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Cloning and Expression of the Ca^{2+} Channel α_{1C} and β_{2a} Subunits from Guinea Pig Heart[1]

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Complimentary DNA clones encoding the α_{1C} and β_{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 α_{1C} and 597 amino acids for the β_{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 α_{1C} and β_{2a} subunits was 8.0 and 3.5/4.0 kb, respectively. RT-PCR analysis revealed that the α_{1C} subunit is expressed exclusively in the heart, while the β_{2a} subunit is expressed in the heart, cerebellum, whole brain, and stomach. The α_{1C} and β_{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^{2+}. In cells expressing α_{1C} alone, the Ba^{2+} current was activated at -30 mV and more positive potentials and peaked at about 10 mV. The co-expression of β_{2a} with α_{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 α_{1C} and rabbit $\beta_1+\alpha_2/\delta$, a Ba^{2+} 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^{2+} current, suggesting that cAMP-mediated modulation is not easily reproduced in transfected cells, unlike that seen in native cardiac myocytes.

Key words: α_{1C} subunit, β_2 subunit, Ca^{2+} channel, cloning, guinea pig heart.

The cDNA encoding the voltage-dependent Ca^{2+} channel α_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^{2+} channels into T, L, N, P/Q, R types (*3*). Most of these types comprise three or four subunits termed α_1, β, α_2/δ, and γ. The α_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^{2+} channel α_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^{2+} channel α_1 genes with site-directed mutagenesis have demonstrated that the S4 segments are the voltage sensor of channel gating, P loops (linker at S5-S6 of each repeat) form a pore for ion permeation, and a domain on the I-II loop interacts with β subunits (*5*). The β subunit comprises four isoforms, β_{1-4}, which are expressed in a tissue-specific manner (*6*). The β subunits, when co-expressed with the α_1 subunits, increase the α_1 mediated current and/or alter the kinetics of activation and inactivation (*7-9*). Other auxiliary subunits, α_2/δ, and γ, have been reported to increase the Ca^{2+}-channel current when co-expressed with α_1.

The majority of Ca^{2+} current in the heart is mediated by the dihydropyridine (DHP) sensitive L-type channel, the α_1 subunit of which is encoded by the α_{1C}. DHP derived Ca^{2+}-channel blockers bind to a site close to the pore formed by the α_{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 α_{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, α_1-subunit interaction domain; BID, β-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 α_{1C} and β subunits from guinea pig heart and compared the properties of reconstituted Ca^{2+} channels in a mammalian cell line BHK with those of native channels. The voltage-dependent, DHP-sensitive Ba^{2+} currents were recorded in cells transfected with α_{1C} and co-transfection with the β 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^{2+} 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; [^{32}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 α_{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)$^+$ RNA was purified from total RNA using oligo-(dT)-cellulose and the Quick prep micro mRNA purification kit.

Cloning of α_{1C} and β 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 α_{1C} and β 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 α_{1C} subunit, *pGA1* (−21-978), *pGA2* (806-3256), *pGA3* (3110-4498), *pGA4* (4318-6571), and four PCR fragments for β_{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 [^{32}P]dCTP and *Δ*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)$^+$ 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×SSC buffer containing 0.1% (w/v) SDS and 5% (w/v) dextran sulphate, and hybridized with 10 ng of the appropriate probe (for α_{1C}, nucleotides 3110-4490; for β_{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 α_{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 β_{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^{2+} Channels in BHK Cells—BHK (subclone BHKtk$^-$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% CO_2 at 37°C in 35 mm culture dishes at a density of $2\text{-}4\times10^5$ 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 α_{1C} and β_{2a} cDNAs subcloned into the expression vectors (3 μg of α_{1C}, 9 μg of β_{2a}, and 1 μg 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 α_{1C} or α_{1C}/β_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^{2+} current mediated by Ca^{2+} channels expressed in BHK cells. The external solution contained: 30 mM $BaCl_2$, 82 mM NaCl, 1 mM $MgCl_2$, 20 mM tetraethylammonium-Cl, 5 mM HEPES, and 10 mM glucose, pH 7.4. The pipettes were filled with 110 mM CsCl, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM EGTA, and 3 mM Na_2ATP (pH 7.4 with CsOH) and had a resistance of 3-8 MΩ. The Ba^{2+} 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^{2+}-channel currents were detected in 60-70% of cells showing GFP fluorescence. No difference in the expression rates of cells expressing α_{1C} alone and $\alpha_{1C}+\beta_{2a}$ was observed. Experiments were carried out at room temperature.

RESULTS

cDNA Clones Encoding the α_{1C} and β_{2a} Subunits from Guinea Pig Heart—cDNA clones encoding fragments of the Ca^{2+} channel α_1 and β_2 subunits were obtained by RT-PCR using poly(A)$^+$ RNA from guinea pig heart. The amino acid sequence of α_{1C} and β_{2a} are shown in Fig. 1. The α_{1C} clone contains an open reading frame of 2,169 amino acids, yielding a protein with a calculated molecular mass of 243

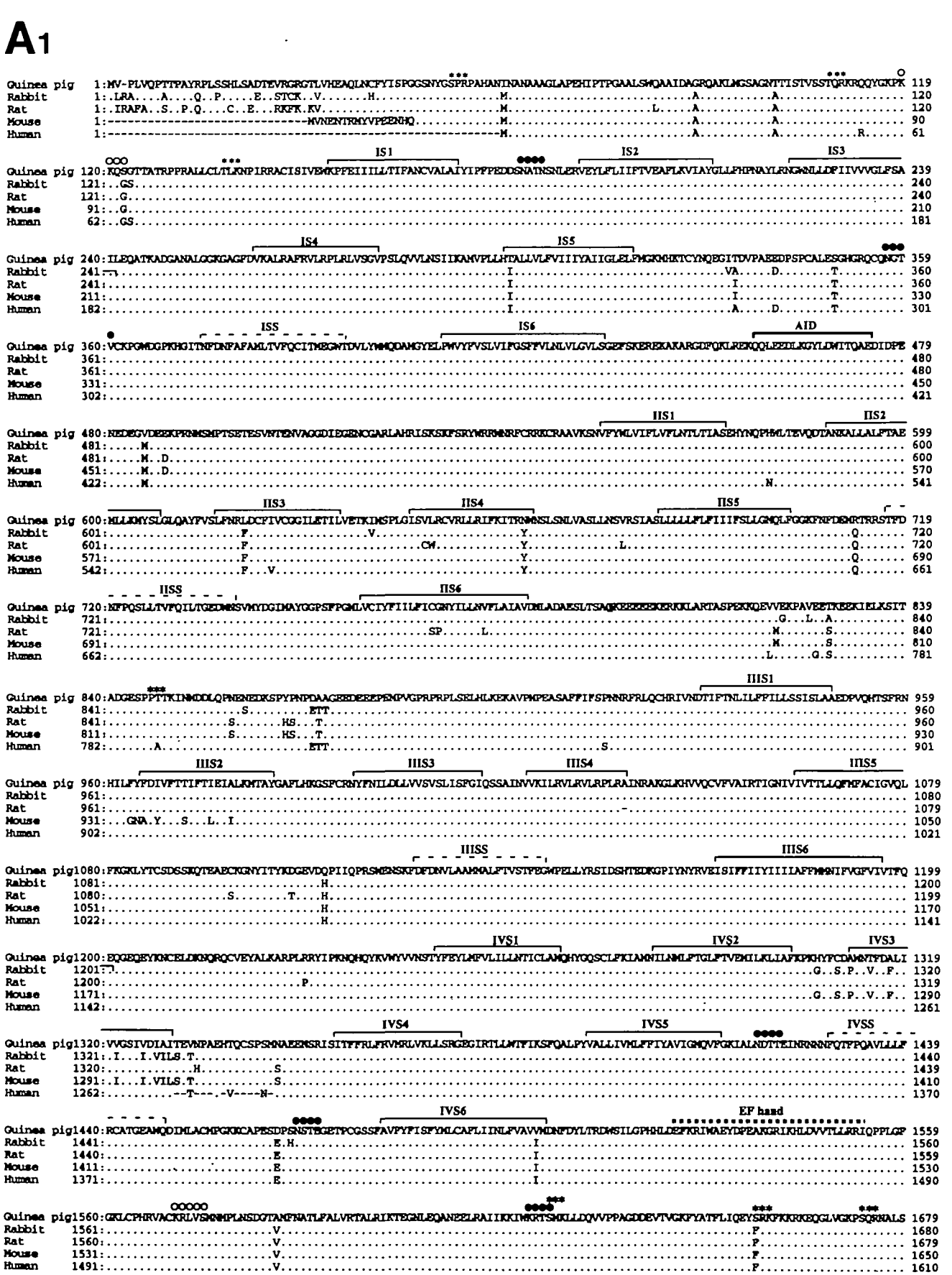

Fig. 1 (continued on next page)

kDa. The length of the α_{1C} from guinea pig is shorter than that from rabbit by 2 amino acids. The predicted structure of the α_{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 α_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

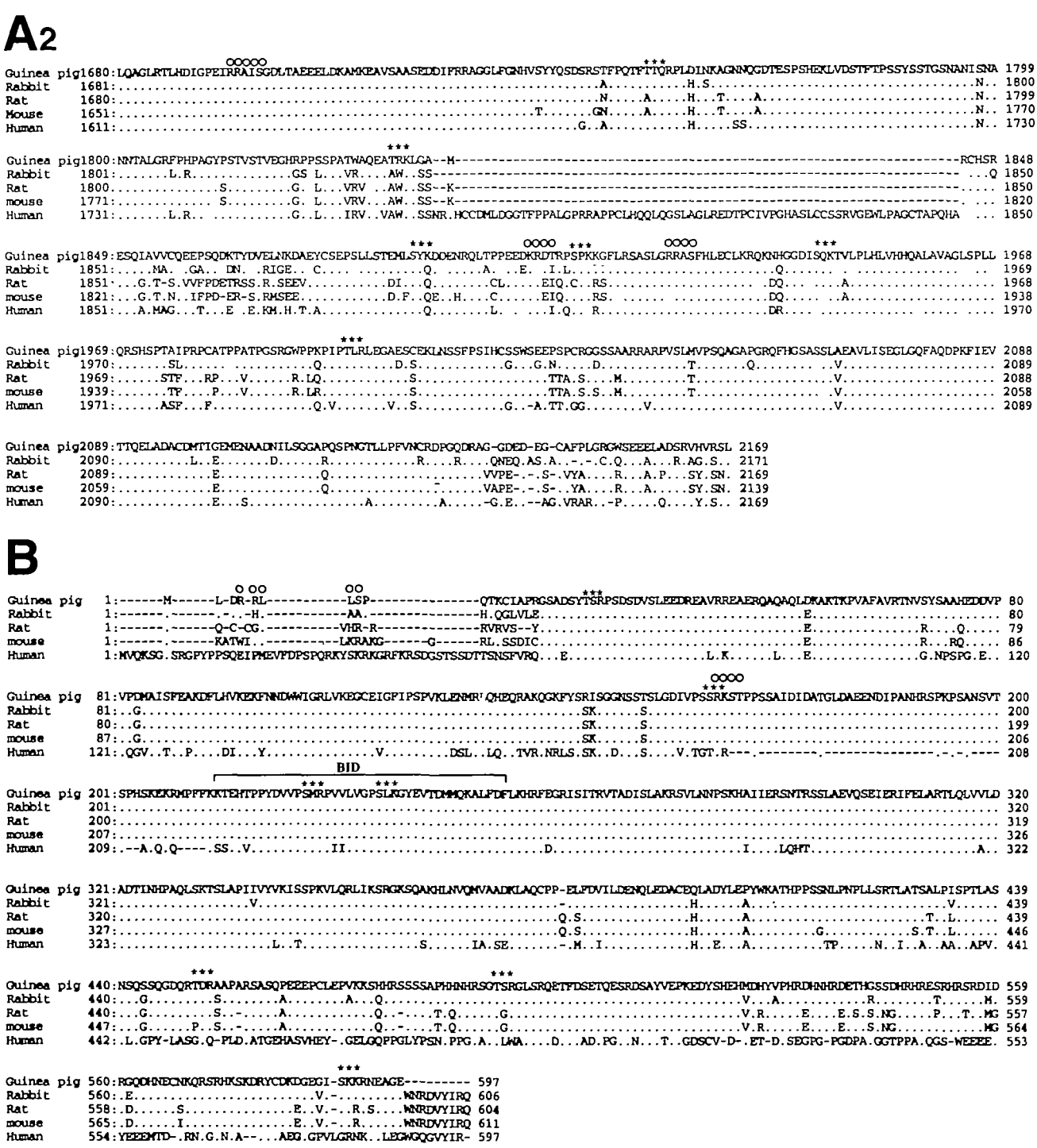

Fig. 1. **Alignment of the deduced amino acid sequences of guinea-pig cardiac α_{1C} (A) and β_{2a} (B) subunits.** Amino acid sequences of rabbit, rat, mouse, and human α_{1C} are aligned for comparison. Amino acid residue numbering is shown at the right end of the individual lines. The putative transmembrane 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 (●), cAMP-dependent phosphorylation (○), protein kinase C phosphorylation (*), putative α_1 interaction domain (AID), and β 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^{2+} channels, are conserved here as well. The α_{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 β subunit, the α_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 α_{1C} (*23*). There are four potential *N*-glycosylation sites in the putative regions facing the extracellular space.

The β_2 clone, identified as β_{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 β_{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 β_{2a} is 9 amino acids earlier than in the rabbit, rat, and mouse subunits. The β_{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 α_1 subunit, the β interaction domain (BID) (*24*), is identified in the figure.

Expression of α_{1C} and β_{2a} in Various Tissues—The expression of the mRNA for α_{1C} and β_{2a} was examined by Northern hybridization (Fig. 2). The α_{1C} mRNA of 8 kb and the β_{2a} mRNA of 4.0 and 3.4 kb were detected in the heart. The 4.0 kb band for the β_{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 α_{1C} and β_{2a}, respectively (Fig. 2B). The β_{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 α_{1C} and β_{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^{2+} and examined for an inward Ba^{2+} current. In cells transfected with α_{1C} alone, depolarizing steps from a holding potential of -80 to 50 mV during test pulses of 200 ms duration evoked small inward currents ($\sim$50 pA). Figure 3A illustrates a set of currents in the cell that showed the largest current amplitude. The currents inactivated either very slowly or not at all during the test pulse. The current-voltage (I-V) curve for the Ba^{2+} current suggested a threshold potential at around -30 mV and a potential for the maximum current at approximately 10 mV (Fig. 3B). The average of the maximum current and current density (normalized by cell capacitance) in cells expressing α_{1C} alone were -35.8 ± 5.4 pA and -0.41 ± 0.07 pA/pF ($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^{2+} current was also evoked by test pulses at -20 mV or greater. The Ba^{2+} 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 ± 2.0 pA and -0.76 ± 0.11 pA/pF ($n=4$), indicating that the current density was significantly larger than that seen in

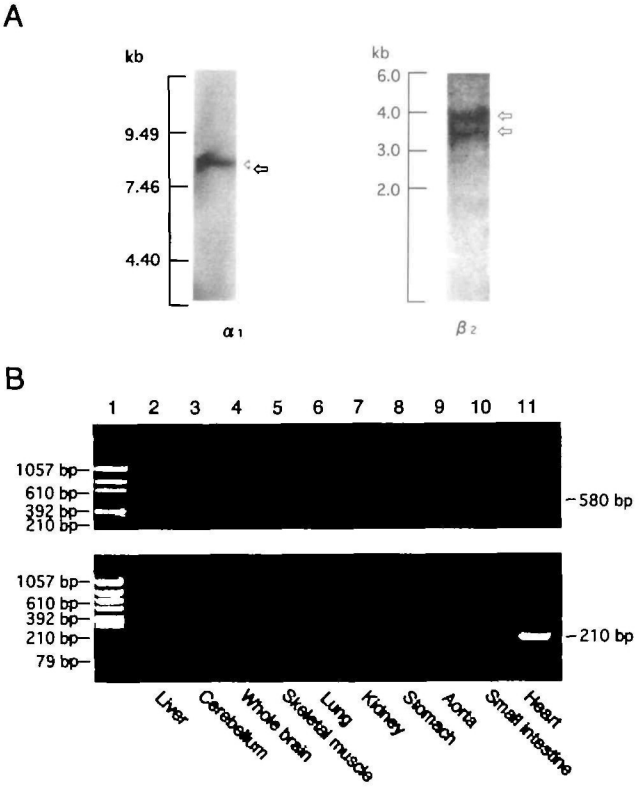

Fig. 2. **Northern blot and PCR analysis of guinea-pig cardiac α_{1C} and β_{2a} subunits.** mRNA from guinea pig heart was analyzed by Northern blot as described in "MATERIALS AND METHODS." A: An 8 kb band (left) was hybridized with a 1,384 bp cDNA probe coding between IIIS4 and IVS6 of the α_{1C} subunit. The 800 bp cDNA probe of the β_{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 α_{1C} and β_{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 $\sim$580 bp was detected for α_{1C}, (upper photograph) and a $\sim$210 bp product was detected for β_{2a} (lower photograph). Primers were designed based on sequences in the N-terminal region of α_{1C} and in the C-terminal region of β_{2a}.

cells expressing α_{1C} alone ($p<0.01$).

The amplitude of the Ba^{2+} current mediated by $\alpha_{1C}+\beta$ has been reported to increase further by co-expression of the α_2/δ subunit. To examine whether the α_2/δ subunit affects the current amplitude mediated by the guinea pig α_{1C} clone or not, the α_{1C} subunit from guinea pig was introduced into BHK6 cells, which stably express rabbit skeletal muscle β_{1a}, and α_2/δ (Y. Mori, personal communication) and the expressed currents obtained were compared. As illustrated in Fig. 4, BHK6 cells expressing guinea pig α_{1C} showed a relatively large Ba^{2+} current. On average, the maximum current was $-2{,}370\pm770$ pA ($n=4$) and the current density was -5.5 ± 1.8 pA/pF ($n=4$). For comparison, the rabbit cardiac α_{1C} subunit was expressed in BHK6 cells (Fig. 4C), resulting in the maximum current of -717 ± 95 pA ($n=4$) and the current density of -8.2 ± 2.7 pA/pF ($n=4$). Thus, the current density for guinea pig α_{1C} and rabbit $\beta_{1a}+\alpha_2/\delta$ was significantly larger than that for α_{1C} alone or $\alpha_{1C}+\beta_2$. The late current level was 43% for guinea pig, while it was 70% for the rabbit α_{1C}. This suggested that the current decay is faster in guinea pig than in rabbit. The I-V curve for the Ba^{2+} current mediated by guinea pig and rabbit α_{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 α_{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 μM Bay K 8644 to cells expressing both α_{1C} and β_{2a} subunits. The Ba^{2+} current was increased about 3-fold (Fig. 5A). The current in cells expressing α_{1C} alone was also increased to a similar extent (not shown). Nifedipine (3 μM) completely blocked the Ba^{2+} current in BHK cells expressing α_{1C} and β_{2a}.

We then examined the effect of phosphorylation on the Ba^{2+} 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^{2+} the currents in cells expressing α_{1C} and β_{2a} were recorded in the presence or absence of 5 μM okadaic acid in the pipette solution, and then, 5 μM forskolin was extracellulary applied. The Ba^{2+} current was not affected, regardless of the presence of okadaic acid. Application of forskolin with/without okadaic acid had little effect on the Ba^{2+} current in 7 other cell preparations expressing α_{1C} and β_{2a} and 4 cell preparations expressing α_{1C} alone. Forskolin was also ineffective on the Ba^{2+} current in BHK cells expressing rabbit α_{1C} (data not shown). Thus, unlike in native cardiac myocytes, modula-

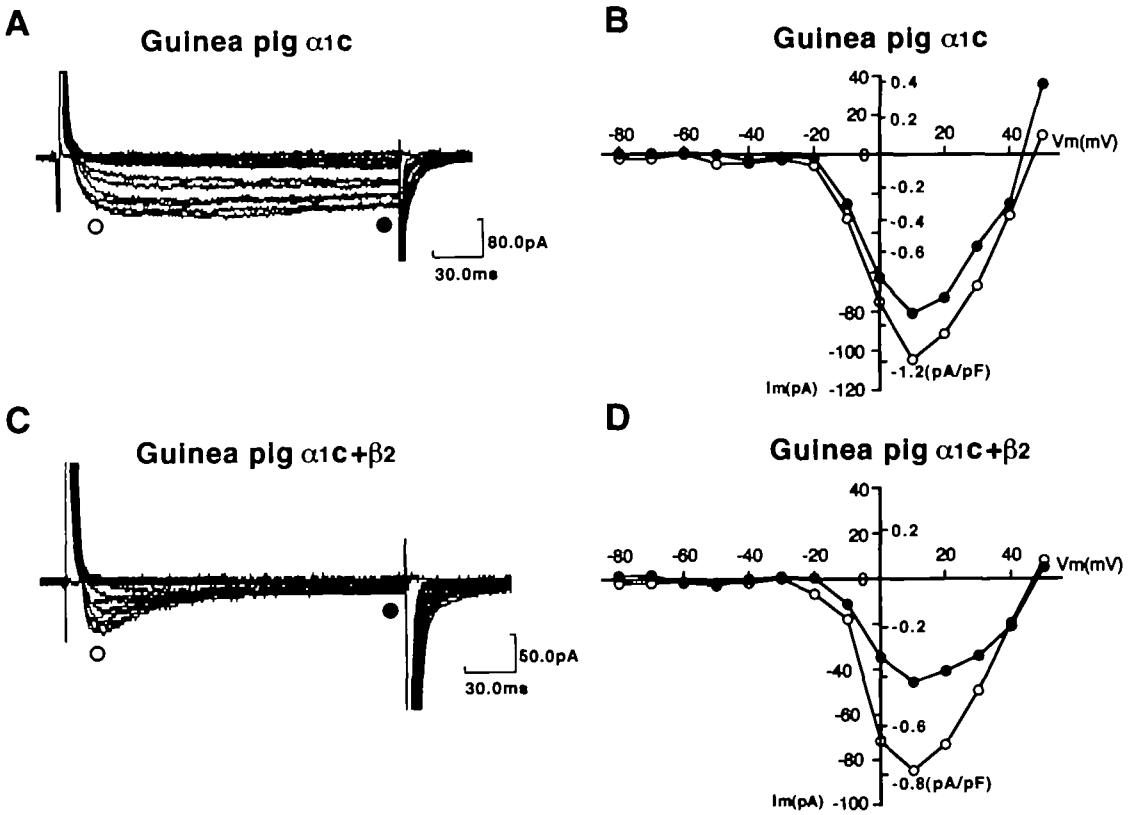

Fig. 3. **Whole-cell Ba^{2+} currents in BHK cells expressing the guinea pig Ca^{2+} channel.** A and C: Ba^{2+} current mediated by α_{1C} alone (A, an example showing the largest current) or $\alpha_{1C}+\beta_{2a}$ (C). The extracellular solution contained 30 mM Ba^{2+}. Currents were elicited by 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^{2+} 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 (○) and the late current (●).

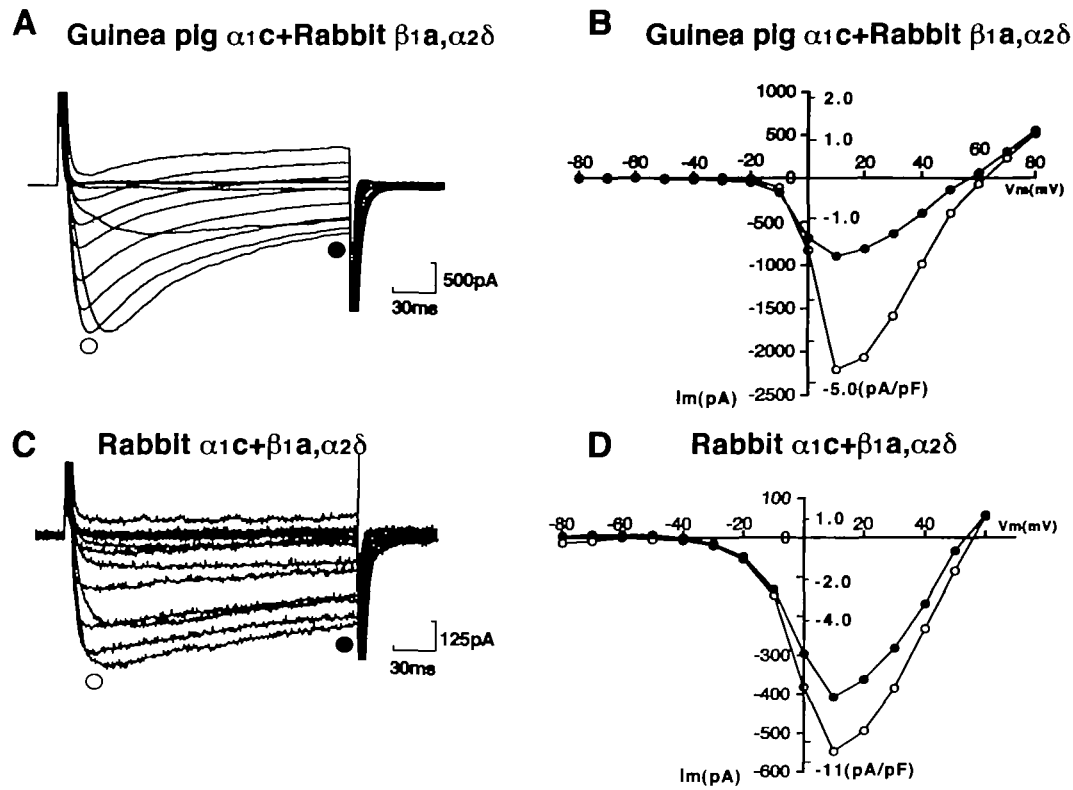

Fig. 4. **Whole-cell Ba^{2+} current in BHK6 cells expressing the guinea pig α_{1C} or rabbit α_{1C} subunit.** BHK6 cells express rabbit β_{1a} stably. A and B: The Ba^{2+} current mediated by guinea pig α_{1C} (A) and its I-V relationship (B) for the maximum current (○) and the late current (●). C and D: The Ba^{2+} current mediated by rabbit α_{1C} (C) and 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 Ba^{2+}.

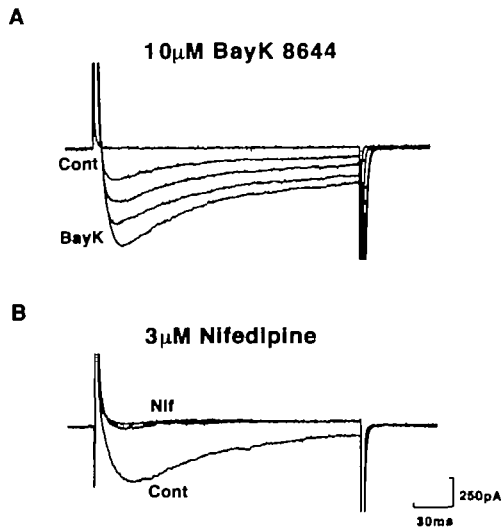

Fig. 5. **Effects of dihydropyridines on the Ba^{2+} current in BHK cells expressing guinea pig α_{1C} and β_{2a} subunits.** A: Increasing effect of 10 μM Bay K 8644, a Ca^{2+}-channel activator, on the Ba^{2+} current elicited by consecutive step pulses from −80 to 10 mV. The current at control (Cont) 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 μM nifedipine, a Ca^{2+}-channel blocker, on the Ba^{2+} current was elicited as in A. The current at control (Cont) and in the presence of nifedipine (Nif, two traces superimposed).

tion of the Ca^{2+}-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 α_{1C} and β_2 subunits of Ca^{2+} channels from guinea pig heart. Our results demonstrate that α_{1C}, alone or together with β_2, mediates a DHP-sensitive high voltage-activated, long-lasting inward current, properties typical of L-type Ca^{2+} channels. BHK cells lack an endogenous Ca^{2+}-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 α_{1C} is smaller compared to those reported for rabbit α_{1C}, the current density normalized by the cell capacitance (~0.4 pA/pF) was comparable to those for the rabbit.

The primary structure of guinea pig α_{1C} shows a high similarity to α_{1C} subunits from other mammalian species. The amino acid sequence shows >90% homology to rabbit, rat, and mouse α_{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

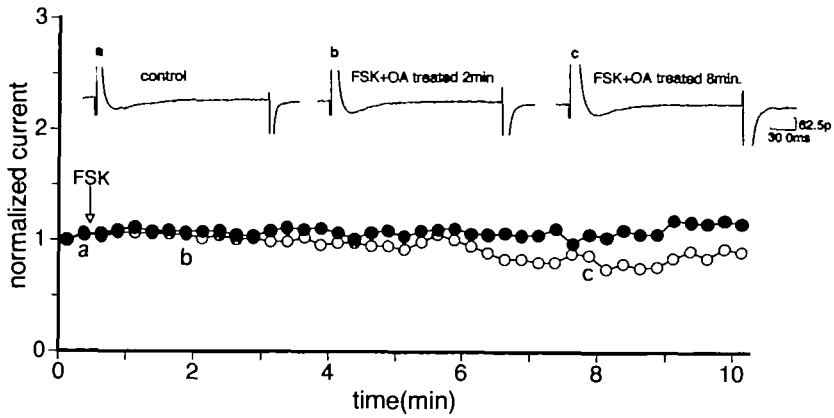

Fig. 6. **Time course of the Ba^{2+} current in BHK cells expressing guinea pig α_{1C} and β_{2a} subunits.** Currents were recorded with or without 5 μM okadaic acid, a protein phosphatase inhibitor, in the pipette, and then 5 μM forskolin, an adenylyl cyclase activator, was applied at the time indicated by the arrow. The Ba^{2+} 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 β 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 β subunit in other species, the clone shows 95% identity in amino acid sequence with the β_{2a} isoform (except for human), and is thus identified as the counterpart of guinea pig β_{2a}. Co-expression of β_{2a} increases the α_{1C} mediated current about 2-fold. In the case of rabbit, rat, and human α_{1C}, the current is increased 3- to 20-fold by co-expression of the β_1 or β_{2a} subunit (*2, 30-32*). Thus, the extent of the effect of guinea pig β_{2a} seems to be smaller than the effect in other species. The Ba^{2+} current for guinea pig α_{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^{2+} channels occurs by two mechanisms, one voltage-dependent and the other Ca^{2+}-mediated. When there is a Ba^{2+} current flowing through the Ca^{2+} channels, the rate of inactivation is greatly slowed, presumably due to a low affinity of Ba^{2+} for the site of Ca^{2+}-mediated inactivation (*33*). In this study, the Ba^{2+} current mediated by guinea pig α_{1C} alone was barely or very slowly inactivated, while that mediated by α_{1C} and β_2 showed a clear acceleration in the rate of inactivation. This is in line with previous studies, in which β subunits affect the inactivation of the Ca^{2+}-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 β 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 α_{1C} might interact with the β subunits, as reported for α_{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 α_{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^{2+} 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^{2+} current in cells expressing guinea-pig α_{1C} alone or $\alpha_{1C}+\beta_{2a}$. The drugs also had no effect on the Ba^{2+} current of rabbit α_{1C}. These results are in contrast to previous findings that the current in BHK cells transfected with rabbit α_{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^{2+} channels by phosphorylation is variable in the heterologous expression system, possibly dependent on the metabolic state of the cells.

The α_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 α_{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 α_{1C} as exist on that of the rabbit. Recently, Gao *et al.* (*45*) reported that in HEK293 cells transfected with rabbit cardiac α_{1C}, the PKA phosphorylation site is located in the C-terminus and a PKA anchoring protein, AKAP79, regulates the phosphorylation. An α_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 α_{1C} (Ser1927), it is

relevant to examine whether this site is involved in PKA-mediated channel modulation.

The β subunit is also thought to be phosphorylated by PKA. The phosphorylation site of the β subunit is conserved in guinea pig β_{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 α_{1C} (and perhaps β) are involved in channel regulation remains to be examined.

The electrophysiological and pharmacological properties of Ca^{2+} 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^{2+}-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 α_{1C} and β_{2a} subunits will provide a useful tool for further studies on the cardiac Ca^{2+} channel.

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