# Cloning and Expression of the Ca<sup>2+</sup> Channel $\alpha_{1C}$ and $\beta_{2a}$ Subunits from Guinea Pig Heart<sup>1</sup>

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Complimentary DNA clones encoding the  $a_{1c}$  and  $\beta_{2a}$  subunits of guinea-pig cardiac L-type  $Ca^{2+}$  channels were isolated using the PCR method. The open reading frame encoded 2,169 amino acids for the  $\alpha_{1c}$  and 597 amino acids for the  $\beta_{2a}$  subunit. The proteins showed 94.2 and 94.8%, respectively, identity to the respective subunit of the rabbit protein. The message size of the guinea pig  $\alpha_{1c}$  and  $\beta_{2a}$  subunits was 8.0 and 3.5/4.0 kb, respectively. RT-PCR analysis revealed that the  $\alpha_{1c}$  subunit is expressed exclusively in the heart, while the  $\beta_{2a}$  subunit is expressed in the heart, cerebellum, whole brain, and stomach. The  $\alpha_{1c}$  and  $\beta_{2a}$  subunits are transiently expressed in BHK (baby hamster kidney) cells, and the channel currents were studied using the whole-cell patch clamp technique in medium containing 30 mM Ba<sup>2+</sup>. In cells expressing  $\alpha_{1C}$  alone, the Ba<sup>2+</sup> current was activated at -30 mV and more positive potentials and peaked at about 10 mV. The co-expression of  $\beta_{2a}$  with  $\alpha_{1c}$  did not affect the voltage-dependence of the current, but increased the peak current and accelerated current decay. In cells transfected with guinea pig  $a_{1c}$  and rabbit  $\beta_1 + a_2/\delta$ , a Ba<sup>2+</sup> current comparable to those in native myocytes was observed. The  $Ba^{2+}$  current can be blocked completely by nifedipine and is enhanced 3-fold by Bay K 8644. On the other hand, neither forskolin nor okadaic acid affects the Ba<sup>2+</sup> current, suggesting that cAMP-mediated modulation is not easily reproduced in transfected cells, unlike that seen in native cardiac myocytes.

Key words:  $a_{1C}$  subunit,  $\beta_2$  subunit,  $Ca^{2+}$  channel, cloning, guinea pig heart.

The cDNA encoding the voltage-dependent Ca<sup>2+</sup> channel  $\alpha_1$ subunit was first isolated from skeletal muscle (1) and then from heart (2). Electrophysiological and pharmacological studies have let to the classification of Ca<sup>2+</sup> channels into T, L, N, P/Q, R types (3). Most of these types comprise three or four subunits termed  $\alpha_1$ ,  $\beta$ ,  $\alpha_2/\delta$ , and  $\gamma$ . The  $\alpha_1$  subunit is the pore-forming main subunit and consists of four homologous regions, I, II, III, and IV, each of which is composed of six transmembrane segments. Several types of Ca<sup>2+</sup> channel  $\alpha_1$  subunits have been cloned from the neural tissues, muscles, and secretory cells of various animals, and have been classified into seven classes, S, C, B, A, D, E, and G (4). Studies using Ca<sup>2+</sup> channel  $\alpha_1$  genes with site-directed mutagenesis have demonstrated that the S4 segments are the voltage sensor of channel gating, P loops

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(linker at S5-S6 of each repeat) form a pore for ion permeation, and a domain on the I-II loop interacts with  $\beta$ subunits (5). The  $\beta$  subunit comprises four isoforms,  $\beta_{1-4}$ , which are expressed in a tissue-specific manner (6). The  $\beta$ subunits, when co-expressed with the  $\alpha_1$  subunits, increase the  $\alpha_1$  mediated current and/or alter the kinetics of activation and inactivation (7-9). Other auxiliary subunits,  $\alpha_2/\delta$ , and  $\gamma$ , have been reported to increase the Ca<sup>2+</sup>-channel current when co-expressed with  $\alpha_1$ .

The majority of Ca<sup>2+</sup> current in the heart is mediated by the dihydropyridine (DHP) sensitive L-type channel, the  $\alpha_1$  subunit of which is encoded by the  $\alpha_{1c}$ . DHP derived Ca<sup>2+</sup>-channel blockers bind to a site close to the pore formed by the  $\alpha_{1C}$  subunit and thereby block the channel activity of L-type channels (10, 11). The cardiac L-type channel is known to be regulated by phosphorylation mediated by cAMP-dependent protein kinase. Although the electrophysiological effect produced by phosphorylation is well documented in native cardiac cells, the molecular basis of this regulation has not been fully elucidated. Furthermore, although  $\alpha_{1C}$  subunits have been cloned from cardiac muscle (2), smooth muscle (12, 13), and brain (14), their primary structures do not account for the diversity of the electrophysiological and pharmacological properties of the channels distributed in these tissues.

Although the electrophysiological properties of the cardiac L-type  $Ca^{2+}$  channel have been studied most exten-

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Abbreviations: BHK, baby hamster kidney; DHP, dihydropyridine; GFP, green fluorescent protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; CK2, casein kinase 2; AID,  $\alpha_1$ -subunit interaction domain; BID,  $\beta$ -subunit interaction domain; I-V, current voltage.

sively in guinea pig myocytes, the molecular structure of the channel has not yet been clarified. Therefore, we cloned cDNAs encoding the  $\alpha_{1c}$  and  $\beta$  subunits from guinea pig heart and compared the properties of reconstituted Ca<sup>2+</sup> channels in a mammalian cell line BHK with those of native channels. The voltage-dependent, DHP-sensitive Ba<sup>2+</sup> currents were recorded in cells transfected with  $\alpha_{1c}$  and co-transfection with the  $\beta$  subunit increases the amplitude of the current and accelerates the current decay. However, the effect of phosphorylation by cAMP-dependent protein kinase in the transfected cells seemed to differ from that seen in native myocytes. The cDNA clones encoding the guinea-pig cardiac Ca<sup>2+</sup> channel will provide a useful tool in studies of the molecular mechanisms of the kinetics and regulation of the channel.

### MATERIALS AND METHODS

Materials-Taq DNA polymerase was purchased from Takara; Quickprep micro mRNA purification kit was from Pharmacia Biotech; random hexamer primers, reverse transcriptase, and Dulbecco's modified Eagle medium were from Gibco BRL; pGEM-T vector system I was from Promega; Sequencing PRO from Toyobo; expression vector plasmids pZeoSV2(+) and pcDNA3.1(+) and Zeocin from Invitrogen; Hybond-N membranes and Gene Images random prime labeling module from Amersham; fetal bovine serum and forskolin were from Sigma; G418 from Nakarai; nifedipine and Bay K 8644 from Bayer; [<sup>32</sup>P]dCTP was from New England Nuclear. Okadaic acid was a generous gift from Dr. A. Takai, Department of Physiology, Nagoya University, Nagoya. The rabbit  $\alpha_{1c}$ cDNA clone (pSP72) was kindly provided by Dr. Y. Mori, National Institute for Physiological Sciences, Okazaki. Green fluorescent protein (GFP) expression vector (GFP S65A/CAG) was a generous gift from Dr. K. Moriyoshi, Department of Biological Science, Kyoto University.

RNA Isolation—Total RNA was prepared from a variety of freshly excised and/or frozen guinea pig tissues using a modified procedure of the guanidium thiocyanate method. Poly(A)<sup>+</sup> RNA was purified from total RNA using oligo-(dT)-cellulose and the Quick prep micro mRNA purification kit.

Cloning of  $\alpha_{1c}$  and  $\beta$  Subunit cDNAs—DNA synthesis and RT-PCR were carried out according to the recommended protocols of the manufacturers (Pharmacia and Takara). The PCR primers for the  $\alpha_{1c}$  and  $\beta$  subunits were designed based on the highly conserved nucleotide sequence in rabbit, rat, and mouse (2, 15-18). Four PCR fragments covering the entire coding region of the guinea pig  $\alpha_{1c}$ subunit, pGA1 (-21-978), pGA2 (806-3256), pGA3(3110-4498), pGA4 (4318-6571), and four PCR fragments for  $\beta_{2a}$ , pGB1 (-21-181), pGB2 (57-1071), pGB3 (1009-1808), and pGB4 (1726-1936), were subcloned into pGEM-T vector. Then, full size cDNAs for each subunit were constructed using appropriate restriction enzymes, and subcloned into mammalian expression vectors, pcDNA3.1-(+) and pZeoSV2(+), respectively. At least three of the fragments were sequenced using the dideoxy nucleotide chain method with [<sup>32</sup>P]dCTP and ⊿Tth polymerase, and PCR errors were eliminated.

Northern Blot Analysis and RT-PCR Analysis-Northern blot analysis was carried out using Gene Images according to the manufacturer's protocol. Ten micrograms of poly(A)<sup>+</sup> RNA from guinea pig tissues was denatured and separated on a 1.2% agarose gel containing 7.4% formaldehyde, and then transferred to Hybond-N membranes. The RNA was fixed by baking the blots for 2-3 h at 80°C. The blots were prehybridized with blocking reagent (Liquid block) for 30 min at 65°C in  $5 \times SSC$  buffer containing 0.1% (w/v) SDS and 5% (w/v) dextran sulphate, and hybridized with 10 ng of the appropriate probe (for  $\alpha_{1C}$ , nucleotides 3110-4490; for  $\beta_{2a}$ , nucleotides 1009-1808), prelabeled with fluorescein, for 16 h at 65°C. The hybridized probes were then visualized by an enzymatic reaction with alkaline phosphatase-labeled anti-fluorescein antibody.

RT-PCR was carried out using primers for  $\alpha_{1c}$  (sense: 5'-CCT TGT TCA GCC CAC CAC ACC TGC A, antisense: 5'-TGA GAA AGA GAT ATT CCA CTC GTT C, based on the sequence of the N-terminus) and  $\beta_{2a}$  (sense: 5'-TCC AAG GAT CGC TAC TGT GA, antisense: 5'-TGA GCA ACA GCA ATA CAA AA, based on the C-terminus). The PCR reaction was performed by 20 cycles of amplification.

Expression of Ca<sup>2+</sup> Channels in BHK Cells-BHK (subclone BHKtk<sup>-</sup>ts13) and BHK6 were obtained from Dr. T. Yoshinaga, Eisai Tsukuba Research Laboratories, Ibaraki, and Dr. Y. Mori, National Institute for Physiological Sciences, Okazaki, respectively. Cells were maintained in culture under 5% CO2 at 37°C in 35 mm culture dishes at a density of  $2-4 \times 10^5$  cells/dish in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Plasmids of  $\alpha_{1C}$  and  $\beta_{2a}$  cDNAs subcloned into the expression vectors (3  $\mu$ g of  $\alpha_{1C}$ , 9  $\mu$ g of  $\beta_{2a}$ , and  $1 \mu g$  of GFP cDNAs) were transfected into BHK or BHK6 cells using the calcium phosphate precipitation method (19, 20). Successfully transfected cells were identified by GFP fluorescence 3-7 days after transfection. The transfection efficiency of the  $\alpha_{1C}$  or  $\alpha_{1C}/\beta_2$  subunits, estimated by the fluorescence, was 60-70%.

Electrophysiology of BHK Cells—The whole-cell patch clamp technique (21) was used to record the Ba<sup>2+</sup> current mediated by Ca<sup>2+</sup> channels expressed in BHK cells. The external solution contained: 30 mM BaCl<sub>2</sub>, 82 mM NaCl, 1 mM MgCl<sub>2</sub>, 20 mM tetraethylammonium-Cl, 5 mM HE-PES, and 10 mM glucose, pH 7.4. The pipettes were filled with 110 mM CsCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 10 mM EGTA, and 3 mM Na<sub>2</sub>ATP (pH 7.4 with CsOH) and had a resistance of 3-8 m $\Omega$ . The Ba<sup>2+</sup> currents were evoked by depolarizing steps from a holding potential of -80 mV. Currents were sampled at 3.3 kHz after filtering at 1 kHz and stored on a computer for later off-line analysis. Ca<sup>2+</sup>channel currents were detected in 60-70% of cells showing GFP fluorescence. No difference in the expression rates of cells expressing  $\alpha_{1C}$  alone and  $\alpha_{1C} + \beta_{2a}$  was observed. Experiments were carried out at room temperature.

## RESULTS

cDNA Clones Encoding the  $\alpha_{1c}$  and  $\beta_{2a}$  Subunits from Guinea Pig Heart—cDNA clones encoding fragments of the Ca<sup>2+</sup> channel  $\alpha_1$  and  $\beta_2$  subunits were obtained by RT-PCR using poly(A)<sup>+</sup> RNA from guinea pig heart. The amino acid sequence of  $\alpha_{1c}$  and  $\beta_{2a}$  are shown in Fig. 1. The  $\alpha_{1c}$  clone contains an open reading frame of 2,169 amino acids, yielding a protein with a calculated molecular mass of 243

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Fig. 1 (continued on next page)

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kDa. The length of the  $\alpha_{1c}$  from guinea pig is shorter than that from rabbit by 2 amino acids. The predicted structure of the  $\alpha_{1c}$  subunit consists of four repeating domains I to IV, each domain comprised of five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment, S4 (Fig. 1A), suggesting the same transmembrane topology as described for other  $Ca^{2+}$  channel  $\alpha_1$  subunits (1, 2). There is great similarity between the guinea pig and rabbit, rat, mouse, or human subunits, with identities at the amino acid level of 95.3, 93.9, 94.3, and 96.6% (comparison between

# **A**2

1.0111068	$ni\sigma 1$	680 · LOAGLETT HDIGPETRATSCH, TAEFFI DKAMKEAVSAASEDDIFRRAGGLEGINVSYYOSDSRSTFPOTFTTORPLDINKAGNNOGDIESPSHERLVDSTFTPSSYSSTGSNANISNA	1799
Rabbit	1	601:	1800
Rat	1	680:N	1799
Mouse	1	651:	1770
Human	1	611:	1/30
		***	
Guinea	pig1	800: NYTALGRPPHPAGYPSTVSTVECHRPPSSPATWAQEATRKLGA-HRCHSR	1848
Rabbit	1	801:L.RGS LVRAWSSQ	1850
Rat	1	800SSG. LVRVAWSS-K	1850
mouse	1	771:	1820
Human	1	731:L.R	1820
Guinea	nial		1968
Rabbit	1		1969
Rat	ī	851	1968
mouse	1	821:G.T.N. IFPD-ER-S.RHSEED.F. QE. HCEIQRS	1938
Human	1	851:A.MAGTE .E.KM.H.T.AQQLI.Q RDRDR	1970
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Guinea	pigi	969 : QRSHSPTATPRCATPPATPGSRGwPPKPTPTLRLEGAESCEKLNSSFPSTHCSSWSEEPSPCRGGSSAARRARPVSLHVPSQAGAPGRQFHGSASSLAEAVLTSEGLGQFAQDPKPTEV	2088
RADDIC	1	970°	2089
mouse	1	939 TP P V R.LP S TTASSM T V	2058
Human	î	971: ASF F. O.V. V.S. GATTRG V. V.	2089
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Guinea	pig2	089: TTQELADACDMTIGEMENAADNILSQGAPQSPNGTLLPFVNCRDPGQDRAG-GDED-EG-CAFPLGRGWSEEELADSRVHVRSL 2169	
Rabbit	2	090:L.EDRRRRRQNEQ.AS.AC.QAR.AG.S 2171	
Rat	2	089:EQQVVPESVYARA.PSY.SN. 2169	
mouse	2	059:	
Human	2	090:ESAAG.EAG.VRARPQY.S 2169	
B			
Guinea	pig	1:MLDR-RLLSPSPOTKCIAPRGSADSYTSPSDSDVSLEEDREAVRREAERQAQAQLDRAKTKPVAFAVRTNVSYSAAHEDDVP	80
Rabbit		1:H.QCLVLEE	80
Rat		1:Q-C-CGVHR-RRVRVS-Y	70
mouse		-	13
		1:KATWIKATWI	86
Human		1:KATWILKAAKGGRL.SSDIC	86 120
Human		1:RL.SSDIC	86 120
Human		1: KATWI	86 120
Human Guinea Rabbit	pig	1:RL, SSDIC	86 120 200
Human Guinea Rabbit Rat	pig	1:KATWILARANGGRL.SSDIC	86 120 200 200
Human Guinea Rabbit Rat	pig	1: KATWI	86 120 200 200 199 206
Human Guinea Rabbit Rat mouse Human	pig	1:R.TWIT	86 120 200 200 199 206 208
Human Guinea Rabbit Rat Mouse Human	pig	1:KATWIKATKI	86 120 200 200 199 206 208
Human Guinea Rabbit Rat Mouse Human	pig	1: KATWI	86 120 200 200 199 206 208
Human Guinea Rabbit Rat mouse Human Guinea	pig	1:KATWIKATANGGRL.SSDIC	200 200 200 199 206 208
Human Guinea Rabbit Rat mouse Human Guinea Rabbit	pig pig	1:KATWIKATANGGRL.SSDIC	200 200 199 206 208 320 320
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat	pig pig	1:KATWILKRANGGRL.SSDIC	200 200 199 206 208 320 320 319
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse	pig pig	1:KATWI	200 200 199 206 208 320 320 319 326
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig	1:KATWIKATANGGRL.SSDIC	86 120 200 199 206 208 320 320 320 319 326 322
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat Human	pig pig	1:KATWIKARAKGGRL.SSDIC	200 200 199 206 208 320 320 319 326 322
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig	1:KATWIKARAKGGRL.SSDIC	200 200 199 206 208 320 320 319 326 322
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea	pig pig pig	1:KATWILARANGGRL.SSDIC	200 200 199 206 208 320 320 329 326 322 439
Human Guinea Rabbit Rat Human Guinea Rabbit Rat Guinea Rabbit Rat	pig pig pig	1:KATWILKRANGGRL.SSDIC	36           120           200           200           199           206           208           320           319           326           322           439           439
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat Human Ouinea Rabbit Rat mouse	pig pig pig	1:	36           120           200           200           199           206           208           320           319           326           322           439           439           439           446
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig	1:KATWI	36         120         200         200         199         206         208         320         319         326         322         439         439         434
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig	1:	320           320           320           320           320           320           320           439           439           446           441
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Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Ouinea	pig pig pig	1:	200 200 199 206 208 320 319 326 322 319 326 322 439 449 444 441 559
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rat mouse Human	pig pig pig	1:	200 200 199 206 208 320 320 320 320 320 322 439 439 439 439 444
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig	1:	200 200 200 206 208 320 320 320 320 322 439 439 445 441 559 555 555
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Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig	1: KUTWI	86 120 200 200 206 208 320 319 326 322 439 439 439 439 439 439 439 559 557 557 555
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Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig	1:KATWI	3200           2000           2000           1399           2066           208           3200           3199           326           3203           3204           3205           3220           3231           324           325           326           327           328           329           439           439           439           559           555           555           553
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig pig	1:KATWI	320           2000           2001           2002           2003           3200           3200           3201           3202           3203           3204           3205           3206           3207           3208           3209           3201           3202           4339           4439           4439           559           5557           564           5533
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat	pig pig pig pig	1:	320           320
Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human Guinea Rabbit Rat mouse Human	pig pig pig pig	1::	320           320           320           320           320           320           320           320           320           321           322           439           441           559           557           564           553

Fig. 1. Alignment of the deduced amino acid sequences of guinea-pig cardiac  $\alpha_{1c}$  (A) and  $\beta_{2a}$  (B) subunits. Amino acid sequences of rabbit, rat, mouse, and human  $\alpha_{1c}$  are aligned for comparison. Amino acid residue numbering is shown at the right end

of the individual lines. The putative transmembrane segments S1-S6

and P-loop (SS segments) in each of the repeats I to IV are shown by

brackets. The potential sites for N-glycosylation ( $\bullet$ ), cAMP-dependent phosphorylation ( $\bigcirc$ ), protein kinase C phosphorylation (\*), putative  $\alpha_1$  interaction domain (AID), and  $\beta$  interaction domain (BID) are shown with bold brackets. The EF-hand-motif-like regions are indicated by the broken bracket.

guinea pig 58-1852 and human 1-1788), respectively. Regions showing relatively low homology among species were found in the N-terminus, a portion of IVS3 and the following S3-S4 loop, and the C-terminus.

The P loop region (SS segment) of four repeats, which is supposed to form the pore, is well conserved. The four glutamic acids (392, 735, 1144, and 1445) in each P loop, which have been shown to be important for ion selectivity and hence conserved in all known voltage-dependent Ca<sup>2+</sup> channels, are conserved here as well. The  $\alpha_{1C}$  subunit has 6 potential phosphorylation sites for PKA on the cytoplasmic side, one site on the N-terminal side (Ser122), and 5 sites in the C-terminal region (Ser1574, 1626, 1699, Thr1908, and Ser1927). It also has potential phosphorylation sites for PKC at 13 sites (Fig. 1A) and for CK2 at 18 sites (not shown). A consensus sequence for the interaction with the  $\beta$  subunit, the  $\alpha_1$  interaction domain (AID) (22), is conserved in loop I-II. It has been suggested that a consensus sequence similar to the Ca binding motif (EF hand) is located in the C-terminal tail in  $\alpha_{1c}$  (23). There are four potential N-glycosylation sites in the putative regions facing the extracellular space.

The  $\beta_2$  clone, identified as  $\beta_{2a}$  by its homology to the rabbit sequense, contains an open reading frame of 597 amino acids with a calculated molecular mass of 67.1 kDa (Fig. 1B). Comparing the guinea pig  $\beta_{2a}$  subunit with that of the rabbit, rat, mouse, and human subunits, the clone showed 94.8, 93.1, 91.0, and 58.5% identity at the amino acid level, respectively. It was noticed that the stop codon in  $\beta_{2a}$  is 9 amino acids earlier than in the rabbit, rat, and mouse subunits. The  $\beta_{2a}$  subunit contains two potential phosphorylation sites for PKA (Ser8 and Thr165), 7 potential sites for PKC, and 10 potential sites for CK2. A consensus sequence for interaction with the  $\alpha_1$  subunit, the  $\beta$  interaction domain (BID) (24), is identified in the figure.

Expression of  $\alpha_{1c}$  and  $\beta_{2a}$  in Various Tissues—The expression of the mRNA for  $\alpha_{1c}$  and  $\beta_{2a}$  was examined by Northern hybridization (Fig. 2). The  $\alpha_{1c}$  mRNA of 8 kb and the  $\beta_{2a}$  mRNA of 4.0 and 3.4 kb were detected in the heart. The 4.0 kb band for the  $\beta_{2a}$  subunit was also detected in the brain at low levels (not shown). Tissue distribution was examined by RT-PCR. First strand cDNA was isolated from guinea pig heart, brain, cerebellum, liver, skeletal muscle, kidney, stomach, aorta, and small intestine. After electrophoresis, specific products of 580 and 210 bp were detected for  $\alpha_{1c}$  and  $\beta_{2a}$ , respectively (Fig. 2B). The  $\beta_{2a}$ subunit is expressed highly in heart and weakly in cerebellum and whole brain, but is not detected in skeletal muscle, lung, aorta, or the small intestine.

Properties of  $Ca^{2+}$  Channel Currents Expressed in BHK Cells—The properties of ion currents mediated by the guinea pig cardiac  $\alpha_{1c}$  and  $\beta_{2a}$  subunits were studied in the transient expression system. The cDNAs encoding each subunit were cloned in mammalian expression vectors and heterologously expressed in BHK cells. The cells were perfused with an extracellular solution containing 30 mM Ba<sup>2+</sup> and examined for an inward Ba<sup>2+</sup> current. In cells transfected with  $\alpha_{1c}$  alone, depolarizing steps from a holding potential of -80 to 50 mV during test pulses of 200 ms duration evoked small inward currents (~50 pA). Figure 3A illustrates a set of currents in the cell that showed the largest current amplitude. The currents inactivated either very slowly or not at all during the test pulse. The current-voltage (I-V) curve for the Ba<sup>2+</sup> current suggested a threshold potential at around -30 mV and a potential for the maximum current at approximately 10 mV (Fig. 3B). The average of the maximum current and current density (normalized by cell capacitance) in cells expressing  $\alpha_{1c}$  alone were  $-35.8\pm5.4$  pA and  $-0.41\pm$ 0.07 pA/pF (n=4), respectively. The current level at end of the pulse at 10 mV (late current) was 92% of the peak current, indicating little voltage-dependent inactivation of the current. In BHK cells transfected with  $\alpha_{1c} + \beta_{2a}$ , a Ba<sup>2+</sup> current was also evoked by test pulses at -20 mV or greater. The Ba2+ current, however, was inactivated as seen in inactive cardiac myocytes (Fig. 3C). The late current level was 28% of the peak current. The I-V curve suggested a threshold potential of about -30 mV and a maximum peak potential of about 10 mV (Fig. 3D). The average of the maximum current and the current density were  $-63.9\pm$ 2.0 pA and  $-0.76\pm0.11$  pA/pF (n=4), indicating that the current density was significantly larger than that seen in



Fig. 2. Northern blot and PCR analysis of guinea-pig cardiac  $\alpha_{1c}$  and  $\beta_{2a}$  subunits. mRNA from guinea pig heart was analyzed by Northern blot as described in "MATERIALS AND METHODS." A: An 8 kb band (left) was hybridized with a 1,384 bp cDNA probe coding between IIIS4 and IVS6 of the  $\alpha_{1c}$  subunit. The 800 bp cDNA probe of the  $\beta_{2a}$  subunit, coding between 1009-1808 in the C-terminal region, detected two bands of 4.0 and 3.5 kb (right). The size of the RNA markers is indicated on the left. B: The PCR products of  $\alpha_{1c}$  and  $\beta_{2a}$  cDNA from skeletal liver (lane 2), cerebellum (lane 3), brain (lane 4), muscle (lane 5), lung (lane 6), kidney (lane 7), stomach (lane 8), aorta (lane 9), small intestine (lane 10), and heart (lane 11). A PCR product of ~580 bp was detected for  $\alpha_{1c}$ , (upper photograph) and a ~210 bp product was detected for  $\beta_{2a}$ .

cells expressing  $\alpha_{1c}$  alone (p < 0.01).

The amplitude of the Ba<sup>2+</sup> current mediated by  $\alpha_{1C} + \beta$ has been reported to increase further by co-expression of the  $\alpha_2/\delta$  subunit. To examine whether the  $\alpha_2/\delta$  subunit affects the current amplitude mediated by the guinea pig  $\alpha_{1C}$  clone or not, the  $\alpha_{1C}$  subunit from guinea pig was introduced into BHK6 cells, which stably express rabbit skeletal muscle  $\beta_{1n}$ , and  $\alpha_2/\delta$  (Y. Mori, personal communication) and the expressed currents obtained were compared. As illustrated in Fig. 4, BHK6 cells expressing guinea pig  $\alpha_{1C}$  showed a relatively large Ba<sup>2+</sup> current. On average, the maximum current was  $-2,370\pm770$  pA (n=4) and the current density was  $-5.5 \pm 1.8 \text{ pA/pF}$  (n=4). For comparison, the rabbit cardiac  $\alpha_{1c}$  subunit was expressed in BHK6 cells (Fig. 4C), resulting in the maximum current of  $-717\pm95$  pA (n=4) and the current density of  $-8.2\pm2.7$  pA/pF (n=4). Thus, the current density for guinea pig  $\alpha_{1C}$  and rabbit  $\beta_{1a} + \alpha_2/\delta$  was significantly larger than that for  $\alpha_{1C}$  alone or  $\alpha_{1C} + \beta_2$ . The late current level was 43% for guinea pig, while it was 70% for the rabbit  $\alpha_{1c}$ . This suggested that the current decay is faster in guinea pig than in rabbit. The I-V curve for the Ba<sup>2+</sup> current mediated by guinea pig and rabbit  $\alpha_{1C}$  suggest that the voltage-dependence of activation is not significantly different between the two clones. The results showed that expression of the guinea pig  $\alpha_{1C}$  clone was comparable to that of the rabbit.

Effects of Dihydropyridines and Phosphorylation on  $Ba^{2+}$ Current—In order to determine the pharmacological properties of the  $Ba^{2+}$  current, we examined the effects on  $Ba^{2+}$ current of dihydropyridine derivatives known to activate and to block L-type  $Ca^{2+}$  channels. We first applied 10  $\mu$ M Bay K 8644 to cells expressing both  $\alpha_{1c}$  and  $\beta_{2a}$  subunits. The  $Ba^{2+}$  current was increased about 3-fold (Fig. 5A). The current in cells expressing  $\alpha_{1c}$  alone was also increased to a similar extent (not shown). Nifedipine (3  $\mu$ M) completely blocked the  $Ba^{2+}$  current in BHK cells expressing  $\alpha_{1c}$  and  $\beta_{2a}$ .

We then examined the effect of phosphorylation on the Ba<sup>2+</sup> current in BHK cells. It is well established that forskolin, an activator of adenylyl cyclase, and okadaic acid, an inhibitor of protein phosphatases 1 and 2A, enhance the  $Ca^{2+}$  channel current in native cardiac myocytes (25). In the experiments shown in Fig. 6, Ba<sup>2+</sup> the currents in cells expressing  $\alpha_{1c}$  and  $\beta_{2a}$  were recorded in the presence or absence of 5  $\mu$ M okadaic acid in the pipette solution, and then, 5  $\mu$ M forskolin was extracellulary applied. The Ba<sup>2+</sup> current was not affected, regardless of the presence of okadaic acid. Application of forskolin with/without okadaic acid had little effect on the Ba<sup>2+</sup> current in 7 other cell preparations expressing  $\alpha_{1C}$  and  $\beta_{2a}$  and 4 cell preparations expressing  $\alpha_{1C}$  alone. Forskolin was also ineffective on the Ba<sup>2+</sup> current in BHK cells expressing rabbit  $\alpha_{1c}$  (data not shown). Thus, unlike in native cardiac myocytes, modula-



Fig. 3. Whole-cell Ba<sup>2+</sup> currents in BHK cells expressing the guinea pig Ca<sup>2+</sup> channel. A and C: Ba<sup>2+</sup> current mediated by  $\alpha_{1c}$  alone (A, an example showing the largest current) or  $\alpha_{1c} + \beta_{2a}$  (C). The extracellular solution contained 30 mM Ba<sup>2+</sup>. Currents were elicited by step pulses ranging from -70 to 50 mV for 200 ms from a holding

potential of -80 mV. B and D: The I-V curve for Ba<sup>2+</sup> currents in the same cells as in A and C. Current densities (normalized by cell capacitance) are indicated in the graphs. Symbols are for the maximum current ( $\bigcirc$ ) and the late current ( $\bigcirc$ ).

#### В Α Guinea pig $\alpha_1$ c+Rabbit $\beta_1$ a, $\alpha_2\delta$ Guinea pig $\alpha_1$ c+Rabbit $\beta_1$ a, $\alpha_2\delta$ 1000 T 2.0 500 1.0 20 a -500 -1000 500pA -1500 30m -2000 -5.0(pA/pF) Im(pA) -2500 J С Rabbit $\alpha_1 c + \beta_1 a, \alpha_2 \delta$ D Rabbit $\alpha_1 c + \beta_1 a, \alpha_2 \delta$ 1.0 20 40 20 20 125pA -300 30ma -400 -500

Fig. 4. Whole-cell Ba<sup>2+</sup> current in BHK6 cells expressing the guinea pig  $\alpha_{1C}$  or rabbit  $\alpha_{1C}$  subunit. BHK6 cells express rabbit  $\beta_{1n}$ stably. A and B: The Ba<sup>2+</sup> current mediated by guinea pig  $\alpha_{1C}$  (A) and its I - V relationship (B) for the maximum current (O) and the late current ( $\bullet$ ). C and D: The Ba<sup>2+</sup> current mediated by rabbit  $\alpha_{1C}$  (C) and



Fig. 5. Effects of dihydropyridines on the Ba2+ current in BHK cells expressing guinea pig  $a_{1c}$  and  $\beta_{2a}$  subunits. A: Increasing effect of 10  $\mu$ M Bay K 8644, a Ca<sup>2+</sup>-channel activator, on the Ba<sup>2+</sup> current elicited by consecutive step pulses from -80 to 10 mV. The current at control (Cont) was increased 3-fold. Three current traces recorded in the presence of Bay K 8644 at different times (BayK) and the trace recorded at -80 mV (for zero level) are superimposed. B: The blocking effect of 3  $\mu$ M nifedipine, a Ca<sup>2+</sup> channel blocker, on the Ba<sup>2+</sup> current was elicited as in A. The current at control (Cont) and in the presence of nifedipine (Nif, two traces superimposed).

its I - V relationship (D) with symbols as in B. Currents were elicited by step pulses ranging from -70 to 50 mV for 200 ms from the holding potential of -80 mV. The extracellular solution contained 30 mM Ba2+.

-11(pA/pF)

-600

Im(pA)

Vm(mV)

tion of the Ca<sup>2+</sup>-channel current by PKA-mediated phosphorylation was not reproduced in BHK cells.

#### DISCUSSION

In this study, we describe the cloning and primary characterization of the  $\alpha_{1C}$  and  $\beta_2$  subunits of Ca<sup>2+</sup> channels from guinea pig heart. Our results demonstrate that  $\alpha_{1c}$ , alone or together with  $\beta_2$ , mediates a DHP-sensitive high voltageactivated, long-lasting inward current, properties typical of L-type Ca<sup>2+</sup> channels. BHK cells lack an endogenous Ca<sup>2+</sup>channel current, so they have been successfully employed as a heterologous expression system for  $Ca^{2+}$  channels (26, 27). Although the amplitude of the  $Ba^{2+}$  current in BHK cells transfected with guinea pig  $\alpha_{1c}$  is smaller compared to those reported for rabbit  $\alpha_{1c}$ , the current density normalized by the cell capacitance ( $\sim 0.4 \text{ pA/pF}$ ) was comparable to those for the rabbit.

The primary structure of guinea pig  $\alpha_{1C}$  shows a high similarity to  $\alpha_{ic}$  subunits from other mammalian species. The amino acid sequence shows >90% homology to rabbit, rat, and mouse  $\alpha_{1C}$ : The transmembrane segments, which are important for gating, pore formation, and ion selectivity of the channel, are highly conserved, while the cytoplasmic domains near the N- and C-terminus are more variable (amino acid identity 68-77%), consistent with previous reports (28). It is also notable that the identity score for the region around IVS3 is relatively low (58%). It has been reported that IIIS3-S4 and IVS4-S5 are subject to sequence



Fig. 6. Time course of the Ba<sup>2+</sup> current in BHK cells expressing guinea pig  $\alpha_{1c}$  and  $\beta_{2a}$ subunits. Currents were recorded with or without  $5 \mu$ M okadaic acid, a protein phosphatase inhibitor, in the pipette, and then  $5 \mu$ M forskolin, an adenylyl cyclase activator, was applied at the time indicated by the arrow. The Ba<sup>2+</sup> current elicited by depolarizing pulses from -80 to 10 mV was not significantly changed. Insert figures show the current traces recorded as indicated by the respective letters (a, b, c).

variation by alternative splicing in the human gene (29). Thus, it is possible that the low similarity of IVS3 between guinea pig and rabbit is due to splice variations.

The guinea pig  $\beta$  subunit we cloned contains an open reading frame of 597 amino acids with a calculated molecular mass of 67 kDa. Compared with known isoforms of the  $\beta$  subunit in other species, the clone shows 95% identity in amino acid sequence with the  $\beta_{2a}$  isoform (except for human), and is thus identified as the counterpart of guinea pig  $\beta_{28}$ . Co-expression of  $\beta_{28}$  increases the  $\alpha_{1C}$ mediated current about 2-fold. In the case of rabbit, rat, and human  $\alpha_{1c}$ , the current is increased 3- to 20-fold by co-expression of the  $\beta_1$  or  $\beta_{2a}$  subunit (2, 30-32). Thus, the extent of the effect of guinea pig  $\beta_{2a}$  seems to be smaller than the effect in other species. The Ba<sup>2+</sup> current for guinea pig  $\alpha_{1C}$  and rabbit  $\beta_{1a} + \alpha_2/\delta$  was comparable to that for rabbit  $\alpha_{1C} + \beta_{1a} + \alpha_2/\delta$ . Although the current density is smaller than that reported for rabbit  $\alpha_{1c} + \beta_1 + \alpha_2/\delta$  (26), our result suggest that the effects of  $\beta + \alpha_2/\delta$  on the current amplitude are consistent with those reported in other species.

Inactivation of the Ca<sup>2+</sup> channels occurs by two mechanisms, one voltage-dependent and the other Ca<sup>2+</sup>-mediated. When there is a Ba<sup>2+</sup> current flowing through the Ca<sup>2+</sup> channels, the rate of inactivation is greatly slowed, presumably due to a low affinity of Ba<sup>2+</sup> for the site of Ca<sup>2+</sup>-mediated inactivation (33). In this study, the  $Ba^{2+}$  current mediated by guinea pig  $\alpha_{1c}$  alone was barely or very slowly inactivated, while that mediated by  $\alpha_{1c}$  and  $\beta_{2}$  showed a clear acceleration in the rate of inactivation. This is in line with previous studies, in which  $\beta$  subunits affect the inactivation of the Ca<sup>2+</sup>-channel current (34). A mutation study has suggested that a region around IS6 is important for the rate of inactivation (35). This region is located just before the I-II loop, the position of the domain that interacts with the  $\beta$  subunit (AID). The C-terminal tail has also been reported to play a critical role in channel inactivation (36). Although the mechanism by which the C-terminal tail affects channel inactivation is entirely unknown, this part of  $\alpha_{1C}$  might interact with the  $\beta$  subunits, as reported for  $\alpha_{1A}$ (37).

The mechanism for  $Ca^{2+}$ -mediated inactivation is less specified. It has been reported that an EF hand, a consensus  $Ca^{2+}$ -binding motif, is located on the C-terminus of the  $\alpha_{1c}$ subunit and contributes to  $Ca^{2+}$ -mediated inactivation (23). Another study, however, suggested that  $Ca^{2+}$ -mediated inactivation depends on a short sequence in the C terminus that does not include an EF-hand-like domain (38). Since the amino acid sequence of the EF-hand-like domain is identical among known mammalian species including guinea pig, this region may play an important role in channel function.

It is well known that the L-type Ca<sup>2+</sup> channel is regulated by the phosphorylation by PKA in cardiac myocytes. In expression systems using cloned channel subunits, however, the effect of PKA activation is highly variable (39). In this study, forskolin and okadaic acid failed to have a reproducible enhancing effect on the Ba<sup>2+</sup> current in cells expressing guinea pig  $\alpha_{1c}$  alone or  $\alpha_{1c} + \beta_{2a}$ . The drugs also had no effect on the Ba<sup>2+</sup> current of rabbit  $\alpha_{1C}$ . These results are in contrast to previous findings that the current in BHK cells transfected with rabbit  $\alpha_{1C}$  is modulated by activation of endogenous PKA (26, 27). There are several possible explanations for this. First, PKA activity might be so high in BHK cells that the channels are already up-regulated (40). Second, the PKA activity might be very low, even when forskolin does not activate PKA sufficiently to modulate the channels. Third, the region in the channel important for regulation was cleaved by proteases (41). Finally, some component required for phosphorylation (such as the cytosketelal component or anchoring protein) may have been absent from BHK cells. Whatever the explanation, the present results suggest that the modulation of Ca<sup>2+</sup> channels by phosphorylation is variable in the heterologous expression system, possibly dependent on the metabolic state of the cells.

The  $\alpha_1$  subunit is shown to be phosphorylated in vitro and in intact cells (42-44). Although the underlying molecular mechanisms have not been fully clarified, the C-terminal region of  $\alpha_{1C}$  is suggested to play a crucial role. There are 5 potential phosphorylation sites for PKA on the C-terminal tail of the guinea pig  $\alpha_{1c}$  as exist on that of the rabbit. Recently, Gao et al. (45) reported that in HEK293 cells transfected with rabbit cardiac  $\alpha_{1c}$ , the PKA phosphorylation site is located in the C-terminus and a PKA anchoring protein, AKAP79, regulates the phosphorylation. An  $\alpha_1$ subunit mutant (S1928A), in which Ser1928 is replaced by Ala, demonstrated a loss in PKA-mediated modulation of the expressed channels, indicating the importance of Ser1928 in that kind of regulation (43, 46, 47) (but see Leach et al. (48)]. Since the corresponding phosphorylation site is conserved in guinea pig  $\alpha_{1c}$  (Ser1927), it is relevant to examine whether this site is involved in PKA-mediated channel modulation.

The  $\beta$  subunit is also thought to be phosphorylated by PKA. The phosphorylation site of the  $\beta$  subunit is conserved in guinea pig  $\beta_{2a}$ . While the electrophysiological effects of PKA activation are well explained by postulating dual sites of phosphorylation (49), the possibility that two or more sites on  $\alpha_{1c}$  (and perhaps  $\beta$ ) are involved in channel regulation remains to be examined.

The electrophysiological and pharmacological properties of Ca<sup>2+</sup> channels in guinea pig myocytes have been studied for a long time, and much knowledge has accumulated. In this study, we reconstituted guinea-pig cardiac Ca<sup>2+</sup>-channel currents in a heterologous expression system, with properties comparable to those seen in the native myocytes (except for modulation by phosphorylation). Thus, the guinea pig  $\alpha_{1c}$  and  $\beta_{2a}$  subunits will provide a useful tool for further studies on the cardiac Ca<sup>2+</sup> channel.

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