphorylated serines only in a PKA consensus site (arginine-arginine-x-serine, where x can be any amino acid). The second antibody was an anti-phosphoserine antibody that can detect phosphorylated serines regardless of the consensus site. Fig. 3 shows a Western blot using the two antibodies. The N terminus and proximal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel were phosphorylated (Fig. 3, *A* and *B*). The distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel showed phosphorylation when probed with the anti-PKA substrate (Fig. 3*A*) and minimal when probed with anti-phosphoserine antibodies (Fig. 3*B*). These experiments were repeated four times, each giving iden-

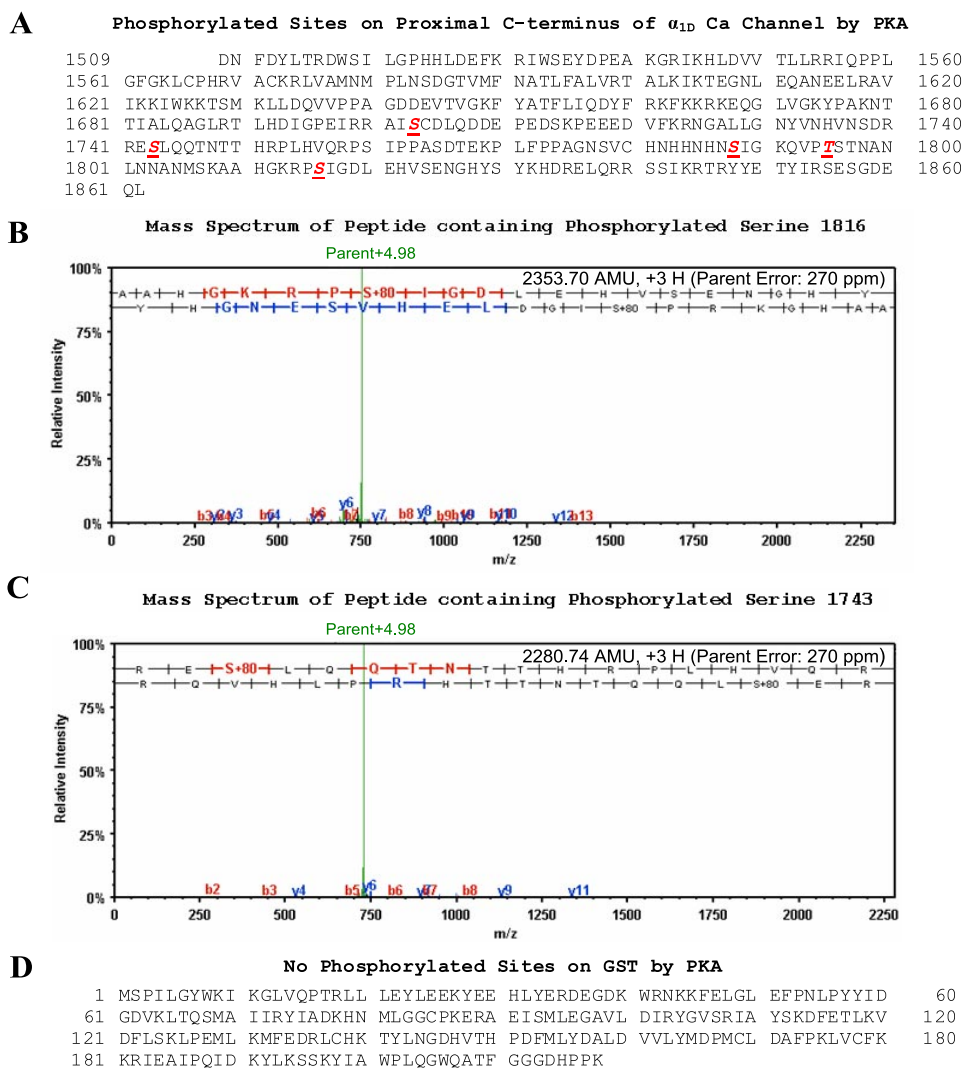paired or unpaired Student's *t* test was performed. Differences were deemed significant at a *p* value of <0.05.

RESULTS

Generation of GST Fusion Proteins—Scansite showed that the α_1 subunit of the α_{1D} Ca^{2+} channel has a number of PKA consensus sites, mainly on the C terminus (Fig. 1*A*). To identify which of the potential PKA consensus sites is phosphorylated by PKA, constructs of GST fusion protein were designed in

A

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          Phosphorylated Sites on Proximal C-terminus of α1D Ca Channel by PKA

1509        DN FDYLTRDWSI LGPHHLDEFK RIWSEYDPEA KGRIKHLDVV TLLRRIQPPL  1560
1561 GFGKLCPHRV ACKRLVAMNM PLNSDGTVMF NATLFALVRT ALKIKTEGNL EQANEELRAV  1620
1621 IKKIWKKTSM KLLDQVVPPA GDDEVTVGKF YATFLIQDYF RKFKKRKEQG LVGKYPAKNT  1680
1681 TIALQAGLRT LHDIGPEIRR AISCDLQDDE PEDSKPEEED VFKRNGALLG NYVNHVNSDR  1740
1741 RESLQQTNTT HRPLHVQRPS IPPASDTEKP LFPPAGNSVC HNHHNHHNSIG KQVPTSTNAN  1800
1801 LNNANMSKAA HGKRPSIGDL EHVSENGHYS YKHDRELQRR SSIKRTRYYE TYIRSESGDE  1860
1861 QL
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B

Mass Spectrum of Peptide containing Phosphorylated Serine 1816

C

Mass Spectrum of Peptide containing Phosphorylated Serine 1743

D

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                    No Phosphorylated Sites on GST by PKA

  1 MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL EFPNLPYYID   60
 61 GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL DIRYGVSRIA YSKDFETLKV  120
121 DFLSKLPEML KMFEDRLCHK TYLNGDHVTH PDFMLYDALD VVLYMDPMCL DAFPKLVCFK  180
181 KRIEAIPQID KYLKSSKYIA WPLQGWQATF GGGDHPPK
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FIGURE 4. **Phosphorylation of serine 1743 and serine 1816 identified by mass spectrometry on the proximal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel.** Trypsinized proximal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel was subjected to mass spectrometry. *A*, amino acid sequence of proximal C terminus of α_1 subunit of α_{1D} Ca^{2+} channel with phosphorylated amino acid residues. Five sites were found to be phosphorylated by PKA (amino acids in *red*). Mass spectrometry covered 82% of the proximal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel. *B* and *C*, mass spectra for peptides containing serines 1816 and 1743, respectively. *D*, amino acid sequence of GST. No phosphorylated sites were detected on GST. Mass spectrometry covered 92% of GST.

tical results. In contrast, the intracellular loops of the α_1 subunit of α_{1D} Ca^{2+} channel were found to be minimally phosphorylated, both with the anti-PKA substrate and with the anti-phosphoserine antibodies (Fig. 3, *C* and *D*), while GST protein (negative control) was not found to be phosphorylated (Fig. 3, *E* and *F*).

Mass Spectrometry Identified Phosphorylated Sites on GST Fusion Proteins—To identify the phosphorylated PKA consensus sites on the α_1 subunit of α_{1D} Ca^{2+} channel, mass spectrometry was performed on GST fusion proteins phosphorylated by PKA. The phosphorylated GST fusion proteins were run on a polyacrylamide gel, stained with Coomassie Blue, and the corresponding bands were excised. We performed an in-gel digestion with trypsin and chymotrypsin. Mass spectrometry identified two PKA consensus sites phosphorylated on the proximal C terminus of the α_1 subunit of the α_{1D} Ca^{2+} channel: serines 1743 and 1816 (Fig. 4, *A–C*). Mass spectrometry identified

three more non-PKA consensus sites not identified by scansite and these were: serine 1703, serine 1788, and threonine 1795 (Fig. 4*A*). Mass spectrometric analysis did not detect any phosphorylated residues on GST (Fig. 4*D*) in agreement with our prior findings (Fig. 3, *E* and *F*).

The same approach applied for the proximal C terminus (mass spectrometry), we found that serine 1964 was phosphorylated. This residue is a PKA consensus site, phosphorylated in trypsinized digests of the distal C terminus of the α_1 subunit of α_{1D} Ca^{2+} channel. In addition, from our mass spectrometry studies, we identified a number of non-PKA consensus sites that were phosphorylated on the N terminus and distal C terminus of the α_1 subunit of the α_{1D} Ca^{2+} channel. These included serines 1944, 2152, 2165, and threonine 2147 phosphorylated on the distal C terminus (Fig. 5*A*). Likewise, we found that serines 45, 46, 52, 121, and threonine 49 were all phosphorylated on the N terminus (Fig. 5*B*). These findings suggest that there are non-PKA consensus sites that can be phosphorylated by PKA on the N terminus and distal C terminus of the α_1 subunit of the α_{1D} Ca^{2+} channel.

We also found non-PKA consensus sites on intracellular loop 1 of the α_1 subunit of the α_{1D} Ca^{2+} channel (Fig. 5*C*). On the other hand, we did not detect any phos-

phorylated residues on double digests of the other two loops. However, we identified phosphorylated non-PKA consensus sites on trypsinized digests of intracellular loop 2 of the α_1 subunit of α_{1D} Ca^{2+} channel. The phosphorylated residues on intracellular loop 1 were: serines 517, 519 and threonines 443, 504. For trypsinized digests of intracellular loop 2 the phosphorylated amino acid residues were: serines 857, 923, 929, and threonines 863.

Negative Controls of Intracellular Loop 1, N Terminus, Proximal and Distal C Terminus Were Not Phosphorylated—To test the antibodies and the mass spectrometric approaches, we performed an *in vitro* kinase assay without the catalytic subunit of PKA. No phosphorylated peptides were detected by Western blot (Fig. 6) nor by mass spectrometry.

Site-directed Mutagenesis and Patch Clamp Studies Showed That Serine 1816 Is Functionally Important—Of the nine PKA sites identified by scansite, 3 were found to be phosphorylated

A

Phosphorylated Sites on Distal C-terminus of α₁D Ca Channel by PKA

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1863   PTIFREDP EIHGYFRDPR CFGEQEYFSS EECCEDDSSP TWSRQNYSYY NRYPGSSMDF  1920
1921   ERPRGYHHPQ GFLEDDDSPI GYDSRRSPRR RLLPPTPPSH RRSSFNFECL RRQNSQDDVL  1980
1981   PSPALPHRAA LPLHLMQQQI MAVAGLDSSK AQKYSPSHST RSWATPPATP PYRDWTPCYT  2040
2041   PLIQVDRSES MDQVNGSLPS LHRSSWYTDE PDISYRTFTP ASLTVPSSFR NKNSDKQRSA  2100
2101   DSLVEAVLIS EGLGRYARDP KFVSATKHEI ADACDLTIDE MESAASTLLN GSVCPRANGD  2160
2161   MGPISHRQDY ELQDFGPGYS DEEPDPGREE EDLADEMICI TTL
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B

### Phosphorylated Sites on N-terminus of α₁D Ca Channel by PKA

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  1   MMMMMMMKKM QHQRQQQEDH ANEANYARGT RLPISGEGPT SQPNSSKQTV LSWQAAIDAA   60
 61   RQAKAAQTMS TSAPPPVGSL SQRKRQQYAK SKKQGNSSNS RPARALFCLS LNNPIRRACI  120
121   SIVDWK
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C

Phosphorylated sites on Intracellular Loop 1 of α₁D Ca Channel by PKA

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                                               GEFS KEREKAKARG  420
421   DFQKLREKQQ LEEDLKGYLD WITQAEDIDP ENEEEGGEEG KRNTSMPTSE TESVNTENVS  480
481   GEGETQGCCG SLWCWWKRRG AAKTGPSGCR RWGQAISKSK LRSHGAREAL CVCRCSLESL  540
541   VKLWTSRFSA HLQAAYVRPY SRRWRRWNRF NRRRCRAAVK SV
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FIGURE 5. **Mass spectrometry-identified phosphorylated sites on intracellular loop 1, N terminus, and distal C terminus on the α₁ subunit of α₁D Ca²⁺ channel.** *A,* amino acid sequence of distal C terminus of the α₁ subunit of α₁D Ca²⁺ channel with phosphorylated amino acid residues. Four sites were found phosphorylated on distal C terminus of the α₁ subunit of α₁D Ca²⁺ channel by mass spectrometry (amino acids in *gray* and *underlined*). *B,* amino acid sequence of N terminus of the α₁ subunit of α₁D Ca²⁺ channel with phosphorylated amino acid residues. Four sites were found phosphorylated on the N terminus of the α₁ subunit of α₁D Ca²⁺ channel by mass spectrometry (amino acids in *gray* and *underlined*). Mass spectrometry covered 67% for both N terminus and distal C terminus of α₁ subunit of α₁D Ca²⁺ channel. *C,* amino acid sequence of intracellular loop 1 of the α₁ subunit of α₁D Ca²⁺ channel with phosphorylated amino acid residues. Four sites were found phosphorylated on intracellular loop 1 of the α₁ subunit of α₁D Ca²⁺ channel by mass spectrometry (amino acids in *gray* and *underlined*). Mass spectrometry covered 35% of intracellular loop 1 of the α₁ subunit of α₁D Ca²⁺ channel.
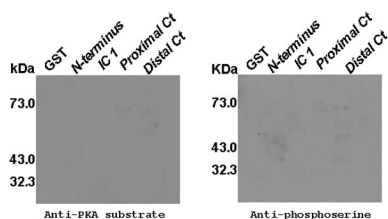
FIGURE 6. **Negative controls of GST fusion proteins were not phosphorylated.** Western blot of GST fusion proteins of intracellular loop 1, N terminus, proximal and distal C terminus of the α₁ subunit of α₁D Ca²⁺ channel after they have been subjected to an *in vitro* PKA kinase assay without PKA catalytic subunit. Western blots with either anti-PKA substrate or with anti-phosphoserine antibodies are shown with no bands. All of these fusion proteins were subjected to mass spectrometry, and no phosphorylated sites were detected.

by mass spectrometry. These were serines 1743, 1816, and 1964. To test for their functional relevance, deletion mutants, and site-directed mutagenesis combined with electrophysiology experiments were performed.

We first prepared two deletion mutants of the C terminus of the α₁D Ca²⁺ channel and expressed them in tsA201 cells. We then measured the basal current of these cells with or without the presence of 50 μM 8-Br-cAMP, which activates PKA in tsA201 cells (2). To minimize the variation of the current measurements, all current comparisons were made under similar conditions, which included using the same set of tsA201 cells, same amount of plasmids and same post transfection recording time.

The first deletion mutant with a stop codon at amino acid residue 1517 of the C terminus of the α₁D Ca²⁺ channel resulted in the abolition of α₁D I_{Ca-L} (data not shown). The second deletion mutant had a stop codon at amino acid residue 1917 of the C terminus of α₁D Ca²⁺ channel. Cells expressing this deletion mutant showed an increase of α₁D I_{CaL} in response to 50 μM 8-Br-cAMP similar to that observed in cells expressing the wild-type (WT) α₁D channel. These findings further support the unique importance of the proximal region of the C terminus of α₁D Ca²⁺ channel.

We finally examined the modulation of single site mutants in response to 8-Br-cAMP (50 μM) (3). While treatment of tsA201 cells expressing WT α₁D Ca²⁺ channels with 8-Br-cAMP caused a significant increase in α₁D I_{Ca-L} amplitude (43.64 ± 9.6%) (Fig. 7A), substitution of serine 1743 by alanine reduced α₁D I_{Ca-L} increase by PKA activation to 22.0 ± 4.2% (Fig. 7B). However, substitution of serine 1816 by alanine almost completely abolished 8-Br-cAMP-induced current increase (10.70 ± 3.3%) (Fig. 7C). Substitution of serine 1964 by alanine did not show any alteration in 8-Br-cAMP-induced enhancement of α₁D I_{Ca-L} (data not shown). Averaged data for WT α₁D I_{CaL}, α₁D/S1743A and α₁D/S1816A are shown in Fig. 7D.

DISCUSSION

Here we provide a systematic biochemical and proteomic approach for identifying phosphorylated PKA consensus sites on the α₁ subunit of the α₁D Ca²⁺ channel. We have identified serines 1743 and 1816 as two major functional PKA consensus sites. We also showed that serine 1816 has a major functional role in the α₁D Ca²⁺ channel response to PKA as compared with serine 1743.

We also showed that the distal C terminus is phosphorylated by PKA, but no functional site was identified. We found that serine 1964, a PKA consensus site identified by scansite and mass spectrometry, was not functionally relevant.

Several phosphorylated non-PKA consensus sites on the proximal and distal C terminus were identified. However, we were mainly interested in characterizing PKA consensus sites identified by scansite and phosphorylated by PKA. We did not identify any PKA consensus sites on intracellular loops of the α₁ subunit of the α₁D Ca²⁺ channel. Although scansite identified several PKA consensus sites on intracellular loops 1 and 2 of α₁D Ca²⁺ channel, none of these sites was phosphorylated by PKA. The data obtained with the site-directed mutants with S1816A and S1743A of α₁D Ca²⁺ channel argue against the functional relevance of other PKA consensus sites present on the α₁D Ca²⁺ channel. In fact, when serine 1816 was mutated to
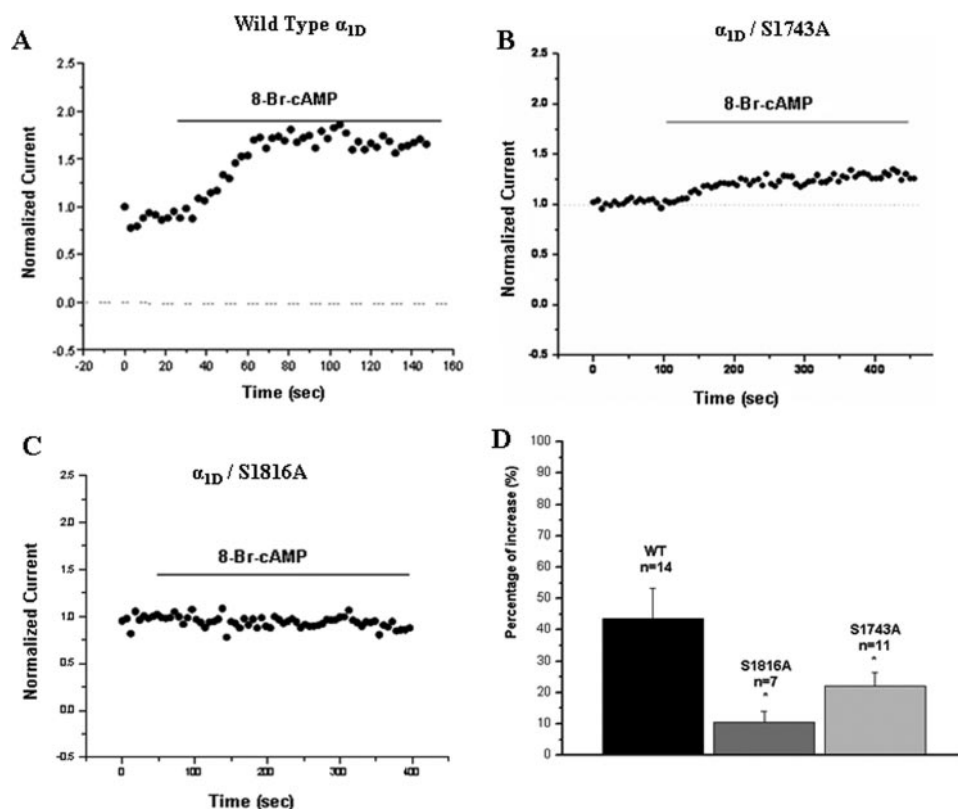
FIGURE 7. α_{1D}/S1743A and α_{1D}/S1816A mutants had a decreased response to 8-Br-cAMP in tsA201 cells. Time course for the effect of 8-Br-cAMP (50 μM) on I_{Ca-L} of wild-type α_{1D} Ca^{2+} channel, α_{1D}/S1743A and α_{1D}/S1816A expressed in tsA201 cell, respectively. *A*, 8-Br-cAMP increased I_{Ca-L} of wild-type α_{1D} Ca^{2+} channel. *B*, response to 8-Br-cAMP was decreased in mutant α_{1D}/S1743A Ca^{2+} channel. *C*, response to 8-Br-cAMP was almost completely abolished in mutant α_{1D}/S1816A Ca^{2+} channel. *D*, averaged data of the increase of α_{1D} I_{Ca-L} by 8-Br-cAMP (50 μM) in tsA201 cells. Differences were deemed significant at a *p* value of < 0.05.

the SA node is heavily innervated by the sympathetic nervous system (SNS) (29), stimulation of the SNS in response to exercise or stress insults results in a rapid and dramatic increase in heart rate. cAMP-dependent PKA is the point of convergence for the regulation of Ca^{2+} channels by many neurotransmitters and hormone receptors including the β1- and β2-adrenergic receptors and the serotoninergic 5-HT$_4$ receptor, all of which are present in atrial cells (27, 28). PKA is a serine/threonine kinase that can be stimulated by extracellular signals that elevate intracellular cAMP concentrations which, in turn, can induce phosphorylation of the α_{1D} L-type Ca^{2+} channel, leading to increased channel activity (30). Identifying consensus PKA phosphorylation sites α_{1D} L-type Ca^{2+} channel is important to the understanding of cardiac rhythm regulation by the SNS.

A—In summary, the present work establishes that serines 1743 and 1816, two PKA consensus sites, are phosphorylated by PKA and are functionally relevant. The present data are the first to identify serine 1816 as a PKA consensus site that can be phosphorylated by PKA and is functionally relevant. Not all PKA consensus sites are functionally relevant, *i.e.* phosphorylation of these sites does not alter the activity of α_{1D} Ca^{2+} channel in response to PKA activation and phosphorylation.

alanine the response of the α_{1D} Ca^{2+} channel to 8-Br-cAMP was almost abolished.

The weak phosphorylation seen for intracellular loop 3 on Western blots can be accounted for by the presence of serines 1263 and 1264, two non-PKA consensus sites that were not detected by mass spectrometry. This occurred because treatment of the intracellular loop 3 peptide with trypsin or chymotrypsin generates small peptides that are of low mass to be detected. For example, tryptic digestion of the intracellular loop 3 peptide results in a small peptide, KFWYVVNSS, which has a molecular mass close to the detection threshold for mass spectrometry. Chymotrypsin generates peptides that are even smaller than the tryptic peptides.

Physiological Significance—The α_{1D} Ca^{2+} channel has a more negative activation voltage (between -60 to -40 mV) than the α_{1C} Ca^{2+} channel. This enables the channel to play an important role in phase 4 diastolic depolarization of the pacemaker (12, 15, 19, 25, 26). The restricted expression of the α_{1D} Ca^{2+} channel to atria, SA, and AV nodes supports such a notion (2, 3, 10). Sinus bradycardia and second degree AV block seen in the α_{1D} Ca^{2+} channel knock-out mice, shows that deletion of α_{1D} Ca^{2+} channel seems to affect heart rate and conduction (12, 16, 17, 19). Furthermore, the α_{1D} Ca^{2+} channel knock-out mice were prone to atrial fibrillation (12, 16, 17, 19).

The phosphorylation process constitutes one of the major regulatory pathways for cardiac L-type Ca^{2+} channels. Because

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