Ion Channels in the Cardiovascular System:

Function and Dysfunction

edited by

Peter M. Spooner
Arthur M. Brown
William A. Catterall
Gregory J. Kaczorowski
Harold C. Strauss

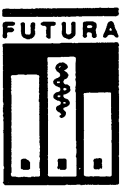

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Table of Contents

List of Contributors

Peter H. Backx, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

Joel C. Barrish, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

Kurt Beam, Department of Physiology, Colorado State University, Ft. Collins, CO

Bruce Bean, Department of Neurobiology, Harvard Medical School, Boston, MA

Paul B. Bennett, Departments of Pharmacology and Medicine, Vanderbilt School of Medicine, Nashville, TN

Wolfgang Berger, Institut für Biochemische Pharmakologie, University of Innsbruck, A-6020 Innsbruck, Austria

M. Biel, Institut für Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40, Germany

Shawn C. Black, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

E. Bosse, Institut für Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40 Germany

A. P. Braun, Department of Medical Physiology, University of Calgary School of Medicine, Calgary, Alberta, Canada

Arthur M. Brown,* Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX

Donald L. Campbell, Department of Pharmacology, Duke University Medical Center, Durham, NC

A. C. Campos de Carvalho, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, and Federal University of Rio de Janiero, Rio de Janiero, Brazil

Agustin Castellanos, Division of Cardiology, Department of Medicine, University of Miami School of Medicine and the, Miami VA Medical Center, Miami, FL

William A. Catterall,* Department of Pharmacology, University of Washington, Seattle, WA

*Member of the Research Assessment Panel.

Todd Chapman, Department of Physiology, University of Nevada School of Medicine, Reno, NV

Liguo Chi, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

G. J. Christ, Department of Urology, Albert Einstein College of Medicine, Bronx, NY

R. B. Clark, Department of Medical Physiology, University of Calgary School of Medicine, Calgary, Alberta, Canada

Thomas J. Colatsky, Division of Cardiovascular and Metabolic Disorders, Wyeth-Ayerst Research, Princeton, NJ

Karen K. Deal, Department of Pharmacology, Vanderbilt Medical School, Nashville, TN

Mariella De Biasi, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX

Henry J. Duff, Department of Pharmacology, University of Washington, Seattle, WA

G. I. Fishman, Departments of Molecular Genetics and Medicine, Albert Einstein College of Medicine, Bronx, NY

V. Flockerzi, Institüt fur Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40, Germany

David M. Floyd, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

Harry A. Fozzard, Cardiac Electrophysiology Laboratories, Departments of Pharmacological & Physiological Sciences and Medicine, and University of Chicago, Chicago, IL

Gregory S. Friedrichs, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

Jesús García, Department of Physiology, Colorado State University, Ft. Collins, CO

Maria L. Garcia, Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, NJ

Alfred L. George, Jr., Departments of Pharmacology and Medicine, Vanderbilt School of Medicine, Nashville, TN

W. R. Giles, Department of Medical Physiology, University of Calgary School of Medicine, Calgary, Alberta, Canada

Hartmut Glossmann, Institut für Biochemische Pharmakologie, University of Innsbruck, A-6020 Innsbruck, Austria

Alan L. Goldin, Department of Microbiology and Molecular Genetics, University of California–Irvine, Irvine, CA

Jack Z. Gougoutas, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

Pádraig J. Hart, Department of Physiology, University of Nevada School of Medicine, Reno, NV

Steffen Hering, Insitut für Biochemische Pharmakologie, University of Innsbruck, A-6020 Innsbruck, Austria

Peter Hess, Department of Cellular and Molecular Physiology, Program in Neuroscience, Harvard Medical School, Boston, MA

F. Hofman, Institut für Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40, Germany

Burton Horowitz, Department of Physiology, University of Nevada School of Medicine, Reno, NV

Melissa H. House, Department of Molecular Physiology and Biophysics, Vanderbilt Medical School, Nashville, TN

Joseph R. Hume, Department of Physiology, University of Nevada School of Medicine, Reno, NV

John T. Hunt, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

N. K. Jurkiewicz, Department of Pharmacology, Merck Research Laboratories, West Point, PA

Gregory J. Kaczorowski,* Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, NJ

Robert S. Kass, Department of Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY

Arnold M. Katz,* Cardiology Division, Department of Medicine, University of Connecticut, Farmington, CT

Kenneth M. Kessler, Division of Cardiology, Department of Medicine, University of Miami School of Medicine and the Miami VA Medical Center, Miami, FL

S. David Kimball, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

Timothy J. Knittle, Department of Molecular Physiology and Biophysics, Vanderbilt Medical School, Nashville, TN

Wan F. Lau, Bristol-Myers Squibb, Pharmaceutical Research Institute, New York, NY

John H. Lawrence, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

Paul C. Levesque, Department of Physiology, University of Nevada School of Medicine, Reno, NV

Benedict R. Lucchesi, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

William C. Lumma, Jr., Merck Research Laboratories, West Point, PA

Peter J. Manley, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

Eduardo Marban, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

Rafael Mejía-Alvarez, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

A. P. Moreno, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY

Issam F. Moubarak, Division of Cardiovascular and Metabolic Disorders, Wyeth-Ayerst Research, Princeton, NJ

Robert J. Myerburg, Division of Cardiology, Department of Medicine, University of Miami School of Medicine, and the Miami VA Medical Center, Miami, FL

Randal Numann, Department of Pharmacology, University of Washington, Seattle, WA

H. Bradley Nuss, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

Jeanne Y. Oh, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

Brian O'Rourke, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

L. A. Pardo, Max-Planck-Institut für Experimentelle Medizin, D-3400 Gottingen, FRG

D. Earl Patton, Department of Microbiology and Molecular Genetics, University of California–Irvine, Irvine, CA

Sunny Po, Department of Pharmacology, Vanderbilt School of Medicine, Nashville, TN

Yusheng Qu, Department of Pharmacology, Duke University Medical Center, Durham, NC

Randall L. Rasmusson, Department of Biomedical Engineering, Duke University Medical Center, Durham, NC

Steven L. Roberds, Department of Pharmacology, Vanderbilt School of Medicine, Nashville, TN

M. B. Rook, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY

P. Ruth, Institut für Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40, Germany

J. C. Sáez, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY

M. C. Sanguinetti, Department of Pharmacology , Merck Research Laboratories, West Point, PA

Todd Scheuer, Department of Pharmacology, University of Washington, Seattle, WA

Peter J. Schwartz, Department of Medicine, University of Pavia , Pavia, Italy, and Istituto di Clinica Medica II, University of Milan, Milan, Italy

Robert S. Slaughter, Membrane Biochemistry and Biophysics, Merck Research Laboratories, Rahway, NJ

Thomas W. Smith,* Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA

Dirk J. Snyders, Departments of Pharmacology and Medicine, Vanderbilt School of Medicine, Nashville, TN

Walter Spinelli, Division of Cardiovascular and Metabolic Disorders, Wyeth-Ayerst Research, Princeton, NJ

Peter M. Spooner,* Cardiac Functions Branch, Division of Heart and Vascular Diseases, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

David C. Spray, Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY

Harold C. Strauss,* Departments of Pharmacology and Medicine, Duke University Medical Center, Durham, NC

Jörg Striessnig, Institut fur Biochemische Pharmakologie, University of Innsbruck, A-6020 Innsbruck, Austria

W. Stühmer, Max-Planck-Institut für Experimentelle Medizin, D-3400 Gottingen, FRG

Michael M. Tamkun, Departments of Pharmacology, and Molecular Physiology and Biophysics, Vanderbilt Medical School, Nashville, TN

Gordon Tomaselli, Division of Cardiology, Department of Medicine, The Johns Hopkins University, Baltimore, MD

Stephen S. Tsung, Department of Physiology, University of Nevada School of Medicine, Reno, NV

Jan Tytgat, Department of Cellular and Molecular Physiology, Program in Neuroscience, Harvard Medical School, Boston, MA

Andrew C. G. Uprichard, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI

Carol A. Vandenberg, Department of Biological Sciences and Neuroscience Research Institute, University of California—Santa Barbara, Santa Barbara, CA

M. P. Walsh, Department of Medical Biochemistry, University of Calgary School of Medicine, Calgary, Alberta, Canada

August M. Watanabe,* Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, IN

A. Welling, Institut für Pharmakologie und Toxikologie, Technische Universität Munchen, D-8000 Munich 40, Germany

James W. West, Department of Pharmacology, University of Washington, Seattle, WA

Douglas P. Zipes, Department of Medicine, Krannert Insitute of Cardiology, Indiana University School of Medicine and, Roudebush VA Medical Center, Indianapolis, IN

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Functional Expression of Cardiac and Smooth Muscle Calcium Channels

F. Hofmann, M. Biel, E. Bosse, V. Flockerzi, P. Ruth, and A. Welling

Hormones and neurotransmitters play an important role in regulating the force of contraction in heart and smooth muscle. The force of contraction of the heart is primarily controlled by the calcium influx across the cell membrane during the action potential, whereas that of smooth muscle is initially controlled by the release of calcium from intracellular stores followed by a calcium influx from the extracellular space through voltage-dependent calcium channels. The best-characterized pathway for calcium entry in both cell types are voltage-dependent L-type calcium channels, which activate at membrane potential around −30 mV, inactivate slowly, and are expressed in many different cell tissue (1). These channels are readily blocked by classical organic calcium channel blockers (CaCBs) nifedipine [a 1,4-dihydropyridine (DHP)], verapamil [a phenylalkylamine (PAA)], and diltiazem (a benzothiazepine) (2–4).

Cardiac and smooth muscle calcium channels have common electrophysiological characteristics, yet they are regulated distinctly by CaCBs and hormones. The β-adrenergic receptor agonist isoproterenol increases cardiac calcium current 3- to 7-fold either by cAMP-dependent phosphorylation of the channel (5, 6), or through the activated α-subunit of the trimeric glutamyl transpeptidase (GTP) binding protein G_s (7, 8), or by the combined action of $G\alpha_s$ and the catalytic subunit of cAMP kinase (9). The L-type calcium current of isolated tracheal smooth muscle cells is stimulated also by the activation of the β-adrenergic receptor (10). This β-receptor effect is not mediated by cAMP kinase, but by the direct effect of a G-protein. These and further results (11) lead to the conclusion that the smooth and cardiac muscle calcium channels may be regulated in vivo by the α-subunit of a G-protein, but only the cardiac channel by cAMP-dependent phosphorylation. The primary sequences of the cardiac and a putative smooth muscle calcium channel have been identified by cloning their

cDNAs. The sequences of these clones are very similar, and they direct the expression of L-type calcium channels of very similar properties.

Molecular Identity of Cardiac and Smooth Muscle Calcium Channels

General Composition of the Calcium Channel

Initially, the L-type calcium channel was purified from skeletal muscle. The purified complex contains four proteins (Fig. 1): the α_1-subunit (212,018 Da), which contains the binding sites for all known CaCBs and the calcium conducting pore; the intracellular located β-subunit (57,868 Da); the transmembrane γ-subunit (25,058 Da); and the α_2-/δ-subunit, a disulfide-linked dimer of 125,018 Da (2–4 and references cited there). Reconstitution of the purified complex into phospholipid bilayers resulted in functional calcium channels that are reversibly blocked by CaCBs and

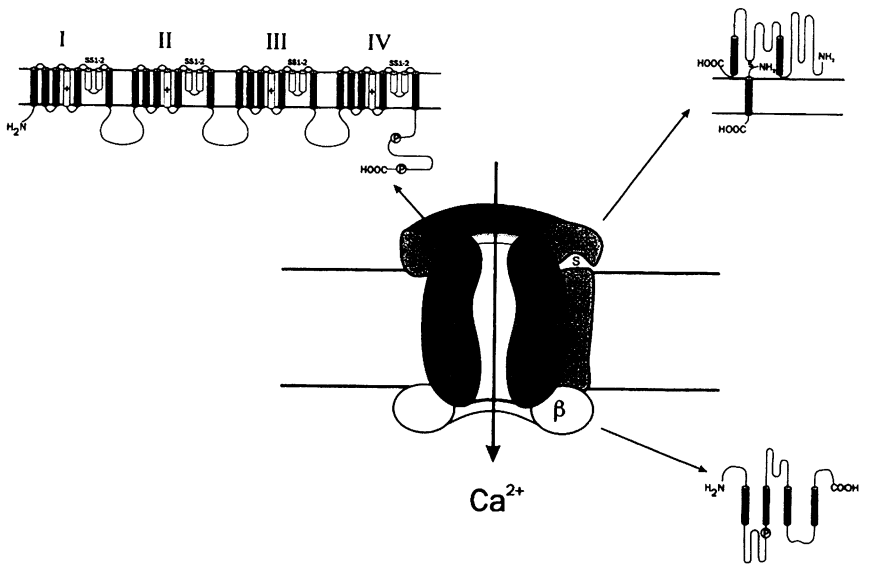

Figure 1. Proposed structure of the cardiac calcium channel. The putative transmembrane configuration of individual subunits is based on hydropathicity analysis of the deduced primary sequences, I, II, III, and IV, proposed repeats of calcium channel α_1-subunit; +, transmembrane amphipathic α-helix, respectively, the proposed voltage-sensing helix of the channel; Ⓟ, putative cAMP kinase phosphorylation sites; only two of several phosphorylation sites are shown in the sequence of the α_1-subunit; s, disulfide bridge between the transmembrane δ and the extracellular-located α_2-subunit; SS1–SS2, suggested part of the channel pore. The extracellular space is above the horizontal lines. Note that a γ-subunit that is present in the skeletal muscle calcium channel has not been identified in cardiac tissue.

are modulated by cAMP-dependent phosphorylation (12–15). The primary sequences of these proteins have been deduced by cloning their corresponding cDNAs from rabbit skeletal muscle (16–20). Using these cDNAs as probes, different α_1- and β-subunits have been cloned from heart and smooth muscle.

The Calcium Conducting α_1-Subunit

The L-type calcium channel α_1-subunits are encoded by three different genes (CaCh1–3) (see refs. 21 and 22 for nomenclature, and Fig. 2). The product of the CaCh1 gene occurs in skeletal muscle; the product of the CaCh2 gene is expressed in heart, smooth muscle, endocrine, and neuronal cells, and that of the CaCh3 gene is present in neuroendocrine and neuronal cells. The α_1-subunits from cardiac (CaCh2a) (23) and smooth muscle

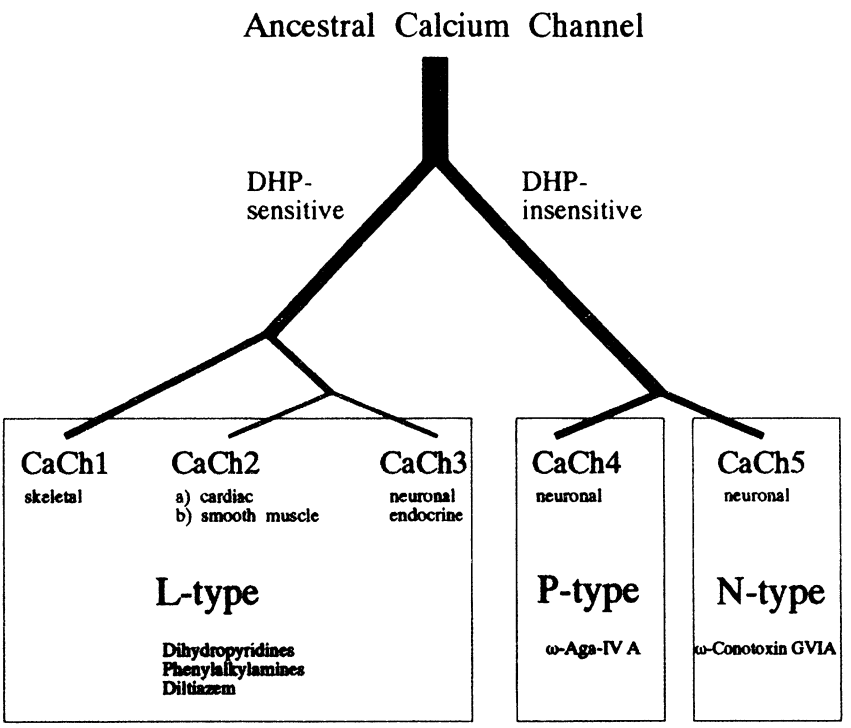

Figure 2. Developmental tree of cloned and functionally expressed calcium channel α_1-subunits. The identification of the calcium channel α_1-subunits follows the order of publication and includes only those clones that have been expressed functionally (21, 22, 25). The Snutch nomenclature (52) for the brain calcium channels does not include the CaCh1 gene. The CaCh2, CaCh3, CaCh4, and CaCh5 genes correspond to Snutch genes C, D, A, and B, respectively.

(CaCh2b) (24) are splice products of the second gene (CaCh2) (Fig. 3). The CaCh2b sequence is 95% identical with the CaCh2a sequence and differs only at four sites. The use of alternative exons (25) results in different IVS3 segments (Fig. 3, site D). Diebold and coworkers (26) reported that the two exons are differentially expressed during cardiac development. Northern blots using the CaCh2a and CaCh2b specific 5′-sequences as probes, and polymerase chain reaction analysis of the nonidentical sequences, showed that the sequence of cardiac site A is present only in cardiac muscle polyA$^+$ mRNA, whereas the sequences of the smooth muscle sites A, B, and C are present in tracheal, lung, and cardiac polyA$^+$ mRNA (27). This distribution of the CaCh2a and CaCh2b sequences strongly favors the conclusion that the CaCh2a protein is a cardiac muscle-specific α_1-subunit, whereas the CaCh2b protein is a smooth muscle-specific α_1-subunit (27).

cDNA of the third gene (CaCh3) was isolated from neuronal and endocrine tissues and represents a neuroendocrine specific L-type calcium channel (28, 29), whereas the gene products of the fourth and fifth gene (CaCh4 and CaCh5) have been found exclusively in neuronal tissues. Calcium channels transiently expressed from cRNA of CaCh4 induce high-voltage–activated, calcium currents insensitive to nifedipine and ω-conotoxin, but inhibited by a mixture of toxins from the funnel web spider, thus characterizing this channel as a P-type calcium channel (30). The gene product of CaCh5 binds and is irreversibly blocked by picomolar concentrations of ω-conotoxin identifying the CaCh5 protein as a neuronal N-type calcium channel (31, 32).

Additional Subunits

α_2-/δ-Subunit

The skeletal muscle α_2-/δ-subunit is a glycosylated membrane protein of 125,018 Da (17) that is apparently high conserved in most tissues. In

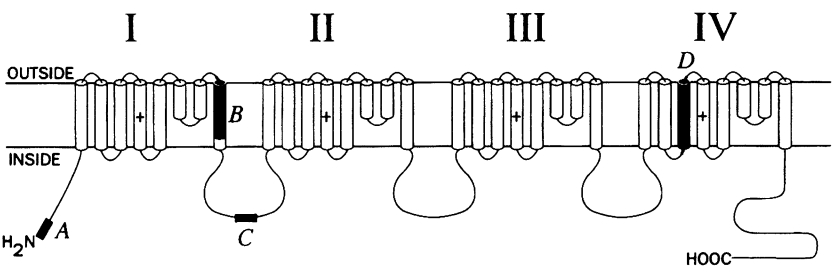

Figure 3. Sequence differences between the CaCh2a (cardiac) and CaCh2b (smooth muscle) α_1-subunit. The sequences that are different in the smooth muscle clone are shown in black and are identified by A, B, C, and D.

skeletal muscle the primary protein product of the α_2-/δ-gene is processed post-translationally by proteolysis resulting in an α_2-protein containing amino acids 1 through 934, and a δ-protein containing amino acids 935 through 1080 (33) (see Fig. 1). The transmembrane δ-subunit anchors the extracellular located α_2-protein by disulfide bridges to the plasma membrane (34). Immunoblot (35) and Northern blot (17, 27) show that similar or identical α_2-/δ-subunits exist in skeletal muscle, heart, brain, vascular, and intestinal smooth muscle. Coexpression of the cardiac α_1-subunit with the skeletal muscle α_2-/δ-protein in *Xenopus* oocytes increases slightly the current density (23, 26) and decreases 2-fold channel activation time (36). It is not clear whether these modulatory effects are physiologically relevant properties of the α_2-/δ-protein or restricted to the *Xenopus* oocyte expression system.

γ-Subunit

Northern blots and screening of cardiac cDNA libraries failed to identify the presence of the γ-subunit in cardiac or smooth muscle polyA$^+$ mRNA, suggesting that this protein may be specific for skeletal muscle.

β-Subunit

The skeletal muscle β-subunit (CaB1) is an intracellular located membrane protein consisting of 524 amino acids (18). Its deduced amino acid sequence contains stretches of heptad repeat structure that are characteristic of cytoskeletal proteins. Two other genes (CaB2 and CaB3) encoding β-proteins different from the skeletal muscle β-subunit have been isolated from a cardiac cDNA library (37). Their deduced amino acid sequences show an overall homology to CaB1 of 71% (CaB2) and 66.6% (CaB3). Differential splicing of the primary transcript of CaB1 results in at least four isoforms: CaB1a through CaB1d (18, 32, 38). CaB1a is expressed in skeletal muscle, whereas the other isoforms are expressed in brain. Four different splice variants have been characterized for the CaB2 gene (CaB2a through CaB2d); CaB2a and CaB2b have been isolated from a rabbit cardiac cDNA library, whereas CaB2c and CaB2d have been cloned from rabbit and rat brain libraries (37, 39). Like the CaB1 gene, the CaB2 and CaB3 genes are expressed tissue specifically, with transcripts of CaB2 existing abundantly in heart and to a lower degree in aorta, trachea, and lung. Transcripts of CaB3 genes are expressed in brain and smooth muscle-containing tissues, such as aorta, trachea, and lung (37). This suggests that the CaB3 gene product may be expressed predominantly in neuronal and smooth muscle cells. The deduced amino acid sequence of the skeletal muscle β-subunit (CaB1) contains several phosphorylation sites. Two of these sites, Ser-182 and Thr-205, are phosphorylated in vitro by cAMP-dependent protein kinase (18, 40). The equivalent to Thr-205 is conserved in the "cardiac" β-subunit (Thr-165 in CaB2a and Thr-191 in CaB2b), but

is not present in the "smooth muscle" β-subunit, CaB3. The sequence following this potential phosphorylation site is highly variable and determines several splice variants (37). The absence of a putative cAMP kinase phosphorylation site in the variable region of the "smooth" muscle β-subunit and its presence in the "cardiac" muscle β-subunit may be responsible for the tissue-specific regulation the L-type calcium currents by cAMP kinase.

Expression of the Cloned Cardiac and Smooth Muscle Calcium Channel α_1-Subunits

The two alternative splice variants CaCh2a and CaCh2b have been expressed transiently in *Xenopus* oocytes (23, 24, 36) and stably in Chinese hamster ovary (CHO) cells (41, 42). In either system they direct the synthesis of functional L-type calcium channels. CHO cells transfected with either CaCh2a or CaCh2b cDNA express 1,4-dihydropyridine binding sites that bind isradipine stereospecifically with an affinity of 0.1 nM (Table 1). Binding of isradipine is modulated allosterically by *d-cis*-diltiazem (41). Nontransfected cells, or CHO cells transfected with a cDNA unrelated to

Table 1. Electrophysiological properties of the expressed cardiac (CaCh2a) and smooth (CaCh2b) muscle α_1-subunit of the calcium channel.

cell line	CaCh2a (heart)	CaCh2b (smooth muscle)
DHP binding sites (fmol/mg)	73.5 ± 5.4 (3)	141 ± 3 (3)
Affinity (nM)	0.2	0.1 ± 0.04 (3)
Current density (μA/cm²)		
−Bay K 8644	−15.2 ± 4.4 (9)	−13.3 ± 1.9 (11)
+Bay K 8644	−68.2 ± 16.6 (8)	−46.7 ± 5.4 (14)
Increase with Bay K 8644 at 10 mV (fold)	9.3 ± 2.6 (8)	5.5 ± 0.8 (11)
90% ttp (msec)	7.9 ± 0.8 (9)	12.5 ± 1.5 (12)*
dec_{100} (%)	87.1 ± 2.7 (9)	84.7 ± 1.8 (11)
$V_{0.5}$inact (mV)	+5.2 ± 3.6 (9)	+5.1 ± 4.0 (5)
$V_{0.5}$act (mV)	+8.3 ± 0.8 (9)	+9.5 ± 0.8 (5)

CHO cells were stably transfected and inward currents were measured as described in Fig. 4 legend. The current is the maximum current from the current−voltage relation divided by the cell capacitance. The increase in I_{Ba} caused by Bay K 8644 was calculated for each cell at a membrane potential of 10 mV. 90% ttp, time for I_{Ba} to reach 90% of its peak amplitude at 10 mV; dec_{100}, decrease in I_{Ba} from peak to the level observed 100 msec after the beginning of depolarization to 10 mV; $V_{0.5}$inact, half-maximal inactivation voltage, $V_{0.5}$act, half-maximal activation voltage. Values are given as mean ± SEM, with the number of cells in parentheses. The DHP binding sites were determined with (^{3}H)isradipine.

*Significantly different from the CaCh2a value at $P < 0.05$.

the calcium channel, do not possess DHP binding sites or L-type calcium current, whereas cells expressing the cardiac or smooth muscle α_1-subunit have L-type calcium current (Fig. 4). The basic electrophysiological characteristics of these two splice variants of the CaCh2 gene are almost identical (Table 1). Both channels are blocked by isradipine and are stimulated 5- to 10-fold by Bay K 8644 at 10mV (Table 1). Activation of the channel by Bay K 8644 shifts the maximal inward current by 10 mV to 10 mV. In the presence of Bay K 8644 and 30 mM $BaCl_2$, half-maximal activation ($V_{0.5act}$) occurred at 9.5 and 8.3 mV for the smooth and cardiac muscle, respectively. Under the same conditions, half-maximal steady-state inactivation ($V_{0.5inact}$) occurred at 5.1 and 5.2 mV, respectively. Both channels inactivate faster in the presence of Ca^{2+} than Ba^{2+} (i.e., inactivation of the CaCh2 channel is voltage- and calcium-dependent as known for the native L-type calcium channel). The only difference noted was faster activation for cardiac than for smooth muscle channels in the absence and presence of the Bay K 8644. Functional expression of chimeras of the skeletal and the cardiac muscle α_1-subunit showed that repeat I determines the activation time of the chimeric channel; that is, a slow activation upon membrane depolarization with the repeat from skeletal muscle, and rapid activation with that from cardiac muscle (43). It is possible that the difference in activation times between the cardiac and smooth muscle channels is caused by the difference in the IS6 sequence (site B in Fig. 3). The open probability of the expressed smooth muscle channel increases with membrane depolarization. The channel has a single-channel conductance of 26 pS in 80 mM $BaCl_2$ (41). These data show that the α_1-subunit alone is sufficient to form a physiologically relevant calcium channel that has the properties of a smooth or cardiac muscle L-type calcium channel.

Stable expression of the CaCh2b channel with the skeletal muscle β-gene (CaB1), increased in parallel the number of DHP binding sites and the amplitude of whole-cell barium current suggesting that the amplitude of the inward current is directly related to the density of expressed α_1-protein (35). In addition the coexpression of the β-subunit decreased channel activation time 2-fold and shifted the voltage dependence of steady-state inactivation by 18 mV, to −13 mV, without affecting sensitivity to the calcium channel agonist Bay K 8644.

Modulation of the Expressed Calcium Channel

In heart, β-adrenergic stimulation leads to activation of cAMP kinase and an increase in L-type calcium current. Two subunits of the purified skeletal muscle calcium channel, the α_1- and β-subunits, are substrates for cAMP-kinase in vitro (4, 18, 40, 44–46). Similar potential cAMP kinase phosphorylation sites are present in the CaCh2 calcium channel sequence of both splice variants. The potential importance of these phosphorylation sites is supported by experiments that showed that the L-type calcium

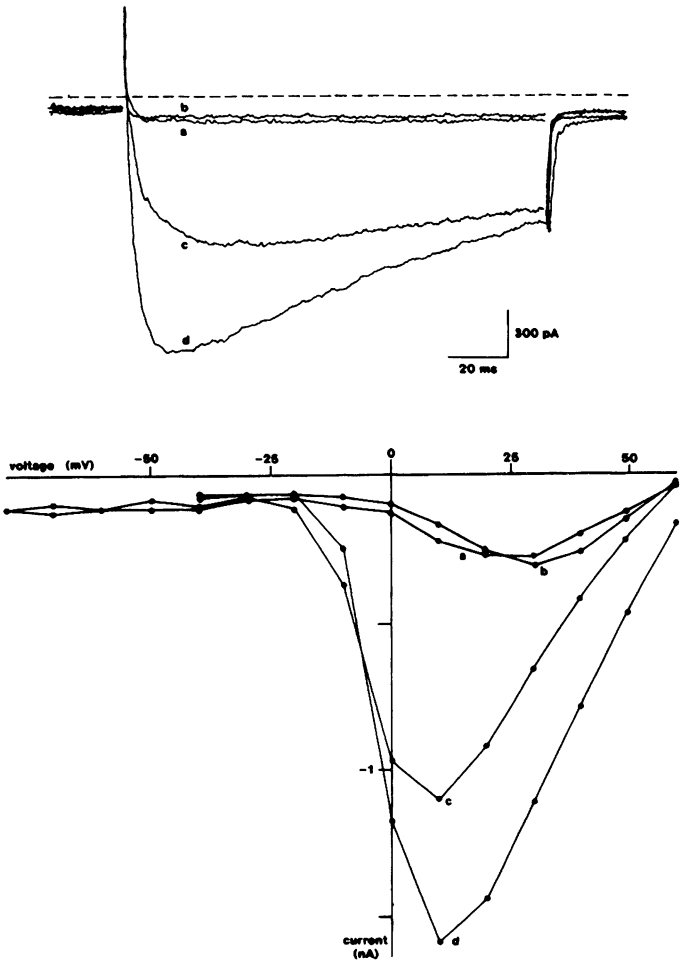

Figure 4. Electrophysiology of expressed smooth **(a, c)** and cardiac **(b, d)** muscle α_1-subunit of the calcium channel. **(Top)** Barium currents (I_{Ba}) were elicited by a 140-msec depolarization pulse from a holding potential of −80 mV to 10 mV before (a, b) and during the exposure to 2 μM Bay K 8644 (c, d). The dashed line represents the 0 current level. **(Bottom)** Current-voltage relationships were determined by stepping the membrane potential from −80 mV to the potentials indicated. I_{Ba} was measured in the absence (a, b) and presence (c, d) of 2 μM Bay K 8644. CHO cells were stably transfected (41) with the cDNA of the smooth muscle α_1-subunit of the calcium channel (24) in a p91023(B) expression vector or with the cDNA of the cardiac α_1-subunit (23) in a pKNH expression vector (53). Positive clones were selected for the expression of dihydrofolate reductase or with the neomycine derivative G418. Whole-cell current was recorded at room temperature in a solution containing (in mM) 82 NaCl, 20 tetraethylommonium-chloride, 30 $BaCl_2$, 5.4 CsCl, 1 $MgCl_2$, 5 HEPES, and 10 glucose, pH 7.4 (NaOH), with a pipette solution containing (in mM) 112 CsCl, 1 $MgCl_2$, 3 Na_2ATP, 10 EGTA, and 5 HEPES, pH 7.4 (CsOH).

current expressed in *Xenopus* oocytes after the injection of rat cardiac poly(A^+) RNA is modulated by cAMP-dependent phosphorylation (47). However, perfusion of a CHO cell expressing the CaCh2b gene with 8Br-cAMP for 5 min did not increase significantly the inward current (Fig. 5), although CHO cells contain a functional adenylyl cyclase, G_S, and cAMP-dependent protein kinase. The inward barium current was not affected significantly when the pipette solution contained 3mM ATPγSalme; 3 mM ATPγS + 10; or 100 μM 8Br-cAMP or 3 mM ATPγS + 10 μM GTPγS. Isoproterenol failed to increase the barium current in a CHO cell line that expressed the β_2-adrenergic receptor at a concentration of 1 pmol/mg protein and the CaCh2b calcium channel. These negative findings suggest that the α_1-subunit alone is not sufficient to restore hormonal regulation of the native calcium channel. Similar conclusions were reached by Klöckner et al. (48), who injected the cardiac α_1-subunit alone or together with the skeletal muscle β-subunit into *Xenopus* oocyctes. These authors reported

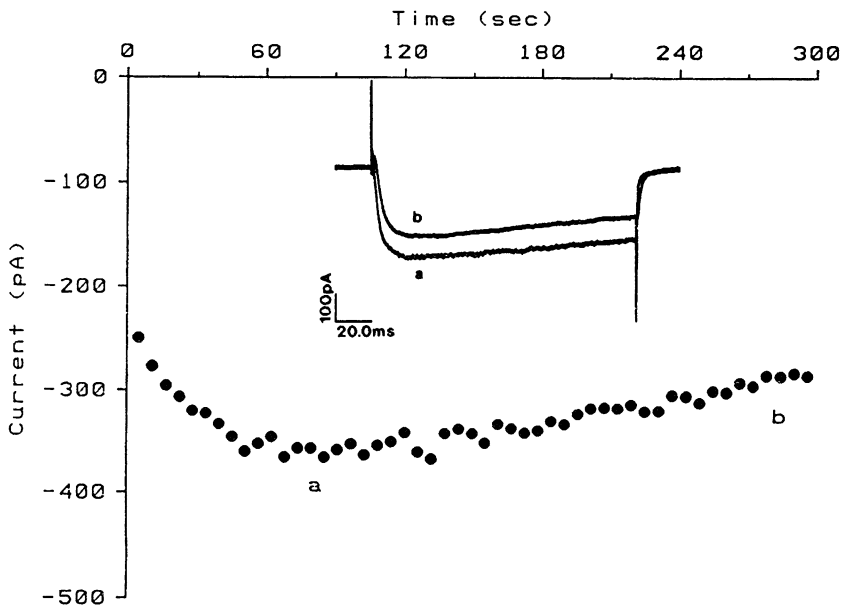

Figure 5. 8Br-cAMP has no effect on the barium current. The cell was transfected stably with the CaCh2b clone. The barium inward current was measured as described in Table 1 footnote. Cells were superfused with (in mM) 79.0 NaCl, 20 tetraethylammonium, 30 $BaCl_2$, 5.4 CsCl, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4. The pipette solution contained (in mM) 107 CsCl, 3 $MgCl_2$, 10 EGTA, and 10 HEPES, pH 7.4, 3 mM ATPγS, and 10μM 8Br-cAMP. The inward current at 10 mV is shown. The inset shows two current traces taken at time points a and b. The small increase in inward current occurring within the first minute was observed also in all cells perfused in the absence of ATPγS and 8Br-cAMP and is caused most likely by the chelation of calcium by EGTA.

that cAMP increased barium currents only in *Xenopus* oocytes expressing the cardiac α_1- and the skeletal muscle β-subunit (48). However, the reported inward currents were small and their sensitivity toward Bay K 8644 or a 1,4-dihydropyridine blocker was not tested. Therefore, these authors did not exclude the possibility that the β-subunit associated with the endogenous *Xenopus* oocyte calcium channel (36, 49), which is insensitive to the 1,4-dihydropyridines, is stimulated by cAMP in the presence of the skeletal muscle subunits (50). Perfusion of CHO cells expressing the CaCh2 α_1-subunit and the skeletal muscle β-subunit with cAMP or 8Br-cAMP had no effect on the size of the inward current, suggesting that at least in CHO cells the combination of these two subunits, which are not expressed in vivo in the same tissue, does not restore hormonal control of the cardiac calcium channel. These negative results are unexplained, so far. The cAMP kinase activity of *Xenopus* oocytes is higher than in most cells and could phosphorylate constantly the α_1-subunit in vivo.

Different results were obtained with protein kinase C. The cardiac L-type calcium current is enhanced and subsequently inhibited by the activation of protein kinase C (51). Activation of protein kinase C has been proposed to mediate the potential effects of angiotensin II on the calcium current in heart. The skeletal muscle α_1-subunit is rapidly phosphorylated by protein kinase C (44). Currents through the CaCh2a α_1-subunit expressed in *Xenopus* oocytes were biphasically modulated by activation of protein kinase C (49). Initially the current increased followed by a marked inhibition. The biphasic modulation was not modified significantly by coexpression of the cardiac α_1-subunit with the α_2-/δ-, β-, and γ-subunits from skeletal muscle, suggesting that protein kinase C affected the current by phosphorylation of the α_1-subunit.

Conclusions

The cardiac and the smooth muscle, high-voltage–activated, L-type calcium channels are oligomeric complexes of three different subunits: α_1, α_2/δ, and β. The α_1-subunits are splice products of the CaCh2 gene. The α_2-/δ-subunit may be identical with the skeletal muscle subunit. The β-subunits are encoded by different genes. The expression of a tissue-specific subunit combination most likely results in the differences in pharmacology and function of the channel. This genetic polymorphism may explain also the different regulatory mechanisms and possibly offers a chance for refined drug therapy in the future.

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